STUDIES ON RECEPTOR ACTIVATION IN INTACT AND PERMEABILIZED SMOOTH MUSCLE.

A Thesis presented for the degree of

Doctor of Philosophy

by

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ABBREVIATIONS

ACh	Acetylcholine
ATP	Adenosine Triphosphate
CrP	Creatine Phosphate
cAMP	Cyclic Adenosine Monophosphate
DG	Diacylglycerol
EDTA	Ethylenediaminetetraacetic ACID
EGTA	Ethylene Glycol-bis(ß-aminoethyl Ether) N,N,N',N'-Tetraacetic Acid
GDP	Guanosine Diphosphate
GDP-ß-S	Guanosine 5'-O-(2-thiodiphosphate)
GTP	Guanosine Triphosphate
GTP-γ-S	Guanosine 5'-O-(3-thiodiphosphate)
Ins(4,5)P_2	Inositol(4,5)bisphosphate
Ins(1,4,5)P_3	Inositol(1,4,5)triphosphate
Ins(1,3,4,5)P_4	Inositol(1,3,4,5)tetrakisphosphate
MLCK	Myosin Light Chain Kinase
NA	Noradrenaline
PhE	Phenylephrine
PtdIns	Phosphatidylinositol
P_i	Inorganic Phosphate
PKC	Protein Kinase C
Tmax	Maximum calcium-activated force
5-HT	5 Hydroxytrptamine

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DECLARATION

The experimental work contained within this thesis was undertaken wholly by myself. None of the material has been previously presented for any other degree. Some of the results have been published during the period of this study, details of which are given below.

PUBLICATIONS

CRICHTON, C.A., SMITH, G.L., MILLER, D.J. & McGRATH, J.C. (1989) The modulation of force in isolated rat EGTA-treated anococcygeus muscle by phosphate, cyclic AMP and noradrenaline. *Quarterly Journal of Experimental Physiology* **74**, 943-945

SMITH, G.L., CRICHTON, C.A., McGRATH, J.C. & MILLER, D.J. GTP diminishes the noradrenaline response in isolated rat anococcygeus muscle permeabilized by alpha-toxin treatment. *Journal of Physiology* **424**, 39P

CRICHTON, C.A., SMITH, G.L., MILLER, D.J. & McGRATH, J.C. The effect of ATP on the response of intact and toxin-permeabilized isolated rat anococcygeus muscle to noradrenaline and acetylcholine. *Journal of Molecular and Cellular Cardiology* (in press)

CRICHTON, C.A., SMITH, G.L., McGRATH, J.C. & MILLER, D.J. The effect of GTP and noradrenaline on Ca-activated force in rat anococcygeus muscle and longitudinal muscle from guinea-pig portal vein after alpha-toxin treatment.
Journal of Physiology **429** 112P

CRICHTON, C.A. & SMITH, G.L. Effects of GTP on noradrenaline-induced force in isolated toxin permeabilized rat anococcygeus and guinea-pig portal vein. *Journal of Physiology* (submitted)

SUMMARY

The aim of this thesis was to study agonist-induced contractions in smooth muscle. The majority of the work was undertaken on rat anococcygeus, although some has been performed on guinea pig portal vein longitudinal muscle. The agonists which were examined (noradrenaline, phenylephrine and acetylcholine) all cause contraction in rat anococcygeus. They are thought to have their effect mediated by a G-protein which in turn stimulates the phosphatidylinositol cycle and promotes contraction. Work was carried out therefore, to analyse the effect of receptor activation at two levels: (a) the G-protein; and (b) the phosphatidylinositol cycle. To examine the intracellular effects of receptor activation the muscle was permeabilized. Part of this thesis comprises an investigation into the effectiveness of the three putative permeabilizing techniques.

ANALYSIS OF THREE DIFFERENT PERMEABILIZATION TECHNIQUES

The three permeabilization techniques examined were:
(a) saponin-treatment; (b) EGTA- treatment; and (c) alpha-toxin-treatment. To analyse the effectiveness of each treatment several tests were carried out and the behaviour of the permeabilized smooth muscle after the three different treatments was compared with that of an intact muscle. The muscles were examined to see: (i) if they contracted readily to calcium; (ii) if they produced rigor crossbridges when ATP and CrP were removed from the bathing medium;

(iii) how they responded when two factors known to have a direct effect on the contractile proteins, cyclic AMP and inorganic phosphate, were added to the bathing medium; and (iv) how they responded to the application of noradrenaline at a low calcium concentration.

It was found that calcium-activated force decayed with time in saponin- and alpha-toxin-treated muscle. Possible mechanisms for the decay were examined. These included: (a) loss of calmodulin; (b) reduced or increased ionic strength; and (c) reduced or increased pH. These were all examined in saponin-treated muscle. The effect of lowering ionic strength was examined in toxin-treated muscle.

RECEPTOR ACTIVATION AND CONTRACTION

Once a suitable permeabilization technique had been established, further experimentation was undertaken to examine the effect of receptor activation on contraction in intact and permeabilized muscle. The permeabilization technique adopted was alpha-toxin treatment. Originally it had been hoped that this method would allow the analysis of receptor activation by noradrenaline and acetylcholine. However, it was found that alpha-toxin-treated rat anococcygeus muscle was unable to contract in response to acetylcholine. Possible reasons for this is examined in Chapter 4.

(a) G-protein

Kitazawa et al (1989) had already reported that GTP had to be included in the bathing medium before phenylephrine could produce a contraction in

alpha-toxin permeabilized guinea pig portal vein. This led these workers to conclude that a G-protein was involved in receptor activation. The rest of Chapter 4 aims to assess the involvement of G-proteins in receptor activation in alpha-toxin-treated rat anococcygeus and guinea pig portal vein. Receptor activation was examined by looking at its effect on calcium release and calcium-activated force. The involvement of G-proteins was examined by using GTP, GTP-γ-S (a non-hydrolysable analogue of GTP) and GDP-β-S (a non-hydrolysable analogue of GDP).

A striking feature of the noradrenaline-activated contractions in both tissues was that their amplitude decayed upon repeated exposure. Several factors were examined to ascertain the cause of this decay. These included examining the effect of GTP on the decay, the accessibility of the calcium store and the sensitivity of the calcium store to Ins(1,4,5)P_3 before and after the decay in noradrenaline-activated force.

(b) phosphatidylinositol cycle

The fifth chapter attempts to assess the role of the phosphatidylinositol cycle in receptor activation. This was examined by chronically treating rats with lithium chloride which is known to block the phosphatases which normally are responsible for the breakdown of Ins(1,4,5)P_3. This blockade eventually causes the rundown of inositol within the cell and, therefore, eventually of PtdIns(4,5)P_2, the precursor of Ins(1,4,5)P_3 and of diacylglycerol (DG).

The effects of chronic lithium treatment of rats on (i) the concentration response curves to different agonists; and (ii) the intracellular component

produced by different agonists were examined in intact anococcygeus muscle. In the second set of experiments, chronic lithium treatment was undertaken at the same time as the animals were being given myo-inositol in their drinking water. The concentration response curves and the intracellular component in these tissues were compared with those form chronic lithium treated animals to see whether the inclusion of myo-inositol in the treatment was able to prevent the effect of lithium. Lithium was found to depress the response to noradrenaline and to the other agonists. To discover if this was a direct effect on the contractile proteins or the calcium store, the acute effects of lithium exposure were examined, in alpha-toxin permeabilized anococcygeus muscle strips, on caffeine-, calcium- and noradrenaline-activated force. The effect of acute myo-inositol and lithium exposure was also examined in each of these situations.

It was discovered that all three permeabilizing treatments were effective, but because of the profound decay in calcium-activated force in saponin-treated preparations and the small amount of force produced by EGTA-treated preparations these were not used again in the following studies. Instead alpha-toxin-treated smooth muscle was used. This type of muscle had the added advantage that it retained functional membrane bound receptors. Both rat anococcygeus and guinea pig portal vein displayed characteristics which indicated that adrenoreceptor activation is mediated through a G-protein. However, in rat anococcygeus there appeared to be some other process functioning which to some extent masked the involvement of GTP. No concrete conclusions can

be drawn from the LiCl experiments, although the results tentatively suggest that, if LiCl does indeed block the PtdIns cycle, NA at least is linked to this cycle to promote contraction.

CHAPTER 1

GENERAL INTRODUCTION

Smooth muscle is widespread throughout the body of vertebrates and its contractile activity is vital for normal functioning of the body. The contractile parts of the walls of hollow vessels and cavities, such as blood vessels, the alimentary canal and the urinogenital tract, are formed from layers of smooth muscle cells which propel, mix and retain the contents. Elsewhere smooth muscle is less intimately associated with other tissues and their contraction tends to move one structure relative to another. These include ciliary muscles and nictating membranes of the eye, pilo-erector muscles of the skin and muscle which is attached at one end to bone, for example anococcygeus.

Smooth muscle varies widely in its pattern of activity and this is associated with the muscle's position and function. For example, at one extreme, a continuous maintained activity may be required of the muscle, as in the iris, blood vessels or ureter; whereas at the other extreme, occasional bursts of activity occurs in muscle involved in defaecation or pregnancy. Again, tissue such as the urinary bladder or iris contract as a unit, whereas, in the ureter or intestine, waves of contraction pass from one end to the other. Localised contraction of sphincters and arterioles also occur. The activity of any muscle depends on a number of factors, including the properties of the individual smooth muscle cells, the interactions between cells and the influence of external agents such as nerves, hormones and the physical environment.

Hormones and neurotransmitters exert their action on smooth muscle cells by reacting with specific targets on the cell membrane, the receptors. The question of how these membrane events are coupled to the contractile response has been one of the most intriguing problems in smooth muscle research in recent years. As in skeletal and cardiac muscle, excitation-contraction coupling involves the release and entry of calcium ions into the cytoplasm. However, the rise in $[Ca^{2+}]$ promotes an increase in force in a very different manner from that in skeletal and cardiac muscle. Calmodulin is generally accepted to be the calcium receptor and with Myosin Light Chain Kinase (MLCK) activates tension. The details of this mechanism will be discussed later. This chapter will deal with:

(1) the basic types of smooth muscle;

(2) the diversity of receptors;

(3) the mechanisms of calcium release and entry;

(4) the site of the stores of intracellular calcium; and

(5) mechanism of force production in smooth muscle.

SMOOTH MUSCLE DIVERSITY

Smooth muscle can be broadly divided into three categories on the basis of its inherent plasma membrane electrical activity and its electrical response to stimulation. The categories are:

(a) Spontaneously active, highly excitable muscle;

(b) Quiescent, but highly excitable muscle;

(c) Quiescent, poorly excitable muscle (reviewed by Creed, 1979; Bulbring & Tomita, 1987).

Smooth muscle can also be divided on the basis of the type of contraction produced by the muscle, that is, whether it is either phasic or tonic. Phasic contractions are short lived and usually produced in spontaneously active and highly excitable muscles in response to an action potential. These phasic contractions can be fused together to produce a maintained contraction or waves of contraction and relaxation. Examples of muscles which produce this type of contraction are taenia coli (Shimo & Holland, 1966), intestinal smooth muscle (Kuriyama & Suzuki, 1975), stomach (Shino, 1976), myometrium (Gabella, 1978), portal vein (Gabella, 1978), ureter (Sunano, 1976) and bladder (Sunano & Miyazaki, 1975). Tonic contractions are slower to form than phasic contractions and are not always preceded by an action potential. These contractions are maintained. They are, therefore, more usually found in quiescent, poorly excitable muscles *e.g.* trachea (Kirkpatrick, 1975) aorta (Bohr, 1963), rabbit ear artery (Bevan & Waterson, 1971) and rat anococcygeus (Gillespie, 1980).

(a) Spontaneously active, highly excitable muscles: Examples of muscles included in this group are the taenia coli, the small and large intestine, the myometrium, bladder and the portal vein. These muscles have all been shown to have spontaneous action potentials (Bulbring, 1954; Nagai & Prosser, 1963; Kuriyama 1967; Kuriyama & Mekata; 1971). These are mostly spike-like, but can have a plateau. They may occur in bursts or at regular intervals and propogate along the tissue at conduction velocities of 10-80mm/s. This activity

originates within the muscle and can spread along the muscle length. Spontaneous activity arises from simultaneous depolarisation of many cells. The pattern of these depolarisations, called slow waves or pacemaker potentials, varies in different smooth muscles. However, the basic mechanism appears to involve an unstable membrane potential. Transmitters released by nerves act on a large number of cells and modify the basic activity, increasing or decreasing spike frequency.

In summary, these muscles have an unstable membrane potential and give rise to all-or-none action potential which produce phasic contraction. The action potential is able to propagate throughout the muscle via of low-resistance pathways between cells and the spontaneous activity can be modified by nerves.

(b) Quiescent, but highly excitable muscle: Examples of muscles included in this group are vas deferens and ureter. These muscles are normally quiescent, but do occasionally show spontaneous activity and produce phasic contractions. The vas deferens is not spontaneously active and normally contracts in response to nerve stimulation. This gives rise to excitatory junction potentials which sum with each other so that, at a critical value, a spike is initiated. In guinea pig vas deferens the spikes are all-or-none with overshoots of 20mV (Burnstock & Holman, 1961). In the mouse, however, the spikes are graded in amplitude, varying from 'humps' to overshoots of 5mV (Furness & Burnstock, 1969). In these tissues with graded spikes, propagation tends to be decremental. This could be due to insufficient current spread between cells or to poor electrical excitability of the membrane.

(c) Quiescent, poorly excitable muscle: Examples of muscle included in this group are stomach, trachea, anococcygeus, arteries and veins. This muscle has no spontaneous, rhythmic activity. Rat anococcygeus for example has a resting membrane potential of -51mV to -75mV and no slow waves or spontaneous depolarisation (Creed, Gillespie & Muir, 1975). Furthermore the membrane cannot be excited electrically by either intracellular current injection or by external polarization. Field stimulation of the excitatory nerves, however, produces depolarisation of graded amplitude.

The anococcygeus is densely innervated by adrenergic nerve fibres, but close neuromuscular contacts are absent, the minimum separation being 55nm (Gillespie & Lullmann-Rauch, 1974). Co-ordinated activity of the whole muscle depends on synchronous or sequential nerve discharge. In rat anococcygeus spikes are small and rarely seen and contraction apparently occurs in their absence. Even with nerve stimulation at less that 1Hz, contraction occurs and this is associated with depolarisations of less than 20mV. An action potential is, therefore, not essential for activation of the muscle.

Records of the membrane potential suggest that, in arterial and tracheal smooth muscle, contraction need not be preceded by a spike. The carotid, main pulmonary and ear arteries of the rabbit resemble the rat anococcygeus in being quiescent (Mekata & Niu,1972; Casteels *et al*, 1977). Low concentrations of noradrenaline and adrenaline (10μM) can evoke contractions without depolarising the membrane, though at higher concentrations some depolarisation occurs. The membrane potentials of tracheal smooth muscle of cattle and dog

is also stable and there is no spontaneous electrical or mechanical activity (Kirkpartrick, 1975; Suzuki, Morita, & Kuriyama, 1976). The mechanical response is triggered by a depolarisation of about 5mV. Histamine, acetylcholine and nerve stimulation induce contraction and depolarise the membrane, sometimes with superimposed slow potential oscillations, but no spikes.

Therefore, the rat anococcygeus muscle, certain arteries and tracheal smooth muscles cannot be excited directly electrically and have a steady membrane potential with no evidence of spontaneous electrical activity. These muscles produce tonic contractions.

RECEPTOR DIVERSITY

A 'receptor' is a protein in the surface membrane which acts as a switch turning a biochemical response either on or off within the cell when the receptor becomes bound to a 'substrate'. These 'substrates' are normally hormones and/or neurotransmitters and are termed as agonists. Receptors are usually named after the agonist or family of agonists which activate them. For example the receptors which respond to adrenaline and noradrenaline are termed adrenoreceptors; activation of these receptors and the consequences are the main concern of this thesis. Adrenoreceptors can be further subdivided into two categories α and β. The original subdivision was made by Ahlquist (1948) on the basis of the potency of both synthetic and natural compounds to activate the receptor. Ahlquist (1948) laid the ground rules for further classification by

recognising that receptors should be classified by their pharmacological properties rather than their location. This was particularly important because these two adrenoreceptor subtypes have inhibitory or excitatory properties depending on their location, but the same agonist potency regardless of their location.

In the 1960's it became clear that β-adrenoreceptors in different tissues showed different pharmacological properties. Lands and colleagues (1967) proposed that these receptors be further subdivided into β_1- and β_2-adrenoreceptors. β_1-Adrenoreceptors were found predominantly in the heart, while β_2-adrenoreceptors were found predominantly on smooth muscle. However, both β-receptor types can coexist on the same tissue (Minneman, Pittman & Molinoff, 1981). β-Adrenoreceptor subtypes show many similarities and only a few differences: both β_1- and β_2-adrenoreceptors stimulate formation of cyclic AMP as their primary mechanism for signal transduction in cells. Very few drugs show more than a 20- to 50-fold difference in potency in binding to the two different subtypes.

In a similar way during the 1970's it became clear that α-adrenoreceptors in different tissues did not have identical pharmacological properties. Based on differences in the potency of phenoxybenzamine in blocking presynaptic increases in noradrenaline release and postsynaptic increases in contractility in cat spleen, Langer, (1974) proposed that postsynaptic α-adrenoreceptors be referred to as α_1, and presynaptic receptors be referred to as α_2. However, it soon became obvious that α_1- and α_2-adrenoreceptors existed postjunctionally on

smooth muscle (Docherty, MacDonald & McGrath, 1979 ; Drew & Whiting, 1979; Timmermans, Kwa & van Zweiten, 1979; Docherty & McGrath, 1980; Langer *et al*, 1980; Starke, 1981; Timmermans & van Zweiten, 1981) and activate contraction. It subsequently also became clear that α_1-adrenoreceptors could exist on presynaptic nerve terminals (Kobinger & Pichler, 1982; Docherty, 1983; Story, Standford-Starr & Rand, 1985; McDonough, Wetzel & Brown, 1986). Unlike the β-adrenoreceptor subtypes, the α-adrenoreceptor subtypes are much less alike. Antagonist selectivities of two or three orders of magnitude are often seen between the two α-adrenoreceptor subtypes, whereas a difference in potency of one order of magnitude is more likely between the β-adrenoreceptor subtypes. The α-adrenoreceptors appear also not to use the same second messenger system. There is a large body of evidence to suggest that α_1-adrenoreceptors produce their intracellular effects via the phosphatidyl inositol cycle (Berridge & Irvine, 1984, 1989) whereas α_2-adrenoreceptors are thought to work by suppressing cyclic AMP production (Fain & Garcia-Sainz, 1980; Exton, 1985).

MECHANISMS OF CALCIUM RELEASE AND ENTRY

Once the receptor has been activated it promotes a rise in intracellular $[Ca^{2+}]$. This increase in intracellular $[Ca^{2+}]$ can be brought about by calcium entry from the extracellular space, calcium release from the intracellular store or by a combination of both. Calcium entry can be brought about by (a) membrane depolarisation which opens voltage operated calcium channels

(VOCs); (b) receptor activation which can directly open calcium channels through receptor operated calcium channels (ROCs); and (c) production of second messengers, which in turn might open calcium channels (SOCs). Calcium release can be brought about by (a) release of intracellular calcium from the stores by second messengers and (b) release of calcium from the stores by a small increase in the calcium concentration of the cytosol - termed calcium induced calcium release (CICR).

Calcium Entry

(a) Voltage Operated Channels: Two types of voltage-operated calcium channel have been identified in smooth muscle. These are:

(i) 'T'-type channels; these need only a weak deplorisation to open and cause short lived inward transient current;

(ii) 'L'-type channels which are characterised by the requirement for a strong depolarisation to open and an inward current which decays very slowly (Nowychy, Fox & Tsien, 1985).

(b) Receptor Operated Channels: Up until recently, only indirect evidence existed to suggest that agonists might cause calcium entry without depolarising the membrane. To do so the agonist was thought to act directly on the calcium channel causing it to open (Droogmans, Raeymayers & Casteels, 1977; Bolton, 1979; Meisheri, Hwang & van Breemen, 1981). Recently Benham (Benham & Tsien, 1987; Benham, 1989) presented direct evidence that, in voltage clamped single cells from the smooth muscle of the rabbit ear artery, ATP opens a

calcium channel in the membrane. There is also a report in the literature that acetylcholine opens a non-selective cation channel in isolated cells from guinea pig ileum (Inoue & Isenber, 1990)

(c) Second Messenger Operated Channels: There is considerable uncertainty concerning the mechanism and control of these channels and there is no direct evidence for them in smooth muscle. However, there is evidence in mast cells and lymphocytes that cAMP and $Ins(1,4,5)P_3$ can increase channel opening (Kuno & Gardener, 1987; Penner, Mathew & Neher, 1988).

There is some evidence to suggest that $Ins(1,4,5)P_3$ might not have a direct effect on calcium entry by activating a channel on the plasma membrane, but instead release the calcium from the endoplasmic reticulum which then causes calcium to enter the cell. This hypothesis is based on work with endothelial cells, where calcium entry is preceded by $Ins(1,4,5)P_3$-induced calcium release from the endoplasmic reticulum (Hallam, Jacob & Merritt, 1988). There is evidence from mouse lacrimal cells that $Ins(1,3,4,5)P_4$ might also be involved in promoting calcium entry. This work showed that a sustained calcium current could be only activated when both $Ins(1,3,4,5)P_4$ and $Ins(1,4,5)P_3$ were present (Morris *et al*, 1987). Diacylglycerol (DG) has also been implicated as a second messenger which promotes calcium entry, but not in smooth muscle. It has been shown to facilitate calcium entry in Aplysia neurones (Deriemer *et al*, 1985), Herissenda photoreceptors (Farley & Auerbach, 1986) and rat adrenal medulla (Wakade, Malhotra & Wakade, 1986).

Calcium Release

(a) Release of Intracellular Calcium by Second Messengers: The two known second messengers in smooth muscle which are produced on receptor activation are Ins(1,4,5)P_3 and cAMP. Alpha-adrenoreceptor activation has been shown to produce Ins(1,4,5)P_3. Its best understood function is to release calcium from the sarcoplasmic and endoplasmic reticulum. This was first reported by Streb and colleagues (1983) in rat pancreatic acinar cells and subsequently in smooth muscle (Suematsu *et al*, 1984; Somlyo *et al*,1985; Hashimoto *et al*, 1986). None of the other compounds produced in the phosphatidylinositol (PtdIns) cycle have been shown to release calcium to the same extent as Ins(1,4,5)P_3. The PtdIns cycle is discussed in more detail in the introduction to Chapter 5 and reviewed by Berridge and Irvine (1984, 1989). There are no reports that cAMP influences mobilisation of calcium from the intracellular store. Instead it is thought to have its effect by influencing myosin light chain kinase (MLCK) (Ruegg & Paul, 1982) decreasing its ability to phosphorylate the myosin light chain and hence to interact with actin, thus causing relaxation.

(b) Calcium Induced Calcium Release: It has already been shown in skeletal and cardiac muscle that a rise in [Ca^{2+}] in the vicinity of the sarcoplasmic reticulum (SR) induces a further regenerative release of calcium from the SR (Endo, Tanaka & Ogawa, 1970; Fabiato & Fabiato, 1975; Barsotti *et al*, 1988). However, no direct evidence of a similar nature has been shown in smooth muscle. Itoh, Kuriyama and Suzuki (1981) and Obaro, Ito and Yabu

(1987) have both shown that the calcium content of the SR was reduced when the bathing [Ca^{2+}] was increased. Thus calcium induced calcium release might have a role in the modulation of the calcium content of the smooth muscle sarcoplasmic reticulum. It has yet to be shown, however, that calcium induced calcium release is involved in the calcium release mechanism in smooth muscle.

Calcium induced calcium release has been postulated as the mechanism by which Ins(1,4,5)P_3 causes a uniform calcium release within non-excitable cells (Gilkey *et al*, 1987; Busa *et al*,1985; Miyazaki *et al*, 1986; Lakatta *et al*, 1989). However, it has been dismissed as the mechanism by which Ins(1,4,5)P_3 releases calcium in smooth muscle by Somlyo *et al* (1990) because procaine (a known blocker of calcium induced calcium release) does not block Ins(1,4,5)P_3 induced calcium release.

STORES OF INTRACELLULAR CALCIUM

A rise in cytoplasmic [Ca^{2+}] is usually triggered by a surface membrane event. For the majority of the work contained in this thesis this has meant activation of a membrane bound receptor by its agonist. How this activation is propagated from the receptor to the intracellular source of calcium is discussed in detail in the introductions of Chapters 4 and 5. Briefly the agonist binds to its receptor which activates a G-protein in membrane. The G-protein in turn activates the enzyme phospholipase C which splits phosphatidylinositol (4,5)bisphosphate to produce inositol(1,4,5)triphosphate and diacylglycerol. Both of these compounds then have effects on calcium release from the SR,

entry across the sarcolemma and calcium sensitivity of the contractile proteins. The intracellular sites for calcium storage previously thought to be involved include: (a) sarcoplasmic reticulum; (b) mitochondria; and (c) surface membrane.

(a) Sarcoplasmic reticulum: It has now been universally accepted that the source of intracellular calcium is the sarcoplasmic reticulum (SR). This has not always been the case even although there is an obvious parallel with skeletal and cardiac muscle (Endo, 1977). This was mainly due to an early work by Peachy and Porter (1959) who reported that smooth muscle had a scarcity of SR. This finding was backed by Somlyo and Somlyo (1968) and prompted the search for an alternate source of calcium. Peachy and Porter's findings were later discovered to be in error. The primary fixative that they had used partially destroyed the smooth muscle and in addition they had been unfortunate in choosing types of smooth muscle that had a small amount of SR (Devine, Somlyo & Somlyo, 1972; Gabella, 1981). In fact, an SR that occupies no more that 2% of the cell volume can store enough calcium to activate maximal contraction (Bond *et al*, 1984a; Kowarski *et al*, 1985).

The SR is an intracellular membrane system of tubules (Somlyo *et al*, 1971). These tubules exclude extracellular markers such as ferritin, horseradish peroxidase or collodial lanthanum. The Na and Cl concentration in the SR, measured with electron probe analysis (Kowarski *et al*, 1985) is similar to the cytoplasmic but not the extracellular concentrations, indicating that the SR is not in direct ionic communication with the extracellular space. The SR can occupy

about 1.5 to 7.5% of the total cell volume depending on the smooth muscle type, being most extensive in the large elastic arteries (e.g. rabbit aorta and main pulmonary artery) and in pregnant uterus (Somlyo & Somlyo, 1975; Garfield & Somlyo, 1985). The longer persistence of contractions in nominally calcium-free solutions in large elastic arteries, compared to taenia coli and portal vein, was originally related to the smaller volume of SR in the latter types of (phasic) smooth muscle. However, it has since been demonstrated that guinea pig portal vein smooth muscle, which contains only 2% SR can contract maximally in nominally calcium-free solutions (Bond *et al*, 1984a).

The SR's ability to accumulate calcium has been demonstrated using strontium. Calcium and strontium are taken up by the SR using the same transport mechanism. Strontium is more electron-dense than calcium which allows it to be visualised using an electron microscope. Strontium was accumulated in both quiescent and activated cells (Somlyo & Somlyo, 1971). Electron probe analysis was used to demonstrate that calcium was accumulated by smooth muscle SR (Somlyo *et al*, 1979; Popescu & Diculescu, 1975; Heumann, 1976; Somlyo *et al*, 1982). Physiological experiments demonstrating the ATP-dependence of such calcium uptake also identified the SR as the calcium store (Endo *et al*, 1982). The calcium content was measured with electron probe micro analysis in relaxed vascular smooth muscle (Bond *et al*, 1984a; Kowarski *et al*, 1985). It was estimated to be 28mmol/kg dry weight in the junctional SR of the guinea pig portal vein and 15-18mmol/kg dry weight in the central SR of the rabbit main pulmonary artery. These concentration are

considerably higher than those found in the cytoplasm and provide further evidence for the accumulation of calcium by the SR.

It has been known for a considerable time that smooth muscle can contract in the virtual absence of extracellular calcium and this was taken as evidence for the release of calcium from intracellular stores (Devine *et al*, 1972). The fact that this calcium was stored in the SR was harder to prove. Changes in the calcium content of the SR could be examined using electron probe micro analysis (Bond *et al*, 1984a; Kowarski *et al*, 1985). These latter two groups examined two different vascular smooth muscle preparation. One was spontaneously active and highly excitable muscle, guinea pig portal vein, and the other a quiescent and poorly excitable muscle, main pulmonary artery. It was discovered that these two muscle preparations had different amounts of SR, 2 and 7.5% of total cell volume respectively, but that each could release enough calcium to cause a maximal contraction. However, they both released their calcium from different SR sites within the muscle. Guinea pig portal vein released it from the junctional SR and main pulmonary artery released it from the central SR.

(b) Mitochondria: Mitochondria have a relatively low affinity, but very large capacity for accumulating calcium. The apparent Km of mitochondria isolated from smooth muscle for calcium is only 10-17μM (Vallieres, Scarpa & Somlyo, 1975; Wikstrom, Ahonen & Luukkaine, 1975). In smooth muscles that are not damaged or experimentally exposed to unphysiological high free cytoplasmic [Ca^{2+}], the mitochondrial [Ca^{2+}] ranges from 0 to 3 mmol/Kg dry

weight (Somlyo, Somlyo & Shuman, 1979; Bond *et al*, 1985) This [Ca^{2+}] is comparable with the estimate made for cardiac mitochondria (Denton & McCormack, 1980; Hansford & Castro, 1982). These latter workers also suggested that this range of free intra-mitochondrial [Ca^{2+}] would regulate certain important mitochondrial enzymes (e.g. dehydrogenase). The Somlyo's claim that mitochondria do not have any role in calcium accumulation has been disputed (Miller, 1985). This author suggests that relaxation can be induced in smooth muscle if the mitochondiria sequesters 1mM [Ca^{2+}] which is a concentration taht is below the detection level of X-ray probe microanalysis and that mitochondria might only be transiently accumulating calcium before transferring it to the SR.

(c) Surface Membrane: The bidirectional transport of calcium across the plasma membrane and the role of membrane-bound calcium itself are the two major functional aspects of the participation of the surface membrane in cellular calcium metabolism. There is much evidence based on measurement of radioactive calcium isotope flux (Jones, 1980; Fleckenstein, 1983), electrophysiological (Somlyo & Somlyo, 1971) and electron probe microanalytical (Bond *et al*, 1984a) studies showing that excitation by potassium depolarisation or noradrenaline can increase calcium influx across the surface membrane. If this is a maintained influx, calcium accumulates in the SR (Somlyo & Somlyo, 1971, 1975; Bond *et al*, 1984b; Somlyo *et al*, 1985) and binds to calcium-cytoplasmic binding proteins (Bond *et al*, 1984b). The amount of calcium bound to the surface membrane is lower than that contained in the lumen of the SR

(Bond *et al*, 1984b) and it is unknown whether calcium from this site can be released into the cytoplasm.

MECHANISM OF FORCE PRODUCTION IN SMOOTH MUSCLE

Smooth muscle cells are spindle-shaped, about 5-50μm wide and up to 0.5mm long. They contain double the concentration of actin and tropomyosin as skeletal muscle, but a four to five times lower concentration of myosin (Murphy, Heruhy & Megerman, 1974). This difference has a structural correlate. Within smooth muscle cells, actin and myosin filaments are organised in bundles in such a way that each myosin filament is surrounded by up to 10 or 15 actin filaments. The latter may be several μm long and 8nm thick (Small, 1974). Myofilament bundles are obliquely orientated within the spindle-shaped smooth muscle cells as they run diagonally from an attachment point on the cell membrane through the cell and insert at an attachment point on the opposite side of the cell. Quite often, the position of the attachment patches of neighbouring cells match. Since the narrow gap between these patches is bridged by electron dense material, the cells are evidently mechanically coupled. In this way, smooth muscle cells form a contractile network to which collagen fibres may be attached (Gabella, 1984).

At the molecular level, the mechanism of contraction in all muscles is believed to be the same (Marston & Taylor, 1980). Crossbridges arising from the myosin filaments bind actin and MgATP, the MgATP is hydrolysed on the enzymic site and the energy released is used to produce a conformational

change in the actin-myosin complex which results in a relative movement of the two filaments (Tregear & Marston, 1979).

For smooth muscle, as for skeletal muscle, it is generally accepted that it is calcium ions which link excitation with contraction. The first demonstration of calcium regulation in smooth muscle was given by Filo, Bohr and Ruegg (1965). They found that glycerol extracted muscle from pig carotid artery required Mg^{2+}, ATP and Ca^{2+} for contraction. Tension production was absolutely dependent on Ca^{2+}.

A calcium-dependent MgATPase was subsequently demonstrated in smooth muscle homogenates from various sources (Murphy *et al*, 1969; reviewed by Marston, 1982) but attempts at isolating the contractile proteins usually produced preparations with low MgATPase activity and no calcium sensitivity (Needham & Williams, 1963; Barany *et al*, 1966; Murphy *et al*, 1969). The first successful smooth muscle actomyosin preparation was developed by Sparrow *et al* (1970) using pig carotid artery. Once a satisfactory method for isolating actomyosin had been developed Bremel (1974) was able to show that, unlike striated muscle, the myosin activity of chicken gizzard smooth muscle was regulated by calcium. These experiments failed to show any calcium regulation by the actin filaments and isolated actin filaments exhibited no calcium-binding (Sobieszek & Small,1976; Sobieszek, 1977b). Consequently attention became focused on myosin linked calcium regulation. At first it was thought that calcium activated smooth muscle myosin by binding to it directly (Sobieszek & Small, 1976). However, because it was already known that purified smooth

muscle myosin had a reduced actin activated MgATPase activity (Barany *et al*, 1966; Yamaguchi, Miyazawa & Sekine, 1970, Driska & Hartshorne, 1975) it became apparent that components in addition to actin were required for activation of the myosin-MgATPase activity in smooth muscle. It seemed unlikely, therefore, that calcium would be having its effect on myosin directly.

In 1977 Sobieszek observed that there was a calcium-dependent phosphorylation of the 20,000 Mol.wt. light chain component of myosin in chicken gizzard actomyosin preparations. The calcium dependence of phosphorylation was close to that of the activation of myosin-MgATPase activity and both high ATPase activity and phosphorylation persisted after the removal of calcium. These observations were quickly confirmed in most smooth muscle types; gizzard (Sobieszek, 1977a; Aksoy *et al*, 1976), blood vessels (di Salvo, Gruenstein & Silver, 1978), uterus (Lebowitz & Cooke, 1979), vas deferens (Chacko, Contim & Adeldstein, 1977) and stomach (Small & Sobieszek, 1977a). Further studies in several laboratories led to the phosphorylation model of smooth muscle regulation (Sobieszek, 1977a; Small & Sobieszek, 1977; Adelstein *et al* 1977; Adelstein, 1978; Hartshorne *et al*, 1977). This model states that actin cannot activate myosin MgATPase activity unless the 20,000 Mol.wt. myosin light chain is phosphorylated. Phosphorylation is catalyzed by a specific myosin light chain kinase (MLCK) which is activated by calcium at concentrations similar to those required to activate striated muscle actomyosin MgATPase. Dephosphorylation of myosin and hence inhibition of actomyosin MgATPase activity, is catalyzed by a specific phosphatase which is active irrespective of the

[Ca^{2+}]. Subsequent purification of phosphorylated and unphosphorylated myosin, the myosin light chain kinase and the phosphatase has supported this model, although it has not excluded the possibility of direct calcium regulation of myosin also occurring under some conditions (Chacko *et al*, 1977).

When the MLCK was further purified, in an effort to discover how it was controlled by calcium, it lost its activity. Dabrowska and colleagues (1977) showed that the kinase could be reactivated by the calcium-dependent regulator of cyclic nucleotide diesterase, discovered by Cheung (1970) and later termed 'calmodulin'. Calmodulin is a highly coiled globular molecule with a Mol.wt. of 17,000, containing 148 amino acid residues organized in four calcium binding domains (Babu *et al*, 1985). Under physiological conditions, calmodulin is capable of binding Ca^{2+} in the physiologically important concentration range of 0.1μM to 10μM. When occupied with four calcium ion, calmodulin changes its shape such that the hydrophobic sites from the interior of the molecule become exposed to the outside. This increases the affinity of calmodulin for target proteins including MLCK (Adelstein *et al*, 1981).

