SOME MECHANISMS OF NEUROMUSCULAR TRANSMISSION

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by

Thomas Christopher Cunnane

Department of Pharmacology

University of Glasgow

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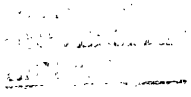

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SUMMARY

This thesis is divided into three main areas of research: (A) an electrical study of neuromuscular transmission in the vas deferens of the guinea-pig; (B) an examination of the electrical responses of the rabbit recotocccygeus to extrinsic parasympathetic nerve stimulation; (C) the effects of d-tubocurarine on skeletal neuromuscular transmission.

(A) Excitatory junction potentials were recorded intracellularly in the guinea-pig vas deferens following stimulation of the hypogastric nerves. The effects of a number of drugs which block the effects of adrenergic nerve stimulation in other tissues were investigated. It is shown that the excitatory junction potential in the guinea-pig vas deferens is unlikely to be generated by noradrenaline acting on α or β-adrenoceptors.

Excitatory junction potential amplitude was increased by α-adrenoceptor antagonists and decreased by α-adrenoceptor agonists and these drugs interacted in a competitive manner. A prejunctional α-adrenoceptor was therefore shown to influence transmitter release. A model for the study of the effects of drugs on transmitter release to single or short trains of stimuli was developed. The characteristics of transmitter regulation by the prejunctional α-adrenoceptor during short trains of stimuli were discussed.

During the course of the study, it was possible to study the mechanism of release of transmitter from individual varicosities. Differentiation of the rising phase of the excitatory junction potential showed them to be made up of transient peaks in the rate of depolarisation, the 'discrete event'. In any one cell discrete events occurred at one or several latencies, intermittently, the frequency of occurrence varying between 1 in 1.8 to 1 in 45 stimuli. Intermittence was not an artifact due to the use of submaximal stimulation nor the result of a ganglionic

relay between the hypogastric and vas deferens nerve. Discrete events occurring with a single latency had amplitudes that were multimodally distributed. In some cells the preferred values of amplitude were simple whole number multiples of the smallest preferred value. The time course of discrete events varied from cell to cell and at different latencies. The discrete event had a time to peak of 5.3 ± 1.9 msec, n = 220 (Mean ± S.D.) and a time to half decay of 8.3 ± 3.6 msec, n = 220. Discrete events in a cell could be matched for amplitude and time course by spontaneous excitatory junction potentials in the same cell and both probably represent the release of a single packet of transmitter. The excitatory junction potential is made up of: (1) discrete events which represent the release of transmitter from a single varicosity; (2) a non intermittent slow component which represents the electronic spread of activity from smooth muscle excited from distant release sites. It is concluded that transmitter release from individual varicosities is packeted and the number of packets liberated per stimulus from a single varicosity is small, varying between zero and ten.

(B) The responses of the rabbit rectococcygeus muscle to stimulation of the extrinsic pelvic nerves have been investigated using intracellular micro-electrode recording techniques. Submaximal pelvic nerve stimulation evoked a depolarisation (excitatory junction potential) which was graded with stimulus strength and abolished by atropine (10^{-6}g/ml) and tetrodotoxin. Single supramaximal stimuli evoked action potentials associated with muscle contraction. In the presence of atropine, to abolish the excitatory junction potentials, supramaximal stimulation of the pelvic nerves evoked hyperpolarisations (inhibitory junction potentials) which were graded with stimulus strength and abolished by tetrodotoxin. The transmitter responsible for the inhibitory junction

potentials is unknown. Phentolamine (10^{-5}g/ml) and propranolol (3×10^{-5}g/ml) in concentrations which block respectively α and β adrenoceptors and the adrenergic neurone blocking agent guanethidine (10^{-6}g/ml) were ineffective in blocking the inhibitory response. It is concluded that stimulation of the pelvic nerves to the rectococcygeus releases two transmitters, acetylcholine, responsible for the excitatory junction potential (and muscle contraction) and a non-adrenergic non-cholinergic transmitter which is the basis for the mechanical relaxation.

(C) The final section of this thesis comprises a study of the effects of d-tubocurarine on skeletal neuromuscular transmission. The question asked was whether d-tubocurarine exerts a prejunctional effect on evoked transmitter release in addition to the classic view of post-junctional receptor blockade. Subthreshold end-plate potentials were recorded intracellularly from intact rat hemi-diaphragm preparations exposed to d-tubocurarine. The transmission process in intact hemi-diaphragms was compared with that in cut muscle preparations when neuromuscular transmission was studied in the absence of blocking agents. End-plate potential amplitude was well maintained in cut muscle preparations at all frequencies of stimulation (1 Hz - 50 Hz) in contrast to intact hemi-diaphragms exposed to d-tubocurarine. Two possible explanations of the results were discussed. First, that d-tubocurarine exerts a prejunctional effect on transmitter release and second that the 'apparent' decline in end-plate potential amplitude has a post-junctional origin, due to a shift to the right of the acetylcholine dose response curve.

Similar experiments were performed on neuromuscular junctions of the frog sartorius and differences in the transmission process were observed.

One explanation discussed is that there is a species difference in the pharmacological response to d-tubocurarine of frog and rat skeletal muscle. It is concluded that d-tubocurarine does not exert a prejunctional effect on transmitter release in the frog.

INTRODUCTION

The modern concept of the 'autonomic nervous system' is based to a large extent on the anatomical and physiological studies of Gaskell and Langley. Gaskell (1886) was the first to give a clear account of the origin of the cranial, thoracolumbar and sacral outflows from the spinal cord. In 1916, Gaskell grouped these outflows together with the sympathetic trunks and the prevertebral and other ganglia as the 'involuntary nervous system'. This term, as the name implies, described "a system of motor nerve cells to involuntary structures". Gaskell designated the terms 'sympathetic' and 'enteral' nervous system to these nerves after he had shown that stimulation of the vagus and sympathetic, for example, produced inhibitory and excitatory functions respectively.

A new impetus to the study was provided when Langley & Dickinson (1890) discovered the paralysing action of nicotine on cells in sympathetic ganglia. By the selective use of nicotine it became possible to determine the position of 'cell stations' or synapses, and the distribution of 'preganglionic' and 'post ganglionic' fibres, terms first used by Langley in 1893.

In 1893, Langley suggested that the term autonomic nervous system should describe the cranial, thoracolumbar and sacral outflows. About this time Oliver & Schäfer (1895) discovered epinephrine and experiments by Langley (1901) established its sympathomimetic effect. It was known following the work of Gaskell, Langley and others that, in general, stimulation of fibres in the craniosacral and thoracolumbar outflows produced opposite effects. These facts and the parallel discovery that pilocarpine produced effects like those resulting from stimulation of the fibres in the craniosacral outflow, led Langley in 1905 to suggest that the latter fibres should be grouped as 'parasympathetic' and the older term 'sympathetic' should be reserved for fibres in the thoracolumbar outflow.

Langley's classification was widely adopted and autonomic nerves are regarded as the motor nerves of the parasympathetic and sympathetic systems.

THE INNERVATION OF THE GUINEA-PIG VAS DEFERENS

According to the classical concept of the anatomical organisation of the autonomic nervous system, postganglionic sympathetic fibres emerge from cell bodies located some distance from the effector organ in the paravertebral ganglia or in the ganglia of the abdominal plexa. In contrast, the cell bodies of parasympathetic fibres lie in peripheral ganglia close to or within the effector organ. It is now known that some sympathetic ganglia, particularly in the pelvic viscera, lie close to the effector organ. The term 'short' adrenergic neuron was introduced by Owman & Sjöstrand (1965) to describe this special kind of post-ganglionic sympathetic neuron.

A major part of this thesis is concerned with a study of the mechanism of release of neurotransmitter from the short intact sympathetic nerves innervating the vas deferens of the guinea-pig. For this reason, it will be useful at this point to describe in some detail the anatomy and innervation of this organ.

The left and right hypogastric nerves originate from the inferior mesenteric ganglion. The hypogastric nerves may occasionally diverge during their course into secondary branches before ending in the pelvic plexus (see Sjöstrand, 1965). This plexus is situated in connective tissue close to the internal genital organs, the bladder and rectum, and supplies these organs. The vas deferens nerves originate from the pelvic plexus and divide into numerous fine branches as they enter the vas deferens with blood vessels at its prostatic end. The vas deferens

nerves consist predominantly of non-myelinated fibres (0.2-1.75 μ diameter) embedded in Schwann cells (Merrillees, Burnstock & Holman, 1963; Merrillees, 1968). When the nerve branches pass into the muscle coats, they split up into smaller bundles of 2 to 8 axons. The varicose regions (about 0.5 to 1 μ diameter) of these axons are packed with vesicles and mitochondria and in these regions the Schwann sheath is often incomplete leaving them naked (see Burnstock, 1970). Single varicose nerve fibres leave the bundles, run separately, lose their Schwann sheath and may end in shallow depressions in muscle cells. Sometimes they form several 'en passage' close contacts with the same or different muscle cells before they end (Merrillees *et al.*, 1963; Merrillees, 1968). The separation of nerve and muscle membranes at close neuromuscular junctions in the guinea-pig vas deferens is about 20 nm (Merrillees *et al.*, 1963). There is little evidence of consistent post-junctional specialization of muscle membranes at all close neuromuscular junctions (close contact varicosities). Three main kinds of post-junctional structures have been described, namely, aggregations of caveolae intracellulares in the smooth muscle cell membrane opposed to nerve varicosities, areas of increased electron density of the post-junctional membrane, and subsurface cisternae in the smooth muscle where it is closely (20 nm) apposed to nerve terminals in the vas deferens (see Burnstock & Costa, 1975).

Quantitative measurements of the number of close (20 nm) neuromuscular contacts relative to muscle cell profiles seen in one plane of section in the vas deferens suggests the occurrence of individual innervation of all the smooth muscle cells in the vas deferens of the adult rat (Richardson, 1962) and mouse (Lane & Rhodin, 1964; Yamauchi & Burnstock, 1969) but not of the guinea-pig (Merrillees *et al.*, 1963). About 20% of the smooth muscle cells of the guinea-pig vas deferens receive a direct innervation (Merrillees, 1968).

There is evidence also of a separate cholinergic nerve supply to the vas deferens of the guinea-pig (Bell, 1967a; Burnstock & Robinson, 1967; Robinson, 1969).

THE EXCITATORY TRANSMITTER OF THE GUINEA-PIG VAS DEFERENS

The bulk of the morphological and pharmacological evidence indicates that the main motor transmitter of the guinea-pig vas deferens is noradrenaline (Sjöstrand, 1965; Swedin, 1971). The vas deferens has the highest noradrenaline content of any tissue, varying between 5 and 20 μg/g in several different species (Sjöstrand, 1965; Blakeley, Dearnaley & Harrison, 1970). A dense adrenergic innervation has been demonstrated by the use of histochemistry (Falck, Owman & Sjöstrand, 1965). It has, however, been known for some time that the motor response to nerve stimulation is resistant to α-adrenoceptor antagonists (Boyd, Chang & Rand, 1960).

Ambache & Zar (1971) made a systematic pharmacological study of motor transmission in the guinea-pig vas deferens and considered several lines of evidence which they felt negated the conventional view that motor transmission in the vas is adrenergic (Huković, 1961; Burnstock & Holman, 1961; Birmingham & Wilson, 1963; Sjöstrand, 1965). Ambache & Zar (1971) demonstrated the presence of two tetrodotoxin susceptible components in the motor response of the field stimulated desheathed vas deferens exposed to a ganglion blocker. The two components were differentiated by altering the pulse width of the stimulus, one responding maximally to pulses of 0.1 to 0.4 msec duration, the other to pulses of 2 msec. These differences were attributed to possible differences in the fibre diameter of the two postulated groups of nerve fibres. Cooling selectively potentiated the more excitable component, but no pharmacological distinction could be made between the two components.

The contractions evoked by pulses of 0.1 or 1.0 msec duration were not affected by the α-adrenoceptor antagonist phentolamine or the β-adrenoceptor antagonist propranolol or a combination of these drugs in doses that produced a thousandfold reduction in the sensitivity of the muscle cells to exogenous noradrenaline. Tyramine, amphetamine, tranylcypramine and prostaglandin E_2 inhibited the twitches elicited by nerve stimulation but potentiated the contractile effect of exogenous noradrenaline. In preparations from animals pretreated with reserpine, the motor response to field stimulation was unaffected and exogenous noradrenaline inhibited the twitch responses of the field stimulated vas deferens. The adrenergic neurone blocking agents, bretylium and guanethidine, however, reduced or abolished the motor response to field stimulation. Ambache & Zar (1971) concluded that motor transmission was 'non adrenergic'. This view has also been taken by von Euler & Hedqvist (1975). Ambache, Dunk, Verney & Zar (1972) postulated that the noradrenaline liberated by the known adrenergic nerves in the guinea-pig vas deferens might act prejunctionally on the 'non adrenergic' nerves to inhibit the release of the unknown transmitter.

Swedin (1971) demonstrated that in rat and guinea-pig vasa the motor response to field stimulation is complex, consisting of two phases, first, an initial rapid 'twitch' response which declines to be replaced by a slower, better maintained 'secondary' response. Swedin (1971) proposed that the neurotransmitter is noradrenaline and the 'twitch' response is due to receptors within the junctional cleft while the secondary response is due to extrajunctional receptors. Stjärne (1976) proposed that there were two transmitters at one junction. McGrath (1978) has shown that the response of the rat vas deferens to a single stimulus is biphasic. By bissecting the vas and recording responses to

single stimuli, the two components were clearly separated. The prostatic portion exhibits predominantly a non-adrenergic twitch component and the epididymal portion predominantly a slower second component. The second component is blocked by α-adrenoceptor antagonists and potentiated by drugs which inhibit neuronal uptake suggesting that the transmitter responsible for the second component is noradrenaline. The initial twitch component is resistant to blockade by drugs known to block the effects of adrenergic nerve stimulation in other preparations. The first component of the twitch survives the chemical destruction of the nerve terminals by 6-hydroxydopamine and reserpine but the second component is lost (Booth, Connell, Docherty & McGrath, 1978). Two components have been observed in the response of vasa to single pulses in several species (Anton, Duncan & McGrath, 1977; Anton & McGrath, 1977) including that of the guinea-pig (Hotta, 1969). In this thesis a detailed examination of the membrane response of the guinea-pig vas deferens to hypogastric nerve stimulation in the presence and absence of some drugs was carried out.

MECHANISM OF RELEASE OF TRANSMITTER

Sir Bernard Katz (1977) in an introduction to a symposium on the synapse commented "that transmitter substances are discharged from many, and possibly from most, chemical nerve terminals in the form of discrete multi-molecular packets", a view which Katz felt was no longer questioned. The aim of this section of the introduction is to review the evidence which has led to the belief that transmitter release from sympathetic nerve terminals occurs in multimolecular packets.

Much information about the mechanism of release of neurotransmitters has come from electrophysiological studies using amphibian nerve muscle

preparations. When muscle fibres are penetrated with micro-electrodes at the end-plate region, small random spontaneous depolarisations are recorded. These spontaneous potentials were first discovered by Fatt & Katz (1952) who named them miniature end-plate potentials. Miniature end-plate potentials arise from the random impact of multimolecular packets of acetylcholine upon the nicotinic receptors on the surface of the muscle fibre at the end-plate.

The exact relationship of the spontaneously released miniature end-plate potential to the nerve evoked end-plate potential was first demonstrated by del Castillo & Katz (1954a). Experiments were carried out on the neuromuscular junctions of the fourth toe of the frog. It was found that the number of components contributing to an end-plate potential at the neuromuscular junction could be varied by altering the concentrations of magnesium and calcium in the Ringer bathing the preparations. The amplitude of the end-plate potential was gradually decreased but in steps. When the probability of release was further reduced, the number of units in a given end-plate potential was small. End-plate potentials evoked by successive impulses showed a marked random fluctuation in amplitude. A proportion of the nerve impulses released no transmitter. These fluctuations in end-plate potential amplitude were not associated with any corresponding variation in the nerve terminal action potential (Katz & Miledi, 1965a). A large number of miniature end-plate potentials were recorded from the same junction to allow an accurate determination of their mean amplitude and standard deviation.

A statistical analysis of the data showed that the distribution of amplitudes of the end-plate potentials is fitted accurately by a '*Poisson series*', whose 'unit class' is identical with the spontaneously

occurring miniature end-plate potentials. The statistical analysis was based on the hypothesis that there was a fixed population of n units each capable, on the arrival of a nerve action potential, of releasing a single packet of transmitter of the same kind as that secreted spontaneously. For a given junction the average probability ($\bar{p}$) of a single unit releasing a packet of transmitter remained constant from stimulus to stimulus. The expected value for the mean number (m) of quanta released per stimulus is equal to $n\bar{p}$. If $\bar{p}$ is less than about 0.1 the expected proportions of stimuli which release 0 (i.e. the proportion of failures) 1, 2, 3 .... packets of transmitter are the successive terms of the Poisson distribution (del Castillo & Katz, 1954a). The value of m is given by the ratio of the mean evoked end-plate potential amplitude (including failures which are regarded as zeros) to the mean amplitude of the miniature end-plate potentials. Having been calculated thus, the value m was used to test for a Poisson distribution by comparing the theoretically expected number of zeros with the actual proportion of failures observed. In ten series of nerve stimuli at six different junctions, 939 failures were predicted and 948 observed (del Castillo & Katz, 1954a). The observed distribution of amplitudes of the end-plate potentials was compared with that predicted on the basis of the Poisson terms (supra vide) and excellent agreement was observed.

A further test of the quantal hypothesis involved direct counts of the number of quanta released per stimulus. Nerve muscle preparations were bathed in a calcium free Ringer at low temperature. A focal external electrode containing calcium chloride was placed close to an active spot on the motor nerve terminal. The rate of release of calcium from the focal electrode was just sufficient to ensure that only a small fraction of the nerve terminal in the vicinity of the electrode released quanta in

response to nerve stimuli. Since the experiments were carried out at low temperature the release of quanta was highly asynchronous and they were counted individually. A close agreement was found between the observed and expected distribution (Katz & Miledi, 1965b). Various other statistical tests have confirmed that the transmission process is quantal, e.g. variance analysis of a series of end-plate potentials. Details of such tests can be found in a recent review of the statistics of transmitter release (McLachlan, 1978).

It thus became a widely held view that the spontaneous miniature end-plate potential and the underlying membrane conductance change is the basic unit of transmitter action and that the end-plate potential is made up of an integral multiple of such unit components (Katz, 1969). The idea that evoked transmitter release occurs in standard 'packets' of large multimolecular size which are identical with the spontaneously occurring units and whose size is independent of the event which causes the release became known as the Quantal Hypothesis (del Castillo & Katz, 1954a). Katz was criticised for using the term 'quantum', which is not applicable to something of variable size. Katz (1969), however, felt that this criticism was based on a misconceived analogy to the physical energy quantum since it is not the energy quantum $h\nu$, but its coefficient h which is a universal constant. Nevertheless, the quantal hypothesis was widely accepted.

Much of the present knowledge of the mechanism of storage and release of the sympathetic transmitter is based on biochemical experiments and morphological observations. The majority of noradrenaline in sympathetic neurons is located in the varicosities of the terminal axon (Geffen & Livett, 1971) although noradrenaline may be synthesised

throughout the cell body and axon (Iversen, 1967). The majority of the total noradrenaline in the axon is stored in a heterogeneous vesicle store comprised of both small (25-60 nm) and large (70-160 nm) dense cored granular vesicles. There is convincing evidence that nerve impulses release only noradrenaline which is bound or stored in vesicles and not extravesicular transmitter. Reserpine depletes adrenergic nerves of their noradrenaline content by blocking the uptake or binding of the amine by the storage granule. In the presence of drugs which inhibit monoamine oxidase, the enzyme responsible for the intraneuronal degradation of noradrenaline, reserpinised tissues can take up and retain noradrenaline. Most of the evidence suggests that the noradrenaline taken up in these circumstances is not bound or taken up by vesicles but is free in the cytosol. The noradrenaline is taken up into the nerve terminals where it remains 'free' in the cytosol, prevented from entering the vesicles by reserpine and prevented from oxidation by inhibition of monoamine oxidase. Potter (1967) concluded that radioactive noradrenaline taken up by the adrenergic nerves of the reserpinised rat vas deferens treated with a monoamine oxidase inhibitor cannot be released by nerve stimulation. Häggendal & Malmfors (1969) also demonstrated, using the adrenergically innervated rat iris, that noradrenaline confined to the cytosol, cannot be released by nerve stimulation.

It is considered that all varicosities within the terminal arborisation of adrenergic nerves are capable of discharging transmitter following invasion by an action potential. Malmfors (1965) demonstrated that the noradrenaline fluorescence of the varicosities of the rat iris was greatly reduced following prolonged nerve stimulation when transmitter synthesis was inhibited. Stimulation of the splenic nerve with 500

supramaximal stimuli releases about 20% of the total noradrenaline content of the cat spleen (Brown, 1965; Haefely, Hürlimann & Thoenen, 1965). These results are consistent with the view that all varicosities release transmitter. It is unlikely that such a large fraction of transmitter could be liberated by a selected proportion of varicosities along the terminal axons to produce such gross changes.

An important function of vesicles apart from storage of transmitter is that they are directly involved in the biosynthesis of the transmitter (see Smith, 1972; Smith & Winkler, 1972). At physiological frequencies, sympathetic nerves maintain their output of transmitter by uptake and re-use of what is liberated. When transmitter loss is brought about by any means, transmitter release is maintained at a lower level by synthesis (Brown, 1965). The vesicle is known to play a vital role in the storage, and conservation of released noradrenaline recaptured from the neuroeffector junction and is the site from which noradrenaline is released in response to nerve stimulation.

It is likely that transmitter release from sympathetic nerves occurs in multimolecular packets by the process of exocytosis. In the adrenal glands, catecholamines are secreted from chromaffin cells together with all the soluble components of the storage granule but not the cytoplasmic proteins such as lactate dehydrogenase or phenylethanolamine-N-methyltransferase (Schneider, Smith & Winkler, 1967). Furthermore adrenaline, adenosine triphosphate and its metabolites are found in the perfusates from adrenal glands stimulated via the splanchnic nerves, in a molar ratio identical to that found in the intact granule (Douglas, 1968); there are four moles of catecholamine for each mole of adenosine triphosphate (Blaschko, Born, D'Iorio & Eade, 1956; Falck, Hillarp and Högberg, 1956) in the perfusate and in the intact storage granule.

These observations were primarily responsible for the proposal of an exocytotic release mechanism for catecholamines from chromaffin cells of the adrenal medulla.

The enzyme dopamine-β-hydroxylase and the protein chromogranin A, which are found only in vesicles in sympathetic nerve terminals, are released together with noradrenaline following sympathetic nerve stimulation (de Potter *et al.*, 1969). This result strongly indicates that the release mechanism involves an exocytotic process. The fact that unique constituents of the vesicle are released during sympathetic nerve stimulation demonstrates unequivocally that it is noradrenaline sequestered within vesicles which is released by nerve action potentials rather than noradrenaline 'free' within the cytosol. Electron microscopical studies have shown illustrations of vesicles fusing with the axoterminal membrane in varicose regions of adrenergic fibres providing further evidence for an exocytotic mechanism (Fillenz, 1971). It is interesting also that drugs capable of disrupting microtubules such as colchicine, vinblastine and cytochalasin B prevent the release of noradrenaline and dopamine-β-hydroxylase from nerves following stimulation (Axelrod, 1972). These results may indicate that a contractile process is involved in the exocytotic release mechanism, perhaps in vesicle transport.

Thus, in sympathetic nerves, there is good evidence that transmitter is stored in multimolecular packets which are released by an exocytotic mechanism. In somatic motor nerves, there is excellent evidence that transmitter release occurs in multimolecular packets of uniform size, i.e. quanta. The quantal theory of chemical transmission was widely accepted in the early 1950s and about this time, subcellular organelles termed vesicles were discovered in the presynaptic terminals

of neurons (Sjöstrand, 1953; Palade, 1954; Palay, 1954; de Robertis & Bennett, 1955). The suggestion naturally arose that a single quantum could be equated with the amount of acetylcholine sequestered within a single vesicle. This concept became known as the vesicle hypothesis (del Castillo & Katz, 1956). In contrast to the sympathetic nerve, however, the question of whether acetylcholine release occurs by exocytosis is still open.

Several lines of evidence favour the view that transmitter is secreted from somatic motor nerves by exocytosis. Electron micrographs have shown vesicles fused with the nerve terminal membrane and apparently open to the junctional cleft (Nichol & Potter, 1970).

The morphological studies of Heuser & Reese (1973) showed that during stimulation of motor nerves, vesicles which normally lie in close contact with the axolemmal membrane of the nerve terminal disappear while pits and dimples in the membrane develop. This change was interpreted as indicating that vesicles coalesce with the nerve terminal membrane and presumably discharge their contents into the neuromuscular junction by an exocytotic process. At this time the nerve terminal is depleted of vesicles but the number of coated vesicles and irregularly shaped cisternae is increased. When stimulation was followed by a period of rest there was a gradual restoration of the morphology of the nerve terminal to the resting state indicating that vesicles are reformed. When stimulated nerve muscle preparations were bathed in solutions containing horseradish peroxidase, a substance which does permeate membranes, Heuser & Reese (1973) found that the peroxidase was located within cisternae in the axonal terminal and when the period of stimulation was followed by rest, peroxidase was demonstrated within the vesicles. Furthermore, when nerve terminals were stimulated after vesicles had

been labelled with horseradish peroxidase there was a marked depletion of labelled vesicles. Heuser & Reese (1973) proposed that release of neurotransmitter at the skeletal neuromuscular junction is accomplished by fusion of the vesicle and nerve terminal (axolemmal) membrane and that there is a cycle of membrane retrieval and neutralisation resulting in the formation of new vesicles. This paper has been interpreted as decisive evidence for the exocytotic mechanism of transmitter release. It should be pointed out however that the results have effectively shown only the possibility of endocytosis of the horseradish peroxidase.

Furthermore, vesicle numbers decrease and the prejunctional nerve terminal membrane area increases during enhanced acetylcholine release at the skeletal neuromuscular junction, a result interpreted as a temporary expansion of the terminal nerve membrane due to the incorporation of membranes of vesicles (Clark, Hurlbut & Mauro, 1972; Heuser & Reese, 1973).

At present, perhaps the strongest argument for an exocytotic release mechanism at the skeletal neuromuscular junction is the analogy with the adrenergic neuroeffector junction. Conversely, the strongest arguments for quantal secretion of transmitter from autonomic nerves is by analogy with electrophysiological results obtained at the somatic motor nerve.

Electrophysiological studies of the mechanism of release of transmitter at the autonomic neuroeffector junction have been less successful than studies at the skeletal neuromuscular junction. Skeletal muscle fibres in general are innervated by a single nerve terminal. All the post-junctional activity recorded when these cells are penetrated with micro-electrodes can therefore be attributed to the effects of transmitter released form a single nerve terminal.

At the skeletal neuromuscular junction, the amplitude distribution of spontaneous miniature end-plate potentials is normally used to determine the average size of the quantum. The amplitude distribution of spontaneous excitatory junction potentials at the autonomic neuroeffector junction cannot be used in this way for the following reasons. The amplitude distribution of spontaneous excitatory junction potentials recorded from individual smooth muscle cells has a continuous size distribution down to the noise level of the recording system. Interpretation of such data is complicated by multiple innervation and a lack of electrical isolation between adjacent cells. As a result the exact relationship of the spontaneous excitatory junction potential to the excitatory junction potential is not known. Thus, there is a lack of electrophysiological knowledge of the characteristics of transmitter release from individual varicosities. Bennett (1972) explained the amplitude distribution and temporal characteristics of the spontaneous excitatory junction potentials in the guinea-pig vas deferens with reference to the three-dimensional syncytial properties of this organ. The amplitude distribution is skewed due to severe spatial attenuation of an electrotonic potential generated in a smooth muscle cell in the muscle. Thus, spontaneous excitatory junction potentials originating in a smooth muscle cell distant from the recording micro-electrode, will appear very much attenuated compared to potentials generated close to the recording micro-electrode. Large numbers of small spontaneous excitatory junction potentials will therefore always be recorded in a syncytial structure. The time course of the spontaneous potentials will however remain unaltered by this electrotonic spread in the muscle, as the time constant of decay of an electrotonic potential generated at a point in the muscle is less than 2 msec. This time constant is very much shorter than the time constant of decay of the spontaneous excitatory

junction potential which is about 30 msec. The spontaneous excitatory junction potentials therefore spread in the muscle with severe spatial attenuation, but with little change in the time course (Bennett, 1972). Variation in amplitude may also be attributed to variation in the transmitter content of the spontaneously released packet, and in the concentration of transmitter at the smooth muscle membrane. This latter variable will depend on the geometry of the neuroeffector junction.

The duration of transmitter action which gives rise to the spontaneous excitatory junction potential is also unknown as is the relationship between the spontaneous excitatory junction potential and the excitatory junction potential. The time course of the spontaneous excitatory junction potential is much shorter than that of the excitatory junction potential. Furthermore the spontaneous exictatory junction potential can be larger than the excitatory junction potential evoked by supra-maximal nerve stimulation (Holman, 1970). Thus, while there is electro-physiological evidence for the packeted release of transmitter, as evidenced by spontaneous excitatory junction potentials, it is not possible to state with any confidence whether the packets are uniform in size and therefore quantal (Holman, 1970). It is assumed however that the excitatory junction potential is due to the release of many packets of transmitter from many nerve endings.

Furness (1970) has reported stepwise variation in the amplitudes of excitatory junction potentials in the mouse vas deferens evoked by nice adjustment of the stimulating voltage. Kuriyama (1964), when studying the effects of magnesium and calcium ions on neuromuscular transmission in the guinea-pig vas deferens, reported stepwise variation in the amplitudes of excitatory junction potentials during a train. It has not been possible, however, to analyse the characteristics of

transmitter release from individual varicosities by recording the excitatory junction potential.

It has been discussed often whether catecholamine secretion from the adrenal medulla and sympathetic nerve terminals is graded or quantal in nature. Viveros, Arqueros & Kirshner (1969) concluded that adrenal medullary secretion is quantal in nature and that the size of the secretory quantum is determined by the entire catecholamine content of individual vesicles. It was further shown that the fractional release of catecholamine secreted per splanchnic nerve impulse from rabbit adrenal medulla corresponded to about 3×10^{-5} of the total organ content (Kirshner & Viveros, 1970) and 3.5×10^{-5} of the cat adrenal medulla (Folkow, Häggendal & Lisander, 1967). Since the cat adrenal medullary cell has been reported to contain on average about 13,000 catecholamine vesicles (Kirshner & Viveros, 1970), if all adrenal medullary cells respond to each splanchnic nerve stimulus, the size of the secretory quantum could not be greater than one third of the contents of a single vesicle. Alternatively, if the amine granules secrete catecholamines in an all or none manner, then the above results would indicate that only one in three of the medullary cells responds to each impulse in the splanchnic nerve (Stjärne, 1970). Similar arguments have been put forward for transmitter secretion from adrenergic nerve terminals.

Folkow, Häggendal & Lisander measured the release of noradrenaline from the vasoconstrictor nerves of the cat calf muscle. They found that the fraction of the total noradrenaline released by a nerve impulse was about 1/50,000 of the total noradrenaline content of the vasoconstrictor nerve endings. Folkow *et al.* (1967) made two assumptions; first, that most of the noradrenaline in the tissue is contained in

vesicles in the varicosities; second, the majority of varicosities release transmitter when excited by a nerve impulse. Estimates were made of the number of vesicles per varicosity (about 1000) and the number of molecules of noradrenaline per vesicle (about 15,000). These figures were based on calculations of the number of varicosities in the rat peripheral adrenergic neuron and the total noradrenaline content of the corresponding tissue (Dahlström, Häggendal & Hökfelt, 1966). In addition, the calculations of Dahlström *et al.* (1966) assume that the concentration of amines in the vesicles of both the chromaffin cells and sympathetic nerve terminals is the same. The calculation of the fractional release of the noradrenaline store per nerve impulse depends on the assumption that all the transmitter released is from vesicles and that the fractional release figure holds for all adrenergic nerve terminals. If the fractional release per nerve impulse is 2×10^{-5}, then only 1-3% of the transmitter contents of a vesicle are released per nerve impulse. An alternative explanation proposed by Folkow & Häggendal (1970) is that not all varicosities are capable of discharging transmitter when invaded by an action potential. This hypothesis requires that adrenergic varicosities, when invaded by an action potential, discharge transmitter only once each 30th or 100th impulse. This latter hypotehsis was rejected since there was no evidence that evoked transmitter release from sympathetic nerves occurred intermittently. Folkow & Häggendal (1970) felt that their results were compatible with the presence of a small pool of readily available noradrenaline within the vesicle, a view for which there was already considerable pharmacological evidence (see Carlsson, 1965; Iversen, 1967).

Beven, Chesher & Su (1969) applied another approach to this problem which did not involve making assumptions of the amount of noradrenaline

contained in a varicosity. They estimated the amount of noradrenaline released per varicosity in the rabbit pulmonary artery by counting the number of varicosities per unit of tissue and determining the amount of noradrenaline released per unit. The results of Beven *et al.* (1969) suggest that the contents of one vesicle may be released on average every 7-8 pulses and are consistent with the hypothesis that the contents of one vesicle are released from each varicosity every few pulses (see Smith & Winkler, 1972).

In the present study, an electrophysiological study of the mechanism of release of transmitter from the sympathetic nerves of the guinea-pig vas deferens has been carried out.

LOCAL REGULATION OF TRANSMITTER RELEASE

The prejunctional α-adrenoceptor

The amount of transmitter released from sympathetic nerves depends mainly on the centrally determined frequency of impulse traffic in these nerves. However, evidence has been accumulating over the past twenty years which suggests that transmitter can regulate its own release locally at the nerve terminal. Transmitter release may also be modified by locally formed substances such as the prostaglandins or by various factors which may circulate in the blood (see reviews, Westfall, 1977; Starke, 1977).

The general hypothesis that noradrenaline regulates its own release locally at the nerve terminal can be summarised briefly. Previously released noradrenaline acting on prejunctional α-adrenoceptors located on or near nerve terminals inhibits release by subsequent nerve impulses. Evidence for this hypothesis has come mainly from studies which have

measured directly the amount of noradrenaline overflowing from a tissue after sympathetic nerve stimulation in the presence of various drugs.

The first demonstration that drugs could increase evoked transmitter release was provided by Brown & Gillespie (1956; 1957) using the blood perfused spleen of the cat. The amount of noradrenaline appearing in the venous blood was related to the frequency of splenic nerve stimulation. The amount of noradrenaline overflowing at a frequency of stimulation of 30 Hz was greater than at 10 Hz. When the α-adrenoceptor antagonists dibenamine or phenoxybenzamine were added to the blood perfused spleen, a large increase in the stimulation evoked noradrenaline overflow occurred. The increase was greatest at 10 Hz with a smaller but significant increase at 30 Hz. At that time monoamine oxidase was thought to be the enzyme responsible for the inactivation of released transmitter. Monoamine oxidase inhibitors however did not change, significantly, the amount of noradrenaline overflowing in the venous blood. Since Brown & Gillespie knew that dibenamine and phenoxybenzamine blocked α-adrenoceptors, they concluded that noradrenaline secreted by adrenergic nerves was taken up by receptor sites on the smooth muscle before the process of transmitter inactivation occurred. When these receptor sites were occupied by drugs, much of the released noradrenaline overflowed into the venous blood. In the presence of α-adrenoceptor antagonists, a true estimate of the quantity of noradrenaline released by the nerves was obtained.

Paton (1960) observed similar effects of phenoxybenzamine on adrenaline release from the adrenal medulla, and argued that post-junctional receptor blockade could hardly account for these affects. Paton (1961) prophetically suggested that "the uptake process described by Sir Lindor is not by or not only by, the recipient tissue, but may

involve the nerve endings themselves" and went on to speculate that the released noradrenaline is "sucked back, recovered, returned to store, when the events of excitation are over". Brown retorted that "this is a most attractive heresy" and congratulated Professor Paton on his courage in suggesting such a hypothesis since the idea that a blocking agent of any sort should act on the nerve endings, Brown regarded "as having gone out rather earlier this century".

The existence of a neuronal uptake mechanism (uptake 1) was soon demonstrated and its characteristics established (see Iversen, 1967). Briefly, uptake is saturable and obeys Michaelis-Menton kinetics. It is stereospecific, the naturally occurring l-isomer of noradrenaline having a higher affinity constant than the d-isomer. The uptake process is sodium and temperature dependent, inhibited by metabolic poisons such as ouabain and by drugs which may or may not be structurally related to the natural transmitter. A second uptake process (uptake 2) into smooth muscle was also characterised.

It was suggested that phenoxybenzamine increased transmitter release by blocking neuronal uptake (Rosell, Kopin & Axelrod, 1963; Thoenen, Hürlimann & Haefely, 1964). Phenoxybenzamine and other α-adrenoceptor antagonists, however, can increase the release of noradrenaline in concentrations which do not inhibit neuronal or extraneuronal uptake (see Starke, 1977; Westfall, 1977).

In the early 1970s, several groups of workers put forward the hypothesis that noradrenaline regulates its own release by activating α-adrenoceptors located on the nerve terminal (Farnebo & Hamberger, 1971; Kirpekar & Puig, 1971; Langer, Adler-Graschinsky, Enero & Stefano, 1971; Starke, 1971). Several lines of evidence support this view. Many

α-adrenoceptor antagonists increase the release of noradrenaline to sympathetic nerve stimulation in concentrations which do not inhibit neuronal or extraneuronal uptake (Langer, 1970; Starke, Montel & Schumann, 1971; Cripps & Dearnaley, 1972). Drugs which block neuronal uptake cause a small increase only in the overflow of noradrenaline following nerve stimulation. In contrast, α-adrenoceptor antagonists produce a much larger increase in transmitter overflow. Furthermore α-adrenoceptor antagonists cause an additional increase in the amount of transmitter overflowing in tissues in which neuronal and extra-neuronal uptake has previously been inhibited. Phenoxybenzamine will increase the overflow of noradrenaline and dopamine-β-hydroxylase, a granular component of molecular weight, 290,000 which is not inactivated by uptake or degradative enzymes, but inhibitors of neuronal uptake increase the release of noradrenaline only. The increased overflow of dopamine-β-hydroxylase therefore probably results from an increased release from the amine granules in the nerve terminals (Johnson, Thoa, Weinshilboum, Axelrod & Kopin, 1971; de Potter, Chubb, Put & de Schaepdryver, 1971). This result separates the effects of α-adrenoceptor antagonists and uptake blockers on evoked transmitter release.

α-adrenoceptor agonists such as clonidine, phenylephrine and noradrenaline produce a concentration dependent decrease in the stimulation evoked overflow of noradrenaline in many tissues including the rabbit heart (Starke, Wagner & Schümann, 1972; Starke & Altmann, 1973), rabbit pulmonary artery (Starke, Endo & Taube, 1975), cat spleen (Kirpekar, Furchgott, Wakade & Prat, 1973), rabbit ear artery (Rand, Story, Allen, Glover & McCulloch, 1973), guinea-pig vas deferens (Stjärne, 1974) and mouse vas deferens (Farnebo & Malmfors, 1971). The inhibition of stimulation evoked noradrenaline release by α-adrenoceptor agonists

is distinct from that due to local anaesthetic or adrenergic neurone blocking agents such as guanethidine (Starke & Altmann, 1973). Furthermore, α-adrenoceptor agonists can antagonize the effect of α-adrenoceptor antagonists in increasing noradrenaline release following sympathetic nerve stimulation.

Recent studies have indicated that there may be qualitative differences between the pre- and post-junctional α-adrenoceptors. Such studies are normally based on the selectivity of action of a number of α-adrenoceptor agonists and antagonists (Borowski, Starke, Ehrl & Endo, 1977; Cambridge, Davey & Massingham, 1977; Doxey, Walker & Smith, 1977). Langer (1977) has suggested that the adrenoceptors may be classified as either α_1 - (post-junctional) or α_2 - (prejunctional) subtypes.

Much of the evidence which has established the hypothesis of local regulation of transmitter release, has been based on deductions from studies which have measured changes in transmitter overflow after drugs. There is little electrophysiological evidence to support the hypothesis that previously released transmitter inhibits subsequent release. Hottá (1969) was the first to demonstrate, using the sucrose gap technique, that noradrenaline was inhibitory on the excitatory junction potential in the guinea-pig vas deferens. Although Hottá (1969) concluded that the depressant effect of noradrenaline on the excitatory junction potential was due to desensitisation, he pointed out that an alternative explanation would be that "noradrenaline acts on the prejunctional nerve terminal to depress the transmitter release following depolarization". These experiments were carried out at room temperature (23-25^{o}C) however, making interpretation difficult since cooling is known to effect the excitatory junction potential (Kuriyama, 1963; Blakeley & Cunnane, 1977).

Furthermore there is doubt as to whether the excitatory transmitter in the guinea-pig vas deferens is noradrenaline (Ambache & Zar, 1971).

In the present study, the effects of α-adrenoceptor agonists and antagonists on the excitatory junction potential of the guinea-pig vas was examined. A model to study the characteristics of prejunctional α-adrenoceptor activation during single or short trains of pulses was developed.

Prostaglandins

The prostaglandins - first discovered and named by von Euler (1933) - are a group of chemicals which are usually 20-carbon unsaturated lipids containing a 5-membered ring. It has been suggested that prostaglandins formed locally during sympathetic stimulation, can regulate transmitter release, by a mechanism independent of the prejunctional α-adrenoceptor (Hedqvist, 1969; Wennmalm, 1971; Smith, 1972; Hedqvist, 1973; Horton, 1973).

The principal evidence supporting this hypothesis can be summarised briefly. Prostaglandins E_1 and E_2 depress the output of noradrenaline in response to sympathetic nerve stimulation in a wide range of tissues including the cat spleen (Hedqvist, 1969), and vasa deferentia of the guinea-pig and rat (von Euler & Hedqvist, 1969; Hedqvist & von Euler, 1972). Johnson *et al.* (1971) showed that prostaglandin E_2 and to a lesser extent prostaglandin E_1, inhibited the release of both noradrenaline and dopamine-β-hydroxylase after stimulation of the hypogastric nerves of the isolated vas deferens of the guinea-pig. This result demonstrates unequivocally that prostaglandins of the E series can interfere with the secretion of transmitter from sympathetic nerves. Prostaglandins E_1 and E_2 have been detected in the perfusates of many tissues after

stimulation of the sympathetic innervation (Dubocovich & Langer, 1975; see Hedqvist, 1977).

When the biosynthesis of prostaglandins is inhibited by agents such as aspirin and indomethacin (Ferreira, Moncada & Vane, 1971; Smith & Willis, 1971; Vane, 1971), the synthetic analogues, eicosa-8-cis-12-trans-14 cis trienoic acid (Van Dorp, 1971) and eicosa-5, 8, 11, 14 tetraynoic acid (Downing, Ahern & Bachta, 1970) noradrenaline release in response to nerve stimulation is increased in the cat spleen (Hedqvist, Stjärne & Wennmalm, 1971) and guinea-pig vas deferens (Fredholm & Hedqvist, 1973). When prostaglandin biosynthesis is promoted by increasing the availability of the natural precursor of prostaglandins, arachadonic acid, noradrenaline release in response to sympathetic nerve stimulation is decreased.

Together this evidence favours the hypothesis first proposed by Hedqvist (1969) that prostaglandins formed locally during sympathetic nerve stimulation, could act on the nerve terminals and depress subsequent release by a negative feedback loop. This hypothesis is not generally accepted however.

Prostaglandins of the E series do not inhibit the mechanical response, nor transmitter release, following stimulation of all sympathetic nerves (see Hedqvist, 1977; Westfall, 1977). Inhibitors of prostaglandin biosynthesis such as indomethacin do not increase the noradrenaline overflow following splenic nerve stimulation in the isolated perfused cat spleen (Dubocovich & Langer, 1975) or dog spleen (Davies & Witherington, 1971). The former authors concluded that prostaglandins did not play an important role in the regulation of adrenergic neurotransmission in the cat spleen. The latter authors' conclusion is in

agreement with the observation of Hedqvist & Brundin (1969) who found that the amount of prostaglandins appearing in the perfusate from the cat spleen during splenic nerve stimulation was very low. Such results indicate that species differences may exist. For example, prostaglandin E_1 and E_2 has no effect on the initial twitch response of the rat vas deferens but has a marked effect on the mechanical response of the vas deferens of the guinea-pig (Ambache & Zar, 1971; Sjöstrand, 1965).

Nevertheless in many species, prostaglandins of the E series are potent inhibitors of adrenergic transmission. The depressant effects of prostaglandin on transmitter overflow are frequency dependent. In the guinea-pig vas deferens, the response to hypogastric nerve stimulation at low frequencies ($\lesssim$ 5 Hz) being most effected.

In the guinea-pig vas deferens the inhibitory effects of prostaglandin E_1 and E_2 on evoked noradrenaline release varies inversely with the calcium concentration in the saline bathing the preparations. Kinetic analysis of this data has indicated that the apparent V_{max} is depressed, and the K_m enhanced of calcium dependent noradrenaline release (Stjärne, 1973). Prostaglandins may inhibit noradrenaline release by interfering with the availability of calcium for the release process, possibly by blocking calcium channels in the nerve terminal membrane. The inhibitory effect of prostaglandin E_2 on nerve evoked noradrenaline release from the guinea-pig vas deferens is reduced by increasing the frequency of nerve stimulation. Shortening the interval between nerve impulses is considered to leave more residual calcium at the active releasing sites in the nerve terminal (Katz & Miledi, 1968). Prolongation of the nerve action potential by prejunctional polarisation or by the administration of tetraethylammonium reduces significantly the inhibitory effect of prostaglandin E_1 on noradrenaline release from

the vas deferens of the guinea-pig (Hedqvist, 1976). There is no direct evidence that prostaglandins interfere with the propagation of the nerve action potential in the terminal varicosity for technical reasons. Prostaglandins of the E series do not, however, affect the compound action potential in the nerve trunk of the splenic nerve (Hedqvist, 1970). Furthermore, prostaglandins readily inhibit the release of noradrenaline evoked by potassium in the heart and vas deferens of the guinea-pig (Stjärne, 1973).

Together these results indicate that prostaglandins inhibit transmitter secretion from sympathetic nerves by an effect on electro-secretory coupling specifically on the availability of calcium for the release mechanism. For detailed reviews of the effects of other agents on transmitter release such as dopamine, angiotensin and acetylcholine, see reviews by (Hedqvist, 1977; Westfall, 1977; Starke, 1977). In the present study, the effects of prostaglandin E_1 on neuromuscular transmission in the guinea-pig vas deferens have been examined.

RABBIT RECTOCOCCYGEUS

The rabbit rectococcygeus is an accessory smooth muscle of the intestine and is derived from the longitudinal layer of the muscularis externa, with which it is continuous. Like the anococcygeus (Gillespie, 1972), the rectococcygeus is unusual in possessing a point of bone insertion. The rectococcygeus anchors the rectum to the coccygeal vertebrae and may assist in defaecation by pulling the rectum further into the pelvic cavity.

The innervation of the rabbit rectococcygeus was first described by Langley & Anderson (1895) and more recently by McKirdy (1972), Ambache, Killick & Zar (1974) and Davey, Gibbs & McKirdy (1975). Langley & Anderson (1895) observed that, following weak tetanizing induction shocks of nerves in the pelvic region, "a distinct action on the recto-coccygeal (rectococcygeus) muscle was only observed with the sacral nerves". At that time the term *nervus erigens* (nerves of erection) was used commonly to describe the visceral fibres of the sacral nerves. Langley & Anderson (1895) felt that this term was inappropriate for a nerve which causes, amongst other effects, contraction of the rectum and bladder. Since it lies in the pelvic cavity they called it the pelvic visceral nerve, or more briefly the pelvic nerve.

The rectococcygeus receives a parasympathetic motor innervation from the posterior and postero-median branches of the pelvic nerve. This division of the pelvic nerve arises "from a considerable ganglion situated on the posterior branch of the pelvic nerve soon after the anterior strand has emerged" (Langley & Anderson, 1896).

Davey *et al.* (1975) clarified in some detail the relationship of the nerves to the smooth muscle fibres. Electron microscopic examination

showed that the rectococcygeus consists exclusively of smooth-muscle fasciculi, forming a flat thin sheet of muscle. Individual cells were about 300 nm long and about 3 μ in transverse diameter. Small axon bundles enveloped by a single Schwann cell, and usually containing 3-10 axons run longitudinally along the fasciculi. Axon varicosities containing synaptic vesicles were naked of Schwann cell and were separated from the nearest muscle fibre by a distance of about 150 nm. Close contact varicosities were observed rarely. Intercellular gap-junctions were observed frequently between adjacent muscle fibres and it was suggested that these represent sites of electrical coupling. However, not all smooth muscles show gap junctions e.g. the guinea-pig ileum (Gabella, 1972), yet the spread of electrotonic potential can be demonstrated clearly (Bolton, 1971; 1972).

Davey *et al.* (1975) observed no ganglia in the rectococcygeus. Ambache *et al.* (1974) concluded from pharmacological evidence that the rectococcygeus was free from imbedded ganglion cells. The ganglion stimulant, nicotine, in concentrations which excite ganglion cells in the intestine and elsewhere, failed to contract the muscle. Furthermore, a number of ganglion-blocking agents did not reduce the contraction evoked by stimulation of the extrinsic motor nerves in the mesentery, up to 2 cm from the muscle (Ambache *et al.*, 1974). They concluded that most if not all the pre-ganglionic parasympathetic fibres relay in the 'considerable ganglion' described by Langley & Anderson (1896). This conclusion was strengthened by the observation that the topical application of nicotine in high concentrations to this ganglion blocked the motor response of the rectococcygeus to stimulation of the pelvic nerves at their origin (Langley & Anderson, 1896). The pelvic nerves innervating the rectococcygeus are therefore unusually long post-ganglionic parasympathetic fibres.

Stimulation of the parasympathetic nerves to the rabbit rectococcygeus produces both excitatory (Langley & Anderson, 1895; Ambache *et al.*, 1974; King, McKirdy & Wai, 1977) and inhibitory (King *et al.*, 1977; King & Muir, 1978) mechanical responses.

Excitatory responses are abolished by tetrodotoxin, botulinum toxin A and atropine and are mediated therefore by acetylcholine released from cholinergic nerves and acting on muscarinic receptors. When muscle tone is raised with the muscarinic agonist carbachol, contractions in response to extrinsic parasympathetic (pelvic) nerve stimulation are abolished or converted to small inhibitory responses. The inhibitory responses are abolished by tetrodotoxin (King *et al.*, 1977) but are insensitive to drugs which block the effects of sympathetic nerve activity in other tissues. This result suggested that the rectococcygeus is also innervated by nerves which are neither adrenergic nor cholinergic. In the last decade Burnstock has put forward evidence which suggests that adenosine triphosphate may be an inhibitory transmitter in the gastro-intestinal tract, the so called purinergic nerves (see Burnstock, 1975). Cocks, Crowe & Burnstock (1979) have examined the possibility that adenosine triphosphate may be the unknown inhibitory transmitter in the rabbit rectococcygeus. They employed three techniques: (1) Fluorescence histochemistry of quinacrine, which binds to adenosine triphosphate (Irvin & Irvin, 1954); (2) Assay of adenosine triphosphate released by field stimulation of the rectococcygeus using the sensitive firefly assay (McElroy & Seliger, 1963; Strehler, 1968); (3) Pharmacological experiments. Cocks *et al.* (1979) reported the presence of quinacrine labelled nerves, the release of adenosine triphosphate by field stimulation and the ability of adenosine triphosphate to mimic the inhibitory response to field stimulation. The results from these experiments led

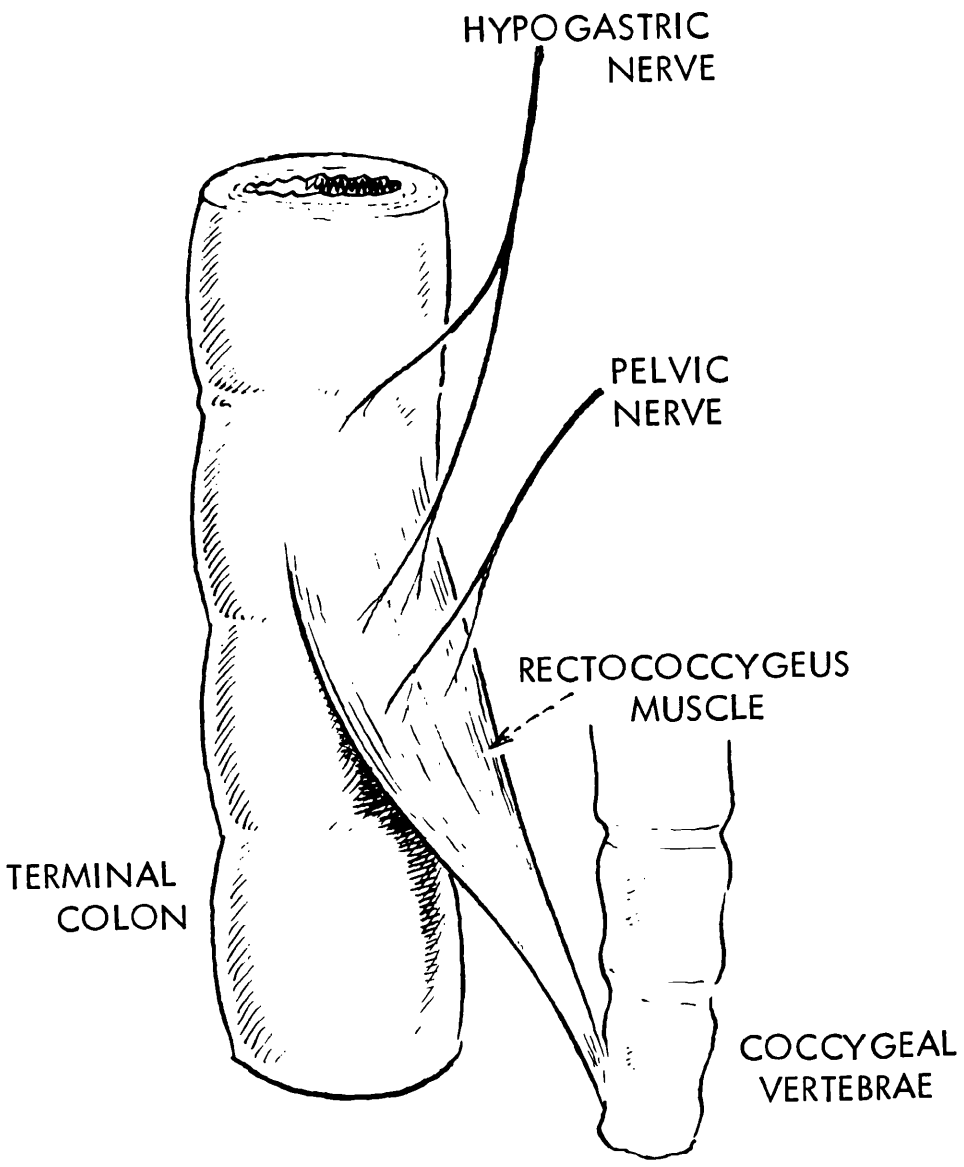

Fig. 1.1. Schematic representation of the relationship of the rabbit rectococcygeus to the terminal colon.

