PHARMACOLOGY OF ADENOSINE RECEPTORS OF THE RAT ISOLATED SUPERIOR CERVICAL GANGLION

by

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I dedicate this thesis to my parents for their help, understanding and love.

DECLARATION

All of the work presented in this thesis are solely the results of my own experimentation.

GERALD P. CONNOLLY

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SUMMARY

This study has examined the effect of purines and pyrimidines on the rat superior cervical ganglion in vitro. Using an extracellular recording technique it was found adenosine and its analogues produced concentration dependent hyperpolarisations. In contrast pyrimidines such as UTP and the P2-purinoceptor agonist alpha,beta-methylene adenosine triphosphate depolarised, via a methylxanthine independent mechanism.

A mechanism for the uptake and degradation of adenosine in the ganglion was evident as the effects of adenosine were enhanced in the presence of adenosine uptake inhibitors or in the presence of an adenosine deaminase inhibitor. The deaminated metabolite of adenosine, i.e. inosine was inactive.

The ionic mechanism for the adenosine-induced hyperpolarisation was examined by altering the extracellular cation concentrations and the use of selective ion channel antagonists. The hyperpolarisation to adenosine was not antagonised by inorganic or dihydropyridine calcium channel antagonists, suggesting adenosine did not alter Ca^{2+} entry. The response to adenosine was resistant to antagonism of sodium and chloride channels and the inhibition of Na^+/K^+ ATPase and $Na^+/K^+/Cl^-$ co-transporters, but was sensitive to changes in extracellular K^+ concentration. The hyperpolarisation to adenosine was enhanced in reduced external potassium solution or the presence of low concentrations of TEA suggesting adenosine may increase potassium conductance.

The response to muscarinic agonists and not other agonists was selectively reduced by adenosine but not by all purines or pyrimidines. The depression of muscarine was selective for the depolarisation and not the hyperpolarisation.

Both the relative order of potency of adenosine and its analogues to hyperpolarise and depress the response to muscarine were indicative of the presence of purinoceptors of the A1 subtype on the rat SCG. Studies to confirm the nature of the adenosine receptor using two selective A1-adenosine receptor antagonists. 1,3-dipropyl-8-cyclopentylxanthine and xanthine amine congener produced an apparently non-competitive inhibition. This may indicate the presence of two receptor subtypes. However, a selective A2 adenosine receptor antagonist 3,7-dimethy-1-propargyl-xanthine did not alter the inhibition of muscarinic responses or the hyperpolarisation caused by purines.

The biochemical mechanism responsible for the hyperpolarisation to adenosine remains elusive. Cyclic adenosine monophosphate phosphodiesterase inhibitors Ro 20-1724 and denbufylline and an adenylate cyclase inhibitor, SQ 22,536, reduced the hyperpolarisation to purines but not the depression of the response to muscarine. However, agents known to increase cAMP levels in the ganglion did not dramatically alter the response to adenosine suggesting the responses to adenosine are not mediated by a change in cyclic nucleotide levels. The ability of phorbol esters to enhance the response to adenosine suggests that protein kinase C is involved in the interaction of adenosine with muscarine. An interaction with the arachidonic acid cascade pathway was suggested by the inhibition of the hyperpolarisation to adenosine by a selective lipoxygenase inhibitor.

LIST OF SYMBOLS AND ABBREVIATIONS

All drugs and chemicals used in this study are listed in table 2.2

A1	Adenosine receptor subtype 1
A2	Adenosine receptor subtype 2
[A]	Concentration of drug A, subscripts: e = extracellular and i = intracellular
AA	Arachidonic acid
ANS	Autonomic nervous system
AP	Action potential
AC	Adenylate cyclase
ACh	Acetylcholine
AChE	Acetylcholine esterase
AD	Adenosine
AHP	After-hyperpolarisation
ATP	Adenosine triphosphate
Ba^{2+}	Barium
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CA	Catecholamine
CAP	Compound action potential
CRC	Concentration response curve
Cd^{2+}	Cadmium
ChE	Cholinesterase
Conc.	Concentration
CPCA	5'-Cyclopropyl-carboxamidoadenosine
CR	Concentration ratio: ratio of the concentration of agonist required to produce a given response in the presence of a competitive antaonist to that required in its absence
CST	Cervical sympathetic trunk

4DAMP	4-diaphenyl acetoxy-N-methylpiperidine methiodide
d.c.	Direct current
DHP	Dihydropyridine
DRG	Dorsal root ganglion
EC_{50}	Concentration which produces 50% of the maximal effect
ECN	External carotid nerve
E_i	Equilibrium potential for an ion (i)
E_k	Potassium equilibrium potential
E_{Na}	Sodium equilibrium potential
e.p.p.	end plate potentials
EPSP	Excitatory postsynaptic potential
fEPSP	fast EPSP
sEPSP	slow EPSP
FURO	Furosemide
G	Guanosine e.g., guanosine triphosphate (GTP) binding proteins i.e., G-proteins
g	Conductance
gM	Neuronal membrane conductance
Gi	Inhibitory guanine nucleotide regulatory protein
Gs	Stimulatory guanine nucleotide regulatory protein
^{3}[H]	Tritiated hydrogen
HETE	Hydroxyeicosatetraenoic acid
HHSD	Hexahydrosiladifenidol
I_{ahp}	Calcium activated potassium current
I_{KCa}	Membrane potassium current gated by calcium
I_K	Potassium current
Im	Membrane potassium current controlled by

	muscarinic receptor
IBMX	Isobutylmethylxanthine
ICN	Internal carotid nerve
IP1	Inositol monophosphate
IP2	Inositol bisphosphate
IP3	Inositol trisphosphate
IPSP	Inhibitory postsynaptic potential
IC_{50}	Concentration which causes 50% inhibition of the maximal effect
Ki	Inhibition constant
Km	Michaelis constant
La^{3+}	Lanthanium
Li^{+}	Lithium
log	Logarithm to base 10
log P	Logarithm of the octanol:water partition coefficient
LN	Late negative potential
LT	Leukotriene
LTE	Long term enhancement
LTP	Long term potential
mAChR	Muscarinic acetylcholine receptor
mV	millivolt
mM	millimolar
M1	Muscarinic receptor subtype 1
M2	Muscarinic receptor subtype 2
M-current (I_m)	Membrane potassium current controlled by muscarinic receptor
nAChR	Nicotinic acetylcholine receptor
NA	Noradrenaline
NANC	Nonadrenergic-Noncholinergic
NDGA	Nordihydroguaiaretic acid
NMJ	Neuromuscular junction

N-MeAtr	N-methyl-atropine
NITR	Nitrendipine
Pa	Phosphatidate
pA_2	Negative logarithim of the concentration of antagonist which shifts agonist concentration-response curve two fold to the right
p.d.	potential difference
PDE	Phosphodiesterase
PDBu	Phorbol 12,13-dibutyrate
PG	Prostaglandin
Pi	Inorganic orthophosphate
PI	Phosphatidylinositol
PIP	Phosphatidylinositol-4-phosphate
PIP	Phosphatidylinositol-4,5-bisphosphate
PIT	Phosphatidylinositol turnover
PK	Protein kinase
PKC	Protein kinase C
PLC	Phospholipase C
(+)PN200-110	Isradipine
PSS	Physiological salt solution
PTX	Pertussis bordatella toxin
Rm	Neuronal membrane resistance
RMP	Resting membrane potential
SCG	Superior cervical ganglion
SEM	Standard error of the mean
SIF	Small imunofluorescent cell
Tx	Thromboxane
uV	microvolts
uM	micromolar

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CHAPTER ONE

GENERAL INTRODUCTION

Chapter 1

INTRODUCTION

1. THE RAT SUPERIOR CERVICAL GANGLION.

The autonomic nervous system (ANS) is an efferent outflow from the brain and spinal cord having a thoracolumbar or sympathetic and a parasympathetic division, the latter comprised of cranial and sacral components. In part the sympathetic nervous system of the rat consists of a series of ganglia on each side of the vertebral column. At the cervical level the ganglia are distinguished by their positions, namely the superior, middle and inferior ganglia. The superior cervical ganglion (SCG) provides sympathetic innervation to the pineal gland, median eminance, cephalic blood vessels, the choroid plexus, carotid body, the neurohypophysis, and thyroid and parathyroid glands. In keeping with the homeostatic role of the autonomic nervous system the SCG is involved in neuroendocrine intergration, and removal of the SCG causes several neurodendocrine and behavioural changes in mammals (Cardinali, Vacas & Gejman, 1981). Several effects arise from the interruption of neuronal connections originating in the SCG including modified water balance, drinking and feeding behaviour in rats, alteration of photoperiodic control of reproduction in mammals and modifications to adenohypophysial, thyroid, parathyroid and calcitonin release in rats (Cardinali et al., 1981; Cardinali, Romeo & Vacas, 1987).

The input to the ganglion cells is nearly all unmyelinated cholinergic axons having their origin in the spinal cord, and preganglionic sympathetic fibres project to adrenergic cell bodies and dendrites of the rat SCG (Hedger & Weber, 1976). The ganglion is complicated in structure and the

nerve cells that give rise to the postganglionic fibres are multipolar with numerous dendrites, of varying sizes and staining characteristics, and are almost entirely enclosed by satellite cells. Most autonomic ganglia have a variable number of small cells which have a high content of catecholamines, as detected by formaldehyde-induced fluorescence, i.e. small intensely fluorescent (SIF) cells (for review see Williams & Jew, 1983) and chromaffin type cells are often in close contact to ganglion cells or capillaries.

It is unlikely that the SCG exist solely for relaying nerve inpulses as anatomical and electrophysiological studies have shown complicated interactions between different cells (Williams & Jew, 1983). In the guinea-pig each postganglionic neurone is multiply-innervated by an average of four spinal cord segments (Nja & Purves, 1977) and the number of axons leaving the ganglion is greater than those innervating the tissue. The pre- to postganglionic fibre ratios of the SCG is reported to be species dependent and to be between 1-11 and 1-30. There is good evidence that SCG may be subdivided into regions of neurones that project to specific target organs (for review see Dail & Barton, 1983). Nja & Purves (1977) demonstrated that stimulation of the ventral roots produces an effector response, with the upper spinal cord segments (T1 to T3) controlling pupillary dilation and more caudal cord segments (T3 to T5) controlling piloerection on the face and neck and vasoconstriction of the ear.

A number of substances including acetylcholine (ACh), dopamine (DA), noradrenaline (NA), 5-hydroxytryptamine (5HT), gamma-aminobutyric acid (GABA), hormones and purines have been reported to function as neurotransmitters or neuromodulators of the SCG. The

presence of catecholamines and in particular DA, in SCG has led to the suggestion by some researchers that they could be the neurotransmitter released by interneurones, and may generate the slow inhibitory postsynaptic potential (sIPSP) (Eccles & Libet, 1961). This hypothesis has been the subject of much controversy, and in the rat SCG little evidence for a catecholamine mediated hyperpolarisation has been found (Newberry,Priestley & Woodruff 1987). However the presence of a wide range of other substances within ganglion cells, such as histamine (Christian, Undem & Weinrech, 1989) and 5HT (Hadjiconstantinou, Potter & Neff, 1982), implies that they may have a neuromodulatory role consistent with the concept of integration of neuronal signals by the SCG. Therefore signals entering the SCG, instead of being relayed to effector organs via monosynaptic processes, may be modulated at both pre- and postsynaptic sites in a highly complex manner (Kuba & Koketsu, 1978).

1.1 Role of cholinergic transmission in the rat SCG

Synaptic transmission has been investigated in considerable detail in sympathetic ganglia. Both the rabbit and rat SCG are well established and popular preparations for the study of the cholinergic synapse and are of particular interest because three distinct cholinergic potentials can be evoked in rabbit (Eccles, 1952; Eccles & Libet, 1961) and rat SCG (Dunant & Dolivo, 1967; Dunn & Karczmar, 1980; Newberry & Connolly, 1989). In order of latency, the potentials are the fast excitatory postsynaptic potential (fSPSP), the sIPSP and the slow excitatory postsynaptic potential (sEPSP). The fEPSP is mediated by nicotinic receptors, as shown by its sensitivity to d-tubocuarine (dTC) and hexamethonium (C6), and the slow potentials are both muscarinic, since they are antagonised by atropine (Eccles & Libet, 1961). It is

thought the fEPSP provides a mechanism for the rapid transfer of frequency-coded information through the synapse (Brown, 1983; Birks & Iscoff, 1988) whereas the major role of the muscarinic processes appears to be a facilitation or reinforcement of neuronal activity over prolonged periods (Libet, Kobayashi & Tanka, 1975).

The actions of muscarinic agents on isolated rat SCG are similar to the effects produced by ACh in the presence of nicotinic receptor antagonists. The predominant effect seen is a slow muscarinic depolarisation recorded as a corresponding extracellular late negative (LN) surface potential (see Eccles, 1952; Eccles & Libet, 1961). The LN potential, termined the sEPSP by Libet (1964; 1967), was recorded with intracellular microelectrodes in the rabbit SCG in 1968. Muscarinic stimulation can also produce a slow muscarinic hyperpolarisation (corresponding to the sIPSP, see Eccles, 1952) and/or postsynaptic repetitive firing both of which are clearly seen upon repetitive stimulation in the presence of an anticholinesterase (antiChE) agent (Volle, 1966).

The results of many pharmacological and biochemical techniques suggest that muscarinic receptors are heterogenous as discriminated by pirenzepine (PIR) (Hammer, Bernie, Birdstall, Burgen & Holme, 1980; Hammer & Giachetti, 1982, see also Levine, Birdstall, North, Holman, Wantanabe & Iversen, 1988). There are at least three different pharmacologically identifiable types of muscarinic receptors and at least five different molecular forms have been discovered (Bonner, 1988) and two concomitant nomenclatures are used to describe these receptors. The three pharmacologically defined muscarinic receptors are represented by a capital M and include M1, which has a high affinity for pirenzepine; M2 which has a high affinity for AFDX-116 (full chemical name: 11-((2-

(diethylamino)methyl)-1-piperidinyl) acetyl)-5,11-dihydro-6H-pyrido(2,3-b)benzodiazepine-2-one) or gallamine and M3, which has a high affinity for 4-diaphenyl acetoxy-N-methylpiperidine methiodide (4DAMP) or hexahydro-siladifenidol (HHSD). The five molecular forms are denoted by Bonner and colleagues (Bonner, Buckley, Young & Brann, 1987; Bonner, Young, Brann & Buckley, 1988) as m1, m2, m3, m4 and m5. It has been found that the properties of m1, m2 and m3 receptors show a good correlation with M1, M2 and M3 subtypes. Four of the five muscarinic receptors identified by Bonner in the rat (m1, m2, m3 and m4) are homologous with four muscarinic receptors (Hm1, Hm2, Hm3 and Hm4) described by Peralta, Ashkenazi, Winslow, Smith, Ramachandran & Capon (1987) in human brain. However the nomenclature used by these investigators is different and Hm4 and Hm3 correspond to m3 and m4 respectively.

It is believed that stimulation of muscarinic receptors activates intracellular responses due to the interaction with guanosine triphosphate (GTP) binding proteins (G-proteins), which bind guanine nucleotides and act as intermediaries in the transduction of the agonist receptor binding and the intracellular signal that produces the response.

It has been suggested that the different muscarinic receptor subtypes are functionally specialised so that M1 receptors and M2 receptors are preferentially coupled to the activation of phosphatidylinositol turnover (PIT) and the inhibition of adenylate cyclase (AC) respectively (Peralta et al., 1987) and that m1, m3 and m5 receptors couple primarily with Gp and in some cases with Gs, whereas M2 and M4 receptors are mainly coupled to Gi and Gk (Peralta et al., 1988; Kim, Lewis, Graziadei, Neer, Bar-Sagi & Clapham, 1989). However other research groups

have argued there is considerable overlap between the receptor subtypes both in terms of coupling to G proteins and intracellular messengers, and these differences may account for the unreliability of any classification of muscarinic receptors based purely on the basis of G-protein coupling or the production of a second messenger (see Peralta et al., 1987; and Chapter 5, for further discussion).

Concomitant with the development of M1 and M2 selective muscarinic antagonists these compounds have been applied to the SCG to discover if the sEPSP and sIPSP are mediated via M1 and M2 receptors respectively. Pirenzepine has been shown to be a potent antagonist of ganglionic depolarisation produced by muscarinic agonists in the rat SCG (Brown, Forward & Marsh, 1980) and selectively depressed the sEPSP in rabbit SCG (Ashe & Yarosh, 1984). In contrast the M2 receptor antagonists, gallamine selectively reduced the sIPSP of the rabbit (Ashe & Yarosh, 1984) and rat SCG (Newberry & Connolly, 1989). Further support for an M2 receptor mediated cholinergic hyperpolarisation was provided by the selective antagonism of the sIPSPs of both rabbit and rat SCG by a selective M2 antagonist, AFDX-116 (see Mochida & Kobayashi, 1988 and Newberry & Connolly, 1989 respectively). Thus the slow synaptic depolarising potential of the rat SCG is mediated by the interaction of ACh with pharmacologically distinct muscarinic binding sites which are similar to those of the cerebral cortex (McCormick & Prince, 1986; North, 1986) are mediated by M1 receptors.

The ionic mechanism of the fEPSP is similar to that for the end plate potential at the neuro-muscular junction (for comparison see Fig. 4 in Kuba & Koketsu, 1978), and involves increases in the membrane conductance to sodium (gNa), potassium (gK) and calcium (gCa). In contrast the

ionic mechanism of the slow potentials are not fully resolved (Kobayashi and Libet, 1970) but studies on voltage-clamped amphibian neurones have revealed that the inward (depolarising) current occurs primarily due to an inhibition of a time and voltage dependent potassium current termed the 'M-current' (Im, see Adams & Brown, 1982). Im has been found to be present in mammalian sympathetic neurones and Im of the rat SCG is reduced by a number of substances including ACh, angiotensin (Constanti & Brown, 1981), luteinizing hormone releasing factor (LHRH) and uridine triphosphate (UTP) (Adams, Brown & Constanti, 1982a).

It is believed that Im the predominant current involved in depolarisation of the rat SCG to muscarine (Brown, 1988) although other currents may also be involved. Studies by Brown & Selyanko (1985a,b) have revealed at least three components of the response to muscarinic agonists; an inward current resulting from Im inhibition, and a simultaneous outward current (Ix) due to voltage independent resting current, apparently carried in part by Cl^- and an occasional late inward current associated with an increased membrane conductance. Other researchers have found the responses recorded via extra- and intracellular electrodes to muscarine are complex and depends upon the concentration of muscarine applied (Hashiguchi, Kobayashi, Tosaka & Libet, 1982; Mochida & Kobayashi, 1986a,b; personal observation; Newberry & Gilbert, 1989a), the extracellular concentration of calcium ($[Ca^{2+}]e$) (Tokimasa & Akasu, 1990) and the neuronal resting membrane potential (RMP) (Adams et al., 1982; Jones, 1989).

The sIPSP is considered to be mediated by an opening of K^+ channels (Horn & Dodd, 1981; Dodd & Horn, 1983; Cole & Shinnick-Gallagher, 1984), although the ionic movements involved in the hyperpolarisation of the rat SCG by

muscarinic agents do not appear to have been determined, but are likely to involve an increased K+ efflux.

1.2 PURINES

1.2.1 Historical perspective and overview

In 1929 Drury and Szent-Györgi reported on the profound physiological properties of purine nucleotides which included hypotension and bradycardia of dog and guinea-pig hearts and these effects were attributed to the adenosine monophosphate (AMP) content of the extracts used. In further experiments the injection of adenosine extracted from yeast produced identical but more potent effects when injected into animals. During hypoxia or post-ischaemic hyperaemia, adenosine 5'-triphosphate (ATP) may have a dominant role as a more potent coronary vasodilator to dilate blood vessels directly or after metabolism to adenosine. The important role of adenosine in the regulation of the cardiovascular system has been reviewed by Berne (1964; 1980).

Since the seminal work of Drury and Szent-Györgi (1929) many physiological roles have been proposed for adenosine and adenine nucleotides of which the modulation of synaptic transmission is probably the most validated. The concept of purines as neuromodulators and neurotransmitters has developed over the last two or three decades, and has been reviewed by Burnstock (1978, 1985) and Stone (1981a, 1989). The physiological and pharmacological effects of adenine nucleotides are often different to those of the parent molecule adenosine (Fig. 1), and separate receptors for each compound have been distinguished (Burnstock, 1978).

Most cell types have been found to respond to adenosine, and a search for drugs that can selectively modify the actions of adenosine has resulted in intensive pharmaceutical research to discover new therapeutic agents. The potential use of these agents include the treatment of asthma and apnoea as respiratory stimulants; as cognitive enhancers and as cardiotonics and renal modulators (Daly, 1982; Williams & Jarvis, 1988; Bowmer & Yates, 1989).

1.2.2 Role of ATP as a neurotransmitter

It is believed that adenosine may function as a general modulator of cell physiology, while ATP functions as a neurotransmitter for some tissues. The first suggestion that ATP might be a neurotransmitter came from Holton & Holton in 1954, who presented evidence for release of ATP from collateral branches of primary afferent sensory fibres during antidromic stimulation, to cause vasodilatation of constricted rabbit ear vessels. Subsequent studies showed that antidromic stimulation of the great auricular nerve was accompanied by ATP release (Holton, 1959).

In the early 1960's, Burnstock and his colleagues recorded inhibitory junction potentials in intestinal smooth muscle in response to stimulation of non-adrenergic non-cholinergic (NANC) nerves. In 1972 Burnstock proposed that the principal active substance released from these NANC nerves was ATP and he proposed the term "purinergic nerve". Subsequent experiments have produced evidence in favour of the release of ATP with neurotransmitters to function as a co-transmitter including observations on the synthesis, storage, release, receptor interaction and metabolism of ATP (see Burnstock 1985). Evidence from other groups has supported the concept that ATP can be

released from synaptic vesicles by itself or with ACh or noradrenaline (Fedan, Hogaboom, O'Donnell, Colby & Westfall, 1981; Sneddon, Westfall & Fedan, 1982; Stjarne & Astrand, 1985).

The direct effects of ATP on postjunctional sites i.e. the P2 purinoceptor, have been reviewed by Burnstock (1972); Stone (1981a,b) and Gordon (1986). As well as being a neurotransmitter it has been postulated that ATP is a neuromodulator because it influences responses produced by classical neurotransmitters such as ACh in the rat diaphragm (Ewald, 1976), the frog skeletal muscle end-plate (Akasu, Hirai & Koketsu, 1981), sympathetic ganglia (Silinsky & Ginsborg, 1983; Akasu, Hirai & Koketsu, 1982, 1983a), noradrenaline in the rat vas deferens (French & Scott, 1983) and GABA in the bullfrog spinal ganglion (Morita, Katayama, Koketsu & Akasu, 1984).

1.2.3 Purine receptor classification

Burnstock in 1978 suggested that the actions of ATP and AD could be attributed to different subtypes of purinergic receptor i.e. those preferring adenosine and sensitive to antagonism by methylxanthines such as caffeine and theophylline (THEO), namely the P1 purinoceptors, and the other preferring adenine nucleotides and insensitive to methylxanthines, the P2 purinoceptors, (ATP receptors). Since then a more elaborate classification of both P1 and P2 receptor subtypes has developed with the introduction of more selective agonists and antagonists. Table 1 summarises the classification, properties and nomenclature that will be used here.

1.2.3.1 Properties of P2 purinoceptors.

P2 receptors have been subclassified into four main categories (Gordon, 1986) of which the P2x and P2y are fairly well characterised (Burnstock & Kennedy, 1985). This subdivision is based on the potency of ATP agonists, antagonism by an arylazidoaminopropionyl-ATP (ANAPP3), receptor distribution and the physiological consequences of receptor occupation (table 1). P2 purinoceptors are located largely postjunctionally. These receptors are more sensitive to ATP, are not antagonised by methylxanthines, do not in general alter adenylate cyclase activity and can lead to the generation of prostaglandin (PG).

The majority of the research on P2 purinoceptors has been carried out on smooth muscles where the stimulation of P2x purinoceptors tends to produce a contraction whereas stimulation of P2y purinoceptors generally has an inhibitory or relaxant effect on smooth muscle. The excitatory P2x purinoceptor appear to be involved in cotransmission of ATP and NA from sympathetic perivascular nerves. These receptors are selectively antagonised by ANAPP3 or suramin and desensitised by alpha,beta-methylene-ATP.

From a study of many smooth-muscle preparations it is thought that the activation of P2 receptors by ADP and ATP alters potassium or calcium ion flux. Activation of P2y-receptors can stimulate G-protein coupled phospholipase C (PLC) activation, while P2x-receptor activation mediates calcium (Ca^{2+}) influx into cells. Until very recently (see Williams & Jarvis, 1988; Williams & Cusack, 1990) P2 receptor antagonists have proven rather unselective and the classification of P2 receptors, like that of P1 purinoceptors is dependant on the development of selective

antagonists. Putative selective P2 purinoceptor antagonists include a photoaffinity analogue of ATP, ANAPP3 (selective for the P2x purinoceptor) and more recently competitive antagonists suramin and beta, gamma-dichloromethylene-ATP.

In some tissues exogenous ATP is rapidly degraded to active compounds such as ADP, AMP and adenosine, which can then interact with other purine receptors and complicate the interpretation of experimental results. To avoid such complexities, slowly degradable analogues of ATP have been synthesised, by joining the bridge oxygens to a phosphate in ATP by a methylene group, producing a methylenephosphonate linkage that is relatively stable to enzymatic hydrolysis. The incorporation of the methylene group into ATP between the innermost phosphates produces alpha, beta-methylene-ATP (α,β-MeATP). This is slowly degraded to α,β-MeADP, which is not degraded further. The incorporation of a methylene group between the outer phosphates produces beta, gamma-metylene-ATP (β,γ Me-ATP), which is reported to be completely resistant to dephophorylation by ectoenzymes such as 5'-nucleotidase (Hourani, Welford & Cusack, 1985; Welford, Cusack & Hourani, 1986).

1.2.3.2 Properties of P1 purinoceptors

Most responses to adenosine have been attributed to an action on extracellular adenosine receptors, which are located on the outer face of the plasma membrane (Huang & Daly, 1974). It has been found with only a few exceptions (see Collis & Pettinger, 1982; Moody, Meghj & Burnstock, 1984) that the adenosine receptor has no significant affinity for adenine nucleotides (Bruns, 1980a; Satchell, 1984), and conversely that ADP and ATP receptors have little or no affinity for adenosine (Burnstock, 1972). It is reported that all adenosine receptors are antagonised

by theophylline, although some require rather high concentrations, whereas the responses mediated by ADP and ATP i.e., P2 purinoceptors are unaffected by theophylline.

1.2.3.3 The "P-site" an intracellular site of action for adenosine

In addition to an action at extracellular adenosine receptors, adenosine can inhibit adenylate cyclase by interacting with an intracellular "P-site", that appears to be located on the catalytic subunit of adenylate cyclase (Premont, Guillon & Bockaert, 1979). The P site has a low affinity for adenosine, in the high micromolar range and is not blocked by methylxanthines (Bruns, 1980b).

The ability of adenosine to stimulate cAMP production in some cells and inhibit cAMP production in others, gave rise to a subdivision of P1 purinoceptors into Ri and Ra receptors, by Londos, Cooper & Wolff (1980) and into A1 and A2 respectively, by Van Calker, Muller & Hamprecht (1979). In many instances P1 receptors control adenylate cyclase activity but not all, and the subdivision of the P1 receptors has been adopted not on the interaction with a secondary messenger, but on the relative order of potency of a series of adenosine analogues (for discussion see Stone, 1985a, and Hamprecht & Van Calker, 1985). The classification of any receptor only in terms of agonist potency can produce anomalies and complicate the interpretation of experimental data (for discussion see Collis, 1985) but it is a simple and effective method for categorising a physiological response to a particular adenosine receptor, until truly selective antagonists are available.

From the data derived from extensive binding studies by Bruns, Lu & Pugsley (1986) and other researchers a detailed view of the adenosine receptor environment has been constructed and these structure activity relationships, have proved useful in classifying the actions of adenosine agonists on a particular response e.g., the correlation of hypomobility effects of adenosine analogues in mice by Durcan & Morgan (1989) and the binding of A2 receptor ligands. More recently it has been demonstrated that the A1 and A2 receptors for adenosine are distinct proteins of different molecular weights (Mr 38000 and 45000 respectively) and that each displays the appropriate pharmacological profile (Stiles & Jacobson, 1988; Barrington, Jacobson, Hutchinson, Williams & Stiles, 1989). Research is currently in progress to purify the A1 adenosine receptor in order to study its properties and obtain amino acid sequence data and clone the receptor gene. As with other 'cloned receptors' these experiments should allow the transfection, and expression of the adenosine receptor into a suitable cell line, to study of the intracellular transduction mechanism.

There is increasing evidence suggesting purines can modulate cholinergic transmission in the autonomic nervous system (Siggins, Gruol, Padjen & Forman, 1977; Burnstock, Cocks, Crowe & Kasakov, 1978 ,; DeGroat & Booth 1980; Akasu et al., 1981, 1982, 1983a,b; Silinsky & Ginsborg, 1983) and these actions are discussed in detail in chapter 5. The presence of purinergic nerves has been described in peripheral systems including perivascular nerves and ATP rather than adenosine is considered to be the neurotransmitter in such nerves (Burnstock, 1978).

1.2.4 The actions of purines in sympathetic ganglia

Consistent with the idea that purinergic nerves release ATP was the finding by Silinsky & Ginsborg (1983) who reported ATP and not adenosine inhibited the release of ACh from preganglionic nerves of frog lumbar sympathetic ganglia. As the inhibition of ACh release by ATP was antagonised by 2mM theophylline and ACh release was unaffected by the P2x selective agonist alpha,beta-MeATP, Silinsky & Ginsborg (1983) concluded the receptor responsible for the inhibitory effects of ATP was not a P1 or P2 purinoceptor. Perhaps this ganglion contains a theophylline sensitive P2 receptor, however with hindsight it would be useful to study the inhibition of ACh using more selective agonists e.g. 2-methylthioadenosine triphosphate (2MeS-ATP), and more selective antagonists e.g. 8-phenyltheophylline (8PT) to determine if the effects of theophylline are due to receptor antagonism or indirect via an increase in intracellular calcium ($[Ca^{2+}]i$) and/or cAMP.

In contrast to Sklinisky & Ginsborg (1983), Akasu and his colleagues published an elegant set of experiments showing that P1 purinergic transmission can occur in some autonomic ganglia. Akasu, Shinnick-Gallagher & Gallagher (1984), were able to mimic the slow hyperpolarising synaptic potentials i.e. the sIPSP of cat vesical parasympathetic ganglia, by applying exogenous adenosine. Both the sIPSP and adenosine hyperpolarisation were depressed by caffeine and adenosine deaminase (ADA), and potentiated by dipyridamole (DIP), an adenosine uptake inhibitor. The sIPSP and the hyperpolarisation induced by adenosine had a similar reversal potential of about -93 mV suggesting the activation of an outward K^+ current. A study by Kato, Katz & Collier (1974), failed to detect the release of tritiated ($[^3H]$) ATP ($[^3H]$ATP) during

preganglionic stimulation of cat SCG prelabelled with tritiated adenosine (3[H]-AD), suggesting ATP is not released upon electrical stimulation of these nerves. However the incorporation, release and metabolism of 3[H]-AD by cat SCG was not determined in the above study. Thus the possiblity that in vivo cat SCG neurones release adenosine and/or ATP which is rapidly metabolised to adenosine which modulates synaptic transmission has yet to be assessed.

Release of adenosine may occur via presynaptic (Silinsky, 1975) or postsynaptic sites (Fredholm, Fried & Hedqvist, 1982) and it has been suggested that adenosine may be a retrograde inhibitor of synaptic transmission (Israel, Lesbats, Manaranche, Menunier & Franchon, 1980). Thus ACh released alone or with a cotransmitter could activate the postsynaptic region to release adenosine, which may inhibit further ACh release.

The spontaneous release of purines has been observed when ganglia are labelled with tritiated adenosine (^{3}[H]-AD) both in culture (Wolinsky & Patterson, 1985), and for intact rat SCG (McCaman & McAfee, 1986) and frog paravertebral ganglia (Rubio, Bencherif & Berne, 1988). Purine release was increased by brief exposure to high potassium (Wolinsky & Patterson, 1985; McCaman & McAfee, 1986; Rubio et al., 1988). Intact rat SCG also increased their release of 3[H]-purines during presynaptic electrical stimulation and hypoxia, suggesting purines may be involved in the physiological response to stress.

It is interesting that Bencherif, Rubio & Berne (1986) found orthodromic stimulation of both frog and rabbit sympathetic ganglia, caused concurrent release of both ^{3}[H]-AD and ^{3}[H]-ACh. Even more interesting is the report that ^{3}[H]-purines, of which 60% was ^{3}[H]-AD, are spontaneously released from the postsynaptic structures of the frog ganglion. The rate of release of ^{3}[H]-purines was increased by both antidromic stimulation and the application of carbachol, but the relative proportion of

^{3}[H]-AD was constant, suggesting ^{3}[H]-AD was released directly and not as a consequence of the extracellular metabolism of ^{3}[H]-AD. Rubio and colleagues concluded that the primary release of adenosine was from the postsynaptic structures during synaptic activation.

It is tempting to regard the results of Rubio and colleagues as evidence for a role of adenosine as a retrograde inhibitor of synaptic transmission (Israel et al., 1980), but the results of Silinksy & Ginsborg (1983) are at variance with this idea. Silinsky & Ginsborg (1983) found ATP and not adenosine inhibited the release of ACh from preganglionic nerves of the frog lumbar sympathetic ganglia. An alternative mode of action of postsynaptically released adenosine would be to depress the postsynaptic response to ACh. During sustained nerve activity the level of adenosine would rise dramatically, due to both the degradation of ATP to adenosine and the postsynaptic release of adenosine upon depolarisation, leading to the activation of postsynaptic adenosine receptors and depression of the postsynaptic response to ACh.

1.2.4.1 Can purines alter the postsynaptic response of ganglia to neurotransmitters?

There are a few reports that provide good evidence that purines can alter both pre- and postsynaptic events of sympathetic ganglia. ATP was found to modulate the postsynaptic response of bullfrog sympathetic neurones to ACh (Akasu & Koketsu, 1985) and GABA (Morita et al., 1984) and may arise from an increased sensitivity of the nicotinic receptors due to an action of ATP on an allosteric site of the ACh receptor-ion channels (Akasu & Koketsu, 1985).

1.2.4.2 Effect of purines on the rat SCG.

Both Henon & McAfee (1983b) and Alkadi et al. (1984) reported that adenosine and its analogues decreased synaptic transmission in the isolated rat SCG by reducing the height of the compound action potential (CAP). Henon & McAfee (1985) concluded "these agents are presumed to act presynaptically since they do not affect postsynaptic responses to exogenous cholinergic agonists". At high frequencies of stimulation adenosine facilitated ganglionic transmission during repetitive stimulation of the preganglionic nerve (Henon & McAfee, 1983a,b). It was postulated that adenosine functions as a "high pass filter", inhibiting single or weak synaptic inputs but facilitated transmission of higher frequency events, (Henon & McAfee, 1985) and this action would be appropriate as a mechanism for boosting synaptic transmission in times of stress.

Using intracellular electrodes, Henon & McAfee (1983a) found that adenosine (1-200uM) produced small (1-4 mV) transient hyperpolarisations, which were not dose-related and inconsistent in different trials on the same cell. In contrast the application of adenosine during the intracellular generation of postganglionic action potentials inhibited three Ca^{2+} dependent potentials, namely, a) a shoulder on the falling phase of the action potential (AP); b) an after-hyperpolarisation (AHP) and c) a regenerative Ca^{2+} spike (recorded in tetrodotoxin (TTX) and tetraethylammonium (TEA)). The potency order for inhibition of Ca^{2+} currents was R(-)-Phenylisopropyl-adenosine (R-PIA) > 2 chloro-adenosine (2CA) > adenosine (AD) > cyclic adenosine-5′-monophosphate (cAMP). The effects of adenosine and 2CA were antagonised by theophylline, suggesting an external P1 purinoceptor, but Henon & McAfee did not assign a receptor subtype. These

researchers concluded that the adenosine hyperpolarisation "does not appear to be an important mechanism in sympathetic ganglia" and that adenosine directly inhibits postganglionic inward Ca^{2+} currents. These results have been cited and interpreted by Ribeiro & Sebastiao (1986) to provide support to the concept of a third adenosine receptor, which is linked to decreased calcium entry.

1.3 AIMS OF THESIS

The studies reviewed above suggest that purines such as adenosine and ATP may function to modulate the postsynaptic sensitivity of the rat SCG. It is the aim of this thesis to answer the following questions:

1. Do purines and pyrimidines alter the membrane potential of the rat SCG and what receptors are responsible for any potential changes?

2. Do purines and pyrimidines modulate the postsynaptic sensitivity of the rat SCG to other agents?

3. Does adenosine alter the membrane potential of the rat SCG by inhibiting the entry of extracellular calcium.

4. What biochemical events mediate the actions of adenosine? Does adenosine function via an intracellular messenger to affect cyclic nucleotide metabolism.

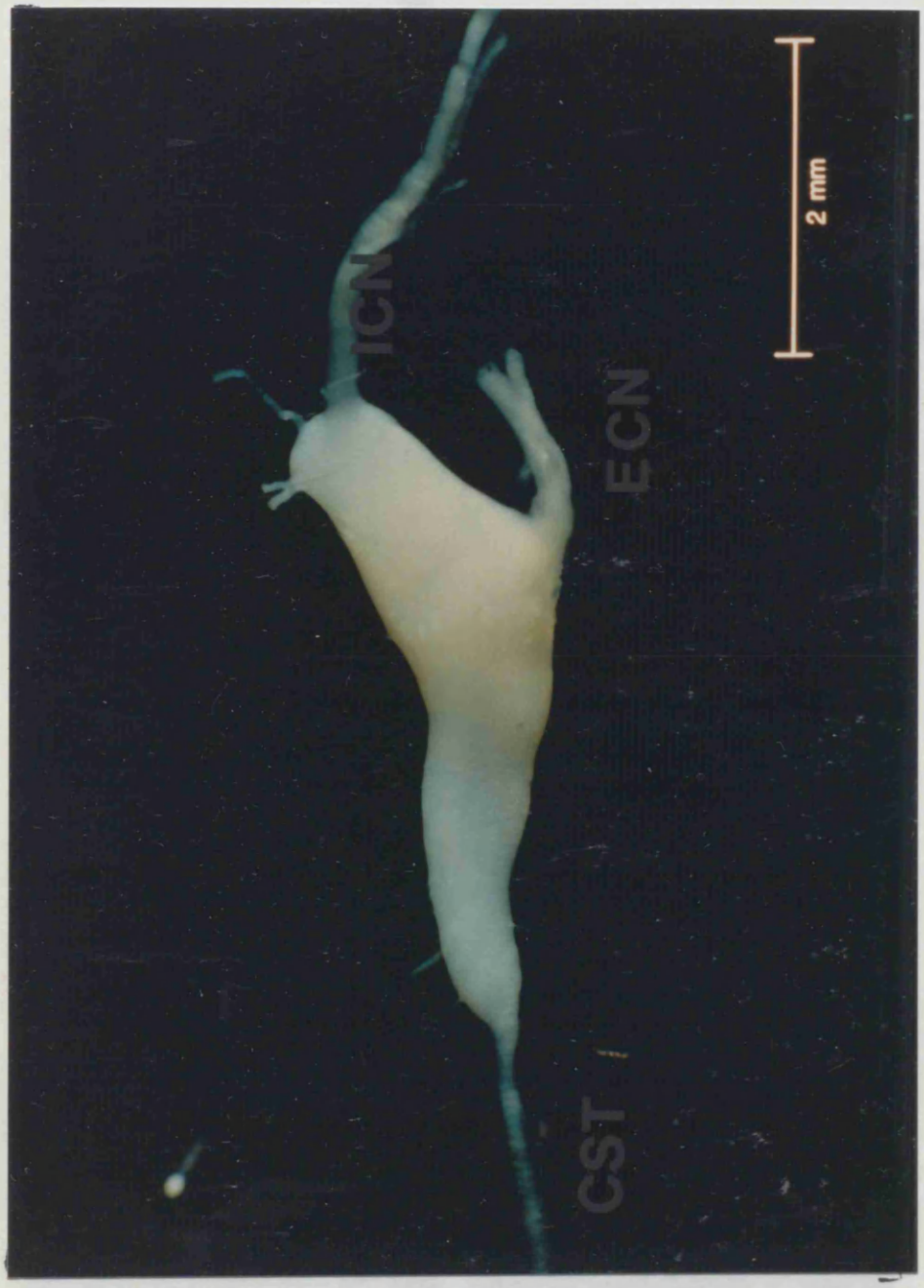
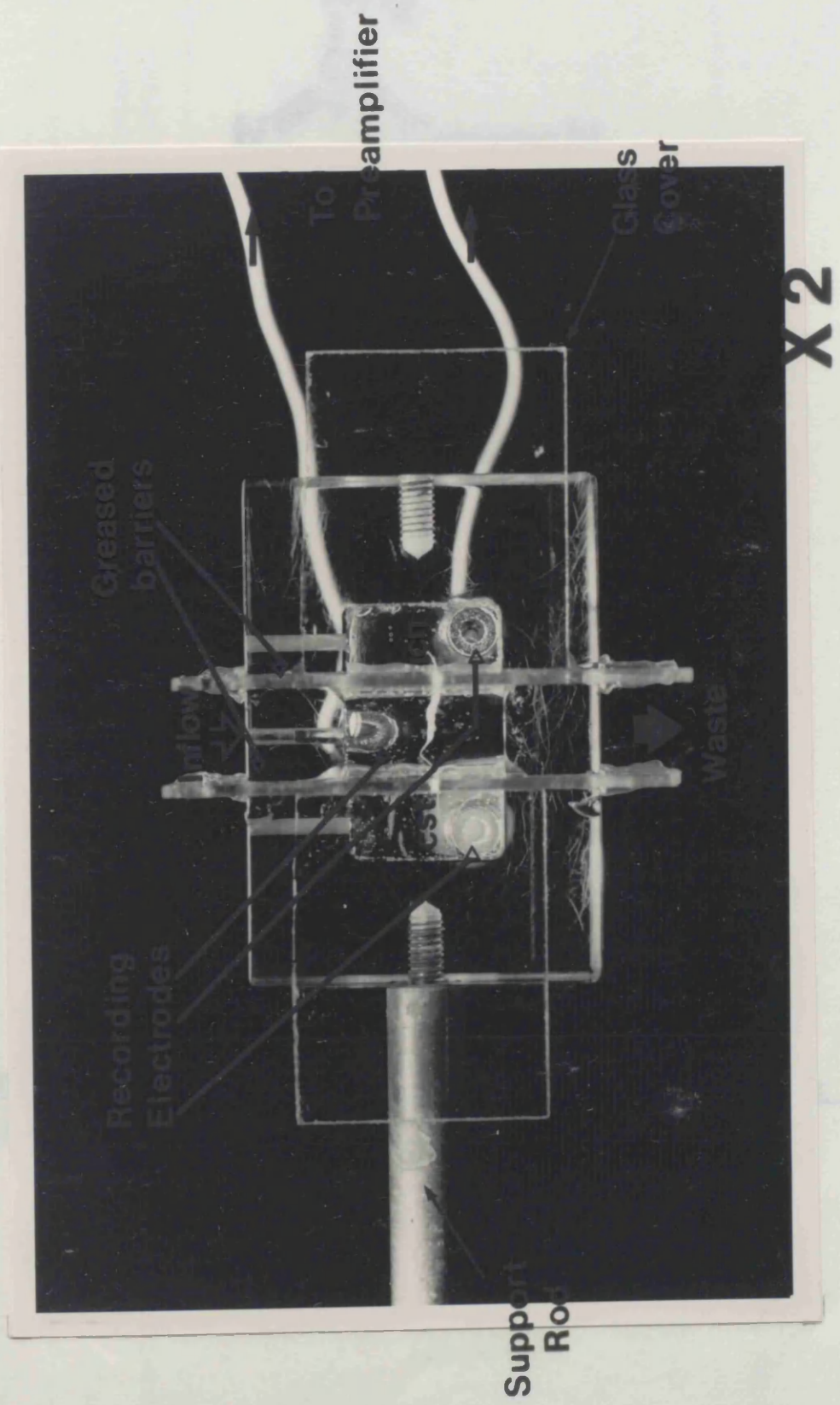


Plate 1. Photograph of an isolated superior cervical ganglion of the rat showing the major nerve trunks.

The postganglionic nerve trunks are denoted by ICN for the internal carotid nerve and ECN for the external carotid nerve. The cervical sympathetic trunk (CST) forms the preganglionic nerve input.

Plate 2. Photograph of an isolated superior cervical ganglion of the rat in a 'Perspex' recording chamber as used for recording direct current potentials between the perfused central chamber, containing the ganglion body and the right hand postganglionic chamber, containing the internal carotid nerve (ICN).



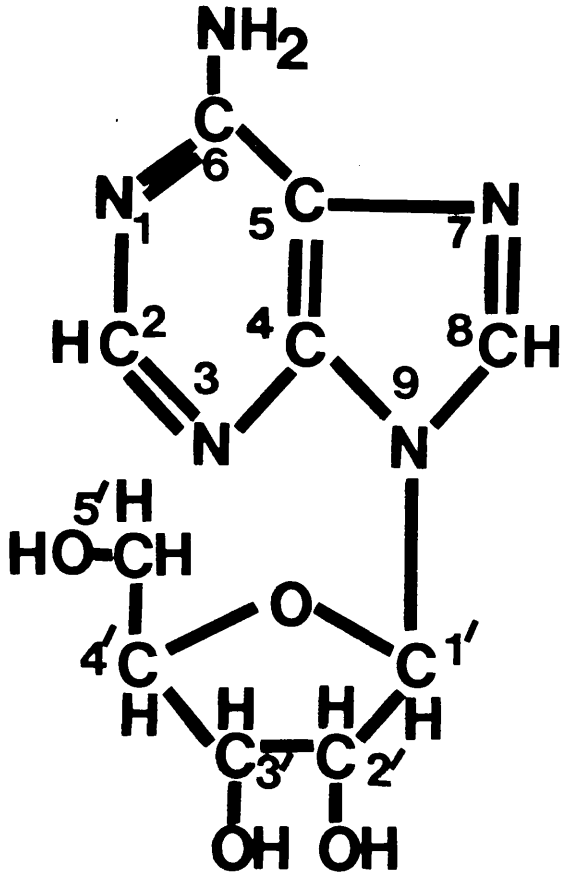


Fig. 1 Structural formula of adenosine illustrating the nomenclature used in this thesis.

Table 1 CLASSIFICATION OF PURINERGIC RECEPTORS

TYPES		SUBDIVISIONS			
Symbol	Selectivity for purine agonists		Affinity for adenosine (uM)	Receptor subtype	Selectivity for antagonists
	All P1 purinoceptors are antagonised by alkylxanthines e.g. 8-phenyltheophylline				
P1	AD > AMP > ADP >= ATP	R-PIA > NECA >> S-PIA	0.01 - 0.1	A1	DPCPX
		NECA > R-PIA > S-PIA		A2	DMPX, CGS 15943A
			0.1 - 1	A2a	
			2 - 20	A2b	
		R-PIA = CHA = NECA > 2CA > S-PIA		A3	
P2	ATP >= ADP > AMP > AD	a,b-MeATP, b,g-MeATP > ATP = 2MeSATP		P2x	ANAPP3, Suramin
		2MeS-ATP >> ATP > a,b-MeATP, b,g-MeATP		P2y	
		2MeS-ATP > ADP		P2t	ATP, AMP, AD
P site	2,5-DDA > AD >> 2-CA		>10		5-MTA

For key to abbreviations see ABBREVIATIONS or Table 2.1

CHAPTER TWO

EXPERIMENTAL METHODS

Chapter Two EXPERIMENTAL METHODS

Introduction

The rat SCG preparation has been extensively studied both iv vivo and in vitro and the pre- and post-ganglionic nerves are easily identified. Classically the ganglion is left in situ and is perfused through its normal vasculature and this method is most suited to large animals such as the cat or rabbit (for review see Collier & Kwok, 1982). In vivo the high metabolic activity of the rat SCG is maintained by the passage of nutrients and oxygen into a capillary plexus from its rich blood supply via the neck into both pre- and postganglionic nerve trunks.

The isolated superfused SCG has been described as the 'poor mans microelectrode' by which it is meant that some indication of the effect of drugs on the resting membrane potential (RMP) can be inferred from changes in the polarity of the ganglion i.e., the change in direct current (d.c.) potential recording. The major difference between intracellular and extracellular recording techniques, is that an intracellular electrode records mainly the membrane potential from a single cell body, whereas both the sucrose and grease gap methods record the local field potential generated by the somas of the ganglion cells that span the gap and their initial axon segments and a part of the proximal axons.

The method adopted here was a modification of the sucrose-gap technique (Kosterlitz & Wallis, 1966; McAfee & Greengard, 1972) where a grease-gap is used instead of sucrose as the later technique requires the precise control of flow rates during the recording and changing the perfusate, to reduce flow artifacts. The grease gap

as described here had similar properties to those reported by McAfee & Greengard (1972) but without the complications and confounding factors of the sucrose gap method. The grease-gap method can be used to record both the basal d.c. potential and synaptic potentials of superfused ganglia. The neurones of the rat SCG are isolated from their environment by a thick continuous capsule of connective tissue and this must be removed for in vitro experiments, to allow full penetration of drugs, oxygen and superfusate (see Watson, 1969). Desheathed rat SCG can be maintained in vitro for hours, with no change in morphological integrity (Sansone, McIsaac & Tomasulo, 1982) and qualitatively similar responses to some drugs can be obtained even after overnight storage (Brown & Caulfield, 1979; personal observation). Thus the isolated rat SCG is fairly robust and useful ganglion for the analysis of the actions of drugs on neuronal tissue.

The major difference between intracellular and extracellular recording techniques, is that an intracellular electrode records mainly the membrane potential from a single cell body, whereas the grease gap records the local field potential generated by the membrane current generated from the somas of the ganglion cells that span the gap but also from their initial axon segments and a part of the proximal axons. The sucrose gap requires the precise control of flow rates during the recording and changing the perfusate, to reduce flow artifacts. The grease gap as described here had similar properties to those reported by McAfee & Greengard (1972) but without some of the complications and confounding factors of the sucrose gap.

A recent development has been the introduction of the isolated perfused rat sympathetic ganglion, as described by Birks & Isacoff (1988). The isolated perfused rat SCG

appears to have some distinct advantages over the grease gap method as it allows the rapid access and equilibration of drugs and ions at the synaptic site and minimises the accumulation of potassium in the intracellular space, during electrical stimulation. However as this new technique was unreported until a substantial part of the experimental work for this thesis was completed, the results presented here are from experiments using the grease-gap method.

2.1 Drugs and solutions used

The compounds, names of chemical suppliers and abbreviations used in this study are listed in table 2.1. All other chemicals were obtained from British Drug Houses (BDH) Ltd or Fisons Ltd and were of "Analar" quality.

I am most grateful for the generous gifts of: methylfurmethide (MeF) and dihydro-beta-erthroidine (DHBE) from Merck Sharp & Dohme, USA; pirenzepine and dipyridamole (DIP) from Thomae-Biberach/Riss, DGRM; AFDX-116 from Boeringer Ingelheim, Bracknell, U.K.; 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) from Roche Products Ltd, Welwyn Garden City, U.K.; Dr. Barraclough of The Wellcome Foundation Ltd for erythro-6-amino-9-(2-hydroxy-3-nonyl) purine hydrochloride (EHNA); Dr. R. Marshal of University of Wales, for Suramin, Dr. J.W. Daly of NIH for 1-propargyl-3,7-dimethylXanthine (DMPX); Dr. N.J. DeSouza of Hoechst, India for forskolin (FSK); Dr. Lucania of the Squibb Institute for Medical Research, NJ, USA for SQ 22-536; May & Baker Ltd., Dagenham, U.K., for M&B 22948 and Professor Stone for 6-(2-hydroxy-5-Nitrobenzyl-thioguanosine) (HNBTG), A23,187 and N6-(9H-fluorenylmethyl) adenosine (PD 117,413).

2.1.1 Composition of the physiological salt solution (PSS)

SCG preparations were normally perfused with a PSS which had the following composition (mM) - NaCl, 125; $NaHCO_3$, 25; KCl, 5; KH_2PO_4, 1; $MgSO_4$, 1; glucose, 10; $CaCl_2$, 2.5; and all procedures were carried out using this PSS unless stated differently in the text. Nominally calcium free PSS ($0Ca^{2+}$ PSS) contained no added $CaCl_2$. It is expected that a PSS without added calcium chloride, i.e. 0mM Ca^{2+} PSS could have as much as 30uM free Ca^{2+} in solution, due to Ca^{2+} leaching from glassware and contamination from chemicals used (Campbell, 1983). To reduce the calcium concentration to submicromolar concentration PSS was prepared without added calcium chloride and with 2mM EGTA, and is referred to as $0Ca^{2+}$/EGTA PSS. For a nominally potassium free PSS ($0K^{+}$ PSS), KCl was omitted and KH_2PO_4 was replaced with an equimolar concentration of Na_2HPO_4 (1mM). For nominally magnesium free PSS ($0Mg^{2+}$ PSS), Mg_2SO_4 was omitted and the magnesium concentration present was estimated from the manufacturer's data as less than 11uM. Phosphate and sulphate free PSS were made by replacing Mg_2SO_4 by $MgCl_2$ (1mM) and KH_2PO_4 by KCl (1mM). This PSS was used when calcium chloride was replaced by cobalt (CO^{2+}) chloride, cadmium (Cd^{2+}) chloride, or barium (Ba^{2+}) chloride.

HEPES PSS was prepared by replacing the $NaHCO_3$ of the phosphate and sulphate PSS with 5mM HEPES. NaCl was replaced by LiCl (125mM) for Lithium-HEPES (Li-HEPES). Calcium was replaced by lanthanum for lanthanum-HEPES (La-HEPES). Sucrose at 20mM in PSS was used when comparing the response of adenosine to that in Li-PSS (lithium chloride 10mM added to PSS) to avoid changes in osmolarity (Sebastiao, 1989). Rubidium (Rb^{2+}) at 1mM was added to PSS to give a PSS containing 1mM Rb. Rb-PSS was prepared

by replacing 6mM potassium chloride with 6mM rubidium chloride. Low Cl^- PSS containing 8.5mM $[Cl^-]e$ was made by modifying PSS by replacing sodium chloride with sodium isethionate (at 125mM) and replacing potassium chloride by potassium hydrogen carbonate (5mM).

PSS were made fresh each day in double glass distilled water or milli-q (Millipore systems) water to prevent the precipitation of trace impurities. PSS solutions were oxygenated with 5% carbon dioxide/95% oxygen and HEPES solutions with pure oxygen for at least 30 minutes before use. Random checks of PSS with a pH meter or blood gas analyser indicated that a pH of 7.4 was maintained.

When necessary drugs were dissoved in PSS as 10mM stock solutions in 10 to 100 uL of hydrochloric acid (1 or 10 M) or sodium hydroxide (1 or 10 M) and stored as aliquots at -20°C. Drugs diluted in PSS did not alter the pH of the PSS perfused on to the ganglion. Cromakalim was made up as a stock solution (10mM) in 70% ethanol and lemakalim as a 10mM stock solution in 50% polyethylene glycol.

Where drugs were dissolved in solvents control responses for vehicles were determined and are described in table 2.2. Changing from PSS to a new PSS often altered the d.c. potential and/or the response to adenosine (Table 2.2) and therefore all experiments to examine the effect of drugs were performed using suitable control PSS of the same composition as the PSS containing drugs.

The effect of Ca^{2+} channel antagonists on the response to adenosine and the depression of muscarine was assessed by changing from the appropriate PSS containing metal ions or Ca^{2+} channel antagonists. Nickel (Ni^+) chloride at 500uM or lanthanum (La^{3+}) chloride at 20uM was added directly to PSS and for a 1mM Ni-PSS nickel chloride replaced 2.5mM

calcium chloride in PSS. Experiments using dihydropyridines (DHPs) were carried out with the tissue bath in the dark. The perfusate and associated equipment were kept in darkness apart from a safety lamp (Wratton series filter number 1, 60 watt bulb) placed about 1 meter and at 45 degrees to the tissue bath. DHPs were dissolved in absolute ethanol as 10mM stock solutions and diluted at 100ul stock solution per litre PSS. Ethanol did not alter the d.c. potential but did significantly reduce the response to adenosine (Table 2.2); the effects of incubation with DHPs were compared to control responses in PSS + ethanol (2mM).

2.2 DISSECTION AND ISOLATION OF THE SUPERIOR CERVICAL GANGLION

Male Wistar rats (120 to 390g) obtained from the animal houses of St. George's Hospital Medical School (SGHMS) or SKB, were maintained at 22 $\pm$ 1^{o}C, on a 12 hour light/dark cycle, with food and water available ad libitum.

In a few preliminary experiments carried out at SGHMS rats were killed by a blow to the head and cervical dislocation rather than with a lethal dose of urethane. In all other experiments rats were injected with urethane (1.5-2.0g/kg), given intraperitoneally as a 25% w/v solution in saline. Rats in terminal anaesthesia were pinned, ventral side up. The thoracic cavity was opened and the aorta was cut to drain off the blood from the head and neck region. The skin of the neck region was cut longitudinally from the mid-thorax to the jaw, and removed. The overlying fatty tissue and underlying muscle was dissected away to expose the trachea and oesophagus, which were removed. The common carotid arteries and vagus nerves were separated from the preganglionic cervical sympathetic

trunk (CST). The ganglia were located by following the preganglionic sympathetic trunk to the carotid bifurcation. Under a binocular microscope (x 10), the preganglionic nerve was separated from the vagus and carotid artery, using watch makers No.5 forceps. The ganglia was cut away from the carotid artery at the level of the bifurcation by holding the connective tissue surrounding the ganglion or the external carotid nerve (ECN) and cutting underneath the ganglion body with iridectomy scissors. The postganglionic internal carotid nerve (ICN) which lies next to the internal carotid artery was gently cut free with iridectomy scissors and followed down the bony projection of the hyoid arch, as far as possible before cutting. At least 25mm of CST and 5mm of ICN was obtained by this method. The contralateral ganglion was removed from the animal within 5 minutes, while the first ganglion was maintained in about 30 mL PSS in a glass petri dish, kept in a moist saturated oxygenated chamber. The first ganglion was then placed in fresh PSS in a petri dish and the connective tissue sheath removed, with the aid of a dissecting microscope (x40), and the ganglion placed in the incubating chamber while the other ganglion was desheathed.

2.3 ELECTRICAL RECORDINGS

2.3.1. Preparation of electrodes

Preformed disc electrodes (product code E202, 2mm dia x 4mm deep, Clark Electromedical Instruments) were inserted into the top of a cut down plastic 1mL Luer syringe. The electrodes were glued in place overnight, and were ready for use the next day. The tissue bath was designed to allow the electrodes to be easily removed for replacement and cleaning of the bath and electrodes (plate 2.2).

Electrodes were either kept in place overnight, or when not used for a few days placed in a 20mL glass vial containing sterile saline. Before use each day electrodes were washed with PSS and allowed to equilibrate while the dissection was performed. The recordings of the d.c. potential taken at this time showed little drift in the base line, indicating the electrodes were satisfactory.

2.3.2 Extracellular d.c. recording

The tissue bath was based on a modification of the sucrose gap technique first described for use with rabbit SCG by Kosterlitz, Lees & Wallis (1968), and modified for use with the rat SCG, using insulating grease-sealed partitions by Brown & Marsh (1978) and Newberry, Priestley & Woodruff (1987).

Desheathed ganglia were mounted in a three compartment bath, with the ganglion body situated in the central chamber (bath volume about 500 uL) and the pre- and post-ganglionic trunks protruding through lightly greased (High vacuum silicon grease, BDH) slots into the outer chambers as shown in plate 2.1. This was achieved by placing the nerve trunks above the slot of each of the lower barriers and lightly pressing down pregreased upper barriers onto the nerve trunks, in order to form an efficient electrical seal. All chambers were filled with PSS and perfusion of the central chamber started. The pre- and post-ganglionic chambers were covered with a piece of greased glass and the junction between the glass and the barriers greased to prevent differential evaporation of PSS. Pre-oxygenated perfusate passed through a heating coil to arrive at the tissue bath at 25 $\pm$ 1^{o}C (measured with a small thermocouple). Perfusate was run to waste via fibre wicks (Boots 3 star nappy liner) placed in between the barriers.

The potential difference (p.d.) between the central chamber (earthed to reduce electrical noise) and the adjacent chamber was recorded i.e., for the SCG between the ganglion body and the ICN or ECN. The potential difference was filtered through a 1M ohm resistor and a 2.2 uF capacitor in parallel, to allow only d.c. potentials to be recorded. Preliminary monitoring of the recordings on an oscilloscope of the electrodes before and after filtering confirmed that 50Hz was removed by the filter. No difference in the time course of the response to drugs was seen with or without the filter but the basal noise was dramatically reduced. The resistance of the ganglia recorded in situ was about 60K ohms and changes in d.c. potential were amplified via d.c. pre-amplifiers (Electromed, model 3461/3460 with 10M ohms input impedance) and recorded on a two channel Electromed pen recorder (model MX216).

2.3. Application of drugs

A maximum of up to 300 uL of concentrated drug solution was diluted with 10 or 20 mL PSS for superfusion on to the ganglion. Drugs were superfused by changing the flow from a reservoir containing PSS to a glass vial containing drug/s in PSS. The flow rate was constant for each tissue bath and averaged around 2.5 mL per minute, ensuring the bathing fluid was exchanged 4 to 5 times each minute. The time required for the drug to reach the ganglion, i.e., the 'dead time' was estimated from preliminary experiments, by measuring the time taken for; (a) a dye to reach the central chamber or (b) a small air bubble to arrive at the chamber and (c) a ganglion to response to 12mM potassium. All three methods were in good agreement with each other and the 'dead time' was estimated to be around 25 seconds.

2.4. Presentation of results

In each experimental record a bar shows the point where a drug reached the ganglionic chamber and a downward deflection of the recording pen represents a hyperpolarsiation of a ganglion. Responses were recorded so that a 40mm pen deflection was equivalent to a change in potential difference of 500 microvolts (uV). The concentration of the drug in the perfusate is shown under the bar.

2.5. Calculations and evaluation of data

Results from different experiments are summarised as the mean $\pm$ standard error of the mean (SEM), and the number of ganglia studied (N). A minimum of two experiments and animals were used to obtain each set of results. Data are presented either as response in uV or as a % change from control response on the same ganglion. Statistical analysis was performed on untransformed data (values in uV) using a paired or unpaired t-test (2 tailed), and values were considered statistically significant if P values of less than 0.05 were obtained. Where data was expressed as a % depression of muscarine, responses were compared for statistical significance ($P < 0.05$) using the Wilcoxon signed rank test.

Table 2.1. Compounds used and their suppliers and abbreviations used throughout the text.

COMPOUND	ABBREVIATION
Sigma Chemical Corporation:	
2-chloroadenosine	2CA
acetylcholine chloride	ACh
adenosine aminohydrolase, Type III	ADA
adenosine hemisulphate	AD
adenosine 5'-triphosphate disodium	ATP
adenosine 3':5'-cylic monophosphate monosodium	cAMP
bovine serum albumin, fraction V (product code A-7638)	BSA
8-bromo-adenosine 3':5'-cylic monophosphate monosodium	8BrcAMP
N6-2'-O-dibutryladenosine 3':5'-cylic monophosphate monosodium	dbcAMP
4-aminopyridine	4AP
alpha-beta-methylene-adenosine-5' triphosphate sodium	a,b-MeATP
beta-gamma-methylene-adenosine-5' triphosphate sodium	b,g-MeATP
atropine methyl nitrate	N-Me-ATR
apamin	Apamin
carbachol chloride	CCh
cytidine 5'-triphosphate, Type III sodium salt	CTP
cobaltous chloride	CoCl
3,4-diaminopyridine	3,4DAP
1,1 dimethyl-4-phenyl piperazinium iodide	DMPP
ethyl carbamate (urethane)	
ethylene glycol-bis(b-aminoethylether)-N,N,N',N', tetra acetic acid	EGTA
eledoisin related peptide dihydrochloride	ERP
furosemide	FUR
gamma-aminobutyric acid	GABA
hexamethonium bromide	HEX
N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid	HEPES
5-hydroxytryptamine	5HT
indomethacin	INDO
inosine	I
isoprenaline	ISO
1-(5-(isoquinolinylsulfonyl)-2-methylpiperazine dihydrochloride	H7
isethionate sodium	
lanthanum chloride	LaCl
5'-deoxy-5'-methyl-thioadenosine	MTA
5'-N-ethyl-carboxamidoadenosine hydrate	NECA
D-Ala-6-luteinising hormone releasing hormone	LHRH
noradrenaline d-bitartrate	NAdr
nordihydroguaiaretic acid	NDGA

pilocarpine hydrochloride	PILO
pentobarbitone sodium	PB
2-phenylaminoadenosine	PAA
phorbol 12,13-dibutyrate	PDBu
sodium carbonate	$NaCO_3$
sucrose	sucrose
tetraethylammonium	TEA
theophylline	THEO
tetrodotoxin	TTX
trifluoperazine	TFP
d-tubocuarine chloride	d-TC
uridine 5'-monophosphate disodium	UMP
uridine 5'-triphosphate sodium	UTP

Research Biochemical Incorporated (Semat, UK)

N6-benzyladenosine	BZA
cyclopentyladenosine	CPA
8-cyclopentyl-1,3-dipropylxanthine	DPCPX
dipyridamole	DIP
5'-N-ethylcarboxamidoadenosine	NECA
muscarine hydrochloride	MUSC
8-phenyltheophylline	8-PT
8-para-sulfophenyl-theophylline	8psPT
2-methyl-thioadenosine	2-MeS-ATP
methoctramine hydrochloride	MTO
oubain octahydrate	OU
oxotremorine-M	OXO-M
N6-(2-phenylisopropyl)adenosine R(-)-isomer	R-PIA
N6-(2-phenylisopropyl)adenosine S(+)-isomer	R-PIA
xanthine amine congener	XAC

British Drug Houses Ltd

cadmium chloride	CdCl
ethyl alcohol	EtOH
nicotine hydrogen (+) tartrate	NICO
potassium acetate	K^+Ac

Aldrich Chemical Company Ltd

lithium chloride	LiCl

May & Baker

5-hydroxytryptamine creatine sulphate	5-HT

Fisons Ltd

barium chloride	BaCl
nickel chloride	NiCl

I am indebted to SKB and in particular the antihypertension and chronic organic brain disease research groups for supplying the following:

cromakalim	BRL 34915
lemakalim	BRL 38227
denbufylline	BRL 30892
5-carboxytryptamine	5-CT
(+) isradipine	(+) PN 200-110
nitrendipin	NITR

Table 2.2. Effect of different drug vehicles and physiological salt solutions on the response of the rat SCG to adenosine

The statistical significance (paired t-test) between normal PSS and the new PSS is denoted by * for $P<0.05$.

PSS	N	Response to adenosine (100uM, 2 minutes PSS	new PSS
PSS + BSA (80mg/L)	4	-69 ± 14	-45 ± 10*
PSS + ethanol (2mM)	8	-79 ± 7	-69 ± 9*
PSS + sucrose (20mM)	4	-110 ± 12	-79 ± 4*
Ca^{2+} HEPES	6	-60 ± 7	-50 ± 6
PO4/SO4 free	3	-65 ± 3	-53 ± 3

Changing from PSS to PSS + sucrose (20mM) produced a transient depolarisation in two out of four ganglia (+40 ± 23uV, n=4) but no change in the response to adenosine or muscarine.

There was no significant difference in the depression of the response to 100nM muscarine by 100uM adenosine in PSS -26 ± 4% and in HEPES-PSS was -19 ± 3% (n=6).

Changing from PSS to PO_4/SO4 free PSS did not alter the basal d.c. potential or the response to adenosine. Changing from PSS to HEPES-PSS produced a transient depolarisation (205 ± 77uV, n=6) that returned to the original baseline within 20 minutes, with no significant effect on the responses to adenosine or the response to 12mM K^+ (response in PSS = 278 ± 36 and Ca^{2+} HEPES-PSS 308 ± 49uV (N = 3)).

CHAPTER THREE

EFFECTS OF PURINES AND SOME PYRIMIDINES

ON THE RAT SCG

Chapter Three

INTRODUCTION

While studying the effects of catecholamines on the rat SCG, Brown, Caulfield & Kirby (1979) noted that exogenous cyclic AMP (10-100uM) and related compounds hyperpolarised. The relative potency of the compounds was AD > cAMP = AMP > ADP > ATP > dibutyryl cyclic AMP (dbcAMP). These results are consistent with the presence of P1-purinoceptors on the rat SCG as defined by Burnstock (1978). However the order of potency was based upon results for a single ganglion kept overnight before use and the maximum response to each compound was not obtained.

In order to determine if the rat SCG has receptors for adenosine and other purines a more detailed investigation was performed. Firstly the ability of adenosine and some purines to produce reproducible hyperpolarisations of the rat SCG was determined and quantified. Secondly the site of action of purines was elucidated in experiments to determine if adenosine indirectly activates the SCG e.g. via the release some mediator or if purines have an action on the nerve trunks. Thirdly it has been reported that the response of various tissues to a number of different transmitters is altered by the actions of adenosine (see review by Stone, 1989) and the ability of adenosine to alter the response of the rat SCG to muscarine and other agonists was examined.

3.1 VALIDATION OF EXPERIMENTAL METHODS AND THE EFFECTS OF ADENOSINE ON THE RAT SCG

The sympathetic ganglion presents various problems when quantitative measurements are required and to obtain

consistent and reproducible responses the following factors were studied.

3.1.1 The effect of anaesthetic on the response to drugs

No qualitative differences in the responses of the rat SCG (n=12) in PSS to adenosine, muscarine, potassium and carbachol were found between animals killed with urethane or those killed by spinal dislocation. This is in keeping with the report by Larabee (1961) that urethane 'washes out' of ganglia very quickly and does not alter the level of ganglionic ACh or choline levels (Consolo, Peri, Prigioni, Ladinsky & Perri, 1978). Therefore ganglia were routinely removed from rats killed with urethane.

3.1.2 Dissection of ganglia

Although particular attention was paid during the preparation of each ganglion, there was probably some variation between individual preparations. This is predictable both in terms of the variable anatomy of the ganglion, where it has been reported that the ICN has a variable number of postganglionic fibres from animal to animal (Haefely, 1972) and in terms of variation in the dissection and recording of the ganglion. The predominant cause of variability is likely to arise from missing or damaged nerve fibires and from the positioning of the ganglion across the barriers. This would alter the number of fibres that pass through the slot in the greased barrier and thus the number of ganglion cells that are recorded.

3.1.3 Tissue Bath

Many of the problems encountered with the grease gap are similar to those of the sucrose gap, and have been discussed by Wallis, Lees & Kosterlitz (1975). However the grease gap replaces the high resistance sucrose chamber and the need for a constant flow rate, and has the advantage of reducing junction potential drift and solution change artifacts. With the method employed here only the central chamber is perfused. Perfusion of both central and postganglionic chambers resulted in qualitatively similar results but recordings with excessive noise and drift were obtained. The disadvantage of not perfusing the outer chambers, was that they were at room temperature (between 18 to 25^{o}C) and not necessarily of the same ionic composition. The responses to potassium and adenosine were unaltered by increasing the potassium concentration of the postganglionic chamber (table 3.1). When appropriate the PSS in the outer chambers was changed to the same solution as the new perfusate.

3.1.4 Measurement and consistency of responses

The grease gap method of recording the SCG is subject to some base line drift which was minimised by sealing the unperfused chambers with silicone grease. Some ganglia displayed a positive increase in p.d. during the first hour of perfusion which may have arisen due to an increased resistance as the ganglion swells around the grease gap to create a better seal. In initial pilot experiments, a downward drift in the base line occurred, but did not significantly alter the results, because of the large signal size/drift ratio. Downward drift may have occurred due to poor preparation of the ganglia or due to leakage across the barrier, and was detected by increased basal noise and intererence. The results from

these ganglia were not included in the analysis of the results.

Responses to individual agents were defined as the deviation from baseline which was measured by extrapolating the trace preceding the response. For the depression of agonist responses by purines, the agonist response was measured from it's start to the peak amplitude from the extrapolated baseline.

3.1.5 The effect of time on the response to agonists.

All drug regimes used were derived from initial experiments so that consistent results were obtained throughout an experiment (maximum of 12 hours). The sensitivity of isolated tissue preparations to agonists often changes during the course of an experiment. The results of experiments presented here suggest that the rat SCG showed a marked increase in sensitivity to muscarine during the first 2 to 3 hours of being placed in the tissue bath. Therefore all ganglia were allowed to pre-equilibrate for at least two hours and preliminary responses to drugs were assessed. No ganglia exhibited any form of spontaneous activity.

Before studying the effects of purines on the response to agonists on the ganglion it was necessary to determine the reproducibility of the response to an agonist during the course of an experiment. Responses to repeated applications of an agonist were reproducible over many hours (Figs 3.1 and 3.2). In some experiments one control ganglion was run in parallel with test tissues to exclude any changes in sensitivity with time. When constructing concentration response curves the effect of time on the

response of control ganglia to agonists was examined by repeating the responses after a minimum of 30 minutes.

3.1.6 The effects of purines on the response to agonists

To determine the effect of a purine on the response to an agonist, two or three reproducible responses to the agonist were obtained and the last response used as the control i.e., the 'pretest response'. For the test response, a ganglion was perfused with a purine for 5 minutes, and the agonist applied for one minute, a minute from the start of perfusion with purine. A minimum of 15 to 20 minutes later the response to the agonist alone was repeated (post-test).

When studying the effect of purines on agonists each sequence of responses i.e., pretest/test/post-test took at least an hour and often only three or four concentrations of agonist could be evaluated in a day. Therefore some results are presented as a composite of responses obtained from randomised dose schedules. Because of the time taken to obtain data points and in order to reduce day to day variability the test responses are expressed as a % of the pretest responses. The pre and post test responses were within 20% of each other. When studying the effect of antagonists on the depression of muscarine responses it was only possible to study two concentrations of antagonist on the same ganglion, as each pair of determinations took about 3 hours.

To avoid desensitisation and inconsistent responses to purines a minimum of 20 minutes was allowed between applications. Concentration response curves (CRC) for non purine agonists were determined by applying single concentrations of drugs and washing for a minimum of

twenty minutes unless reported differently. To construct concentration response curves agonists were applied at near or subthreshold concentrations, and then increased by threefold or tenfold, until the response size was maximal. Concentrations refer to those in contact with the ganglion. When changing the superfusate for a modified PSS, changes in d.c. potential generally reached a stable level within the 15 to 20 minutes and a minimum of 30 minutes equibration time was allowed before the next response.

3.2 DETERMINATION OF THE SITE OF ACTION OF ADENOSINE ON THE SCG

There are several mechanisms by which purines may exert their actions including; altering the conduction of impuses in preganglionic and postganglionic neurones (Ribeiro & Sebastiao, 1984); by depressing the postjunctional activation of the ganglion cells to agonists (Gustaffson, 1981; Cox & Walker, 1987); by altering the release of a neurotransmitter from presynaptic nerve terminals (Silinsky & Ginsborg, 1983); by altering the interaction between neurones and other cells (El-Etr, Cordier, Glowinski & Premont, 1989).

The principle site of action of purines may be the postsynaptic soma-dendritic regions of the ganglion cells. However, one other site of action of purines may be on the preganglionic CST as an ATP-activated cation conductance has been found on mammalian neurones (Krishtal, Marchenko & Pidoplichko, 1983) and adenosine receptors have been located on axon terminals of excitatory neurones of cerebellar granule cells (Goodman, Kuhar, Hester & Snyder, 1983) and in several white matter areas of the rat brain (Weber, Jones, Loshe & Palacios, 1990). In addition

Ribeiro & Sebastiao (1987) reported adenosine enhanced and ATP decreased the inhibitory effect of TTX on action potentials recorded from frog sciatic nerves suggesting these nerves have functional receptors for adenosine. The function of these purine receptors is unknown but they may regulate neuronal conduction and arise from the axonal transport of adenosine receptors to the presynaptic terminal (Stone, 1981a). The presence of receptors for purines on axonal membranes of the rat SCG preparation may contribute to the change in p.d. recorded and the ability of purines to activate the axonal membrane of the CST of the rat was assessed.

Anatomical studies of the SCG have shown that the rat SCG comprises between 15,000 to 40,000 neurones that are divided into a caudal group of about 45% of the total that leave the ganglion via the ECN, and a rostral group comprising of about 35% of SCG neurones, leave the ganglion via the ICN (Bowers & Zigmond, 1979). The major input to the SCG is via the CST with a much smaller input via the ECN. Both the adrenergic cell bodies and dendrites of the SCG receive branching fibres from the cholinergic input of the CST. It is unknown if these anatomical differences are reflected in the responses obtained when recording the rat SCG via different postsynaptic nerve trunks. A few experiments were performed to decide if these anatomical differences are reflected in the ability of the ganglion to response to purines and muscarine.

In addition to hyperpolarising neuronal tissue adenosine can alter the response to a number of neurotransmitters and other agents (reviewed by Stone, 1989). In chapter 5 the effect of adenosine on the response to different agonists is described in detail, but in summary adenosine was found to selectively depress the response to

muscarinic agonists and this interaction was used to study the effect of purines and pyrimidines on the rat SCG.

METHODS. Modification

To examine the action of drugs on the CST, ganglia were dissected and the preganglionic CSTs desheathed and cut away from the ganglion body, 5mm distal from the body of the ganglion. About 20mm of CST was mounted in the tissue bath and maintained as for the SCG. The effect of drugs on the d.c. potential was measured by applying purines for 2 minutes and muscarine (MUSC), potassium (K^+), 5HT and GABA for 1 minute. In addition to recording ganglia via the ICN an additional five SCG were recorded between the ECN and the ganglion body and responses to muscarine or muscarine in the presence of adenosine were recorded.

RESULTS

3.2.1 Effect of adenosine on the d.c. potential of the SCG.

Application of 10 to 1000uM adenosine for 2 or 5 minutes produced hyperpolarisations with a maximum amplitude of -100 to -280uV in different preparations. The mean hyperpolarisation produced by 100uM adenosine on 42 ganglia was -73 $\pm$ 6uV (range -25 to 200uV). Figure 3.3 shows hyperpolarisations produced by increasing concentrations of adenosine for 2 minute periods every 20 minutes. The onset of the response was immediate, reaching a maximum 30 to 50 seconds after beginning application. Responses to adenosine at upto 100uM were maintained throughout a five minute superfusion. At 300uM adenosine or higher, the responses peaked and stabilised at lower level. On washing out adenosine, there was a rapid return to the basal level, and apparent recovery

within 5 minutes from the start of washing. The responses to adenosine appeared to be smaller when applied at less than 15 minute intervals, and hence more time was allowed between responses. Using two minute applications, reproducible responses were obtained every 20 to 40 minutes throughout an experiment (maximum of 12 hours). Using this protocol, the hyperpolarisations to adenosine were dose related with an EC_{50} of 10uM and a maximal response at 300uM (Fig. 3.3).

3.2.2 Effect of tetrodotoxin (TTX) and pirenzepine (PIR) on the response of the SCG to adenosine.

In both PSS and 0mM Ca^{2+}/0mM Mg^{2+} PSS the hyperpolarisations to 100uM adenosine were not significantly altered after pretreatment, for a minimum of 30 minutes with TTX (Table 3.2). In 0mM Ca^{2+}/0mM Mg^{2+} adenosine produced both a hyperpolarisation and an after depolarisation. The after depolarisation to adenosine in 0mM Ca^{2+}/0mM Mg^{2+} was abolished by TTX (Table 3.2). 1uM TTX also completely blocked the generation of action potentials, in two different ganglia (results not shown). In PSS pirenzepine abolished the response to 100nM muscarine but did not alter the hyperpolarisation to 100uM adenosine.

3.2.3 Effect of drugs on the CST

The results are summarised in figure 3.4 and application of either purines or muscarine had no significant effect on the resting d.c. potential of the CST. In contrast one minute applications of K^+, 5HT and GABA produced dose related depolarisations.

3.2.4 Effect of adenosine on the response to muscarine recorded via the ICN or ECN of the SCG

The response to 100nM muscarine recorded via the ICN was reduced in a concentration dependent manner by increasing concentrations of adenosine. In the presence of 100uM adenosine the response to 100nM muscarine was reduced by 26 $\pm$ 2% (Table 3.5). The hyperpolarisation to adenosine measured in the first minute of application, recorded from the ECN were smaller than that recorded from the ICN (Fig. 3.5a). The depolarisations to muscarine recorded from the ECN (334 $\pm$ 37uV, n=5) were about half the size of those recorded from the ICN (532 $\pm$ 59uV, n=9). There was no significant difference between the depression of muscarine by adenosine recorded via the ICN or the ECN (Fig. 3.5b).

DISCUSSION

The inactivity of purines and muscarine on the CST suggests the receptors for these drugs are either absence or inactive. The ability of GABA, 5HT and a non specific depolarising agent, potassium to depolarise the CST is consistent with the identification of receptors for GABA and 5HT on sympathetic neurones as described by Ireland (1987), Round & Wallis (1986) respectively.

The spontaneous generation of action potentials in the rat SCG could cause the release of ACh, to produce an endogenous cholinergic "tone", which would be abolished in the presence of TTX, a sodium (Na^{2+}) voltage dependent channel antagonist, or in the presence of a selective M1 receptor antagonist, such as pirenzepine. The lack of effect of TTX, at a concentration found to abolish the generation of action potentials suggests the hyperpolarisation to adenosine is not the result of excitation of an inhibitory synaptic input from other

neurones and adenosine does not activate voltage gated Na^+ channels. A similar finding was reported by Henon & McAfee (1983b) who found the depression of calcium spikes by adenosine on the rat SCG was not blocked in the presence of 1uM TTX. Further evidence against an action of adenosine on a cholinergic "tone" is provided by the inability of pirenzepine to alter the hyperpolarisation to adenosine.

It has been reported that activation of muscarinic receptors can hyperpolarise the ganglion via an indirect mechanism (discussion: chapter 1 and Ashe & Yarosh, 1984), but the ability of the pirenzepine to abolish the responses to muscarine and not adenosine, suggests the response to muscarine is via an action on muscarinic receptors of the M1 subtype.

If adenosine releases a putative hyperpolarising substance, then the release would most likely occur via a calcium dependent mechanism, as has been demonstrated for the release of ACh from SCG by Harvey & MacIntosh (1940) and would be abolished in low Ca^{2+}/high Mg^{2+} media. The enhancement of the response to adenosine in low calcium PSS suggests adenosine does not require a $[Ca^{2+}]e$ dependent process such as the release of ACh. It has been suggested that the hyperpolarisation to muscarinic agents on the rabbit SCG is partially mediated via the release of catecholamines from SIF interneurones of the ganglion (Christ & Dun, 1986), and it is possible although unlikely that adenosine could cause the release of catecholamines. Given that the release of catecholamines is calcium dependent the results presented do not support this hypothesis. Indirect evidence for a lack of involvement of catecholamines in the response to adenosine comes from the inability of phentolamine (Henon & McAfee, 1983b; Elliott, Marsh & Brown, 1989), and yohimbine and prazosin

(Elliott et al., 1989) to antagonise the effects of adenosine on the rat SCG.

These results are consistent with the existence in the rat SCG of receptors for adenosine that hyperpolarise the ganglion via a direct action on postganglionic neurones. Experiments are described in chapter 4 to determine the ionic basis for the effects of adenosine and to determine if as with many tissues the actions of adenosine are linked to the actions of calcium.

Although two anatomically distinct populations of neurones have been described in the rat SCG there was no evidence to suggest these populations are qualitatively different in their response to adenosine and muscarine. As the responses recorded via the ECN to muscarine and adenosine were similar although smaller, and the literature reports are confined to ICN, in subsequent experiments only recordings via the ICN were made.

The depression of the response to muscarine is consistent with the idea that adenosine may modulate the postganglionic response of the neuroneal tissue to cholinergic agonists. The application of cholinergic agonists to the rat SCG may produce a number of effects including depolarising and hyperpolarisation and the ability of adenosine to alter these different responses is examined in detail in chapter 5.

3.3 METABOLISM OF ADENOSINE BY THE SCG

It is well-documented that many tissues metabolise adenosine (Arch & Newsholme, 1978) and the effects of adenosine are mediated via adenylate cyclase (Stone, 1981a, 1989). Thus it might be expected that the rat SCG may metabolise adenosine and some of the actions of adenosine may be mimicked by cyclic nucleotides.

The inhibitory effect of adenosine on synaptic transmission is known to be enhanced by adenosine uptake inhibitors (Phillis & Wu, 1982) and it is curious that Alkadhi et al (1984) reported "that the uptake mechanism is not important in controlling the extracellular adenosine concentration in the superior cervical ganglion of the rat". This conclusion was based on the observation that 2CA was equipotent with adenosine in inhibiting ganglionic transmission and dipyridamole at 1 and 100uM failed to potentiate the inhibitory action of adenosine. In contrast Henon & McAfee (1983a) reported that 10uM dipyridamole displaced the concentration response curve for the inhibition of the AHP by adenosine, so that the IC_{50} was about the same as that for 2CA. Alkahdi and colleagues do not adequately explain the reason for this discrepancy, and supported their argument for a lack of uptake by referring to the results of Henon & McAfee (1983a), who were unable to potentiate the accumulation of ^{3}H-AD from ^{3}H-cAMP labelled ganglia in the presence of dipyridamole. It is important to note that there are many routes for the inactivation of adenosine (for excellent reviews see Arch & Newsholme (1978) and Gordon (1986)) and adenosine may be metabolised by enzymes such as ADA, adenosine kinase, and S-adenosylhomocysteinase, all of which maintain low levels of endogenous adenosine.

Schubert (1988) has provided some evidence for the presence of a physiological concentration of adenosine, about 1uM, which had a depressive 'tonus' on synaptic transmission. One criterion that should be met to ascribe a physiological role to adenosine i.e., as a neuromodulator is to show that a mechanism exists for the efficient removal and control of adenosine in the extracellular space and the synapse. The rapid response to adenosine (Fig. 3.1a) may indicate the receptors for adenosine are more closely linked to ion channels than those for the response to muscarine, which is thought to require the accumulation of intracellular messengers (Brown, 1983). Alternatively, the short-lived action of adenosine may result from the rapid degradation and elimination of adenosine from the medium, by ganglionic uptake and extracellular degradation to inactive metabolites. The ability of the rat SCT to degrade and take up adenosine, and the possibility of an adenosine 'tonus' were examined.

3.3.1 Effects of ADA on the response to adenosine

ADA has been reported to reduce the inhibition of the AHP of the rat SCG produced by cAMP and adenosine (Henon & McAfee, 1983a). ADA, which catalyses the conversion of adenosine to inosine, reduced the hyperpolarisation of the rat SCG to adenosine (Fig. 3.6). These results suggest that inosine may be inactive on the rat SCG. This conclusion was confirmed by the lack of effect of inosine, at up to 1mM on both the d.c. potential and the response to muscarine (Fig. 3.8, Table 3.3) and this is consistent with reports that inosine is a very weak activator of the adenosine receptors (Linden, 1989). Thus the ability of purines to interact with the binding sites for adenosine and produce a hyperpolarisation and depression of

muscarine may require the presence of a nitrogen or an amine, in the 6 position of the purine ring (see Fig. 1).

Erythro-6-amino-9-(2-hydroxy-3-nonyl)purine hydrochloride (EHNA) is a competitive inhibitor of ADA which has been found to have a Ki for ADA at nanomolar concentrations (Agarwal, Spector & Parks, 1977) and the presence of 10uM ENHA would be expected to completely antagonise the actions of any endogenous ADA. The presence of ADA in the rat SCG is indirectly inferred from the ability of EHNA, to enhance the hyperpolarisations to low concentrations of adenosine (Fig. 3.7). Some support for an extracellular location of ADA is provided by the reports by Henderson (1985) that ADA is released by damaged cells. ADA has been suggested to have hydrophobic domains that span the membrane of human erythrocytes (Bielat & Tritsch, 1989). In addition, Henon & McAfee (1983a) have reported that the rat SCG is able to degrade exogenous cAMP to adenosine and the inability of dipyridamole to potentiate the accumulation of ^{3}H-AD from exogenous ^{3}H-cAMP.

3.3.2 Inhibition of transport of adenosine

The nucleotide uptake inhibitor, dipyridamole has been reported to inhibit adenosine uptake of rat brain tissues with an IC_{50} of 0.45uM (Phillis & Wu, 1982) and 1.3uM (Davies & Hambely, 1983) but also has very poor binding to rat brain membranes compared to other species. In order to ensure blockade of the uptake of adenosine, a high concentration of dipyridamole i.e., 10uM was employed which also allowed comparison to the results obtained by Henon & McAfee (1983a).

Dipyridamole did not alter the depolarisation to potassium suggesting this concentration of dipyridamole does not affect general excitability of the ganglion. The ability

of both dipyridamole and HNBTG to shift the dose response curve for the inhibition of muscarine to the left (Fig. 3.9) is consistent with the presence of an uptake mechanism for adenosine in ganglia and with the presence of external adenosine receptors. The potentiation of the depression of muscarinic responses by adenosine in dipyridamole and HNBTG was similar (cf. Figs. 3.9a, 3.9b).

The level of endogenous adenosine can alter the apparent potency of exogenous purines, and membranes are known to continuously produce adenosine (see Linden, 1989). The ability of dipyridamole to produce a small but significant (Table 3.3) reduction of the response to muscarine which corresponds to an equivalent reduction of muscarine by about 1uM adenosine (see chapter 5), suggests the presence of endogenous adenosine in the rat SCG. Caution is necessary in this interpretation as the depression of muscarine is near to the limits of detection. Theophylline also significantly reduced the response to muscarine (Table 6.2). However it has been reported that both theophylline and dipyridamole can also affect intracellular calcium sequestration and phosphodiesterase (PDE) activity, and these effects may account for the reduction of muscarine responses. The equipotency of HNBTG and dipyridamole in enhancing the hyperpolarisation to adenosine, but lack of any effect of HNBTG on the response to muscarine does not support the idea of endogenous adenosine release from the rat SCG.

The non-metabolised analogue of adenosine, 2CA produced slowly developing and persisent hyperpolarisations of the rat SCG, suggesting that the rapid return of the response of adenosine to the basal level is due to uptake and metabolism of adenosine. It is reported that 2CA is a substrate for the adenosine uptake mechanism in dog erythrocytes (Jarvis, Martin & Ng, 1985) and the

possibility that 2CA is transported by the rat SCG was determined. The inability of dipyridamole to alter the response to 2CA (Table 3.4) suggests 2CA is not a substrate for the adenosine uptake mechanism of the rat SCG. In contrast the responses to adenosine were significantly enhanced in the presence of dipyridamole (Fig. 3.8). The degree of potentiation of adenosine by dipyridamole is comparable to that reported by Henon & McAfee (1983a), on the inhibition of the AHP of the rat SCG by adenosine.

Marangos, Deckert & Bisserbe (1987) has suggested that "the uptake mechanism is probably the neurobiologically relevant process, since adenosine deaminase is largely intracellular in its distribution." The results reported here do not support this hypothesis as the potentiation of the hyperpolarisation to adenosine by EHNA was similar to the potentiation by dipyridamole (cf. Figs. 3.7 & 3.9). The potentiation of the hyperpolarisation to adenosine by dipyridamole and EHNA on the rat SCG are similar to those found by Sebastiao & Ribeiro (1988). Using S-(p-nitrobenzyl)-6-thioinosine (NBI) and EHNA these authors reported the potency of adenosine on the inhibition of twitch responses of the isolated rat diaphragm was enhanced 2.6 fold in 5uM NBI and by 2.2 fold in 25uM EHNA (Sebastiao & Ribeiro, 1988).

Both Hopkins & Goldie (1971) and Stone (1983) have reported that little adenosine uptake could be detected in the rat heart and the rat anococcygeus muscle preparations, respectively and suggested the lack of adenosine uptake may be species dependent. Alternatively the lack of uptake reported by these workers could reflect the distribution of uptake sites, and indicate there is greater uptake of adenosine at postsynaptic sites.

In summary these results are in agreement with the results of Henon & McAfee (1983a) and not with those of Alkadhi et al (1984) that the rat SCG contains an uptake mechanism for the removal of adenosine. At physiological concentrations i.e., micromolar adenosine, it is suggested both deamination and uptake would have equivalent roles in the metabolism of adenosine in the rat SCG. Both the selective uptake inhibitor HNBTG and the adenosine receptor antagonist, 8PT did not alter the d.c. potential or the response to muscarine and do not provide evidence for the presence of endogenous adenosine 'tonus' in the isolated perfused rat SCG preparation.

3.4 RESPONSE OF THE SCG TO CYCLIC NUCLEOTIDES

The cyclic nucleotides of cyclic 3',5'-AMP (cAMP) and cyclic 3',5'-GMP (cGMP) are known to be involved in the actions of neurotransmitters, and in altering the sensitivity of neurones. cAMP is known to exert an excitatory action in neuronal cell bodies of rat ganglia (Libet et al., 1975) and Greengard (1976) has proposed that cyclic nucleotides play a major postsynaptic role in the ganglionic transmission, cAMP and cGMP being involved in the slow inhibitory and excitatory postsynaptic potentials respectively. It has been suggested that cAMP and cGMP operate in opposite directions (the 'Yin-Yang hypothesis') to exert long term control over neuronal excitability in sympathetic ganglia (McAfee & Greengard, 1972). An increase in cAMP has been reported to produce a prolongation of the response of the rat SCG to methacholine (Libet et al., 1975). The ability of cAMP, cGMP and 8-bromo cAMP (8BrcAMP) to alter the d.c. potential and the response of the rat SCG to muscarine was investigated to discover if these compounds mimic the actions of adenosine.

Cyclic AMP produced slowly developing concentration related hyperpolarisations with a maximum response above 1mM (Fig. 3.10). Both the slowness of the hyperpolarisation to cAMP and lower potency of cAMP compared to adenosine are consistent with results reported Brown et al. (1979) who found hyperpolarisations to cAMP were antagonised by theophylline, and are therefore likely to be due to degradation of cAMP and activation of adenosine receptors.

In order to activate intracellular cAMP-dependent processes a lipid soluble cAMP analogue, 8BrcAMP was tested. 8BrcAMP was chosen because it penetrates the plasma membranes and is deacylated to serve as a source of intracellular cAMP (Bletcher & Hunt, 1972). Over the same concentration range as cAMP, 8BrcAMP produced dose related depolarisations, which were not altered by the presence of 8PT at a concentration which significantly reduced the hyperpolarisation to adenosine (Fig. 3.11), suggesting the depolarisation to 8BrcAMP may be via an increase in intracellular cAMP concentration and not via the activation of P1 purinoceptors. Compared to adensoine, 8BrcAMP was less potent at depressing the response to muscarine, and may indicate that the depression of muscarine and the hyperpolarisation to adenosine do not involve an increase in cAMP, although a decrease in cAMP cannot be excluded. Experiments to examine the role of cAMP in the response to adenosine are described in chapter 5.

The low potency of cGMP (Fig. 3.11) is similar to the observations of Brown et al. (1979) who found 1mM cGMP and 1mM 8BrcAMP produced very small depolarisations of 2 rat SCG and was ineffective on another 8 ganglia. Along with the inability of cGMP to alter the response to muscarine (Table 3.4) these results indicate that the selective

depression of muscarinic responses and the hyperpolarisation of the rat SCG by adenosine and adenine nucleotides is not shared by guanosine nucleotides.