Since calmodulin's affinity for myosin light-chain kinase is calcium-dependent, it can act both as a calcium sensor and a calcium switch for the regulation of the enzymic phosphorylation of smooth muscle myosin; it is the ternary complex of calcium-calmodulin-MLCK that represents the active enzymic moiety. The concentration of this complex, therefore, depends on both the concentration of free calcium and free calmodulin. It has been estimated that smooth muscle contains 30-50μM calmodulin although most of this is bound

to calmodulin binding proteins (Grand & Perry, 1979) so that only a very small fraction of calmodulin is available and responsible for smooth muscle activation (Ruegg *et al*,1984).

The phosphorylation hypothesis for smooth muscle contraction and relaxation is shown diagramatically in Figure 1.1 (taken from Ruegg, 1988). When the $[Ca^{2+}]$ rises sufficiently calmodulin binds four calcium ions and can then react with myosin light chain kinase to a form an active ternary enzyme complex. This complex catalyzes the phosphorylation of myosin. Actin can then interact with the phosphorylated myosin which increases its ATPase activity and the muscle 'contracts'.

Lowering the $[Ca^{2+}]$ to an appropriate level depresses the myosin light chain kinase activity. The myosin then tends to be dephosphorylated by a myosin phosphatase (Di Salvo *et al*, 1983) resulting in an inhibition of actomyosin ATPase. This causes muscle to relax. The activity of the phosphatase is independent of the $[Ca^{2+}]$. Thus during contraction it is a balance of kinase and phosphatase activity which controls the level of phosphorylation and hence the extent of activation of the muscle.

Dephosphorylated myosin has a low ATPase activity and is less able to interact with actin. Under these conditions, the rate limiting step of the ATP-splitting mechanism, presumably the dissociation of the actomyosin-ADP state, appears to be inhibited (Marston, 1982; Sellers, 1985). It is possible, therefore, that the dephosphorylated light chain is an inhibitor of actin-myosin

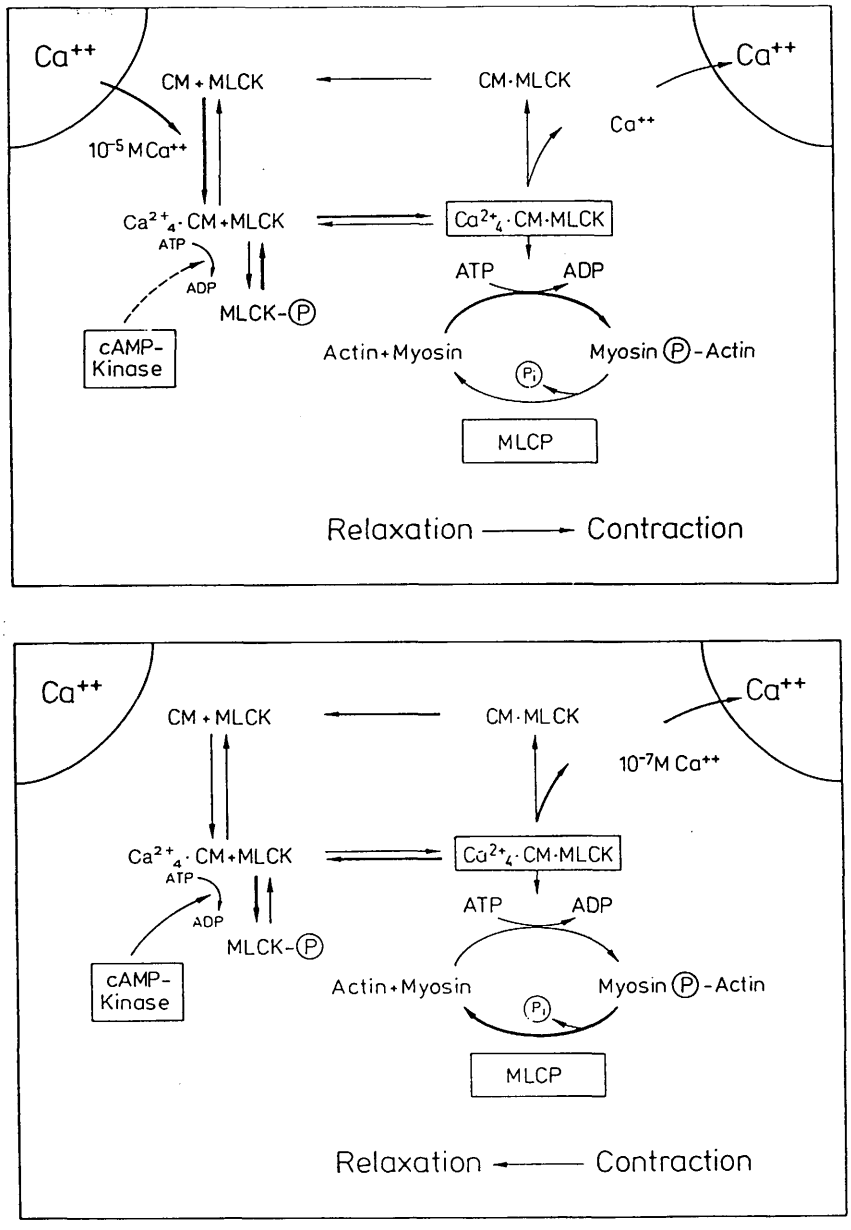


Figure 1.1 Myosin phosphorylation regulates smooth muscle contraction. Activation is initiated by Ca^{2+}, myosin light chain kinase and calmodulin forming the active ternary comlex which catalyzes the phosphorylation of the myosin light chain kinase. When Ca^{2+} is lowered the calmodulin-myosin light chain kinase complex decomposes and myosin is dephophorylated by myosin light chain phosphatases. Only phophorylated myosin interacts with actin to form 'cycling', ATP-hydrolysing contractile crosslinkages.

interaction in smooth muscle. Indeed, myosin subfragment S-1 is fully enzymically active in the presence of actin when the regulatory light chain has been removed (Mrwa & Ruegg, 1977).

Although the phosphorylation hypothesis is the most widely accepted explanation of how smooth muscle contracts, there are other theories. There are at least three other different mechanisms which have been proposed to account for smooth muscle activation in the absence of additional phosphorylation of myosin: (1) calcium binding to myosin; (2) activation by leiotonin; and (3) regulation by the thin-filament proteins caldesmon and tropomyosin.

(1) Calcium Binding to Myosin: Chacko and Rosenfeld (1982) showed that actin binding to myosin could be increased once it had been phosphorylated by increasing the $[Ca^{2+}]$ in the bathing medium. These workers suggested that calcium was having this effect by binding to the myosin light chains.

(2) Activation by Leiotonin: Ebashi (1980) described the properties of leiotonin, a calcium-dependent activator of smooth muscle contraction; it binds calcium, requires tropomyosin as a cofactor and increases actomyosin-ATPase activity without any increase in myosin phosphorylation.

(3) Regulation by the Thin-filament proteins Caldesmon and Tropomyosin: Marston and Smith (1984) isolated, from vascular smooth muscle, a preparation of actin filaments that activated myosin ATPase in a calcium-dependent manner. These results suggested that the thin filament muscle proteins include regulatory

proteins conferring calcium sensitivity on the contractile system. It was suggested that these proteins were (i) caldesmon and (ii) tropomyosin.

(i) Caldesmon can bind both actin and calmodulin and it has been postulated to be the calcium regulatory component of the thin filament (Marston & Lehman, 1985). In the absence of calcium ions, caldesmon binds actin rather than calmodulin and possibly prevents the protein from interacting with myosin. In the presence of calcium, it tends to bind to calmodulin rather than actin relieving its possible inhibitory action (Sobue *et al*, 1982). There are reports that caldesmon has to be phosphorylated by a calcium/calmodulin-dependent protein kinase before it is able to bind calmodulin (Nagai & Walsh, 1984, 1985).

(ii) Tropomyosin has been reported (Merkel, Meisheri & Pfitzer, 1984) to increase the calcium sensitivity of a hybrid actomyosin made up of skeletal muscle actin and supplemented with myosin light chain kinase and calmodulin. At an intermediate $[Ca^{2+}]$, total muscle myosin is partly phosphorylated and its ATPase partly activated, whereas at the same calcium concentration addition of tropomyosin increases myosin-ATPase activity without increasing the extent of phosphorylation. It appears, therefore, that tropomyosin improves phosphorylation-contraction coupling and this allows the contractile system to operate at a lesser degree of myosin phosphorylation.

AIMS

The introduction has discussed the factors involved in smooth muscle contraction. The aim of this thesis was to obtain a better understanding of the inolvement of G-proteins and the phosphatidylinositol cycle in receptor activation in rat anococcygeus. Their involvement was examined in both intact and permeabilized muscle. Special interest was paid to their role in modulating calcium release from the sarcoplasmic reticulum and caclium-activated force.

CHAPTER 2

MATERIALS AND METHODS

A. INTACT MUSCLE

PREPARATION

Male rats (250 - 300g) were killed by a blow to the back of the head followed by exsanguination. The two 'legs' of the anococcygeus muscle were rapidly removed and placed in a petri dish filled with warm Krebs solution (composition shown in Table 2.1, column 1).

POSITION

The two 'legs' of the anococcygeus muscle arise from the coccygeal vertebrae close to one another in the midline of the pelvic cavity. The muscles pass caudally, lying first behind and then to one side of the colon, finally joining together to from a ventral bar in front of the colon a few millimetres from the anus

HARVESTING

The abdomen was opened at the midline. The testes, bladder and seminal vesicles were removed. Two incisions were then made from the abdomen into the scrotum, the penis was cut at its base and the skin pulled back. The pelvis was split and the colon cut and carefully pulled back to reveal the anococcygeus muscle. Any connective tissue around the muscle was carefully removed, the tissue was tied with cotton thread just above where the muscle inserts into the coccygeal vertebrae and at the point where it meets with the colon. Hence the

TABLE 2.1.

Compound	Krebs (mM)	Low Calcium Krebs (mM)	Tyrode (mM)
NaCl 118.4	118.4	150	
KCl 4.7	4.7	5	
$MgSO_4$	1.2	1.2	-
$MgCl_2$	-	-	2
$CaCl_2$	1.25	0.25	1.25
$NaHCO_3$	15	15	-
Hepes	-	-	25
KH_2PO_4	1	1	1
EDTA	0.0023	-	0.0023
EGTA	-	0.5	-
Cocaine	0.003	0.003	0.003
Glucose	11.1	11.1	10

of muscle used in these experiments did not include the 'bar' of tissue around the colon, but only the 'legs' of the muscle, as shown in Figure 2.1.

EQUIPMENT

Once the muscle had been removed from the animal, it was suspended in a 10ml organ bath as shown in Figure 2.2. The muscle was attached at one end to a fixed point in the bath by cotton thread. The other end was also attached, by cotton thread, to an isometric force transducer (Harvard, Grass FT03). The solution in the organ bath was aerated with 95% O_2 and 5% CO_2 and was kept at 37°C by circulating water from a heated water bath. The pH buffer used in these experiments was usually bicarbonate and the pH was approximately 7.4. The signal from the transducers was amplified and displayed on a Grass Polygraph.

SETTING UP

Once the muscle had been attached to the transducer and the fixed point as described previously a resting tension of 1N was applied. The muscle was allowed to relax and resting tension was restored twice over a 15 minute period. The bathing medium was replaced before each new application of tension. The muscle was then allowed to relax for a further 30 minutes. At the end of this period, the bathing medium was replaced and a 'priming dose' of noradrenaline (30μM) was added to the bath. The muscle was allowed to contract for 5 minutes and then the bathing medium was replaced every 5 minutes for 15

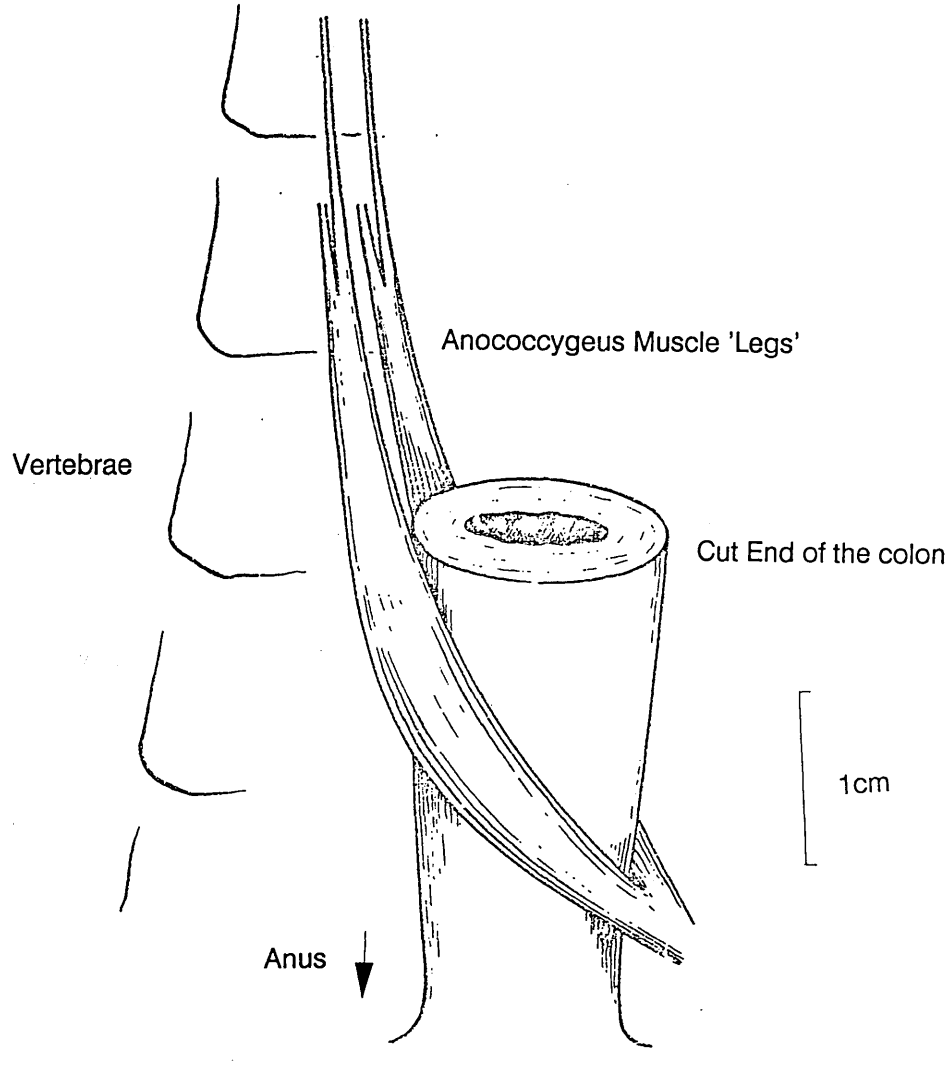


Figure 2.1 Position of the anococcygeus muscle in the male rat. The muscle lies deep to the colon in the pelvic cavity, originating from a tendinous contact with the coccygeal vertebrae and running around either side of the colon to join together on its ventral surface just short of the anal margin. This diagram has been taken from Prof. J.C. McGrath's PhD thesis.

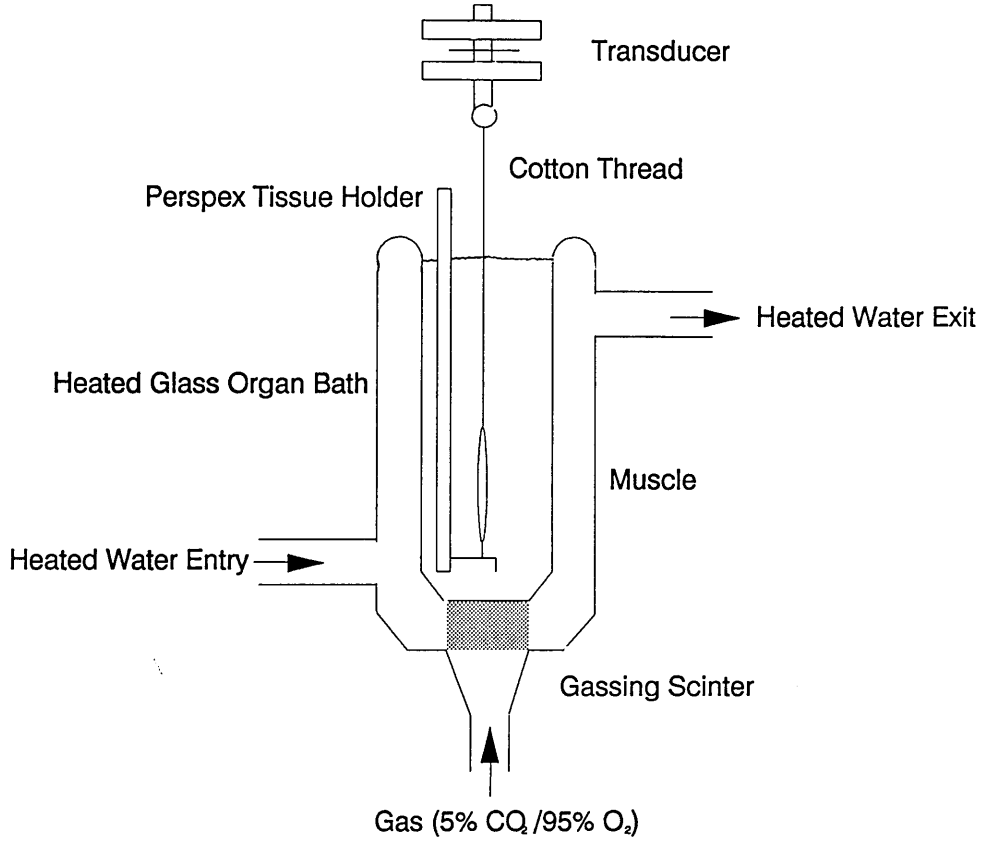


Figure 2.2 The experimental set-up used in the intact muscle experiments. The diagram is a representation of an organ bath and shows how the muscle is attached to the transducer and positioned in the organ bath.

minutes. The muscle was then allowed to relax for a further 30 minutes or until the resting tension had returned to control levels. The muscle was now ready for experimentation. This same protocol was used to prepare muscles which were subsequently bathed in low calcium Krebs (0.1μM) to examine intracellular release of calcium. The composition of this bathing medium is shown in Table 2.1, column 2.

SOLUTIONS

The solutions used are detailed in Table 2.1. These solutions were made up fresh each day.

DRUGS

The drugs used are listed in Table 2.2.

TABLE 2.2.

COMPOUND	*SUPPLIER*
ATP (disodium salt)	Sigma Chemicals, Poole, Dorset, England
CrP (disodium salt)	"
EDTA	"
EGTA	"
cAMP	"
GTP	"
GTP-y-S	"
GDP-ß-S	"
Hepes	"
Saponin	"
Caffeine	"
Myoinositol	"
Methansulphonic acid	"
Propionic acid	"
$KH_2PO_4.2H_2O$	"
Noradrenaline	"
Acetylcholine	"
5-Hydroxytryptamine	"
Phenylephrine	"
KCl	BDH Chemicals, Poole, England
NaCl	"
LiCl	"
KOH (1M titration standard)	"
NaOH (1M titration standard)	"
CaCl2 (1M titration standard)	"
$MgCl_2$ (1M titration standard)	"
Atropine sulphate	"
Glucose	Formachem, Strathaven, Scotland
Sucrose	"
IP_3	Calbiochem, California, USA
UK14304	Gift - Pftizter, Sandwich, Kent
Prazosin	"
Nifedipine	Gift - Bayer, West Germany
Crude alpha-toxin	Dept of Cell Biology, Glasgow University

B. PERMEABILIZED MUSCLE

PREPARATIONS

Adult male Wistar rats (250 - 270g) and adult female Dunkin Hartley guinea pigs (300 - 350g) were killed by a blow to the back of the head followed by exsanguination. Rat anococcygeus was removed as described previously. Guinea pig portal vein was found by opening the abdomen along the midline and displacing the intestines to one side revealing the vein. A piece of tissue of about 1cm in length was removed below the bifurcation where the vein enters the liver. This was placed in a beaker of Tyrode solution (composition shown in Table 2.1, column 3) and then transferred to the dissecting dish. Any connective tissue and fat were carefully removed under the microscope using fine scissors and the adventitia was removed. Once it was clean the vein was opened along its length and pinned out. The anococcygeus muscle was cleaned up in a similar manner after its removal. Small strips (100μm wide by 2-3mm long) of anococcygeus and of the longitudinal muscle of the portal vein were cut using fine scissors.

SETTING UP

The muscle was then mounted between a fixed point and a transducer. The fixed point, as shown in Figure 2.3, comprises a brass rod which has three pieces of hollow stainless steel tubing with an outside diameter of 200μm and an inside diameter of 100μm (Goodfellows) glued to it (Super glue, Loctite).

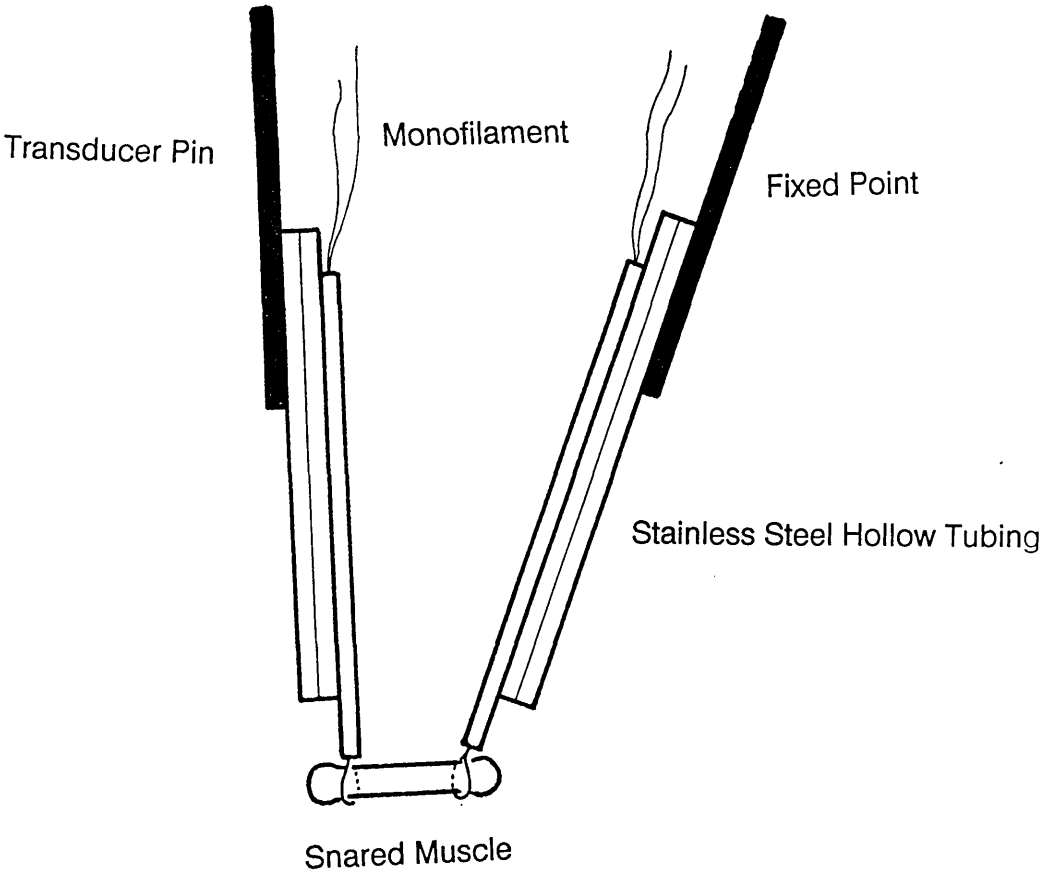


Figure 2.3 diagram (components not to scale) of the experimental arrangement used to measure tension in permeabilized muscle. The muscle is snared between the transducer and a fixed point.

The piece of tubing extending farthest has been threaded (nylon monofilament) such that a loop of thread extends from the bottom to form a snare. A similar arrangement exists on the transducer. The transducer and fixed point are mounted on a magnetic block and Narashige MM3 micromanipulator. This allows them to be moved relative to each other as well as together in 3 planes. The muscle is mounted under a dissecting microscope by passing it through both snares which are tightened when the muscle is in place to hold it securely. The mounted preparation is then transferred to the bath system. Once in the bath system the muscle has a resting tension of 0.5mN (rat anococcygeus) or 0.1mN (guinea pig portal vein) applied. The muscle is allowed to relax for 30 minutes before the experiment begins.

EQUIPMENT

The bath system comprises of a series of wells which have been cut out of a perspex block. Each well holds either 0.95 or 4.65mls of solution. The bath system fits into a perspex holder which can be moved in two planes by a horizontal and vertical stepper motors. These motors are controlled by an Apple II microcomputer. To change solutions the bath system is lowered moved along and then raised again under the preparation. This system and accompanying software allow large numbers of accurately timed solution changes to be preprogrammed. Solutions were continuously stirred by a stainless steel paddle

TENSION MEASUREMENT

The output of the tension transducer was amplified, filtered at 25Hz and displayed on a chart recorder (Linseis 1800). It was also simultaneously digitised. The signal was recorded on videotape via an A/D VCR adaptor (PCM 4/8, Medical systems corp. Greenvale, N.Y.) and video recorder. The data were then transferred from the videotape to an IBM computer (via a data translation board) for further analysis.

SOLUTIONS

The solutions used daily were made from stock solutions. The stocks were made at regular intervals and stored in the 'fridge'. These were 1M KCl and $MgCl_2$, 100mM CaEGTA, EGTA and KH_2PO_4, and 500mM Hepes. The amount of EGTA added to the stock solution was calculated with reference to recent reports regarding the purity of EGTA obtained from Sigma (Bers, 1982, Miller & Smith, 1984) The appropriate concentrations (Table 2.3) of these stock solutions were mixed together and the appropriate amounts (Table 2.3) of Na_2ATP, Na_2CrP and glucose were added. When KCH_3SO_3 was used it was either made up fresh from methansulphonic acid and potassium hydroxide or prepared then stored in the freezer and defrosted on the day of use.

There are three basic solutions '10 activating' (which contains 10mM CaEGTA), '10 relaxing' (which contains 10mM EGTA) and '0.2 relaxing' (which contains 0.2mM EGTA). The full composition of these solutions are shown in Table 2.3. The strongly calcium-buffered solutions -'10 activating' and '10 relaxing' - are

TABLE 2.3.

SOLUTION	10 ACTIVATING	10 RELAXING	0.2RELAXING
	(mM)	(mM)	(mM)
KCl or KCH_3SO_3	120	120	120
Hepes	25	25	25
Na_2CrP	15	15	15
EGTA	-	10	0.2
CaEGTA	10	-	-
$MgCl_2$	7.0	7.0	7.0
Na_2ATP	5.0	5.0	5.0

(pH 7.0 - adjusted with KOH, Ionic strength 0.2M)

used to examine calcium-sensitivity. To obtain the appropriate free [Ca^{2+}] in the bath, these solutions were mixed together. The free [Ca^{2+}] was calculated using a computer programme (REACT) written by G. L. Smith and D. J. Miller. The affinities of EGTA and the other ligands for calcium and the other metal ions have been incorporated. REACT can provide a complete profile of the free metal ion concentrations and ligand-mental concentrations. For details of the binding constants and correction for pH, ionic strength and temperature which were employed, see Smith and Miller, (1985). The weakly calcium buffered solution ('0.2 relaxing') was used to examine calcium release from the sarcoplasmic reticulum. The [Ca^{2+}] of the '0.2 relaxing' solution was increased to allow store loading by addition of $CaCl_2$. As the EGTA in the '0.2 relaxing' solution is not saturated, addition of calcium results in the net release of approximately 2 hydrogen ions per calcium-bound at pH 7.0 (Smith & Miller, 1985). Consequently, the appropriate amount of KOH was added to maintain the solution at pH 7.0. All the chemicals and drugs used are listed in Table 2.2.

pH

This is a very important factor since it is in the nature of the experimentation that the solutions directly bathe the contractile proteins and intracellular organelles. These organelles naturally work at a constant pH of 7.0 and are affected by changes in pH. Furthermore, the apparent binding constants of EGTA and ATP for Ca^{2+} and Mg^{2+} are highly pH sensitive. The pH buffer Hepes (25mM) was used to minimise pH changes during contracture.

The useful buffering range of Hepes is pH 6.5-7.5.

The pH of the experimental solutions was adjusted using a null point method. A standard solution was prepared, which was calculated to have the same pH (activity) and ionic strength (0.2M) as the solutions. The electrodes were allowed to equilibrate in the pH-standard and then all other solutions were adjusted to the pH by the addition of KOH. The pH electrodes used in this study consisted of separate reference (Corning 003116029) and pH electrodes (Corning 0031101J). For details of the composition of the pH standard solution see Harrison et al, (1988).

IONIC STRENGTH

Ionic strength (I) in this study has been calculated using the equation: $I_e = 1/2\ C_j.z_j$. Ionic strength is defined as the total of the ionic equivalents (I_e) where C_j is the concentration of the jth ionic species and z_j is its valency. Ionic strength for the large majority of this study has been set at 0.2M. It was adjusted by changing the concentration of KCl in the solutions.

PERMEABILIZATION TECHNIQUES

These are discussed in detail in the Materials and Methods section of Chapter 3.

CHAPTER 3

A COMPARISON OF THREE DIFFERENT METHODS USED TO PERMEABILIZE SMOOTH MUSCLE

INTRODUCTION

The 'skinned' muscle preparation has been in existence for over 35 years. A 'skinned' muscle is one in which the surface membrane has been removed or permeabilized to such an extent that there is free diffusion of small ions and other solutes between the extracellular and intracellular media. Hence by changing the extracellular medium, the intracellular medium and therefore the environment surrounding the contractile proteins and intracellular organelles can be controlled. Over the 35 years a number of skinning methods have been developed. These have had varying degrees of success in the different muscle types. The majority of the techniques were developed for skeletal muscle and have subsequently been used on cardiac and most recently, smooth muscle. The different methods will be discussed in this chapter.

Szent-Gyorgyi (1949) was the first to describe and use a method of preparing glycerinated skeletal muscle fibres. This method, which retains the native structure of the myofilaments, has been applied to visceral and vascular smooth muscle (Briggs, 1963; Filo, Bohr & Ruegg, 1965; Bozler, 1968). In these studies the time course of tension development induced by the removal of ATP and by the increase of free $[Ca^{2+}]$ was extremely slow in comparison with that of the living state. It was thought that the long exposure to glycerol (up to 3 months in some instances) was damaging the internal organisation of the muscle such that it could no longer respond in the same manner as the intact muscle.

Natori (1954) introduced a new technique which involved the mechanical removal of the surface membrane of skeletal muscle fibres. The technique had an advantage over the glycerinated fibres in that the intracellular organelles were left intact. This, however, is technically a very difficult method for skinning, especially in smaller muscle cells, such as cardiac and smooth muscle cells. It has, however, been successfully adapted by Fabiato and Fabiato (1973) for use in single cardiac muscle cells, but has not yet been applied to smooth muscle cells.

Another method of permeabilizing muscle was introduced in 1971 by Winegrad. This method involved exposing the muscle, in this instance frog ventricular muscle, to a solution which contained 3mM EDTA for 15 mins. The muscle was thereafter:

(1) responsive to raising the $[Ca^{2+}]$;

(2) had a membrane potential between +10 and +15 mV; and

(3) could no longer initiate an action potential.

These effects could be reversed by raising the $[Ca^{2+}]$. Winegrad postulated that the muscle was made permeable by the low $[Ca^{2+}]$ and $[Mg^{2+}]$ present in the EDTA solution. This technique was also used by two groups (Mrwa, Archtig, & Ruegg, 1974; Baguet & Marchand-Dumont, 1975) to permeabilize smooth muscle. Both these groups found that, following this treatment, the muscle was as sensitive to calcium as a muscle treated with glycerol. However, they both reported that the muscle showed slower rates of contraction and relaxation than the intact muscle. Baguet and Marchant-Dumont (1975)

reported that the anterior byssus retractor muscle (ABRM) took twice as long to contract as a glycerol-treated muscle and six times longer to relax. This group also reported that the muscle responded in a similar manner whether it is EDTA- or Triton-treated. However, the exposure to Triton X-100 (see later) lasted only 10-20s and might have been inadequate to ensure that the preparation had been permeabilized through its entire radius. In an effort to reduce the reversibility of the EDTA technique and to achieve a more profound permeabilization, McClellan and Winegrad (1978) used EGTA (3mM) to reduce the $[Ca^{2+}]$ and incubated rat cardiac muscle for 12-18 hours at 0-4°C. Rat cardiac muscle treated in this manner was still responsive to calcium. However, this responsiveness was increased further after the muscle was treated with the non-ionic detergent Triton X-100. McClellan and Winegrad (1978) explained this discrepancy as a result of the removal of membrane bound enzymes by Triton X-100 and indeed when the phosphodiesterase inhibitor theophylline was added the calcium sensitivity of the muscle was similar to that of the Triton X-100 permeabilized preparation. However, the effect that theophylline would be having directly on the contractile proteins was not explored.

There is much evidence for the ineffectiveness of the EGTA-skinning technique for cardiac (Miller, 1979; Kentish & Jewell, 1984; Miller, Elder & Smith, 1985; Miller & Smith, 1985) and smooth muscle (Cornelius 1980). Using ultrastructural and microanalytical techniques, Miller *et al* (1985) showed in rat trabeculae that La and LaEGTA could gain entry to muscles which had been permeabilized with Triton X-100 and Saponin but not EGTA permeabilized

muscle. Kentish and Jewell (1984) and Miller and Smith (1985) reported that EGTA-treated and Triton X-100-treated preparations had different response times to calcium and responded very differently when ATP and CrP were removed. Both sets of authors reported that EGTA-treated preparations had a lower apparent diffusion coefficient for calcium than Triton X-100-treated preparations. In addition, Miller and Smith (1985) reported that EGTA-treatment was not dependent on a low extracellular [Ca^{2+}]. That is, the muscle performed in the same manner regardless of whether the disruption solution had a low [Ca^{2+}] or not. There is evidence that it is ATP which causes the permeabilization (Gomperts, 1983). Tatham and Lindau (1990) have shown that it is the ATP^{4-} form of ATP which causes the formation of the pores in mast cells. After exposure to ATP^{4-} at a concentration as low as 4μM ethidium bromide and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene can gain access to the intracellular medium.

Another chemical skinning technique which has been developed involves the use of agents that insert into the membrane and disrupt the structure. The two main methods used are: (1) the non-ionic detergent Triton X-100 and (2) Saponins. Triton X-100 dissolves all membranes including the internal membranes (mitochondria and sarcoplasmic reticulum). This essentially leaves only the contractile proteins and collagen-elastic networks. Triton X-100 was first used to skin smooth muscle by Gordon (1978). It has since been used to permeabilize many types of smooth muscle quite successfully. An advantage of

this technique is that the tension produced by the muscle is equal to that by the intact muscle which is not the case in glycerol or EDTA-treated muscle.

Saponins are alkaloids some of which are extracted from plants. The saponin available commercially (Sigma and BDH) is derived from the sea cucumber *Actinopyga agassiza*, another which is widely used is derived from the alpha-pha plant. Ohtsuki *et al* (1978) reported that saponin was able to form pores in red blood cell ghosts. Saponin acts on cholesterol and, at certain concentrations, will selectively remove the cholesterol in the sarcolemma and not affect the intracellular membranes. This is believed to be due to the fact that these internal membranes contain less cholesterol than the sarcolemma (Martonosi, 1968; Waku, Uda & Nakazawa, 1971). This method was applied to smooth muscle almost simultaneously by Endo et al (1977) and Saida and Nonomura (1978). Both these groups of workers showed that the maximum calcium-activated force produced was equal to the maximum tension produced by a potassium contracture in the intact muscle. Endo *et al* (1977) also showed that caffeine could release calcium from the intracellular store indicating that the treatment had left the sarcoplasmic reticulum intact and functional. Although the internal stores of calcium are left intact after this method of permeabilizing they cannot be stimulated by activating the surface membrane bound receptors (Itoh, Kuriyama & Suzuki, 1983).