Cocks *et al.* (1979) to conclude that the rectococcygeus is innervated by inhibitory purinergic nerves.

Recently, by the use of a histofluorescence technique a sparse adrenergic innervation of the rectococcygeus from sympathetic branches of the hypogastric nerve has been demonstrated (King *et al.*, 1977).

When muscle tone was raised with carbachol, extrinsic sympathetic nerve stimulation evoked inhibitory mechanical responses which were abolished by tetrodotoxin and guanethidine (King *et al.*, 1977) and reduced by β-adrenoceptor antagonists (King & Muir, 1978) suggesting that they are mediated by noradrenaline released from adrenergic nerves and acting on β-adrenoceptors.

It has further been shown that some ganglion cells are present on the surface of, and within, the rectococcygeus. These ganglia are associated with the sympathetic nerve fibres (King *et al.*, 1977; Cocks *et al.*, 1979) but the functional significance of these ganglia is unknown.

From this review of the literature, it is clear that the rectococcygeus receives a complex innervation. In the present study, the electrical correlate of mechanical responses following stimulation of the extrinsic parasympathetic nerves has been examined using intracellular recording techniques. In this way one can study the membrane effects of transmitter(s) liberated following discrete stimulation of the post-ganglionic parasympathetic nerves innervating the rectococcygeus.

D-TUBOCURARINE

The principal action of d-tubocurarine is the paralysis of skeletal muscle. By competing with acetylcholine for post-junctional nicotinic receptors, the amplitude of the end-plate potential is reduced below the level necessary to trigger the muscle action potential. The use of d-tubocurarine has helped in the elucidation of fundamental mechanisms of synaptic and neuromuscular transmission. Curare or d-tubocurarine has been used extensively over the last 50 years as an experimental tool, often under the assumption that it paralyses neurotransmission by antagonising the effects of released transmitter at the post-junctional membrane. It is important to establish beyond reasonable doubt the precise location of its site of action.

Although there can be no doubt that d-tubocurarine prevents the access of acetylcholine to receptor sites on the end-plate, the question is often raised whether this action can entirely account for its neuromuscular blocking action. There have been persistent reports in the literature over the last two decades which indicate that d-tubocurarine may reduce the amount of acetylcholine released in response to motor nerve stimulation. It has even been suggested that the principal action of d-tubocurarine is prejunctional (Galindo, 1971). Much of the experimental evidence for a post-junctional mechanism has come from studies using amphibian nerve preparations, whereas many of the reports indicating a prejunctional mechanism have come from studies using mammalian tissues. It is possible that the frog and mammalian skeletal neuromsucular junction differ with regard to the action of d-tubocurarine. This question will be investigated in the present study.

The experimental evidence for the mechanism of action of d-

tubocurarine can be divided conveniently into two broad areas, biochemical and electrophysiological. This literature review will cover each area in turn.

The classic view of d-tubocurarine blockade of neuromuscular transmission as a post-junctional mechanism evolved from the studies of Dale, Feldberg & Vogt (1936) who stated that "when transmission of excitation to the perfused muscle is prevented by curarine, stimulation of the motor nerves causes the usual release of acetylcholine". They concluded that d-tubocurarine had principally a post-junctional action and this view was soon supported by Cowan (1936) and Kuffler (1942). Further evidence against a prejunctional effect of d-tubocurarine was found by Cheymol, Bourillet & Ogura (1962) who found that d-tubocurarine did not affect the amount of acetylcholine released following motor nerve stimulation at frequencies up to 25 Hz but that in similar experiments, an inhibitor of choline uptake, hemicholinium did. Fletcher & Forrester (1975) also found that d-tubocurarine in concentrations which caused paralysis of indirectly stimulated rat diaphragms did not affect the release of acetylcholine.

There are, however, some reports in the literature which show that d-tubocurarine decreases the nerve evoked release of acetylcholine from skeletal muscle preparations. Beani, Bianchi & Ledda (1964) found that d-tubocurarine could produce up to 50% reduction in acetylcholine outflow at 28 and 33^{o}C and low frequency of stimulation. However at 38^{o}C and low frequency stimulation, d-tubocurarine had no effect on acetylcholine release. A decrease in acetylcholine release was only observed at 38^{o}C when the frequency of stimulation was increased to 50 Hz. It should be noted that acetylcholinesterase was inhibited in this study by di-isopropylfluorophosphonate. A high concentration of the anti-

cholinesterase was given and the drug subsequently washed out.

Gergis, Dretchen, Sokoll & Long (1971), using isolated frog sciatic nerve gastrocnemius preparations found that d-tubocurarine caused a dose-related decrease in muscle twitch height paralleled with an inhibition of acetylcholine release and suggested that the main site of action of d-tubocurarine could be prejunctional. Experiments which measure directly the amount of acetylcholine overflow from skeletal muscle have inherent difficulties of interpretation. One of the difficulties in interpreting results of this kind is that drugs, added to prevent the breakdown of acetylcholine, may themselves influence the experimental system. Furthermore, there is doubt as to the precise localisation and origin of acetylcholine overflowing spontaneously and in response to nerve stimulation from skeletal muscle preparations. Mitchell & Silver (1963) have shown that when innervated skeletal muscle is depolarised with excess potassium (20-30 mM), there is a three fold increase in acetylcholine release, but a two to three hundred fold increase in miniature end-plate potential frequency. These results show that spontaneous released miniature end-plate potentials represent only a small fraction (1-3%) of the total acetylcholine release. Similar conclusions were reached by Fletcher & Forrester (1975) who calculated that only two percent of spontaneously released acetylcholine could contribute to post-junctional events at the end-plate, the remainder possibly having a non-neuronal origin.

Evidence for a non-neuronal origin of acetylcholine comes from studies which show no difference in the resting release of acetylcholine from acutely denervated and muscles which have been chronically denervated for one week (Straughan, 1960). This resting release was too great to be explained by spontaneous quantal release or leakage from the cut nerve

and indicates that some of the acetylcholine may be released from the muscle (Straughan, 1960).

Additional evidence is provided by the demonstration that the resting release of acetylcholine persists in the presence of botulinum toxin (Burgen, Dickens & Zatman, 1949) which prevents the release of the cholinergic transmitter (Brooks, 1956). Botulinum poisoning reduced evoked acetylcholine release to five to ten percent of controls, and miniature end-plate potentials and end-plate potentials were abolished (Brooks, 1956). Finally, there is no detectable difference between the amount of acetylcholine released upon direct muscle stimulation between innervated and 10-day chronically denervated rat diaphragms (Hayes & Riker, 1963).

When similar experiments were carried out by Krnjević & Straughan (1964) they found that the resting release from denervated diaphragm was fifty percent lower than that from innervated muscle. There was a similar difference between evoked acetylcholine outflow from innervated and chronically denervated muscles suggesting that the bulk of the acetylcholine released during direct muscle stimulation comes from the motor nerves. In view of the conflicting reports, the question remains as to the source of the acetylcholine. Evans & Saunders (1974) suggested that most of the acetylcholine comes from the pre-terminal region of motor nerves and is not involved in synaptic activity. It has been postulated also that a steady leakage of acetylcholine from the nerve terminal cytoplasm occurs. This cytoplasmic acetylcholine may constitute the bulk of the resting release but be distinct from quantal acetylcholine (Mitchell & Silver, 1963; Katz & Miledi, 1977). An effect of d-tubocurarine on quantal acetylcholine secretion may be masked by the large background release of acetylcholine from non-neuronal sources.

A second way of studying the effects of d-tubocurarine on nerve evoked release of acetylcholine is by the use of electrophysiological methods which measure primarily quantal rather than cytoplasmic acetylcholine release (Katz & Miledi, 1977). The most important of these is intracellular recording from vertebrate nerve skeletal muscle preparations. The particular advantage of these preparations is that there is generally only one nerve terminal per muscle fibre. Since the muscle fibres are electrically isolated from one another, all the post-junctional activity recorded when these fibres are penetrated with micro-electrodes at the end-plate region, can be attributed to the effects of transmitter released by a single nerve terminal. Thus the muscle end-plate can be used as a sensitive detector for quantal acetylcholine release from individual nerve terminals.

The investigations of Katz and his colleagues have established that the miniature end-plate potential represents the post-junctional response to a spontaneously released multimolecular packet or quantum of acetylcholine. It was shown that the end-plate potential is the post-junctional response to the synchronous release of several hundred quantal components, identical to the miniature end-plate potential, by a nerve action potential. The number of miniature end-plate potentials observed per unit time and the number of quantal components of the end-plate potential (quantal content) must always reflect prejunctional events. A rapid alteration of the amplitude of miniature end-plate potentials usually reflects a change in the post-junctional sensitivity to acetylcholine. As a result it is possible to distinguish between pre- and post-junctional actions of drugs affecting this system. Thus a micro-electrode can faithfully record changes in quantal transmitter release from a single nerve terminal.

When skeletal muscle preparations are exposed to increasing concentrations of d-tubocurarine, there is a progressive parallel reduction in the amplitude of miniature end-plate potentials, end-plate potentials and potentials evoked by iontophoretic acetylcholine. Neuromuscular block begins when the miniature end-plate potentials are about twenty percent of their normal amplitude (Quastel & Hackett, 1973). Since miniature end-plate potential amplitude serves as a sensitive indicator of end-plate responsiveness to acetylcholine and as d-tubocurarine has no effect on miniature end-plate potential frequency or quantum size, it was concluded that neuromuscular block occurs as a result of receptor occupation by d-tubocurarine.

The recent investigations of Katz & Miledi (1972) who studied statistical fluctuations of membrane potential during acetylcholine induced depolarisation (acetylcholine noise) confirm this view. Noise recorded from the end-plate region of the frog sartorius muscle is related to the collision of acetylcholine molecules with post-junctional nicotinic receptors. They derived the values 'a' and 'τ' which correspond to the amplitude and time course of these 'elementary shot effects'. Addition of d-tubocurarine in a concentration which reduced miniature end-plate potentials to one tenth their normal size did not alter 'a' or 'τ' but did reduce the number of shot effects recorded. This experiment shows that the classical view of d-tubocurarine as a post-junctional antagonist of acetylcholine at the end-plate receptor is correct but does not exclude, however, the possibility that d-tubocurarine may in addition have a prejunctional effect on acetylcholine secretion by the motor nerves, since 'acetylcholine noise' is exclusively a post-junctional phenomenon.

Most of the evidence purporting to show a prejunctional effect comes from experiments using mammalian preparations. If d-tubocurarine

has a prejunctional action then it would be expected that this drug would affect miniature end-plate potential frequency and the quantal content of the end-plate potential. Galindo (1971), using stretched rat hemi-diaphragm preparations to inactivate the muscle action potential generating mechanism, demonstrated a preferential action of d-tubocurarine on miniature end-plate potential frequency without an alteration of their amplitude distribution. Depolarisation of the motor nerve terminal with focal electrodes increases miniature end-plate potential frequency whereas depolarising the muscle affects only miniature end-plate potential amplitude (del Castillo & Katz, 1954b; Liley, 1956). Miniature end-plate potential frequency is thus a sensitive and unequivocal index of nerve terminal activity. If the results of Galindo (1971) are correct, then d-tubocurarine must affect the phrenic nerve terminals innervating the rat diaphragm.

The analysis of end-plate potentials allows one to study the effects of drugs on evoked transmitter release. However it is necessary to block the action potential generating mechanism in the muscle and thereby prevent contraction from dislodging the micro-electrode. There are a number of techniques available including (a) reducing transmitter release by decreasing or increasing the calcium and magnesium concentrations respectively in the saline bathing skeletal muscle preparations (Boyd & Martin, 1956); (b) reducing post-junctional sensitivity with nicotinic receptor antagonists; (c) producing a cathodal block of the muscle action potential generating mechanism by transverse cutting of muscle fibres (Barstad, 1962; Hubbard, Wilson & Miyamoto, 1969; Hubbard & Wilson, 1973); (d) disruption of excitation-contraction coupling with glycerol-Ringer solutions (see Miyamoto, 1978).

Lilleheil & Naess (1961) observed a rapid decline in end-plate

potential amplitude during a train with increasing rates of stimulation, in curarized rat diaphragm preparations exposed to increasing concentrations of d-tubocurarine. They concluded that this effect was due to d-tubocurarine exerting a prejunctional effect at high rates of stimulation but supported Dale *et al.* (1936) in the view that at low frequencies of stimulation there is unlikely to be anything other than the classical post-junctional action.

However, the decline in end-plate potential amplitude during a train implies a reduction of transmitter output since end-plate sensitivity is unchanged during the end-plate potential rundown (Hutter, 1952; Otsuka & Endo, 1960).

Beránek & Vyskočil (1967) reported that d-tubocurarine reduces the depolarising action of iontophoretically applied acetylcholine much less than it reduces the amplitude of end-plate potentials evoked by nerve stimulation indicating a possible prejunctional action of d-tubocurarine on transmitter release. These results may not be strictly comparable since the iontophoretic potential may be generated by acetylcholine stimulating a different population of receptors. Furthermore, the inactivation characteristics of neuronal transmitter and iontophoretically applied acetylcholine may be different under these experimental conditions.

Beránek & Vyskočil (1967) further investigated a possible prejunctional effect of d-tubocurarine in preparations paralysed by high Mg^{2+} concentrations in the physiological saline. Under these conditions, d-tubocurarine did not affect transmitter release. In Mg^{2+} poisoned frog skeletal muscle, d-tubocurarine has no effect on m (m is equal to the amount of acetylcholine released and is the product of the number of

quanta available (n) and the mean probability of release (p) (del Castillo & Katz, 1954) at a frequency of stimulation of 1 Hz (Steinberg & Volle, 1972). Similar results were obtained using rat diaphragm preparations blocked with excess Mg^{2+} where it was found that d-tubocurarine had no effect on n in concentrations which decreased the amplitude of the end-plate potential by 50%. In the presence of concentrations of Mg^{2+} that were sufficient to paralyse the muscle, many agents which might otherwise affect release may not be able to do so. Agents which have been used to enable one to study evoked acetylcholine release may themselves have modified the transmission process.

Barstad (1962), Hubbard *et al.* (1969) and Hubbard & Wilson (1973) developed a preparation whereby the muscle action potential generating mechanism was inactivated by transverse cutting of the muscle fibres of rat diaphragm. This procedure leaves the end-plate potential generating mechanism unaltered. In contrast to results obtaining from curarized hemi-diaphragms it was found that end-plate potentials were well maintained during trains of stimulation at all frequencies. In the presence of d-tubocurarine there was a marked decline in end-plate potentials during a train. This result prompted Hubbard *et al.* (1969) to suggest that the increase in Wedensky inhibition observed in the presence of d-tubocurarine indicated that d-tubocurarine caused a reduction in acetylcholine release during a train. Similar results were obtained by Blaber (1970) who found that a stimulation frequency of 200 Hz was necessary before a decline of end-plate potential amplitude was detectable in the cat tenuissimus cut muscle preparation.

Auerbach & Betz (1971) criticised the work of Hubbard *et al.* (1969) on the grounds that end-plate potential amplitude would be distorted due to alteration of the space constant in cut muscle fibres.

Hubbard & Wilson (1973), however, answered this criticism by providing evidence that the alteration of the space constant was not a critical factor in the analysis of end-plate potentials. They further showed that in the presence of d-tubocurarine (4×10^{-7}g/ml), a fall in quantum content of subsequent end-plate potentials compared to the first in a train occurred.

There is clear evidence for the existence of prejunctional nicotinic receptors located on or near somatic motor nerve terminals possible at the first node of Ranvier. Acetylcholine can depolarise the motor nerve terminal membrane and this effect can be antagonised by d-tubocurarine (see Miyamoto, 1978). Whether the prejunctional receptors play any part in the normal physiological transmission process is still a matter of controversy. There can be no doubt that in amphibian nerve muscle preparations, d-tubocurarine exerts its effects solely by competition with the natural transmitter at post-junctional receptor sites. The evidence in the mammal is somewhat less convincing. Some possible mechanisms whereby d-tubocurarine could exert a prejunctional effect on transmitter release are discussed below.

Bhatnager & MacIntosh (1967) demonstrated that d-tubocurarine could inhibit acetylcholine synthesis in homogenized brain and proposed that this inhibition might be due to d-tubocurarine weakly inhibiting the enzyme choline acetylase. This enzyme is involved in the synthesis of acetylcholine from choline and acetyl Co-enzyme A. Matthews (1966) found however that d-tubocurarine did not significantly reduce the synthesis of acetylcholine in concentrations which were adequate to completely block synaptic transmission in the ganglion. Even if d-tubocurarine produced a significant inhibition of acetylcholine synthesis it is doubtful whether this effect would be of any immediate importance

in d-tubocurarine induced muscle paralysis because of the relatively long time course that would be necessary to deplete the stores of acetylcholine already present in the motor nerve terminal.

Martin (1968) has shown that d-tubocurarine (2×10^{-4}M) is a potent inhibitor of choline uptake. This effect is unlikely to account for the prejunctional effects of d-tubocurarine again, due to an inappropriate time course. It is necessary to repeatedly stimulate motor nerves in the presence of hemicholinium, at high frequencies, for relatively long time periods, before a reduction in the size of the quantal components of the end-plate potential is observed (Elmqvist & Quastel, 1965). D-tubocurarine also produces inhibition of acetylcholine and choline binding to synaptic vesicles (Kuriyama, Roberts & Vos, 1968). At a concentration of 5×10^{-4}M, d-tubocurarine inhibited acetylcholine and choline binding by 77.5% and choline binding by 63.4%. The importance of these actions at the skeletal neuromuscular junction is questionable.

Coleman & Vrbová (1969) investigated whether d-tubocurarine or compounds which act on the same receptor could displace calcium from membranes. D-tubocurarine was found to produce a marked inhibition of calcium uptake into membranes of human erythrocytes. This inhibitory effect was concentration dependent, fifty percent inhibition of calcium uptake being observed with approximately equimolar concentrations of d-tubocurarine and calcium, at a calcium concentration of 2×10^{-4}M. This form of antagonism between d-tubocurarine and calcium is competitive. The inhibition of calcium uptake was reversed by increasing the calcium concentration in the medium. Such an action of d-tubocurarine on calcium entry into the motor nerve terminal could modify evoked transmitter release.

Standaert (1964) has shown that the repetitive activity that occurs in the cat soleus nerve after high frequency stimulation (post-tetanic repetitive activity) depends on changes produced in the motor nerve terminals. It was demonstrated that d-tubocurarine in concentrations which could abolish post-tetanic repetitive activity by an action on the motor nerve terminal, had no effect on the isometric twitch tension of the indirectly stimulated muscle. Standaert (1964) suggested that the first action of d-tubocurarine was on the motor nerve terminal, and a second action on the post-junctional membrane at higher concentrations. Standaert (1964) asserted that "if d-tubocurarine prevents the nerve action potential from reaching the motor nerve terminal, transmission will be blocked by a prejunctional process, regardless of the intensity of the post-junctional effects". Katz & Miledi (1965a) and Chang, Cheng & Chen (1967) could not, however, detect any effect of d-tubocurarine on nerve terminal spikes.

Galindo (1971) suggests that low concentrations of d-tubocurarine act on the transmitter release mechanism. This suggestion is based on the facts that d-tubocurarine produced a reduction in the frequency of miniature end-plate potentials, an increase in the refractory period of motor nerve terminals, an increase in failure of junctional transmission when the rate of nerve stimulation is high and a reduction in the available pool of transmitter and its rate of mobilisation. He concludes that small concentrations of d-tubocurarine reduce the amplitude of end-plate potentials more probably by reducing transmitter output, than by post-junctional receptor antagonism. Galindo (1971) further states, "since d-tubocurarine is normally used in low concentrations as a coadjuvant to general anaesthesia, it is unlikely that its post-junctional effect plays any significant role in the maintenance of muscular relaxation during

surgical anaesthesia". It is interesting that Paton & Waud (1967) have shown that there can be up to 75% of post-junctional receptor sites occupied by d-tubocurarine before any neuromuscular block occurs.

There is still controversy as to the precise location of the action of d-tubocurarine. Many workers hold the opposite view to Galindo (1971). Although not denying the possibility that d-tubocurarine may have a pre-junctional effect, they feel that if such an effect occurs, then it is only of secondary importance to the post-junctional effect.

The aim of this present study was to try and determine the relative importance of pre- and post-junctional receptor blockade as a mechanism of d-tubocurarine induced muscle paralysis using intracellular recording techniques. The transmission process in frog and rat preparations will be compared with respect to the actions of d-tubocurarine.

METHODS

SMOOTH MUSCLE

GUINEA-PIG VAS DEFERENS

Male guinea-pigs (200-650 g) were killed by cervical dislocation and bled. The left vas deferens and attached hypogastric nerve was removed and pinned at approximately resting length in a 3 ml organ bath which was perfused continuously at a rate of 0.75 ml/min with oxygenated (95% O_2 : 5% CO_2) Krebs solution pH 7.4 of the following composition: mM NaCl, 118.4; $NaHCO_3$, 25.0; KCl, 4.7; $CaCl_2$, 2.7; $MgCl_2$, 1.3; NaH_2PO_4, 1.13; glucose, 11.1. The temperature of the organ bath was maintained at 35-36^{o}C ± 0.1^{o}C. The hypogastric nerve was pulled through bipolar platinum ring stimulating electrodes, the cathode of which was close up to the prostatic end of the vas deferens in order to stimulate mainly post-ganglionic fibres (Ferry, 1967). In some experiments the vas deferens was excited by preganglionic stimulation of the hypogastric nerves. Constant current stimulation was delivered from a Digitimer isolated stimulator unit (NL800).

In most experiments the intensity of stimulation was adjusted so that it was just subthreshold for the initiation of muscle contraction with a frequency of stimulation of 0.91 Hz. This strength of stimulation was some 80-90% of that required for a maximum response.

In some experiments, the intramural nerves of the vas deferens were stimulated through bipolar platinum ring electrodes, separation 2 mm, placed round the prostatic end of the vas.

ELECTRICAL RECORDING

The membrane potentials of smooth muscle cells on or near the surface of the vas deferens were measured with intracellular glass micro-

electrodes (resistances 15-40 MΩ filled with 3 M KCl). Cell penetrations were accepted only if the following criteria were satisfied: (1) the cell penetration was abrupt, (2) the resting membrane potential sealed to a more negative value, (3) spontaneous excitatory junction potentials were recorded and (4) following submaximal hypogastric nerve stimulation, the membrane potential returned to the same or more negative value (see Blakeley & Cunnane, 1977). The membrane potential was recorded photographically together with the differential of it (dv/dt) which was produced electrically.

Slips of nerves were dissected from the vas deferens nerve which, on supramaximal stimulation of the hypogastric nerve, gave a constant amplitude potential and on submaximal stimulation, responded in an all or none manner, suggesting either a single active nerve fibre or a single unit of a few fibres had been isolated (Blakeley, 1968). Electrical activity in the vas deferens nerve was recorded extracellularly with bipolar platinum hook electrodes of separation 0.3 mm.

APPARATUS

Preparations were pinned out on a Sylgard gel (Dow Corning Corporation) base in a 3 ml or 5 ml perspex organ bath and trans-illuminated. The organ bath was heated by a modified Tempette circulator (TE 7). The Tempette was modified to operate via a proportional temperature control circuit (Fig. 2.1). At the desired temperature, just sufficient power was applied to the heater to make up the system losses. This resulted in extremely accurate temperature control. With the components shown in Fig. 2.2, temperature control in the region of 35-45^{o}C was achievable by means of the potentiometer control. The circulator passed warm liquid paraffin to an elevated 500 ml insulated reservoir.

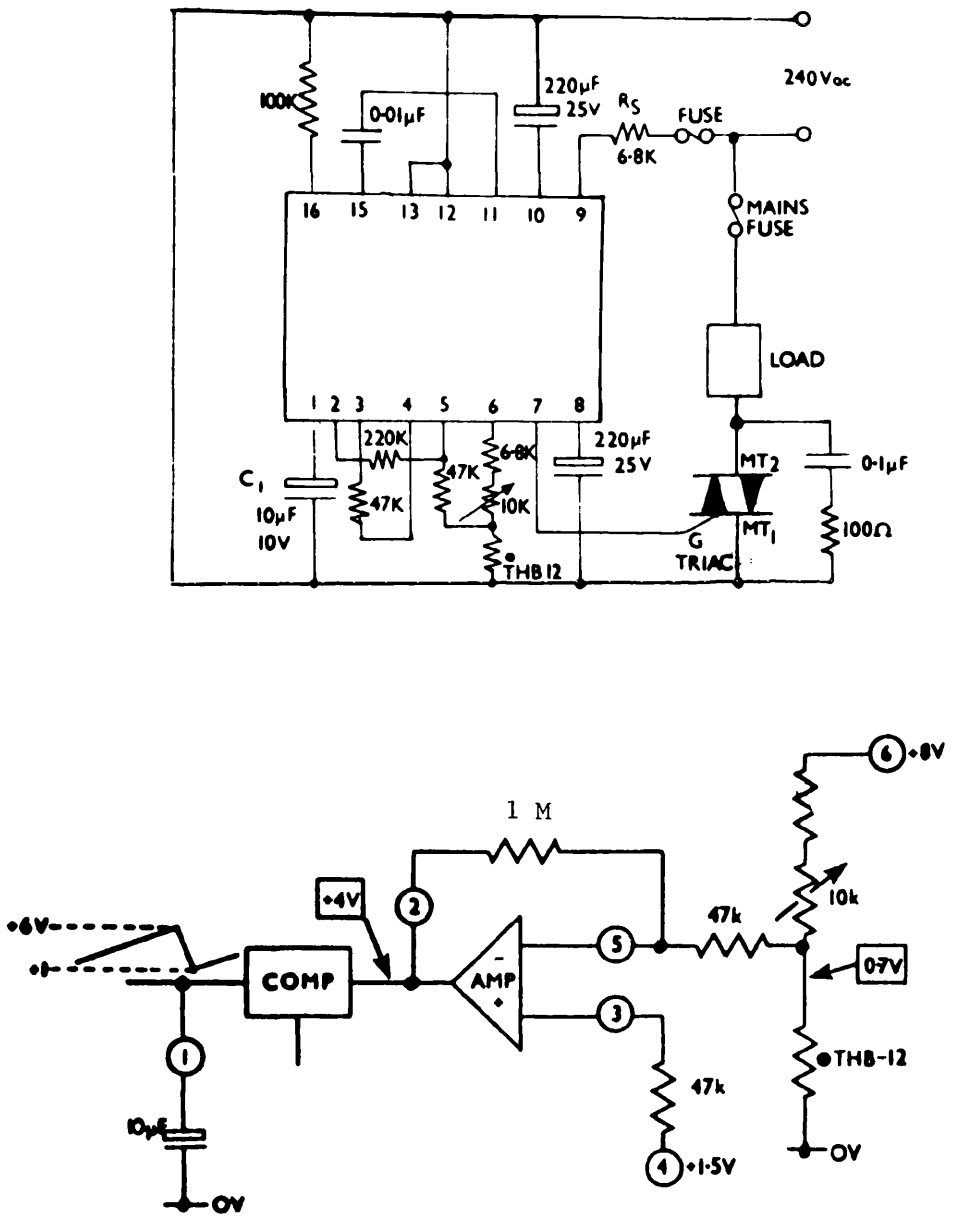

Fig. 2.1. Proportional temperature control circuit used to modify the Tempette circulator (TE7).

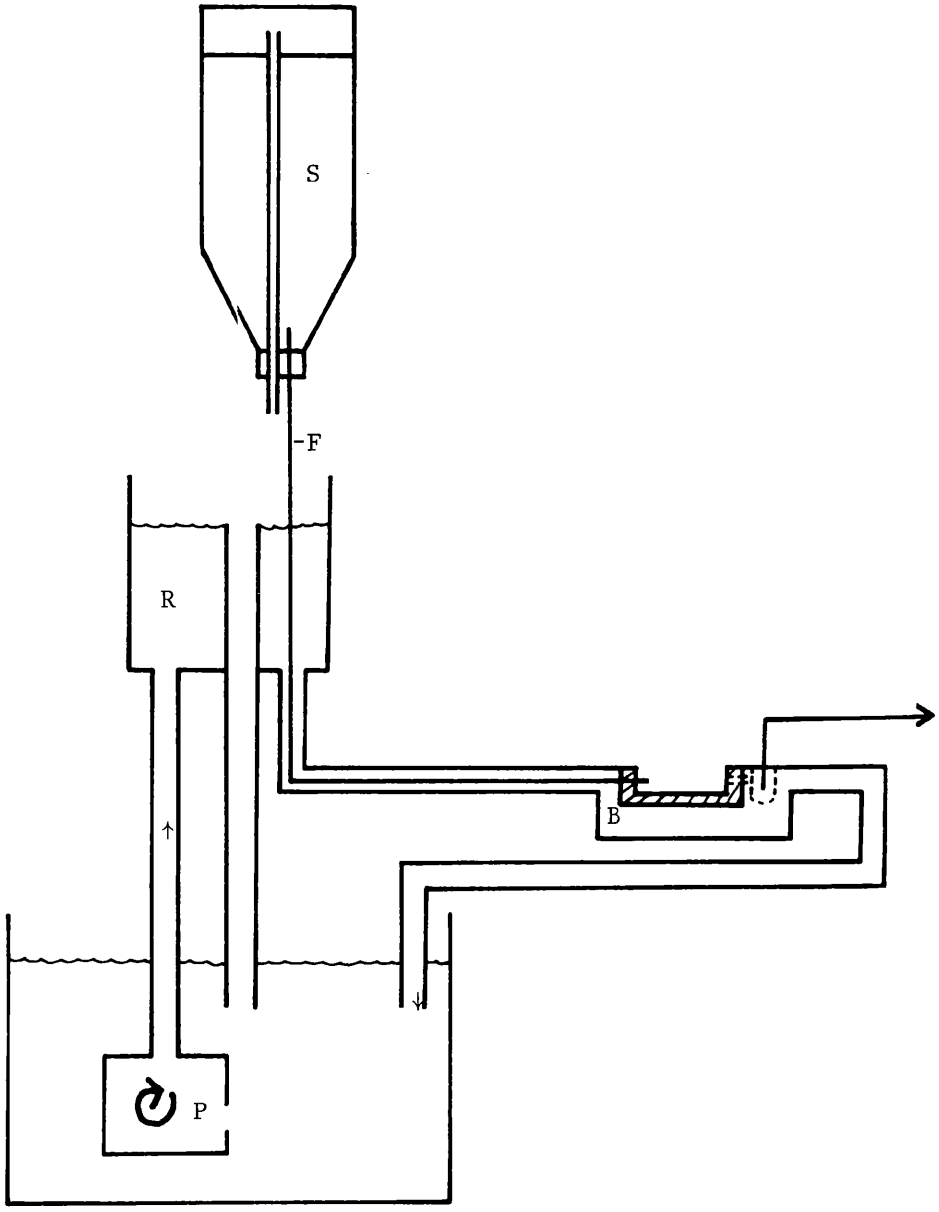

Fig. 2.2. Schematic representation of organ bath and heater circuit. P = modified Tempette circulator, R = reservoir of heated liquid paraffin, S = oxygenated physiological saline, B = perspex organ bath, F = saline drip feed to organ bath.

The reservoir served two purposes: (a) to provide a constant flow of liquid paraffin around and beneath the organ bath to heat it, (b) to isolate the vibration of the circulator pump from the organ bath. Oxygenated Krebs solution from a bottle was passed via a plastic cannula (Portex, PP90) to the bath at a rate of 0.75 ml/min by gravity drip feed. The Krebs solution was heated by routing the cannula along the liquid paraffin pipe between the reservoir and the organ bath. The Krebs solution was drawn across the bath and excess removed by syphoning. A temperature differential of 3-4^{o}C existed between the liquid paraffin in which the circulator was immersed and the temperature of the saline in the organ bath. The temperature of the bath was measured with a glass bead thermistor. Fig. 2.2 shows a schematic representation of the apparatus.

RECORDING APPARATUS

The organ bath was securely fastened to T-slots cut in a 506 lb soft iron, goat myograph stand (Brown & Harvey, 1939). The myograph stand was mounted on Muffelite vibro dampers (K150, K300 Cementation Muffelite) to isolate the myograph stand from vibration generated in and around the laboratory. Intracellular recordings were made with glass micro-electrodes pulled by gravity from glass (outside diameter 1.2 mm) with fused inner filaments (Clark EMI) and filled by immersion in 3 M KCl.

To estimate tip diameter, a sine wave of constant amplitude was passed through the recording circuit (Fig. 2.3) and its frequency adjusted until 30% attenuation of the signal was obtained. Micro-electrode resistance was read off from a standard curve constructed with resistors of known value (Fig. 2.3).

The micro-electrode was connected to a unity gain, high impedance

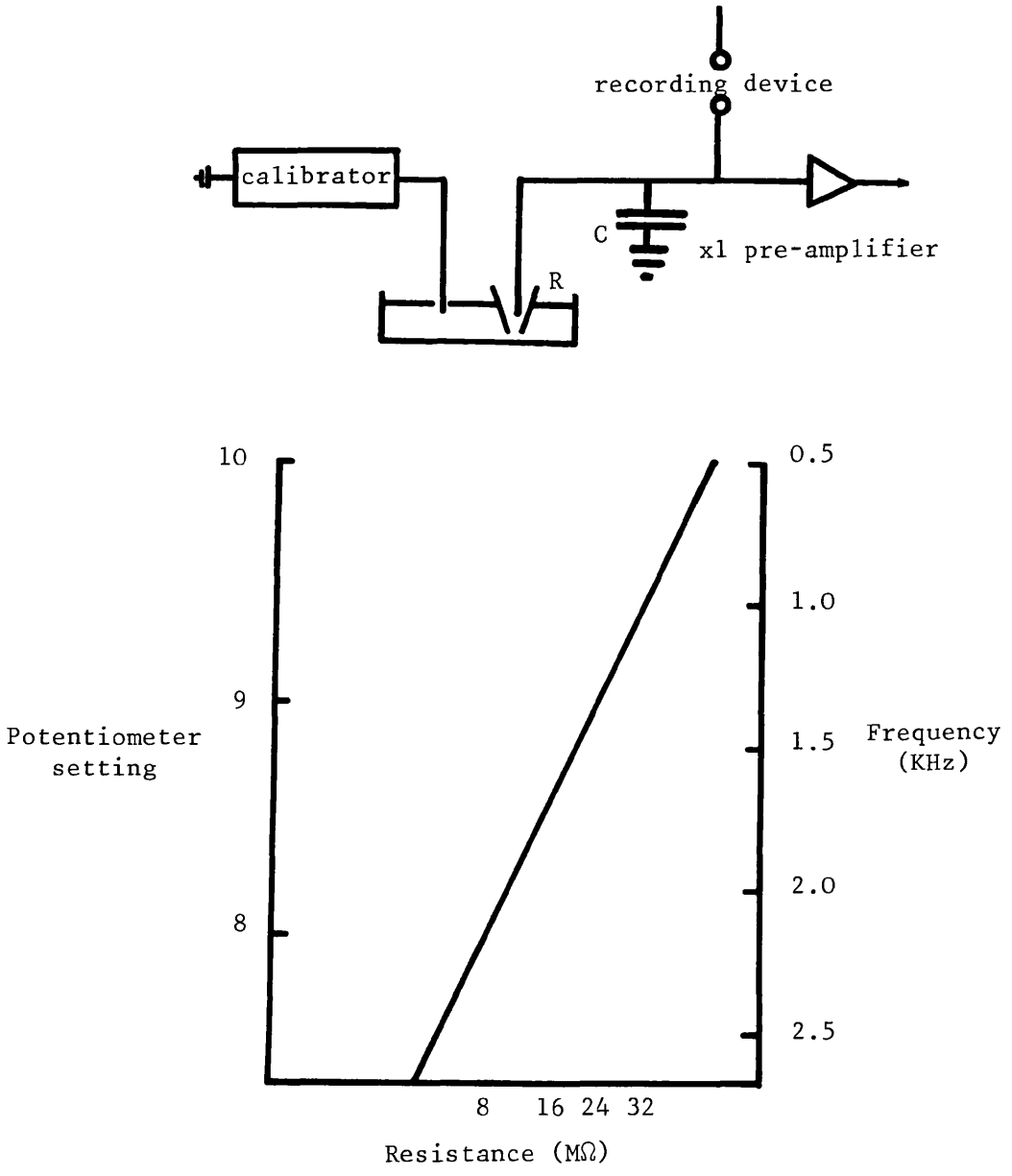

Fig. 2.3. Micro-electrode calibration circuit and curve. R = micro-electrode resistance; C = capacity to earth of input.

amplifier (HA 2005) via a sintered Ag : AgCl pellet attached to a silver wire. A similar Ag : AgCl pellet immersed in the Krebs solution in the organ bath served as the indifferent electrode.

Fine movements of the micro-electrode in three directions at right angles were made possible by a Huxley micromanipulator (Huxley, 1961).

The electrical signals from the amplifier passed directly to a dual beam cathode ray oscilloscope (Tektronix 502A) and also via a preamplifier (Neurolog NL 103), with a gain of 100 or 1000 and frequency cut off controls set at 0.1 Hz and 1 KHz. The signal could also be passed through a differentiator.

The frequency response of the differentiator was severely limited to reduce its noise level. The square wave response of the differentiator to a triangular wave signal had a rise time of 0.35 msec to 60% of the maximum amplitude. This time constant was an order of magnitude less than the time to peak of the events recorded. The noise level of the differentiator and preceding micro-electrode follower was equivalent to 0.025 V/s.

All electrical signals could be displayed on a storage cathode ray oscilloscope (Tektronix 5103N) and on an ultraviolet recorder (S.E. 3006) with a frequency response of 2.5 KHz.

The unit gain amplifier also drove a digital voltmeter (Bell & Howell, VI-920). The background noise of the system with a 10 MΩ micro-electrode in the recording mode was usually less than 0.1 mV and with a 20 MΩ micro-electrode (for smooth muscle recording) less than 0.5 mV. The noise level of the differentiator with a 20 MΩ micro-electrode was about 0.1-0.2 V/s.

MECHANICAL RESPONSE OF GUINEA-PIG VAS DEFERENS

Vasa deferentia were removed from guinea-pigs and threads attached at either end. Vasa were mounted between platinum ring and hook electrodes (Anton *et al.*, 1977) and set up in twin 25 ml organ baths containing oxygenated Krebs solution at 36°C ± 0.1°C. The initial resting tension was 1 g. Isometric responses to trains of stimuli at 10 Hz (0.7 msec, supramaximal) were recorded via strain gauges (Grass FT03) coupled to a Devices M2 hot wire pen recorder.

RABBIT RECTOCOCCYGEUS

ISOLATION AND REMOVAL OF THE RABBIT RECTOCOCCYGEUS

The dissection follows that originally described by Gillespie (1955) and King *et al.* (1977).

Dutch rabbits (1.5-3.5 kg) of either sex were killed by cervical dislocation and bled. The skin over the abdomen was cut from the pubis to the xiphoid. Surface tissue over the pubic symphysis was removed and a longitudinal incision made along the symphysis. The abdominal cavity was opened and the pelvic girdle sprung open. From this stage in the dissection onwards, chilled Krebs solution was applied to keep tissues moist and reduce tissue metabolism, thus minimizing possible effects due to anoxia during the dissection (≃ 30 min). The bladder was ligated at its neck and removed together with all soft structures lying ventral to the terminal large intestine. Both inferior haemorrhoidal arteries and veins were located; these blood vessels are a useful index of the position of the pelvic nerves. Spasm of the blood vessels was

induced by compression and gentle stretch of the vessels with forceps. The artery and its accompanying vein were then severed to reveal the pelvic nerves which originate from the sacral region of the spinal cord (S2-S4). Each pelvic nerve was ligated close to the anus, care being taken to ensure preservation of the nerve tracts held within the meso-colonic wall. The rectum was cut and reflected together with the meso-colon. By pulling on the rectal ligature, the rectococcygeus was identified and cut near the point of bone insertion. The preliminary dissection was completed by ligating and cutting the colon proximal to the inferior mesenteric arteries. The colonic/rectococcygeus preparation with attached pelvic nerves was transferred to a petri dish containing chilled oxygenated Krebs.

With the aid of a Zeiss dissecting microscope, the rectococcygeus and attached pelvic nerve was separated from the terminal colon and pinned out, at approximately resting length in a 3 ml organ bath continuously perfused with oxygenated Krebs solution at $36^{o}C \pm 0.1^{o}C$.

ELECTRICAL RECORDING

The extrinsic parasympathetic (pelvic) nerves on each side were pulled through separate bipolar platinum ring electrodes (separation 2 mm) and stimulated as described previously.

Changes in membrane potential were measured intracellularly with glass micro-electrodes (resistances 15-40 MΩ) filled with 3 M KCl.

The activity in a fine bundle of the extrinsic parasympathetic nerves was recorded extracellularly with platinum electrodes (separation, 2 mm). Data was recorded as described previously.

SKELETAL MUSCLE

Neuromuscular transmission was studied by intracellular recording and examination of the twitch response of rat and frog muscle in response to stimulation of the motor nerves.

RAT DIAPHRAGM PREPARATIONS

Hemi-diaphragm and attached phrenic nerve

Wistar rats (180-250 g) of either sex were anaesthetised with chloroform and killed by exsanguination. The left hemi-diaphragm and attached phrenic nerve was excised by the method of Bulbring (1946) and pinned out flat in a 5 ml organ bath which was perfused continuously at a rate of 0.75 ml/min with oxygenated Krebs solution, at $37^{o}C \pm 0.1^{o}C$.

Cut muscle preparation

The muscle action potential was blocked by transverse cutting of muscle fibres on either side of the point of insertion of the phrenic nerve. This procedure produces a strip of muscle with attached phrenic nerve in which neuromuscular transmission can be studied in the absence of blocking agents (Barstad, 1962; Hubbard & Wilson, 1973). Local circuit (injury) currents generated at the cut ends of the muscle fibres produce a persistent depolarisation of the muscle cell membrane, preventing initiation of the action potential but leaving the end-plate acetylcholine receptors and end-plate potential generating mechanism unaltered.

High magnesium Krebs

In magnesium-enriched solutions the quantal content of the end-plate potential is greatly reduced (del Castillo & Katz, 1954a; Liley, 1956). End-plate potentials were studied in intact hemi-diphragm preparations

when the magnesium concentration of the Krebs was increased to 10 mM.

FROG SARTORIUS PREPARATIONS

Sartorius and attached ischid nerve

Frogs were killed by decapitation and the sartorius and attached ischid nerve removed and pinned out flat in a 5 ml organ bath perfused continuously at a rate of 0.75 ml/min with oxygenated modified Ringer solution pH 6.8 of the following composition: mM Na, 133; Cl, 120; K, 2; Ca, 3.6; PO_4, 1 (Fatt & Katz, 1951). The temperature of the Ringer solution was 18-22°C (room temperature).

Cut muscle preparation

Muscle fibres were cut transversely to block the action potential generating mechanism. Muscle contraction in response to motor nerve stimulation normally ceased 20-40 min after cutting the muscle fibres.

APPARATUS

The apparatus was the same as that described previously in the smooth muscle methods section.

INTRACELLULAR RECORDING

Nerve terminals were located with the aid of a Zeiss dissecting microscope. End-plate potentials evoked by trains of cathodal pulses applied to the nerve trunks (0.2 msec, supramaximal voltage) were recorded with intracellular glass micro-electrodes (resistances 5-15 MΩ).

MECHANICAL RESPONSES

Left and right hemi-diaphragms with attached phrenic nerves were

mounted on Palmer electrode assemblies in twin 100 ml organ baths containing oxygenated (95% O_2 : 5% CO_2) Krebs solution at 37°C ± 0.1°C. Isometric responses to phrenic nerve and occasionally direct stimulation were recorded via Devices strain gauges coupled to a Devices M2 hot wire pen recorder. After they were set up, preparations were allowed to equilibrate for 15 min when they were bubbled vigorously with 95% O_2 : 5% CO_2. Each preparation was exposed to one concentration of d-tubocurarine only. No preparation was used for longer than 75 min following dissection to minimise effects due to anoxia.

STATISTICS

DOSE RESPONSE CURVE

The results of individual experiments were plotted as log dose response curves. Points obtained from the experiments lying between 20% and 80% of the maximum response, *i.e.* on the linear part of the log dose response curve, were used to calculate the line of best fit, using linear regression analysis. The line was calculated using the formula: $y = aX + b$, where y is the percentage maximum response, X is the log dose of agonist, a the gradient and b the intercept. From the line, a value for the ED_{50} and slope of the line could be obtained. The 95% confidence limits of the ED_{50} were calculated (Walpole, 1968).

COMPARISON OF DATA

Where appropriate, sets of data were expressed as the mean ± standard error of mean (S.E.M.) or ± standard deviation (S.D.) and the significance of the difference between sets of means assessed using the Student's t-test. All or none data was compared using a χ^2 test (Crow, Davis & Maxfield, 1960).

RESULTS

S E C T I O N I

GUINEA-PIG VAS DEFERENS

GENERAL FEATURES OF NEUROMUSCULAR TRANSMISSION IN THE GUINEA-PIG VAS DEFERENS

Neuromuscular transmission in the guinea-pig vas deferens was first examined with intracellular micro-electrodes by Burnstock & Holman (1961). The essential features of the transmission process are outlined below.

The guinea-pig vas deferens has a high stable resting membrane potential ranging from -45 to -76 mV (61.9 ± 5.0, Mean ± S.D., n = 635). The membrane response of a smooth muscle cell of the vas deferens recorded with an intracellular glass micro-electrode following hypogastric nerve stimulation (10 stimuli, 1 ms, 2 Hz, supramaximal voltage) is shown in Fig. 3.1. A single stimulus evoked a transient depolarisation of the cell membrane, the excitatory junction potential. The excitatory junction potentials in response to the first few stimuli showed a progressive increase in amplitude (facilitation). At frequencies greater than 1 Hz, successive excitatory junction potentials arose from the falling phase of the preceding excitatory junction potential (summation). As a result of facilitation and summation the cell was depolarised to threshold for initiation of a muscle action potential and the vas deferens contracted. Spontaneous excitatory junction potentials of varying degrees of prominence were recorded in every cell impaled.

In order to prevent muscle contraction from dislodging micro-electrodes from cells, subsequent experiments were carried out at a stimulation frequency of 0.91 Hz using submaximal stimulation (about

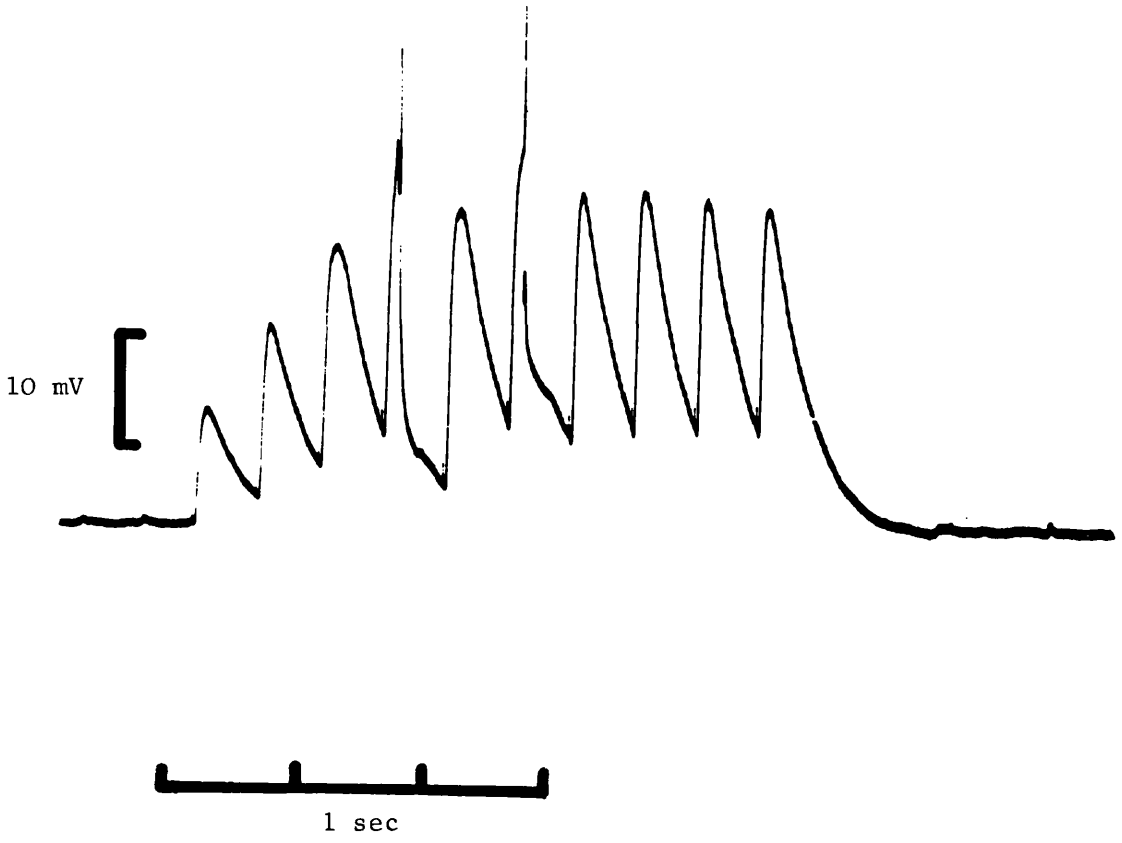

Fig. 3.1. Intracellular record of excitatory junction potentials and action potentials in a smooth muscle cell of the guinea-pig vas deferens. The hypogastric nerve was stimulated with a train of 10 stimuli at 2 Hz (1 msec, supramaximal voltage).

80-90% of maximum), when summation did not occur.

Initially, experiments were carried out to determine whether variation of the experimental conditions would alter the membrane response of the vas deferens to nerve stimulation.

THE EFFECT OF COOLING

The effect of cooling on the resting membrane potential

Cooling the vas deferens from 35°C to room temperature produced a slight ~~hyper~~depolarisation of the resting membrane potential. At 35°C, the resting membrane potential was 64.0 ± 0.4°C, n = 120 and at 22°C the resting membrane potential was 60.9 ± 0.5°C, n = 39. At room temperature, many more cells failed to seal (criterion 2, see Methods) after penetration by the micro-electrode. In one preparation, at 35°C,36 attempts were required to obtain 10 cells which fulfilled the criteria for successful cell penetration. When the vas was cooled to 22°C, 100 attempted impalements produced 7 cells which satisfied the criteria. Cooled cells commonly depolarised slowly after an initial rapid fall in potential following impalement by a micro-electrode. In these cells with lower resting membrane potentials the amplitude of the excitatory junction potentials recorded was often smaller than control excitatory junction potentials at 35°C.

The effect of cooling on the excitatory junction potential

At 35°C, the amplitude of excitatory junction potentials evoked by single submaximal stimuli to the hypogastric nerve was 5.1 ± 0.2 mV, n = 120. When the vas deferens was cooled to 22°C the amplitude of single excitatory junction potentials increased to 15.9 ± 0.5 mV, n = 29. The increase in excitatory junction potential amplitude was accompanied

by a marked change in the time course of the excitatory junction potential. The time to peak was 70.2 ± 1.7, n = 101 at 35°C and 118.3 ± 3.9, n = 30 at 22°C. The time to decay to half amplitude was prolonged from 241 ± 12, n = 30 at 35°C to 621 ± 18, n = 21 at 22°C. The effects were reversible by rewarming. Time course data expressed in msec.

The effects of cooling on trains of excitatory junction potentials

At 35°C, excitatory junction potentials were evoked by stimulation of the hypogastric nerve with trains of 10 stimuli at 0.91 Hz. The amplitude of the excitatory junction potentials characteristically showed facilitation to the first 6 to 8 stimuli and reached a plateau value of 17.5 mV by the eighth pulse of the train (Fig. 3.2). Summation did not occur.

At room temperature, summation of excitatory junction potentials always occurred with a stimulation frequency of 0.91 Hz and action potentials were initiated after two or three stimuli (Fig. 3.3).

When excitatory junction potentials were recorded from a single cell, over a temperature range of 35°C to 24°C, excitatory junction potential amplitude was increased at all points in the train. The time course of the first excitatory junction potential in a train was prolonged to a greater extent than that of subsequent excitatory junction potentials (Fig. 3.4).