3.5 RESPONSE OF THE RAT SCG TO PYRIMIDINES

There is now considerable evidence that ATP and adenosine modulate neuronal function (Stone, 1989) and more recently that pyrimidine nucleotides can modulate cellular functions via pyrimidine receptors rather than purine receptors (Seifert & Schult, 1989). In fact extracellular uridine and uracil nucleotides are effective activators of many cell functions including contraction or relaxation of blood vessels, changes in liver metabolism, aggregation of platelets and neutrophils (Seifert, Burde & Schultz, 1989). It was therefore of interest to examine the action of some pyrimidines on the rat SCG as it has been shown by Siggins et al. (1977) that bullfrog sympathetic neurones in explant culture showed pronounced depolarisations to the di- and triphosphoribose derivatives of several pyrimidine and purine bases. The effect of UTP, UMP and CTP on the d.c. potential and the effects of UTP on the response of the rat SCG to muscarine were examined.

The order of potency for depolarising the rat SCG was UTP > CTP >> UMP (Fig. 3.12) and is similar to the order for pyrimidine receptors described on most cells studies i.e., UTP is more effective than UDP, UMP and uridine, and TTP and CTP are relatively weak agonists (Seifert & Schultz, 1989). The ability of UTP to depolarise the rat SCG reported here is consistent with the initial report by Adams and colleagues (1982a) that UTP inhibits the M-current of rat SCG. The ability of UTP to depolarise the rat SCG is also consistent with the report of Gruol,

Siggins, Padjen & Forman (1981) who reported that explanted adult bullfrog ganglion cells were depolarised by UTP and UDP and that CTP and DCP were inactive and of Siggins & colleagues who found uridine nucleotides were the most postent of all the compounds tested. The possibility that UTP depolarises the rat SCG by activating the same mechanism as ATP has not been excluded. The inability of UTP to alter the response to muscarine (Table 3.4) suggests that the depression of muscarine by adenosine is restricted to adenine or adenine nucleotides.

The low potency of CTP is in agreement with the reports of the action of cytosine and it's phosphates on both the toad spinal cord (Phillis & Kirkpatrick, 1978) and their effects on rat cortical neurones (Phillis, Kostpoulous & Limachet, 1974).

A detailed characterisation of pyrimidine receptors on the rat SCG was outside the scope of this thesis and would require the use of more specific and stable analogues of UTP such as the phosphorothioate analogues of uracil nucleotides to eliminate the effects of uptake and metabolism of pyrimidines. Studies to produce selective desensitisation and antagonism of the purine and pyrimidine receptors would be useful to confirm the separate nature of these receptors. In conclusion these preliminary results suggest that the SCG has receptors for UTP that activate the ganglion but are not involved in the depression of the response to muscarine.

Fig. 3.1. Responses of the rat SCG to three consecutive applications of adenosine at 100uM for two minutes every 20 minutes.

(a) Recording of a single ganglion to three 2 minute applications of adenosine showing reproducible hyperpolarisations; (b) responses of ten ganglia to 3 applications of adenosine (100uM, 2 minutes) displayed as a histogram. The column height and vertical bars for this and all subsequent histograms show the mean response and the standard error of the mean (SEM) respectively. There was no significant difference between any of the columns (two-tailed paired t-test).


a
250
µV
5min
100µM
AD
100µM
AD
100µM
AD

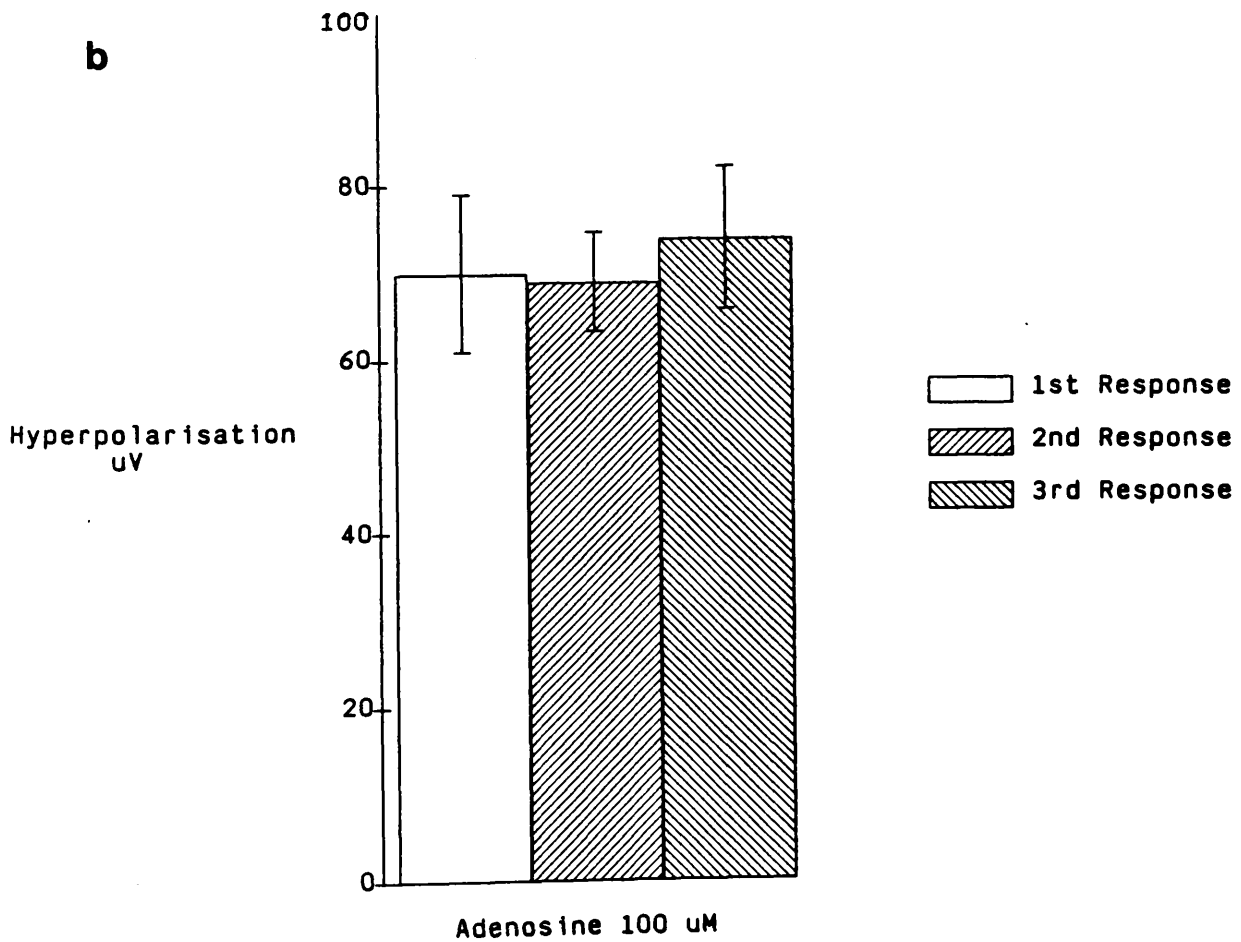

b
100
80
60
40
20
0
Hyperpolarisation
uV
1st Response
2nd Response
3rd Response
Adenosine 100 uM

Fig. 3.2. Response of the rat SCG to three consecutive applications of muscarine at either 30nM, 100nM or 300nM.

The column height and vertical bars show the mean ± SEM for N=4 to 19 ganglia. There was no significant difference (two-tailed t test) between any of the columns at each concentration of muscarine.

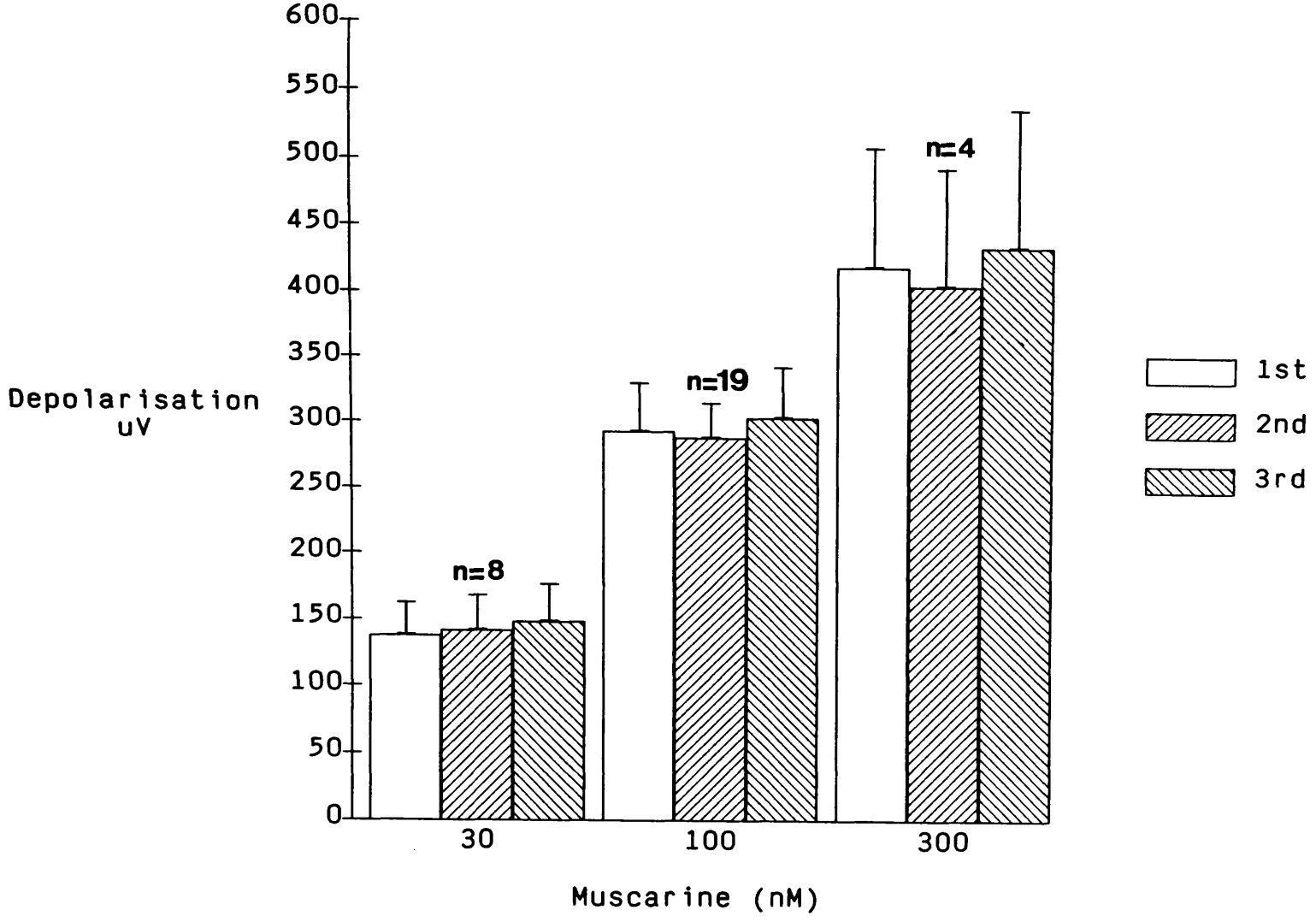

600
550
500
450
400
350
300
250
200
150
100
50
0
Depolarisation
uV
n=8
n=19
n=4
30
100
300
Muscarine (nM)
1st
2nd
3rd

Fig. 3.3. Response of the isolated rat SCG to adenosine.

(a) Response of a single ganglion to increasing concentrations of adenosine applied for 2 minutes, every 20 minutes. (b) Log concentration-response curve for the rat SCG to 2 minute application of increasing concentrations of adenosine applied every 20 minutes. In this and all subsequent graphs the symbols indicate the mean and the vertical lines the SEM of the results from four ganglia unless indicated otherwise by a number alongside the symbol, or where the number of ganglia (N) is indicated in the figure legend. When no SEM is indicated it lies within the area of the symbol.

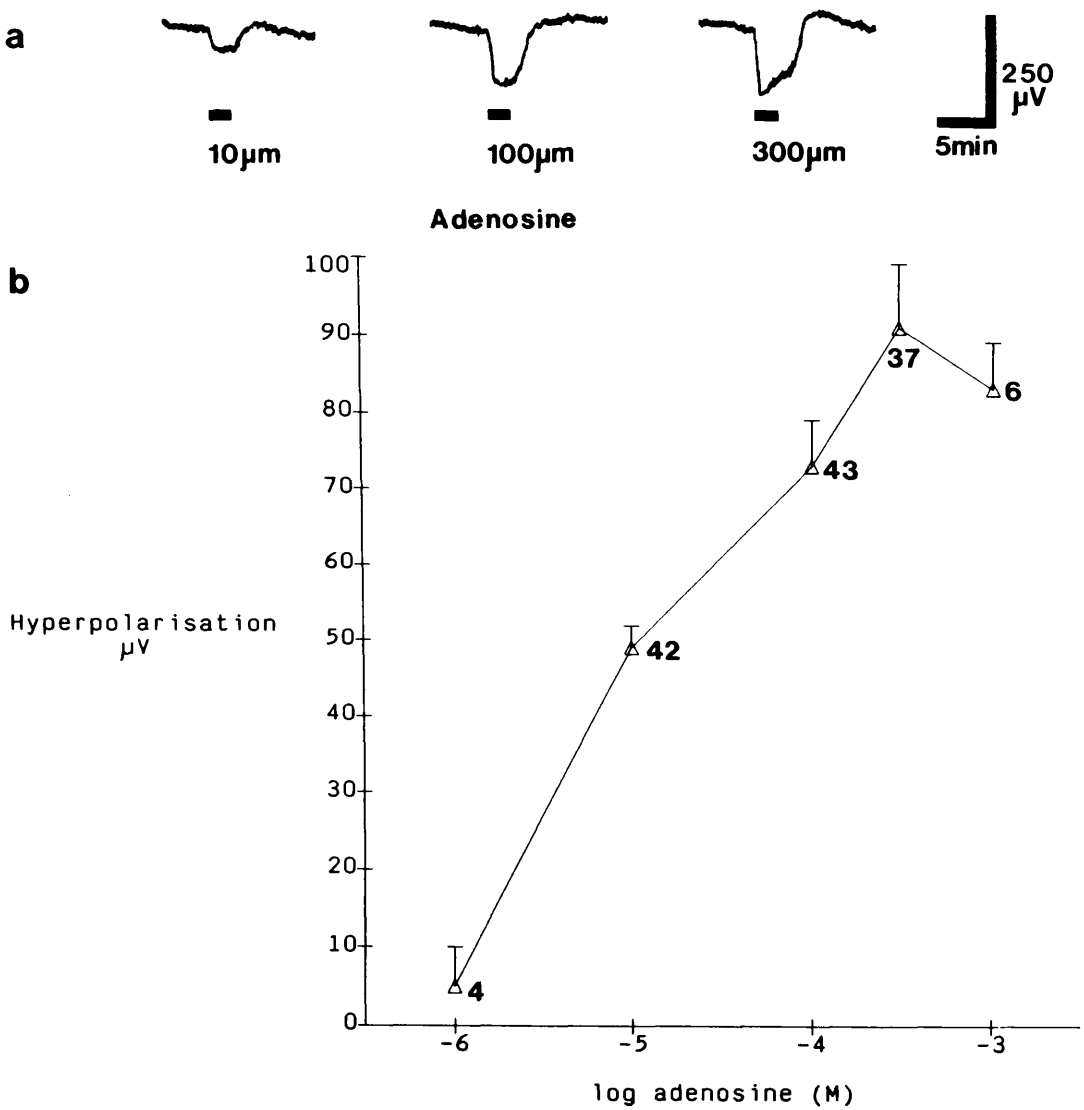

a
10µm
100µm
300µm
250 µV
5min
Adenosine
b
Hyperpolarisation µV
100
90
80
70
60
50
40
30
20
10
0
37
6
43
42
4
-6
-5
-4
-3
log adenosine (M)

Fig. 3.4. The magnitude of the responses of the isolated rat cervical preganglionic sympathetic nerve trunk (CST) to purines and other drugs.

The response to one minute applications of 5-hydroxytryptamine (5HT), gamma-aminobutyric acid (GABA), potassium and muscarine and two minute applications of adenosine triphosphate (ATP), cyclopentyladenosine (CPA), adenosine (AD), phenylaminoadenosine (PAA) and cyclic adenosine-5'-monophosphate (cAMP) were measured. Each point is the mean, and vertical bars the SEM for n=2 to 4 CSTs. A significant difference (two tailed t test) from basal d.c. potential is indicated by a * for $P < 0.05$.

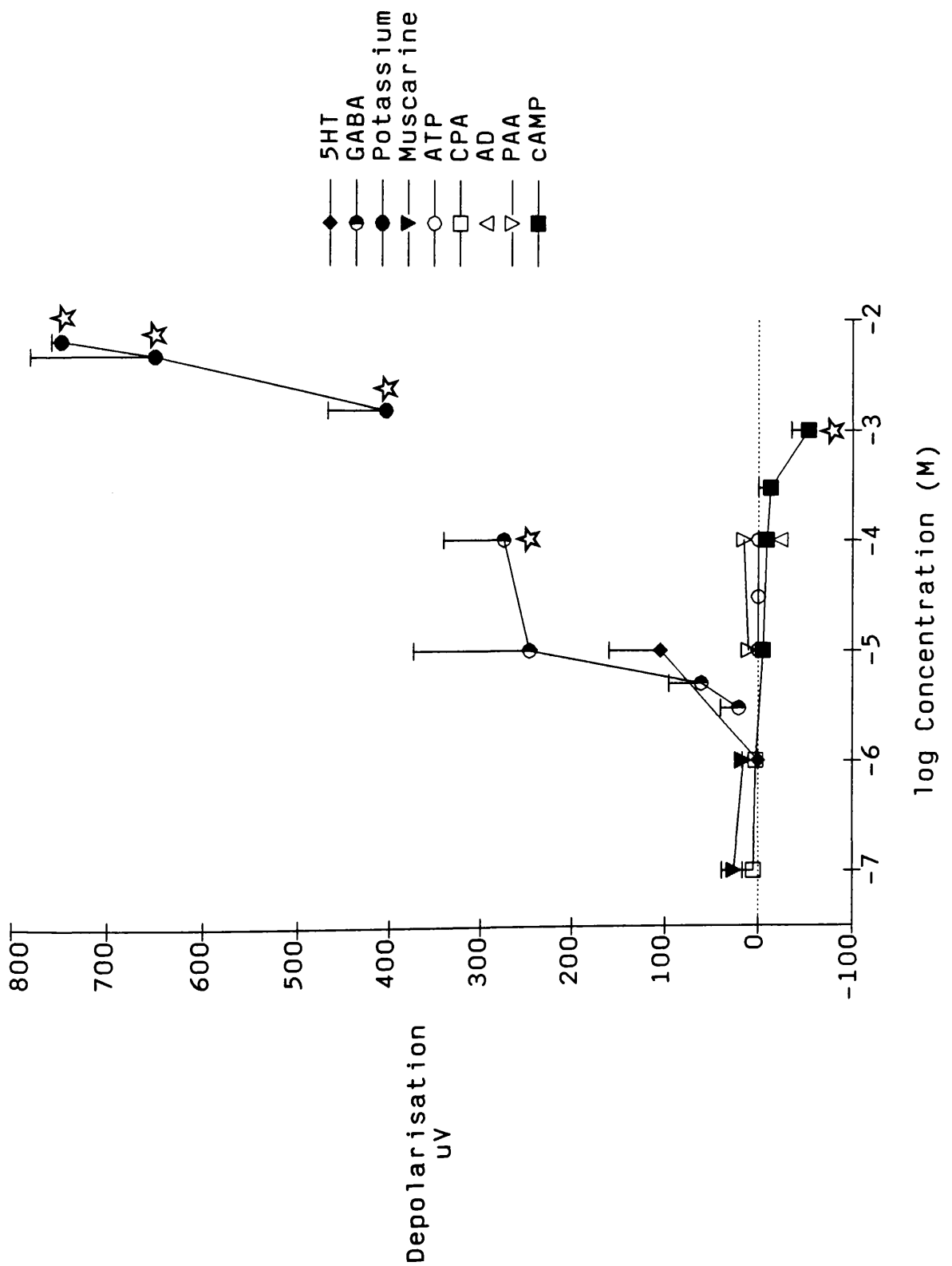
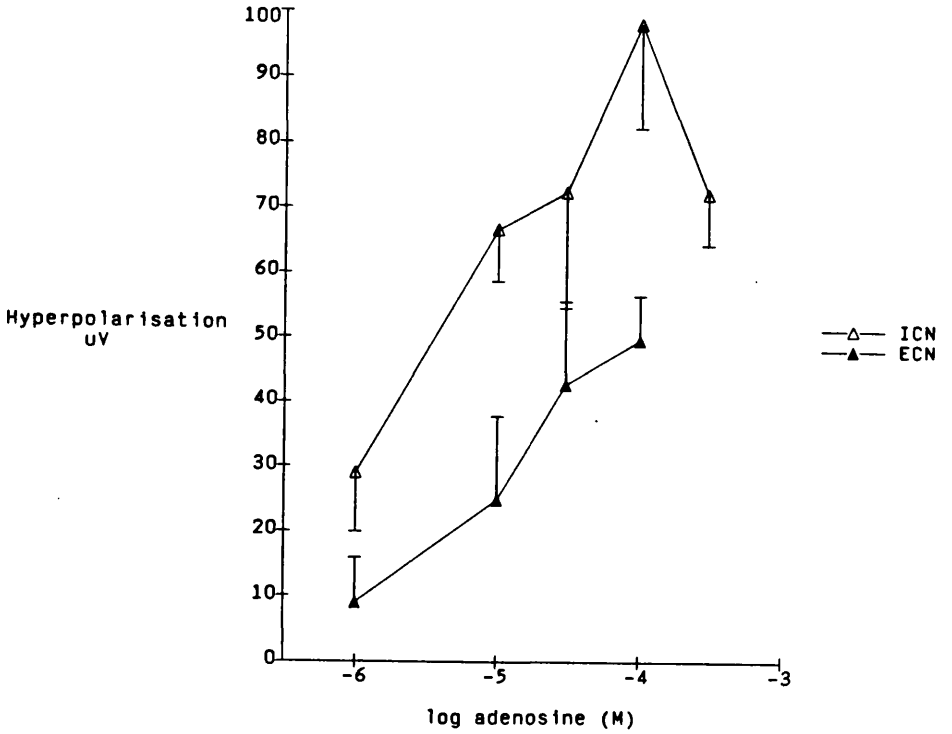

5HT
GABA
Potassium
Muscarine
ATP
CPA
AD
PAA
cAMP
Depolarisation
uV
800
700
600
500
400
300
200
100
0
-100
-7
-6
-5
-4
-3
-2
log Concentration (M)

Fig. 3.5. Comparison of the responses recorded from the internal carotid nerve and external carotid nerve of the isolated rat SCG.

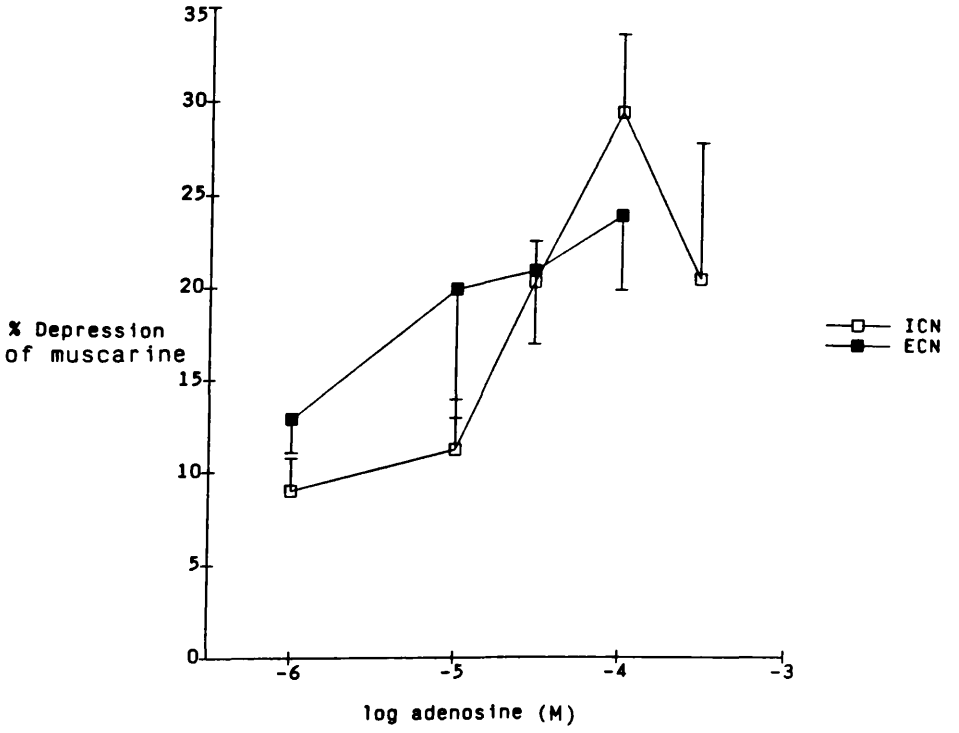
(A) for the hyperpolarisation to increasing concentrations of adenosine (2 minute application at minimum of 20 minutes between applications) and (B) for the % depression of the response to 100nM muscarine by increasing concentrations of adenosine.

ICN = internal carotic nerve; ECN = external carotid nerve

A)


100
90
80
70
60
50
40
30
20
10
0
Hyperpolarisation
uV
-6
-5
-4
-3
log adenosine (M)
ICN
ECN

B)


35
30
25
20
15
10
5
0
% Depression
of muscarine
-6
-5
-4
-3
log adenosine (M)
ICN
ECN

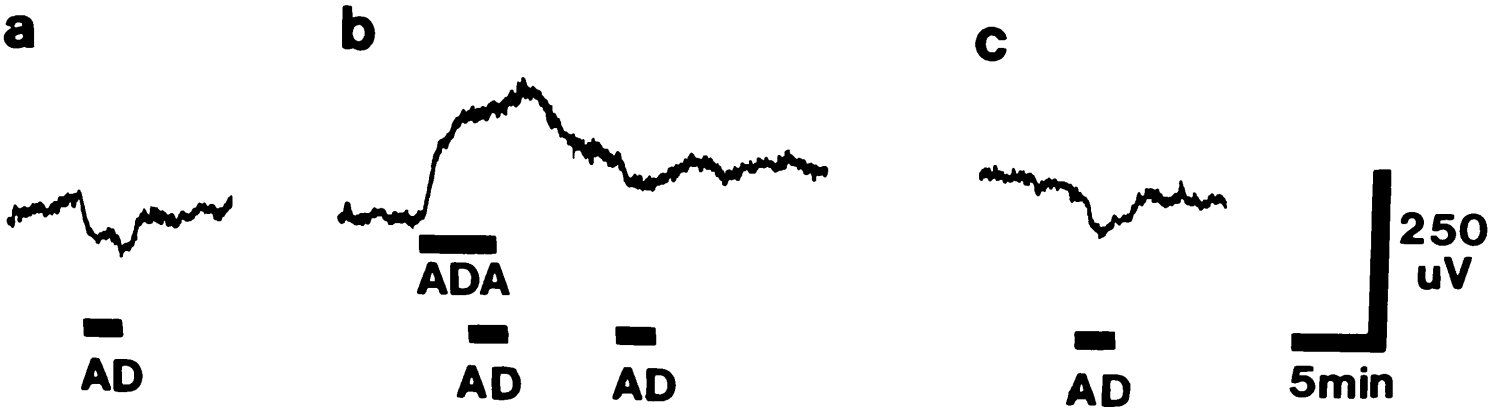


Fig. 3.6. The effect of adenosine deaminase on the response of the isolated rat SCG to adenosine.

(a) Response to adenosine (AD, 100uM, 2 minutes) in normal physiological salt solution and (c) after 30 minutes washout of adenosine deaminase (ADA), (b) response to AD during the application of ADA (2 units/ml). NB: the hyperpolarisation to adenosine was abolished. Partial recovery of the response to adenosine was seen 5 minutes after the end of perfusion with ADA.

Fig. 3.7. Log concentration-response curves of the isolated rat SCG to adenosine in the absence and presence of the adenosine deaminase inhibitor, erythro-9(2-hydroxy-3-nonyl) adenine (EHNA).

The hyperpolarisation to adenosine was determined by applying increasing concentrations of adenosine (1-300uM) for 2 minutes every 20 mins and then incubating the ganglia with 10uM EHNA for 30 mins and repeating the same sequence of adenosine applications. A significant difference (two tailed t test) from untreated ganglia in physiological salt solution (PSS) and PSS + EHNA is indicated by a * for $P < 0.05$.

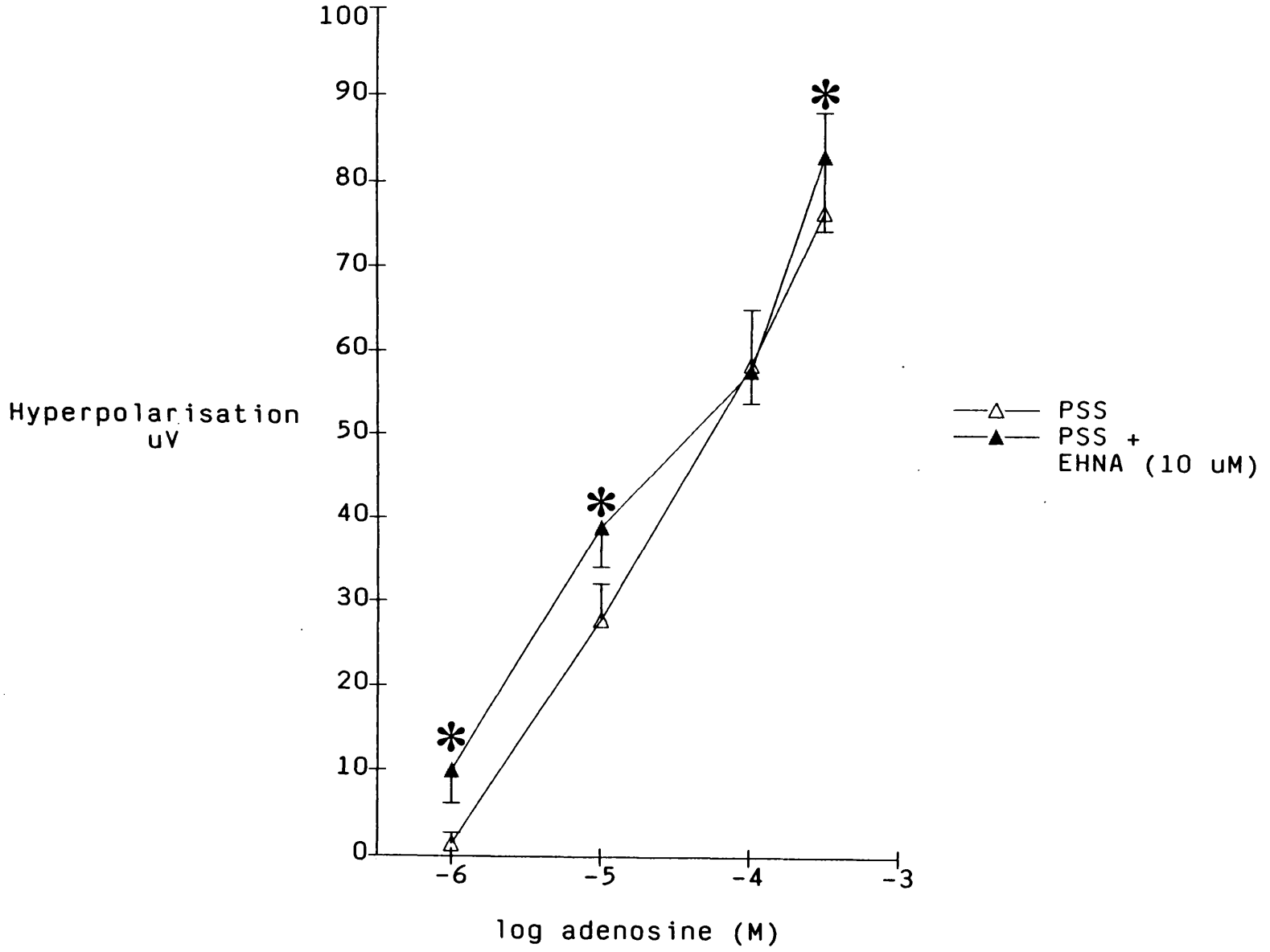

Hyperpolarisation
uV
100
90
80
70
60
50
40
30
20
10
0
-6
-5
-4
-3
log adenosine (M)
PSS
PSS +
EHNA (10 uM)

Fig. 3.8. Log concentration-response curves of the isolated rat SCG to inosine and to adenosine in the absence and presence of dipyridamole.

The effects of adenosine (AD) and inosine on the d.c. potential were tested by applying agonists for 2 minutes in increasing concentrations every 20 minutes. After incubation of the ganglia with 10uM dipyridamole (DIP) for 30 minutes the sequence of adenosine applications was repeated on the same ganglia. Dipyridamole significantly ($P<0.05$ = *, paired t test) enhanced the hyperpolarisation to adenosine at 1, 100 and 300uM.

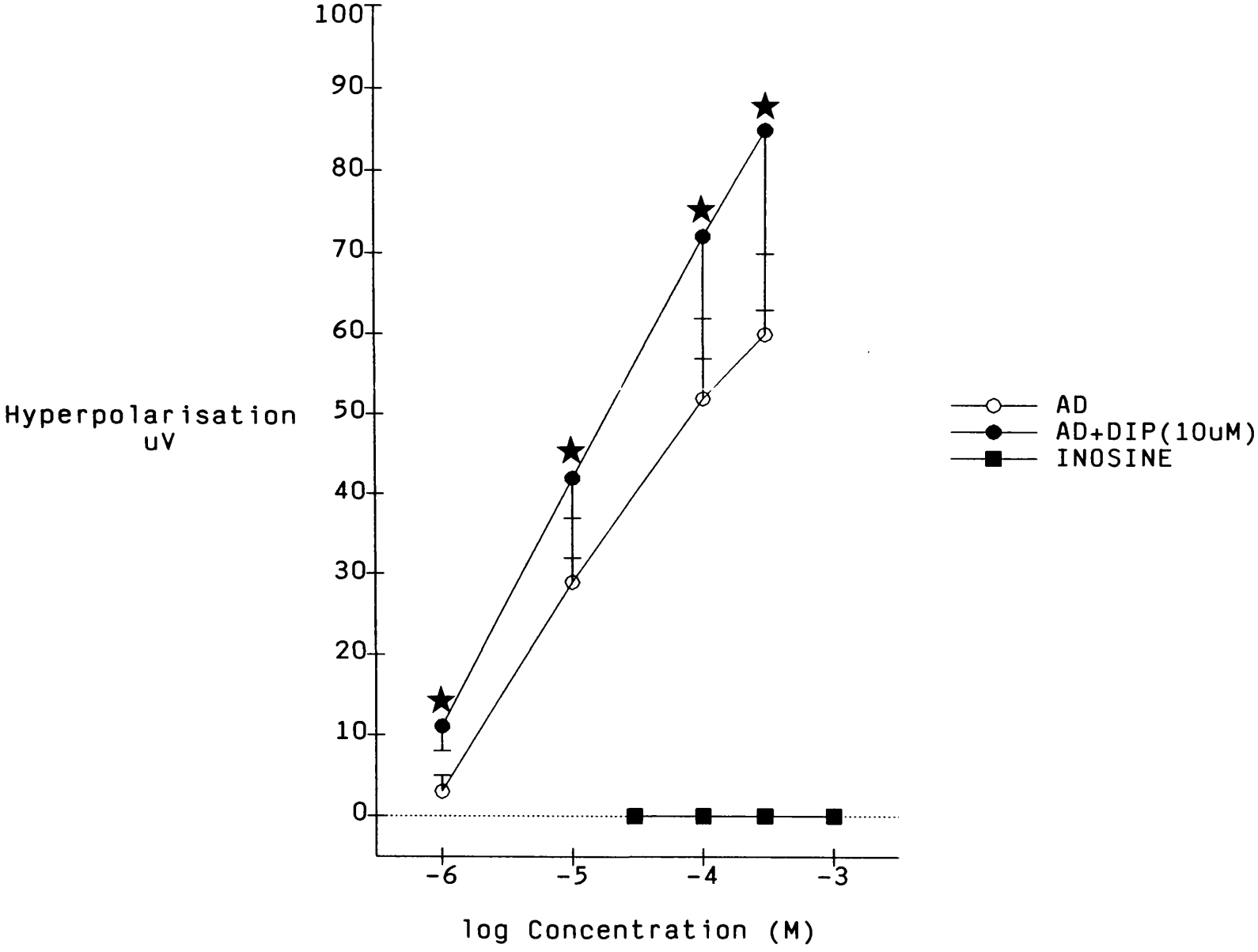
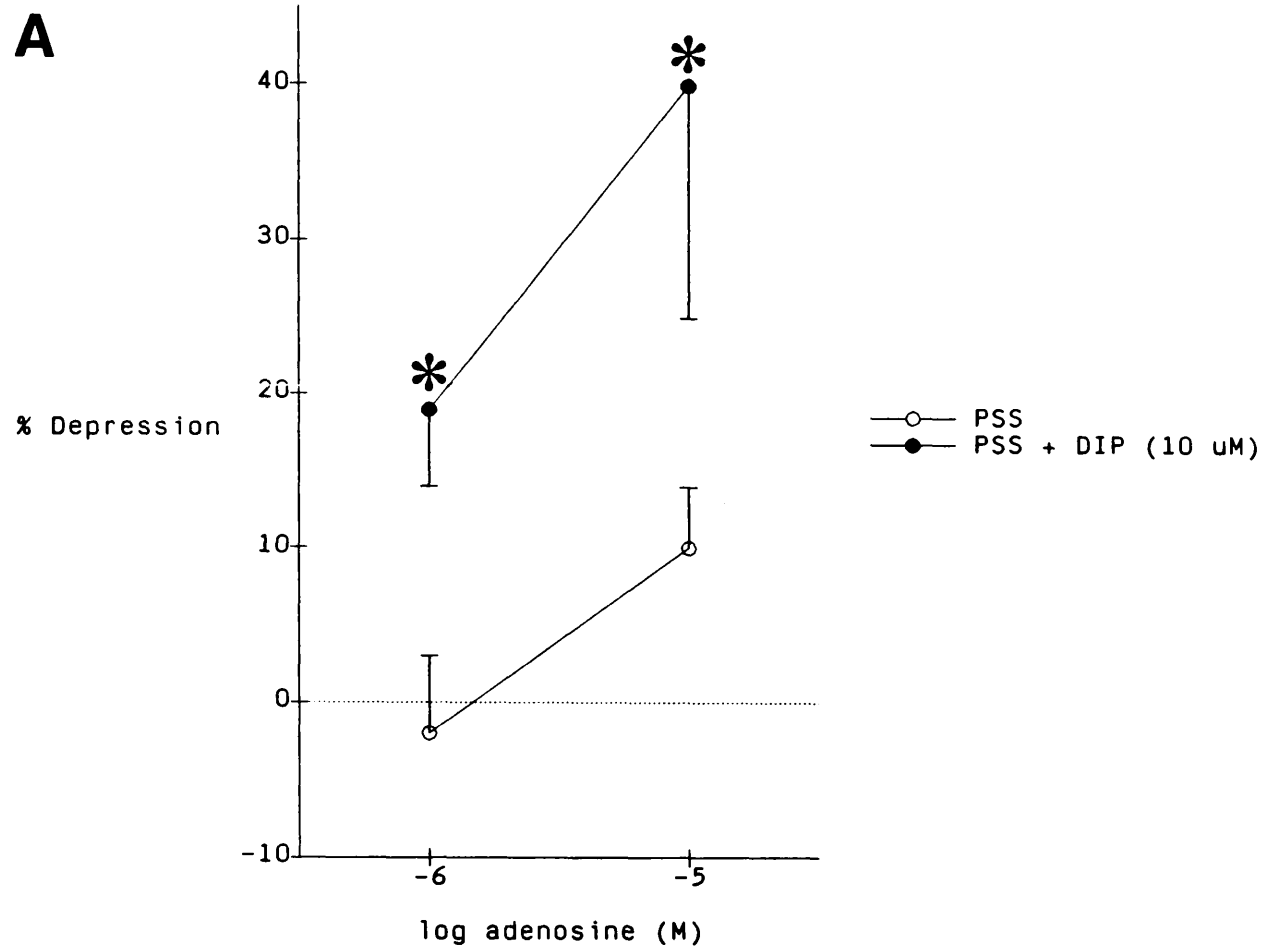
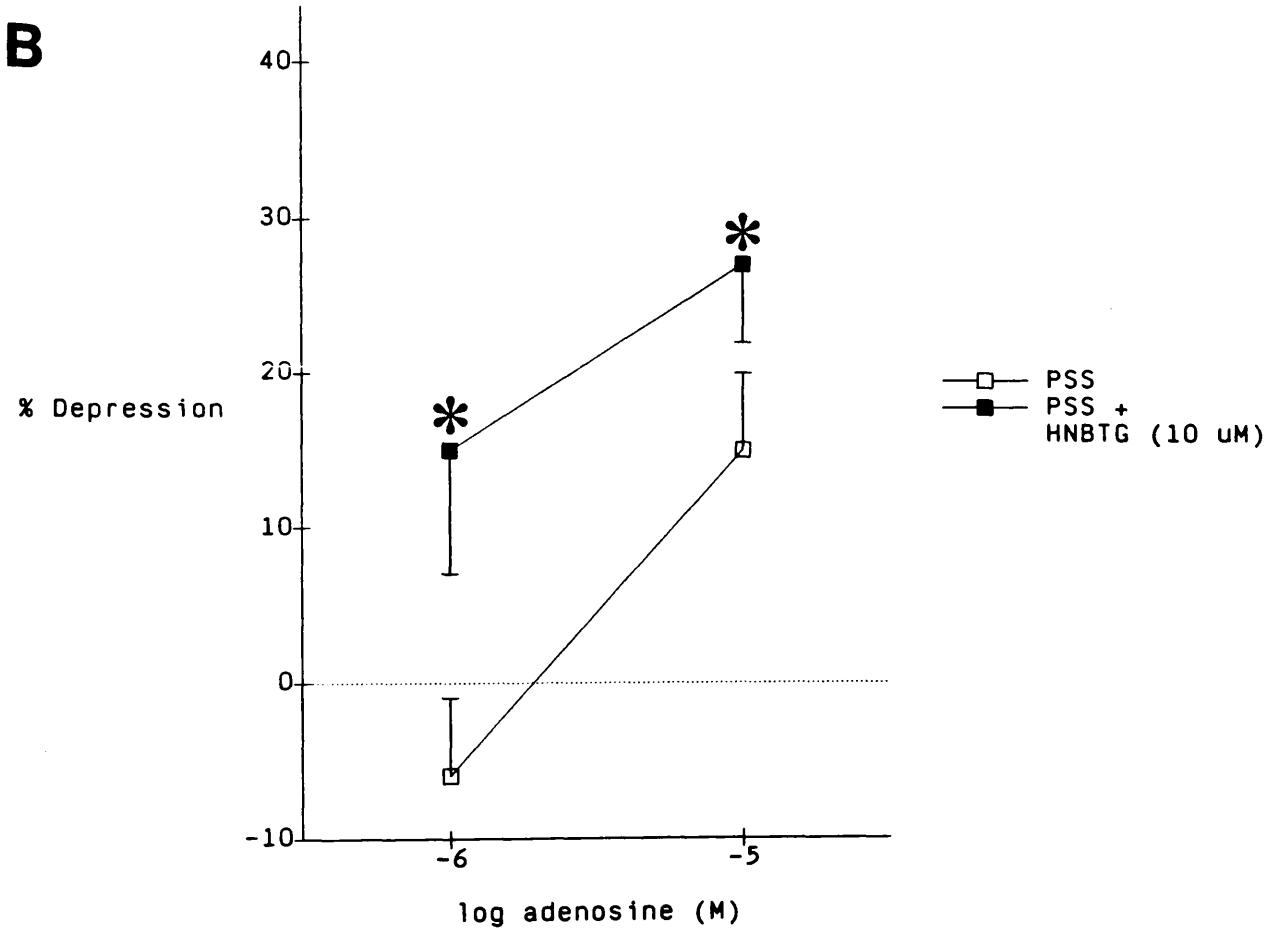

Hyperpolarisation
uV
100
90
80
70
60
50
40
30
20
10
0
-6
-5
-4
-3
log Concentration (M)
AD
AD+DIP(10uM)
INOSINE

Fig. 3.9. % depression of the response to muscarine by adenosine on the isolated rat SCG in presence of dipyridamole or S-(4-nitrobenzyl)-6-thioguanosine.

The depression of 100nM muscarine (1 minute application) by adenosine at 1uM and 10uM was determined. A significant difference (two tailed t test) from untreated ganglia in physiological salt solution (PSS) and PSS + dipyridamole (DIP) or PSS + S-(4-Nitrobenzyl)-6-thioguanosine + (HNBTG) is indicated by a * for $P < 0.05$.


A
40
30
20
10
0
-10
% Depression
*
*
-6
-5
log adenosine (M)
PSS
PSS + DIP (10 uM)


B
40
30
20
10
0
-10
% Depression
*
*
-6
-5
log adenosine (M)
PSS
PSS +
HNBTG (10 uM)

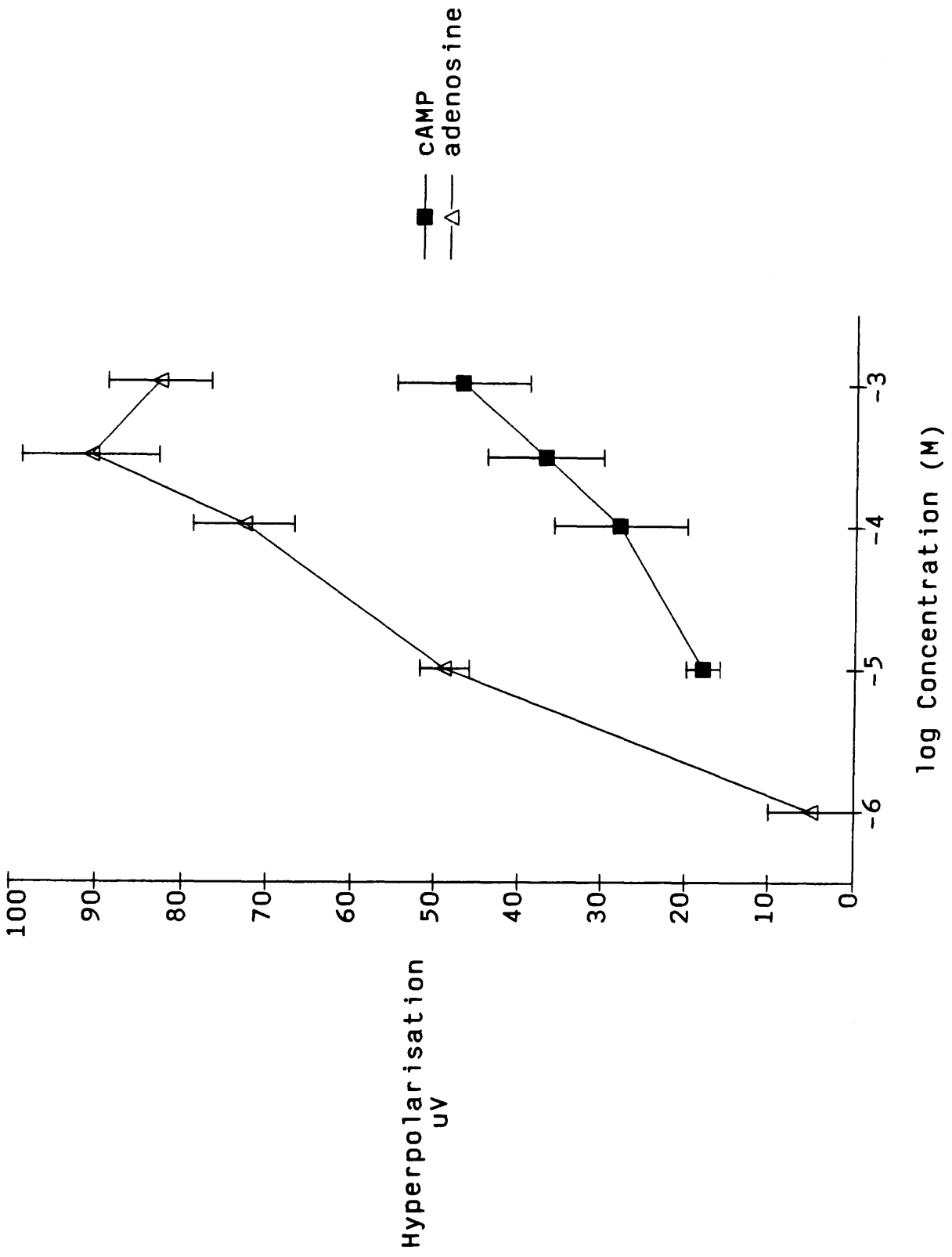


Fig. 3.10. Log concentration response curves of the isolated rat SCG to two minute applications of adenosine (AD) or cyclic 3',5'-adenosine monophosphate (cAMP). Both AD and cAMP were applied with a minimum of 20 minutes between each application of agonist.

Fig. 3.11. Comparison of the log concentration-response curves of the isolated rat SCG to 8 bromo-cyclic adenosine monophosphate, cyclic adenosine monophosphate and cyclic guanosine monophosphate and 8BrcAMP in the presence of 8-phenyltheophylline.

8-Bromo-cyclic adenosine monophosphate (8BrcAMP) produced dose related depolarisations and at 1000uM a small hyperpolarisation preceded the depolarisation to 8BrcAMP in 2 out of 4 ganglia (-30 and -20uV). In the presence of 10uM 8-phenyltheophylline (8PT) the depolarisations to 8BrcAMP were not significantly changed whereas the hyperpolarisations to 100uM adenosine on the same ganglia were significantly reduced (adenosine control = -55 $\pm$ 7uV; adenosine + 8PT (10uM) = -15 $\pm$ 9uV, $P<0.01$, $n=4$).

All purines were applied for 2 minutes and ganglia were incubated in 8PT for a minimum of 40 minutes before repeating the response to 8BrcAMP.

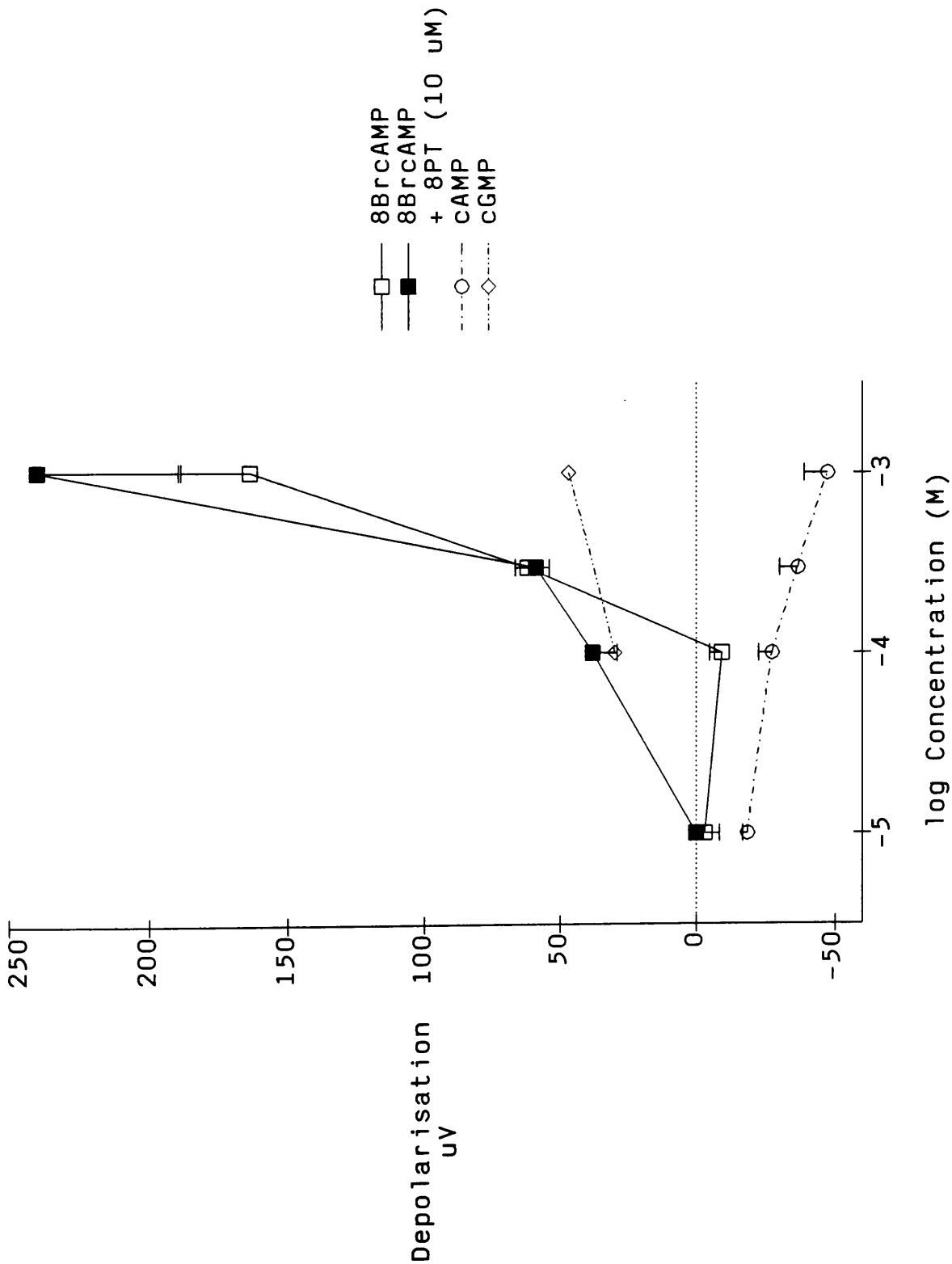

8BrcAMP
8BrcAMP
+ 8PT (10 uM)
cAMP
cGMP
250
200
150
100
50
0
-50
Depolarisation
uV
-5
-4
-3
log Concentration (M)

Fig. 3.12. Log concentration response curves of the isolated rat SCG to 2 minute application of uridine triphosphate, uridine monophosphate and cytosine triphosphate in physiological salt solution, and the response to uridine monophosphate in low potassium (2mM) and calcium (0.1mM) physiological salt solution.

In PSS a 2 minute application of uridine triphosphate (UTP) at 1 to 1000uM resulted in concentration related depolarisations whereas 1000uM cytosine triphosphate (CTP) produced small but significant depolarisations (34 ± 2uV, n=4, $P<0.001$). In contrast uridine monophosphate (UMP) was inactive at 100uM and produced small and prolonged hyperpolarisations at 1000uM (n=7, $P<0.05$), which were about half those produced by a two minute application of 100uM adenosine (-71 ± 7uV, n=7). In low K^+/Ca^{2+} physiological salt solution (PSS) UMP at 100uM did not significantly (-23 ± 9uV, n=4, NS) alter the d.c. potential and at 1000uM hyperpolarised rat SCG by -53 ± 15uV (n=4, $P<0.05$).

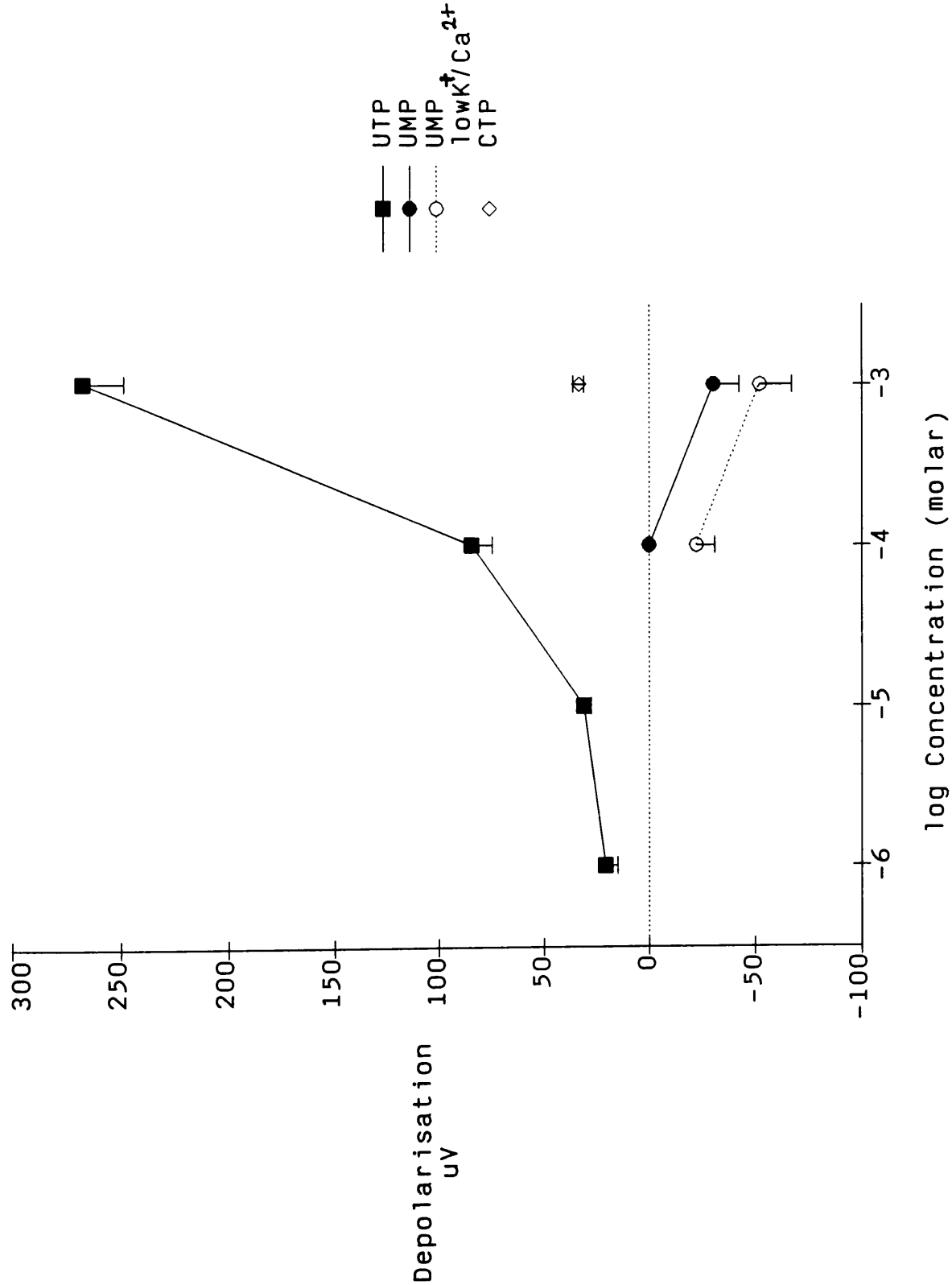

UTP
UMP
UMP low K+/Ca2+
CTP
Depolarisation uV
300
250
200
150
100
50
0
-50
-100
-6
-5
-4
-3
log Concentration (molar)

Table 3.1. Effect of altering the concentration of potassium in the postganglionic chamber of the grease gap tissue bath, on the response of the isolated rat SCG to adenosine and potassium

There was no significant difference (two-tailed paired t-test) in the response of the ganglia to adenosine (2 minutes) or potassium (1 minute) with different concentrations of potassium in the postganglionic chambers. Responses are uV (mean ± SEM) for three ganglia.

	Potassium concentration in postganglionic chamber			
	6mM	12mM	66mM	6mM
Agonist	Pre-test Control	Test	Test	Post-test Control
ADENOSINE 100uM	-80 ± 12	-78 ± 6	-107 ± 3	-65 ± 3
POTASSIUM 9mM	230 ± 60	242 ± 32	253 ± 41	263 ± 45

Table 3.2. Effect of tetrodotoxin and pirenzepine on the response of isolated rat SCG to adenosine

The effect of 1uM tetrodotoxin (TTX) and 0.3uM pirenzepine (PIR) on the response of the isolated rat SCG to a two minute application of 100uM adenosine in physiological salt solution (PSS) and calcium and magnesium free PSS was determined There was no significant difference between responses in PSS containing TTX or PIR treated ganglia and those in PSS (paired t test, where N = number of ganglia tested).

PSS	N	Response to 100uM adenosine (uV, mean + sem) Control	+ TTX (1uM)	+ PIR (0.3uM)
Normal	6	-83±11	-77±10	
Normal	4	-69±11		-48±11
0mM Ca^{2+}/ 0mM Mg^{2+}	3a	-163±29	-127±27	
	3b	90±38	0±0	

a = hyperpolarisation to adenosine
b = after depolarisation to adenosine

Table 3.3. Effect of pirenzepine, inosine, dipyridamole and hydroxy-nitrothiobenzylguanosine on the response of the isolated rat SCG to muscarine

Ganglia were incubated in pirenzepine (PIR), dipyridamole (DIP) and hydroxynitrobenzylguanosine (HNBTG) for a minimum of 20 minutes and muscarine was applied for one minute.

CONTROL PSS uV ± SEM	N	PSS + PIR 0.3uM	PSS + INOSINE 1000uM	PSS + DIP 10uM	PSS + HNBTG 10uM
220 ± 49	4	11 ± 12*			
185 ± 31	3		182 ± 37		
227 ± 23	12			199 ± 16*	
219 ± 29	7				232 ± 39

Responses are means ± standard error of the mean in uV. N = number of ganglia tested. Statistical significance (paired t-test) is indicated by * for $P < 0.05$.

Table 3.4. The effect of dipyridamole on the response of the isolated rat SCG to 2-chloroadenosine and potassium

The effect of a two minute application of 2-chloroadenosine (2CA) and a one minute application of potassium (K^+) was determined in normal physiological salt solution.

AGONIST	CONCENTRATION uM	N	CONTROL	PSS + DIP (10uM)
2-CA	1	4	-53 ± 10	-48 ± 14[a]
2-CA	10	3	-57 ± 15	-70 ± 21[a]
K^+	12000	3	600 ± 35	647 ± 55[b]

Responses are means ± standard error of the mean in uV. N = number of ganglia tested. Minimum incubation time in DIP was (a) 40 minutes and (b) 100 minutes. Responses to 2-CA and K^+ in presence of DIP were not significantly different to values obtained in PSS (paired t test).

Table 3.5. The effect of purines and pyrimidines on the response of the rat isolated SCG to muscarine

The effect of 8-bromo-cyclic-adenosine monophosphate (8BrcAMP), cyclic guanosine-monophosphate (cGMP), uridine triphosphate (UTP) on the response to a one minute application of 100nM muscarine in physiological salt solution (PSS) was determined. The responses in the absence and the presence of test agonists were compared by a paired t test, where N = number of ganglia tested, and *** = $P < 0.001$.

COMPOUND	Conc uM	N	Response to 100 muscarine (uV) PRETEST	TEST	POST-TEST	% Change in response to muscarine
Adenosine	100	61	312±20	228±16	313±21	-26±2***
8BrcAMP	100	2	273±28	230±30	223±3	-16±3
cGMP	100	3	307±12	287±13	318±6	-6±4
UTP	100	7	251±32	277±36	229±25	10±6

CHAPTER FOUR

IONIC MECHANISMS OF ACTION OF ADENOSINE ON THE RAT SCG

Chapter Four IONIC MECHANISM OF ACTION OF ADENOSINE ON THE RAT SCG

INTRODUCTION

Many different ionic mechanisms have been proposed to account for the pharmacological effects of adenosine, of which the most commonly reported are a decrease in calcium conductance (gCa) and/or an increase in potassium conductance (gK) (Fig. 4.1).

Calcium ions have a number of important roles in neurone physiology including entry into presynaptic nerve terminals via voltage operated calcium channels during depolarisation to release neurotransmitters (Levitan & Kaczmarek, 1987), and as secondary messengers to control cell physiology and the entry of Ca^{2+} into postsynaptic neurones in normal and pathological states (Schubert, 1987; Dragunow & Faull, 1988).

Henon & McAfee (1983a,b) have reported that adenosine inhibited postsynaptic Ca^{2+} dependent potentials of the rat SCG and thus it would be predicted that the inhibitory effects of adenosine on muscarinic responses and the adenosine-induced hyperpolarisation of the rat SCG reported in this study, may also be mediated via a change in Ca^{2+} dependent potentials.

Role of Ca^{2+} in the presynaptic actions of adenosine

Adenosine has been suggested to inhibit Ca^{2+} influx (Fredholm & Hedqvist, 1980) and/or impair the internal mobilisation of Ca^{2+} in presynaptic nerve terminals (Silinsky, 1986). However the results of experiments on the effect of adenosine on calcium fluxes and in

particular the depression of the uptake of Ca^{2+} into brain synaptosomes are equivocal. Ribeiro and colleagues (1979) and Wu, Phillis & Thierry (1982) reported that adenosine inhibited depolarisation induced uptake of Ca^{2+} . Bartrup, Wyllie & Stone (1988) found adenosine at 10uM did not significantly alter the uptake of $45Ca^{2+}$, whereas CPA, 2CA and NECA produced a slight but significant reduction in flux, and attributed this effect to an action at A2 receptors. This latter finding requires further clarification as the presynaptic inhibitory actions of adenosine are thought to be via an action on A1 purinoceptors (Stone, 1989).

In contrast it has been reported that adenosine does not affect Ca^{2+} uptake (Barr, Daniell & Leslie, 1985; Garritsen, Ijzerman & Soudijn, 1989). In a comprehensive study, Garritsen et al. (1989) studied the displacement of the selective A1 purinoceptor antagonist, tritiated 8-cyclopentyl-1,3-dipropylxanthine ($[^3H]$-DPCPX) binding in rat synaptosomes and estimated that at 0.1uM, R-PIA occupied most of the A1 receptors. Both R-PIA and 5'-N-ethylcarboxamidoadenosine (NECA) at up to 10uM did not alter basal or KC1-stimulated $45Ca^{2+}$ uptake. Even after altering a considerable number of incubation conditions, including the incubation time, the addition of uptake inhibitors (a factor not controlled or acknowledged by many of the preceding reports), addition of ADA, adenosine and R-PIA still lacked any effect on the basal or stimulated $45Ca^{2+}$ uptake. The results of Garritsen et al. (1989) provide considerable weight to the idea that adenosine may influence the sensitivity for calcium rather than the calcium concentration as proposed by Silinsky (Silinsky, 1986).

Effect of adenosine on postsynaptic neuronal calcium currents

In 1986 Ribeiro & Sebastiao reviewed experimental evidence for the interactions between adenosine and calcium and suggested an additional adenosine receptor subtype, the A3 receptor. The evidence in favour of an A3 purinoceptor was based on an extensive review of reported purine literature where experimental results could not easily be classified as A1 or A2 receptor mediated. In addition Ribeiro & Sebastiao (1984) suggested the actions of purines on the frog neuromuscular junction (NMJ) (Ribeiro & Sebastiao, 1984) were linked to an adenosine receptor controlling Ca^{2+} ions with a relative potency order for adenosine analogues unlike the A1 or A2 order i.e. the A3 receptor (see Table 1). A similar potency order on the mammalian heart was taken as evidence for an A3 receptor mediating the presynaptic inhibitory actions of adenosine.

There is some evidence that in neuronal tissue adenosine causes a depression of Ca^{2+} currents accompanied by an increase in membrane resistance as shown by MacDonald, Skerritt & Werz (1986) using voltage clamped sensory neurones in culture. Similar results were reported by Dolphin, Forda & Scott (1986), who found 2CA depressed barium (Ba^{2+}) currents of dorsal root ganglia. In contrast Halliwell & Scholfield (1984) found purines were unable to inhibit directly evoked calcium currents, as defined by their blockade by cadmium (Cd^{2+}) in both guinea-pig hippocampal and olfactory cortex neurones.

A depression of calcium spikes was reported in ganglionic (Henon & McAfee, 1979, 1983a) and rat CA1 hippocampal neurones (Proctor & Dunwiddie, 1983) and attributed to a direct inhibition of the Ca^{2+} conductance rather than an increase in potassium current such as calcium activated

potassium current (IK_{Ca}). Henon & McAfee (1983a,b) concluded that adenosine inhibited a voltage dependent calcium current of the isolated rat SCG, resulting in a reduction of the IK_{Ca} current responsible for the AHP. In contrast to Henon & McAfee (1983a,b), Haas & Green (1984) found that adenosine potentiated the AHP and accommodation of rat hippocampal pyramidal cells, via an enhancement of an IK_{Ca} current.

In reviewing the role of calcium in the actions of adenosine, Stone (1989) suggested that the ability of adenosine to enhance or reduce the AHP "may suggest a difference between central and peripheral (ganglionic) neurones". However, Palmer, Wood & Zafirov (1987) reported adenosine enhanced the AHP recorded from peripheral nerves of the guinea-pig myenteric plexus, and the reduction of the AHP of the rat SCG may be particular to this preparation.

Schubert (1987) has criticised many of the above results by suggesting that "all of these experimental approaches ..... did not allow a differentiation between pre- and postsynaptic calcium fluxes". This criticism appears unwarranted as many of the responses were recorded from postsynaptic neurones. Schubert has studied the calcium fluxes in hippocampal slices by employing ion selective calcium electrodes (Schubert, Heinemann & Kolb, 1986) and found adenosine and R-PIA induced a decrease in extracellular calcium levels. This decrease in calcium has been attributed to an influx of calcium into presynaptic terminals (Schubert & Kreutzbert, 1987).

Ribeiro & Sebastiao (1986) cited the results obtained by Henon & McAfee (1986a) in support of the presence of A3 receptors in the rat SCG. This conclusion was based on the ability of adenosine, R-PIA and NECA to alter calcium

spikes, but the order of potency given by Henon & McAfee (1986a) does not agree with that proposed for an A3 receptor. Irrespective of whether the adenosine receptor/s involved in the reduction of the calcium spikes of the rat SCG (Henon & McAfee, 1983a) are classified as an A3 or an A1 subtype, it is possible to examine if the effects of adenosine reported here are due to an interaction with Ca^{2+}.

To determine if the response of the rat SCG to adenosine was dependent upon extracellular calcium ($[Ca^{2+}]e$) and/or intracellular calcium ($[Ca^{2+}]i$) the effects of adenosine was determined using different ionic manipulations of the PSS and various calcium channel antagonists.

RESULTS

Effect of changes in $[Ca^{2+}]e$ and $[Mg^{2+}]e$ on the response of the rat SCG to adenosine and its analogues

4.1 Effects of altering $[Ca^{2+}]e$ on;

(1) The hyperpolarisation to purines

The size of the response to adenosine varied according to the different concentrations of Ca^{2+} and Mg^{2+} in the PSS (Table 4.1). Increasing the Ca^{2+} concentration to 5mM depressed the adenosine-induced hyperpolarisation, moving the log concentration response curve to the right (Fig. 4.2). Conversely a reduction of $[Ca^{2+}]e$ to 0.1mM increased the hyperpolarisations to adenosine (Fig. 4.3) displaced the log concentration response curve to the left (Fig. 4.2).

The concentration-response curve to adenosine, PAA and 2CA in 0.1mM Ca^{2+} PSS were all potentiated to a similar extent (Fig. 4.4) e.g. at 30uM the ratio of the responses to PAA and adenosine in 0.1mM Ca^{2+} and 2.5mM Ca^{2+} PSS were the same (2.1). Responses to adenosine in 0.1mM Ca^{2+} (Fig. 4.4) and 0mM Ca^{2+}/EGTA PSS (Fig. 4.8) were reproducible during superfusion for up to eight hours.

(2) The depression of the response to muscarine by adenosine

The depression of the response to 100nM muscarine by adenosine in PSS containing 2.5mM Ca^{2+} was concentration dependent and the log concentration response curve for the depression of muscarine was displaced to the left in 0.1mM Ca^{2+} PSS (Fig. 4.6). In 0.1mM Ca^{2+} the maximum depression of muscarine was potentiated although not significantly and the ED_{50} reduced ten fold. On two ganglia the depression of 100nM muscarine by 100uM adenosine was -39% and -46% in PSS and -41% and -42% in 0mM Ca^{2+}/EGTA PSS.

4.2 Effect of altering $[Mg^{2+}]e$ on the response to adenosine and 2CA

4.2.1 Comparison of the response to adenosine in;

4.2.1.1. The presence and absence of 1mM magnesium

In the presence of normal PSS containing 1mM magnesium both adenosine and 2CA produced concentration related hyperpolarisations. The absence of magnesium (0mM Mg^{2+}) from the PSS did not significantly alter the hyperpolarisations to either adenosine or 2CA (Fig. 4.7).

In 0mM Ca^{2+} PSS both the responses to adenosine and potassium were enhanced in the absence of Mg^{2+} (Fig. 4.9, cf a & b) and at 100uM adenosine or higher the response was biphasic with an after depolarisation as shown in Fig. 4.9a. The presence of 1uM TTX in 0mM Ca^{2+} and 0mM Mg^{2+} medium reduced the enhancement of the potassium depolarisation to values similar to the control responses in 0.1mM Ca^{2+}/1mM Mg^{2+} (313 $\pm$ 70uV, n=3) and abolished the after-depolarisation to 1mM adenosine.

In contrast to the responses to adenosine in 0mM Mg^{2+} and 0mM Ca^{2+} PSS were reduced in the absence of Mg^{2+} in Ca^{2+}/EGTA PSS (Fig. 4.8).

4.2.1.2. The presence of 1mM and 10mM magnesium

Increasing the extracellular concentration of magnesium from 1 to 10mM resulted in a significant reduction in the response to adenosine in PSS containing 0mM Ca^{2+} (Fig. 4.9, cf b & c) or 0.1mM Ca^{2+} but not in PSS containing 2.5 or 5mM Ca^{2+} (Fig. 4.9, cf d & e; Table 4.1).

Discussion

The potentiation of the hyperpolarisation to adenosine in reduced Ca^{2+} PSS, the attentuation of the response to adenosine in 5mM Ca^{2+} PSS (Figs. 4.2 and 4.3) and the enhancement of the depression of muscarine in 0.1mM Ca^{2+} PSS (Fig. 4.6) are similar to the effects reported by Dowdle & Maske (1980) for the inhibition of cholinergic transmission in the guinea-pig ileum by adenine nucleotides, and by Ribeiro (1982) for the depression of neuromuscular transmission of the rat diaphragm by adenosine in low calcium solutions. By themselves the results obtained for the rat SCG are consistent with the

ability of adenosine to alter the influx of calcium. However, the ability of the ganglion to hyperpolarise during prolonged perfusion in 0mM Ca^{2+} PSS with or without EGTA does not support this hypothesis. Likewise the ability of adenosine to depress the response to muscarine in 0.1mM Ca^{2+} PSS (Fig. 4.6) and Ca^{2+} free medium are unlike results reported by Cox & Walker (1987), who reported the inhibitory action of adenosine on ACh induced depolarisations of parietal snail ganglion cells was potentiated by high $[Ca^{2+}]e$.

The potentiation of the hyperpolarisations to 2CA and PAA in low $[Ca^{2+}]e$, was similar to that obtained for adenosine suggesting the increase in sensitivity of the rat SCG in low Ca^{2+} media is unlikely to arise from a decrease in uptake or metabolism of adenosine. The low potency of PAA, a potent and selective P1 purinoceptor agonist on A2-adenosine receptors suggests the hyperpolarisation to adenosine may be mediated by an action on A1 receptors. Further characterisation of the adenosine receptor mediated hyperpolarisation of the SCG is described in chapter 6.

If the hyperpolarisation to adenosine is potentiated by depolarisation and the absence of calcium depolarises the ganglion this would increase the response to adenosine. The potentiation of the hyperpolarisations to adenosine in reduced Ca^{2+} PSS is therefore consistent with this response being dependent on the resting membrane potential (RMP) and is potentiated by depolarisation of the ganglion.

It has been shown that a reduction in $[Ca^{2+}]e$ inhibits presynaptic action potential generation and postsynaptic terminals and nerves become hyperexcitable (Campbell, 1983). The ability of adenosine to hyperpolarise in

reduced or calcium free PSS suggests adenosine does not hyperpolarise the ganglion by inhibiting presynaptic neurotransmitter release.

It is recognised that divalent cations can influence the neuronal activity by screening negative fixed charges on the surface of the neurone. Frankenhaeuser & Hodgkin (1957) using the squid giant axon observed that an increase in $[Ca^{2+}]e$ produced a shift in the activation of both Na^{+} and K^{+} currents to more positive potentials and in low $[Ca^{2+}]$ the neuronal membrane loses much of its selective permeability and a smaller depolarisation is required to increase the sodium conductance to a critical level where the cell depolarises. An increase in the response to adenosine probably arises not because adenosine reduces Ca^{2+} entry but may be due to a change in the total divalent cation concentration which alters the membrane surface potential. An increased excitability of the ganglion is supported by the enhanced response to the non-specific depolarising agent, potassium in low Ca^{2+} and Mg^{2+} solutions (Fig. 4.9). Mg^{2+} ions have a stabilising action like that of Ca^{2+} but are less effective (Frankenhaeuser & Hodgkin, 1957) and some indication of the effect of a change in surface charge on the response to adenosine in low $[Ca^{2+}]e$ may be indicated by the ability of increased $[Mg^{2+}]e$ at 10mM to compensate for the potentiation of the response to adenosine in low $[Ca^{2+}]e$ where the responses to adenosine were similar to those in normal PSS (Table 4.1).

A change in excitability of the neuronal membrane alone may not be the only mechanism that potentiates the response to purines as a number of studies using isolated tissues have demonstrated the dependence of opioid-receptor mediated agonism on $[Ca^{2+}]e$ and an increase in the efficacy of the agonist (Dougall & Leff, 1987). Also

agonists that demonstrated little or no significant agonism under normal conditions exhibit agonism when the $[Ca^{2+}]e$ is reduced (Hayes & Sheehan, 1986) implying a change in efficacy. The increased responsiveness to PAA in 0.1mM Ca^{2+} PSS may be evidence in favour of this hypothesis but as detailed analysis of the data is not possible and a more precise study as described by Dougall & Leff (1987) would be required before making any definite conclusions. However the similar potentiation of the response to adenosine and PAA suggests both actions are altered non-selectively in low $[Ca^{2+}]e$. The effect of low $[Ca^{2+}]e$ on the antagonism of concentration response curves to adenosine is discussed in chapter 6.

Many of the actions of adenosine have been attributed to an action on cAMP levels (Londos et al., 1980). In the presence of IBMX to prevent degradation of cAMP, Lindl (1979) found the incubation of rat SCG in high calcium media (20mM) reduced cAMP accumulation, compared to controls in 2.2mM Ca^{2+}. However the omission of Ca^{2+} did not significantly increase cAMP levels, unless the ganglia were also incubated with 1mM EGTA. The response of the rat SCG to adenosine in 0mM Ca^{2+} with or without EGTA was similar, suggesting the ability of low $[Ca^{2+}]e$ to enhance the response to adenosine may not arise via an increase in cAMP.

If adenosine causes an inhibition of Ca^{2+} influx of the rat SCG, then the two most likely currents to be affected would be calcium activated potassium currents (IK_{Ca}) and the voltage sensitive Ca^{2+} currents. The potential charge resulting when adenosine is applied would then depend upon the relationship between the voltage sensitivity of these currents. Calcium channels are usually highly selective for calcium ions over sodium ions however if the external level of free Ca^{2+} is below micromolar values, e.g. as

with Ca^{2+} free PSS, Ca^{2+} channels can allow the influx of Na^{+} and other ions (Lux & Carbone, 1987). The effect of interaction with $[Mg^{2+}]e$ are discussed in the next section but it is to be expected that Mg^{2+} would antagonise Na^{+} entry. In the event of Na^{+} entry this would be expected to depolarise the ganglion and could potentiate the hyperpolarisation to adenosine. The possibility that adenosine hyperpolarises the rat SCG by altering the movement of Ca^{2+} and Na^{+} ions was examined further.

It has been shown that magnesium ions are required for the inhibitory effects of adenosine on adenylate cyclase (Yeung & Green, 1983). As is the case for several other neurotransmitters the adenosine receptor probably exists in multiple agonist affinity states (Marangos, Patel, Martino, Dilli & Boulenger, 1983; Yeung & Green, 1983; Lohse, Lenschow & Schnabe, 1984; Dunwiddie & Fredholm, 1984) and it is most likely that the receptors affinity will be subject to modulation by external divalent cations. Both the presynaptic depression of adenosine on the guinea-pig olfactory cortex (Scholfield & Steel, 1988) and the inhibition of hippocampal population potentials in vitro (Bartrup & Stone, 1988) were potentiated by increasing $[Mg^{2+}]e$. Bartrup & Stone (1988) have suggested that "the manipulation of magnesium ion concentration may prove to be a simple method for eliminating the A1-receptor mediated actions, and for discriminating between A1 and A2 receptor mediated events".

The results of this study show that the response of the rat SCG to adenosine and 2CA were not affected by the absence of Mg^{2+} from normal PSS (Fig. 4.7).

It is known that magnesium antagonises the actions of calcium for evoked transmitter release (Silinsky, 1985) and the absence of $[Mg^{2+}]e$ would be expected to allow an

increased influx of Ca^{2+} in to the postsynaptic terminal and antagonise the hyperpolarisation to adenosine if it is mediated via an inhibition of Ca^{2+} influx. Thus the size of the adenosine induced hyperpolarisation should be dependent upon the $[Mg^{2+}]e$ concentration if adenosine modulates the site of Ca^{2+} entry.

Although low Ca^{2+} PSS enhanced the response to adenosine, increasing the Mg^{2+} concentration to 10mM counteracted the effect of reduced $[Ca^{2+}]e$ and restored the response size to that obtained in normal PSS, suggesting the increase in the size of the hyperpolarisation to adenosine in reduced Ca^{2+} media may arise from an increased neuronal excitability. The ability of adenosine to hyperpolarise in 0mM Ca^{2+}/10mM Mg^{2+} PSS, where presynaptic release of ACh is abolished suggests adenosine hyperpolarises by a direct action at a postsynaptic site.

In PSS containing different concentration of $[Ca^{2+}]e$ and 1mM $[Mg^{2+}]e$ the hyperpolarisation to 1mM adenosine was complex and consisted of an initial transient hyperpolarisation and a smaller late hyperpolarisation (Fig. 4.9). Interestingly when the $[Mg^{2+}]e$ was increased to 10mM the maximum response to 1mM adenosine was sustained (Fig. 4.9 Cf b & c also d & e). The enhancement of the response to adenosine in 0mM Ca^{2+}/0mM Mg^{2+} PSS and K^+ free PSS compared to 0mM Ca^{2+}/0mM Mg^{2+} PSS + 6mM K^+ (Fig. 4.9 Cf a & f) suggests adenosine may hyperpolarise the rat SCG by increasing K^+ efflux. If adenosine increases gK then one explanation for change in the peak response to a smaller response would be a build up of perineuronal K^+ during the initial response which would then reduce the response to further application of adenosine. Alternatively this complex response to a high concentration of adenosine i.e. 1000uM, may be due to desensitisation as this concentration is supramaximal (see

Fig. 3.3b) and magnesium may prevent this desensitisation. Given the high concentration of adenosine required to see these effects and that there are a variety of other possible explanations such as inhibition of metabolism and/or uptake, or an underlying AHP, or the activation of another receptor subtype (see chapter 6 for further discussion of this phenomenon). The inability of high magnesium to alter the peak response to adenosine in 2.5mM or 5mM Ca^{2+} PSS could arise if there were excess free Ca^{2+} ions to counteract the presence of a high concentration of Mg^{2+} ions or more likely if the effects of adenosine are not linked to Ca^{2+} entry.

In 2.5mM Ca^{2+} the absence of Mg^{2+} in the PSS did not significantly alter the hyperpolarisations to adenosine or 2CA, whereas in 0.1mM Ca^{2+} the removal of Mg^{2+} significantly enhanced the hyperpolarisation to both adenosine and 2CA. It is well known that Ca^{2+} currents through Ca^{2+} channels can be blocked by magnesium and the removal of magnesium could lead to an increased excitability of the ganglion. Some evidence for this conclusion is suggested by the presence of an after-depolarisation in response to high concentrations of adenosine, but this was never observed in 0.1mM Ca PSS containing Mg^{2+}. The possibility that the after depolarisation occurred due to the absence of sufficient Mg^{2+} and Ca^{2+} to stabilise the neuronal membranes was indicated by the ability of TTX to abolish the after-depolarisation. However, it is unlikely the after-depolarisation arose from a presynaptic release of ACh for the following reasons: adenosine generally inhibits ACh release although an action at A2 receptors has been reported to increase release: secondly, the release of ACh requires extracellular Ca^{2+} (Silinsky, 1985) and thirdly the after-depolarisation was unaffected in the presence of 2uM atropine (n=2).

The results of this study suggest that under physiological concentrations of cations the requirement for magnesium is not shared by the adenosine receptor of the rat SCG. However the removal of Mg^{2+} in Ca^{2+} free medium markedly reduced the response to adenosine. The removal of Mg^{2+} ions could conceivably allow residually bound Ca^{2+} to antagonise the hyperpolarisation to adenosine but it would be expected that residually bound Ca^{2+} would be chelated by an excess of EGTA. Alternatively a reduced response in the absence of Mg^{2+} could be interpreted to reflect a requirement for Mg^{2+} in the actions of adenosine (Bartrup & Stone, 1988). A more likely explanation for the results observed here would be that the ganglion becomes depleted of intracellular magnesium ($[Mg^{2+}]i$) during perfusion with 0mM Ca^{2+}/0mM Mg^{2+} PSS. Recently, Nakayama & Tomita (1990) reported that perfusion of guinea-pig caecum with either Mg^{2+} Ca^{2+} free solution for up to two hours resulted in little loss of $[Mg^{2+}]i$, whereas preparations bathed in Mg^{2+} and Ca^{2+} free solution with 1mM EGTA, rapidly lost $[Mg^{2+}]i$ during one to two hours incubation. Assuming the rat SCG behaves in a similar manner to taenia then the incubation in Ca^{2+} free PSS with Mg^{2+} for at least an hour before testing might be sufficient to deplete $[Mg^{2+}]i$ and explain the reduction of the response to adenosine.

These results show the hyperpolarisations of the rat SCG to adenosine and 2CA are independent of $[Mg^{2+}]e$ and the results presented in chapter 6 suggest that the adenosine receptors of the rat SCG are of the A1 subtype. Thus the proposal by Bartrup & Stone (1988) that the removal of Mg^{2+} from PSS provides a method for eliminating the A1 actions of adenosine may only apply to certain effects mediated by adenosine. The reason for the lack of dependence of the response of the rat SCG to adenosine on $[Mg^{2+}]e$ is unclear but Schubert & Kreutzberg (1987),

studying the effect of adenosine on calcium fluxes of rat hippocampus, suggested there are two types of A1 receptors that are Mg^{2+} sensitive and Mg^{2+} insensitive possibly extrasynaptic and synaptic A1 receptors (Tetzlaff, Schubert & Kreutzberg, 1987). Perhaps the former are sensitive to $[Mg^{2+}]e$ (Bartrup & Stone, 1988) and the latter postsynaptic or extrasynaptic adenosine receptors are independent of $[Mg^{2+}]e$. Likewise Fastbom & Fredholm (1990) using autoradiography of the rat brain have suggested that differences in the sensitivity of A1-receptor-G-protein complexes to magnesium may reflect a heterogeneity of the G-proteins to which the A1-receptors are coupled.

In summary, it appears unlikely that (1) the adenosine-induced hyperpolarisation of the rat SCG is mediated by inhibition of Ca^{2+} currents, and (2) is not due to the release of a neurotransmitter or neuromodulator but occurs due to a direct postsynaptic action; (3) the response to adenosine is not Mg^{2+}-dependent.

4.3 Effect of calcium channel antagonists on the response to adenosine

The evidence for multiple types of Ca^{2+} channels has been reviewed by Tsien, Lipscombe, Madison, Bley & Fox (1988) and Bean (1989). Ca^{2+} channels, which open in response to membrane depolarisation admit extracellular calcium into the neurones and have been classified into three types on the basis of their gating properties and pharmacological sensitivities (Nowycky, Fox & Tsien, 1985). T channels are characterised by a transient time-course due to rapid inactivation (20-50 mS) and sensitivity to blockade by nickel ions (Ni^{2+}). L channels slowly inactivate (>500 mS), are selectively antagonised by dihydropyridines

(DHPs) and are more effectively blocked by low concentrations of cadmium (Cd^{2+}) than are T channels. T channels are unaffected by DHPs at concentrations below 3uM (Bean, 1989). The other rapidly inactivated channel, the N channel in contrast to the T channels is sensitive to a snail toxin, omega-conotoxin and Cd^{2+} (Table 4.2).

Cultured rat SCG neurones expressed both L and N type Ca^{2+} channels, which are activated by strong depolarisation, (Perney, Hirning, Leeman & Miller, 1986; Miller, 1987). On the rat SCG the N channels are only partially inactivated (Plummer, Logothetis & Hess, 1989) whereas the L channels do not inactivate even during a steady depolarisation at -20mV (Nowycky et al., 1985). These Ca^{2+} channels are inactive at low membrane potentials but are activated by depolarisation in high potassium PSS (Vidal, Raynaud & Weber, 1989).

The response of postsynaptic muscarinic receptors of the rat SCG can involve the modulation of N-channel gating (Wanke, Ferroni, Malgaroli, Ambrosini, Pozzan & Meldolesi, 1987) and adenosine could depress the muscarinic depolarisation by altering Ca^{2+} influx. This observation is supported by the recent discovery that the inhibition of Im by muscarinic agonists was shown to be $[Ca^{2+}]e$ dependent (Tokimasa & Akasu, 1990b). In addition the presynaptic alpha-adrenergic inhibition of neurotransmitter release from the rat SCG is known to be controlled by N-type Ca^{2+} channels (Hirning, Fox, McCleskey, Olivera, Thayer, Miller & Tsien, 1988; Lipscombe, Kongsamut & Tsien, 1989).

The ability of Ca^{2+} channel antagonists to alter the adenosine-induced hyperpolarisation and the depression of muscarine was examined using non-selective and selective

antagonists of N, L & T channels and are summarised in tables 4.6 to 4.8.

4.3.1 Effects of cobalt

The antagonism of the response to adenosine in Co^{2+} PSS, is a new finding (Table 4.7) and suggests adenosine may hyperpolarisation the ganglion via a Ca^{2+} dependent process. The antagonism of adenosine appeard to be competitive as the response to adenosine in 5mM Co^{2+} could be restored by increasing the concentration of adenosine (Table 4.7).

The ability of Co^{2+} to abolish the response to adenosine invalidates the findings of Henon & McAfee (1983, see Fig. 4) who reported 2CA (10uM) did not alter the current-voltage curve in the presence of 5mM Co^{2+}, as the presence of Co^{2+} would be expected to mask the effects of 2CA.

Co^{2+} at 5mM has been reported to selectively antagonise a Ca^{2+} dependent slow tail current of the rat SCG i.e. the I_{ahp} (Freschi, 1983). Two other currents, a fast tail current (I_c) and another slow current were unaltered by 5mM Co^{2+}. Unfortunately Co^{2+} was the only Ca^{2+} channel antagonist tested on the rat SCG by Freschi (1983) and the selectivity of Co^{2+} for other currents was not determined. A similar effect of Co^{2+} and also Mn^{2+} and Cd^{2+} to antagonise a fast and slow AHP of CA1 pyramidal cells of the rat hippocampus has been reported by Storm (1987). Thus the hyperpolarisations to the rat SCG to adenosine may be induced via an activation of a Co^{2+} sensitive gK_{Ca}. However this hypothesis is not supported by the inability of the more seletive antagonist of $I_{K(ca)}$ channels to alter the hyperpolarisation to adenosine (Table 4.4) or the enhancement of the hyperpolarisation to adenosine in

Ca^{2+} free PSS in the presence or absence of EGTA (Table 4.7) (see section 4.2).

However, the significant reduction of the response to adenosine by Co^{2+} was examined in further detail as the $I_{K(Ca)}$ current reported to be antagonised by Co^{2+} (Freschi, 1983) appears to be similar to the Ca^{2+} current responsible for the shoulder on the falling phase of the action potential that was inhibited by adenosine (Henon & McAfee, 1983b).

If Co^{2+} alters the local membrane field by screening negative fixed charges on the surface of the neurone then the addition of Co^{2+} would have an effect similar to membrane hyperpolarisation and thus could depress the response to adenosine. Such effects are not thought to alter the RMP and should not depend upon the species of divalent cation. The results presented in Tables 4.1 and 4.6 do not support the above hypothesis for the depression of the response to adenosine caused by Co^{2+}. For example at the same concentration 5mM Mg^{2+} was less effective than 5mM Co^{2+} in antagonising the response to adenosine. Because any screening of negative fixed charges would be expected to be very similar in either Co^{2+} or other divalent cations, it is concluded that Co^{2+} does not depress the hyperpolarisation to adenosine by simply screening surface charge.

Replacing Ca^{2+} with Co^{2+} significantly reduced the response to both adenosine and carbachol (Fig. 4.10) suggesting the effects of Co^{2+} may not be non-selective, but are unlikely to be a "general anaesthetic action" because Co^{2+} did not alter the sensitivity to potassium (Table 4.7).

Further studies on the response of the ganglion to muscarine in Co^{2+} PSS, showed Co^{2+} could also reduce the response to muscarine (Table 4.7) and strongly indicate the actions of Co^{2+} were non-selective as both the depolarisation to muscarine (caused by a decrease in gK) and the hyperpolarisation to carbachol (believed to be due to an increase in gK) were antagonised.

4.3.2 Effects of DHPs

Ligand binding studies have demonstrated that somne DHPs reduce adenosine ligand binding to rat brain membranes, while having no effect on other receptors including cholinoreceptors (Morgan, Tamborska, Patel & Marangos, 1987) although other researchers have reported that the DHPs at above 1uM have non-specific interactions including the reduction of muscarinic binding (Gerry, Rauch, Colvin, Adler & Messine, 1987). The ability of (+) PN200 110 to reduce the response of the rat SCG to muscarine although not significantly may be an example of the inhibition of muscarinic binding or an interaction with Ca^{2+} currents of sympathetic ganglia (Tokimasa & Akasu, 1990b). Even though the response to muscarine was reduced in the presence of (+) PN200 110 the inability of (+) PN200 110 to alter the depression of the response to muscarine by adenosine suggests the depression of muscarine is independent of DHP sensitive Ca^{2+} channel activity.

It is reported that some DHPs have both agonist and antagonist activity within the same molecule e.g. nitrendipine on the release of catecholamines from adrenomedullary cells (Castillo, Fonteriz, López, Rosenhelh & Garcia, 1989). The potentiation of the response to adenosine by (+) PN200 110 compared to nitrendipine (Table 4.6) may be due to a mixed

agonist/antagonist action of nitrendipine (Hess, Lansman & Tsien, 1984) compared to the antagonist activity of (+) PN200 110 (Hof, Scholtysik, Loutzenhiser, Vuorela & Neumann, 1984).

Alternatively, it has been reported that some DHPs inhibit the uptake of ^{3}H-adenosine in to rat brain synaptosomes (Phillis, Swanson & Barraco, 1984; Morgan et al., 1987) and inhibited the binding of NBI to dog heart and brain membranes (Marangos, Finkel, Verma, Maturi, Patel & Patterson, 1984). Functional studies have revealed that the ability of nifedipine to enhance the inhibitory action of adenosine on ACh excited cortical neurones (Phillis et al., 1984) and the inhibitory action of adenosine on spontaenous and evoked field potentials of hippocampi in vitro (Bartrup & Stone, 1990) may arise due to the inhibition of the nucleoside transport system. Bartrup & Stone (1990) found the inhibitory effect of adenosine in the presence of dipyridamole was inhibited by nifedipine. The possibility that (+) PN200 110 inhibited the uptake of adenosine and potentiated the hyperpolarisation of the rat SCG cannot be excluded.