These two methods of permeabilizing (Triton X-100 and saponin) have particular problems when used in smooth muscle which do not apply to skeletal and cardiac muscle. Many workers in the field have reported that

calcium-activated force in permeabilized muscle deteriorates with time (Endo *et al* , 1977; Saida & Nonomura, 1978; Gordon, 1978; Ruegg & Paul, 1982; Itoh, Kanmura & Kuriyama, 1986). Ruegg and Paul (1982) reported results obtained with Triton X-100-treated porcine carotid artery. The inclusion of 4μM calmodulin prevented the decline in calcium-activated force. Itoh *et al* (1986) presented similar data from saponin permeabilized mesenteric artery. Contractions induced by repetitive application of 10μM [Ca^{2+}], declined to 78% of the first contraction by the third. This was accompanied by a depression in calcium sensitivity. This decline could be prevented by including 0.1μM calmodulin in the mock intracellular bathing medium. Kossman, Furst and Small (1987) after using biochemical and immunohistochemical analysis of both saponin and Triton X-100 permeabilized taenia coli found changes in the protein content of the muscle and the distribution of the protein within the muscle. Around 50% of myosin and filamin were lost at the permeabilizing stage for both detergents and 30% of actin after Triton X-100 and 15% after saponin skinning. Subsequent cycles of contraction and relaxation resulted in accumulated loss, notably of myosin and filamin, so that after the third contraction as little as 20% and 40% respectively of the original complement of these proteins remained in the muscle strips. These changes in protein composition were accompanied by a drastic redistribution of the proteins in the muscle cells, most notably of myosin.

Another method of permeabilizing, first reported in 1979 by Cassidy, Hoar and Kerrick, has recently become more widely used (Nishimura *et al*; 1988;

Kitazawa *et al*; 1989). This involves alpha-toxin, a transmembrane pore-making exoprotein produced by *Staphylococcus aureus*. Alpha-toxin (Mol. wt. 33 000) binds to cell surfaces and forms hexamers with other toxin molecules which insert into the plasma membrane to form pores of 2-3 nm diameter. The limited pore size allows equilibration of the cytoplasm with inorganic ions and small molecules such as ATP and EGTA but prevents the passage of proteins (including alpha-toxin itself) into and out of the cell. This technique has the advantage over the other techniques that the membrane bound receptors remain intact. This has allowed these workers to investigate the second messenger and G-protein transduction mechanisms.

The aim of the study undertaken in this chapter was to contrast and compare treatment with (1) saponin; (2) EGTA and (3) alpha-toxin as methods for permeabilizing the sarcolemma of rat anococcygeus muscle strips.

MATERIALS AND METHODS

PREPARATION

The preparation used throughout was the anococcygeus muscle of the rat. This was harvested and mounted on the tension measurement system as described in Section B (Permeabilized Muscle) of Chapter 2.

SOLUTIONS

The composition of the solutions used and the method used to prepare them are detailed in Section B Chapter 2. The solution composition is given in Table 2.3. Ionic strength was altered by varying the amount of KCl in the solution.

CALMODULIN

It has already been stated in the introduction that it is essential to have calmodulin present in the bathing medium of saponin-treated preparations to obtain reproducible contractions in response to calcium. Calmodulin is, however, very expensive from commercial sources. For this reason the calmodulin used in the experiments detailed in the results section was made at the National Institute of Medical Research, Mill Hill, London under the tutelage of Dr. Katalin Torok.

Calmodulin is extracted in two stages, first all proteins are extracted and secondly all proteins that bind calcium are extracted. The protein is extracted from bovine brain by first homogenizing the brain. The homogenate is then

centrifuged (1400 r.p.m. for 20mins at 4°C). The supernatant is then mixed with cellulose gel. Cellulose binds any proteins which are in the supernatant. Calmodulin and like proteins are removed from the cellulose by running a salt gradient, with a pH of 5.9, through the cellulose which has now been packed into a column. The effluent from the column is collected by a fraction collector. The fractions which contained protein (this was detected by a dual wavelength spectrophotometer scanning at 265nm and 280nm) were then mixed with phenylsepharose which had 0.5mM calcium added to it. This mixture, of fractions, phenylsepharose and calcium, is then added to a column. Any calmodulin present in the fractions from the cellulose column will bind to the calcium within the phenylsepharose. To remove the calmodulin an EGTA containing solution is passed down the phenylsepharose column. EGTA is able to remove calmodulin from the column because of its ability to bind calcium. Again, fractions are collected by a fraction collector and the protein content measure by a dual wavelength spectrophotometer set at 265nm and 280nm. The fractions containing protein are then freeze dried until they become a fine powder.

An assay for calmodulin is used at all the stages of the extraction. Also, at the end of the extraction procedure the protein that is suspected to be calmodulin is tested against a protein known to be calmodulin using gel electrophoresis.

PERMEABILIZATION TECHNIQUES

(1) Saponin: the mounted strip of muscle was exposed in '0.2 relaxing' solution to 50μg/ml saponin for 30 minutes and then washed several times in '0.2 relaxing' solution.

(2) EGTA: the muscle strips were bathed in the following solution for 4 hours at a temperature of 4^{o}C. The solution contained (mM): K, 130; Na, 40; ATP, 5; EGTA, 10 and HEPES, 25. The concentrations of free [Ca^{2+}] and [Mg^{2+}] resulting from contamination was 1nM and 1.2μM respectively (Smith, 1985). The solution had a pH of 7.0. The EGTA-treatment was similar to a procedure developed for cardiac muscle by McClellan and Winegrad (1978). After treatment the muscle was mounted and further treated as described in Chapter 2, Section B.

(3) Toxin: Figure 3.1A shows an example of the tension response during the protocol used to permeabilize the mounted strips of rat anococcygeus muscle. The strip of anococcygeus was initially exposed to 30μM noradrenaline while in Tyrode's solution. This caused a contraction in the muscle which was maintained until the noradrenaline was removed. The preparation was then placed in a mock intracellular solution (63μM Ca^{2+}) which caused a transient contraction. After 10 minutes in this solution, alpha-toxin from *Staphylococcus aureus* (2mg/ml) was added. This caused a slow rise in tension which eventually reached a plateau. Tension presumably rose as a result of the toxin forming

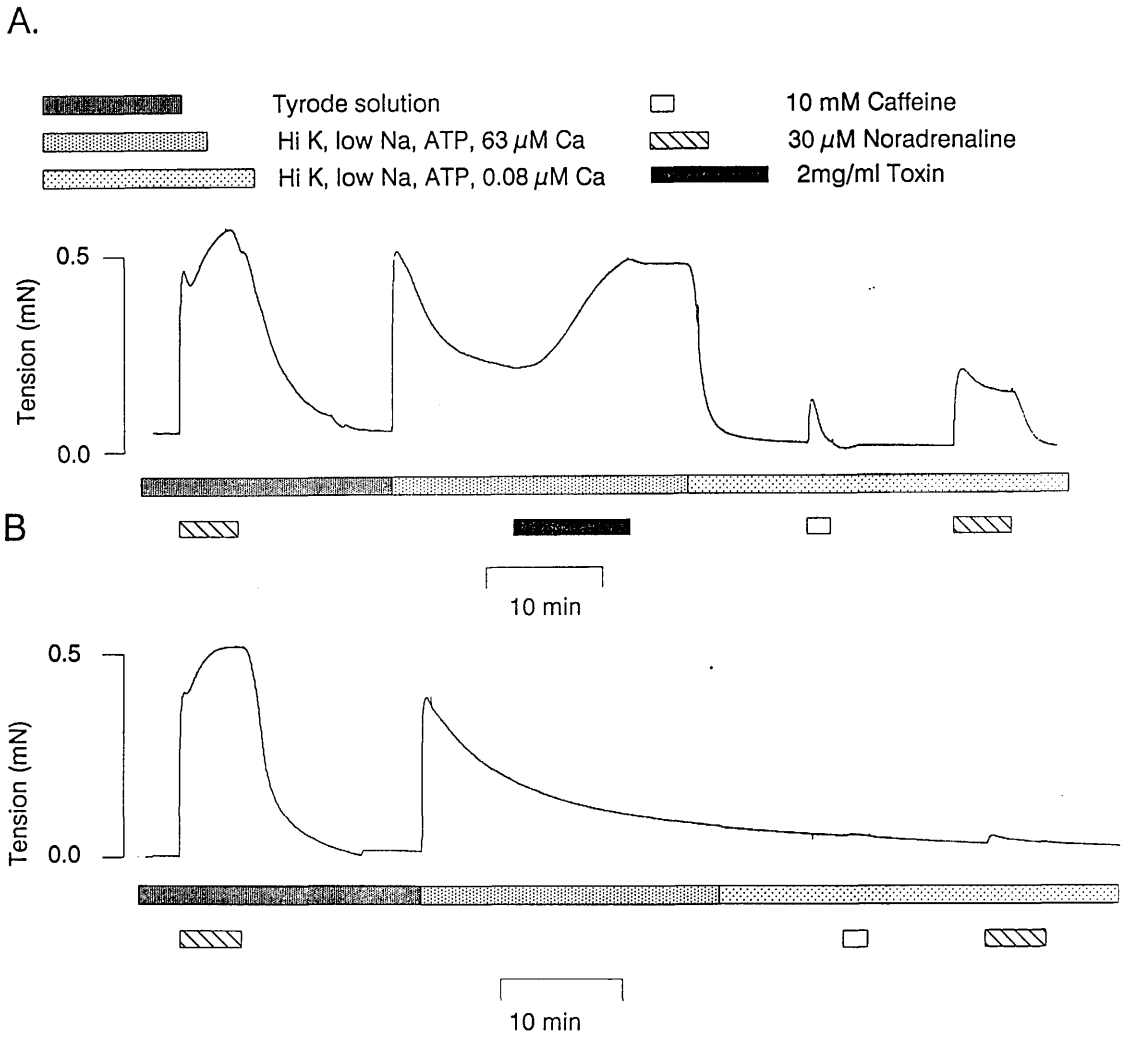


Figure 3.1 shows the solution protocol used to toxin-permeabilize rat anococcygeus muscle. The solution changes are indicated by the hatched bars below each tension record. In Panel A the solution changes include the addition of crude alpha-toxin (2mg/ml) and in Panel B alpha-toxin was omitted from the bathing medium.

pores in the surface membrane enabling calcium to gain access to the contractile proteins. Once tension had reached a plateau the toxin was removed. This produced little change in tension. On the other hand, lowering the $[Ca^{2+}]$ caused a rapid relaxation. Subsequent exposure of the muscle to caffeine or noradrenaline caused a transient contraction. Panel B of Figure 3.1 shows the response of another strip of anococcygeus muscle subjected to a similar protocol, but without the toxin. Under these circumstances lowering $[Ca^{2+}]$ had little effect, and neither caffeine nor noradrenaline caused a significant response compared with that illustrated in panel A.

RESULTS

IS THE MUSCLE PERMEABILIZED?

Reuben and Wood (1979) listed some criteria to help determine whether a muscle was permeabilized. These were:

(1) that development of rigor and relaxation from rigor should occur within seconds of MgATP removal or addition;

(2) the responses of chemically skinned fibres to variations in low MgATP concentrations should be identical to those of mechanically skinned fibres; and

(3) the responses of chemically skinned fibres measured in (1) or (2) should not be quantitatively affected either by mechanical removal of the sarcolemma or by treatment of the preparation with non-ionic detergents that destroy the sarcolemma.

Bearing the first of these three criteria in mind, I examined the effect of removing ATP and CrP from maximally calcium-activated muscles. This was carried out on up to six muscles which had previously been treated in the following way: (A) exposed to '0.2 relaxing' solution without any agents to cause permeabilization, hence the sarcolemma was intact in these muscle strips; (B) saponin-treated; (C) EGTA-treated and (D) alpha-toxin-treated. As shown in Figure 3.2, raising the [Ca^{2+}] from 0.08μM to 63μM caused a maintained contraction in all four types of treated muscle, 63μM Ca^{2+} produces a maximal contraction to calcium. This is demonstrated for alpha-toxin-treated rat anococcygeus in Figure 4.8. Removing ATP and CrP produced a profound

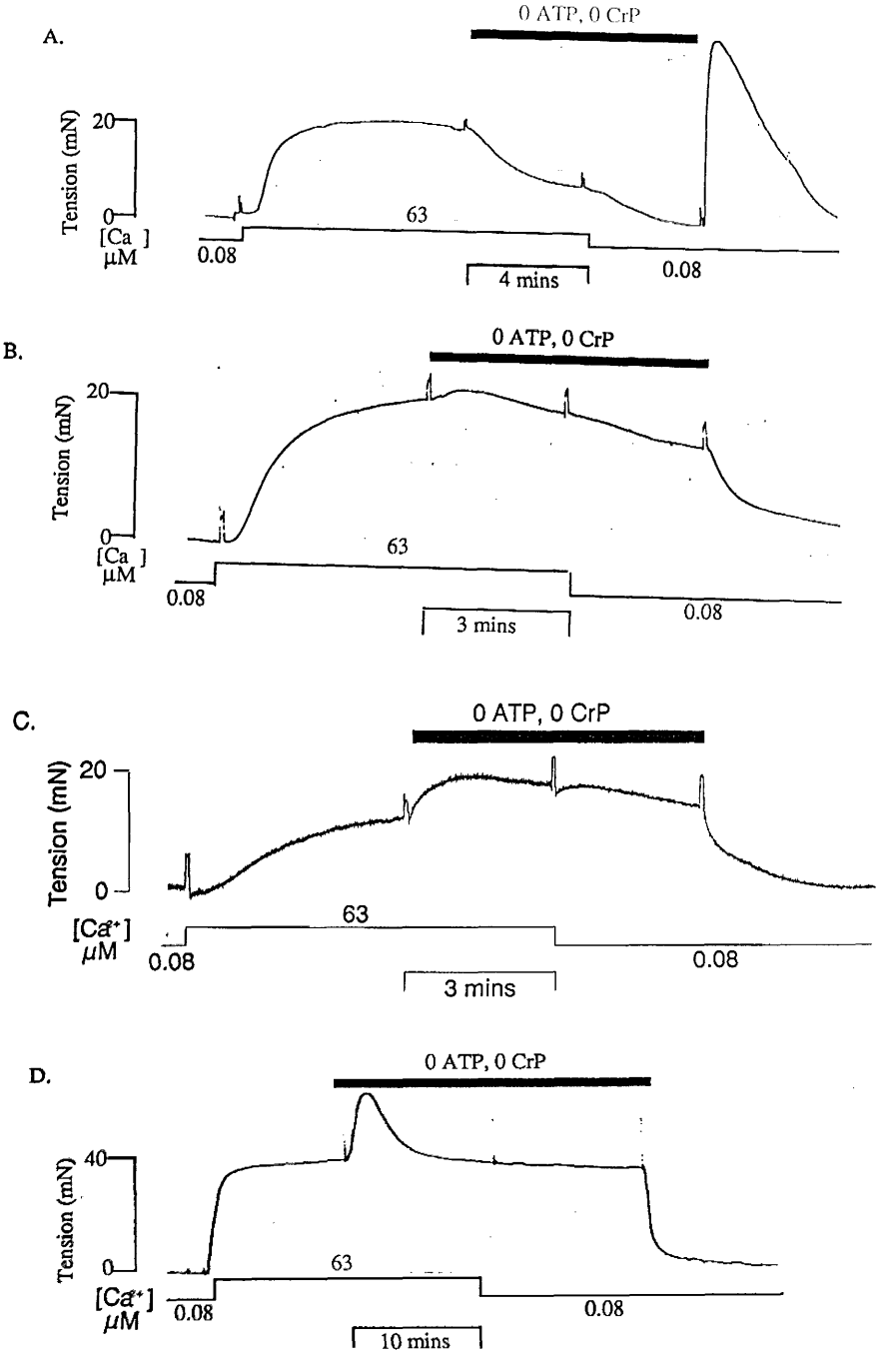


Figure 3.2 shows the effect of removing ATP and CrP (black bar) on maximum calcium-activated force (10mM-total EGTA) in A. intact depolarised muscle; B. saponin-treated muscle; C. EGTA-treated muscle and D. alpha toxin-treated muscle. Changes in $[Ca^{2+}]$ are shown by the step lines under the trace.

relaxation in the depolarised intact muscle (A). However, it caused a further contraction in the other three types of treated muscle. This further contraction was not maintained in any of the treated muscles. The muscles contracted transiently and then relaxed back to the previous tension level. This type of response has also been reported in saponin-treated preparations (Somlyo *et al*, 1988; Itoh *et al*, 1986). The authors suggest that the tension produced in the absence of ATP and CrP is due to attachment of rigor crossbridges. When the $[Ca^{2+}]$ was lowered in the rigor solution the treated muscle did not relax. The muscles only relaxed when they were bathed in a solution containing ATP and CrP and a low $[Ca^{2+}]$. The relaxation upon removal of ATP and CrP observed in the intact depolarised muscle has been observed by other workers and is thought to be the result of removing the purinoreceptor activation of these preparations (Gillespie, 1972; Burnstock et al, 1978).

The success of the various 'skinning' techniques was further examined by measuring the responses of muscles to stimuli which are known to have a direct effect on the contractile proteins. The stimuli chosen were 100μM cAMP and 10mM P_i. Both these compounds are known to suppress force in muscles which have either been skinned using Triton X-100 (Meisheri & Ruegg, 1983; Pfitzer *et al*, 1984; Ruegg & Pfitzer, 1985; Schneider, Sparrow &Ruegg; 1981; Gagelman & Guth; 1987) or saponin (Itoh *et al*, 1981; Itoh, Kanmura & Kuriyama, 1986). Four representative traces (one from up to six examples for each treatment) are shown in Figure 3.3 from (A) an intact muscle, i.e. a muscle which has received no treatment, (B) a saponin-treated muscle,

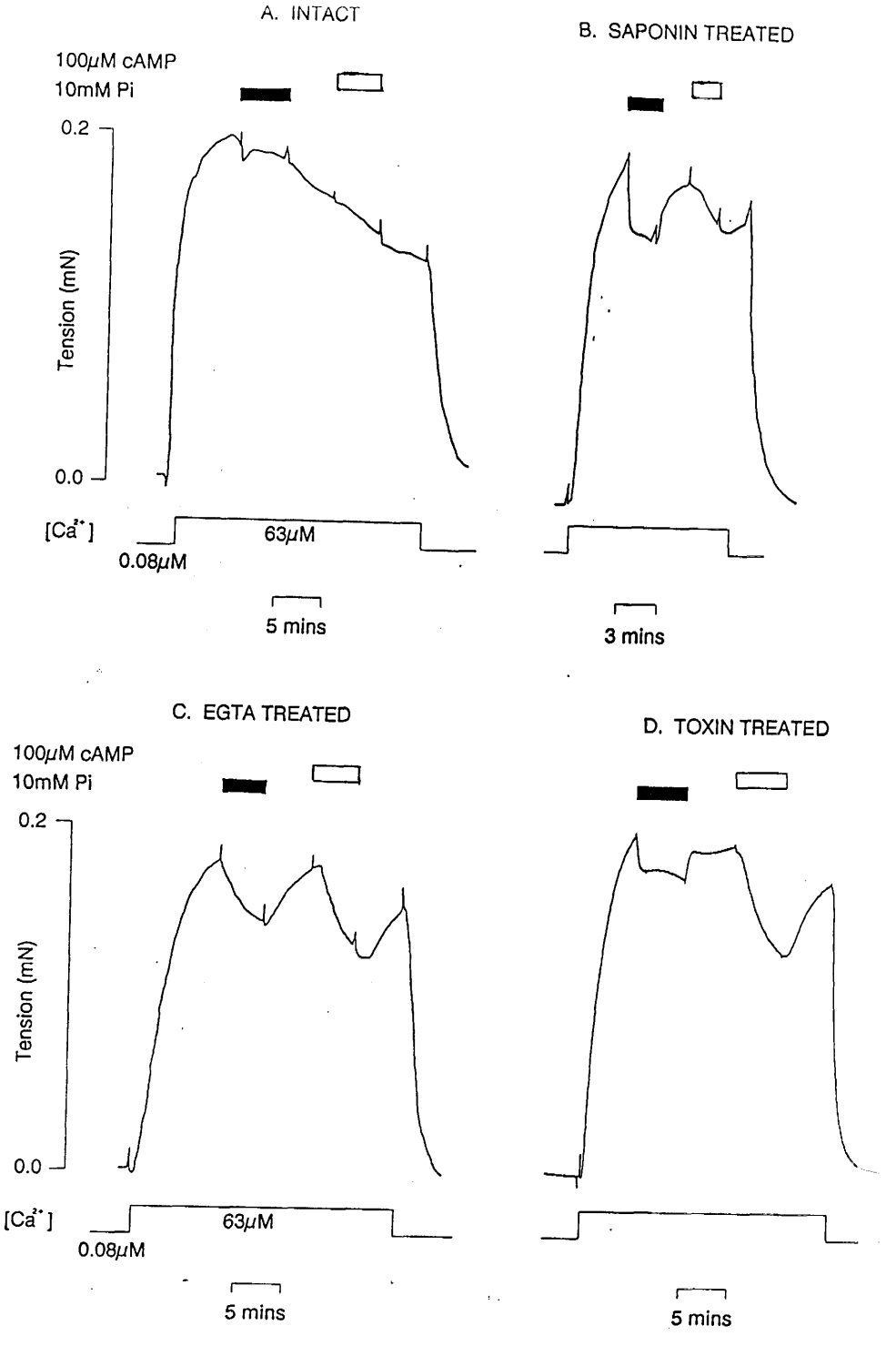


Figure 3.3 shows the effect of 100μM cAMP (unfilled bar) and 10mM phosphate (filled bar) on maximum calcium-activated force (10mM-total EGTA) in A. intact depolarised muscle; B. saponin-treated muscle; C. EGTA-treated muscle and D. alpha toxin-treated muscle. Changes in $[Ca^{2+}]$ are shown by the step lines under the trace.

(C) EGTA-treated muscle and (D) an alpha-toxin-treated muscle. The muscles were first maximally calcium-activated and all four muscles contracted when [Ca^{2+}] was raised. The calcium-channel blocker nifedipine (10μM) inhibited the calcium-activated tension developed by the intact preparation, but had no effect on either saponin- or EGTA-treated preparations (results not shown). Both 100μM cAMP and 10mM P_i cause a fall in tension in all the treated muscles, however, they had no effect in the intact depolarised muscle. The rate of relaxation in response to 100μM cAMP was similar in all the muscles, but the rate of relaxation observed with 10mM P_i was slower in EGTA-treated muscle than in the other two types of treated muscle.

A common method to check if the muscle is completely permeabilized, based on an observation made in saponin-skinned smooth muscle, is to examine whether the muscle is still responsive to agonist stimulation. Itoh, Kuriyama and Suzuki (1983) reported that fully permeabilized rabbit mesenteric artery was no longer able to respond to noradrenaline. Figure 3.4 shows how the different muscles respond to noradrenaline after they have been treated. Only the saponin-treated muscle failed to respond to noradrenaline. The response to noradrenaline seen in EGTA- and toxin-treated muscle was similar to that of the intact muscle and suggests that these treatments leave receptors functional as has been reported by Nishimura *et al* (1988) and Kitazawa *et al* (1989) for alpha-toxin permeabilized vascular smooth muscle.

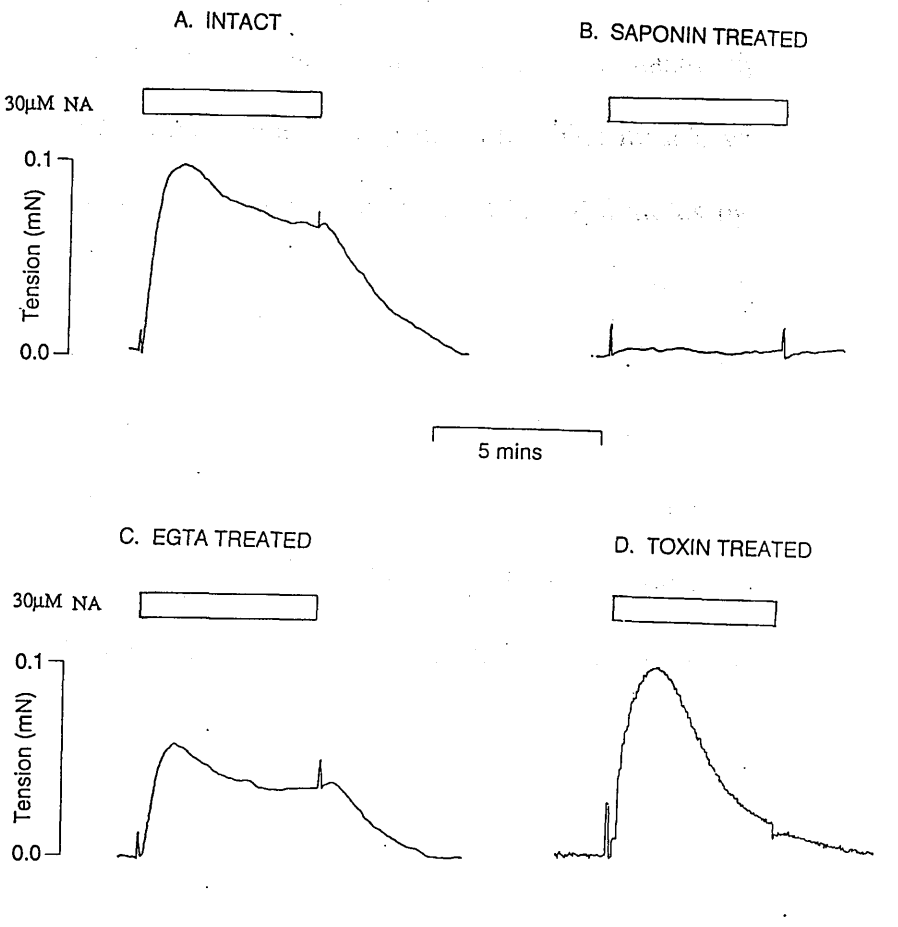


Figure 3.4 shows the response to 30μM noradrenaline (NA, unfilled bar) in the presence of 0.08μM calcium (0.2mM-total EGTA) in A. intact depolarised muscle; B. saponin-treated muscle; C. EGTA-treated muscle and D. alpha-toxin-treated muscle.

AN EXAMINATION OF THE REDUCTION IN MAXIMUM CALCIUM ACTIVATED-FORCE IN SAPONIN-, EGTA- AND ALPHA-TOXIN-TREATED SMOOTH MUSCLE

This part of the study was undertaken to examine and compare the decline in the amplitude of maximum calcium-activated force (Tmax) in rat anococcygeus. An attempt was also made to prevent this decline in saponin- and alpha-toxin-treated muscle. These experiments were all carried out in the same manner: after the muscle had been treated, it was maximally activated by raising the $[Ca^{2+}]$ (63μM) in the bathing medium. The muscle was induced to contract for 10 minutes and then it was relaxed for 10 minutes by lowering the $[Ca^{2+}]$ in the bathing medium. This was repeated either 6 or 10 times. The individual experiments were all carried out on up to 6 different muscle strips. Figure 3.5A shows a series of maximum calcium-activated contractions in an alpha-toxin permeabilized smooth muscle. The $[Ca^{2+}]$ was changed as indicated below the tension record. It can be seen that the time course of the calcium-activated contractions changes as the contraction amplitude decays. This is best exemplified when the contractions are superimposed (Figure 3.5B). This effect was also seen in saponin-permeabilized muscle (data not shown). The change in time course is unlikely to be due to a reduced calcium sensitivity because raising the $[Ca^{2+}]$ above 63μM, after the amplitude of the contractions evoked by calcium had declined, did not produce any more tension.

Figure 3.6 shows the decline in the amplitude of Tmax after the three different permeabilization techniques. The decline is greatest in saponin-treated muscle. Tension has fallen to a mean value of less than 20% by the 6th contraction. Tmax also declines in alpha-toxin-treated muscle, but not to the

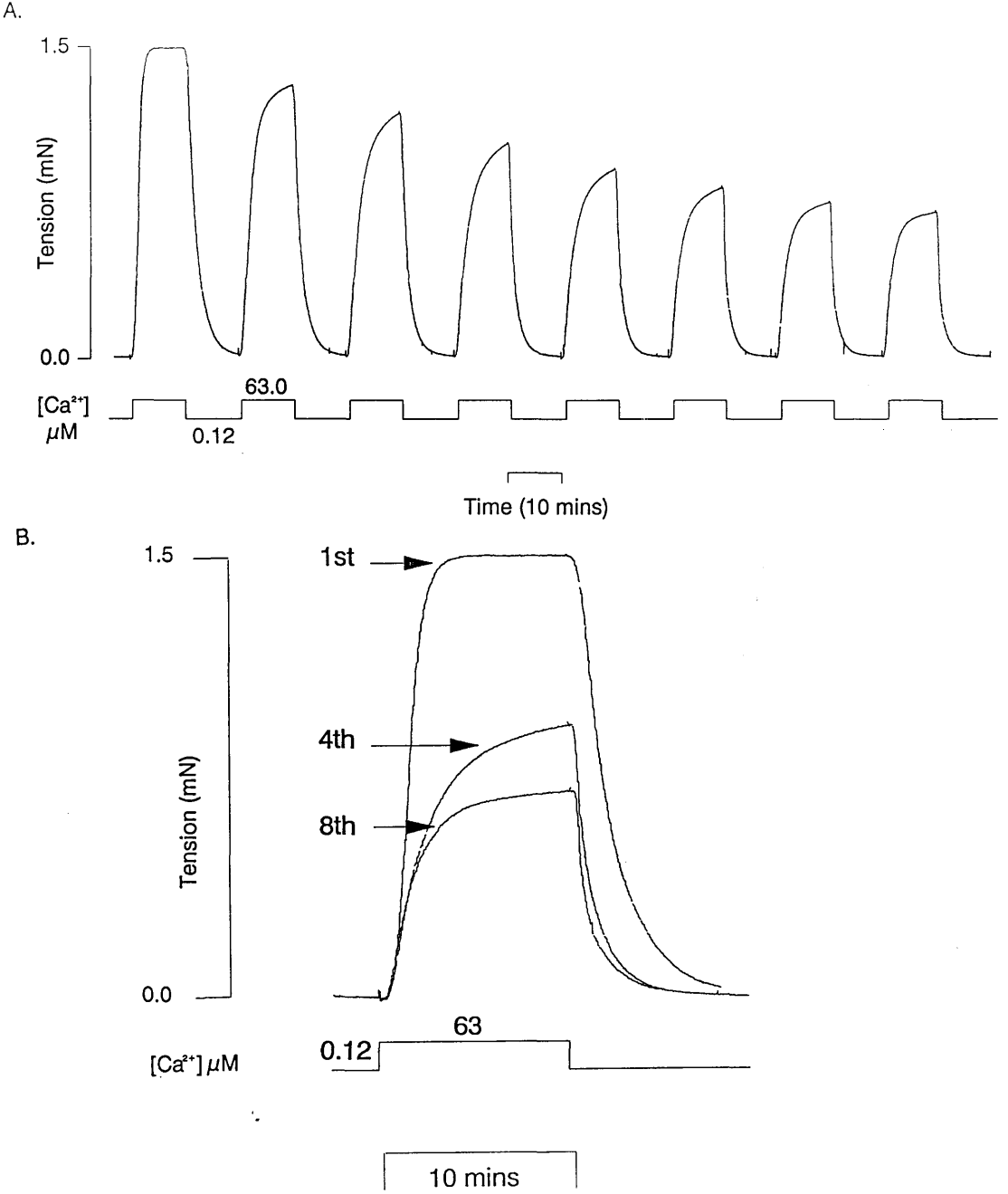


Figure 3.5A shows the decline in the amplitude of maximum calcium-activated force upon repetitive application of calcium in an alpha-toxin permeabilized rat anococcygeus muscle strip. The changes in $[Ca^{2+}]$ are shown as steps underneath the tension record. Figure 3.5B shows three sample tension responses to repetitive exposure to calcium (shown by the step line below the traces) from the same muscle as used in Panel A. 1st, 4th and 8th indicate the number of the contraction in the repeat protocol.

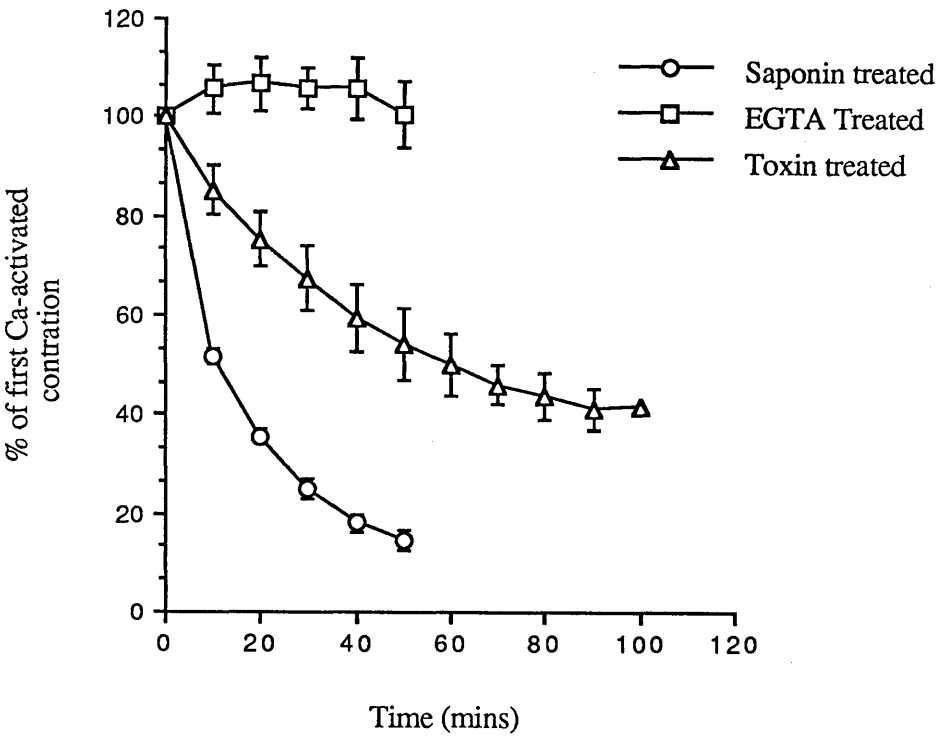


Figure 3.6 shows the decline in maximum calcium-activated force expressed as a percentage of the first maximum calcium-activated contraction for saponin-treated (open circle), EGTA-treated (open square) and alpha-toxin-treated (open triangles) muscle strips. (mean±S.D. n=3-6).

same extent as in saponin-treated muscle. Tension falls to approximately 50% by the 5th contraction and even by the 10th has not fallen below 40%. EGTA-treated muscle shows no reduction in Tmax. However, the mean maximum tension produced by calcium in EGTA-treated muscle was only a third of that produced by saponin-treated muscle. Figure 3.7 shows the individual tensions produced by the first contraction in response to calcium in the three different treated muscles. EGTA-treated muscle produce less tension than either saponin or alpha toxin-treated muscle and saponin-treated muscle produces less than alpha-toxin-treated muscle. In preparing the EGTA-treated muscles the success rate was very low - only one in three muscle strips were responsive enough to calcium to allow further experimentation.

Further experiments were carried out to study the characteristics of the decline in Tmax. The decline might be a function of the number of contractions, or simply the time after saponin-treatment. In order to distinguish between these two possibilities a saponin-treated muscle was activated, allowed to relax, then activated again one hour later. The reduction in the size of the contraction was compared with that in the preparations which had been repeatedly contracted and relaxed for one hour. The results shown in Figure 3.8 indicate that the fall in the size of the contracture was independent of whether the muscle had been activated during this period. This result is consistent with the hypothesis that the fall in force is due to the steady loss of a factor from the preparation with time rather than some accumulating damage as a result of repeated cycles of activation and relaxation.