A graph of the amplitude of the first, third and tenth excitatory junction potentials in a train was plotted against the temperature of the saline bathing the vas deferens. This graph showed that potentiation of excitatory junction potential amplitude was greatest at the beginning of a train (Fig. 3.5). This result was emphasised when the excitatory

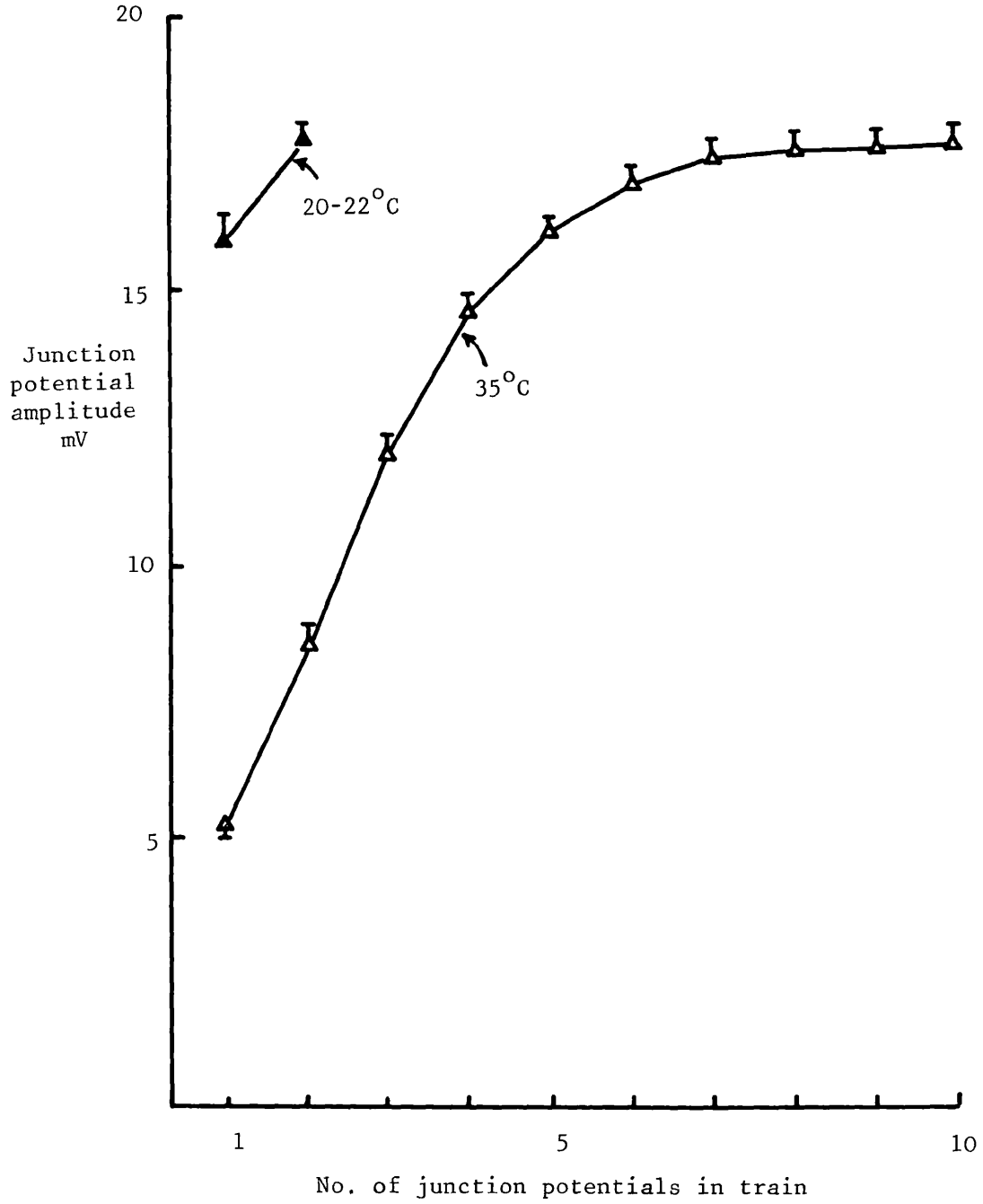

Fig. 3.2. The effect of temperature on excitatory junction potential amplitude in the guinea-pig vas deferens. Excitatory junction potentials were evoked by hypogastric nerve stimulation with trains of 10 stimuli at 0.91 Hz (1 msec, submaximal voltage). Only the first two excitatory junctional potentials in a train at 20-22°C are shown as an action potential was always initiated by the third.

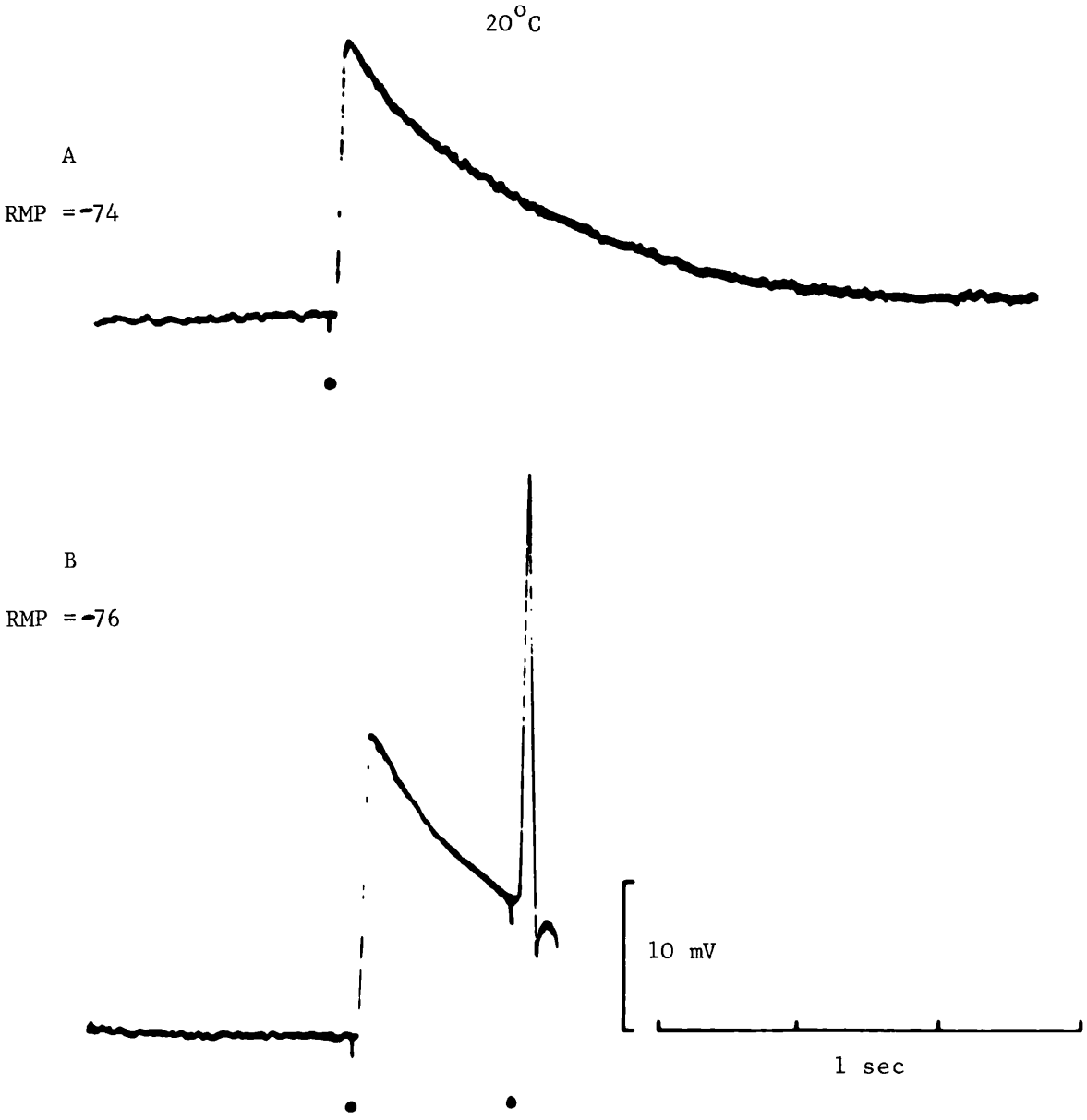

Fig. 3.3. Excitatory junction potentials and action potential evoked at room temperature in the guinea-pig vas deferens. Stimulation parameters A single pulse, B 2 pulses at 0.91 Hz (1 msec, submaximal voltage).

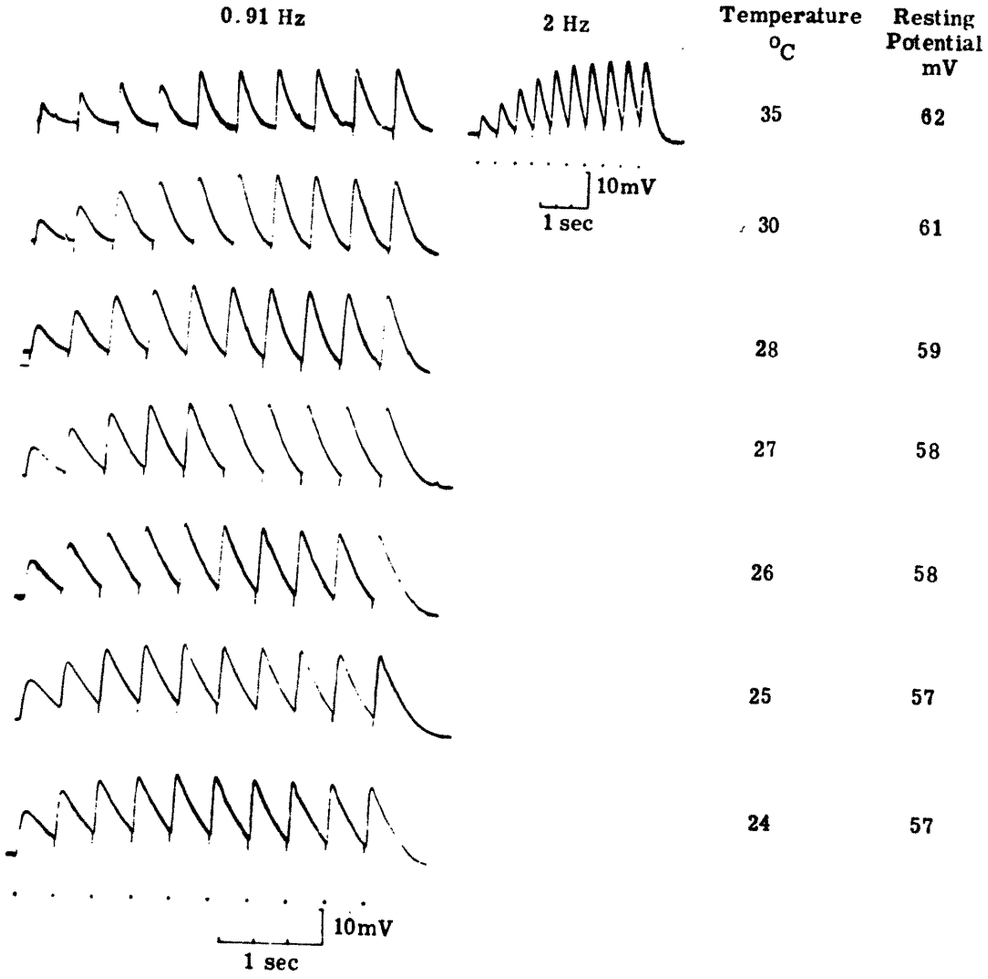

Fig. 3.4. The effect of temperature on excitatory junction potentials in the guinea-pig vas deferens (0.7msec, submaximal voltage).

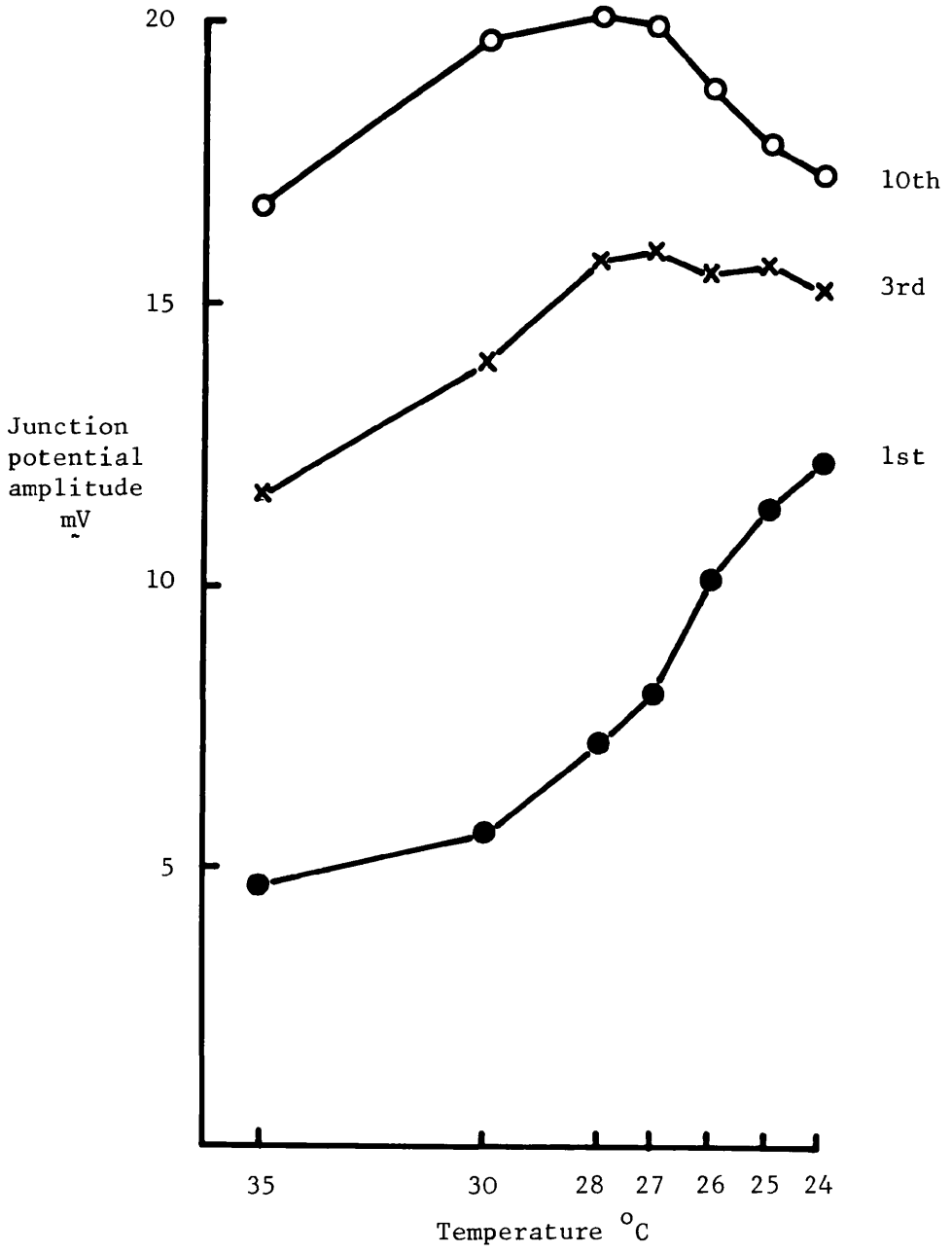

Fig. 3.5. The effect of temperature on the amplitude of the first, third and tenth excitatory junction potential evoked by stimulation of the hypogastric nerve to the guinea-pig vas deferens with a train of 10 pulses at 0.91 Hz (0.7 msec, submaximal voltage).

junction potential amplitude at various temperatures was expressed as a fraction of control excitatory junction potential amplitude at 35°C (Fig. 3.6).

The effect of cooling on facilitation

At 35°C, in response to nerve stimulation at 0.2 Hz to 2 Hz, the amplitude of the first excitatory junction potential in a train was normally smaller than that of subsequent excitatory junction potentials. Facilitation did not occur at temperatures below 22°C and the amplitude of the first excitatory junction potential in a train was often larger than that of subsequent excitatory junction potentials. At frequencies greater than 0.33 Hz, summation always occurred, excitatory junction potentials reached threshold and the vas contracted.

The effect of cooling on nerve action potentials

An explanation for the increased amplitude of the excitatory junction potential may be due to a change in threshold of fibres in the vas deferens nerve as a result of cooling. The electrical activity in a bundle of vas deferens nerve, following stimulation of the hypogastric nerve at 35°C and 22°C was recorded extracellularly. The stimulus strength was varied by altering the pulse width from 0.1 to 4.0 msec. There was an increase in latency on cooling but no detectable change in the number of fibres firing at both temperatures (Fig. 3.7). This result was expressed graphically when the area of each action potential volley was plotted as a fraction of the area of the maximal response (Fig. 3.8).

THE EFFECTS OF SOME DRUGS ON THE EXCITATORY JUNCTION POTENTIAL

The effects of some drugs which block the effects of adrenergic nerve stimulation in other preparations, were studied on trains of

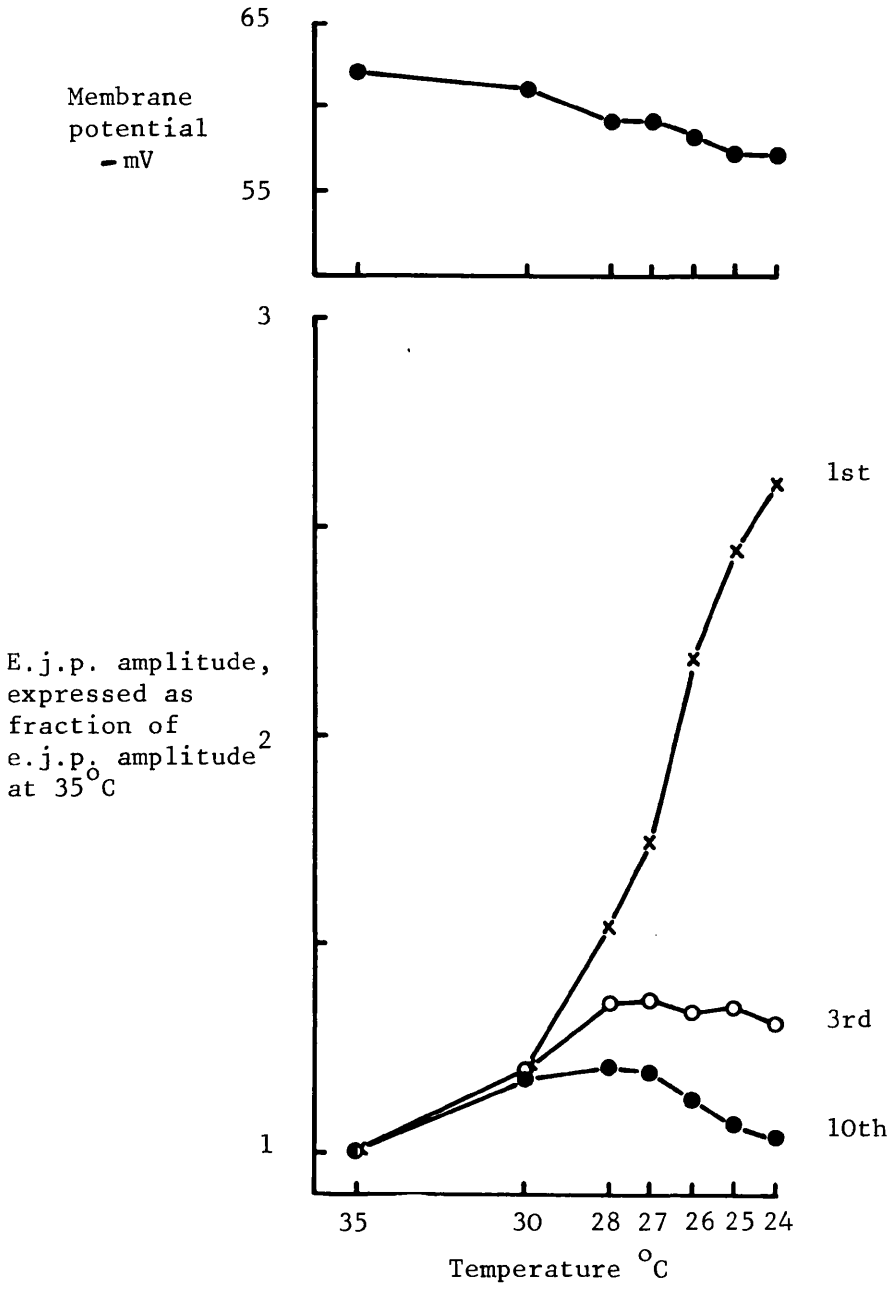

Fig. 3.6. The effect of temperature on the amplitude of the first, third and tenth excitatory junction potential (e.j.p.) evoked by stimulation of the hypogastric nerve to the guinea-pig vas deferens with a train of 10 pulses at 0.91 Hz (0.7 msec, submaximal voltage). E.j.p. amplitude is expressed in terms of the e.j.p. amplitude at 35°C. The top half of the figure shows the resting membrane potential recorded in the same cell.

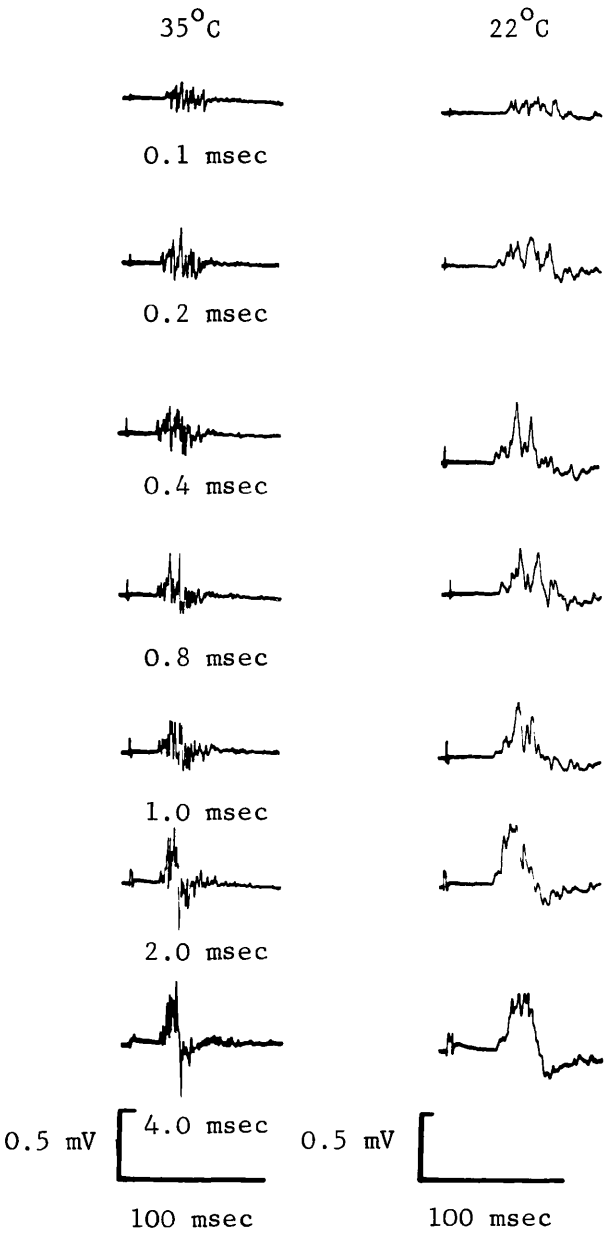

Fig. 3.7. The effects of temperature and pulse width on the activity in a bundle of vas deferens nerve following hypogastric nerve stimulation.

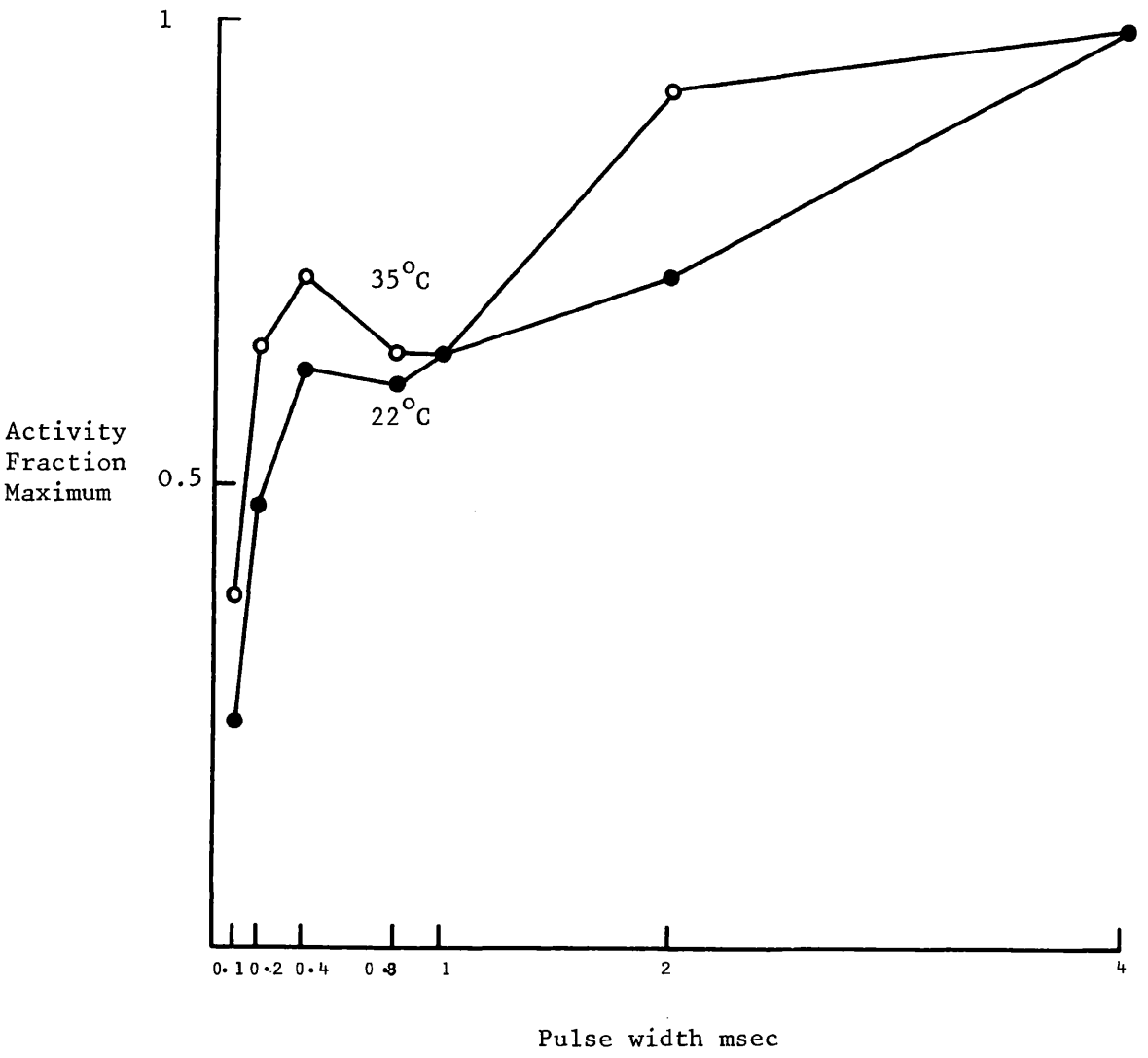

Fig. 3.8. Graph of the activity in a bundle of vas deferens nerve evoked at different stimulus strengths at 35°C and 22°C. The area of each action potential volley is expressed as a fraction of the maximal response at pulse width 4.0 msec.

excitatory junction potentials evoked at low frequency (0.91 Hz).

Effect of the adrenergic neuron blocker, amiodarone

Amiodarone blocks the effects of adrenergic nerve stimulation in the isolated blood perfused spleen of the cat, by prejunctional and post-junctional mechanisms (Blakeley & Summers, 1975).

In concentrations up to 3 x 10^{-5}M, amiodarone did not reduce the amplitude of trains of 10 excitatory junction potentials evoked at 0.91 Hz. There was significant potentiation of the first few excitatory junction potentials in a train (Fig. 3.9).

Effect of preferential post-junctional α-adrenoceptor antagonists

Azapetine and prazosin are preferential post-junctional α-adrenoceptor antagonists (Borowski *et al.*, 1977; Cambridge *et al.*, 1977; Doxey *et al.*, 1977). In concentrations, 10^{-9}M - 10^{-7}M, neither drug had any effect on the amplitude of excitatory junction potentials evoked at 0.91 Hz. In concentrations greater than 10^{-6}M, prazosin potentiated all excitatory junction potentials in a train (Fig. 3.10). Similar results were found using azapetine (10^{-6}M - 10^{-5}M) (Fig. 3.11).

Effect of piperoxan

Piperoxan is an equiactive antagonist at both pre- and post-junctional α-adrenoceptors (Blakeley & Summers, 1977; Starke, 1977). In the concentration range 10^{-8}M - 10^{-5}M piperoxan potentiated all excitatory junction potentials evoked by a train of 10 stimuli at 0.91 Hz. The most effective concentration of piperoxan was 7 x 10^{-7}M and the effects of piperoxan at this concentration were expressed graphically by plotting excitatory junction potential amplitude against the number of stimuli in a

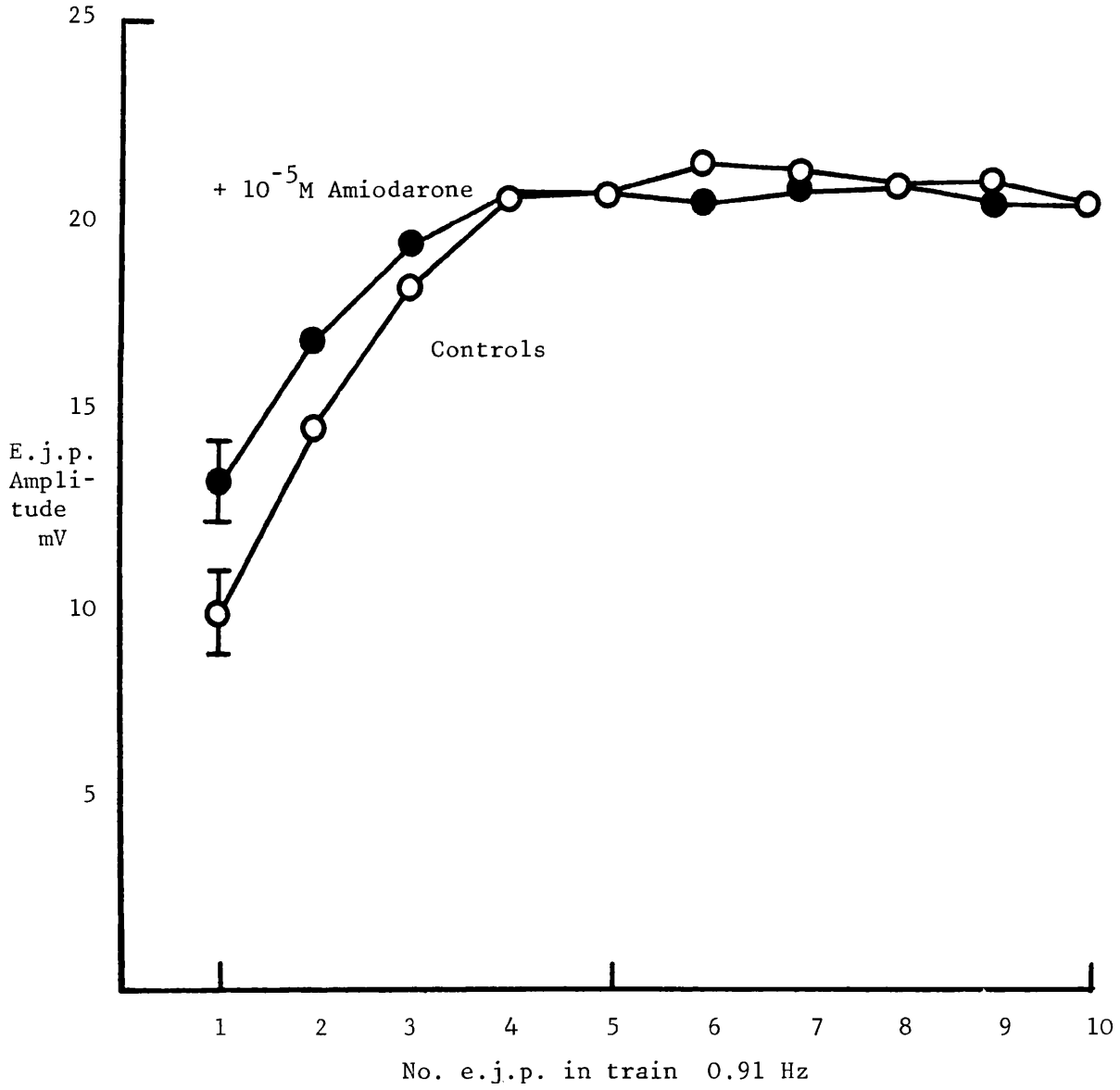

Fig. 3.9. The effects of amiodarone (10^{-5}M) on excitatory junction potentials (e.j.p.) evoked by hypogastric nerve stimulation at 0.91 Hz. o, Control; •, + amiodarone.

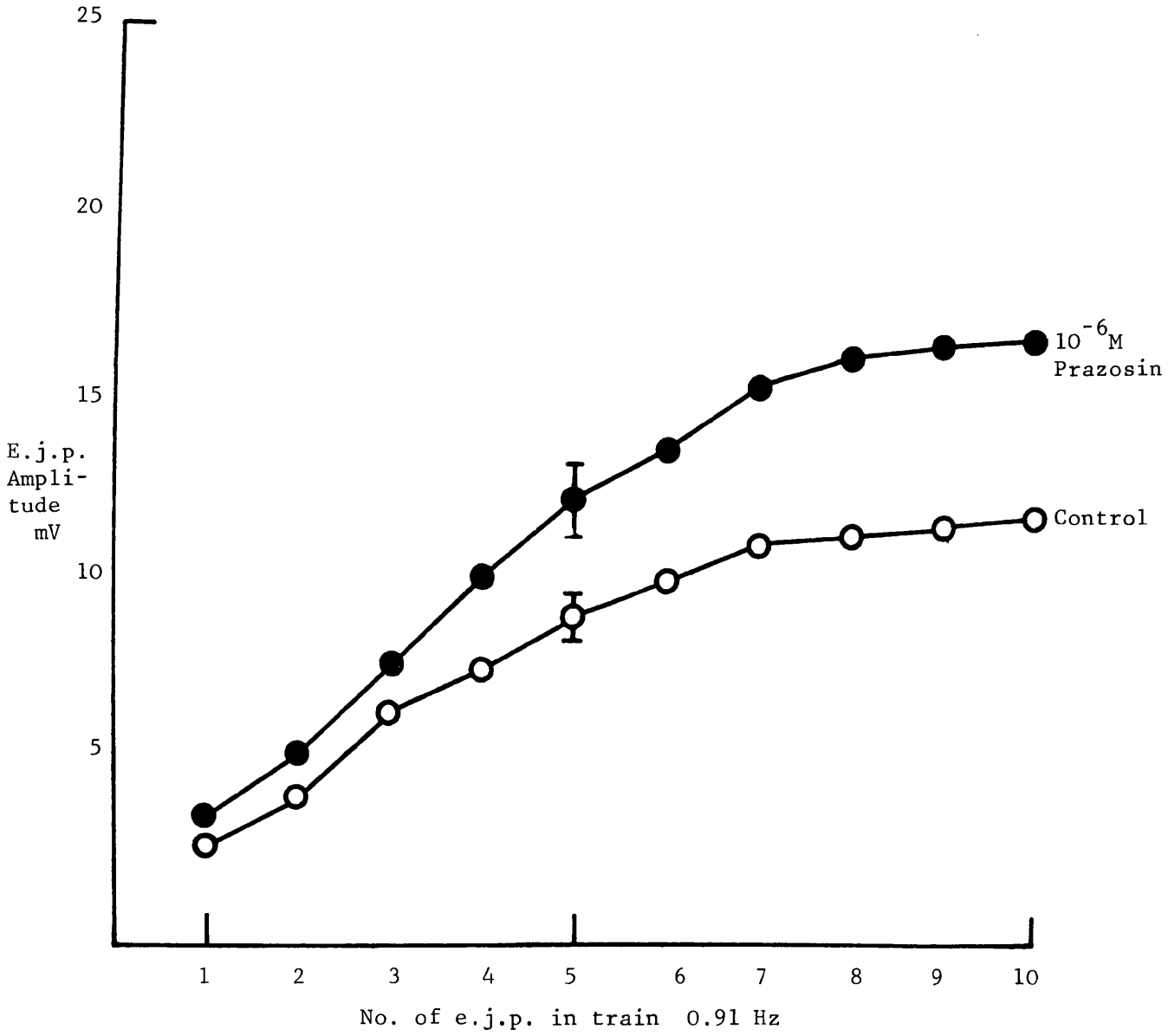

Fig. 3.10. The effects of prazosin on excitatory junction potentials (e.j.p.) evoked by hypogastric nerve stimulation at 0.91 Hz. o, Control; •, + prazosin.

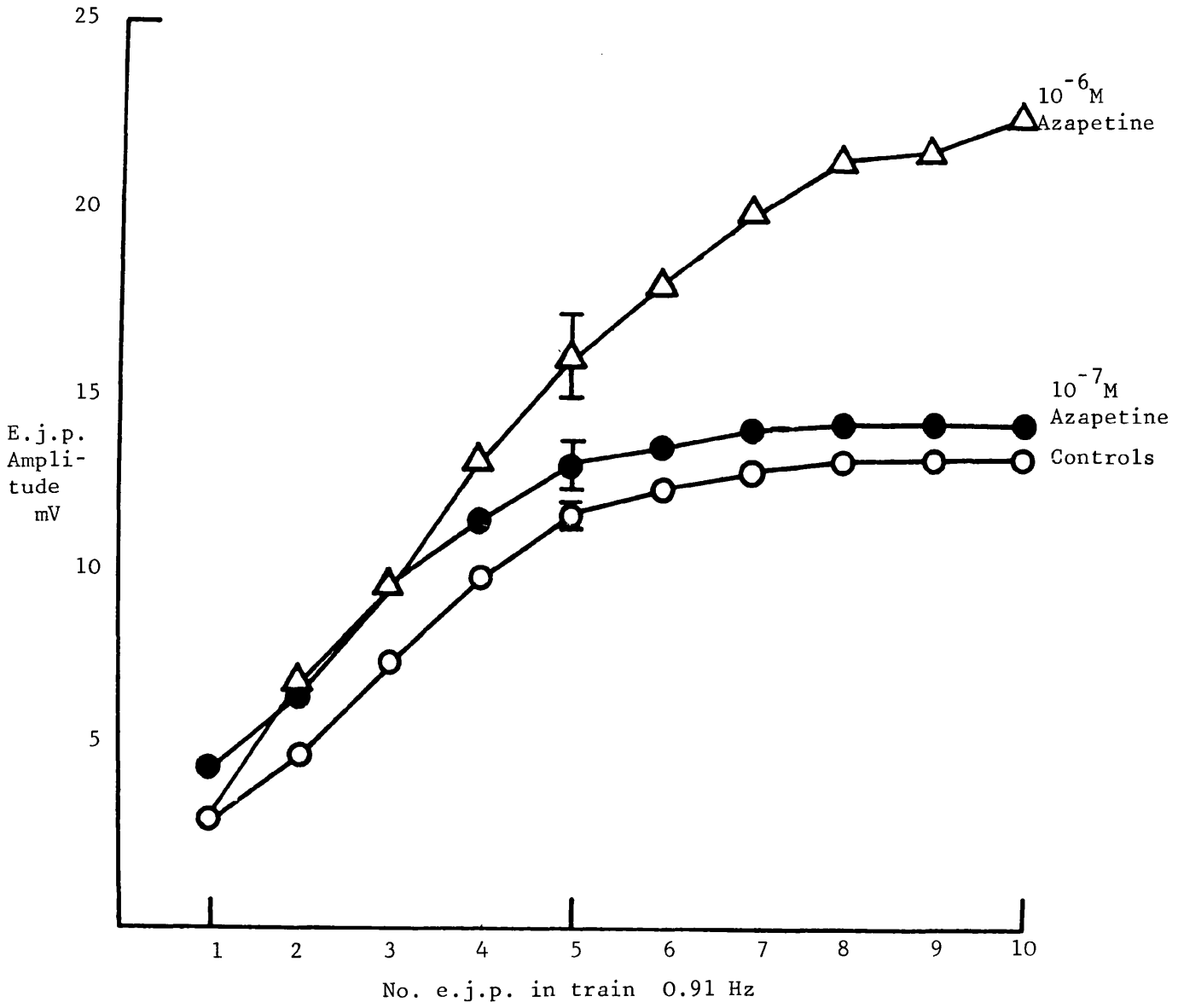

Fig. 3.11. The effects of azapetine (10^{-6}, 10^{-7}M) on excitatory junction potentials (e.j.p.) evoked by hypogastric nerve stimulation at 0.91 Hz. o, Control; •, + 10^{-7}M azapetine; Δ, + 10^{-6}M azapetine.

train at 0.91 Hz. Potentiation of excitatory junction potential amplitude was greatest towards the end of a train but the first excitatory junction potential in a train was potentiated significantly $P < 0.001$ (Fig. 3.12).

Effect of yohimbine

Yohimbine is a selective antagonist of prejunctional α-adrenoceptors (Starke *et al.*, 1975; Drew, 1976). The results obtained using yohimbine in the concentration range 10^{-8}M - 10^{-6}M were similar to those obtained using piperoxan (supra vide).

Effects of phenoxybenzamine

Phenoxybenzamine is an α-adrenoceptor antagonist, an uptake 1 blocker and an inhibitor of extraneuronal uptake (uptake 2) (see Westfall, 1977). Phenoxybenzamine is the most effective drug known at elevating the overflow of adrenergic transmitter following nerve stimulation (see Starke, 1977; Westfall, 1977). The two most important mechanisms of this effect are thought to be inhibition of noradrenaline uptake and α-adrenoceptor blockade. Combinations of other α-adrenoceptor and specific uptake blockers, do not increase stimulation induced release of noradrenaline to the same extent as phenoxybenzamine alone. This suggested that phenoxybenzamine may increase adrenergic transmitter release in response to nerve stimulation by some additional unknown mechanism.

It was of interest to study the effects of phenoxybenzamine on neuromuscular transmission in the guinea-pig vas deferens where the transmitter may not be noradrenaline (Ambache & Zar, 1971).

Trains of excitatory junction potentials were evoked by hypogastric nerve stimulation at 0.91 Hz. In control cells, excitatory junction potentials

showed facilitation to the first 6 to 8 stimuli and reached a plateau value. After a few minutes exposure to phenoxybenzamine (3×10^{-5}M), excitatory junction potentials reached threshold after 3 stimuli and action potentials were initiated. With prolonged exposure (20-50 min) excitatory junction potentials became subthreshold after 6 stimuli and were eventually reduced in amplitude and prolonged in time course with a notable plateau on the excitatory junction potential. These effects of phenoxybenzamine are shown in Fig. 3.13. They cannot be attributed to uptake blockade since inhibitors of neuronal and extra-neuronal uptake have little or no effect on the excitatory junction potential (Bell, 1967b). Since other α-adrenoceptor blockers increase the amplitude of the excitatory junction potential, this effect may be due to α-adrenoceptor block. Unlike other α-adrenoceptor antagonists, phenoxybenzamine did not increase the amplitude of the first excitatory junction potential in a train at 0.91 Hz. The effects of phenoxybenzamine (3×10^{-5}M) in a single cell are shown in Fig. 3.14. Note the maintained firing of action potentials throughout the train after the first few stimuli.

Phenoxybenzamine had one additional action which was quite unique to this compound, the ability to induce repetitive activity on the excitatory junction potential after the first few stimuli in a train at 0.91 Hz (Fig. 3.15). This action may be related to an increase in transmitter release by phenoxybenzamine.

Effect of β-adrenoceptor antagonist

Sotalol, a β-adrenoceptor antagonist without β stimulant activity or membrane stabilising effects (Barrett & Carter, 1970) had no effect on excitatory junction potentials evoked at 0.91 Hz in the concentration range 10^{-8}M - 10^{-5}M.

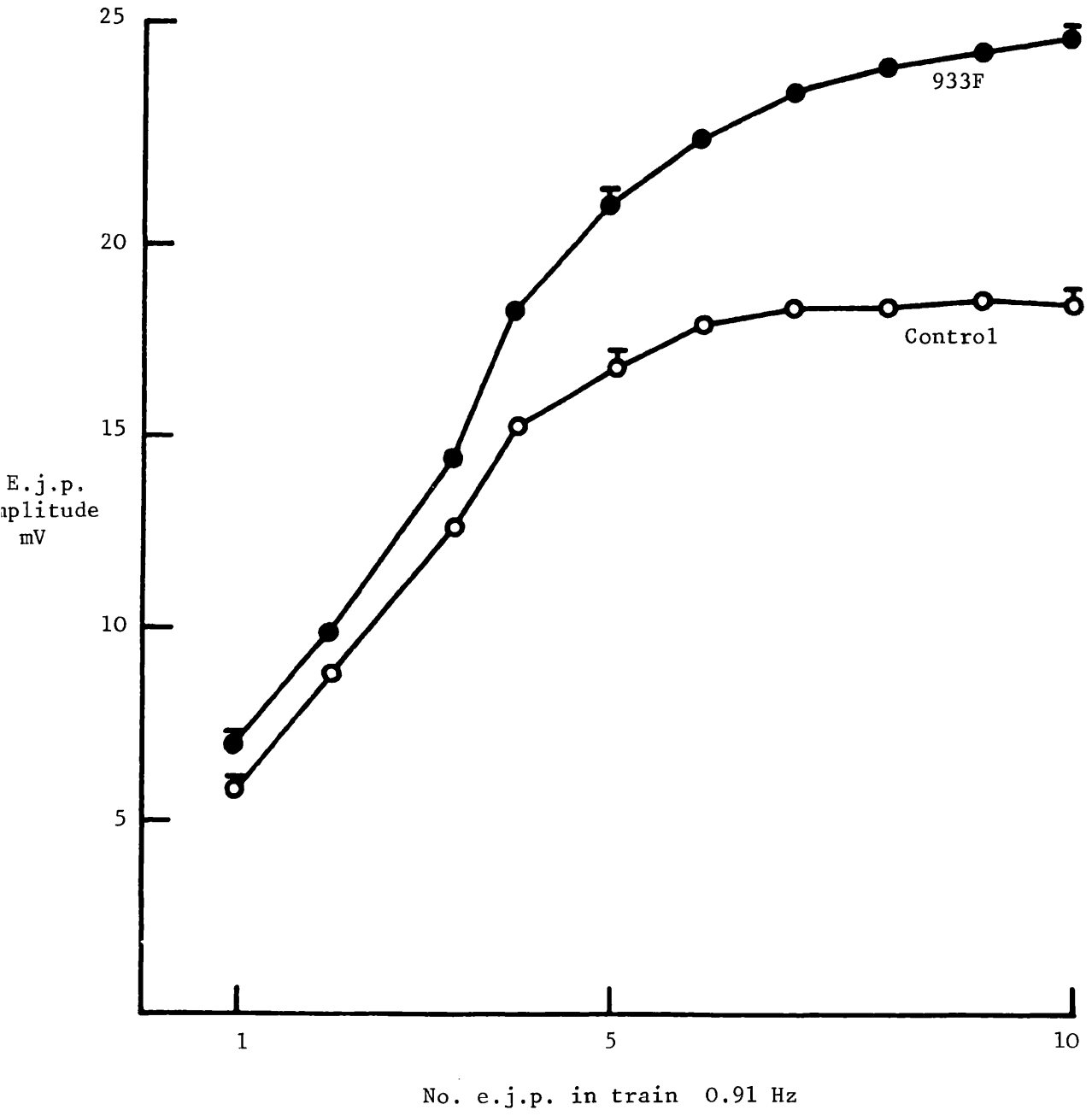

Fig. 3.12. The effect of piperoxan (933F) 7 x 10^{-7}M on excitatory junction potentials (e.j.p.) evoked by hypogastric nerve stimulation at 0.91 Hz. o, Control; •, + 933F.

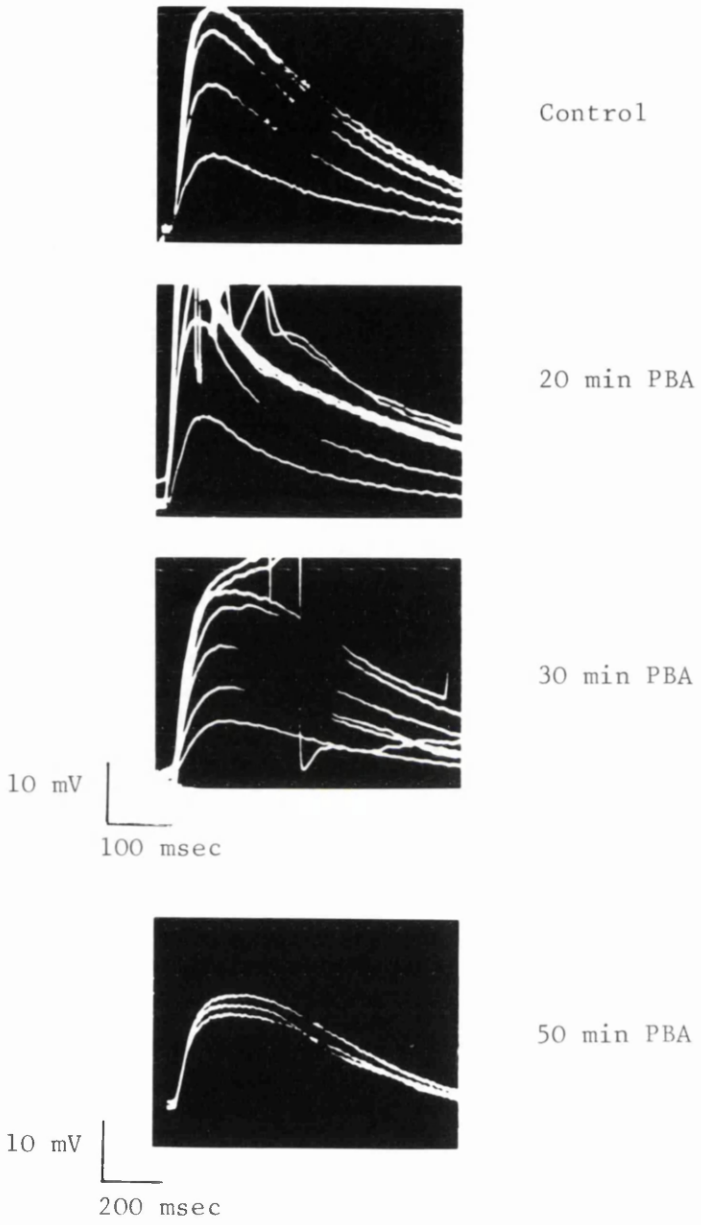

Fig. 3.13. The effects of phenoxybenzamine (PBA) (3×10^{-5}M) on excitatory junction potentials (e.j.p.s) evoked by hypogastric nerve stimulation at 0.91 Hz. Top 3 records show superimposed facilitating e.j.p.s before and after prolonged exposure to PBA. Bottom record shows last 3 e.j.p.s in a train of 10 at 0.91 Hz, 50 min after PBA. Note the change in the calibration time scale.

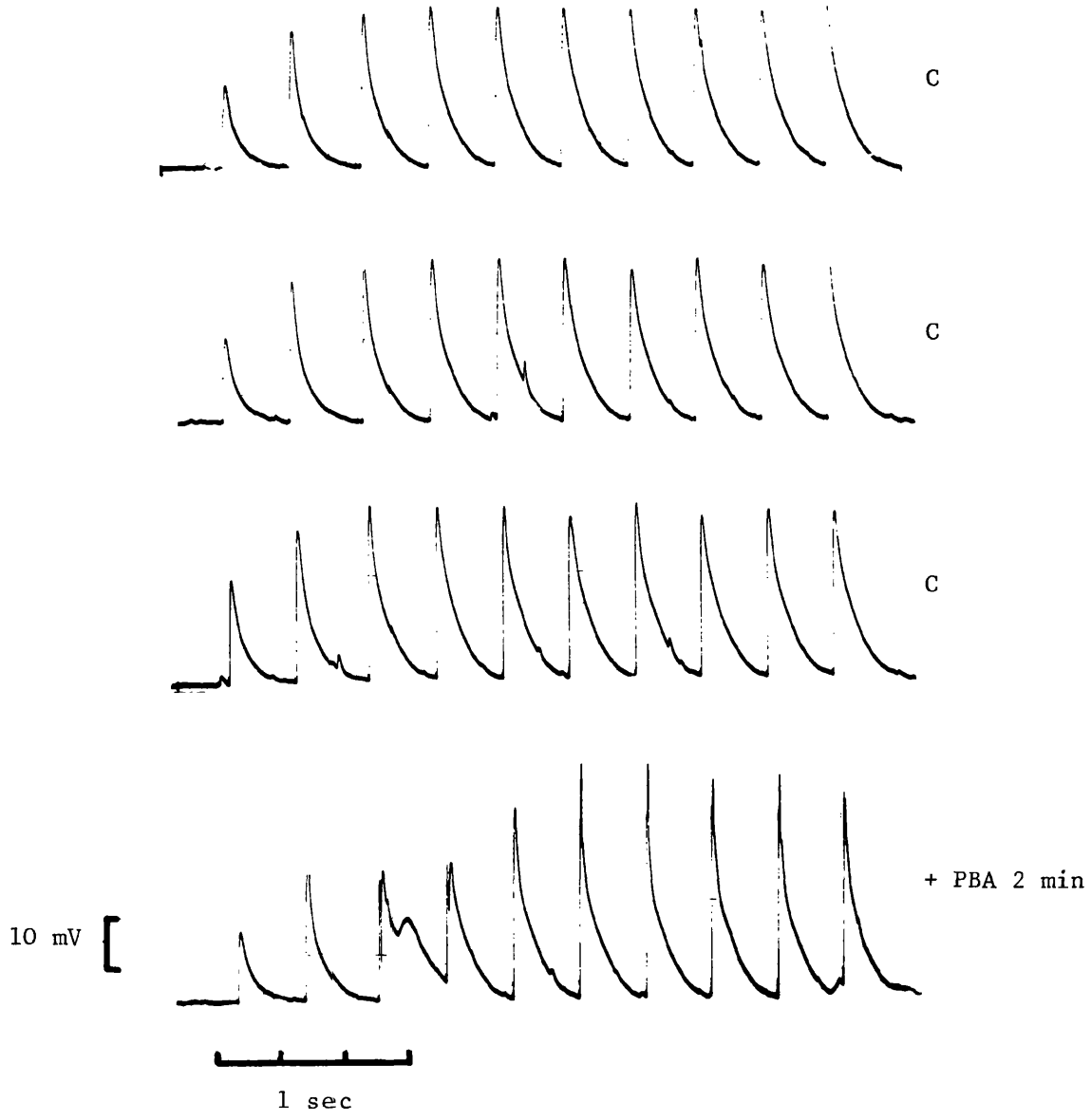

Fig. 3.14. The effects of phenoxybenzamine (PBA) (3×10^{-5}M) on excitatory junction potentials evoked by hypogastric nerve stimulation at 0.91 Hz. C - 3 control trains of 10 stimuli. PBA 2 min - same cell two minutes after phenoxybenzamine.