The effects of the DHPs reported by Bartrup & Stone (1990) suggested the DHP used were weak or inactive at 1uM, in contrast to the potentiation of the hyperpolarisation to adenosine seen in the presence of 1uM (+) PN200 110 (Table 4.6). As the concentrations of DHP used by both Bartrup & Stone (1990) and here are several orders of magnitude greater than that required to saturate the high affinity DHP binding sites (Belleman, Schade & Towart, 1983) their effects are unlikely to be directly on the DHP binding site. The greater potency of (+) PN200 110 may reflect a greater affinity of (+) PN200 110 for neuronal tissue (Weiland & Oswald, 1985), or that (+) PN200 110 is more

potent than nifedipine on the inhibition of adenosine uptake.

Irrespective of the mode of action of the DHPs the inability of either compound to reduce the adenosine-induced hyperpolarisation of the rat SCG and the similar depression of the response to muscarine in the presence of (+) PN200 110 suggests that the actions of adenosine are not mediated by a change in calcium influx through voltage sensitive L-type channels. In addition it is reported that DHPs antagonise CaM mediated events such as the activation of CaM dependent cAMP PDE (Minocherhomjee & Roufogalis, 1984, cited by Phillis et al., 1984) and the inability of (+) PN200 110 to antagonise the adenosine mediated hyperpolarisation of the rat SCG supports the idea that this response is independent of CaM (see Section 4.4).

4.3.3 Effects of nickel

Nickel ions block T-type Ca^{2+} currents approximately ten times more potently than other Ca^{2+} currents and even in the presence of high $[Ca^{2+}]e$, 10 to 100uM Ni^{2+} selectively antagonises low threshold T-type Ca channels and at higher concentrations Ni^{2+} antagonises most Ca^{2+} currents, e.g. the Ca^{2+} currents of the dorsal root ganglion (DRG) were abolished by 5mM Ni^{2+} (Carbone, Swandulla & Lux, 1988). The small reduction of the hyperpolarisation to adenosine of the rat SCG in PSS containing Ni^{2+}(Table 4.6) and the lack of any evidence for the presence to T-type channels in this ganglion (Wanke et al., 1987; Hirining et al., 1988) is consistent with the suggestion that T-type Ca^{2+} currents are not involved in the response of the rat SCG to adenosine.

4.3.4 Effects of cadmium

As well as inhibition of Ca^{2+} currents Cd^{2+} has also been found to inhibit the binding of [^{3}H]CHA to A1 receptors in the rat brain membranes with an IC_{50} of 70uM (Marangos et al., 1983, 1987). The inability of Cd^{2+} to alter the response to adenosine suggests Cd^{2+} does not alter the affinity of adenosine for the adenosine receptor of the rat SCG. In addition these results confirm the response to adenosine was independent of an endogenous "ACh tone" as the antagonism of presynaptic N-type Ca^{2+} channels by micromolar concentrations of Cd^{2+} would be expected to inhibit the release of ACh and reduce the response to adenosine.

Cadmium is a potent antagonist of both L- and N-type Ca^{2+} chanels with an IC_{50} of about 10uM on DRG neurones and is about ten fold weaker on T-type channels (Miller, 1987). At higher concentrations Cd^{2+} is not that selective on the rat SCG and at 200uM antagonises both the I_{ahp} and also a Cl^- current (Selyanko, 1984) and at 500uM Cd^{2+} abolished an inward calcium current (Galvan & Adams, 1982). The antagonism of an inward Cl^- current would be predicted to decrease the RMP and enhance the response to adenosine, as it would start from a greater RMP. If the hyperpolarisation was due to the activation of the inward chloride current, it should have been reduced by 500uM Cd^{2+} instead the response was larger (Table 4.6) suggesting the hyperpolarisation of the rat SCG is independent of an increase in gCl^- or decrease in gCa^{2+}.

4.4 The effect of Ca^{2+} channel antagonists on the response to muscarine

The ability of muscarine to depolarise and adenosine to depress the response to muscarine in Ca^{2+} free PSS is consistent with both responses occurring via a Ca^{2+} independent mechanism. The ability of Co^{2+}, La^{3+}, Ni^{2+} and Ba^{2+} to reduce the response to muscarine suggests a part of the response to muscarine could be Ca^{2+} dependent. Even so the inability of these calcium channel antagonists to alter the depression of muscarinic responses by adenosine suggests this effect of adenosine is independent of Ca^{2+} influx. As well as inhibition of Ca^{2+} channels divalent cations can cause a depolarisation shift in the steady state activation curve for Im (Mayer & Sugiyama, 1988). A reduction in the amplitude of Im by these metal ions would be expected to reduce the depolarisation to muscarine and is consistent with the reductions reported in Table 4.8. Adams, Brown & Constanti (1982a) reported that not only Ic and Ik but also Im of the rat SCG was inhibited by divalent cations with the following potency:

$$Ba^{2+} >= Ni^{2+} > Co^{2+} > Mn^{2+} > Cd^{2+}$$

It is interesting that Ba^{2+} caused a potentiation of the depression by adenosine of muscarinic responses. This may have occurred due to one or other of the many actions of Ba^{2+} including passing readily through Ca^{2+} channels into neurones (Hagiwara & Byerly, 1981) or the antagonism of various outward K^{+} currents including Ca^{2+} dependent K^{+} currents (Connor, 1979; Segal, 1982) and Im (Constanti et al., 1981b). The results of other experiments reported here suggest many of these actions of Ba^{2+} on the hyperpolarisation to adenosine can be disregarded, and the slight increase in the depression of the muscarinic response by adenosine in the presence of some metal ions

reported here may be a consequence of the inhibition of Im. The depolarisation of the ganglion by Ba^{2+} was probably due to the inhibition of Im (see Constanti et al., 1981b) and the ability of Ba^{2+} to enhance the response to adenosine suggests adenosine may activate closed M channels. This hypothesis was tested and is described in section 5.3.

4.4.1 Relative potency of Ca^{2+} channel antagonists on the response of the rat SCG to adenosine

An extensive study of the blocking effect of a number of polyvalent cations was performed on the Ca^{2+} component of the action potential of the barnacle muscle by Hagiwara & Takahashi (1967) and similar orders of potency have been confirmed for other tissues (Blaustein & Goldman, 1968; Hagiwara & Byerly, 1981; Nachshen, 1984; Lansman et al., 1986). The order of Ca^{2+} channel antagonism was:

$$La^{3+} > Co^{2+} > Mn^{2+} > Ca^{2+} > Mg^{2+}$$

Although Cd^{2+} was not tested on barnacle muscle it has been found to be a potent Ca^{2+} channel antagonist on neurones, particularly on presynaptic Ca^{2+} channels of the rat SCG (Hirning et al., 1988; IC_{50} for N and L-type channels of about 2uM) where it was the most potent compound to inhibit Ca^{2+}-spikes of the rat SCG (Elliott et al., 1989), the order of the potency being:

Cation	Cd^{2+}	> La^{3+}	> Ni^{2+}	> Co^{2+}	> Mn^{2+}	> Mg^{2+}
IC_{50} uM	3	10	55	510	1070	15000

The observed reduction in the response to adenosine by divalent cations on the rat SCG, was somewhat different to that found for the blockade of the Ca^{2+} currents described

above. Greater concentrations of divalent cations were required to produce antagonism of the hyperpolarisation to adenosine than used to block the Ca^{2+} spikes of the rat SCG (Elliott et al., 1989) or other tissues. For example, 500uM Cd^{2+} virtually eliminates Ca^{2+} current in nodose ganglia (Ikeda, Schofield & Weight, 1986), whereas 500uM Cd^{2+} did not significantly alter the hyperpolarisation of the rat SCG to adenosine. La^{3+} is thought to block all Ca^{2+} movements and the inability of La^{3+} and also Cd^{2+} and Ni^{2+} to alter the hyperpolarisation of the rat SCG to adenosine or of La^{3+} to reduce the effectiveness of adenosine in depressing the response to muscarine is inconsistent with the effects of adenosine being mediated by a reduction in Ca^{2+} influx.

At the concentrations used to antagonise Ca^{2+} channels the inorganic cations used in this study are believed to have a number of other effects on the membrane including changing surface charge potential, inactivating a range of ionic conductances (Galvan & Sedlmeir, 1983), and altering the binding of specific ligands to adenosine (Marangos et al., 1987) and muscarinic receptors (Birdsall, Burgen, Hulme & Wells, 1979). Although changes in the RMP of the ganglion could bias the conclusions obtained from the experiments reported here, overall these results with different metal cations indicate both the hyperpolarisation and the depression of muscarine responses by adenosine remained unaltered by Ca^{2+} channel antagonists, with the exception of Co^{2+}, which produced non-specific reductions in the sensitivity of the rat SCG.

Marangos & colleagues (1983) have reported that the binding of adenosine to A1-receptors is reduced by some Ca^{2+} channel antagonists whereas other researchers have reported the binding of ligands to adenosine receptors including cyclohexyladenosine (CHA) to cerebral membranes

is enhanced by magnesium and calcium ions (Goodman, Cooper, Gavish & Snyder, 1981; Yeung & Green, 1983; Yeung, Fossom, Gill & Cooper, 1985). Likewise Co^{2+}, Ni^{2+} and La^{3+} are reported to enhance binding. However in the present studies the functional response to adenosine was decreased in high Ca^{2+} PSS (Table 4.7), unaltered by the absence of Mg^{2+} (Table 4.1) and not significantly altered by many Ca^{2+} channel antagonists (Table 4.6), suggesting the results of these binding studies may have little functional significance.

4.5 Effect of A23187 and trifluoperazine on the response to adenosine

The studies in the previous section have shown that the effect of adenosine on the rat SCG are independent of $[Ca^{2+}]e$ and to complement these studies some experiments were done to examine the possibility that adenosine produces its effects via an action on $[Ca^{2+}]i$.

The ionophore A23187 has been shown to raise the $[Ca^{2+}]i$ by causing a large uptake of extracellular calcium (Campbell, 1987) and would be expected to antagonise the effects of adenosine on the rat SCG if they are mediated through a decrease in Ca^{2+} entry. The results of Dowdle & Maske (1980) support such a hypothesis, as they found that A23187 at 0.1uM antagonised the inhibitory effect of adenosine on the response of the guinea-pig ileum. The fact that A23187 did not significantly alter the basal d.c. potential (Table 4.3) is consistent with the idea that A23187 is a neutral charge carrier without a direct effect on membrane potential (Campbell, 1987). It has been reported that as well as facilitating Ca^{2+} transport, A23187 may also stimulate the release of $[Ca^{2+}]i$ and/or alter intracellular pH (Campbell, 1987). It is unknown if

these effects occurred in the experiments described here but with these caveats the lack of effect of A23187 (Table 4.3) suggests adenosine does not hyperpolarise the rat SCG by inhibiting calcium entry and the presumed increase in $[Ca^{2+}]i$ by A23187 did not alter the hyperpolarisation to adenosine.

The maintenance of $[Ca^{2+}]i$ is controlled by a number of proteins including CaM and there is considerable evidence that CaM mediates many Ca^{2+} dependent processes and can regulate both pre- and postsynaptic events (Roufogalis, 1980). As CaM can regulate the metabolism of cAMP and is itself regulated by cAMP an increase in $[Ca^{2+}]i$ due to influx or intracellular release of Ca^{2+} could initiate the activation of CaM dependent PDE's to decrease cAMP and act as the transduction signal for the actions of adenosine. The inability of TFP to alter the response to adenosine (Table 4.3) suggests that CaM may not be involved in the hyperpolarisation of the rat SCG to adenosine and is consistent with the lack of effect of A23187 on the response to adenosine.

The evidence for the mechanism of action of adenosine occurring via an interaction with Ca^{2+} in some tissues, e.g. atrial and blood vessel muscle is not conclusive. For example it is known that adenosine inhibits both Ca^{2+} uptake and the Ca^{2+} mediated action potential plateau in atrial muscle (Schrader, Rubio & Berne, 1975) but effects on other conductances including sodium and potassium have not been conclusively excluded. More recently, Cerbal, Klöckner & Isenberg (1988) reported that adenosine and ACh blocked about one third of the L-channels and not T-channels of patch clamped myocytes recorded with blocked K^+ channels and suggested adenosine and ACh may reduce the number of functional L-type Ca^{2+} channels. However, more important was the observations made using myocytes with

unblocked K^+ channels, where physiological concentrations of adenosine reduced the calcium current (I_{ca}) by 30% but enhanced the potassium current (I_K) by 300% suggesting the "K-agonistic" properties of adenosine are responsible for the hyperpolarisation, shortening of the action potential and negative inotropy reported.

Preceding evidence is strongly against the actions of adenosine on the rat SCG arising from an interaction with Ca^{2+} currents and an examination of figure 4.1 suggests that the ionic mechanism responsible for the actions of adenosine is most likely generated a change in membrane permeability to either Na^+, K^+ and/or Cl^- ions. Each of these possibilities was examined.

4.6 Effects of low $[Cl^-]e$ and furosemide on the response to adenosine

Chloride is the most abundant anion in PSS and another way adenosine may hyperpolarise the ganglion would be to increase the permeability of ganglionic neurones to Cl^- ions (Fig. 4.1). Adenosine has been reported to enhance labelled Cl^-efflux of many tissues including Aplysia neurones (Chenoy-Marchais, 1982), rabbit colon (Gras & Turheim, 1984) and canine tracheal epithelium (Pratt, Clancy & Welsh, 1986). A similar Cl^- current to that described by Chenoy-Marchais (1982) has been found in the rat SCG and was inhibited by 200uM Cd^{2+} (Selyanko, 1984). The results presented in table 4.6 show that Cd^{2+} at 500uM did not significantly alter the response to adenosine suggesting adenosine does not enhance Cl^- efflux.

Another aspect of the role of Cl^- in the response to adenosine is the ability of muscarine to inhibit not only the outward K^+ current (Im), but also the reduction of a

steady state voltage-independent inward current, termed Ix (Brown & Selyanko, 1985a). Ix is believed to be carried by Cl^- ions and the depression of muscarine by adenosine could occur due to activation of Ix.

Depolarisation of the rat SCG by GABA increases Cl^- conductance (Adams & Brown, 1975) and K^+ efflux (Ballanyi & Grafe, 1985) and upon repolarisation produces an AHP due to redistribution of Cl^- by the co-transport of Cl^- into the neurones in exchange for Na^+ or K^+ i.e. the $Na^+/K^+/Cl^-$ co-transporter (Ballanyi & Grafe, 1985). Furosemide a Na^+/Cl^- or K^+/Cl^- co-transport inhibitor is known to antagonise the reuptake of Cl^- and K^+ in a variety of tissues including the rat SCG (Ballanyi, Grafe, Reddy & ten Bruggencate, 1984; Ballanyi & Grafe, 1985).

If a change in gCl is responsible for the production of the hyperpolarisation of the SCG to adenosine, the amplitude of this hyperpolarisation would be affected by replacing the $[Cl^-]e$ with other anions, which would not be permeable through the neuronal membrane, such as isethionate ions. Low $[Cl^-]e$ will reverse the Cl^- gradient so that there will be an efflux or considerable reduction in the influx of Cl^- and should decrease a putative muscarinic gCl, i.e. Ix. The ability of low $[Cl^-]e$ to abolish the AHP to GABA (Table 4.2) is indicative that the $Na^+/K^+/Cl^-$ co-transport mechanism was antagonised and the inability of low $[Cl^-]e$ to alter the response to adenosine (Table 4.2) suggests adenosine is unlikely to alter Ix or the $Na^+/K^+/Cl^-$ co-transporter.

Likewise the inability of low $[Cl^-]e$ to enhance the response to muscarine suggests that Cl^- efflux contributes very little to the response to muscarine at the concentration used and most of the response to muscarine probably occurred via some other mechanisms: an inhibition

of Im. Therefore it is unlikely that the interaction of adenosine and muscarine occurs at the level of Ix.

4.6.1 Effect of furosemide on the hyperpolarisation to adenosine

In 10uM furosemide the AHP to GABA (10uM) was abolished whereas a comparable hyperpolarisation to adenosine was reduced to a lesser extent (Table 4.14). Increasing the concentration of furosemide to 100uM abolished the AHP produced by a ten fold higher concentration of GABA (100uM) but did not produce a significantly greater reduction in the response to adenosine. The ability of furosemide to partially inhibit the hyperpolarisation to adenosine may be indicative of an interaction of adenosine with the $Na^+/K^+/Cl^-$ co-transporter or alternatively a requirement of the active transport of Cl^- in to neurones. Further evidence against adenosine altering the $Na^+/K^+/Cl^-$ co-transport is provided by the fact that the response to adenosine was unaltered during the activation of the $Na^+/K^+/Cl^-$ co-transporter by GABA (Fig. 4.12b). However, the lack of a significant effect of low $[Cl^-]e$ on the adenosine hyperpolarisation or the activation of the $Na^+/K^+/Cl^-$ co-transporter by GABA on the response to adenosine suggests the hyperpolarising effect of adenosine is not coupled to Cl^- currents.

The Ki reported for the $Na^+/K^+/Cl^-$ co-transport for furosemide is probably around 5uM (Aronson, 1984) and at concentrations above 100uM furosemide has been reported to affect the Na^+ pump and other exchange processes. As the specificity of furosemide on the response to adenosine was not assessed in this study and this possibility cannot be ruled out. A 10 to 20 minute application of furosemide at 500uM caused a longlasting depletion of Cl^- and complete

blockade of K^+ and Cl^- uptake in the rat SCG (Ballanyi et al., 1984). As a reduction of $[Cl^-]i$ may inhibit regulatory G-protein activity (Higashijima, Ferguson & Sternweis, 1987) and the hyperpolarisation of the rat SCG may be linked to G-proteins (Newberry & Gilbert, 1989b) this may be the cause of the inhibition of the response to adenosine. Alternatively as furosemide strongly delays or completely inhibits the reuptake of K^+, the build up of $[K^+]e$ due to inhibition of the $Na^+/K^+/Cl^-$ co-transporter would be expected to decrease the response to adenosine if it is produced by an increase in gK.

4.7 Effect of inactivation of the electrogenic Na^+ pump on the response to adenosine

There are certain similarities in the actions of catecholamines and purines on the rat SCG as both agents inhibited Ca^{2+} currents (McAfee & Yarowsky, 1979; Henon & McAfee, 1983a) and hyperpolarise postganglionic SCG neurones (Brown et al., 1979). Catecholamines and purines may promote depressant effects on neuronal activity and catecholamines can enhance the rate of ATP hydrolysis by activating Na^+/K^+ ATPases (Phillis & Wu, 1981). Thus the activation of the sodium/potassium ATPase transporter (here after referred to as the electrogenic Na^+ pump) would lead to the exchange of $[Na^+]i$ for $[K^+]e$ and a hyperpolarisation of the SCG.

The ability of adenosine to activate the electrogenic Na^+ pump may account for the potentiating effects of adenosine in Ba^{2+}-PSS, as Ba^{2+} antagonises many K^+ currents but does not antagonise the electrogenic Na^+ pump (Padjen & Smith, 1983). In fact Ba^{2+} enhanced the potential changes resulting from electrogenic Na^+ pumping by increasing membrane resistance (Rm), and potentiated the AHP produced

by the application of ACh to bullfrog ganglia (Smith & Dombro, 1985).

The possibility that adenosine hyperpolarises the rat SCG by activating the electrogenic Na^+ pump was investigated in a series of experiments which in addition provided information about other ionic mechanisms that may be involved in the response of the ganglion to adenosine.

The role of electrogenic Na^+ pumping in sympathetic ganglia can be studied by examining the AHP which follows the application of high concentrations of ACh (Brown et al., 1972; Smith, 1984). Na^+ enters during nicotinic depolarisation and the accumulated Na^+ load is extruded electrogenically during the AHP. The stimulation of Na^+ extrusion by K^+ results from the activation of Na^+/K^+ ATPase, and this activation is inhibited by cardiac glycosides e.g., ouabain. The depressant effects of adenosine on the cerebral cortex (Sastry & Phillis, 1977) and amphibian ganglia (Koketsu & Nakamura, 1976) were selectively depressed by the cardiac glycoside, ouabain. Thus the ability of the rat SCG to hyperpolarise in oubain was assessed.

In other experiments both the ability of adenosine to activate the electrogenic Na^+ pump and/or increase a gK was assessed by determining the effect of K^+ free PSS ($0K^+$ PSS) on the response to adenosine, a condition where the electrogenic Na^+ pump is believed to be inactive. Another method used to inactivate the electrogenic Na^+ pump was to replace extracellular Na^+ by Li^+. These experiments also provided information about the effect of Na^+ free PSS on the response to adenosine. The substitution of $[Na^+]e$ by Li^+ in many tissues including bullfrog ganglia (over short incubation periods, Koketsu & Yamamoto, 1974) allows the maintenance of membrane excitation, but in mammalian

ganglia Li^+ does not substitute for Na^+ at the internal site of the sodium pump and thus inhbits the electrogenic Na^+ pump (Pappano & Volle, 1966a, 1967).

4.7.1 Effect of ouabain on the response to adenosine

The hyperpolarisation to adenosine persisted in the presence of 1 and 10uM ouabain, and was enhanced in 10uM oubain (Table 4.10), suggesting adenosine does not activate electrogenic Na^+ pumping. Both the depolarisation and the AHP response to DMPP were unaltered in PSS containing 10uM oubain (Table 4.13), although inhibition of the electrogenic Na^+ pump would have been expected to potentiate depolarisation and attenuate the AHP to DMPP.

The ability of ouabain to produce a slowly developing depolarisation of the ganglion suggests ouabain altered the RMP, and the low concentration of DMPP employed may have produced such a small change in $[Na^{2+}]i$ that it was restored by a partially inactivated Na^+/K^+ ATPase pump. It was reported by Brown et al. (1972) that the EC_{50} for the inhibition of the AHP to DMPP by ouabain was about 50uM. It would be predicted that K^+ free media would inactivate the electrogenic Na^+ pump, however Smith (1984) reported that a brief AHP to ACh remained in K^+ free Ringer's and suggested this was due to the efflux of $[K^+]i$. Therefore in PSS and ouabain or K^+ free PSS an accumulation of $[K^+]e$ during the experiment (Galvan, ten Bruggencate & Senekowitsch, 1979) could serve to maintain Na^+ pumping. Jones (1989) reported that the electrogenic Na^+ pump may be responsible for maintaining the ionic gradients to Na^+ and K^+ and the RMP of sympathetic neurones (Jones, 1989). Ouabain increases the resting efflux of K^+ (Galvan et al., 1979) and the potentiation of

the hyperpolarisation to adenosine in ouabain is consistent with an increase in gK.

4.7.2 Effect of Rubidium (Rb^+) on the response to adenosine

The substitution of Rb^+ for K^+ in PSS enhanced the hyperpolarisation to adenosine. Since Rb^+ moves into cells almost as quickly as K^+ and the permeability of nerve membranes to Rb^+ is much less than its permeability to K^+ the enhanced response to adenosine in Rb^+ PSS is consistent with greater Rm in Rb^+.

4.7.3 Effect of lithium on the response to adenosine

The response to adenosine in PSS containing 10mM Li^+ was unaltered after incubation for almost 2 hours (Table 4.10). The difference between the response in Li-HEPES and 10mM Li PSS may have arisen due to the low permeability of mammalian SCG to Li^+ (Papanno & Volle, 1966a,b; 1967).

Normally Na^+ ions carry the inward current to depolarise ganglion cells, but when Na^+ ions are not available for this purpose other ions including Ca^{2+} make a major contribution as carriers of inward current. Calcium ions are only capable of supporing the depolarisation to ACh for a short time in medium completely lacking Na^+ ions (Pappano & Volle, 1966b). The inhibition of the AHP to DMPP (Table 4.13) is consistent with Li-HEPES inhibiting the Na^+ pump as reported by Brown et al. (1972). The ability of adenosine to hyperpolarise in Li-HEPES suggests adenosine does not hyperpolarise the rat SCG by activating the Na^+ pump. Indirect evidence to support this idea comes from

the reduction or abolition of the AHP to carbachol in Ca^{2+} free PSS (Brown et al., 1972), and the ability of adenosine to hyperpolarise in K^+ free PSS, a condition where the activity of the electrogenic Na^+ pump would be inhibited.

Lithium has been found to discriminate between muscarinic receptors and abolished the response produced by stimulation of M1 receptors in the rat hippocampus (Müller, Brunner & Misgeld, 1989). Thus the abolition of the response to muscarine in Li-HEPES (Figs. 4.13 & 4.14) may be due to the blockade of phosphoinositol turnover (PIT), which is coupled to M1 receptors (Fisher, 1986; Batty & Nahorski, 1987) and/or Li^+ may inhibit the coupling of muscarinic receptors to G-proteins (Avissar, Schreiber, Danon & Belmaker, 1988).

In contrast the response to adenosine in Li-HEPES was initially enhanced during a two hour incubation in Li-HEPES returning to the pre Li treatment level (Fig. 4.13b,c & Fig. 4.14). Short term treatment of tissues with Li^+ enhances responses linked to inositol breakdown (Drummond & Raeburn, 1984) possibly by antagonising the metabolic cascade of inositol phosphates (IP) after the activation of protein kinase C (PKC) at the monophosphatase level (Allison & Blisner, 1976). Although Li-HEPES did not completely inhibit the response to adenosine, the response after more than two hours incubation was shorter than the previous control responses suggesting Li^+ inhibits part of the response to adenosine. The effects of Li^+ may be due to an effect on PIT or on the coupling of adenosine receptors to PTX sensitive G-proteins (Avissar et al., 1988).

4.8 The role of potassium channels in the response to adenosine

There is good evidence that adenosine enhances a variety of potassium currents in various cell types including Xenopus oocytes (Lotan, Dascal, Cohen & Lass, 1982), heart muscle (Hartzell, 1979; Belardinelli & Isenberg, 1986; Kurachi, Nakajima & Sugimoto, 1986), parasympathetic ganglia (Akasu et al., 1984), hippocampal pyramidal neurones (Segal, 1982; Haas & Greene, 1984; Greene & Haas, 1985; Schubert & Lee, 1986; Trussell & Jackson, 1987, 1989) and striatal and cortical neurones (Trussell & Jackson, 1987, 1989).

Diversity of potassium channels in neuronal tissues

Many agents that hyperpolarise neurones have been found to increase gK (North, 1989) through a great diversity of potassium channels. At least eleven different types of K^+ channel have been described, based on their voltage gating and sensitivity to calcium (see: Cook, 1990; Castle et al., 1989). Many types of K^+ channel have been found to exist within the same cell and up to six different K^+ currents have been identified in the rat SCG (Freschi, 1983; Galvan & Sedlmeir, 1983) including a delayed outward rectifier (I_K); a transient outward current (I_A); three Ca activated currents (I_C, I_{ahp}) and the M-current (Im). The presence of these K^+ channels in SCG neurones allows the maintenance of the RMP and the termination of excitation by repolarisation or hyperpolarisation of the neuronal membrane (Belluzzi, Sacchi & Wanke, 1985a,b).

To discover if adenosine hyperpolarised the rat SCG by increasing gK experiments were done using drugs known to alter potassium channel activity.

4.8.1 Effects of altered $[K^+]e$ on the response to adenosine

The response to adenosine in 0mM Ca^{2+}/10mM Mg2+ PSS was comparable to that in normal PSS, and the reduction of $[K^+]e$ from 6mM to 2mM in 0mM Ca^{2+}/10mM Mg2+ PSS significantly enhanced the response to 100uM adenosine (Table 4.11).

The potentiation of the response to adenosine in 2mM K^+ PSS in a solution that has been shown by others to block synaptic transmission (0mM Ca^{2+}/10mM Mg2+ PSS) (Table 4.11) is consistent with adenosine increasing gK via a direct postganglionic response. The leftward shift in the concentration response curve to adenosine in $0K^+$ PSS and antagonism of the response to adenosine in 12mM K^+ PSS (Fig. 4.15) is consistent with adenosine increasing gK as reduction of $[K^+]e$ would inhibit the Na^+ pump and increase the concentration gradient for K^+ efflux. If the adenosine induced hyperpolarisation is generated by an increase in gK, the driving force for the outward movement of K^+ will depend on the difference between the RMP (Em) and the potassium equilibrium potential (Ek) i.e. Em-Ek. Intracellular microelectrode recordings suggest Em varies from about -40 to -60mV (McAfee & Yarowsky, 1979; Galvan & Sedlmeir, 1983) and Ek is about -80 mV (Constanti & Brown, 1981) (the low value of Em could be explained by a high Na^+ permeability) and the antagonism of electrogenic Na^+ pumping would tend to move Ek and ENa towards zero mV. The absence of $[K^+]e$ will augment $[Na^+]i$ due to the inhibition of the electrogenic Na^+ pump, and this will stimulate Na^+/Ca^{2+} exchange which may elevate $[Ca^{2+}]i$. Thus if the effects of low K^+ or ouabain arise from an increase in $[Ca^{2+}]i$ this would be more indirect evidence that an increase in $[Ca^{2+}]i$ is not inhibitory for the response to adenosine. It is unlikely that reduction in

the response to adenosine in 12mM K^+ PSS is due to an increase in $[Ca^{2+}]e$ as Galvan and co-workers (1979), using ion sensitive electrodes, found the extracellular calcium activity was unaltered by doubling the $[K^+]e$ to 12mM.

Although an increased response to adenosine in the presence of low or K^+ free PSS indicates adenosine increases gK^+ these results may also indicate a decrease in gNa may contribute to the effects of adenosine. If adenosine reduced Na^+ entry this may also cause a reduction in passive outward Cl^- movement, and both effects would tend to hyperpolarise ganglion cells. These possibilities can be best studied using conventional microelectrode techniques or ion sensitive electrodes. However, a change in gNa^+ is unlikely as Henon & McAfee (1983a) reported adenosine does not inhibit the rate of rise or amplitude of the Na^+ AP spike and TTX did not antagonise the response to adenosine (Henon & McAfee, 1983a) (Table 4.9) and Na^+ free PSS i.e. Li-HEPES did not alter the peak response to adenosine.

4.8.2 Effect of K^+ channel activators cromakalim (BRL 34915) and diazoxide on the d.c. potential

Several compounds have been identified that increase the permeability of smooth muscle cells to K^+, to hyperpolarise them. Two of these compounds, cromakalim and diazoxide are thought to owe their effects to activation of ATP-sensitive K^+ channels ($I_{K(ATP)}$) and are potent vasodilators but more recently cromakalim has been reported to have anticonvulsant activity (Alzheimer & ten Bruggencate, 1988), to enhance a voltage dependent K^+ current in the rat hippocampus (Politi, Suzuki & Rogawaski, 1989) and antagonised a behavioural response to pilocarpine (Tricklebank, Flockhart & Freedman, 1988).

$K_{(ATP)}$ channels have also been detected in neonatal cerebral cortex cultures (Ashford, Struges, Trout, Gardner & Hales, 1988). The presence of $K_{(ATP)}$ channels in neuronal tissues may not be restricted to the CNS and could mediate the response of adenosine on the rat SCG.

The ability of cromakalim and diazoxide to alter the d.c. potential and the response of the rat SCG to muscarine and pilocarpine was investigated. As the biological activity of cromakalim resides primarily in the (-) enantiomer i.e. lemakalim (BRL 38227) (Buckingham, Clapham, Coldwell, Hamilton & Howlett, 1986; Hof, Ouast, Cook & Blarer, 1988) the effect of cromakalim and lemakalim were studied in some experiments.

The inability of diazoxide and lemakalim to alter the d.c. level of the ganglion (Tables 4.15 and 4.16) suggests $K_{(ATP)}$ channels are absent or inactive on the rat SCG. This conclusion was further strengthened by the observation that both lemakalim and diazoxide did not significantly alter the depolarisation to muscarine (Tables 4.15 and 4.16) suggesting $K_{(ATP)}$ channels are not involved in the response to muscarine or its interaction with adenosine.

Cromakalim has been reported to antagonise the induction of purposeless mouth movements of mice treated with pilocarpine with no effect on muscarinic binding or phospholipid hydrolysis (Tricklebank et al., 1988). The inability of lemakalim to alter the response to both muscarine and pilocarpine suggests the behavioural interactions of cromakalim and pilocarpine are indirect.

It is possible that the SCG has few ATP-sensitive K^+ channels as reported for cultured neuronal cells (Ashford et al., 1988) and/or neuronal ATP-sensitive K^+ channels

are located presynaptically (Hall & MaClagan, 1988; for discussion see Ben-Ari, 1990). The inactivity of ATP sensitive K^+ channel activators on the SCG makes it unlikely that the actions of adenosine or muscarine are mediated via these channels.

4.8.3 Effect of calcium activated potassium channel antagonists on the response to adenosine

Under physiological conditions the electrochemical gradient for passive diffusion of K^+ is outward (see Fig. 4.1), and it is predicted that the opening of calcium activated K^+ channels ($K_{(Ca)}$)by adenosine would allow the efflux of K^+ to hyperpolarise the cell. Thus one mechanism of action of adenosine could be to potentiate a Ca^{2+} activated K^+ current ($I_{K(Ca)}$) as reported for the effect of adenosine on the rat hippocampus by Haas & Greene (1984) and on the myenteric neurones of the guinea-pig by Palmer et al. (1987).

$K_{(Ca)}$ channels have been identified in a number of neurones: including guinea-pig enteric plexus neurones (North & Tokimasa, 1987; Akasu & Tokimasa, 1989), rabbit parasympathetic neurones (Nishimura, Tokimasa & Akasu, 1988), bullfrog sympathetic neurones (Constanti, Adams & Brown, 1981a; Adams, Constanti, Brown & Clark, 1982b; Tokimasa, 1984) and rat SCG in culture (Smart, 1987; Gurney, Tsien & Lester, 1987) and may contribute to the RMP. In sympathetic neurones an increase in $K_{(Ca)}$ channel conductance can prolong the spike AHP (McAfee & Yarowsky, 1979; Brown et al., 1982; Belluzzi & Sacchi, 1989) to regulate repetitive firing (Kuba, Morita & Nohmi, 1983) or accelerate spike repolarisation (Brown, Constanti & Adams, 1983; Belluzzi & Sacchi, 1989) allowing the neurone to

pass high frequency discharge during muscarinic excitation.

Before discussing the $K_{(Ca)}$ channels described for the rat SCG it is useful to summarise the properties of $K_{(Ca)}$ channels. $K_{(Ca)}$ channels are selective for K^+ and are activated by micromolar concentrations of $[Ca^{2+}]i$ and classified on the differences in conductance and sensitivity to calcium and pharmacological antagonists (for reviews see Blatz & Magleby, 1987; Castle et al., 1989; Latorre, Oberhauser, Labarca & Alvarez, 1989). There are three main classes namely: big (BK), intermediate (IK) and small (SK) conductance channels (Castle et al., 1989) (Table 4.2). SK channel currents (I_{ahp}) are selectively antagonised by apamin, an octadecapeptide found in bee venom (reviewed by Baidan & Zholos, 1989), whereas BK channel currents are antagonised by tetraethylammonium (TEA) (Cook & Quast, 1990; Castle et al., 1989).

Apamin was reported as ineffective in bullfrog sympathetic ganglion cells by Brown et al (1983), but further study by Pennefather, Lancaster, Adams & Nicoll (1985) showed there were two distinct components of AHP, of which the slow component was apamin sensitive. A slow $K_{(Ca)}$ current similar to that described in bullfrog ganglia has been found in adult rat SCG dissociated in culture (Marrion, Smart, Marsh & Brown, 1989) and Kawai & Wantanabe (1986) reported 50nM apamin significantly reduced a slow AHP of the rat SCG (I_{ahp}). Apamin was highly selective for SK and after 20 minutes incubation the IC_{50} of apamin for the inhibition of synaptically activated AHP was 15nM (Kawai & Wantanabe, 1986). The $K_{(Ca)}$ channels that are insensitive to apamin are antagonised by TEA (Smart, 1987; Marrion et al., 1989). Ic current of the rat SCG is activated by both a rise in $[Ca^{2+}]i$ and depolarisation and is

insensitive to muscarine (10uM), apamin (100-300nM) and 4-aminopyridine (4AP, at 1-3mM) (Smart, 1987). In contrast I_{ahp} is antagonised by muscarine or substance P in frog (Tokimasa, 1984; Pennefather et al., 1985; Akasu & Tokimasa, 1989) and rat ganglia (Brown, 1988).

The AHP of the rat SCG consists of two components, a primary voltage dependent gK which contributes substantially to the peak amplitude of the AHP and a Ca^{2+} dependent gK ($gK_{(Ca)}$) (McAfee & Yarowsky, 1979). The magnitude of the AHP is proportional to the amount of Ca^{2+} entry during the spike (McAfee & Yarowsky, 1979) and was antagonised in the presence of adenosine (Henon & McAfee, 1983a) suggesting adenosine decreases, not increases an $I_{K(Ca)}$. The AHP identified by Henon & McAfee (1983a) was characterised as being both TTX and TEA (up to 10mM) resistant but sensitive to blockade by Co2+ and manganese (Mn2+) (McAfee & Yarowsky, 1979) suggesting it may be due to activation of SK channels. Thus it was of interest to examine the actions of adenosine on the rat SCG when either or both Iahp and Ic are inhibited to see if antagonism of these channels altered the adenosine-induced hyperpolarisation of the ganglion. In some experiments the response to adenosine in apamin PSS was measured in the presence of bovine serum albumin (BSA) to prevent the peptide sticking to the glassware. However BSA in PSS significantly reduced the response to adenosine (Table 2.2), perhaps due to some adenosine binding to BSA, and control responses to adenosine were measured in the presence of BSA and compared to apamin and BSA, PSS. Irrespective of the presence or absence of BSA, the inability of apamin to alter the response to adenosine at up to five times the concentration reported to block Ic of the rat SCG (Kawai & Wantanabe, 1985) suggests adenosine does not activate Ic. The inability of dTc to alter the

response to adenosine is also consistent with a lack of effect of adenosine on Ic.

It is expected that both Ic and I_{ahp} would be reduced or abolished in low $[Ca^{2+}]e$ and the augmentation of the adenosine-induced hyperpolarisation in low $[Ca^{2+}]e$ and the inability of apamin and dTc to alter the response to adenosine both in normal PSS (Table 4.4) and in 0mM Ca^{2+}/10mM Mg2+ + 1mM 4AP PSS in the absence (adenosine, 100uM = -50, -110uV) or presence of dTc 100uM (-60, -110uMV respectively) indicates adenosine does not hyperpolarise the rat SCG by an increase in $gK_{(Ca)}$.

4.8.4 Effect of tetraethylammonium (TEA), 4-aminopyridine (4AP) and 3,4-diaminopyridine (3,4-DAP) on the response to adenosine

The development of various K^+ channel antagonists as pharmacological tools with high selectivity for different K^+ channels (see Cook, 1988; Castle et al., 1989) has allowed the differentiation of individual K^+ currents which are selectively antagonised by TEA and 4AP, and these drugs have been used to discover if the actions of adenosine on neurones are via K^+ channels (Perkins & Stone, 1980; Schubert & Lee, 1986). TEA antagonises K^+ currents and some calcium activated currents, with a low potency against I_A, Ic and I_{ahp}, although higher concentrations of TEA antagonise the majority of K^+ channels including I_K, I_A, inward rectifiers (I_R), $I_{K(ATP)}$ and Im (Cook, 1990; Castle et al., 1989; Cook & Quast, 1990). Likewise 4AP can also function as a non-selective K^+ channel antagonist, but I_A is particularly sensitive to antagonism by aminopyridines including 4AP and 3,4-diaminopyridine (3,4DAP). The ability of TEA and 4AP to alter the response to adenosine was investigated to

discover if the hyperpolarisation to adenosine occurs due to the activation of a K^+ channel.

The ability of 2mM TEA to reduce the response to adenosine (Table 4.9) is indicative that adenosine may hyperpolarise the rat SCG by activating an increase in gK.

A likely site of action of adenosine might be to activate delayed rectifier K^+ current, Ik as Galvan & Sedlmeir (1983) and Belluzzi et al. (1985b) studying the rat SCG found 1 to 3mM TEA abolished Ik. There are at present no more selective antagonists of I_K (Anikoztejn & Ben-Ari, 1991; Cook, 1990) and other currents such as Im and Ic could also be antagonised by TEA. Although TEA is known to antagonise SK channels with an IC_{50} of 3 to 10mM (Cook & Quast, 1990), BK channels of the rat SCG (Smart, 1987) and bullfrog ganglia at 1mM (Adams et al., 1982), it is unlikely these channels are involved in the response to adenosine (see section 4.4).

The inability of TEA at 10mM to antagonise the hyperpolarisation to adenosine is perplexing as TEA might be expected to be more effective at 10mM. However, TEA at 10mM antagonises most classes of K^+ channels (Cook & Quast, 1990) including those found to present in the rat SCG neurones, including Ik and Ic (Constanti et al., 1981b; Adams et al., 1982) and Im (Adams et al., 1982).

The depolarisations to 2mM and 10mM TEA, 1mM 4AP (Table 4.9) and 8mM K^+ (Table 4.10) in PSS were about equal but only 2mM TEA depressed the response to adenosine, suggesting the level of depolarisation of the ganglion is unlikely to account for difference between the effects of these K^+ channel antagonists on the response to adenosine. The potentiation of the response to potassium in 10mM TEA indicated that there was a general increase in

excitability of the ganglion, possibly via the release of ACh or a direct postsynaptic action. An increased blockade of Im by 10mM TEA could potentiate the hyperpolarisation to adenosine if the hyperpolarisation is mediated by an increase in gK e.g. Im, and reduce the overall antagonism of the response to adenosine. However, the lack of antagonism of the response to adenosine by 10mM TEA in PSS containing either atropine or TTX (Table 4.9) does not support this hypothesis.

TEA at 10mM may unmask an effect of adenosine on another conductance as described for the actions of TEA on the response of the guinea-pig atria to carbachol and adenosine (De Biasi, Froldi, Ragazzi, Pandolfo, Caparrotta & Fassina, 1989). In addition to antagonising K^+ channels TEA is known to produce a variety of pharmacological actions including mimicking the actions of ACh on nicotinic receptors. The activation of nicotinic receptors by TEA was not determined but nicotinic depolarisation did not alter the response to adenosine (see Chapter 5) thus the ability of TEA to activate nicotinic receptors would not be expected to alter the response to adenosine.

The ability of 4AP to enhance the response to adenosine may arise from an inhibition of operative I_A channels. 4AP may block these outward K^+ currents due to neuronal depolarisation and allow the activation by adenosine of an outward 4AP resistant current. In addition to blocking K^+ channels from the exterior K^+ channel antagonists may have had both internal and external sites of action as these agents could enter through cut nerve endings, e.g. ECN to depolarise the ganglion. 4AP may cause the release of ACh, although these seem unlikely as in PSS containing TTX, 4AP still potentiated the responses to adenosine (Table 4.9).

It was suggested that adenosine hyperpolarised the rat neocortex (Perkins & Stone, 1980) and hippocampus (Okada & Ozawa, 1980; Schubert & Lee, 1986) by enhancing I_A as the response to adenosine was antagonised by 4AP. The inability of 4AP to antagonise the hyperpolarisation of the rat SCG to adenosine indicates that adenosine is unlikely to be working via a change in I_A. The ability of 4AP to increase the hyperpolarisation to adenosine in contrast to the actions of TEA is intriguing and may have arisen due to the ability of TEA to produce a hyperpolarising shift of the K^+ channel activation curve and 4AP to shift the activation in the opposite direction (Ruff, 1986).

The results suggest that adenosine activates an increase in gK such as I_K and I_M to hyperpolarise the rat SCG.

GENERAL DISCUSSION

The results presented in this chapter indicate adenosine directly hyperpolarises the rat SCG. The most likely mechanism of action of adenosine is via an increase in gK and not via a change in gCa as reported for some actions of adenosine on the rat SCG (Henon & McAfee, 1983a,b). There was no evidence to support the hypothesis that adenosine causes a decrease in gNa, an increase in gCl^-, or activates an electrogenic pump for Na^+ or Cl^- ions.

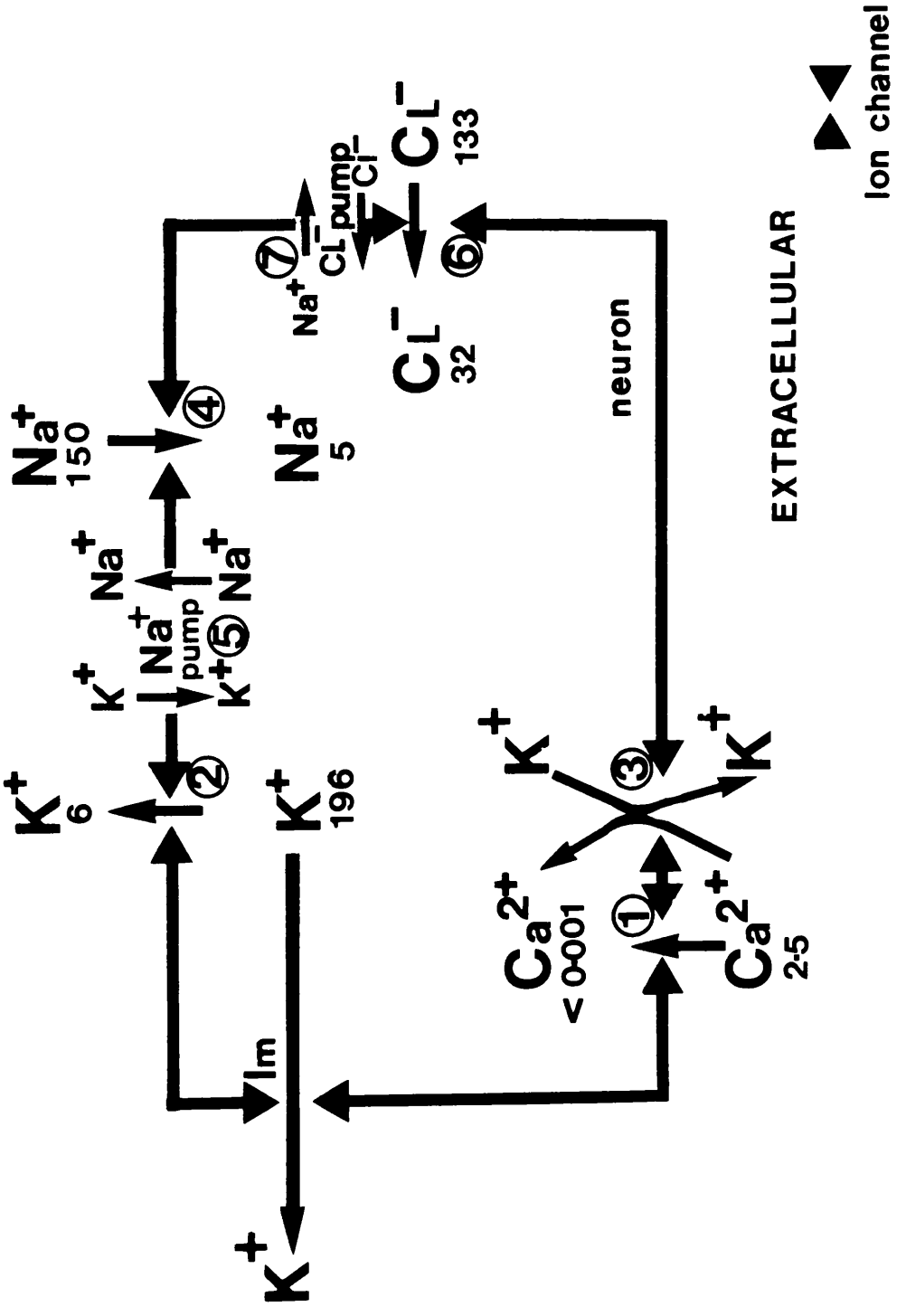
Both the potentiation and the attenuation of the response to adenosine in low and high K^+ containing PSS respectively, suggest adenosine hyperpolarises the rat SCG via an increase in gK. Further analysis of this hypothesis was complicated by the ability of K^+ channel antagonists to depolarise ganglia and therefore indirectly alter the response to adenosine. Thus the response to

adenosine in the presence of a K^+ channel antagonist may have been due to a primary or secondary consequence of drug effect on membrane potential change. However, the ability of TEA and not 4AP to antagonise the response to adenosine indicates a potential role of an I_K like channel in the actions of adenosine. The inability of other K^+ channel antagonists to alter the response to adenosine suggests the hyperpolarisation of the ganglion was not due to an increase in I_A, $I_{K(ATP)}$ or $I_{K(Ca)}$, although changes in other gK's e.g. a K^+ leak current of the type described by Jones (1989) in amphibian ganglia, has not been excluded.

Similarly the depression of the response to muscarine by adenosine was not due to a change in gCa, but is consistent with an increase in gK by adenosine to antagonise the inhibition of Im by muscarine.

One approach to examining the hypothesis that adenosine increases gK would be to use intracellular recording techniques such as those described by Belluzz and colleagues (1985a,b) to determine both the voltage sensitivity and the reversal potential of the response to adenosine. Further information could be derived from single channel studies of patch clamped dissociated SCG neurones (Gurney et al., 1987) to discover the channel properties and if the response to adenosine and carbachol occurs via the same K^+ channels (Belardinelli & Isenberg, 1983; Andrade, Malenka & Nichol, 1986) and/or if common intracellular messengers and G-proteins are involved in the transduction of the hyperpolarisation to adenosine.

Fig. 4.1. A schematic diagram summarising the various ionic concentrations (numbers under chemical symbols refer to mM concentration: estimates of intracellular ions concentration taken from Galvan et al., 1984) of the major anions and cations and the conductances that may be altered by adenosine in the postganglionic neurone of the rat SCG. The following ionic mechanisms have been implicated in the effects of adenosine on the rat SCG; (1) an increase in the entry of calcium ions (Ca^{2+}) e.g. Ribeiro, Sa'-Almeida & Namorado, 1979; (2) an increase in the efflux of potassium ions (K^{+}) e.g. Segal, 1982; Trussel & Jackson, 1987, 1989; (3) an increase in the efflux of K^{+} via Ca^{2+} activated K^{+} channels e.g. Proctor & Dunwiddie, 1987; (4) a decrease in the entry of sodium ions (Na^{+}) e.g. Imai & Takeda, 1967; (5) activation of the electrogenic Na^{+}/K^{+} exchange mechanism (Smith, 1986) and/or (6) an increase in chloride influx via Cl^{-} channels or Cl^{-} exchange mechanism (Palmer & Wood, 1987).


Ion channel
EXTRACELLULAR
neuron
Na^+ 150
Na^+ 5
K^+ 6
K^+ 196
Cl^- 32
Cl^- 133
Ca^{2+} < 0·001
Ca^{2+} 2·5
Na^+ pump
Cl^- pump
Im
1
2
3
4
5
6
7

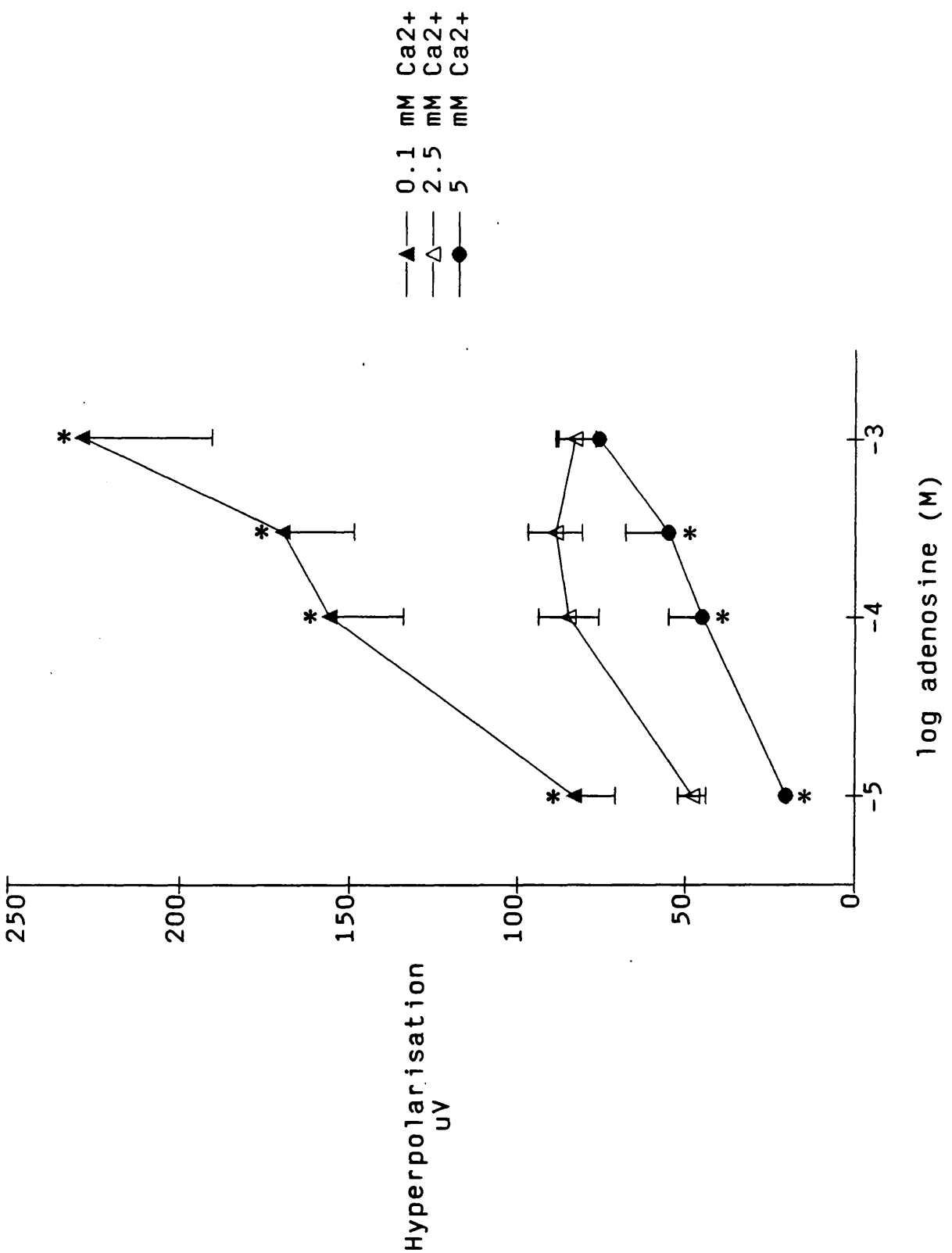


Fig. 4.2. Log concentration-response curve of the rat SCG to two minute applications of adenosine in normal physiological salt solution (PSS) containing 2.5mM Ca^{2+}, 5mM Ca^{2+} (high calcium PSS) or 0.1mM Ca^{2+} (low calcium PSS).

Fig. 4.3. Response of the rat SCG to increasing concentrations of adenosine at 10, 100 and 300uM, applied for 2 minutes, every 20 minutes in physiological salt solution (PSS) containing (a) 2.5mM Ca^{2+}/1mM Mg2+ and (b) 0.1mM Ca^{2+}/1mM Mg2+.

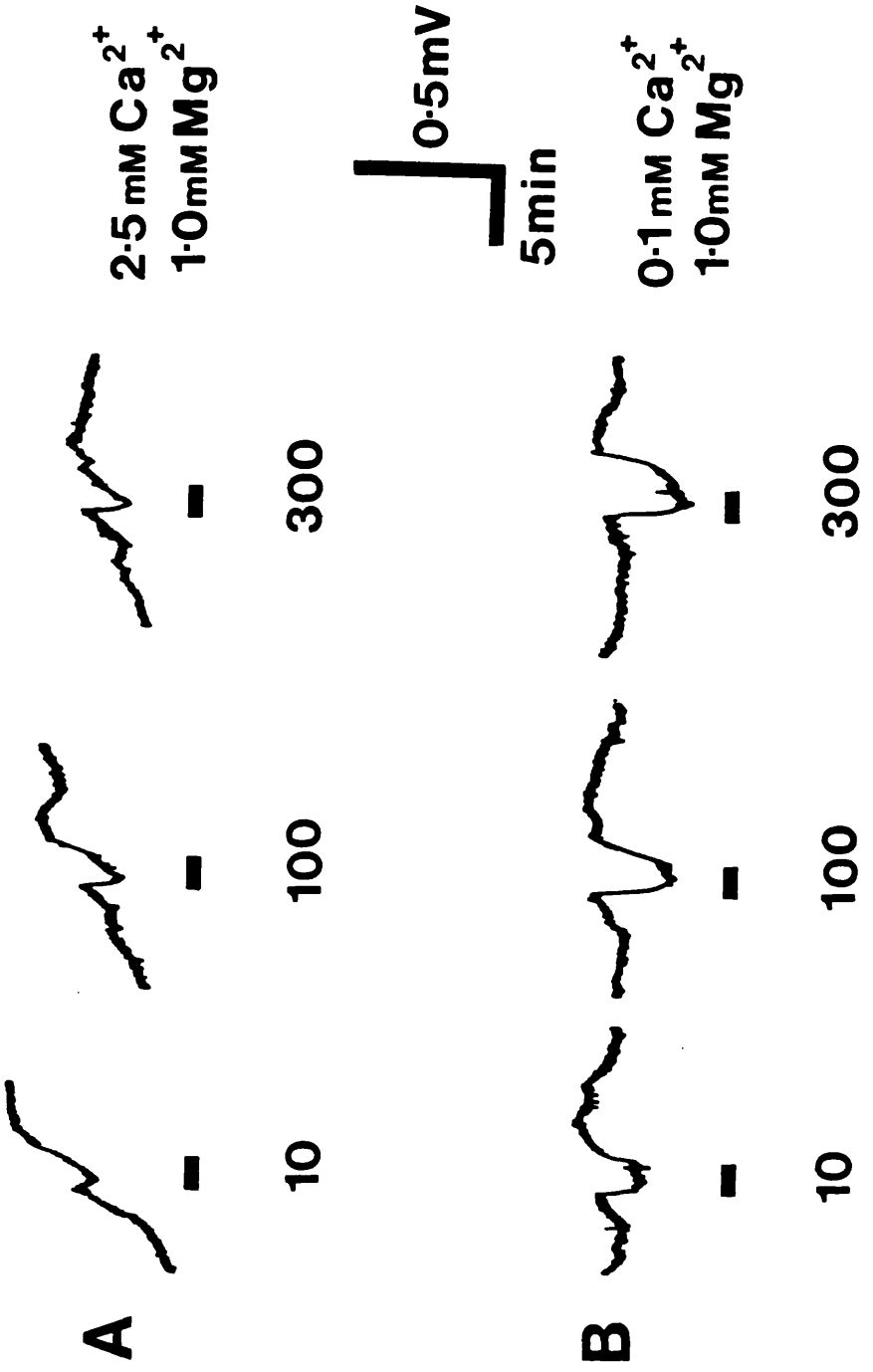


Fig. 4.4. Effect of low calcium physiological salt solution on the response of the rat isolated SCG to adenosine and phenylamino-adenosine

Comparison of log concentration-response curves to adenosine (AD) and phenylaminoadenosine (PAA) in physiological salt solution containing 2.5mM calcium (2.5mM Ca^{2+}) or low calcium (0.1mM Ca^{2+}). The responses to adenosine in 0.1mM Ca^{2+} containing PSS (1st DRC) were significantly (* = $P < 0.05$) enhanced at 10 and 100uM and reproducible when compared to responses obtained 2 hours later (2nd DRC).

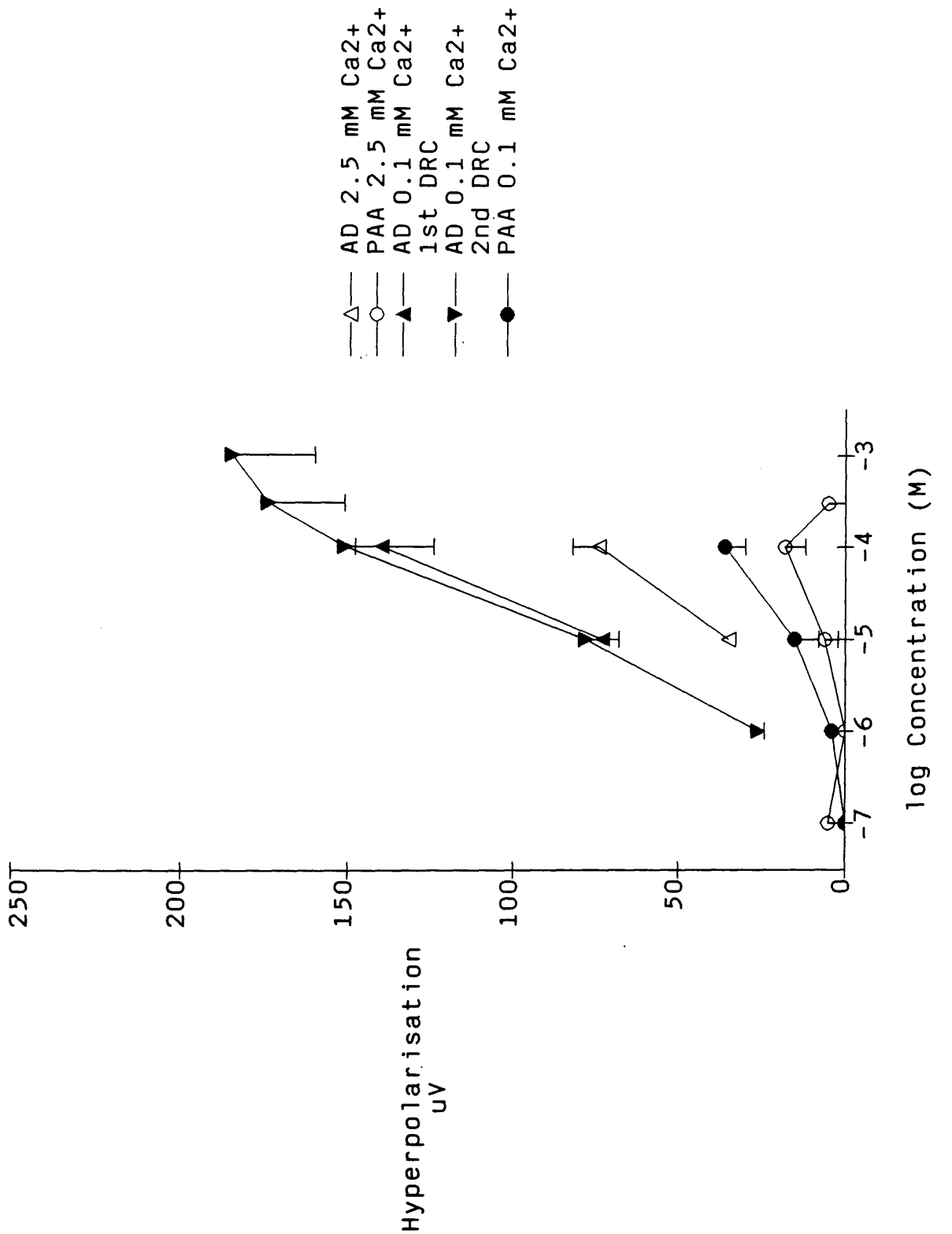

AD 2.5 mM Ca2+
PAA 2.5 mM Ca2+
AD 0.1 mM Ca2+
1st DRC
AD 0.1 mM Ca2+
2nd DRC
PAA 0.1 mM Ca2+
Hyperpolarisation
uV
250
200
150
100
50
0
-7
-6
-5
-4
-3
log Concentration (M)

Fig. 4.5. Effect of low calcium physiological salt solution on the response of the rat isolated SCG to 2-chloroadenosine

Comparison of the log concentration-response curves to a 2 minute application of 2-chloroadenosine (2CA) in physiological salt solution (PSS) containing 2.5mM calcium (2.5mM Ca^{2+}) or PSS containing low calcium PSS (0.1mM Ca^{2+}). Low calcium significantly (* = $P < 0.05$) enhanced the hyperpolarisation to 2CA at 0.3uM and 10uM.

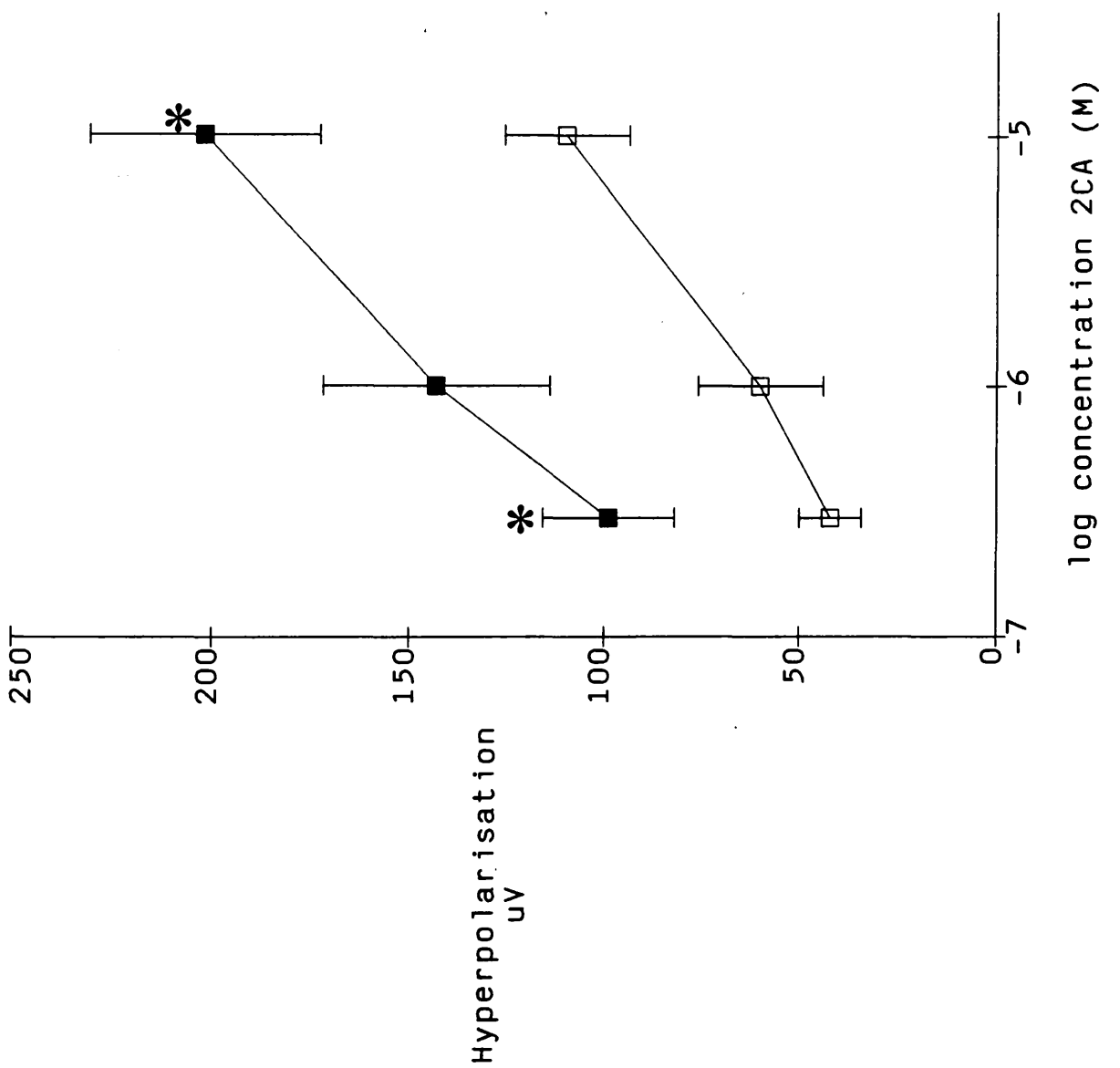

2.5 mM Ca2+
0.1 mM Ca2+
Hyperpolarisation
uV
250
200
150
100
50
0
-7
-6
-5
log concentration 2CA (M)

Fig. 4.6. % depression of the response to muscarine by adenosine on the rat SCG in the presence of 2.5mM calcium or 0.1mM calcium.

The % depression of muscarine for a given concentration of adenosine was compared using an unpaired t-test (as the responses in different concentrations of calcium (Ca^{2+}) and different adenosine concentrations were randomised and recorded from different ganglia), and expressed as statistically significant from responses in 2.5mM Ca^{2+} by * for $P < 0.05$ and *** for $P < 0.01$.

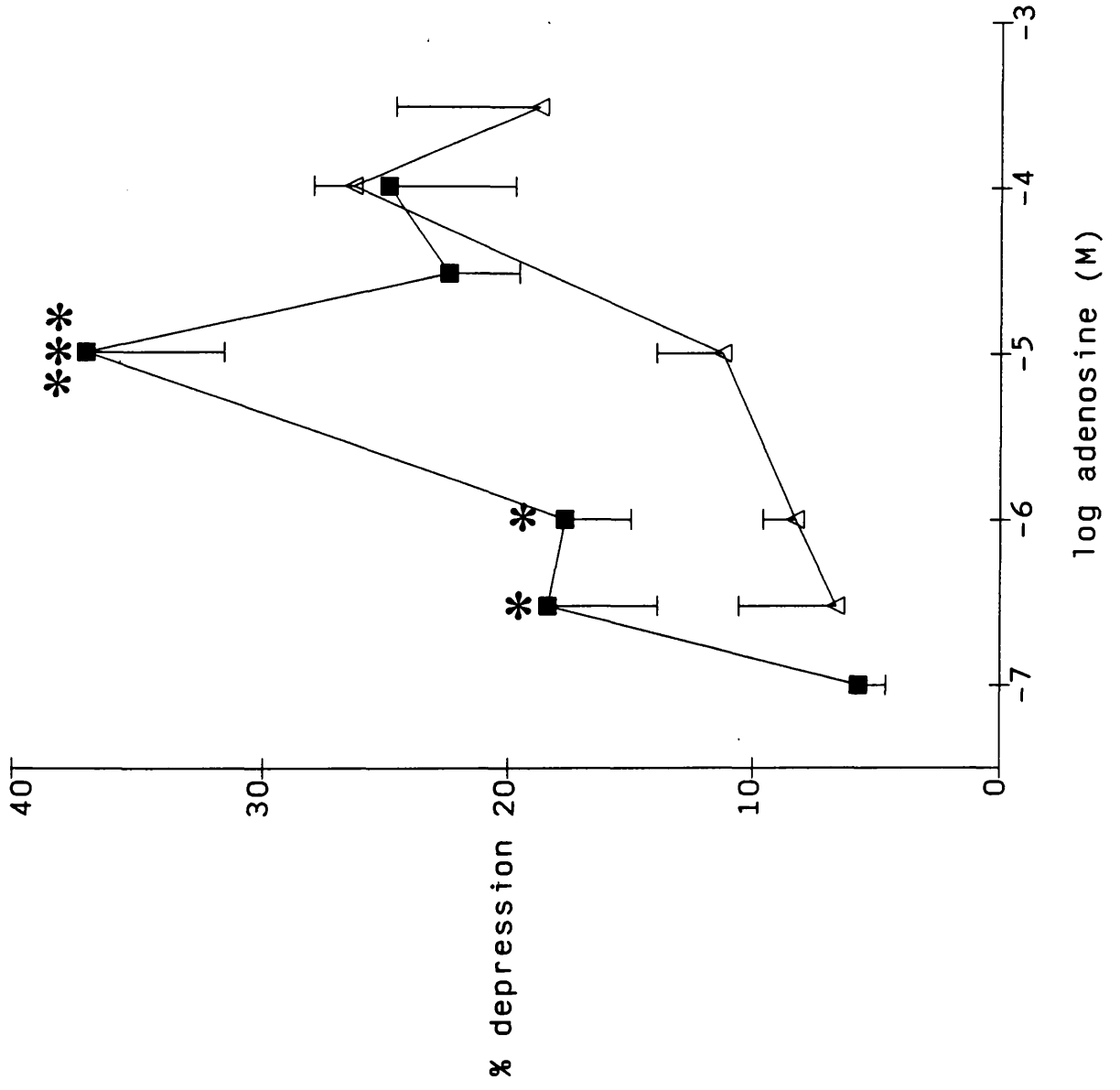

0.1 mM Ca2+
2.5 mM Ca2+
% depression
40
30
20
10
0
-7
-6
-5
-4
-3
log adenosine (M)
***
*
*

Fig. 4.7. Effect of magnesium free solution on the response of rat isolated SCG to 2-chloroadenosine and adenosine

Log concentration-response curves to two minute applications of 2 chloroadenosine (2CA) and adenosine (AD) in physiological salt solution in the absence (0mM $Mg2^{+}$) and presence of 1mM magnesium (1mM $Mg2^{+}$). There was no significant difference (paired t-test) between the responses to 2CA or AD in the absence or presence of Mg^{2+}.

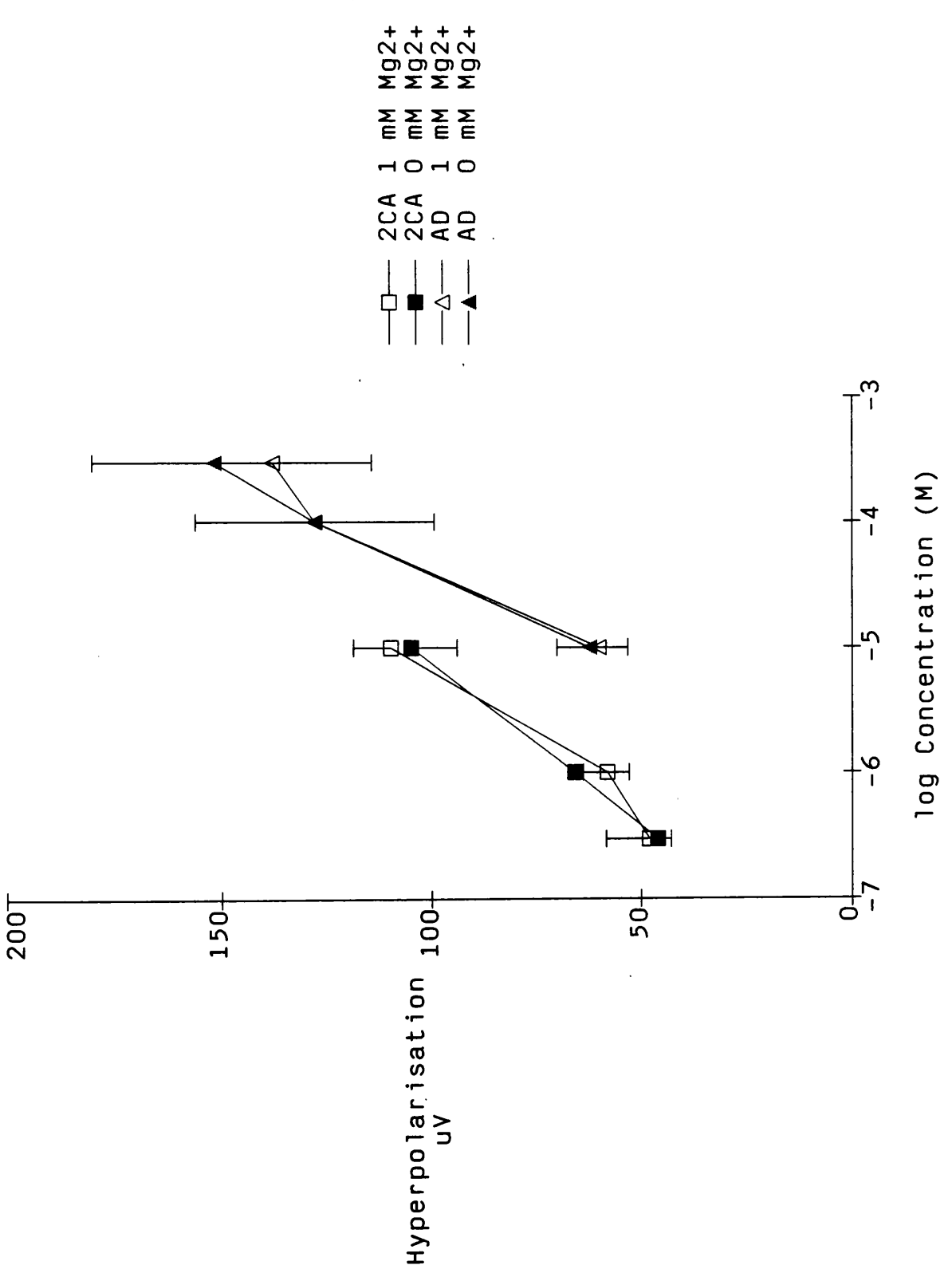

2CA 1 mM Mg2+
2CA 0 mM Mg2+
AD 1 mM Mg2+
AD 0 mM Mg2+
Hyperpolarisation uV
200
150
100
50
0
-7
-6
-5
-4
-3
log Concentration (M)

Fig. 4.8. Effect of magnesium free solution on the response of rat isolated SCG to adenosine

Log concentration-response curves to two minute applications of adenosine (AD) in calcium (Ca^{2+}) free physiological salt solution (PSS) in the presence or absence of 1mM magnesium (0mM Ca^{2+}/1mM Mg^{2+} and 0mM Ca^{2+}/0mM Mg^{2+} respectively). Also log concentration-response curves to adenosine in 0mM Ca^{2+} PSS and ethylene-glycolamino-ethylether-tetra-acetic acid (EGTA) in the presence or absence of 1mM magnesium (0mM Ca^{2+}/1mM Mg^{2+} + EGTA (2mM) and 0mM Ca^{2+}/0mM Mg^{2+} + EGTA (2mM) respectively.

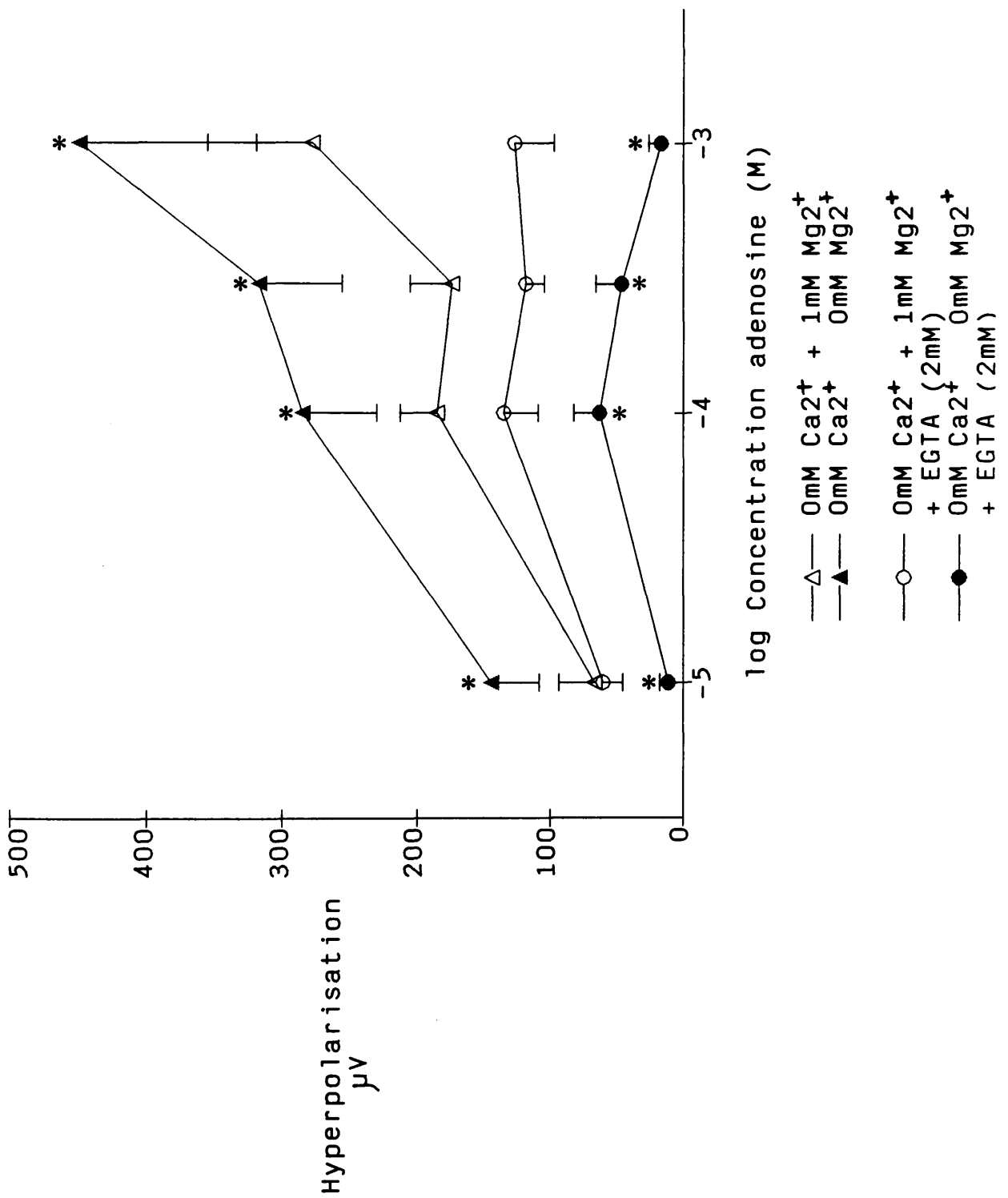

Hyperpolarisation
μV
500
400
300
200
100
0
-5
-4
-3
log Concentration adenosine (M)
0mM Ca2+ + 1mM Mg2+
0mM Ca2+ 0mM Mg2+
0mM Ca2+ + 1mM Mg2+
+ EGTA (2mM)
0mM Ca2+ 0mM Mg2+
+ EGTA (2mM)

Fig. 4.9. Response of an isolated rat SCG to potassium and adenosine in different concentrations of calcium and magnesium.

The first two columns refer to the concentration of calcium (Ca^{2+}) and magnesium (Mg^{2+}) in the physiological salt solution (PSS) and the next two columns an example of a trace obtained from a single ganglion; response to 12mM potassium (K^{+}) applied for 1 minute about 25 minutes prior to the application of adenosine (AD), 1mM, 2 minutes) in each example. The results in the last two columns refer to the mean $\pm$ standard error of the mean for the response to potassium and adenosine respectively and the number of ganglia tested is indicated by a figure in the box.

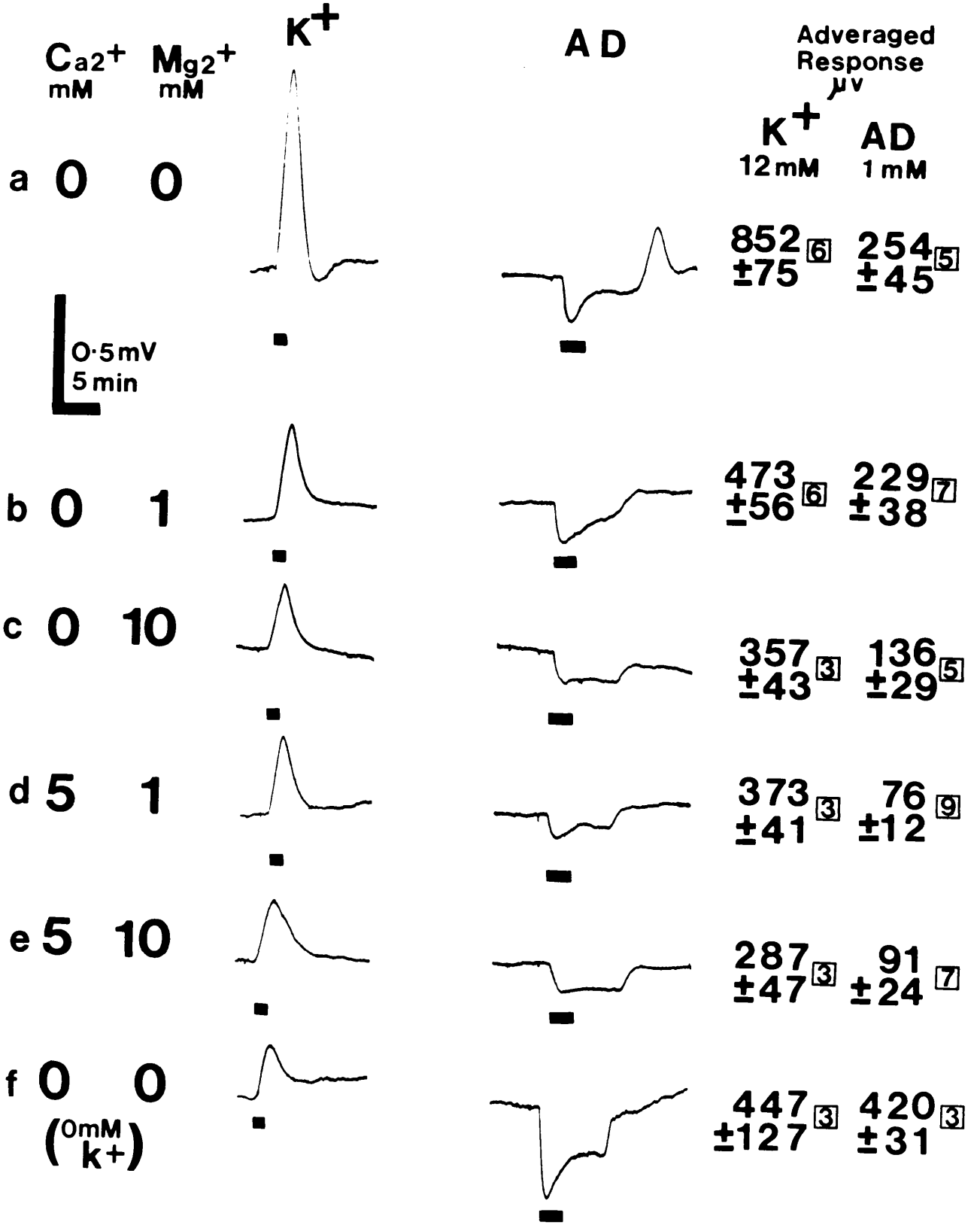

Ca2+ mM
Mg2+ mM
K+
AD
Adveraged Response μv
K+ 12mM
AD 1mM
a 0 0
0·5mV
5 min
852 ±75 [6]
254 ±45 [5]
b 0 1
473 ±56 [6]
229 ±38 [7]
c 0 10
357 ±43 [3]
136 ±29 [5]
d 5 1
373 ±41 [3]
76 ±12 [9]
e 5 10
287 ±47 [3]
91 ±24 [7]
f 0 0
(0mM k+)
447 ±127 [3]
420 ±31 [3]

Fig. 4.10. Effect of cobalt on the response of the rat isolated SCG to carbachol and adenosine

Log concentration-response curves of four rat SCG to a two minute application of adenosine (AD) and one minute application of carbachol (CARB) in the presence of 0.5mM calcium (Ca^{2+}) or 0.5mM cobalt (Co2+). In a PO4/SO4 free physiological salt solution (PSS) containing 0.3uM pirenzepine (PIR) and 2mM K^{+} replacing 0.5mM Ca^{2+} with 0.5mM Co2+ reduced the hyperpolarisation to both adenosine (AD) and carbachol (CARB). A significant difference (two tailed t test) between 0.5mM Ca^{2+} PSS and 0.5mM Co2+ is indicated by a * for $P < 0.05$ and ** for $P < 0.01$. Second concentration response curves to both AD and CARB repeated 30 minutes after washing out Ca^{2+} for 30 minutes were not significantly different to the first curves.

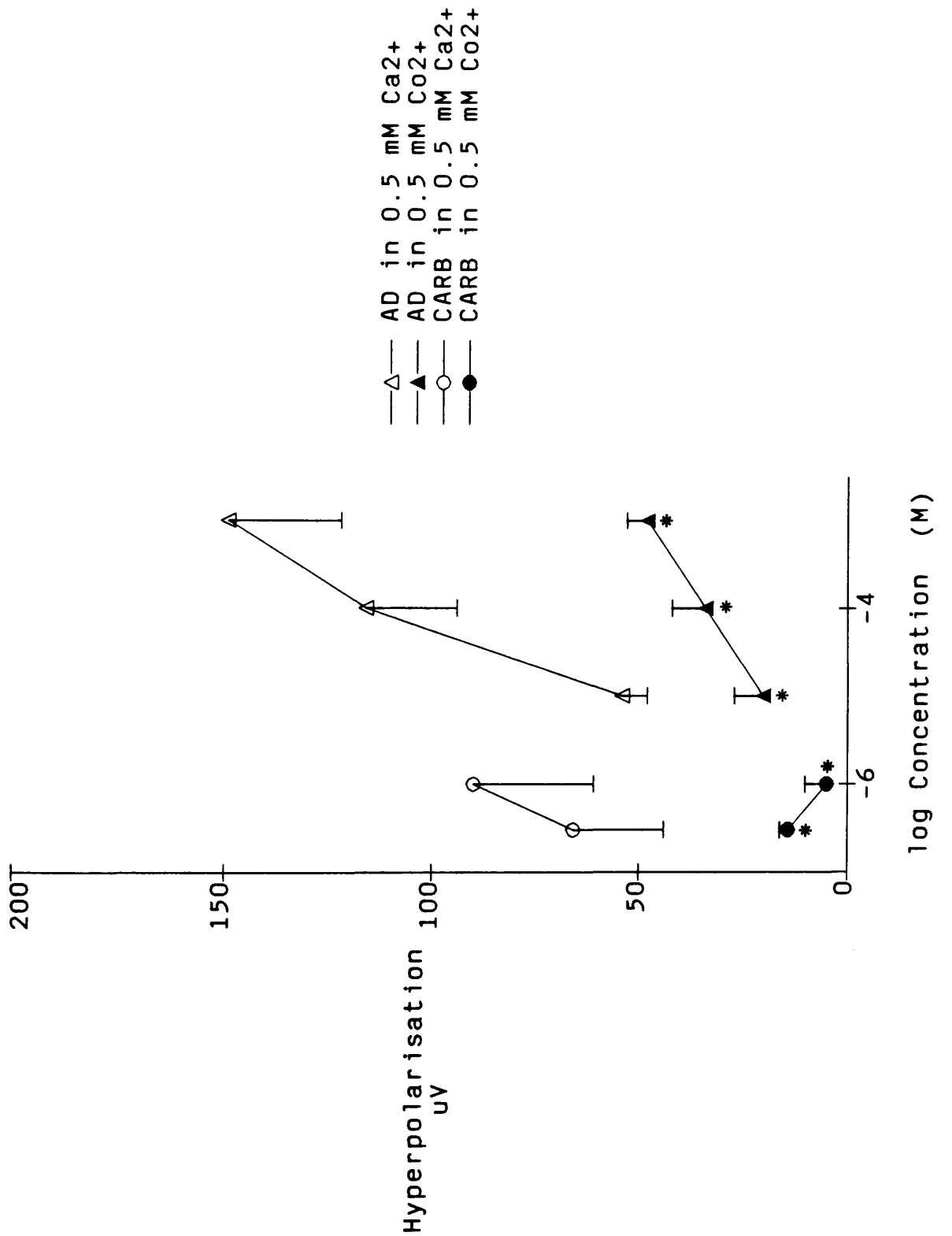

AD in 0.5 mM Ca2+
AD in 0.5 mM Co2+
CARB in 0.5 mM Ca2+
CARB in 0.5 mM Co2+
Hyperpolarisation uV
200
150
100
50
0
-6
-4
log Concentration (M)

Fig. 4.11. Log concentration response curve of the rat SCG to gamma-amino butyric acid

One minute applications of gamma-aminobutyric acid (GABA) were applied every twenty minutes. GABA produced both a depolarisation followed by an after hyperpolarisation (AHP). Each point is the mean, and vertical bars the standard error of the mean for four ganglia. A significant difference (two tailed paired t-test) from basal d.c. potential is indicated by a * for $P < 0.01$.

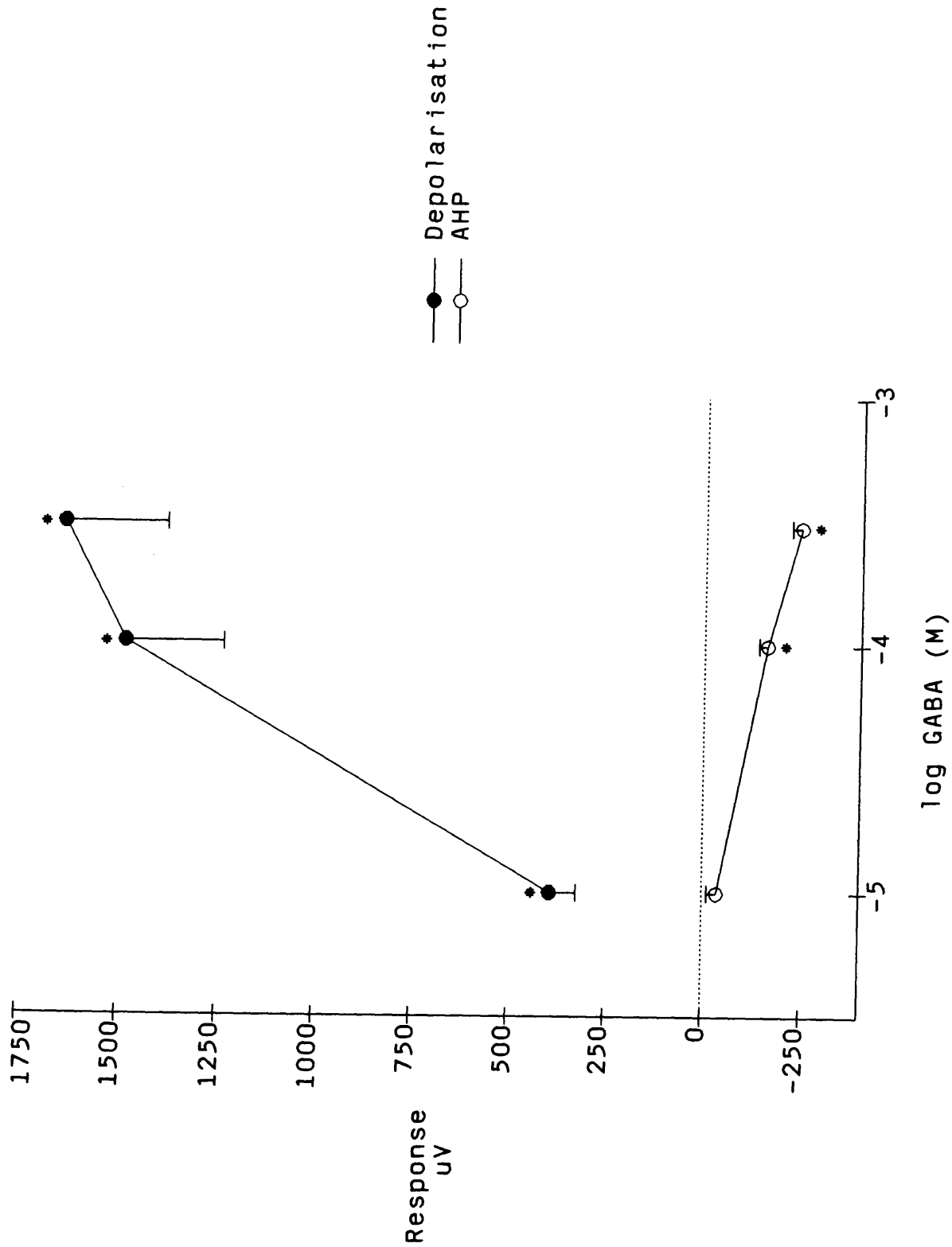

Depolarisation
AHP
Response uV
1750
1500
1250
1000
750
500
250
0
-250
-5
-4
-3
log GABA (M)

Fig.4.12. Response of a single ganglion to gamma-amino butyric acid, adenosine in the absence and presence of furosemide

(A) Responses of an isolated rat SCG in normal physiological salt solution (PSS) produced by one minute applications of 10uM and 300uM of gamma-aminobutyric acid (GABA) at twenty minute intervals and (B) responses to 100uM adenosine (AD) and GABA 10uM and the response to 100uM AD applied during the after hyperpolarisation (AHP) of the response to 10uM GABA, followed by incubation in furosemide (FURO, 10uM, for 1 hour) and the response to 10uM GABA and 100uM AD. In all traces GABA and AD were applied for one and two minutes respectively. Scale for (a) 2.5mV and (b) 0.5mV, note in (b) the depolarisation to GABA is truncated.