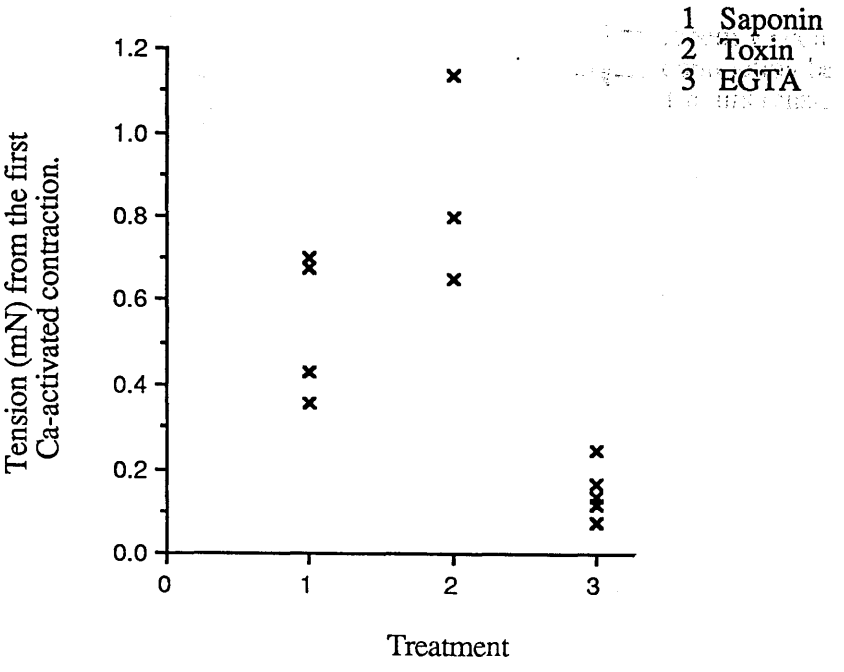


Figure 3.7 shows the peak tension (mN) produced by the initial maximum calcium-activated contraction in saponin-treated, EGTA-treated and alpha-toxin-treated muscle strips.

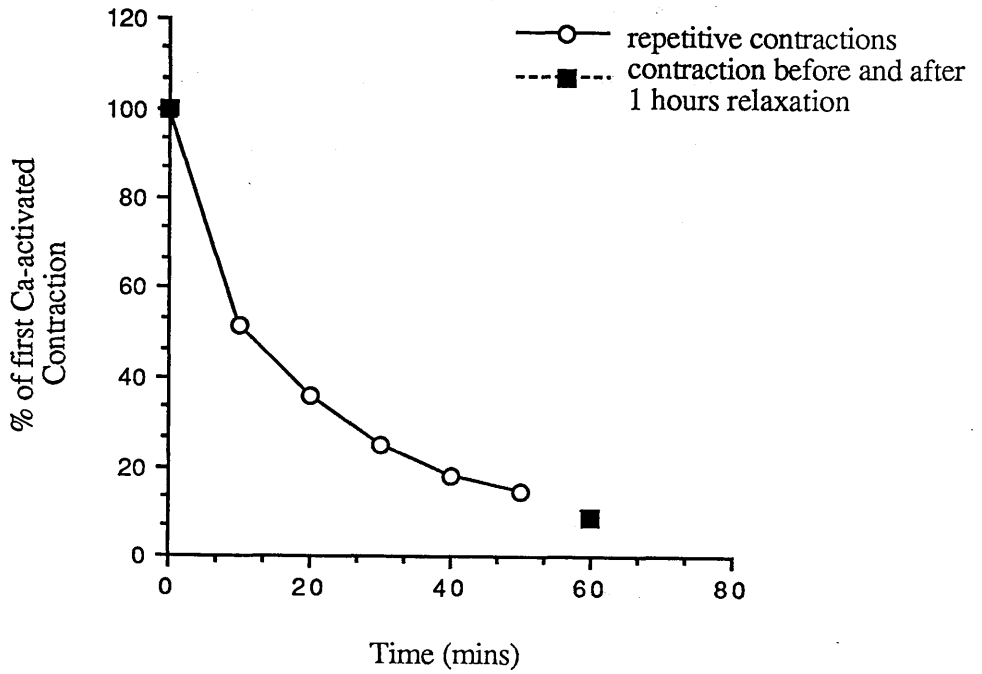


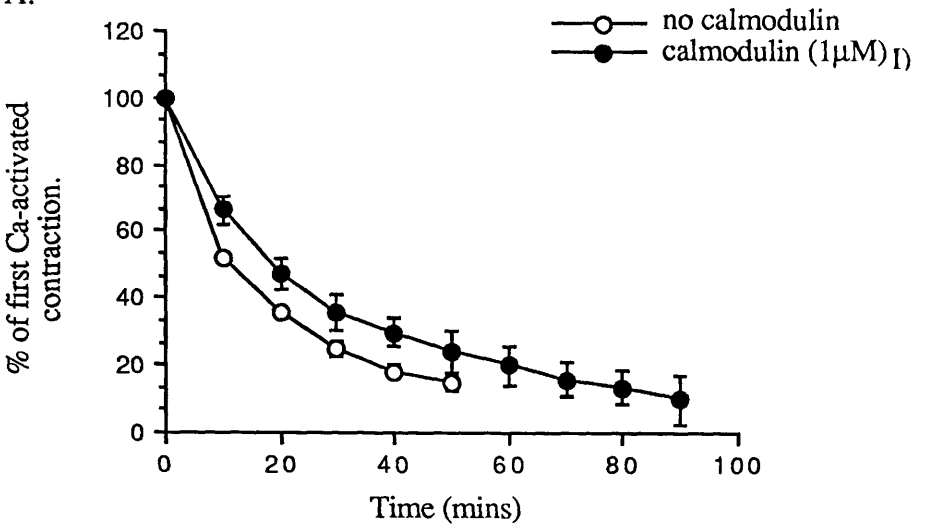
Figure 3.8 compares the decline of maximum calcium-activated force in saponin-treated preparations during repetitive contractions in response to calcium (open circles) or which have contracted once, allowed to relax and then contracted again (filled squares) one hour later. (mean±S.D. n=3-6)

As mentioned in the introduction to this chapter, the cytosolic protein calmodulin had been reported to enhance calcium-activated force and its loss from the preparation might be responsible for the decay in calcium-activated force. The decline in Tmax in saponin-treated rat anococcygeus was, therefore, examined in the presence of 1μM calmodulin. Figure 3.9A shows that calmodulin only had a small effect on the decline. These experiments were carried out at an ionic strength of 0.2M. Since calcium's affinity for calmodulin is increased at lower ionic strengths (Wolff *et al*, 1977) the next set of experiments was carried at an ionic strength of 0.07M. The results in figure 3.9B show that this procedure slows the decline, but does not prevent it. If calmodulin (1μM) (Figure 3.9C) is present in the solutions with a lower ionic strength, the decline is still slower. Other interventions were tried which failed to have any effect on the decline. These included experiments using solutions with (1) a pH of 6.8 or 7.4 to examine the pH used by other workers and (2) a higher ionic strength - (0.27M) to examine whether this had the opposite effect to low ionic strength.

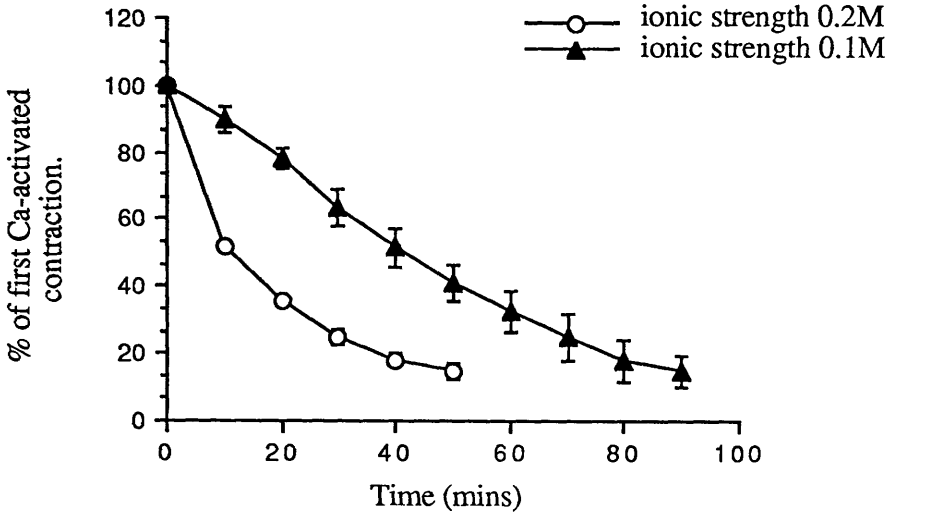
The decline in the amplitude of Tmax toxin-treated muscle was less than that in saponin-treated muscle. The decline in alpha-toxin-treated muscle was prevented at an ionic strength of 0.07M. This is shown in figure 3.10.

Figure 3.9 A, B & C show the decline in maximum calcium-activated force expressed as a percentage of the first maximum calcium-activated contraction in saponin-treated muscle. Panel A shows the decline in muscles bathed in solutions with (closed circles) and without (open circles) calmodulin (1μM) present in the bathing solution (mean±S.D. n=3-6). Panel B shows the decline in muscles bathed in solutions with an ionic strength of 0.2M (open circles) and 0.07M (closed triangles) (mean±S.D. n=3-6. Panel C shows the decline in muscles bathed in solutions with (i) an ionic strength of 0.07M which contain calmodulin (1μM)(closed squares) or (ii) an ionic strength of 0.2M which do not contain calmodulin (1μM) (open circles) (means±S.D. n=3-6).

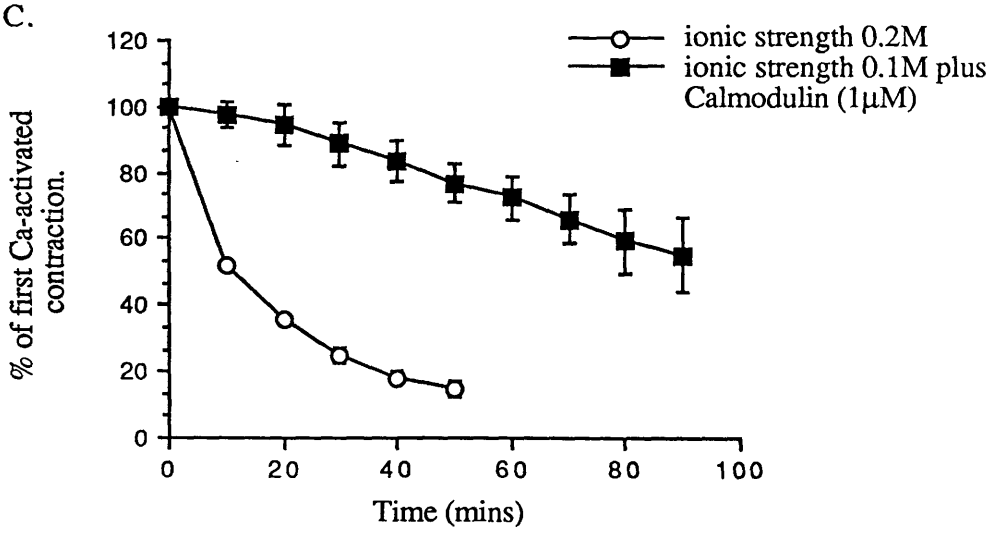
A.


no calmodulin
calmodulin (1μM)
% of first Ca-activated contraction.
120
100
80
60
40
20
0
0
20
40
60
80
100
Time (mins)

B.


ionic strength 0.2M
ionic strength 0.1M
% of first Ca-activated contraction.
120
100
80
60
40
20
0
0
20
40
60
80
100
Time (mins)

C.


ionic strength 0.2M
ionic strength 0.1M plus Calmodulin (1μM)
% of first Ca-activated contraction.
120
100
80
60
40
20
0
0
20
40
60
80
100
Time (mins)

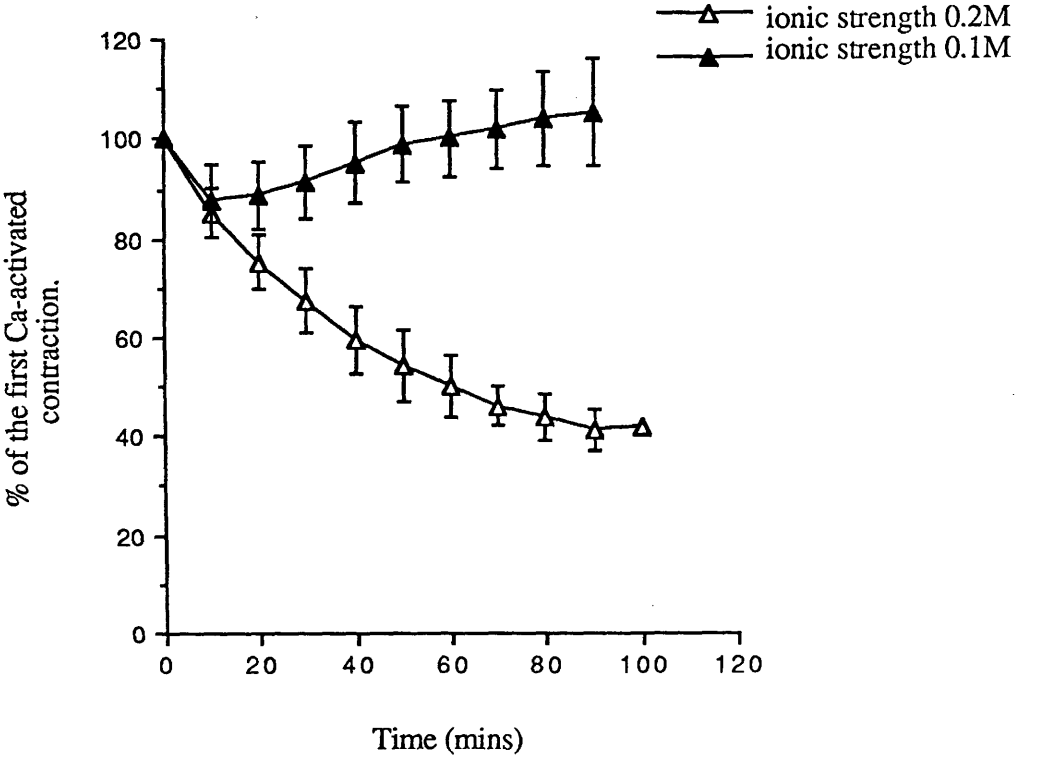


Figure 3.10 shows the decline in maximum calcium-activated force expressed as a percentage of the first maximum calcium-activated contraction in alpha-toxin-treated muscle bathed in solutions with an ionic strength of 0.2M (open triangles) and an ionic strength of 0.07M (closed triangles). (mean±S.D., n=3-6).

The size of the initial maximum calcium-activated contraction altered as the conditions used to examine the repetitive calcium-activated force were changed. These are summarised in Figure 3.11. Mean tension produced at low ionic strength (0.07M) was higher than that produced at the higher ionic strength (0.2M) (0.5mN to 0.8mN). Calmodulin increased this further to 1.0mN. Toxin-treated muscle had a mean tension of 0.8mN and this was increased to 1.0mN at an ionic strength of 0.07M.

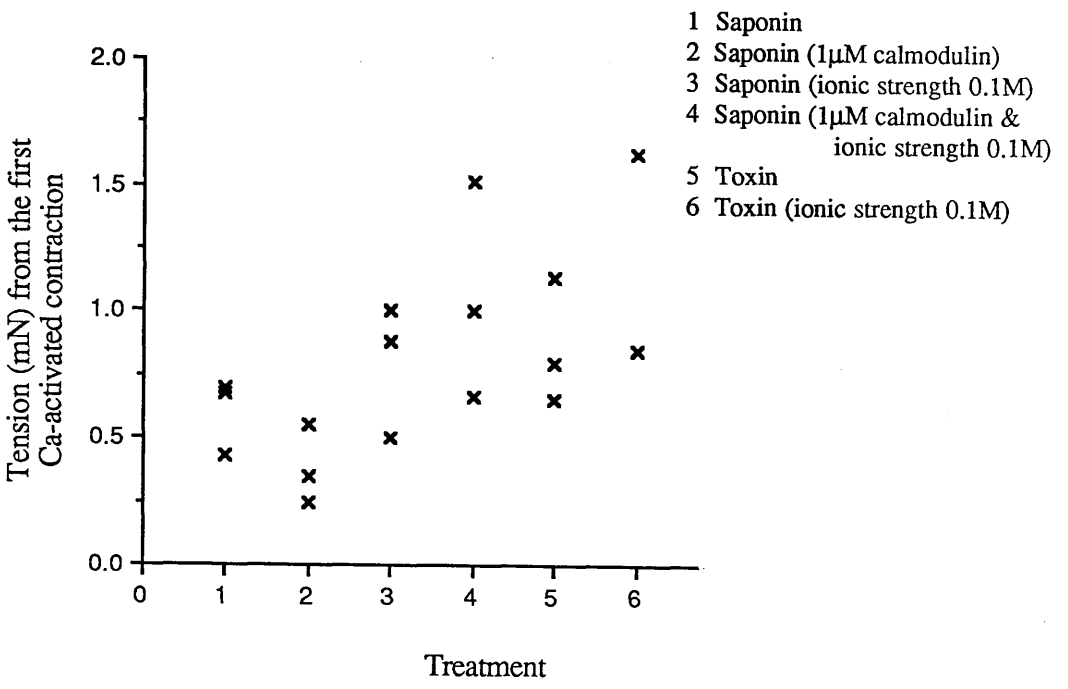


Figure 3.11 shows the peak tension (mN) produced by the initial maximum calcium-activated contraction after saponin-treatment (1-4) and alpha-toxin-treatment (5 & 6) with the following alterations to the skinning and activating solutions: (2) 1μM calmodulin; (3) ionic strength of 0.07M; (4) 1μM calmodulin and an ionic strength of 0.07M; and (6) ionic strength of 0.07M.

DISCUSSION

The aim of the work described in this section was to compare three potential permeabilization techniques and to decide which was best for further use. The first problem was to test whether the different techniques actually permeabilized the muscle. The effectiveness of the three permeabilizing techniques was established by comparing the responses from the permeabilized muscles with those from the intact muscle. Bathing the intact muscle in a mock intracellular solution depolarised the muscle. The muscle now contracted in response to extracellularly applied calcium. The 'treated' muscles also contracted in response to externally applied calcium. However, unlike the other muscles the intact muscle only contracted in response to a $[Ca^{2+}]$ which maximally contracted the treated muscles i.e. 63μM. This contraction could be blocked by application of 1μM Nifedipine which is known to block voltage operated calcium channels (Spedding, 1987). The results imply that the major route for calcium entry in intact preparations is via voltage operated calcium channels. This is in contrast to the maximum calcium-activated force produced by the other three permeabilizing techniques, which was not affected by nifedipine.

All three of the 'treated' muscles responded to removal of ATP and CrP in a manner which satisfies the criteria of Reuben and Woods' (1979) for a 'skinned' muscle. That is, they all contracted when ATP and CrP were removed from the bathing medium and remained contracted when the $[Ca^{2+}]$ was lowered in the rigor solutions. This indicated that the muscles had formed

rigor crossbridges. Again this was very different to the way in which the intact muscle responded; where removal of ATP and CrP caused relaxation and addition of ATP caused a transient contraction. The 'treated' muscles also responded very differently to cAMP and P_i from the intact depolarised muscle. Both agents are known to act on the contractile proteins and cause a reduction of maximum calcium-activated force. No relaxation was evoked in intact preparations, but did occur in the permeabilized muscles. The EGTA-treated muscle did, however, respond differently from the others, as shown in Figure 3.2, being slower to respond to P_i and calcium than the others. This might be explained if the apparent diffusion coefficient was lower after EGTA-treatment. Similar behaviour was reported in EGTA-treated cardiac muscle (Kentish & Jewell, 1984; Miller & Smith, 1985). The lower diffusion coefficient in EGTA-treated muscle to P_i might also explain why it is slower to respond to calcium and produced less tension than saponin- or toxin-treated preparations, the muscle may be accumulating P_i which will depress calcium-activated force.

Another method to determine the extent of permeabilization in saponin-treated muscle was to examine the response to noradrenaline. Itoh *et al* (1983) noted that once the muscle was completely permeabilized (i.e. the permeabilized muscle was producing the same degree of tension in response to Ca^{2+} as the intact muscle in response to K^+) that the response to noradrenaline was abolished. They used this as a measure of the degree of permeabilization. In the present study the response to noradrenaline was abolished only after saponin-treatment, but persisted after both EGTA- and

alpha-toxin-treatments. Nishimura *et al* (1988) and Kitazawa *et al* (1989) had both previously reported that alpha-toxin permeabilized vascular smooth muscle retains its ability to respond to noradrenaline. The present study is the first report that EGTA-treated muscle also retains functional membrane bound receptors.

The fact that alpha-toxin-treated anococcygeus smooth muscle retains functional membrane bound receptors is not evidence that the muscle is not permeabilized. Unlike the intact muscle, alpha-toxin-treated muscle can contract repeatedly upon noradrenaline exposure (e.g. Figure 4.5). The intact muscle cannot contract more than once, probably because the bathing $[Ca^{2+}]$ is too low to allow the intracellular stores to load. This experiment of evoking repeat contractions in response to noradrenaline was not attempted in EGTA-treated muscle. Hence it is more difficult to claim that the muscle is permeabilized even although it can respond to noradrenaline. However, the evidence from the ATP, cAMP and P_i experiments suggest that the muscle is permeabilized

It is not known why saponin-treated muscle loses its ability to respond to noradrenaline and other agonists. There are several possibilities:

(1) as saponin removes the cholesterol from the surface membrane this might dislodge the protein receptors or inactivate them;

(2) the second messengers produced by the agonists which promote the signal internally, for example $Ins(1,4,5)P_3$, diacylglycerol (DG) and cAMP, are produced from membrane-bound precursors which might be lost during saponin-treatment.

The different responses of these three types of 'treated' muscle to

noradrenaline could be connected with the differences in the rate and extent of the decline in Tmax. The larger the 'pores' produced by the permeability treatment, the greater the chance of a loss of receptor function and the more extensive the loss of essential cytosolic proteins and thus of calcium-activated force. The change in time course of Tmax might also be explained by the gradual loss of cytosolic enzymes and other proteins. The amplitude of Tmax declines more slowly in toxin-treated muscle and not at all in EGTA-treated muscle and toxin-treated muscle at low ionic strength. The decline in saponin-treated preparations is reduced by including calmodulin in the bathing media, indicating that it is being lost from the muscle during this permeabilizing technique. Employing conditions which improve the affinity of calmodulin for calcium (i.e. lowering ionic strength) has a similar beneficial effect. Kossman *et al* (1987) have already shown that saponin-treatment allows the loss of proteins from the muscle. The slower decline observed in toxin-treated muscle can probably be attributed to the fact that it produces smaller 'pores' in the membrane than saponin. This interpretation has been proposed by Thelestam and Mollby (1979) who examined 38 cytolytic agents, including saponin and alpha-toxin from *Staphylococcus aureus*. They attempted to classify the different agents on the basis of their effect upon the leakage pattern of three different labels; nucleotides, alpha-aminoisobutyric acid and RNA. Saponin was found to produce 'pores' in the membranes of human fibroblasts which allowed equal leakage of the high molecular weight RNA and the lower molecular weight nucleotides and alpha-aminoisobutyric acid. Alpha-toxin-treatment, on the other

hand, only resulted in the leakage of the lower molecular weight molecules (nucleotides and alpha-aminoisobutyric acid) and retained the higher molecular weight RNA. This would indicate that saponin produces larger 'pores' in the membrane than alpha-toxin. The 'pores' produced by alpha-toxin have been measured to be 2-3nm in diameter (Fussle *et al*, 1981; Ikigai & Nakae, 1987) which would allow the free diffusion of molecules with a molecular weight of less than 4000. Consequently, the toxin itself cannot enter and the contractile proteins and calmodulin cannot be lost (Hohman, 1988).

Using a lower ionic strength of 0.07M helped to minimise the decline in the amplitude of Tmax. This could indicate that an ionic strength of 0.2M was higher than the muscle's natural ionic strength or it might mean that some other factor which normally increases calmodulin's affinity for calcium has also been lost from toxin- and saponin-treated muscle. At low ionic strength (0.07M), toxin-treated muscle behaved like EGTA-treated muscle which might indicate that any such factor lost which naturally increases calmodulin's affinity for calcium is not lost from EGTA-treated muscle. Other factors obviously need to be restored to saponin-treated preparations to prevent completely the decline in the size of Tmax. Some possible candidates are myosin, actin, phosphatases and protein kinase C.

In conclusion, all three techniques appear to permeabilize muscle. However, they have different properties and suitability for further experimentation. These different properties arise because each technique produces different sized 'pores' in the surface membrane. Saponin appears to

produce the largest. This results in the loss of agonist-induced responses and a rapid decline in the amplitude of Tmax. The reduction can only be partially prevented by including the (expensive) protein calmodulin to the bathing solutions and using low ionic strength to favour its affinity for calcium. Toxin-treatment produces the next largest 'pores'. Such muscles retain their ability to respond to agonist stimulation and low ionic strength is sufficient to prevent the decline in the amplitude of Tmax. EGTA-treatment of muscle appears to produce the smallest 'pores' and also to retain its ability to respond to agonist stimulation, showing no decline in the amplitude of Tmax. However, EGTA-treated muscle only produces a third of the tension produced by equivalent saponin- or toxin-treated muscles. Overall, alpha-toxin-treated muscle was considered to be the most suitable permeabilization technique and was used throughout the rest of the study.

CHAPTER 4

THE EFFECT OF GTP ON NORADRENALINE- AND CALCIUM-ACTIVATED FORCE IN ALPHA-TOXIN PERMEABILIZED SMOOTH MUSCLE FROM GUINEA PIG PORTAL VEIN AND RAT ANOCOCCYGEUS.

INTRODUCTION

The involvement of guanosine triphosphate (GTP) in transmembrane signalling is a fairly recent discovery. It was in 1971 that Rodbell *et al* reported that GTP was required for hormonal activation of adenylyl cyclase. Shortly after this Harwood, Low and Rodbell (1973) showed that GTP in the presence of several hormones could both stimulate and inhibit adenylyl cyclase and that this effect could also be produced by Gpp(NH)p, a non-hydrolysable analogue of GTP. It was discovered that a protein was involved when Pfeuffer (Pfeuffer & Helmriech, 1975; Pfeuffer, 1977) using GTP-sepharose chromatography was able to partially resolve a protein fraction that conferred regulatory properties on adenylyl cyclase. At around the same time Ross and Gillman (1977, 1980) showed that a protein extracted from plasma membranes would confer guanine nucleotide-mediated regulation of adenylyl cyclase to membranes devoid of this activity. This protein has since been purified (Northup *et al*, 1980; Stenweis *et al*, 1981) and is termed the G-protein. Cassel and Selinger (Cassel & Selinger, 1977, 1978; Cassel, Levkowitz & Selinger, 1977) demonstrated that hormones working through adenylyl cyclase stimulate the exchange of GTP for GDP through a cholera toxin-sensitive GTPase. This has led to the confirmation that activation of adenylyl cyclase involved the concerted action of three proteins, the stimulatory hormone receptor, the catalytic protein and a G-protein. It was proposed that the activated agonist receptor complex forms a ternary complex with the G-protein (Kent, Delean & Lefkowitz, 1980; Stadel, Delean &

Lefkowitz, 1982) that results in the exchange of GTP for GDP. The activated G-protein then combines with the inactive enzyme to form the active enzyme species. The lifetime of this active complex is determined by the GTPase activity that is inherent in the G-protein. Conversion of GTP to GDP by the GTPase returns the active enzyme and G-protein to an inactive one (Cassel *et al*, 1977). That is, stimulatory hormones promote an 'on' reaction that involves the exchange of GTP for GDP, and GTPase activity catalyses an 'off' reaction. The marked activation of adenylyl cyclase afforded by stable analogues of GTP (Rodbell *et al*, 1971) can therefore be explained by their resistance to hydrolysis by the GTPase which prolongs the steady-state level of the activated enzyme.

G-proteins exist as α-β-γ heterotrimer (Northup *et al*, 1980; Sternweis *et al*, 1981; Bokoch *et al*, 1983,1984; Codina *et al*, 1983). The α-subunit contains the GTP-binding site and the GTPase activity. The β-subunit and γ-subunit are usually found in tight association even under activating conditions. It is the α-subunit which varies in size amongst the different G-proteins. On the whole the β- and γ- subunits are essentially the same in the different G-proteins. In the non-activated cell the G-protein consists of GDP bound to an associated heterotrimer ($G\alpha$-β-γ.GDP). When the hormone binds to the receptor (HR) it causes the dissociation of $G\alpha$-β-γ.GDP (Brandt & Ross,1986) and association of GTP to give HR.$G\alpha$.GTP + $G\beta$-γ. The HR binding also causes an increase in the intrinsic GTPase activity. The dissociation of the G-protein subunits promotes the dissociation of the HR (Hekman *et al*, 1984). The $G\alpha$.GTP complex binds to and activates the enzyme. This activation is switched off by

the conversion of GTP to GDP and reconstitution of the G-protein subunits is promoted (Rojas & Birnbaumer, 1985).

There are at least four different G-proteins which appear to control several enzyme systems. More than one agonist cooperates with each G-protein. The four main functions regulated by G-proteins are:

(1) activation of adenylyl cyclase;

(2) inhibition of adenylyl cyclase;

(3) stimulation of retinal cyclic GMP phosphodiesterase and;

(4) stimulation of phosphoinositide hydrolysis.

These functions are reviewed by Gillman (1987). For the purposes of this chapter I will concentrate on the G-protein interaction with the last of these functions - stimulation of phosphoinositide hydrolysis.

Many hormones mobilise intracellular stores of calcium by virtue of their ability to stimulate the phosphodiesteratic cleavage of phosphatidylinositol(4,5) bisphosphate (PtdIns(4,5)P_2) to yield inositol(1,4,5)triphosphate (Ins(1,4,5)P_3) and diacylglycerol (DG) (reviewed by Berridge & Irvine, 1984; 1989). The phosphatidylinositol cycle will be discussed in Chapter 5. By 1985 it was evident that a G-protein was involved in controlling the enzyme (phospholipase C) which splits PtdIns(4,5)P_2 to give Ins(1,4,5)P_3 and DG. The first reports of GTP's involvement was made by Gomperts (1983) who demonstrated that, in permeabilized mast cells, GTP stimulated a calcium-dependent secretion of histamine. He proposed that a guanine nucleotide binding protein was involved in calcium-dependent secretory events. Haslam and Davidson (1984a;b) showed

that guanine nucleotides reduced the calcium requirement for thrombin stimulation of secretion in permeabilized platelets and enhanced the response to thrombin with respect to both secretion and diacylglycerol formation.

Litosch, Wallis and Fain (1985) demonstrated that, in membranes prepared from blowfly salivary glands, 5-hydroxytyptamine (5-HT) stimulated the breakdown of endogenous [^{3}H] inositol-labelled phosphoinositides by phospholipase C. This stimulatory effect of the hormone was dependent on the addition of guanine nucleotides. GTP, Gpp(NH)p and GTP-γ-S potentiated the effect of 5-HT. The non-hydrolysable analogues of GTP, GTP-γ-S and Gpp(NH)p, activated basal phospholipase C activity. Cockcroft and Gomperts (1985) also found that breakdown of endogenous polyphosphoinositides, in human neutrophil membranes, was stimulated by GTP-γ-S. Subsequently, guanine nucleotide stimulation of membrane phospholipase C activity utilizing labelled endogenous substrate has been demonstrated for a number of different tissues and cells including GH_3 pituitary cells (Lucas *et al*, 1985), hepatocytes (Wallace & Fain, 1985a,b), polymorphonuclear leucocytes (Smith *et al*, 1985,1986,1987), cerebral cortex (Gonzales & Crew, 1985) and pig coronary artery (Sasaguri, Hirato & Kuriyama, 1985).

The reported characteristics for guanine nucleotide activation of membrane phospholipase C are strikingly similar in the studies cited. GTP itself was found to be a weak activator, even at high concentrations (Cockcroft & Gomperts, 1985; Litosch *et al* , 1985; Lucas *et al*, 1985; Smith, Cox & Snyderman, 1986) and GDP, GMP, ATP, ADP and AMP were completely ineffective. In contrast,

non-hydrolysable guanine nucleotides were potent activators of phosphoinositide hydrolysis. GTP-γ-S was found to be the most effective nucleotide tested (Cockcroft & Gomperts, 1985; Litosch *et al* , 1985; Lucas *et al*, 1985). GTP activation of phospholipase C activity was found to be competitively inhibited by a stable GDP analogue, GDP-β-S (Cockcroft, 1986; Uhing *et al*, 1986; Martin *et al*, 1986; Cockcroft & Taylor, 1987), which suggests that activation of phospholipase C is associated with the binding of GTP, with the GDP-bound state being inactive.

Since the discovery that GTP has a regulatory role in releasing calcium, much interest has been shown in its effect on calcium release and calcium-activated force. Dawson (1985) reported in liver microsomes, which were normally insensitive to Ins(1,4,5)P_3, that preincubating the microsomes with GTP and polyethylene glycol (PEG) (3%) increased the preparation's sensitivity to Ins(1,4,5)P_3. However, GTP alone caused a release of calcium via a mechanism that was independent of Ins(1,4,5)P_3. This effect was abolished if PEG was omitted from the solution. Gill and Mullaney have further developed these results (Gill *et al*, 1986; Chueh *et al*, 1987; Mullaney *et al*, 1988 and reviewed in Gill, Mullaney & Ghosh, 1988) in permeabilized neuronal and smooth muscle cell lines. These workers showed that the calcium which was released by GTP alone appears to have come from the Ins(1,4,5)P_3-sensitive store. This effect of GTP cannot be mimicked by GTP-γ-S or any of the other nonhydrolysable analogues of GTP. In fact GTP-γ-S blocks the response to GTP. GTP was also found to increase calcium uptake in the presence of

oxalate in permeabilized neuronal and smooth muscle cell lines at the same concentration as it promotes release. Finally they concluded that GTP has a role to play in transmembrane conveyance of calcium and, since this involves the Ins(1,4,5)P_3-releasable pool GTP may control the size of this calcium store. The one drawback to this hypothesis is the need for PEG to obtain the effect of GTP. Thomas (1987) showed in permeabilized hepatocytes, without PEG, that GTP did not release calcium alone but did potentiate the calcium released by Ins(1,4,5)P_3. However, again this effect was not mimicked by GTP-γ-S.

The work carried out in permeabilized smooth muscle has largely disagreed with the work of Dawson and Gill. In saponin-, alpha-toxin- and β-escin-treated muscle, GTP alone does not cause any substantial release of calcium (Kobayashi, Somlyo & Somlyo, 1988a; Kitazawa *et al*, 1989; Nishimura, Kobler & van Breemen,1988: Kobayashi *et al*, 1989). However, GTP-γ-S has been reported to release intracellular calcium in both saponin and alpha-toxin permeabilized muscle (Nishimura *et al*,1988; Kobayashi *et al*,1988a;b). This is also in contradiction to Gill and colleague's work where GTP-γ-S inhibited the release of calcium. Saida and van Breemen (1987) and Saida *et al* (1988) have reported similar results to Thomas (1987) in which GTP was required before Ins(1,4,5)P_3 could release calcium from the intracellular store. This calcium was also mobilised from the same store as the calcium released by caffeine. However, unlike Thomas (1987), Saida and van Breemen (1987) and Saida, Twort and van Breemen (1988) reported that GTP-γ-S could mimic the effect of GTP. These results by Saida *et al* (1987,1988) have not been found to be the universal case.

Other workers using different smooth muscle preparations, both vascular and visceral, have shown that Ins(1,4,5)P_3 can release calcium in the absence of GTP (Suematsu *et al*, 1984; Yamamoto & van Breemen, 1985; Kobayashi *et al*,1988a;b; 1989; Kitazawa *et al*,1989).

Two important methodological developments for permeabilizing smooth muscle have been: (1) alpha-toxin from *Staphylococcus aureus* and (2) β-escin. These methods retain functional membrane bound receptors (Nishimura *et al*,1988; Kitazawa *et al*, 1989; Kobayashi *et al*,1989), allowing these workers to analyse the role of GTP in agonist activation. Again, however, there have been conflicting results. Nishimura *et al* (1988) report that the response to noradrenaline can be obtained without the presence of GTP, whereas Kitazawa *et al* (1989) and Kobayashi *et al* (1989) reported that, to obtain reproducible responses to phenylephrine and carbachol, GTP was required. Although Nishimura *et al* (1988) showed that noradrenaline could produce a contraction without GTP being present, they did not investigate whether the response could be potentiated by GTP. The response initiated by GTP-γ-S could be partially blocked by heparin (Kobayashi *et al*, 1988) which is known to antagonise the Ins(1,4,5)P_3 receptor (Worley *et al*, 1987). The response to Ins(1,4,5)P_3 (Kobayashi *et al*, 1988b), phenylephrine and carbachol (Kobayashi *et al*, 1989) were abolished by heparin. Where GTP has been shown to increase the response to the agonists GDP-β-S has blocked this effect. Despite these differences in results, the bulk of the evidence suggests that G-proteins are involved in receptor signal transduction, leading to intracellular release of

calcium and contraction in smooth muscle.