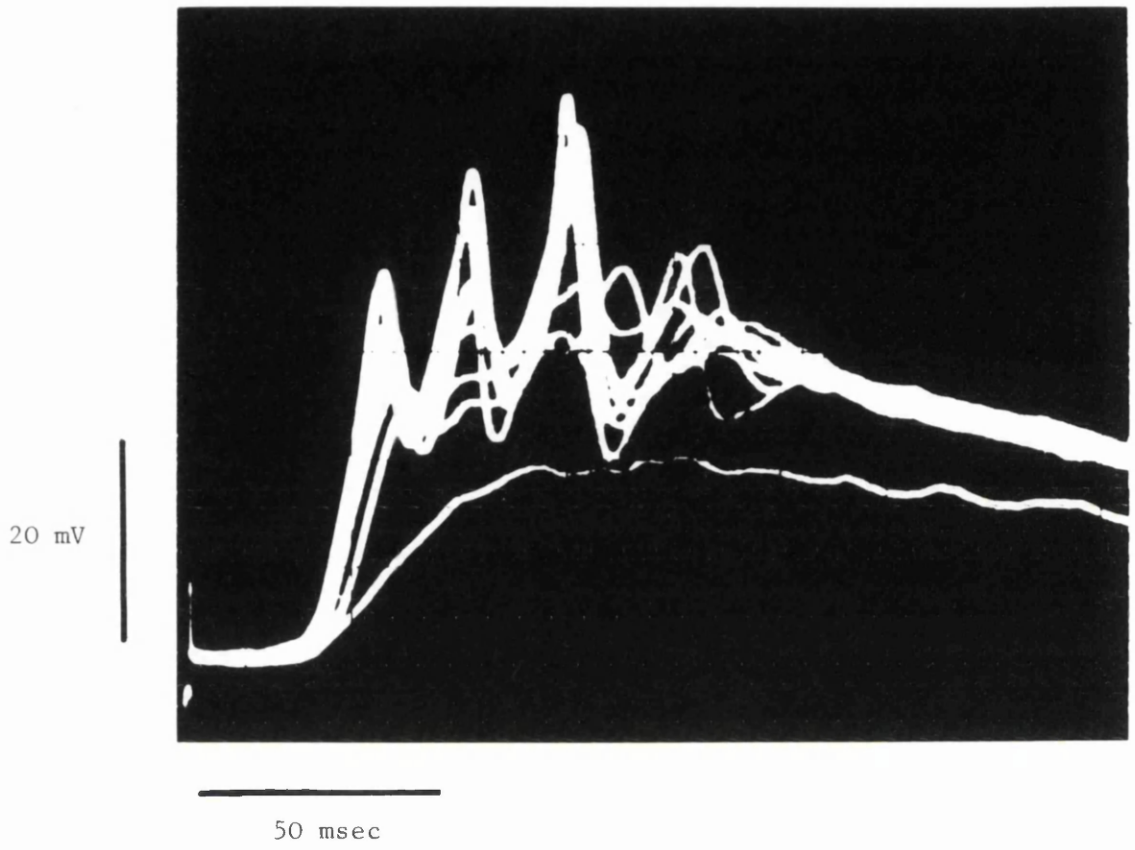

Fig. 3.15. The effects of phenoxybenzamine (3×10^{-5}M, 20 min) on the membrane response of a smooth muscle cell of the guinea-pig vas deferens. Record shows 8 superimposed excitatory junction potentials (e.j.p.) evoked by hypogastric nerve stimulation at 0.91 Hz (first two e.j.p.s in train not shown). Note repetitive electrical activity on e.j.p.s after the first few stimuli.

Effect of piperoxan on nerve action potentials

All drugs which potentiated excitatory junction potential amplitude had at least one property in common, the ability to block α-adrenoceptors. As submaximal stimulation was used it was possible that these drugs changed the threshold in the nerves thus explaining excitatory junction potential augmentation as a change in the number of nerve fibres activated per stimulus.

Piperoxan was chosen for a detailed study of the mechanism of enhancement of the excitatory junction potential. The effects of piperoxan (7×10^{-7}M) on the activity recorded extracellularly in a fine bundle of vas deferens nerve following hypogastric nerve stimulation was investigated. There was no detectable change in the fractional activity of nerve fibres responding after piperoxan (Fig. 3.16).

Effects of preferential prejunctional α-adrenoceptor agonists

If augmentation of the excitatory junction potential by α-adrenoceptor antagonists is due to α-adrenoceptor blockade, then one might expect the excitatory junction potential to be susceptible to alteration by drugs which stimulate α-adrenoceptors. Clonidine, a preferential prejunctional α-adrenoceptor agonist (Starke, 1977; Westfall, 1977) produced a concentration dependent (10^{-9}M - 10^{-6}M) inhibition of all excitatory junction potentials in a train at 0.91 Hz. This result was expressed graphically by plotting excitatory junction potential amplitude against the number of stimuli in a train at 0.91 Hz, in the presence of different concentrations of clonidine (Fig. 3.17).

At all concentrations of clonidine, when excitatory junction potential amplitude was depressed, facilitation still occurred at 0.91 Hz and at

higher frequencies summation also occurred. Clonidine had no effect on the resting membrane potential in the concentrations used.

Clonidine was more effective at reducing excitatory junction potential amplitude at the beginning of a train of stimuli at 0.91 Hz than at the end. The ED_{50} for clonidine for the first, third and tenth excitatory junction potential in a train evoked at 0.91 Hz was 7.7 x 10^{-8}M, 9.2 x 10^{-8}M and 27.7 x 10^{-8}M respectively. The first, third and tenth excitatory junction potentials were examined so that the neurotransmission could be studied in response to a single stimulus, during facilitation, and when the excitatory junction potential reached a plateau during a train of stimuli.

At a concentration of 3 x 10^{-9}M clonidine potentiated the first few excitatory junction potentials in a train evoked at 0.91 Hz. At all other concentrations clonidine had no effect or reduced excitatory junction potential amplitude.

When excitatory junction potentials were abolished by high concentrations of clonidine (10^{-7}M - 10^{-6}M) it was still possible to record spontaneous excitatory junction potentials in many cells.

The effects of lysergic acid diethylamide, which has been shown to be a selective prejunctional α-agonist in the vas deferens (Drew, 1977; Doxey *et al.*, 1977) was also investigated. Lysergic acid diethylamide like clonidine produced a concentration dependent (10^{-8}M - 10^{-6}M) inhibition of all excitatory junction potentials in a train at 0.91 Hz. Unlike clonidine, the amplitude of excitatory junction potentials towards the end of a train 'escaped' the block. The ED_{50} for the first excitatory junction potential was 4.6 x 10^{-8}M and 34.2 x 10^{-8}M for the tenth excitatory junction potential in a train.

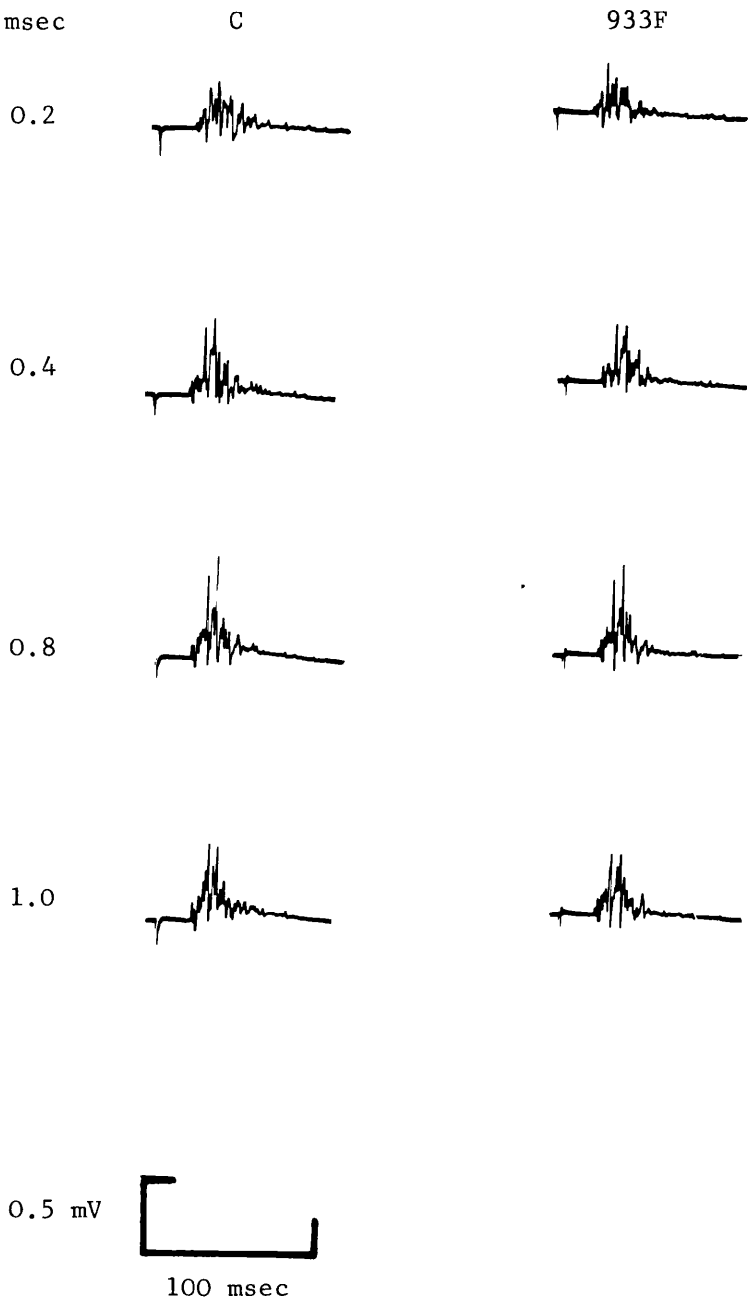

Fig. 3.16. The effects of piperoxan (933F) on the activity in a bundle of vas deferens nerve following hypogastric nerve stimulation. The stimulus strength was varied by altering the pulse width.

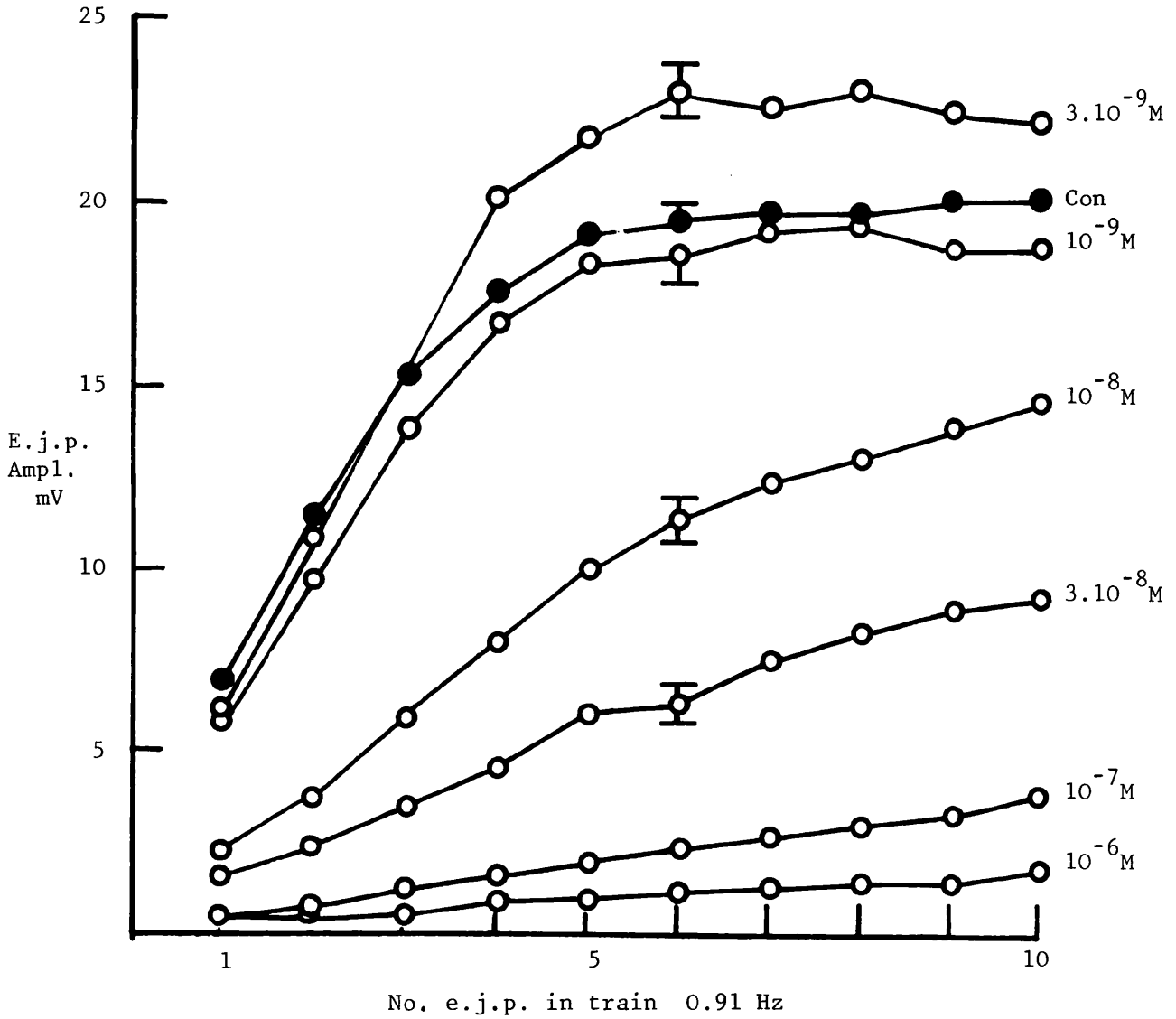

Fig. 3.17. The effects of increasing concentrations of clonidine on excitatory junction potentials (e.j.p.) evoked by hypogastric nerve stimulation at 0.91 Hz. ●, Control; o, + clonidine.

When excitatory junction potential amplitude was reduced by lysergic acid diethylamide or clonidine, the time course of the excitatory junction potential was altered; the excitatory junction potential was rounded and prolonged. This effect of clonidine at a concentration of 3×10^{-8}M can be observed in the top panel of Fig. 3.18.

Interaction of α-adrenoceptor agonists and antagonists

If α-adrenoceptor agonists reduce and α-adrenoceptor antagonists enhance excitatory junction potential amplitude via α-adrenoceptors then one would expect such drugs to interact.

The interaction of clonidine (α-adrenoceptor agonist) and piperoxan (α-adrenoceptor antagonist) was studied by observing the effects of piperoxan on excitatory junction potentials depressed by the prior administration of clonidine. At a concentration of 3×10^{-8}M clonidine reduced to about 40% of control, the amplitude of excitatory junction potentials evoked at 0.91 Hz. Piperoxan (7×10^{-7}M) reversed clonidine induced depression of excitatory junction potentials to all stimuli in the train. As early as 30 sec after perfusion of the preparation with piperoxan has begun, a change in the time course of the excitatory junction potential was observed. Excitatory junction potentials became larger and spikey with a progressive shortening of the time to peak during the train. After 2 min exposure to piperoxan, excitatory junction potential amplitude returned to and often exceeded the original control value. The results in a single cell of one such experiment are shown in Fig. 3.18.

It was not always possible to obtain data from single cells. Excitatory junction potentials were recorded therefore from series of cells before, during and after the administration of drugs. Results

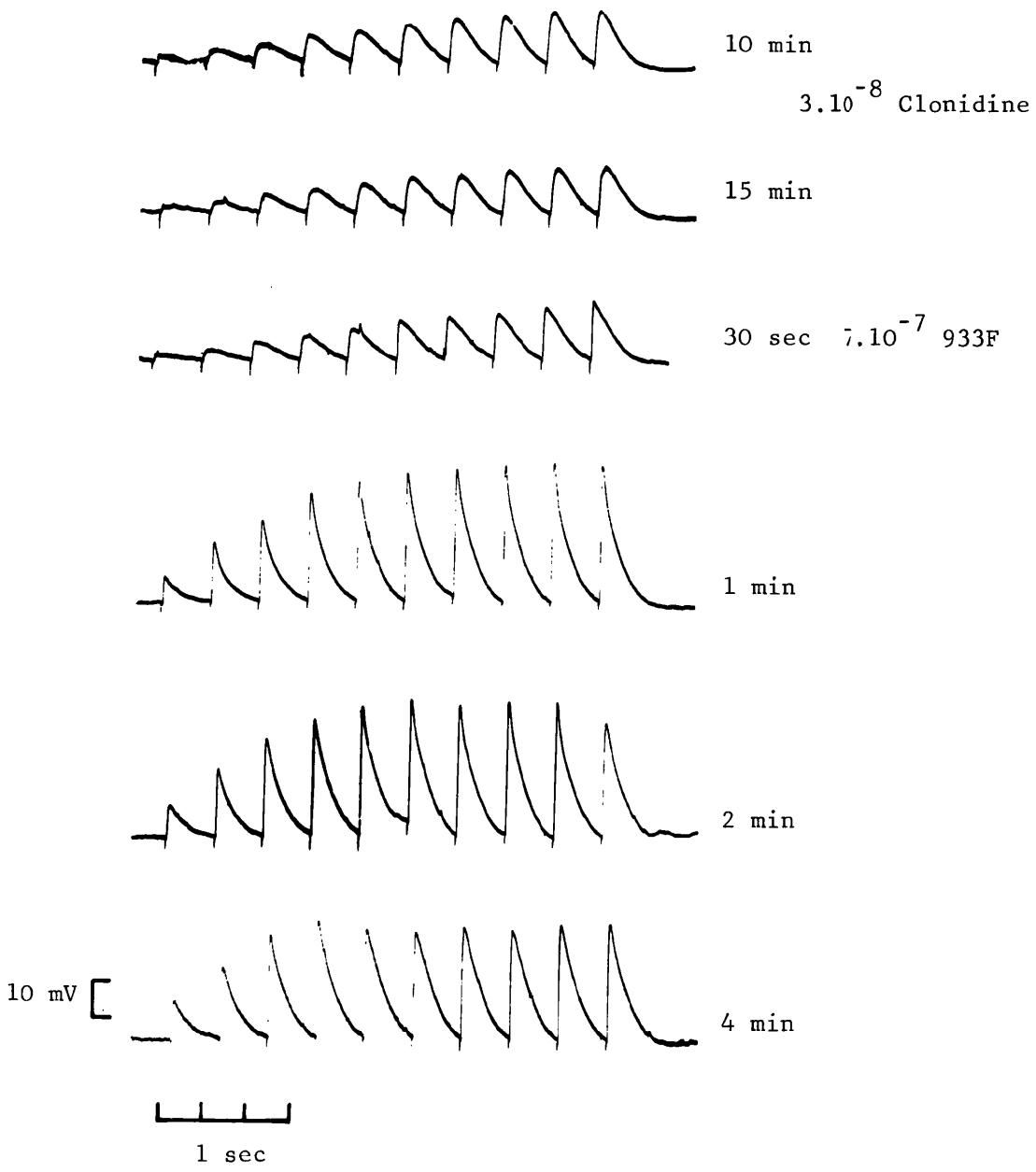

Fig. 3.18. Intracellular records from a single cell of the guinea-pig vas deferens showing the effects of clonidine and clonidine plus piperoxan (933F) on trains of 10 excitatory junction potentials (e.j.p.) evoked at 0.91 Hz. Top two records show e.j.p.s whose amplitude has been reduced to about 40% of control by clonidine (3×10^{-8}M). Bottom four records show the time course of the reversed effects of 933F on e.j.p.s depressed by clonidine.

were expressed graphically as mean excitatory junction potential amplitude versus the position of the excitatory junction potential in a train at 0.91 Hz. The results of a clonidine/piperoxan and lysergic acid diethylamide/azapetine interaction are shown graphically in Figs. 3.19 and 3.20 respectively.

The mechanism of interaction of α-adrenoceptor agonists and antagonists

The mechanism of interaction was investigated using the classical pharmacological technique of a shift in the dose response curve.

The effects of increasing concentrations of clonidine on trains of 10 excitatory junction potentials evoked at 0.91 Hz, before and in the presence of piperoxan (7×10^{-7}M) was examined. Dose response curves for the first, third and tenth excitatory junction potential in a train were constructed as follows; the effects of increasing concentrations of clonidine on trains of 10 excitatory junction potentials, evoked at 0.91 Hz, before and in the presence of piperoxan (7×10^{-7}M) was examined. Excitatory junction potential amplitude was expressed as percentage depression of control excitatory junction potential amplitude by clonidine and percentage depression of excitatory junction potential amplitude by clonidine in the presence of piperoxan.

Piperoxan induced a shift to the right of the dose response curve without a change in the maximum for all excitatory junction potentials in a train at 0.91 Hz. This result indicated that the interaction was competitive and that a common receptor was involved.

The ED_{50} for depression of the first excitatory junction potential with clonidine was 0.77×10^{-8}M and in the presence of clonidine and piperoxan 2.8×10^{-8}M, for the third excitatory junction potential, 1.1×10^{-8}M and 4.0×10^{-8}M and tenth excitatory junction potential

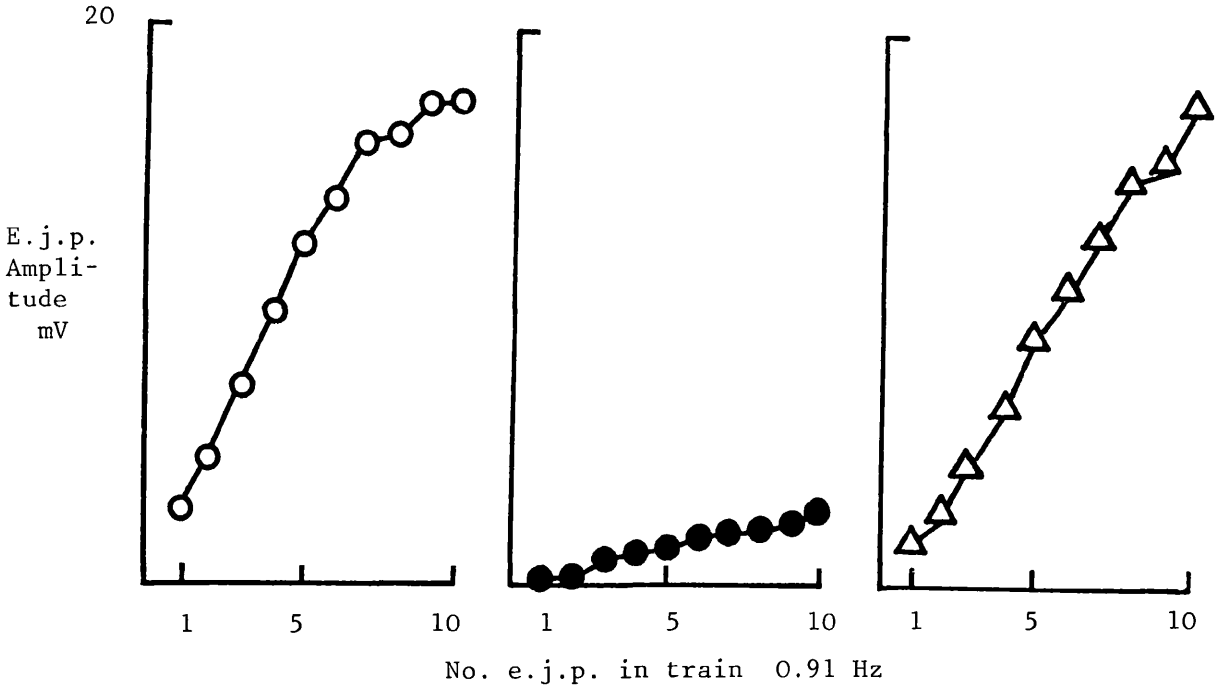

Fig. 3.19. The effect of clonidine (10^{-7}M) and clonidine in the presence of piperoxan (7×10^{-7}M) on excitatory junction potentials (e.j.p.s) evoked by hypogastric nerve stimulation at 0.91 Hz.
o, Control; •, + clonidine; Δ, + clonidine and piperoxan.

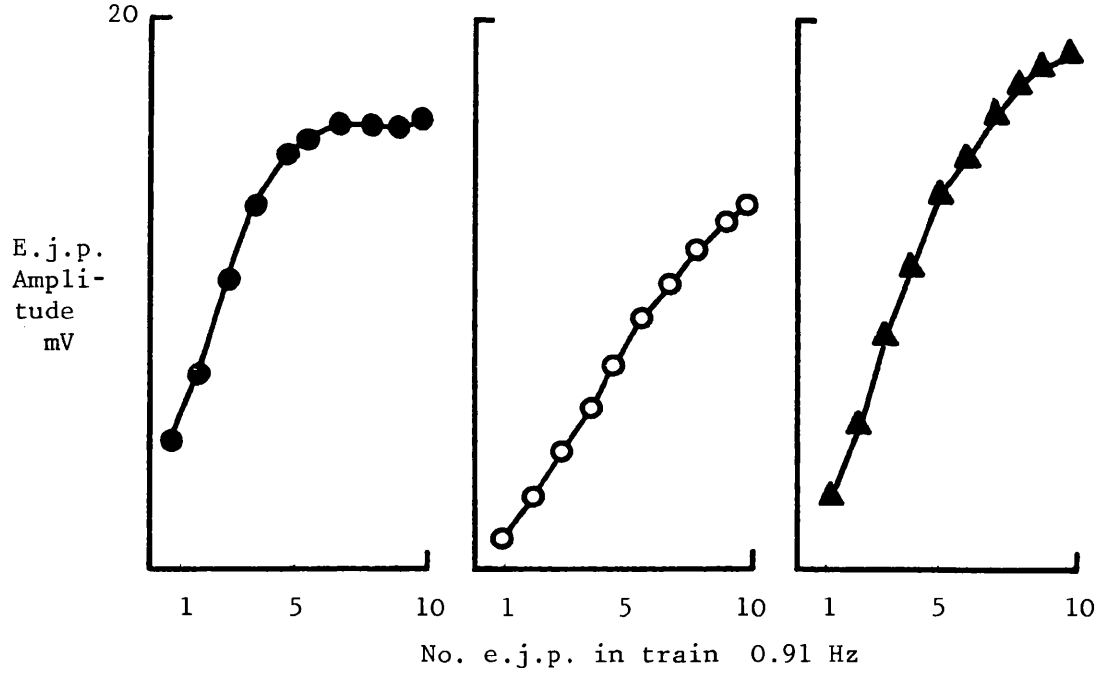

Fig. 3.20. The effect of lysergic acid diethylamide (10^{-7}M) and lysergic acid diethylamide in the presence of azapetine (10^{-6}M) on excitatory junction potentials (e.j.p.s) evoked by hypogastric nerve stimulation at 0.91 Hz. ●, Control; o, + lysergic acid diethylamide; ▲, + lysergic acid diethylamide and azapetine.

2.5×10^{-8}M and 6.9×10^{-8}M respectively. There was no significant change in the slope of the lines. Dose response curves for the first and tenth excitatory junction potential are shown in Fig. 3.21.

The depressant effects of clonidine and lysergic acid diethylamide and the depressant effect of these drugs in the presence of piperoxan (7×10^{-7}M) on the first, third and tenth excitatory junction potentials in a train at 0.91 Hz are summarized in Table 1.

The effects of prostaglandin E_1

Prostaglandin E_1 (1-10 ng/ml) had no effect on the resting membrane potential of smooth muscle cells but produced a concentration dependent inhibition of excitatory junction potentials. This result was expressed graphically by plotting the amplitudes of 10 excitatory junction potentials evoked at 0.91 Hz against the number of stimuli in the train in the presence of increasing concentrations of prostaglandin E_1 (Fig. 3.22). Prostaglandin E_1 (10 ng/ml) completely abolished excitatory junction potentials but spontaneous excitatory junction potentials were still recorded in the same cell. The effects of prostaglandin E_1 were reversible by washing (Fig. 3.23).

The time course of excitatory junction potential block by prostaglandin E_1 in a single cell was also studied. Trains of 10 excitatory junction potentials at 0.91 Hz were evoked at one minute intervals. Prostaglandin E_1 (10 ng/ml) was then added to the saline perfusing the vas deferens. Depression of excitatory junction potentials in the train occurred progressively, complete abolition occurring after ten minutes exposure to prostaglandin E_1. The effects of prostaglandin E_1 (10 ng/ml) in a single cell are shown in Fig. 3.24.

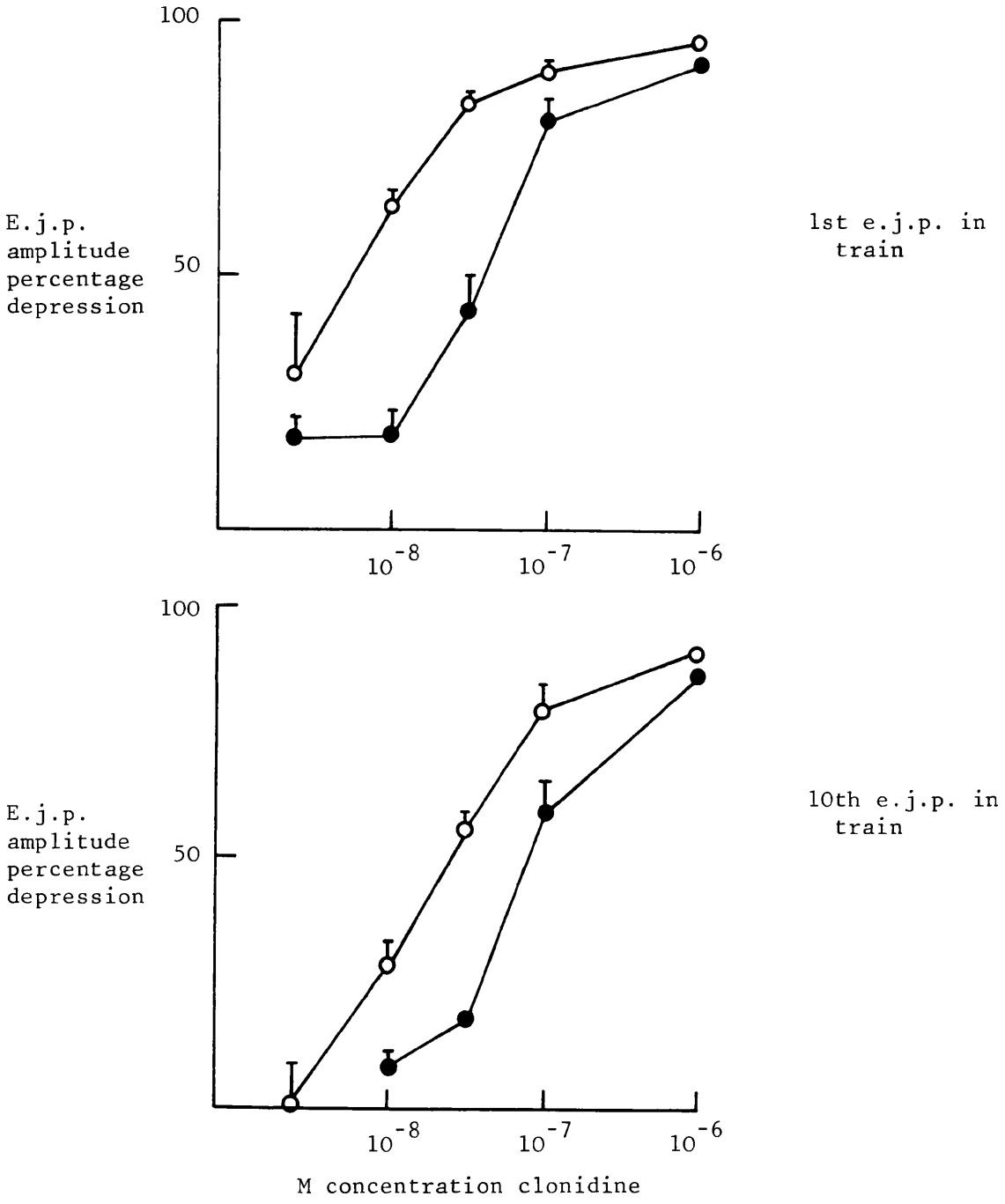

Fig. 3.21. Antagonism by piperoxan (7×10^{-7}M) of the effects of clonidine (3×10^{-9}M - 10^{-7}M) on the amplitude of the first and tenth excitatory junction potential (e.j.p.) in a train at 0.91 Hz. E.j.p. amplitude is expressed as percentage depression of the control amplitude.

Table 1. Depression of excitatory junction potentials (e.j.p.s) by clonidine and lysergic acid diethylamide (L.S.D.) and the effects of piperoxan (933F) (7×10^{-7}M) on the depression. Data is expressed as the concentration of drug which produced 50% inhibition of control e.j.p. amplitude (ED_{50}), with the appropriate confidence limits at $P < 0.05$ level of significance (C.L.).

Position of e.j.p. in train at 0.91 Hz	Clonidine		Clonidine + 933F		L.S.D.		L.S.D. + 933F	
	ED_{50}	C.L.	ED_{50}	C.L.	ED_{50}	C.L.	ED_{50}	C.L.
1st	0.77	1.6	2.8	1.2	4.6	1.9	22.9	2.0
3rd	1.1	1.4	4.0	1.5	7.4	2.0	29.2	2.0
10th	2.5	1.3	6.9	1.5	34.2	3.4	66.7	4.2

ALL DATA x 10^{-8}M

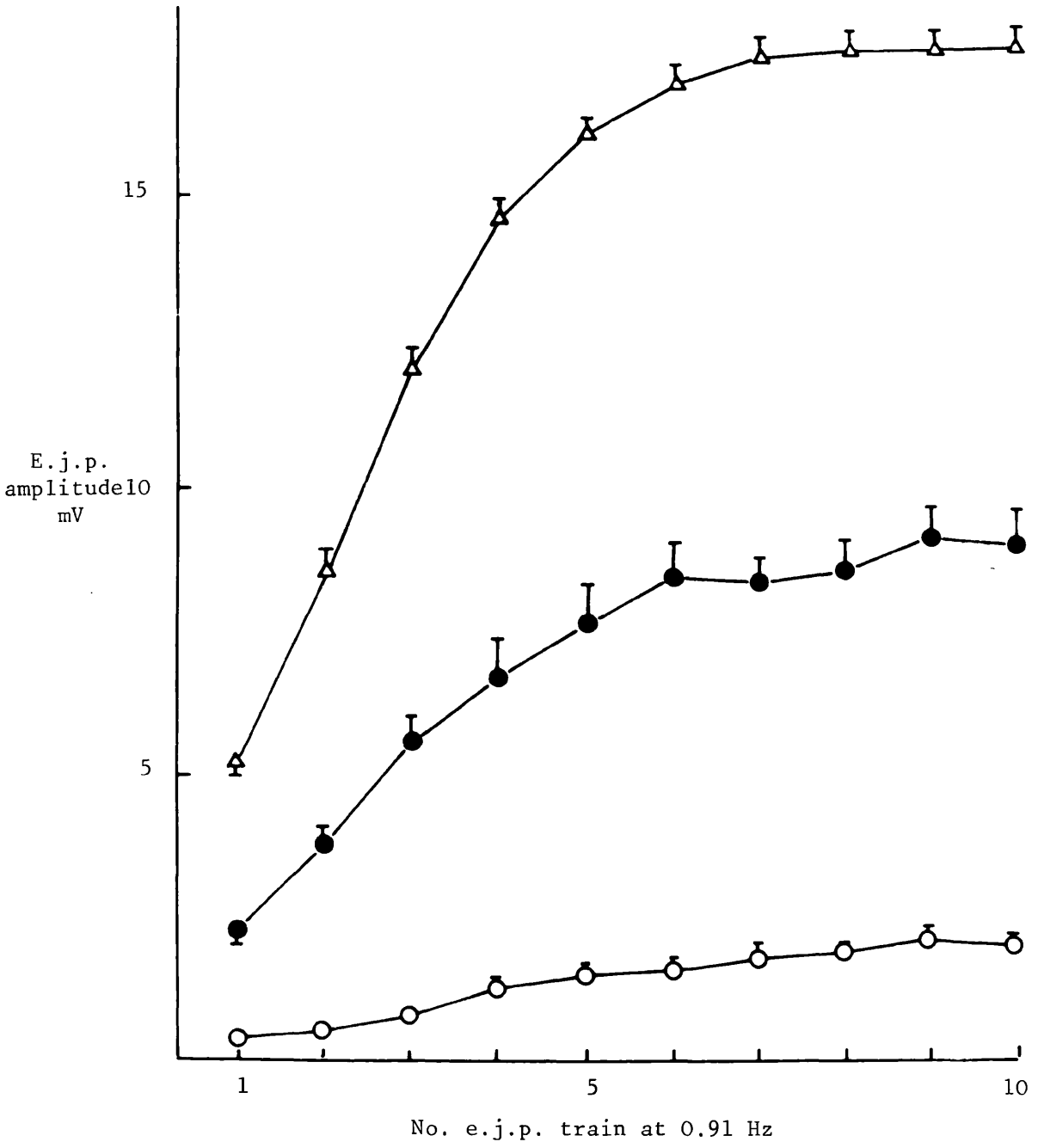

Fig. 3.22. The effects of increasing concentrations of prostaglandin E_1 on the amplitude of excitatory junction potentials evoked by hypogastric nerve stimulation at 0.91 Hz. Δ, Control; •, PGE_1 0.3 ng/ml; o, PGE_1 3 ng/ml.

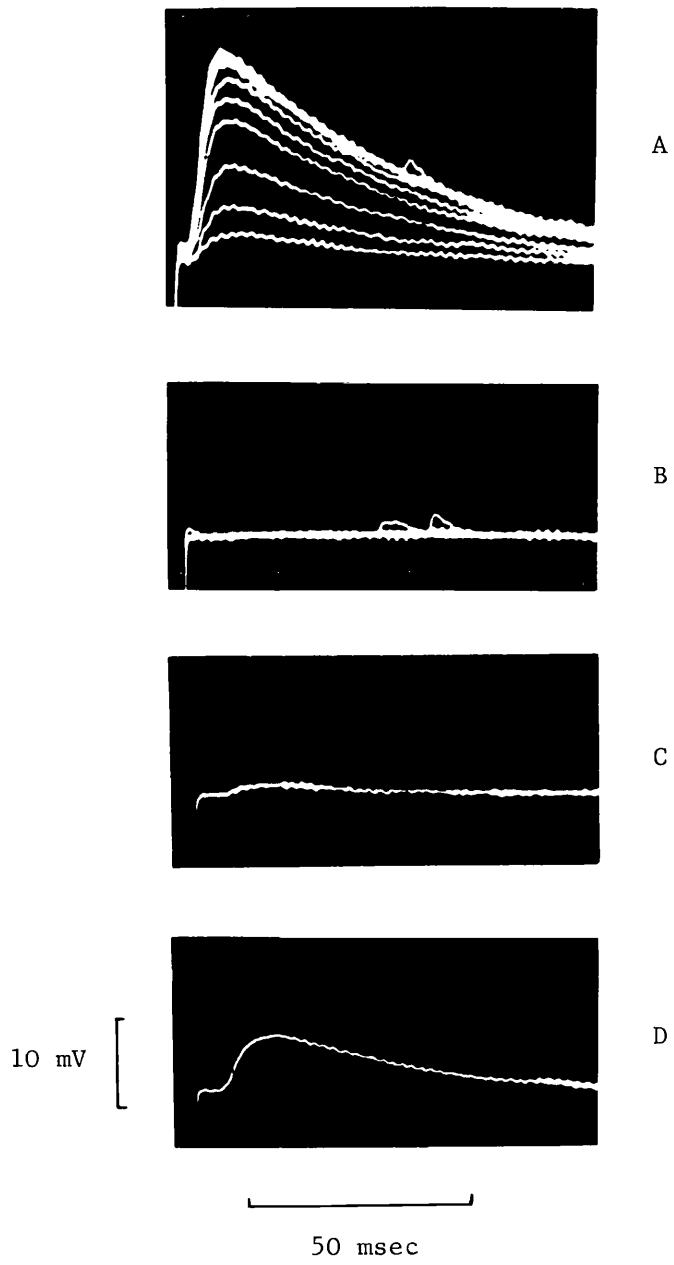

Fig. 3.23. The effect of prostaglandin E_1 (PGE_1) in a single cell on excitatory junction potentials (e.j.p.) evoked by hypogastric nerve stimulation at 0.91 Hz. A, Control showing 10 superimposed facilitating e.j.p.s; B, Same cell, 7 min after PGE_1 (10 ng/ml); C,D, Last two e.j.p.s in a train of 10 at 0.91 Hz, 5 min and 15 min after wash.

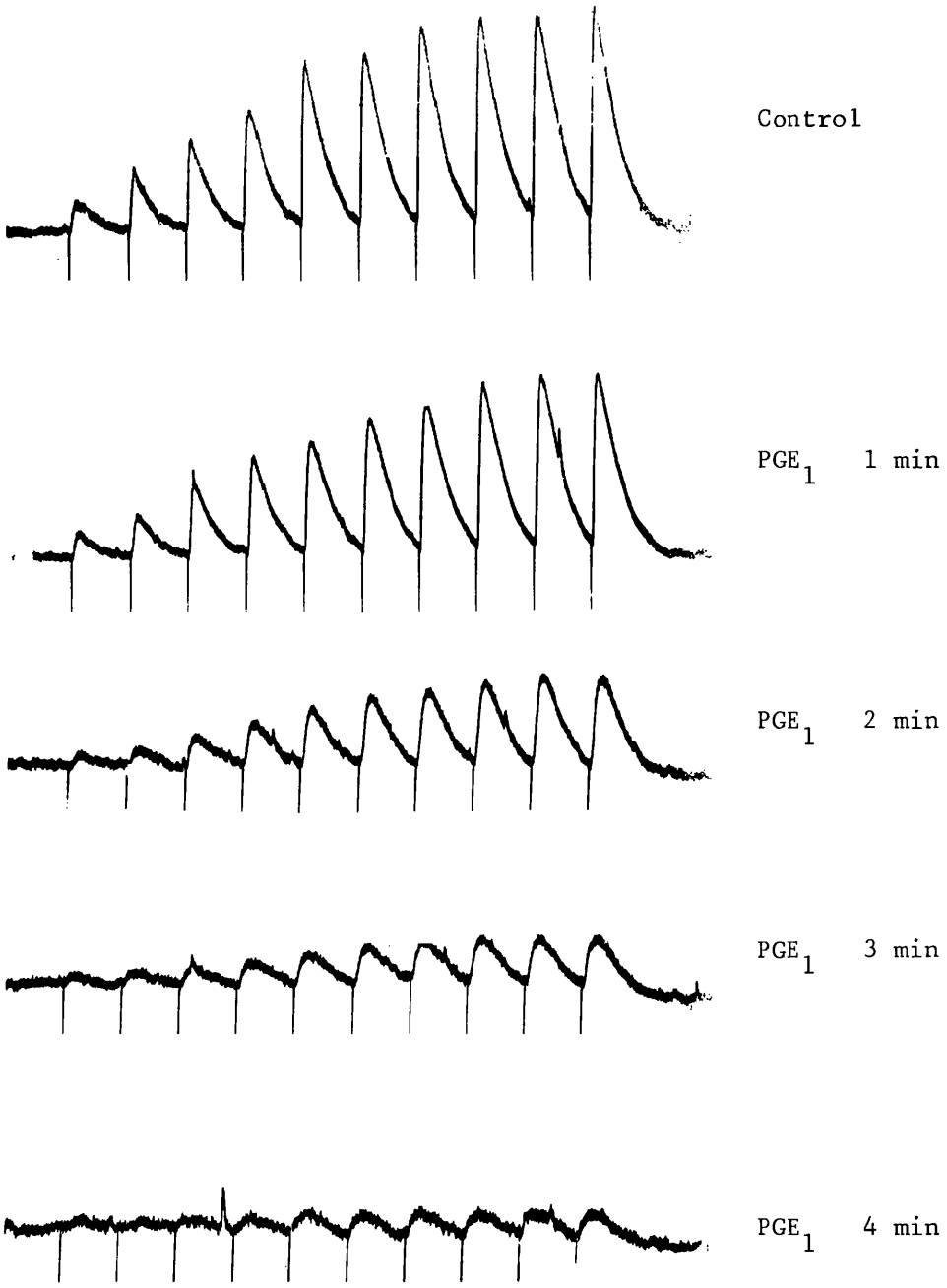

Fig. 3.24. The time course of the depressant effect of prostaglandin E_1 (PGE_1) on excitatory junction potentials (e.j.p.s) in a single cell of the guinea-pig vas deferens. The hypogastric nerve was excited at 1 min intervals with trains of 10 submaximal stimuli at 0.91 Hz. The saline perfusing the preparation was replaced with saline containing PGE_1 10 ng/ml after one set of control stimuli. There was no change in the resting membrane potential which was 64 mV. Downward stroke preceding each e.j.p. is the stimulus artifact.

The effect of increasing the frequency of hypogastric nerve stimulation but keeping the number of stimuli (10) constant in a cell where excitatory junction potentials were partially blocked by the prior administration of prostaglandin E_1 (1 ng/ml) was studied. Although the amplitude of the first excitatory junction potential in a train was reduced, facilitation and summation of excitatory junction potentials occurred. At frequencies of stimulation of 10 Hz, excitatory junction potentials reached threshold and the vas contracted (Fig. 3.25). The inhibitory effects of prostaglandin E_1 on the excitatory junction potential cannot be prevented by the prior administration of, or reversed by, the α-adrenoceptor antagonists, yohimbine or piperoxan.

The effect of prostaglandin E_1 on the mechanical response of the guinea-pig vas deferens

Prostaglandin E_2 is more effective at blocking the mechanical response of the guinea-pig vas elicited by short rather than long trains of stimuli (Ambache & Zar, 1971). This has been interpreted as a selective action of prostaglandin E_2 on an unknown excitatory transmitter which is responsible for the early 'twitch' part of a response.

Vasa deferentia were stimulated via the hypogastric nerve with either a train of 10 or 100 pulses at 10 Hz. Prostaglandin E_1 was added to the organ bath in a final concentration of 10 ng/ml. Prostaglandin E_1 markedly reduced the twitch amplitude elicited by 10 stimuli (Fig. 3.26 A) but had no effect on the maximum amplitude evoked by 100 stimuli (Fig. 3.26 B). Reference to Fig. 3.26 A & B shows that an early fast component of the mechanical response has been blocked by prostaglandin E_1.

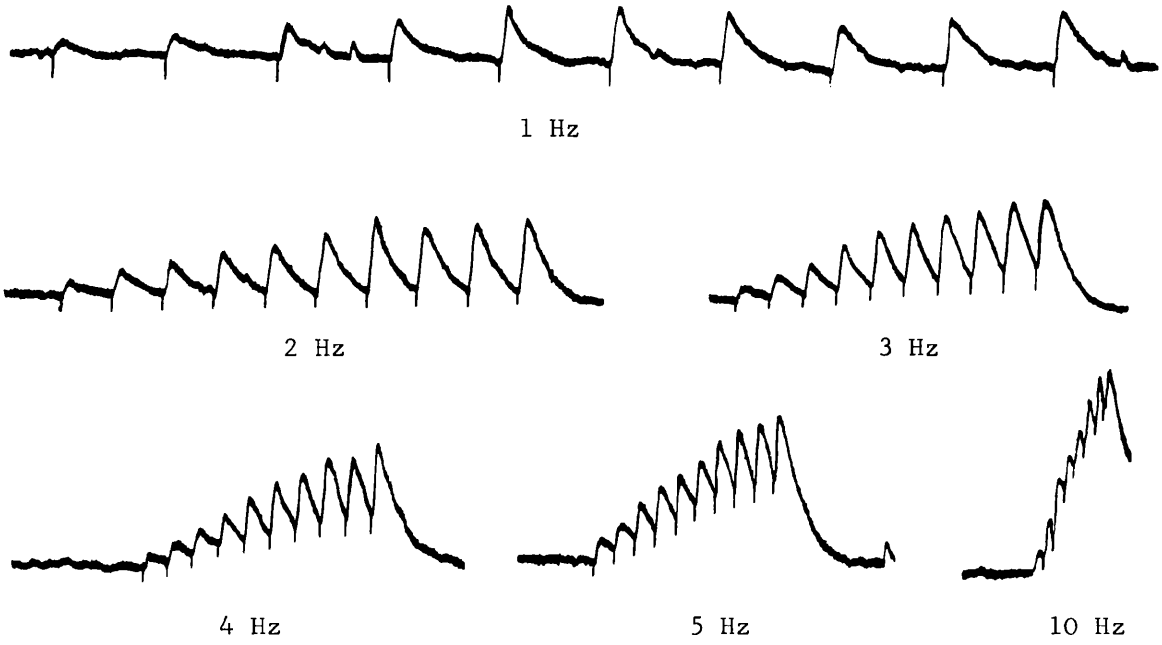

Fig. 3.25. The effect of prostaglandin E_1 (3 ng/ml) on the membrane response of a single cell of the guinea-pig vas deferens to increasing frequency of hypogastric nerve stimulation (10 pulses, supramaximal voltage). Downward stroke preceding each excitatory junction potential is the stimulus artifact.

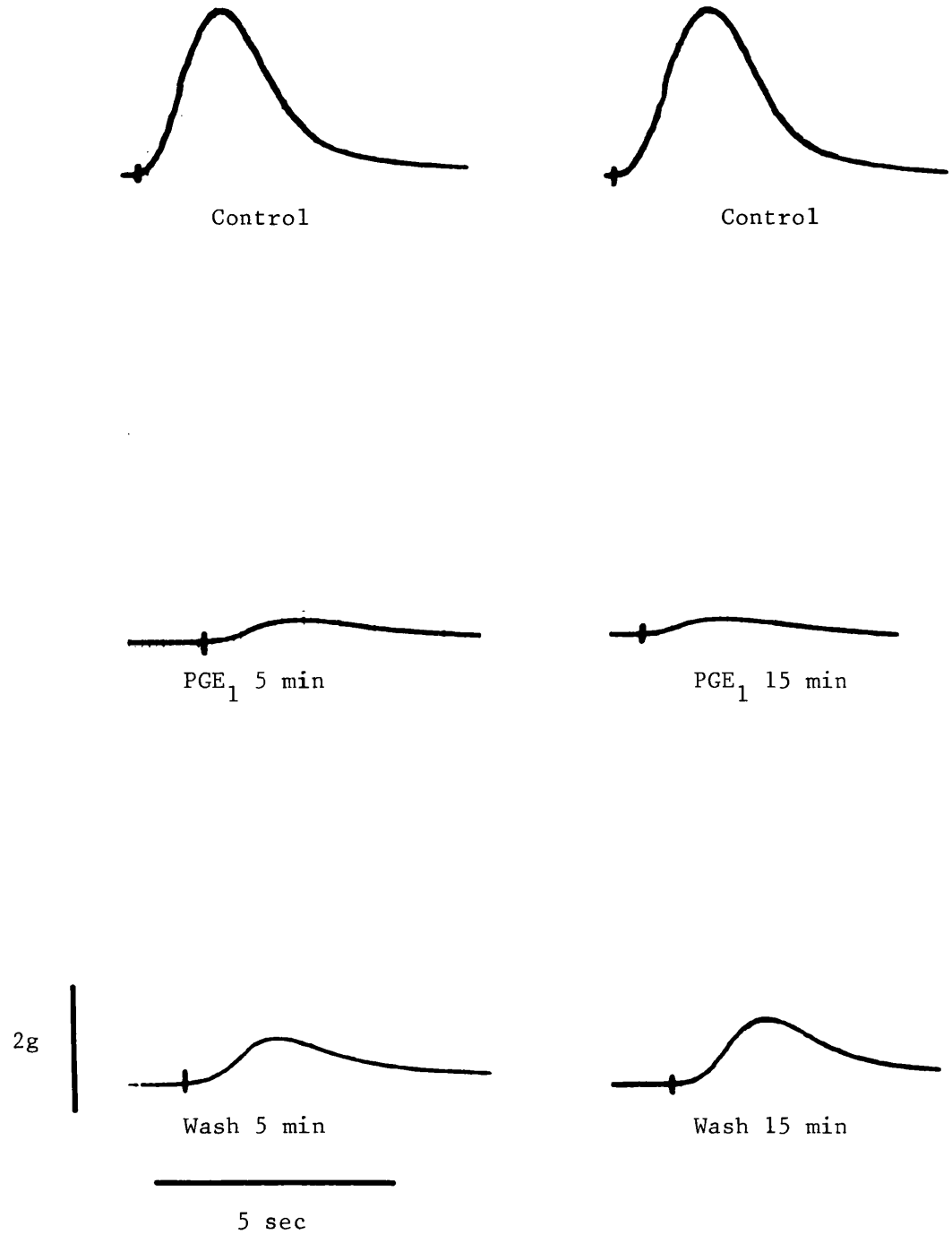

Fig. 3.26 A. The effect of prostaglandin E_1 10 ng/ml (PGE_1) on the mechanical response of the guinea-pig vas deferens in response to hypogastric nerve stimulation. The vas deferens was excited by trains of 10 stimuli, (pulse width 0.7 msec,supramaximal voltage), at 10 Hz.