The AHP to 10uM GABA was submaximal (cf: The AHP to 10uM and 300uM GABA, $P<0.01$, $n=4$, paired t-test), suggesting incomplete activation of the $Na^+/K^+/Cl^-$ cotransporter and this concentration of GABA was chosen to gauge the interaction between the GABA-induced AHP and the hyperpolarisation to adenosine. At 10uM GABA the AHP was maximal 3.5 minutes and the response to adenosine was timed to occur 3.5 minutes after the application of GABA, i.e. during the peak of the AHP to GABA. On four ganglia the response to adenosine in the presence of the AHP to GABA (-60 $\pm$ 7uV) was not significantly different from the response before (-68 $\pm$ 13uV) or after GABA (-71 $\pm$ 12uV). On three ganglia the response to adenosine in both 10uM and on three ganglia 100uM furosemide was significantly reduced (see Table 4.14) and showed a partial recovery when 100uM furosemide was washed out for 20 minutes (-50 $\pm$ 10uV).

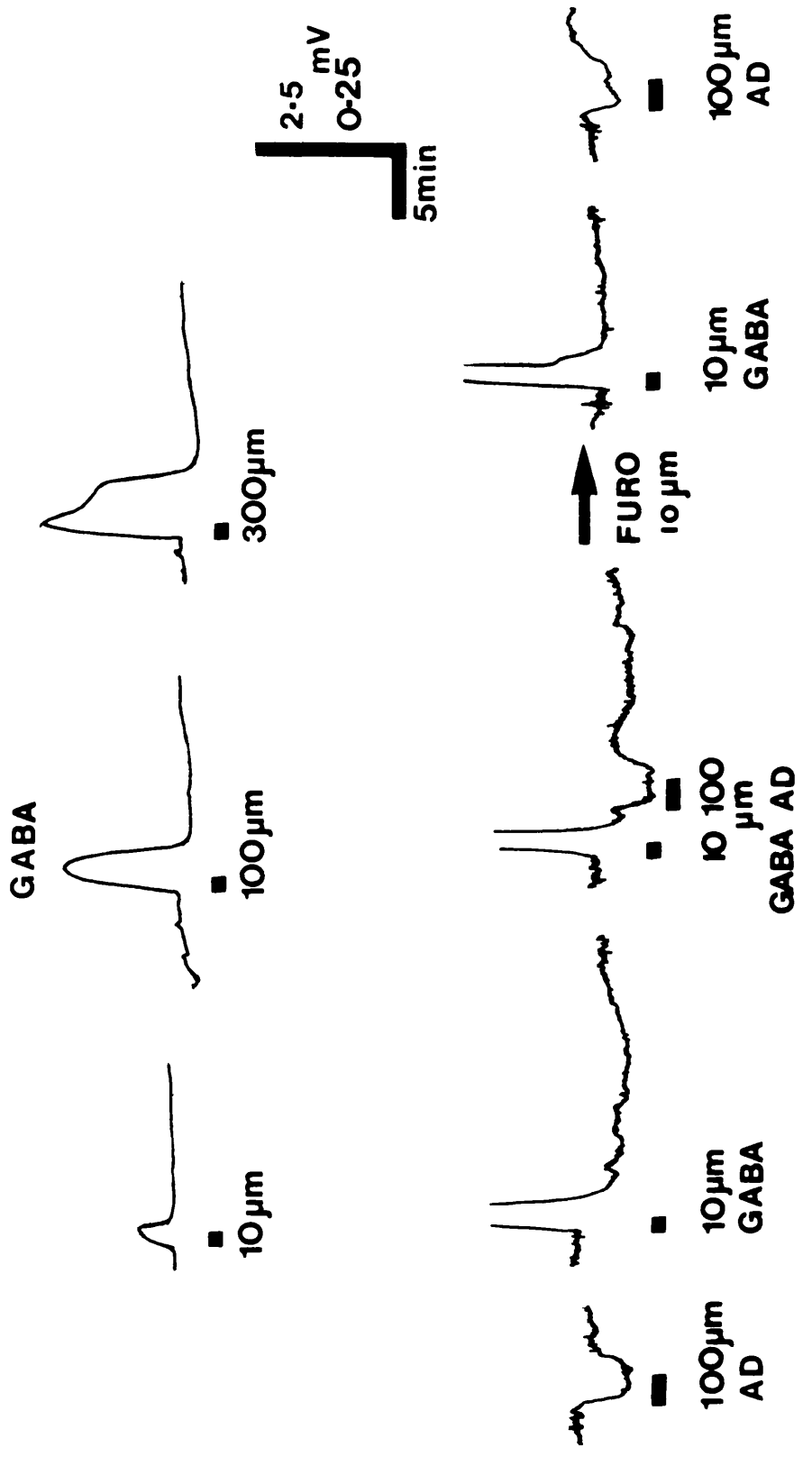

a
GABA
10µm
100µm
300µm
2·5 mV
0·25
5min
b
100µm AD
10µm GABA
10 100 µm GABA AD
FURO 10 µm
10µm GABA
100µm AD

Fig. 4.13. Effect of lithium on the response of isolated rat SCG to adenosine, muscarine and dimethylphenylpiperazinium

(a) The response of an isolated rat SCG in normal physiological salt solution (PSS) to 100nM muscarine (M) and 100uM adenosine (AD) applied for 1 and 2 minutes respectively; and (b) after 30 minutes incubation in sodium-HEPES PSS (Na^+-HEPES), the response to 100nM muscarine in the absence (M) and presence of 100uM adenosine (5 minutes application, AD and M), and 100uM adenosine for 2 minutes; (c) the response to 100uM adenosine applied for 2 minutes after incubation in lithium-HEPES PSS (Li^+-HEPES) for 40 (i), 108 (ii) and 144 (iii) minutes, followed by the response to a one minute application of muscarine at 100nM (M) and muscarine at 1uM and dimethylphenyl piperazinum (DMPP) at 10uM.

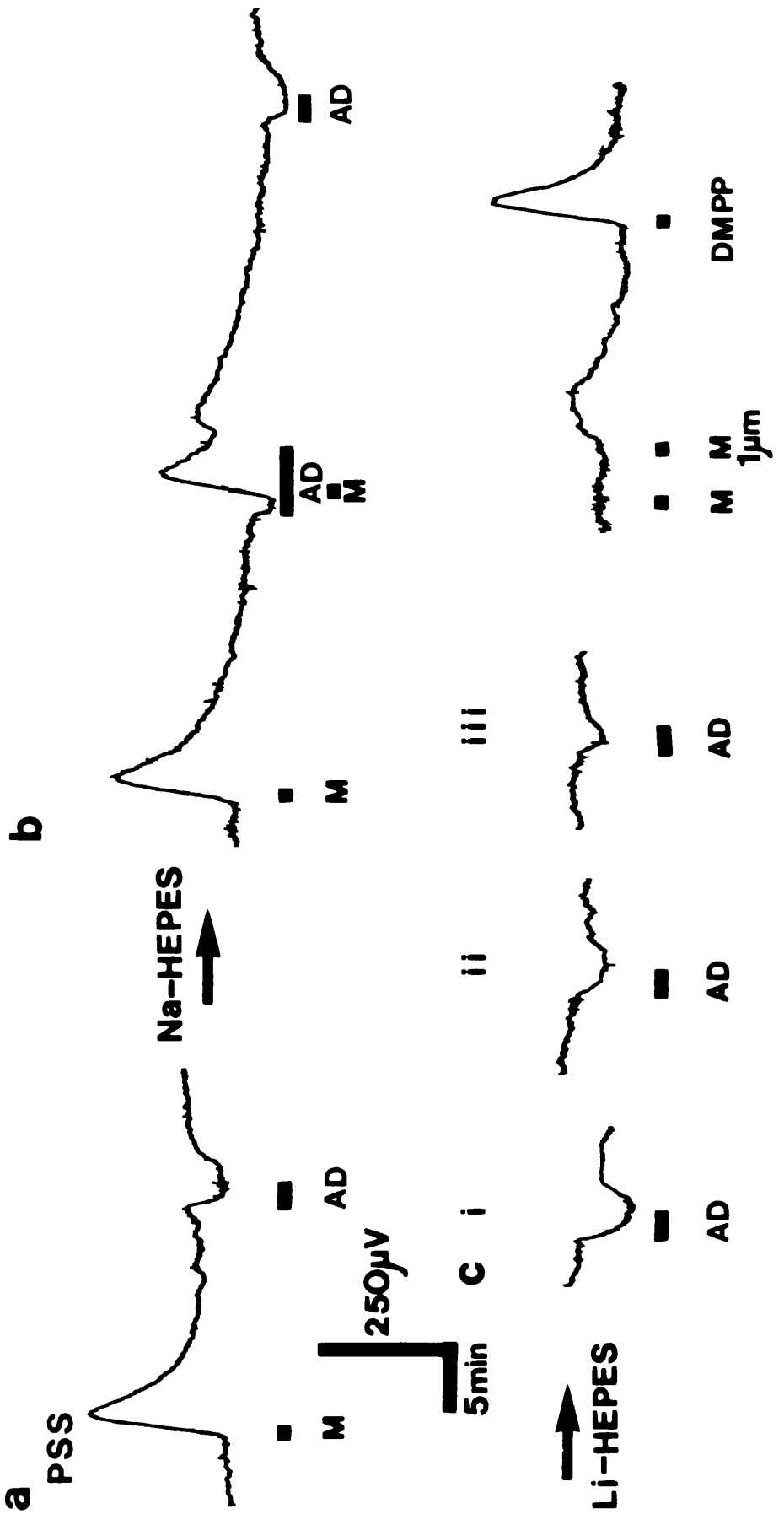
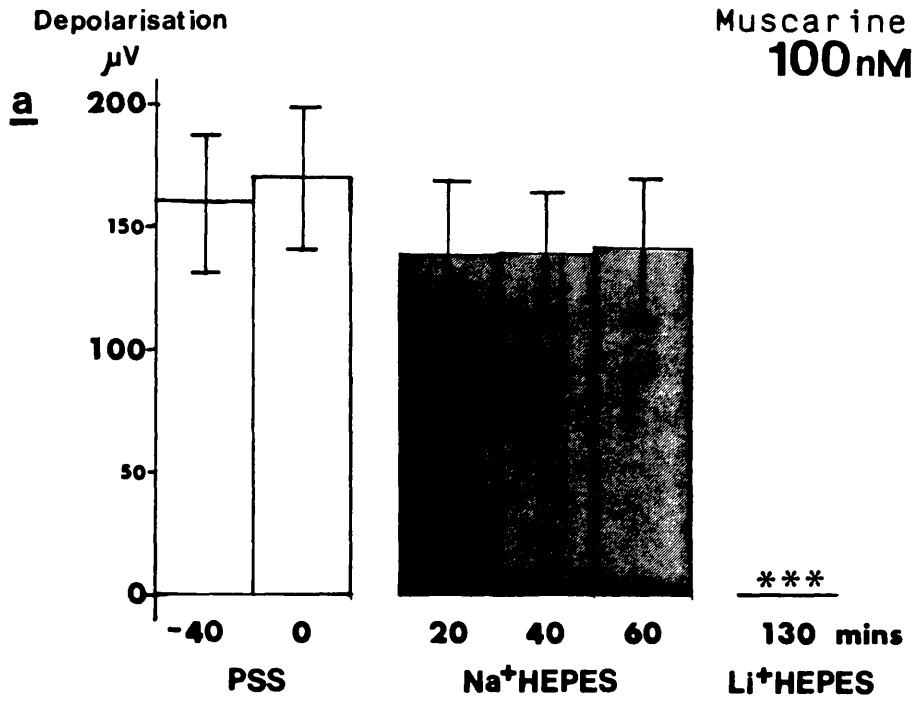

a
PSS
M
AD
250μV
5min
C i
ii
iii
Na-HEPES
Li-HEPES
b
M
AD
AD
M
AD
AD
M
M
1μm
DMPP

Fig. 4.14. Response of four rat SCG to muscarine and adenosine in the absence and presence of lithium

(a) The depolarisation to 100nM muscarine (1 min applications) was not significantly altered by changing from normal physiological salt solution (PSS) to Na+ containing HEPES-PSS to lithium containing HEPES-PSS (Li^+-HEPES) significantly ($P<0.001$ = ***) reduced the response to muscarine.

(b) The hyperpolarisation to a two minute application of adenosine (100uM) were reduced in the presence of Na^+ HEPES-PSS. Changing from Na^+-HEPES to Li^+-HEPES produced a transient increase in the response to adenosine with time for the first hour of incubation in Li^+-HEPES.

Statistical significance was determined using paired t-tests - and are indicated by a * for $P<0.05$, ** for $P<0.01$ and *** for $P<0.001$.


Depolarisation
μV
a
200
150
100
50
0
Muscarine
100nM
***
-40
0
20
40
60
130 mins
PSS
Na+HEPES
Li+HEPES

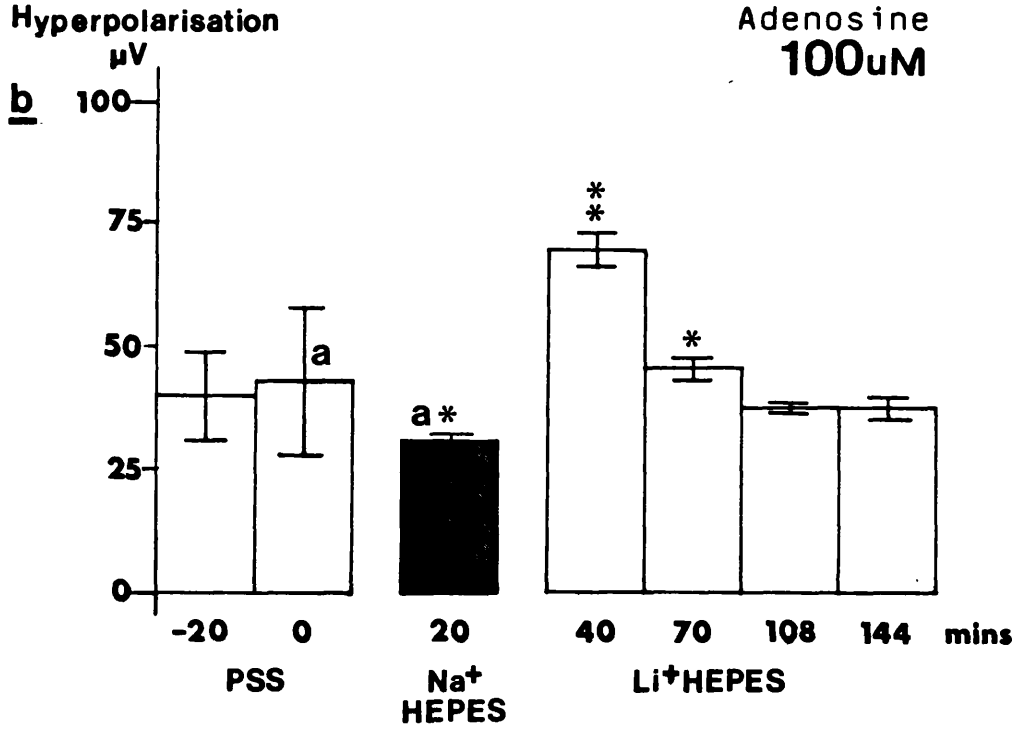

Hyperpolarisation
μV
b
100
75
50
25
0
Adenosine
100uM
a
a*
**
*
-20
0
20
40
70
108
144
mins
PSS
Na+
HEPES
Li+HEPES

Fig. 4.15. Response of the rat SCG to adenosine in potassium free and high potassium containing physiological salt solution

A histogram of the response of isolated rat SCG to adenosine (10, 100 and 300uM, 2 minute applications every 40 minutes) in nominally K^+ free (0mM K^+), normal (6mM K^+) and high (12mM K^+) potassium medium. Bars represent the mean values, the SEM for at least six ganglia. Test for statistical significance by paired t-test of responses in 0mM K^+ or 12mM K^+ relative to 6mM K^+ control, and is indicated by * for $P < 0.05$ and ** for $P < 0.01$.

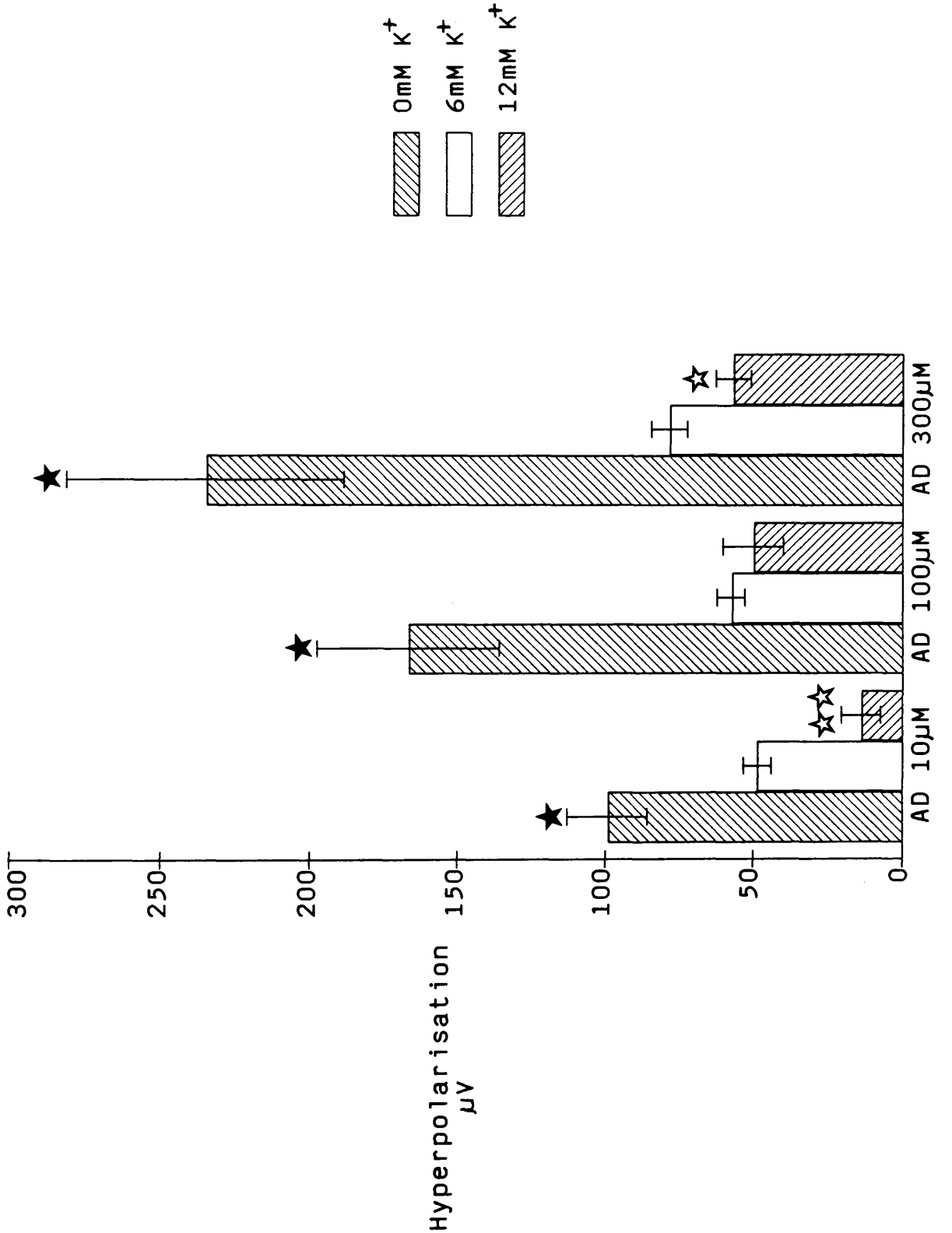

0mM K+
6mM K+
12mM K+
AD 10µM
AD 100µM
AD 300µM
Hyperpolarisation µV
0
50
100
150
200
250
300

Table 4.1. Comparison of the response of the isolated rat SCG to adenosine in physiological salt solutions containing different concentrations of calcium and magnesium

I. Amplitude of hyperpolarising response to 10uM adenosine in uV ± standard error of the mean response and number of ganglia tested in brackets.

Magnesium (mM)	Calcium (mM) 5	2.5	0.1	0	0 + EGTA (2mM)
0		55 ± 6 (16)[a]	61 ± 6 (8)[a]	144 ± 36 (6)[f]	11 ± 7 (8)
1	20 ± 4 (10)[e]	46 ± 3 (43)[de]	86 ± 10 (12)[bd]	93 ± 15 (12)[cf]	60 ± 15 (8)
10	35 ± 5 (8)	37 ± 9 (3)	60 ± 11 (4)[b]	60 ± 11 (6)[c]	

Statistical significance (paired t-test) is indicated by (a) & (e) for $P < 0.05$, and (b) for $P < 0.01$, and (c) & (d) for $P < 0.001$.

II. Amplitude of hyperpolarising response to 100uM adenosine in uV ± standard error of the mean response and number of ganglia tested in brackets.

Magnesium (mM)	Calcium (mM) 5	2.5	0.1	0	0 + EGTA (2mM)
0		82 ± 15 (13)[c]	121 ± 22 (8)[c]	228 ± 55 (6)	63 ± 19 (10)[a]
1	45 ± 6 (10)[b]	73 ± 6 (42)[bf]	156 ± 18 (12)[df]	155 ± 15 (12)[e]	135 ± 26 (10)[a]
10	32 ± 16 (8)	83 ± 11 (6)	48 ± 11 (4)[d]	53 ± 9 (16)[e]	

Statistical significance (paired t-test) is indicated by (a) for $P < 0.05$, and (b) to (e) for $P < 0.01$, and (f) for $P < 0.001$

Table 4.1. Comparison of the response of the isolated rat SCG to adenosine in physiological salt solutions containing different concentrations of calcium and magnesium

III. Amplitude of hyperpolarising response to 300uM adenosine in uV ± standard error of the mean response and number of ganglia tested in brackets.

Magnesium (mM)	Calcium (mM) 5	2.5	0.1	0	0 + EGTA (2mM)
0		99 ± 20 (14)[a]	150 ± 22 (8)[a]	318 ± 62 (6)	46 ± 20 (10)
1	61 ± 8 (9)[c]	89 ± 8 (40)[dc]	183 ± 19 (10)[bd]	172 ± 18 (12)[e]	118 ± 14 (10)
10	58 ± 15 (6)	89 ± 11 (6)	75 ± 20 (4)[b]	80 ± 13 (6)[e]	

Statistical significance (paired t-test) is indicated by (a) (b) & (c) for $P < 0.01$, and (d) & (e) for $P < 0.001$

IV. Amplitude of hyperpolarising response to 1000uM adenosine in uV ± standard error of the mean response and number of ganglia tested in brackets.

Magnesium (mM)	Calcium (mM) 5	2.5	0
0		110 ± 30 (2)	254 ± 45 (5)
1	76 ± 12 (9)	83 ± 6 (6)[a]	229 ± 38 (7)
10	91 ± 24 (7)	103 ± 13 (6)[a]	136 ± 29 (5)

Statistical significance (paired t-test) is indicated by (a) $P < 0.05$ compared with 2.5mM calcium

Table 4.2. Calcium and potassium channel antagonists used in the investigation of the actions of adenosine on the rat SCG

TYPE	ABBREVIATION	ANTAGONISTS (for abbreviations see Table 2.1 or text)
Potassium channels		
Delayed rectifier	I_K	TEA/4AP/Ba^{2+}
Transient outward	IA	4AP/THA
ATP sensitive	$I_{K(ATP)}$	gilbenclamide/TEA
M-current	Im	muscarine/TEA
G-protein	$I_{K(ACh)}$	muscarine
Calcium activated K^+ channels	$I_{K(Ca)}$	
Big/large conductance (BK)	Ic	dTc/TEA
Small conductance	I_{ahp}	Apamin/4AP/Ba^{2+}
Calcium channels		
Transient	T	Ni^+, La^{3+}
Intermediate	N	Cd^{2+}, La^{3+}
Long	L	(+)PN-210-100, La^{3+}

Table 4.3. Effect of the calcium ionophore A23187 and trifluroperazine on the d.c. potential and the hyperpolarisation of isolated rat SCG to adenosine.

The response of ganglia to a 2 minute application of 100uM adenosine in physiological salt solution (PSS) was compared to the response to adenosine after a minumum of 20 minutes incubation in PSS containing 0.1 or 1uM A23187. In separate experiments, four ganglia were incubated in PSS and the response to a two minute application of 100uM adenosine determined and the response to 100uM adenosine determined after 20 and 60 minutes in 50uM trifluroperazine (TFP). Responses are means ± SEM in uV and number of ganglia tested = N. There was no significant difference (paired t-test) between the responses to adenosine for treated and untreated ganglia in A23187 or TFP at any incubation time.

Time of incubation minutes	Compound	Conc uM	N	Effect of compound on d.c. potential	Response to 100uM adenosine
0	A23187	0.1	4		-58 ± 9
0	A23187	1	6		-65 ± 9
0	TFP	50	4		-48 ± 7
20	A23187	0.1	4	18 ± 8	-67 ± 11
20	A23187	1	6	27 ± 23	-53 ± 7
20	TFP	50	4	10 ± 10	-50 ± 9
60	TFP	50	4		-55 ± 15

Table 4.4. Effect of apamin and d-tubocurarine on the response of the isolated rat SCG to adenosine.

The response to a two minute application of adenosine in physiological salt solution (PSS) was compared to that in PSS + antagonist after a minimum of 20 minutes incubation in the antagonist. There was no statistical difference (paired t-test) between any of the control and test groups. Responses are means ± SEM and number of ganglia (N). BSA = Bovine serum albumin at 80mg per litre of PSS.

ANTAGONIST			Response to					
			100uM adenosine		1000uM adenosine		12mM potassium	
Compound	Concentration uM	N	in PSS	in antagonist	in PSS	in antagonist	in PSS	in antagonist
Apamin	0.05	3	-83 ± 12	-77 ± 9				
Apamin + BSA	0.05	4	-56 ± 13	-53 ± 6	-111 ± 18	-86 ± 11		
Apamin + BSA	0.15	4	-56 ± 13	-49 ± 9				
Apamin	0.25	3	-77 ± 9	-73 ± 9			396 ± 42	447 ± 38
d-tubocurarine	100	3	-57 ± 3	-60 ± 12				

Table 4.6. Effect of different calcium channel antagonists on the response of isolated rat SCG to adenosine.

A minimum of 40 minutes incubation in physiological salt solution (PSS) + calcium channel antagonists was employed except for cadmium at 0.5mM where the response to adenosine was measured after 20 minutes incubation. The response to adenosine (2 minute applications) in PSS was compared to that in PSS + calcium antagonist and the statistical significance between control and test responses determined using a paired t-test and is denoted by * for $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$ where N = number of ganglia tested.

Antagonist	Conc mM	N	Response to antagonist uV	Response to 100uM adenosine in PSS uV	Response to 100uM adenosine in PSS + antagonist uV
Cobalt	2.5	4	125 ± 22	-38 ± 6[a]	-26 ± 8
"	5.0	8	170 ± 22	-41 ± 6[a]	-3 ± 3**
Lanthanum	0.02	2	20 ± 20	-57 ± 9[b]	-62 ± 9
"	1.0	6	1298 ± 245 (5)	-50 ± 6[b]	-45 ± 6
Cadmium	0.01	7	163 ± 47	-59 ± 3[a]	-74 ± 8
"	0.5	6	73 ± 66	-52 ± 10[a]	-63 ± 10
Nickel	0.5	3	293 ± 49	-60 ± 0	-58 ± 6
"	1.0	7	351 ± 64	-50 ± 4	-33 ± 6
Barium	2.5	4	518 ± 107	-58 ± 9[a]	-73 ± 5
(+)PN200 110	0.001	4	0 ± 0	-56 ± 6[c]	-81 ± 8*
Nitrendipine	0.001	4	0 ± 0	-80 ± 17[c]	-80 ± 23

a PO4/SO4 free PSS used for control PSS when using Co^{2+}, Cd^{2+} or Ba^{2+} added to PO4/SO4 free PSS for Co2+ PSS, Cd2+ PSS and Ba2+ PSS respectively.
b HEPES-PSS used for both control and La^{3+} containing PSS.
c Control PSS contained ethanol (2mM) and test PSS contained either (+) isomer of PN200 110 or nitrendipine in 2mM ethanol.

Table 4.7. Effect of cobalt on the response of the rat SCG to adenosine, minute potassium and muscarine.

Adenosine was applied for 2 minutes and potassium and muscarine were applied for one minute. The effect of cobalt on the response to adenosine was at least partially reversible as the response to 100uM adenosine recovered to 87 ± 33% (n=8) of the control response size after washing out 5mM Co2+ for 30 minutes. The statistical significance (paired t-test) is denoted by a * for $P < 0.05$ and ** for $P < 0.01$. N = number of ganglia examined.

Agonist	Concentration uM	N	Response (uV) to agonist in 2.5mM Co2+ PSS	5mM Co2+ PSS
Adenosine	100	4	-38 ± 6	-26 ± 8	-31 ± 13
"	100	8	-41 ± 6		-3 ± 3**
"	1000	4	-44 ± 11		10 ± 9
Potassium	12000	4	503 ± 59		515 ± 84
Muscarine	0.1	4	188 ± 48	60 ± 18	
"	0.1	8	164 ± 26		42 ± 6**

Table 4.8. The response of isolated rat SCG to muscarine and the effect of adenosine on the response to muscarine in the presence of metal cations, sucrose and calcium free PSS + EGTA.

All ganglia were bathed for a minimum of 40 minutes in the test compound except for a 20 minute incubation for 0.5mM cadmium. The response to muscarine (one minute) in physiological salt solution (PSS) was compared to that in PSS + test compound and the statistical significance between control and test responses determined using a paired t-test and is denoted by * for $P < 0.05$, ** $P < 0.01$ and *** for $P < 0.001$ where N = number of ganglia tested. NB: The response to 1uM muscarine in 2.5mM Ba^{2+} (= 150 ± 38uV) was greater than the response to 100nM muscarine.

TEST COMPOUND	Concentration mM	N	RESPONSE TO MUSCARINE (100nM) Control uV	Test uV	% DEPRESSION OF MUSCARINIC RESPONSE BY ADENOSINE (100uM) Control uV	Test uV
Cobalt	2.5	4	188 ± 48[a]	60 ± 18*		
"	5.0	3	140 ± 25[a]	45 ± 10**	-19 ± 0	+8 ± 22
Lanthanum	1.0	3	249 ± 28[b]	63 ± 9***	-26 ± 4	-33 ± 3
Cadmium	0.5	4	230 ± 19[a]	174 ± 29		
Nickel	1.0	4	243 ± 22	133 ± 27*		
Barium	2.5	4	228 ± 17[a]	70 ± 12**	-27 ± 7	-54 ± 11
0 Ca2+ + EGTA	2.0	2	200 ± 80	275 ± 55	-43 ± 3	-42 ± 1
(+)PN200-110	0.001	4	308 ± 23[c]	244 ± 37	-22 ± 10	-17 ± 4
Sucrose	20.0	4	234 ± 30	233 ± 17		
Li2+	10.0	4	205 ± 13[d]	205 ± 29	-31 ± 7	-34 ± 10

a,b,c - for solutions used see Key to Table 4.6; d - control PSS contained 20mM sucrose in normal PSS, and Li PSS contained 10mM LiCl in PSS.

Table 4.9. Effect of TEA and 4AP on the response of the isolated rat SCG to adenosine and potassium.

The response to a two minute application of adenosine and a one minute application of potassium in physiological salt solution (PSS) was compared to that in PSS + TEA or 4AP and the statistical significance (paired t-test) is indicated by * for $P < 0.05$ and ** for $P < 0.01$. Responses are means ± SEM and number of ganglia (N) and where a different number of ganglia were measured the number of ganglia recorded is given in brackets.

ANTAGONIST				Response to			
				Adenosine (100uM)		Potassium (12mM)	
Compound	Conc. uM	N	Change in d.c. potential by antagonist (uV)	in PSS	in PSS + antagonist	in PSS	in PSS + antagonist
4AP	100	2		-100, -40	-120, -50		
4AP	500	2		-100, -40	-100, -50		
4AP	1000	8	206 ± 21	-59 ± 3	-116 ± 13**		
4AP + TTX	(3000 + 1)	3		-77 ± 9	-133 ± 15*		
3,4- DAP	1000	4	109 ± 16	-55 ± 5	-78 ± 3*		
TEA	2000	4	324 ± 25[a]	-80 ± 0	-60 ± 4[b]*		
TEA	10000	6	328 ± 27 (3)[c]	-87 ± 13	-98 ± 16	543 ± 74	596 ± 77*
TEA + ATR	(10000 + 2)	5	170 ± 30 (2)	-44 ± 5	-52 ± 18		
TEA + TTX	(10000 + 1)	3		-63 ± 13	-72 ± 14		

a TEA (2mM) produced a rapid and sustained depolarisation which was maximal within 2 minutes and about twice the size of the response to a one minute application of 100nM muscarine (muscarine in PSS = 140 ± 10uV, N=4).

b The response to adenosine recovered to control values within an hour of the start of washing out TEA (adenosine 100uM after 50 minutes washout = -88 ± 13uV, N=4).

c TEA at 10mM caused a rapid depolarisation followed by a decline to a new steady level.

Table 4.10. Effect of potassium, ouabain, lithium and rubidium on the response of the isolated rat SCG to adenosine.

All ganglia were bathed for a minimum of 20 minutes in physiological salt solution (PSS) containing the test compound. The response to adenosine (2 minute application) in PSS was compared to that in PSS containing test compound and the statistical significance (paired t-test) is indicated by * for $P < 0.05$ and ** $P < 0.01$ and N = number of ganglia tested.

Test Compound	Conc. uM	N	Response (uV ± SEM) to Adenosine (100uM) in Test Compound	PSS	PSS + test compound
Potassium	8000	7	277 ± 28	-67 ± 5	-60 ± 5
Oubain	1000	3	100 ± 16	-98 ± 16	-133 ± 24
	10000	6	113 ± 45	-93 ± 17	-150 ± 8**
Lithium	10000	4	120 ± 9	-79 ± 4[a]	-88 ± 6
Rubidium	1000	4[b]	220 ± 83	-78 ± 21	-100 ± 20*
	6000	4[b]		-78 ± 21	-196 ± 45*

a = response to adenosine in PSS + 20mM sucrose.

b = Same ganglia used for recording responses to adenosine in 1mM and 6mM rubidium PSS.

Table 4.11: Effect of different concentrations of potassium on the response of the isolated rat SCG to adenosine

PSS = physiological salt solutions and responses to a two minute application of adenosine are uV mean ± SEM. The statistical significance (n = 3) was determined by unpaired t-test for group (a) and group (b), both not significantly different and paired t-test for group (c), not significant and group (d) $P < 0.05$.

Adenosine uM	Response to adenosine in PSS containing 2.5mM Ca^{2+}/ 1mM Mg^{2+} 6mM K^{+}	0mM Ca^{2+}/10mM Mg^{2+} 6mM K^{+}	2mM K^{+}
10	-33 ± 18[a]	-27 ± 15[a,c]	-21 ± 12[c]
100	-80 ± 10[b]	-80 ± 31[b,d]	-110 ± 25[d]*

Table 4.12. Effect of low chloride physiological salt solution on the response of the isolated rat SCG to adenosine, muscarine, dimethylphenylpiperazinium and gamma-aminobutyric acid

An attempt to measure the response to drugs in physiological salt solution (PSS) containing 2.5mM Cl^- was unsuccessful due to excessive drift in the base line. The base line in a low Cl^- PSS containing 8.5mM Cl^- was variable but the response to a two minute application of adenosine and one minute applications of muscarine, dimethylphenylpiperazinium (DMPP) and gamma-aminobutyric acid (GABA) were compared after a minimum of 1 hour in low Cl^- PSS. The response (uV, mean + SEM) of the agonist in normal PSS was compared to the response in low Cl^- PSS and the statistical significance (paired t-test) is indicated by a * for $P < 0.05$, N = number of ganglia tested., dep = depolarisation to GABA or DMPP and ahp = after hyperpolarisation to GABA.

Agonist	Concentration uM	N	PSS Normal (127.5mM Cl^-)	PSS Low Cl^- (8.5mM Cl^-)
Adenosine	100	3	-40 ± 6	-40 ± 12
Muscarine	0.1	3	183 ± 30	180 ± 35
10uM DMPP (dep)	10	2	420, 540	490, 320
GABA dep	10	3	817 ± 184	707 ± 109
GABA ahp	10	3	-127 ± 33	0 ± 0

Table 4.13. Effect of physiological salt solution containing either lithium, rubidium or ouabain on the response of isolated rat SCG to dimethylphenylpiperazinium.

The response to a two minute application of 10 uM dimethylphenyl piperazinium (DMPP) was recorded after a minimum of 20 minutes incubation in test physiological salt solution (PSS) and after 160 mins incubation in 10 uM ouabain. Responses are mean ± SEM, and N = number of ganglia tested. The response in PSS was compared to that in Li-HEPES, 1mM Rb-PSS or ouabain by a paired t-test (or unpaired t-test for a) and the statistical significance is indicated by * for $P < 0.05$.

		PSS			
Response to DMPP	N	Normal	Li-HEPES	1 mM Rb-HEPES	10 uM Ouabain
Depolarisation	4	343 ± 28	134 ± 37[a]	305 ± 31	
	4	378 ± 65			365 ± 78
Hyperpolarisation	4	-220 ± 54	0 ± 0[a*]	-310 ± 19	
	4	-158 ± 51			-145 ± 12

Table 4.14. Effect of furosemide on the response of the isolated rat SCG to gamma-aminobutyric acid and adenosine

Both the after hyperpolarisation produced by a one minute application of gamma-aminobutyric acid (GABA) and the response to a two minute application of adenosine in normal physiological salt solutions (PSS) were recorded. Responses are mean ± SEM, and N = number of ganglia tested. The response in normal PSS was compared to that in furosemide (paired t-test) and the statistical significance is indicated by * for $P < 0.05$ and ** for $P < 0.01$.

Agonist	Concentration uM	N	PSS	PSS + furosemide at 10uM	100uM
GABA	10	4	-58 ± 6	0 ± 0**	
	100	4		-78 ± 11	0 ± 0**
Adenosine	100	3	-75 ± 11	-55 ± 10*	-44 ± 13**

Table 4.15. Effect of cromakalim and lemakalim on the d.c. potential and lemakalim on the response of rat isolated SCG to muscarine

Cromakalim and lemakalim were applied for 2 to 5 minutes and muscarine (100nM) was applied for 1 minute in the presence of a 5 minute application of lemakalim. Values are responses of individual ganglia (uV).

Concentration uM	Change in d.c. potential in		% Change of response to muscarine in lemakalim
	Cromakalim	Lemakalim	
0.01	0, -30		
0.03	-20, -30, 0		
0.1	0		
0.3	0		
1	0	0	0, 10
3	0		
10	0		
30		-40, -10, 10	
100	0	0, 0, 0	8, 22

The application of lemakalim at 30uM or 100uM for 2 minutes during a depolarisation to pilocarpine (1uM, 1 minute) on three ganglia, did not alter the response to pilocarpine. In contrast the response to adenosine (100uM, 2 mins) in the presence of pilocarpine was potentiated (PSS = -73 + 9uV; -87 $\pm$ 35uV and during response to pilocarpine -102 $\pm$ 12uV, -153 $\pm$ 64uM, N = 3, respectively.

Table 4.16. Effect of diazoxide on the d.c. potential and the response to muscarine of the rat isolated SCG

Responses are mean ± standard error of the mean (SEM) and number of ganglia measured (N). At all concentrations of diazoxide tested there was no significant effect on the d.c. potential or on the response to muscarine.

A. Change in basal d.c. potential by diazoxide on the isolated rat SCG.

Application time (mins)	N	Concentration of diazoxide (uM) 10	100	300
2	3	0 ± 0	0 ± 0	0 ± 0
3	4		-5 ± 5	

B. Effect of diazoxide on the depolarisation produced by a one minute application of 100nM muscarine on the isolated rat SCG.

	Concentration of diazoxide (uM) 1	10	100	300
% Change in muscarinic response (mean ± SEM (N))	4 ± 7(7)	-6 ± 3(8)	-4 ± 3(8)	7 ± 9(6)
Response to diazoxide during 1st minute of application (uV, N = 8)	0 ± 0	0 ± 0	-30 ± 20	-40 ± 40

CHAPTER FIVE

INTERACTION OF ADENOSINE WITH DIFFERENT AGONISTS

Chapter 5: INTERACTION OF ADENOSINE WITH DIFFERENT AGONISTS

In part, the results of the preceeding chapters have relied on the ability of adenosine to depress the response to muscarine. In this chapter the selectivity of adenosine for other agonists and the possible intracellular messengers involved in the interaction with adenosine are discussed. There is evidence that ACh (Birks & MacIntosh, 1961), catecholamines (Brown & Caulfield, 1979), 5HT (Wallis, Williams & Wali, 1978), GABA (Wolff, Joo, Kasa, Storm-Mathiesen, Toldi & Balcar, 1986; Eugene, 1987), and vasointestinal polypeptide (VIP) (Volle & Patterson, 1982; Durroux, Barberis & Jard, 1987) have either a neuromodulatory or neurotransmitter function in the rat SCG and employ different ionic and biochemical mechanisms to mediate their effects. Thus an interaction or lack of interaction between these agonists and adenosine has provided useful clues for the identification of the mechanism of action of adenosine on the rat SCG. A range of agonist responses were examined in the presence of adenosine and the response to adenosine in the presence of some of these agonists was assessed.

5.1 Concentration response curves to different agonists.

The response to increasing concentrations of ACh, muscarine, 2-methyl-5-hydroxytryptamine (2Me5HT), vasoactive intestinal polypeptide (VIP) and oxotremorine-M (OXO-M) are summarised in figure 5.1. Each agonist produced concentration related depolarisations and DMPP, GABA and ACh also produced AHPs (Figs 5.2; 4.16). High concentrations of ACh were required to evoke a response of the ganglion, with muscarine being over 1,000 fold more potent than ACh on the same ganglia. The low potency of ACh on the rat SCG most probably was due to the presence

ADDENDUM CHAPTER 5- Please see insert in back pocket of thesis.

of a significant amount of acetylcholinesterase (AChE). The presence of AChE which predominates the presynaptic terminal is responsible for the destruction of liberated ACh (Koelle & Koelle, 1959) and the spontaneous release of ACh in the rat SCG can only be detected in the presence of an AChE inhibitor (Briggs, McAfee & McCaman, 1988). To avoid using high concentrations of ACh and selectively activate muscarinic receptors, AChE resistant analogues of ACh including muscarine, carbachol and methylfurmethide were used. The response to muscarine was comparable to that obtained in other laboratories and was abolished by N-methyl-atropine and pirenzepine (Table 5.1) suggesting the response to muscarine is due to the activation of M1 muscarinic receptors (Brown et al., 1980; Newberry & Priestley, 1987).

5.1.1 Effect of adenosine on the response to different agonists

The ability of adenosine to alter the response to submaximal responses of different agonists producing about equivalent sized responses was examined and the results are summarised in tables 5.2 and 5.3.

5.1.1.1 Effect of adenosine on the response to potassium

Increasing the total $[K^+]e$ to 8 or 12mM would be expected to cause a non-specific depolarisation of the ganglion. The inability of adenosine to alter the response to K^+ (Fig. 5.2a, Table 5.3) suggests the adenosine-induced hyperpolarisation does not necessarily alter the response of the ganglion to depolarising agonists.

5.1.1.2 Effect of adenosine on the response to GABA

The depolarisation of rat SCG to GABA and the ability to record a fast non-cholinergic depolarising postsynaptic potential from the rat SCG (Eugene, 1987) which is antagonised by bicuculline is good evidence for the existence of functional postsynaptic receptors for GABA in the rat SCG. Postsynaptic depolarisations of the rat SCG by GABA are accompanied by a small efflux of K^+ and a larger change in Cl^- efflux due to opending of Cl^- channels to decrease intracellular Cl^- activity (Ballanyi et al., 1984; Ballanyi & Grafe, 1985; Adams & Brown, 1975). During and after the GABA depolarisation a $Na^+/K^+/Cl^-$ cotransporter is activated to restore and maintain the $[Cl^-]i$ (see Chapter 4). The AHP to GABA was not significantly altered in the presence of adenosine.

The slight but significant potentiation of low concentrations of GABA by adenosine would be consistent with an increase in efflux of Cl^- and/or K^+ ions. As the response to adenosine was unaltered by the low chloride PSS (see Chapter 4), a condition which would favour the efflux of Cl^-, an increase response to GABA in the presence of adenosine may have occurred if adenosine increased the GABA mediated K^+ efflux. Overall the effects of adenosine on the response to GABA were moderate (Fig. 5.2b) and it appears unlikely that adenosine would significantly alter GABA-ergic transmission of the rat SCG in vivo.

5.1.1.3 Effect of adenosine on the response to nicotinic agonists

Adenosine can inhibit sympathetic neuroeffector transmission and it has been reported that adenosine at physiological concentrations potentiates pressor responses

to nicotinic agonists in vivo apparently by enhancing the effects of nicotine on sympathetic ganglia (von Borstel, Renshaw, Wurtman, 1984; Von Borstel, Evoniuk & Wurtman, 1984; 1986). The order of potency for the potentiation of the response to nicotine by adenosine suggests an action via an A2 receptor as the order of potency was:- N-cyclopropylcarboxamidoadenosine (CPCA) > 2CA > R-PIA > S-PIA with an R/S ratio of about 10. In partial agreement with von Borstel & colleagues, Henon & McAfee (1983b) found 2CA reduced single EPSP but facilitated ganglionic transmission during repetitive stimulation. The reduction in the single EPSP amplitude occurred without a change in the sensitivity of postganglionic neurones to bath applied carbachol (0.5uM), suggesting a presynaptic site of action for adenosine. The mechanism of these effects was not discussed but an inhibition of the Ca2+ activated K^+ current by adenosine (Henon & McAfee, 1983a) could increase repetitive firing to facilitate repetitive synaptic transmission and presynaptic inhibition of ACh release by adenosine (Briggs et al., 1988) would reduce the effect of a single CAP. Therefore the effects of adenosine reported in vivo experiments may have occurred due to an indirect action and not as a direct consequence of nicotinic stimulation.

The results presented in tables 5.2 and 5.3 and Fig. 5.2c, show adenosine did not alter the depolarisation to DMPP, a selective nicotinic agonist. Also the "fast" nicotinic depolarisation produced by carbachol at 10 and 30uM was unaltered by 100uM adenosine (Table 5.4). These findings are consistent with the lack of effect of adenosine on the sensitivity of the nicotinic receptor reported by Akasu and colleagues (Akasu et al., 1981; 1983a; 1985) using bullfrog sympathetic ganglia.

In vivo nicotine and adenosine may interact at several loci to modify autonomic activity and the effect of adenosine on ganglia and/or the end organs was not differentiated by von Borstel and colleagues (1986). The inability of adenosine to alter the response of the rat SCG to nicotinic depolarisations suggests the actions of adenosine in vivo are unlikely to have occurred at the end organs. Alternatively it may be argued that the use of a nonphysiological concentration of adenosine may have inhibited the potentiation of nicotinic responses of the ganglion. There is some evidence in favour of this hypothesis as adenosine at plasma concentrations of 3 to 4uM potentiated the response to nicotine but at higher concentrations of adenosine the potentiation was diminished. Even if the concentration of adenosine chosen was too high to record potentiation of DMPP it is clear from the results (Tables 5.2 and 5.3) that the major action of adenosine is to depress the response to muscarinic agonists. In addition the results presented in the next chapter suggest the adenosine receptors of the rat SCG are not of the A2 subtype as selective A2 agonists were weak or inactive on the SCG. If the potentiation of nicotinic responses in vivo is mediated via A2 receptors as judged from the data of von Borstel & colleagues (1986) then it is difficult to understand how adenosine can potentiate the nicotinic responses of the SCG. One mechanism that may have been recorded as an increase in the response to nicotine is an increase in the presynaptic release of ACh. However this seems unlikely as it has been reported by Briggs et al. (1988) that adenosine reduced ACh release from rat SCG in vitro.

Consistent with the inability of ouabain and Li-HEPES PSS to antagonise the response to adenosine (see chapter 4) adenosine did not alter the AHP produced by DMPP, suggesting adenosine does not hyperpolarise the rat SCG by

activating the electrogenic Na^+ pump. The inability of adenosine to alter the depolarisation to nicotinic agonists is also consistent with a lack of effect of adenosine on the response in low Na^+ or low Ca^{2+} PSS as carbachol is known to increase the influx of Na^+ and Ca^{2+} ions during nicotinic depolarisation.

5.1.1.4 Effect of adenosine on the response to 5HT

It is well documented that 5HT exerts excitatory actions on sympathetic ganglia and depolarisation of the ganglionic cell membrane by 5HT is reported to facilitate synaptic transmission (Eccles & Libet, 1961). The fast depolarisation of the rat SCG to 5HT is believed to be mediated by $5HT_3$ receptors (Fortune, Ireland & Tyers, 1985), and the AHP is mediated via the activation of the electrogenic Na^+ pump (Wallis & Woodward, 1975). In addition to these responses, 5HT also hyperpolarises the rat SCG due to an action mediated by $5HT_1$ receptors (Ireland, 1987; Ireland & Jordan, 1987; Gilbert & Newberry, 1987) and accumulates 5HT via an avid uptake system (Ireland, Staughan & Tyers, 1987). The ability of adenosine to alter the response to 5HT may thus be complicated by these factors. Using a submaximal concentration of 5HT only a depolarisation was observed (Fig. 5.2d), which was significantly enhanced by 100uM adenosine. The reason for this potentiation is unknown, but is unlikely to have been due to a potentiation of the $5HT_3$ mediated depolarisation as the depolarisation of the selective $5HT_3$ agonist, 2Me5HT was unaltered by the presence of 100uM adenosine (Cf. Fig. 5.2 d & e, Table 5.3). Results presented in chapter 4 and in this section it suggests that adenosine has no effect on responses mediated via activation of the electrogenic Na^+ pump and

it is therefore most unlikely that adenosine enhanced the response to 5HT by interacting with the Na^+ pump.

5.1.1.5 Effect of adenosine on the response to isoprenaline (ISO)

Among the agents known to increase the cAMP content of the rat SCG, isoprenaline is one of the most potent (Cramer, Johnson, Hanbauer, Silberstein & Kopin, 1973; Quenzer, Yahn, Alkadhi & Volle, 1979; Quenzer, Patterson & Volle, 1980; Briggs et al., 1988) and at 1uM the application of isoprenaline for 4 minutes to the rat SCG increased the cAMP content by ten fold (Quenzer et al., 1980). Adenosine at 10uM produced a small but significant reduction in the response to 100nM isoprenaline and at 100uM adenosine produced a similar reduction of the response to isoprenaline which was not significant. Given that isoprenaline would be expected to produce a significant increase in the cAMP content of the SCG it is difficult to understand why the response to isoprenaline was not dramatically reduced by adenosine (Fig. 5.2e), if adenosine activates A1-adenosine receptors to reduce cyclic AMP levels.

5.1.1.6 Effect of adenosine on the response to muscarinic agonists

From the results presented in tables 5.2 & 5.3 and Fig. 5.2g,h,i, it can be seen that adenosine selectively depressed the response of the rat SCG to muscarinic agonists. The response to 100nM muscarine was reduced by 10 and 100uM adenosine in a concentration-dependent manner (Figs. 5.3 and 5.6).

The ability of adenosine to depress the response to muscarinic agonists was dependent both on the agonist and the level of depolarisation. Both the depression of the response to 100nM muscarine by 100uM adenosine (Fig. 5.4a) and the depression of increasing concentrations of muscarine by adenosine (Fig. 5.4b) was biphasic and maximal at about 100nM muscarine and 100uM adenosine declining at higher concentrations of muscarine or adenosine.

The ability of adenosine to reduce the response to submicromolar concentrations of muscarine is postulated to arise from an action of adenosine on a specific component of the response to muscarine. Some reports indicate the effects of muscarine are concentration-dependent with the induction of different ionic conductances at different concentrations of muscarine. Whatever the explanation for the depression of muscarinic agonists by adenosine, it was clearly complex and not directly related to the degree of depolarisation as the response to non-muscarinic agonists was similar but these responses were either unaltered or enhanced in the presence of adenosine (Fig. 5.2, Tables 5.2 and 5.3).

The depression of muscarinic responses by adenosine was agonist dependent and similar depolarisations did not necessarily produce the same depression of the agonist response (Fig. 5.2a-i; Tables 5.3 and 5.4). In addition to muscarine, adenosine depressed the response to carbachol and MeF (Fig. 5.2h-i; Table 5.2) to similar extent at 10uM adenosine, although the depression of carbachol was not significant. At 100uM adenosine, the depression of muscarine was about twice that produced by adenosine on carbachol and MeF (Table 5.3). The depression of carbachol by adenosine was variable and at some concentrations of carbachol, adenosine was weak or

ineffective (Table 5.4). Given muscarinic agonists not only depolarise the rat SCG but also hyperpolarise the ganglion via M2 muscarinic receptors the low potency of adenosine on the response to carbachol may arise from the ability of adenosine to alter both the M2 mediated hyperpolarisation and the M1 depolarisation. Enhancement of the M2-mediated hyperpolarisation would increase the depolarisation to antagonise the depression of the response to carbachol by adenosine.

It is possible that the inability of MeF to generate a hyperpolarisation to combat increased antagonism of the depression of the depolarisation to MeF would reduce the effectiveness of adenosine. Whereas an increase in the M2 mediated hyperpolarisation to increasing concentrations of carbachol and muscarine may potentiate the depression by adenosine.

5.2 Effect of adenosine on the muscarinic hyperpolarisation of the SCG

As there are no M2 receptor selective agonists the ability of adenosine to alter the M2 receptor mediated response to muscarinic agonists was assessed in two sets of experiments. In the first series the depression of the response to muscarinic agonists was measured in the presence of an M2 muscarinic receptor antagonist, methoctramine (MTO). In a second series of experiments the ability of adenosine to alter the hyperpolarisation to carbachol in low calcium PSS containing pirenzepine, an M1 receptor antagonist was determined.

5.2.1 Effect of adenosine on the response to muscarinic agonists in the presence of MTO

It is reported that MTO selectively antagonises the muscarinic hyperpolarisation of the rat SCG in vitro by Field & Newberry (1988). Using the same concentration (0.3uM) of MTO reported by Field & Newberry (1989) to selectively reduce the hyperpolarising response to muscarine it was found that the response to MeF was significantly reduced (Table 5.5). This result was unexpected as MeF is reported to be a highly selective M1 receptor agonist on the rat SCG and does not hyperpolarise the rat SCG (Newberry & Connolly, 1989; see below). Thus these results suggest that MTO antagonises the hyperpolarisation to muscarine but may also produce a small but significant depression of the depolarisation to muscarinic agonists i.e. the M1-mediated response. An examination of a more detailed account of the actions of MTO on the response of the rat SCG to muscarine (see Fig. 1 in Field & Newberry, 1989) suggests a reduction of the depolarisation to muscarine by MTO at 0.3uM. The depression of both M1 and M2 mediated responses in MTO may complicate the interpretation of the results presented here in that the depression of muscarinic agonists by adenosine was found to be dependent on the degree of depolarisation by these agonists (Table 5.4). However the inability of MTO to alter the response to adenosine is consistent with the inactivity of pirenzepine on the response to adenosine suggesting that the hyperpolarisation to adenosine is independent of any presynaptic release of ACh. The inability of MTO to antagonise the depression of muscarinic responses by adenosine indicates the adenosine-mediated depression was not due to an increase in the M2-receptor mediated hyperpolarisation.

5.2.2 Effect of adenosine on the carbachol-induced hyperpolarisation of the SCG

The similar potency of the depression of different muscarinic agonists by adenosine in MTO suggests the presence of an M2-mediated hyperpolarisation may antagonise the actions of adenosine. The ability of adenosine to alter the M2-receptor mediated response to carbachol was investigated. If the presence of an M2-mediated hyperpolarisation is responsible for the low potency of adenosine on the response to carbachol then it would be expected that carbachol would be the most potent muscarinic agonist to hyperpolarise the ganglion. The results presented in Fig. 5.8 show that the relative potency order for muscarinic agonists to hyperpolarise the SCG was:-

Agonist: Carbachol > OXO-M > muscarine >> methylfurmethide

estimated
EC_{50} (nM) > = 210 > 300 > 800 >> 10000

Carbachol at up to 1uM produced concentration dependent hyperpolarisations (Fig. 5.9a) and at 10uM the hyperpolarisation was followed by a fast nicotinic depolarisation. Although the fast depolarisation was antagonised by dihydrobetaerthroydine (DHBE), a selective nicotinic antagonist, the carbachol hyperpolarisation was also reduced. The poor selectivity of nicotinic antagonists versus the nicotinic and muscarinic responses of the rat SCG to electrical stimulation has been reported by Newberry & Connolly (1989) and a nicotinic antagonist was not used to avoid any adverse effects on the responses to carbachol and adenosine.

The inability of adenosine to alter submaximal of near maximal hyperpolarisations to carbachol (Table 5.6) is illustrated in Fig. 5.9b, where adenosine reduced the depolarisation and not the hyperpolarisation to 3uM carbachol (n=2) (Fig. 5.9c). The inability of adenosine to reduce the M2-mediated hyperpolarisation indicates that the depression of muscarinic responses is due to the selective reduction of the M1-mediated muscarinic depolarisation.

Further evidence in favour of this hypothesis is provided by the ability of both pilocarpine, an effective M1 agonist on the rat SCG (Caulfield & Stubley, 1982) and N-methyl-N-(1-methyl-4-pyrrolidino-2-butynyl)acetamide (BM-5) an oxotremorine analogue with presynaptic antagonist and postsynaptic agonist properties (Casomenti, Cosi & Pepeu, 1986) to enhance the hyperpolarisation to adenosine (Table 5.8). These results indicate that the potentiation of adenosine occurs via an interaction with postsynaptic M1 receptors.

If adenosine and carbachol act upon the same intracellular mechanism then once fully activated by carbachol no greater effect would be obtained. The inability of 1uM carbachol and adenosine (10uM) to attain the same response size as the sum of the individual responses suggests both agonists may hyperpolarise the rat SCG via a common mechanism. Recently it has been reported that many inhibitory neurotransmitters activate an inwardly rectifying K^+ channel by a mechanism that involves a pertusis toxin sensitive G-protein (Trussell & Jackson, 1989; Newberry & Gilbert, 1989). Given that muscarinic hyperpolarisation of amphibian ganglia increases gK (Gallgher, Shinnick-Gallagher, Cole, Griffith & Williams, 1980) and that adenosine mediates the sIPSP of cat parasympathetic ganglia (Akasu et al., 1984), both the

adenosine and carbachol-induced hyperpolarisation of the rat SCG may be mediated by a common mechanism i.e., increased gK (Chapter 4).

5.3 Competitive depression of the response to muscarine by adenosine

Adenosine at 10uM and 100 uM produced parallel shifts in the concentration response curve to muscarine (Fig. 5.6) suggesting adenosine is a competitive antagonist of muscarine. It seems unlikely that adenosine is a functional antagonist of the depolarisation to muscarine as adenosine would have been expected antagonise the response to other agonists to the same extent and this was not the case (Table 5.1). However it may be argued that some form of functional antagonism of muscarine occurred as there was a good correlation between the change in uV between the response to muscarine in the presence and absence of adenosine and the size of the hyperpolarisation to adenosine, as recorded during the first minute of application (Fig. 5.4a).

If the depression of the response to muscarine by adenosine is simply due to functional antagonism then it would be expected that any other compound that hyperpolarise the ganglion would also reduce the response to muscarine. It has been reported that the effect of the $GABA_B$ agonist baclofen (BAC) and adenosine are similar in a number of tissues, e.g. both compounds decrease synaptic excitability of isolated hippocampal cells (Newberry & Nicoll, 1985) and hyperpolarised the rat SCG (unpublished observations: Newberry & Gibert, 1989). In order to examine the possibility that the two effects of adenosine to hyperpolarise the ganglion and depress the response to muscarinic agonists are directly interelated, the effects

of adenosine were compared to the activity of (-)baclofen using the same paradigm described for adenosine. As can be seen from figure 5.5 (-)baclofen produced hyperpolarisations comparable to adenosine but was less potent on the depression of the response to muscarine, producing a maximal reduction of muscarine of 11 $\pm$ 3% ((-)baclofen 10uM, $P<0.05$, $n=7$) about a third of that achieved by adenosine. These results suggest the hyperpolarisation by adenosine and the depression of the response of the rat SCG to muscarine by adenosine may be concomitant but hyperpolarisation of the ganglion may not be the cause of the depression of muscarinic responses.

Further support for this view point is provided by the data shown in Fig. 5.4b which illustrates the hyperpolarisation to 100uM adenosine was consistent but the depression of the response to muscarine was dependent upon the concentration of muscarine.

5.4 Effect of depolarisation with various agonists on the response of the SCG to adenosine

5.4.1 Effect of potassium on the adenosine-induced hyperpolarisation

The RMP (Em) of the rat SCG has been measured by several research groups and is around -50 to -70mV being predominantly determined by the K^+ transmembrane gradient (Blackman, Ginsborg & Ray, 1963; Kosterlitz et al., 1968). When $[K^+]e$ is raised, the K^+ concentration gradient is decreased, and the neurones will be depolarised (the reduction of the RMP can be estimated from substituting values for $[K^+]i$ and $[K^+]e$ in to the Nernst equation) to generate an increased "driving force (Em-Ek)" for potassium. The response to adenosine during the

depolarisation to K^+ was unaltered suggesting depolarisation per se does not enhance the hyperpolarisation to adenosine.

5.4.2 Effect of isoprenaline on the adenosine-induced hyperpolarisation

Adenosine has been reported to have no effect on a gCa of the SA-node of the rabbit heart unless these cells were first activated by isoprenaline (Belardinelli, Giles & West, 1988). If a similar phenomenon exists in rat SCG neurones then it is possible that the lack of effects of calcium antagonists on the response to adenosine and its interaction with muscarine reported in Chapter 4 may not have been studied under conditions which would produce an influx of Ca^{2+} into postganglionic neurones. However the inability of the isoprenaline to alter the response of ganglia to adenosine (Table 5.7) does not support this hypothesis.

Depolarisation of the rat SCG by isoprenaline increases ganglionic cAMP (Quenzer et al., 1980; Petit, Barberis & Jard, 1988) and if adenosine receptor activation modulates cAMP as has been reported for arginine vasopression of the rat SCG (Petit et al., 1988) then it is surprising that isoprenaline did not alter the response to adenosine. These results are consistent with the lack of effect or small reduction of the response to isoprenaline by adenosine and indicate the effects of adenosine may not be mediated via a change in cAMP.

5.4.3 Effect of VIP on the adenosine-induced hyperpolarisation

At 0.1uM VIP is reported to increase the cAMP content of the rat SCG by about four fold (Volle & Patterson, 1982; but see Audigier, Barberis & Jard, 1986) and at higher concentrations (> 1uM) potentiate the response to muscarinic stimulation by carbachol (Kawatani, Rutigliano & De Groat, 1985). In the present study 0.1uM VIP did not alter the response to adenosine or muscarine (Table 5.1) suggesting an increase in cAMP may not facilitate these responses.

5.4.4 Effect of Ba2+ on the adenosine-induced hyperpolarisation

There are several possible mechanisms that may be responsible for the potentiation by Ba2+ of the response to adenosine. Firstly Ba2+ might cause the release of ACh from presynaptic terminals, as it does in cholinergic motor neurones. However the preceeding results suggests that the actions of adenosine are due to a direct action on postganglionic neurones. Furthermore, the rapid response of sympathetic ganglia to Ba2+ recorded by both Takeshige & Volle (1964) and Tashiro & Nishi (1972) was antagonised by dTC to leave a slow depolarisation which was not antagonised by atropine (Adams et al., 1982a), suggesting the effects of Ba2+ are directly on the postganglionic neurones. The second possibility is that Ba2+ penetrates in to ganglion cells via Ca2+ channels (Tashiro & Nishi, 1972) and this would be expected to decrease neuronal RMP and depolarise the ganglion and could potentiate the response to adenosine. It is likely that the ability of Ba2+ to decrease the permeability of the ganglion cell membrane to K^+, i.e. K^+ channel

antagonism inhibits Im as PSS containing Ba2+ has been reported to substantially reduce the M-current (Constanti et al., 1981a; Tsuyi & Kuba, 1988). Fourth, Ba2+ might activate protein kinase C (PKC) in the ganglion as suggested for the C-kinase purified from heart (Wise, Raynor & Kuo, 1982). If PKC is involved in the mechanism of the slow muscarinic excitation (see section 5.4) an increase in the activated PKC by Ba^{2+} would result in the potentiation of the adenosine hyperpolarisation. Finally, 4mM Ba^{2+} has been shown to block Im of amphibian ganglia (Tsuji & Kuba, 1988) and enhance an outward current (I_D). If I_D is present in the rat SCG an enhancement of I_D would be predicted to augment the response to adenosine.

The application of Ba^{2+} for long periods can lead to unexplained changes in rat SCG neurones (Adams et al., 1982a). Adams & colleagues reported that brief applications of 4mM Ba^{2+} produced effects fully compatible with inhibition of Im, comprising of a membrane depolarisation accompanied by an increased input resistance and increased excitability. However, prolonged application of Ba^{2+} resulted in irreversible depression of large outward currents, due to entry of Ba^{2+} through Ca^{2+} calcium channels and a progressive intracellular accumulation of Ba^{2+} (Hagiwara & Byerly, 1981). It is possible that prolonged treatment of the rat SCG with Ba^{2+} antagonised an outward current activated by adenosine, and may account for the loss of the potentiation of the hyperpolarisation to adenosine.

Even though the potentiation of the hyperpolarisation to adenosine by Ba^{2+} disappeared within 30 minutes incubation in Ba^{2+}-PSS, Ba^{2+} still potentiated the depression of the response to muscarine by adenosine, suggesting the actions of Ba^{2+} on the rat SCG are linked to the interactions of adenosine and muscarine. As reported for many divalent

cations, the actions of Ba^{2+} may not be specifically on Im and other potassium currents such as I_{KCa} (Connor, 1979), and Ik may be inhibited as well (Constanti et al., 1981b). The potentiation of Im and experiments in the next section examine this possibility.

5.4.5 Effect of nicotinic depolarisation on the adenosine-induced hyperpolarisation

In contrast to the results of Smith & Zidichouski (1985) where the effect of noradrenaline was enhanced during a nicotinic depolarisation, the response to adenosine in the presence of nicotine was unaltered (Fig 5.7). Smith & Zidichouski concluded that the response to noradrenaline was mediated by an increase in gK in a manner similar to that suggested for the reported effects of adenosine during the application of muscarinic agonists.

5.4.6 Effect of muscarinic agonists on the adenosine-induced hyperpolarisation

The hyperpolarisation to adenosine was augmented in the presence of muscarine or pilocarpine but not during depolarisations to potassium, isoprenaline, forskolin or nicotine (Tables 5.7 and 5.8). These results suggest that the potentiation of the response to adenosine is unlikely to have occurred simply due to a voltage shift to enhance the driving force on the adenosine mediated ionic conductance. The potentiation of the response to adenosine may occur through various mechanisms which could be common to both of these agonists or via an independent mechanism.

A similar potentiation of the hyperpolarisation to NA when applied during a muscarinic depolarisation of the rat SCG has been reported by Brown & Caulfield (1979). Based on an investigation of the ionic conductances of single cells Brown & Caulfield suggested that muscarine and NA produced their effects via separate voltage-sensitive components, i.e. via an action of muscarine on Im and NA on I_{KCa} or gNa (Brown & Caulfield, 1981). In contrast it has been reported by Selyanko, Smith & Zidichouski (1990) that muscarine and adrenaline can have opposite effects on M-channels recorded from amphibian sympathetic ganglia.

5.5 Effect of compounds reported to suppress the M-current on the response of the SCG to adenosine

Complex and concentration-dependent effects of muscarine on sympathetic ganglia have been reported by many research groups and involve a variety of ionic conductances including Im, I_X, I_D and other currents (Brown & Selyanko, 1985a,b; Brown et al., 1989; Mochida & Kobayashi, 1986a,b; Tokimasa, 1984; Tokimasa & Akasu, 1990a; Tsuji & Kuba, 1988). However, on the rat SCG the predominant effect of muscarine is to increase neuronal excitability to produce a 'slow' depolarisation mediated by a decrease in gK (Weight & Voltava, 1970; Kuba & Koketsu, 1974, 1976, 1978; Brown & Adams, 1980) as a consequence of inhibiting the outwardly rectifying K^+ current, Im (Brown & Constanti, 1980).

The results discussed in Chapter 4 suggest the effects of adenosine could be due to an increase in gK and given the reported decrease in gK by muscarine, it would be predicted that adenosine and muscarine would strongly interact so that the adenosine induced hyperpolarisation would be enhanced during a muscarinic depolarsation and

the muscarinic depolarsation would be depressed in the presence of adenosine. The results of experiments presented in this chapter and chapter 4 support this hypothesis.

Many chemically diverse compounds suppress Im and Brown (1988) suggested the mechanism for the transduction of receptor activation and the closure of M-channels in the rat SCG is indirect. Subsequently, it was reported that the inhibition of Im requires the activation of a G-protein as the addition of non-hydrolysable GTP analgoues, GTP-beta-S or Gpp(NH)p to dialysed rat SCG neurones either inhibited Im or potentiated and prolonged the inhibition produced by muscarine, LHRH or substance P (Brown, Marrion & Smart, 1989). Conversely addition of GDP-beta-S into amphibian or rat sympathetic neurones reduced the inhibition of Im by muscarine (Pfaffinger, Leibowitz, Bosma, Almers & Hille, 1988b; Brown et al., 1989).

There are a number of compounds including muscarine (Brown & Adams, 1980; Constanti & Brown, 1981) that inhibit Im including LHRH (Adams & Brown, 1980; Jones, 1987), ATP but not adenosine (Akasu, Hirai & Koketsu, 1983a,b) and adrenaline (Akasu, 1989)), substance P (Adams, Brown & Jones, 1983) on bullfrog ganglia and LHRH and UTP (Adams et al., 1982a) on the rat SCG. The effect of some of these compounds reported to inhibit Im on the response of the rat SCG to adenosine is summarised in table 5.8. Unfortunately most of these agonists produced small depolarisations (UTP and LHRH) and the concentrations employed may not have been sufficient to inactivate sufficient M-current to alter the response to adenosine. ERP produced a similar depolarisation and potentiation of the adenosine hyperpolarisation (although not significant) and may indicate inhibition of Im potentiates the hyperpolarisation to adenosine.

5.6 The role of secondary messengers in the response of the SCG to adenosine and muscarine

Two most frequently proposed biochemical transduction systems used to explain the actions of adenosine are the adenylate cyclase (AC)/cAMP system, and the PI/PKC system.

5.6.1 The adenylate cyclase/cAMP system

One of the first indications that the actions of adenosine were related to modifications of adenylate cyclase activity was described by Sattin & Rall (1970) who showed adenosine increases cAMP accumulation in the guinea-pig cerebral cortex. Further evidence was provided by Van Calker et al. (1979) who demonstrated that adenosine and its analogues can stimulate or inhibit cAMP accumulation in cultured brain cells and proposed that two different receptors are involved in the increase in cAMP (A2) and decrease (A1) in cAMP. However, since these early studies it has become apparent this classification of P1 purinoceptors may be too restricted and not all adenosine receptors are coupled to a change in cAMP concentration.

Greengard (1976) proposed that cyclic nucleotides have a major role in postsynaptic transmission of sympathetic ganglia, and that cAMP and cGMP exert long term control of neuronal excitability (McAfee & Greengard, 1972). Both the response to muscarinic agonists and the response to adenosine in some tissues is mediated via a change in the level of cyclic nucleotides (Stone, 1981, 1989) and a change in the cyclic nucleotides content of the rat SCG may be responsible for the interaction of muscarinic agents with adenosine.

It has been proposed that muscarinic depolarisation of the SCG is mediated through an increase in cGMP (Weight, Petzold & Greengard, 1974; Kobayashi, 1982; Kebabian, Steiner & Greengard, 1975). This idea was strengthened by the observation that cGMP mimicked the excitatory effects of ACh on pyramidal neurones (Stone & Taylor, 1977). Complex effects of cGMP and derivatives have been reported, and on rabbit SCG, McAfee & Greengard (1972) found dibutyryl-cyclic-guanosine 3':5'-monophosphate (DbcGMP) produced a small transient hyperpolarisation followed by a larger depolarisation. Dun and colleagues confirmed that DbcGMP depolarised postganglionic neurones but also produced a long acting and variable AHP (Dun, Kaibara & Karczmar, 1978). It has, however, also been reported that cGMP and its derivatives had no consistent effect on the RMP when iontophoresed or microinjected in to rabbit SCG neurones (Buiss, Weight & Smith, 1978).

In support of the hypothesis that muscarinic agonists depolarise the rat SCG by increasing cGMP both Volle, Quenzer, Patterson, Alkadhi & Henderson (1981) and Briggs, Whiting, Ariano & McAfee (1982) reported muscarinic agonists increased cGMP two fold. Carbachol has also been found to increase cGMP levels of rabbit SCG by two fold (McAfee -in Volle et al., 1981), and by six to eight fold in guinea-pig SCG (Wamsley, West, Black & Williams, 1979) and in both cases the increase in cGMP was antagonised by atropine. An increase in cGMP in response to muscarinic agents has also been reported using bovine SCG (Kebabian et al., 1975) and rabbit SCG (Takahashi, Mochida & Kobayashi, 1988). The ability to immunohistologically localise cyclic nucleotides and the increase in cGMP by carbachol to the postganglionic neurones of the rat SCG (Ariano, Briggs & McAfee, 1982) strengthens the hypothesis that the muscarinic depolarisation is mediated by cGMP.

However other biochemical and electrophysiological studies do not support this association. Brown et al. (1980) reported that muscarine at 1 or 100uM applied for 2 to 5 minutes did not alter resting levels of cGMP of the rat SCG. Also an increase in cGMP by eight fold by sodium azide was shown by Volle and colleagues not to alter the resting d.c. potential or the response to bethanechol (Volle et al., 1981). It has also been noted that when DbcGMP is reported to depolarise rabbit or rat SCG, the depolarisation was accompanied by an increase in membrane conductance (gM) (Dun et al., 1977, 1978; Gallagher & Shinnick-Gallagher, 1978) and in contrast there is either no change or a decrease in gM during the response to the sEPSP (Kuba & Koketsu, 1978), an observation incompatible with the hypothesis that cGMP-mediated the sEPSP. However Hashiguchi, Ushiyama, Kobayashi & Libet (1978) also found an increase in gM, but when the membrane of the rabbit SCG was voltage clamped there was no change in gM and it was concluded these effects are compatible with the mediation of the sEPSP by cGMP. Thus cyclic GMP appears to mediate at least one of the components in the mechanism underlying the sEPSP of the rabbit SCG (Kobayashi, 1982) and more recent studies by Kobayashi and colleagues suggest that muscarine and cGMP and its derivatives phosphorylate the same endogenous protein, and that cGMP appears to be involved in a part of the sEPSP (Takahashi et al., 1988).

The results of this study suggest cGMP depolarises the rat SCG, although the responses to cGMP were smaller than those to muscarine (Fig. 3.10), but are in agreement with Gallagher & Shinnick-Gallagher (1978) who found exogenous DbcGMP (250uM) depolarised the rat SCG, and in partial agreement with Brown & colleagues who found cGMP and 8BrcGMP at 1mM depolarised two out of ten rat SCG and was ineffective on the other eight (Brown et al., 1980). Thus an increase in cGMP may mediate some part of the response

of the rat SCG to muscarine but not the majority of the response.

For many cell types stimulation of muscarinic receptors has two consequences: an increase in PIT and a change in intracellular cGMP or cAMP, e.g. the hydrolysis of PIT due to the stimulation of M1 receptors in a transfected cell line stimulates adenylate cyclase to increase cAMP (Felder, Kanterman & Axelrod, 1989) and muscarine increases cAMP in NE-115 cells (Tsunoo & Narahashi, 1987).

Alternatively muscarinic stimulation can cause a reduction of the cAMP content of tissues, e.g. in 1321N1 human astrocytoma cells muscarinic stimulation reduces cAMP via the activation of a Ca^{2+}/CaM sensitive PDE (Meeker & Harden, 1982). Muscarinic agonists are reported to increase the cAMP content of the rabbit SCG (Kalix, McAfee, Shorderet & Greengard, 1974) and cAMP has been reported to mediate a dopamine induced long term enhancement (LTE) of the muscarinic response of rabbit SCG (Libet et al., 1975).

The intracellular injection of cAMP into postganglionic cells caused a sustained potentiation of the sEPSP of rabbit SCG (Kobayashi, Hashiguchi & Ushiyama, 1978) and thus would be expected to counteract the depression of muscarine by adenosine, if LTE occurs in the rat SCG. The evidence for the change in cAMP upon muscarinic stimulation in the rat SCG is conflicting. Briggs & colleagues found an increase in cAMP (Briggs et al., 1982) where as Volle and co-workers (Volle et al., 1981) found no such change in intracellular cAMP. Likewise the application of extracellular or intracellular cAMP and its derivatives has been found to depolarise (Akasu & Koketsu, 1977; Hsu & McIsaac, 1978; Brown & Dunn, 1983; Gallagher & Shinnick-Gallagher, 1977), hyperpolarise (McAfee &

Greengard, 1972; Machova & Kristofova, 1973; Brown et al., 1979; Brown & Dunn, 1983) or have no effect on SCG (Dun et al., 1977; Dun & Karczmar, 1977; Gallagher & Shinnick-Gallagher, 1977; Busis et al., 1978).

The results of experiments described in this thesis show that compounds known to increase cAMP such as 8BrcAMP, isoprenaline, forskolin and theophylline depolarised the rat SCG. In addition a depolarisation by forskolin of similar magnitude to that produced by muscarine enhanced the hyperpolarisation to adenosine (Table 5.7), suggesting an increase in cAMP enhances the response to adenosine and its effects may result from a decrease in cAMP. Thus the depression of muscarinic responses by adenosine may be due to the antagonism by adenosine of an increase in cAMP generated during muscarinic stimulation.

Some evidence for the dual regulation of Im by agents that increase cAMP to stimulate Im and for muscarinic stimulation to decrease Im has been described for both smooth muscle cells of the toad (Sims, Singer & Walsh, 1988) and adrenaline has been found to increase Im in sympathetic ganglia of the frog (Selyanko et al., 1990) although the application of extra- or intracellular cAMP has been reported not to alter Im of sympathetic ganglia (Adams et al., 1982a; Brown & Adams, 1987; Brown et al., 1989). However other effects of cAMP have not been excluded, including the antagonism of the muscarinic depolarisation via the activation of a conductance distinct from Im and similar to that recently described H-current of bull-frog sympathetic neurones (Tokimasa & Akasu, 1990a).

In an analogous manner to that found in rat or guinea-pig brain the action of adenosine on the rat SCG may occur due to a change in cAMP levels via an effect on adenylate

cyclase activity (Dunwiddie & Fredholm, 1984; Yeager, Nelson & Storm, 1986; Donaldson, Brown & Hill, 1988) or PDE activity (Smellie, Davis, Daly & Wells, 1979; De Mazancourt & Giudicelli, 1984).

The response of the rat SCG to muscarine may be a composite of an increase in cAMP and/or cGMP in order to discover if these cyclic nucleotides are involved in the response of the rat SCG to adenosine or the depression of muscarinic responses the following experiments were performed. Firstly the response to adenosine was assessed in the presence of selective inhibitors of the breakdown of cAMP and cGMP, i.e. the cAMP PDE inhibitors 4-(3,4-dibutoxybenayl)-2-imidazolidinone (Ro 20-1724) and denbufylline (BRL 30892), and a cGMP PDE inhibitor, M&B 222,948. Secondly the depression of muscarinic responses by CPA was tested in the presence of an adenylate cyclase inhibitor SQ 22,536 or Ro 20-1724. The proposed sites of action of these compounds is shown in Fig. 5.11.

5.6.1.1 Effect of adenylate cyclase inhibition on the response to adenosine

One model for sympathetic neurones are PC12 cells which respond to adenosine by the activation of purinoceptors and an increase in cAMP due to the activation of adenylate cyclase (Guroff, Dickens, End & Constantin, 1981). If the PC12 cell line is representative of the rat SCG, which hyperpolarises to A1 receptor agonists (Chapter 6) then the inhibition of adenylate cyclase would be predicted to reduce both the response to muscarine and its depression by CPA.

SQ 22,536 is a potent inhibitor of adenylate cyclase (Harris, Asaad, Phillips, Goldenberg & Antonaccio, 1979)

and at 100uM blocked the increase in cAMP generated by the application of isoprenaline to the rat SCG (Brown & Dunn, 1983). However the same concentration of SQ 22,536 did not alter the depression or the response to muscarine (Table 5.10) indicating the depression of muscarinic agonists by adenosine is unlikely to be due to an interaction with adenylate cyclase. Likewise the inability of SQ 22,536 to alter the depolarisation to muscarine, suggests the increase in cAMP reported upon muscarinic stimulation does not arise from an indirect stimulation of adenylate cyclase via an increase in $[Ca^{2+}]i$ due to the stimulated hydrolysis of inositol lipids. SQ 22,536 is reported to be a "P-site" selective adenosine agonist (Fredholm & Lingren, 1984) (Chapter 1, table 1) and as such the inability of SQ 22,536 to alter the d.c. potential of the rat SCG indicates the hyperpolarisation of the ganglion is unlikely to occur via a decrease in cAMP content.

5.6.1.2 Effect of PDE inhibitors on the response to adenosine and depression of muscarinic responses

The effects of PDE inhibitors on the response of the rat SCG may be complicated by the presence of multiple forms of cyclic nucleotide PDE.

Four major isozymes have been described: one with Ca^{2+}/CaM stimulated activity (PDE I), one having activity with a preference for cGMP (PDE II) and two preferring cAMP, with different Michaelis constants (Km) for cAMP and sensitivity to cGMP (PDE III and PDE IV) (Thompson & Appleman, 1971; Weishaar, 1987; Beavo, 1988; Beavo & Reifsnyder, 1990).

Histological studies have indicated a postsynaptic localisation of cAMP PDE in central cerebral cortex (Florendo, Barnet & Greengard, 1971) and the rat SCG (Vente, Garssen, Tilders, Steinbusch & Schipper, 1987) and a cGMP PDE in rabbit SCG (Quenzer, Yahn, Alkadi & Volle, 1979). Furthermore two forms of cAMP PDE have been characterised from crude enzymes isolated from the guinea-pig SCG by Capuzzo, Biondi, Borasio, Ferretti & Fabbri (1986), one with a high affinity for cAMP (Km 1uM) which was weakly inhibited by the non-selective PDE inhibitor, IBMX (Ki 90uM) and may be similar to the isozyme, PDE IV extracted from the mammalian brain. The second PDE had a low affinity for cAMP (Km 110uM) and was stimulated by Ca^{2+} and CaM, an effect completely antagonised by TFP at 60uM.

The inactivity of TFP on the adenosine-induced hyperpolarisation of the rat SCG (Table 4.3) suggests the actions of the isozyme of the PDE I type, if present in rat SCG neurones is unlikely to be involved in the response to adenosine.

Oleshansky (1980) reported that adenosine inhibits cGMP stimulation of cAMP hydrolysis, i.e. PDE II activity, with an IC_{50} of 80uM in the rat striatum to raise the level of neuronal cAMP. However, the inability of the PDE I and II isozyme inhibitor, M&B 22,948 (Souness, Brazdil, Diocee & Jordan, 1989) to alter the response of the rat SCG to adenosine (Table 5.9) suggests adenosine does not stimulate a similar PDE and the depression of muscarine by adenosine is not mediated by a decrease in cGMP.

The Ca^{2+}/CaM-independent low Km cAMP PDE isozyme, (PDE IV) is selectively inhibited by denbufylline (Nicholson, Jackman & Wilke, 1989) and Ro 20-1724 (Sheppard & Wigan, 1970; Sheppard, Wigan & Tsien, 1972) and is thought to

regulate the cAMP concentration in both the rat cerebrum and guinea-pig hippocampus (Stanley, Brown & Hill, 1989; Challiss & Nicholson, 1990). Denbufylline, an analogue of theophylline is a potent inhibitor of PDE IV and almost abolished the in vitro PDE activity of of rat cerebral cortex at 10uM (Nicholson & Wilkie, 1987), and Ro 20-1724 at 180uM increased both the basal and carbachol stimulated increase in cAMP levels of the rabbit SCG (Kalix et al., 1974). It would be expected that the incubation of the rat SCG with either Ro 20-1724 or denbufylline would augment the response to adenosine if it is due to a decrease in cAMP.

Ro 20-1724, denbufylline and SQ 22,536 were either ineffective or reduced (denbufylline at 100uM) the depolarisation of the rat SCG to muscarine (Table 5.9). These results suggest the muscarinic response does not occur via an increase in cAMP as it would be predicted that the PDE inhibitors would potentiate inhibition of adenylate cyclase and reduce the response to muscarine. Likewise, the inability of Ro 20-1724, denbufylline (up to 10uM) to significantly alter the depression of the response to muscarine by adenosine or CPA suggests an action of adenosine on PDE IV activity is unlikely.

The inability of denbufylline or Ro 20-1724 to potentiate the response to adenosine suggests the hyperpolarisation of the rat SCG by adenosine is independent of cAMP. At 1uM and 10uM denbufylline caused a small ($P<0.05$ at 10uM) reduction of the hyperpolarisation to adenosine with an estimated ED_{50} (36uM) for the antagonism of the hyperpolarisation of the rat SCG which was similar to the Ki (20uM) reported for the inhibition of the A1 radiolabelled ligand binding of [^{3}H] cyclohexyl adenosine (CHA) by denbufylline (Nicholson & Jackman, 1988). Thus the antagonism by denbufylline of the hyperpolarisation of

the rat SCG to adenosine may be due to receptor antagonism.

In contrast to the methylxanthine PDE inhibitors Ro 20-1724 has been used extensively in experiments where there is a need for a PDE inhibitor which is structurally unrelated and thus will not interfere with the binding of adenosine and related ligands. However, it has been reported that at 100uM Ro 20-1724 inhibited the binding of [^{3}H]-PIA to rat brain membranes by 20% (Schwabe & Trost, 1980) and it is possible that Ro 20-1724 at 200uM strongly antagonised the binding of adenosine to the rat SCG. Both Ro 20-1724 and purinoceptor antagonists caused an apparently non-parallel antagonism of the concentration-response curve to adenosine (Cf Figs. 5.10 and 6.20) and Ro 20-1724 may also act as an adenosine receptor antagonist.

Furthermore, there is circumstantial biochemical evidence to suggest that adenosine does not affect the response of agonists that stimulate cAMP formation in SCG. Roch & Kalix (1975) studying blocks of bovine SCG found the cAMP content was not altered by incubation in 100uM adenosine for 9 minutes in either untreated, or SCG treated with theophylline or Ro 20-1724. Adenosine was reported not to alter the increase in cAMP induced by high potassium medium, the latter effect of high [K^+]e was attributed to the release of catecholamines, which presumably activated beta-adrenoceptors to stimulate cAMP formation. Similarly Kalix and colleagues reported that in rabbit SCG adenosine had no effect on cAMP levels at rest or during cholinergic stimulation (Kalix et al., 1974). Unfortunately both of these reports lack sufficient experimental details in that the concentrations of agonists and antagonists, and conditions employed are unknown but support the results

presented, in that the effects of adenosine on the rat SCG are cAMP-independent.

The ability of forskolin to enhance the response to adenosine may be mediated through some other effect rather than a direct stimulation of adenylate cyclase (Laurenza, Sutkowski & Seaman, 1989) such as an increase in open channel activity as suggested by Akagi & Kudo (1985) for the rat SCG.

5.6.2 Interaction of adenosine with PIT in the rat SCG

As the effects of adenosine and muscarine on the rat SCG appear independent of cyclic nucleotide metabolism, then the interaction between adenosine and muscarine may occur via some other secondary messenger. Recent molecular characterisation of muscarinic receptors has revealed at least five subtypes of receptor (outlined in chapter 1) which may be selectively coupled with different effector systems, but not exclusively (Fukuda, Higashida, Kubo, Maeda, Akiba, Bujo, Mishina, & Numa, 1988). Muscarinic receptors coupled to phospholipase C (PLC) have been demonstrated in many neuronal tissues (Fisher & Agranoff, 1987) and based on the sensitivity to pirenzepine it was suggested that M1 receptors are coupled to PLC activation while pirenzepine insensitive M2 receptors are linked to adenylate cyclase inhibition (Gill & Wolfe, 1985).

One consequence of the stimulation of cholinergic receptors by muscarine is the hydrolysis by PLC of phosphatidylinositol 4,5-bisphosphate (PIP2) to yield two second messengers, inositol 1,4,5-triphosphate (IP3) and 1,2-diacylglycerol (DAG) (Berridge, 1987; Fisher & Agranoff, 1987; Fig. 5.9). It has been reported that adenosine may interact with PIT in a variety of cell types

including rat striatum (Petcoff & Cooper, 1987), mouse cerebral cortex (Kendall & Hill, 1988), cultured GH3 pituitary tumour cells (Delahunty, Cronin & Linden, 1988), frog NMJ (Sebastiao, 1989), and frog sympathetic ganglia (Rubio, Bencherif & Berne, 1988) and rat aorta (Long & Stone, 1987), and thus represents a potential target for the selective interactions of adenosine and muscarine on the rat SCG.

IP3 stimulates the release of intracellular Ca^{2+} while DAG can activate a family of Ca^{2+} and phospholipid dependent protein kinases, referred to as protein kinase C (PKC) (Berridge, 1987; Nishizuka, 1989; Huang, 1989), which may in turn phosphorylate specific proteins. Ca^{2+} mobilisation may activate phospholipase A_2(PLA_2) and trigger the release of arachidonic acid (AA) and the formation of cyclo-oxygenase or lipoxygenase products to cause hyperpolarisation (Higashida & Brown, 1986; Kim & Clapham, 1989; Kim, Lewis, Graziadei, Neer, Bar-Sagi & Clapham, 1989; Kurachi, Ito, Sugimoto, Shimizu, Miki & Ui, 1989a,b; Fig. 5.15). Likewise the PI response of various tissues has been linked to a hyperpolarisation produced by activation of I_{Kca}, e.g. in the hippocampus and NG108-15 cells. Higashida & Brown (1986) have shown that stimulation of NG108-15 cells with bradykinin has two sequential effects on gM:- the first is a hyperpolarisation attributed to the activation of an I_{Kca} and was mimicked by the application of IP3 or increased $[Ca^{2+}]i$ and the second, a subsequent depolarisation resulting from inhibition of a voltage dependent K^+ current (Im), which was inhibited by DAG activators.

In hippocampal neurones (Fig. 5.12), Dutar & Nicoll (1988) concluded IP3 is responsible for Im suppression as agonists that activate PLC suppress Im. Whereas phorbol ester activation of PKC has no effect (Malenka, Madison, Andrade

& Nicholl, 1986) and the intracellular injection of IP3 results in the loss of Im via Ca^{2+} independent process, suggesting a direct effect of IP3 or an IP3 metabolite on Im. In contrast the underlying biochemical mechanisms responsible for the inhibition of Im and hence the depolarisation of the rat SCG are not fully understood. It is known that muscarinic stimulation of the rat SCG increases the incorporation of 32Pi into phospholipids and the accumulation of IP3 (Hokin, 1965; Lapetina, Brown & Michell, 1975; Horwitz, Tsymbalov & Perlman, 1984; Bone & Michell, 1985). Muscarine at 100uM caused a 6 to 15 fold increase in PI labelling which was not mimicked by 1mM 8BrcGMP, 8BrcAMP or dopamine (Horwitz et al., 1984). Low $[Ca^{2+}]e$ increased the basal levels of PI and did not alter the accumulation of PI by muscarine and PIT hydrolysis was independent of $[Ca^{2+}]e$. In contrast, Tokimasa & Akasu (1990) have reported that Im of amphibian ganglia is subject to regulation by the $[Ca^{2+}]e$ and up to 90% of Im was antagonised by the application of Ca^{2+} antagonists. In contrast Brown & colleagues found Im of the rat SCG is independent of both $[Ca^{2+}]e$ and $[Ca^{2+}]i$ (Adams et al., 1982a; Brown et al., 1989), although the possibility that Im requires a minimum level of $[Ca^{2+}]i$ for its activation has not been discounted.

In the absence of receptor-induced PIP2 hydrolysis the tumour promoting phorbol esters, such as phorbol 12,13-dibutyrate (PDBu) can substitute for DAG to activate PKC (Berridge, 1987) and the addition of PDBu or unsaturated DAGs partially suppressed the M-current (Pfaffinger, Leibowitz, Subers, Nathanson, Almers & Hille, 1988a; Brown et al., 1989). Im was not altered by the intracellular injection or extracellular application of agents that increase cAMP, e.g. db-cAMP, 8BrcAMP, theophylline or forskolin (Adams et al., 1982a; Moore, Madamba, Joels & Siggins, 1988) suggesting Im is independent of cAMP.

Neither forskolin nor cAMP increased Im or reversed a partial blockade of Im by muscarine in frog ganglion cells (Adams et al., 1982; Brown & Adams, 1987; Moore et al., 1988). However, perhaps more importantly the suppression of Im was not enhanced by raising either $[Ca^{2+}]i$ to > 200nM or the level of IP3 to 100nM (Pfaffinger, Leibowitz, Bosma, Almers & Hille, 1988b). Additional studies by Brown & colleagues using Li^{+} at a concentration (2mM) that would be expected to inhibit PIT, did not alter Im (Brown et al., 1989). All these results strongly indicate that the inhibition of Im and hence the depolarisation of the rat SCG occurs via an IP3-independent mechanism.

Alternatively DAG has been proposed as the mediator of the muscarinic depolarisation of the rat SCG based on two observations, namely (1) muscarinic agonists stimulate PIT and the production of DAG (Bone, Fretten, Palmer, Kirk & Michell, 1984; Patterson & Volle, 1984; Horwitz et al., 1984) and (2) PDBu mimics the depolarisation of muscarine by partially suppressing Im and a leak current of rat SCG neurones (Brown et al., 1989).

In contrast Bosma & Hille (1989) found the PKC inhibitors H7 and staurosporine had no effect on LHRH suppression of Im but blocked the response to phorbol esters. A similar effect of PKC inhibitors on the response of the rat SCG was very recently reported by Grove & colleagues who found PDBu mimicked the muscarinic depolarisation of the rat SCG. Staurosporine abolished the response to PDBu but only slightly reduced the response to muscarine, probably via a non-specific action (Grove, Caulfield & Evans, 1990).

Furthermore, studies by Hille and his colleagues have produced evidence that agonist-induced suppression of Im in frog sympathetic neurones is independent of the PLC

second messenger cascade as phorbol esters caused only partial suppression of Im and the addition of LHRH following phorbol ester application suppressed the remaining current, suggesting LHRH can inhibit Im in PKC-independent manner (Pfaffinger et al., 1988a). Thus there may be a PLC independent and dependent mechanism of action of muscarine on the rat SCG.