Much interest has also been paid to GTP's effect on calcium-activated force. The first report was made by Kobayashi *et al* (1988a) who demonstrated in freeze-glycerinated rabbit main pulmonary artery that GTP or GTP-γ-S had no effect on the pCa tension curve. This, however, has been the only report of GTP and GTP-γ-S failing to affect calcium-activated force. All other studies have been made in muscle which has either been saponin-, alpha-toxin- or β-escin-treated. It has been universally agreed that, in these muscles, GTP or GTP-γ-S increases calcium sensitivity (Nishimura *et al*, 1988; Kobayashi *et al*, 1988b; 1989; Kitazawa *et al*,1989; Fujiwara *et al*, 1989). This effect is similar to increases in calcium-activated force produced by phenylephrine (Kitazawa *et al*,1989), and the effect of GTP on calcium-activated force is potentiated by noradrenaline (Nishimura *et al*,1988). However, there is disagreement about what happens to maximum calcium-activated force (Tmax). Nishimura *et al* (1988) and Fujiwara *et al* (1989) report that GTP has no effect on Tmax, whereas Kitazawa *et al* (1989) and Kobayashi *et al* (1989) found that GTP-γ-S increases Tmax. There is much speculation about what causes the increase in calcium-activated force. The effect can be blocked by GDP-β-S, indicating that a G-protein is involved. However, it cannot be blocked by heparin indicating that $Ins(1,4,5)P_3$ release of calcium is not a prerequisite for the effect. This suggests that GTP-γ-S activates phospholipase C via a G-protein producing $Ins(1,4,5)P_3$ and DG from $PtdIns(4,5)P_2$. It is DG that then activates protein kinase C which in turn phosphorylates the contractile proteins, increasing their

sensitivity to calcium. Fujiwara *et al* (1989) have shown that GTP-γ-S promotes increased phosphorylation of the myosin light chain and increases shortening velocity. However, the authors were not convinced that this was due to protein kinase C because, although activation of protein kinase C by a phorbol ester increases calcium sensitivity it depresses Tmax, this is unlike the effect of GTP-γ-S, which either has no effect on Tmax or increases it. Another possible explanation of GTP-γ-S increase in Tmax is that it inhibits the inhibitory phosphatases which normally dephosphorylate the myosin light chain (Somlyo *et al*,1989).

To summarise, from this work on permeabilized smooth muscle: There seems reasonable agreement that a G-protein is involved in the agonist transduction mechanism. This appears to be mediated through the PtdIns cycle. There is some evidence that GTP-γ-S causes calcium release via Ins(1,4,5)P_3 and increases calcium-activated force via DG.

The aim of this study was to compare and contrast the effect of GTP on noradrenaline- and calcium-activated force in an alpha-toxin permeabilized visceral (rat anococcygeus) and a vascular (guinea pig portal vein) smooth muscle.

MATERIALS AND METHODS

PREPARATIONS

Rat anococcygeus and guinea pig portal vein were isolated and prepared as described in Chapter 2. Each was set up in the experimental apparatus as described in Chapter 2 - Permeabilized Muscle. The muscle strips were permeabilized using alpha-toxin from *Staphylococcus aureus* as described and demonstrated in Chapter 3 and Figure 3.1.

SOLUTIONS

The solutions used were prepared as described in Chapter 2 - Permeabilized Muscle. The solution compositions are given in Table 2.3 and the drugs used are detailed in Table 2.2. The potassium salt used was potassium methansulphonate. The pH was set at 7.1 and the ionic strength at 0.2M.

RESULTS

PROBLEMS ASSOCIATED WITH ALPHA-TOXIN PERMEABILIZATION IN RAT ANOCOCCYGEUS

Permeabilization with alpha-toxin from *Staphylococcus aureus* retains functional membrane bound receptors (Nishimura *et al*,1988; Kitazawa *et al*; 1989). Kitazawa showed that both alpha adrenoreceptors and muscarinic receptors were retained. These features were tested for in rat anococcygeus. The results are shown in Figure 4.1. The muscle was permeabilized as described in Chapter 3 and thereafter proved responsive to calcium, noradrenaline and caffeine. The calcium stores were loaded in the experimental muscle by raising the $[Ca^{2+}]$. This caused a contraction, the end portion of which can be seen in Figure 4.1. The $[Ca^{2+}]$ was lowered to 0.08μM and the muscle relaxed. The muscle was then exposed to 30μM noradrenaline and the muscle contracted transiently. However, the muscle did not respond when exposed to acetylcholine (3mM). The response of the intact muscle to acetylcholine was then investigated (Figure 4.2). The muscle was bathed in Tyrode solution and was exposed to 30μM noradrenaline, 3mM acetylcholine and 5mM ATP. All three agonists caused a phasic contraction which only relaxed when the agonist was withdrawn. This indicates that the adreno-, muscarinic and purino- receptors are functional in the intact muscle bathed in Tyrode solution. The factor inhibiting the muscarinic response after alpha-toxin-treatment, therefore, must be connected with either the permeabilization technique or the composition of the mock intracellular solution.

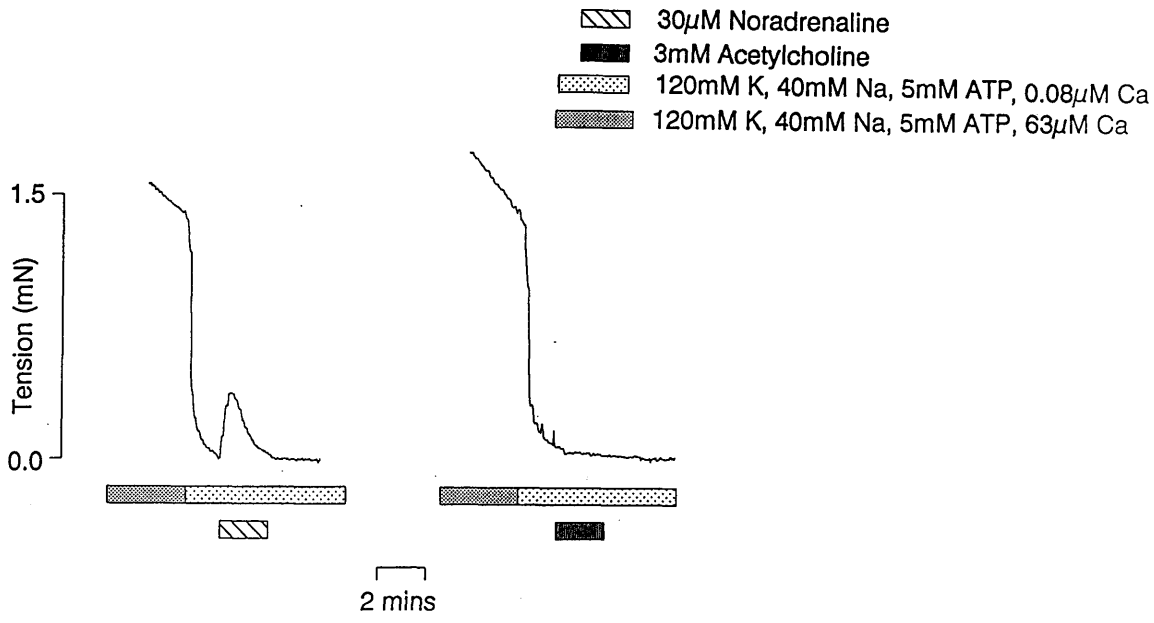


Figure 4.1 shows the response of alpha-toxin permeabilized rat anococcygeus muscle to 30μM noradrenaline (hatched bar) and 3mM acetylcholine (heavily stippled bar). The bathing solution was changed from one containing 63μM calcium to one of 0.8μM. This is indicated by the change in the stipple pattern of the larger of the bars beneath the two traces.

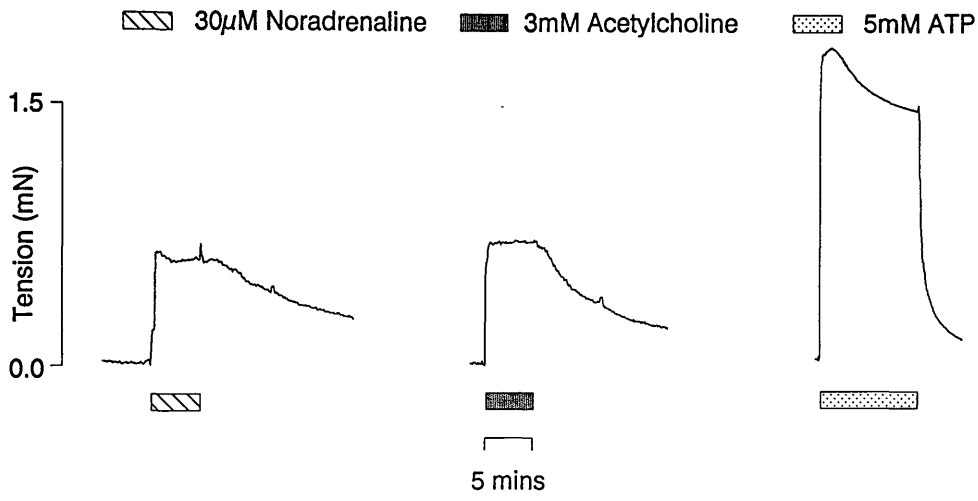


Figure 4.2 shows contractions produced by intact rat anococcygeus muscle to 30μM noradrenaline (hatched bar), 3mM acetylcholine (heavily stippled bar) and 5mM ATP (lightly stippled bar), each in Tyrode solution.

To examine this, Tyrode (with added ATP) was replaced by normal mock intracellular bathing medium in an intact strip of anococcygeus muscle. This caused a transient contraction (Figure 4.3). Application of NA, but not of ACh caused a transient contraction. Figure 4.4 shows that, when the bathing medium around the muscle was changed from Tyrode to a mock intracellular solution with no added ATP or CrP (40mM NaCl has been added to prevent changes in ionic strength) that the muscles contracted transiently and were responsive to noradrenaline, acetylcholine and ATP. This inhibitory effect of ATP cannot be reversed by adding 1μM alpha-beta methylene ATP. Alpha-beta-methylene ATP is a non-hydrolysable analogue of ATP. It works by desensitising P_{2x} receptors (Kasakoz & Burnstock, 1983; Delebro *et al*, 1985). The effect of ATP does, however, prove to be a problem. ATP has to be present in the mock intracellular solutions to fuel contraction. The results from Chapter 3 (Figure 3.2) show that removing ATP from the solutions causes the muscle to go into rigor. During any further analysis of findings made using this preparation this inhibitory effect of ATP on the muscarinic response must be kept in mind.

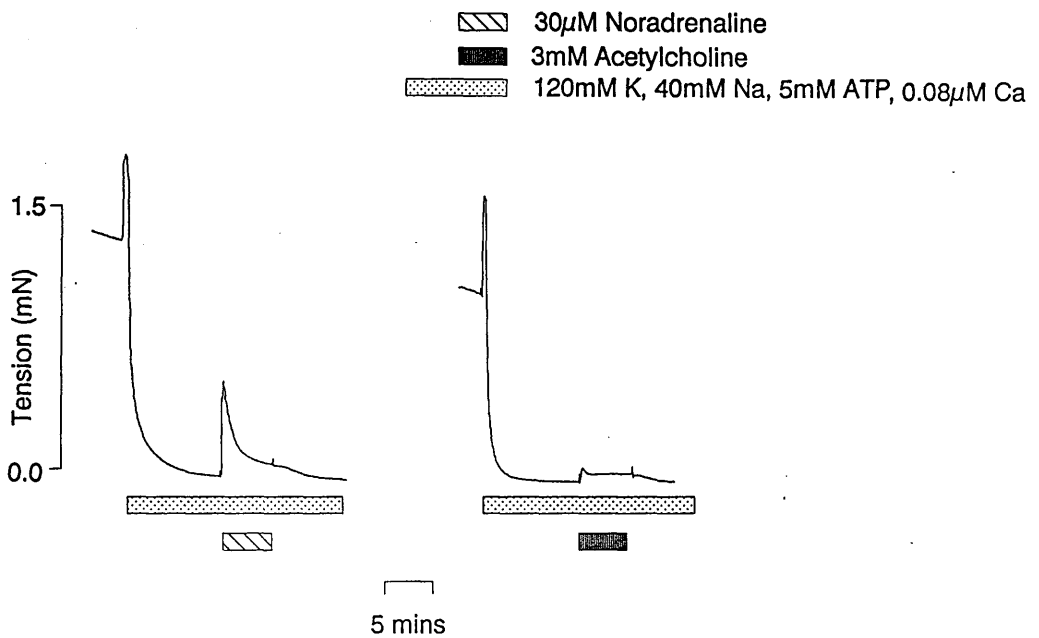


Figure 4.3 shows contractions of intact rat anococcygeus muscle induced by 30μM noradrenaline (hatched bar) and 3mM acetylcholine (heavily stippled bar) in a mock intracellular solution with 5mM ATP. Prior to the solution change indicated by the lightly stippled bar underneath the tension record the muscle had been bathed in Tyrode solution with added ATP.

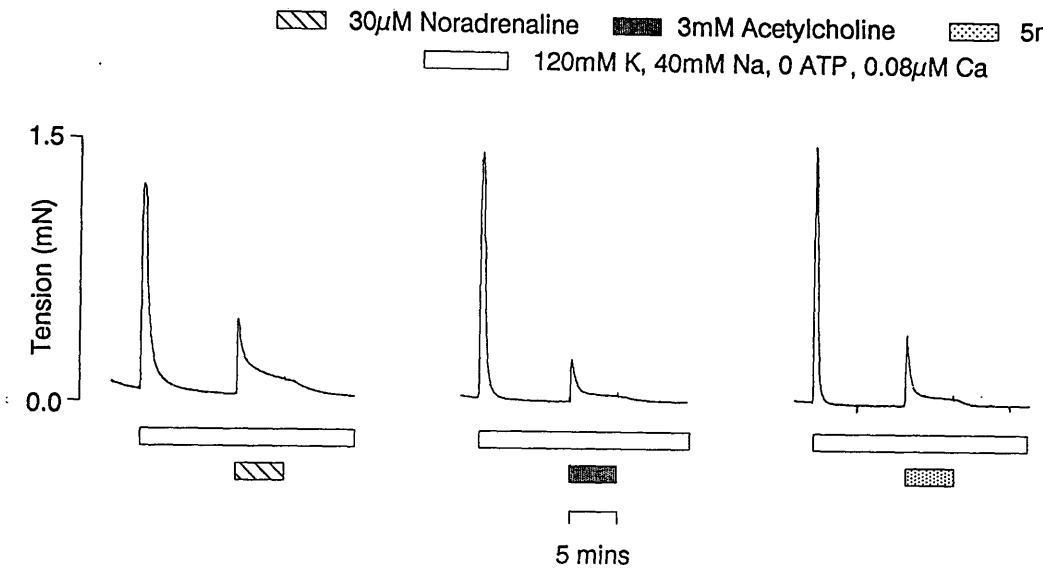


Figure 4.4 shows contractions of intact rat anococcygeus muscle induced by 30μM noradrenaline (hatched bar), 3mM acetylcholine (heavily stippled bar) and 5mM ATP (lightly stippled bar) in a mock intracellular solution which does not contain ATP. This is indicated by the open bar underneath the tension record. Prior to the solution change indicated the muscle had been bathed in Tyrode solution.

All the muscles used were stabilized by repetitively evoking contractions with 10mM caffeine. The SR was allowed to accumulate calcium for 10 minutes in a solution with a fixed $[Ca^{2+}]$ below the threshold for contraction. During each calcium load and release cycle caffeine (10mM) was applied for 2 minutes, washed out and the muscle allowed to accumulate calcium for 10 minutes and so on. This was continued until the muscle produced reproducible contractions in response to caffeine. Once this had been achieved 30μM noradrenaline was applied which produced a transient contraction in rat anococcygeus (Figure 4.5) and guinea pig portal vein (Figure 4.7). Noradrenaline is thought to produce its response by stimulating phospholipase C to produce Ins(1,4,5)P_3 and DG. Ins(1,4,5)P_3 then releases calcium from the intracellular store (Streb *et al.* 1983) which causes the muscle to contract. Ins(1,4,5)P_3 is rapidly broken down (Berridge and Irvine, 1989) which might explain why the contraction observed here is transient.

(a) Rat Anococcygeus

Figure 4.5 consists of two panels showing the effects of GTP on noradrenaline-activated force when applied at different times in the experimental protocol. Figure 4.5A shows the stabilized contractions in response to caffeine followed by a series of transient contractions in response to noradrenaline. The amplitude of the noradrenaline-activated contraction declines with time. Exposing the muscle to 100μM GTP causes a contraction itself which on average

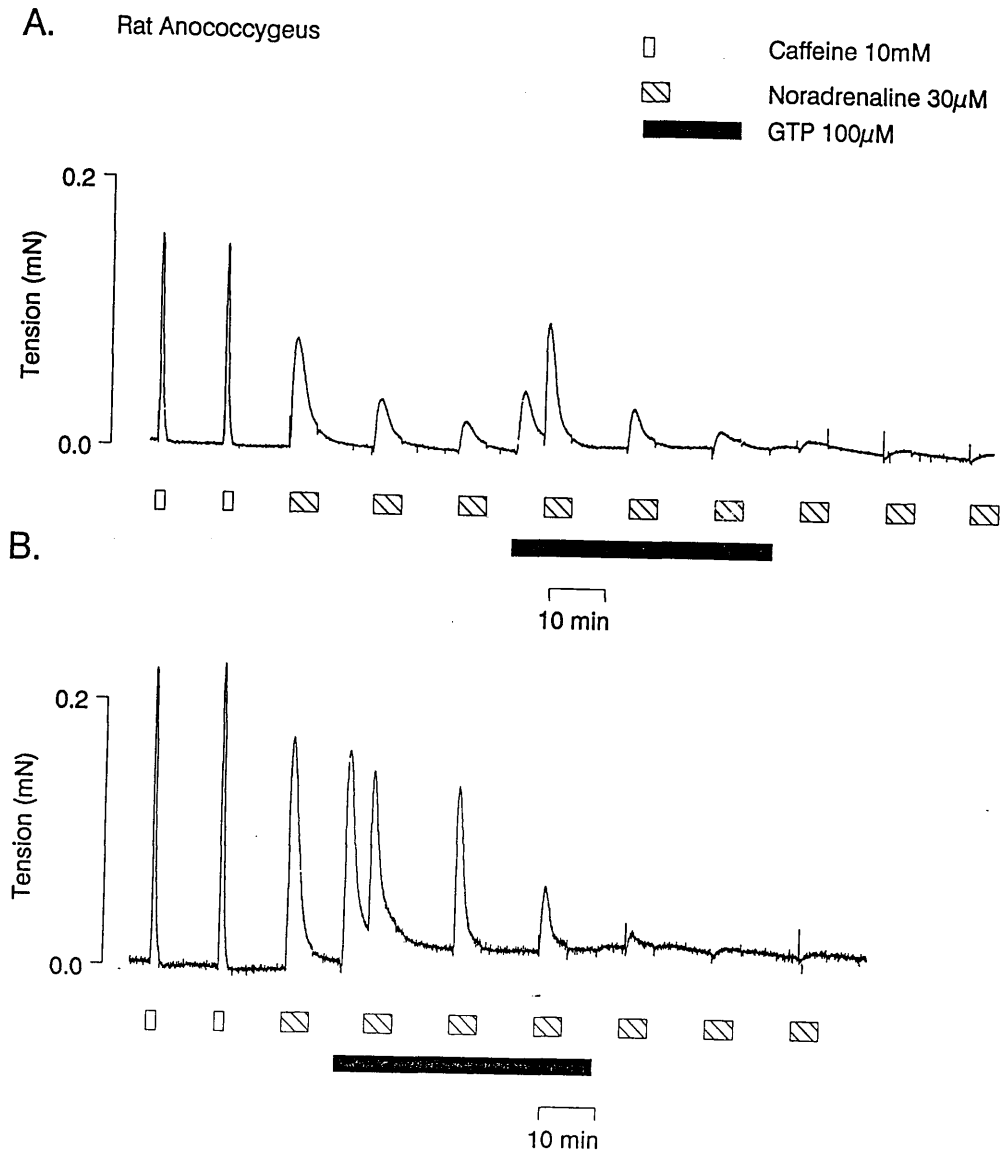


Figure 4.5 shows the effect of 100μM GTP on the response to noradrenaline in permeabilized rat anococcygeus muscle. The application of 10mM caffeine (open bar), 30μM noradrenaline (hatched bar) and 100μM GTP (filled bar) are indicated. Panel A shows the effect of exposing the muscle to GTP after three previous contractions in response to noradrenaline. Panel B shows the effect of exposing the muscle to GTP after only one response induced by noradrenaline.

was 64% (±14%, n=3) of the first contraction in response to noradrenaline. The presence of 100μM GTP transiently potentiates the next response to noradrenaline, but it does not reverse the decline in peak tension. Application of 100μM GTP after the first noradrenaline-activated contraction also causes a contraction by itself (Figure 4.5B). This on average was marginally larger than the first noradrenaline-activated contraction (114%±22, n=6) although not in the example shown here. The peak of the next contraction in response to noradrenaline had not declined as much as it would have had there been no GTP present in the solution. However, the presence of GTP did not stop the decline in the amplitude of the contractions evoked by noradrenaline. Removal of GTP in both protocols had no obvious effect on the response. The contractions to noradrenaline in both panels gradually became smaller until eventually only a small tonic component was visible. Figure 4.6 shows the average noradrenaline-activated contraction expressed as a percentage of the first contraction in response to noradrenaline for the two different protocols just described for rat anococcygeus. This figure shows that GTP (100μM) does not reversibly halt the decline in the amplitude of noradrenaline-activated contractions. The peak of the response to noradrenaline, directly after application of GTP, was potentiated but only to 67% and 68% of the first noradrenaline-activated contraction. This potentiation by GTP appears to be independent of what has occurred in the muscle before its addition.

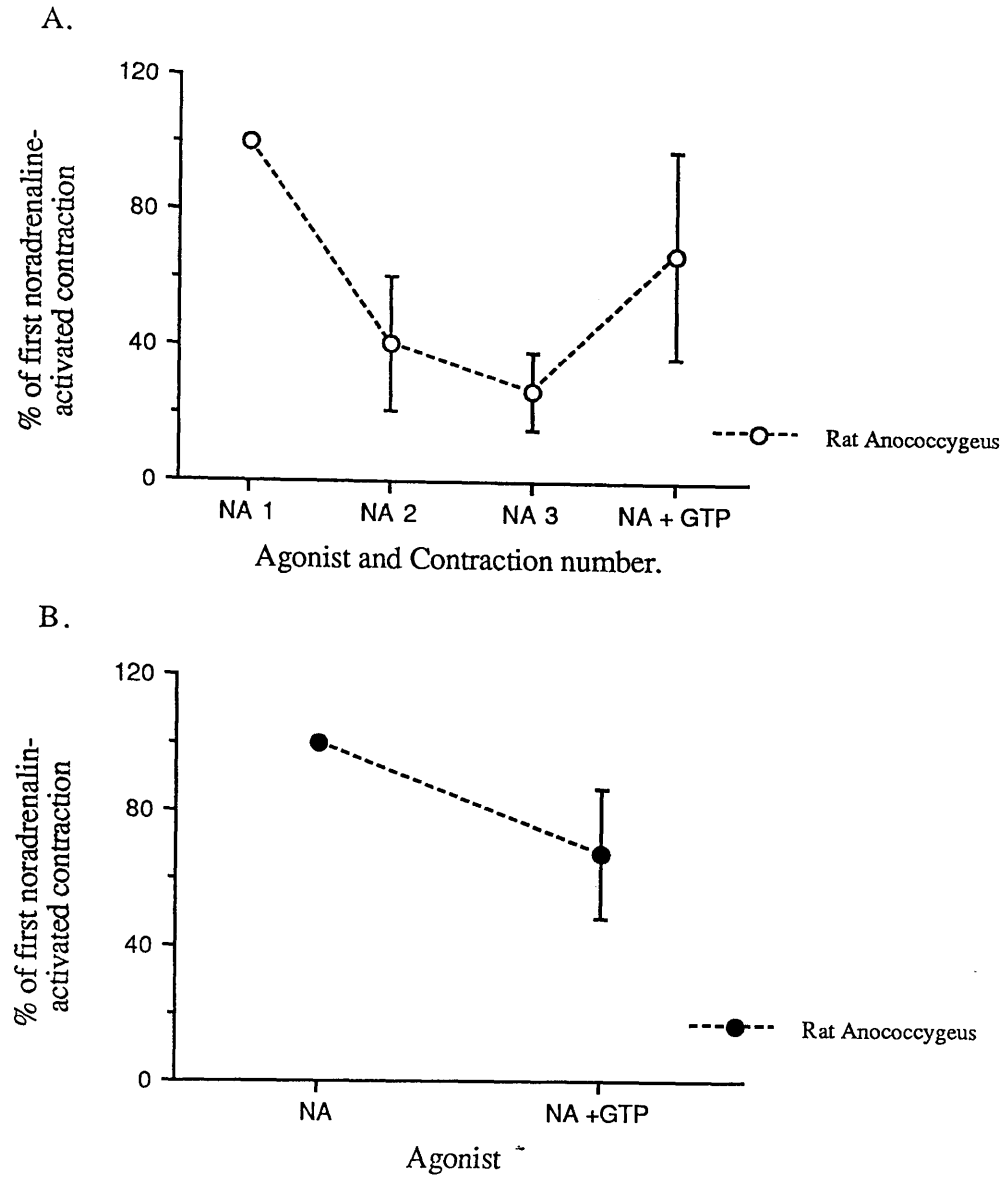


Figure 4.6 shows the average data for the decline in the amplitude of the response induced by noradrenaline in permeabilized rat anococcygeus and how this is affected by GTP. Each point has been expressed as a percentage of the amplitude of the first noradrenaline-activated contraction. The points represent the mean±S.D. (n=3-8).

(b) Guinea Pig Portal Vein

Figure 4.7 also has two panels which contrast the effects of exposing the muscle to GTP at different times in the experimental protocol. Both the muscles have been stabilized using repetitive exposures to caffeine. Once the muscle had stabilized a series of exposures to noradrenaline were undertaken. The amplitude of successive noradrenaline-activated contractions in portal vein also decline with time. However, this decline in peak tension in response to noradrenaline was slower than in rat anococcygeus. Exposing the muscle to 100μM GTP did not in itself cause a contraction. However, the peak of the response to noradrenaline was potentiated and this potentiation lasted as long as the muscle was exposed to GTP. On removal of GTP the amplitude of the noradrenaline-activated contractions declined once again. Panel B shows that applying GTP (100μM) after the first noradrenaline-activated contraction also does not cause a contraction, but does potentiate the amplitude of the noradrenaline-activated contractions which followed. Again, the size of the response to noradrenaline were maintained for the period that the muscle was exposed to GTP. On removing GTP peak tension evoked by noradrenaline declined. Figure 4.8 shows the mean peak tension in response to noradrenaline expressed as a percentage of the peak tension of the first contraction in response to noradrenaline for the two different protocols just described. GTP (100μM) potentiated the amplitude of noradrenaline induced contractions to the extent that they were greater (148% and 144%) than the first noradrenaline induced contraction. This potentiation appears to be

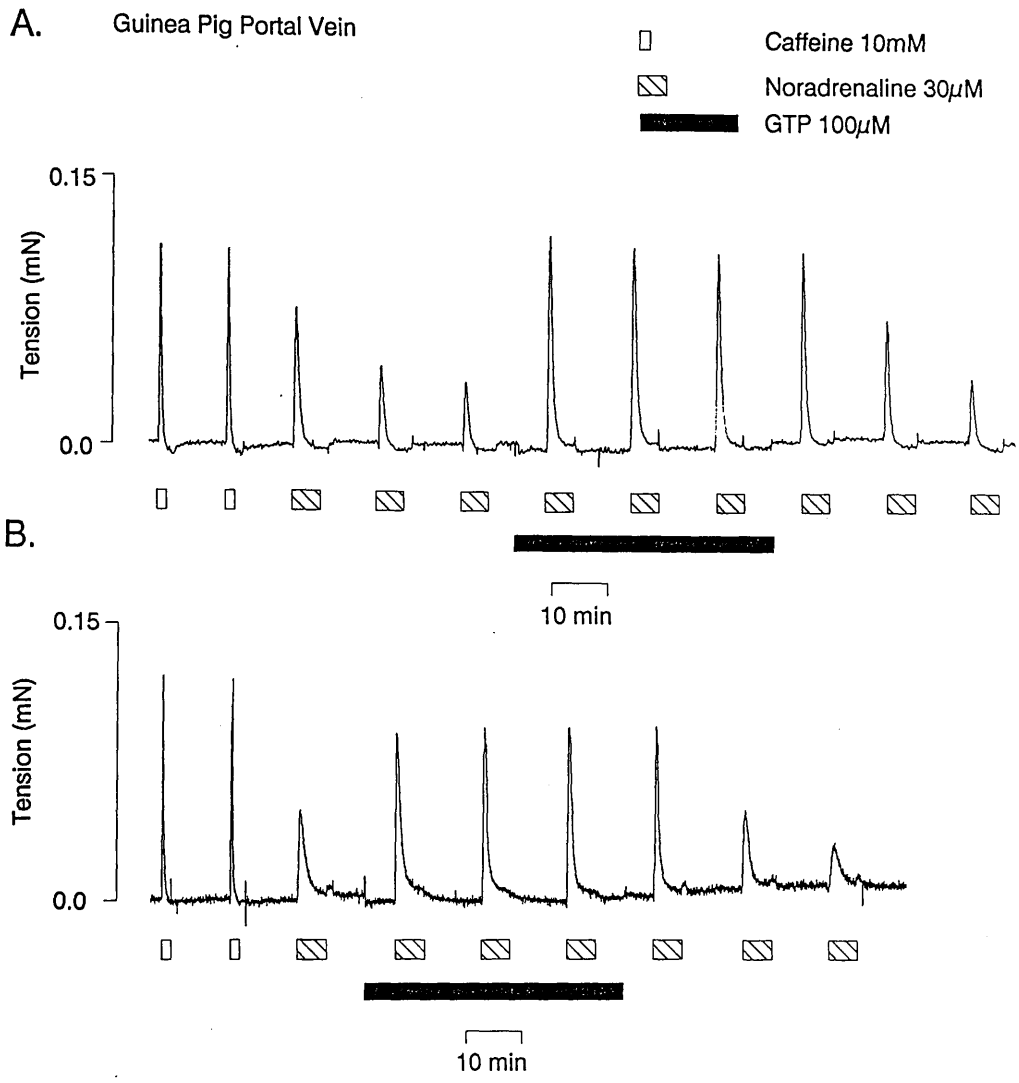


Figure 4.7 shows the effect of 100μM GTP on noradrenaline-activated force in permeabilized guinea pig portal vein longitudinal muscle. The application of 10mM caffeine (open bar), 30μM noradrenaline (hatched bar) and 100μM GTP (filled bar) are indicated. Panel A shows the effect of exposing the muscle to GTP after three previous contractions in response to noradrenaline. Panel B shows the effect of exposing the muscle to GTP after only one response induced by noradrenaline.

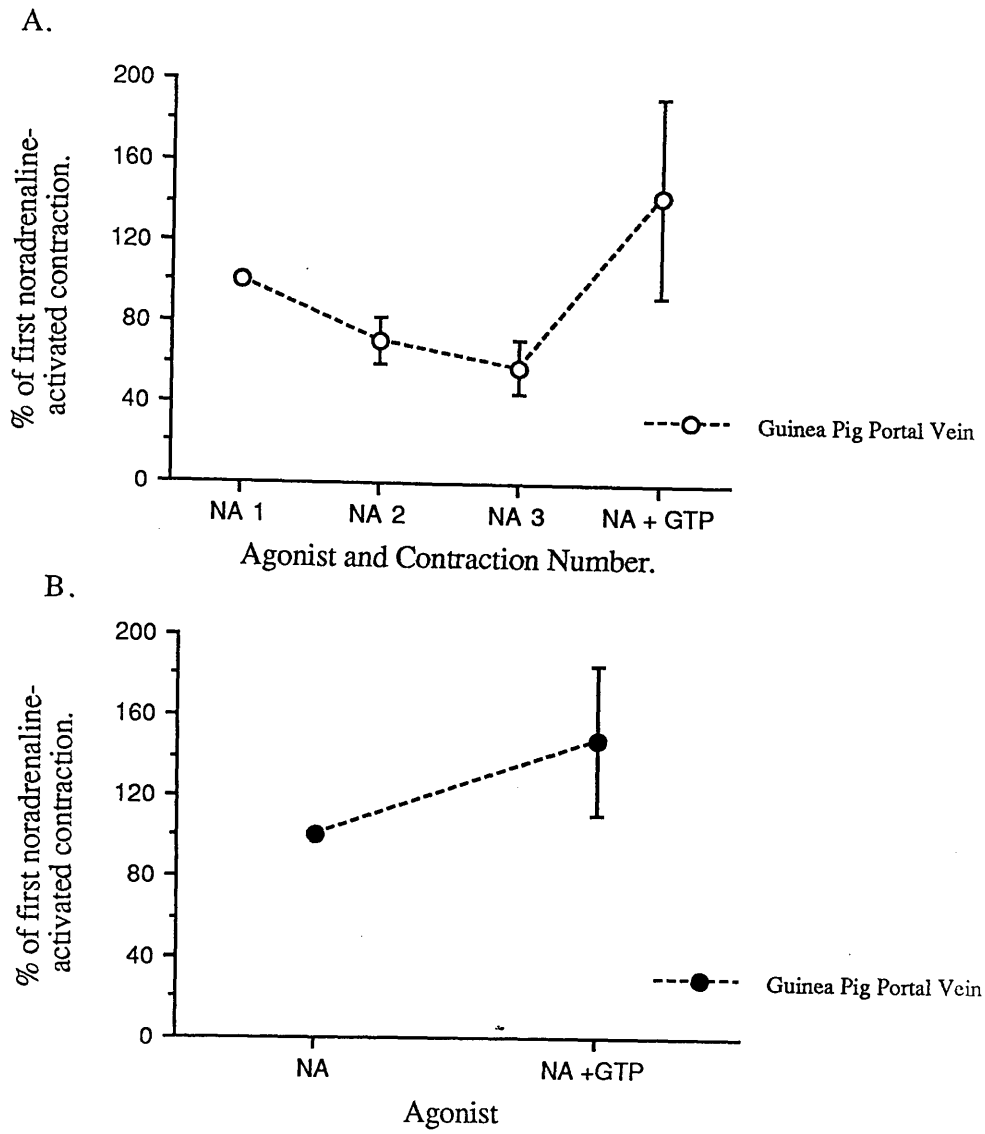


Figure 4.8 shows the average data for the decline in the amplitude of the response induced by noradrenaline in permeabilized guinea pig portal vein longitudinal muscle and how this is affected by GTP. Each point has been expressed as a percentage of the amplitude of the first contraction induced by noradrenaline. The points represent the mean±S.D. (n=3-8).

independent of the time in the protocol that the muscle was exposed to GTP.

In summary, the amplitude of successive noradrenaline-activated contractures in both rat anococcygeus and guinea pig portal vein declines with time. The decline is greater, however in rat anococcygeus. The decline can be reversed in guinea pig portal vein by exposing it to GTP (100μM). This is not the case in rat anococcygeus where the decline is only transiently arrested by GTP. Finally GTP caused a transient contraction in rat anococcygeus, but not in guinea pig portal vein.

THE EFFECT OF GTP ON CAFFEINE- AND Ins(1,4,5)P_3-ACTIVATED CONTRACTIONS.