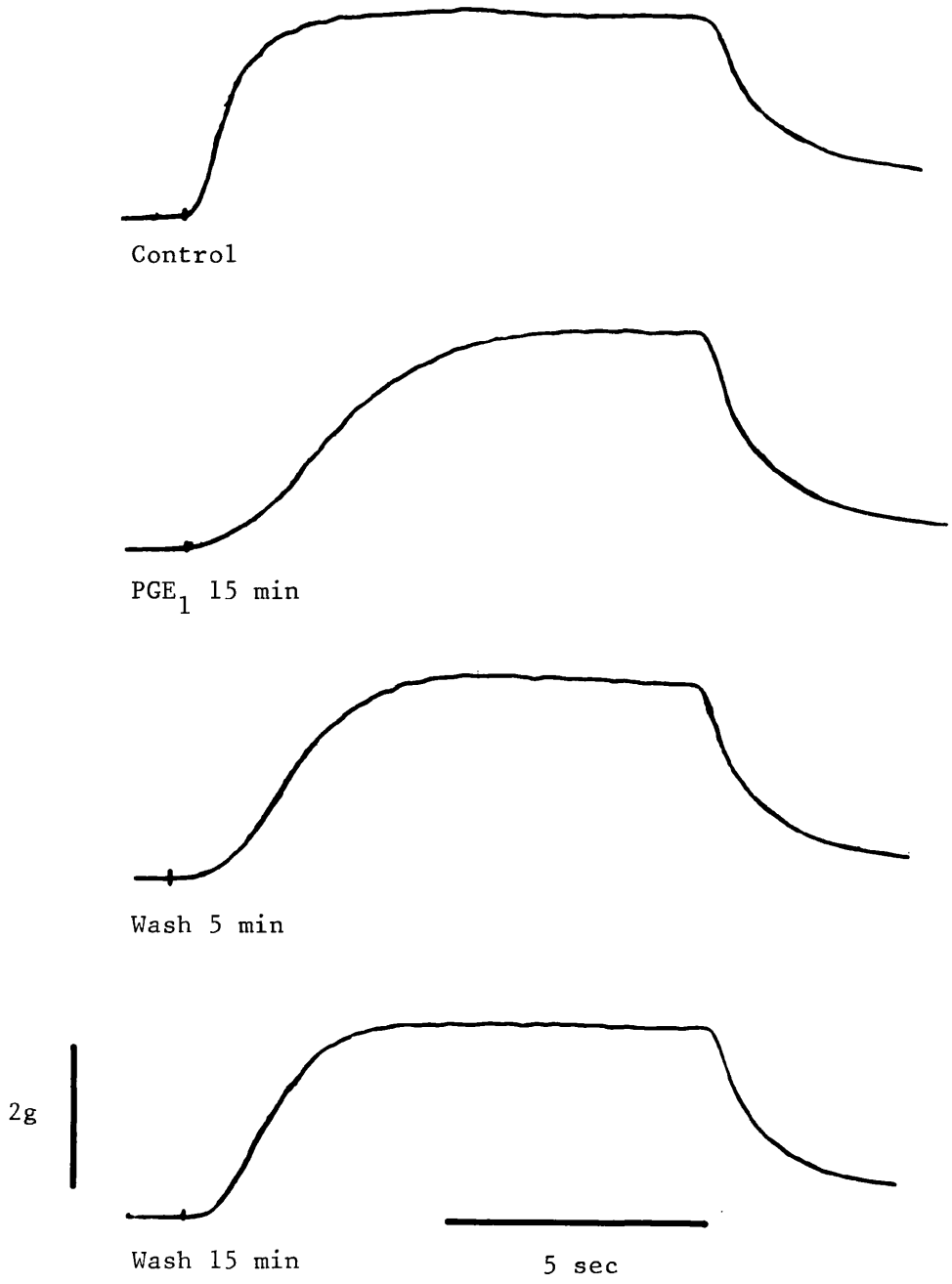

Fig. 3.26 B. The effect of prostaglandin E_1 10 ng/ml (PGE_1) on the mechanical response of the guinea-pig vas deferens in response to hypogastric nerve stimulation. The vas deferens was excited by trains of 100 stimuli (pulse width 0.7 msec, supramaximal voltage) at 10 Hz.

CLOSE EXAMINATION OF THE RISING PHASE OF THE EXCITATORY JUNCTION POTENTIAL

When the rising phases of excitatory junction potentials in the guinea-pig vas deferens, evoked at 0.91 Hz by submaximal stimuli to the hypogastric nerve, were examined on a fast time base, occasional discontinuities were observed. Closer examination of this portion of the excitatory junction potential by differentiation of the signal revealed these as occasional peaks in the rate of depolarisation which have been termed 'discrete events'. The characteristics of these discrete events are as follows (see Fig. 3.27 I, II). (1) In any cell, discrete events occur at one or a few distinct latencies after the stimulus. (2) They are not constantly present in a series of excitatory junction potentials. At one latency discrete events never, in the absence of drug treatment, occur to every stimulus but to 1 in 1.8 to 1 in 45 stimuli. (3) They vary in amplitude at all latencies. (4) Their time course varies from latency to latency. (5) They occur with varying degrees of prominence in different cells. Prominent discrete events have short rise times. Prominent discrete events were found in about 20% of cells examined. Cells with prominent discrete events generally have obvious large spontaneous excitatory junction potentials.

Since the discrete events are stimulus locked it is assumed that they are the result of the release of transmitter from one or more release sites with a single conduction delay from the point of stimulation of the hypogastric nerve.

The discrete events arise from a slower non-intermittent component of the response. This component on the differentiated signal has a time to peak of 26.6 ± 7.4 msec, n = 200 and a time to half decay of

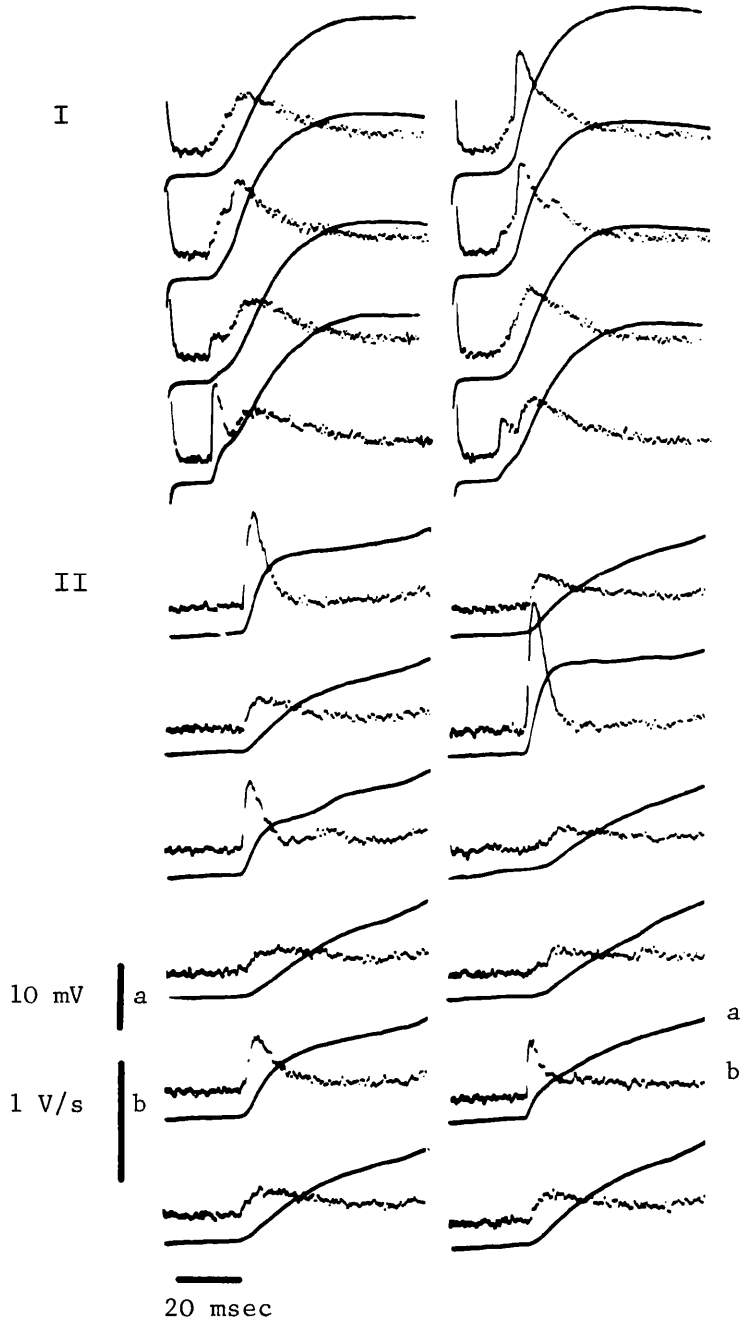

Fig. 3.27. Intracellular records of the membrane potential of two smooth muscle cells (I, II) of the guinea-pig vas deferens. Excitatory junction potentials (e.j.p.) were evoked by trains of submaximal stimuli (1 msec, 0.91 Hz). Records show e.j.p.s after facilitation has occurred. (a) E.j.p., (b) $^{dV}/_{dt}$ of (a). Resting membrane potential I, 68 mV, II 65 mV.

42.6 ± 13.3 msec, n = 200. The slower component represents the electrotonic spread in a functional syncytium of transmitter induced activity in other cells distant from the micro-electrode.

THE CAUSES OF INTERMITTENT TRANSMITTER RELEASE

In order to prevent the excitatory junction potentials from reaching threshold for a muscle action potential it was necessary to use submaximal stimuli (80-90% maximum) to the hypogastric nerve. I have therefore investigated the cause of the intermittence of the discrete events at a single latency to exclude the possibility that this intermittence was simply an artifact due to the use of submaximal stimuli, so that all the varicosities whose transmitter could reach the muscle were not always invaded by an action potential and therefore did not contribute a discrete event to each excitatory junction potential.

The activity induced by the submaximal stimulus in single 'all or none' units dissected from the vas deferens nerve was recorded (see Fig. 3.28). At the low frequency of 0.91 Hz intermittence was very difficult to produce and could only be produced by nice adjustment of the stimulus. Most units fired continuously or not at all.

Secondly, I have never been able, by increasing the stimulus strength to convert an intermittently occurring discrete event into a continuously active one or to increase its frequency of occurrence. This check has been customarily made whenever possible.

Thirdly, in experiments with trains of supramaximal stimuli at 0.1 Hz which do not cause contraction of the vas deferens, the discrete events still occurred with intermittence similar to that seen at 0.91 Hz. Unfortunately this low frequency of stimulation could not be used routinely

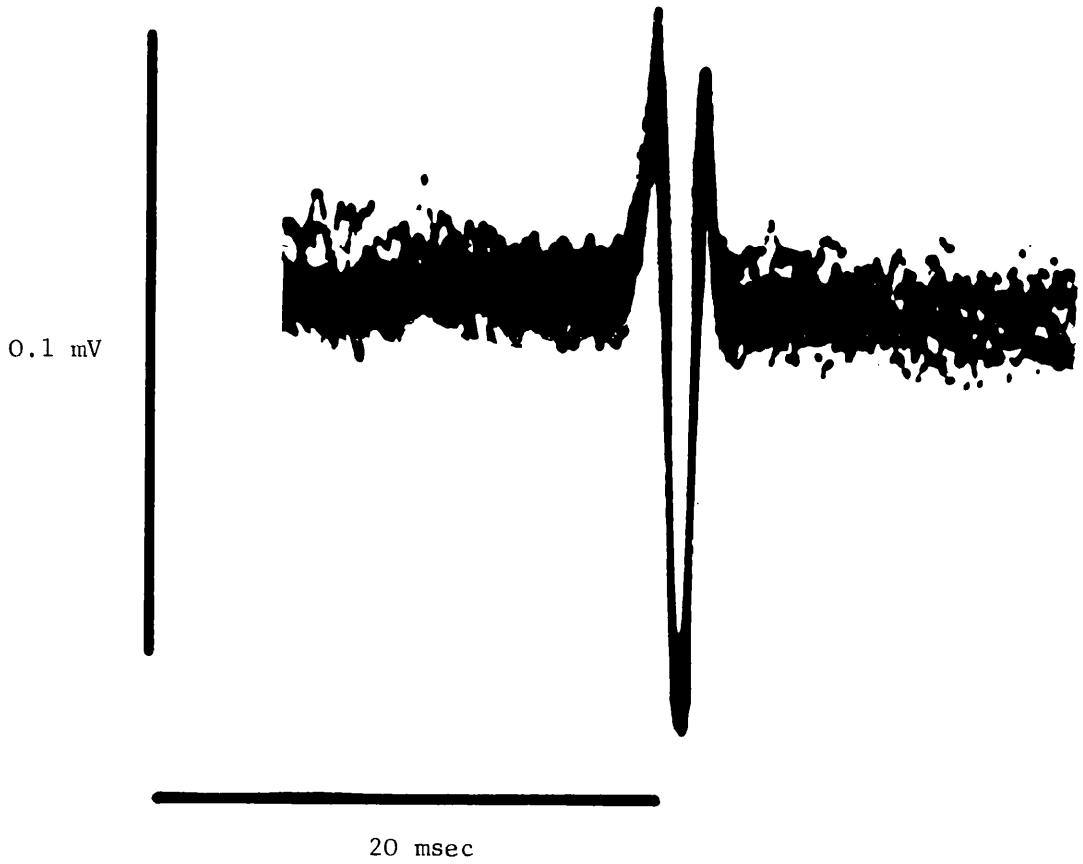

Fig. 3.28. 10 consecutive superimposed action potentials of a single active unit dissected from the vas deferens nerve, recorded extracellularly. The unit was excited by submaximal stimulation (0.2 msec, 0.91 Hz) (similar to that used in other experiments) of the hypogastric nerve 1 cm from the recording site and had a latency of 22 msec.

because of the difficulty in remaining in any single cell for long enough with stimuli at only 1 every 10 sec to collect records of the large number of consecutive excitatory junction potentials needed for analysis.

Finally, when the frequency of stimulation with submaximal pulses was raised from 0.1 to 0.91 Hz the probability of occurrence of discrete events at a single latency to each stimulus was increased rather than decreased.

From these experiments it is concluded that intermittent stimulation of the units in the hypogastric nerve at the stimulating electrodes is unlikely to be the cause of the observed intermittent occurrence of the discrete events.

As a similar pattern of intermittently occurring discrete events was recorded following transmural stimulation of the desheathed vas deferens at frequencies between 0.1 and 2 Hz, one can exclude the ganglia as the site of intermittence.

THE PACKETED NATURE OF TRANSMITTER RELEASE BY THE AUTONOMIC INNERVATION OF THE VAS DEFERENS

Observation of short trains of stimuli at 0.91 Hz showed that, at a single latency the discrete events did not vary in size continuously but varied in a stepwise manner (Fig. 3.29).

When a histogram was constructed of the amplitudes of discrete events evoked at one latency by long trains of stimuli, it was found that in all cells these amplitudes had several preferred values. In some cells the preferred values were not simple whole multiples of the smallest preferred value (Fig. 3.30 I) but in other cells the larger preferred values were whole number multiples of the smallest preferred value

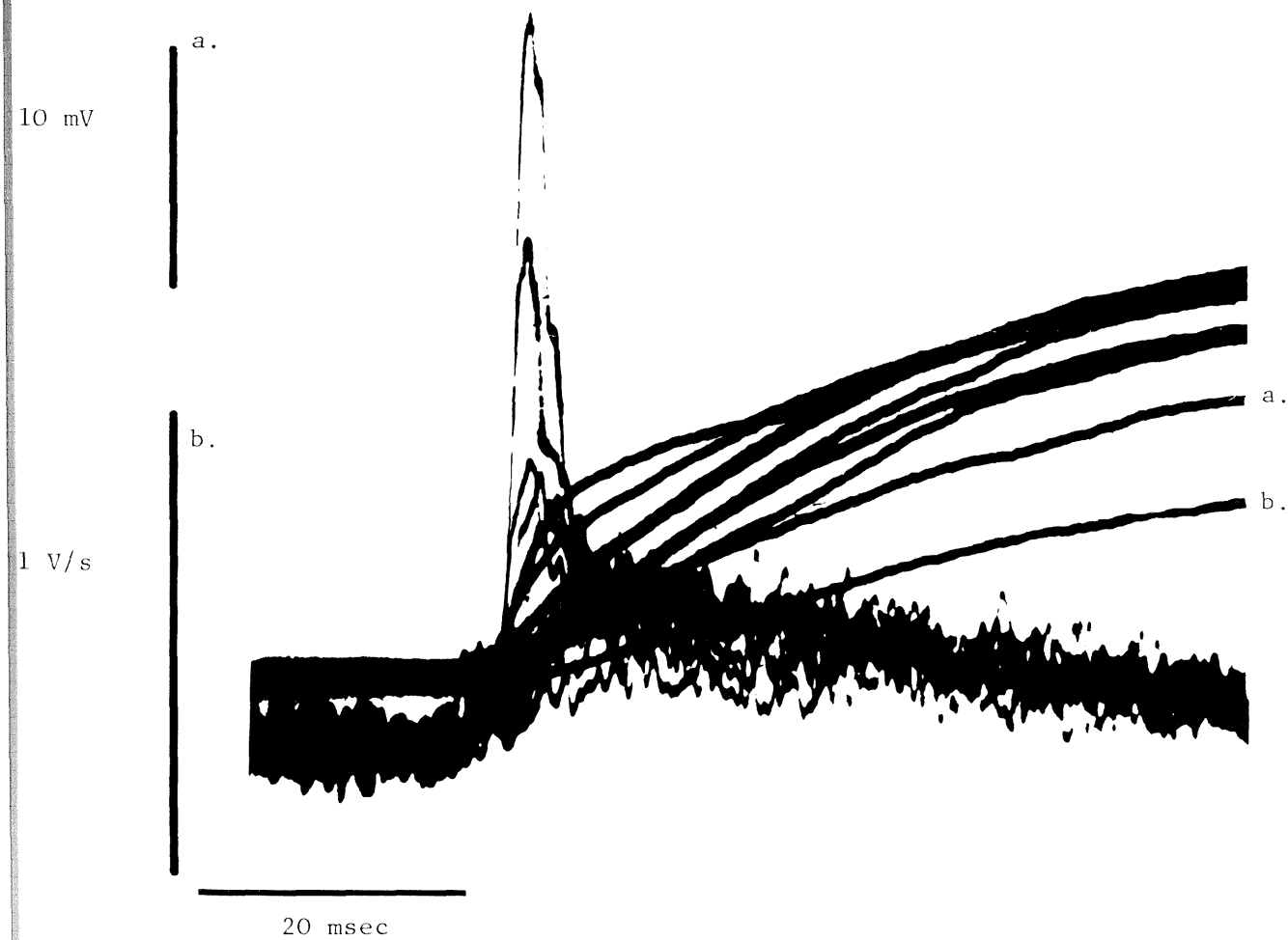

Fig. 3.29. Intracellular records of the membrane potential of a smooth muscle cell. Excitatory junction potentials (e.j.p.) were evoked by a train of submaximal stimuli (1 msec, 0.91 Hz). Record shows 10 superimposed e.j.p.s exhibiting facilitation to the first few stimuli (a) e.j.p. (b) $^{dV}/_{dt}$ of (a) (upstrokes retouched), showing stepwise change in discrete event amplitude at a single latency. No discrete events occurred to 6 stimuli in the train. Resting membrane potential 67 mV.

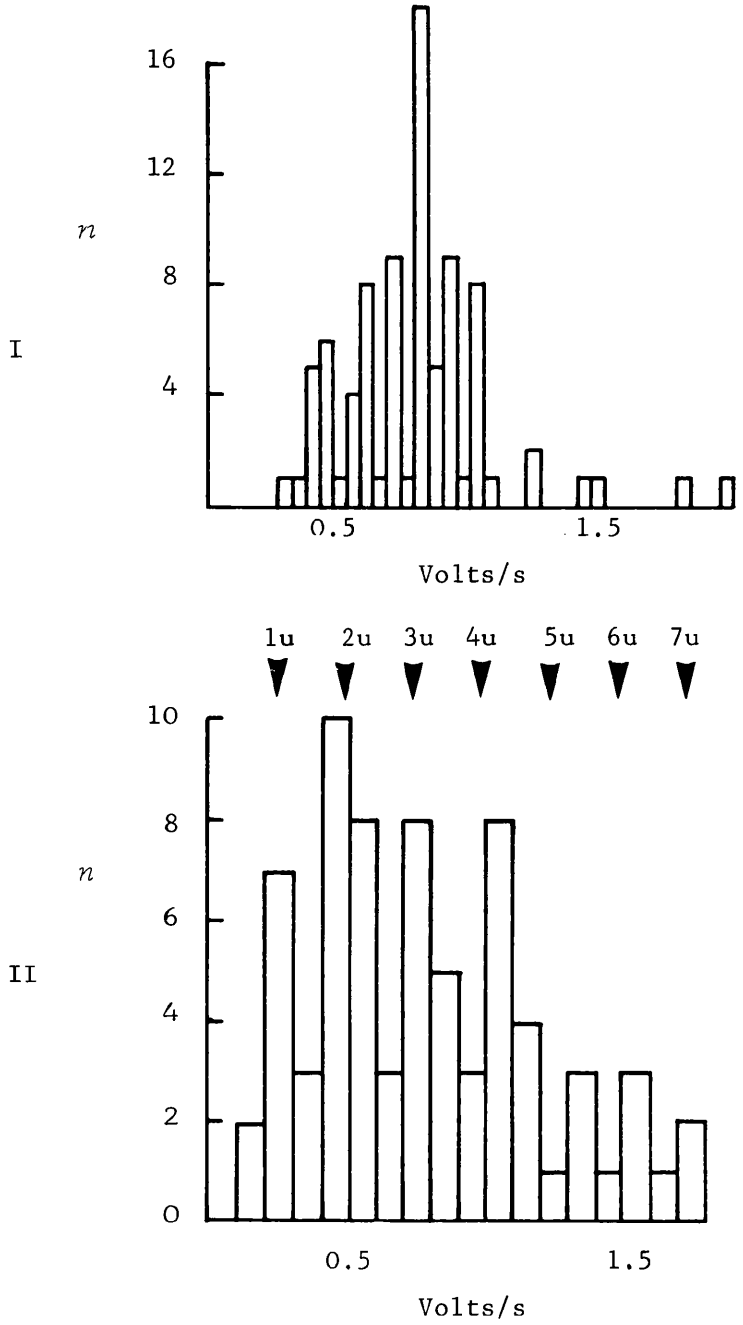

Fig. 3.30. I. Histogram of the amplitude of discrete events in a cell where the distribution was multimodal but the preferred values are not obviously simple multiples of the smallest.

II. Histogram of the amplitude of discrete events in a cell where the distribution was multimodal with peaks of preferred values which are whole multiples of the smallest preferred value (u). The number of failures was 200.

(Fig. 3.30 II). This latter distribution is similar to that seen in Mg^{2+} blocked skeletal muscle (Boyd & Martin, 1956) and suggests that it may be due to a similar phenomenon, i.e. the release by each stimulus, from any one release site, of only a few (or no) packets of transmitter.

In those cells where the preferred values at a single latency did not follow a simple whole number multiple relationship to the smallest preferred value one may have been observing the effect of transmitter release from more than one release site, due to an inability as yet to separate and identify transmitter release from two or more sites with the same conduction delay.

THE TIME COURSE OF THE DISCRETE EVENTS AND SPONTANEOUS EXCITATORY JUNCTION POTENTIALS

The time course of the discrete events as recorded by the differentiator is much shorter than that of the excitatory junction potential. The discrete events reached a peak in 5.3 ± 1.9 msec, n = 220 and decayed to 50% of maximum in 8.3 ± 3.6 msec, n = 220 compared with the excitatory junction potential which reached a peak in 70.3 ± 10.1, n = 37 and decayed to 50% of maximum in 241 ± 66 msec, n = 30.

In a smaller population of cells the time courses of the decay of the discrete event and the spontaneous excitatory junction potential were compared. The spontaneous excitatory junction potential decayed with a time constant of 32.7 ± 7.7 msec, n = 13. Its differential decayed with a time constant of 4.2 ± 1.6 msec, n = 17 which was similar to that of the discrete event, 4.4 ± 1.4 msec, n = 17.

In any one cell the discrete events at different latencies sometimes had clearly different time courses (see Fig. 3.31). These different

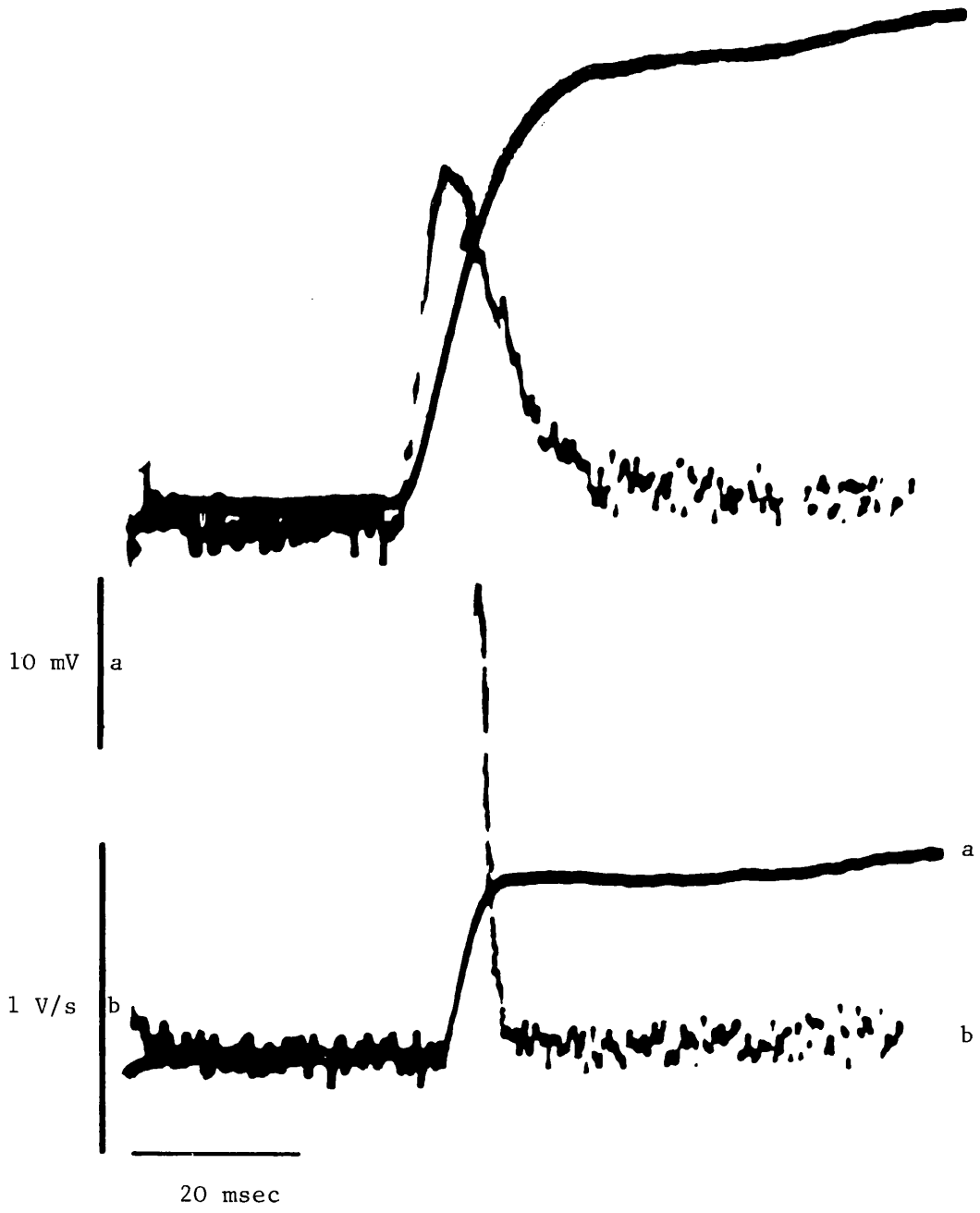

Fig. 3.31. Comparison of discrete events in the same cell at two latencies showing different time course. Excitatory junction potentials (e.j.p.) were evoked by trains of submaximal stimuli (1 msec, 0.91 Hz). Records were selected from train after facilitation had occurred (a) E.j.p., (b) $^{dV}/_{dt}$ of (a). Resting membrane potential 64 mV. The faster response may represent one or a small number of packets close to the recording site and the slower response many packets released at some distant site.

time courses probably represent events which occurred at varying distances from the recording micro-electrode.

THE RELATIONSHIP BETWEEN THE DISCRETE EVENT OF THE EXCITATORY JUNCTION POTENTIAL AND THE SPONTANEOUS EXCITATORY JUNCTION POTENTIAL

In skeletal muscle the miniature end-plate potential is thought to have the same anatomical basis as the quantum of transmission; they are the effects of a single multimolecular packet of transmitter. It was of interest, therefore, to compare the discrete event with the spontaneous excitatory junction potential. In any one cell, spontaneous excitatory junction potentials occur with a continuous size distribution down to the noise level of the system (Burnstock & Holman, 1962). It was possible to select from this population, spontaneous excitatory junction potentials that corresponded in amplitude and time course with discrete events in the same cell (Fig. 3.32). That this comparison can be exact is illustrated in Fig. 3.33. It is therefore assumed that the discrete event and the spontaneous excitatory junction potential have the same basis.

THE EFFECT OF STIMULATION SITE UPON THE SHAPE OF THE EXCITATORY JUNCTION POTENTIAL

The excitatory junction potential recorded from the same single muscle cell was of different shape depending upon the site of nerve stimulation (see Fig. 3.34). When the excitatory junction potential was elicited by stimulation of the hypogastric nerve the conduction delay was 43 msec and the time to peak 80 msec. Evoked by transmural stimulation with a conduction delay of only 22 msec the response was sharper with a time to peak of only 54 msec. The later stages of the recovery phase of the two excitatory junction potentials were similar.

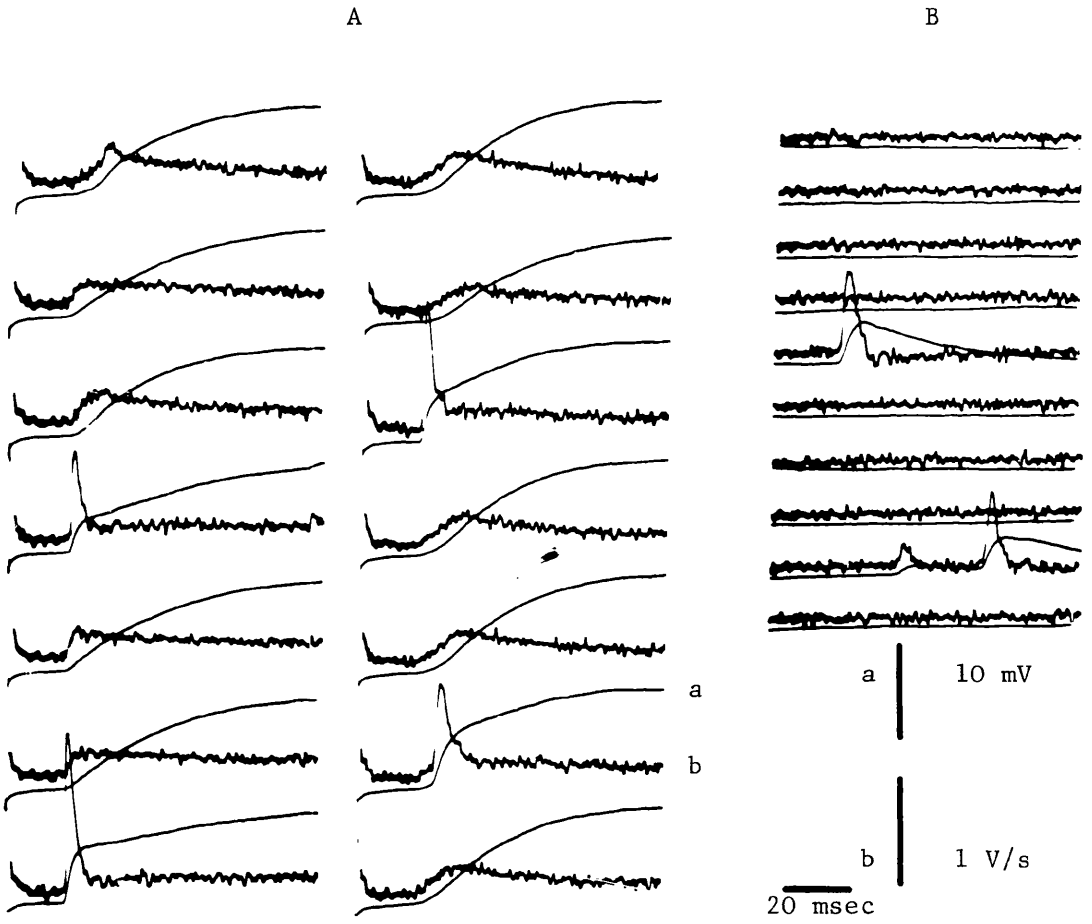

Fig. 3.32. Intracellular records of the membrane potential of a single smooth muscle cell comparing discrete events and spontaneous excitatory junction potential (s.e.j.p.). Excitatory junctional potentials (e.j.p.) were evoked by trains of submaximal stimuli (1 msec, 0.91 Hz). A e.j.p.s after facilitation has occurred (a) e.j.p. (b) $^{dV}/_{dt}$ of (a). B S.e.j.p.s.

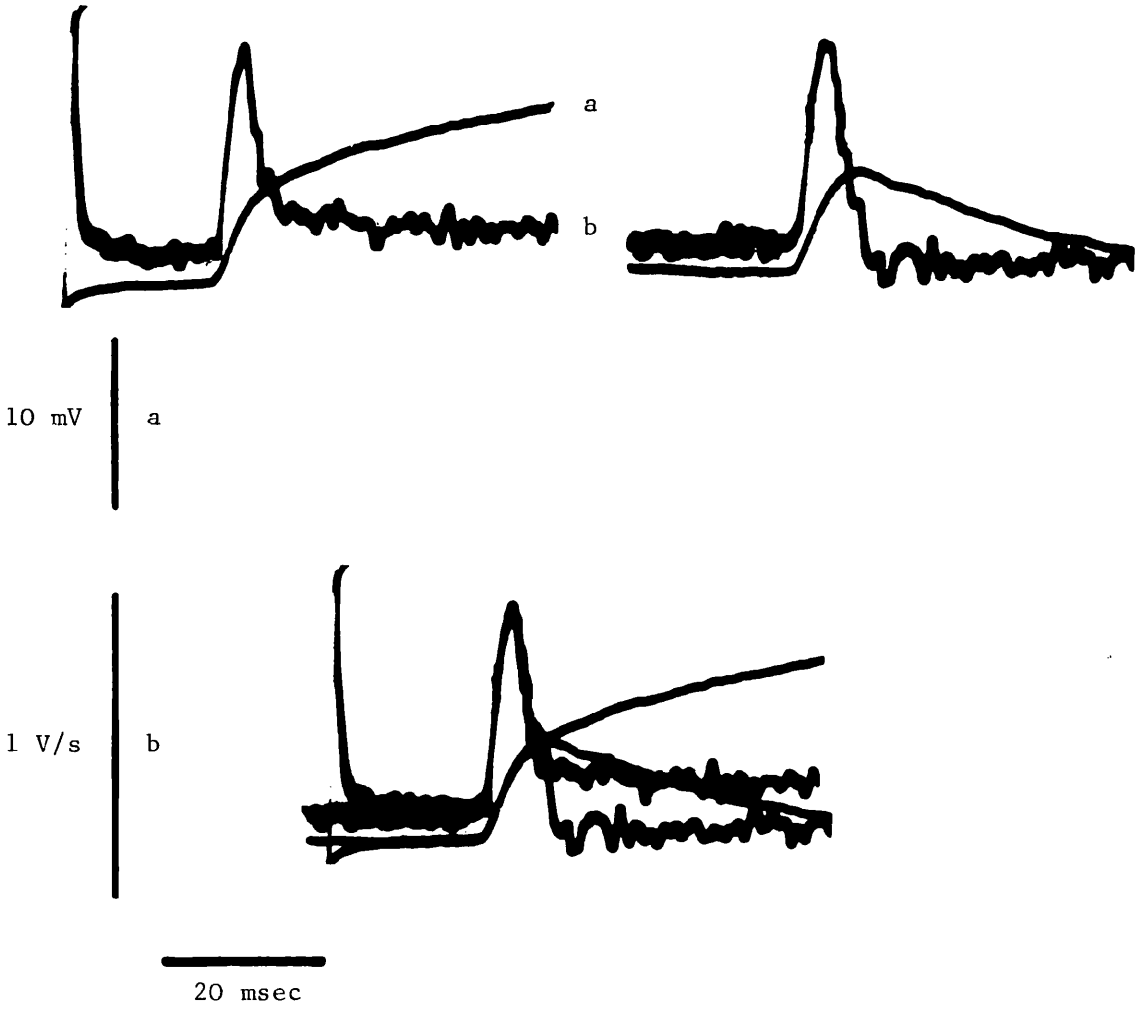

Fig. 3.33. Intracellular records of the membrane potential of a single smooth muscle cell comparing a selected discrete event and spontaneous excitatory junction potential (s.e.j.p.). The excitatory junction potential (e.j.p.) was selected from a train evoked at 0.91 Hz (1 msec, submaximal stim.). Top left record, e.j.p. and discrete event, top right, s.e.j.p. Bottom record, discrete event and s.e.j.p. superimposed photographically showing them to be identical. RMP 69 mV. (a) e.j.p., (b) discrete event.

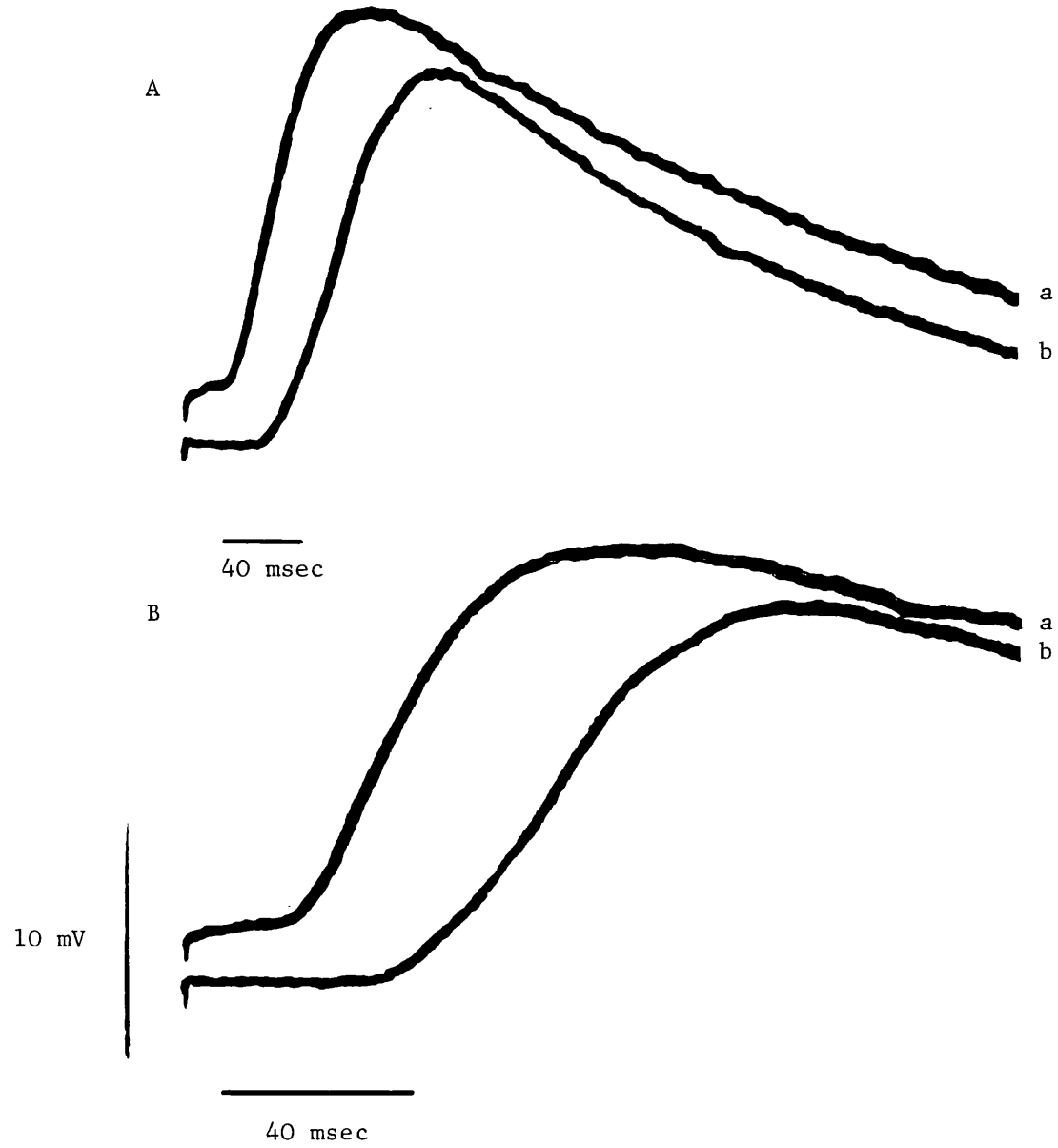

Fig. 3.34. Comparison in the same cell, of the time course of excitatory junction potentials (e.p.p.) evoked by stimulation of the intramural nerve fibres of the hypogastric nerve. A. (a) E.j.p. evoked by single stimulus (1 msec, supramaximal) to intramural nerve fibres. (b) E.j.p. evoked by single stimulus (1 msec, supramaximal to hypogastric nerve. B. Same records as A recorded on faster time base. Resting membrane potential (RMP) 69 mV.

The time for the excitatory junction potential to decline to 1/e of maximal amplitude following hypogastric and transmural nerve stimulation was 109 and 117 msec respectively. Since the two excitatory junction potentials evoked by the two stimuli were of similar amplitude one must explain their difference in shape in terms of a greater degree of temporal dispersion of the action potential volley in the vas when it is evoked at a greater distance.

Since the discrete event and the spontaneous excitatory junction potential are identical the discrete event probably represents the release of one or more packets of transmitter from a single varicosity. One can investigate therefore, the effects of α-adrenoceptor agonists and antagonists on transmitter release from single release sites of the autonomic ground plexus by studying the discrete event.

THE EFFECTS OF PIPEROXAN ON THE DISCRETE EVENT

Piperoxan had a number of actions on the discrete event evoked by hypogastric nerve stimulation at 0.91 Hz. The most characteristic action of piperoxan was on the frequency of occurrence of discrete events. In the absence of piperoxan, discrete events occurred intermittently. In the presence of piperoxan after the first few stimuli, discrete events occurred to every stimulus in a train (Fig. 3.35).

The second action of piperoxan was on the number of discrete events observed on the rising phase of any one excitatory junction potential. In the absence of drugs, the latencies of discrete events were normally closely grouped. In the presence of piperoxan, stimulus locked discrete events occurred over a wider latency range, and occurred in response to nearly every stimulus, at the longer latencies particularly. An example

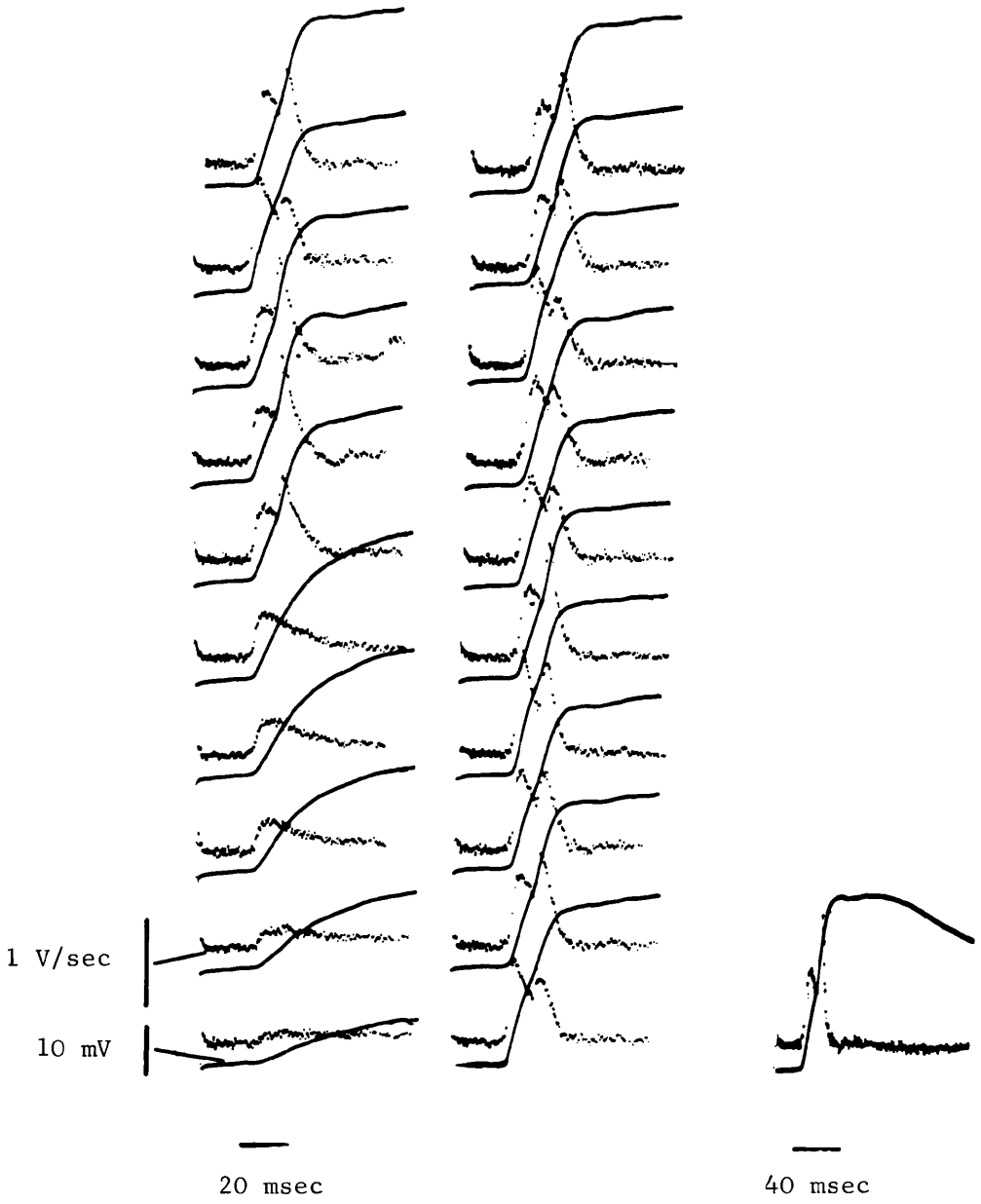

Fig. 3.35. Intracellular records of the membrane response of a single smooth muscle cell showing the effect of piperoxan (7×10^{-7}M) on the discrete event. Excitatory junction potentials were evoked by stimulation of the hypogastric nerve with a train of 20 submaximal stimuli (1 msec, 0.91 Hz).

of this action of piperoxan can be seen in Fig. 3.36. One explanation may be that blockade of α-adrenoceptors by piperoxan allowed more varicosities to release transmitter.

When the effects of piperoxan were studied on single cells, there was an increase in the frequency of occurrence and amplitude of discrete events in response to the first stimulus in a train. This was associated with an increase in the amplitude of the first excitatory junction potential in a train. The effects of piperoxan in a single cell are shown in Fig. 3.37.

THE EFFECTS OF CLONIDINE ON THE DISCRETE EVENT

Clonidine (10^{-8}M - 10^{-6}M) reduced the frequency of occurrence of discrete events to very low levels, less than one discrete event in 50 to 200 stimuli. The amplitude of the discrete event was also reduced. These effects were accompanied by a parallel reduction in excitatory junction potential amplitude. There was no change in the resting membrane potential.

EFFECT OF TRANSMITTER RELEASE ON SUBSEQUENT TRANSMITTER RELEASE

The discrete event allows one to study transmitter release from single varicosities. It was of interest therefore, to investigate the effects of previous transmitter release on subsequent transmitter release from the same varicosity. Trains of excitatory junction potentials were evoked at 0.91 Hz and the mean probability (p) at which discrete events occurred was calculated as follows: $p = \frac{\text{number of discrete events}}{\text{number of stimuli}}$. Thus a discrete event occurring 20 times in 100 stimuli had a control probability value p of 0.2. The p value was recalculated in the same way, one, two and three stimuli (at 0.91 Hz) after a discrete event occurred during a long train of excitatory junction potentials. The time

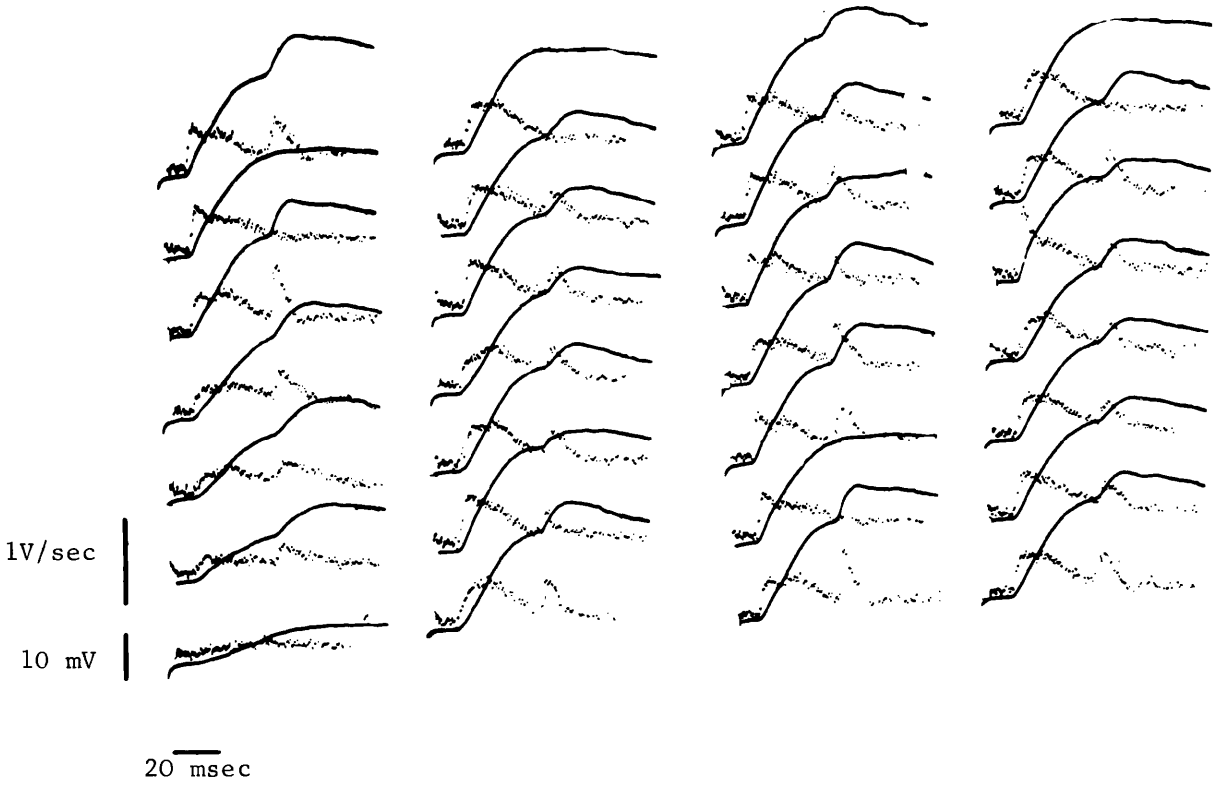

Fig. 3.36. Intracellular records of the membrane response of a single smooth muscle cell showing the effects of piperoxan (7×10^{-7}M) on the discrete events. Excitatory junction potentials were evoked by stimulation of the hypogastric nerve with a train of 28 submaximal stimuli (1 msec, 0.91 Hz). Note the almost continuous occurence of discrete events at the longer latency.