5.6.2.1 Effect of phorbol esters and the PKC inhibitor H7 on the response to adenosine

When applied to the rat SCG (Fig 5.14c) PDBu produced a slowly developing depolarisation, which probably reflects the time needed for this compound to cross the cell membrane and act upon PKC. The slow recovery to PDBu would be expected to be dependent on its degradation and/or diffusion from the ganglion. The comparable depolarisation and potentiation of the hyperpolarisation to adenosine by PDBu and muscarine is indicative of a common site of action for adenosine, at or beyond the activation of PKC. The inability of the PKC-inhibitor H7 to alter the response to PDBu is in contrast to the reported action of another PKC inhibitor, staurosporine to inhibit the depolarisation of the rat SCG to PDBu (Grove et al., 1990). It has been reported PKC inhibitors are not completely specific in that all of them are reported to inhibit other PKs, e.g. H7 also inhibits cAMP dependent PK at 3uM and cAMP dependent PK at 6uM (Hidaka, Inagaki, Kawamoto & Sasaki, 1984), and polymixin B inhibits both PKC and Ca^{2+}/CaM dependent PK but not the cyclic nucleotide dependent PKs. The inactivity of H7 on the response to adenosine (Tables 5.10 & 5.11) further supports the hypothesis that cAMP is not involved in the response to adenosine as H7 would be expected to inhibit PKA.

One possibility is that the concentration of H7 (50uM) employed was insufficient to block PKC. Some evidence for the need for a higher concentration to inhibit PKC was suggested by Linden & Routtenberg (1989) who in reviewing the literature on long term potentiation (LTP) reported Malinow, Madison & Tsien (1988) found the LTP of the hippocampus was inhibited by 300uM H7, whereas another group of investigators found no inhibition of hippocampal LTP at 100uM H7. In contrast a concentration of 50uM H7 may be sufficient to inhibit PKC as the depression of Ca^{2+} dependent components of the CAP of isolated cultured rabbit SCG neurones by ACh, an effect mimicked by phorbol esters was abolished by a minimum of 10 minutes incubation in 50uM H7 (Mochida & Kobayashi, 1988).

Similarly, Sebastiao (1989) reported 60uM H7 inhibited the evoked endplate potential (e.p.p.) of frog sartorius muscle, but did not modify the excitatory action of 100nM phorbol diacetate (PDAc) on e.p.p. amplitude, whereas polymixin B antagonised the excitatory effect of PDAc. The apparent ineffectiveness of H7 was attributed to the ability of polymixin B to inhibit the binding of PKC in an indirect manner, i.e. to inhibit the binding of phospholipid to PKC.

PKC inhibitors can also act at two different sites, i.e. the regulatory domain of PKC, which binds Ca^{2+}, phospholipid and DAG or phorbol ester to unmask the second active site, the catalytic domain. Sphingosine and polymixin B compete with phosphatidylserine, Ca^{2+} and DAG at the regulatory site, whereas H7 competes with ATP at the catalytic phosphotransferase site. Thus these differences in the sites of action between H7 and spingosine may account for the depression of the response of the rat SCG to PDBu by sphingosine but not by H7.

The results presented here suggest that the interaction of adenosine with muscarine and PDBu is at a site distinct to the ATP binding site of PKC. Adenosine may alter the activation by PKC at the regulatory site although this seems unlikely as TFP has been shown to antagonise the binding of Ca^{2+} to the regulatory domain of PKC (Huang, 1989) but not the hyperpolarisation of the rat SCG by adenosine.

It is possible that a neurotransmitter such as ACh whose principle biochemical effect is to increase PIT can also increase cAMP formation via an indirect action (Fredholm, Longren, Lindstrom & Norstedt, 1987; Karbon, Shenolikar & Enna, 1986).

Tumour-promoting phorbol esters have been reported to augment drug and neurotransmitter-induced second messenger production, e.g. cAMP, by an unknown mechanism in a variety of tissues including brain (for references see Karbon et al., 1986). However, it is unlikely that the enhanced response of the rat SCG to adenosine in the presence of PDBu is due to an increase in cAMP as reported for the effects of 2CA on rat brain slices (Karbon et al., 1986) as the alteration of cyclic nucleotide metabolism in PSS did not enhance the response to adenosine.

Whatever the mechanism of interaction between muscarine and adenosine the ability of muscarine and PDBu to enhance the response to adenosine during the persistent depolarisation to muscarine, suggests the effector mechanism is not transient, and is unlikely to be a depletable secondary messenger. In addition the ability of PDBu to augment the response to adenosine suggests that adenosine modifies a second messenger response beyond the level of muscarinic receptor.

The ability of muscarine to potentiate subthreshold responses, i.e. at ≤ 1uM adenosine (Fig. 5.13c) is indicative that the effect of adenosine on the rat SCG would be greater in vivo during normal and pathophysiological situations. The difference in the response shape at millimolar concentrations (Fig. 5.13c, cf 100uM and 1000uM adenosine) is discussed further in Chapter 6. Possible explanations for such an effect include; desensitisation to adenosine, an interaction at two different receptors or the efflux of K^+ in to the extracellular space.

Muscarinic agonists differ considerably in their ability to enhance PIT in brain (Fisher & Agranoff, 1987; Baumgold & White, 1989) an effect which depends on agonist structure since ACh and carbachol activate PIT to a much greater extent than pilocarpine (Fisher, Klinger, Agranoff, 1983; Brown & Brown, 1984). The ability of different muscarinic agonists to produce different degrees of PIT in the rat SCG is indicated by the results of Horwitz et al. (1984) who found muscarine was more effective than bethanechol at stimulating phospholipid metabolism. Using rat cerebral cortex Freedman, Harley & Iversen (1988) suggested that methylfurmethide is not considered to be a "full" agonist at stimulating the PI system.

The different susceptibility of muscarinic agonists to adenosine reported here (Table 5.4) may be associated with the coupling of these agonists to the muscarinic receptor and their respective responses via secondary messengers. The most potent effects of adenosine were found on MeF which is reported to be poorly coupled to PIT and the smallest depression by adenosine was of carbachol an agonist that may be strongly coupled to PIT.

The coupling of muscarinic receptors to two different biochemical pathways could also explain the biphasic nature of the depression of muscarinic responses, i.e. with increasing concentrations of muscarine a second biochemical mechanism is activated and antagonises the depression of the response of the muscarinic agonist by adenosine.

5.7 The role of prostaglandins and leukotriens on the response of the SCG to adenosine

It is possible that the G-protein that couples muscarinic receptors to PLC second messenger cascade may also couple in an independent manner, to the suppression of Im, perhaps via a direct mechanism. Alternatively the suppression of Im in the rat SCG could occur via DAG which is hydrolysed by DAG-lipase to form monoacylglycerol (MAG) and AA (Irvine, 1982). In support of this idea is the ability of A9L cells to release AA upon stimulation of M1 receptors (Irwin, 1982) and the activation of muscarinic receptors of some cell lines (De George, Morell, McCarthy & La Petina, 1986). Recently the products of AA metabolism, i.e. prostaglandins (PG) and leukotrienes (LT) (Fig. 5.15) have been found to modulate K^+ channels (Higashida & Brown, 1986; Kim & Clapham, 1989; Kim et al., 1989; Kurachi et al., 1989a,b).

A second pathway for the synthesis of AA is via the hydrolysis of phospholipids by PLA_2 to release lysophospholipid and free fatty acid, usually AA, which can directly activate or inhibit PKC (Huang, 1989). In addition AA in neurons can be metabolised by lipoxygenase, cyclooxygenase or epoxygenase enzymes to form LTs, hydroxyeicosatetraenoic acids (HETEs), PGs, thromboxanes

(TXs) and epoxides respectively (Axelrod, Burch & Jelsema, 1988) (Fig. 5.15).

Most electrophysiological effects of adenosine so far identified appear to result from its ability to alter cAMP or PIT metabolism and arachidonate pathway products do not appear to be a major second messenger system for adenosine. and ATP arose from the ability of ATP rather its metabolically stable, phosphate-modified analogues to stimulate the synthesis and release of PG's (Brown & Burnstock, 1981b; Needleman, Minkes & Douglas, 1974). Recently this dogma has been challenged by two reports of the activation of PG synthesis by adenosine in guinea-pig atria (Caparrota, Fassina, Froldi & Poja, 1987) and the rabbit heart (Karwatowska-Prokocsuk, Ciabattoni & Wennmalm, 1988).

Both intact and homogenated rat SCG have the ability to synthesise PGs. Ganglia spontaneously released PGE and indomethacin at 55uM inhibited the synthesis of PGE in intact ganglia, with an IC_{50} for rat SCG homogenates of 11uM (Webb, Saelens & Halushaka, 1978). Homogenates of rat SCG were also reported to produce PGE_2 and 6-keto-PGF_{1alpha} (Gonzales, Goldyne, Taiwo & Levine, 1989) and the ability of adenosine to activate the LT and PG pathways of the rat SCG was tested.

At 50uM indomethacin produced a small non-significant rightward shift in the concentration-response curve for the hyperpolarisation of the rat SCG to adenosine (Fig. 5.16). The concentration of indomethacin chosen is likely to be supramaximal for the inhibition of PG synthesis and was reported to inhibit the formation of PGs in the rat SCG (Webb et al., 1978), suggesting PGs are not involved in the response of the ganglion to adenosine. If PLA_2 activation is necessary for the hyperpolarisation to

IN FACT ONE REASON FOR POSTULATING SEPERATE RECEPTORS FOR ADENISINE

adenosine then the effect of a PLA_2 inhibitor such as nordihydroguaiaretic acid (NDGA) should be to decrease the response to adenosine. In combination with indomethacin to inhibit PG synthesis, NDGA produced a significant antagonism of the hyperpolarisation of the rat SCG to adenosine, suggesting either the activation of PLA_2 and/or the lipoxygenase pathway may mediate a significant part of the response to adenosine.

The results reported here appear to be similar to those effects on the opening of S-channels of Aplysia sensory neurones. The opening of these K^+ channels was imitated by exogenous AA and blocked by inhibitors of phospholipase and lipoxygenase, but not by indomethacin (Piomelli, Volterra, Dale, Siegelbaum, Kandel, Schwartz & Belardetti, 1987).

Other similar actions of AA metabolites, i.e. the lipoxygenase products have been found to be intracellular modulators of G-protein-gated muscarinic K^+ channels of guinea-pig atrial cells (Kurachi et al., 1989a,b) and neonatal rat atrial cells (Kim & Clapham, 1989; Kim et al., 1989).

Given the broad spectrum of actions of PGs and LTs the results reported here must be considered as preliminary findings and suitable controls will need to be included before any specific claims can be made for the role of AA in the actions of adenosine on the rat SCG. However, a recent report has suggested that the A1 receptors of rat striatal glial cells may be coupled to the PLA_2-PG synthesis pathway (Caciacli, Ciccarelli, Di Iorio, Tacconelli & Ballerni, 1989) and a similar coupling of adenosine receptors to the PLA_2 secondary messenger system may occur in rat SCG neurones.

It is proposed that adenosine may increase the production of arachidonic acid and the production of lipoxygenase products which can then activate K^+ channels to hyperpolarise the rat SCG. To determine whether the 5-lipoxygenase pathway is involved in the response to adenosine in the presence of a specific inhibitor of LT synthesis such as nafazatrom could be tested. Muscarinic depolarisation would thus augment the response to adenosine due to an increased activity of PLC that would increase the concentration of DAG and AA. An increase in free AA may be sufficient to inhibit the activation of PKC and cause a subsequent depression of the muscarinic response.

Discussion

An interaction of purines with postsynaptic responses to ACh and cholinergic agents has been reported by several research groups although both the receptors and the mechanisms of action are unknown or poorly characterised (Table 5.12).

It has been shown that 5'-isobutylthio-adenosine (SIBA) and its analogues at 100uM antagonised ACh-induced contractions of the guinea-pig ileum (Pankaskie, Kachur, Itoh, Gordon & Chiang, 1985). It is reported that SIBA and related agonists can alter the binding of muscarine to muscarinic receptors (Smejkal, Ibrahim, Pankaskie & Chiang, 1989). It seems unlikely that adenosine directly inhibited the binding of muscarine to muscarinic receptors as methroctramine and pirenzepine did not alter the response of the ganglion to adenosine. In addition the Ki for the inhibition of specific binding of ^{3}H-pirenzepine to the rat cerebral cortex was 160uM (Smejkal et al.,

1989), a value 10 fold greater than the IC_{50} for the depression of muscarinic responses on the rat SCG.

Opposite effects to those described here have been reported by Worley & colleagues (Worley, Baraban, McCarren, Snyder & Alger, 1987; Worley, Heller, Snyder & Baraban, 1988). Both phorbol esters and cholinomimetics were shown to prevent the inhibitory effects of adenosine on orthodromically-induced synaptic potentials and the adenosine-induced hyperpolarisation of CA1-pyramidal cells (Worley et al., 1987; 1988). Carbachol was substantially more effective than oxotremorine in blocking the adenosine elicited outward current (Worley et al., 1987), whereas on the rat SCG carbachol was the most resistant to the depressant by effects of adenosine. It is interesting to speculate that the stimulatory and inhibitory activity of phorbol esters and cholinomimetics arises from a difference in the role of PKC in the ganglion and hippocampus (Fig. 5.12).

In agreement with the results reported here Brooks & Stone (1988) found that adenosine reduced the activity of cholinomimetics at concentrations that did not alter the actions of excitatory amino acids on the hippocampus. The mechanism of action was not studied but both the potency and relative order of potency of the purines tested i.e. R&S-PIA, NECA and adenosine was similar to that reported for these agonists on the rat SCG (r^2 = 0.95) suggesting a similar mechanism may be responsible for both actions of adenosine. Both of these studies appear to be different from the reports by Worley & colleagues as the actions of adenosine on the rat SCG and hippocampus (Brooks & Stone, 1988) were postsynaptic and the concentration of adenosine achieved at the cell surface by Worley & colleagues is predicted to be much higher than that used in this study and that used by Brooks & Stone (1988).

The effects reported here and by Brooks & Stone (1988) are in sharp contrast to other studies where purines enhanced the nicotinic or muscarinic sensitivity of other preparations (Table 5.12). These results are in partial agreement with studies on the central neurones of Helix (Cox & Walker, 1987) where low concentrations potentiated and higher concentrations depressed the ACh response. The adenosine receptor responsible for the depressant activity of adenosine on Helix neurones may be of the A3-receptor subtype given the inactivity of NECA and the Ca^{2+} dependent nature of the depression.

A bell-shaped depression of the response of the rat SCG to muscarine by increasing concentrations of purines was observed in both normal and low Ca^{2+} PSS. The application of adenosine at increasing time intervals did not alter the size or shape of the response, suggesting receptor desensitization may not account for the shape of the concentration-response curves. A second possibility is that higher concentrations of purines activate a second receptor or another process that antagonises the effects of lower concentrations of adenosine. The possibility of a second adenosine receptor is addressed in the next chapter.

Thus there are many potential interactions between purines and responses to cholinomimetics which may provide the capacity for the modulation of both central and peripheral neurones.

In summary the results presented in this chapter suggest that adenosine selectively depressed the response of the rat SCG to muscarinic agonists via a reduction of the M1-mediated depolarisation and that these actions were competitive and readily reversible.

Furthermore, the results presented point to adenosine sensitive and adenosine insensitive components in the response to muscarine and may reflect the multiple actions of muscarine reported (Mochida & Kobayashi, 1986a,b). Many agonsits that are reported to increase intracellular cAMP depolarised the rat SCG but did not alter the response to adenosine suggesting an increase in cAMP is not the mechanism responsible for the potentiation of the hyperpolarisation to adenosine in the presence of muscarinic agents. The potential for an interaction of adenosine at a number of intracellular sites has been reviewed and it is suggested that adenosine may alter the activity of these sites to counteract the inhibition of Im by muscarine.

Fig. 5.1. Log concentration response curves for 2 methyl-5-hydroxytryptamine, acetylcholine, muscarine, vasoactive intestinal polypeptide and oxotremorine-M on rat isolated SCG

Concentration-response curves for the depolarising responses induced by 2-methyl-5-hydroxytryptamine (2Me-5HT), acetylcholine (ACh), muscarine, vasoactive intestinal polypeptide (VIP) and oxotremorine-M (OXO-M) were constructed with one minute applications and a minimum of 15 minutes between applications. The responses to agonists were reproducible. Each data point represents the mean of 4 to 12 ganglia with vertical or downward bars for S.E.M.

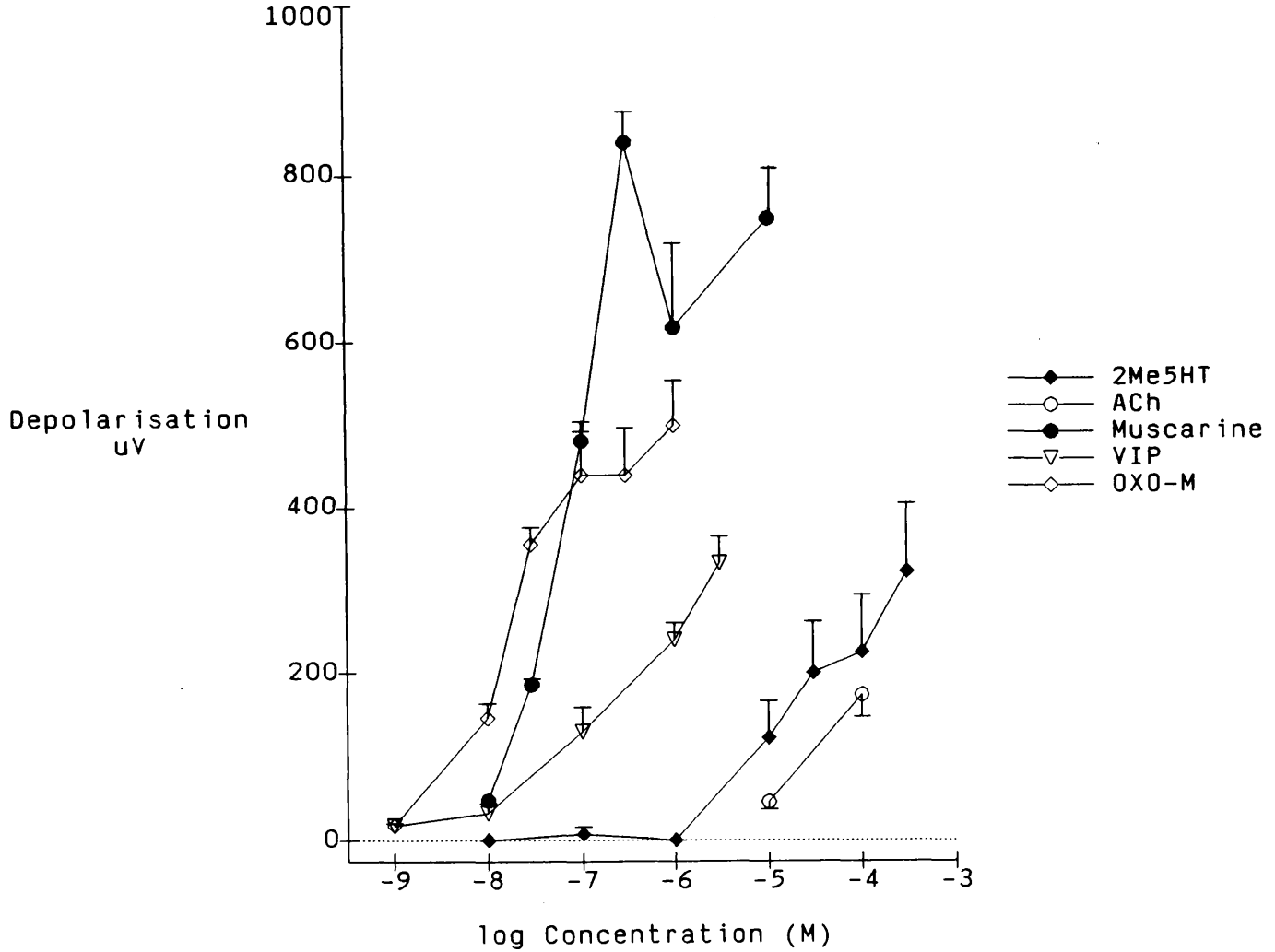
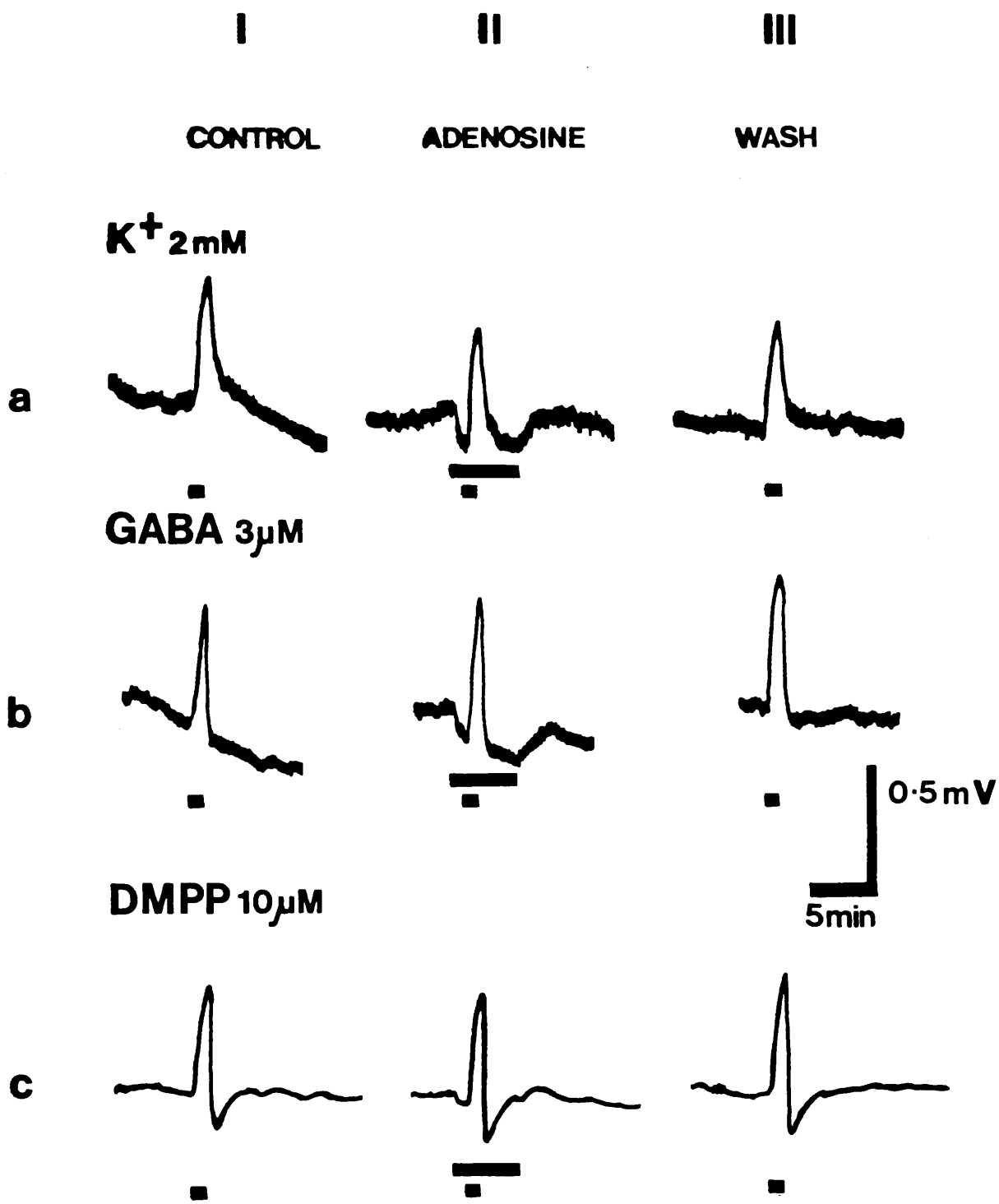
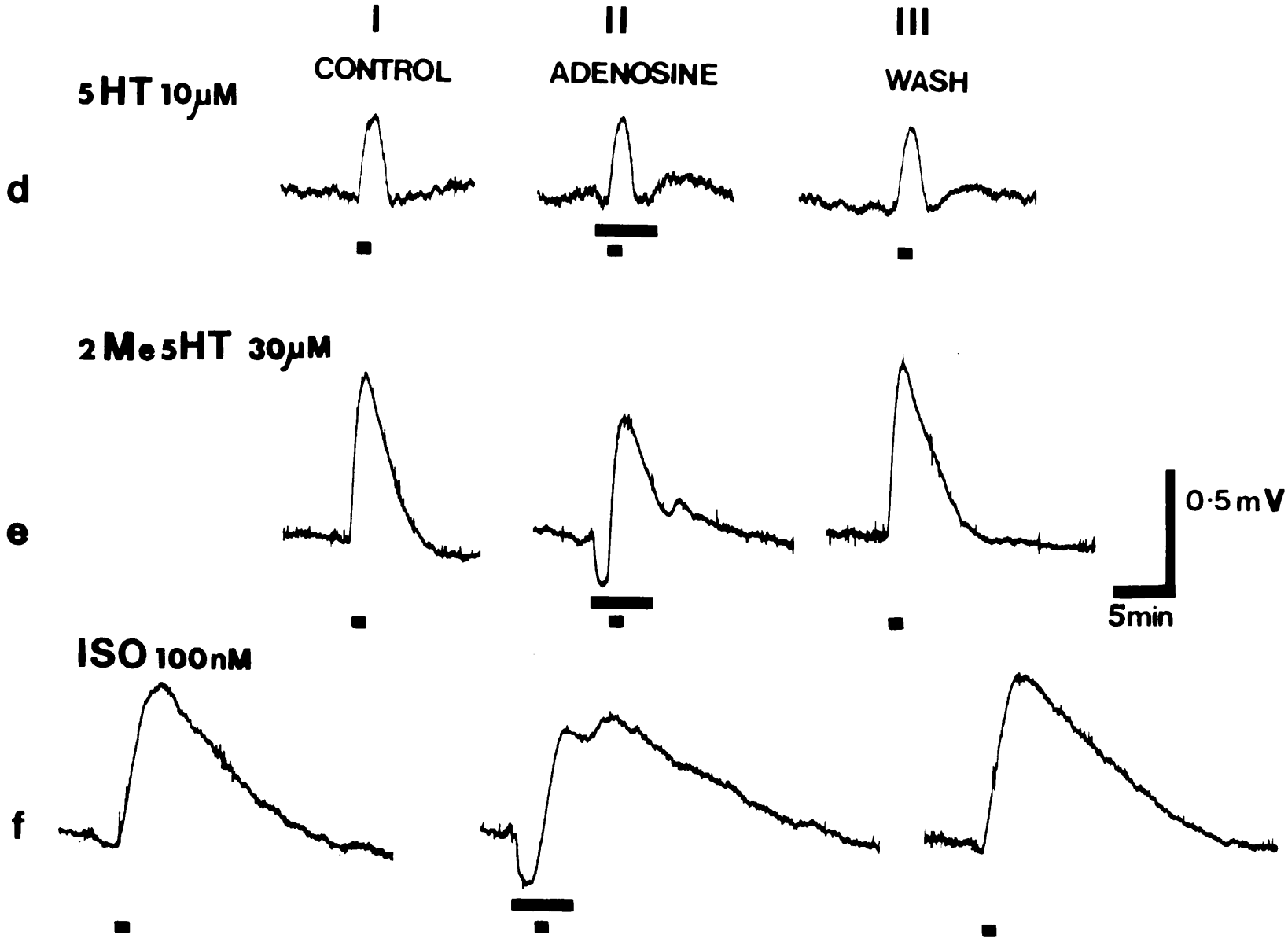

1000
800
600
400
200
0
Depolarisation
uV
-9
-8
-7
-6
-5
-4
-3
log Concentration (M)
2Me5HT
ACh
Muscarine
VIP
OXO-M

Fig. 5.2. Effect of adenosine on the response of isolated SCG of the rat to cholinomimetics and other depolarising agents.

The effect of adenosine (AD) on the response to one minute applications (solid bars) of (a) potassium (K^+), (b) gamma-aminobutyric acid (GABA), (c) dimethyl-piperadinium (DMPP), (d) 5-hydroxytryptamine (5HT), (e) 2-methyl-5-hydroxytryptamine (2Me-5HT), (f) isoprenaline (ISO), (g) muscarine (Musc), (h) carbachol (Carb) and (i) methylfurmethide (MeF) is shown.

For each trace (I) pretest response before adenosine (CONTROL), (II) response to agonist during a five minute application of 100uM adenosine and (III) response to agonist after washing out adenosine.


I
II
III
CONTROL
ADENOSINE
WASH
K+ 2mM
a
GABA 3μM
b
0·5mV
5min
DMPP 10μM
c


I
II
III
CONTROL
ADENOSINE
WASH
5HT 10μM
d
2Me5HT 30μM
e
0·5mV
5min
ISO 100nM
f

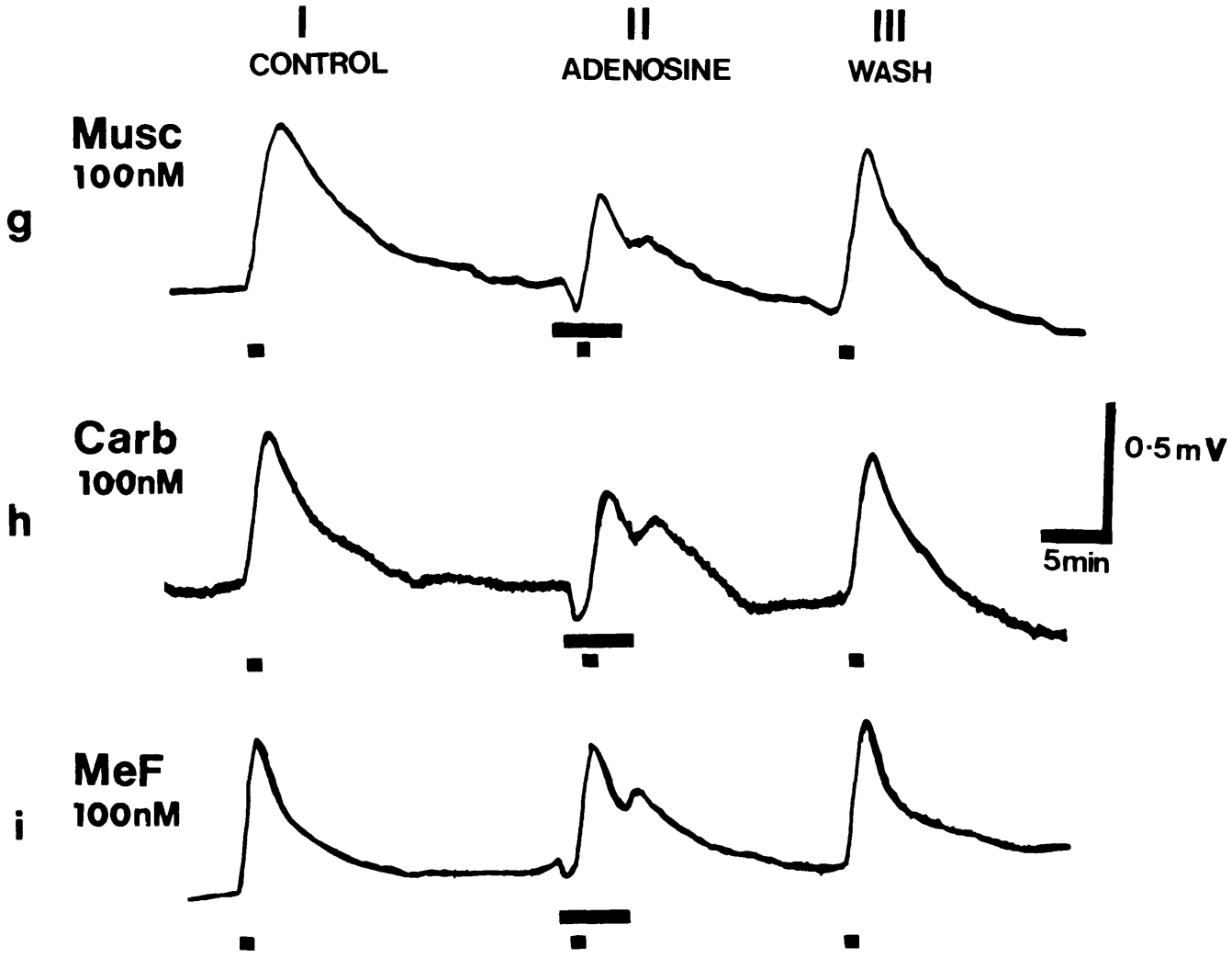

I
CONTROL
II
ADENOSINE
III
WASH
g
Musc
100nM
h
Carb
100nM
i
MeF
100nM
0·5mV
5min

Fig. 5.3. Depression of the responses of a single rat superior cervical ganglion to muscarine produced by increasing concentrations of adenosine

Responses to muscarine (100nM, 1 minute applications at a minimum of 20 minute intervals) before adenosine (CONTROL), during a five minute application of adenosine (ADENOSINE) and after washing out of adenosine for a minimum of 20 minutes (WASH) were recorded sequentially. The values in the right hand column refer to the % reduction (mean $\pm$ SEM) of the response to muscarine at the respective concentrations of adenosine used. The value in brackets refers to the number of ganglia tested.

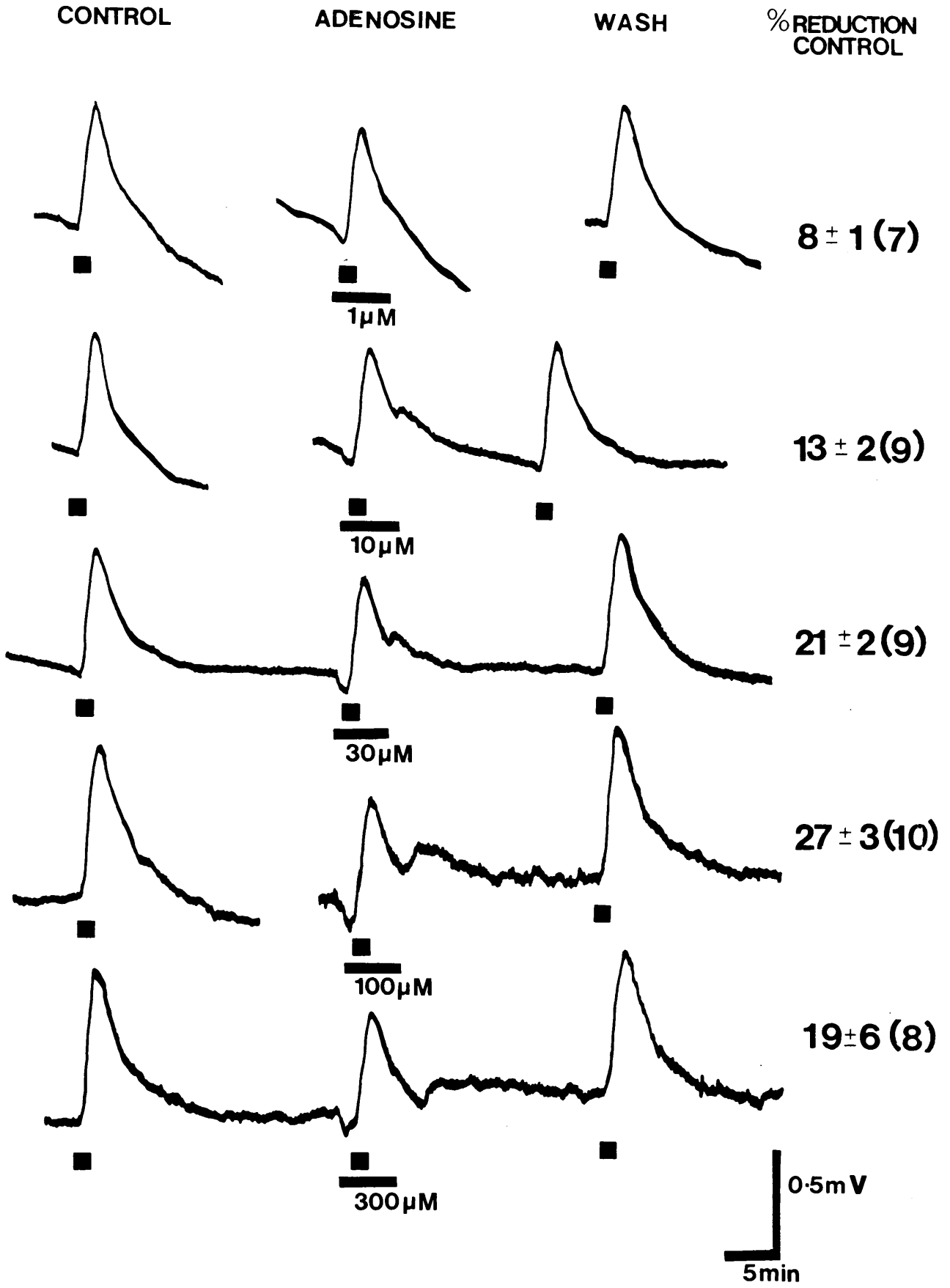
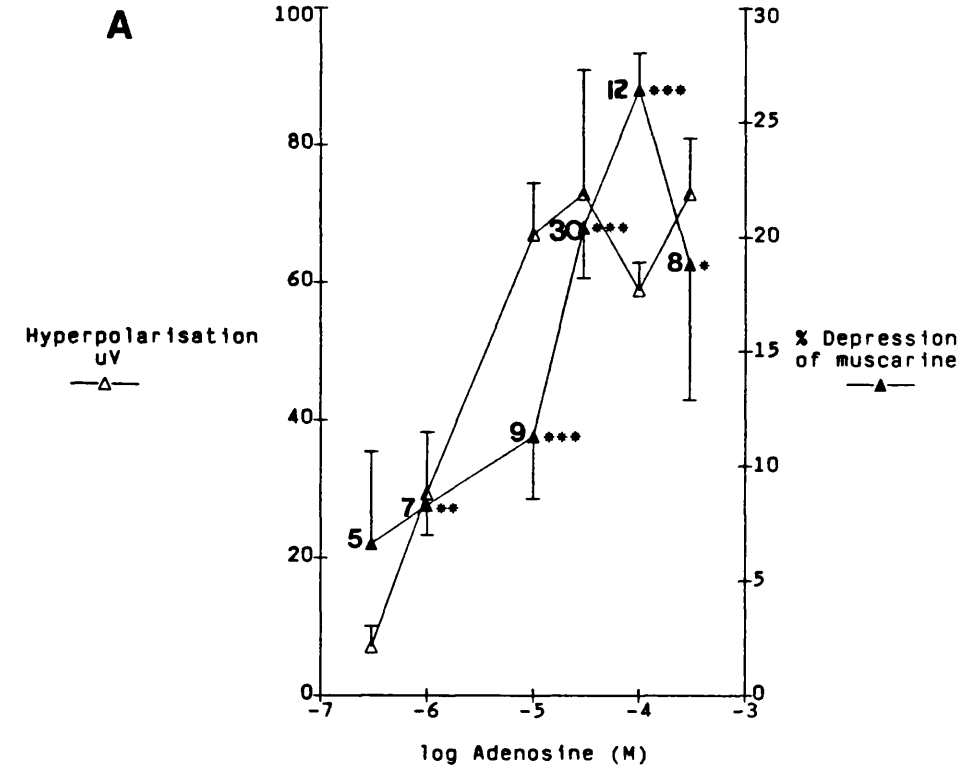
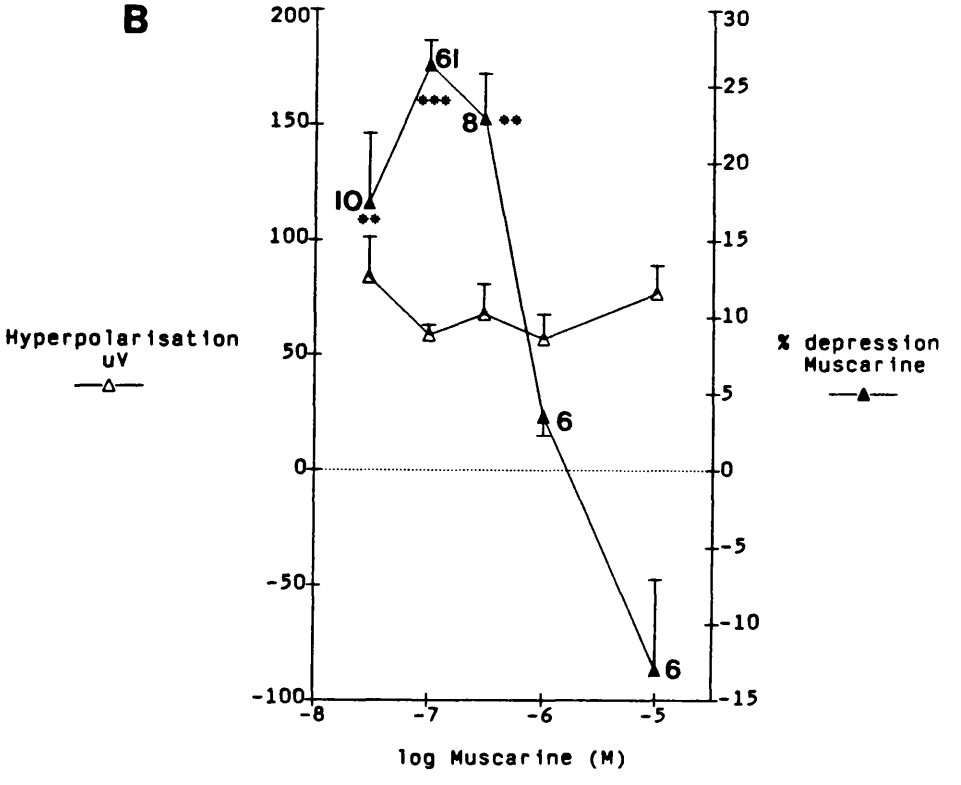

CONTROL
ADENOSINE
WASH
%REDUCTION CONTROL
8 ± 1 (7)
1μM
13 ± 2 (9)
10μM
21 ± 2 (9)
30μM
27 ± 3 (10)
100μM
19 ± 6 (8)
300μM
0·5mV
5min

Fig. 5.4. Depression of the response of the rat SCG to (A) increasing concentrations of adenosine and (B) effect of 100uM adenosine on different concentrations of muscarine by 100uM adenosine.

Statistical significant depressions of the response to muscarine were determined using a paired t-test on the responses in uV and is indicated by * for $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$.

The depression of the muscarinic response was fully reversible at all concentrations of adenosine (up to 1mM), upon washing out adenosine, and allowing up to an hour for recovery.


A
Hyperpolarisation
uV
% Depression
of muscarine
log Adenosine (M)
100
80
60
40
20
0
30
25
20
15
10
5
0
-7
-6
-5
-4
-3
12
30
8
9
7
5


B
Hyperpolarisation
uV
% depression
Muscarine
log Muscarine (M)
200
150
100
50
0
-50
-100
30
25
20
15
10
5
0
-5
-10
-15
-8
-7
-6
-5
61
8
10
6
6

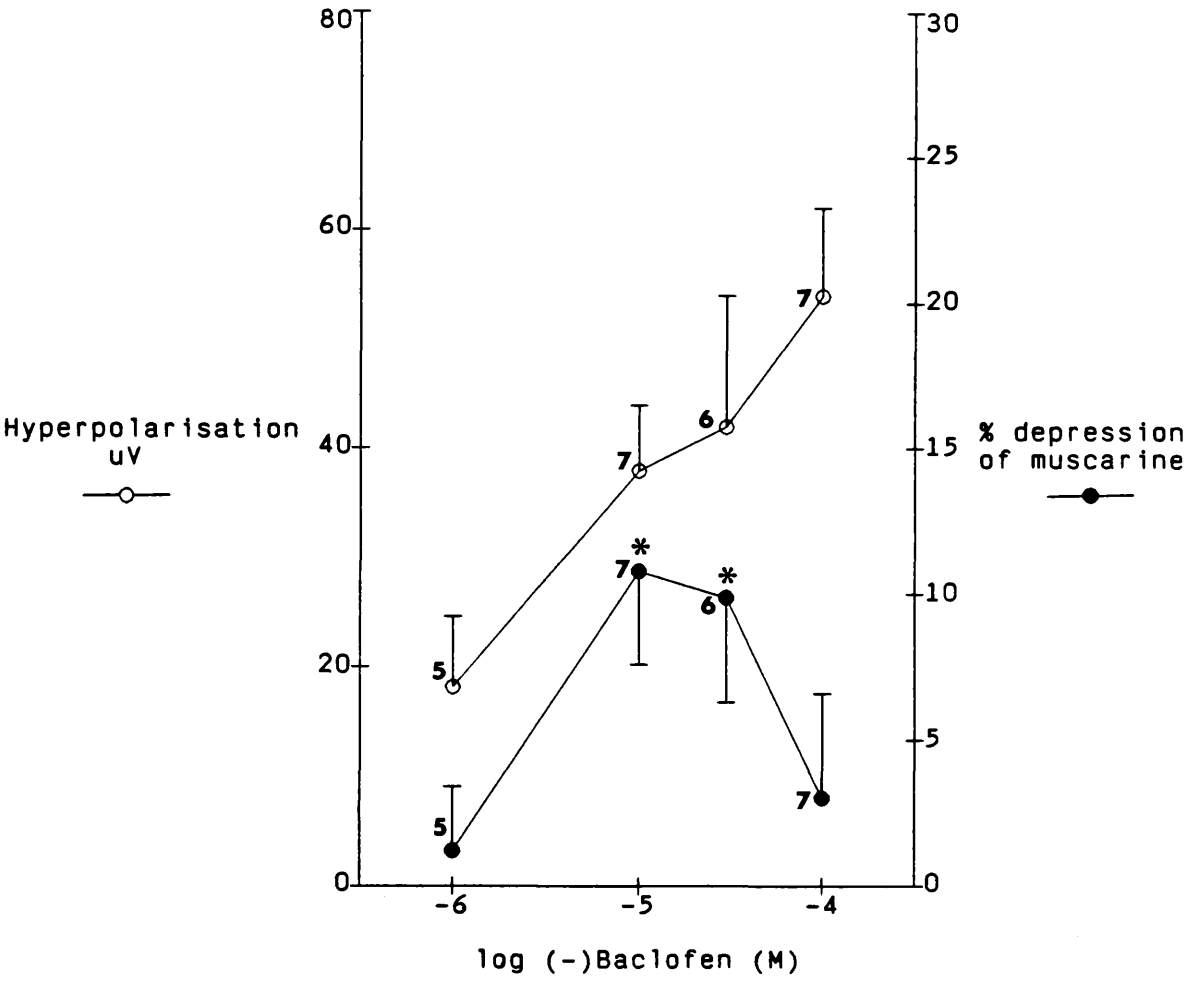
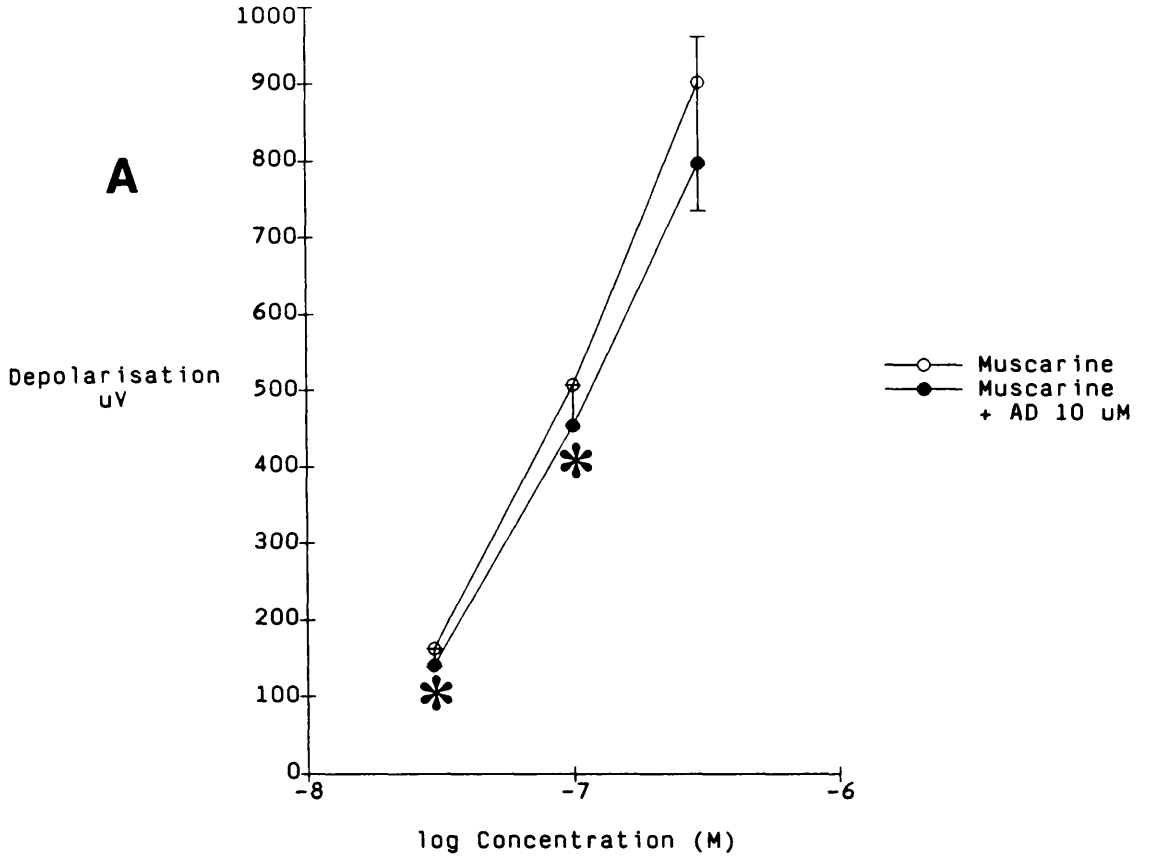


Fig. 5.5. Effect of (-) baclofen on the response of the rat SCG to muscarine

The response to muscarine (100nM, 1 min application) was assessed in the absence and presence of increasing concentrations of (-) baclofen. Statistical signficant depressions of the response to muscarine were determined using a paired t-test on the responses in uV and is indicated by a * for $P < 0.05$.

Fig. 5.6. Effect of adenosine on the concentration-response curve to muscarine

Log concentration response curve of the rat SCG to muscarine in the presence of (A) 10uM adenosine (muscarine + AD 10uM) and (B) 100uM adenosine (muscarine + AD 100uM). Statistical significant depressions of the response to muscarine were determined using a paired t-test on the responses in uV and is indicated by a * for $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$. A minimum of six ganglia were used at each concentration of muscarine.


A
Depolarisation uV
1000
900
800
700
600
500
400
300
200
100
0
-8
-7
-6
log Concentration (M)
Muscarine
Muscarine + AD 10 uM

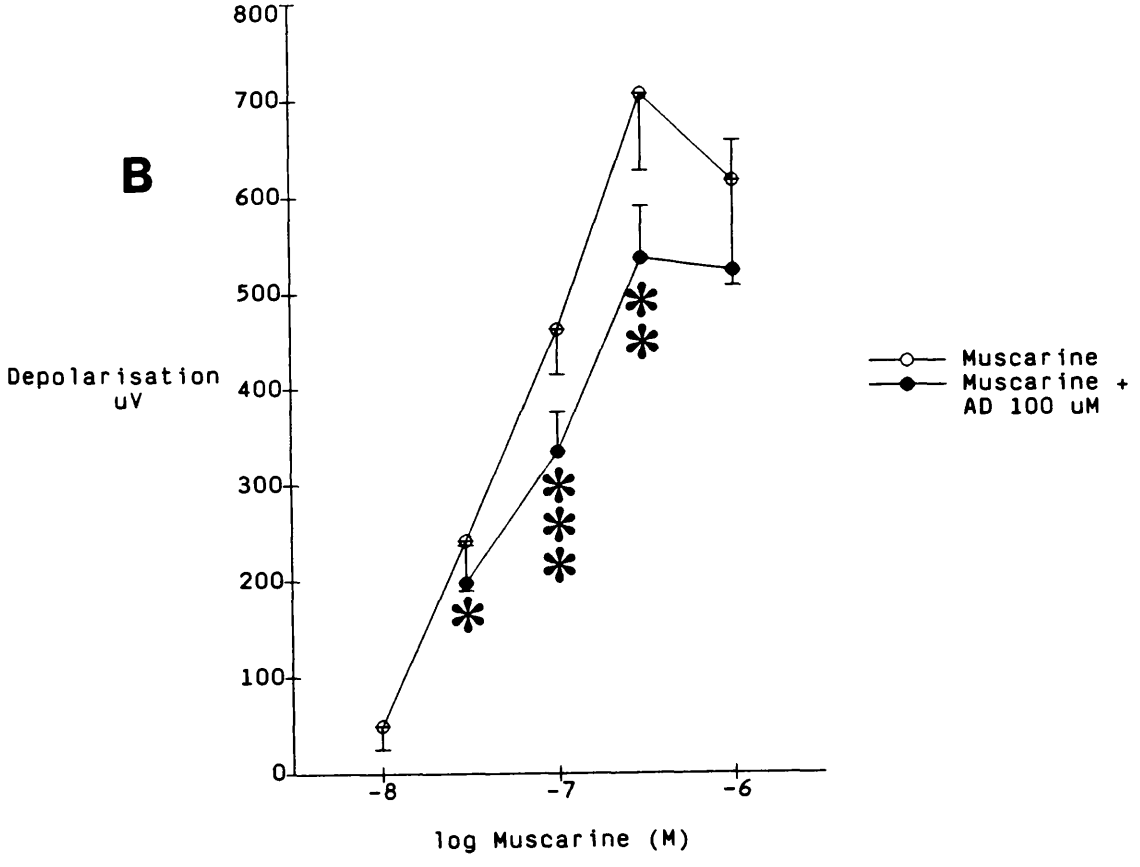
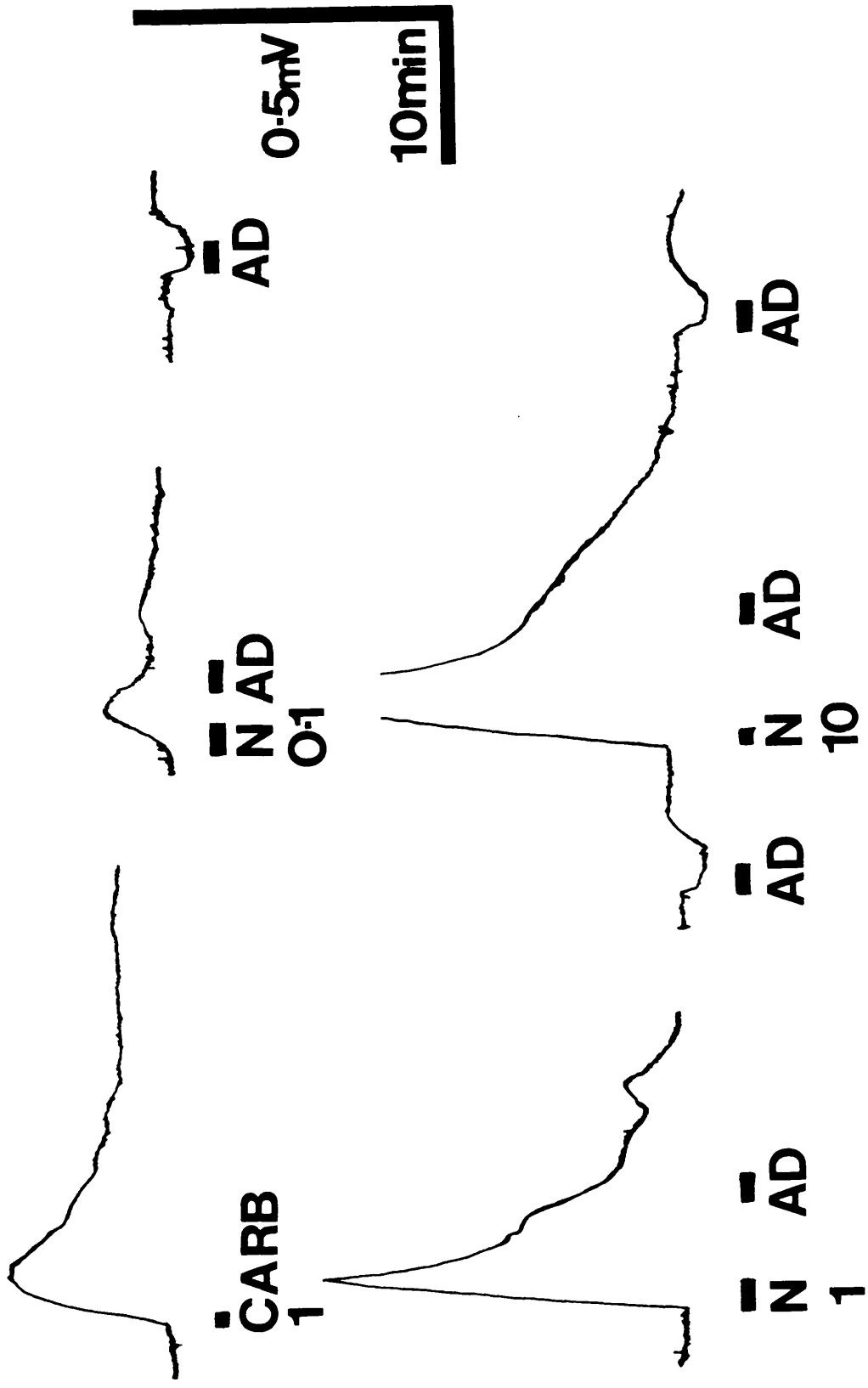

B
Depolarisation uV
800
700
600
500
400
300
200
100
0
-8
-7
-6
log Muscarine (M)
Muscarine
Muscarine + AD 100 uM

Fig. 5.7. Response of a single rat SCG to adenosine in the absence and presence of increasing concentrations of nicotine.

(A) The response to a one minute application of 1uM carbachol (CARB), (B&C) The response to two minute applications of nicotine (N) at 0.1uM, 1uM and (D) application of 10uM nicotine for 1 minute and the response to adenosine (AD, 100uM, 2 minutes) during nicotinic depolarisations and after washout of nicotine. NOTE: In (D) the maximum response to 10uM nicotine was trunkcated by the chart recorder.


CARB
1
N AD
0·1
AD
0·5mV
10min
N AD
1
AD
N AD
10
AD

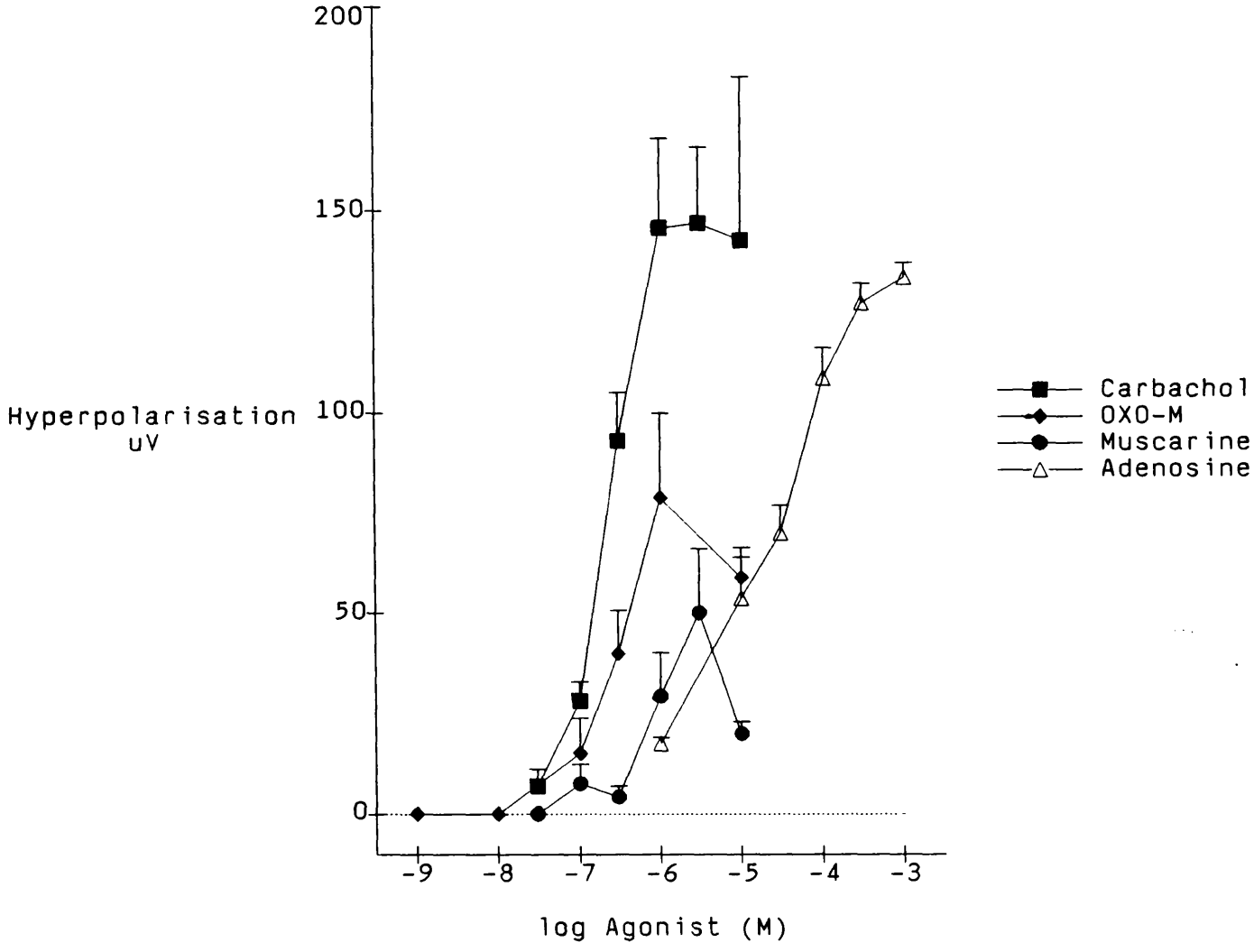
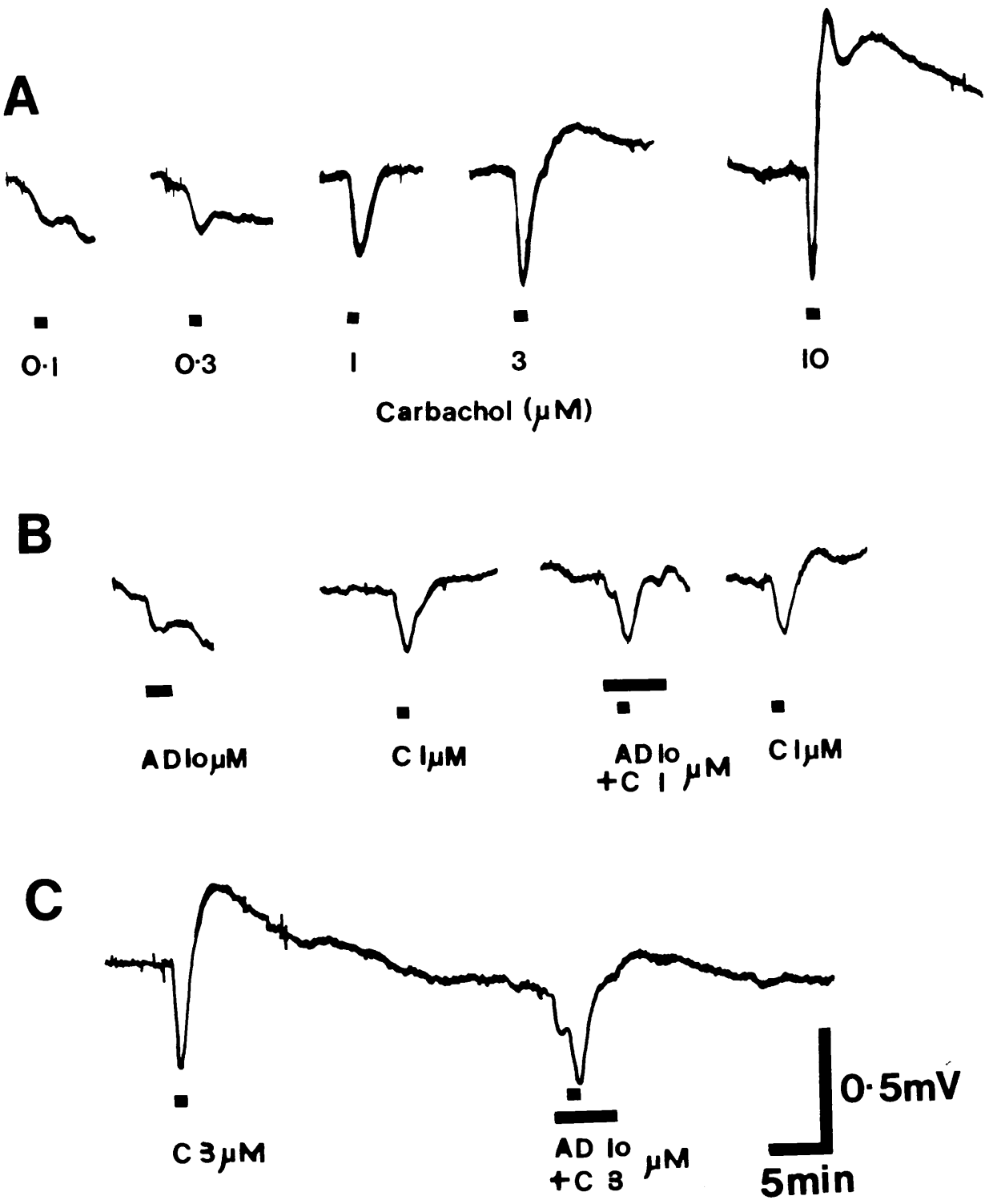


Fig. 5.8. Log concentration-response curves to carbachol, oxotremorine-M, muscarine, methylfurmethide and adenosine in physiological salt solution containing 0·1mM calcium and 300nM pirenzepine. N=4 to 15 ganglia.

Fig. 5.9. Response of a single SCG to increasing concentrations of carbachol and the effect of adenosine on the response to carbachol

The ganglion was recorded in physiological salt solution containing low calcium (0.1mM) and pirenzepine (300nM). (A) Effect of increasing concentrations of carbachol, (B) response to adenosine (AD) (2 minutes), control response to 1uM carbachol (C), followed by response to 1uM carbachol during the hyperpolarisation to adenosine and the response to 1uM carbachol recorded 20 minutes after the removal of adenosine, (C) response to 3uM carbachol and response to 3uM carbachol during a hyperpolarisation to adenosine. Note: Reduction of the amplitude of the depolarisation produced after the initial hyperpolarisation to carbachol.


A
0·1
0·3
1
3
10
Carbachol (μM)
B
AD 10μM
C 1μM
AD 10 +C 1 μM
C 1μM
C
C 3μM
AD 10 +C 3 μM
0·5mV
5min

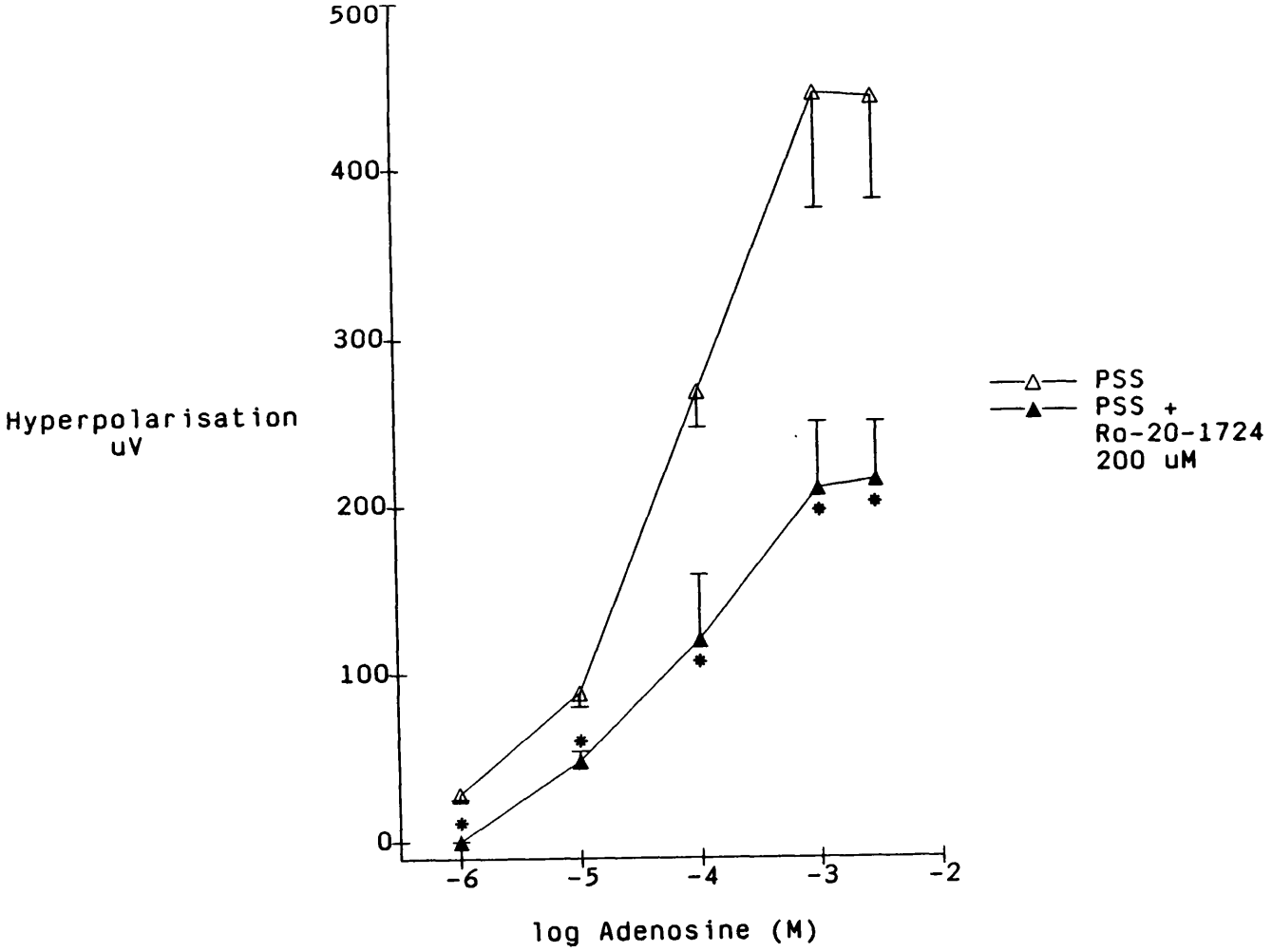


Fig. 5.10. Concentration response curves for the hyperpolarisation response of isolated rat SCG produced by adenosine in normal physiological salt solution (PSS) and PSS-containing Ro20-1724.

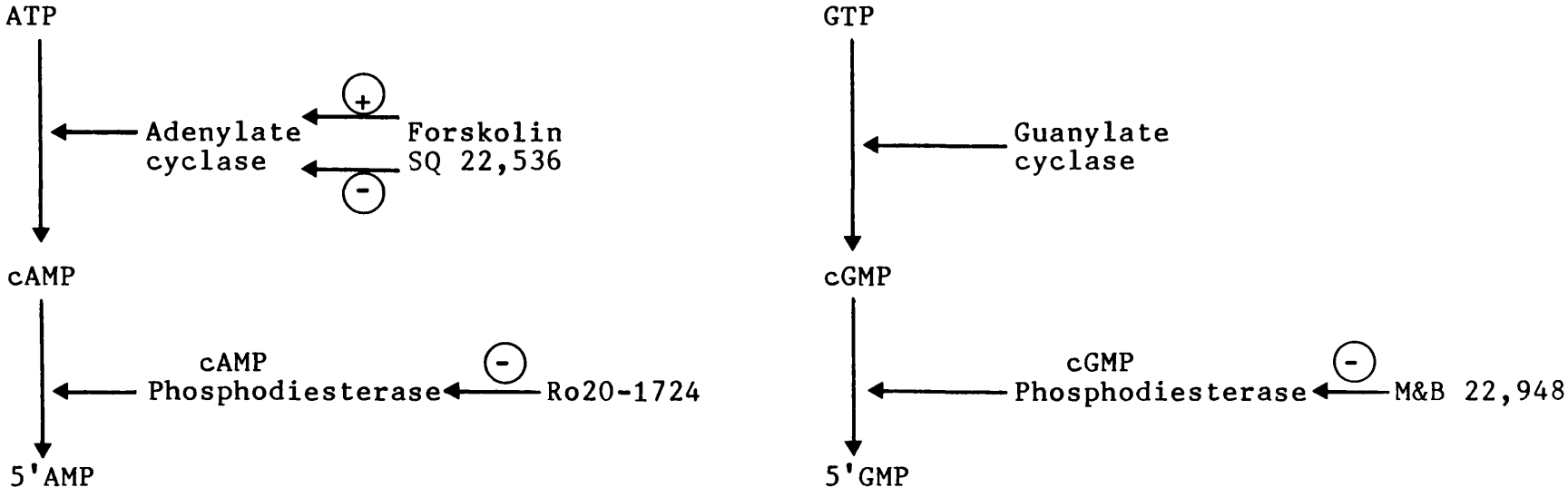


Fig. 5.11. Pathways for the metabolism of ATP and GTP to form cAMP and cGMP and the proposed sites of action of SQ 22,536, Ro20-1724 and M&B 22,948 on the cyclic nucleotide metabolism. (+) = stimulation and (-) = inhibition of enzyme.

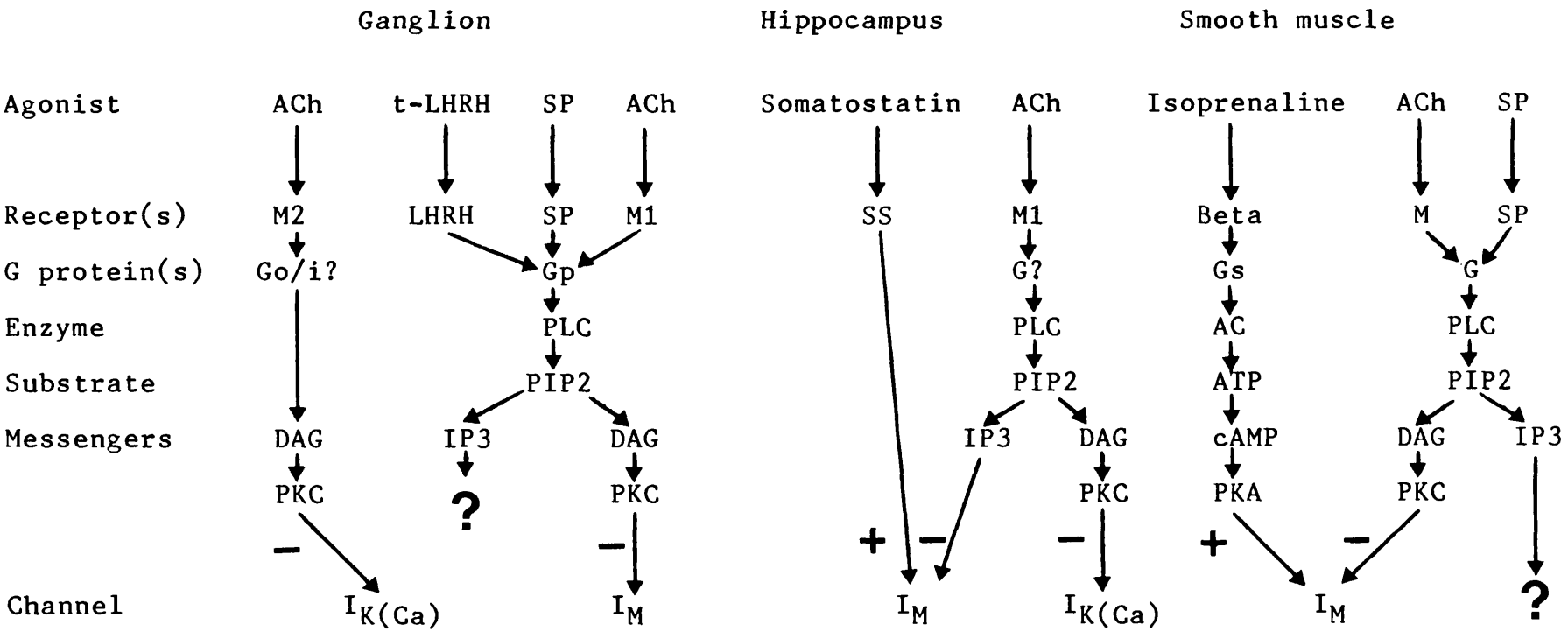


Fig. 5.12. Some suggested pathways for M-current control of the rat SCG, hippocampus and smooth muscle cells (adapted from Brown, 1988).

Abbreviations: ACh, acetylcholine; SP, substance P; PLC, phospholipase C; AC, adenylate cyclase; PIP2, phosphatidylinositol-2,3-bisphosphate; IP3, inositol-1,4,5-triphosphate; DAG, diacylglyerol; PKA, protein kinase A; PKC, protein kinase C.

Fig. 5.13. Response of a single rat SCG to muscarine, adenosine and increasing concentrations of adenosine during the continuous application of muscarine

(A) a one minute application of 100nM muscarine (B) a two minute application of 100uM adenosine followed by the continuous application of 100nM muscarine and of 100uM adenosine (5 minutes) and (C) continuous trace of the same ganglion as in a & b, and the response to increasing concentrations of adenosine (1-1000uM) during continuous the application of muscarine. Note: The response to adenosine (100uM) during the application of muscarine (c) is over twice that obtained in the absence of muscarine (b).

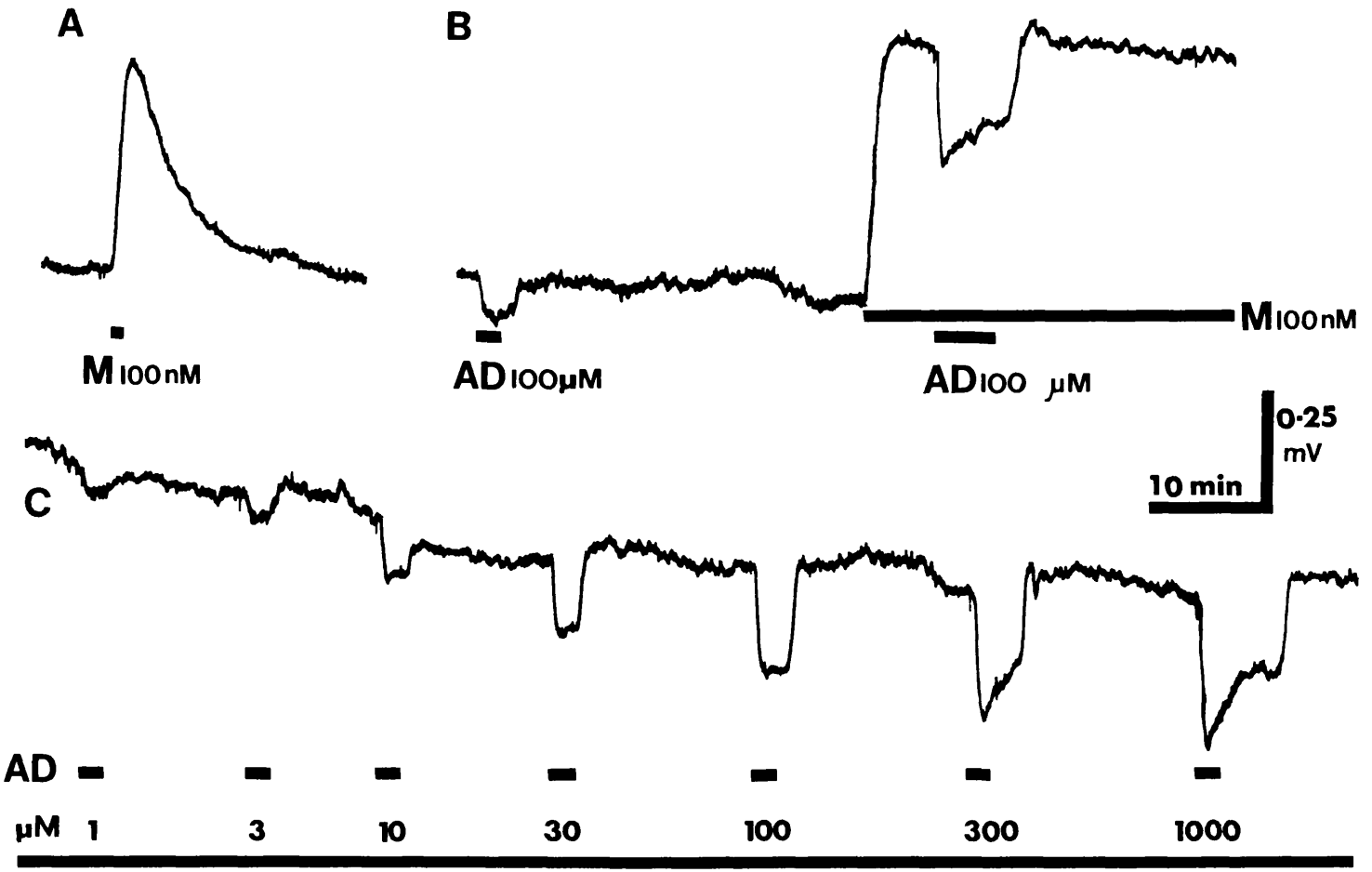

A
B
M 100nM
AD 100µM
M 100nM
AD 100 µM
0·25
mV
10 min
C
AD
µM 1
3
10
30
100
300
1000

Fig. 5.14. Response of an isolated rat SCG to adenosine in the absence and presence of muscarine, pilocarpine or phorbol dibutyrate.

The response to 100uM adenosine (AD, 2 minutes) is shown before the depolarising response to (A) muscarine (musc), (B) pilocarpine (10uM, 1 minute) and (C) phorbol dibutyrate (PDBu, 200nM) and during the depolarisations to these agonists. NB: Phorbol dibutyrate (PDBu) produced a concentration-dependent sustained depolarisation of the SCG. The arrow denotes displacement of chart recorder pen to allow continuous recording of response. Responses to 20 and 200nM began 1 to 2 minutes after application and developed slowly over at least 15 minutes and took over 2 hours to washout. At 20nM PDBu produced a comparable peak depolarisation to that produced by 100nM muscarine (1 min application) and a similar potentiation of the hyperpolarisation to 100uM adenosine (AD) (Tables 5.7 & 5.8).

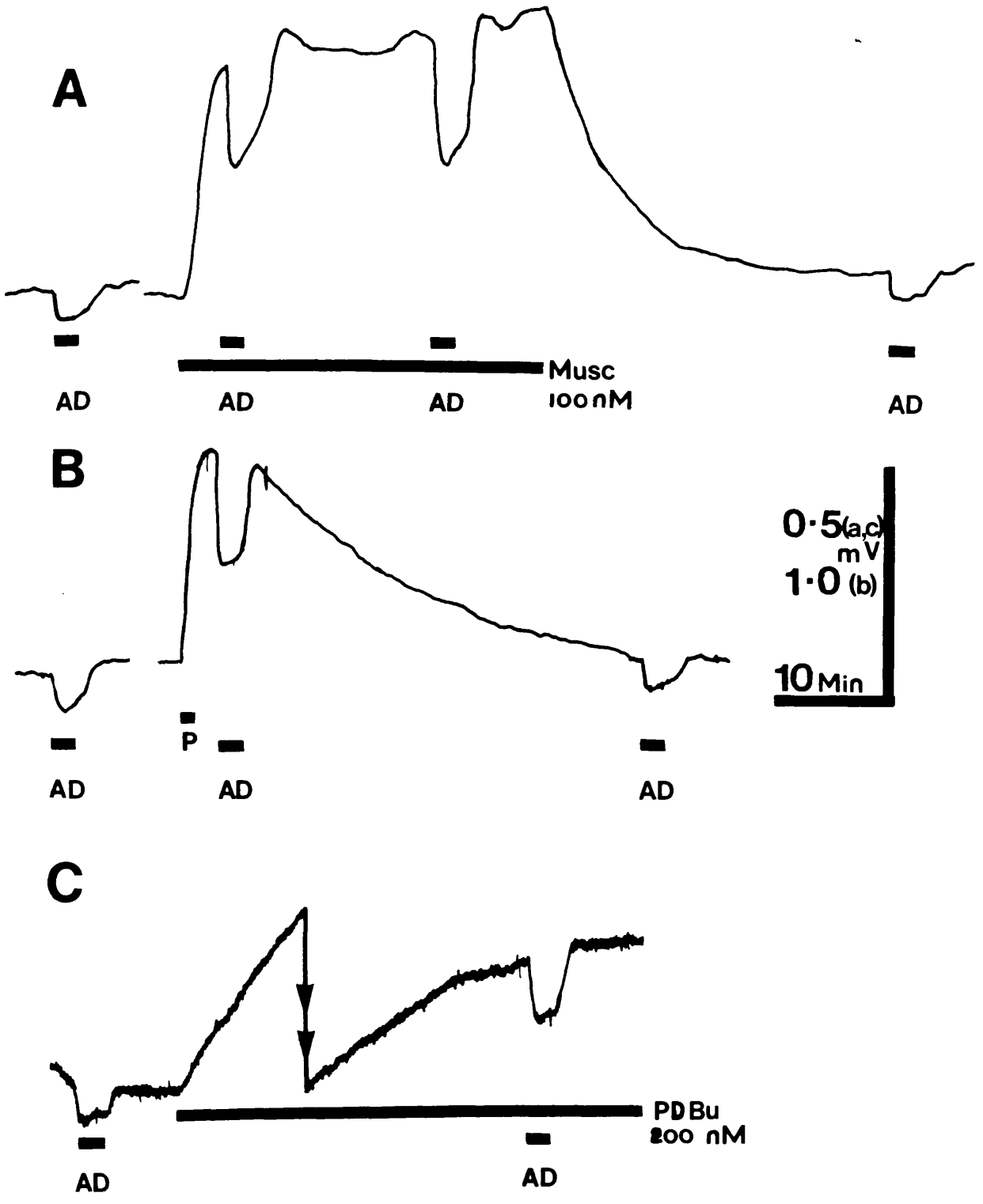

A
Musc
100nM
AD
AD
AD
AD
B
0·5(a,c)
mV
1·0 (b)
10 Min
P
AD
AD
AD
C
PDBu
200 nM
AD
AD

Fig. 5.15. Pathways for the generation and metabolism of arachidonic acid (adapted from Cook, 1990).

The activation of a receptor (R) can activate G proteins (G) to release arachidonic acid directly from phospholipids through the action of phospholipase A_2 (PLA_2) or from the prior action of phospholipase C (PLC), followed by the action of diglyceride lipase. Alternatively the diglyceride may be phosphorylated to phosphatidic acid by the action of diglyceride kinase, and arachidonate may be released through the action of PLA_2. Arachidonic acid may then be metabolised by 5 or 12-lipoxygenase to produce 5 or 12-hydroperoxyeicosatetranoic acids (5-HPETE, 12-HPETE respectively), by cyclooxygenase to prostaglandins (PGs) and thromboxanes (TXs) or by epoxygenase to epoxides. The arrows, broken lines indicate inhibition of synthesis of PGs and TXs by indomethacin and inhibition of 5 and 12-lipoxygenase products by nordihydroguaiaretic acid (NDGA).

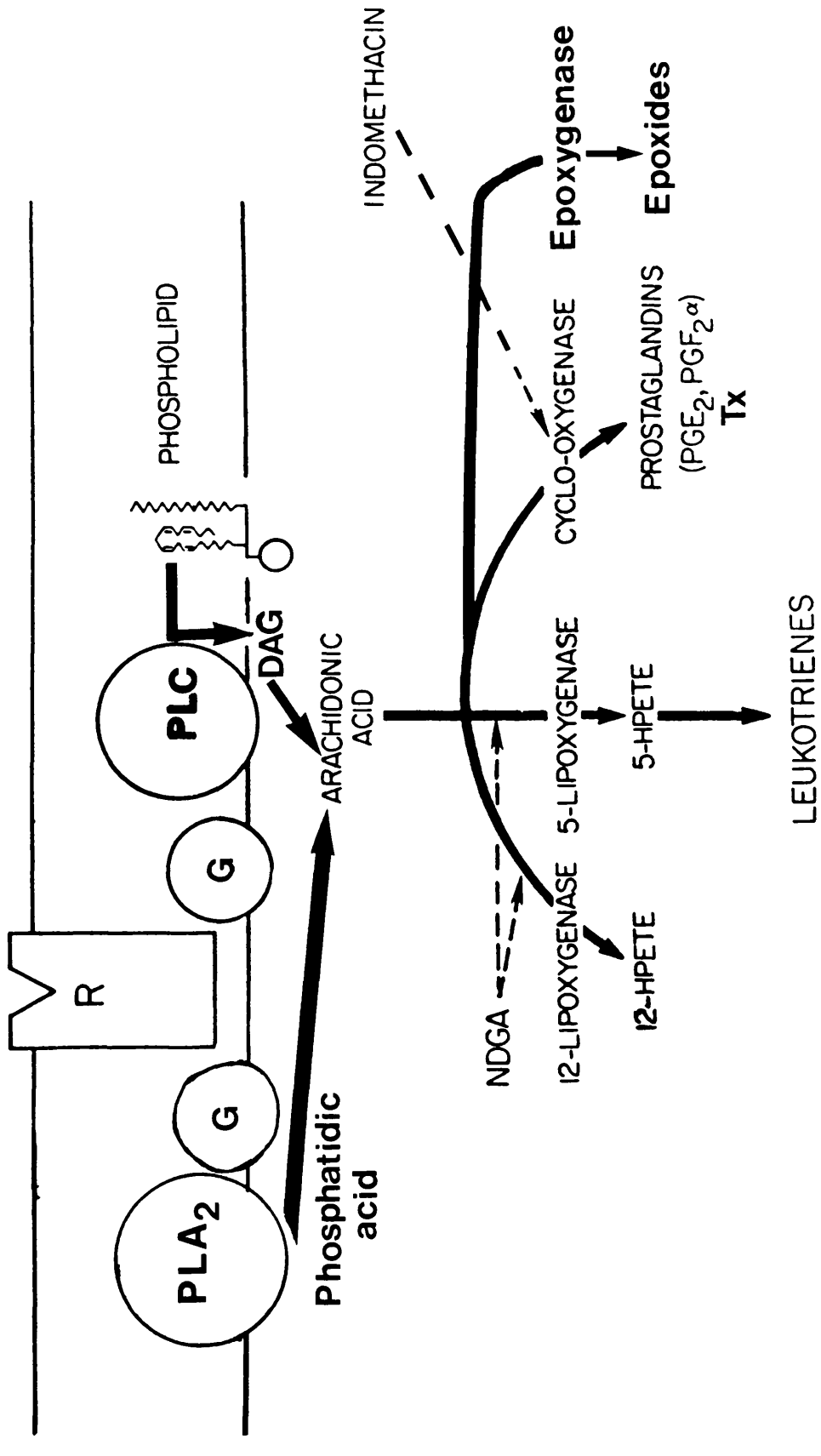

R
G
PLC
PHOSPHOLIPID
DAG
G
PLA2
Phosphatidic acid
ARACHIDONIC ACID
INDOMETHACIN
Epoxygenase
Epoxides
CYCLO-OXYGENASE
PROSTAGLANDINS
(PGE2, PGF2α)
Tx
5-LIPOXYGENASE
5-HPETE
LEUKOTRIENES
NDGA
12-LIPOXYGENASE
12-HPETE

Fig. 5.16. Effect of indomethacin and nordihydroguaiaretic on the log concentration-response curve of the rat SCG to adenosine.

The response to adenosine (AD, 2 minutes applciation) was recorded in the absence and presence of 10uM indomethacin (INDO) and then 10uM INDO + 50uM nordihydroguairaretic acid (NDGA). The statistical significance was determined by paired t-tests with respect to control responses and is

indicated by a ☆ for controls vs. INDO and by ★

for INDO vs. INDO + NDGA.

A stock solution of indomethacin was freshly prepared for each experiment and used within 4 hours. This stock solution contained indomethacin, 10mg and anhydrous sodium carbonate 5mg dissolved into 10ml distilled water and diluted to achieve a final concentration of 10uM INDO in PSS.

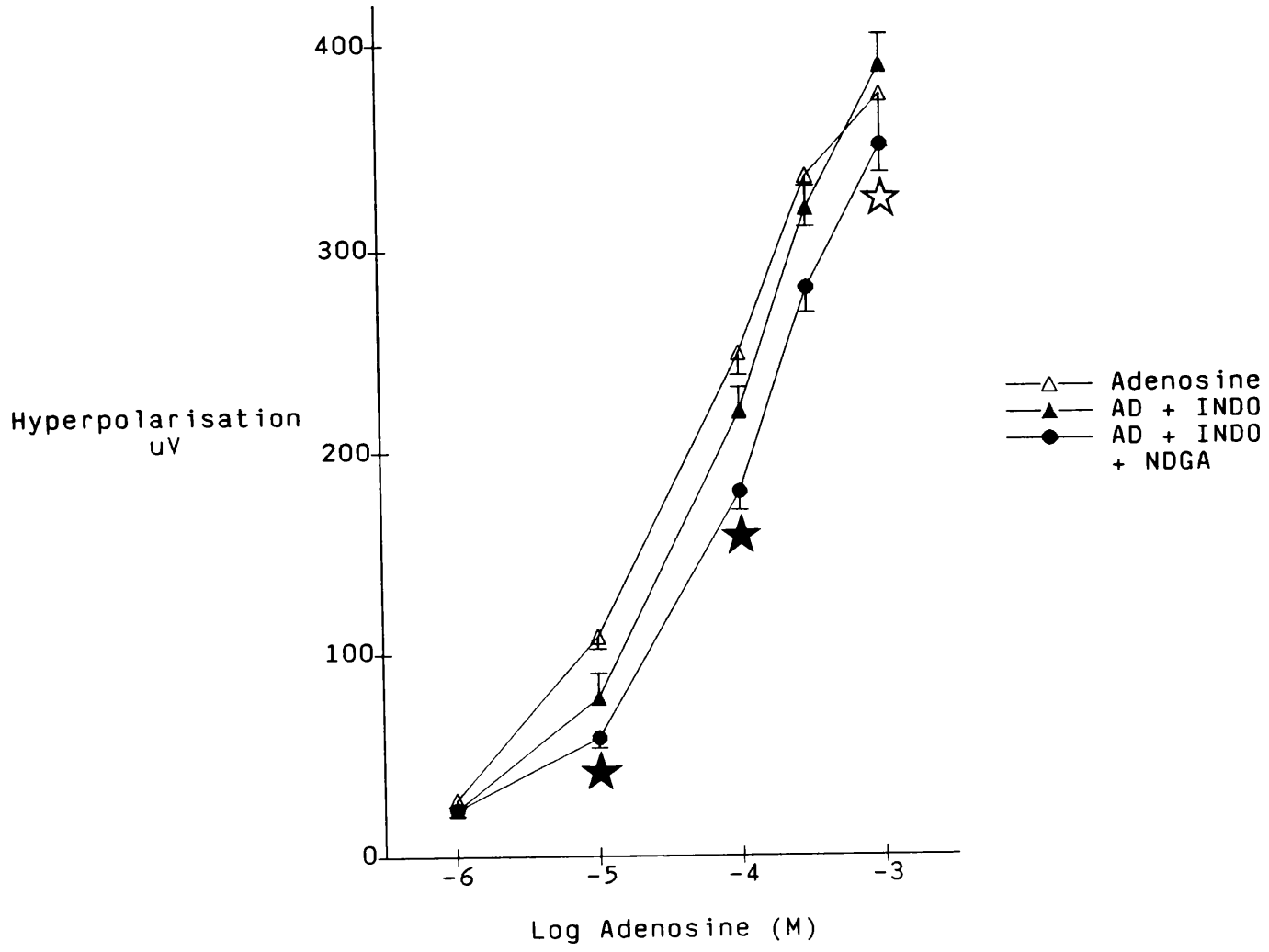

Hyperpolarisation
uV
400
300
200
100
0
-6
-5
-4
-3
Log Adenosine (M)
Adenosine
AD + INDO
AD + INDO
+ NDGA

Table 5.1. The effect of N-methylatropine, pirenzepine and vasoactive intestinal polypeptide (VIP) on the response of the rat SCG to adenosine in physiological salt solution (PSS).