Figure 4.9 examines the effect that GTP (100μM) has on caffeine induced contractures in permeabilized rat anococcygeus. The muscle was stabilized by evoking repeated contractions with caffeine. The first contraction in the trace is representative of these. Noradrenaline, followed by GTP was applied as shown. GTP caused a transient contraction and the amplitude of the subsequent contraction in response to noradrenaline was potentiated initially, but then declined successively. The amplitude of the response to caffeine, however, actually increased with time possibly indicating that there was more calcium available for release. This suggests that the amplitude of the noradrenaline response does not decay because of inadequate loading of the intracellular store in the presence of 100μM GTP.

The decay in the amplitude of the response to noradrenaline could be explained if the intracellular store became less sensitive to Ins(1,4,5)P_3 in the

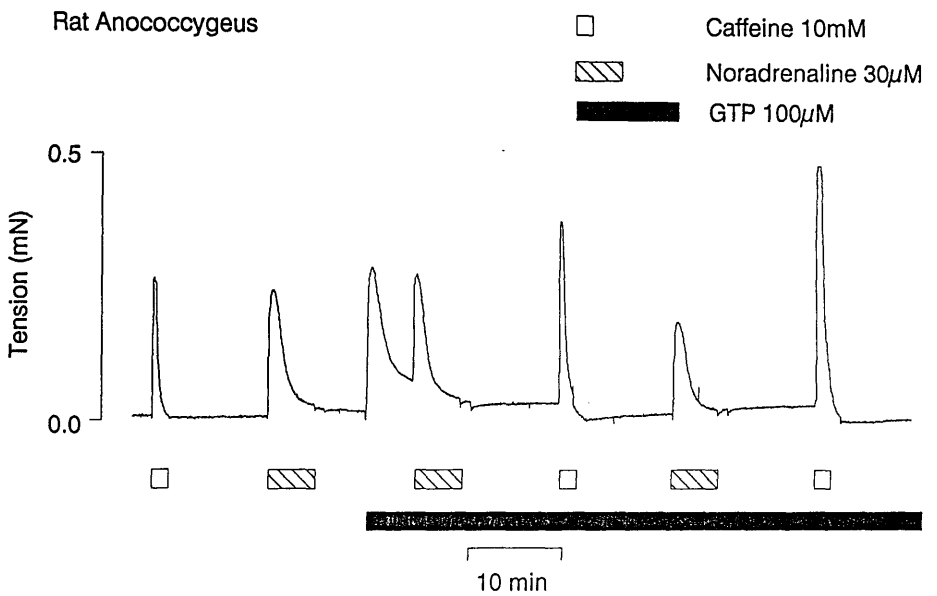


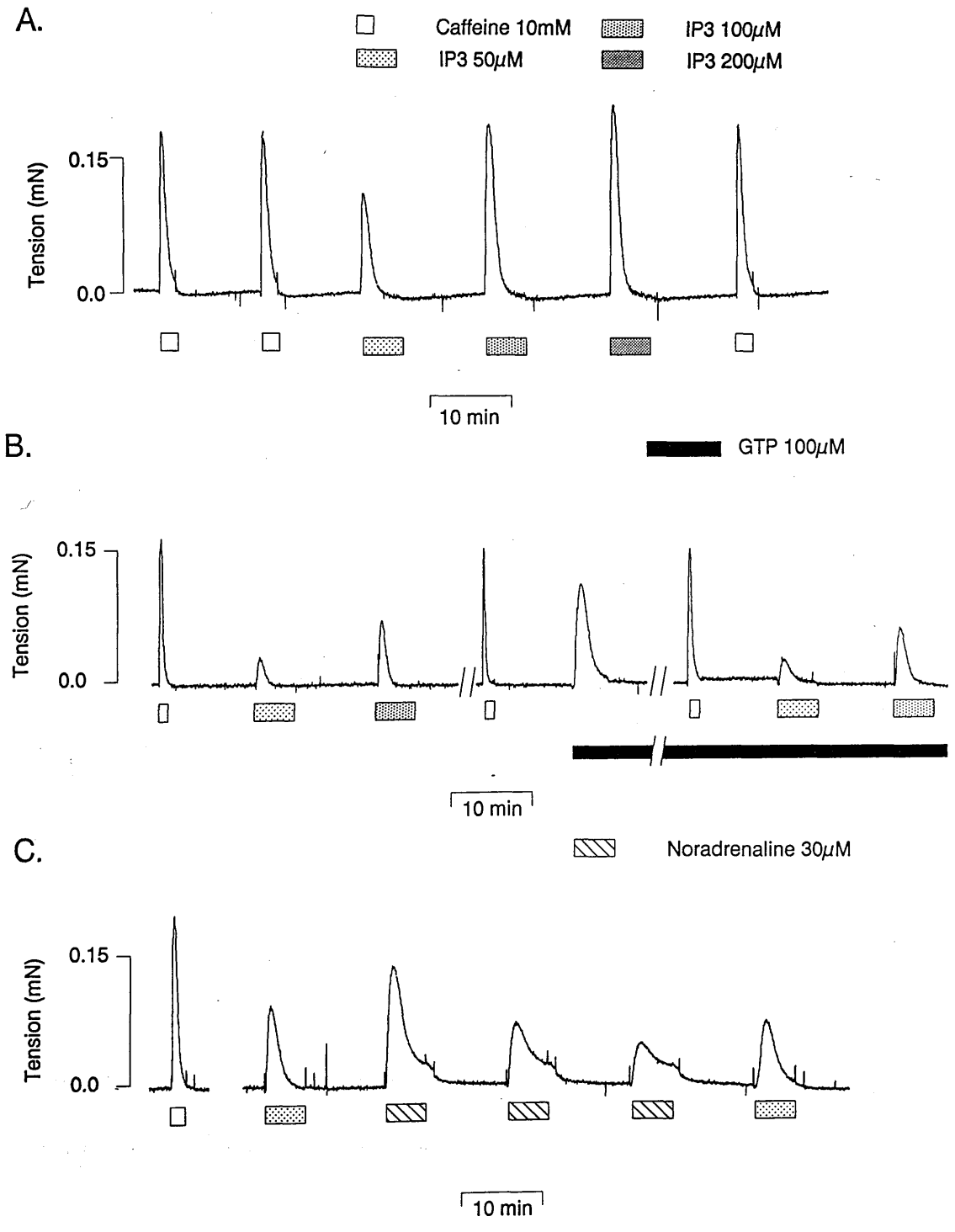
Figure 4.9 shows the effects of 100μM GTP on the response induced by noradrenaline and caffeine in permeabilized rat anococcygeus muscle. The application of 10mM caffeine (open bar), 30μM noradrenaline (hatched bar) and 100μM GTP (filled bar) are indicated.

presence of GTP. Figure 4.10 has three panels. Panel A shows responses to a range of Ins(1,4,5)P_3 concentrations by rat anococcygeus. It can be seen that the response to Ins(1,4,5)P_3 approaches a maximum at a concentration of 200μM. Panel B investigates the effect of GTP (100μM) on the size of the Ins(1,4,5)P_3-activated contraction. The effect was examined at two different concentrations, 50 and 100μM. The muscle was stabilized by repeated challenges with caffeine the last of which is shown as the first contraction in Panel B. The muscle was then exposed to Ins(1,4,5)P_3. After applying GTP (100μM) the muscle was exposed for a second time to each of the two different Ins(1,4,5)P_3 concentrations. It can be seen that GTP has no effect on the size of either response to Ins(1,4,5)P_3. Panel C illustrates the amplitude of the response to 50μM Ins(1,4,5)P_3 before and after the now familiar decay in the amplitude of the response to noradrenaline. The amplitude of the response to Ins(1,4,5)P_3 was similar before and after the decay in the amplitude of the response to noradrenaline. These results indicate that the decay in the amplitude of the response to noradrenaline is not due to a reduced sensitivity of the intracellular store to Ins(1,4,5)P_3. It would seem more likely that the decay is due to reduced production of Ins(1,4,5)P_3 by the agonist.

THE EFFECT OF A HIGHER CONCENTRATION OF GTP ON NORADRENALINE-ACTIVATED CONTRACTIONS

One possible explanation for the failure of 100μM GTP to cause a contraction in guinea pig portal vein is that this muscle is less sensitive to GTP than rat anococcygeus. Figure 4.11 shows that, in both rat anococcygeus and

Figure 4.10 shows the effects of noradrenaline and GTP on the contraction produced by Ins(1,4,5)P_3 in permeabilized rat anococcygeus. The application of 10mM caffeine (open bar), 30μM noradrenaline (hatched bar), 100μM GTP (filled bar) and Ins(1,4,5)P_3 (stippled bars) are indicated. Panel A shows the effect of increasing concentrations of Ins(1,4,5)P_3 on the tension response. Panel B shows the effect of GTP on the response induced by Ins(1,4,5)P_3. Panel C shows the effect of the decline in the amplitude of the response induced by noradrenaline on the amplitude of the response to Ins(1,4,5)P_3.


A.
Caffeine 10mM
IP3 100μM
IP3 50μM
IP3 200μM
Tension (mN)
0.15
0.0
10 min
B.
GTP 100μM
Tension (mN)
0.15
0.0
10 min
C.
Noradrenaline 30μM
Tension (mN)
0.15
0.0
10 min

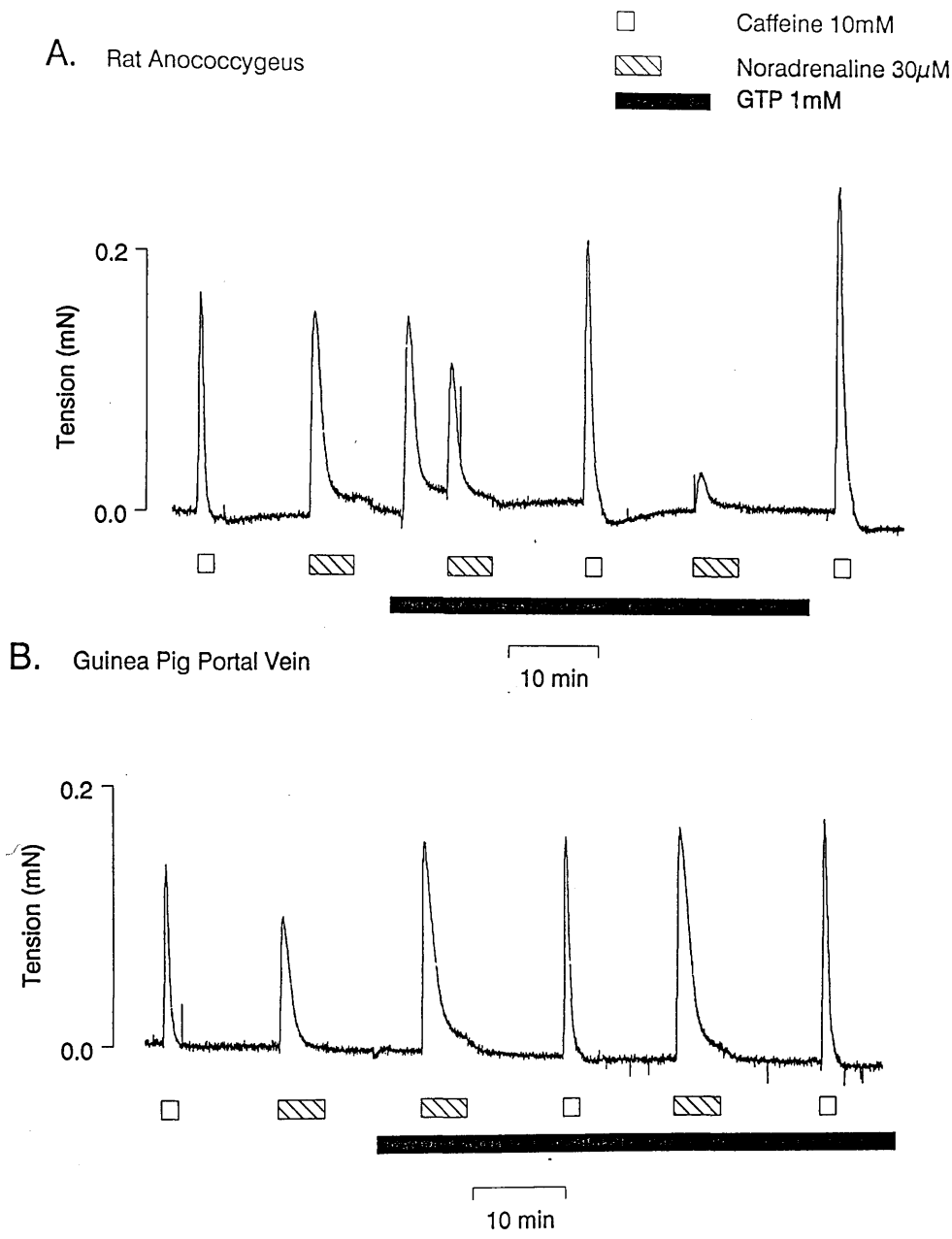


Figure 4.11 shows the effects of 1mM GTP on the response induced by noradrenaline and caffeine in permeabilized rat anococcygeus (Panel A) and permeabilized guinea pig portal vein (Panel B). The application of 10mM caffeine (open bar), 30μM noradrenaline (hatched bar) and 100μM GTP (filled bar) are indicated.

guinea pig portal vein, 100μM and 1mM GTP had a similar effect on noradrenaline-activated force. This higher concentration of GTP (1mM) still failed to promote a contraction in guinea pig portal vein or inhibit the decay in the amplitude of the response to noradrenaline in rat anococcygeus. The contraction produced by GTP in the latter tissue averaged 116% ($\pm$43%,n=3) of the first contraction in response to noradrenaline, which is essentially the same as the contraction produced by 100μM GTP (114%$\pm$22%, n=6).

THE EFFECT OF GTP-γ-S ON NORADRENALINE-ACTIVATED CONTRACTIONS

GTP-γ-S is a non-hydrolysable analogue of GTP. The effect of 100μM GTP-γ-S on the response to noradrenaline was investigated in permeabilized rat anococcygeus and guinea pig portal vein. Figure 4.12A shows the effect observed in rat anococcygeus. GTP-γ-S was applied after the now familiar decline in the amplitude of the response to noradrenaline. GTP-γ-S (100μM) caused a large transient contraction and blocked all subsequent noradrenaline-activated contractions. This effect of GTP-γ-S was not reversible. Figure 4.12B shows a typical tension record obtained from guinea pig portal vein using the same protocol as described for rat anococcygeus. GTP-γ-S caused a transient contraction and thereafter the response to noradrenaline was blocked. This effect of 100μM GTP-γ-S was not reversible. This experiment was repeated using 10μM GTP-γ-S in both tissues and similar results were obtained (data not shown). In summary, 10 and 100μM GTP-γ-S alone cause contraction in both rat anococcygeus and guinea pig portal vein. In the presence of

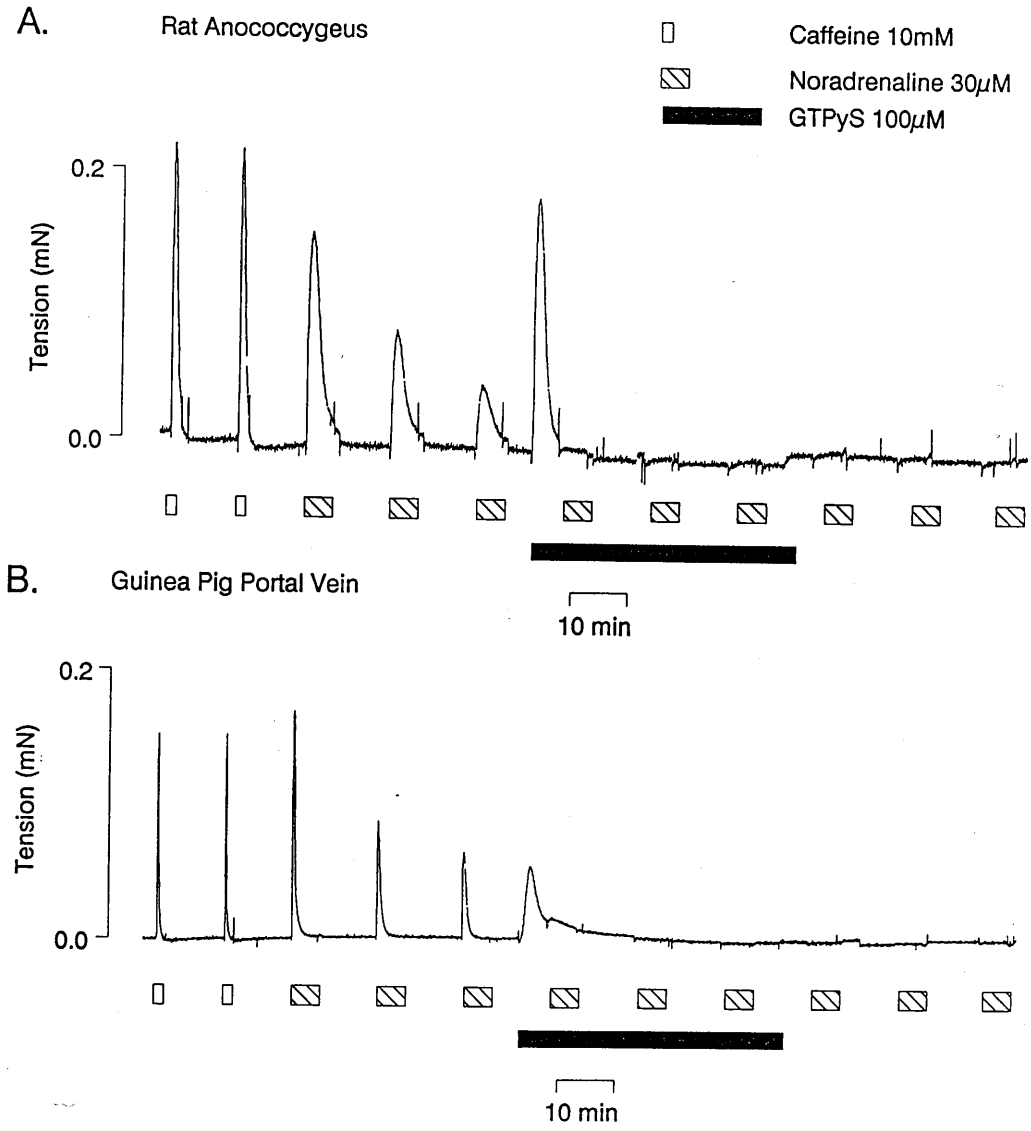


Figure 4.12 shows the effects of 100μM GTP-γ-S on the response induced by noradrenaline in permeabilized rat anococcygeus (Panel A) and permeabilized guinea pig portal vein (Panel B). The application of 10mM caffeine (open bar), 30μM noradrenaline (hatched bar) and 100μM GTP-γ-S (filled bar) are indicated.

GTP-γ-S both muscles proved unresponsive to noradrenaline and this effect was not reversible.

THE EFFECT OF GDP-β-S ON NORADRENALINE-ACTIVATED CONTRACTIONS

GDP-β-S is a non-hydrolysable analogue of GDP. GDP-β-S binds to the G-protein and inactivates the protein (Williamson, 1986). Figure 4.13 illustrates the effect of GDP-β-S on noradrenaline-activated force in rat anococcygeus and guinea pig portal vein. GDP-β-S (100μM) did not produce a contraction and, in rat anococcygeus it did not immediately abolish the noradrenaline-activated contractions. However, it slowed the time course of the contraction in response to noradrenaline immediately after its application and abolished all subsequent responses to noradrenaline. This effect was not reversible. Figure 4.13B shows the effect of GDP-β-S on guinea pig portal vein. GDP-β-S (100μM) did not produce a contraction, but it did inhibit subsequent contractions in response to noradrenaline. This effect was at least partially reversible when GDP-β-S was removed. In summary, GDP-β-S does not produce a contraction on its own in either type of permeabilized muscle. However, it does inhibit further contraction to noradrenaline. This is immediate and reversible in guinea pig portal vein and after one contraction to noradrenaline in rat anococcygeus. The effect of 100μM GDP-β-S was not reversible in rat anococcygeus and only partially in guinea pig portal vein, however, the irreversible effect on anococcygeus might be a result of the irreversible decay in the amplitude of the response to noradrenaline.

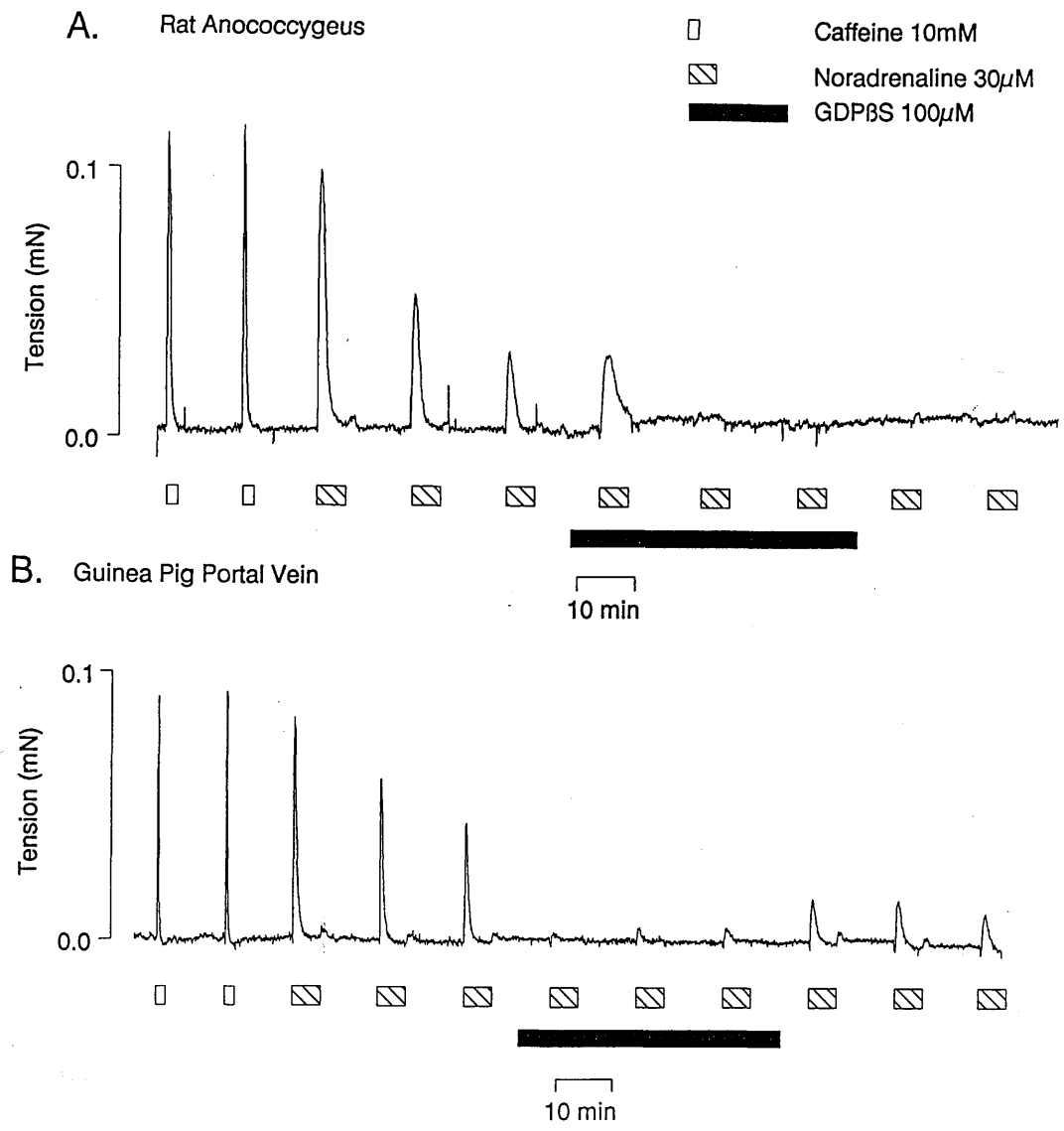


Figure 4.13 shows the effect of 100μM GDP-β-S on the response to noradrenaline in permeabilized rat anococcygeus (Panel A) and permeabilized guinea pig portal vein (Panel B). The application of 10mM caffeine (open bar), 30μM noradrenaline (hatched bar) and 100μM GDP-β-S (filled bar) are indicated.

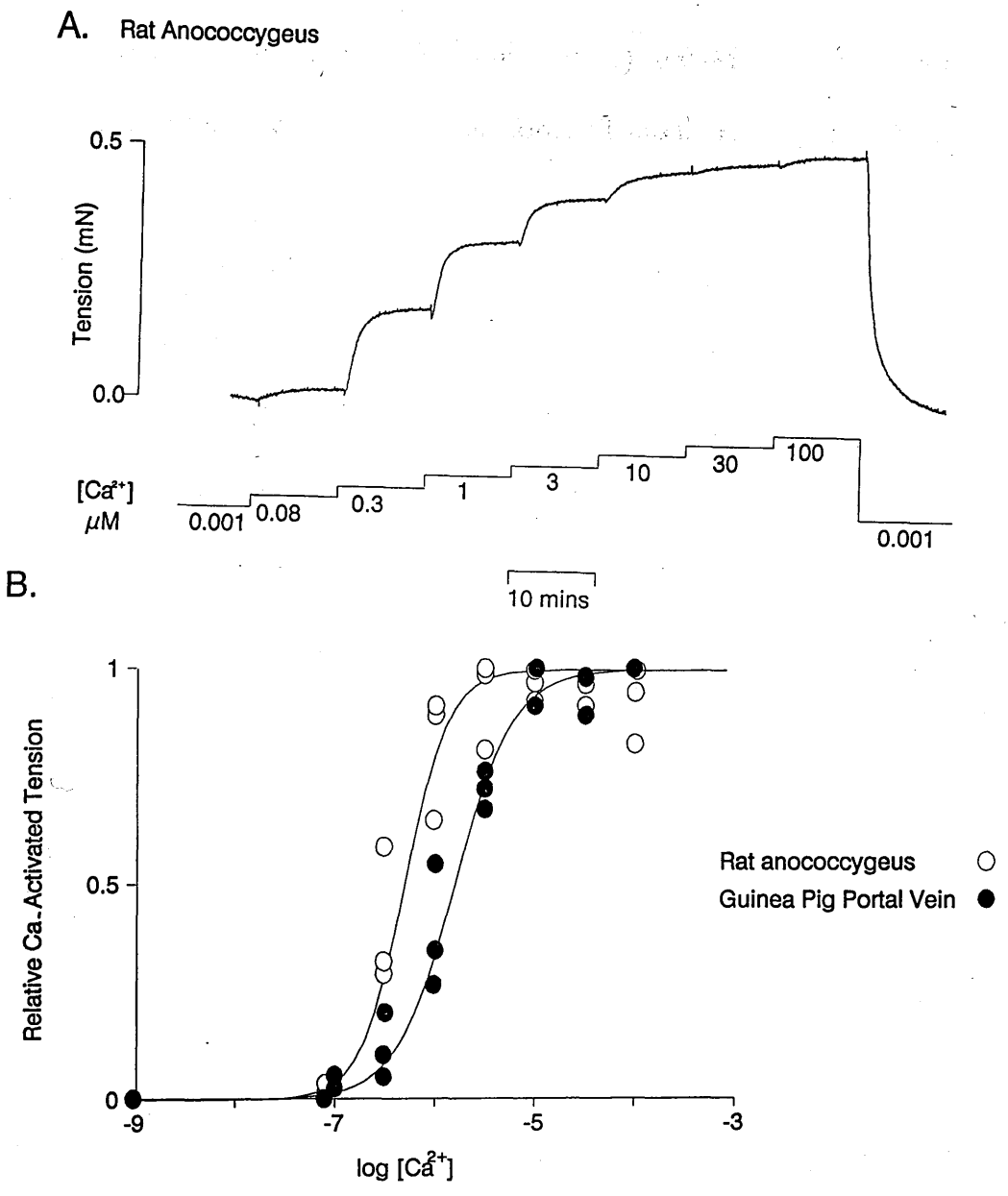
The experiments reported so far have investigated the effect of GTP on the release of calcium from the intracellular store. This next set of experiments examines the effect of GTP and noradrenaline on calcium-activated force. This line of investigation was stimulated by reports in the literature that GTP and agonists which activate the PtdIns cycle increase calcium sensitivity of the contractile proteins (Nishimura *et al*,1988; Kitazawa *et al*,1989; Fujiwara *et al*, 1989; Kobayashi *et al*,1989). Figure 4.14A shows a cumulative concentration response curve to calcium in permeabilized rat anococcygeus. The $[Ca^{2+}]$ was raised in the bathing solution as indicated by the steps underneath the tension record. Tension was allowed to reach a plateau before the next concentration of calcium was added. Figure 4.14B shows the best fit curves for results from three different experiments in permeabilized rat anococcygeus and guinea pig portal vein. Each curve was expressed as a percentage of its own maximum. It can be seen that the two permeabilized muscle types have a similar calcium-sensitivity. However, rat anococcygeus is more sensitive to calcium than portal vein. The mean maximal calcium-activated force produced by muscle strips (approximately 100μM in diameter) from rat anococcygeus muscle was 1.16mN ($\pm$0.35, n=5), compared with only 0.25mN ($\pm$0.14, n=8) in guinea pig portal vein.

Figure 4.15A and B shows the effect of noradrenaline and GTP on rat anococcygeus and guinea pig portal vein respectively whilst the muscle was calcium-activated. The muscles are assumed to be calcium-activated when they

Figure 4.14 shows the sensitivity of toxin-permeabilized smooth muscle to calcium in the presence of 10mM EGTA. Panel A shows a tension record from rat anococcygeus muscle. The $[Ca^{2+}]$ was raised in steps as indicated below the tension record. Panel B shows a graph of $\log_{10}[Ca^{2+}]$ against normalised tension for both permeabilized rat anococcygeus muscle and guinea pig portal vein longitudinal muscle. The data plotted are the steady state tensions from three experiments carried out on each preparation. The solid line represents the best fit curve of the following form of the Hill equation:

$$T/Tmax = (K_m \times [Ca^{2+}])^n / [1 + (K_m \times [Ca^{2+}])^n]$$

Where T/Tmax is a fraction of maximal calcium-activated force (Tmax); K_m is the apparent affinity constant of the myofilaments for calcium. For guinea pig portal vein data, n=2.0 and $K_m = 10^{6.3}$. For rat anococcygeus data, n=1.5 and $K_m = 10^{5.8}$.


A.
Rat Anococcygeus
0.5
Tension (mN)
0.0
[Ca²⁺]
μM
0.001
0.08
0.3
1
3
10
30
100
0.001
10 mins
B.
1
0.5
0
Relative Ca.Activated Tension
-9
-7
-5
-3
log [Ca²⁺]
Rat anococcygeus
Guinea Pig Portal Vein

contract when [Ca^{2+}] is raised in the bathing medium and relax when it is lowered. The effect of noradrenaline and GTP were examined at three different [Ca^{2+}] concentrations: (1) 0.08μM - a concentration just below the threshold for tension; (2) 0.3μM - a concentration which produces about half maximal calcium-activated force in rat anococcygeus and (3) 100μM - a concentration which produces maximal calcium-activated force (Tmax). Each panel shows a typical tension record from an experiment performed in a different muscle for each [Ca^{2+}]. Each muscle was exposed to the [Ca^{2+}] and allowed to equilibrate to it before noradrenaline or GTP were applied.

Figure 4.15A shows that, at the two [Ca^{2+}] which produce tension (0.3 and 100μM), noradrenaline increases calcium-activated force. Tension was observed to fall when the agonist was removed. GTP (100μM) also caused an increase in calcium-activated force at the two concentrations which produced tension. Noradrenaline in the presence of 100μM GTP produces only a further small increase in calcium-activated force.

Figure 4.15B shows that in permeabilized guinea pig portal vein exposure to noradrenaline also causes an increase in calcium-activated force. Initially this is at all three [Ca^{2+}] but, after the first application of noradrenaline increases in calcium-activated force were observed only at the [Ca^{2+}] which produced significant tension. GTP (100μM) causes only a small increase in calcium-activated force in comparison to the effect of noradrenaline. Subsequent noradrenaline produced large effects on calcium-activated force in addition to the effect that GTP has on calcium-activated force.

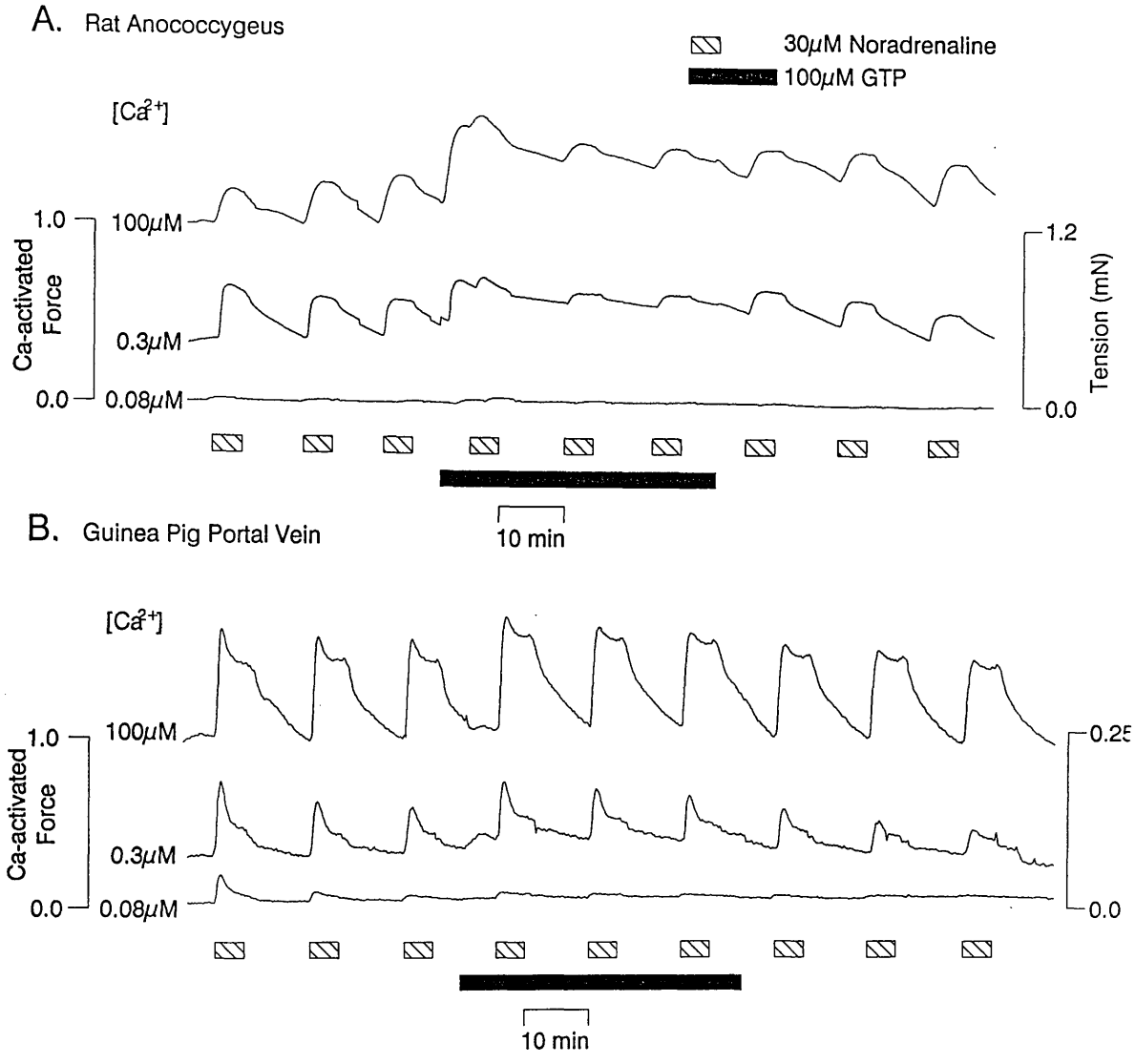


Figure 4.15 shows the effects of GTP and noradrenaline on calcium-activated force in toxin-permeabilized rat anococcygeus muscle (Panel A) and guinea pig portal vein longitudinal muscle (Panel B). The application of 30μM noradrenaline (hatched bar) and 100μM GTP (filled bar). The [Ca^{2+}] that each muscle was equilibrated to is indicated at the side of the experimental tension record.