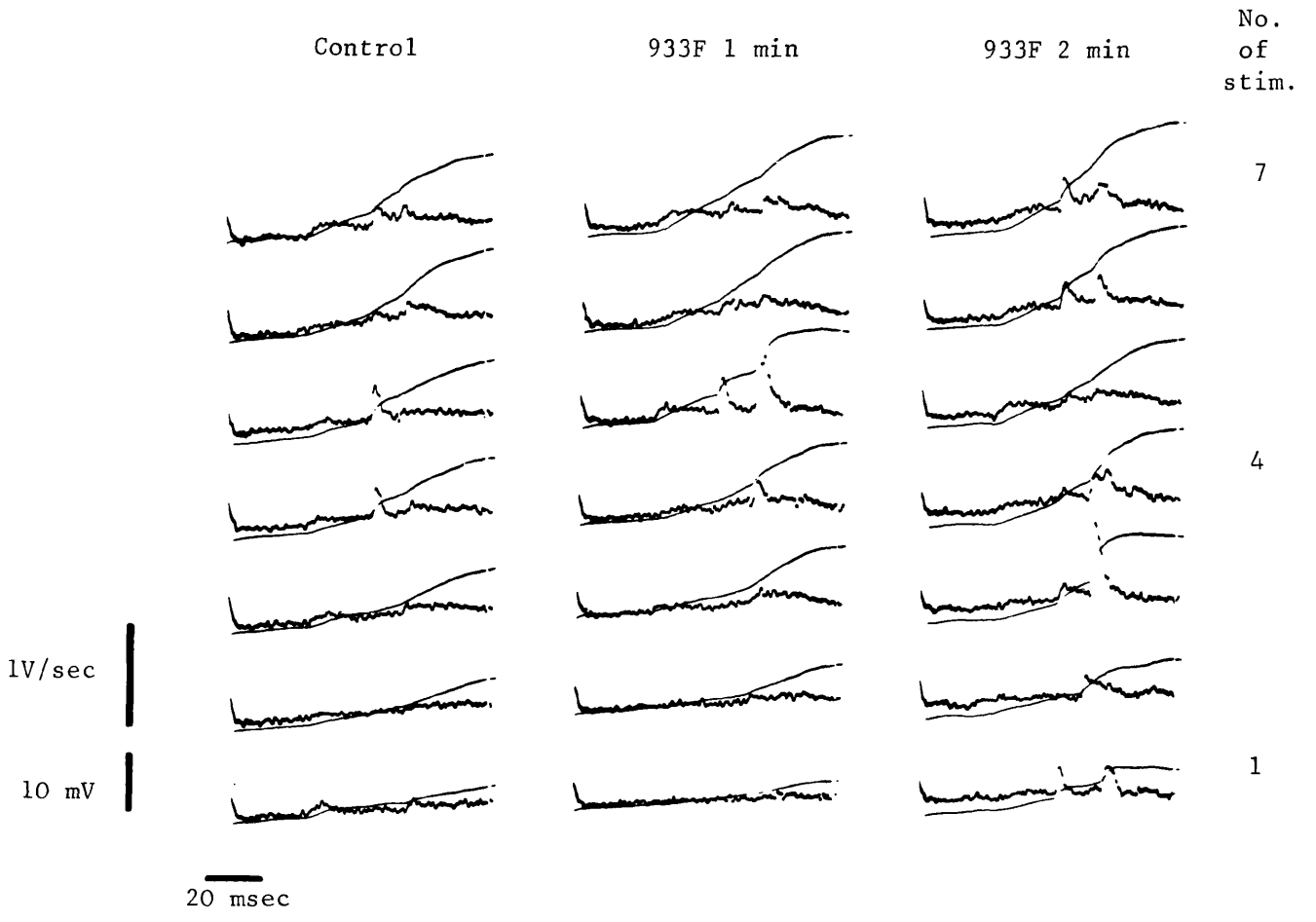

Fig. 3.37. Intracellular records of the membrane response of a single smooth muscle cell showing the effects of piperoxan (7×10^{-7}M) on the discrete event. Excitatory junction potentials were evoked by stimulation of the hypogastric nerve at 1 min intervals with trains of 7 submaximal stimuli (1 msec, 0.91 Hz).

course of any effect of previous transmitter release could thus be determined. A change in p following a discrete event would indicate that previous transmitter release altered the probability of subsequent transmitter release occurring. It was found that previous transmitter release enhanced the probability of subsequent transmitter release occurring (Table 2). This augmentation of release returned to control value over a time course of 1 to 3 sec. When 5 cells were investigated augmentation of transmitter release was found in 2 cells and no effect in 3. In no cells was a negative effect found.

Table 2. EFFECT OF TRANSMITTER RELEASE ON SUBSEQUENT TRANSMITTER RELEASE DURING A TRAIN OF STIMULATION AT 0.91 Hz.

	P	
Mean Overall Frequency	0.14	
1 sec	0.30	$p < 0.001$
2 sec	0.19	n.s.
3 sec	0.16	n.s.

SECTION II

RABBIT RECTOCOCCYGEUS

In this section of the thesis, the electrical correlate of the mechanical responses of the rabbit rectococcygeus to stimulation of the extrinsic parasympathetic (pelvic) nerves is described. The aim of the investigation was to determine (1) the electrical correlate of excitation and inhibition; (2) to investigate the mechanism of release of neurotransmitter from parasympathetic nerve terminals by differentiation of the excitatory junction potential.

RESTING ACTIVITY

The rabbit rectococcygeus was normally quiescent but spontaneous tone and rhythmic activity developed in about 10% of the preparations examined. This activity was seen even in muscles set up under a tension of 1 g or less. The spontaneous activity took the form of large fluctuations of tone repeated every 2-3 min. Atropine (10^{-5} g/ml) which blocks the effects of nerve stimulation in this preparation had no effect on the spontaneous mechanical activity. Preparations which were spontaneously active were discarded since it was impossible to hold microelectrode penetrations for any length of time.

In the absence of spontaneous mechanical activity, the resting membrane potential ranged from -45 to -70 (56.9 ± 5.2 mV mean ± S.D., n = 284). In the presence of spontaneous activity the membrane was depolarised to a varying degree and action potentials occurred.

In 5-10% of cells examined, without tone or rhythmic mechanical activity, rhythmic fluctuations in membrane potential with a periodicity

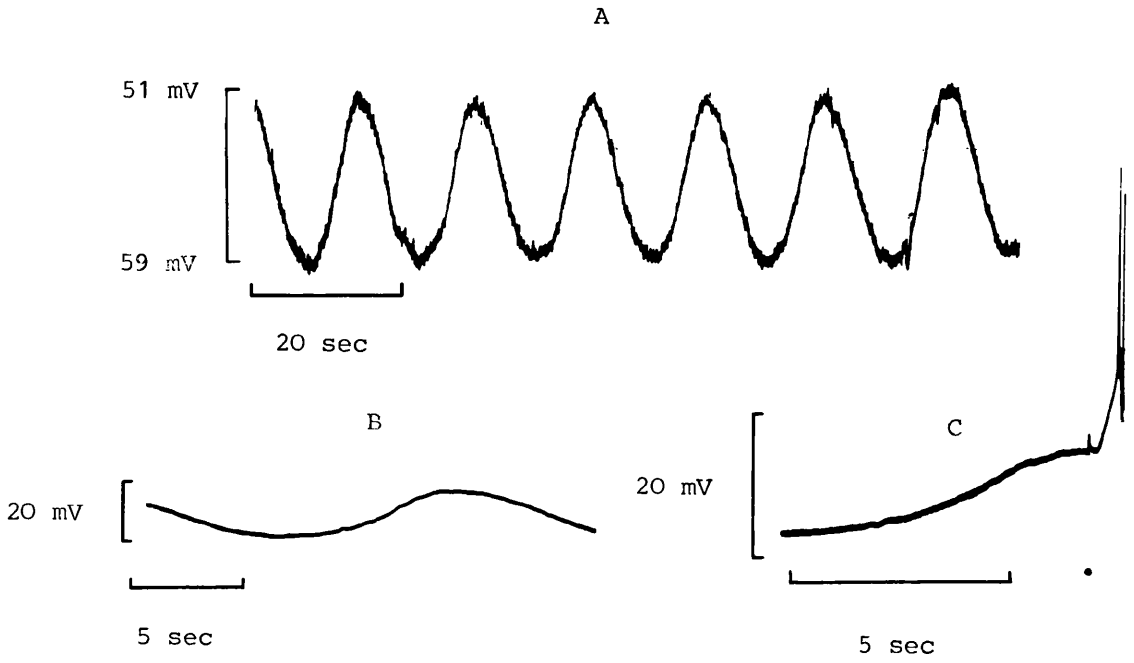

Fig. 4.1. Records from three spontaneously active smooth muscle cells. A, rhythmic fluctuations in membrane potential unaccompanied by spontaneous spikes; B, rhythmic fluctuation in membrane potential with a periodicity of about 20 sec; C, excitatory junction potential and muscle action potential evoked by nerve stimulation (• single pulse, 0.1 msec, supramaximal voltage).

between 15 and 20 sec were observed (Fig. 4.1). The rhythmicity was not associated with the generation of muscle action potentials or spontaneous mechanical activity in the muscle. The slow waves were unaffected by atropine (10^{-6} g/ml) or phentolamine (10^{-6} g/ml).

Prominent spontaneous excitatory junction potentials were not normally observed but were seen occasionally following stimulation of the pelvic nerves (Fig. 4.2).

THE RESPONSE TO EXTRINSIC PARASYMPATHETIC NERVE STIMULATION

Each of the two heads of origin of the rectococcygeus is separately innervated by fibres from the posterior and posterio-median branches of the pelvic nerves (Fig. 4.3).

Stimulation of the pelvic nerves evoked excitatory junction potentials only from muscle cells on the same head of the muscle as the stimulated nerve. Recordings from cells on the head contralateral to that being stimulated showed only small excitatory junction potentials and conducted action potentials. The rectococcygeus muscle was excited easily by pelvic nerve stimulation; action potentials were evoked by single stimuli (0.1-0.5 msec, submaximal voltage). Single maximal stimuli evoked one or more muscle action potentials with a threshold at between -45 and -35 mV. Muscle action potentials exhibited overshoot potentials which reached +15 mV and were associated with mechanical contractions. Single sub-threshold stimuli evoked excitatory junction potentials which were graded with stimulus strength and could reach 25 mV (Fig. 4.2). The latency of the excitatory junction potential was approximately 250 msec with a conduction distance of 1.5 cm, the time to peak 298 ± 10 msec (n = 74) and the time-to-decay to half-amplitude 533 ± 31 msec (n = 74).

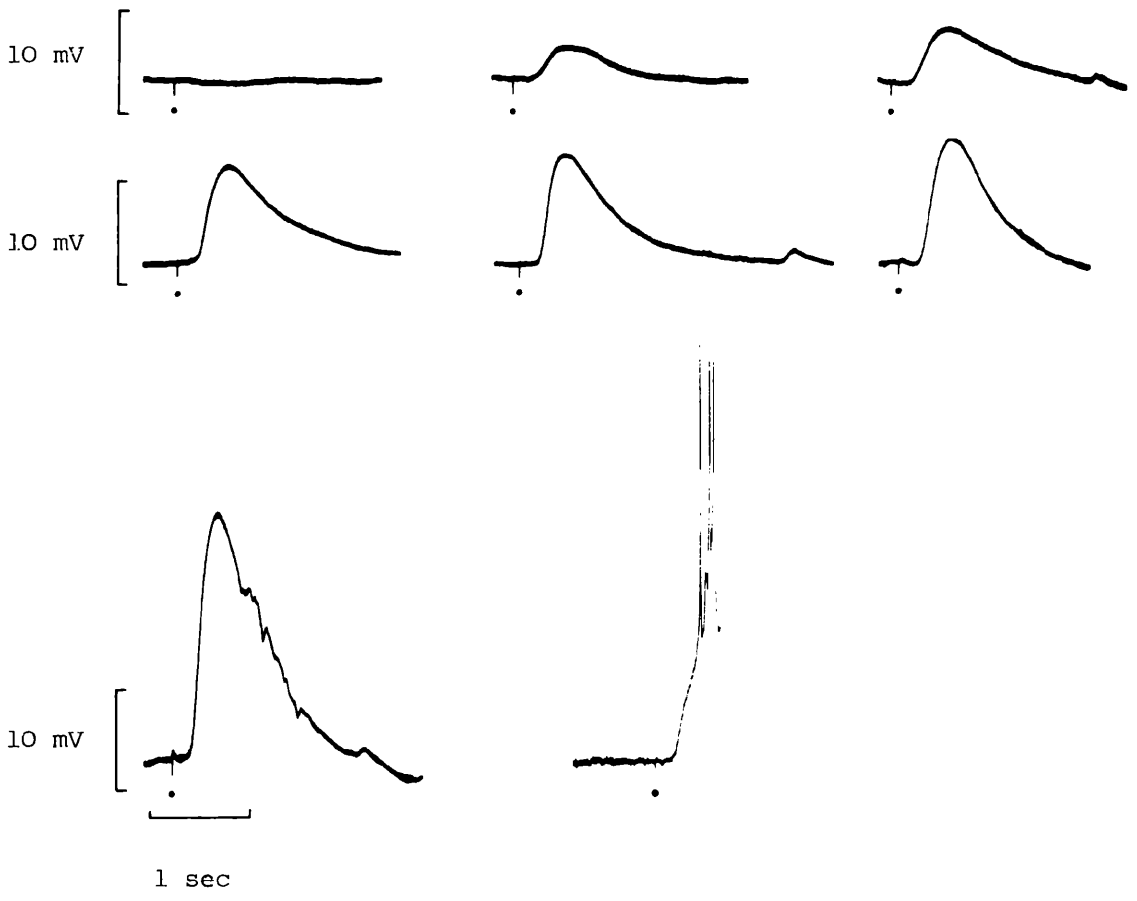

Fig. 4.2. Excitatory junction potentials (e.j.p.) recorded in a single smooth muscle cell of the rabbit rectococcygeus in response to increasing strength of pelvic nerve stimulation (• Single stimuli, every 30 sec). E.j.p.s were graded with stimulus strength and reached 25 mV or more. Prominent spontaneous junction potentials were occasionally observed, particularly following nerve stimulation. The resting membrane potential was 69 mV. Single supramaximal stimuli evoked one or more spikes and muscle contraction. The bottom right hand record was taken from another cell; resting membrane potential 56 mV.

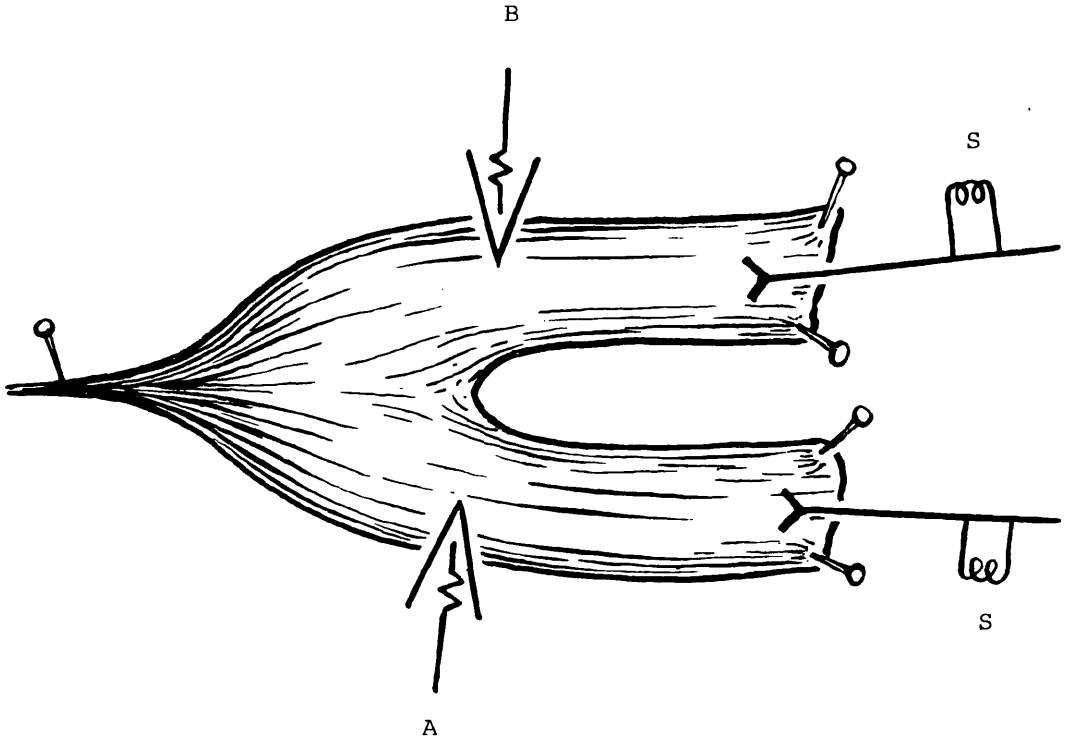

Fig. 4.3. Schematic representation of the rectococcygeus showing the stimulating and recording arrangement. When recordings were made at either A or B following ipsilateral stimulation (S) large excitatory junction potentials and spikes were recorded in all cells impaled. When recordings were made at either A or B following contralateral stimulation only small excitatory junction potentials and spikes following a long latency were recorded.

Trains of submaximal stimuli (0.1-5 Hz) produced excitatory junction potentials which did not facilitate throughout the train but summated at about 1 Hz or above (Fig. 4.4).

The amplitude of the first excitatory junction potential was always larger than that of subsequent excitatory junction potentials. Excitatory junction potentials were abolished by tetrodotoxin (1 to 2 x 10^{-7}g/ml) and reduced or abolished by atropine 10^{-8}-10^{-5}g/ml in a dose dependent manner, suggesting that they are due to cholinergic nerve activity (Fig. 4.5).

The conduction velocity in a fine bundle of pelvic nerves was found to be 0.3 m/sec.

INHIBITORY RESPONSES

In the rectococcygeus muscle inhibition of tone was best demonstrated in the presence of carbachol (King & Muir, 1978). To demonstrate the electrical changes underlying the inhibitions, carbachol was unnecessary. When atropine (10^{-6}g/ml) was added to the Krebs solution perfusing the muscle, supramaximal nerve stimulation at 0.1-30 Hz evoked slow hyperpolarising junction potentials which were graded with stimulus strength and abolished by tetrodotoxin (1 to 2 x 10^{-7}g/ml).

The hyperpolarisation following single supramaximal stimuli ranged from base line noise to 5 mV (1.58 ± 0.05 mV, n = 24). The time to peak was 850 ± 50 msec, n = 24 and time-to-decay to half-amplitude 1357 ± 125 msec, n = 15. There was little or no facilitation of the response during trains of stimuli at 0.25-2 Hz but summation occurred at frequencies greater than 0.3 Hz (Fig. 4.6). At moderate stimulation frequencies (2-5 Hz), maximal inhibition was achieved after 7-11 stimuli.

The time course of the inhibitory junction potentials in response

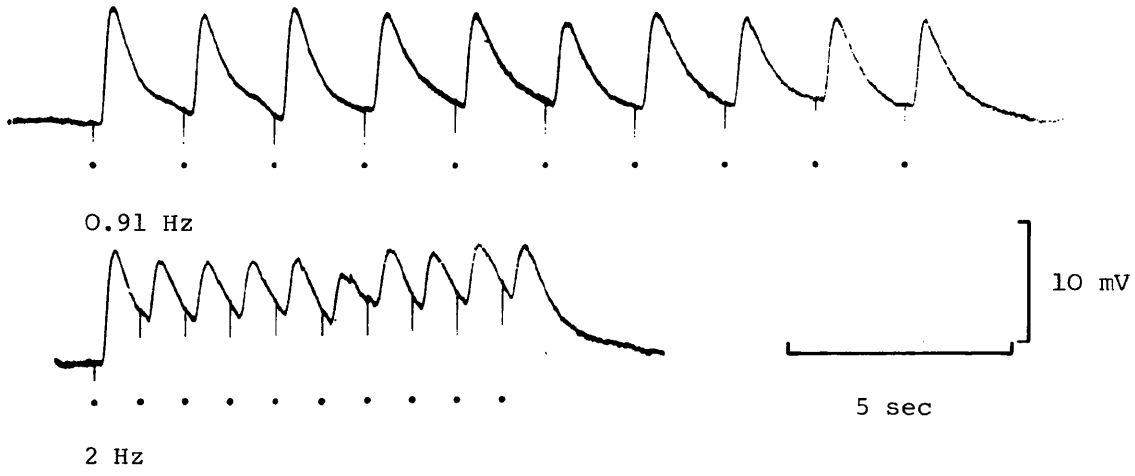

Fig. 4.4. The effect of increasing the frequency of pelvic nerve stimulation (•) on the excitatory junction potentials (e.j.p.) recorded in a single cell of the rabbit rectococcygeus (resting membrane potential 65 mV). In both records, the amplitude of the first e.j.p. in the train was the largest; subsequent e.j.p.s did not facilitate but summation occurred. The amplitude of subsequent depolarisations at each frequency was approximately equal to that of the first e.j.p. Top record, 10 stimuli at 0.91 Hz, bottom record, 10 stimuli at 2 Hz. (0.2 msec, sub-maximal voltage). The resting membrane potential was 65 mV.

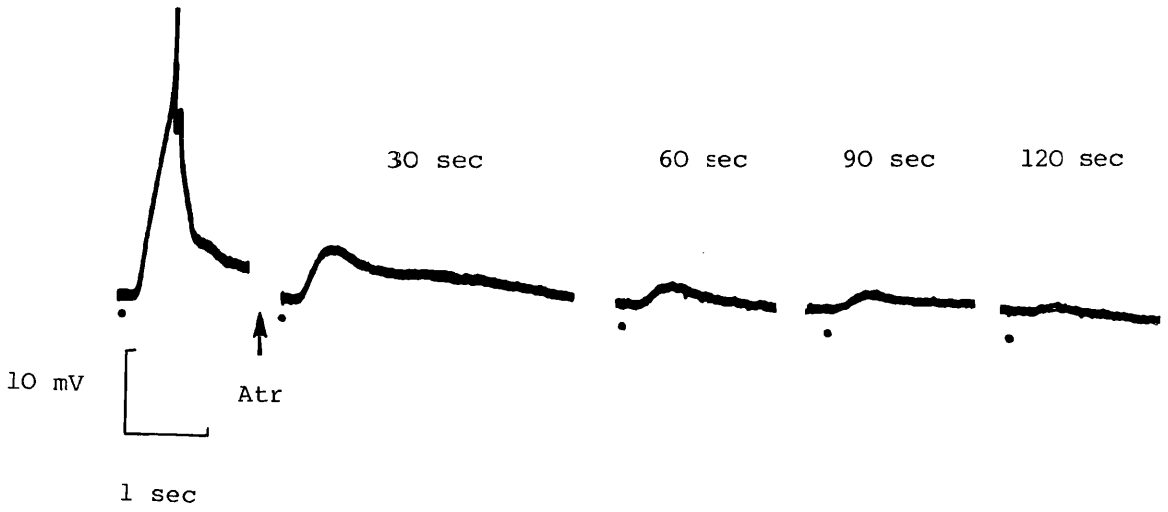

Fig. 4.5. The effect of atropine (↑ Atr, 10^{-6} g/ml) on excitatory junction potentials (e.j.p.) recorded in a smooth muscle cell of the rabbit recto-coccygeus in response to pelvic nerve stimulation. Single stimuli (• 0.5 msec, supramaximal voltage) were delivered every 30 sec. The control e.j.p. and action potential (left hand record) were rapidly replaced by a subthreshold e.j.p. which was progressively reduced and eventually abolished 150 sec (not shown) after perfusion of the muscle with atropine.

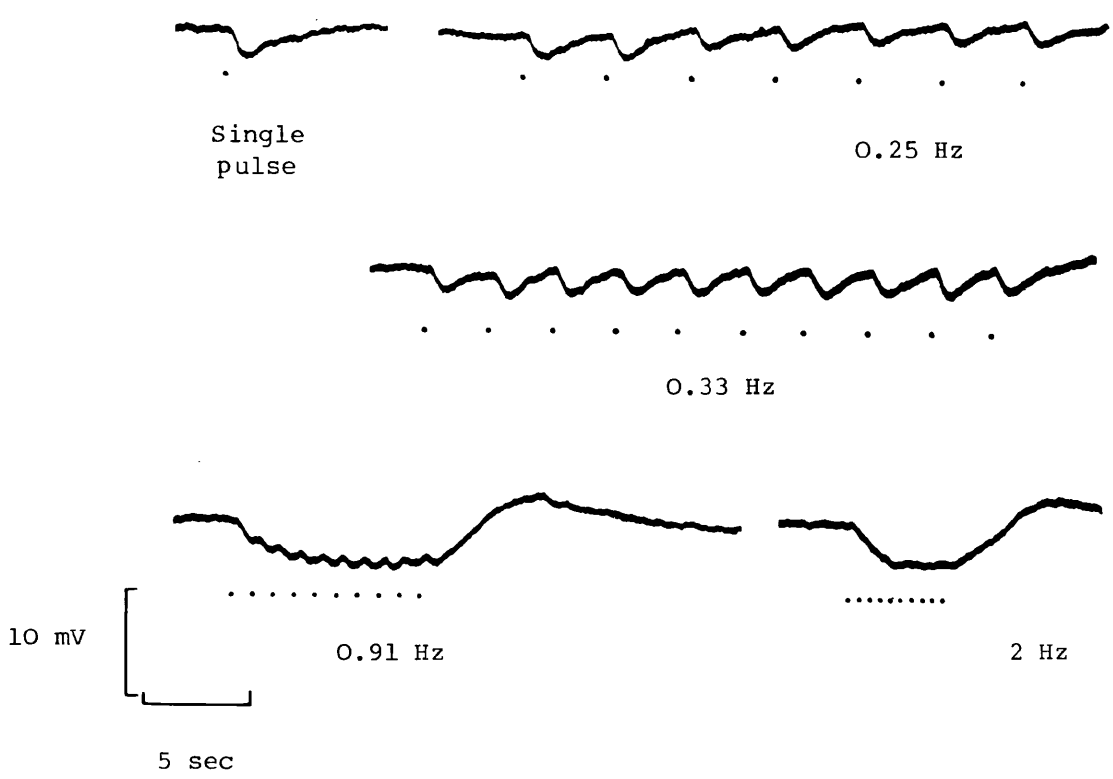

Fig. 4.6. The effect of increasing frequency of pelvic nerve stimulation (•) on the inhibitory junction potentials recorded in a single smooth muscle cell of the rabbit rectococcygeus in the presence of atropine, 10^{-6}g/ml. Single stimuli and low frequencies (up to 0.33 Hz) produced discrete hyperpolarisations of the membrane potential which summated at higher frequencies (0.91 Hz) and plateaued (2 Hz). Above 0.33 Hz a rebound depolarisation occurred when stimulation was stopped. The resting membrane potential was 59 mV.

to short trains of stimuli (10 pulses, 5-10 Hz) was unusually long, the time to peak was 2640 ± 190 msec, n = 22 and the time-to-decay to half-amplitude 3560 ± 120 msec, n = 22. The latency of the inhibitory junction potential (200-340 msec) was similar to that of the excitatory junction potential. The rate of hyperpolarisation increased with increasing frequency and increased with increasing numbers of pulses at a given frequency up to the optimal (5-10 Hz). Spontaneous inhibitory junction potentials were observed rarely. When the frequency of stimulation or the number of stimuli at one frequency was increased a rebound excitation was observed after the stimulation had stopped (Fig. 4.7).

The inhibitory response to pelvic nerve stimulation was well maintained over prolonged periods (up to 50 sec) at low frequencies (up to 2 Hz) but waned at frequencies above 3 Hz (Fig. 4.8).

THE EFFECT OF POTENTIAL ANTAGONISTS OF THE INHIBITORY RESPONSE

The dopamine antagonists haloperidol (10^{-5}g/ml), lysergic acid diethylamide (10^{-5}g/ml), the α (phentolamine 10^{-5}g/ml) and β (propranolol 3×10^{-5}g/ml) adrenoceptor antagonists, the adrenergic neurone blocker, guanethidine (10^{-6}g/ml) and an inhibitor of prostaglandin synthesis (indomethacin 10^{-6}-10^{-5}g/ml) were investigated. None altered the membrane potential significantly or reduced the inhibitory response to pelvic nerve stimulation. The effects of indomethacin (10^{-5}g/ml) and phentolamine (10^{-5}g/ml) are shown in Fig. 4.9 and the effects of guanethidine in Fig. 4.10. Characteristically, guanethidine induced oscillations of the membrane potential similar to those reported in the rat anococcygeus by Creed, Gillespie & Muir (1975).

When high concentrations of either phentolamine (5×10^{-4}g/ml) or propranolol (10^{-5}g/ml) were employed the inhibitory junction potentials

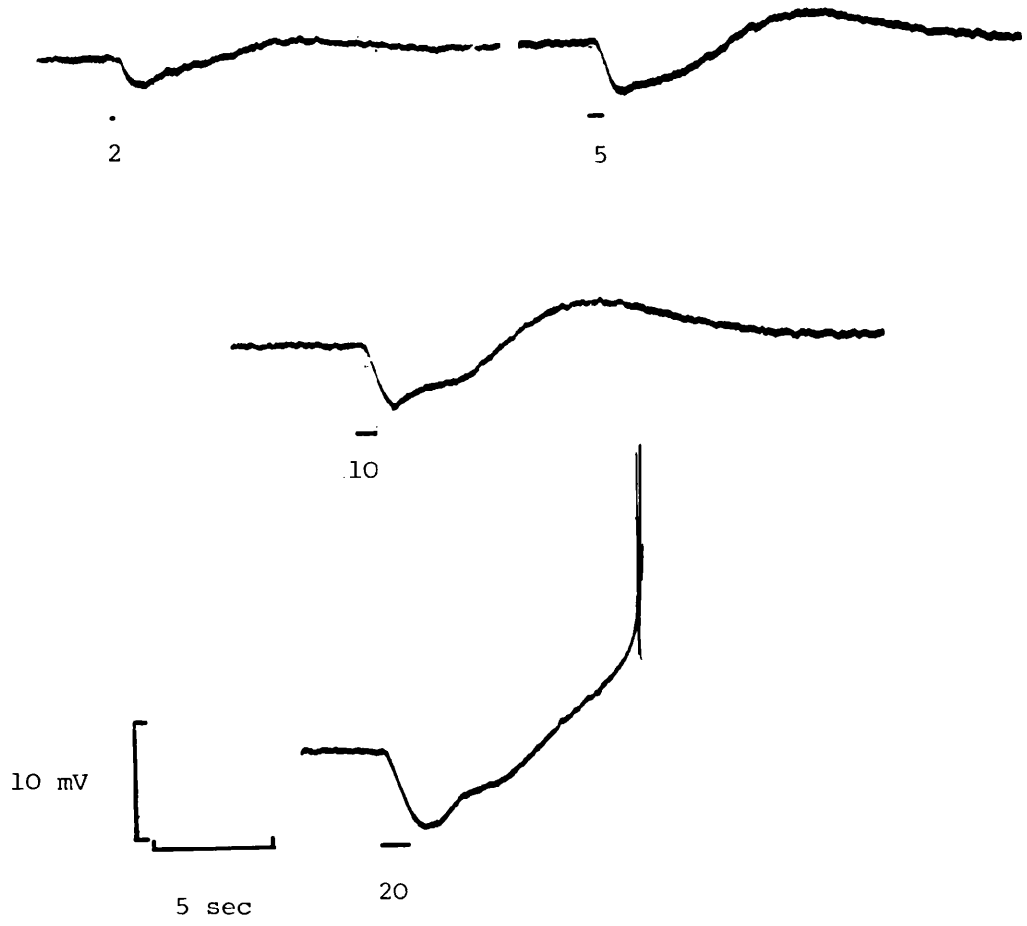

Fig. 4.7. Inhibitory junction potentials recorded in a smooth muscle cell of the rabbit rectococcygeus in response to pelvic nerve stimulation in the presence of atropine (10^{-6} g/ml). Trains of 2, 5, 10 and 20 stimuli at 10 Hz were delivered at 1 min intervals (0.5 msec, supramaximal voltage). The continuous line indicates the period of stimulation. Note the long latency and slow time course of summated inhibitory junction potentials, and the rebound depolarisation when stimulation was stopped. The magnitude of the inhibition and the rebound depolarisation increased with the number of stimuli in the train. With a train of 20 stimuli, the rebound depolarisation triggered an action potential and muscle contraction.

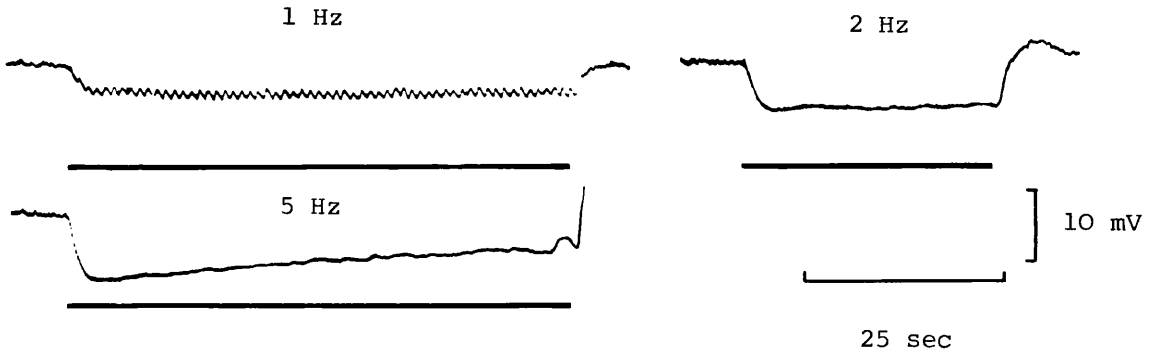

Fig. 4.8. The effect of prolonged pelvic nerve stimulation on the inhibitory junction potentials recorded in a single smooth muscle cell of the rabbit rectococcygeus in the presence of atropine, 10^{-6} g/ml. Trains of stimuli at 1 Hz, 2 Hz and 5 Hz were delivered at 2 minute intervals (0.5 msec, supramaximal voltage). The continuous line indicates the period of stimulation. Note that the inhibitory responses are well maintained at low frequencies (1 Hz & 2 Hz) but wane at higher frequencies (5 Hz) in spite of continuing stimulation.

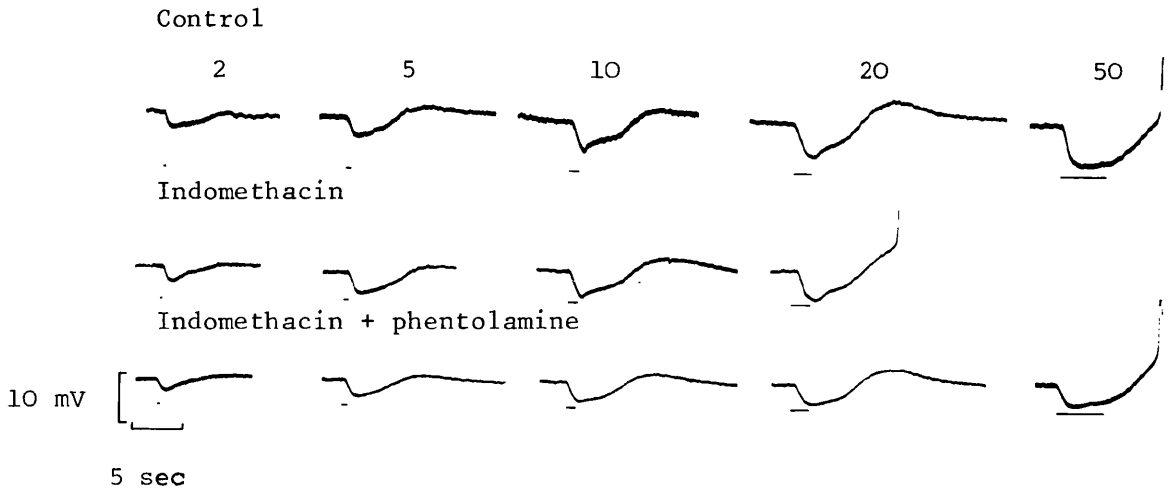

Fig. 4.9. The effect of indomethacin (5×10^{-6} g/ml) alone and with phentolamine (10^{-5} g/ml) on the inhibitory responses of a single cell of the rabbit rectococcygeus to trains of 2, 5, 10, 20 and 50 stimuli (0.5 msec supramaximal) of the pelvic nerves (10 Hz) in the presence of atropine (10^{-6} g/ml). The continuous line indicates the period of stimulation. Neither drug alone or in combination altered significantly either the inhibitory response to nerve stimulation or the rebound depolarisation when stimulation was stopped.

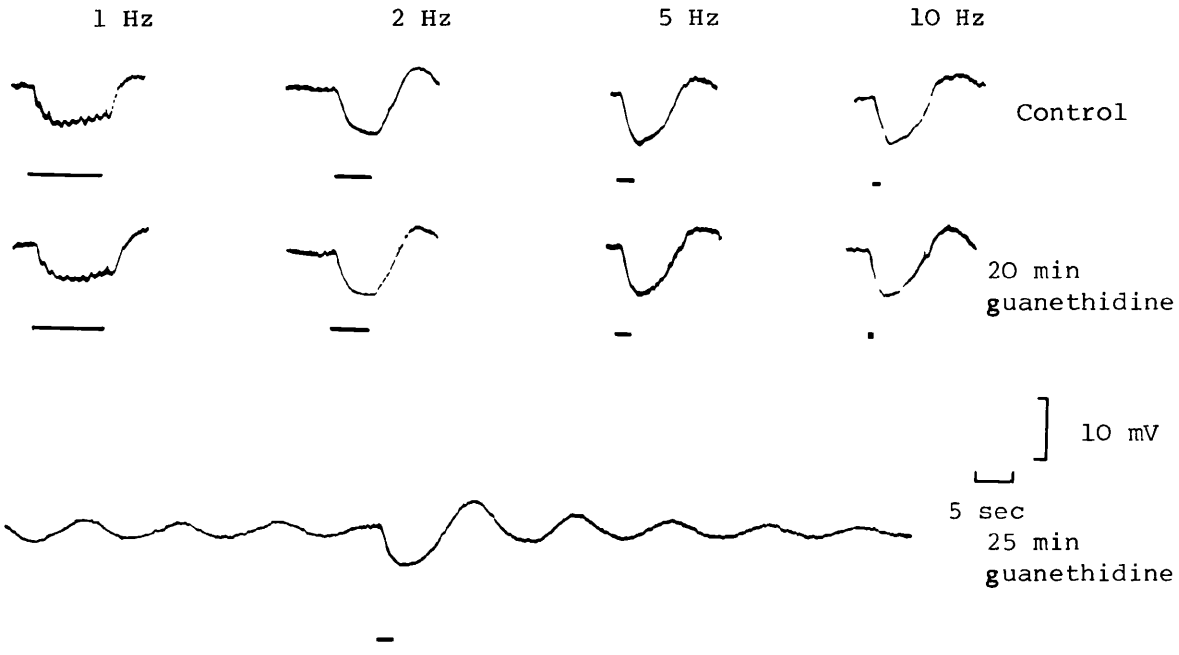

Fig. 4.10. The effect of guanethidine (10^{-6}g/ml) on the inhibitory junction potentials recorded in a single cell of the rabbit recto-coccygeus in response to pelvic nerve stimulation in the presence of atropine, 10^{-6}g/ml. Trains of 10 stimuli at 1 Hz, 2 Hz, 5 Hz and 10 Hz were delivered at 1 min intervals. The continuous line indicates the period of stimulation. Guanethidine had no effect on the inhibitory junction potentials or the rebound depolarisation. Note the characteristic oscillation (lower record) in membrane potential produced by guanethidine.

were reduced or abolished. The abolition of inhibitory junction potentials by phentolamine was accompanied by spontaneous fluctuations in membrane potential, not reversed by washing. The effects of propranolol were reversible by prolonged washing (45 min). The effects of phentolamine and propranolol in high concentrations may be attributable to actions other than adrenoceptor blockade, e.g. local anaesthetic action.

THE EFFECT OF NORADRENALINE

The action of noradrenaline was examined on the inhibitory response produced by pelvic nerve stimulation. The membrane response to the catecholamine was not repeatable. In some preparations the initial dose of noradrenaline hyperpolarised the muscle membrane significantly, the extent of the hyperpolarisation often exceeding the maximal hyperpolarisation evoked by pelvic nerve stimulation and could reach 20 mV. During the noradrenaline induced hyperpolarisation, the inhibitory response to nerve stimulation was reduced (Fig. 4.11 A). The reduction of the inhibitory response to nerve stimulation varied with the degree of hyperpolarisation. The hyperpolarisation in response to subsequent doses of noradrenaline was reduced presumably due to desensitisation of the muscle membrane to the catecholamine although the inhibitory junction potential in response to nerve stimulation was unaltered (Fig. 4.11 B). These effects were reversible on washing. In other preparations, noradrenaline did not alter the muscle membrane potential nor affect the inhibitory junction potentials evoked by nerve stimulation.

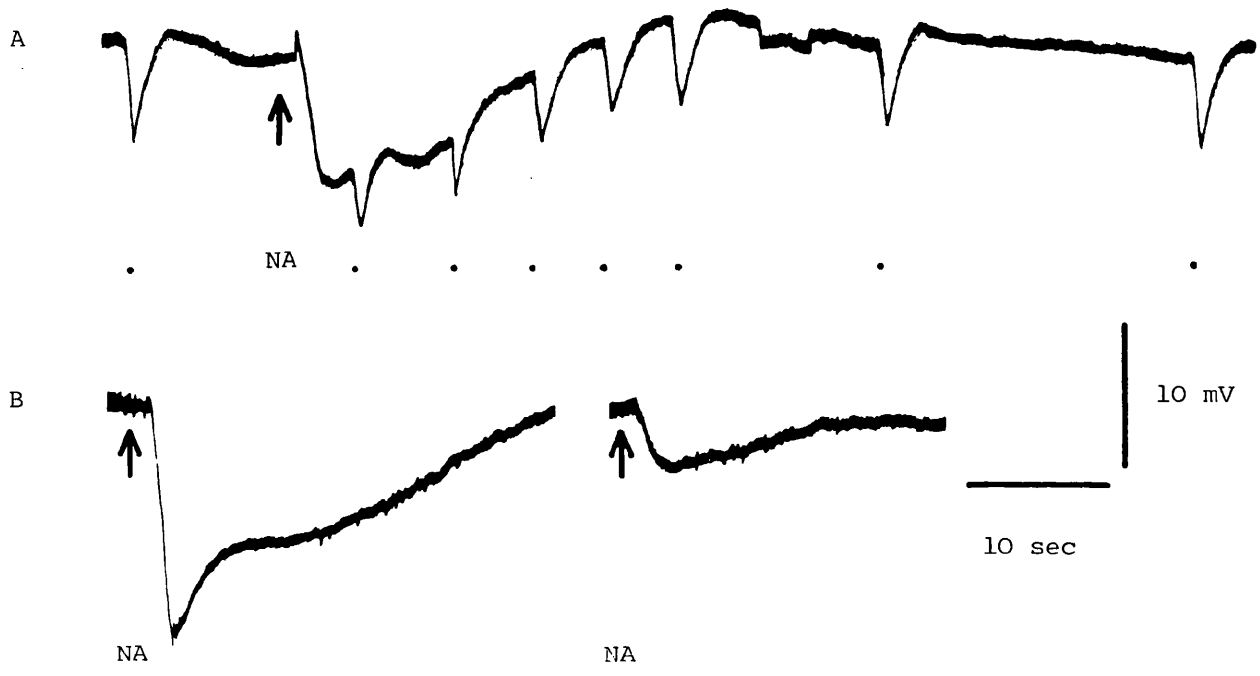

Fig. 4.11. A. The effects of noradrenaline (NA) (10 µl of 10^{-4}M) on the resting potential and the inhibitory junction potentials in response to pelvic nerve stimulation. Trains of 10 stimuli at 10 Hz were delivered during the NA-induced hyperpolarisations (• 0.5 msec, supra-maximal voltage). The inhibitory junction potentials were reduced in parallel with the membrane hyperpolarisation.

B. The effect of a subsequent dose of NA (10 µl of 10^{-4}M) in same cell 1 min after first dose showing desensitisation of membrane to the catecholamine.

SECTION III

SKELETAL MUSCLE

ELECTROPHYSIOLOGY

Innervation of skeletal muscle fibres

Vertebrate nerve voluntary muscle preparations are composed of several thousand muscle fibres each of which usually receives a single nerve terminal. Since the muscle fibres are electrically isolated from one another, all the post-junctional activity recorded when these cells are penetrated with micro-electrodes at the end-plate represents the activity of neurotransmitter liberated from a single nerve ending.

In the present study, using rat diaphragm, occasional cells were encountered which received a multiple or polyneuronal innervation. These fibres were observed rarely, once in every two hundred and fifty cells impaled. Nice alteration of the stimulus strength produced step-wise changes in the size of the end-plate potential indicating activation of one or more nerve fibres with different thresholds. Dual and triple (Fig. 5.1) innervated cells were observed. It is well known that the developing somatic motor nervous system makes many superfluous synaptic connections which are eliminated with maturation (O'Brien, Östberg & Vrbová). The cells described above presumably represent occasional fibres in which the inappropriate nerve terminals have not been eliminated, with maturation.

In this section of the thesis, the effects of d-tubocurarine on neuromuscular transmission at the skeletal neuromuscular junction is described. The principle aim of the investigation was to determine

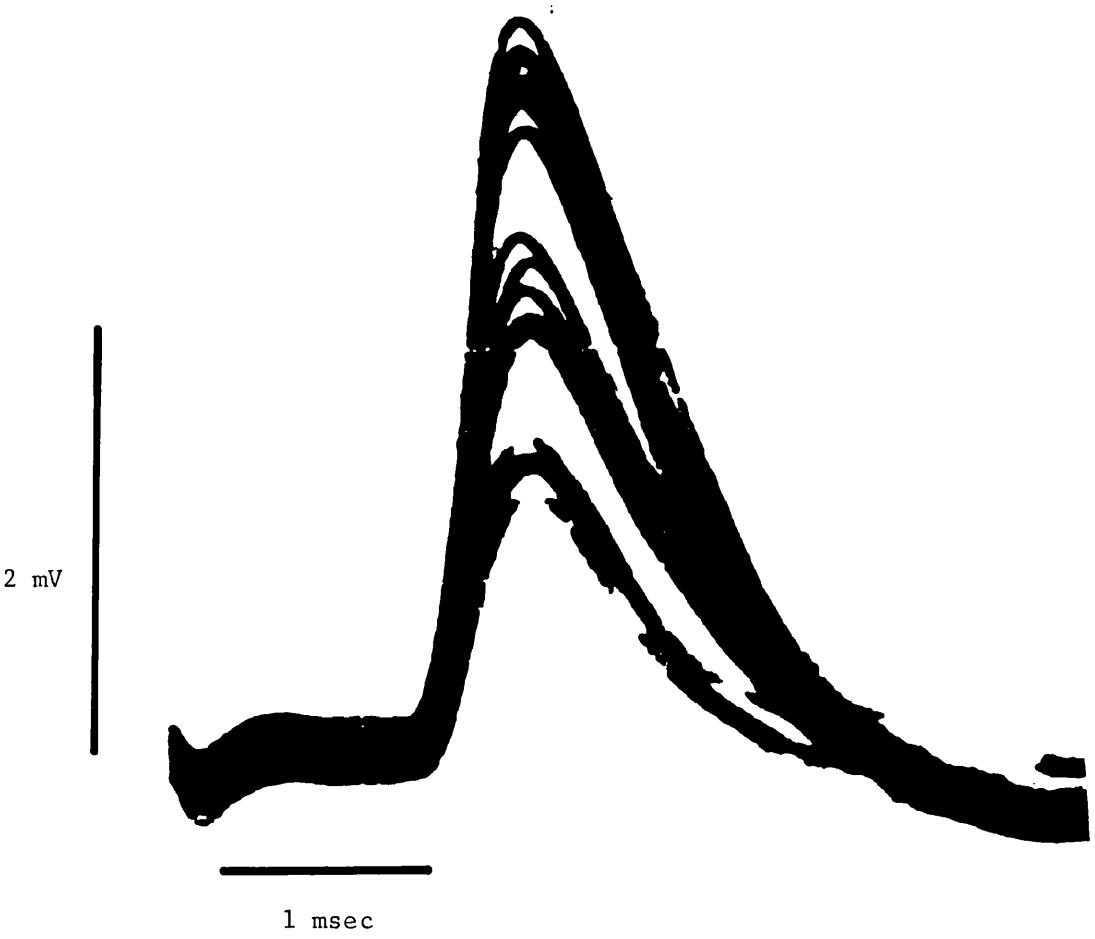

Fig. 5.1. Multiple innervation of a cut muscle fibre in the rat diaphragm. End-plate potentials (e.p.p.s) were evoked by raising and lowering the strength of the stimulus applied to the phrenic nerve. Record shows $\geqslant$ 6 superimposed e.p.p.s evoked at 3 different stimulus strengths in the same cell.

whether d-tubocurarine paralyses skeletal muscle by a prejunctional action on somatic motor nerves in addition to its well documented action on post-junctional nicotinic receptors. Much of the work describing a prejunctional effect of d-tubocurarine came from studies using mammalian tissue while most of the classical work on the post-junctional action of d-tubocurarine was carried out with amphibian nerve muscle preparations.

In the present study the transmission process in frog and rat muscle was investigated using electrical recording and examination of the muscle contractions following nerve stimulation. In this way the transmission process in both species was examined and compared with respect to the action(s) of d-tubocurarine.

RAT DIAPHRAGM

Comparison of end-plate potentials recorded from cut muscle and intact hemi-diaphragm preparations at 37°C and 22°C

The use of the cut muscle preparation with attached phrenic nerve allows one to study neuromuscular transmission in the absence of drugs. End-plate potentials recorded in cut muscle fibres were compared to subthreshold end-plate potentials recorded from intact hemi-diaphragm preparations exposed to d-tubocurarine (10^{-6}g/ml). Trains of end-plate potentials were evoked at increasing frequencies with 10 supramaximal stimuli applied to the phrenic nerve. The amplitude of the last end-plate potential in a train was expressed as a percentage of the amplitude of the first end-plate potential at each frequency of stimulation (1 Hz, 2 Hz, 5 Hz, 10 Hz). In this way one could quantify the relative decline in end-plate potential amplitude during a train (% depression first end-plate potential) at different frequencies of stimulation. The decline in

end-plate potential amplitude may reflect a decrease in the amount of transmitter liberated per stimulus.

End-plate potential amplitude was better maintained in cut muscle fibres than in uncut fibres exposed to d-tubocurarine at all frequencies of stimulation (Fig. 5.2). The higher the frequency of stimulation the greater the end-plate potential depression by the 10th pulse in a train. One explanation is that in the presence of d-tubocurarine successive pre-junctional action potentials release smaller amounts of neurotransmitter.

When similar experiments were carried out at room temperature ($22^{o}C$) depression of end-plate potential amplitude during a train was more marked than at $37^{o}C$ in both control cut muscle cells and in hemi-diaphragms exposed to d-tubocurarine (Fig. 5.3).

The effect of d-tubocurarine on end-plate potentials recorded in cut muscle fibres

Maintenance of end-plate potential amplitude during trains of stimuli at increasing frequency may be a special property of the transmission process in cut muscle preparations. This possibility was investigated by observing the effects of d-tubocurarine on trains of 10 end-plate potentials evoked at a low (1 Hz) and high (10 Hz) frequency in cut muscle fibres.

In control cut muscle fibres, end-plate potentials were well maintained during trains of stimuli at 1 Hz and 10 Hz. In cut muscle preparations exposed to d-tubocurarine (5×10^{-7} g/ml) there was marked depression of end-plate potential amplitude during the train at both frequencies. The degree of depression was greater at 10 Hz than at 1 Hz. The effects were reversible by washing (Fig. 5.4).

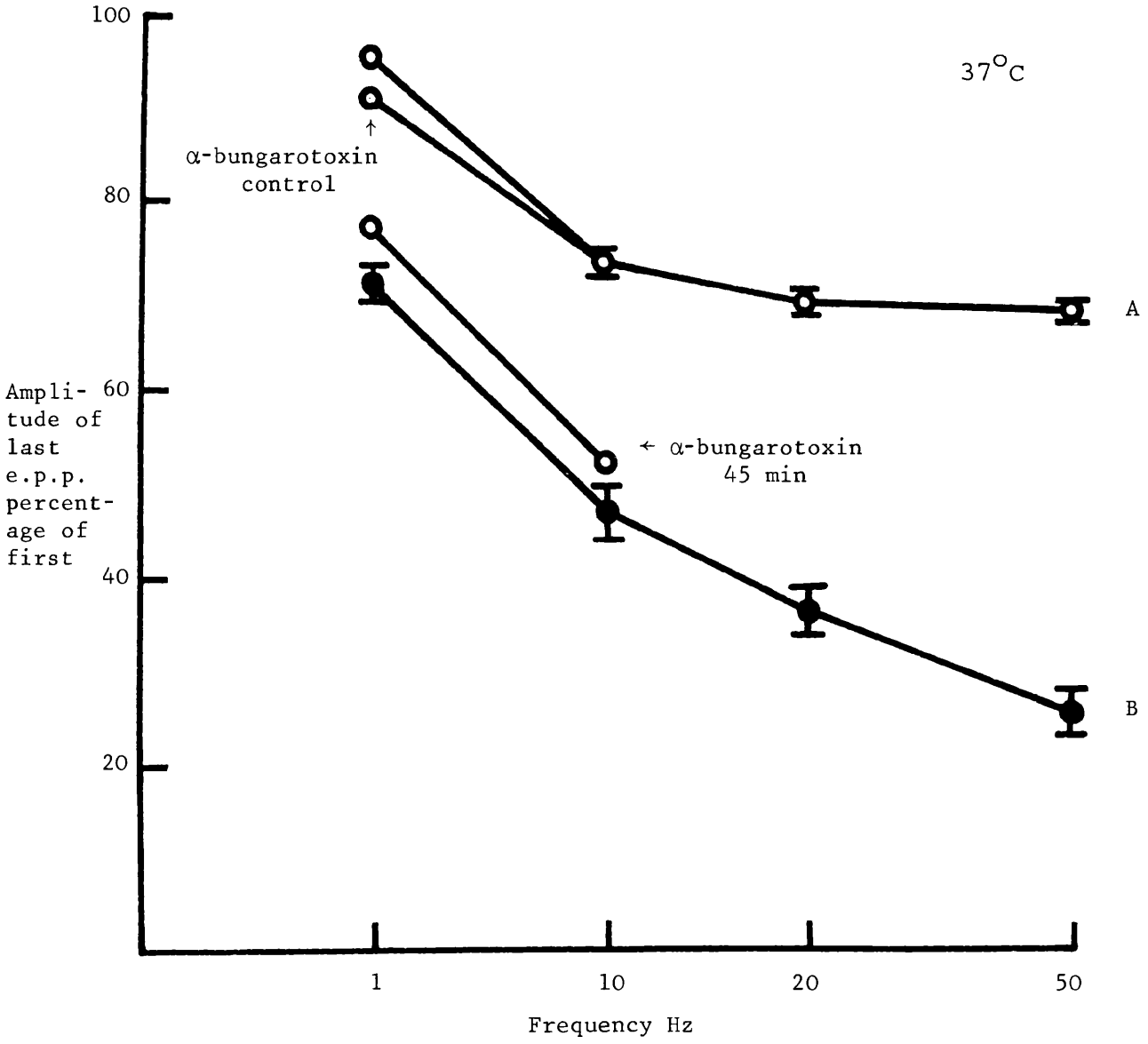

Fig. 5.2. The maintenance of end-plate potential (e.p.p.) amplitude at 37°C in response to indirect stimulation of rat diaphragm preparations and the effect of α-bungarotoxin. E.p.p.s were recorded in cut muscle fibres. Trains of 10 e.p.p.s were evoked at 1 Hz, 10 Hz, 20 Hz and 50 Hz. The amplitude of the last e.p.p. in a train is plotted as a percentage of the first. In one cell the effect of α-bungarotoxin was followed for 45 min.