The responses to adenosine (100uM, 2 minute application) and muscarine (100nM, 1 minute) in PSS, were compared to the response after a minumum of 20 minutes in the test compound and the statistical significance between control and test responses were determined using a two tailed paired t-test on the uV values where * = $P < 0.05$ and N = number of ganglia tested.

TEST COMPOUND	Concentration uM	N	RESPONSE TO MUSCARINE Control uV	RESPONSE TO MUSCARINE Test uV	RESPONSE TO ADENOSINE Control uV	RESPONSE TO ADENOSINE Test uV
N-Methylatropine	2	2	90, 115	0, 0		
Pirenzepine	0.3	4	220 ± 19	11 ± 12*	-69 ± 11	-48 ± 11
Low Ca^{2+} (0.1mM) PSS & N-methylatropine	2	3	343 ± 97	0 ± 6*		
VIP	0.1	3	263 ± 63	275 ± 51	-52 ± 7	-60 ± 27

Table 5.2. The response of the rat SCG to various agonists and the effect of 10uM adenosine on the response to these agonists. The responses (uV) to agonists in the presence of 10uM adenosine (test) were compared to the response before adenosine (pre-test) using a two tailed paired t-test. The level of significance is indicated by a * for $P < 0.05$; ** for $P < 0.01$ and *** for $P < 0.001$. DMPP produced a depolarisation (dep) followed by an after hyperpolarisation (hype) and the response to DMPP was assessed in 30uM adenosine (a).

AGONIST Compound	Conc. uM	N	Response (uV mean ± SEM) PRETEST	TEST	POSTTEST	% Change in agonist in 10uM AD
GABA	1	6	90 ± 17	90 ± 21	89 ± 18	-3 ± 11
〃	3	6	204 ± 75	213 ± 83	218 ± 82	-1 ± 2
〃	10	6	482 ± 67	475 ± 62	503 ± 69	1 ± 6
5HT	10	5	320 ± 27	341 ± 39	376 ± 42	3 ± 5
DMPP[a]	10 dep	6	927 ± 262	877 ± 281	940 ± 305	-10 ± 5
〃	10 hype	6	-635 ± 218	-643 ± 272	-703 ± 263	-6 ± 7
ISOPRENALINE	0.1	7	277 ± 31	266 ± 27*	255 ± 29	-8 ± 3
CARBACHOL	0.1	6	301 ± 42	266 ± 42**	309 ± 43	-12 ± 3
MUSCARINE	0.1	9	508 ± 53	454 ± 54***	532 ± 61	-11 ± 3
METHYLFURMETHIDE	0.1	6	572 ± 134	514 ± 125*	559 ± 132	-12 ± 4

Table 5.3. The response of the rat SCG to various agonists and the effect of 100uM adenosine on the response to these agonists. The responses (uV) to agonists in the presence of 100uM adenosine (test) were compared to the response before adenosine (pre-test) using a two tailed paired t-test. The level of significance is indicated by a * for $P < 0.05$; ** for $P < 0.01$ and *** for $P < 0.001$. Both GABA at 100uM and DMPP produced a depolarisation (dep) followed by an after hyperpolarisation (hype) and the response to DMPP was assessed in 30uM adenosine (a).

AGONIST Compound	Conc. uM	N	Response to uV mean ± SEM PRETEST	TEST	POSTTEST	% Change in agonist in 100uM AD
POTASSIUM	8000	3	203 ± 38	203 ± 43	217 ± 55	-1 ± 4
//	12000	7	580 ± 114	579 ± 111	586 ± 103	1 ± 2
BARIUM	1000	6	350 ± 18	309 ± 21*	322 ± 21	-12 ± 4*
GABA	1	6	91 ± 32	97 ± 32*	83 ± 31	11 ± 4
//	3	6	292 ± 82	313 ± 78	298 ± 76	11 ± 7
//	10	7	594 ± 137	643 ± 144*	623 ± 111	9 ± 4
//	100 dep	7	994 ± 111	1037 ± 100	1010 ± 112	5 ± 3
	100 hype	7	-131 ± 12	-167 ± 23	-163 ± 21	37 ± 25
5HT	10	6	312 ± 63	358 ± 79*	308 ± 79	14 ± 5
2Me5HT	30	4	223 ± 68	221 ± 65	228 ± 65	1 ± 3
DMPP	10 dep	8	855 ± 265	830 ± 265	851 ± 253	-4 ± 3(a)
	10 hype	8	-576 ± 225	-498 ± 202	-561 ± 225	-5 ± 9(a)
ISOPRENALINE	0.01	4	141 ± 11	136 ± 17	154 ± 13	-5 ± 9
	0.1	4	268 ± 29	261 ± 52	284 ± 44	-8 ± 6
CARBACHOL	0.1	12	206 ± 29	165 ± 20	218 ± 31	-14 ± 7
MUSCARINE	0.1	61	312 ± 20	228 ± 16***	313 ± 21	-26 ± 2
METHYLFURMETHIDE	0.1	5	653 ± 109	554 ± 99*	554 ± 116	-18 ± 5

Table 5.4. The effect of 10uM and 100uM adenosine on the response of isolated rat SCG to carbachol, muscarine and methylfurmethide.

Compound	AGONIST Conc. nM	Response uV	% depression by 10uM AD	Response uV	% depression by 100uM AD
CARBACHOL	30	95 ± 14	-10 ± 6 (9)	129 ± 16	-6 ± 7 (6)
	100	301 ± 42	-12 ± 3 (7)**	206 ± 29	-14 ± 7 (12)
	300	289 ± 61	-5 ± 2 (10)*	378 ± 33	-17 ± 2 (7)***
	1000			411 ± 73	-3 ± 3 (4)
	10000n			876 ± 62	-2 ± 8 (5)
	30000n			1162 ± 215	-7 ± 9 (5)
MUSCARINE	30	168 ± 23	-14 ± 4 (8)**	242 ± 52	-17 ± 5 (10)*
	100	508 ± 53	-11 ± 3 (9)***	312 ± 20	-26 ± 2 (61)***
	300	903 ± 167	-12 ± 4 (6)*	708 ± 79	-23 ± 3 (8)***
	1000			584 ± 75	-4 ± 6 (6)
	10000			587 ± 92	+13 ± 6 (6)
MeF	30	230 ± 37	-14 ± 5 (12)*	208 ± 33	-30 ± 3 (9)**
	100	572 ± 134	-12 ± 4 (7)*	653 ± 109	-18 ± 5 (7)*
	300	892 ± 143	-7 ± 3 (11)*	916 ± 174	-19 ± 4 (5)*
	1000			800 ± 103	-12 ± 4 (5)*

The peak responses (uV) to muscarine, carbachol and methylfurmethide (MeF) in the presence of adenosine were compared to the peak response before adenosine using a two tailed paired t-test. The level of significance is indicated by a * for $P < 0.05$; ** for $P < 0.01$ and *** for $P < 0.001$. n = N-methylatropine (2uM) resistance component i.e., a fast "nicotinic" response which occurred before the slower muscarinic responses.

Table 5.5. The effect of methoctramine (MTO, 0.3uM) on the depression by 100uM adenosine (AD) of the response of the rat SCG to carbachol, muscarine and methylfurmethide (MeF)

AGONIST Compound	Conc. uM	N	CONTROL (PSS) Response to Agonist	CONTROL (PSS) Response to AD	CONTROL (PSS) % depression by 100uM AD	MTO (0.3uM in PSS) Response to Agonist	MTO (0.3uM in PSS) Response to AD	MTO (0.3uM in PSS) % depression by 100uM AD
CARBACHOL	0.3	9	372 ± 39	-68 ± 10	26 ± 7	371 ± 29	-56 ± 8	35 ± 6**(b)
MUSCARINE	0.1	6	240 ± 34	-64 ± 12	29 ± 12	198 ± 36***(a)	-58 ± 15	39 ± 5
MeF	0.03	6	183 ± 33	-53 ± 13	19 ± 6	143 ± 31*	-40 ± 12	34 ± 4

(a) The peak response (in uV) to agonists or adenosine in the presence of MTO was compared to the peak responses obtained in PSS using a paired t-test. (b) The % depression of agonist responses by adenosine in the presence of MTO was compared to the % depression of agonist responses using the Wilcoxon matched pairs signed ranks test. For both statistical tests the significance is indicated by a * for $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$, where N is the number of ganglia tested.

Table 5.6. The effect of 1uM and 10uM adenosine (AD) on the response of isolated rat SCG to carbachol in physiological salt solution containing 0.1mM calcium and 300uM pirenzepine. The responses (uV) to carbachol in the presence of adenosine were compared to the response in the absence of adenosine using a two-tailed paired t-test. The level of significance is indicated by a * for $P < 0.05$. N = number of ganglia tested.

Carbachol Conc. uM	Carbachol response uV	Adenosine response (uV) 1uM	Adenosine response (uV) 10uM	N	Response to co-administration of carbachol + AD 1uM	Response to co-administration of carbachol + AD 10uM
0.3	-124 ± 21	-34 ± 9		6	-130 ± 27	
0.3	-48 ± 4		-29 ± 9	7		-78 ± 9
1.0	-176 ± 42	-29 ± 8		6	-192 ± 43	
1.0	-151 ± 34		-56 ± 11	8		-161 ± 34*

Table 5.7. Effect of different depolarising agonists on the isolated rat SCG and the response to adenosine (100uM, 2 min). The response to adenosine in the absence and presence of agonist was compared using paired t-test of values and * = $P < 0.05$, ** = $P < 0.01$, where N = number of ganglia tested.

AGONIST	N	Conc. uM	Application time (min)	Depolarisation uV	Response to 100uM adenosine PRETEST	TEST
Potassium	7	2000	C	277 ± 28	67 ± 5	60 ± 6
Rubidium	4[a]	1000	C	220 ± 83	78 ± 21	100 ± 20*
	4[a]	6000	C		78 ± 21	196 ± 45*
Lithium	4	10000	C	120 ± 9	79 ± 4	88 ± 6
Ouabain	3	1	C	78 ± 28	98 ± 16	133 ± 24
	7	10	C	100 ± 23	95 ± 11	150 ± 14**
Forskolin	6	1	C	184 ± 21	71 ± 14	110 ± 9*
PDBu	8	0.2	C	381 ± 60	55 ± 7	133 ± 17**
VIP	3	0.1	1	140 ± 50	52 ± 7	60 ± 21
Nicotine	4	0.1	2	203 ± 59	90 ± 25	73 ± 24**
〃	4	1	2	>800 ± 70	68 ± 14	43 ± 24
〃	3	10	1	>710 ± 10	62 ± 14	77 ± 23
2Me5HT	4	300	1	323 ± 82	93 ± 25	93 ± 26
Isoprenaline	7	1	2	193 ± 28	84 ± 17	85 ± 20
	7	10	2	127 ± 13	83 ± 6	73 ± 5

a = same ganglia used for response to adenosine in 1mM and 6mM rubidium
C = continuous application

Table 5.8. Effect of some agonists reported to inhibit the M-current of sympathetic ganglia on the d.c. potential of the rat SCG and the response to adenosine (100uM, 2 min). The statistical difference between the response to adenosine before agonist (PRETEST) and during the response to the agonist (TEST) was determined using a paired t-test and denoted by a * = $P < 0.05$, ** $P < 0.01$ and *** = $P < 0.001$, where N = number of ganglia tested.

AGONIST	N	Conc. uM	Application time (min)	Depolarisation of agonist uV	Response to 100uM adenosine (-uV) PRETEST	TEST
Muscarine	4	0.1	1	140 ± 10	83 ± 3	120 ± 11*
〃	4	0.1	2	299 ± 11	71 ± 5	148 ± 8**
〃	4	0.1	C	535 ± 104	69 ± 8	134 ± 28*
Pilocarpine	6	1	2	360 ± 63	57 ± 11	128 ± 31*
BM5[a]	4	0.1	1	171 ± 24	66 ± 8	109 ± 11*
BM5	4	1	1	300 ± 63	66 ± 8	183 ± 37*
ERP	8	1	2	297 ± 81	78 ± 7	177 ± 18**
LHRH	2	5	2	36 ± 19	60, 75	50, 65
LHRH	4	30	0.7	26 ± 2	53 ± 3	60 ± 4
UTP	4	100	2	64 ± 1	83 ± 5	79 ± 11
Barium	3	3000	1	428 ± 137	68 ± 7	120 ± 7*
	4	2500	C	518 ± 107	58 ± 9	73 ± 5

a = same ganglia used for both 0.1 and 1uM BM5
C = continuous application of agonist

Table 5.9. The effect of M&B 22,948, denbufylline, Ro20-1724 and SQ 22,536 on the response of the rat SCG to adenosine (AD), cyclopentyladenosine (CPA), muscarine and the depression of the response to muscarine (100nM, 1 minute) by adenosine or CPA. The response in physiological salt solution (PSS) to AD, CPA or muscarine (CONTROL) was compared to the response in PSS containing a test compound using a paired t-test. The depression of muscarine by CPA or AD in PSS with or without test compound was compared using the Wilcoxon matched pairs signed ranks test where N > 6, and N is the number of ganglia. For both statistical tests the significance is indicated by a * for P < 0.05 and ** for P < 0.01.

				TEST PSS CONTAINING							
							Denbufylline				
Agonist Response	Conc.	N	Control PSS	M&B 22,948 10uM	M&B 22,948 100uM	1uM	(BRL 30982) 10uM	100uM	Ro20-1724 200uM	SQ 22,536 10uM	SQ 22,536 100uM
Adenosine	100uM (2')	7	-74 ± 21			-69 ± 15	-65 ± 20**				
	100uM (2')	4	-69 ± 24					-15 ± 10*			
	100uM (2')	7	-76 ± 12						-33 ± 2*		
	100uM (2')	7	-53 ± 10	-49 ± 7	-53 ± 4						
CPA	100nM (2')	3	-77 ± 23						-37 ± 7		
Muscarine	100nM (1')	7	275 ± 41			249 ± 34	268 ± 56				
	100nM (1')	4	253 ± 51					116 ± 26*			
	100nM (1')	6	284 ± 31						286 ± 25		
	100nM (1')	7	304 ± 41							311 ± 35	289 ± 41
% depression of response to 100nM muscarine by											
Adenosine	100uM	7	20 ± 4			23 ± 5	24 ± 5				
	100uM	4	29 ± 7					11 ± 9			
	100uM	10	24 ± 3						31 ± 4		
CPA	**100nM**	**7**	**22 ± 3**							24 ± 3	25 ± 3

Table 5.10. Response of three isolated rat SCG in physiological salt solution (PSS) to adenosine and carbachol in 0.1mM Ca^{2+} and pirenzepine (0.3uM) in the absence (CONTROL) and presence of 1-(5-isoquinolinyl-sulfonyl)-2-methyl-piperzine (H7). The values in brackets are the responses of a ganglion used as a time control. There was no significant difference between the responses to both adenosine and carbachol in the absence (PSS) or presence of H7 (PSS + H7, 50uM) at any incubation time (paired t-test, uV responses).

AGONIST	CONTROL PSS	PSS + H7 50uM Incubation time (minutes) 10	20	30
Adenosine 100uM (2 minute application)	-127 + 12 (-60)	-117 + 13 (-60)	-	-137 + 15 (-95)
Carbachol 300nM (1 minute application)	-90 + 13 (-55)	-	-77 + 7 (-55)	-

Table 5.11. The response of three rat SCG in normal physiological salt solution (PSS) to muscarine, phorbol dibutyrate (PDBu), adenosine and the depression of the response to muscarine by adenosine in the absence (CONTROL) and presence of 1-(5-isoquinolinyl-sulfonyl)-2-methyl-piperazine (H7).

Incubation times in H7 (a) = 10 minutes, (b) = 22 minutes, (c) = 36 minutes. There was no significant difference (paired t-test) between the responses to muscarine, PDBu and adenosine recorded in the presence and absence of H7. The values in brackets (uV response) are the response of a single ganglion used as a time control i.e., not treated with H7. The response to PDBu for the time control ganglion increased with time (t) but the ratio of the responses to muscarine/PDBu was similar, suggesting a general increase in sensitivity of this ganglion with time and not a specific change in sensitivity to PDBu with time.

AGONIST	Conc. uM	Application time (minutes)	Response to agonist in PS (CONTROL)	PSS + H7 (50uM)
Muscarine	0.1	1	247 + 42 ($\bar{135}$)	187 + 34[a] ($\bar{150}$)
PDBu	0.02	5	90 + 6 ($\bar{75}$)	113 + 18[c] ($\bar{165}^{t}$)
Adenosine	100	2	-50 + 12 ($\bar{-40}$)	-63 + 15[b] ($\bar{-40}$)
% depression of muscarinic response (100nM) by 100uM adenosine			-37 + 3 ($\bar{-21}$)	-40 + 5[b] (-27)

Table 5.12. Interaction of purines with postsynaptic responses to acetylcholine on muscarinic and nicotinic receptors of various preparations

PREPARATION	CHOLINERGIC [1] RESPONSE	PURINE [2]	CHANGE IN CHOLINERGIC [3] SENSITIVITY	REFERENCE
Rat diaphragm	M	AD ATP	0 +	Ewald (1986)
Bullfrog sympathetic ganglion cells	N	ATP	+	Akasu et al. (1981) Akasu & Koketsu (1985)
Cat blood pressure	N	AD	+	Von Borstel et al. (1984, 1986)
Rabbit SCG	M	cAMP cGMP	+ -	Kobayashi et al. (1978)
Rabbit gastric muscle	M	AD	+	Gustafsson (1981)
Helix central neurones	M	AD	+ or -	Cox & Walker (1985)
Secretion of insulin (rat pancreas)	M	ATP (P_2)	+	Bertrand, Chapal & Loubatieres-Mariani (1986)
Xenopus oocyte	M	AD	-	Stinnakre & Van Renterghem (1986)
Cat heart rate, eyelid tension, vas deferens perfusion pressure	M/N?	AD	+	Von Borstel et al. (1986)
Hippocampus CA3	M	AD	-	Worley et al. (1987, 1988)
Hippocampal CA1 excitation	M	AD	-	Brooks & Stone (1988)
Guinea pig ileum	M2	Isobutylthio-adenosine	-	Pankaskie et al. (1985) Smejkal et al. (1989)

(1) N = nicotinic & M = muscarinic receptor. (2) AD = adenosine, ATP = adenosine triphosphate, cAMP = cyclic adenosine-3',5'-triphosphate, cGMP = guanosine-3',5'-triphosphate. (3) 0 - no effect, + = increase in sensitivity, - = decrease in sensitivity

CHAPTER SIX

CHARACTERISATION OF THE PURINOCEPTORS OF THE RAT SCG

CHARACTERISATION OF THE PURINOCEPTORS OF THE RAT SCG

The classification of purine receptors, for the P1 receptor has been mainly based on the relative order of potency of adenosine and its analogues, and for the P2 receptor on the order of potency of ATP analogues (Chapter One, table 1). By themselves these results are not conclusive evidence for the existence of two separate receptors and need to be substantiated by the study of the effects of selective antagonists on these responses.

6.1 Characterisation of the purinoceptors of the rat SCG

To discover if the rat SCG contains P2-purinoceptors, the effects of ATP were compared to slowly degradable analogues of ATP, namely alpha, beta-methylene ATP (α,β-MeATP) and beta, gamma-methylene ATP and a relatively selective P2 purinoceptor agonist, 2 methylthio-ATP (2MeS-ATP). In addition the effect of 8PT on the responses to ATP and β,γ-MeATP were examined.

6.1.1 The effect of ATP on the d.c. potential of the rat SCG

ATP produced small and often insignificant changes in the d.c. potential unless the responses to ATP were recorded from ganglia bathed in low K^+/Ca^{2+} PSS (Fig. 6.1). In low K^+/Ca^{2+} PSS, ATP produced concentration-dependent hyperpolarisations, however the response to other agonists including adenosine and carbachol were also enhanced.

It has been proposed that some of the effects of ATP are mediated by adenosine due to the enzymatic (5'-nucleotidase) hydrolysis of the nucleotide (Bruns, 1980b; Zimmermann, Grondal & Keller, 1985) e.g. in the central nervous system many effects of nucleotides are antagonised

by methylxanthine analogues and there is little evidence for the presence of P2-purinoceptors in the hippocampus (Stone & Cusack, 1989). The lower potency of ATP compared to adenosine on the rat SCG may be indicative of the degradation of ATP to adenosine or may reflect other factors such as differences in the efficacy of numbers of receptors on the ganglion.

6.1.2 Effect of 8-phenyltheophylline on the response to ATP and beta,gamma-MeATP

The ability of 8PT to antagonise the hyperpolarisation of the ganglion by ATP (Figs. 6.1 and 6.2; table 6.1) and convert the response into a depolarisation suggests ATP may hyperpolarise and depolarise the rat SCG via P1- and P2-purinoceptors respectively. The former action would be predicted to occur if ATP was metabolised to adenosine and subsequently activates adenosine-receptors. The opposing nature of the P1- and P2-purinoceptor mediated responses to ATP (Fig. 6.1) may thus account for the relatively lower potency of ATP compared to adenosine (Fig. 6.3).

The ability of ATP to depolarise sympathetic ganglia has also been observed by two different research groups led by Siggins and by Akasu (Siggins, Gruol, Padjen & Forman, 1977; Akasu, Hirai & Koketsu, 1981). In contrast Brown and colleagues reported ATP hyperpolarised the rat SCG by upto 150uV in a PSS very similar to normal PSS used in this study (see Figures 5a & b, Brown et al., 1979). It is possible that the larger hyperpolarisations reported by Brown & colleagues occurred because of the storage of ganglia at 4^{o}C overnight, resulting in a loss of sensitivity to the P2-purinoceptor mediated depolarisation. A similar phenomenon was reported by Brown & Caulfield (1979) for the absence of the

depolarisation to isoprenaline by rat SCG stored overnight at 4°C.

In low K^+/Ca^{2+} PSS, the P2x-purinoceptor agonist β,γ-MeATP produced concentration related hyperpolarisations. Because of the likelihood of degradation of β,γ-MeATP to adenosine (Cusack, Hourani & Welford, 1988), the effect of 8PT on the response to β,γ-MeATP was examined. 8PT produced a parallel rightward shift in the concentration-response curve over the limited concentrations of β,γ-MeATP tested (Fig. 6.4). Adenosine and β,γ-MeATP probably activate the same receptor, i.e. a P1-purinoceptor as the response to β,γ-MeATP was clearly antagonised by 8PT. The greater CR produced by β,γ-MeATP in the presence of 8PT may simply have arisen from the lower concentration of adenosine generated from the degradation of β,γ-MeATP. Alternatively, other mechanisms may be responsible for the larger CR, including; (1) the response to β,γ-MeATP was composed of an 8PT sensitive hyperpolarisation and an 8PT insensitive depolarisation to β,γ-MeATP, and the presence of 8PT would then enhance the relative potency of 8PT, and/or (2) β,γ-MeATP may activate an ATP sensitive P1-purinoceptor (Collis & Pettinger, 1982) or a P3-purinoceptor (Westfall, Shinozuka & Bjur, 1990). Further investigation of the effects of β,γ-MeATP would necessitate the use of a greater range of concentrations of antagonist to obtain an accurate pA_2 value (by the method of Arunlakshana & Schild, 1959).

6.1.3 Effect of 2-methylthio-adenosine-5'-triphosphate on d.c. potential and response to muscarine

The introduction of 2-substituents on to methylene phosphonates enhances their potency on P2-purinoceptors (Cusack, Welford & Hourani, 1988) and the effect of a

relatively selective P2y purinoceptor agonist, 2 methylene thio ATP (2MeS-ATP) (Burnstock, Cusack, Hills, MacKenzie & Meghj, 1983) on the rat SCG was examined.

Although metabolised by some tissues 2MeS-ATP is a potent P2y purinoceptor agonist at nanomolar to low micromolar concentrations and the small depolarisations in response to 100uM 2MeS-ATP (Fig. 6.3, c.f. Figures 6.3 & 6.6, response of 2MeS-ATP and α,β-MeATP) suggest there are few if any P2y purinoceptors on the rat SCG. The inability of 100uM 2MeS-ATP to alter the response to muscarine (Table 6.1) supports this view.

Thus the actions of ATP and β,γ-MeATP are most likely to occur either due to an action on P2x-purinoceptors or via their metabolism to adenosine. Of the nucleotides tested, it would be expected that α,β-MeATP is the most resistant to enzymatic hydrolysis to adenosine and does not activate P1-purinoceptors. Support for this hypothesis is provided by the lack of antagonism of the response to α,β-MeATP in the presence of 8PT (Fig. 6.6). Given the low potency of 2MeS-ATP and insensitivity of α,β-MeATP-mediated depolarisations to 8PT, it is suggested that the effect of ATP on the ganglion is due to a mixutre of a P2x-mediated depolarisation and a P1-mediated hyperpolarisation. Further evidence to support this conclusion was obtained using suramin P2x-purinoceptor antagonist.

6.1.4 Effect of suramin on the response of the ganglion to adenosine, ATP, β,γ-MeATP and α,β-MeATP

The classification of P2-purinoceptors is limited by the lack of selective competitive antagonists. The trypanocide suramin has recently been suggested to be a

specific antagonist of the P2x-purinoceptor in the mouse vas deferens (Dunn & Blakely, 1988) and antagonises the effects of α,β-MeATP and NANC transmission of the rat vas deferens (Mallard, Marshall & Spriggs, 1989). In contrast Den Hertog, Nelemans & Van den Akker (1989) reported suramin antagonised the relaxation of taenia caeci, an effect believed to be mediated by P2y-purinoceptors. Leff and colleagues reported that suramin is a slowly equilibrating but competitive P2x-receptor antagonist on the purinoceptor of the rabbit ear artery (Wood, O'Connor, Fear & Leff, 1990). Recently the first report of the effects of suramin on non-muscle cells was presented by Nakazawa, Fujimori, Takanaka & Inoue (1990) who found suramin reversibly and selectively antagonised an ATP-receptor operated membrane current of PC12 phaeochromocytoma cells.

The results of incubation with suramin on the response of the rat SCG to ATP and its analogues are shown in figure 6.7. The ability of suramin to selectively antagonise the depolarisation of the rat SCG to α,β-MeATP suggests the depolarisation occurs via an activation of P2x-purinoceptors. Suramin enhanced the hyperpolarisations to ATP and β,γ-MeATP, indicating the response to ATP and β,γ-MeATP comprised of a hyperpolarisation and depolarisation the latter of which functionally antagonised the former response (see also Fig. 6.1). A tenfold increase in the concentration of α,β-MeATP to 1,000uM in the presence of suramin produced a greater response than at 100uM, suggesting the antagonism by suramin was competitive. The reason for the enhancement of the hyperpolarisation to adenosine in the presence of suramin is unknown but may result from an inhibition of the metabolism of adenosine as suramin has been reported to inhibit the extracellular metabolism of ATP (Hourani & Chown, 1989), or via the

modulation of the adenosine-receptor G-protein complex (Huang, Dehaven, Cheung, Diehl, Dixon & Strader, 1989).

6.1.5 Effect of purine nucleotides on the response to muscarine

A postsynaptic interaction between muscarinic receptors and purine nucleotides has been reported by Bertrand, Chapal, Loubatiers-Mariana (1986) where ATP and ADP were synergistic with ACh in increasing the release of insulin from the isolated perfused rat pancreas. Unlike Bertrand et al. (1986) none of the ATP analogues tested on the rat SCG increased the response to muscarine, instead anagloues of ATP were inactive or depressed muscarinic responses (Table 6.1). The similar potency of ATP and adenosine in depressing the response of the rat SCG to muscarine (Fig. 6.5) suggest ATP may activate the same mechanism as adenosine to depress the response to muscarine. The depression of muscarinic responses may occur via the stimulation of P2 purinoceptors by ATP or the degradation of ATP to adenosine and activation of P1 purinoceptors. In contrast to the similar potency of adenosine and ATP to depress muscarinic responses, the hyperpolarisation to ATP (Table 6.1) was considerably less and is consistent with the small hyperpolarisations recorded during a two minute application of ATP in PSS (Fig. 6.1). Thus the degree of hyperpolarisation by an agonist may not directly correlate with the extent of depression of the response to muscarine. This conclusion is strengthened by the ability of β,γ-MeATP to produce significant hyperpolarisations at concentrations that did not depress the response to muscarine. Both the hyperpolarisation to ATP and the depression of muscarinic responses by ATP were reduced in the presence of 8PT (Table 6.2) indicating both actions of ATP were mediated via P1-purinoceptors. Consistent with

this finding is the inability of both the stable ATP isoster α,β-MeATP and 2-MeS-ATP to alter the responses to muscarine (Table 6.1) suggesting P2x and P2y purinoceptors are not involved in the modulation of muscarine. The ability of α,β-MeATP to depolarise but not depress the muscarinic responses of the rat SCG may arise from the depression of Im as Akasu & colleagues have reported that ATP strongly depressed Im of frog sympathetic ganglia (Akasu & Koketsu, 1982; Akasu, Hirai & Koketsu, 1983a,b; Morita, Katayama, Koketsu & Akasu, 1984), although effects on gNa or a non-selective effect on cationic channels could also occur.

6.1.6 Effect of ATP and β,γ-MeATP on the responses to GABA

Akasu & colleagues in addition to showing the slow depolarisation of bullfrog sympathetic spinal ganglion cells in response to ATP was via an inactivation of gK, it was discovered that ATP augmented the responses to GABA (Morita et al., 1984). The effect of GABA on the rat SCG is complex and the depolarising action of GABA occurs with an accumulation of $[K^+]_o$ and an increase in gCl^- and an outward movement of Cl^- (Ballanyi & Grafe, 1985). The ability of β,γ-MeATP to potentiate the response of the rat SCG to GABA (Table 6.1) may arise from a potentiation of the chloride efflux as reported by Ferrero & Frischknecht (1983) or may be due to a change in the GABA-receptor ion channel complex as described for bullfrog ganglia by Morita et al (1984).

6.1.7 Summary of effects of ATP and its analogues on the rat SCG

Ideally a study of the P2 purinoceptors of the rat SCG should be performed using stable nucleotides that are not hydrolysed to adenosine, such as those synthesised and tested by Cusack on both peripheral (Cusack & Hourani, 1984) and central neurones (Stone & Cusack, 1989). However, these analogues were unavailable and the effects of ATP were compared to the slowly degradable analogues of ATP, α,β-MeATP and β,γ-MeATP (Moody & Burnstock, 1982). The results of this study were complicated by the hyperpolarisation of the ganglion by ATP and its analogue via P1-purinoceptors, but the potency of producing a depolarisation indicates a predominance of P2x-purinoceptors rather than P2y-purinoceptors on the rat SCG.

6.2 The Classification of the Adenosine Receptors of the Rat SCG

The 5' and N6 substituted analogues of adenosine were used to classify adenosine receptors in to A1 and A2 subtypes by Van Calker et al. (1979) and Londos et al. (1980). More recently the relative potency of N6-substituted adenosine analogues as agonists was examined in a variety of tissues (Daly, Padgett, Thompson, Kosachi, Bugni & Olsson, 1986; Ukena, Olsson & Daly, 1987) and it was proposed on the basis of these binding studies that out of 145 analogues, 31 compounds might be useful in classifying the adenosine receptor, and a further subset of 7 analogues, namely 5'-N-ethylcarboxamideadenosine (NECA) and it's N6-cyclohexyl derivative, cyclohexyladenosine (CHA), R or S-N^6-phenylisopropyladenosine (R-PIA and S-PIA), 2CA and phenylaminoadenosine (PAA) might be

particularly valuable for use in functional studies. This study has employed these compounds and some other agonists and antagonists that are reported to show selectivity for A1- and A2-adenosine receptor subtypes. Both the order of potency of agonists to hyperpolarise and depress the response of the rat SCG to muscarine was used to determine the P1-purinoceptor subtype of the ganglion.

6.2.1 The potency of adenosine and its analogues on the d.c. potential of the SCG

Adenosine and its analogues hyperpolarised the ganglion in a concentration-dependent manner. The response to analogues of adenosine developed more slowly than the response to adenosine and were maximal within two minutes. The adenosine receptor responsible for the hyperpolarisation of the rat SCG appears to be an A1-adenosine receptor. This conclusion is based on the following observations.

The order of potency to hyperpolarise the ganglion was firstly the selective A1 agonist cyclopentyladenosine (CPA) (Bruns et al., 1986) which was 5 fold more potent than 2CA, an agonist with equal affinity for A1 and A2 receptors: about 80 fold more potent than adenosine and 140 times more potent than PAA, a selective A2 agonist (Fig. 6.8, Table 6.3). In PSS the maximal response to PAA was considerably less than that for other agonists and 100uM the hyperpolarisation to PAA was not altered by increasing the application time to 5 minutes. The low potency of both PAA and PD 117,413 (N^6-(9-fluorenylmethyl)adenosine) a potent A2-adenosine receptor agonist (Trivedi, Bristol, Bruno, Haleen, & Steffen, 1988) indicates functionally active A2-adenosine receptors do not alter the d.c. level of the rat SCG.

The potency of adenosine is likely to have been underestimated because of tissue uptake and metabolism. If 2CA and adenosine have similar affinities as has been predicted by Bruns et al. (1986), who suggested 2CA is a good substitute for adenosine under conditions where breakdown of adenosine is a problem; then a purely additive effect of inhibition of uptake and metabolism by ADA may not account for the higher potency of 2CA verses adenosine. However, uptake and deamination may not be the only mechanisms for the inactivation of adenosine and/or their combined effects may be supra-additive.

Purines may have multiple actions on some preparations and there are several examples of biphasic responses to ATP e.g. the contractile response of the vas deferens (Fedan et al., 1982a,b, 1986), smooth muscle cells in culture (Molleman et al., 1989) and a curious feature of the log concentration-response curves to adenosine analogues shared a reduced response at higher agonist concentrations (e.g. Figs. 6.5, 6.8 and 6.11) and are similar to the findings of Henon & McAfee, 1983a; see Figs. 2 and 7). It is possible that high concentrations of adenosine may disturb the balance between endogenous purine metabolites (Stone, 1981). However the inhibition of uptake (Chapter 3; Henon & McAfee, 1983a, Fig. 7) or deamination (Chapter 3) did not dramatically alter the maximal effects of adenosine suggesting the reduced response to adenosine is not due to the increased metabolism of adenosine. One possibility suggested by Henon & McAfee (1983a) was that more than one adenosine receptor subtype is present on the ganglion. Thus an opposing inhibitory response may occur at high concentrations of adenosine and its analogues to activate A2-adenosine receptors and this could account for the "bell-shaped" agonists concentration-response curves obtained (Figs. 6.8 and 6.10). Such an effect, i.e. functional antagonism has been reported for the effects of

submicromolar concentrations of CHA to inhibit renin secretion and higher (micromolar) concentrations of CHA stimulate secretion (Churchill, Jacobson & Churchill, 1987). However, the low potency of PAA and inability of 100uM, PD 117,413 to depress the response to muscarine does not support this hypothesis.

NECA is about equipotent on A1 and A2 receptors (table 6.5) and if the depression of muscarine is mediated by both A1 and A2 receptors, it would be expected that the maximal depression of muscarine would be greater for compounds that are equipotent on A2 and A1 receptors. It seems unlikely that A2 receptors increase the response to muscarine as the two selective A2 agonists, PAA and PD 117,413, depressed or did not significantly alter the response to muscarine (Fig. 6.10).

It is unlikely that the adenosine receptors of the rat SCG were desensitised by high concentrations of adenosine (>100uM) as they were not altered by increasing the interval between applications (20 to 60 minutes) or the application time from 2 to 5 minutes for adenosine. An alternative explanation that would explain the bell-shaped concentration response curves for the hyperpolarisation of the ganglion and depression of the response to muscarine by purines, would be if these purines activate a single receptor subtype, but stimulate different affinity states of the receptor at various concentrations of agonist.

Multiple affinity states of a single receptor subtype and different receptor coupling for these states have been described for both the muscarinic receptor (Birdsall & Burgen, 1979) and adenosine receptors (Lohse et al., 1984; Marangos et al., 1983, 1984, 1987; Patel et al., 1982; Nakata & Fuijisawa, 1983; Dunwiddie & Fredholm, 1984), and these receptors may be present on the rat SCG preparation.

6.2.2 The effect of 5'-deoxy-5'-methylthioadenosine on the d.c. potential of the SCG

5'-Deoxy-5'-methylthio adenosine (MTA) is an agonist of A1 receptors of the rat cerebellum and a competitive antagonist of A2 receptor mediated stimulation of fibroblasts (Bruns, 1980) and neuroblastoma adenylate cyclase (Munshi, Clanachan & Baer, 1988). MTA was a weak agonist on A1 receptors and an A2 adenosine receptor antagonist on the isolated rabbit jejunum (Munshi et al., 1988). On the rat SCG in normal PSS or low K^+/Ca^{2+} PSS, MTA was less potent than adenosine and produced a shallower concentration-response curve (Fig. 6.9). The weak agonist effect of MTA could result from the metabolism of the nucleotide due to the action of MTA phosphorylase to form adenine which would be expected to be inactive on the adenosine receptors of the rat SCG. The metabolism of MTA via uptake to deamination is unlikely as MTA did not bind to uptake sites in the cerebellum and is resistant to ADA (Munshi et al., 1988). Alternatively, Bruns (1980) has reported that modifications to the 5'-position of nucleotides had drastic effects on both the affinity and efficacy of binding to adenosine receptors. The low potency and low efficacy of MTA on the rat SCG may thus result from metabolism and/or via low affinity of MTA for the adenosine receptor. The substituion of the oxygen at the 5'-position with sulphur may reduce the affinity of MTA

for the adenosine receptor of the rat SCG as has been reported for the loss of substrate specificity for ADA, adenosine kinase, S-adenonyl-homocysteine hydrolase, where MTA does not substitute for adenosine as a substrate (Carrera, Willis, Chilcote, Kubota & Carson, 1988; personal communication J. Duley).

The order of potency for hyperpolarising the rat SCG;

CPA > 2CA > AD > PAA > PD 117,413

is consistent with the classification of the adenosine receptors responsible for the hyperpolarisation of the ganglion as a P1-purinoceptor of the A1-adenosine receptor subtype.

6.2.3 The potency of adenosine and its analogues to depress the response of the SCG to muscarine

The response to muscarine was reduced in a concentration-dependent manner by adenosine and all adenosine analogues tested except for PD 117,413 which was inactive at upto 100uM (Fig. 6.10; see also Figures 6.11 & 6.12). The evidence for a depression of muscarine responses by adenosine receptors of the A1-subtype is based upon the following observations.

In binding studies it has been found that N^6-Benzyl-adenosine (BZA) shows little selectivity for A1 or A2 receptors and has similar physio-chemical properties to CPA (Bruns et al., 1986; Bridges et al., 1988). Therefore in functional studies it would be predicted that in tissue containing predominantly A1 recpetors, CPA would be considerably more potent than BZA and about equipotent for a preparation containing predominantly A2-adenosine

receptors. An examination of figure 6.10 and table 6.4 shows that both CPA and BZA produced equivalent and large depressions of muscarine and a CR of 0.002, indicating CPA was 500 fold more potent than BZA, and the adenosine receptor induced depression of muscarine is mediated via an action on the A1 receptor subtype.

A1 and A2 receptors have been reported to differ in their stereoselectivity with respect to the isomers of PIA, with the A1 receptor having a 40 to 100 fold preference for the R over the S diastereo-isomer and the A2 receptor exhibiting only a 5 fold preference for the R-isomer (Smellie, Daly, Dunwiddie & Hoffer, 1979; Bruns, 1980; Bruns, Daly & Snyder, 1980). For the rat SCG, the S-isomer of PIA was less potent with a R/S potency ratio of about 5 (Fig. 6.11), suggesting the presence of A2 receptors. However a R/S potency ratio of 11 has been reported for A1 receptors by Trost & Schwabe (1981), and an R/S ratio of 15 was reported for the A2a high affinity receptor, and is intermediate between the classically accepted ratios for A1 and A2 sites. Likewise for rat liver preparations the rank order was clearly A1 but the R/S ratio was only 1.7 (Schutz, Tuisl & Kraupp, 1982). Stone (1989) has shown "the R/S ratio spans a very wide and arguably quite meaningless range". As more tissues have been studied and concluded to contain A1 or A2 receptors; the range of the R/S ratios reported has become broader and although the ratio is still used by some researchers (Paton, 1988), others have concluded that this ratio is probably not a reliable criterion for the sub-classification of adenosine receptors (see Bruns et al., 1986; Stone, 1989).

In an A2 adenosine receptor binding study Bruns and colleagues (Bruns et al., 1986) found PAA possesses about 10,000 fold selectivity for the high affinity A2 receptor

(A2a) over the low affinity A2 receptor (A2b). The low potency of PAA (Figs. 6.8 and 6.10) is indicative of the absence or low efficacy of A2 receptors on the rat SCG. Likewise it seems unlikely that A2 receptors increase the response to muscarine as the two selective A2 agonists, PAA and PD 117,413, depressed or did not significantly alter the response to muscarine (Fig. 6.10).

Three agonists, 2CA, NECA and BZA, which are reported to bind to A1 and A2a receptors with approximately equal affinity (Table 6.5) were less efficacious or had a similar efficacy for depressing the response to muscarine (Figs. 6.10 & 6.12), compared to CPA suggesting the depression of muscarinic responses is not mediated by A2 receptors, as it would be expected that the maximal depression of muscarine would be greater for agonists that are equi-active on both A1 and A2-adenosine receptors.

The hyperpolarisation produced by adenosine analogues was slow to develop and may arise from their highly lipophilic nature, resulting in slow equilibration with neuronal tissue (Dunwiddie & Fredholm, 1984). It has been reported that PIA is a more lipid-soluble compound than NECA, and this may account for its greater potency on many isolated tissues and its reduced potency in vivo (Fastbom, Post & Fredholm, 1990). It is evident from table 6.4 that the potency of NECA was intermediate between that of R-PIA and S-PIA, and as stereoisomers have the same physiochemical properties a difference in lipid solubility is unlikely to account for the reported potency order. If differences in lipid solubility are responsible for the differneces in the maximal depression of muscarine, then they should be a good correlation between logarithm partition coefficient (log P) and the maximal depression of muscarine. Both NECA, R- and S-PIA were of a similar efficacy on the rat SCG suggesting these compounds function in a similar

manner and the greater potency of R-PIA relative to NECA is indicative of the presence of A1 receptors on the rat SCG.

Lipophilicity has been found to be an essential factor governing potency of all kinds of CNS agents. Often the correlations obtained for potency versus log P show a parabolic nature with an optimum lipophilicity near 2 $\pm$ 0.3 and this is the ideal log lipophilic character in a neutral molecule for passive penetration into the CNS (Gupta, 1989). The hydrophobicity of a compound is related not only to *in vivo* penetration and distribution but also to the interaction with receptor sites, which will increase the binding of adenosine derivatives to its receptor. CPA and benzyladenosine (BZA) were the most efficacious at depressing the response of the ganglia to muscarine and have a similar log P value of about 1.6. It is postulated that the adenosine receptor of the SCG may be optimally activated by compounds with a log P value of about 1.6. However, many other factors, such as the ability to form hydrogen bonds, charge distribution and polarisability may affect the binding of ligands to the adenosine receptor.

From the results presented in table 6.4 and figures 6.10, 6.11 and 6.12, the rank order of potency for the depression of muscarinic responses by adenosine and its analogues was:-

CPA > R-PIA = 2CA > NECA > S-PIA > BZA > AD > PAA > PD 117,413

The relative order of potency for the depression of muscarinic responses by adenosine and adenosine analogues is similar to the potency series for the hyperpolarisation of the ganglion, suggesting the same receptor subtype is responsible for both effects.

The potency for the depression of muscarinic responses by purines was about 10 fold higher than that reported for the inhibition of $I_{K(Ca)}$ of the rat SCG (Henon & McAfee, 1983a) suggesting these events may be mediated by two different mechanisms or adenosine receptors.

Both the relative order of potency for the hyperpolarisation and the depression of the response to muscarine are indicative of the involvement of an A1-adenosine receptor. Likewise the absolute potencies of adenosine agonists is consistent with those published for the A1-adenosine receptor in other systems (Daly, 1982; Burnstock & Buckley, 1985; Williams, 1989).

6.3 Correlation of the depression of muscarinic responses and the inhibition of binding of adenosine to rat brain membranes

Using the extensive and elegant binding data presented by Bruns and colleagues for the inhibition of the binding of [^{3}H]-CHA to rat brain and [^{3}H]-NECA to rat striatal membranes (Bruns et al., 1986) various studies have found good correlations between the binding of agonists and the effects studied, e.g. Fig. 6.13 shows the correlation obtained from a scatter diagram of the IC_{50} for the depression of the response of the rat SCG to muscarine by adenosine analogues and the inhibition of the binding of these analogues to rat brain membranes (Bruns et al., 1986). There was a good correlation for the effects of adenosine and its analogues on the rat SCG and the binding to A1-receptors (Fig. 6.13) but a poor correlation of the former values and the inhibition of binding to A2-receptors supporting the hypothesis that the adenosine receptors of the rat SCG are of the A1-subtype.

6.4 Effect of purinoceptor antagonists on the effects of adenosine

To examine the actions of adenosine in more detail and establish a pA_2 for the adenosine receptor antagonist and the effects of selective A1 and A2 antagonists were studied.

6.4.1 The effect of pentobarbitone on the response to adenosine

It has been proposed that barbiturates might be adenosine receptor antagonists based on the ability of barbiturates to displace [^{3}H]-PIA and 1,3-diethyl-8-[^{3}H]-phenylxanthine ([^{3}H]-DPX) binding of rat brain synaptosomal membranes (Lohse, Lenschow & Schwabe, 1984). Further work by Lohse and colleagues supported this view and pentobarbitone inhibited in a competitive manner [^{3}H]-PIA binding to solubilised A1-adenosine receptors with a Ki of around 90uM (Lohse, Klotz, Jakobs & Schwabe, 1985; Lohse, Boser, Klotz & Schwabe, 1987) without any agonist activity.

The ability of pentobarbitone to enhance the response of the rat SCG to GABA (Little, 1987) was confirmed (Table 6.6, Fig. 6.14a) although the response to adenosine in 50uM or 100uM pentobarbitone was unaltered (Table 6.6, Fig. 6.14a,b) suggesting the adenosine receptor of the rat SCG may not be subject to modulation by barbiturates. Recently Stone (1988) examined the actions of three anti-convulsants on the response of rat hippocampal slices to adenosine; carbamazepine (>20uM) reduced the inhibitory response to adenosine, whereas chlormethiazole and pentobarbitone were without effect.

The reason for the difference between the functional studies reported here and by Stone (1988), and the binding

studies of Lohse and colleagues (1984, 1985, 1987) is unknown. However, it has been reported that another barbiturate phenobarbitone did not alter the binding of PIA and CHA to brain membranes (Skerritt, Davies & Johnston, 1982; Weir, Padgett, Daly & Anderson, 1984). Further studies of the effects of phenobarbitone and pentobarbitone will be required to see if, as suggested by Stone (1988) the results obtained by Lohse and colleagues (1984, 1985, 1987) are unique to their experimental preparation or reflect a difference between the two barbiturates studied.

6.4.2 Effect of methylxanthines on the response to muscarine, adenosine and the depression of muscarinic responses by purines

To examine if the responses to adenosine are due to an action on external P1-purinoceptors, the effects of adenosine on the d.c. potential, response to muscarine and the depression of muscarinic responses by adenosine and ATP were examined in the absence and presence of purine receptor antagonists, i.e. theophylline (THEO) and 8-phenyltheophylline (8PT), and the results summarised in table 6.2.

Both theophylline and 8PT have been used to show the effects of purines are mediated by an action on P1-purinoceptors and the ability of theophylline or 8PT to antagonise both the hyperpolarisation to adenosine and the depression of muscarine by adenosine supports this view. Likewise the reportedly non membrane permeable analogue of theophylline 8-parasulphotheophylline (8psPT) also reduced the hyperpolarisation and the depression of muscarine by adenosine (Table 6.2), strengthening the view that these

effects of adenosine are mediated via an external P1-purinoceptor.

It is tempting to postulate that theophylline depolarises and reduces the response to muscarine (Table 6.2) via an action unrelated to purinoceptor antagonism, e.g. inhibition of Im; Hille & colleagues reported that high concentrations of caffeine depolarised frog sympathetic neurones by suppressing Im via an action on G proteins (Pfaffinger, Leibowitz, Subers, Nathanson, Almers & Hille, 1988). However other sites of action including an increase in $[Ca^{2+}]i$ and/or PDE inhibition may also occur. Interestingly the reduction of muscarinic responses may not be restricted to theophylline as Fredholm & Duner-Engstrom (1989) found propentofylline reduced the activity of muscarinic autoreceptors by an unknown mechanism and these results may represent a general effect of methylxanthines on muscarinic responses.

In contrast the more selective purinoceptor antagonist 8PT, which may have some PDE inhibitory activity at 10uM did not alter the d.c. potential or the response to muscarine suggesting the depolarisation to theophylline is unlikely to be due to an antagonism of purinoceptors. These results also indicate that there was no detectable endogenous release of adenosine from the rat SCG.

6.4.2.1 Effect of 8PT on the hyperpolarisations of the rat SCG to adenosine

The ability of 8PT to produce parallel and rightwards shift the concentration response curve to adenosine and for adenosine to produce a similar maximal response in the presence of 8PT (Fig. 6.15) suggests 8PT competitively antagonised the adenosine mediated hyperpolarisation of the rat SCG. Substituting the concentration ratios (CR)

obtained at the EC_{50} values for adenosine in the absence and presence of 8PT into the Schild equation (Arunlakshana & Schild, 1959, equation (1)),

$$pA_2 = \log (CR-1) - \log [8PT]$$

where [8PT] is the molar concentration of 8PT, pA_2 is the negative logarithm of the concentration of antagonist which shifts the agonist concentration-response curve two fold to the right.

From the CR derived from the data presented in figure 6.16 the calculated mean pA_2 value were 6.44 $\pm$ 0.09 (n=3) and 5.68 $\pm$ 0.12 (n=7) for 8PT and 1uM and 10uM respectively.

Other researchers have studied the antagonistic potency of 8PT in isolated tissue preparations and pA_2 values of between 6.1 to 6.6 have been reported. Collis and colleagues reported pA_2 values of 6.39 to 6.6 for the purinoceptors of the guinea-pig atrium and aorta (Collis & Pettinger, 1982). Stone (1985) reported pA_2 values for 8PT against adenosine and β,γ-MeATP of 6.08 and 6.36 respectively (not significantly different from each other) for the rat vas deferens. Likewise pA_2 values of 6.46 and 6.5 were reported for 8PT as an antagonist of the purinoceptors of cat cerebral arteries and guinea-pig trachea (Edvinson & Fredholm, 1983; Farmer, Canning & Wilkins, 1988 respectively). The mean pA_2 for antagonism of the hyperpolarisation of the ganglion by adenosine by 8PT was in broad agreement with the values obtained by other researchers cited above. The ability of the ganglion to transport adenosine (Chapter 3; Henon & McAfee, 1983a) may have decreased the apparent potency of 8PT, i.e. at 10uM 8PT (Clanachan & Muller, 1980). Confirmation of the presence of P1-purinoceptor would be best established by the use of a non-metabolised analogue

of adenosine. The present results however, are consistent with the presence of P1-purinoceptors on the rat SCG.

6.4.2.2 Effect of 8PT on the depression of the response to muscarine by adenosine and CPA

In order to determine if the receptors responsible for the depression of the response to muscarine are the same as those receptors responsible for the hyperpolarisation of the ganglion the effect of 8PT on these responses were examined. The time necessary for these experiments made it impractical to construct detailed concentration-response curves before and after the antagonist. Consequently, CR's were determined at the level of 20% depression of the response to muscarine, i.e. approximately the IC_{50} level. The pA_2 value for 8PT was calculated using the equation described (section 6.4.2.1) assuming the antagonist was competitive. 8PT antagonised the depression of muscarinic responses by adenosine (Fig. 6.16, Table 6.2), CPA (Fig. 6.17) and 2CA (Table 6.2). The pA_2 values for 8PT's antagonism of the depression of the response to muscarine by CPA were estimated to be 6.45 and 6.75 at 1uM and 10uM respectively. These results are consistent with an action of adenosine, 2CA and CPA on P1-purinoceptors to depress the response to muscarine.

The ineffectiveness of adenosine-receptor antagonists on the response to muscarine alone suggests that endogenous adenosine does not contribute to the response to muscarine.

6.4.3 The effect of 3,7-dimethyl-1-propargylxanthine (DMPX) on the depression of muscarine by CPA and the response of the rat SCG to adenosine, CPA and PAA

It is not possible to define the adenosine subtype by using 8PT as although 8PT is a competitive P1 purinoceptor antagonist, it does not show any selectivity for A1 and A2 adenosine receptor subtypes (Collis, Palmer & Saville, 1985). In order to discover if the adenosine receptors responsible for the effects of adenosine and its analogues are of the A2-adenosine receptor subtype the effects of DMPX, an analogue of caffenine, were studied. DMPX has both good *in vitro* (Daly, Padgett & Shamim, 1986; Ukena, Shamim, Padgett & Daly, 1986) and *in vivo* (Seale, Abla, Shamim, Carney & Daly, 1988) selectivity for A2-adenosine receptors, compared to A1-adenosine receptors, was investigated on the response of the rat SCG to adenosine, CPA and PAA, and the depression of the response to muscarine by CPA.

Daly & colleagues (Daly et al., 1986) reported the Ki for the inhibition of CHA binding to the A1-adenosine receptor of the rat cerebral cortex was 6uM. DMPX at a concentration of upto 50uM had no effect on the response of the rat SCG to adenosine and its analogues (Table 6.7a,b; Fig. 6.18) or the depression of muscarinic responses by CPA (Table 6.7a) and on the basis of these results it suggested that A2-adenosine receptors are not involved in these responses.

6.4.4 Effect of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) on the depression of muscarinic responses by CPA and the response to adenosine and its analogues

DPCPX competitively antagonised both the inhibition of adenylate cyclase activity via A1-adenosine receptors and the stimulation via A2-adenosine receptors (Lohse, Klotz, Lindenborn-Fotinos, Reddington, Schwabe & Olsson, 1987). The Ki-values of this antagonism were 0.45nM at the A1-receptor of fat cells, and 330nM at the A2 receptor of human platelets, giving a more than 700-fold A1 selectivity. A similar A1-selectivity was determined in radioligand binding studies (Lohse et al., 1987; Bruns, Fergus, Badger, Bristol, Santay, Hartman, Hays & Huang, 1987) and 150 fold A1 selectivity was reported by Martinson, Johnson & Wells (1986). DPCPX has been used as a pharmacological tool to demonstrated the effects of adenosine receptor agonists are mediated by A1-adenosine receptors in a number of studies including the negative chronotropic activity of R-PIA on the heart (Haleen, Steffen & Hamilton, 1987) and the detection of A1-receptors in tissues with very low receptor density (Lohse et al., 1987).

6.4.4.1 The effect of DPCPX on the response to adenosine and its analogues

The actions of CPA, NECA, adenosine and PAA were reduced in the presence of 1nM DPCPX (Fig. 6.19). The ability of DPCPX to antagonise the hyperpolarisation to PAA and convert it into a small depolarisation suggests the response to PAA may be combination of an A1-adenosine receptor receptor mediated hyperpolarisation and an A2-adenosine receptor mediated depolarisation. The pA_2

values for CPA and adenosine in DPCPX (1nM) were calculated to be 9.95 and 9.61 respectively suggesting CPA and AD hyperpolarise the ganglion via or action on A1-adenosine receptors.

6.4.4.2 The effect of DPCPX on the depression of the response to muscarine by CPA

CPA produced concentration dependent depression of the response to muscarine and increasing concentrations of DPCPX produced a progressive inhibition of this depression (Fig. 6.20). To avoid high concentrations of agonist which could potentially have an effect on other types of adenosine receptor the effect of low concentrations of DPCPX were studied. DPCPX at 0.1nM did not significantly alter the depression of muscarinic responses by CPA (Fig. 6.20a). In both 0.5 and 1nM DPCPX the maximal depression of the response muscarine by CPA was reduced indicating a non-competitive antagonism of the effects of CPA by DPCPX and that CPA inhibits muscarinic responses via an action on A1-adenosine receptors (Fig. 6.20b,c).

6.4.4.3 The effect of DPCPX on the concentration response curves to adenosine and CPA

A more detailed study of the concentration-dependent hyperpolarisations to adenosine, showed adenosine produced reproducible concentration dependent hyperpolarisations (Fig. 6.18a). However in the presence of increasing concentrations of DPCPX, the concentration-response curves to adenosine were displaced to the right in a non-parallel manner (Fig. 6.21b,c,d). Figure 6.22 illustrates a typical experiment showing the depression of the hyperpolarisation to increasing concentrations of

adenosine by 0.6 and 0.9nM DPCPX. High concentrations of adenosine produced an initial rapid hyperpolarisation followed by a smaller more sustained hyperpolarisation, e.g. 300 or 1,000uM adenosine in Figure 6.22. Both the initial rapid and the more sustained hyperpolarisations were equally reduced in the presence of DPCPX. The non-competitive nature of the antagonism by DPCPX was not restricted to the antagonism of adenosine, as figure 6.23 illustrates the effect of DPCPX on the hyperpolarisations to CPA were also non-parallel. When the responses to CPA in DPCPX were normalised and expressed as a % of the maximal response to each control curve, apparent pA_2-values of 9.37, 9.56 and 9.70 were obtained for 0.3, 0.5 and 0.9nM DPCPX respectively. The mean pA_2 value of 9.54 $\pm$ 0.1 indicates an apparent affinity of 0.3nM for the adenosine receptor of the rat SCG, a value consistent with the involvement of A1-adenosine receptors (Kd < 0.5nM Bruns et al., 1987; Lohse et al., 1987; Kiotz, Lohse, Schawbe, Cristalli, Vittori & Grifantini, 1989). Likewise a Ki of 0.45nM was reported for DPCPX for the A1-receptor of the rat hippocampus (Sebastiao, Stone & Ribeiro, 1990).

One factor that may have influenced the concentration-response curve to adenosine and its antagonism is the $[Ca^{2+}]e$. It has been shown that a reduction in $[Ca^{2+}]e$ can alter the affinity of receptors for their agonist (Dougall & Leff, 1987) and hence may affect the affinity of the adenosine receptor of the rat SCG to adenosine. In 2.5mM Ca^{2+} PSS, DPCPX produced a 'non-competitive' antagonism of the hyperpolarisations to adenosine (Fig. 6.20a) suggesting low calcium does not alter the affinity of DPCPX for the adenosine receptor (cf 6.21a,b).

It is unlikely that the non-competitive actions of DPCPX recorded on the rat SCG are due to the inhibition of cyclic nucleotide phosphodiesterase as DPCPX at up to

690nM had very little effect on PDE activity producing about 2% inhibition at 69 and 690nM (Martinson et al., 1986).

The non-competitive action of DPCPX on the depression of muscarine responses by CPA may be due to the nature of the "bell-shaped" concentration response curves. Over a narrow range of concentrations of DPCPX the antagonism may appear competitive. Higher concentrations of DPCPX may then reduce the maximal inhibition achieved by CPA and DPCPX would thus appear to be a non-competitive antagonist. This phenonomenon would also explain the non-competitive antagonism of the hyperpolarisations to adenosine or CPA.

Often the low potency of purines on isolated tissues compared to the potency on purified rat brain receptor membrane preparations is lower and attributed to the 'poor' access to the receptor in the isolated tissue. The potency of adenosine agonists and antagonists on the rat SCG was closely correlated with the reported binding affinities (Tables 6.5 and 6.8) suggesting adequate access to the adenosine receptors of the ganglion. However it has also been reported that nicotine is concentrated within SCG neurones (Brown, Halliwell & Scholfield, 1971) and likewise it is possible that DPCPX was concentrated within SCG neurones. A log P value for DPCPX of 3.6 (Bruns et al., 1987) may predispose this compound towards sequestration into hydrophobic sites (not present in the studies of receptor binding) and could lead to an over estimation of the apparent potency and apparent non-competitive antagonism.

Recently the antagonistic profiles of a number of substituted xanthines as functional antagonists of the responses mediated by adenosine receptors have been

described (Ribeiro & Sebastiao, 1989; Sebastiao, Stone & Ribeiro, 1990). 1,3-dipropyl-8-(4-((2-aminoethyl)amino)-carbonylmethyloxyphenyl) xanthine, i.e. XAC, has been reported to be more potent on the A2-adenosine receptors of human platelets and the A3-adenosine receptors of the frog NMJ (Ribeiro & Sebastaio, 1989) than DPCPX, whereas DPCPX is more potent than XAC on the A1-adenosine receptors of rat brain membranes, fat cells (Ribeiro & Sabastiao, 1989) and the rat NMJ and hippocampus (Sebastaio et al., 1990). XAC was around ten fold less potent than DPCPX (cf Figs 6.21 c & d) indicating the adenosine receptors of the rat SCG are A1-adenosine receptors. Interesting XAC also produced a "non-parallel" competition of the concentration-response curve to adenosine (Fig. 6.21d).

There is evidence to suggest that A1 and A2-adenosine receptors may be differently affected by temperature (Broadley, Broome & Paton, 1985) however the pA_2 for XAC on the rat hippocampal A1-adenosine receptor was not significantly altered by temperature between 22-30°C (Sebastiao et al., 1990) suggesting the reduced temperature used in these studies, i.e. 25°C may not be a critical factor influencing the antagonism of the hyperpolarisation to CPA and adenosine by DPCPX or XAC.

There are many mechanisms which may account for the biphasic response to adenosine including an action on two separate receptors, e.g. A1 and another adenosine receptor (NB: but not the A2 adenosine receptor - see section 6.4.3). The existence of multiple coupling states which differ in their affinity for agonists has been described for the A1-adenosine receptor (Goodman et al., 1981; Yeung & Green, 1983; Lohse et al., 1984; Green, 1984; Bruns et al., 1987). CPA preferentially binds to a high affinity agonist-preferring coupling state of the A1 receptor whereas DPCPX labels both coupling states (Bruns et al.,

1987). Even within the same tissue Marangos & colleagues have demonstrated two binding sites for CHA which had affinities of 0.4 and 4nM respectively (Marangos et al., 1984a,b). Thus the ability of DPCPX and XAC to antagonise the hyperpolarisations to adenosine and CPA in an apparently competitive manner at low concentrations of antagonist and in a "non-competitive" manner at higher concentrations of antagonist could reflect the existence of multiple affinity states of the adenosine receptor.

Another possibility that may explain the effects of DPCPX may be that reported by Gurden, Brialla & Kennedy (1987) who have questioned the A1-selectivity of DPCPX. DPCPX antagonised the effect of adenosine on the guinea-pig aorta, a tissue reported to contain A2-adenosine receptors (Collis & Brown, 1983) with a similar pA_2 value to that of the guinea-pig left atria, which contains A1-adenosine receptors (Collis, 1983) and had pA_2 values of 6.72 and 7.25 respectively. These pA_2 values are closer to the range of Kds reported for the A2-adenosine receptors in binding assays (Kd, 330-340nM) by Bruns et al., 1987; Lohse et al., 1987). The pA_2 value obtained on the rat right atria, an A1-adenosine receptor was significantly different from the values obtained for the guinea-pig aorta and left atria being 8.74 and is closer to that reported for other tissues. This report has yet to be confirmed in a more extensive study by these authors or repeated by other researchers.

It is interesting that other substituted xanthines including (1,3-dipropyl-8-(2-amino-4-chlorophenyl)xanthine (PACPX), a potent A1 selective adenosine receptor antagonist (Bruns et al., 1983; Daly et al., 1985; Schwabe et al., 1985) and the triazoloquinazoline, CGS 15943 a selective A2-adenosine receptor antagonist (Williams, Francis, Ghai, Braunwalder, Psychoyos, Stone & Cash,

1987a) have both been found to behave competitively in one A1 or A2 system but non-competitively in other systems (Burnstock & Hoyle, 1985; Williams, Ferkany, Jarvis, Stills & Braunwalder, 1987b). CGS 15943A was a non-competitive inhibitor of rat brain A2-adenosine receptors and a competitive antagonist in peripheral tissues (Ghai, Francis, Williams, Zimmerman, Goodman, Hopkins, Cote & Dotson, 1987 in Williams et al., 1987a; Farmer, Canning & Wilkins, 1988). A similar paradox has been noted for PAPCX, which is non-competitive in the A1-adenosine receptor driven guinea-pig left atrial model and competitive at A1-receptors in brain tissues (Burnstock & Hoyle, 1985). It is thus possible that the non-competitive nature of DPCPX on the response of the rat SCG to adenosine and CPA versus the competitive antagonism of A1-adenosine receptors of the rat brain, is an example of a similar phenomenon.

In conclusion, DPCPX was demonstrated to be a very potent antagonist of both the hyperpolarisation to adenosine, CPA, BZA and the depression of the response to muscarine by CPA and BZA. The potency of DPCPX and XAC as antagonists of the hyperpolarisation to CPA are comparable with their potencies as antagonists on the A1-adenosine receptor and not A2 -adenosine receptors. Thus both the agonist and antagonist profiles for the adenosine receptor of the rat SCG are consistent with the presence of A1-adenosine receptors in this preparation.

Fig. 6.1. Effect of 8-phenyltheophylline on the concentration response curve to adenosine-5'-triphosphate

In normal physiological salt solution (PSS) adenosine-5'-triphosphate (ATP) produced small and statistically insignificant hyperpolarisations. The response to 1000uM ATP was small and inconsistent on different ganglia: two ganglia depolarised by 30 and 50uV, two more ganglia hyperpolarised by -10 and -25uV.

Four different ganglia were examined in low potassium and low calcium PSS (low K^+/Ca^{2+} PSS), ATP produced dose related hyperpolarisations. The response to 100uM ATP was significantly (62 $\pm$ 8% the response to adenosine, n=4, $P<0.05$) smaller than the hyperpolarisation to 100uM adenosine (-215 $\pm$ 56uV). Upon repeating the concentration response curve to ATP in the presence of 8-phenyltheophylline (8PT), ATP produced concentration dependent depolarisations and at 300uM and 1000uM ATP, a hyperpolarisation appeared superimposed on the depolarisation (see Fig. 6.2). (n=4 for both groups).

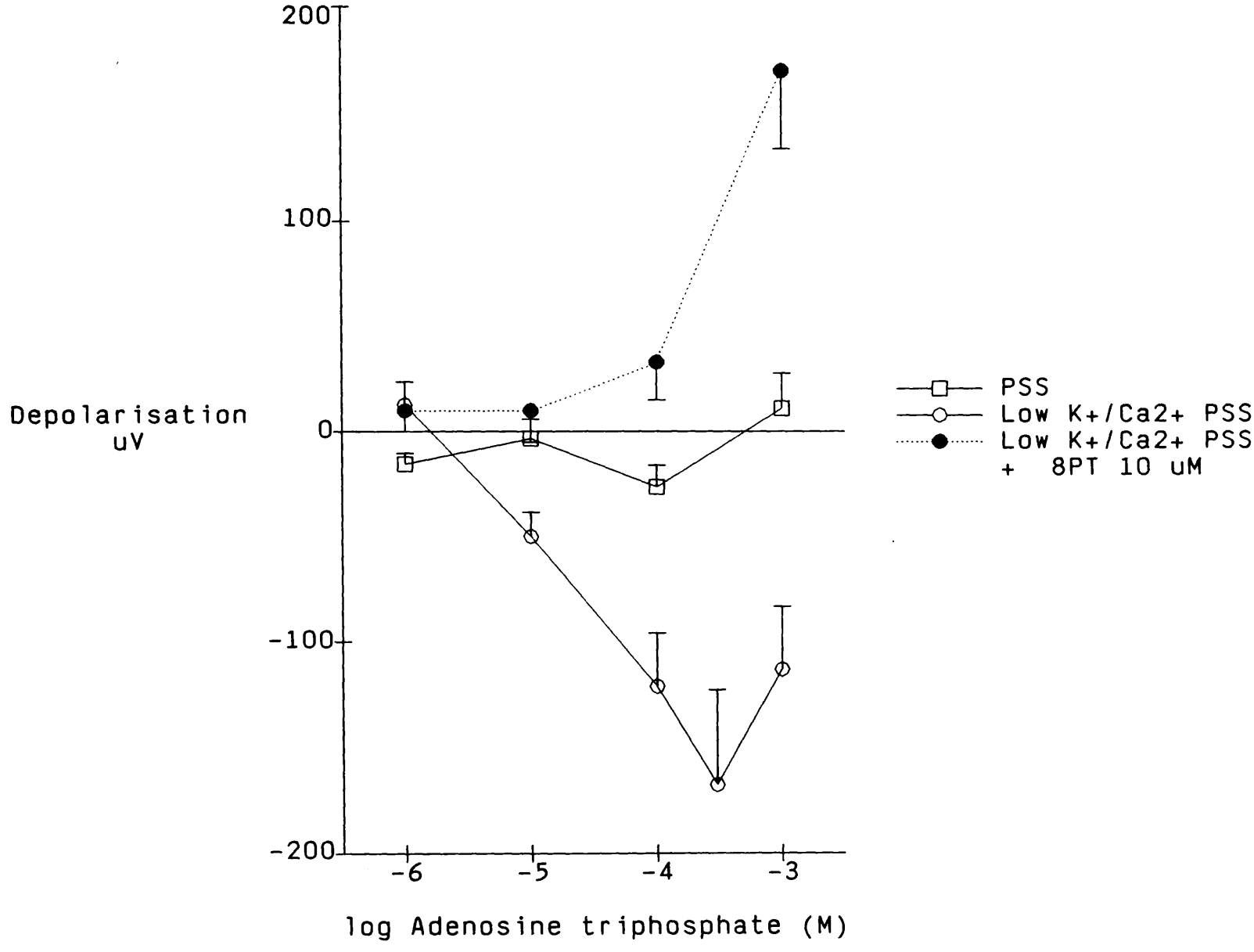

200
100
0
-100
-200
-6
-5
-4
-3
Depolarisation
uV
log Adenosine triphosphate (M)
PSS
Low K+/Ca2+ PSS
Low K+/Ca2+ PSS
+ 8PT 10 uM

Fig. 6.2. Response of a single rat SCG to increasing concentrations of adenosine-5'-triphosphate in the absence and presence of 8-phenyltheophylline

(A) Response to a two minute application of 1, 10, 100, 1000uM and repeated response to 1000uM adenosine-5'-triphosphate (ATP) in physiological salt solution containing 2mM potassium and 0.1mM calcium (low K^+/Ca^{2+} PSS).

(B) Response to two minute applications of 1, 10, 100 and 1000 ATP repeated in low K^+/Ca^{2+} PSS in the presence of 10uM 8-phenyltheophylline (8PT).

In low K^+/Ca^{2+} PSS the response to 300 and 1000uM ATP was complex, consisting of an initial hyperpolarisation, a transient depolarisation and an after-depolarisation. There was no sign of desensitisation to ATP at up to 1000uM (Fig. 6.2) and the responses to 1000uM ATP were reproducible with 2 minute applications every 30 mins (1st application -113 $\pm$ 30uV, and 30 minutes later -129 $\pm$ 23uV, n=4) (Trace A).

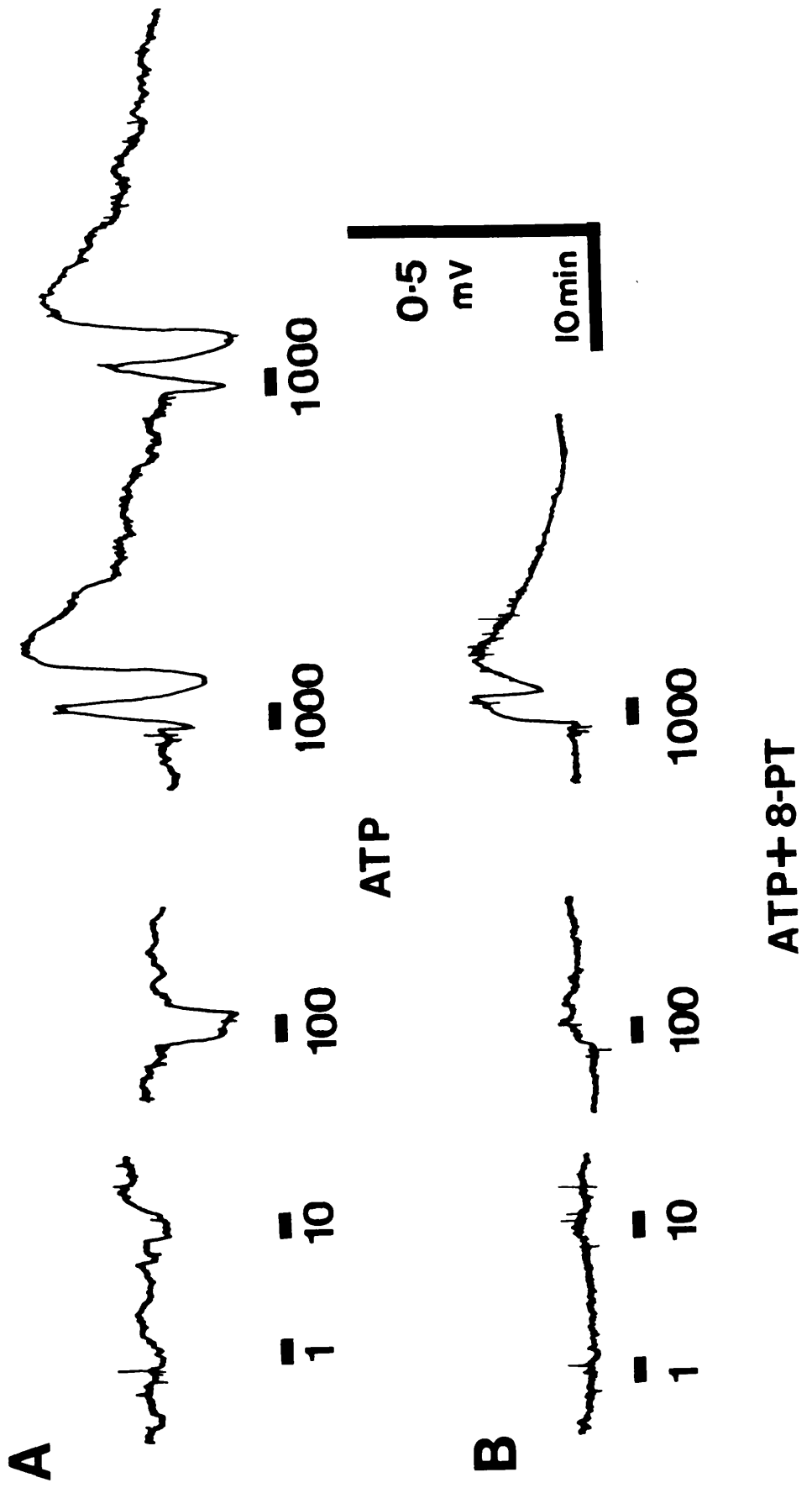

A
1
10
100
1000
1000
ATP
0·5
mV
10min
B
1
10
100
1000
ATP+8-PT

Fig. 6.3. Log concentration-response curves for the effect of adenosine, adenosine-5'-triphosphate, beta,gamma-adenosine-5'-triphosphate and 2-methylthio-adenosine-5'-triphosphate on the isolated rat SCG

Adenosine, adenosine-5'-triphosphate (ATP), beta,gamma-methylene-adenosine-5'-triphosphate (b,g-MeATP) and 2-methylthio-adenosine-5'-triphosphate (2MeS-ATP) were applied in increasing concentrations for 2 minutes, at >20 minutes to ganglia perfused with normal physiological salt solution.

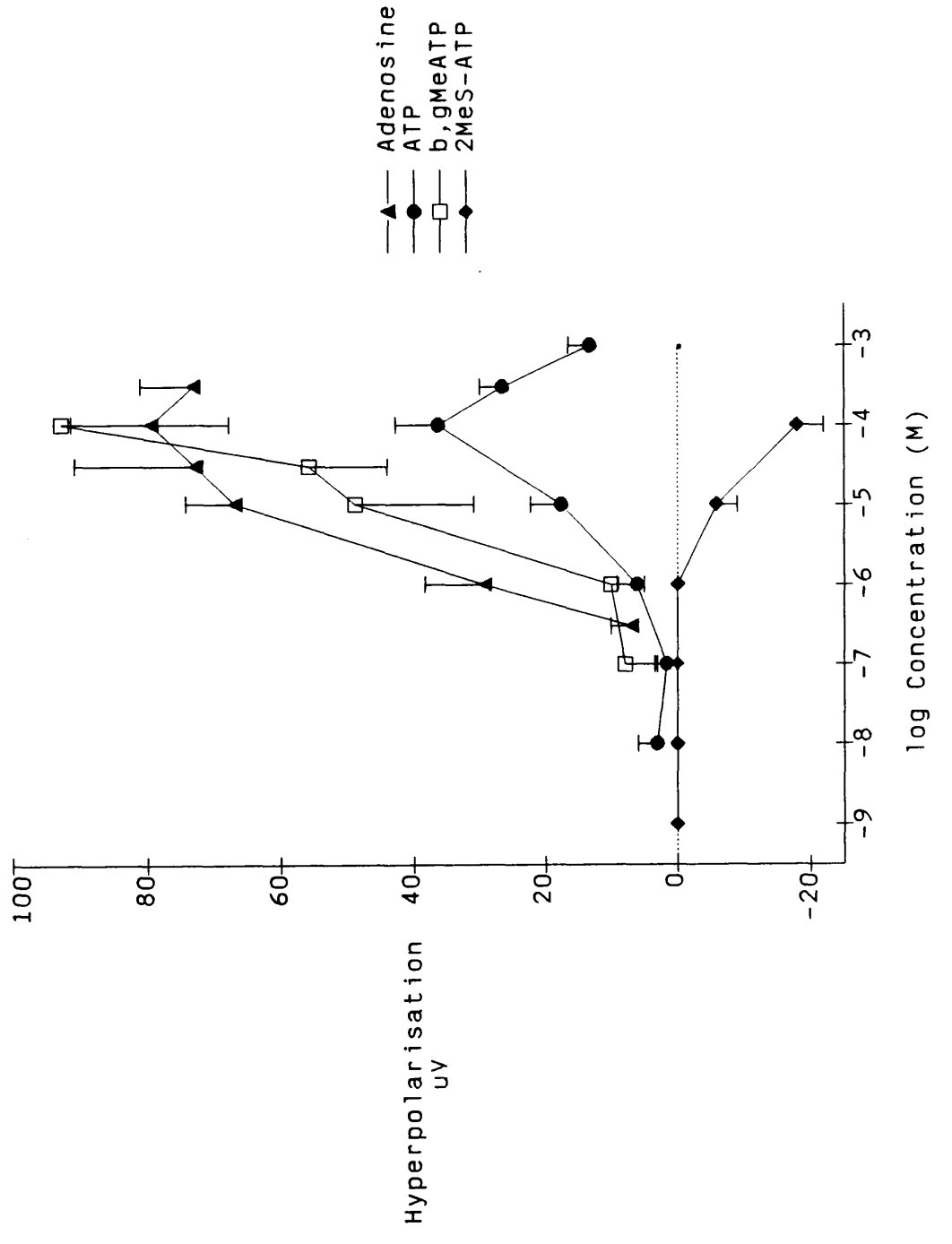

Adenosine
ATP
b,gMeATP
2MeS-ATP
Hyperpolarisation
uV
100
80
60
40
20
0
-20
-9
-8
-7
-6
-5
-4
-3
log Concentration (M)

Fig. 6.4. Log concentration-response curves for adenosine and beta,gamma-methylene-adenosine-5'-triphosphate in the absence and presence of 8-phenyltheophylline

In low potassium and low calcium physiological salt solution (low K^+/Ca^{2+} PSS) both adenosine and beta,gamma-methylene-adenosine-5'-triphosphate (b,g-MeATP) (2 minute applications, minimum of 20 minute intervals) produced concentration related hyperpolarisations with a maximum response at about one millimolar. In the presence of 8-phenyltheophylline (8PT) the responses to adenosine and b,g-MeATP were antagonised and the concentration-response curves displaced rightwards.

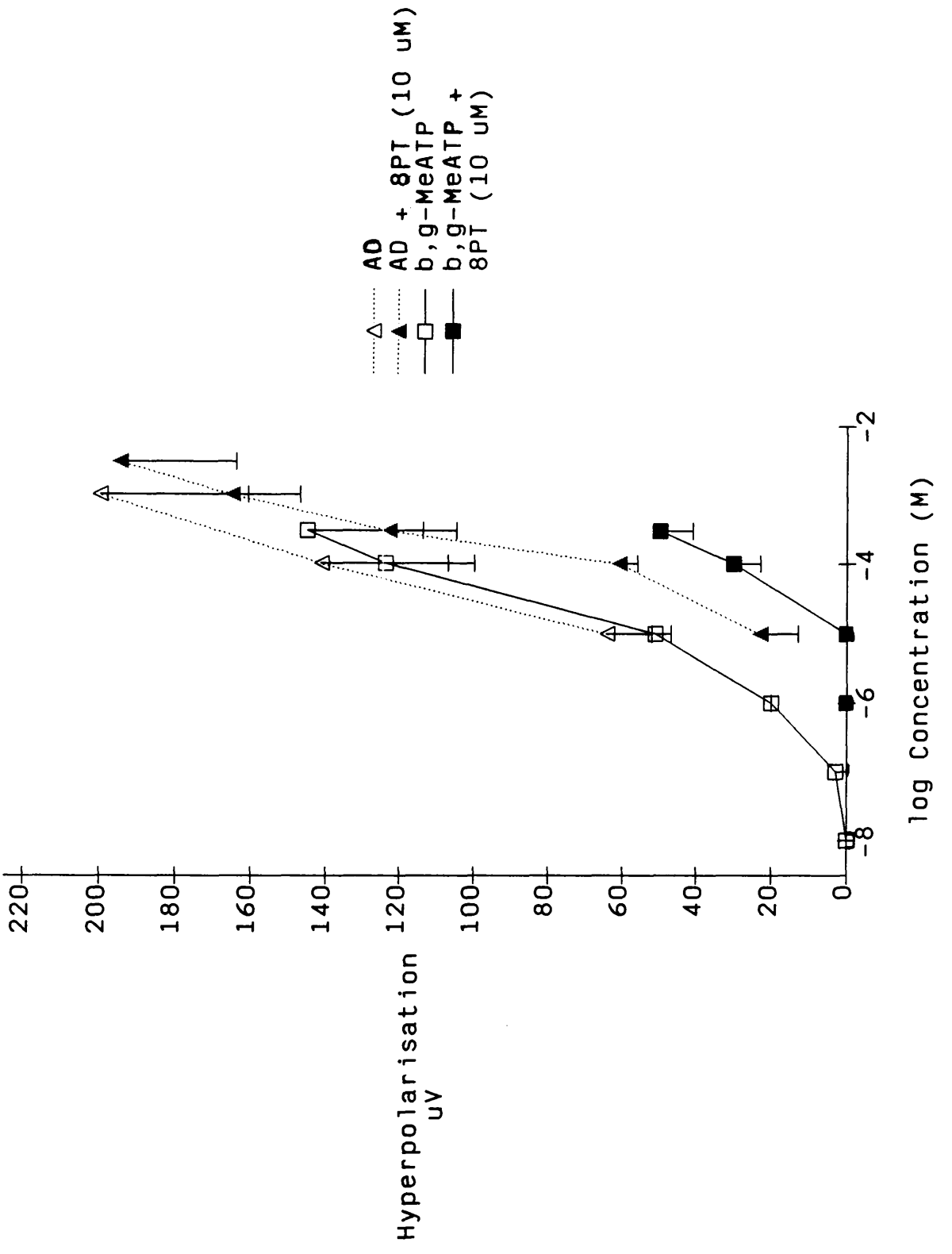

AD
AD + 8PT (10 uM)
b,g-MeATP
b,g-MeATP + 8PT (10 uM)
Hyperpolarisation uV
log Concentration (M)
220
200
180
160
140
120
100
80
60
40
20
0
-8
-6
-4
-2

Fig. 6.5. Effect of adenosine, beta,gamma-methylene-adenosine-5'-triphosphate and adenosine-triphosphate on the response of rat isolated SCG to muscarine

Adenosine (AD), beta,gamma-methylene-adenosine-5'-triphosphate (b,g-MeATP) and adenosine-5'-triphosphate (ATP) reduced the response to 100uM muscarine (1 minute). However b,g-MeATP only significantly reduced the response to muscarine at 30uM (* = $P<0.05$). Minimum number of ganglia used, N = 6 for all compounds.

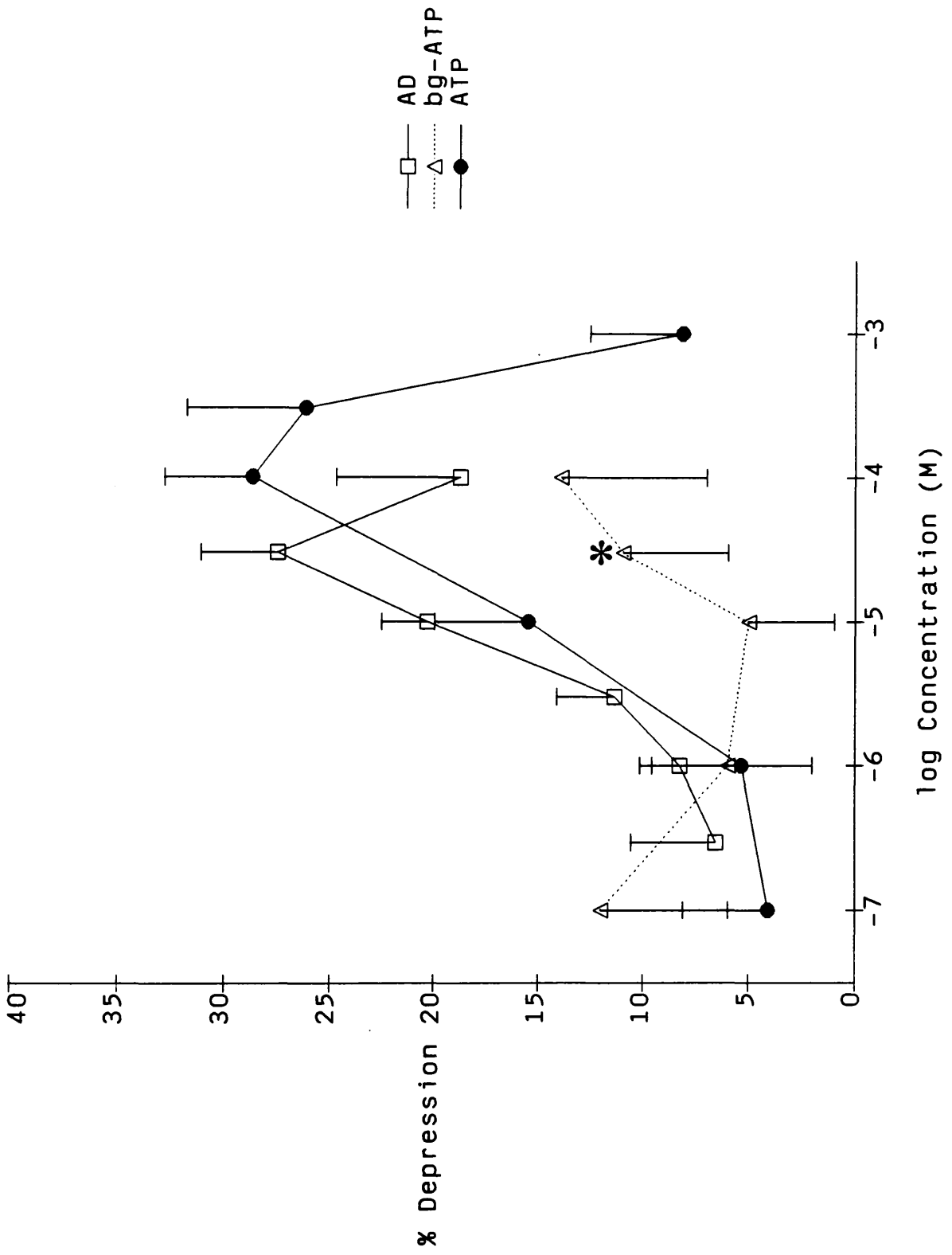

AD
bg-ATP
ATP
% Depression
log Concentration (M)
40
35
30
25
20
15
10
5
0
-7
-6
-5
-4
-3
*

Fig. 6.6. Effect of alpha,beta-methylene ATP on the d.c. potential of rat isolated SCG

In physiological salt solution (PSS) a two minute application of alpha,beta-methylene ATP (a,b-MeATP) resulted in a slow depolarisation of ganglia, compared to adenosine (2 minutes, 100uM) which hyperpolarised (-70 $\pm$ 22uV, n=4). The responses to a,b-MeATP were enhanced in 2mM K^+/0.1mM Ca^{2+} (low K^+/Ca^{2+} PSS) compared to normal PSS, but were not significantly altered by the presence of 8-phenyltheophylline (8PT), whereas the response to adenosine (-263 $\pm$ 41uV, n=3) was significantly reduced to -50 $\pm$ 6uV ($P<0.05$, n=3).

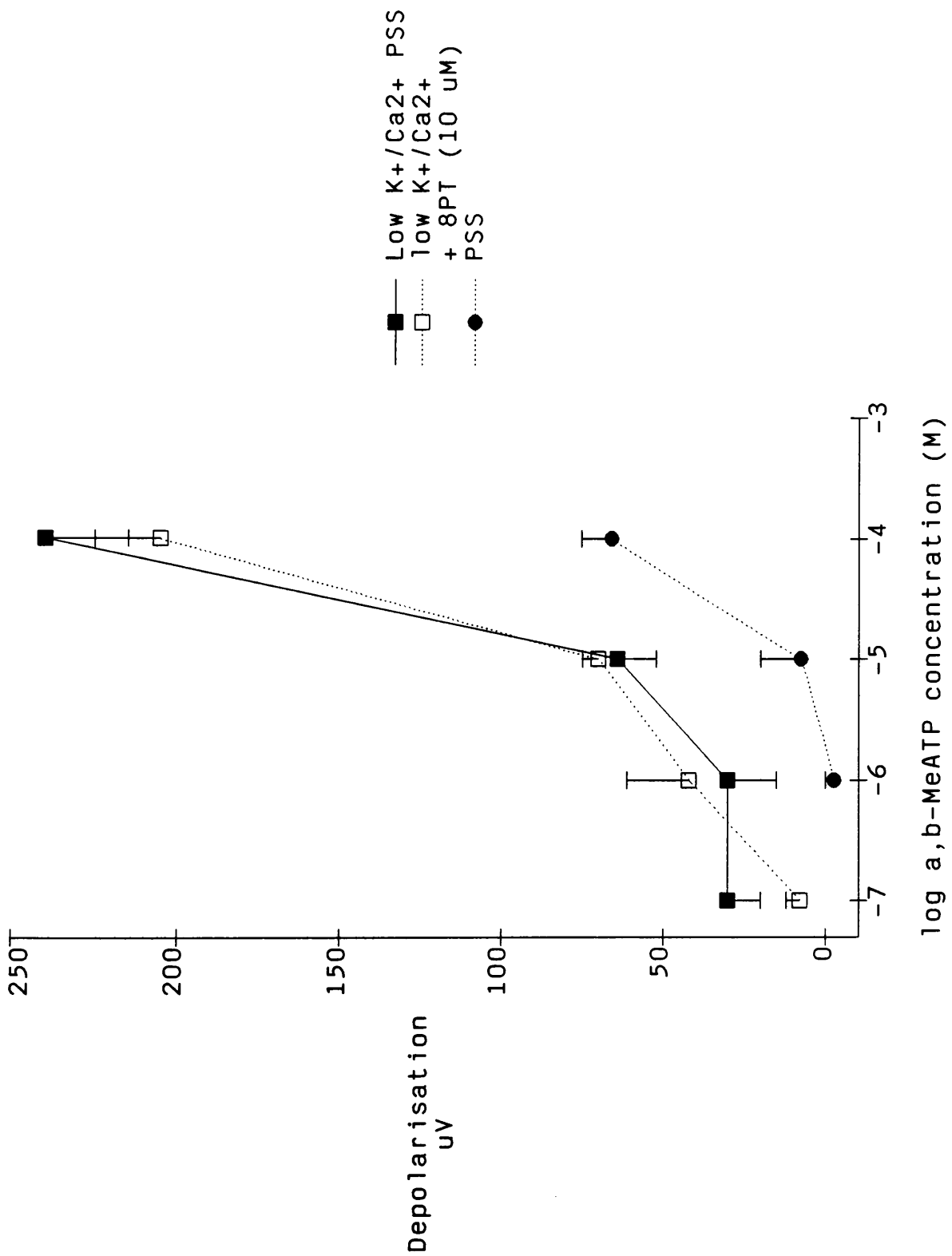

Low K+/Ca2+ PSS
low K+/Ca2+
+ 8PT (10 uM)
PSS
Depolarisation
uV
250
200
150
100
50
0
-7
-6
-5
-4
-3
log a,b-MeATP concentration (M)

Fig. 6.7. Effect of suramin on the response of the isolated rat SCG to adenosine, adenosine-5'-triphosphate, a,b-Methylene-ATP and b,g-Methylene-ATP

In low K^+/low Ca^{2+} physiological salt solution (PSS) adenosine (AD), adenosine-5'-triphosphate (ATP) and b,g-Methylene-ATP (b,g-MeATP) hyperpolarised and a,b-Methylene-ATP (a,b-MeATP) depolarised four ganglia. The effect of suramin on the response of the ganglion to agonists was compared in the presence and absence of suramin (300uM) using a paired t-test and the significance is indicated by a * for $P<0.05$.

Note: When the same concentrations of agonists were applied in the presence of suramin the hyperpolarisations to adenosine and ATP, and the depolarisations to a,b-MeATP were significantly increased or decreased respectively.