In summary, noradrenaline increases calcium-activated force reproducibly at two of the [Ca^{2+}] tested in both types of permeabilized muscle. These were the two [Ca^{2+}] which produced calcium-activated force (0.3 and 100μM). The effect of 100μM GTP on calcium-activated force was greater in rat anococcygeus and the contractions produced by noradrenaline in the presence of GTP were greater in guinea pig portal vein. Noradrenaline increased Tmax in both muscle types. GTP, however, only increased Tmax in rat anococcygeus.

THE EFFECT OF NORADRENALINE AND GTP-γ-S ON CALCIUM-ACTIVATED FORCE

The effect of GTP-γ-S (a non-hydrolysable analogue of GTP) on calcium-activated force was examined at one calcium level (0.3μM). GTP-γ-S binds to and therefore activates G-proteins in competition with GTP. However, because GTP-γ-S cannot be broken down by the inherent GTPase activity of the G-protein it activates the protein for as long as it remains bound. Figure 4.16A shows that noradrenaline repetitively increases calcium-activated force in rat anococcygeus. The calcium-activated tension, shown in the tension traces, produced by both muscles was not in this instance, maintained throughout the experiment. This phenomenon was tissue dependent. The direction of the effect of NA and GTP-γ-S was always in the same direction throughout the experiment. GTP-γ-S (100μM) also increases calcium-activated force. Noradrenaline in the presence of GTP-γ-S can further increases calcium-activated force only by a small amount. Figure 4.16B shows essentially the same effect in guinea pig portal vein.

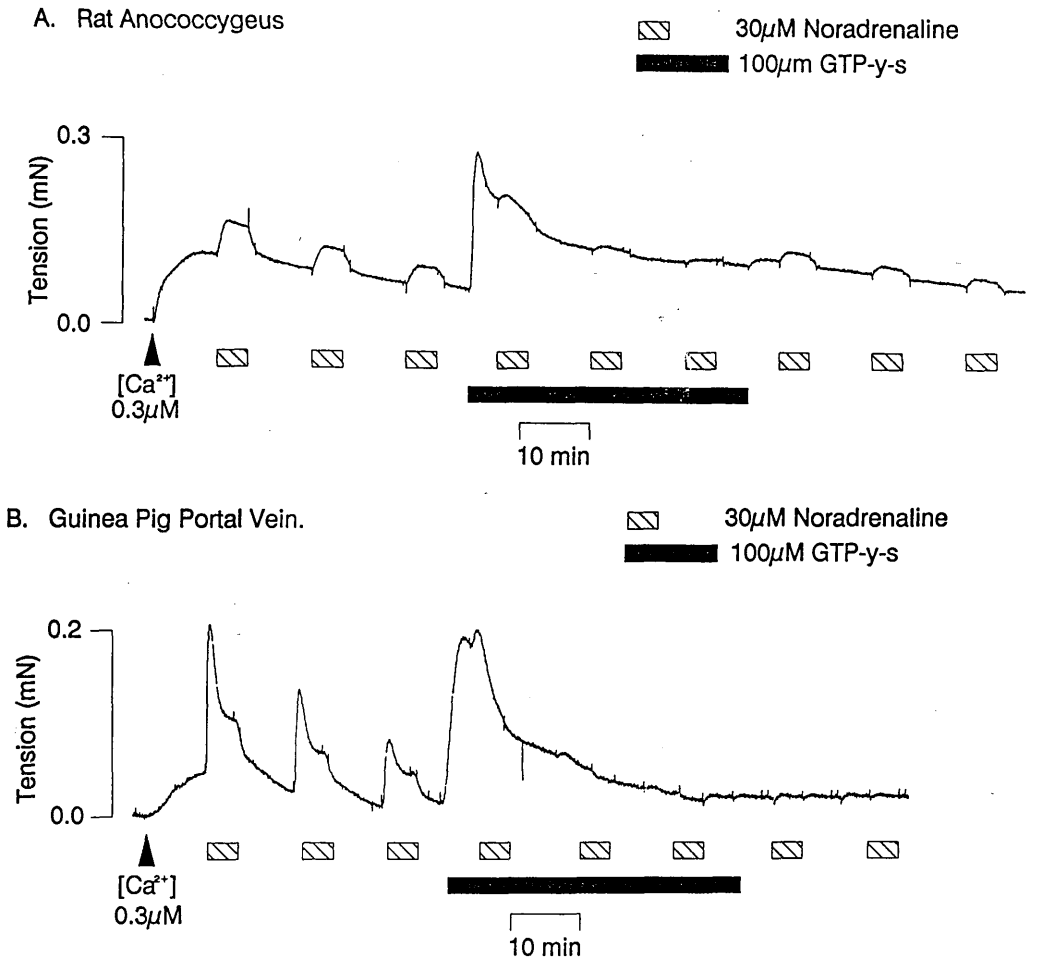


Figure 4.16 shows the effects of GTP-γ-S and noradrenaline on calcium-activated force in toxin-permeabilized rat anococcygeus muscle (Panel A) and guinea pig portal vein longitudinal muscle (Panel B). The application of 30μM noradrenaline (hatched bar) and 100μM GTP-γ-S (filled bar) are indicated. $[Ca^{2+}]$ was raised at the time indicated by the arrow underneath the tension record and was kept at this level throughout the experiment.

In summary, GTP-γ-S appears to increase calcium-activated force to such a extent that there is little further scope to increase the effect by noradrenaline. This is the case for both types of permeabilized smooth muscle.

DISCUSSION

GTP has very different effects on both noradrenaline- and calcium-activated force in the two different types of smooth muscle examined: rat anococcygeus and guinea pig portal vein longitudinal smooth muscle. These differences were:

(1) a different time course of decline in the amplitude of the responses to noradrenaline; furthermore the decline in the response to noradrenaline was reversed in guinea pig portal vein, but not rat anococcygeus;

(2) rat anococcygeus, but not guinea pig portal vein contracted when exposed to GTP;

(3) the decline in the amplitude of the response to noradrenaline was greater in rat anococcygeus than guinea pig portal vein;

(4) GDP-β-S immediately inhibited the response to noradrenaline in guinea pig portal vein, whereas rat anococcygeus was able to contract once more in response to noradrenaline and guinea pig portal vein recovered, but rat anococcygeus did not;

(5) GTP had a greater effect on calcium-activated force in rat anococcygeus than noradrenaline, whereas in guinea pig portal vein the opposite was the case.

Possible reasons for these differences will be discussed below.

POSSIBLE MECHANISMS FOR THE Decline IN THE AMPLITUDE OF THE RESPONSE TO NORADRENALINE

From the experiments that have been undertaken in this study it is known that the decline in the amplitude of the repeated contractions in response to noradrenaline is not due to a reduced calcium content of the intracellular store (Figure 4.9) or a reduced sensitivity of the muscle to Ins(1,4,5)P_3 (Figure 4.10). It is also not likely to be due to a decreased calcium-sensitivity of the contractile proteins, because the responses induced by caffeine and Ins(1,4,5)P_3 are not reduced upon successive exposures. The reduction in the size of the response to noradrenaline could be explained if the amount of Ins(1,4,5)P_3 being released at each challenge of noradrenaline decreased. The amount of calcium being released in response to noradrenaline through Ins(1,4,5)P_3 would thereafter also be decreased. The amount of Ins(1,4,5)P_3 would be reduced if (a) the amount of PtdIns(4,5)P_2 in the membrane was reduced; (b) the supply of GTP was reduced; or (c) if the receptor/G-protein response was reduced.

(a) Reduction in PtdIns(4,5)P_2: The amount of PtdIns(4,5)P_2 would be reduced in the membrane if the permeabilized muscle was unable to reincorporate inositol into the membrane once it had been released as Ins(1,4,5)P_3. A possible protocol to test this hypothesis would be to include myo-inositol in the bathing medium. This mechanism does not explain, however, why the amplitude of the response to noradrenaline declines more quickly in rat anococcygeus than in guinea pig portal vein unless the latter contains higher reserves of PtdIns(4,5)P_2.

(b) Reduced Availability of GTP: Another explanation for the decline in

the size of the response to noradrenaline, which is suggested at least for guinea pig portal vein, is the loss of intrinsic GTP. In guinea pig portal vein the decline was reversed (Figure 4.7) by the inclusion of 100μM GTP in the bathing medium suggesting that it was the lack of this substance which was causing the decline. There is also a similar decline once GTP is removed from the solution. Even in rat anococcygeus, loss of intrinsic GTP might explain its ability transiently to reduce the decline (Figure 4.5). However, as GTP does not completely reverse the effect, then in rat anococcygeus, something other than loss of intrinsic GTP must be responsible for the decline in the amplitude of the response induced by noradrenaline.

(c) Reduction in the receptor/G-protein Activation: The amplitude of the response to repeated agonist challenges would decline if the amplitude of the receptor/G-protein response was reduced after each agonist exposure. That is to say, at each exposure there would be a reduced activation of phospholipase C and hence production of Ins(1,4,5)P_3. There are reports in the literature which suggest that activation of the receptor/G-protein activates PKC (through DG) which phosphorylates the G-protein (Katada *et al*, 1985) and receptor (Berridge, 1987; reviewed by Sagi-Eisenberg, 1989). The phosphorylated receptor/G-protein has a lower activity and, therefore, produces less Ins(1,4,5)P_3 when it is activated *(Baba et al*, 1989). Hence, the amplitude of successive responses to noradrenaline will decline.

A POSSIBLE MECHANISM FOR GTP-INDUCED CONTRACTION

A major difference in the response of the two muscle types was that rat anococcygeus contracted when exposed to GTP, but guinea pig portal vein did not. The response of guinea pig portal vein was as expected. Most other workers using permeabilized smooth muscle preparations have reported that GTP on its own does not cause a contraction (Saida *et al*,1987), but that it potentiates contractions to agonists (Kitazawa *et al*, 1989). For fuller review see the introduction.

Experiments with rat anococcygeus have already shown that this muscle's response to agonists was affected by the presence of ATP in the bathing medium. Figures 4.1 to 4 show that having ATP in the bathing medium blocks the muscle's response to acetylcholine (3mM). ATP is essential to prevent the permeabilized muscle from forming rigor crossbridges. Rat anococcygeus contracts in response to ATP (Gillespie, 1972; Burnstock, Cock & Crow, 1978). P_{2x}-purinoreceptors cause contraction in smooth muscle (Burnstock & Warland, 1987). These can be antagonised by $ANAPP_3$ (Hogaboom *et al*, 1980) or desensitised by alpha-beta-methylene ATP (Kasakov & Burnstock, 1983; Delbro *et al*, 1985).

Alpha-toxin permeabilization preserves functional receptors (Nishimura *et al*, 1988; Kitazawa *et al*,1989) so it has to be assumed that the purinoreceptors remain operational. In the continuous presence of ATP (5mM) the purinoreceptors and the associated G-protein will be chronically activated. Hence, when GTP is present it will provide 'substrate' for the activated

receptor/G-protein which can then cause a contraction by activating phospholipase C and producing Ins(1,4,5)P_3 and DG. Exogenous ATP does not appear to be a significant problem in guinea pig portal vein. This tissue does have purinoreceptors, but they seem to have different characteristics from those in rat anococcygeus.

It has proved to be very difficult to test this hypothesis. This is mainly due to the fact that there are no good antagonists for purinoreceptor responses and alpha-beta-methylene ATP (a desensitising agonist) had no effect on the response. It functions by chronically activating the receptor and eventually desensitizing it to ATP. As described in the results section, a side effect of the presence of ATP appears to be the inhibition of the response to ACh. In a number of experiments alpha-beta methylene ATP was added to the muscle for thirty minutes before the muscle was permeabilized, during the permeabilization procedure and thereafter. Despite this alpha-beta-methylene ATP was not able to block the effect that ATP has on the response to acetylcholine. This might be a consequence of the permeabilization or ATP might be having its effect via another purinoreceptor.

Evidence for the chronic activation of G-proteins in rat anococcygeus muscle comes from the results obtained with GTP-γ-S which activates G-proteins without an agonist present. The responses of permeabilized rat anococcygeus and guinea pig portal vein to this compound are identical and similar to those produced by GTP seen in rat anococcygeus.

POSSIBLE REASONS FOR THE GREATER DECLINE IN THE AMPLITUDE OF THE RESPONSE TO NORADRENALINE IN RAT ANOCOCCYGEUS.

The possible causes of the fall in the size of successive noradrenaline-activated contractures has already been discussed. The most plausible explanation is that the decline is due to desensitisation of the receptor/G-protein as a result of phosphorylation of the proteins by PKC. There are several explanations for the faster decline in the size of the response to noradrenaline in rat anococcygeus compared with guinea pig portal vein: (a) a lack of enzymes which cause the phosphorylation in guinea pig portal vein; (b) a lack of enzymes which cause dephosphorylation in rat anococcygeus; (c) the activation of purinoreceptors by ATP might accelerate the decline in rat anococcygeus, but not in guinea pig portal vein.

(a) Possible lack of enzymes which cause phosphorylation: Perhaps guinea pig portal vein lacks the enzymes which phosphorylate the receptor/G-protein system, but these enzymes are present in rat anococcygeus. Thus the decline observed in guinea pig portal vein could then be explained by the loss of intrinsic GTP alone. This could be partially tested by using a phorbol ester to activate PKC and then examining the effect on the response to noradrenaline. The problem with this experiment would be that activating PKC in this manner not only phosphorylates the receptor/G-protein, but also the contractile proteins, making them more calcium sensitive. Therefore, a more specific agent to phosphorylate the receptor/G-protein would be required.

(b) Possible lack of enzymes which cause dephosphorylation: If rat anococcygeus lacked, or had a reduced amount of, functioning enzymes which caused dephosphorylation, this would also explain the faster decline in the size of the response to noradrenaline in rat anococcygeus compared with guinea pig portal vein. One possible way to examine this would be to study the decline in the presence of okadaic acid, a known blocker of phosphatases (Somlyo *et al*, 1990). This might make the dephosphorylation abilities of the two muscles more comparable.

(c) Possible effect of ATP on the level of phosphorylation: It has already been demonstrated that ATP blocks the response to acetylcholine in rat anococcygeus. ATP might chronically activate a sub-population of G-proteins to enable GTP alone to produce a contraction. The activation of this sub-population of G-proteins might increase basal levels, and subsequently the overall level, of phosphorylation when an agonist is applied. This would imply, however, that the size of the first contraction in response to noradrenaline would be smaller the longer the time that the muscle had spent in the ATP-containing solution. This did not appear to be the case, although it was not systematically studied. Another possibility is that this chronic activation with ATP inhibits the enzymes which normally dephosphorylate the receptor and G-protein.

A POSSIBLE REASON FOR THE EFFECT OF GDP-β-S

Inhibition of an agonist response by GDP-β-S is usually taken as evidence that the agonist mediates its response through a G-protein. Normally this effect is immediate and reversible, as was the case for guinea pig portal vein. The

effect of GDP-β-S on rat anococcygeus, however, was delayed until the muscle had been activated once more by noradrenaline and was not reversible. There is no clear explanation for this effect. However, its basis might lie in the state of the G-protein when GDP-ß-S was added.

The effect of GDP-ß-S is not reversible in rat anococcygeus, but it is in guinea pig portal vein. A possible explanation for this can also be suggested within the frame of the hypothesis. Activation of receptors in both muscle types causes phosphorylation of the receptor/G-protein and so causes the amplitude of successive contractions induced by noradrenaline to decline. Rat anococcygeus appears to have higher rate of phosphorylation which might be related to the chronic activation of the purinoreceptors and associated G-protein. The chronic activation induced by ATP will continue in rat anococcygeus in the presence of GDP-β-S until all the bound GTP has been replaced by GDP-β-S. Hence, phosphorylation of the receptor/G-protein system will also continue making it less likely that this muscle type will recover its ability to respond to noradrenaline when GDP-β-s is removed.

In summary, the basic differences between the two preparations can be explained if their enzymes have different phosphorylation/dephosphorylation activities. Rat anococcygeus's enzymes would appear to have an increased phosphorylation activity or a decreased dephosphorylation activity compared with guinea pig portal vein. This imbalance might be related to the chronic purinoreceptor stimulation. Such chronic stimulation might also explain why the muscle contracts in response to GTP.

A POSSIBLE EXPLANATION FOR GTP's GREATER EFFECT ON CALCIUM-ACTIVATED FORCE IN RAT ANOCOCCYGEUS

The previous section has investigated GTP's effect on calcium release. However, the potentiation of noradrenaline-induced force by GTP could be explained by an increase in the sensitivity of the contractile proteins to calcium. The results from both rat anococcygeus and guinea pig portal vein show that, at the $[Ca^{2+}]$ used in the experiments to examine GTP's effect on calcium release (a $[Ca^{2+}]$ below threshold for tension), noradrenaline and GTP had no significant effect on calcium-activated force. However, at the $[Ca^{2+}]$ s which did cause contraction, noradrenaline and GTP did increase calcium sensitivity and Tmax in both tissues. However, in relative terms the two tissues were affected differently. Calcium-activated force was affected more by noradrenaline in guinea pig portal vein but more by GTP in rat anococcygeus. The result found in guinea pig portal vein was consistent with the published literature (see introduction). The increase in Tmax has been reported only by one other group (Kitazawa *et al*,1989). Phosphorylation of the contractile proteins by PKC has been suggested as the cause of the increased calcium-activated force. Fujiwara *et al* (1989) have reported that, in saponin permeabilized mesenteric artery, there is increased phosphorylation in the presence of GTP-γ-S. However, the authors were not certain that all of the effect could be explained by activation of PKC because TPA (an activator of PKC) has been reported to decrease Tmax (Itoh *et al*, 1986; Itoh *et al*; 1988 and Fujiwara *et al*, 1988) whereas in their hands GTP-γ-S had no effect on maximum calcium sensitivity. Kobayashi *et al* (1989) have proposed another method to account for the increase in

maximum calcium-activated force, suggesting that phosphatases acting on the myosin light chain are inhibited by GTP-γ-S.

Guinea pig portal vein can be made to react in a similar manner to rat anococcygeus by adding GTP-γ-S instead of GTP (Figure 4.16). As has already been discussed, GTP-γ-S is a non-hydrolysable analogue of GTP, which chronically activates the G-protein. This result suggests that, in rat anococcygeus, a sub-population of G-proteins are chronically activated so that GTP acts in a similar manner to GTP-γ-S.

In conclusion, the two tissues, rat anococcygeus and guinea pig portal vein, respond very differently to GTP. This difference includes their response to GTP with respect to both calcium release and calcium-activated force. In guinea pig portal vein GTP does not in itself release calcium, but does potentiate noradrenaline-activated calcium release. In this preparation, GTP has only a small effect on calcium-activated force in comparison with the effect of noradrenaline on calcium-activated force. The results obtained with guinea pig portal vein are consistent with current understanding of the involvement of G-proteins in the agonist transduction system in smooth muscle. Rat anococcygeus, on the other hand, reacted in a novel manner to GTP with respect to both calcium release and calcium-activated force. GTP itself caused a contraction and only transiently potentiated noradrenaline-activated force. It also had a greater effect on calcium-activated force than noradrenaline. Both muscle types could be made to react in the same manner by using GTP-γ-S instead of GTP; that is, both contracted in response to GTP-γ-S itself and

GTP-γ-S had a greater effect on calcium-activated force than noradrenaline. This response is similar to the way GTP affects calcium release and calcium-activated force in rat anococcygeus. This would indicate that permeabilized rat anococcygeus muscle has a population of chronically activated G-proteins in the presence of ATP which affects its response to GTP.

CHAPTER 5

THE EFFECT OF LITHIUM ON AGONIST RESPONSES IN INTACT AND ALPHA-TOXIN PERMEABILIZED SMOOTH MUSCLE FROM RAT ANOCOCCYGEUS.

INTRODUCTION

A. INOSITOL PHOSPHATES

It is over 35 years since Hokin and Hokin (1953) discovered receptor-activated metabolism of inositol lipids. In the following 15-20 years they described most of its key characteristics. However, despite their intense work on this system they were unable to assign a biological function to this widespread biochemical response of cells to stimulation. Michell in 1975 reported that after surveying a large number of tissues showing the phosphatidylinositol (PtdIns) response, he noted that:

(1) the stimulus response mechanism required extracellular calcium, and rises in intracellular [Ca^{2+}] occurred on agonist stimulation;

2) the PtdIns effect was independent or only partially dependent on extracellular [Ca^{2+}] in many tissues; and

(3) in some tissues, calcium ionophores stimulated the physiological response, but not PtdIns breakdown.

These observations indicated that PtdIns is a prerequisite to [Ca^{2+}] elevation in some cells and led to the hypothesis that PtdIns synthesis caused the opening of calcium-gates in the membrane. Further support was given to this hypothesis by Berridge and Fain (1979) who found that stimulation with 5-HT, in blowfly salivary gland, caused PtdIns loss and increased exchange of calcium. At around the same time as this Abdel-Latif, Akhatar and Hawthorne (1977) demonstrated a rapid breakdown of phosphatidylinositol bisphosphate (PtdIns(4,5)P_2) in iris

smooth muscle after muscarinic or alpha-adrenergic stimulation. Subsequently they (Akhatar & Abdel-Latif, 1980) showed that the breakdown was accompanied by the formation of 1-InsP, PtdIns(4,5)P_2 and inositol triphosphate (Ins(1,4,5)P_3). However, these workers did not think that these breakdown products had any significance in calcium mobilization because the response was partially dependent on [Ca^{2+}] and could be mimicked by a calcium ionophore.

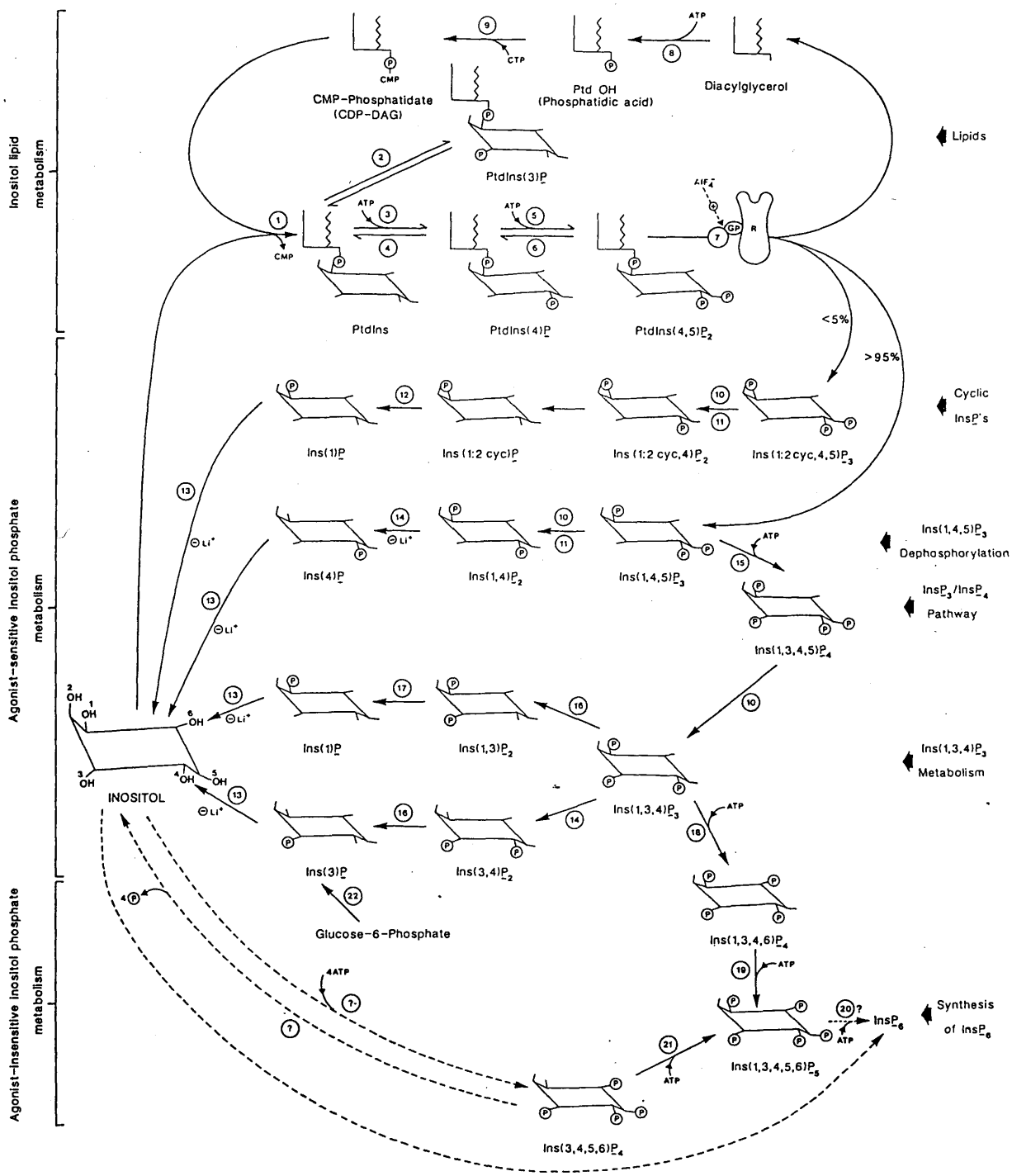
The role of Ins(1,4,5)P_3 was not appreciated until 1983, when Streb *et al* reported that it was able to release calcium from an intracellular, non-mitochondrial store in isolated rat pancreatic acinar cells. They also reported that Ins(1,4,5)P_3 released the calcium from the same store as acetylcholine. Similar observations have since been noted in smooth muscle (Suematsu *et al*, 1984; Somlyo *et al*, 1985; Hashimoto *et al*, 1986). In this tissue Ins(1,4,5)P_3 acts on the sarcoplasmic reticulum to release calcium which subsequently causes contraction.

Much work has been done in trying to understand the PtdIns cycle. There are many breakdown products and branches. These were recently summarised by Berridge and Irvine (1989) and their schematic diagram of the cycle is shown in Figure 5.1. Receptor activation stimulates a G-protein (Gomperts, 1983) which in turn activates phospholipase C which breaks down phosphatidylinositol bisphosphate (PtdIns(4,5)P_2) and gives two main breakdown products:

(1) diacylglycerol (DG) which remains within the membrane plane; and

(2) inositol triphosphate (Ins(1,4,5)P_3) which is released into the cytosol.

Figure 5.1A Summary of known (solid arrows) and suspected (dashed arrows) routes of metabolism of compounds containing inositol and phosphate. Myo-inositol is represented in its chair configuration and all the inositol phosphates are numbered in the D-isomer configuration. The enzymes are (1) PtdIns synthetase (CMP-PA; inositol phosphatidyltransferase); (2) PtdIns-3-kinase; (3) PtdIns-4-kinase (type II); (4) PtdIns(4)P phosphomonoesterase; (5) PtdIns(4)P-5-kinase; (6) PtdIns(4,5)P_2 phosphomonoesterase; (7) phospholipase C; (8) diacylglycerol kinase; (9) CMP-PA synthetase; (10) Ins(1,4,5)P_3/Ins(1,3,4,5)P_4-5-phosphatase; (11) Ins(1,4,5)P_3-5 phosphatase; (12) Ins(1:2cyc)P phosphodiesterase; (13) InsP phosphatase; (14) inositolpolyphosphate-1-phosphatase; (15) Ins(1,4,5)P_3-3-kinase; (16) inositolpolyphosphate-4-phosphatase; (17) Ins(1,3)P_2-3-phosphatase; (18) Ins(1,3,4)P_3-6-kinase; (19) Ins(1,3,4,6)P_4-5-kinase; (20) Ins(1,3,4,5,6)P_5-2-kinase; (21) Ins(3,4,5,6)P_4-1-kinase; (22) Ins(3)P-synthetase. This diagram has been taken from Berridge and Irvine, 1989.


Inositol lipid metabolism
Agonist-sensitive inositol phosphate metabolism
Agonist-insensitive inositol phosphate metabolism
CMP-Phosphatidate (CDP-DAG)
Ptd OH (Phosphatidic acid)
Diacylglycerol
PtdIns(3)P
PtdIns
PtdIns(4)P
PtdIns(4,5)P2
<5%
>95%
Ins(1)P
Ins(1:2 cyc)P
Ins(1:2 cyc,4)P2
Ins(1:2cyc,4,5)P3
Ins(4)P
Ins(1,4)P2
Ins(1,4,5)P3
Ins(1,4,5)P3 Dephosphorylation
InsP3/InsP4 Pathway
Ins(1,3,4,5)P4
Ins(1)P
Ins(1,3)P2
Ins(1,3,4)P3
Ins(1,3,4) P3 Metabolism
INOSITOL
Ins(3)P
Ins(3,4)P2
Glucose-6-Phosphate
Ins(1,3,4,6)P4
Ins(1,3,4,5,6)P5
InsP6
Synthesis of InsP6
Ins(3,4,5,6)P4
Lipids
Cyclic InsP's

Another product formed is cyclic inositol triphosphate (cIP_3). Its function, however, is unclear. The discovery that cyclic inositols are produced in vitro by phospholipase C and the demonstration of its biological activity have been well documented (Majerus *et al*, 1986). Cyclic Ins(1,4,5)P_3 is a more stable compound than Ins(1,4,5)P_3 by the virtue of the fact that it is poorly metabolised by Ins(1,4,5)P_3 phosphatase. It might, therefore, have a role to play in long term control of calcium within cells.

Inositol(1,4,5)triphosphate (Ins(1,4,5)P_3) appears to bind to a receptor on a non-mitochondrial intracellular membrane with the result that a calcium channel opens to release calcium into the cytosol (Streb *et al*, 1983). This has been further verified (Ehrlich & Watras, 1988) and it is now proposed that in smooth muscle Ins(1,4,5)P_3 opens a calcium channel and releases calcium from the sarcoplasmic reticulum. There are two routes for metabolism of this compound. It can either be dephosphorylated by Ins(1,4,5)P_3-5-phosphatase to Ins(1,4)P_2 (Downes, Hawkins & Michell, 1982) or it can be phosphorylated by Ins(1,4,5)P_3-3-kinase to inositol tetrakisphosphate (Ins(1,3,4,5)P_4) (Batty, Nahorski & Irvine,1985). Ins(1,4)P_2 is thought to be inactive, but Ins(1,3,4,5)P_4 is thought to have a calcium regulating role. This compound, however, has little effect on its own. It is thought to act synergistically with Ins(1,4,5)P_3. It was found in sea urchin eggs that Ins(1,3,4,5)P_4 in combination with Ins(2,4,5)P_3 could raise the fertilization envelope. This effect was dependent on both the presence of extracellular calcium and on co-injection with a calcium mobilizing compound (Ins(2,4,5)P_3) (Irvine & Moor, 1986; 1987). Consequently, it was

proposed that $Ins(1,3,4,5)P_4$ was causing calcium entry after $Ins(1,4,5)P_3$ had released the contents of the intracellular store. Morris *et al* (1987) also reported a similar synergism in mouse lacrimal glands. They showed that in single cells, neither $Ins(1,4,5)P_3$ nor $Ins(1,3,4,5)P_4$ alone could activate a sustained calcium current, whereas together they could. Bradford and Irvine (1987) have since demonstrated a specific binding site for $Ins(1,3,4,5)P_4$ on the membrane of HL-60 cells which the authors claim is the intracellular receptor for this compound, thus giving it more credence as an intracellular second messenger.

$Ins(1,3,4,5)P_4$ is metabolised to $Ins(1,3,4)P_3$ by $Ins(1,4,5)P_3$/$Ins(1,3,4,5)P_4$-5-phosphatase. This is also thought to be an inert compound physiologically. It can, however, release calcium from the intracellular store but it is 30 times less potent than $Ins(1,4,5)P_3$ (Irvine *et al*, 1986). Its slow rate of formation and removal after termination of the stimulus (Berridge & Irvine, 1984) suggest that any possible function is over a comparatively long time scale.

Two other inositol phosphates have been recently identified - $InsP_5$ and $InsP_6$. However, it is thought that their metabolism occurs through pathways which are largely separate from the agonist-sensitive pathway. A pathway of synthesis for $InsP_5$ might exist from the agonist-sensitive inositol phosphates through the phosphorylation of $Ins(1,3,4)P_3$ to $Ins(1,3,4,6)P_4$ and thence to $Ins(1,3,4,5,6)P_5$ (Stephens *et al*, 1988). However, the main route of synthesis of this inositol phosphate is probably through $Ins(3,4,5,6)P_4$. The origin of this compound is unknown. It might be formed by phosphorylation of inositol or

from Ins(1,4,5)P_3 (Berridge & Irvine, 1989). $InsP_6$ is probably formed by phosphorylation of inositol. Both these compounds exist within cells at quite high concentrations (millimolar) (Martin *et al*, 1986). However, because their concentration changes only very slowly after receptor activation (Tilly, 1987) it is thought that these compounds do not have an acute second messenger role. There is a possibility that they do have a longer-term role in controlling the intracellular environment.

Diacylglycerol is the other breakdown product of PtdIns(4,5)P_2. It is retained within the membrane and functions as a second messenger by activating protein kinase C (PKC), but only in the presence of calcium (Nishizuka, 1984). Stimulation of protein kinase C by phorbol esters (which are thought to mimic the effect of DAG on protein kinase C) has been shown to trigger contraction in smooth muscle from various arteries (Rasmussen *et al*, 1984; Baraban et al, 1985; Danthuluri & Deth, 1984). Protein kinase C is responsible for phosphorylating proteins, although these have been difficult to identify. It has been shown, however, that protein kinase C does phosphorylate myosin isolated from smooth muscle (Endo, Naka, & Hidaka, 1982) and there is some evidence that, in conjunction with myosin light chain kinase (MLCK), protein kinase C might modulate contraction of smooth muscle (Umekawa *et al* 1985). There have also been reports in skinned smooth muscle that protein kinase C increases the calcium sensitivity of the contractile proteins (Itoh *et al*, 1986; 1988; Fujiwara *et al*, 1988). Protein kinase C has also been identified as the kinase which phosphorylates active agonist receptors (Llano & Marty, 1987) and G-proteins

(Sagi-Eisenberg, 1989) causing them to be inactivated. This would give DG a role in switching off the agonist-second messenger response. Protein Kinase C also appears to have a function in controlling intracellular [Ca^{2+}]. It might interact with voltage dependent calcium channels to facilitate calcium entry. This has been shown in Aplysia neurones (Deriemer *et al*,1985), Hermissenda photoreceptors (Farley & Auerbach, 1986) and rat adrenal medulla (Wakade, Malhotra & Wakade, 1986). Protein kinase C might also activate the pumps that remove calcium from the cytosol (Drummond, 1985).

The breakdown products of DG might also have cellular effects. Diacylglycerol can be converted to arachidonate which can be converted to prostaglandins, leukotrienes and thromboxanes. These are collectively called eicosanoids and are potent regulators of various physiological responses. Arachidonate and its eicosanoids have been shown to increase in concentration after agonist-induced increases of inositides. This topic is reviewed by Rana and Hokin, (1990).

The primary soluble by-products of PtdIns(4,5)P_2 - Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3 - are subsequently dephosphorylated through a number of reactions back to inositol. Diacylglycerol, the insoluble product of PtdIns(4,5)P_2, is converted by diacylglycerol kinase, to phosphoatidic acid and then to CMP-phosphatidate. This can be combined with inositol by PtdIns synthetase to form PtdIns. Further phosphorylation of this compound, which is now within the membrane plane, reforms the original substrate - PtdIns(4,5)P_2.

B. LITHIUM

Lithium was first reported as a treatment for manic depressive patients in 1949 by J.F.J. Cade. However, its method of action was unknown. One possible explanation was suggested by Allison and Stewart (1971) who reported that a single intraperitoneal injection of LiCl (10meq/kg) in rats caused a 30% reduction in cerebral cortex inositol. They later reported that this could be blocked by co-administering atropine or scopolamine with the lithium (Allison & Blisner, 1976). In the same year Allison *et al* (1976) showed that myo-inositol 1-phosphate (Ins1P) was also elevated in rats treated with lithium and to a greater extent than inositol. It was soon discovered that lithium inhibited inositol monophosphatases (Naccarato, Ray & Wells, 1974). This was confirmed at a more physiological concentration (1mM as opposed to 250mM) by Hallcher and Sherman (1980). Lithium treatment, therefore, prevents the conversion of any of the InsP to inositol. Lithium has also been found to inhibit at least one other phosphatase in the breakdown pathway, namely inositolpolyphosphate-1-phosphatase (Berridge *et al*, 1983; Storey *et al*, 1984; Burgess *et al*, 1985). Inhibition of this enzyme prevents the conversion of $Ins(1,4)P_2$ to Ins(4)P and of $Ins(1,3,4)P_3$ to $Ins(3,4)P_2$. Normally there are three main routes for the supply of inositol:

(1) recycling of PtdIns;

(2) de novo synthesis from glucose-6-phosphate; and

(3) the diet.