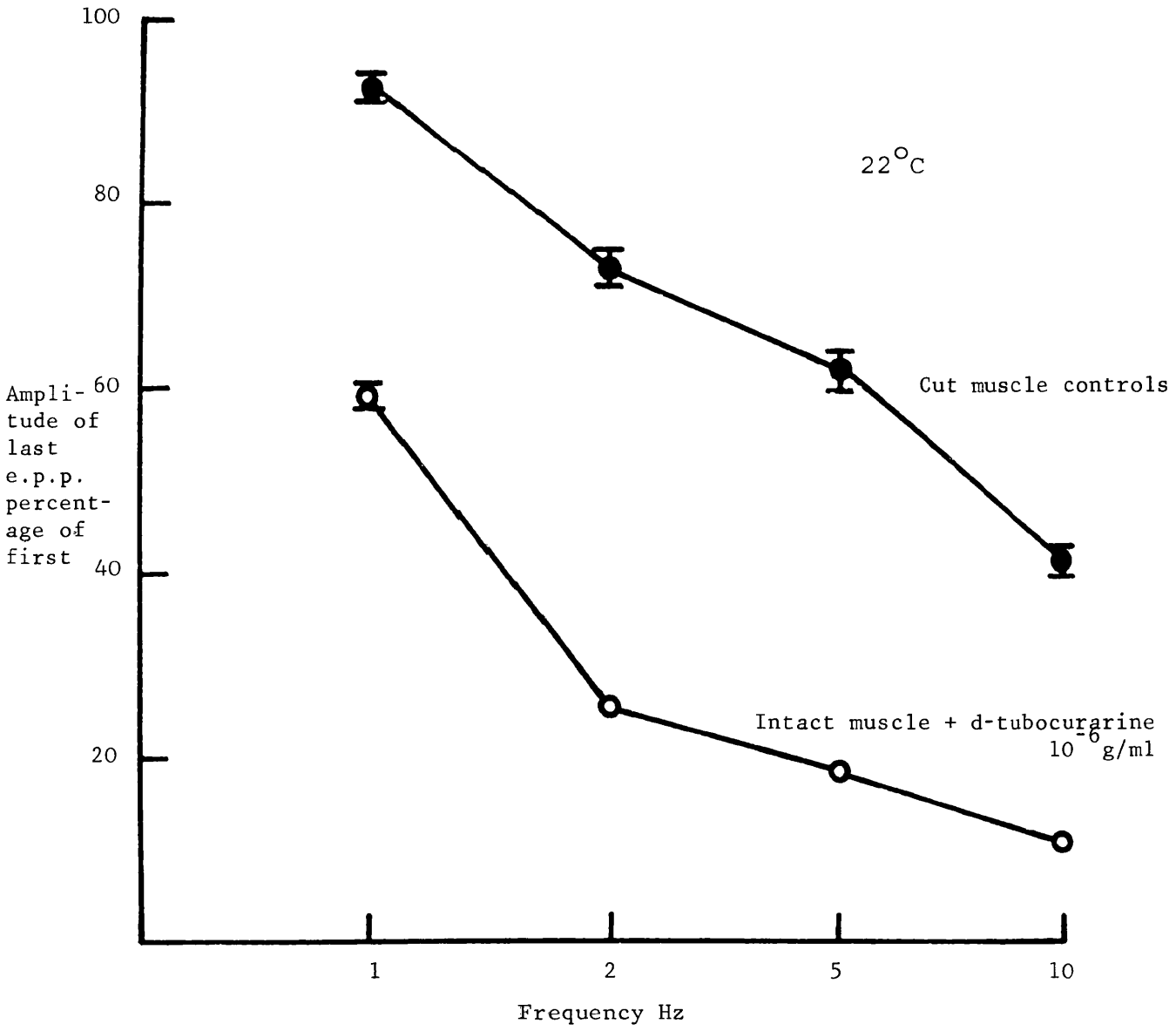

Fig. 5.3. The maintenance of end-plate potential (e.p.p.) amplitude at 22°C in response to indirect stimulation of rat diaphragm preparations. Trains of 10 e.p.p.s were evoked at 1 Hz, 10 Hz, 20Hz and 50 Hz. The amplitude of the last e.p.p. in a train is plotted as a percentage of the first.

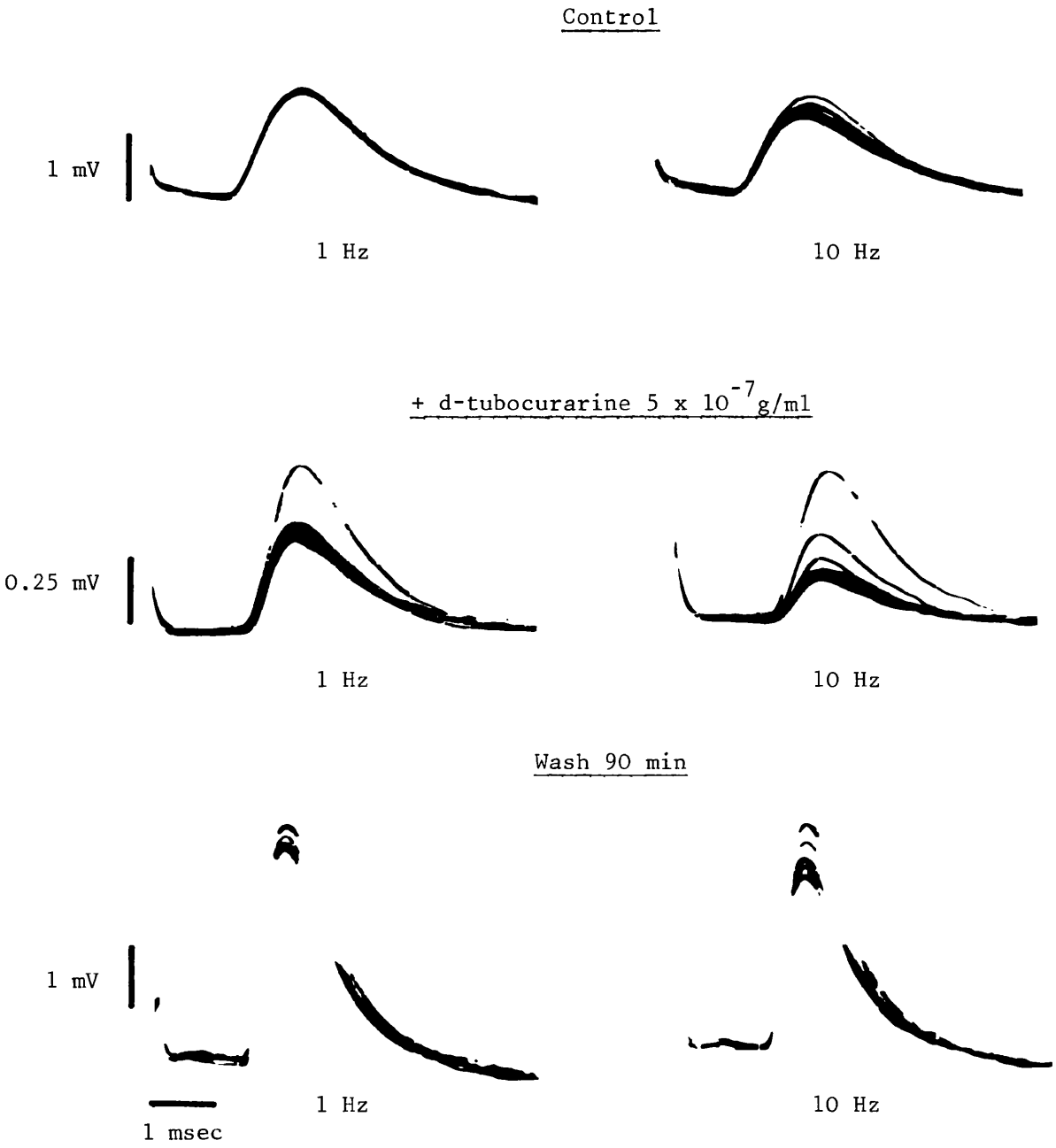

Fig. 5.4. The effects of d-tubocurarine (5×10^{-7} g/ml) on trains of 10 end-plate potentials (e.p.p.s) evoked by repetitive phrenic nerve stimulation at 1 Hz and 10 Hz in cut muscle fibres of the rat diaphragm. The amplitude of the first e.p.p. is always the largest. E.p.p.s progressively decline throughout the train. Records are from 3 different cells in the same preparation.

The effects of d-tubocurarine on end-plate potentials evoked at a low frequency

Workers who accept that d-tubocurarine has a prejunctional effect on transmitter release from somatic motor nerves consider the effect to be important only at high frequencies of stimulation (see Bowman & Webb, 1976). It was of interest therefore to investigate the effects of d-tubocurarine on end-plate potentials evoked at a low stimulation frequency (1 Hz). Experiments were carried out in both cut muscle and intact hemi-diaphragm preparations exposed to d-tubocurarine. Trains of 10 end-plate potentials were evoked by supramaximal nerve stimulation at 1 Hz. The amplitude of the last end-plate potential in the train was expressed as a percentage of the first.

End-plate potential amplitude was well maintained during a train of stimuli at 1 Hz in control cut muscle preparations. A marked decline in end-plate potential amplitude occurred in all diaphragm preparations exposed to d-tubocurarine; the magnitude of the decline being similar in both cut muscle and hemi-diaphragm preparations exposed to d-tubocurarine (Fig. 5.5).

Lowering the temperature from 37°C to 22°C increased the magnitude of decline in end-plate potential amplitude during a train in both control cut muscle preparations and in all preparations exposed to d-tubocurarine. This result may indicate that d-tubocurarine exerts a prejunctional effect at low frequencies of stimulation.

Time course of end-plate potential depression in cut muscle fibres exposed to d-tubocurarine

The time course of depression of end-plate potentials evoked at 1 and 10 Hz in cut muscle exposed to d-tubocurarine (5×10^{-7} g/ml) was

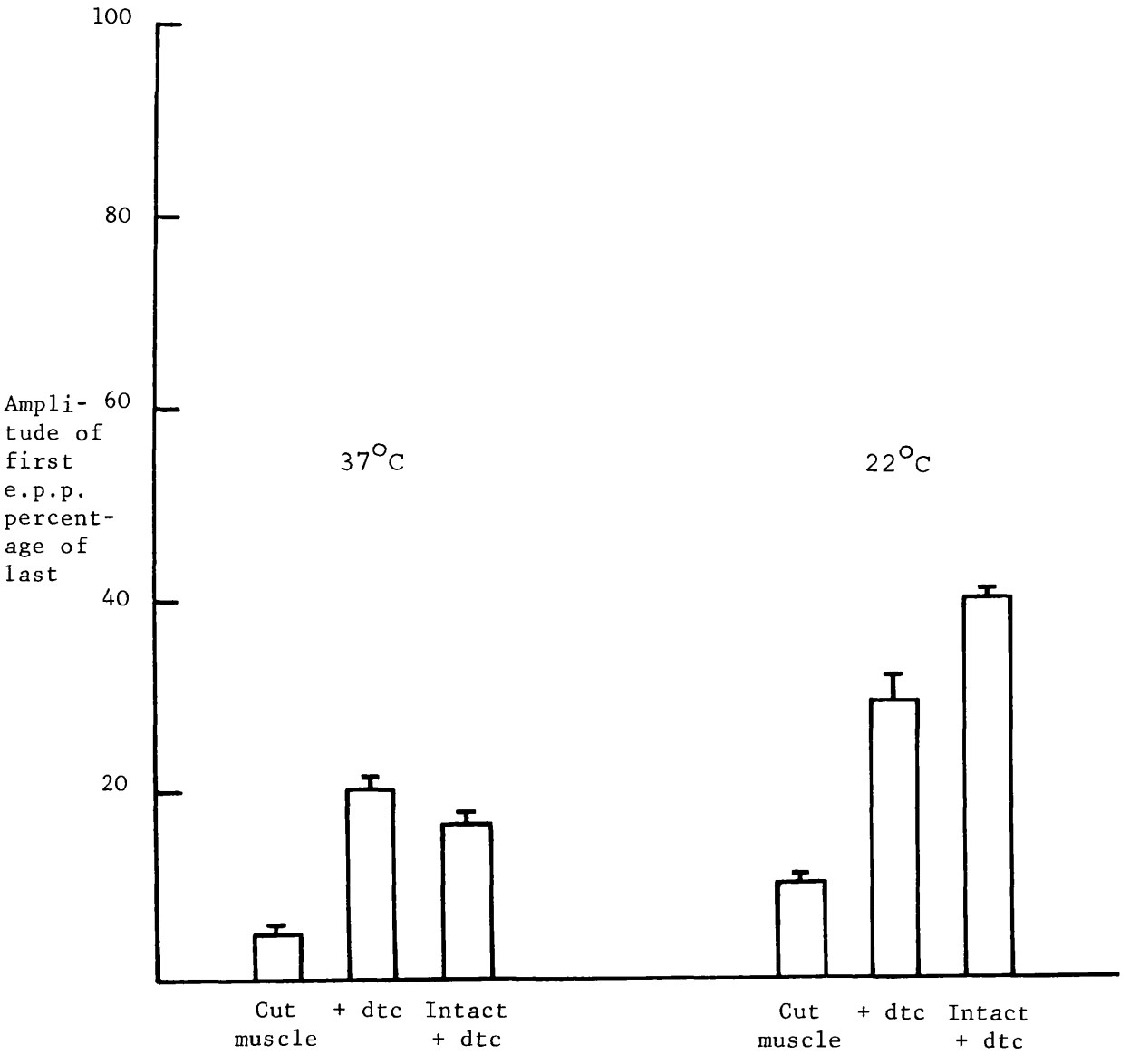

Fig. 5.5. The effect of d-tubocurarine and cooling on the decline of end-plate potential (e.p.p.) amplitude during a train. Trains of 10 e.p.p.s were evoked at 1 Hz. The decline in amplitude of the last e.p.p. in a train was expressed as a percentage of the amplitude of the first. Experiments were carried out at 37°C and at 22°C.

studied. A conditioning train of 10 end-plate potentials was evoked, followed by a second train of end-plate potentials at precise time intervals following the first end-plate potential in the conditioning train. The amplitude of the first end-plate potential in the second train was expressed as a fraction of the first end-plate potential in the conditioning train and the results of the calculation of end-plate potential fraction plotted against time. A decrease in end-plate potential amplitude was evident for up to 5 sec after the conditioning stimuli. The degree of end-plate potential depression was more marked at 10 Hz than at 1 Hz but the time course of recovery was similar at both frequencies of stimulation (Fig. 5.6).

The effects of d-tubocurarine on end-plate potentials evoked in cut muscle preparations exposed to 10 mM Mg^{2+}

There are two explanations for the decline in end-plate potential amplitude during a train. One interpretation is that d-tubocurarine in addition to its post-junctional receptor blocking action, exerts a pre-junctional effect on the motor nerve terminals to depress transmitter release. Alternatively, the observation can be explained without postulating a mechanism other than the classic view of post-junctional receptor blockade. In 'normal' muscle with a 'normal' dose response curve to acetylcholine, d-tubocurarine will produce a parallel shift of that curve to the right. If repetitive nerve stimulation causes a progressive decline in the release of transmitter then the expression of that decline in terms of an end-plate potential will differ, and may differ because of the shift to the right of the dose response curve. Since d-tubocurarine has a competitive post-junctional action then one must expect this to occur and all that is in dispute is the magnitude.

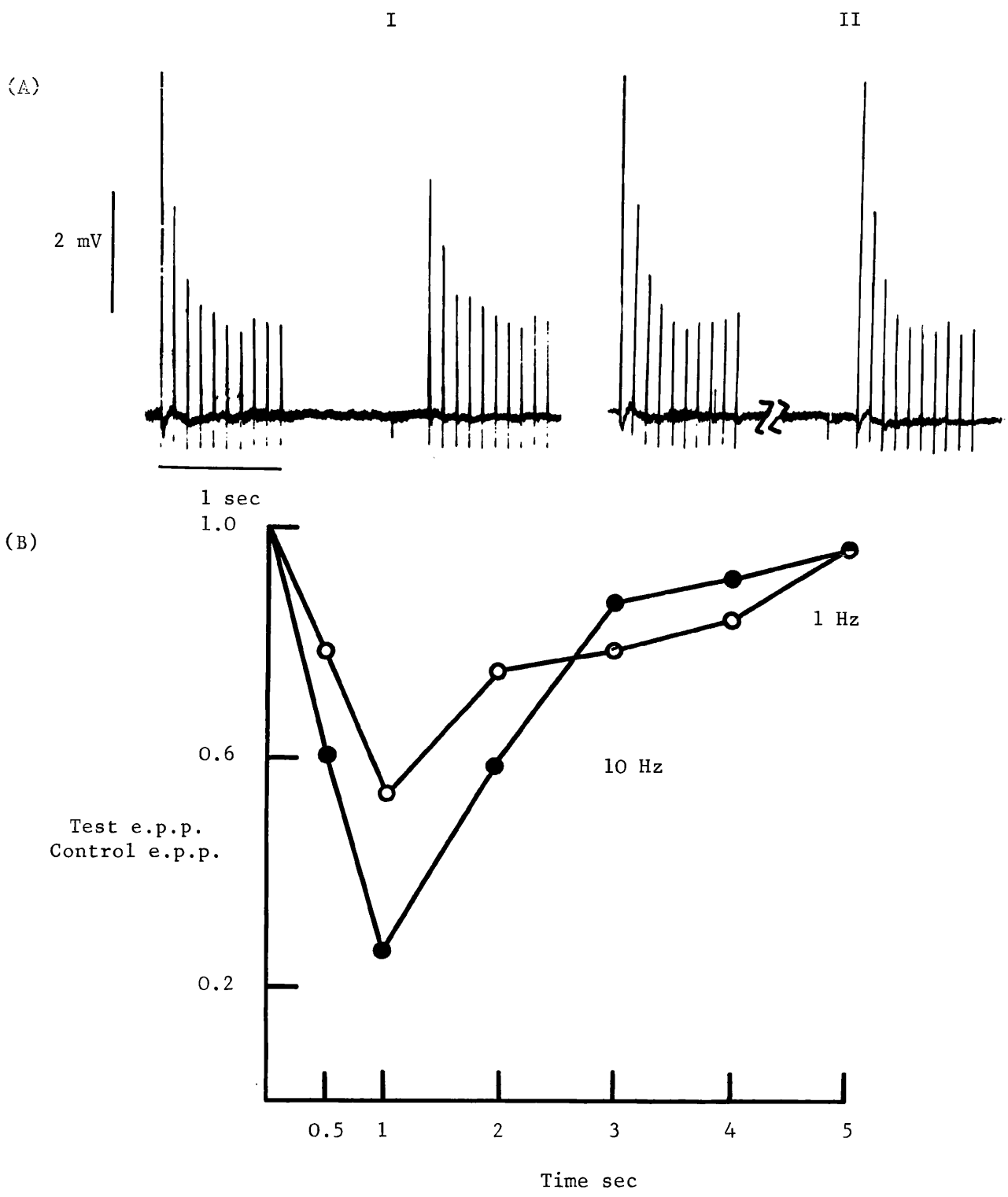

Fig. 5.6. Long lasting depression of neuromuscular transmission in cut muscle fibres exposed to d-tubocurarine (5×10^{-7} g/ml). (A) Conditioning trains of 10 end-plate potentials (e.p.p.s) were evoked at 1 Hz, o, and 10 Hz, •. At precise time intervals after the first pulse in the conditioning train, a second, test train was evoked. (B) The amplitude of the first e.p.p. in the test train was expressed as a fraction of the first e.p.p. in the conditioning train and plotted against time. I and II illustrate the depression of the test e.p.p. at 10 Hz, 2 sec and 4 sec after the conditioning train, respectively.

Also in cut muscle fibres when the tissue is depolarised then the magnitude of the response to a given increment in acetylcholine will differ from normal and successive increments will not be equal (Martin's correction), further arguing that the end-plate potential is well up the dose response curve. One way of testing this hypothesis was to change the position of the end-plate potential on the acetylcholine dose response curve by means other than d-tubocurarine.

The effects of d-tubocurarine on end-plate potentials recorded in cut muscle fibres exposed to 10 mM Mg^{2+}

The number of acetylcholine quanta liberated per stimulus is small when diaphragm preparations are exposed to high magnesium concentrations (10 mM). Any problems due to non linear summation are eliminated as the end-plate potential is composed of small numbers of quanta, and the position of the end-plate potential on the acetylcholine dose response curve will therefore change. Blockade of neuromuscular transmission with Mg^{2+} leads to a change in the character of end-plate potentials evoked by repetitive nerve stimulation.

Facilitation of successive end-plate potentials in a train occurred due to an increase in the number of packets of transmitter liberated by successive impulses (Liley, 1956). At low (1 Hz - 10 Hz) frequencies of stimulation responses were variable (due to a large number of failures). To obtain reproducible responses it was necessary to use a high frequency of stimulation (100 Hz). The results cannot be compared with those described previously due to the appearance of this new phenomenon (facilitation). The point to be made with these experiments is whether d-tubocurarine can influence the process of facilitation which has a prejunctional origin.

Trains of 10 end-plate potentials were evoked at 100 Hz. The amplitude of the first end-plate potential in the train was expressed as 100%. Subsequent end-plate potentials were expressed as a percentage of the first end-plate potential in the train. The mean amplitude of the end-plate potentials increased throughout the train. D-tubocurarine (5×10^{-7} g/ml) reduced or abolished facilitation (Fig. 5.7). Since facilitation has a prejunctional origin, this result suggests that d-tubocurarine can influence events in the motor nerve terminal.

Much of the classical work on the mechanism of action of d-tubocurarine has been carried out at the frog neuromuscular junction. Most of the evidence purporting to show a prejunctional action of d-tubocurarine has come from studies on mammalian tissue. Similar experiments were therefore carried out using frog sartorius preparations (intact and cut muscle) and the results compared to those with rat diaphragms presented earlier. One can thus compare the transmission process in both species with respect to the action of d-tubocurarine. If the effects of d-tubocurarine described previously are due to a dose response curve shift, then a similar decline in end-plate potential amplitude should be observed in both species.

FROG SARTORIUS

Two preparations of sartorius with attached ischid nerve were used: (1) intact sartorius exposed to d-tubocurarine (10^{-6} g/ml) and (2) cut muscle preparation (in absence and presence of d-tubocurarine). Trains of 10 end-plate potentials were evoked by supramaximal nerve stimulation at 1 Hz, 10 Hz, 20 Hz and 50 Hz. The amplitude of the last end-plate potential in a train was expressed as a percentage of the first. At moderate frequencies of stimulation (1 Hz - 10 Hz) end-plate potential amplitude was well maintained in both control and preparations exposed

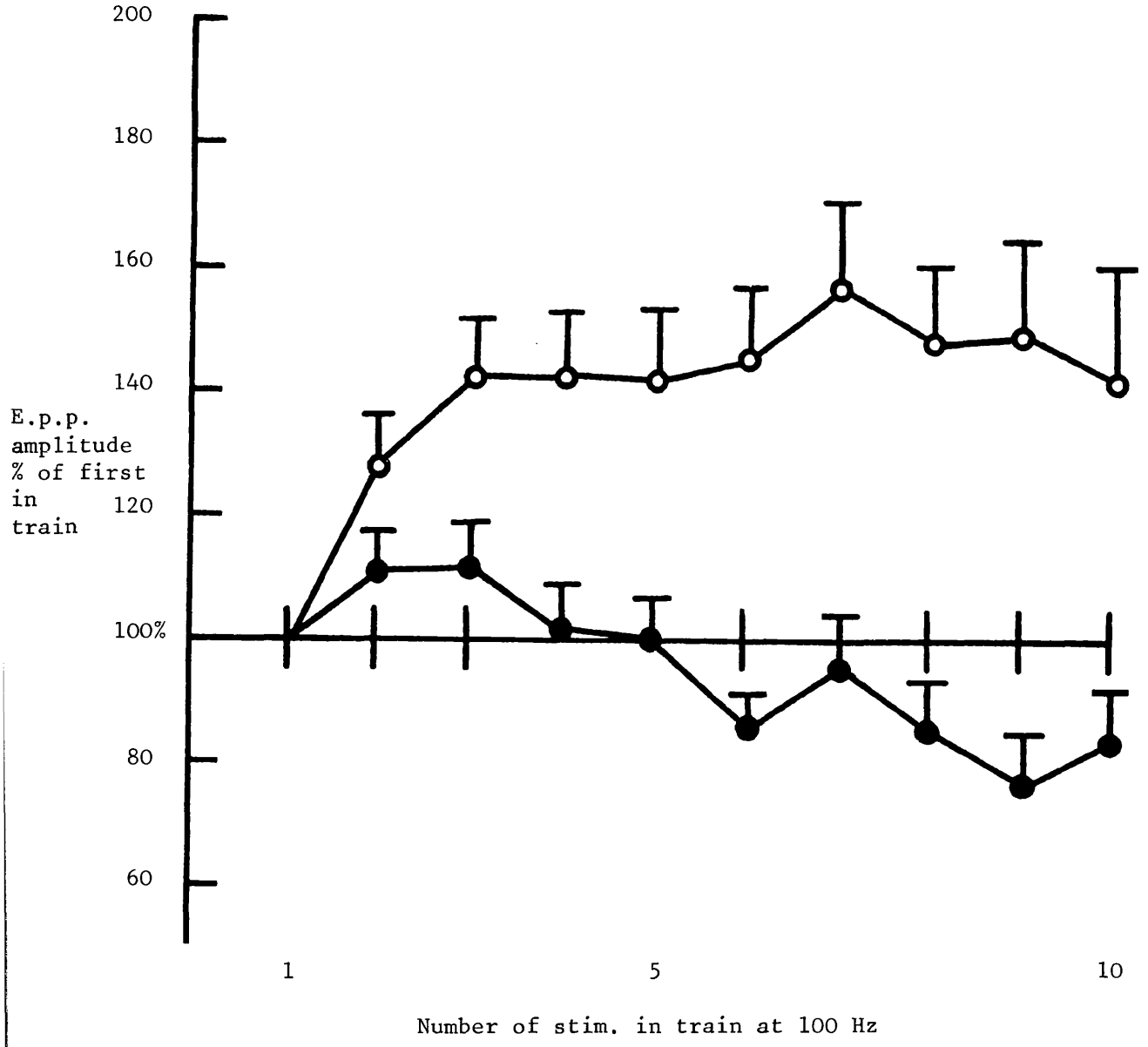

Fig. 5.7. The effects of d-tubocurarine (5×10^{-7} g/ml) on end-plate potentials (e.p.p.s) evoked by phrenic nerve stimulation at 100 Hz in cut muscle fibres of the rat diaphragm. o, 10 mM Mg^{2+} controls; •, + d-tubocurarine.

to d-tubocurarine. At high frequencies of stimulation (20 Hz, 50 Hz) marked potentiation of the first few end-plate potentials in a train occurred, under all experimental conditions. This augmentation was greater at the beginning than at the end of a train. Facilitation of end-plate potential amplitude was not modified by d-tubocurarine. These results are compared with those obtained when similar experiments were carried out in the rat (Fig. 5.8). The results are in marked contrast to those obtained in rat diaphragm preparations (where an increase in the frequency of nerve stimulation intensified the decline in end-plate potential amplitude during a train).

RAT DIAPHRAGM

The effect of d-tubocurarine on the mechanical response of rat hemi-diaphragm following phrenic nerve stimulation

Isometric twitch responses were evoked by trains of 30 supramaximal stimuli at 1 Hz, 2 Hz, 5 Hz and 10 Hz. Control responses were well maintained throughout the train at moderate stimulation frequencies. Some fade was observed at 10 Hz (Fig. 5.9).

In the presence of d-tubocurarine (5×10^{-7} g/ml) there was a small reduction in the amplitude of the first twitch. During a train, there was a decline in twitch amplitude, from the first. The magnitude of the decline increased with increasing frequency of stimulation and reached a plateau after 4-8 stimuli. After prolonged exposure to d-tubocurarine (15 min) the magnitude of decline increased, with little or no further effect on the amplitude of the first twitch.

Since the effects were reversible by washing, they cannot readily be attributed to anoxia in a deteriorating preparation. The record of one such experiment is shown in Fig. 5.9.

The decline in amplitude of responses was expressed graphically.

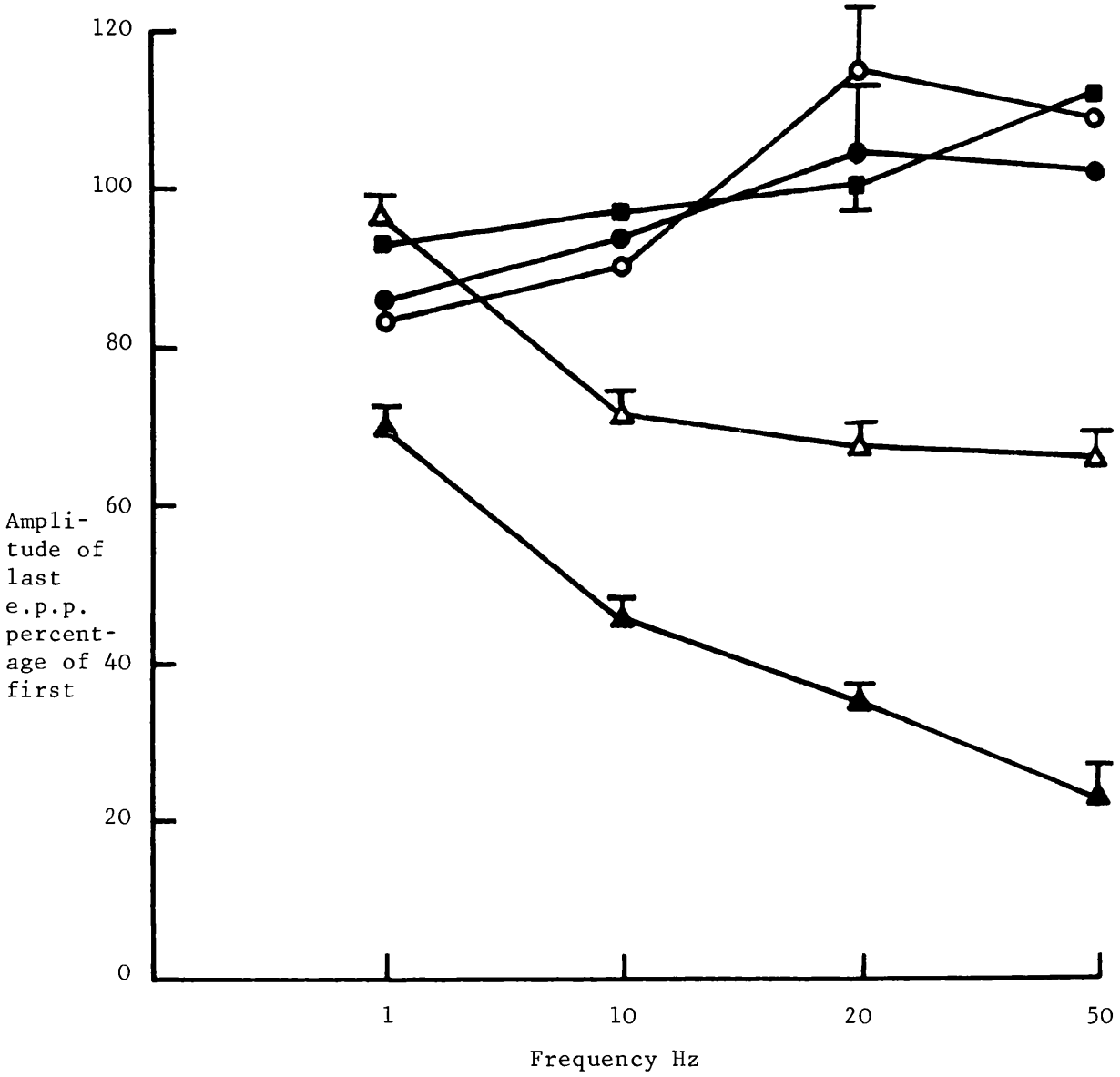

Fig. 5.8. Maintenance of end-plate potentials (e.p.p.s) in frog sartorius and rat diaphragm preparations in response to increasing frequency of nerve stimulation. Trains of 10 e.p.p.s were evoked by indirect nerve stimulation at 1, 10, 20 and 50 Hz. The amplitude of the last e.p.p. in a train is expressed as a percentage of the first. Δ, rat cut hemi-diaphragm control; ▲, rat hemi-diaphragm + d-tubocurarine 10^{-6}g/ml; o, frog sartorius + d-tubocurarine; □, frog cut sartorius control; ■, frog cut sartorius + d-tubocurarine 10^{-6}g/ml.

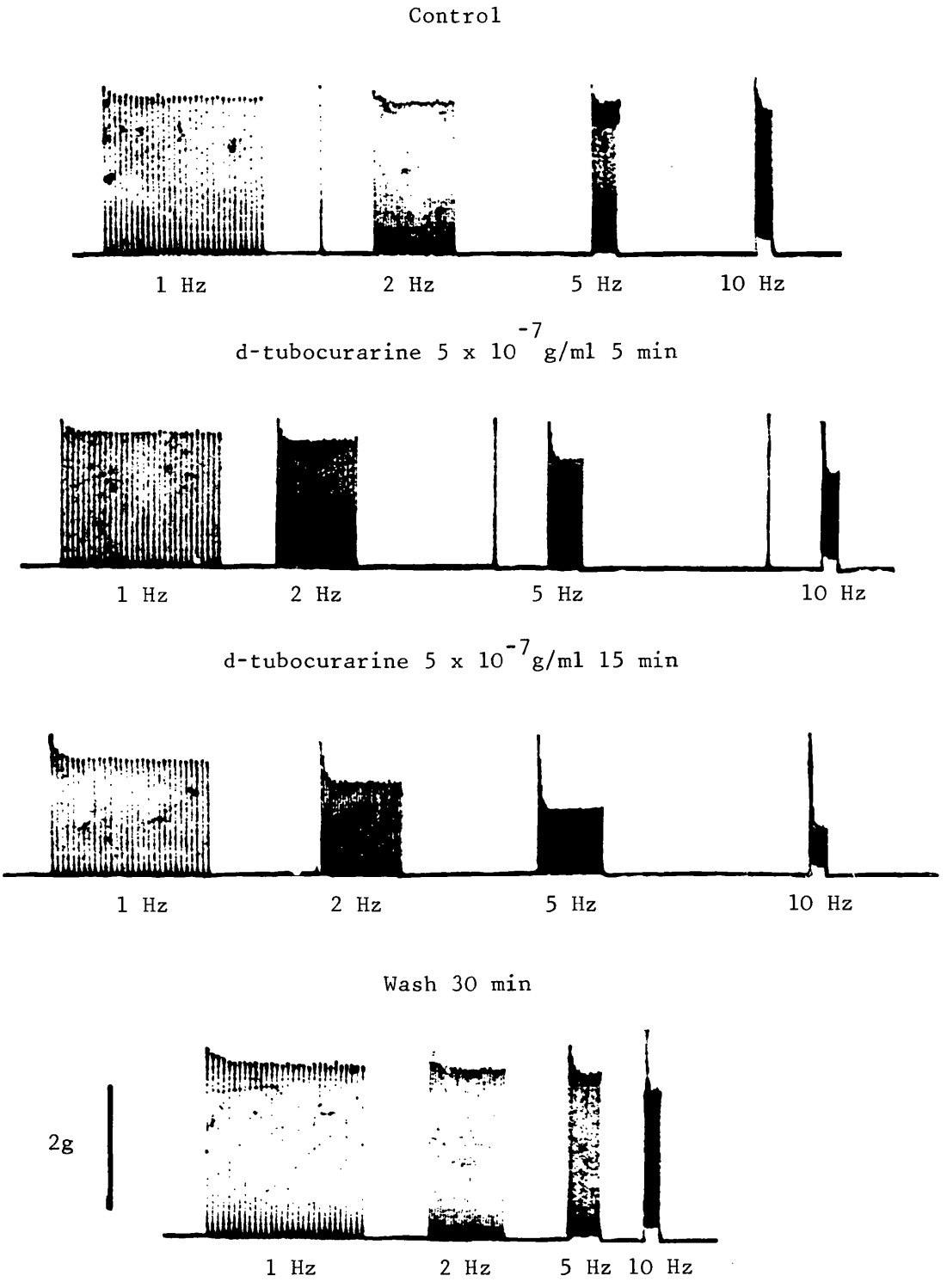

Fig. 5.9. The effects of d-tubocurarine (5×10^{-7} g/ml) on the twitch tension response of a rat hemi-diaphragm preparation. The muscle was excited by phrenic nerve stimulation with trains of 30 supramaximal stimuli at 1 Hz, 2 Hz, 5 Hz and 10 Hz. Isometric recording.

The amplitude of the last twitch in a train was expressed as a percentage of the first, and the resultant percentage depression plotted against the frequency of stimulation (Fig. 5.10).

Complete abolition of all responses occurred when higher (10^{-6}g/ml) concentrations of d-tubocurarine were used. Since there were no responses, it was not possible to investigate any contribution a prejunctional action of d-tubocurarine might have had. To overcome this problem, the time course of recovery of responses during washing was studied.

Trains of 30 twitches were evoked at 1-10 Hz. D-tubocurarine (10^{-6}g/ml) was added to the Krebs bathing the preparation. When responses were abolished the preparation was washed. Trains of 30 twitches at 1-10 Hz were then evoked at 5 min intervals from the onset of the wash. A marked discrepancy in the recovery phase of twitches at different points in a train was found. The amplitude of the first twitch in a train readily recovered to control values (10-15 min). However, during a train there was a marked decline in the amplitude of subsequent responses. The magnitude of the decline in amplitude increased with increasing frequency of stimulation. At 10 Hz, although the amplitude of the first response in a train was similar to that of control value, the amplitude of the fourth response was zero.

With prolonged washing (> 30 mn) the twitch responses at all frequencies returned to control values. The record of one such experiment is shown in Fig. 5.11. These results are quantitatively similar to those obtained when observing the effect of d-tubocurarine on end-plate potentials. However, it must be remembered that the twitch is a composite response due to the synchronous contraction of several thousand muscle fibres.

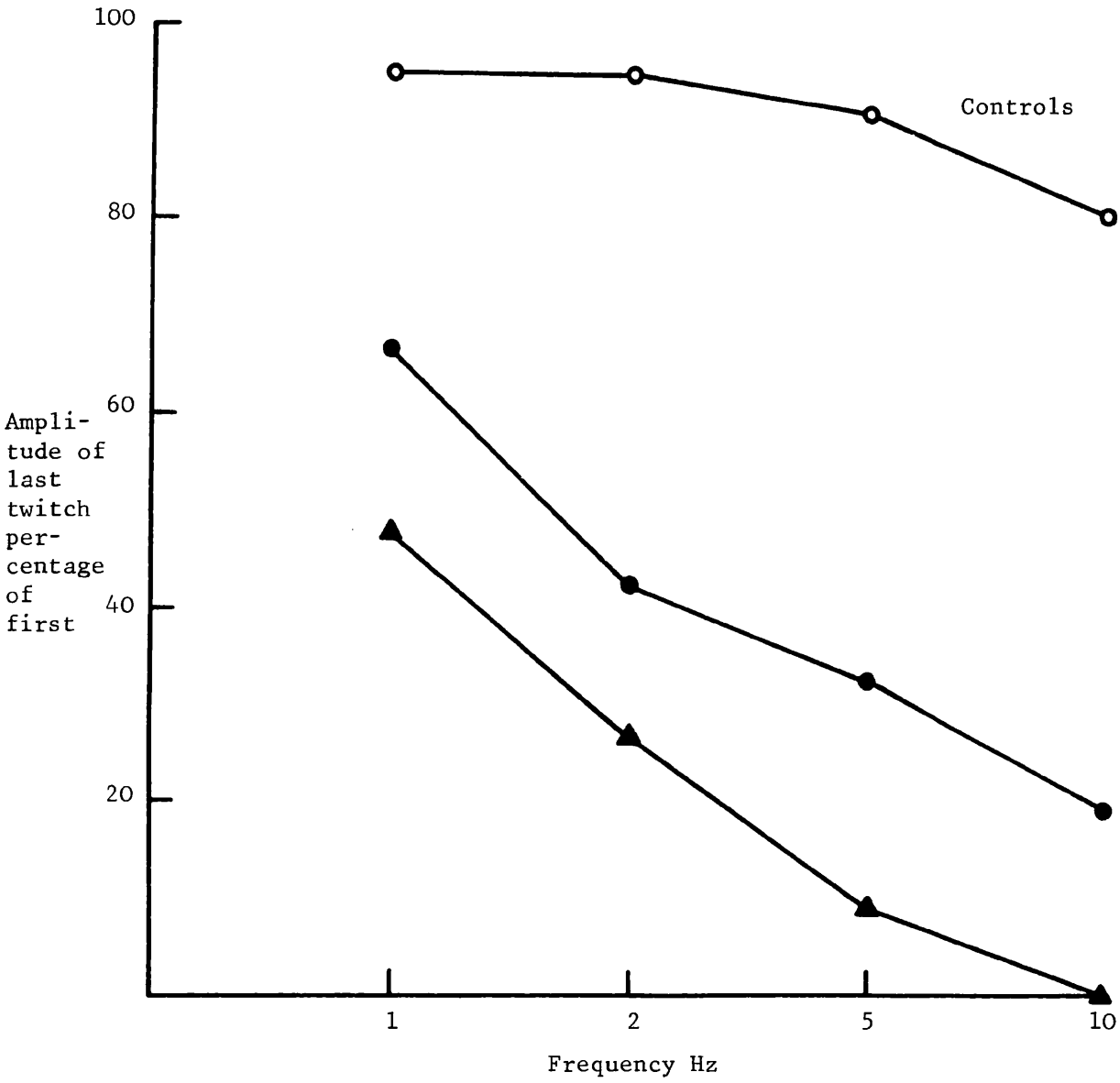

Fig. 5.10. The effect of increasing concentrations of d-tubocurarine on the twitch tension of rat hemi-diaphragms. Preparations were excited by phrenic nerve stimulation with trains of 30 supramaximal stimuli at 1 Hz, 2 Hz, 5 Hz and 10 Hz. The amplitude of the last twitch in a train was expressed as a percentage of the first. Isometric recording. o, Control; •, d-tubocurarine 5 x 10^{-7} g/ml; ▲, d-tubocurarine 10^{-6} g/ml. $n = \geqslant 6$.

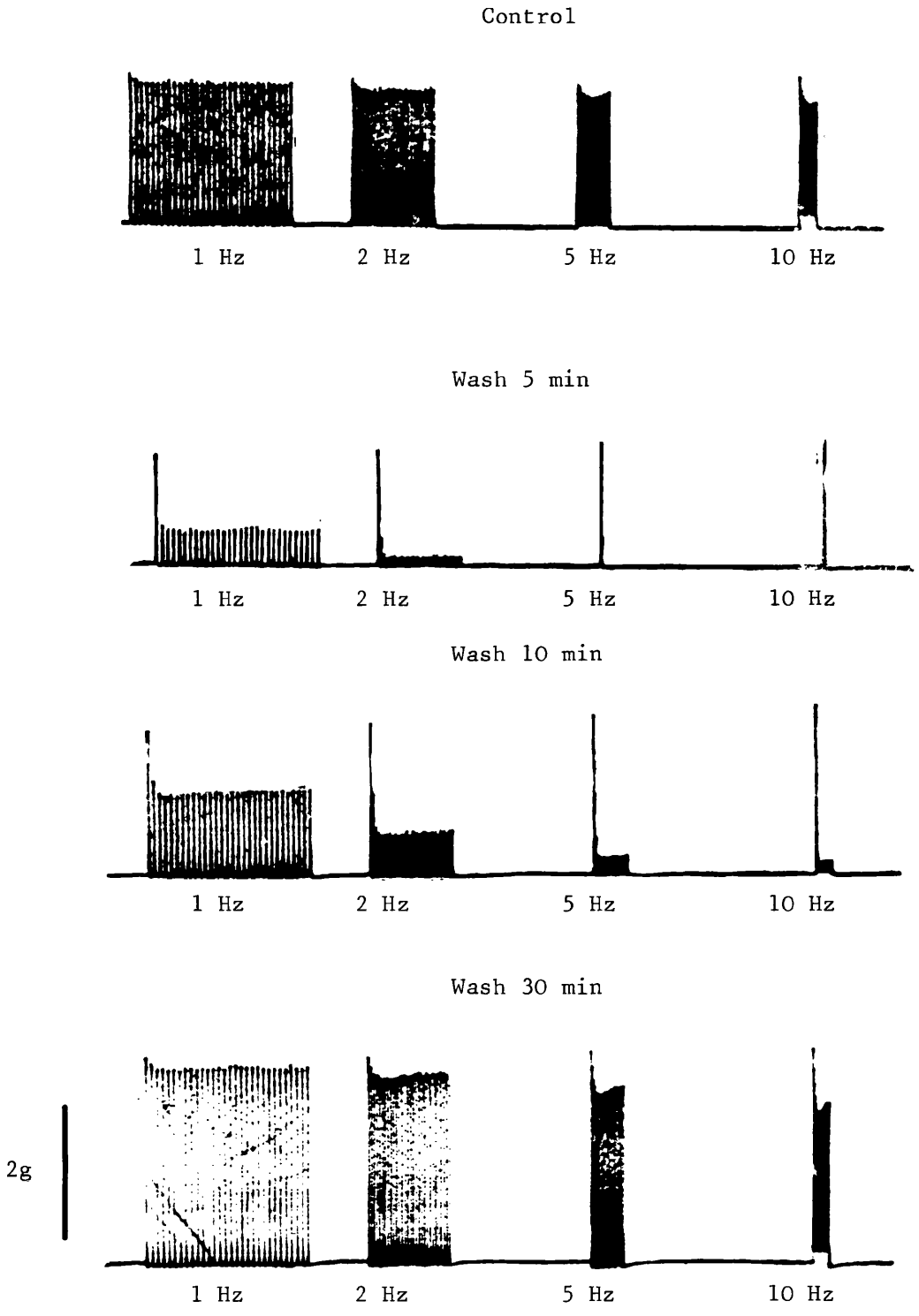

Fig. 5.11. The effects of a high concentration of d-tubocurarine (10^{-6} g/m) on the twitch response of a rat hemi-diaphragm preparation after d-tubocurarine had been washed out. Trains of 30 supramaximal stimuli were delivered to the phrenic nerve at 1 Hz, 2 Hz, 5 Hz and 10 Hz. Isometric recording.

The effect of α-bungarotoxin on end-plate potentials in rat diaphragm cut muscle preparation

α-bungarotoxin irreversibly binds to post-junctional nicotinic receptors at the skeletal neuromuscular junction (Lee, 1972).

In one preliminary experiment α-bungarotoxin induced a frequency dependent decline in end-plate potential amplitude in cut muscle preparations which were similar to that produced by d-tubocurarine (see Fig. 5.2). If the increased decline in end-plate potential amplitude during a train in the presence of d-tubocurarine is due to a shift in the acetylcholine dose response curve, then a similar decline should be observed with any nicotinic receptor antagonistic. Preliminary experiments with the competitive nicotinic receptor antagonists di- and trimethyltubocurarine and pancuronium showed these compounds to have a similar profile of action on trains of end-plate potentials.

D I S C U S S I O N

GUINEA-PIG VAS DEFERENS

Since its introduction by Huković (1961) as a model system, the isolated vas deferens of the guinea-pig has been widely used to study the transmission of excitation from sympathetic nerves to smooth muscle. The bulk of the morphological, and some pharmacological evidence indicates that the main motor transmitter of the vas deferens is noradrenaline. For example, the high noradrenaline content of the guinea-pig vas deferens corresponds with a very dense distribution of adrenergic terminals in the smooth muscle of this organ (Sjöstrand, 1965). Noradrenaline is released upon hypogastric nerve stimulation (Stjärne, 1973), and the mechanical response of the vas deferens is reduced or abolished by the adrenergic neuron blockers, bretylium and guanethidine (Swedin, 1971). However, the contractile response to short trains of stimuli does not behave as if it were elicited by adrenergic nerves (Ambache & Zar, 1971). The two most important lines of evidence indicating that the main motor transmitter in the guinea-pig vas deferens is not noradrenaline can be summarised briefly. Low concentrations of noradrenaline inhibit the nerve mediated contractions of the vas and α-adrenoceptor antagonists inhibit the contractile response to exogenous noradrenaline without reducing the motor response to nerve stimulation (Ambache & Zar, 1971). Furthermore, neuromuscular transmission still occurs after depletion of neuronal stores of noradrenaline by reserpine and chemical destruction of adrenergic nerve terminals by 6-hydroxydopamine. The indirectly acting sympathomimetic amine tyramine also inhibits the motor response to nerve stimulation presumably by displacing noradrenaline from inhibitory noradrenergic nerve terminals, the displaced noradrenaline activating α-adrenoceptors located on or near the excitatory nerve terminals (see Ambache & Zar, 1971; Ambache *et al.*, 1972; 1975; Anton

et al., 1977; McGrath, 1978; Booth *et al.*, 1978).

McGrath (1978) has further shown by recording the isometric response of the rat vas on an unconventionally fast time base, that there are two distinct phases in the mechanical response to a single pulse, an initial rapid 'twitch' and a slower, better maintained 'secondary' contraction. The two phases show differential susceptibility to drugs, the secondary phase behaving as if it were elicited by adrenergic nerves, the initial twitch being resistant to drugs which are known to block the effects of sympathetic nerve stimulation in other organs. Stjärne (1976) has further postulated that two transmitters, i.e. noradrenaline and potassium, can be released from the same varicosity.

The adrenergic neuron blocking agents, guanethidine and bretylium, however, block the excitatory junction potential (Burnstock & Holman, 1962) and the motor response to hypogastric nerve stimulation (Ambache & Zar, 1971). These latter observations have been explained as non specific actions of these drugs since they have been shown to be capable of disrupting cholinergic transmission at the skeletal neuromuscular junction (Dixit, Gulati & Gokhale, 1961).

The complex hypotheses proposed to explain the innervation of the guinea-pig vas deferens are based to a large extent on observations of the mechanical responses of preparations to field stimulation of the intramural nerve terminals. It was hoped that by studying the effects of various drugs on excitatory junction potentials evoked by single or short trains of stimuli, that a contribution to this rather confusing picture could be made. The excitatory junction potential allows one to study the effects of released transmitter with a time resolution not possible with studies of mechanical responses.

The guinea-pig vas deferens hypogastric nerve preparation *in vitro* was chosen for a number of reasons. First, the electrical characteristics of transmission have been established by Burnstock & Holman (1961), but no systematic pharmacological study of the effects of some newer drugs on the excitatory junction potentials had been carried out. Second, regardless of the chemical nature of the transmitter, it was intended to establish a model for the study of the effects of α-adrenoceptor antagonists on short trains of stimuli. Using intracellular recording, one can study transmitter release evoked by a single pulse as measured by the excitatory junction potential.

The first step was to record the excitatory junction potential and see how variation of the experimental conditions could alter the membrane response of the vas.

THE EFFECTS OF COOLING

Although cooling the vas deferens depolarised the muscle cells from 63 mV to 59 mV, the amplitude, time to peak, and time to decay to half amplitude of the excitatory junction potential were increased. Kuriyama (1963) studied the effects of temperature on the excitatory junction potentials of the guinea-pig vas deferens. The present results agree with Kuriyama that the time course of the excitatory junction potential is prolonged and the rate of depolarisation decreased, but differ as to the effects of temperature on the amplitude of the excitatory junction potential. It was possible to obtain quite different values for excitatory junction potential amplitude according to the criteria for cell penetration adopted. It was found that when excitatory junction potentials were recorded from cells which drifted a few millivolts after penetration by micro-electrodes, excitatory junction

potentials were often smaller than those recorded at 36°C. When strictly adhering to the cell penetration criteria described in the Methods section the excitatory junction potentials recorded were larger at the lower temperature and the vas deferens contracted, usually after one or two stimuli. Visual observation through a binocular microscope showed that the contraction was potentiated and prolonged compared to those observed at 36°C. These results confirm the finding of Della Bella, Gandini & Preti (1965) and Ambache & Zar (1971) who found that cooling potentiated the mechanical response of the guinea-pig vas deferens.

It was more difficult to maintain cell penetrations at room temperature than at 36°C and many more cells failed to seal (criterion 2) after penetration by the micro-electrode, and therefore have slightly lower membrane potentials. The inclusion of these cells in Kuriyama's data would explain the difference in mean resting membrane potential recorded at 20-22°C (59 mV in the present study, 48 mV in Kuriyama's) and would explain the discrepancies between our results since unsealed cells had small excitatory junction potentials.