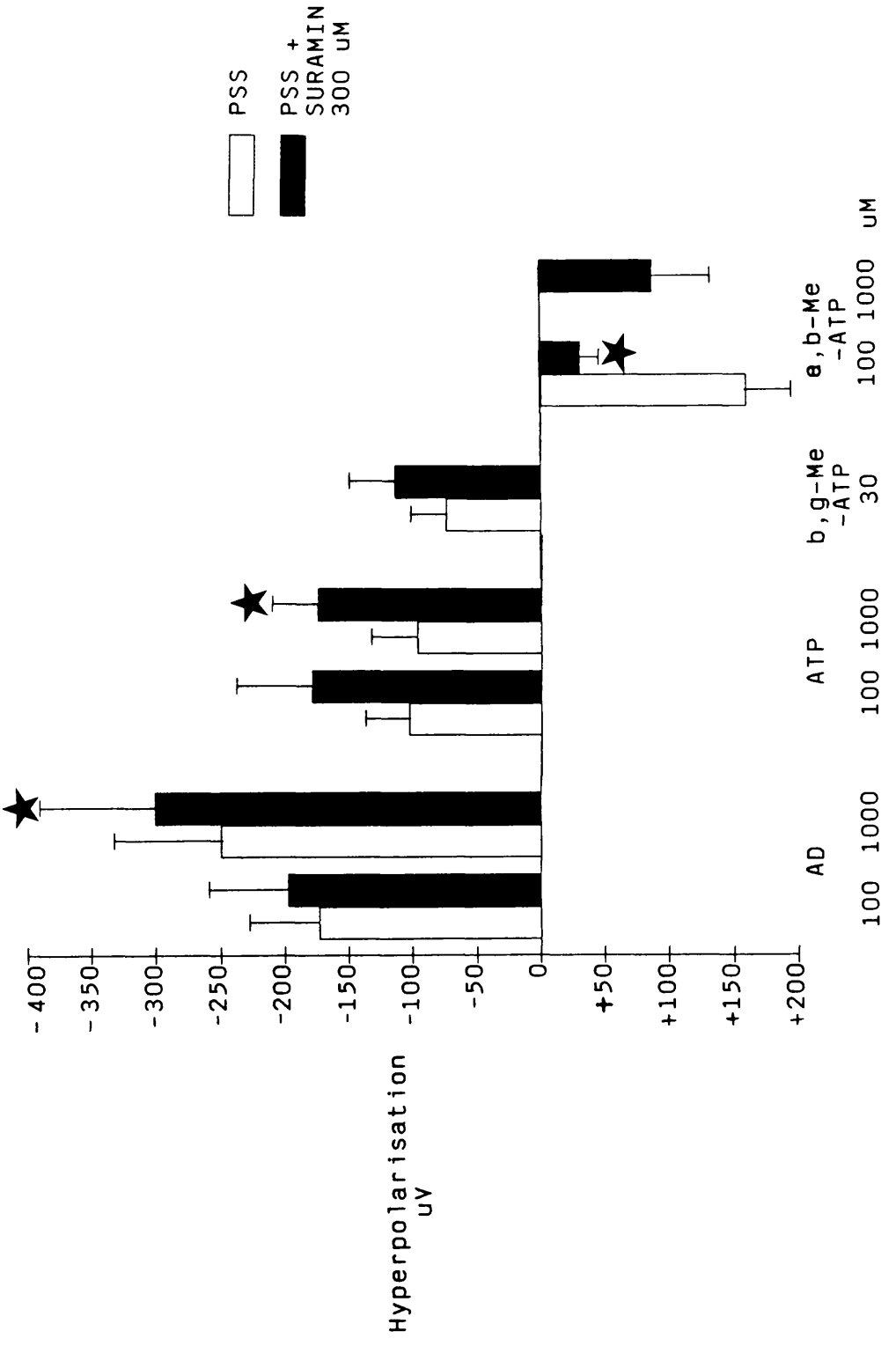

PSS
PSS + SURAMIN 300 uM
Hyperpolarisation uV
-400
-350
-300
-250
-200
-150
-100
-50
0
+50
+100
+150
+200
AD
ATP
b,g-Me -ATP
a,b-Me -ATP
100 1000
100 1000
30
100 1000 uM

Fig. 6.8. Effect of adenosine and some adenosine analogues on the d.c. potential of the rat isolated SCG

Hyperpolarisation to two minute applications (minimum of 20 minutes between applications) of cyclopentyl adenosine (CPA), 2-chloroadenosine (2CA), adenosine, phenylamidoadenosine (PAA) and N6-(9H-fluorenylmethyl)-adenosine (PD 117,413). Each point represents the mean from 6 (2CA) or 3 (other purines) ganglia.

PD 117,413 at up to 100uM did not significantly alter the basal d.c. potential of ganglia tested.

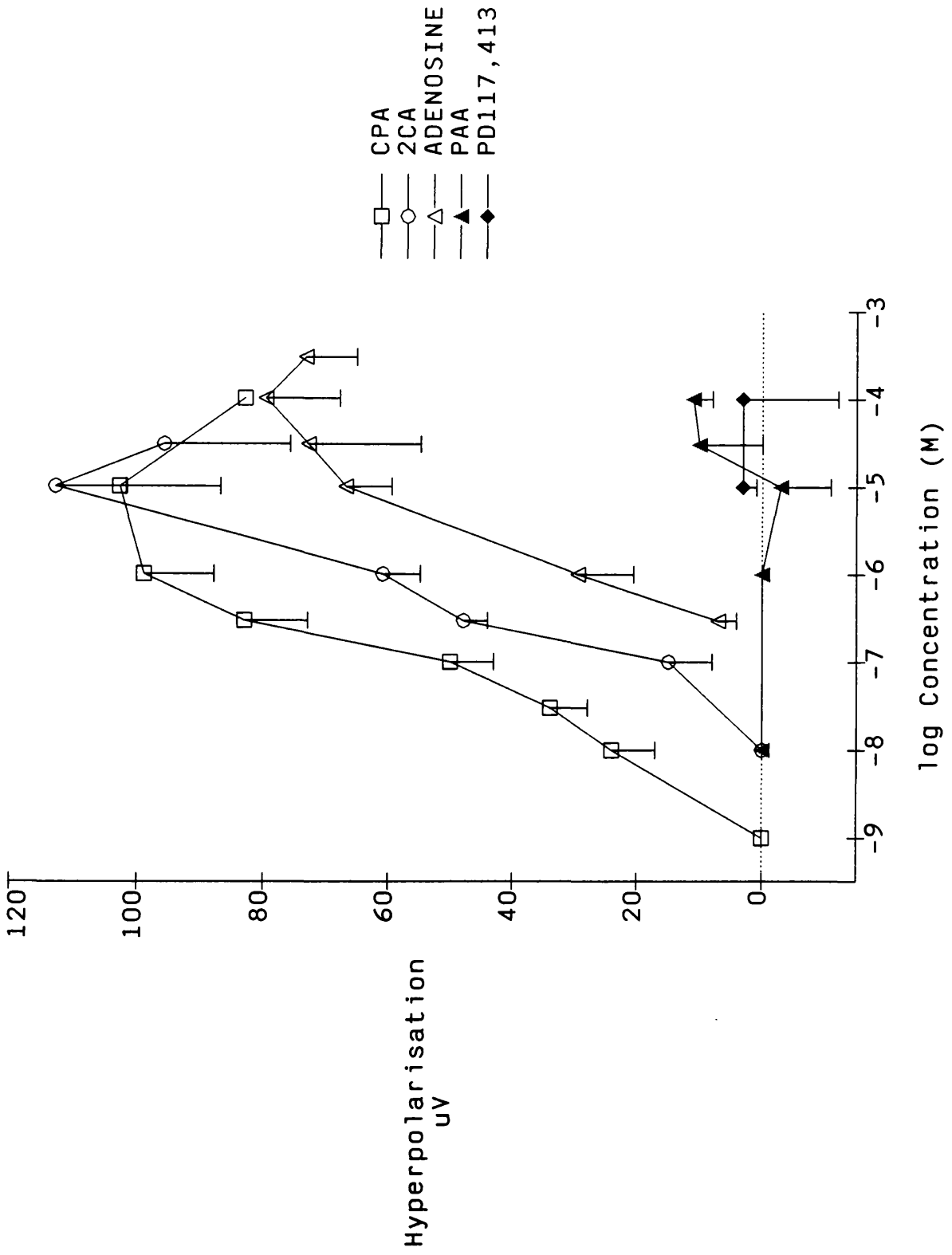

CPA
2CA
ADENOSINE
PAA
PD117,413
Hyperpolarisation
uV
120
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
-3
log Concentration (M)

Fig. 6.9. Comparison of response of rat isolated SCG to adenosine and 5'-methylthioadenosine

The response to both adenosine and 5'-methylthioadenosine (MTA) was tested by applying 2 minute applications at a minimum of 20 minute intervals.

The response to MTA was maximal at 100uM (i.e. no significant difference between responses at 100, 300 or 1000uM, paired t-test, $P>0.05$, $N=4$).

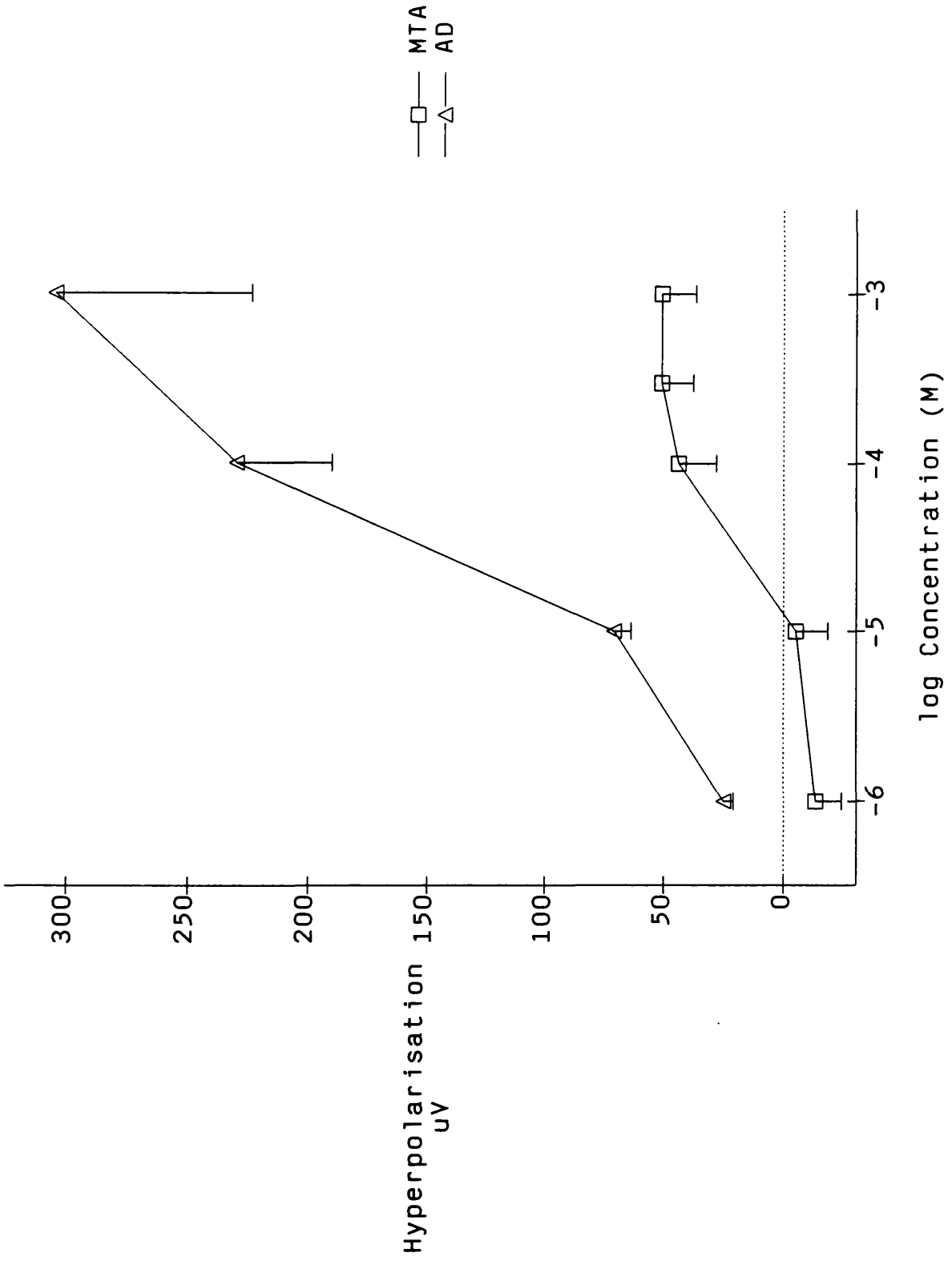

MTA
AD
Hyperpolarisation
uV
300
250
200
150
100
50
0
-6
-5
-4
-3
log Concentration (M)

Fig. 6.10. Effect of adenosine and some adenosine analogues on the response of the rat SCG to muscarine

% Depression of the response to 100nM muscarine (1 minute application) by cyclopenyladenosine (CPA), benzyladenosine (BZA), phenylaminoadenosine (PAA) and N6-(9H-fluoroenylmethyl)adenosine (PD 117,413). There was no significant alteration (paired t-test) of the response to muscarine in the presence of up to 100uM PD 117,413. The ratio of the EC_{50}'s for CPA and BZA was about 500.

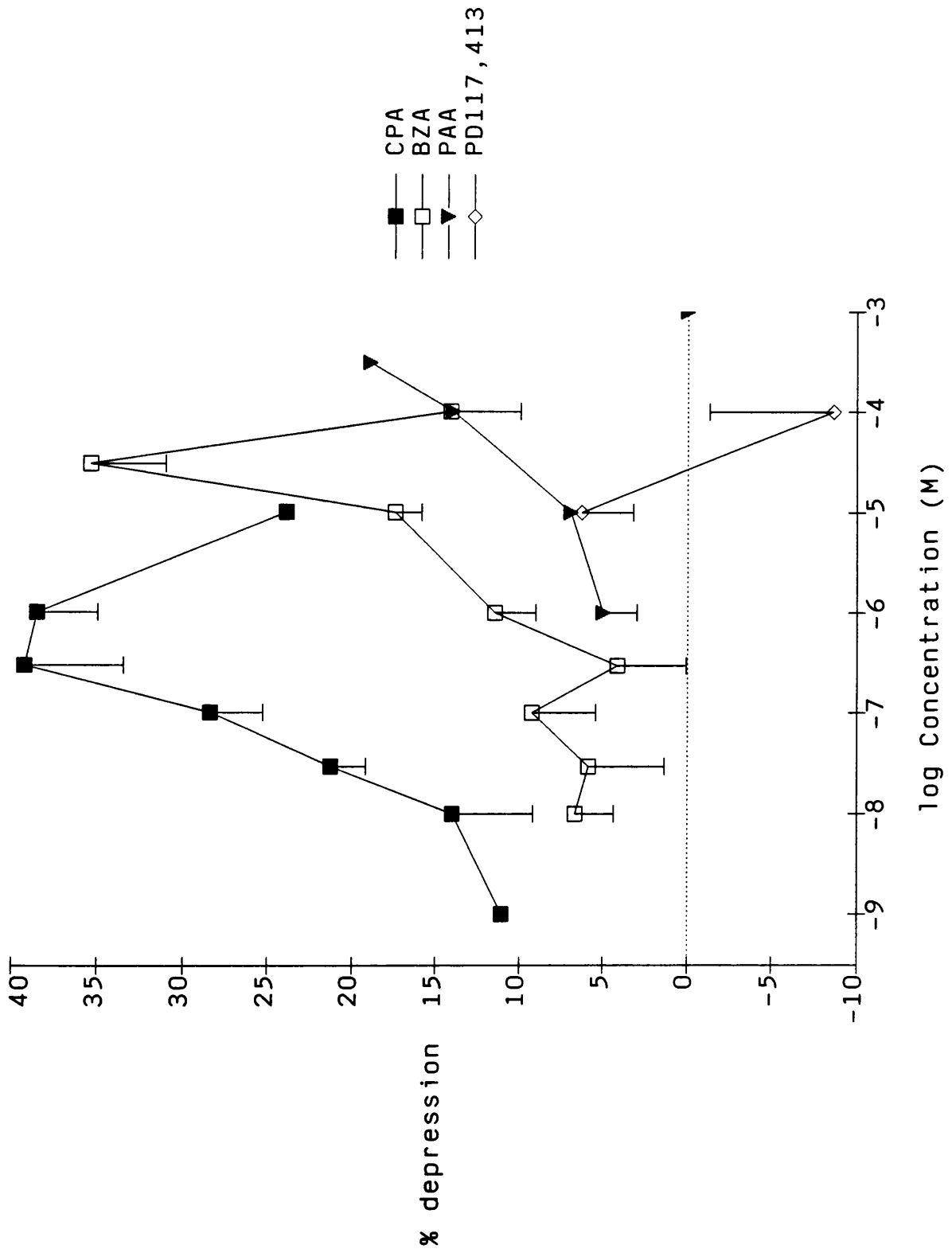

CPA
BZA
PAA
PD117,413
% depression
40
35
30
25
20
15
10
5
0
-5
-10
-9
-8
-7
-6
-5
-4
-3
log Concentration (M)

Fig. 6.11. Effect of cyclopentyladenosine and R and s-isomers of phenylisopropyladenosine on the response of the rat SCG to muscarine

Cyclopentyladenosine (CPA) and R- and S-phenyliso-propyladenosine (R-PIA, S-PIA) produced significant and concentration-dependent depression of the response to 100nM muscarine (1 minute).

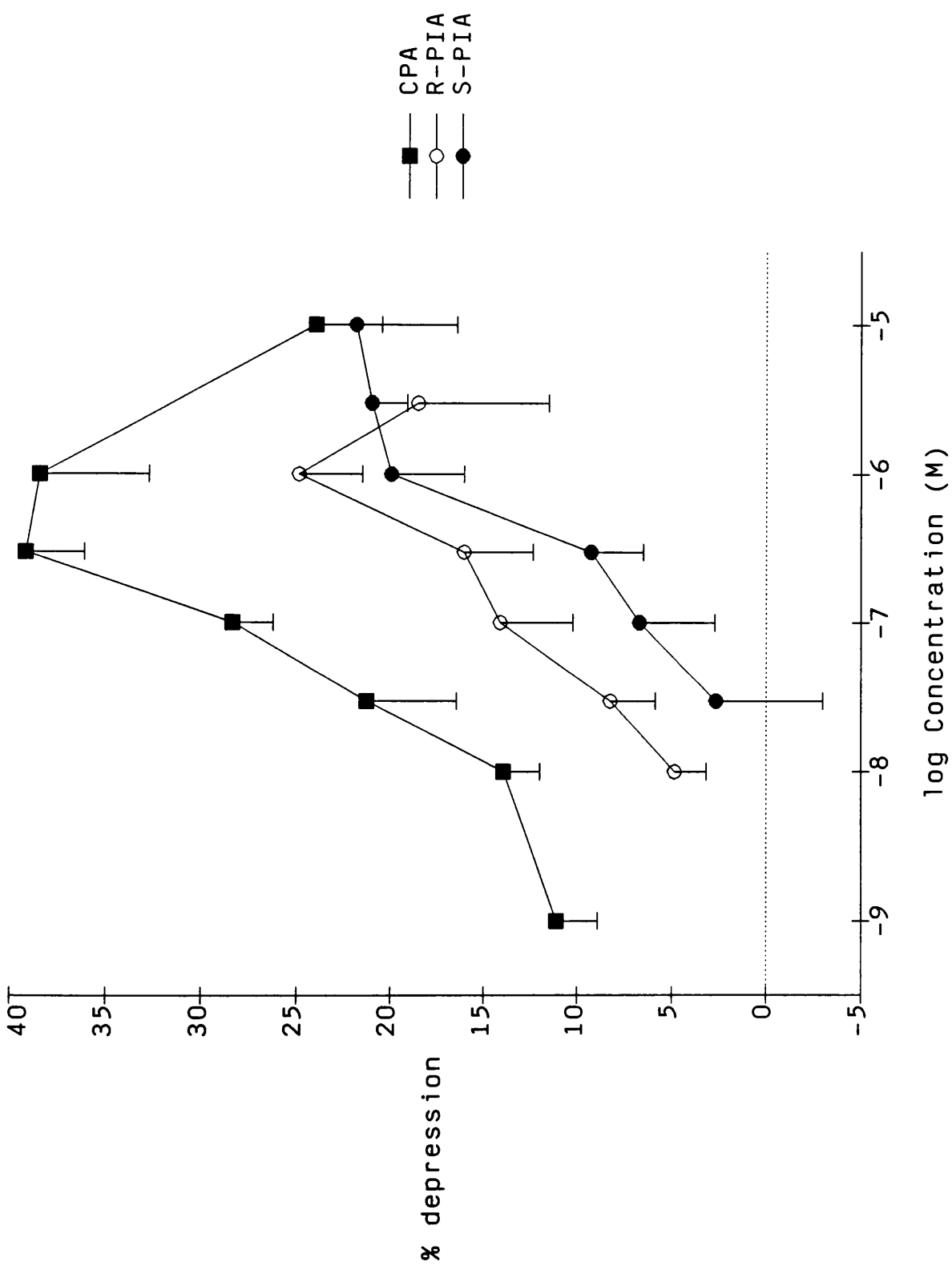

CPA
R-PIA
S-PIA
% depression
40
35
30
25
20
15
10
5
0
-5
-9
-8
-7
-6
-5
log Concentration (M)

Fig. 6.12. Effect of 2-chloroadenosine, 5'-N-ethylcarboxyamidoadenosine and adenosine on the response of the rat isolated SCG to muscarine

2-Chloroadenosine (2CA), 5'-N-ethylcarboxyamido-adenosine (NECA) were more potent than adenosine (AD) at depressing the response of the ganglion to 100nM muscarine (1 minute application).

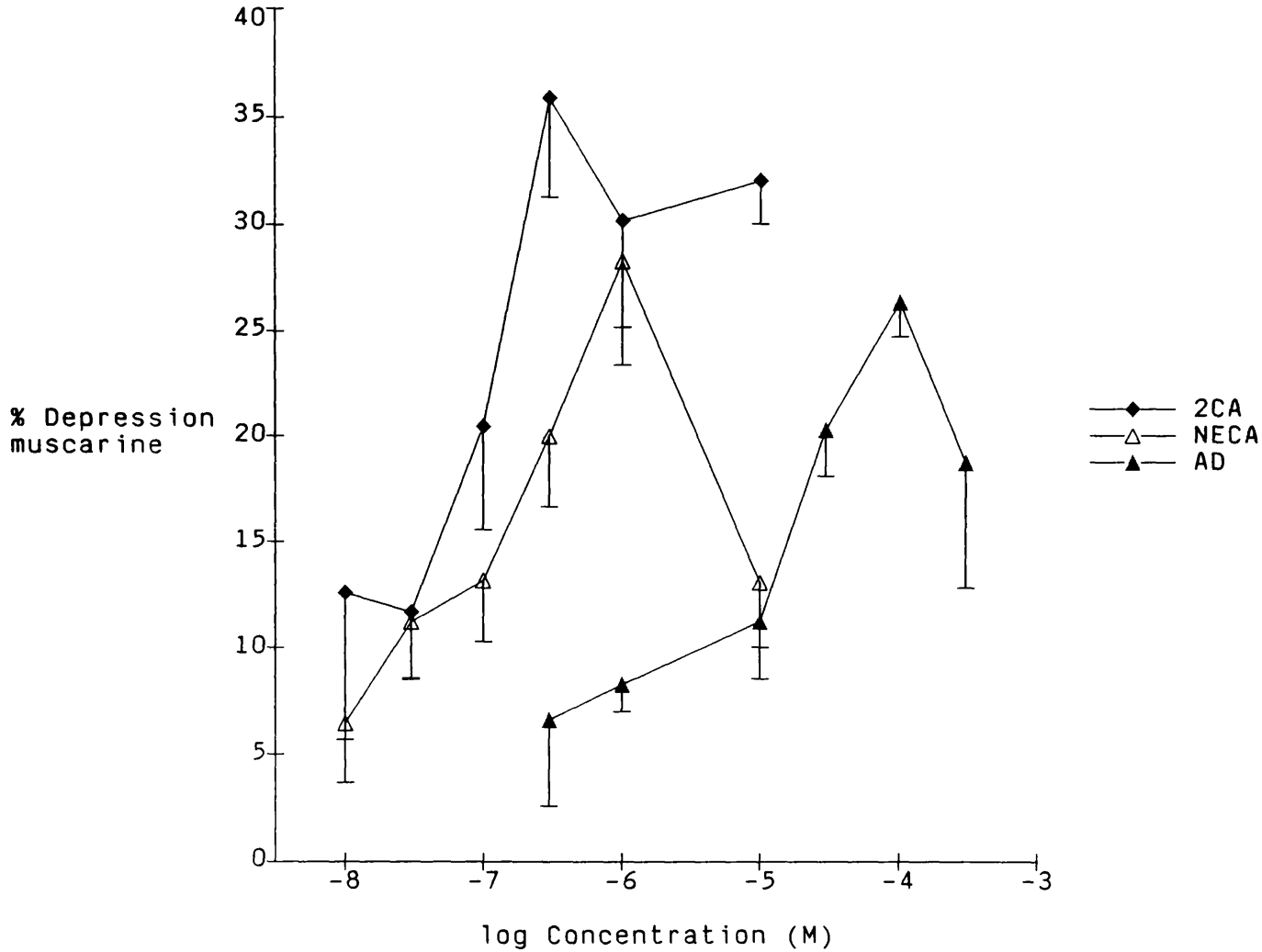
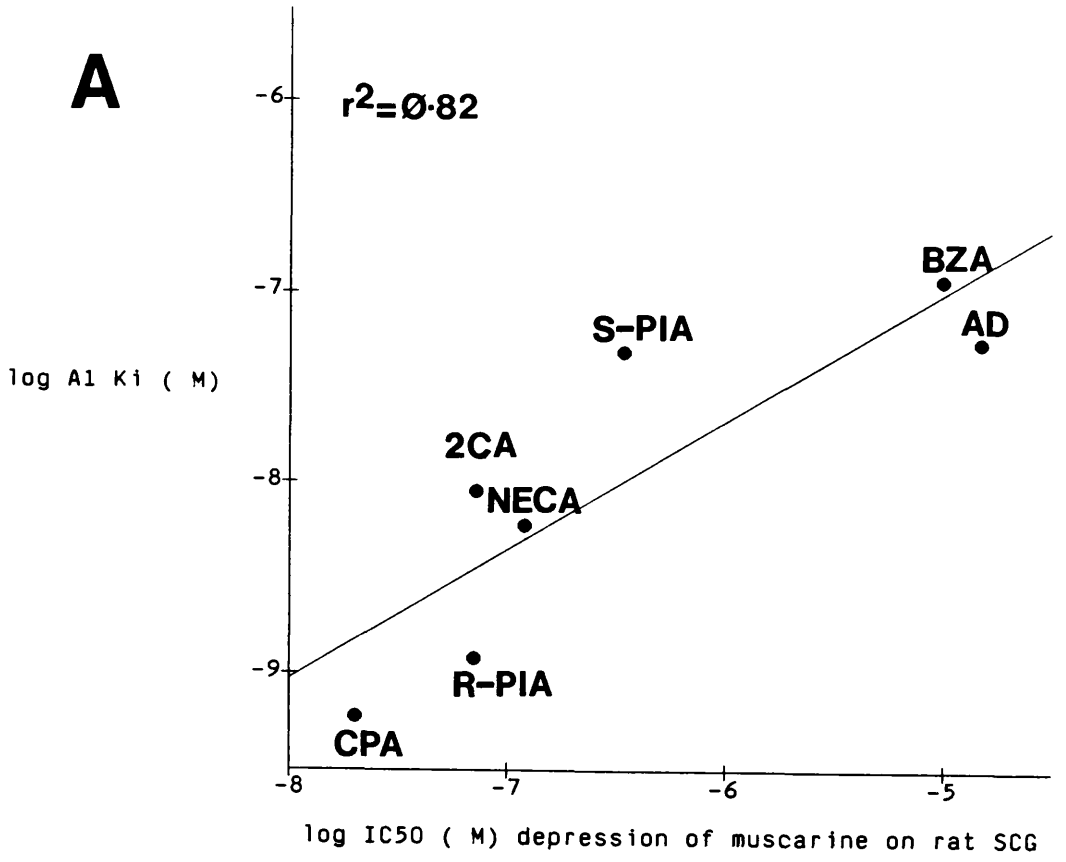

% Depression
muscarine
40
35
30
25
20
15
10
5
0
-8
-7
-6
-5
-4
-3
log Concentration (M)
2CA
NECA
AD

Fig. 6.13. Scatter diagram comparing the affinities of adenosine analogues for the adenosine receptor at the rat SCG (expressed on the abscissae as the IC_{50} values shown in Table 6.5) and for (A) the binding of [^{3}H]-cyclohexyladenosine to A_1-adenosine receptors in whole rat brain membranes minus brainstem and cerebellum (Bruns et al., 1986) and (B) the A_2-adenosine receptors of rat striatal membranes, determined from the binding of [^{3}H]-5'-N-ethylcarboxamidoadenosine in the presence of 50nM CPA to rat striatal brain membranes (Bruns et al., 1986).

The regression line is indicated by the continuous line. The coefficient of determination (r^2) for the IC_{50} values on the rat SCG and (A) the A_1-adenosine receptor binding was 0.82 ($P<0.005$) and for (B) the A_2-adenosine receptor binding, $r^2 = 0.05$.

For key to the abbreviations used see Table 2.1 and ABBREVIATIONS.


A
r2=Ø·82
BZA
AD
S-PIA
2CA
NECA
R-PIA
CPA
-6
-7
-8
-9
-8
-7
-6
-5
log A1 Ki (M)
log IC50 (M) depression of muscarine on rat SCG

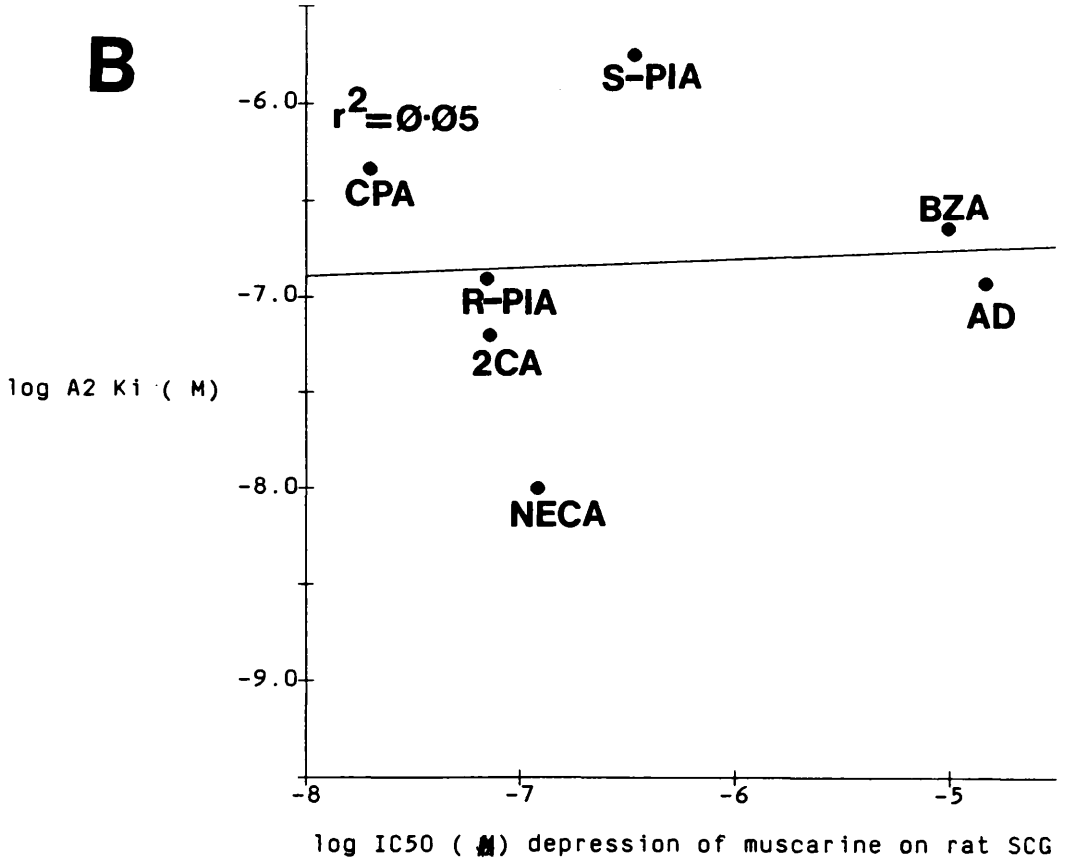
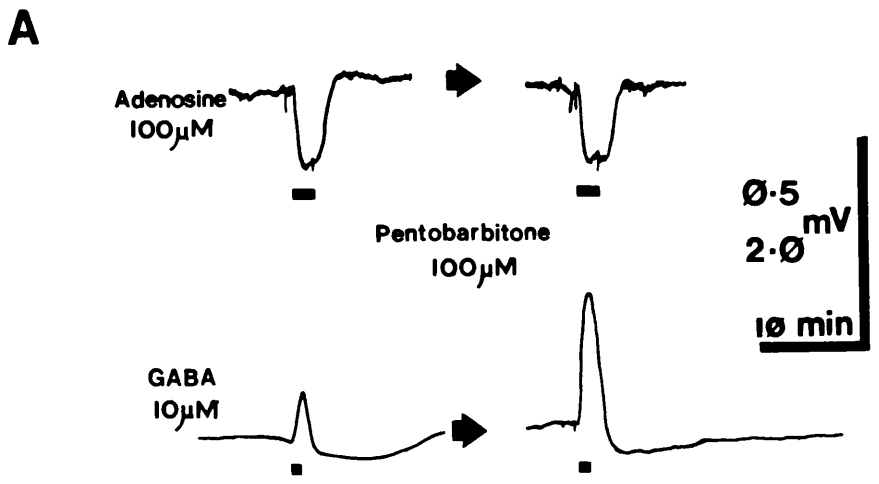

B
S-PIA
r2=Ø·Ø5
CPA
BZA
R-PIA
AD
2CA
NECA
-6.0
-7.0
-8.0
-9.0
-8
-7
-6
-5
log A2 Ki (M)
log IC50 (M) depression of muscarine on rat SCG

Fig. 6.14. Effect of pentobarbitone on the response to gamma-aminobutyric acid and adenosine

(A) Response of a single ganglion to gamma-aminobutyric acid (GABA, 1 minute application) and adenosine (100uM, 2 minutes) in the absence and presence of pentobarbitone (PB, 100uM). The depolarising response to GABA (10uM) was significantly increased from 160 $\pm$ 70uV to 398 $\pm$ 132uV ($n=4$, $P<0.05$) in the presence of PB. In contrast, both the AHP to 10uM GABA (control = 50 $\pm$ 24uV, +PB = -89 $\pm$ 45, $P>0.05$, $n=4$) and the response of adenosine (100uM, 2 minutes) was not significantly altered in the presence of PB (control = 230 $\pm$ 40uV and AD in the presence in PB = 220 $\pm$ 49uV, $n=4$) after a minimum of 20 minutes incubation.

(B) Effect of PB on the concentration-response curve of adenosine in physiological salt solution (PSS) containing 2mM potassium/0.1mM calcium. There was no significant effect of PB (paired t-test) on the response to adenosine at all concentrations of adenosine tested.


A
Adenosine
100μM
Pentobarbitone
100μM
GABA
10μM
Ø·5
2·Ø mV
1Ø min

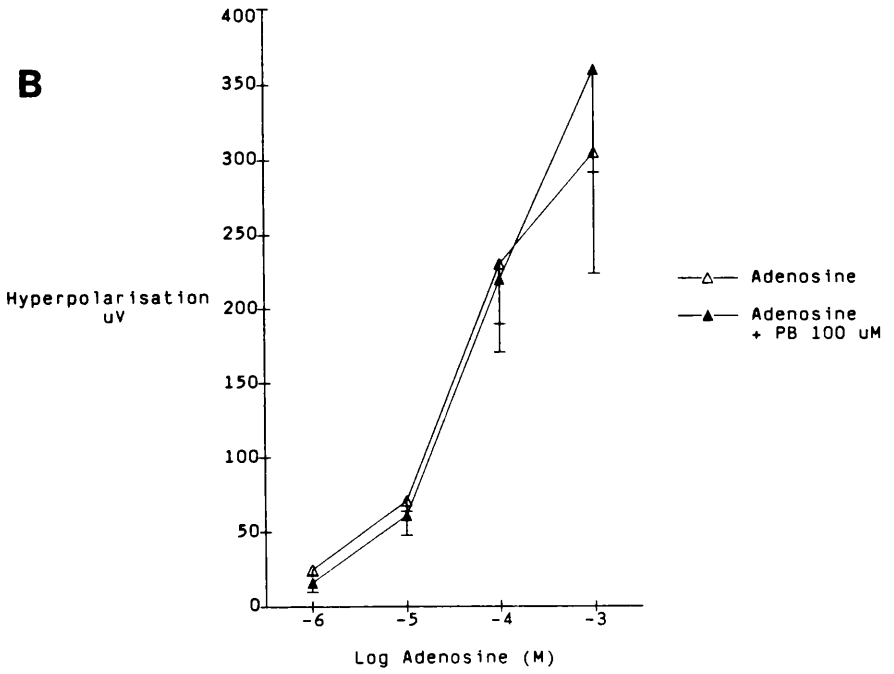

B
Hyperpolarisation
uV
400
350
300
250
200
150
100
50
0
-6
-5
-4
-3
Log Adenosine (M)
Adenosine
Adenosine
+ PB 100 uM

Fig. 6.15. Effect of 8-phenyltheophylline on the log concentration-response curve to adenosine

In physiological salt solution containing 2mM K^+/0.1mM Ca^{2+}, the concentration-response curves to adenosine (AD) were reproducible (cf: 1st and 2nd CRC). In the presence of 8-phenyltheophylline (8PT) the concentration-response curve to adenosine was displaced to the right. N=4 ganglia.

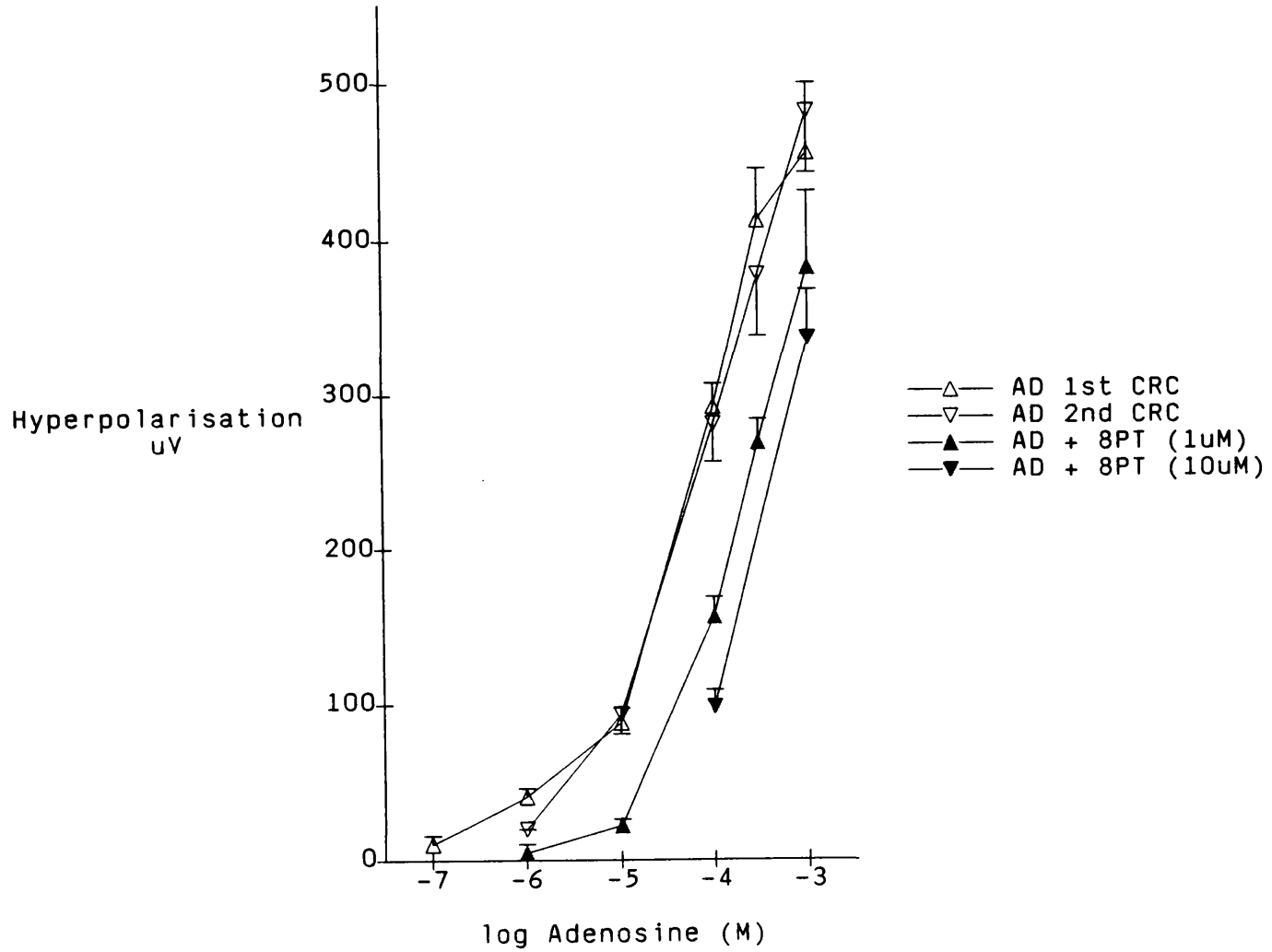

Hyperpolarisation
uV
500
400
300
200
100
0
-7
-6
-5
-4
-3
log Adenosine (M)
AD 1st CRC
AD 2nd CRC
AD + 8PT (1uM)
AD + 8PT (10uM)

Fig. 6.16. Effect of 8-phenyltheophylline on the depression of the response to muscarine by adenosine

The effect of adenosine (100uM) on the response to 100nM muscarine in (A) absence and (B) presence of 8-phenyltheophylline (10uM). NB: In B the reduction of the hyperpolarisation to adenosine and no depression of the response to muscarine.

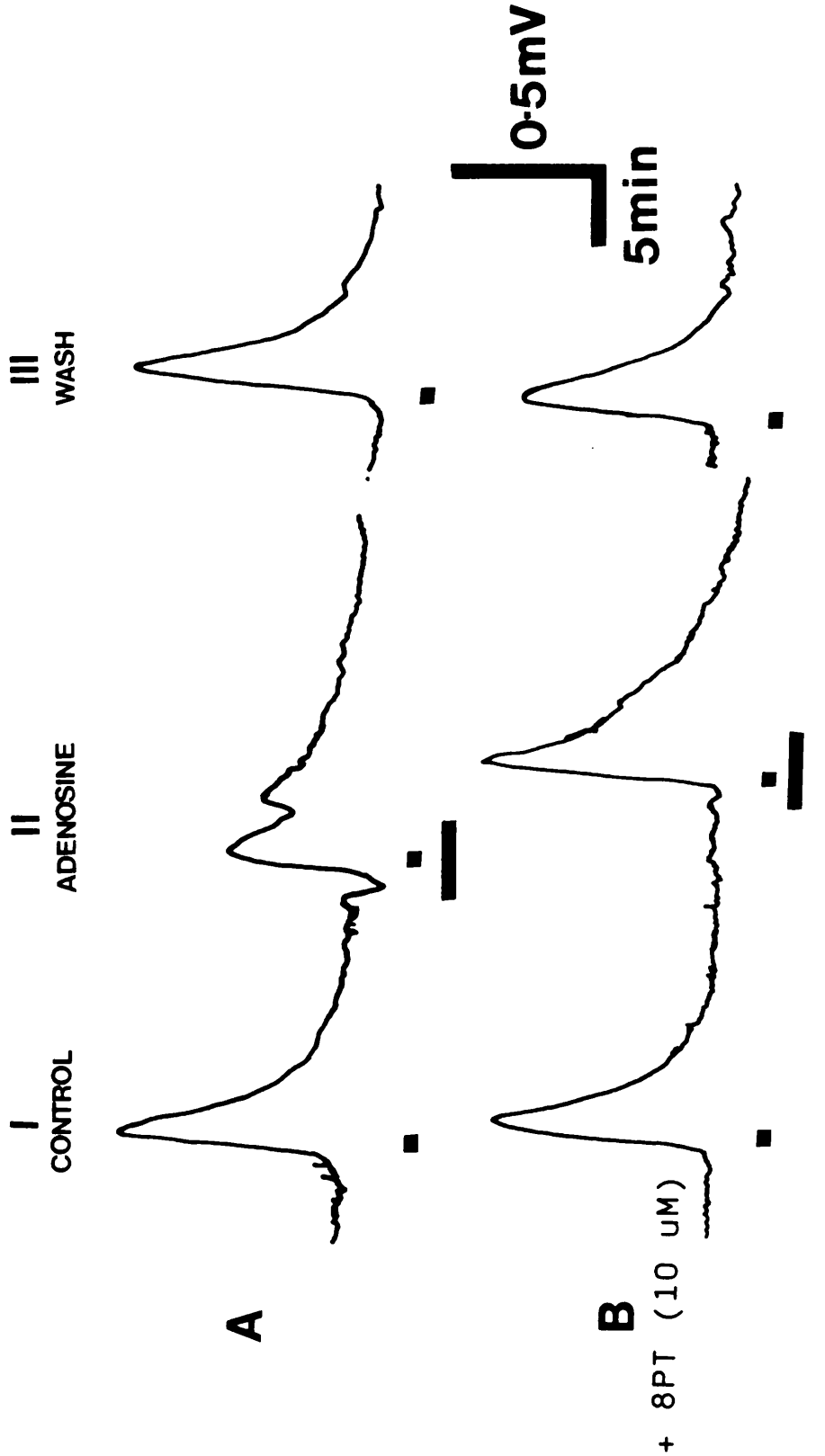
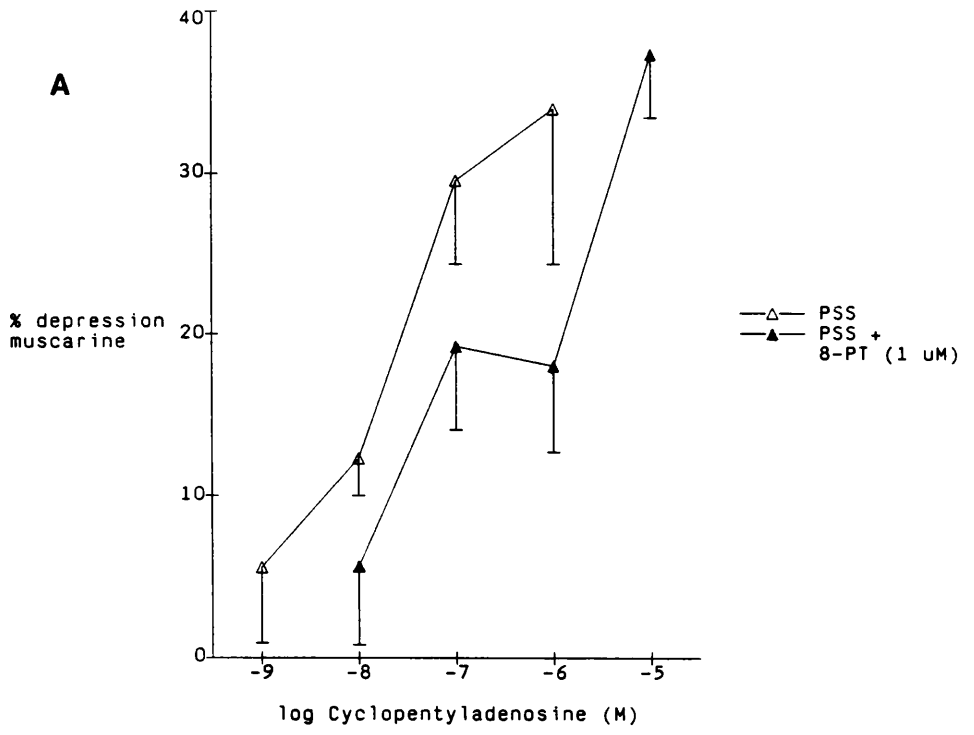

I
CONTROL
II
ADENOSINE
III
WASH
A
B
+ 8PT (10 uM)
0·5mV
5min

Fig. 6.17. Effect of 8-phenyltheophylline on the depression by cyclopentyladenosine of the response of the rat SCG to muscarine

The response to 100nM muscarine (1 minute) was reduced in a concentration-dependent manner by cyclopentyl-adenosine (CPA). 8-phenyltheophylline (8PT) at 1uM (A) and 10uM (B) produced a rightward displacement of the concentration-response curve. N=3 to 8 ganglia.


A
% depression muscarine
40
30
20
10
0
-9
-8
-7
-6
-5
log Cyclopentyladenosine (M)
PSS
PSS + 8-PT (1 uM)

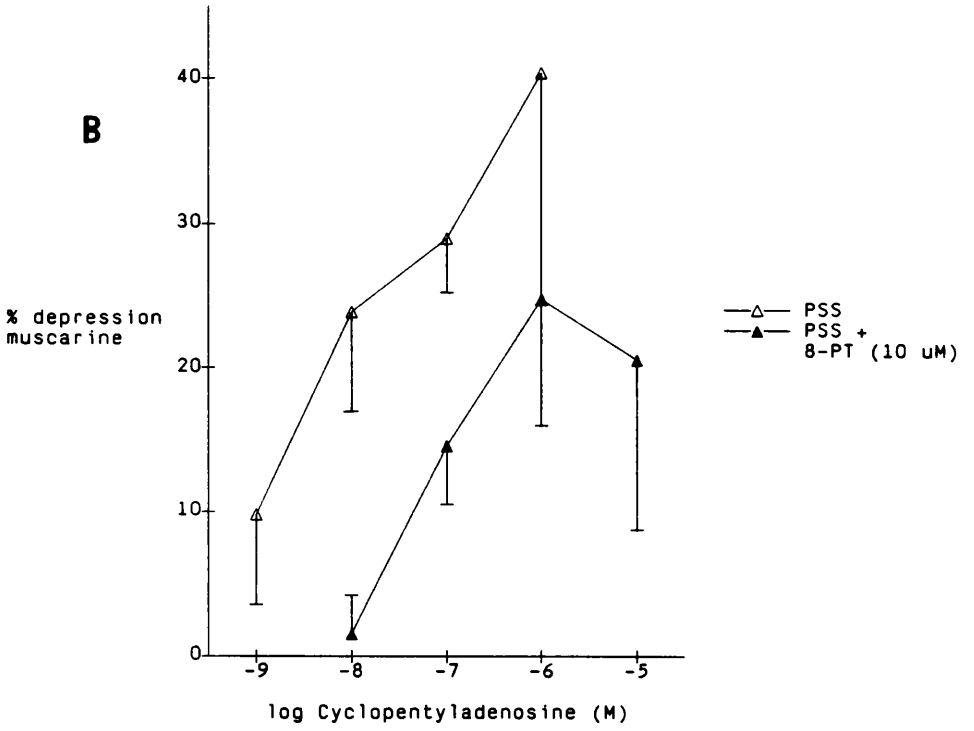
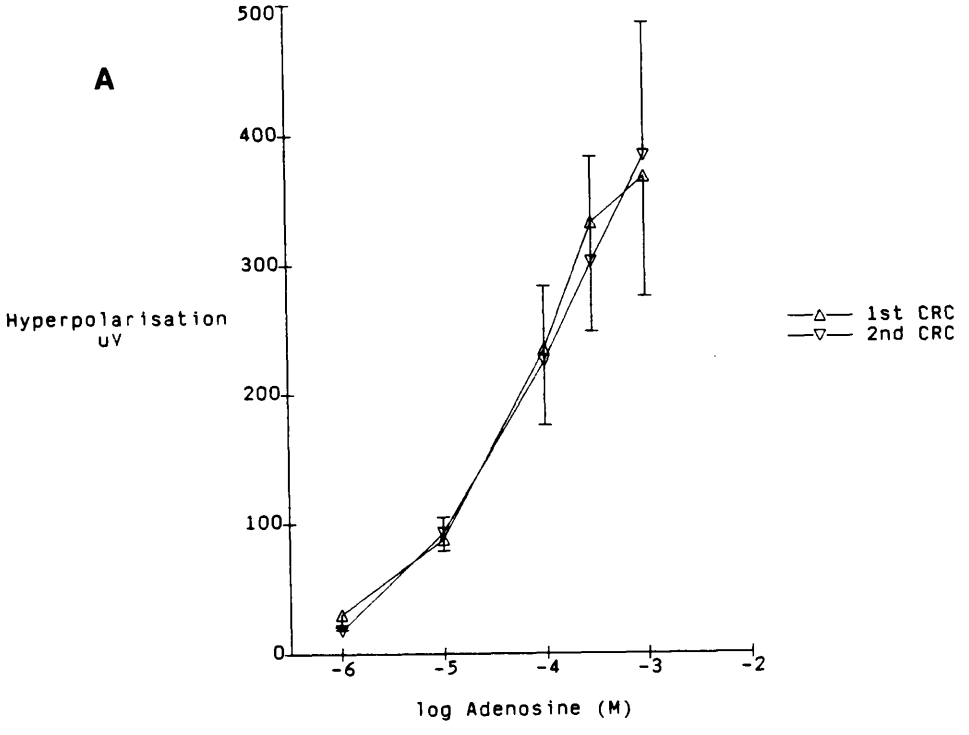

B
% depression muscarine
40
30
20
10
0
-9
-8
-7
-6
-5
log Cyclopentyladenosine (M)
PSS
PSS + 8-PT (10 uM)

Fig. 6.18. Effect of dimethylpropargylxanthine on the concentration response curve of the rat SCG to adenosine

A) Concentration-response curve to adenosine (1st CRC), repeated after 30 minute interval (2nd CRC).
B) Responses to adenosine (AD) in the absence and presence of 50uM dimethylpropargylxanthine (AD + DMPX). N = 3 ganglia. NB: there was no statistically significant difference (paired t-test) between responses in the absence or presence of DMPX.

Physiological salt solution = 2mM K^+/0.1mM Ca^{2+}.


A
500
400
300
200
100
0
Hyperpolarisation
uV
-6
-5
-4
-3
-2
log Adenosine (M)
1st CRC
2nd CRC

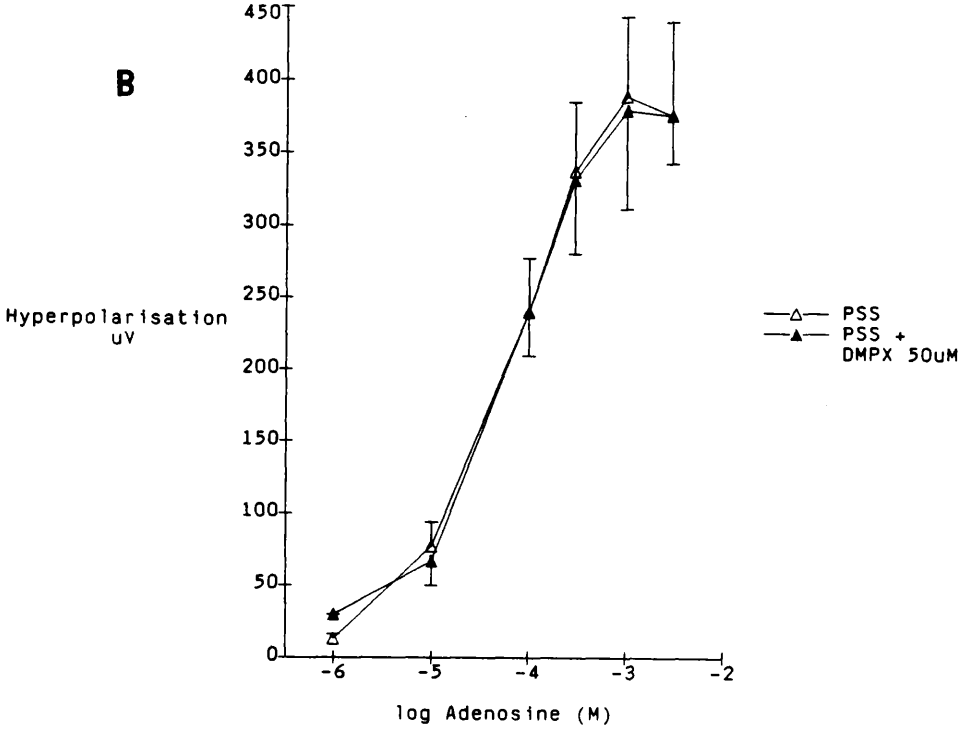
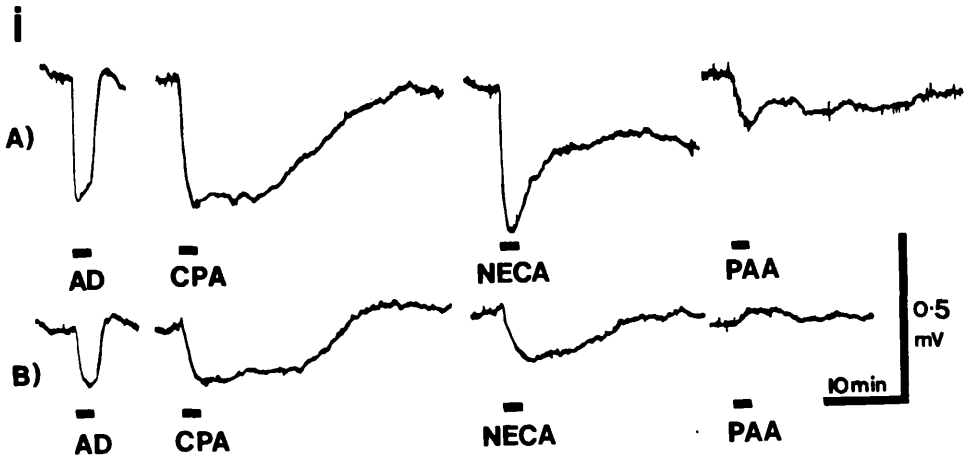

B
450
400
350
300
250
200
150
100
50
0
Hyperpolarisation
uV
-6
-5
-4
-3
-2
log Adenosine (M)
PSS
PSS +
DMPX 50uM

Fig. 6.19. Effect of **8-cyclopentyl-1,3-dipropylxanthine on the** response of the rat SCG to adenosine, cyclopentyl-adenosine, 5'-N-ethylcarboxylamido-adenosine and phenylamidoadenosine

The effect of 50uM 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) on the response to adenosine (AD), cyclopentyl-adenosine (CPA), 5'-ethylcarboxyamidoadenosine (NECA) and phenylamidoadenosine (PAA).

(1) Responses of a single ganglion in 2mM K^+/0.1mM Ca^{2+} physiological salt solution in the absence (A) and presence of 50uM DMPX (B).

(2) Histogram showing responses of four ganglia in the absence and presence of DMPX.


i
A)
AD
CPA
NECA
PAA
B)
AD
CPA
NECA
PAA
0·5
mV
10min

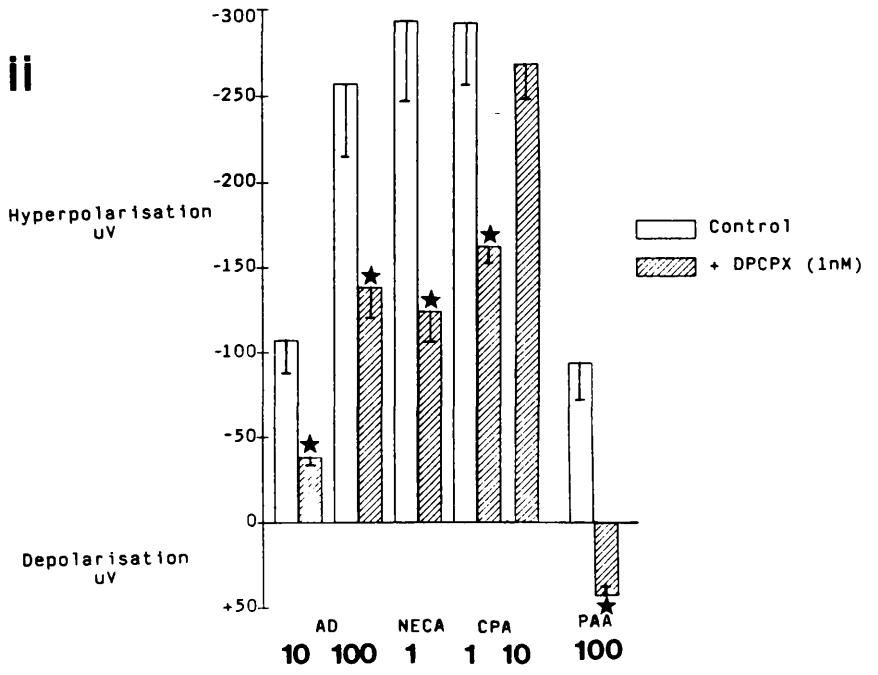

ii
Hyperpolarisation
uV
Depolarisation
uV
-300
-250
-200
-150
-100
-50
0
+50
AD
NECA
CPA
PAA
10
100
1
1
10
100
Control
+ DPCPX (1nM)

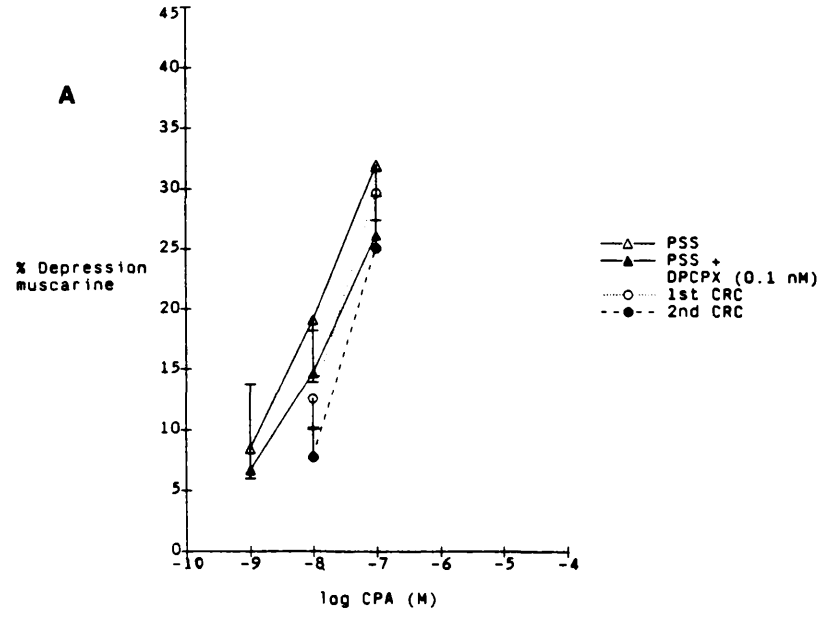
Fig. 6.20. Depression of the response of the rat SCG to muscarine by CPA in the presence of 1,3-dipropyl-8-cyclopentylxanthine

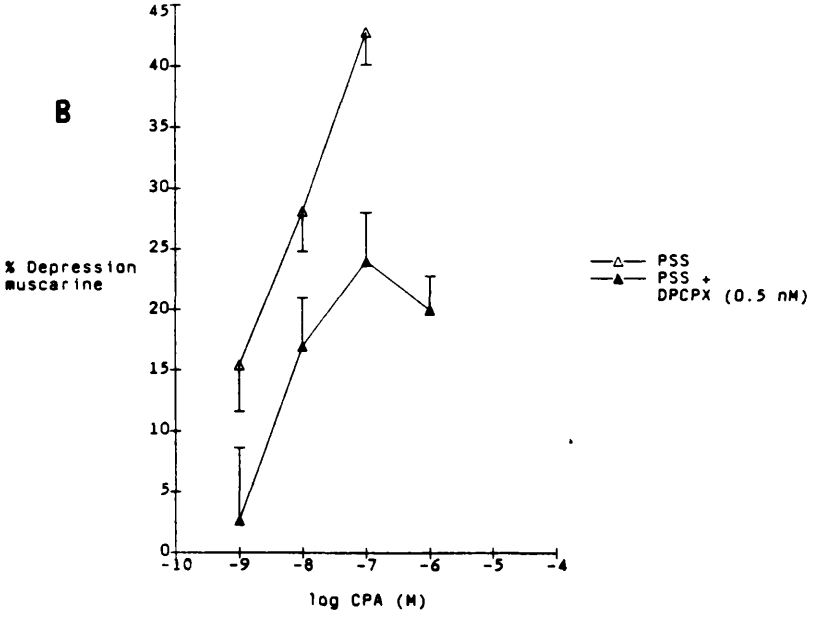
Ganglia were incubated in the presence of different concentrations of 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) for a minimum of 40 minutes before determining the depression of the response to 100nM muscarine by increasing concentrations of cyclopentyladenosine (CPA).

(A) The depression of the response to muscarine by 10 and 100nM CPA was determined (1st CRC) and repejated after a minimum of 80 minutes (2nd CRC) (N=4). On other ganglia (N=7) the effect of 0.1nM DPCPX on the depression of the response to muscarine by CPA was determined. The effect of 0.1nM DPCPX was not discernible within the variability of the control responses.

In further experiments the effect of 0.5nM (B; N=4), 1nM and 10nM (C; N=3 to 11 and N=5 respectively) DPCPX on the depression of the response to muscarine by CPA were examined.

N=number of ganglia studied.


A
% Depression muscarine
log CPA (M)
PSS
PSS + DPCPX (0.1 nM)
1st CRC
2nd CRC


B
% Depression muscarine
log CPA (M)
PSS
PSS + DPCPX (0.5 nM)

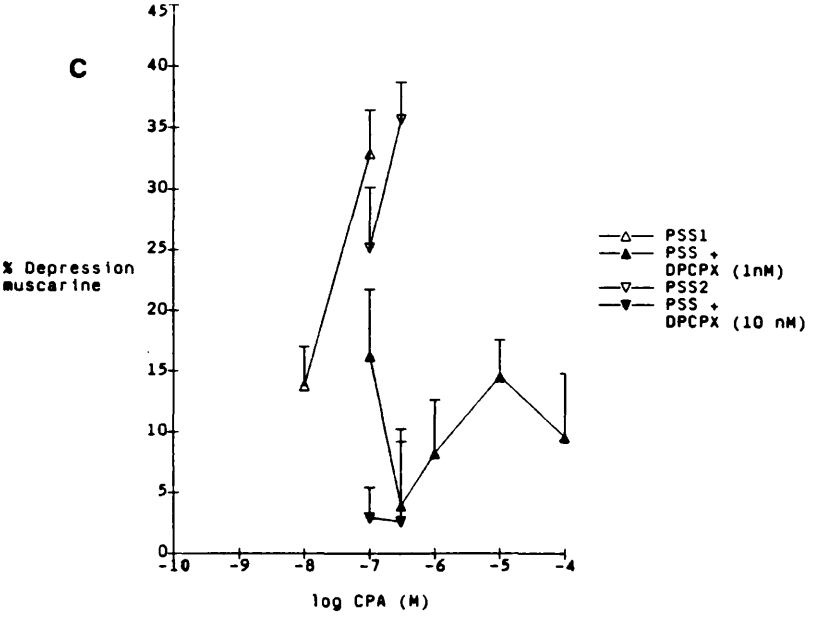
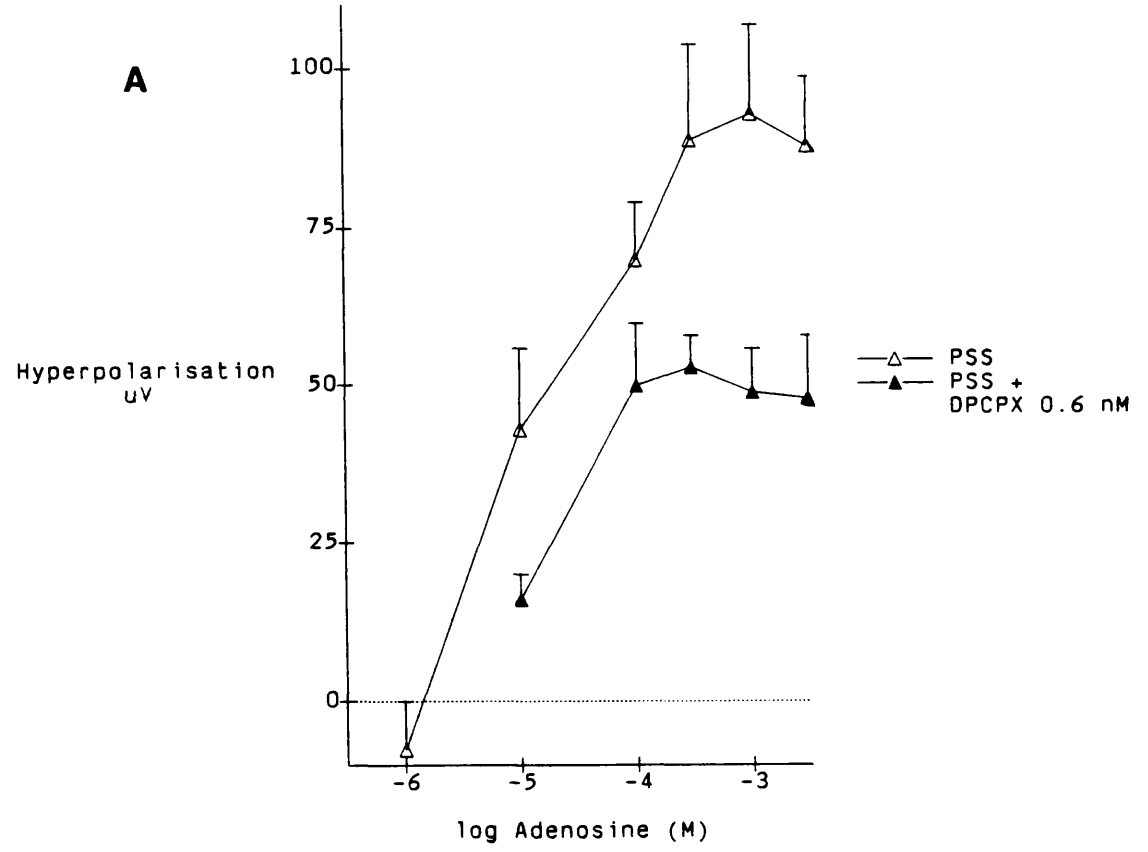
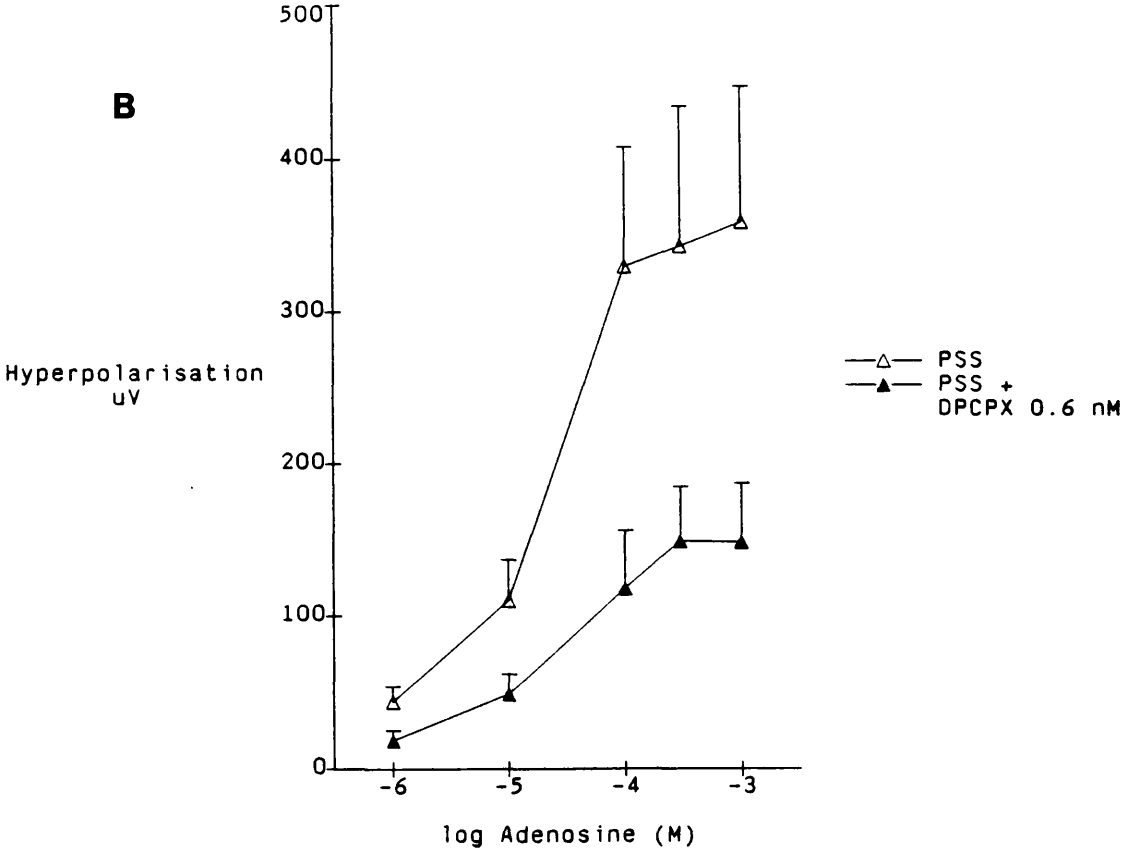

C
% Depression muscarine
log CPA (M)
PSS1
PSS + DPCPX (1nM)
PSS2
PSS + DPCPX (10 nM)

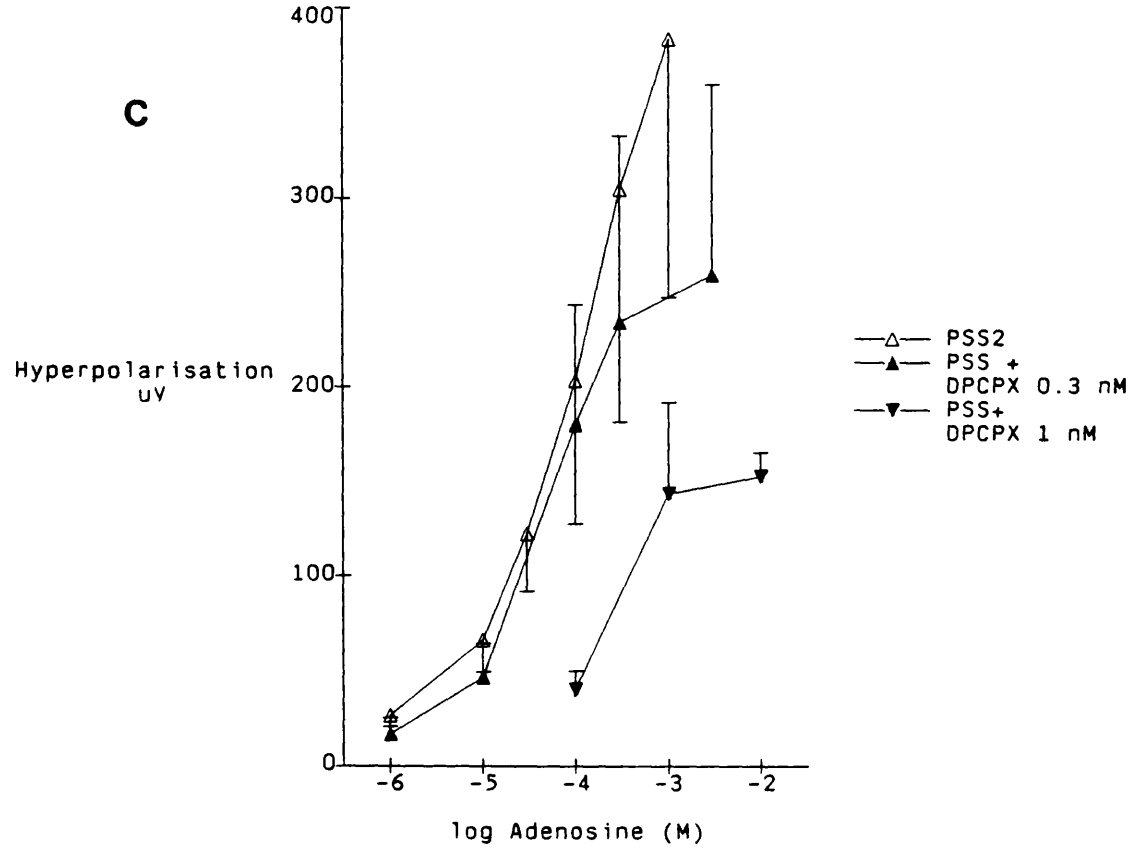
Fig. 6.21. Effect of 1,3-dipropyl-8-cyclopentyl-xanthine and xanthine amine congener on the hyperpolarisation of the rat SCG to adenosine

In physiological salt solution (PSS) containing 2mM potassium and 0.1mM calcium (2mM K^+/0.1mM Ca^{2+} PSS) 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) (Fig. 6.20A-C) and xanthine amine congener (XAC) (Fig. 6.20D) antagonised the response to adenosine (AD).

The hyperpolarisations to AD were smaller in 2mM K^+/2.5mM Ca^{2+} PSS (Fig. 6.20A) compared to 2mM K^+/0.1mM Ca^{2+} PSS (Fig. 6.20B), but the effect of DPCPX was similar.


A
100
75
50
25
0
Hyperpolarisation
uV
-6
-5
-4
-3
log Adenosine (M)
PSS
PSS +
DPCPX 0.6 nM


B
500
400
300
200
100
0
Hyperpolarisation
uV
-6
-5
-4
-3
log Adenosine (M)
PSS
PSS +
DPCPX 0.6 nM


C
Hyperpolarisation
uV
400
300
200
100
0
-6
-5
-4
-3
-2
log Adenosine (M)
PSS2
PSS +
DPCPX 0.3 nM
PSS+
DPCPX 1 nM

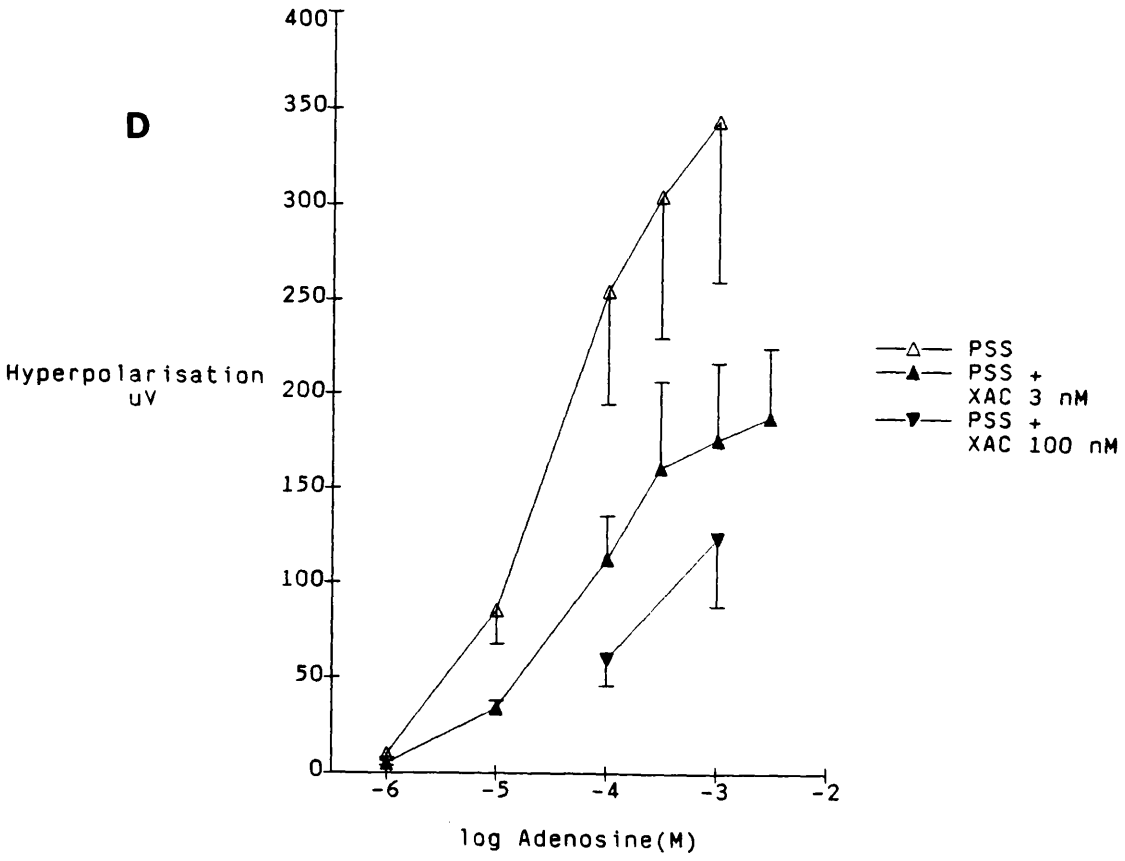

D
Hyperpolarisation
uV
400
350
300
250
200
150
100
50
0
-6
-5
-4
-3
-2
log Adenosine(M)
PSS
PSS +
XAC 3 nM
PSS +
XAC 100 nM

Fig. 6.22. Response of a single ganglion to adenosine in the absence and presence of 1,3-dipropyl-8-cyclopentylxanthine

A) Control responses to 2 minute applications of increasing concentrations of adenosine and (B) in the presence of 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) at 0.6nM and (C) 0.9nM DPCPX.

NB: The response to cyclopentyladenosine (CPA) at 1uM (2 minutes application) in 0.9nM DPCPX is shown for comparison to the responses to adenosine.

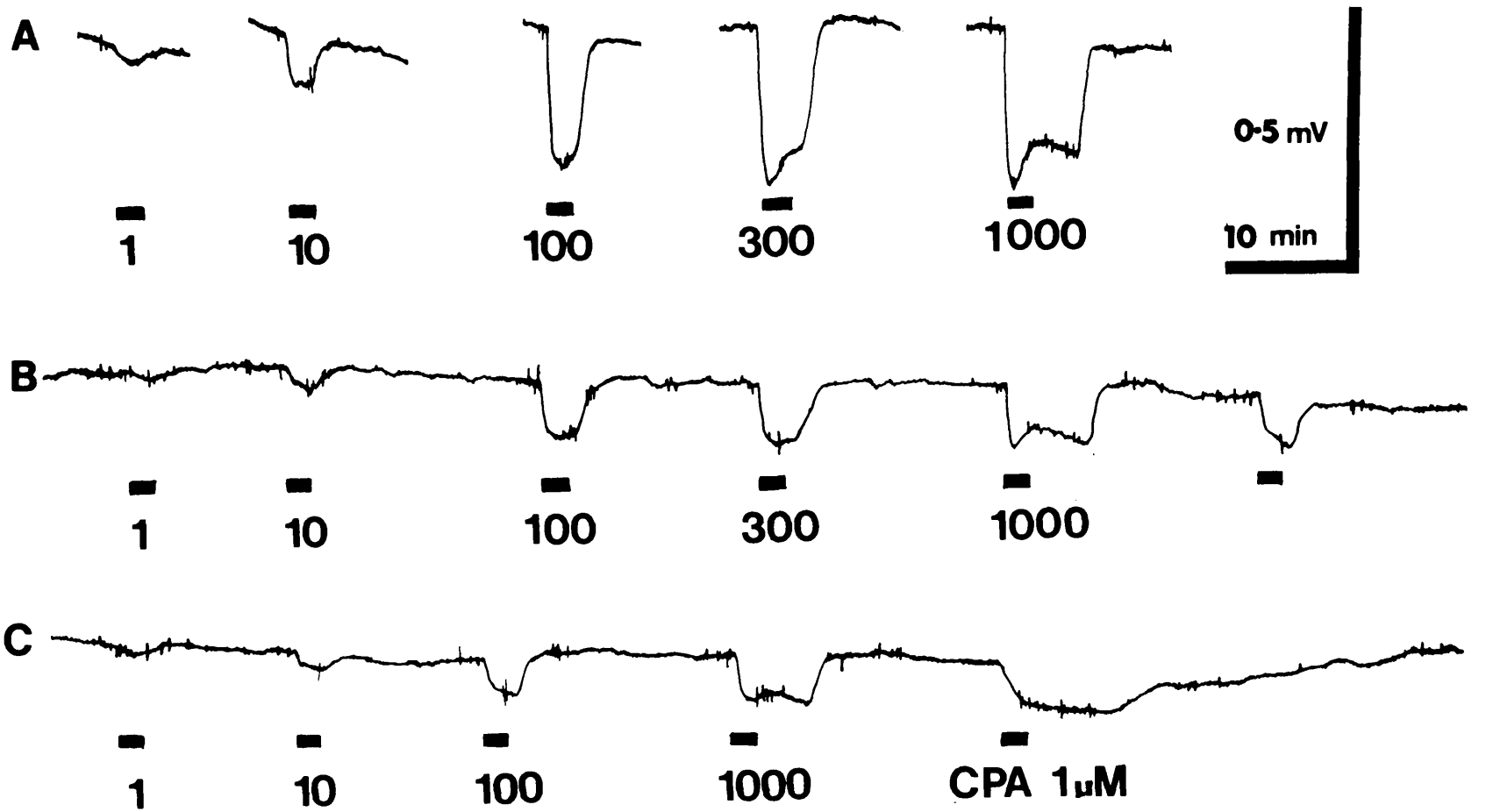
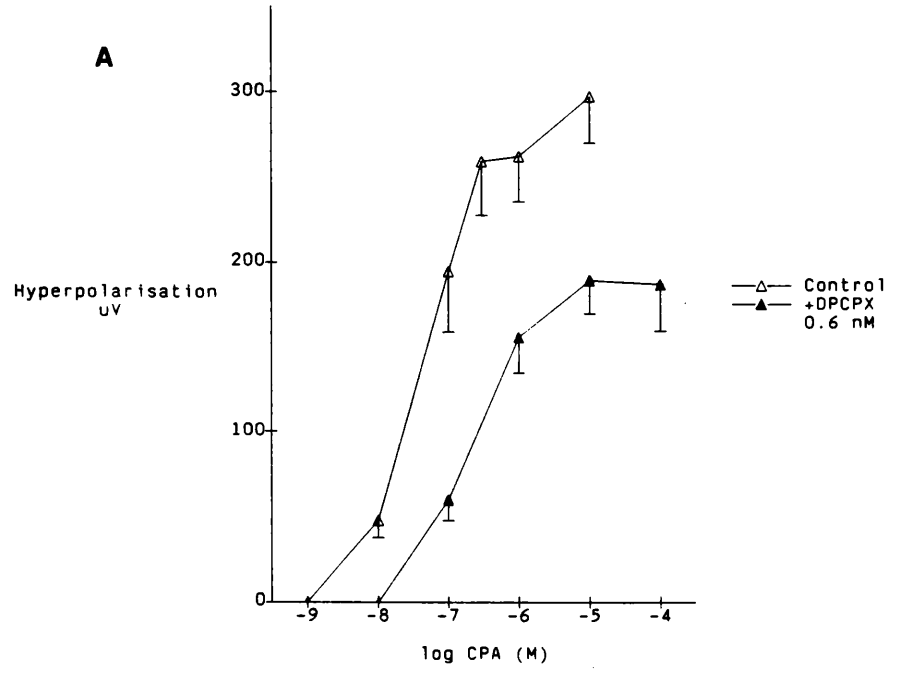

A
1
10
100
300
1000
0·5 mV
10 min
B
1
10
100
300
1000
C
1
10
100
1000
CPA 1μM

Fig. 6.23. Effect of 1,3-dipropyl-8-cyclopentylxanthine on the hyperpolarisation of the rat SCG to cyclopentyladenosine

Log concentration response curves to cyclopentyladenosine (CPA) in the absence and presence of 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) at (A) 0.6nM, (B) 0.3nM and 0.9nM DPCPX.


A
Hyperpolarisation
uV
300
200
100
0
-9
-8
-7
-6
-5
-4
log CPA (M)
Control
+DPCPX
0.6 nM

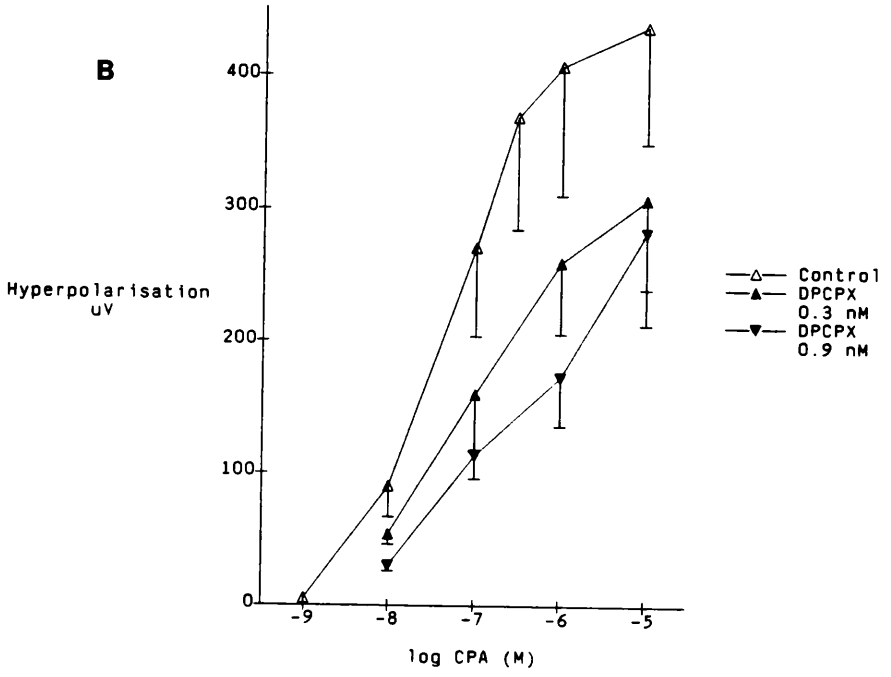

B
Hyperpolarisation
uV
400
300
200
100
0
-9
-8
-7
-6
-5
log CPA (M)
Control
DPCPX
0.3 nM
DPCPX
0.9 nM

Table 6.1. Effect of adenosine-5'-thiophosphate and its structural analogues on the d.c. potential, responses to muscarine and GABA and effect of 8-phenyltheophylline and suramin on the change of d.c. potential of the rat SCG.

	ATP	b,g-MeATP	2MeS-ATP	a,b-MeATP	Adenosine
% change in response to muscarine[a]	-29 ± 4 (12)***	-11 ± 5 (8)*	-9 ± 6 (8)[b]	0 ± 5 (4)	-36 ± 3 (4)*[b]
% change in response to GABA[c]	7 ± 6 (3)	15 ± 5 (8)**			9 ± 4 (7)*
Change in d.c. potential (uV)	-26 ± 10 (4)	-29 ± 6 (9)***	0 ± 0 (4)	+66 ± 9 (4)**	
Change in d.c. potential antagonised by 8PT (10uM)	Yes	Yes		No	Yes
Effect of suramin on change in d.c. potentially purine[d]	Increased	Increased		Decreased	Increased

The effects of adenosine triphosphate (ATP), beta,gamma-methylene adenosine triphosphate, 2-methlthio-adenosine triphosphate (2MeS-ATP), alpha,beta-methylene adenosine triphosphat (a,b-MeATP) and adenosine (AD) were examined at 100uM.

(a) Muscarine (100nM) or GABA (10uM) were applied for 1 minute in the absence and presence of an ATP analogue. Significant differences from control responses were determined as described in Chapter 2 and are indicated by a * for P<0.05, ** for P<0.01 and *** for P<0.001, and expressed as trhe mean ± SEM and number of ganglia. Number of ganglia in brackets.
(b) The effects of 2MeS-ATP and adenosine were tested on the same ganglia.
(c) The change in d.c. potential recorded during the 1st minute of application.
(d) Effect of suramin (300uM) was tested after a minimum of 40 minutes incubation and the response to purines (2 minutes application) compared to responses in the absence of suramin.
8PT = 8-phenyltheophylline

Table 6.2. Effect of methylxanthines on the d.c. potential response to muscarine, adenosine, adenosine-5'-triphosphate, 2-chloroadenosine and the depression of the response to muscarine

The effect of theophylline and 8-phenyltheophylline (8PT) on the basal d.c. potential, response to a one minute application of muscarine, 2 minute applications of adenosine, 2-chloroadenosine (2CA) or adenosine-5'-triphosphate (ATP) and the depression of the response to a one minute application of muscarine by adenosine, 2CA or ATP was determined as described in chapter 2. A minimum of 30 minutes incubation in theophylline or 8PT and 20 minutes between responses to agonists was used. Responses are means $\pm$ SEM, where N = number of ganglia tested. Statistical significance was determined using a paired t-test comparing the response in physiological salt solution (PS) to PSS-containing theophylline, 8PT or 8-parasulpho-phenyltheophylline (8psPT) and is indicated by a * for $P<0.05$.

Table 6.2

	Concentration uM	N	PSS	Response in Theophylline 1000uM	PSS containing 8PT 1uM	8PT 10uM	8psPT 10uM
Change in d.c. potential (uV)							
		4		244 ± 42*			
		6			0 ± 0		
		13				6 ± 6	
		4					10 ± 10
Response (uV) to							
Muscarine	0.1	4	128 ± 24	73 ± 11*			
"	0.1	6	304 ± 38		320 ± 49		
"	0.1	13	310 ± 48			316 ± 43	
Adenosine	10	6	-37 ± 7		-7 ± 4*		
"	100	4	-60 ± 11	-38 ± 9			
"	100	9	-53 ± 10			-18 ± 4*	
"	30	2	-90, -30				0, 0
2CA	0.3	3	-27 ± 7			0 ± 0	
ATP	10	6	-18 ± 5			-5 ± 3	
ATP	100	5	-36 ± 11			-7 ± 4*	
% Change in response to 100nM muscarine by							
Adenosine	10	6	-26 ± 6		-1 ± 8*		
"	100	9	-27 ± 4			-9 ± 6*	
"	30	2	-29, -25				17, -4
ATP	100	5	-33 ± 8			-22 ± 5*	
2CA	0.3	3	-38 ± 2			-11 ± 5*	

Table 6.3. Comparison of the hyperpolarisation of the rat SCG by various adenosine analogues

Compound	Maximum Response uV	EC_{50} uM	Peak of response uM	Potency relative to CPA (a)
CPA	110 ± 12 (4)	0.12	1	1
2CA	112 ± 26 (16)	0.6	10	0.2
Adenosine	88 ± 11 (18)	10	100	0.012
PAA	17 ± 5 (6)	17	100	0.007

Responses are means ± SEM in uV and N in brackets.

(a) Rations of EC_{50} values relative to CPA = 1

Table 6.4. The effect of adenosine and its analogues on the response of the rat SCG to muscarine

Values for the peak depression and the maximum % depression of the response to 100nM muscarine were obtained from the data presented in figures 6.10, 6.11 and 6.12 (minimum of four ganglia).

Compound	Peak depression nM	Maximum % depression of muscarinic response (mean ± SEM)	Relative efficacy[a]	IC_{50} nM[b]	Potency[c] relative to CPA	Potency[c] relative to R-PIA
CPA	300	39 ± 3	1	20	1	3.5
R-PIA	1000	25 ± 3	0.6	70	0.29	1.0
2CA	300	36 ± 5	0.9	72	0.28	0.97
NECA	1000	28 ± 5	0.7	120	0.17	0.58
S-PIA	3000	21 ± 2	0.5	340	0.06	0.21
BZA	30000	36 ± 2	0.9	10000	0.002	0.007
Adenosine	100000	28 ± 4	0.7	15000	0.001	0.005
PAA	>300000[d]	19 ± 4	0.5	>15000	0.001	0.005

(a) Efficacy was estimated as a ratio of the maximal depression of muscarine by a compound relative to the depression produced by CPA.
(b) The IC_{50} for a given compound was estimated as the concentration of compound which produced half the maximum % depression of muscarine for that compound.
(c) Potency relative to CPA and R-PIA are the reciprocals of the molar potency ratios of IC_{50} values relative to CPA and R-PIA respectively.
(d) Maximal concentration tested due to low solubility.

Table 6.5. Comparative potencies of nucleosides for binding to rat brain membranes and depression of the response of the rat SCG to muscarine

Compound	Binding Ki[a]			Ratios binding		IC_{50} for muscarinic response on rat SCG[b]
	A1	A2a	A2b	A1:A2a	A2a:A2b	
Agonists						
CPA	0.6	462		784		20
CHA	1.3	514	160000	392	311	
R-PIA	1.2	124	150000	106	1210	70
2CA	9	63	20000	7	316	72
NECA	6	10		1.6		120
S-PIA	49	1820	750000	37	413	340
BZA	120	285	90000	2.4	316	10000
PAA	561	119		0.2		>15000[c]
MTA	201	1100	8200	3.9	7.4	

(a) Membrane receptor binding data, reported in Bruns et al. (1986), using whole rat brain minus brainstem and cerebellum for A1 and rat striatum for A2 adenosine receptors.
(b) The IC_{50} for the depression of muscarine was calculated from plots of the concentration response curves of compounds at the half maximal effect for that compound.
(c) Maximum concentration tested due to low solubility was 100uM.
All concentrations are in nanomolar

Table 6.6. The effect of pentobarbitone on the response of the rat SCG to gamma-aminobutyric acid and adenosine.