One of the characteristics of the transmission of excitation from sympathetic nerves in the hypogastric nerve to the smooth muscle of the guinea-pig vas deferens is the progressive increase in amplitude of the first 6 to 8 exictatory junction potentials in a train (Burnstock & Holman, 1961; Burnstock, Holman & Kuriyama, 1964). At 36°C facilitation does not normally occur at a stimulation rate of 0.1 Hz but from 0.25 Hz upwards. Kuriyama (1963) reported that cooling the vas deferens suppressed facilitation at all frequencies of stimulation investigated (0.1-1 Hz). In these latter experiments, facilitation was expressed as the ratio of the fifth excitatory junction potential :

first excitatory junction potential in a train. In agreement, when facilitation is expressed in this way, then facilitation does not occur after cooling. However, reference to Fig. 3.2 shows that the amplitude of the first excitatory junction potential in a train at 0.91 Hz is increased on cooling. Furthermore, there was a small increase only in the amplitude of excitatory junction potentials towards the end of a train at 0.91 Hz. The ratio, first : fifth excitatory junction potential, therefore approached one, and by definition, facilitation was suppressed. Kuriyama (1963) found that the first excitatory junction potential in a train was reduced and of similar amplitude to the fifth. On cooling the vas deferens, I have found that the fifth excitatory junction potential in a train remains approximately the same amplitude as the control but the first excitatory junction potential in the train is increased in amplitude by a factor of two or three. Thus facilitation as measured by a progressive increase in the amplitude of excitatory junction potentials during a train, does not occur. These results suggest that transmitter release by the first stimulus is increased, and that the changes in nerve terminals normally associated with facilitation are already maximally activated in the absence of stimulation, rather than the view that facilitation is suppressed by cooling.

There are a number of possible explanations for the augmentation of excitatory junction potential amplitude by cooling. Potentiation of excitatory junction potential amplitude is unlikely to be due to changes in the number of functional nerve terminals contributing to any one excitatory junction potential due to the use of a submaximal stimulus. First, there was no detectable change in the fractional activity in the vas deferens nerve fibres on cooling showing that the same proportion of nerve fibres were activated at any given stimulus strength. Second,

there was a small increase only in the amplitude of excitatory junction potentials towards the end of a train.

Since the amplitudes of the last excitatory junction potentials in a train are only slightly increased and since the amplitude of spontaneous excitatory junction potentials was decreased by cooling (Kuriyama, 1963), changes in excitatory junction potential amplitude are unlikely to represent an increased responsiveness of the post-junctional membrane.

The amplitude and time to peak of excitatory junction potentials was increased by cooling, effects similar to those reported for the end-plate potential at the skeletal neuromuscular junction (Fatt & Katz, 1952; Takeuchi, 1958). Prolongation of the rising phase of the end-plate potential is attributable to at least two mechanisms, namely, prolongation of the duration of transmitter action by inhibition of the enzyme responsible for its destruction, acetylcholinesterase, and prolongation of the transmitter release process from individual nerve endings. In somatic motor nerves, at low temperature, the release of quanta becomes highly asynchronous and the total duration of the rising phase of the end-plate potential is increased therefore (Katz & Miledi, 1965b, c). Furthermore, evoked transmitter release at the skeletal neuromuscular junction is enhanced by cooling from 37°C to 30°C (see MacIntosh & Collier, 1976). It is likely that similar factors may be involved at the autonomic neuroeffector junction upon cooling. The guinea-pig vas deferens is considered by some to be excited by noradrenaline released from the hypogastric nerves (Sjöstrand, 1965; Swedin, 1971). It is generally accepted that neuronal uptake is the main process responsible for the inactivation of transmitter at sympathetic neuroeffector junctions rather than enzymatic inactivation, and cooling

is known to inhibit neuronal uptake (Iversen, 1967). Bell (1967b) carried out a detailed investigation of the effects of neuronal and extra-neuronal uptake inhibitors, monoamine oxidase and catcholamine -o-methyl transferase inhibitors on the excitatory junction potential of the guinea-pig vas deferens and found that they had little or no effect. Prolongation of the rising phase of the excitatory junction potential by cooling is unlikely to be due to inhibition of uptake processes for catecholamines. It is possible, however, that cooling inhibits the process normally responsible for inactivating the excitatory sympathetic transmitter in the guinea-pig vas deferens.

The slower rising and falling phases of the excitatory junction potential compared with the end-plate potential is probably due to a number of factors (see Burnstock & Costa, 1973). In particular, one is recording the activity of transmitter released from several nerve terminals in a functional smooth muscle syncytium. Prolongation of the rising phase of the excitatory junction potential may be attributed to the asynchronous invasion by action potentials of varicosities contributing to any given excitatory junction potential. This would be due to the differing diameters of the nerve fibres in the vas deferens nerves and is evidenced by the spread of electrical activity (latencies) recorded in the vas deferens nerves on cooling (see Fig. 3.7). More experiments are required to fully understand the effects of cooling on the excitatory junction potential of the guinea-pig vas deferens.

EFFECTS OF DRUGS ON THE EXCITATORY JUNCTION POTENTIAL

All α-adrenoceptor antagonists studied potentiated the amplitude of excitatory junction potentials, without altering the resting membrane potential of cells. These results are consistent with the well known observation that the nerve mediated contractile response of the guinea-

pig vas deferens is resistant to block and can be potentiated by α-adrenoceptor antagonists (Boyd *et al.*, 1960; Ambache & Zar, 1971). Various explanations have been put forward to explain this type of result. First, α-adrenoceptor antagonists cannot penetrate in sufficient concentrations to the neuroeffector junction and the excitatory junction potential is resistant to α-adrenoceptor antagonists since the transmitter concentration in the junctional cleft is sufficient to overcome the competitive blockade (Swedin, 1971; Furness, 1974). Furness & Iwayama (1972) showed that noradrenergic axons often lie in grooves or deep invaginations of the muscle cells, and are 'sealed' in by a confluence of basement membrane associated with the axon and muscle cell, which forms a barrier to the free diffusion of drugs (Furness, 1974). Hottá (1969) and Ambache *et al.* (1972), however, concluded that there is no morphological basis for a diffusion barrier.

A second explanation is that α-adrenoceptor antagonists contribute to their own ineffectiveness by increasing the amount of noradrenaline released. α-adrenoceptor antagonists could increase release in at least two ways, one by antagonism of local regulation of transmitter release, and two by blocking neuronal uptake. The first explanation is considered unlikely since the selective post-junctional α-adrenoceptor antagonists, azapetine and prazosin, did not block the exictatory junction potential. The second explanation is considered unlikely since uptake blockers have little or no effect on the amplitude of the excitatory junction potential (Bell, 1967b; Holman, 1970). McGrath (1978) has shown that the 'twitch' response of the rat vas deferens to a single stimulus, is not potentiated by uptake blockers whereas the 'secondary' component of the response is increased in both height and duration. If Swedin's (1971) hypothesis is correct, that the rapid first phase of the mechanical

response to repetitive stimulation, is due to a high concentration of noradrenaline in the neuromuscular cleft, then it might be expected that this component would be susceptible to potentiation by blockade of neuronal uptake, but no such effect was found (McGrath, 1978).

An alternative explanation is that the excitatory transmitter is noradrenaline but that the post-junctional receptor is not α or β. The β-adrenoceptor antagonist sotalol did not depress the amplitude of excitatory junction potentials. One would still, however, expect uptake blockers to potentiate the excitatory junction potential in these circumstances if the motor transmitter was noradrenaline.

An alternative explanation is that the motor transmitter is not noradrenaline. Amiodarone, which, in addition to blocking post-junctional α and β-adrenoceptors, blocks transmitter release from sympathetic nerves by a prejunctional mechanism, potentiated the first excitatory junction potential in a train. Unless the response to amiodarone of short intact sympathetic nerves is different from that of the long postganglionic fibres innervating the cat spleen, the transmitter generating the excitatory junction potential is not noradrenaline acting on α or β-adrenoceptors.

On balance of evidence, I have to conclude that the transmitter generating the excitatory junction potential is not noradrenaline and agree with Ambache & Zar (1971) that the main motor transmitter in the guinea-pig vas deferens is unknown.

Yet, it is clear from the study of the interaction of α-adrenoceptor agonists and antagonists that an α-adrenoceptor is regulating local transmitter release. This conclusion is based on the finding that the

preferential prejunctional α-adrenoceptor agonists, clonidine and lysergic acid diethylamide, produced dose dependent inhibition of excitatory junction potentials, effects reversed by α-adrenoceptor antagonists. Since there was a parallel shift to the right of the appropriate dose response curves, one can conclude that these drugs are acting on the same receptor in a competitive manner.

A number of other possible mechanisms whereby α-adrenoceptor antagonists could potentiate excitatory junction potential amplitude were ruled out. Such drugs may for example, interfere with the propagation of the action potential in the nerves. Since submaximal stimulation was often used in the present study, α-adrenoceptor antagonists may change the number of nerve fibres activated per stimulus and thus increase the size of the excitatory junction potential. This possibility was excluded for piperoxan by recording the activity in a bundle of vas deferens nerves following hypogastric nerve stimulation.

There is no reason to suppose that α-adrenoceptor antagonists interfere with the propagation of impulses in the nerve trunks. It is less certain, however, whether such drugs can exert effects on the spread of excitation throughout the terminal nerve net. Häefely (1972) has indicated that the sudden large increase in surface area of the nerve at the transition between preterminal axon and varicosity may involve conduction problems particularly during hyperpolarisation. Stjärne (1978) has proposed that α-adrenoceptor antagonists may operate by determining the degree of recruitment of varicosities, perhaps by altering the potassium permeability of the nerve membrane. This idea is interesting in view of the fact that intermittent transmitter release has been observed in the present study. Uptake blockade as a mechanism of excitatory junction potential augmentation can also be ruled out

for the reasons presented earlier.

Clonidine and lysergic acid diethylamide have been reported to stimulate prejunctional α-adrenoceptors in the vas deferens and decrease the release of noradrenaline (Drew, 1977; Doxey *et al.*, 1977).

Both clonidine and lysergic acid diethylamide produced dose dependent inhibitions of excitatory junction potentials, but there were some differences between the drugs. Clonidine was more effective at depressing excitatory junction potential amplitude towards the end of a train evoked at 0.91 Hz although both drugs had a similar profile of action on the first excitatory junction potential. Results correlate with the work of Ambache *et al.* (1973) who observed that the effects of lysergic acid diethylamide and clonidine on the mechanical response of the guinea-pig vas deferens to field stimulation differed according to the stimulation parameters chosen. Inhibition of mechanical responses was best seen with short trains (5) of pulses. The effectiveness of lysergic acid diethylamide declined as the train length was increased and became very small at 20-30 pulses. Clonidine on the other hand can markedly depress all responses in high concentrations while responses to high concentrations of lysergic acid diethylamide 'escape' the block (Ambache *et al.*, 1973).

The effects of clonidine in low concentrations deserves a special comment. Clonidine reduces the release of transmitter from a wide variety of tissues (see Starke, 1977; Westfall, 1977). However, Stjärne (1975) has shown that clonidine increases the overflow of noradrenaline from the guinea-pig vas deferens. In contrast, clonidine reduces rather than enhances the contractile response to stimulation in the vas deferens of the rat (Vizi *et al.*, 1973) and the guinea-pig (von Euler & Hedqvist, 1975).

In the present study clonidine in low concentrations (3×10^{-9}M) could increase the amplitude of the first few excitatory junction potentials in a train at 0.91 Hz. The increased amplitude of the excitatory junction potential does not, however, correlate with the data of Stjärne, since the latter author found that concentrations of 10^{-7} - 10^{-6}M were required to enhance release of noradrenaline, whereas these concentrations were found to greatly depress excitatory junction potential amplitude and presumably transmitter release therefore.

One explanation for this discrepancy in the literature is that in Stjärne's experiments, the use of uptake blocking agents led to a high concentration of noradrenaline in the junctional cleft, despite the low stimulation frequency of 1 Hz disclosing a partial agonist character of clonidine (Starke, 1977).

A case can also be made for clonidine preferentially activating prejunctional α-receptors located on noradrenergic nerve terminals which are themselves inhibitory on the excitatory nerves (whose transmitter is unknown), responsible for the generation of the excitatory junction potential. This possibility will be considered elsewhere in the discussion. There is no doubt that an α-adrenoceptor regulates transmitter release from the sympathetic nerves of the guinea-pig vas deferens. The guinea-pig vas deferens is a suitable preparation for determination of the mechanism of action of drugs on prejunctional α-adrenoceptors. The resistance to block of the excitatory junction potential by such agents allows the transmission process to be studied without the complication of a post-junctional alteration of the electrical response. The guinea-pig vas deferens is therefore particularly suitable for a study of the relative potency of a series of α-adrenoceptor antagonists on transmitter release, evoked by single or short trains of stimuli.

PACKETED RELEASE OF TRANSMITTER

Studies of the mechanism of transmitter release at the autonomic neuroeffector junction by electrophysiological techniques have been less successful than similar studies at the skeletal neuromuscular junction since smooth muscle cells are not electrically isolated from one another and are often multiply innervated. It has however been assumed that transmission at the two junctions is similar in many respects. In particular it has been assumed that transmitters are packeted (del Castillo & Katz, 1954a; Burnstock & Holman, 1962). If the parallel exists, then the spontaneous excitatory junction potential in smooth muscle must, like the miniature end-plate potential at the skeletal neuromuscular junction, be due to the spontaneous release of a multi-molecular packet of transmitter. The relationship between the spontaneous excitatory junction potential and the excitatory junction potential is not clear (Holman, 1970). The time course of the spontaneous excitatory junction potential is much shorter than that of the excitatory junction potential and the spontaneous excitatory junction potential can be larger in amplitude than the excitatory junction potential. My results confirm the assumption of packeted release of sympathetic transmitter and clarify the relationship between the spontaneous excitatory junction potential and the excitatory junction potential. Intermittent stimulus-locked, peaks in the rate of depolarisation (discrete events) of the excitatory junction potential, have been observed. These evoked discrete events correspond exactly to spontaneous excitatory junction potentials in the same cell and may therefore be assumed to be the basic packet of transmitter release. The recorded excitatory junction potential is made up of these packets of transmitter. Packets released close to the recording site are evident individually and cause the discrete events.

Those released further from the recording site are not individually evident but sum together to make up the non-intermittent component of the response.

It is clear from these observations that any single varicosity or release site does not release transmitter to every stimulus. Release is intermittent. For the reasons presented earlier, this failure to release transmitter cannot to any significant extent, be due to intermittent failure of the main axons of the vas deferens nerve to be excited by the stimulus. The intermittence does not occur as the result of ganglia in the nerve pathway since similar results were obtained in transmurally stimulated preparations where stimuli were applied to the postganglionic fibres directly. There are two possible causes of this intermittence. First, that the varicosity is always invaded by the nerve action potential but is not always capable of discharging transmitter or secondly there is intermittent failure of the nerve action potential at the bifurcations of an axon or in the thin non-myelinated fibres of the ground plexus. This latter cause cannot definitely be excluded, but because of the resemblance between the spontaneous excitatory junction potential and the discrete event, one favours the former cause.

In some cells the amplitude distribution of discrete events at a single latency was multimodal with preferred values of amplitude that were exact multiples of the smallest preferred value (Fig. 3.30, II). This distribution is similar to that seen at the magnesium blocked skeletal neuromuscular junction where the transmission process is quantal (Boyd & Martin, 1956). Therefore, the mechanism of transmitter release from an individual varicosity in the vas deferens is packeted and the number of packets liberated by a single varicosity per stimulus

is small, varying between zero and less than ten.

It must be emphasised that as there is only, at present, the conduction delay by which to assign the discrete event to any particular release site the data will overestimate the frequency with which transmitter release occurs when a single release site is activated.

Folkow, Häggendal & Lisander (1967) have shown in cat calf muscle that there is a disparity between the number of vesicles per varicosity (about 1000) and the fractional release of transmitter produced by a single maximal stimulus to the adrenergic innervation (1/50,000 of the total transmitter contents). They put forward two hypotheses to explain the results: (1) transmitter release by sympathetic nerves is intermittent and the entire contents of a vesicle is released occasionally, or (2) that transmitter release occurs to every stimulus but the quantity liberated per stimulus is small (1-3% of contents of vesicle). Folkow & Häggendal (1970) favoured the second hypothesis and suggested that the varicosity released transmitter to every stimulus in units which were only small fractions of the total contents of a vesicle. Since it was found that in the guinea-pig vas deferens, the sympathetic postganglionic nerves (whose motor transmitter may not be noradrenaline (Ambache & Zar, 1971)) release transmitter only intermittently, and that the transmitter is packeted, it is suggested that their first hypothesis may be correct and one must therefore agree with Smith & Winkler (1972) and de Potter, Chubb & de Shaepdryver (1972) that the entire vesicle contents of transmitter may be released by the action potential.

One of the difficulties in relating the spontaneous excitatory junction potential to the excitatory junction potential has been the wide variation in time course of the two processes and it has not been

possible to state with any confidence the duration of transmitter action which gives rise to either the spontaneous excitatory junction potential or the excitatory junction potential (Holman, 1970). It has been clearly shown that the excitatory junction potential is made up of components that exactly match the spontaneous excitatory junction potential (see Figs. 3.32; 3.33). The duration of transmitter action during both the spontaneous excitatory junction potential and the excitatory junction potential can therefore be estimated from the spontaneous excitatory junction potential. Hirst & Neild (1978) suggested that the unitary conductance change underlying the excitatory junction potential is brief compared with the total duration of the excitatory junction potential. The results obtained in the present study confirm this view.

Purves (1976) shows that in an infinite continuous model of smooth muscle the time course of a spontaneous junction potential recorded at its site of origin is nearly identical with the time course of transmitter action. In an infinite discrete model he draws an essentially similar conclusion.

The time taken for a spontaneous excitatory junction potential to decay to 1/e of its original amplitude is about 30 msec (cf. Bennett, 1972). This time constant is an order of magnitude larger than the time course predicted for the diffusion of transmitter from a close neuromuscular junction (Ogston, 1955; Eccles & Jaeger, 1958).

One cannot therefore yet define the time course of transmitter action at this junction except to state that its decay must have a time constant of less than 30 msec (if the Purves model is correct and the input impedance is purely resistive) and more than 4 msec (if the input impedance is largely capacitative and for the discrete event, the first

time differential of the spontaneous excitatory junction potential defines the membrane current).

Purves also offers an explanation for the longer time to peak of the excitatory junction potential relative to the spontaneous excitatory junction potential. However, the mean rise of the excitatory junction potential, whilst affected by the spread of current in the smooth muscle cells and by the rate at which transmitter reaches the receptors (see Burnstock & Costa, 1975) is also determined by the degree of temporal dispersion at the smooth muscle of the action potential volley elicited in the nerves by the stimulus. This temporal dispersion will be mainly due to the differing conduction velocities of the nerve axons.

Prominent discrete events can be recorded, on average, in twenty per cent of muscle cells impaled. Merrillees (1968) showed that about one in five cells of the guinea-pig vas deferens had close neuromuscular junctions ($\lesssim$ 20 nm). Prominent discrete events are to be expected in cells that have a direct innervation.

The results of the present study correlate well with the model of innervation of the guinea-pig vas deferens proposed by Burnstock (1970). A variable number of cells are electrotonically coupled to the innervated cells. As a consequence excitatory junction potentials evoked in directly innervated cells can be recorded in coupled cells. Thus, the discrete event represents local transmitter action from a neuromuscular junction on or near the muscle cell impaled, and the slow component (see Fig. 3.27) the electrotonic spread of activity from smooth muscle excited from distant release sites.

The discrete event is thought to represent the packeted release of transmitter from a single release site, presumed to be the varicosity. The mechanism of release of transmitter and the effects of drugs on the transmission process can be studied with a degree of precision not previously possible.

It was assumed in previous discussion that an increase in the amplitude of the excitatory junction potential reflected an overall increase in the amount of transmitter released by many varicosities. The precise mechanism of this effect was elucidated by determining the effects of the α-adrenoceptor antagonist, piperoxan, on the discrete event. The most characteristic action of this drug was to convert the normal intermittent pattern of release from individual varicosities into one of continuous release. This increase in the frequency of occurrence of discrete events was associated with a corresponding increase in the amplitude of the excitatory junction potential. α-adrenoceptor antagonists therefore increase the net amount of transmitter liberated per stimulus from individual release sites.

Piperoxan had a greater effect on long latency release sites than on those of short latency which in its absence released more often. After piperoxan, additional release sites, not previously observed, occurred continuously, later in the excitatory junction potential. This result suggests that inhibitory α-adrenoceptors activated by noradrenaline released from early firing noradrenergic fibres, normally prevents long latency excitatory varicosities from releasing transmitter. If short latency excitatory varicosities release transmitter at the same time or before the inhibitory noradrenergic fibres, then there would be no instantaneous inhibitory effect of noradrenaline on these excitatory

fibres. However, noradrenaline liberated from inhibitory varicosities first activated could depress the probability of transmitter release from neighbouring excitatory nerve terminals. This mechanism requires the α-adrenoceptor to be activated during a time less than the temporal dispersion of the action potential volley in the vas deferens nerves, i.e. 30-70 msec (see Fig. 3.16).

If, on the other hand, one assumes that there is one transmitter, noradrenaline, then one can test the hypothesis that transmitter inhibits its own subsequent release from the same varicosity. By studying the effects of previously released transmitter on subsequent release from the same site as defined by the discrete event, on no occasion was negative feedback observed. Transmitter liberated from a varicosity defined by an individual latency enhanced the probability of subsequent release occurring from the same site, rather than depressing it. Either the excitatory transmitter responsible for the discrete event is not noradrenaline or negative feedback does not occur at a stimulation frequency of 0.91 Hz. These results will be discussed in the next section of discussion in relation to possible models of the innervation of the guinea-pig vas deferens.

Preliminary experiments with the α-adrenoceptor agonist, clonidine, have shown it reducing the probability of any discrete event occurring in response to nerve stimulation. This reflects a change in the number of packets of transmitter liberated from any single varicosity by an action potential. Since both piperoxan and clonidine alter the frequency of occurrence of discrete events it is likely that these events are prejunctional in origin. Spontaneous excitatory junction potentials are not affected by either drug.

Experiments will be carried out to quantify the effects of piperoxan and clonidine in terms of the number of packets of transmitter released per stimulus in the guinea-pig. Preliminary experiments with the mouse vas deferens where the transmitter is thought to be noradrenaline (Bennett & Middleton, 1975) showed a similar intermittent release process. One interesting result obtained was that the α-adrenoceptor antagonist, azapetine, increased the quantal content of the excitatory junction potential (Cunnane, unpublished).

PROSTAGLANDIN E_1

It is well known that prostaglandins of the E series depress the output of noradrenaline following sympathetic nerve stimulation in many tissues including the guinea-pig vas deferens (see Hedqvist, 1977; Starke, 1977; Westfall, 1977). Conclusive evidence that prostaglandins inhibit adrenergic transmission was presented by Johnson *et al.* (1971) who found that prostaglandins E_1 and E_2 inhibit the release of both noradrenaline and dopamine-β-hydroxylase in the guinea-pig vas deferens. Yet, the contractile response to nerve stimulation does not behave as if it were elicited through an adrenergic mechanism (Ambache & Zar, 1971). It has been suggested that noradrenaline is an inhibitory transmitter in the guinea-pig vas deferens (Ambache *et al.*, 1972).

Prostaglandin E_1 produced a dose dependent inhibition of all excitatory junction potentials in short trains without altering the resting membrane potential of smooth muscle cells. This result confirms the observations of Sjöstrand (1972) who, using the sucrose gap technique, obtained similar results. Sjöstrand (1972) found however, that prostaglandin E_1 depolarised the muscle cell membrane, an affect not observed in the present study.

This discrepancy is readily explained by the fact that Sjöstrand used higher concentrations of prostaglandin E_1. The threshold concentration for inhibition in his experiments was of the order of 10 ng/ml and concentrations up to 100 ng/ml were required for depolarisation to occur. The concentration necessary to block excitatory junction potentials, recorded intracellularly was an order of magnitude lower, concentrations of 1-10 ng/ml producing complete abolition of the excitatory junction potential. When excitatory junction potentials were abolished by prostaglandin E_1, spontaneous excitatory junction potentials were recorded in the same cell showing that the inhibitory effect was prejunctional.

The depressant effect of prostaglandin E_1 was frequency dependent and was most marked at low frequencies of stimulation. Similarly in guinea-pig vas deferens, prostaglandin E_1 depressed the motor response to postganglionic sympathetic stimulation in a frequency dependent manner with low frequencies (3-5 Hz) being most affected (Hedqvist & von Euler, 1972). Ambache & Zar (1971) interpreted blockade of mechanical responses of the guinea-pig vas to short trains of pulses by prostaglandin E_2 as evidence for an action of prostaglandin E_2 on nerves whose excitatory transmitter is unknown, the maintenance of the secondary response to prolonged stimulation being due to a lack of effect of prostaglandin E_2 on the adrenergic nerves.

A study of the electrical responses of the vas to hypogastric nerve stimulation has produced no evidence of an electrical correlate of two transmitters. The apparent resistance of long trains of stimuli to the action of prostaglandin E_1 does not require the hypothesis that there are two sets of excitatory nerves. A mechanical response is

obtained when excitatory junction potentials, as a result of facilitation and summation, reach threshold and initiate muscle action potentials. If prostaglandin E_1 produces general depression of transmitter release then clearly, short trains of excitatory junction potentials will more readily become subthreshold (since the safety factor will be smaller for short than for long trains). Since facilitation and summation occur in the presence of prostaglandin E_1, these processes sum together to overcome the prostaglandin E_1 induced block during a long train of stimuli. Thus a single correlate of mechanical and electrical responses is obtained.

It has been postulated that prostaglandins inhibit the output of sympathetic transmitter in the guinea-pig vas deferens by a negative feedback mechanism which results in a lowering of the membrane potential with a consequent reduction of calcium influx in response to the arrival of the nerve action potential (Hedqvist, 1973). The lack of effect of indomethacin, in concentrations which inhibit prostaglandin biosynthesis in this tissue, suggests that prostaglandin E_1 does not modulate the amplitude of the excitatory junction potential *in vitro* (Cunnane, unpublished). Nevertheless, prostaglandin E_1 was the most potent chemical at inhibiting nerve evoked responses, 300 picogms/ml could depress the excitatory junction potential by 50%. The question remains whether this is an interesting pharmacological artifact.

The mechanism of the inhibition was not investigated in detail in the present study. Prostaglandin E_1 does not activate α-adrenoceptors since the prior administration of α-adrenoceptor antagonists does not prevent the excitatory junction potential depression. This result shows that prostaglandin E_1 and α-adrenoceptor agonists depress transmitter release via different receptors.

Prostaglandins do not interfere with the propagation of impulses in the nerve trunk of splenic nerves (Hedqvist, 1970). Most of the evidence (see reviews, Starke, 1977; Hedqvist, 1977; Westfall, 1977) suggests that prostaglandins inhibit noradrenaline release by an action on stimulus secretion coupling and more specifically on the availability of calcium for the release mechanism. Prolongation of the nerve action potential by administering tetraethylammonium decreases the inhibitory effect of prostaglandin E_2 on noradrenaline release induced by nerve stimulation in the guinea-pig vas deferens (Hedqvist, 1976). This observation links the inhibitory action of prostaglandins on noradrenaline release to an effect on calcium availability since prolongation of the action potential is thought to allow the calcium gates in the axonal membrane to remain open longer, and allow more calcium to enter the axon (Katz & Miledi, 1967). An alternative explanation is that prostaglandins depolarise the nerve terminal membrane and therefore reduce the amplitude of the nerve terminal action potential.

The latter mechanism is less likely since high concentrations of prostaglandin are required to depolarise compared with those required to inhibit release.

The effects of prostaglandin E_1 on the excitatory junction potential are consistent with the known effects of this compound on evoked noradrenaline release from the guinea-pig vas deferens. It is not easy to directly compare electrophysiological data with studies which have measured noradrenaline overflow from the guinea-pig vas deferens in view of the uncertainty of the excitatory transmitter. It should be remembered that even if the excitatory junction potential is not generated by the action of noradrenaline on the post-junctional membrane, that noradrenaline will still be released from inhibitory sympathetic nerves and that studies

measuring noradrenaline overflow may be observing effects on this transmitter system.

CORRELATION OF RESULTS WITH POSSIBLE MODELS OF INNERVATION OF GUINEA-PIG VAS DEFERENS

In recent years, Ambache & Zar (1971) and von Euler & Hedqvist (1975) have provided evidence that noradrenaline is not the main motor transmitter in the guinea-pig vas deferens. Swedin (1971), however, concluded that the excitatory innervation was adrenergic. McGrath (1978), considers the vas to be innervated by two sets of excitatory fibres, one noradrenergic and the other releasing an unidentified chemical. In the present study, the transmission process has been examined in some detail using electrophysiological techniques and using some drugs which block the effects of sympathetic nerve stimulation in other organs. It was hoped that the results would clarify some of the pharmacological anomalies which have arisen form studies of mechanical responses.

Initially it was shown that the excitatory junction potential could be modified by drugs which activate or block prejunctional α-adrenoceptors. The excitatory junction potential was resistant to the effects of α and β-adrenoceptor antagonists, selective for post-junctional adrenoceptors, and to the adrenergic neurone blocker, amiodarone. The excitatory junction potential is unlikely to be due to noradrenaline acting post-junctionally on α or β-adrenoceptors. The electrical results correlate well with the resistance of nerve evoked mechanical responses to this type of drug.

During the study the characteristics of transmitter release from individual varicosities was determined. Transmitter release occurred intermittently

from an individual release site following an action potential in the hypogastric nerve. Transmitter release occurred in packets (discrete events) which were identical to spontaneous excitatory junction potentials. The number of packets secreted from one varicosity per stimulus was small, varying between zero and less than ten.

α-adrenoceptor antagonists such as piperoxan converted the pattern of intermittent release from a single varicosity to one of continuous release - i.e. a discrete event was evoked by every action potential in the hypogastric nerve. Conversely, the α-adrenoceptor agonist, clonidine, depressed the frequency of occurrence of discrete events.

Since transmitter release from single varicosities can be followed, the opportunity to test the negative feedback theory of transmitter release on subsequent release from the same nerve terminal, presented itself. Negative feedback was never observed from single release sites. It was clear however, that α-adrenoceptor antagonists suppressed an inhibitory process.

I will now interpret these results by considering possible hypothetical models of the innervation of the guinea-pig vas deferens.

Two models of the innervation of the vas are presented in Fig. 6.1. The classic model is that the adrenergic nerves release noradrenaline and this noradrenaline once released, can modify its subsequent release. When the effects of transmitter release from single varicosities was investigated, it was found that previous transmitter release facilitated subsequent transmitter release rather than depressing it. This result cannot be fitted into model A in Fig. 6.1. Data can be interpreted in another way if the transmitter is noradrenaline but the post-junctional

A B

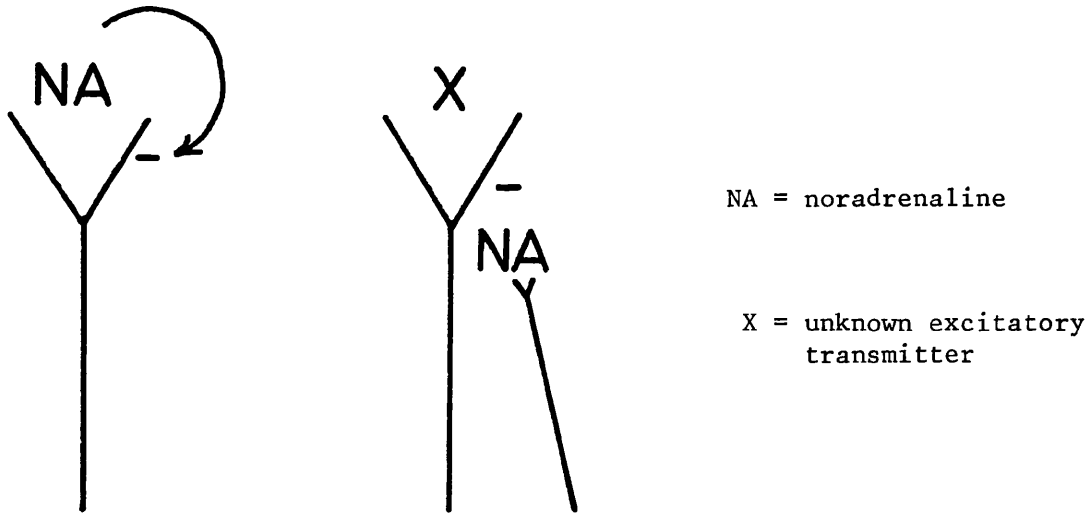

Fig. 6.1. Schematic illustration of hypothetical models of the innervation of the guinea-pig vas deferens.

receptor is not α or β. The rising phase of the excitatory junction potential is long compared with the duration of transmitter action from a single release site. This is due to the asynchronous arrival of nerve action potentials in the terminal arborisation, due, presumably, to different fibre diameter and therefore different conduction velocities. If the transmitter is noradrenaline and as the receptor on the nerve terminal is an α-adrenoceptor then release from the varicosity first activated will reduce the probability of transmitter release from neighbouring varicosities. Since transmitter action is brief (10 msec) compared to the rate of stimulation (1 Hz) transmitter release from one site does not depress its own release. Rather, it affects varicosities close to it. α-feedback inhibition of transmitter release therefore becomes a local phenomenon, limited by the proximity of varicosities to one another and distance. When the noradrenaline concentration falls below a certain level, determined by the distance from one varicosity to another, then the concentration to trigger feedback, falls below threshold. This suggestion is supported by the fact that the probability of a short latency varicosity releasing transmitter is greater than that of long latency ones. Thus piperoxan has a greater effect on varicosities with long latencies. These do not normally express themselves since early transmitter release depresses the probability of their releasing transmitter.

An alternative model of the innervation (model B) of the guinea-pig vas deferens is that the transmitter generating the excitatory junction potential is some unknown chemical x. The dense adrenergic innervation is there to modulate release of this unknown excitatory transmitter x by activating α-adrenoceptors located on the excitatory nerve x. This is the model first proposed by Ambache *et al.* (1972). A number of results obtained in the present study fit well with this model. Drugs which block

the effects of adrenergic nerve stimulation by blocking post-junctional α-adrenoceptors are ineffective on the excitatory junction potential, as the excitatory junction potential is not attributed to noradrenaline acting on post-junctional α-adrenoceptors. The potentiation of the excitatory junction potentials at the beginning of a train by amiodarone and low concentrations of piperoxan may be explained thus: these drugs selectively block the adrenergic nerve which is normally exerting an inhibitory influence on the excitatory nerve. Potentiation of the excitatory junction potential by low doses of clonidine may reflect a preferential action of clonidine on the α-adrenoceptors located on the adrenergic nerves. Potentiation of the excitatory junction potential therefore occurs as a result of the removal of the inhibitory influence of the adrenergic nerves. Adrenergic neuron blockade by amiodarone could produce a similar effect. In higher concentrations, the excitatory junction potential is reduced by clonidine since the drug now activates the α-adrenoceptors located on the excitatory nerves.

Thus the concept of a negative feedback loop operating to modulate transmitter release from the guinea-pig vas is not required. Such a mechanism may operate on the adrenergic nerves, but cannot be investigated, as there is no electrical correlate of noradrenaline action on the post-junctional membrane. Studies of noradrenaline overflow from the guinea-pig vas in response to hypogastric nerve stimulation support the view that noradrenergic nerves have a negative feedback system (Starke, 1977; Westfall, 1977).

It is interesting to consider what the physiological function of the prejunctional α-adrenoceptor in the guinea-pig vas deferens might be. If the excitatory transmitter is noradrenaline then these receptors act to even out the spread of excitation throughout the terminal innervation

of smooth muscle. Transmitter release would thus be locally regulated in a most economical fashion. If the excitatory transmitter is unknown, and the α-adrenoceptor is located on the excitatory nerve terminal, then clearly, these receptors serve to inhibit transmitter release from every excitatory varicosity in close apposition to an inhibitory adrenergic varicosity. This would provide an example of an inhibitory transmitter being released from one nerve fibre whose main function is to modulate the release of excitatory transmitter from another.

RABBIT RECTOCOCCYGEUS

The rectococcygeus muscle is particularly suited to a study of the electrical aspects of autonomic transmission using intracellular recording techniques. It is often free from spontaneous activity and has a high stable resting membrane potential (approx. -60 mV). Anatomically it is composed uniformly of parallel longitudinally arranged smooth muscle fibres unlike the colon to which it is attached and which contains both longitudinal and circular smooth muscle.

The pelvic nerves to the rectococcygeus contain both excitatory and inhibitory fibres. Stimulation of the pelvic nerves releases two transmitters. The effect of the inhibitory is only observed when the effect of the excitatory has been blocked by atropine.

As in the guinea-pig vas deferens (Burnstock & Holman, 1961) excitatory junction potentials in response to nerve stimulation were recorded in every muscle cell impaled. In any one preparation the excitatory junction potentials recorded in different cells showed only small variations in amplitude provided the stimulation parameters remained constant, suggesting that the nerve endings in the rectococcygeus are widely distributed among the smooth muscle bundles. Unlike the vas deferens in which the junction potentials have markedly different time courses in different cells (Bennett, 1972), the time course of excitatory junction potentials in the rectococcygeus in any one preparation was constant.

The slow time course of the excitatory junction potentials and the absence of prominent spontaneous excitatory junction potentials is in keeping with the electron microscopical evidence (Davey *et al.*, 1975) which showed no close contact varicosities and a gap of not less than

150 nm between the axon varicosity and the muscle cell. Two other factors could have contributed to the slow time course of the excitatory junction potential - the presence of ganglia in the pelvic pathway (King *et al.*, 1977) near the muscle itself and the slow activation characteristics of muscarinic receptors, the latency of which is probably at least 100 msec (Purves, 1974; Hill-Smith & Purves, 1978). Neither possibility can be excluded by the present results but it is unlikely that the slow time course arose from a slow rate of conduction in the pelvic nerve which was found to be 0.3 m/sec.

The amplitude of the first excitatory junction potential in the train was greatest and the amplitude of each subsequent depolarisation equalled but never exceeded the response of the first excitatory junction potential. One explanation may be that the release of the inhibitory transmitter may have truncated subsequent excitatory junction potentials. Presumably pelvic nerve stimulation releases both inhibitory and excitatory transmitters concomitantly although the stimulus strength required to demonstrate an inhibitory junction potential experimentally greatly exceeds that required to evoke an excitatory junction potential.

There is no general agreement concerning the response of the rabbit colon (which is continuous with the rectococcygeus) to pelvic nerve stimulation. Evidence from both mechanical (Garry & Gillespie, 1955; Furness, 1969) and intracellular micro-electrode studies (Gillespie, 1968) suggested that the extrinsic pelvic nerves contained only excitatory fibres. On the other hand Julé & Gonnella (1972) observed inhibitory junction potentials in the rabbit colon following pelvic nerve stimulation using extracellular recording methods. The present results from intracellular techniques confirm those obtained by mechanical recordings (King & Muir, 1979) that inhibitory fibres are present in the pelvic nerves

at least in the posterior and posterio-median strands which supply the rectococcygeus. The resistance of inhibitory junction potential to the actions of drugs which antagonize sympathetic nerves (Bennett, Burnstock & Holman, 1966) make it unlikely that the transmitter responsible for the inhibitory junction potential is a catecholamine. Ambache *et al.* (1974) demonstrated noradrenaline induced inhibition of both motor transmission and spontaneous movements was not blocked by either phentolamine, propranolol or a combination of both.

The inhibitory junction potentials obtained in the present investigation showed similarities to those obtained following stimulation of non-adrenergic non-cholinergic nerves (Burnstock, 1975). For example inhibitory junction potentials waned during repetitive stimulation above 4 Hz but were remarkably well maintained at lower frequencies (see Fig. 4.8); when stimulation was stopped a rebound excitation occurred (depolarisation and muscle action potential). The characteristic resistance of responses mediated by non-adrenergic non-cholinergic nerves to pharmacological antagonists in other preparations (Burnstock, 1975) is shared by the rabbit rectococcygeus and no specifically acting antagonist has been found in the present study. The time courses of the inhibitory junction potentials are slow compared with other inhibitory responses so far described (Holman, 1970) and probably reflect the anatomy of the preparation rather than any unique property of the transmitter itself.

The ionic basis for the inhibitory junction potential has not been investigated in this study. However, the inhibitory junction potential was reduced during noradrenaline-induced membrane hyperpolarisation. The reduction in inhibitory junction potential amplitude is probably due to the inhibitory junction potential approaching the null potential for inhibitory transmitter action. The maximum level of

membrane potential at the peak of inhibitory transmitter action observed was approximately -90 mV. Since the potassium equilibrium potential for the taenia coli is about -100 mV it has been suggested that the transmitter responsible for the inhibitory junction potential causes a marked increase in K conductance (Bennett, Burnstock & Holman, 1963). It is tempting to speculate that a similar mechanism underlies the inhibitory junction potential in the rabbit rectococcygeus.

SKELETAL MUSCLE

D-tubocurarine is a competitive antagonist of post-junctional nicotinic receptors at the skeletal neuromuscular junction. Much of the evidence for this mechanism of actions has come from studies using amphibian nerve muscle preparations. The question raised is whether this action can entirely account for neuromuscular block in the mammal. In the present study, trains of end-plate potentials recorded in cut muscle fibres were more resistant to fade than end-plate potentials recorded in intact hemi-diaphragm preparations exposed to tubocurarine. Previously, it was generally believed that quantal transmitter release per stimulus falls off as the frequency of stimulation increased (see MacIntosh & Collier, 1978). Many of the studies which have demonstrated a fall-off in transmitter output are based on the measurement of end-plate potential amplitude in preparations where d-tubocurarine has been added to the physiological saline, to prevent muscle contraction. When neuromuscular transmission is studied in the absence of tubocurarine, using the cut muscle preparations, end-plate potential amplitude is well maintained. These results confirm the work of Hubbard & Wilson (1973), and are similar to those obtained by Blaber (1970) who found that a stimulation frequency of 200 Hz was necessary before a decline of end-plate potential amplitude was detectable in the cat tenuissimus cut muscle preparation.

In the rat diaphragm preparations exposed to d-tubocurarine, a decline in end-plate potential amplitude during a train occurred, even at the low frequency of 1 Hz. Many workers have interpreted the decline in end-plate potential amplitude during a train, as a fall off in transmitter output because of a prejunctional effect of d-tubocurarine. An

explanation can be made for these observations without postulating a mechanism other than the classic view of post-junctional receptor blockade. Suppose, in normal muscle with a normal dose response curve to acetylcholine, that during repetitive nerve stimulation, a small progressive decline in the release of transmitter occurs. The expression of this decline in terms of the end-plate potential will be different in preparations exposed to d-tubocurarine. Since d-tubocurarine is a competitive antagonist, a shift to the right of the acetylcholine dose response curve will occur. The magnitude of the decline in end-plate potential amplitude will be exaggerated, not because of an action of d-tubocurarine on the release mechanism, but because the end-plate potential is now recorded on a different part of the acetylcholine dose response curve. It is possible however that both prejunctional and post-junctional effects occur and all that is in dispute is the magnitude of each.

Also, the 'apparent' maintenance of end-plate potential amplitude in response to increasing frequency of stimulation could be an artifact of the cut muscle preparation. In cut muscle fibres, where the tissue is depolarised, the magnitude of the response to a given increment of acetylcholine will differ from normal and successive increments will not be equal. Small changes in transmitter output are not normally reflected as large declines in end-plate potential amplitude, unless d-tubocurarine is present.

It is not necessary to postulate a prejunctional action of d-tubocurarine on transmitter release since all the observations can be explained by a post-junctional mechanism with an end-plate potential, 'well up' the acetylcholine dose response curve. The pattern of end-

plate potentials recorded in cut muscle fibres may not therefore be representative of the transmission process in intact preparations.

There were a number of ways of testing the hypothesis that the 'apparent' prejunctional effect of d-tubocurarine on transmitter output is due to a change in position of the end-plate potential on the acetylcholine dose response curve. First, the position of the end-plate potential on the acetylcholine dose response curve was altered by perfusing muscles with saline which contained 10 mM magnesium. This procedure greatly reduced the amount of transmitter liberated per nerve action potential. End-plate potentials were recorded therefore, on a different part of the acetylcholine dose response curve. Unfortunately, in order to obtain reproducible results, it was necessary to change the experimental procedure. The frequency of stimulation was increased to 100 Hz and a new phenomenon, facilitation, was observed. Successive end-plate potentials exhibited a random stepwise increase in amplitude throughout a train, due to an increased release of acetylcholine as a consequence of an increased free calcium concentration in the nerve terminal (Katz & Miledi, 1968). It was argued that if tubocurarine could alter the process of facilitation (which has a prejunctional origin), then it must exert effects on the motor nerve terminal. Since tubocurarine abolished facilitation at 100 Hz, the result provides evidence that tubocurarine can influence events in the motor nerve terminal.

Furthermore, if d-tubocurarine only changed end-plate potential amplitude by a post-junctional mechanism, it would be expected that the magnitude of facilitation would have increased rather than the observed decrease. This would have been because the end-plate potential was

shifted to a steeper point on the acetylcholine dose response curve and hence changes in transmitter output, as measured by the end-plate potential, would be faithfully recorded. It is recognized however that the results are complex, with facilitation occurring, and are not strictly comparable with the results previously described.

Much of the evidence purporting to show a prejunctional effect of tubocurarine has come from electrophysiological studies with mammalian nerve-muscle preparations. Similar experiments with amphibian nerve-muscle preparations have failed to show a prejunctional effect of d-tubocurarine and indeed support the view that post-junctional receptor blockade adequately accounts for neuromuscular block (Auerbach & Betz, 1971).

Experiments were therefore carried out with frog sartorius preparations which, when carried out in rat diaphragms, had indicated a possible prejunctional effect. Such experiments provide a second way of testing the dose response curve hypothesis since a similar shift to the right should be observed in both species.

To strictly compare results in two species it was necessary to devise a cut muscle preparation of the frog sartorius. Transverse sectioning of the muscle fibres allowed end-plate potentials to be recorded from frog sartorius fibres in the absence of d-tubocurarine. Some obvious differences in the transmission process of the two species were found. End-plate potential amplitude was well maintained in frog sartorius preparations as the stimulation frequency was increased, in contrast to end-plate potentials recorded in rat diaphragms where there was depression. Furthermore, end-plate potentials showed facilitation in frog sartorius preparations at stimulation frequencies greater than

1 Hz. Facilitation was more marked in the intact frog sartorius exposed to tubocurarine than in cut muscle controls, probably due to post tetanic decurarisation. At higher stimulation frequencies, the increased release of transmitter will displace the acetylcholine-tubocurarine receptor equilibrium. It was important to establish whether the differences between the responses of rat and frog muscles could be attributed to the experimental conditions. The amphibia experiments were carried out at room temperature (18-22^{o}C) and the rat experiments at 37^{o}C, the physiological temperatures of both species. When the experiments on rat diaphragm were repeated at room temperature, the magnitude of end-plate potential depression was increased. One can eliminate temperature as the source of the differences in response of the preparations. The composition of the saline which bathed the two preparations also differed (see Methods). In particular, the calcium concentration of frog Ringer was increased from 2.2 mM to 3.6 mM as advised by Fatt & Katz (1951). Since transmitter release is calcium dependent, an increased calcium concentration results in an increased transmitter release per stimulus, but like other agents which increase transmitter release, calcium increases the depression seen in trains of end-plate potentials (Otsuka, Endo & Ninomura, 1962). The calcium concentration of the Ringer is unlikely to account for the observed differences. More likely there may be a genuine species difference between the rat and frog neuromuscular junction in their response to tubocurarine. Indeed, tubocurarine is more effective in blocking neuromuscular transmission in the rat than in the frog (Bĕranek & Vysočil, 1967) whereas atropine is equipotent in the two.

Galindo (1971) felt that there was unlikely to be a species difference, and concluded that a prejunctional effect of tubocurarine

would be demonstrable in the frog if studied at the 'proper concentration'. In the present study, a range of concentrations of tubocurarine were used but marked differences in the pattern of end-plate potentials recorded in the rat and frog muscles were still found. It is concluded that d-tubocurarine does not exert a prejunctional effect in the frog. It is likely that there is a difference in the response of the rat and frog muscles to tubocurarine. The neuromuscular junctions of the frog and rat are histologically quite different, estimates of quantal content are different, and the pattern of end-plate potentials recorded in response to repetitive stimulation is different. Why therefore should their responses to d-tubocurarine not differ? The question remains as to whether the response to d-tubocurarine is different because in the rat, d-tubocurarine has an effect on transmitter release.

What is the importance in mammals of any prejunctional effect in terms of the neuromuscular blocking potency of tubocurarine? The end-plate potential in mammalian muscle has a large safety factor, about five times that required to initiate a muscle action potential, so it was possible that the effect described was subthreshold to the physiological transmission process. It was of interest to determine whether a prejunctional effect could be demonstrated on twitch responses of rat hemi-diaphragm preparations. In control experiments, twitches evoked by indirect stimulation were well maintained at frequencies up to 10 Hz. When preparations were exposed to d-tubocurarine (5×10^{-7} g/ml), the amplitude of twitch responses at 1 Hz were depressed to a small extent only, but there was a marked decline in twitch amplitude during trains of stimuli at higher frequencies. When the bath concentration of tubocurarine was increased to 10^{-6} g/ml, post-junction receptor blockade was marked, as evidenced by a reduction in the amplitude of the

first twitch in a train. The crucial question is whether the amplitude of the first twitch in a train can be taken as an index of end-plate responsiveness to transmitter. If one accepts this latter argument, when the first twitch is unaffected by low concentrations of d-tubocurarine, a decline in responses during a train must represent a decline in transmitter output. Fatigue of responses cannot be attributed to an effect of d-tubocurarine on the contractile apparatus since muscle twitches were maintained during direct stimulation of the muscle at a time when responses to phrenic nerve stimulation declined. It is considered that 75% of post-junctional receptors sites have to be occupied by d-tubocurarine before neuromuscular block begins (Paton & Waud, 1967). Since tubocurarine is used in low concentrations, as a coadjuvant to general anaesthesia, Galindo (1971) suggested that the post-junctional receptor blocking action of d-tubocurarine is unlikely to play any significant role in the maintenance of muscle relaxation, during surgery, in man.

There is convincing evidence that acetylcholine receptors are located on or near somatic motor nerve terminals (Masland & Wiglon, 1940; Hubbard, Schmidt & Yohoto, 1965; Riker, 1966; Bowman & Webb, 1976; Riker, 1975). The prejunctional receptors are stimulated by suxamethonium and decamethonium, as well as by acetylcholine, and are blocked by acetylcholine antagonists (Standaert & Adams, 1965; Blaber, 1970). The prejunctional receptors must therefore be similar in structure to the post-junctional receptors. Blaber & Karcymar (1967) have postulated that there is a second prejunctional cholinoceptive site close to the nerve terminals which, when stimulated, does not affect the membrane potential, but facilitates the influx of sodium ions into the nerve terminal, and indirectly increases the release of acetylcholine

by increasing the size of the available store of transmitter. Since there are cholinoceptive sites on the motor nerve terminal, it is possible that d-tubocurarine could act on nerve terminals and inhibit transmitter release.

Bowman & Webb (1976) have proposed a hypothesis to encompass all observations, which suggests that cholinoceptive sites on motor nerve terminals are involved in a positive feedback mechanism that helps to maintain transmitter output during a tetanus. The principal features of the hypothesis can be briefly summarised. During low frequency stimulation (e.g. 0.1 Hz) transmitter is mobilized from a stationary store to an available store at a sufficient rate to keep pace with release, without the need for a feedback mechanism. Under these conditions, the effects of acetylcholine antagonists e.g. tubocurarine will arise entirely from post-junctional receptor block. As the frequency of stimulation is increased, some of the released acetylcholine acts on the terminal membrane in a positive feedback mechanism that facilitates the mobilisation of transmitter to the available store, so that transmitter output is maintained. Under these conditions, the effects of the antagonists will arise from their interaction with both pre- and post-junctional receptors. One difficulty with this hypothesis is that drugs which inhibit acetylcholinesterase should allow acetylcholine to enhance its own release, at lower frequencies, but no such affect has been observed. The duration of transmitter action is merely prolonged (see Miyamoto, 1978). In the present study, the frequency of stimulation at which no decline in end-plate potential amplitude during a train was detectable was 0.1-0.2 Hz. Furthermore the time course of end-plate potential depression in the presence of tubocurarine, following a conditioning stimulus was 3-5 sec at a stimulation frequency of either

1 Hz or 10 Hz. Since d-tubocurarine does not affect the nerve terminal action potential (Chang *et al.*, 1967; Katz & Miledi, 1964) this result may be interpreted thus. Tubocurarine may impair the mobilisation of acetylcholine in response to nerve stimulation. If d-tubocurarine interferes with the replenishment of the small available store of transmitter, then subsequent end-plate potentials will be smaller. Such an action would explain the discrepancy between end-plate potentials recorded in the absence of tubocurarine and end-plate potentials recorded in preparations exposed to the drug. In cut muscle preparations there is little or no depression of transmitter mobilisation and end-plate potential amplitude is readily maintained at moderate rates of stimulation. One can also interpret the results by the dose response curve shift hypothesis. One way of distinguishing between the two hypotheses was to use a drug which selectively blocks post-junctional nicotinic receptors. α-bungarotoxin is believed to act as a selective post-junctional receptor blocker (Lee, 1972).

Reference to Fig. 5.2 shows that a similar profile of action was found on end-plate potential amplitude. The magnitude of decline of end-plate potential amplitude was similar to that observed with tubocurarine. This result could be taken as evidence that all the effects described are attributable to post-junctional receptor blockade. Results require however, that α-bungarotoxin exerts no effect on pre-junctional receptors. Since these receptors have a similar pharmacological profile to post-junctional receptors this may be an unreasonable assumption. The experiment with α-bungarotoxin was tried on one occasion only. It will be necessary to repeat this experiment using different concentrations of α-bungarotoxin. Preliminary results with pancuronium, di and trimethylcurare have also shown that these neuromuscular blocking

agents have a similar profile of action on the end-plate potential amplitude as tubocurarine. These results provide further evidence that the decline of end-plate potential amplitude during a train is due to a shift of the acetylcholine dose response curve. However, an intriguing possibility to be borne in mind is that all competitive receptor antagonists may, in addition to post-junctional receptor blockade, modify transmitter release in the mammal.

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