The response of three different groups of ganglia to a one minute application of gamma-aminobutyric acid (GABA) and a two minute application of adenosine in different physiological salt solutions (PSS) in the absence and presence (minimum 20 minutes incubation) of pentobarbitone (PB). Pentobarbitone significantly (paired t-test; *P<0.05) enhanced the response to GABA in 0mM Ca^{2+}/EGTA PSS (a) and low K^{+}/Ca^{2+} PSS (b) but did not significantly alter the after hyperpolarisation to GABA or the hyperpolarisation to adenosine.

Agonist		Physiological salt solution				
		CONTROL			TEST (PSS + PT, 100uM)	
	N	Normal	0mM Ca^{2+}/EGTA	2mM K^{+}/0.1mM Ca^{2+}	Normal +	0mM Ca^{2+}/EGTA	2mM K^{+}/0.1mM Ca^{2+}
GABA 10uM, 1 minute	2	280, 760			740, 1440		
Depolarisation	4	290 ± 120	126 ± 46[a]			470 ± 118*[a]	
	4			160 ± 70[b]			398 ± 132*[b]
GABA 10uM, 1 minute	4	-14 ± 14	-30 ± 27			-55 ± 40	
After hyperpolarisation	4			-50 ± 24			-89 ± 45
Adenosine 100uM, 2 minutes	2	-50, -30			-40, -40		
	4	-48 ± 9	-83 ± 9			-69 ± 5	
	4			-230 ± 40			-220 ± 49

Table 6.7. The effect of 3,7-dimethyl-1-propargylxanthine on the depression of the response of the rat SCG to muscarine by cyclopentyladenosine and the response to adenosine, cyclopentyladenosine and phenylaminoadenosine

(A) % Depression of response to 100nM muscarine by 100nM cyclopentyl adenosine (CPA) in normal physiological salt solution (PSS) and PSS containing 3,7-dimethyl-1-propargylxanthine (DMPX). The ability of CPA to depress the response to muscarine in PSS was repeated in PSS (on three ganglia) or PSS + DMPX (on five ganglia) after a minimum of 60 minutes between applications of CPA i.e. 1st and 2nd control responses or 1st control and 2nd response in DMPX respectively (N = number of ganglia tested).

(B) Response of rat SCG to two minute applications of adenosine, CPA and phenylaminoadenosine (PAA) in the absence (control) or presence of DMPX. There was no statistically significant difference between responses (uV $\pm$ SEM) obtained in the absence or presence of DMPX (paired t-test on three ganglia). The values in brackets refer to the responses obtained from the "time control" ganglion.

Table 6.7

(A)

N	% Depression of response to muscarine by CPA		
	PS		PSS + DMPX (10uM)
	1st response	2nd response	
3	28 ± 3	27 ± 5	
5	27 ± 4		29 ± 5

(B)

AGONIST	Concentration uM	CONTROL PSS (2mM K^+/0.1mM Ca^{2+})	PSS + DMPX (10uM)
Adenosine	10	-198 ± 7 (-140, -180)	-168 ± 17 (-140)
Adenosine	100	-287 ± 15 (-360)	-308 ± 91 (-330)
CPA	0.1	-272 ± 64 (-300)	-262 ± 70 (-310)
PAA	10	-47 ± 17 (-110, -65)	-33 ± 15 (-40)
PAA	100	-112 ± 20 (90)	-83 ± 24 (80)

CHAPTER SEVEN

GENERAL DISCUSSION

CHAPTER SEVEN: GENERAL DISCUSSION

The main aims of this thesis were to characterise the adenosine-induced hyperpolarisation that can be recorded extracellularly from the rat isolated SCG; to study the ionic mechanisms responsible for these effects; to examine any interaction between adenosine and various neurotransmitters and to identify the receptors involved in the actions of adenosine.

The results presented in chapter 3 show that adenosine and its analogues hyperpolarised the SCG in a concentration-dependent manner and that a system for the uptake and deamination of adenosine exists. This uptake system may reflect a requirement for the uptake of purines that are not synthesised by the ganglion and/or represents an efficient method for the removal of circulating adenosine. The effects of adenosine are attributable to a direct action on the ganglion as purines were weak or inactive on isolated nerves (CST) and unaltered in conditions known to inhibit neurotransmitter release.

It has been reported that adenosine altered Ca^{2+}-dependent potentials of the rat SCG (Henon & McAfee, 1983a,b), however no evidence was found to support a role of Ca^{2+} channels in the actions of adenosine reported in this thesis. The response to adenosine was resistant to antagonism of Na^{+}, Ca^{2+} and Cl^{-} conductances, but was sensitive to changes in extracellular K^{+} concentration indicating the response to adenosine is likely to be due to an increase in gK (chapter 4). The pharmacological profile of the adenosine-induced hyperpolarisation was distinct from those gK described for the effects of adenosine on other neurones. The response to adenosine was not antagonised byt the following pharmacological agents: 4AP which antagonises I_A and I_D, high

concentrations of TEA, which antagonises I_r, I_C and Im; apamin and dTc which antagonise I_{ahp} and I_C respectively. Adenosine-induced hyperpolarisations were enhanced in the presence of Ba^{2+}, high concentrations of TEA and in the presence of muscarinic agonists, suggesting the effects of adenosine are mediated via an interaction with Im. In support of this hypothesis the data presented in chapter 5 show that adenosine selectively depressed the response to muscarine but not the response to other agonists which mediate their effects via ionic conductances distinct from Im.

The depression of muscarinic responses by adenosine was not replicated by the pyrimidine UTP or alpha,beta-MeATP, both of which depolarised the ganglion, suggesting the SCG contains receptors for these compounds and the depression of muscarinic responses is selective for adenine or adenine nucleotides.

It is unlikely that the depression of muscarinic responses occurred as a result of a functional antagonism by adenosine as agonists that produced equivalent sized hyperpolarisations compared to adenosine e.g. (-) baclofen, beta,gamma-MeATP, R- and S-PIA were not equipotent at depressing the response to muscarine. Also ATP was weak or ineffective at hyperpolarisating the ganglion but produced equivalent reductions of the response to muscarine compared to adenosine. It would be desirable to confirm that the depression of muscarinic responses in individual ganglionic neurones was independet of the degree of hyperpolarisation of these neurones. Intracellular recording techniques were only available for a short period during the course of the experimental work for this thesis and when intracellular recordings were made by a similar method to that of Henon & McAfee (1983a). The actions of adenosine were inconsistent as

reported by Henon & McAfee (1983a). A similar complication was reported by Smith & Zidichouski (1985) who found adrenaline produced small and inconsistent responses when recorded intracellularly from bullfrog paravertebral ganglia. It was therefore not feasible to confirm the grease gap results using this technique. A more suitable technique such as whole cell patch clamping may provide both conformation and additional information about the results reported here.

The level at which the interaction between adenosine and muscarine occurs is somewhat speculative. One general mechanism that may apply to these studies is the interaction of adenosine and muscarinic receptors with a common population of K^+ channels via GTP-binding proteins (Trussel & Jackson, 1989; Kurachi, Na Kajima & Sugimoto, 1986) (Fig. 7). In contrast to the proposed action of adenosine on the hippocampus (Trussel & Jackson, 1989), the effect of adenosine on sympathetic ganglia may be due to the activation of an outward K^+. The mechanism of interaction proposed is shown in figure 7, where the activation of M1-receptors by ACh or muscarine leads to the closure of M-channels and depolarisation of the ganglion. The activation of A1-adenosine receptors could then open these channels to antagonise the effects of muscarine. It seems unlikely that a direct effect via a common pool of G-protein subtypes occurs as the effects of muscarine and adenosine are PTX insensitive and sensitive respectively. Perhaps more likely is a modification of the intracellular secondary messenger pathways.

To date the biochemical mechanism of muscarine on the rat SCG and the hippocampus are poorly understood (Duttar & Nicholl, 1988; Brown, 1989; Marrion et al., 1990) although there is good evidence to suggest a lack of involvement of cyclic nucleotides. Likewise there are several lines of

evidence to suggest the effects of adenosine on the rat SCG are not mediated by cyclic nucleotide levels, including the inability of adenosine to reduce the response to agonists known to increase the cAMP content of the ganglion (chapter 5). Muscarinic agonists have been shown to activate PIT and in interaction with this intracellular system and the ability of a phorbol ester to enhance the response to adenosine supports a role of PKC in the actions of adenosine.

It is possible that either branch of the inositol phospholipid signal transduction pathway may be involved in the modulation of the muscarinic depolarisation via DAG production and subsequent activation of PKC or via an IP_3 metabolite and/or an action on PLA_2. There was some evidence to suggest arachidonic acid production was involved in the response to adenosine although it remains to be determined whether the depressant effects of adenosine are due to the stimulation of arachidonic acid formation or via another mechanism.

The effects of adenosine and its derivatives were inhibited by adenosine receptor antagonists including, 8PT, DPCPX and XAC (chapter 6) and the response to adenosine was enhanced by dipyridamole (chapter 3) suggesting an action via external adenosine receptors. The similar potency order for the depression of muscarinic responses and the hyperpolarisation to adenosine and its analogues indicates that a common population of adenosine receptors was involved in both these responses.

The high potency and apparent pA_2 values for DPCPX and XAC as antagonists of the effects of adenosine strongly suggest the presence of A1-adenosine receptors on the rat SCG. The apparent non-competitive antagonism of the effects of adenosine may arise from the biphasic nature of

the concentration-response curves. Some support for theis hypothesis is provided by the inability of DMPX, a selective A2-receptor antagonist to alter the concentration response curve to adenosine. Alternatively the adenosine receptors of the rat SCG may be different to those reported for the CNS, i.e. an A1-adenosine receptor subtype. Wiklund, Wiklund & Gustafsson (1989) have reported that the A1-adenosine receptors of the autonomic nervous system may be different from CNS neurone receptors. Preliminary findings suggested CV 1674 (2-(4-methoxyphenyladenosine) is an agonist at CNS but not autonomic nerve adenosine receptors and the latter receptors have a lower affinity for some antagonists. As CV 1674 was unavailable the possibility of Ala receptors on the rat SCG cannot be discounted. However, the high potency of DPCPX and the high correlation between CNS A1-receptor binding and the inhibition of muscarinicic responses suggests the A1 receptors are similar, if not identical to those of the CNS.

General implications and physiological role of adenosine on the rat SCG

In both human (Valli, Zuccia, Seghezzi, Bonifati & Botta, 1985), rabbit (Volle, 1969; Brimble & Wallis, 1974) and rat superior cervical ganglia (Brown, 1983) a significant proportion of the response to stimulation of the ganglion is mediated via muscarinic transmission, which regulates neuronal discharge patterns over prolonged periods by closing M-channels. The opening of K^+ channels by adenosine would provide a suitable mechanism for hyperpolarising the cell by a small amount to decrease the rate of spike discharge. Thus the main effect of adenosine might be a modification of the discharge-frequency characteristics, which would be predicted to be particularly significant under conditions where Im is

suppressed by muscarinic agonists or peptides. Factors such as the frequency of stimulation of the ganglion may influence the effect of adenosine on the sensitivity of the postsynaptic nerves (Cacigli et al., 1985; Henon & McAfee, 1985) and in vivo the effects of adenosine would be dependent upon a complex interaction at both pre- and postsynaptic receptors. Circulating or neuronally released adenosine may serve to inhibit excessive activation of muscarinic receptors.

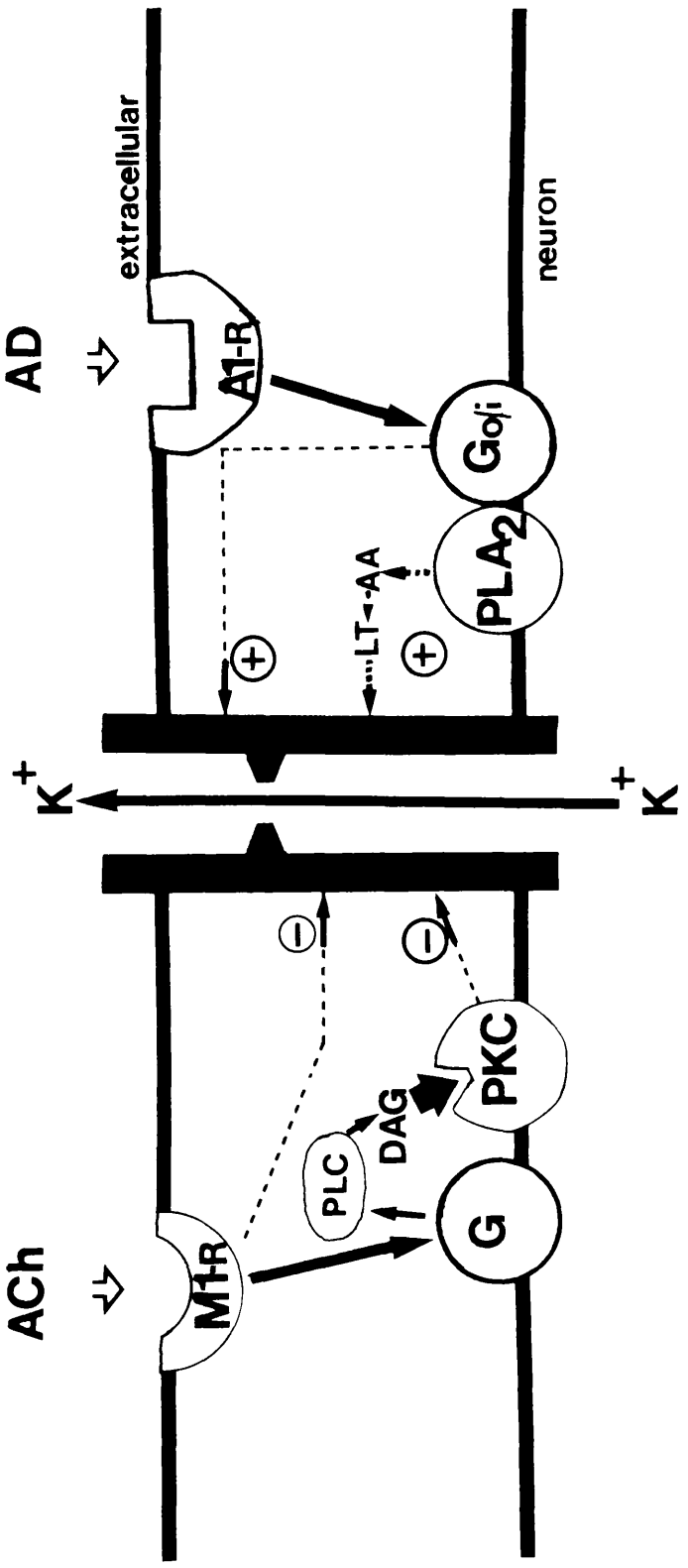
Conclusions

Adenosine is now wide regarded as a modulator of neuronal activity in a variety of tissues and the rat isolated SCG has provided a useful and reasonable model for the study of the actions of adenosine on the peripheral nervous system. There was a significant and selective interaction of adenosine with postsynaptic muscarinic depolarisations which may represent a general phenomenon occuring in a variety of tissues including the CNS, where muscarinic transmission rather than nicotinic transmission predominates excitation of CNS neurones by ACh. However, further experimentation will be required before the physiological implications of this process become clear.

Fig. 7. Schematic diagram of the purinergic and muscarinic activation of a K^+ channel in the rat SCG neuronal membrane. Acetylcholine (ACh) activates a muscarinic receptors (M1-R) to stimulate the formation of diacylglycerol (DAG) via a G-protein (G) and phospholipase C(PLC) to activate protein kinase-C (PKC) which closes the K^+ channel. Adenosine (AD) activates an adenosine 1-receptor subtype (A1-R) to open the K^+ channel via a pertussis toxin sensitive mechanism. The activation of a G-protein may stimulate phospholipase A_2 (PLA_2) to produce arachidonic acid (AA) with the subsequent formation of leukotrienes (LT) which may open the K^+ channel.

This scheme does not represent any quantitative relationships between the various components shown and their exact location within the cell membrane. Solid lines represent pathways known to be involved in the response to ACh and adenosine, whereas the dotted lines represent putative mechanisms.

⊕ = activation; ⊖ = inhibition


extracellular
neuron
AD
A1-R
Gofi
PLA2
LT-AA
K+
K+
ACh
M1R
PLC
DAG
PKC
G

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ADDENDUM CHAPTER FIVE:

INTERACTION OF ADENOSINE WITH DIFFERENT AGONISTS

For figures and tables see pages 199 to 228.

Chapter 5: INTERACTION OF ADENOSINE WITH DIFFERENT AGONISTS.

INTRODUCTION.

The results of the preceding chapters have provided some evidence for the ability of adenosine to depress the response of the rat SCG to muscarine. In this chapter the selectivity of adenosine for muscarinic agonists and the potential intracellular messengers involved in these responses are examined.

Over the last decade it has been reported that adenosine can modulate the activity of numerous tissues including the sensitivity of smooth muscle to noradrenaline (NA) and neuronal tissue to ACh (Table 5.12) (Akasu et al., 1983a; Stone, 1989). A large number of substances have been reported to have a neuromodulatory or neurotransmitter function in the rat SCG, including :- ACh (Birks & MacIntosh, 1961), catecholamines (Brown & Caulfield, 1979), 5HT (Wallis, Williams & Wali, 1978), GABA (Wolff, Joo, Kasa, Storm-Mathiesen, Toldi & Blacar, 1986; Eugene, 1987), and vasoactive-intestinal polypeptide (VIP)(Volle & Patterson, 1982; Dorroux, Barberis & Jard, 1987). The ability of these agents to activate different ionic and biochemical mechanisms has therefore been used here to aid the identification of the mechanism of action of adenosine on the rat SCG.

The results described in chapters 3 & 4 suggest that adenosine depressed the response to muscarine but the relationship been the degree of depolarisation and the depression by adenosine, or the selectivity of adenosine for the muscarinic depolarisation versus muscarinic hyperpolarisation was not examined (see Chapter 1 for discussion of M1 mediated depolarisation & M2 mediated hyperpolarisation, also Brown et al., 1980; Newberry et al, 1987). To examine the first question the effects of a $GABA_B$ agonist, (-) baclofen (BAC) were compared to the effects of adenosine. The rationale for these experiments is provided by the reports that baclofen produces similar effects to adenosine in

a number of tissues, e.g. both compounds decrease synaptic excitability of isolated hippocampal cells (Newberry & Nicholl, 1985) and baclofen hyperpolarises the rat SCG (see Results, Figs 5.4 & 5.5). More recently this latter finding was confirmed by Newberry & Gilbert, 1989).

To address the question of whether adenosine modulates both the M1 and M2 mediated responses of muscarinic agonists, further experiments were performed to study the ability of adenosine to depress the depolarisation or hyperpolarisation produced by muscarinic agonists (Results 5.3, 5.5. & 5.6). As there are no M2 receptor selective agonists the ability of adenosine to alter the M2 receptor mediated response to muscarinic agonists was assessed in two sets of experiments. In the first series the depression of the response to muscarinic agonists was measured in the presence of a an M2 muscarinic receptor antagonist, methoctramine (MTO). In a second series of experiments the ability of adenosine to alter the hyperpolarisation to carbachol in low Ca^{2+} PSS containing an M1 receptor antagonist, pirenzepine (PIR), was determined.

It is of interest that the hyperpolarisation to adenosine was attributed to an increase in gK (Chapter 4), whereas the muscarinic depolarisation of the rat SCG is reported to occur via a decrease in gK (see introduction Chapter 1; Brown & Constanti, 1980; Fig 5.12). Given these findings it would be predicted that adenosine would depress the response to muscarine as described in Chapters 3 & 4, and in addition the adenosine-induced hyperpolarisation of the ganglion would be enhanced during a muscarinic depolarisation. To test the latter hypothesis the response of the rat SCG to adenosine in the presence of muscarinic and non-muscarinic agonists was determined (see Results 5.7).

An interaction of purines with postsynaptic responses to ACh and cholinergic agents has been reported by several research groups although both the receptors and the mechanism of action are

unknown or poorly characterised (Table 5.12). To determine if the interaction of adenosine and muscarine occurs due to an effect on Im, the interaction of adenosine on compounds reported to inhibit Im, including muscarine (Brown & Adams, 1980; Constanti & Brown, 1981), LHRH (Adams & Brown, 1980; Adams et al., 1982a; Jones, 1987), a substance P analogue, ERP (Adams, Brown & Jones, 1983) and UTP (Adams et al., 1982a) was determined.

An increasing number of intracellular messenger, including cyclic nucleotides, PIT and arachidonic acid (AA) are believed to mediate the effects of adenosine. The two most frequently proposed transduction systems that may explain the actions of adenosine are the adenylate cyclase (AC)/cAMP system and the PI/PKC system. In 1976, Greengard proposed that cyclic nucleotides have a major role in postsynaptic transmission of sympathetic ganglia, and that cAMP and cGMP exert long term control of neuronal excitability (McAfee & Greengard, 1972). Thus in an analogous manner to that found in rat or guinea-pig brain the action of adenosine on the rat SCG may occur due to a change in cyclic nucleotides. It is possible that adenosine and/or muscarine alter ganglionic cAMP levels via an effect on adenylate cyclase (Dunwiddie & Fredholm, 1984; Yeager, Nelson & Storm, 1986; Donaldson, Brown & Hill, 1988) and/or a change in PDE activity (Smellie, Davis, Daly & Wells, 1979; De Manzancourt & Giudicelli, 1984) and each of these hypotheses was examined (Results 5.9). The possibility that the actions of adenosine are mediated via an interaction with PIT, PKC and AA was also examined (Results 5.8 & 5.10).

METHODS.

Ganglia were isolated and recorded as described in chapter 2 (methods) and perfused with PSS. CRCs to agonists were constructed with one minute applications and a minimum of 15 minutes between applications. Once the responses to agonists were reproducible the ability of adenosine to alter the response to an agonist was assessed by measuring the peak response to the

agonist applied before (pre-test), during (test) and after (post-test) incubation with adenosine or (-) baclofen, and the change in the agonist response was expressed as a percentage of the control response.

The ability of adenosine to alter the hyperpolarisation to carbachol was determined as for the effect of adenosine on the response to muscarinic agonists except that hyperpolarisations to carbachol were obtained by perfusing ganglia in PSS containing 0.1 mM Ca^{2+} and 0.3 uM pirenzepine (PIR).

The effect of MTO on the responses to muscarinic agonists, adenosine and the depression of muscarinic agonists by adenosine was determined in PSS as described above. Ganglia were incubated in 0.3 uM MTO for a minimum of an hour before reassessing both the response to muscarinic agonists and their interaction with adenosine.

The role of cyclic nucleotide metabolism in the response of the rat SCG to adenosine was assessed using selective inhibitors of the breakdown of cAMP and cGMP i.e. the cAMP PDE inhibitors (PDEI), 4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) and denbufylline (BRL 30892), and a cGMP PDEI, M&B 22,948. The sites of action of these agents are shown in Fig 5.11. In addition the depression of muscarinic responses by CPA was tested in the presence of an adenylate cyclase inhibitor SQ 22,536 or Ro 20-1724.

The effect of indomethacin (INDO) and nordihydroguaiaretic acid (NDGA) were assessed by first constructing CRCs to 2 minute applications of adenosine every 20 minutes in the absence and presence of indomethacin (50 uM), followed by a CRC to adenosine in the presence of freshly prepared indomethacin (50 uM) and NDGA (10 uM) in PSS.

RESULTS.

5.1 Concentration response curves to agonists.

The response to increasing concentrations of ACh, muscarine, 2-methyl-5-hydroxytryptamine (2Me5HT), VIP and oxotremorine-M (OXO-M) are summarised in figure 5.1.

High concentrations of ACh were required to evoke a response of the ganglion, with muscarine being over 1,000 fold more potent than ACh on the same ganglia. To avoid using high concentrations of ACh and selectively activate muscarinic receptors, AChE resistant analogues of ACh including muscarine (Fig 5.1), carbachol (CARB) (Fig 5.2h) and methylfurmethide (MeF) (Fig 5.2i) were used.

The depolarisation to 100 nM muscarine was abolished by incubation with either N-Methylatropine or pirenzepine (Table 5.1), whereas the response to adenosine was unaltered by pirenzepine (Table 5.1).

5.2 Effect of adenosine on the response to non-muscarinic agonists.

The ability of adenosine to alter the response to submaximal responses of different agonists producing about equivalent sized responses is summarised in tables 5.2 and 5.3.

Potassium produced concentration dependent depolarisations which at 8 and 12 mM were not significantly altered by the presence of 100 uM adenosine (Fig 5.2a, Table 5.3).

GABA produced concentration dependent depolarisations followed by AHPs which were easily observed at 100 uM (see Figs 4.11 & 4.12). Adenosine at 10 uM did not alter the response of up to 10 uM GABA (Table 5.2). In the presence of 100 uM adenosine the AHP in response to 100 uM GABA was not significantly altered whereas depolarisations to 1 and 10 uM GABA were significantly enhanced (Table 5.3).

DMPP produced concentration related depolarisations and AHPs (Fig 5.2c) both of which were unaltered by the presence of 30 uM or 100 uM adenosine (Fig 5.2, Tables 5.2 & 5.3). Likewise the 'fast' nicotinic depolarisation produced by carbachol at 10 and 30 uM was unaltered by the presence of 100 uM adenosine (Table 5.4).

The depolarisation to 10 uM 5HT was unaltered in the presence of 10 uM adenosine and significantly enhanced by 100 uM adenosine (Tables 5.2 & 5.3). 2Me5HT produced concentration-dependent depolarisations (Fig 5.1) which were broader than responses to 5HT (Fig 5.2 cf d & e). The response to 30 uM 2Me5HT was similar in shape and size to that produced by 100 nM muscarine (Fig 5.2 cf e & g) but in contrast to muscarine the response to 2Me5HT was unaltered by 100 uM adenosine (Fig 5.2 cf e & g, Table 5.3).

Isoprenaline (ISO) at 100 nM produced a response similar to the response observed for 100 nM muscarine (Fig 5.2 f & g and Tables 5.3 & 5.4). The response to 100 nM isoprenaline was significantly reduced by 10 uM adenosine (Table 5.2) but not by 100 uM adenosine (Table 5.3).

5.3 Effect of adenosine on the response to muscarinic agonists.

The results presented in tables 5.2 & 5.3 and Fig 5.2 g-i, show adenosine depressed the responses to muscarinic agonists. The response to 100 nM muscarine was significantly reduced by 10 and 100 uM adenosine in a concentration dependent manner (Figs 5.3, 5.4 & 5.6, Tables 5.2 & 5.3). Both the depression of the response to 100 nM muscarine by 100 uM adenosine (Fig. 5.4a) and the depression of increasing concentrations of muscarine by adenosine (Fig. 5.4b) were biphasic and maximal at about 100 nM muscarine and 100 uM adenosine declining at higher concentrations of muscarine or adenosine.

The ability of adenosine to depress the response to muscarinic

agonists was dependent both on the agonist and the level of depolarisation (Tables 5.2 & 5.3; Fig 5.2 g-i). At 10 uM adenosine there was no selectivity for carbachol, muscarine or MeF but at 100 uM adenosine the depression of the response to muscarine was about twice as great as that for adenosine on carbachol or MeF (Table 5.4). Adenosine at 100 uM did not significantly alter the response to carbachol and in contrast to muscarine and MeF the depression of the agonist response showed considerably greater variability.

5.4 The effect of (-) baclofen on the response to muscarine.

(-)BAC produced concentration dependent hyperpolarisations and at 10 and 30 uM significantly reduced the response to 100 nM muscarine (Fig 5.5). The response to muscarine was reduced by 10 uM (-)BAC by 11 ± 3 % (response to muscarine = 366 ± 69 uV, response to muscarine in (-)BAC 10 uM = 331 ± 65 uV, $P < 0.05$, n=7).

5.5 Effect of adenosine on the hyperpolarisation to carbachol.

Muscarinic agonists in the presence of PIR in low Ca^{2+} PSS produced concentration related hyperpolarisations (Figs 5.8, 5.9). Carbachol in low Ca^{2+} PSS containing PIR produced concentration dependent hyperpolarisations (Fig 5.9) and at 10 uM produced a hyperpolarisation followed by a fast depolarisation and a slower depolarisation. Both the hyperpolarisation and the slow depolarisation were abolished in 2 uM atropine (N=2), and the rapid depolarisation was reduced by dihydrobetaerythroidine (DHBE). In addition to reducing the fast component of the carbachol response, DHBE also reduced the hyperpolarisation to carbachol. Thus to avoid the use of DHBE the effect of adenosine on the response to carbachol was assessed in low Ca^{2+} PSS containing pirenzepine (Table 5.6). The ability of adenosine to selectively reduce the depolarising response but not

the hyperpolarisation to carbachol is illustrated in Fig 5.9c.

5.6 Effect of adenosine on the response to muscarinic agonists in the presence of MTO.

The response to MeF and muscarine were significantly reduced, in the presence of 0.3 uM MTO and the responses to carbachol and adenosine were not significantly altered (Table 5.5). MTO produced a significant increase in the depression of responses to carbachol in MTO (Table 5.5).

5.7 Effect of depolarisation with different agonists on the response to adenosine.

5.7.1 Effect of depolarisation with potassium on the response to adenosine.

The continuous application of 2 mM potassium produced a sustained depolarisation which did not significantly alter the response to 100 uM adenosine (Table 5.7).

5.7.2 Effect of depolarisation with nicotine on the response to adenosine.

Nicotine produced concentration dependent depolarisations of the ganglia (Fig 5.7 & Table 5.7). The response to adenosine when applied during the depolarisation to 100 nM nicotine was significantly depressed compared to controls (Fig 5.7 & Table 5.7) but was not significantly altered during the depolarisation to 1 or 10 uM nicotine (Table 5.7).

5.7.3. Effect of adenosine on the response to agonists reported to inhibit the M-current.

5.7.3.1 Barium (Ba^{2+})

The depolarisation to 1 mM barium was of a similar magnitude to that produced by 100 nM muscarine, and was significantly reduced in the presence of 100 uM adenosine (Table 5.3). The application of Ba^{2+} at 3 mM for 1 minute to depolarise the ganglion significantly enhanced the subsequent response to 100 uM adenosine during the depolarisation to Ba^{2+}, whereas the continuous application of 2.5 mM Ba^{2+} did not alter the response to adenosine (Table 5.8).

5.7.3.2 Muscarinic agonists.

Pilocarpine (Fig 5.14), BM5 (Table 5.8), muscarine (Figs 5.13 & 5.14) significantly enhanced the hyperpolarisation to adenosine (Table 5.8). The continuous application of 100 nM muscarine produced a persistent depolarisation and an enhancement of the hyperpolarisation to adenosine (Figs 5.13 & 5.14; Table 5.8).

5.7.3.3 LHRH, UTP and ERP.

LHRH and UTP produced small but significant depolarisations of the ganglia but did not significantly alter the response to 100 uM adenosine (Table 5.8). ERP depolarised ganglia and significantly enhanced the response to adenosine (Table 5.8).

5.8 Effect of an activator and inhibitor of PKC.

5.8.1 Response to adenosine during depolarisation by PDBu.

PDBu produced concentration dependent sustained depolarisation of ganglia, with responses to 20 nM and 200 nM beginning within 1 to 2 minutes of application. Responses developed slowly over 15 to 20 minutes and took 1 to 2 hours to washout. At 200 nM

PDBu, depolarised ganglia to a similar degree to 100 nM muscarine (Table 5.7, Fig 5.14) and the response to adenosine was potentiated when applied during the response to PDBu (Fig 5.14, Table 5.8).

5.8.2 The effect of H7 on the response to adenosine, carbachol, muscarine, PDBu and the depression of the response to muscarine by adenosine.

In low Ca^{2+} and PIR containing PSS, 100 uM adenosine and 300 nM carbachol produced similar sized hyperpolarisations which were unaffected by incubation in PSS containing 50 uM H7 (Table 5.10).

Incubation of ganglia in PSS containing H7 did not alter the response to muscarine, PDBu, adenosine or the depression of the response to muscarine by adenosine (Table 5.11).

5.9 Effect of inhibitors of cGMP and cAMP phosphodiesterases and adenylate cyclase on the response to purines, muscarine and depression of the muscarinic response.

Denbufylline at 1 uM did not significantly alter the response to adenosine, muscarine or the depression of the response to muscarine by adenosine (Table 5.9). Denbufylline at 10 uM did not alter either the response to muscarine (100 nM) or it's depression by adenosine, and significantly reduced the hyperpolarisation to adenosine (Table 5.9). At 100 uM denbufylline significantly reduced the responses to adenosine and muscarine and reduced the depression of the response to muscarine by adenosine (Table 5.9).

Ro 20-1724, denbufylline and SQ 22,536 were either ineffective or reduced (denbufylline at 100 uM) the depolarisation of the rat SCG to muscarine (Table 5.9). Ro 20-1724 significantly reduced the response to adenosine (Fig 5.10; Table 5.9) and reduced the

response to CPA but not the response to muscarine or the depression of the response to muscarine by adenosine (Table 5.9). Ro 20-1724 caused an apparently non-parallel antagonism of the CRC to adenosine (cf Figs. 5.10 and 6.20).

SQ 22,536 did not significantly alter the response to muscarine or the depression of the response to muscarine by CPA (Table 5.9).

5.10 Effect of agents that alter AA metabolism.

At 50 uM indomethacin produced a small rightward shift in the CRC for the hyperpolarisation to adenosine (Fig 5.16). In the presence of indomethacin (50 uM) and NDGA (10 uM) responses to adenosine were displaced further to the right and significantly reduced at 10 and 100 uM adenosine (Fig 5.16).

DISCUSSION.

Effect of adenosine on the response to depolarising agonists.

The depression of muscarinic responses by adenosine was agonist dependent and similar depolarisations by different agonists were not depressed to a comparable extent by adenosine (Fig. 5.2a-i; Tables 5.3 and 5.4). The ability of adenosine to modulate the response to some agonists but not others allows inferences to be made as to how adenosine may function during ganglionic transmission.

Interaction with GABA.

One means by which adenosine might interfere with transmission is to modify GABA activity. Both the presence of GABA and the ability of GABA to depolarise ganglia (see Bowery & Brown, 1974; Figs 4.11 & 4.12) together with the existence of a fast non-cholinergic postsynaptic potentials which resemble the effects of GABA (Eugene, 1987) is evidence in favour of functional GABA-

ergic transmission in the rat SCG. However the function of GABA receptors on SCG cells remains unclear and it has been reported that GABA had only slight effects on ganglionic transmission (Adams & Brown, 1975).

The results presented here suggest a small but significant potentiation of low concentrations of GABA by adenosine (Tables 5.2 & 5.3), an effect that would be consistent with an increase in efflux of Cl^- and /or K^+ (see Chapter 4.6, also Ballanyi et al., 1984; Ballanyi & Grafe, 1985; Adams & Brown, 1975). The results presented in chapter 4.6 showed that the response to adenosine was unaltered in low Cl^- PSS, a condition which would be expected to enhance efflux of Cl^-, indicating the potentiation of the response to GABA by adenosine is more likely to have arisen from increased K^+ efflux. This hypothesis is consistent with the potentiation of the response to adenosine in low K^+ PSS (see Chapter 4.8.1).

Interaction with 5HT.

A second potential site of interaction of adenosine in vivo is with tryptaminergic transmission. 5HT exerts excitatory actions on sympathetic ganglia and depolarisation by 5HT is reported to facilitate synaptic transmission (Eccles & Libet, 1961). The fast depolarisation of the rat SCG to 5HT is believed to be mediated by $5HT_3$ receptors (Fourtune, Ireland & Tyres, 1985), and the AHP is mediated via the activation of the electrogenic Na^+ pump (Wallis & Woodward, 1975). In addition to these responses, 5HT also hyperpolarises the rat SCG due to an action mediated by $5HT_1$ receptors (Ireland, 1987; Ireland & Jordan, 1987; Gilbert & Newberry, 1987) and accumulates 5HT via an avid uptake system (Ireland, Staughan & Tyers, 1987). The ability of adenosine to alter the response to 5HT may thus be complicated by these factors. Using a submaximal concentration of 5HT only a depolarisation was observed (Fig 5.2d), which was significantly enhanced by 100 uM adenosine. The reason for this potentiation is unknown, but is unlikely to have been due to a potentiation

of the $5HT_3$ mediated depolarisation as the response to a selective $5HT_3$ agonist, 2Me5HT was unaltered by the presence of 100 uM adenosine (cf Figs 5.2 d & e, Table 5.3). Results presented in chapters 4 and 5 suggest adenosine has no effect on responses mediated via activation of the electrogenic Na^+ pump and it is most unlikely that adenosine enhanced the response to 5HT by interacting with the electrogenic Na^+ pump.

Interaction with acetylcholine.

Interaction with nicotinic receptors.

A third method by which adenosine could alter sympathetic ganglionic transmission in vivo is to modulate the response to cholinergic receptor activation (see Table 5.12) and in particular the response to nicotinic receptor stimulation (Akasu et al, 1981; 1983a,1985) and hence the pressor responses to nicotinic agonists (see von Borstel, Evoniuk and Wurtman, 1984; 1986).

It was not possible to directly determine the effect of adenosine on the response of the SCG to ACh due to it's low potency (Fig 5.1) and non-selectivity at higher concentrations that depolarised ganglia. The low potency of ACh most probably arose from the presence of acetylcholinesterase (AChE) within the ganglion and is responsible for the destruction of both liberated (Koelle & Koelle, 1959) and spontaneously released ACh in the rat SCG (Briggs, McAfee & McCaman, 1988).

Adenosine did not alter depolarisations to a selective nicotinic agonist, DMPP (Tables 5.2 & 5.3 and Fig 5.2c) or the 'fast' nicotinic depolarisation produced by carbachol (Table 5.4). These findings are consistent with the lack of effect of adenosine on the sensitivity of the nicotinic receptor reported by Akasu et al (1981; 1983a; 1985) using bullfrog sympathetic ganglia.

The results presented here are in contrast to those obtained by

von Borstel et al., (1984,1986) who reported that adenosine potentiated the pressor response to nicotinic agonists invivo. The order of potency for the potentiation of the response to nicotine was N-cyclopropylcarboxamidoadenosine (CPCA) > 2CA > R-PIA > S-PIA with an R/S ratio of about 10 (von Borstel et al., 1986) suggesting an action via A2 receptors. In partial agreement with von Borstel et al., (1984,1986), Henon & McAfee (1983b) found 2CA reduced single EPSP but facilitated ganglionic transmission during repetitive stimulation (see Henon & McAfee, 1983b). The reduction in the single EPSP amplitude occurred without a change in the sensitivity of postganglionic neurones to bath applied carbachol (0.5 uM), suggesting a presynaptic site of action for adenosine. The mechanism of these effects was not discussed but it is reasonable to suggest that the inhibition of a Ca^{2+} activated K^{+} current by adenosine (see Henon & McAfee, 1983a) could increase repetitive firing to facilitate synaptic transmission while presynaptic inhibition of ACh release by adenosine (Briggs et al., 1988) would reduce the effect of a single CAP.

Thus nicotine and adenosine may interact at several loci to modify autonomic activity and the effect of adenosine on ganglia and the nature of the effects reported by von Borstel et al., (1984, 1986) requires further study. The inability of adenosine to alter the response of the rat SCG to nicotinic depolarisations (see Tables 5.2 to 5.4) suggests the actions of adenosine, reported by von Borstel et al, are more likely to have occurred at the end organs. Alternatively it may be argued that the use of nonphysiological concentrations of adenosine in this study may have inhibited the potentiation of nicotinic responses of the ganglion. There is some evidence in favour of this hypothesis as adenosine at plasma concentrations of 3 to 4 uM potentiated the response to nicotine but at higher concentrations of adenosine the potentiation was diminished (von Borstel et al., 1984). Even if the concentration of adenosine chosen was too high to record potentiation of DMPP it is clear from the results (Tables 5.2 & 5.3) that the major action of adenosine is to depress the

response to muscarinic agonists.

In agreement with the results of Akasu et al., (1981, 1983a, 1985) adenosine did not alter the response to nicotinic agonists nor did nicotinic depolarisation potentiate the response to adenosine (Fig 5.7, Table 5.7).

Furthermore the results presented in chapter 6 indicate the adenosine receptors of the rat SCG are not of the A2 subtype as selective A2 agonists were weak or inactive on the SCG. If the potentiation of nicotinic responses in vivo is mediated via A2 receptors as judged from the data of von Borstel et al., (1986) then it is difficult to understand how adenosine can potentiate the nicotinic responses of the SCG.

Consistent with the inability of ouabain (Table 4.10) and Li-HEPES PSS (Figs 4.13 & 4.14 & Table 4.13) to antagonise the response to adenosine the AHP produced by DMPP was unaltered by up to 100 uM adenosine (Tables 5.3 & 5.4), suggesting adenosine does not hyperpolarise the rat SCG by activating the electrogenic Na^+ pump. The inability of adenosine to alter the depolarisation to nicotinic agonists is also consistent with a lack of effect of low Na^+ PSS on the response to adenosine as carbachol is known to increase the influx of Na^+ ions during nicotinic depolarisation.

Interaction with muscarinic receptors.

ACh, in addition to activating nicotinic receptors, also activates muscarinic receptors and represents another potential modulatory site for adenosine (see Table 5.12 for reported interactions). An examination of the data presented in this thesis shows that by far the greatest interaction between adenosine and depolarising agonists is the depression of the response to muscarine by adenosine (Fig 5.3). Adenosine at 10 uM and 100 uM produced parallel shifts in the CRC to muscarine (Fig 5.6) suggesting adenosine is a competitive antagonist of

muscarine. The correlation between the change in response to muscarine in the presence of adenosine and the size of the hyperpolarisation to adenosine (Fig 5.4a) may be interpreted to suggest adenosine is a functional antagonist of the depolarisation to muscarine. However adenosine would have been expected to antagonise the response to a variety of other agonists to a similar extent and this was not the case (see Tables 5.2 & 5.3). Likewise the smaller depression of muscarinic responses but similar degree of hyperpolarisation of ganglia by (-)baclofen (cf Figs 5.4a & 5.5) suggests the depression of muscarinic responses was not due to functional antagonism. The results with (-) baclofen support the view that there is a specific and selective interaction of muscarine and adenosine.

Further evidence for a selective interaction between adenosine and muscarinic responses is provided by the lack of effect of adenosine on the depolarisation to potassium (Fig 5.2a, Table 5.3) and isoprenaline (Fig 5.2f, Tables 5.3 & 5.4) and the ability of muscarinic agonists to enhance the hyperpolarisation to adenosine (Results 5.7.3.2 & Table 5.8).

It has been shown that 5'-isobutylthio-adenosine (SIBA) and its analogues at 100 uM antagonised ACh-induced contractions of the guinea-pig ileum (Pankaskie, Kachur, Itoh, Gordon & Chiang, 1985). It is reported that SIBA and related agonists can alter the binding of muscarine to muscarinic receptors (Smejkal, Ibrahim, Pankaskie & Chiang, 1989). It seems unlikely that adenosine directly inhibited the binding of muscarine to muscarinic receptors as MTO and pirenzepine did not alter the response of the ganglion to adenosine (Tables 5.1 & 5.5). In addition the Ki for the inhibition of specific binding of ^{3}H-pirenzepine to the rat cerebral cortex by SIBA was 160 uM (Smejkal et al., 1989), a value 16 fold greater than the IC_{50} for the depression of muscarinic responses on the rat SCG.

Opposite effects to those described here have been reported by Worley & Colleagues (Worley, Baraban, McCarren, Snyder & Alger,

1987; Worley, Heller, Snyder & Baraban, 1988). Both phorbol esters and cholinomimetics were shown to prevent the inhibitory effects of adenosine on orthodromically-induced synaptic potentials and the adenosine-induced hyperpolarisation of CA1-pyramidal cells (Worley et al., 1987; 1988). Carbachol was substantially more effective than oxotremorine in blocking the adenosine elicited outward current (Worley et al., 1987), whereas on the rat SCG carbachol was the most resistant to the depressant effects of adenosine. It is interesting to speculate that the stimulatory and inhibitory activity of phorbol esters and cholinomimetics arises from a difference in the role of PKC in the ganglion and hippocampus (Fig. 5.12).

In agreement with the results reported here Brooks & Stone (1988) found that adenosine reduced the activity of cholinomimetics at concentrations that did not alter the actions of excitatory amino acids on the hippocampus. The mechanism of action was not studied but both the potency and relative order of potency of the purines tested i.e. R & S-PIA, NECA and adenosine was similar to that reported for these agonists on the rat SCG (r^2 = 0.95) suggesting a similar mechanism may be responsible for both actions of adenosine. Both of these studies appear to be different from the reports by Worley et al., (1987, 1988) as the actions of adenosine reported here on the rat SCG and on the hippocampus (Brooks & Stone, 1988) were postsynaptic and the concentration of adenosine achieved at the cell surface by Worley et al., (1987, 1988) may be considerably higher than that reported here or in the study by Brooks & Stone (1988).

The effects reported here and by Brooks & Stone (1988) are in sharp contrast to other studies where purines enhanced the nicotinic or muscarinic sensitivity of other preparations (Table 5.12). These results are in partial agreement with studies on the central neurones of Helix (Cox & Walker, 1987) where low concentrations potentiated and higher concentrations depressed the ACh response. The adenosine receptor responsible for the depressant activity of adenosine on Helix neurones may be of the

A3 purinoceptor subtype given the inactivity of NECA and the Ca^{2+} dependent nature of the depression.

The bell-shaped depression of the response of the rat SCG to muscarine by increasing concentrations of purines was observed in both normal and low Ca^{2+} PSS. The application of adenosine at increasing time intervals did not alter the size or shape of the response, suggesting receptor desensitization may not account for the shape of the CRC. A second possibility is that higher concentrations of purines activate a second receptor or another process that antagonises the effects of lower concentrations of adenosine. The latter explanations may also account for the inability of adenosine to abolish the response to muscarine. However other factors such as differences in the coupling or number of receptors for adenosine and muscarine may also account for these phenomena e.g., if there were an appreciable number of spare receptors for muscarinic agonists (Brown et al., 1980) but few if any spare adenosine receptors, as may be indicated by the non-parallel CRC to adenosine in the presence of a competitive A1 antagonist (see Chapter 6).

In addition to muscarine, adenosine depressed the response to carbachol and MeF (Figs 5.2 h-i; Table 5.2) with the response to muscarine being the most susceptible. It is well documented that muscarine can both depolarise and hyperpolarise the ganglion and the effects of adenosine may result from effects on the M1 depolarisation and/or the M2 mediated hyperpolarisation.

Using the same concentration of MTO reported by Field & Newberry (1988) to reduce selectively the hyperpolarising response to muscarine, the response to MeF was significantly reduced (Table 5.5). This result was unexpected as MeF is reported to be a highly selective M1 receptor agonist on the rat SCG and does not hyperpolarise the rat SCG (see Results and Newberry & Connolly, 1989). The results obtained here suggest that MTO antagonises the hyperpolarisation to muscarine but may also produce a small but significant depression of the depolarisation to muscarinic

agonists. In a more recent report published after the completion of this research, the effects of MTO as illustrated by Fig 1 in Field & Newberry (1989) indicate a reduction of the depolarisation to muscarine by MTO. Thus the depression of both M1 and M2 mediated responses by MTO may complicate the interpretation of the results presented here in that the depression of muscarinic agonists by adenosine was found to be dependent on the degree of depolarisation by these agonists (Table 5.4). Even so the inability of MTO to alter the response to adenosine is consistent with the inactivity of pirenzepine on the response to adenosine (Table 5.1) suggesting that the hyperpolarisation to adenosine is independent of presynaptic release of ACh.

To examine if adenosine alters the M2 mediated hyperpolarisation of the ganglion the effect of adenosine on the hyperpolarisation to carbachol was studied. Carbachol was the most potent muscarinic agonist to hyperpolarise the ganglion, whereas MeF did not hyperpolarise the SCG. The potency order for muscarinic agonists to hyperpolarise was:-

	Carbachol	> OXO-M	> muscarine	>> MeF
ca EC_{50} (nM)	>= 210	> 300	> 800	>> 10000

The response to carbachol was complex and at up to 1 uM produced concentration dependent hyperpolarisations (Fig. 5.9a, whereas at 10 uM the hyperpolarisation was followed by a 'fast' depolarisation. Although the 'fast' depolarisation was antagonised by a nicotinic antagonist, DHBE, the initial hyperpolarisation to carbachol was also reduced. The poor selectivity of nicotinic antagonists versus the nicotinic and muscarinic responses of the rat SCG to electrical stimulation has been reported by Newberry & Connolly (1989) and a nicotinic antagonist was not used to avoid any adverse effects on the responses to carbachol and adenosine.

The inability of adenosine to alter submaximal or near maximal

hyperpolarisations to carbachol (Table 5.6, Fig 5.9b) indicates that the depression of muscarinic responses is due to the selective reduction of M1-mediated depolarisation (see also Fig 5.9c).

Consistent with this conclusion is the ability of both pilocarpine, an effective M1 agonist on the rat SCG (Caulfield & Stubley, 1982) and N-methyl-N-(1-methyl-4- pyrrolidino-2-butynyl)acetamide (BM-5) an oxotremorine analogue with presynaptic antagonist and postsynaptic agonist properties (Casomenti, Cosi & Pepeu, 1986) to enhance the hyperpolarisation to adenosine (Table 5.8). These results indicate that the potentiation of adenosine occurs via an interaction with postsynaptic M1 receptors.

If adenosine and carbachol activate the same intracellular mechanism then when fully activated by carbachol no greater effect by adenosine would be obtained. The inability of a combination of 1 uM carbachol and 10 uM adenosine to attain the same response size as the sum of the individual responses (Table 5.6) therefore suggests both agonists may hyperpolarise the rat SCG via a common mechanism. Recently it has been reported that many inhibitory neurotransmitters activate an inwardly rectifying K^+ channel by a mechanism that involves a pertussis toxin sensitive G-protein (Trussell & Jackson, 1989; Newberry & Gilbert, 1989). Given that muscarinic hyperpolarisation of amphibian ganglia increases gK (Gallagher, Shinnick-Gallagher, Cole, Griffith & Williams, 1980) and that adenosine mediates the sIPSP of cat parasympathetic ganglia (Akasu et al., 1984), both the adenosine and carbachol-induced hyperpolarisation of the rat SCG may be mediated by a common mechanism i.e., increased gK (Chapter 4).

Thus the differential potency of adenosine on the response to muscarinic agonists (Table 5.4) appears to arise from some other effect than a difference in ability to modulate the M2-mediated hyperpolarisation of the ganglion. A difference in biochemical

coupling of the muscarinic receptor and/or in the coupling of intracellular messengers (see discussion below) would also explain the ability of adenosine to reduce the response to submicromolar and not higher concentrations of muscarine (Fig 5.4a).

Effect of ganglionic depolarisation on the response to adenosine.

Complex and concentration-dependent effects of muscarine on sympathetic ganglia have been reported by many research groups and involve a variety of ionic conductances including Im, I_X, I_D and other currents (Brown & Selyanko, 1985a,b; Brown et al., 1989; Mochida & Kobayashi, 1986a,b; Tokimasa, 1984; Tokimasa & Akasu, 1990a; Tsuji & Kuba, 1988). It is unlikely that adenosine interacts with muscarine induced I_X as the response to adenosine was independent of $[Cl^-]_e$ (Chapter 4) and for rat SCG neurones the predominant effect of muscarine is to increase neuronal excitability to produce a 'slow' depolarisation mediated by a decrease in gK (Weight & Voltava, 1970; Kuba & Koketsu, 1974, 1976, 1978; Brown & Adams, 1980). This decreases in gK occurs as a consequence of the activation of M1 receptors (Brown et al., 1989) and the inhibition of the outwardly rectifying K^+ current, Im (Brown & Constanti, 1980). In contrast the effects of adenosine in some preparations have been found to be via an increase in gK (reviewed in Stone 1981, 1989) and this hypothesis is strongly favoured from the results presented here and in Chapter 4. Thus it was predicted and confirmed experimentally that adenosine and muscarine would strongly interact so that the adenosine induced hyperpolarisation would be enhanced during a muscarinic depolarisation (Fig 5.13 & 5.14 & Table 5.7). The difference in the shape of the response to adenosine at millimolar concentrations (Fig. 5.13c) may arise because of desensitisation to adenosine, an interaction at two different receptors or the efflux of K^+ in to the extracelluar space (see Chapter 6 for further discussion).

The RMP (Em) of the rat SCG has been measured by several research

groups to be between -50 to -70 mV and is predominantly determined by the K^+ transmembrane gradient (Blackman, Ginsborg & Ray, 1963; Kosterlitz et al., 1968). When $[K^+]_e$ is raised, the K^+ concentration gradient is decreased, and ganglionic neurones will be depolarised (the reduction of the RMP can be estimated by substituting values for $[K^+]_i$ and $[K^+]_e$ in to the Nernst equation) to generate an increased "driving force (Em -Ek)" for potassium. The response to adenosine during the depolarisation to K^+ was unaltered suggesting depolarisation per se does not enhance the hyperpolarisation to adenosine. Depolarisation with isoprenaline (Table 5.8) or nicotine (Fig 5.7 & Table 5.8) did not enhance the response to adenosine, supporting this view.

It is interesting to note that a similar potentiation of the hyperpolarisation to NA when applied during a muscarinic depolarisation of the rat SCG was observed by Brown & Caulfield (1979). Based on an investigation of the ionic conductances of single cells Brown & Caulfield suggested that muscarine and NA produced their effects via separate voltage-sensitive components by an action of muscarine on Im and NA on IK_{Ca} or gNa (Brown & Caulfield, 1981). In contrast it has been reported by Selyanko, Smith & Zidichouski (1990) that muscarine and adrenaline can have opposite effects on M-channels recorded from amphibian sympathetic ganglia. The results presented in chapter 4 suggest the response of the SCG to adenosine is not via IK_{Ca} channels or a change in gNa and it is therefore unlikely that the effects of adenosine occur via an interaction with these conductances.

A number of compounds apart from muscarine (Brown & Adams, 1980; Constanti & Brown, 1981) inhibit Im including LHRH (Adams & Brown, 1980; Jones, 1987), barium (Constanti et al., 1981a), substance P (Adams, Brown & Jones, 1983) on bullfrog ganglia and LHRH and UTP (Adams et al., 1982a) on the rat SCG. The effect of some of these compounds reported to inhibit Im on the response of the rat SCG to adenosine is summarised in table 5.8. The potentiation of the response to adenosine in the presence of most of these agonists that are reported to inhibit Im (Table 5.8)

supports the hypothesis that adenosine interacts with Im.

Both UTP and LHRH produced small depolarisations which did not significantly alter the response to adenosine (Table 5.8). It is possible that the concentrations employed may not have been adequate to inactivate sufficient M-current to alter the response to adenosine. This conclusion would be in agreement with the results obtained by Jones (1987) where amphibian ganglia required high concentrations of muscarine and low concentrations of LHRH to inhibit Im, whereas the converse situation would be expected in mammalian ganglia, where ACh is the predominant transmitter to mediate inhibition of Im. In addition one uncontrolled factor in the experiments described here (Table 5.8) was the metabolism of LHRH and UTP to inactive or less active metabolites.

In contrast both the substance P analogue, ERP and Ba^{2+} depolarised the ganglion and significantly potentiated hyperpolarisations to adenosine (Table 5.8) supporting the hypothesis that adenosine may interact with Im.

There are several possible mechanisms that may be responsible for the potentiation of the response to adenosine by Ba^{2+}. Firstly Ba^{2+} might cause the release of ACh from presynaptic terminals, as it does in cholinergic motor neurones. The rapid response of sympathetic ganglia to Ba^{2+} recorded by both Takeshige & Volle (1964) and Tashiro & Nishi (1972) was antagonised by dTC to leave a slow depolarisation which was not antagonised by atropine (Adams et al., 1982a), suggesting a direct effect of Ba^{2+} on postganglionic neurones.

A second possibility is that Ba^{2+} penetrates in to ganglion cells via Ca^{2+} channels (Tashiro & Nishi, 1972) and decreases neuronal RMP to depolarise the ganglion and potentiate the response to adenosine. Although the indirect effect of Ba^{2+} to depolarise the ganglion via Ca^{2+} entry was not examined, the results presented in Chapter 4 do not support the hypothesis that adenosine interacts with Ca^{2+} entry and is an unlikely explanation for the

depression of the response to Ba^{2+} by adenosine (Table 5.3). In addition the degree of depolarisation induced by Ba^{2+} (see Table 5.3) would be unlikely to activate voltage operated Ca^{2+} channels.

However the application of Ba^{2+} for long periods can lead to unexplained changes in rat SCG neurones (Adams et al., 1982a). Adams et al., (1982a) reported that brief applications of 4 mM Ba^{2+} produced effects fully compatible with inhibition of Im, comprising a membrane depolarisation accompanied by an increased input resistance and increased excitability. Prolonged application of Ba^{2+} resulted in irreversible depression of large outward currents (Adams et al, 1982a), probably due to entry of Ba^{2+} through calcium channels and a progressive intracellular accumulation of Ba^{2+} (Hagiwara & Byerly, 1981). It is possible that prolonged treatment of the rat SCG with Ba^{2+} antagonised an outward current activated by adenosine, and may account for the loss of the potentiation of the hyperpolarisation to adenosine (Table 5.8).

Even though the potentiation of the hyperpolarisation to adenosine by Ba^{2+} disappeared within 30 minutes incubation (Table 5.8), Ba^{2+} potentiated the depression of the response to muscarine by adenosine (Table 4.8), suggesting the actions of Ba^{2+} on the rat SCG are linked to the interactions of adenosine and muscarine. As reported for many divalent cations, the actions of Ba^{2+} may alter other membrane currents, such as IK_{Ca} (Connor, 1979), and Ik (Constanti et al., 1981b) may be inhibited or potentiated e.g., (I_D) (Tsuji & Kuba, 1988) and interact with adenosine. The most likely explanation of the ability of adenosine to reduce the response to Ba^{2+} (Table 5.3), is due to an interaction with the decrease in permeability of the ganglion cell membrane to K^+ induced by Ba^{2+} due to inhibition of Im (Constanti et al., 1981a; Tsuyi & Kuba, 1988).

Another and not mutually exclusive hypothesis is that Ba^{2+} activates protein kinase C (PKC) in the ganglion as suggested for

the C-kinase purified from heart (Wise, Raynor & Kuo, 1982). Given that PKC is involved in the mechanism of the slow muscarinic excitation (see Brown, 1988 & discussion on PKC below) an increase in the activated PKC by Ba^{2+} would result in the potentiation of the adenosine hyperpolarisation.

The role of secondary messengers in the response of the rat SCG to adenosine and muscarine.

The effect of altered cyclic nucleotide metabolism.

One of the first indications that the actions of adenosine were related to modifications of adenylate cyclase activity was described by Sattin & Rall (1970) who showed adenosine increases cAMP accumulation in the guinea-pig cerebral cortex. Further evidence was provided by Van Calker et al., (1979) who demonstrated that adenosine and its analogues can stimulate or inhibit cAMP accumulation in cultured brain cells and proposed that two different receptors are involved in the increase in cAMP (A2) and decrease (A1) in cAMP. However, since these early studies it has become apparent this classification of P1 purinoceptors may be too restricted and not all adenosine receptors are coupled to a change in cAMP concentration.

Greengard (1976) suggested cyclic nucleotides have a major role in postsynaptic transmission of sympathetic ganglia, and that cAMP and cGMP exert long term control of neuronal excitability (McAfee & Greengard, 1972). It was suggested that muscarinic depolarisation of the SCG is mediated through an increase in cGMP (Weight, Petzold & Greengard, 1974; Kobayashi, 1982; Kebabian, Steiner & Greengard, 1975). This idea was strengthened by the observation that cGMP mimicked the excitatory effects of ACh on pyramidal neurones (Stone & Taylor, 1977). Complex effects of cGMP and derivatives have been reported, and on rabbit SCG, McAfee & Greengard (1972) found dibutyryl-cyclic-guanosine 3':5'-monophosphate (DbcGMP) produced a small transient

hyperpolarisation followed by a larger depolarisation. Dun et al., (1978) confirmed that DbcGMP depolarised postganglionic neurones but also produced a long acting and variable AHP. It has, however, also been reported that cGMP and its derivatives had no consistent effect on the RMP when iontophoresed or microinjected in to rabbit SCG neurones (Buiss, Weight & Smith, 1978).

In support of the hypothesis that muscarinic agonists depolarise the rat SCG by increasing cGMP both Volle, Quenzer, Patterson, Alkadhi & Henderson (1981) and Briggs, Whiting, Ariano & McAfee (1982) reported muscarinic agonists increased cGMP two fold. Carbachol has also been found to increase cGMP levels of rabbit SCG by two fold (McAfee -in Volle et al., 1981), and by six to eight fold in guinea-pig SCG (Wamsley, West, Black & Williams, 1979) and in both cases the increase in cGMP was antagonised by atropine. An increase in cGMP in response to muscarinic agents has also been reported using bovine SCG (Kebabian et al., 1975) and rabbit SCG (Takahashi, Mochida & Kobayashi, 1988). The ability to localise immunohistologically, cyclic nucleotides and the increase in cGMP by carbachol to the postganglionic neurones of the rat SCG (Ariano, Briggs & McAfee, 1982) strengthens the hypothesis that the muscarinic depolarisation is mediated by cGMP.

However other biochemical and electrophysiological studies have not confirmed this association. Brown et al. (1980) reported that muscarine at 1 or 100 uM applied for 2 to 5 minutes did not alter resting levels of cGMP of the rat SCG. Also an increase in cGMP by eight fold by sodium azide was reported by Volle et al., (1981) not to alter the resting d.c. potential or the response to bethanechol. It has also been noted that the depolarisation of rabbit or rat SCG by DbcGMP, is accompanied by an increase in membrane conductance (gM) (Dun et al., 1977, 1978; Gallagher & Shinnick-Gallagher, 1978) whereas there was no change or a decrease in gM during the response to the sEPSP (Kuba & Koketsu, 1978). Theses observations are incompatible with the hypothesis

that the sEPSP is mediated by cGMP. However Hashiguchi, Ushiyama, Kobayashi & Libet (1978) also found an increase in gM, but when the membrane of the rabbit SCG was voltage clamped there was no change in gM and it was concluded these effects are compatible with the mediation of the sEPSP by cGMP. Thus cyclic GMP appears to mediate at least one of the components in the mechanism underlying the sEPSP of the rabbit SCG (Kobayashi, 1982) and more recent studies by Takahashi et al., (1988) suggest that muscarine and cGMP and its derivatives phosphorylate the same endogenous protein, and that cGMP appears to be involved in mediating a part of the sEPSP.

The results of this study show cGMP depolarises the rat SCG, although the responses to cGMP were smaller than those to muscarine (Fig. 3.11), but are in agreement with Gallagher & Shinnick-Gallagher (1978) who found exogenous DbcGMP (250 uM) depolarised the rat SCG, and in partial agreement with Brown et al., (1980) who found cGMP and 8BrcGMP at 1 mM depolarised two out of ten rat SCG and was ineffective on the other eight. Thus an increase in cGMP may mediate some part of the response of the rat SCG to muscarine but not the majority of the response.

The intracellular injection of cAMP into postganglionic cells caused a sustained potentiation of the sEPSP of rabbit SCG (Kobayashi, Hashiguchi & Ushiyama, 1978). If long term enhancement (LTE) occurs in rat SCG neurones then an increase in cAMP levels would be predicted to counteract the depression of muscarine by adenosine. However the evidence for a change in cAMP upon muscarinic stimulation in the rat SCG is conflicting. Briggs et al., (1982) found an increase in cAMP whereas Volle et al., (1981) found no such change in intracellular cAMP. Likewise the application of extracelluar or intracellular cAMP and its derivatives has been found to depolarise (Akasu & Koketsu, 1977; Hsu & McIsaac, 1978; Brown & Dunn, 1983; Gallagher & Shinnick-Gallagher, 1977), hyperpolarise (McAfee & Greengard, 1972; Machova & Kristofova, 1973; Brown et al., 1979; Brown & Dunn, 1983) or have no effect on SCG (Dun et al., 1977; Dun &

Karczmar, 1977; Gallagher & Shinnick-Gallagher, 1977; Busis et al., 1978).

For many cell types stimulation of muscarinic receptors has two consequences: an increase in PIT and a change in intracellular cGMP or cAMP, e.g. the hydrolysis of PIT due to the stimulation of M1 receptors in a transfected cell line stimulates adenylate cyclase to increase cAMP (Felder, Kanterman & Axelrod, 1989) and muscarine increases cAMP in NE-115 cells (Tsunoo & Narahashi, 1987). Alternatively muscarinic stimulation can cause a reduction of the cAMP content of tissues, e.g. in 1321N1 human astrocytoma cells muscarinic stimulation reduces cAMP via the activation of a Ca^{2+}/CaM sensitive PDE (Meeker & Harden, 1982). Muscarinic agonists are reported to increase the cAMP content of the rabbit SCG (Kalix, McAfee, Shorderet & Greengard, 1974) and an increase in cAMP has been reported to mediate a dopamine induced LTE of the muscarinic response of rabbit SCG (Libet et al., 1975). In addition muscarine might increase cAMP formation via an indirect action, via a rise in $[Ca^{2+}]_i$ due to the stimulated hydrolysis of phosphoinositol lipids (PI) (Fredholm, Longren, Lindstrom & Norstedt, 1987; Karbon, Shenolikar & Enna, 1986). This mechanism is unlikely to be responsible for the response of the SCG to muscarine given that SQ 22,536 did not alter the depolarisation to muscarine (Table 5.9).

In an analogous manner to that found in rat or guinea-pig brain the action of adenosine on the rat SCG may occur due to a change in cAMP levels via an effect on adenylate cyclase activity (Dunwiddie & Fredholm, 1984; Yeager, Nelson & Storm, 1986; Donaldson, Brown & Hill, 1988) or PDE activity (Smellie, Davis, Daly & Wells, 1979; De Mazancourt & Giudicelli, 1984). To discover if a change in cyclic nucleotide metabolism is involved in the response of the rat SCG to adenosine or the depression of muscarinic responses, the response to adenosine was assessed in the presence of selective inhibitors of the synthesis of cAMP and breakdown of cAMP or cGMP. The proposed sites of action of these compounds are shown in Fig. 5.11.

One model for sympathetic neurones are PC12 cells which respond to adenosine by the activation of adenylate cyclase to increase intracellular cAMP (Guroff, Dickens, End & Constantin, 1981). If the PC12 cell line is representative of the rat SCG, which hyperpolarises to A1 receptor agonists (Chapter 6) then the inhibition of adenylate cyclase would be predicted to depress the response to muscarine and potentiate its depression by CPA. SQ 22,536 a potent inhibitor of adenylate cyclase (Harris, Asaad, Phillips, Goldenberg & Antonaccio, 1979) at 100 uM blocked the increase in cAMP generated by the application of isoprenaline to the rat SCG (Brown & Dunn, 1983). The same concentration of SQ 22,536 did not alter the depression of the response to muscarine or the response to muscarine itself (Table 5.9) indicating these responses are not due to an interaction with adenylate cyclase. The inability of SQ 22,536 a "P-site" (see Chapter 1, Table 1) selective adenosine agonist (Fredholm & Lingren, 1984) to alter the d.c. potential of the ganglion argues against a decrease in cAMP mediating the adenosine-induced hyperpolarisation of the rat SCG.

Alternatively adenosine may alter cAMP levels of the ganglion by altering PDE activity (Fig 5.11). The effects of PDEI on the response of the rat SCG may be complicated by the presence of multiple forms of cyclic nucleotide PDE. Four major isozymes have been described: one with Ca^{2+}/CaM stimulated activity (PDE I), one having activity with a preference for cGMP (PDE II) and two preferring cAMP, with different Michaelis constants (Km) for cAMP and sensitivity to cGMP (PDE III and PDE IV) (Thompson & Appleman, 1971; Weishaar, 1987; Beavo, 1988; Beavo & Reifsnyder, 1990).

Histological studies have indicated a postsynaptic localisation of cAMP PDE in central cerebral cortex (Florendo, Barnet & Greengard, 1971) and the rat SCG (Vente, Garssen, Tilders, Steinbusch & Schipper, 1987) and a cGMP PDE in rabbit SCG (Quenzer, Yahn, Alkadi & Volle, 1979). Furthermore two forms of cAMP PDE have been characterised from crude enzymes isolated from

the guinea-pig SCG by Capuzzo, Biondi, Borasio, Ferretti & Fabbri (1986), one with a high affinity for cAMP (Km 1 uM) which was weakly inhibited by the non-selective PDE inhibitor, IBMX (Ki 90 uM) and may be similar to the isozyme, PDE IV extracted from the mammalian brain. The second PDE had a low affinity for cAMP (Km 110 uM) and was stimulated by Ca^{2+} and CaM, an effect completely antagonised by TFP at 60 uM. The inactivity of TFP on the adenosine-induced hyperpolarisation of the rat SCG (Table 4.3) suggests the effects of the PDE I type isozyme if present in rat SCG neurones is unlikely to be involved in the response to adenosine.

Oleshansky (1980) reported that adenosine inhibits cGMP stimulation of cAMP hydrolysis, i.e. PDE II activity, with an IC_{50} of 80 uM in the rat striatum to raise the level of neuronal cAMP. However, the inability of the PDE I and II isozyme inhibitor, M&B 22,948 (Souness, Brazdil, Diocee & Jordan, 1989) to alter the response of the rat SCG to adenosine (Table 5.9) indicates adenosine does not stimulate a similar PDE and the depression of muscarine by adenosine is unlikely to be mediated by a decrease in cGMP.

The Ca^{2+}/CaM-independent low Km cAMP PDE isozyme, (PDE IV) is selectively inhibited by a theophylline analogue, denbufylline (Nicholson, Jackman & Wilke, 1989) and Ro 20-1724 (Sheppard & Wigan, 1971; Sheppard, Wigan & Tsien, 1972) and is thought to regulate the cAMP concentration in both the rat cerebrum and guinea-pig hippocampus (Stanley, Brown & Hill, 1989; Challiss & Nicholson, 1990). It would be expected that the incubation of the rat SCG with Ro 20-1724 or denbufylline would depress the response to adenosine if it is due to a decrease in cAMP.

Ro 20-1724, denbufylline and SQ 22,536 were either ineffective or reduced the depolarisation and depression by adenosine of the muscarinic response (Table 5.10). The ability of denbufylline and Ro 20-1724 to antagonise the effects of adenosine may indicate the effects of adenosine are mediated by a reduction in cAMP.

The concentration of denbufylline required to reduce the hyperpolarisation to adenosine by 50% was estimated to be 36 uM and is similar to the Ki (20 uM) reported for the inhibition of A1 radiolabelled ligand binding of [^{3}H] cyclohexyladenosine (CHA) by denbufylline (Nicholson & Jackman, 1988). Thus the antagonism of the effects of adenosine (Table 5.9) reported here may have occurred due to direct receptor antagonism. Given that denbufylline almost completely abolished the PDE (Type IV) activity of the rat cerebral cortex at 10 uM (Nicholson & Wilkie, 1987) it is possible that the reduction of the response to muscarine (Table 5.9) may be due to a nonspecific action.

It has been shown that at 180 uM Ro 20-1724 increased both the basal and carbachol stimulated increase in cAMP levels of the rabbit SCG (Kalix et al., 1974) and it would be expected to have a similar effect on the rat SCG. The reduction of the hyperpolarisation to adenosine but not the depression of the response to muscarine (Table 5.9) indicates the former but not the latter effects of adenosine may be mediated by cAMP.

Ro 20-1724 was chosen as a PDEI as it has been used extensively in experiments where there is a need for a PDEI which is structurally unrelated to the methylxanthines and therefore should not interfere with the binding of adenosine and related ligands. However, it has been reported that at 100 uM Ro 20-1724 inhibited the binding of [^{3}H]-PIA to rat brain membranes by 20% (Schwabe & Trost, 1980) and it is possible at 200 uM Ro 20-1724 strongly antagonised the binding of adenosine to the rat SCG. Thus both the antagonism by denbufylline and Ro 20-1724 of the effects of adenosine on the rat SCG may be due to receptor antagonism. In support of this hypothesis, Ro 20-1724 (Fig 5.10) and an A1 adenosine receptor antagonist (Fig 6.23) caused a similar antagonism of the CRC to adenosine.

There is some circumstantial biochemical evidence to suggest that adenosine does not affect the response of agonists that stimulate cAMP formation in SCG. Roch & Kalix (1975) studying blocks of

bovine SCG found the cAMP content was not altered by incubation in 100 uM adenosine for 9 minutes in either untreated, or SCG treated with theophylline or Ro 20-1724. Adenosine did not alter the increase in cAMP induced by high potassium medium, the latter effect of high $[K^+]_e$ being attributed to the release of catecholamines, which presumably activated beta-adrenoceptors to stimulate cAMP formation. Similarly Kalix et al., (1974) reported that in rabbit SCG adenosine had no effect on cAMP levels at rest or during cholinergic stimulation. Unfortunately both of these reports lack sufficient experimental details in that the concentrations of agonists and antagonists, and conditions employed are unknown, but the results do support those presented here, in that the effects of adenosine on the rat SCG are cAMP-independent.

Among the agents known to increase the cAMP content of the rat SCG, isoprenaline is one of the most potent (Cramer et al., 1973; Quenzer et al., 1979; Quenzer, Patterson & Volle, 1980; Briggs et al, 1988; Petit, Barberis & Jard, 1988), e.g., at 1 uM the application of isoprenaline for 4 minutes to the rat SCG increased the cAMP content ten fold (Quenzer et al., 1980). If adenosine receptor activation modulates cAMP as reported for the actions of arginine vasopressin on the rat SCG (Petit et al., 1988) it is surprising that isoprenaline did not alter the response to adenosine (Table 5.7).

It has been reported that adenosine had no effect on a Ca^{2+} dependent potential in sino atrial node of the rabbit heart unless these cells were first activated by isoprenaline (Belardinelli, Giles & West, 1988). The inability of isoprenaline to alter the response to adenosine reported here (Table 5.7) suggests adenosine does not interact with a current similar to that described by Belardinelli et al., (1988). This result supports the results obtained in Chapter 4 where it was shown that adenosine does not interact with Ca^{2+} dependent potentials.

Likewise VIP, which at 0.1 uM is reported to increase the cAMP content of the rat SCG by about four fold (Volle & Patterson, 1982; but see Audigier, Barberis & Jard, 1986) and at higher concentrations (> 1 uM) potentiated the response to muscarinic receptor stimulation by carbachol (Kawatani, Rutigliano & De Groat, 1985), did not alter the response to adenosine or muscarine (Tables 5.1 & 5.7). These results are consistent with the small reduction or inactivity of adenosine on the depolarising response to isoprenaline (Tables 5.2 & 5.3, Fig 5.2 f) and indicate the effects of adenosine may not be mediated via a change in cAMP. The potentiation of the response to adenosine by forskolin may be mediated through some other effect rather than stimulation of adenylate cyclase (Laurenza, Sutkowski & Seaman, 1989), such as an increase in open channel activity as reported by Akagi & Kudo (1985) for rat SCG neurones.

The dual regulation of Im by agents that increase cAMP to stimulate Im and for muscarinic stimulation to decrease Im has been described for smooth muscle cells of the toad (Sims, Singer & Walsh, 1988) and for adrenaline to increase Im in frog ganglia (Selyanko et al., 1990). In contrast Im was not altered by the intracellular injection or extracelluar application of agents that increase cAMP, e.g. DbcAMP, 8BrcAMP, theophylline or forskolin (Adams et al., 1982a; Moore, Madamba, Joels & Siggins, 1988) suggesting Im is independent of cAMP. Neither forskolin nor cAMP increased Im or reversed a partial blockade of Im by muscarine in frog ganglion cells (Adams et al., 1982a; Brown & Adams, 1987; Moore et al., 1988; Brown et al., 1989). The results presented here support the hypothesis that adenosine interacts with Im but not with cAMP and are in agreement with the findings above.

Role of inositol triphosphate and PKC on the response of the rat SCG to adenosine.

As the effects of adenosine and muscarine on the ganglion appear independent of cyclic nucleotide metabolism, then the interaction

between adenosine and muscarine may occur via some other secondary messenger. Recent molecular characterisation of muscarinic receptors has revealed at least five subtypes of receptor (described in chapter 1) which are often selectively but not exclusively coupled with different effector systems, (Fukuda, Higashida, Kubo, Maeda, Akiba, Bujo, Mishina, & Numa, 1988). Muscarinic receptors coupled to phospholipase C (PLC) have been demonstrated in many neuronal tissues (Fisher & Agranoff, 1987) and based on the sensitivity to pirenzepine it was suggested that M1 receptors are coupled to PLC activation while pirenzepine insensitive M2 receptors are linked to adenylate cyclase inhibition (Gill & Wolfe, 1985).

One consequence of the stimulation of cholinergic receptors by muscarine is the hydrolysis by PLC of phosphatidylinositol 4,5-bisphosphate (PIP2) to yield two second messengers, inositol 1,4,5-triphosphate (IP3) and 1,2-diacylglycerol (DAG) (Berridge, 1987; Fisher & Agranoff, 1987; Fig 5.12). It has been reported that adenosine may interact with PIT in a variety of cell types including rat striatum (Petcoff & Cooper, 1987), mouse cerebral cortex (Kendall & Hill, 1988), cultured GH3 pituitary tumour cells (Delahunty, Cronin & Linden, 1988), frog NMJ (Sebastiao, 1989), frog sympathetic ganglia (Rubio, Bencherif & Berne, 1988) and rat aorta (Long & Stone, 1987), and thus represents a potential target for the selective interactions of adenosine and muscarine on the ganglion.

IP3 stimulates the release of intracellular Ca^{2+} while DAG can activate a family of Ca^{2+} and phospholipid dependent protein kinases i.e. PKC (Berridge, 1987; Nishizuka, 1989; Huang, 1989), which may in turn phosphorylate specific proteins. Ca^{2+} mobilisation may activate phospholipase A2 (PLA_2) and trigger the release of arachidonic acid (AA) and the formation of cyclo-oxygenase or lipoxygenase products to cause hyperpolarisation (Higashida & Brown, 1986; Kim & Clapham, 1989; Kim, Lewis, Graziadei, Neer, Bar-Sagi & Clapham, 1989; Kurachi, Ito, Sugimoto, Shimizu, Miki & Ui, 1989a,b; Fig. 5.15). Likewise

the PI response of various tissues has been linked to a hyperpolarisation produced by activation of IK_{Ca}, e.g. in the hippocampus and NG108-15 cells. Higashida & Brown (1986) have shown that stimulation of NG108-15 cells with bradykinin has two sequential effects on gM:- the first is a hyperpolarisation attributed to the activation of an IK_{Ca} and was mimicked by the application of IP3 or increased $[Ca^{2+}]_i$ and the second, a subsequent depolarisation resulting from inhibition of Im, which was inhibited by DAG activators.

In hippocampal neurones (Fig. 5.12), Dutar & Nicoll (1988) concluded IP3 is responsible for Im suppression as agonists that activate PLC suppress Im. Phorbol ester activation of PKC had no effect on Im and the intracellular injection of IP3 resulted in the loss of Im via a Ca^{2+} independent process (Malenka, Madison, Andrade & Nicholl, 1986), suggesting a direct effect of IP3 or an IP3 metabolite on Im. In contrast the underlying biochemical mechanisms responsible for the inhibition of Im and hence the muscarinic depolarisation of the rat SCG are not fully understood. It is known that muscarinic stimulation of the rat SCG increases the incorporation of ^{32}Pi into phospholipids and the accumulation of IP3 (Hokin, 1965; Lapetina, Brown & Michell, 1975; Horwitz, Tsymbalov & Perlman, 1984; Bone & Michell, 1985). Muscarine at 100 uM caused a 6 to 15 fold increase in PI labelling which was not mimicked by 8BrcGMP, 8BrcAMP or dopamine (Horwitz et al., 1984). Low $[Ca^{2+}]_e$ increased the basal levels of PI and did not alter the accumulation of PI by muscarine and PIT hydrolysis was independent of $[Ca^{2+}]_e$. In contrast, Tokimasa & Akasu (1990) have reported that Im of amphibian ganglia is subject to regulation by $[Ca^{2+}]_e$ and up to 90% of Im was antagonised by the application of Ca^{2+} antagonists. Brown et al., (1989) found Im of the rat SCG is independent of both $[Ca^{2+}]_e$ and $[Ca^{2+}]i$ (see also Adams et al., 1982a), although the possibility that Im requires a minimum level of $[Ca^{2+}]_i$ for its activation has not been discounted.

Using amphibian ganglia the suppression of Im was not enhanced

by raising either $[Ca^{2+}]_i$ to > 200 nM or the level of IP3 to 100 nM (Pfaffinger, Leibowitz, Bosma, Almers & Hille, 1988b). Additional studies by Brown et al., (1989) using Li^+ at a concentration (2 mM) that would be expected to inhibit PIT, did not alter Im. Lithium at 10 mM did not alter the response of the rat SCG to adenosine or muscarine (Tables 4.8 & 4.10) suggesting PIT is not involved in these responses.

Muscarinic agonists differ considerably in their ability to enhance PIT in brain (Fisher & Agranoff, 1987; Baumgold & White, 1989) an effect which depends on agonist structure since ACh and carbachol activate PIT to a much greater extent than pilocarpine (Fisher, Klinger, Agranoff, 1983; Brown & Brown, 1984). The ability of different muscarinic agonists to produce different degrees of PIT in the rat SCG is indicated by the results of Horwitz et al. (1984) who found muscarine was more effective than bethanechol at stimulating phospholipid metabolism. The different susceptibility of muscarinic agonists to adenosine reported here (Table 5.4) may be associated with the differential coupling of these agonists to the muscarinic receptor and their respective responses via secondary messengers. The most potent effects of adenosine were found on MeF which is reported to be poorly coupled to PIT in the rat cerebral cortex (Freedman, Harley & Iversen, 1988) and the smallest depression by adenosine was of carbachol, an agonist that may be strongly coupled to PIT (Freedman et al, 1988). Both of these results indicate that the interaction of muscarine and adenosine may occur via an IP3-independent mechanism.

The coupling of muscarinic receptors to two different biochemical pathways could also explain the biphasic nature of the depression of muscarinic responses, i.e. with increasing concentrations of muscarine a second biochemical mechanism is activated and antagonises the depression of the response of the muscarinic agonist by adenosine.

DAG has been proposed as the mediator of the muscarinic

depolarisation of the rat SCG based on two observations. Firstly muscarinic agonists stimulate the production of DAG (Bone, Fretten, Palmer, Kirk & Michell, 1984; Patterson & Volle, 1984; Horwitz et al., 1984). Secondly in the absence of receptor induced PIP2 hydrolysis the tumour promoting phorbol esters, such as phorbol 12,13-dibutyrate (PDBu) can substitute for DAG to activate PKC (Berridge, 1987) and the addition of PDBu or unsaturated DAGs partially suppressed Im (Pfaffinger, Leibowitz, Subers, Nathanson, Almers & Hille, 1988a; Brown et al., 1989). Likewise PDBu mimics the depolarisation of muscarine by partially suppressing Im and a leak current of rat SCG neurones (Brown et al., 1989).

In contrast Bosma & Hille (1989) using amphibian ganglia found the PKC inhibitors H7 and staurosporine had no effect on LHRH suppression of Im but blocked the response to phorbol esters. A similar effect of PKC inhibitors on the response of the rat SCG was very recently reported by Grove, Caulfield & Evans (1990) who found PDBu mimicked the muscarinic depolarisation of the rat SCG and staurosporine abolished the response to PDBu but only slightly reduced the response to muscarine, probably via a non-specific action.

Furthermore, studies by Pfaffinger et al., (1988a) have produced evidence that agonist-induced suppression of Im in frog sympathetic neurones is independent of the PLC second messenger cascade as phorbol esters caused only partial suppression of Im and the addition of LHRH following phorbol ester application suppressed the remaining current, suggesting LHRH can inhibit Im in PKC-independent manner (Pfaffinger et al., 1988a). Thus there may be both a PLC independent and dependent mechanism of action of muscarine on the rat SCG.

When applied to the rat SCG (Fig 5.14c) PDBu produced a slowly developing depolarisation, which probably reflects the time needed for this compound to cross the cell membrane and activate PKC. The slow recovery to PDBu would be expected to be dependent

on its degradation and/or diffusion from the ganglion. The comparable depolarisation and potentiation of the hyperpolarisation to adenosine by PDBu and muscarine (Table 5.7, Fig 5.14) is indicative of a common site of action for adenosine, at or beyond the activation of PKC. The inability of the PKC-inhibitor H7 to alter the response to PDBu is in contrast to the reported action of another PKC inhibitor, staurosporine to inhibit the depolarisation of the rat SCG to PDBu (Grove et al., 1990).

These findings can be explained in variety of ways. One possibility is that the concentration of H7 employed was insufficient to antagonise PKC. Some evidence for the need for a higher concentration to inhibit PKC was suggested by Linden & Routtenberg (1989) who in reviewing the literature on long term potentiation (LTP) reported Malinow, Madison & Tsien (1988) found the LTP of the hippocampus was inhibited by 300 uM H7, whereas another group of investigators found no inhibition of hippocampal LTP at 100 uM H7. In contrast the concentration of H7 used on the rat SCG may have been sufficient to inhibit PKC as the depression of Ca^{2+} dependent components of the CAP of cultured rabbit SCG neurones produced by phorbol esters was abolished by a minimum of 10 minutes incubation in 50 uM H7 (Mochida & Kobayashi, 1988). Similarly, Sebastiao (1989) reported 60 uM H7 inhibited the evoked endplate potential (e.p.p.) of frog sartorius muscle, but did not modify the excitatory action of 100 nM phorbol diacetate (PDAc) on e.p.p. amplitude, whereas polymixin B antagonised the excitatory effect of PDAc. The apparent ineffectiveness of H7 was attributed to the ability of polymixin B to inhibit the binding of PKC in an indirect manner, i.e. to inhibit the binding of phospholipid to PKC.

The conclusions of Sebastiao (1989) are in agreement with current knowledge as to how inhibitors of PKC may inhibit two different sites, i.e. the regulatory domain of PKC, which binds Ca^{2+}, phospholipid and DAG or phorbol ester to unmask the second active site, the catalytic domain. Sphingosine and polymixin B compete

with phosphatidylserine, Ca^{2+} and DAG at the regulatory site, whereas H7 competes with ATP at the catalytic phosphotransferase site. Thus these differences in the sites of action between H7 and sphingosine may account for the depression of the response of the rat SCG to PDBu by sphingosine (Grove et al., 1990) but not by H7 (Tables 5.10 & 5.11). Thus the results presented here (Tables 5.10 & 5.11) suggest the interaction of adenosine with muscarine and PDBu is at a site distinct to the ATP binding site of PKC. Adenosine may alter the activation by PKC at the regulatory site although this seems unlikely as TFP has been shown to antagonise the binding of Ca^{2+} to the regulatory domain of PKC (Huang, 1989) but did not alter the hyperpolarisation of the rat SCG by adenosine (Table 4.3).

Tumour-promoting phorbol esters have been reported to augment drug and neurotransmitter-induced second messenger production, e.g. cAMP, by an unknown mechanism in a variety of tissues including brain (for references see Karbon et al., 1986). However, it is unlikely that the enhanced response of the rat SCG to adenosine in the presence of PDBu is due to an increase in cAMP as reported for the effects of 2CA on rat brain slices (Karbon et al., 1986) as the alteration of cyclic nucleotide metabolism in PSS did not enhance the response to adenosine.
In addition it is known that PKC inhibitors are not completely specific in that all of them are reported to inhibit other PKs, e.g. H7 also inhibits cAMP dependent PK at 3 uM and cAMP dependent PK at 6 uM (Hidaka, Inagaki, Kawamoto & Sasaki, 1984), and polymixin B inhibits both PKC and Ca^{2+}/CaM dependent PK but not the cyclic nucleotide dependent PKs. The inactivity of H7 on the response to adenosine (Tables 5.10 & 5.11) further supports the hypothesis that cAMP is not involved in the response to adenosine as 50 uM H7 would be expected to inhibit PKA.

<u>The role of arachidonic acid on the response of the SCG to adenosine.</u>

It is possible that the G-protein that couples muscarinic receptors to PLC second messenger cascade may also couple in an

independent manner, to the suppression of Im, perhaps via a direct mechanism. Alternatively the suppression of Im in the rat SCG could occur via DAG which is hydrolysed by DAG-lipase to form monoacylglycerol (MAG) and AA (Irvine, 1982). In support of this idea is the ability of A9L cells to release AA upon stimulation of M1 receptors (Irvine, 1982) and the activation of muscarinic receptors of some cell lines (De George, Morell, McCarthy & La Petina, 1986). Recently the products of AA metabolism, i.e. prostaglandins (PG) and leukotrienes (LT) (Fig. 5.15) have been found to modulate K^+ channels (Higashida & Brown, 1986; Kim & Clapham, 1989; Kim et al., 1989; Kurachi et al., 1989a,b).

A second pathway for the synthesis of AA is via the hydrolysis of phospholipids by PLA_2 to release lysophospholipid and free fatty acid, usually AA, which can directly activate or inhibit PKC (Huang, 1989). In addition AA in neurons can be metabolised by lipoxygenase, cyclo-oxygenase or epoxygenase enzymes to form LTs, hydroxyeicosatetraenoic acids (HETEs), PGs, thromboxanes (TXs) and epoxides respectively (Axelrod, Burch & Jelsema, 1988) (Fig. 5.15).

Most electrophysiological effects of adenosine so far identified appear to result from its ability to alter cAMP or PIT metabolism and arachidonate pathway products do not appear to be a major second messenger system for adenosine. In fact one reason for postulating separate receptors for adenosine and ATP arose from the ability of ATP rather its metabolically stable, phosphate modified analogues to stimulate the synthesis and release of PGs (Brown & Burnstock, 1981b; Needleman, Minkes & Douglas, 1974). Recently this dogma has been challenged by two reports of the activation of PG synthesis by adenosine in guinea-pig atria (Caparrota, Fassina, Froldi & Poja, 1987) and the rabbit heart (Karwatowska-Prokocsuk, Ciabattoni & Wennmalm, 1988).

Both intact and homogenated rat SCG have the ability to synthesise PGs. Ganglia spontaneously released PGE and indomethacin inhibited the synthesis of PGE with an IC_{50} for rat

SCG homogenates of 11 uM (Webb, Saelens & Halushaka, 1978). Homogenates of rat SCG have also been reported to produce PGE2 and 6-keto-PGF1α (Gonzales, Goldyne, Taiwo & Levine, 1989). Given the ability of the rat SCG to synthesise PGs, the interaction of adenosine with the LT and PG pathways of the rat SCG was tested.

At 50 uM indomethacin produced a small non-significant rightward shift in the CRC to adenosine (Fig. 5.16). At this concentration, indomethacin is likely to be supramaximal for the inhibition of PG synthesis as it was reported to inhibit the formation of PGs in intact rat SCG at 55 uM (Webb et al., 1978). Thus it appears PGs are not involved in the response of the ganglion to adenosine. If PLA_2 activation is necessary for the hyperpolarisation to adenosine then the effect of a PLA_2 inhibitor such as nordihydroguaiaretic acid (NDGA) should be to decrease the response to adenosine. In combination with indomethacin to inhibit PG synthesis, NDGA produced a significant antagonism of the hyperpolarisation of the rat SCG to adenosine (Fig 5.16), suggesting either the activation of PLA_2 and/or the lipoxygenase pathway may mediate a significant part of the response to adenosine.

The results reported here appear to be similar to those effects on the opening of S-channels of Aplysia sensory neurones. The opening of K^+ channels in Aplysia neurones was imitated by exogenous AA and blocked by inhibitors of phospholipase and lipoxygenase, but not by indomethacin (Piomelli, Volterra, Dale, Siegelbaum, Kandel, Schwartz & Belardetti, 1987). Other similar actions of AA metabolites have been reported, i.e. some lipoxygenase products have been found to be intracellular modulators of G-protein-gated muscarinic K^+ channels of guinea-pig atrial cells (Kurachi et al., 1989a,b) and neonatal rat atrial cells (Kim & Clapham, 1989; Kim et al., 1989) and thus the interaction of muscarine and adenosine on the rat SCG may occur via one of these secondary messengers.

Given the broad spectrum of actions of PGs and LTs the results

reported here must be considered as preliminary findings and suitable controls will need to be included before any specific claims can be made for the role of LTs in the actions of adenosine on the rat SCG. However, a recent report has suggested that the A1 receptors of rat striatal glial cells may be coupled to the PLA_2-PG synthesis pathway (Caciacli, Ciccarelli, Di Iorio, Tacconelli & Ballerni, 1989) and a similar coupling of adenosine receptors to the PLA_2 secondary messenger system may occur in rat SCG neurones.

With these caveates it is proposed that adenosine may increase the production of arachidonic acid and the production of lipoxygenase products which can then activate K^+ channels to hyperpolarise the rat SCG (Fig 7). It is hypothesised that muscarinic depolarisation augments the response to adenosine due to an increased activity of PLC that can increase the concentration of DAG and AA. An increase in free AA may be sufficient to inhibit the activation of PKC and cause a subsequent depression of the muscarinic response. To determine whether the 5-lipoxygenase pathway is involved in the response of the rat SCG to adenosine, the effect of a specific inhibitor of LT synthesis such as nafazatrom could be tested.

CONCLUSION.

There are many potential interactions between purines and responses to cholinomimetics which may provide the capacity for the modulation of both central and peripheral neurones (Table 5.12). The results presented in this chapter suggest that adenosine selectively depressed the response of the rat SCG to muscarinic agonists via a reduction of the M1-mediated depolarisation and that these actions were competitive and readily reversible. Furthermore, the results indicate there was an adenosine sensitive and adenosine insensitive component to the response to muscarine and may reflect the multiple actions of muscarine reported (Mochida & Kobayashi, 1986a,b).
Many agonists that are reported to increase intracellular cAMP depolarised the rat SCG but did not alter the response to adenosine suggesting a change in cAMP is not responsible for the potentiation of the hyperpolarisation to adenosine in the presence of muscarinic agents. The potential for an interaction of adenosine at a number of intracellular sites has been reviewed and it is suggested that adenosine may alter the activity of these sites to counteract the inhibition of Im by muscarine possibly via an interaction with PKC. Whatever the mechanism of interaction between muscarine and adenosine the ability of muscarine and PDBu to enhance the response to adenosine during the persistent depolarisation to muscarine, suggests the effector mechanism is beyond the level of the muscarinic receptor and is unlikely to be a depletable secondary messenger.

From the experimental data obtained in chapters 4 and here it is proposed that opposing changes in gK underlie the depression of muscarinic responses by adenosine. This may occur via a direct action of adenosine on G-proteins to activate a K^+ channels (Fig 7), as reported for the heart (Pffaninger et al., 1988a,b) or via a secondary messenger/s to alter Im. It is known that muscarine depolarises the rat SCG via a G-protein that is insensitive to pertussis toxin (Brown, Marrion & Smart, 1989, Newberry & Gilbert, 1989b) and the actions of several compounds that

hyperpolarise the rat SCG, including adenosine were antagonised by treatment with PTX (Newberry & Gilbert, 1989b). However the specific G-proteins responsible for each of these effect have not been characterised and their interaction has not been studied. Intracellular recording techniques were made by a similar method to that of Henon & McAfee (1983a) on intact ganglia, but the actions of adenosine were inconsistent as reported by Henon & McAfee (1983a). Therefore to address the role of intracellular messengers and G-protein coupling in these responses, it is recommended that primary cultured SCG neurones are studied by a patch clamp technique.

Muscarine and MeF are known to augment and produce a prolonged response to submaximal preganglionic stimulation (for references see Brown et al., 1980). The ability of adenosine to selectively depress the depolarising response to muscarine would therefore be predicted to depress muscarinic facilitation of synaptic transmission in vivo. In addition the ability of muscarine to potentiate subthreshold responses to adenosine should result in a potentiation of adenosine-induced hyperpolarisations of the rat SCG in vivo.