The first two of these possible routes are inhibited by lithium. Thus when the PtdIns cycle is repetitively or continually stimulated in the presence of LiCl the level of inositol will decrease within the cell and the response to the agonist will, therefore, also decrease. It cannot be regained by de novo synthesis because this involves the monophosphatases which are blocked by lithium. The only means of maintaining inositol is for it to come from the diet. Berridge *et al* (1983) proposed that, because the brain does not have access to dietary inositol, this might explain why lithium treatment is selective for the brain in manic depressive patients.

There have been reports of lithium affecting other sites in the agonist transduction mechanism. Lithium has been reported to decrease the number of new agonist receptors incorporated into skeletal muscle membranes (Pestronk & Drachman, 1987). However, this process is also influenced by the intracellular [Ca^{2+}] and so might be a secondary effect of the inhibition of the PtdIns cycle. Avissar *et al* (1988) have also reported that lithium blocks the G-proteins involved in activating both the PtdIns cycle and adenylyl cyclase.

C. PURPOSE OF STUDY

The aim of this study was to examine the effect of chronic lithium treatment on different agonist responses in intact rat anococcygeus to try and analyse if these agonists were producing their effect via the PtdIns cycle. The effect of lithium was examined on:

(1) concentration response curves to noradrenaline, phenylephrine, acetylcholine and 5-HT;

(2) noradrenaline, phenylephrine and acetylcholine responses in low calcium Krebs solution (0.1μM); and

(3) finally the effect of short term application of LiCl on responses to noradrenaline-, caffeine- and calcium-activated force was investigated in alpha-toxin permeabilized anococcygeus muscle.

As has already been stated, lithium is thought to have its effect on the PtdIns cycle by blocking the enzymes which breakdown Ins(1,4,5)P_3 to inositol. This, therefore, runs down the available inositol within the cell. To try and prevent this effect of lithium, myo-inositol was fed to the rats at the same time as they were being injected with lithium. The response to the different agonists in these muscles was also examined. The acute effects of lithium and myo-inositol were also examined in alpha-toxin permeabilized rat anococcygeus muscle.

MATERIALS AND METHODS

A. INTACT MUSCLE

ANIMAL PRETREATMENT

Lithium chloride (6.8mmoles/kg) was injected intraperitoneally on a daily basis for four consecutive days. Eighteen hours after the final injection the rats were sacrificed and the tissue isolated. During the four day course of injections the animals were given 0.45% saline ad libitum. The 'LiCl plus myo-inositol' treated group was injected daily, as before. In addition, myo-inositol 10mM was administered in the 0.45% saline. This is essentially the same protocol as that used by Eglen *et al* (1987).

EXPERIMENTAL PROCEDURE

The rat anococcygeus muscles were isolated and set up for experimentation as described in Chapter 2 (intact muscle section). The solutions used are detailed in Table 2.1 in Chapter 2.

PROTOCOL FOR EXAMINING INTRACELLULAR RELEASE USING LOW CALCIUM (0.1μM) KREBS

In an attempt to examine the intracellular release of calcium the muscle was bathed in a low calcium Krebs solution (buffered with EGTA to approximately 0.1μM calcium). This external [Ca^{2+}] is below the level of calcium needed to permit calcium entry, hence any contraction produced by the

agonist is thought to be due to release of intracellular calcium. The following protocol has been devised to examine this release which has been called the intracellular component (ITC). Step 1 is proposed to allow the intracellular store to load; if this step is missed out then no contraction can be induced. Step 2 lowers the extracellular $[Ca^{2+}]$ to 0.1μM. Step 3 is the agonist challenge step and Step 4 lowers the $[Ca^{2+}]$ again and washes out the agonist before the store is reloaded.

(1) Wash with 1.25mM calcium Krebs (10 minutes): twice at 0 minutes and once at 5 minutes.

(2) Wash with low calcium Krebs (0.1μM) (10 minutes): twice at 0 minutes, once at 3 minutes and once at 9 minutes.

(3) Challenge with agonist in low calcium Krebs (0.1μM) (5 minutes)

(4) Wash with low calcium Krebs (0.1μM) (10 minutes): twice at 0 minutes, once at 3 minutes, once at 5 minutes and once at 7 minutes.

B. PERMEABILIZED MUSCLE

EXPERIMENTAL PROCEDURE

Strips of rat anococcygeus muscle were isolated and set up for experimentation as described in Chapter 2 (Permeabilized Muscle). The muscle was permeabilized as described in Chapter 3 (Materials and Methods). The solutions used are detailed in Table 2.3. The potassium salt used was potassium propionate. Lithium chloride was added from a 1M stock to the solutions to obtain a final concentration of 10mM. The effect that this might have on ionic

strength was examined by looking at the effect of adding 10mM sodium chloride. Myo-inositol and sucrose were added as solids to the solutions.

Figure 5.2A shows a typical example of a concentration response curve (CRC) obtained with noradrenaline. All the CRCs reported in this chapter were performed in the same manner: the muscle was allowed to equilibrate to each new concentration before the next concentration was added in a cumulative fashion. The agonist concentration was increased by half logarithmic units. Figure 5.2B shows a typical response to a maximal dose of noradrenaline (30μM) in a muscle which has been incubated in low calcium Krebs (0.1μM). The calcium buffer EGTA was used to maintain the [Ca^{2+}] in the solution at 0.1μM. This concentration is low enough to prevent entry of extracellular calcium playing a significant role in producing a contraction. Thus any contraction produced in response to noradrenaline is assumed to be due to release of intracellular calcium. This contraction is called the intracellular component (ITC). Figure 5.3A shows the mean of six experiments for six repeat contractions to noradrenaline in the low calcium Krebs (0.1μM). It is only after the third contraction that the muscle contracts reproducibly. These same six muscles were examined to determine whether the left and right 'legs' of the tissues produced comparable sized contractions (Figure 5.3B). Each point is the mean of four consecutive contractions. It can be seen that in only one out of six cases the paired muscles are significantly different (Student's t-test). This

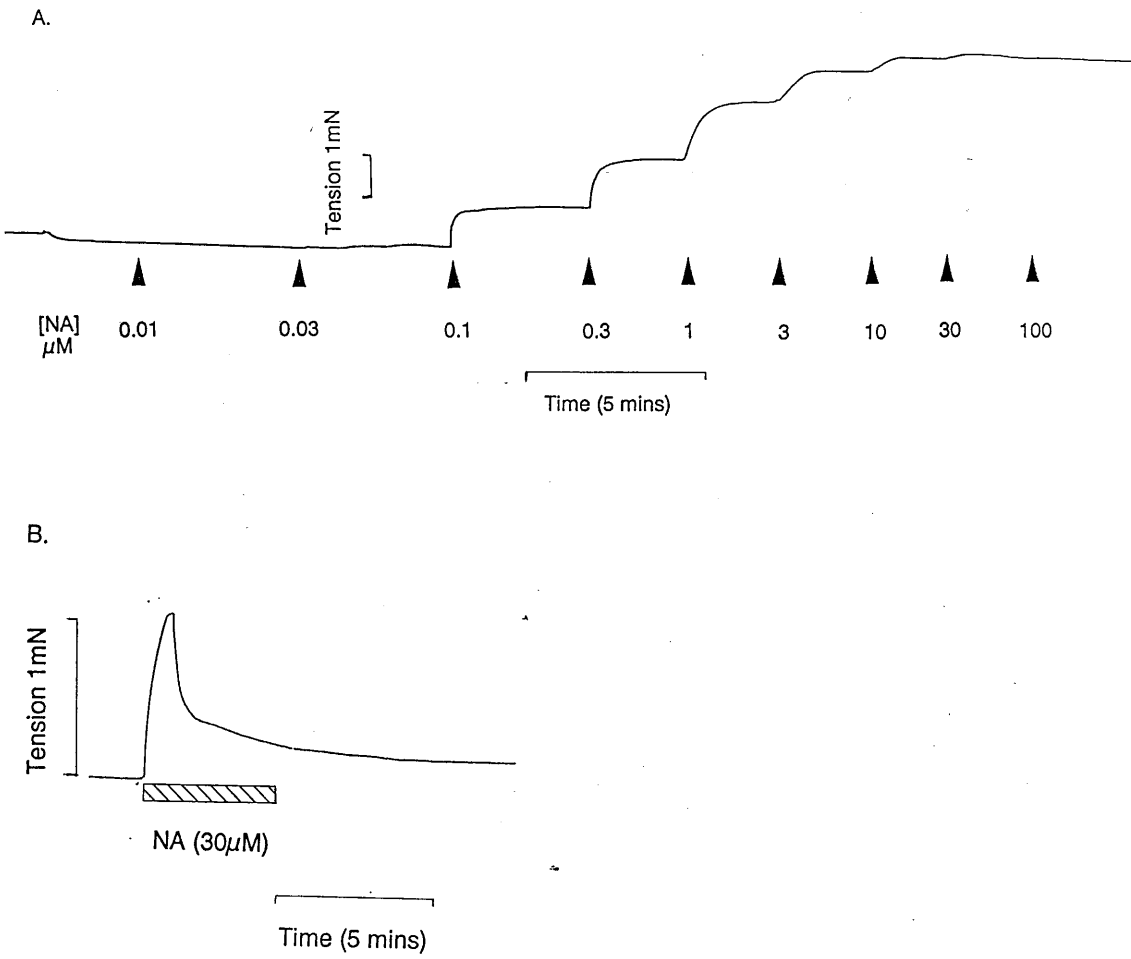


Figure 5.2A. shows a typical cumulative concentration response curve (CRC) in response to noradrenaline (NA) in rat anococcygeus. The agonist concentration is increased in half logarithmic units. The next concentration of agonist was added when the muscle had equilibrated to the previous concentration. Figure 5.2B. shows a typical effect of 30μM noradrenaline from rat anococcygeus bathed in a low calcium Krebs (0.1μM) solution (composition is detailed in Chapter 2, Table 2.1).

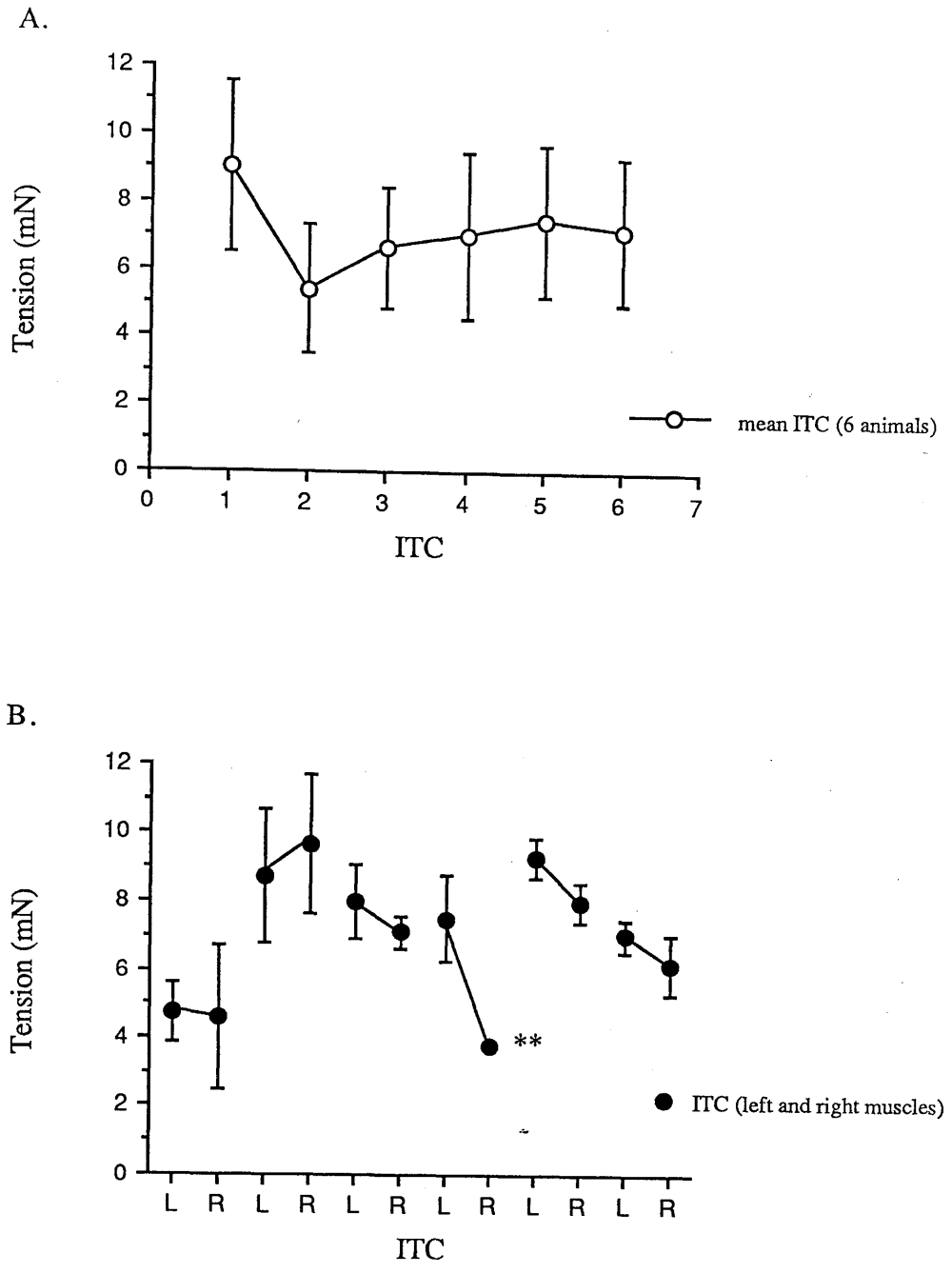


Figure 5.3A. shows the average size of six consecutive contractions in low calcium Krebs (0.1μM) from six animals and therefore twelve tissues. Figure 5.3B. shows the average size of the last four contractions shown in Figure 5.3A as a comparison of the contraction size in the Left (L) and right (R) 'legs' of the muscle. The results are shown for each of the six animals as the mean±S.D. of the four tests. (**p=0.05)

result is important because, when the lithium experiments were undertaken, one 'leg' was examined for LiCl's effect on the CRC and the other for the effect on the ITC.

The magnitude of the ITC was found to be variable. This was especially so when the time in low calcium Krebs (0.1μM) was altered (this is after the muscle had been bathed in 1.25mM calcium Krebs for a fixed time to load the calcium stores). Figure 5.4 compares within one muscle the effect on the ITC of varying the time in low calcium Krebs (0.1μM) after the stores have been allowed to load. Leaving the muscle for 15 minutes dramatically reduces the ITC and leaving it for 5 minutes increases its size. However, not only is the size increased, but a second component is introduced. The second component can be abolished if the muscle is incubated in nifedipine (1μM). Figure 5.5 shows the size of the ITC after 10 minutes in low calcium Krebs (0.1μM), 5 minutes in low calcium Krebs (0.1μM) and 5 minutes in low calcium Krebs (0.1μM) in the presence of nifedipine (1μM). In the five muscles examined, incubation for five minutes increased the ITC and introduced an extra component in three of the muscles. This extra component was abolished by nifedipine indicating that it was due to calcium entry via voltage operated calcium channels (Spedding, 1987). The size of the peak was also reduced making it more like the ITC obtained after 10 minutes incubation. It would appear, therefore, that incubating in EGTA for 5 minutes is not a long enough time to reduce the extracellular [Ca^{2+}] to such a level that the ITC is due only to intracellular

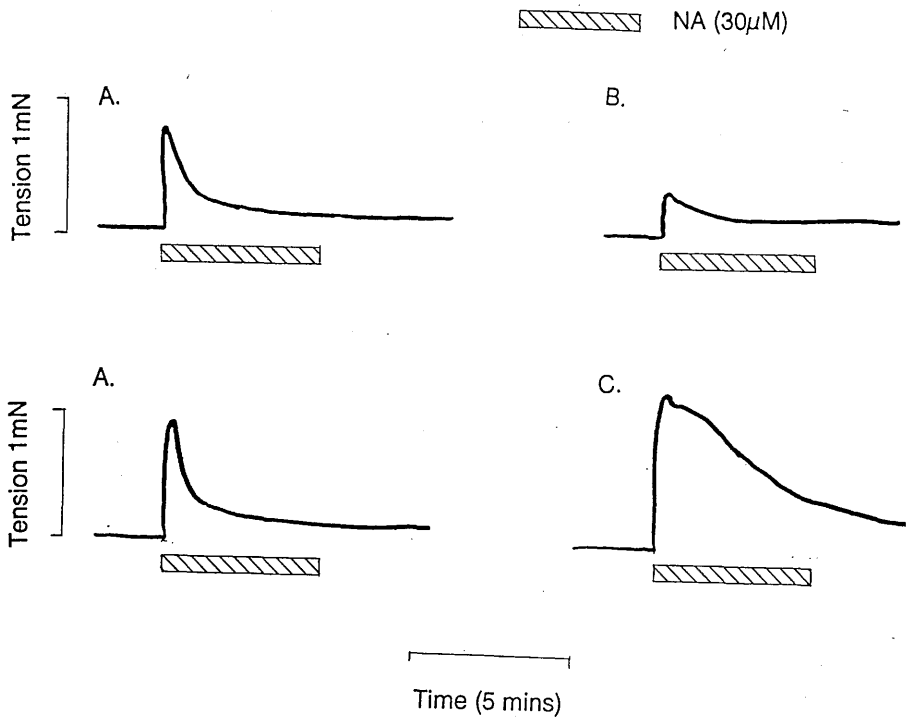


Figure 5.4 shows typical examples from one animal of responses to 30μM NA in low calcium Krebs (0.1μM). The contractions are compared after different incubation times in low calcium Krebs (0.1μM) and hence various times since the intracellular store was loaded: (A) 10 minutes; (B) 15 minutes; (C) 5 minutes.

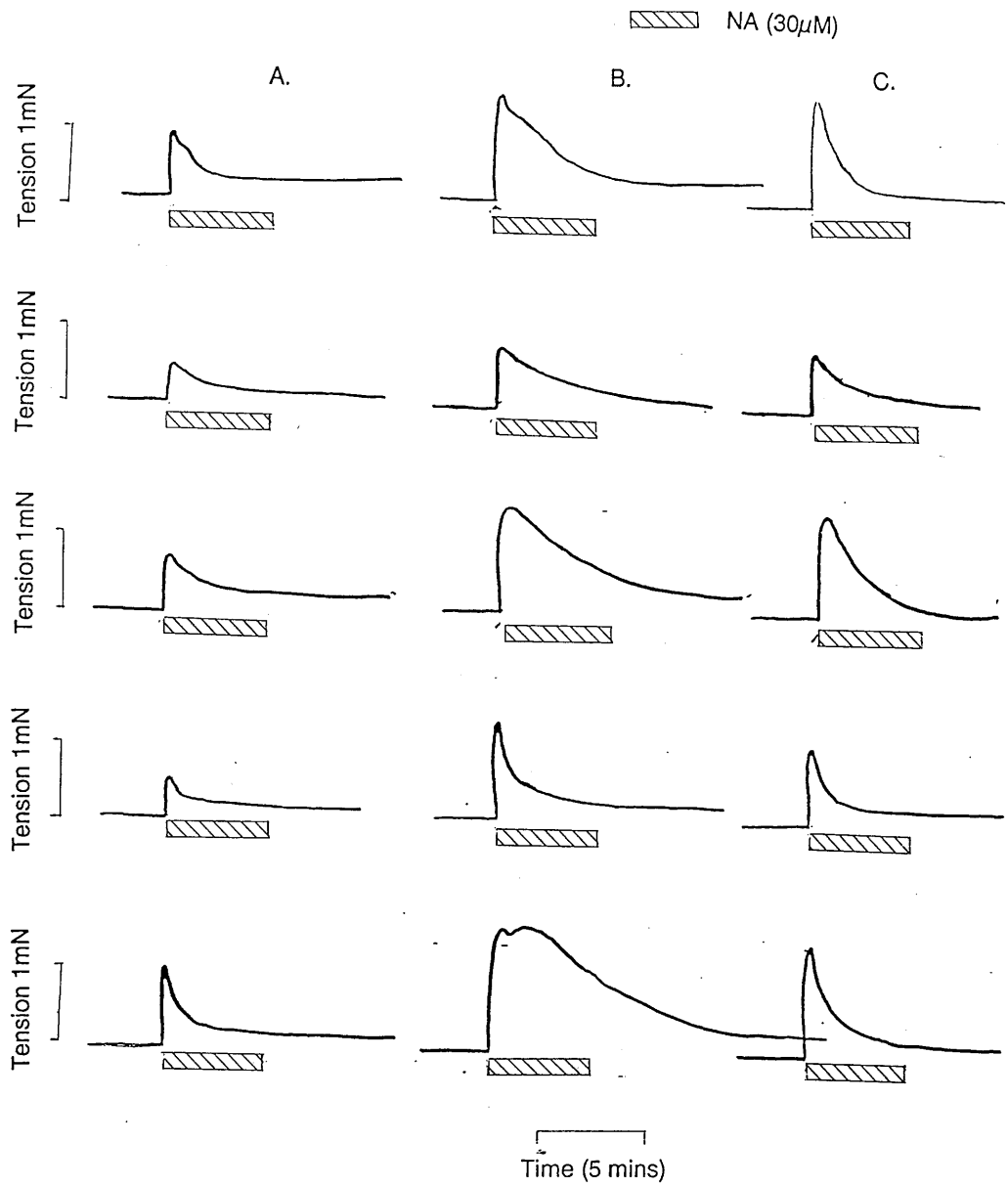


Figure 5.5 shows the contractile response to NA in low calcium Krebs (0.1μM) in five different muscles. The contractions are compared after different incubation times in low calcium Krebs (0.1μM) and hence various times since the intracellular store was loaded: (A) 10 minutes; (B) 5 minutes, and (C) 5 minutes with 1μM nifedipine throughout the loading period.

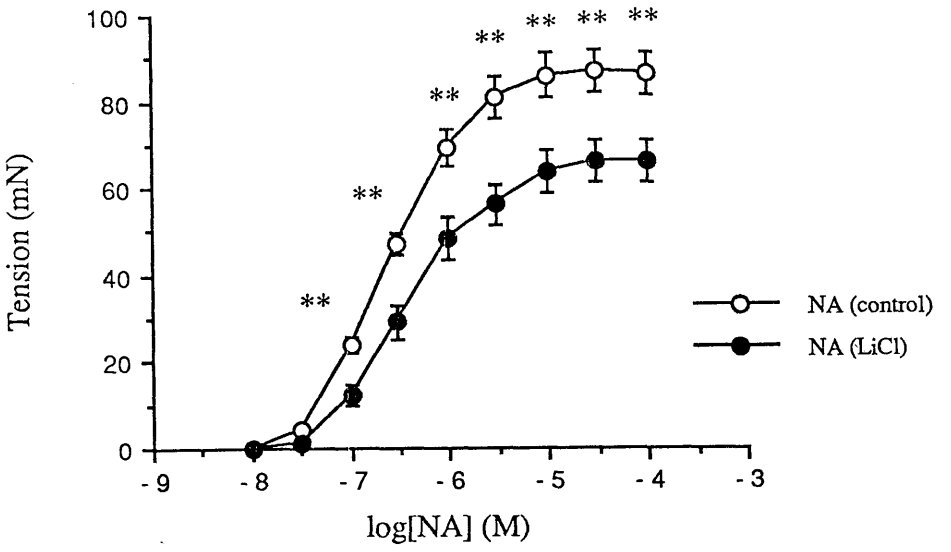
release. Hence, for the rest of the experiments examining the ITC the incubation time was set at 10 minutes.

It has been proposed that lithium blocks the PtdIns cycle. In an attempt to examine this proposal the responses of rat anococcygeus to a variety of agonists were examined and compared with those obtained in rats chronically treated with LiCl.

EFFECTS OF LITHIUM ON THE CRC

Figure 5.6 shows the effect of chronic treatment with LiCl on: (A) noradrenaline CRC, (B) phenylephrine CRC, (C) Acetylcholine CRC and (D) 5-HT CRC. The curves were undertaken in the following order 5-HT, ACh, NA and PhE. LiCl treatment appears to significantly suppress the responses to each of the agonists. However, the only agonist in which the sensitivity is affected is PhE and even in this case there is no significant difference in the PD_2 values (Student's t-test) (Figure 5.7). Sensitivity was examined after the curves had been normalised to their own maximum. Only tissues which had reached a clearly defined maximum were examined for an effect on sensitivity. Hence 5-HT CRC's were not analysed for the effect of LiCl on sensitivity.

A.



B.

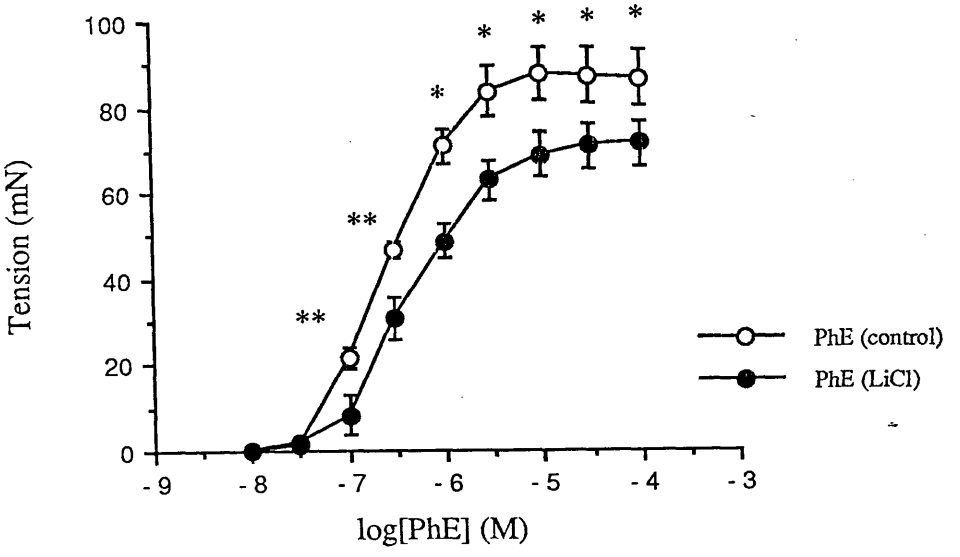
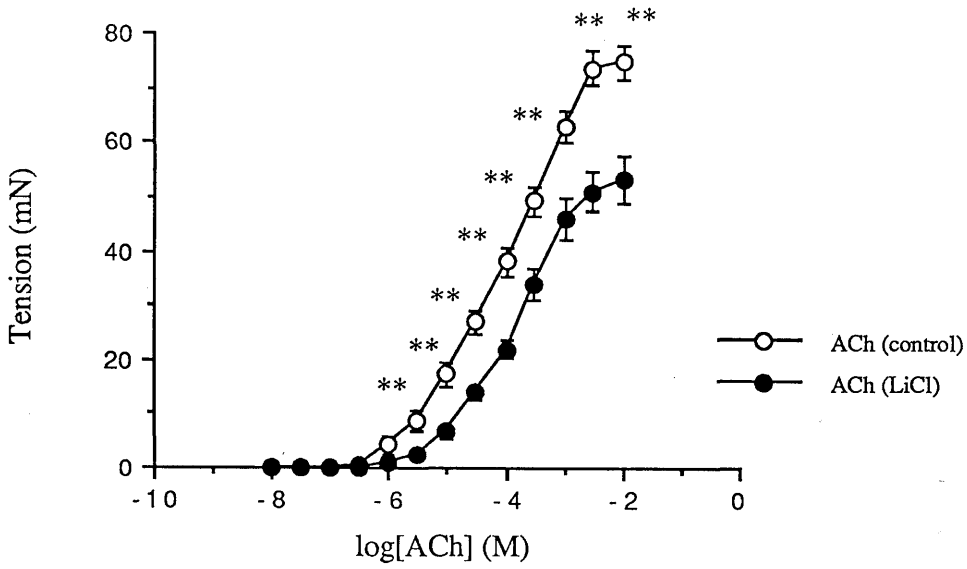


Figure 5.6 shows mean CRCs in response to (A) NA, (B) PhE, (C) ACh and (D) 5-HT. The CRCs have been performed in tissues taken from rats which have (closed circles) and have not (open circles) been chronically treated with LiCl. Details of the LiCl treatment are give in the Materials and Methods of this chapter. Results shown are the mean±s.e.m. (n=6-12). Significance was tested using an unpaired Student's t-test. (* $p<0.05$ **$p<0.01$).

C.


Tension (mN)
80
60
40
20
0
-10
-8
-6
-4
-2
0
log[ACh] (M)
**
ACh (control)
ACh (LiCl)

D.

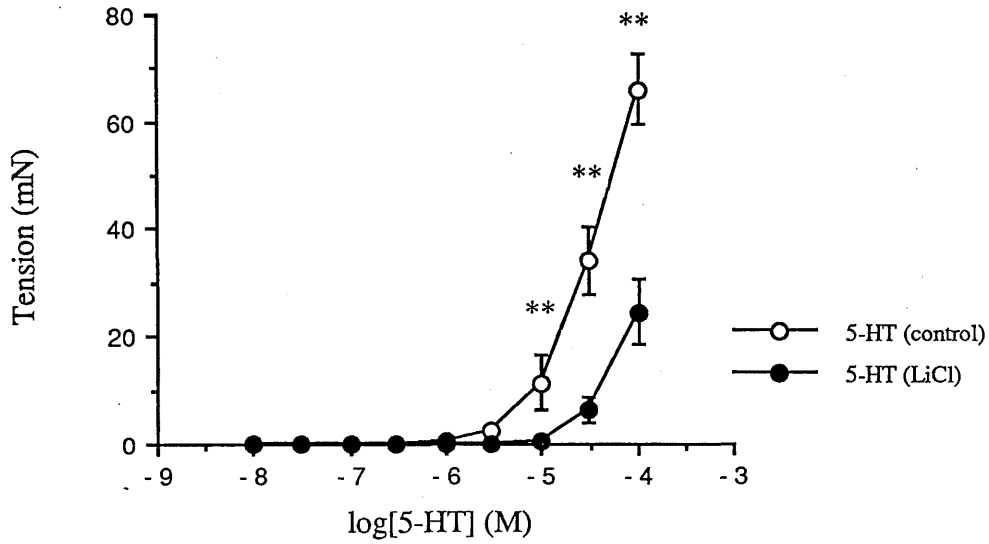
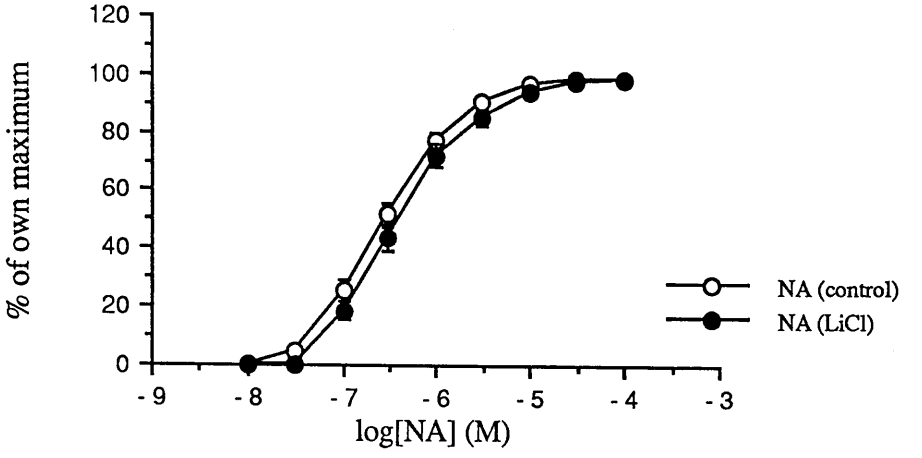
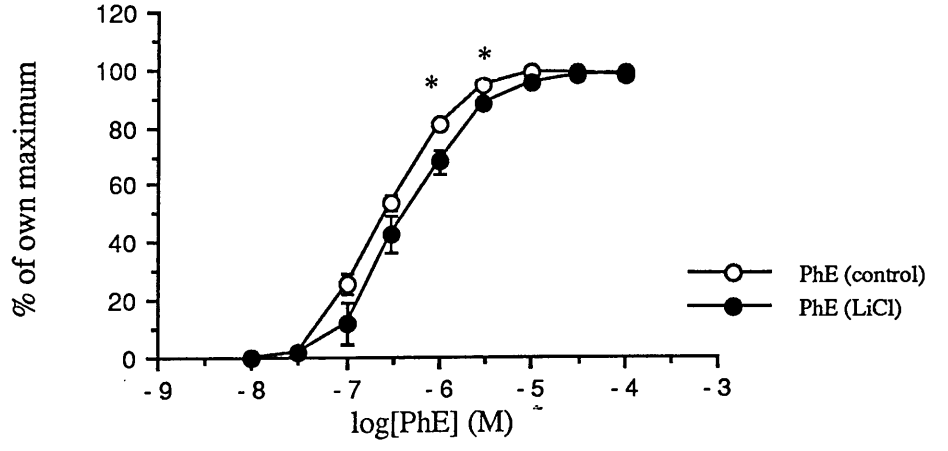

Tension (mN)
80
60
40
20
0
-9
-8
-7
-6
-5
-4
-3
log[5-HT] (M)
**
5-HT (control)
5-HT (LiCl)

Figure 5.7 shows the normalised results from the CRCs to (A) NA, (B) PhE and (C) ACh. Only curves from muscles which reached a clearly defined maximum were included and each curve was expressed as a percentage of its own maximum. Closed circles denote muscles which have been harvested from rats which were chronically LiCl treated and the open circles muscles from untreated rats. The PD_2 values were calculated for the three agonists under each of the conditions and were found not to be significantly different. (mean±s.e.m., * $p<0.05$)

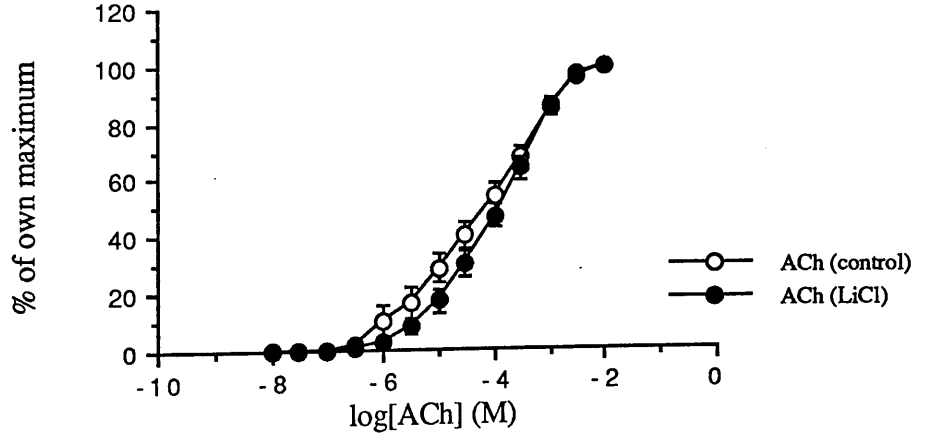
A.


% of own maximum
120
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
-3
log[NA] (M)
NA (control)
NA (LiCl)

B.


% of own maximum
120
100
80
60
40
20
0
*
*
-9
-8
-7
-6
-5
-4
-3
log[PhE] (M)
PhE (control)
PhE (LiCl)

C.


% of own maximum
120
100
80
60
40
20
0
-10
-8
-6
-4
-2
0
log[ACh] (M)
ACh (control)
ACh (LiCl)

Since LiCl treatment is supposed to cause the run down of inositol in the cytosol (Berridge,1983) the effect of administering myo-inositol in the water of the rats at the same time as they are being treated with LiCl was examined. The rats freely drank the saline with the myo-inositol since the LiCl has a natriuretic action. Figure 5.8 shows that co-administering the myo-inositol significantly blocks the inhibitory action of LiCl on (A) NA, (B) PhE and (C) ACh. The effect was not examined for 5-HT because the contractile response proved to be unreliable in all the tissues examined. Co-administering myo-inositol at the same time as LiCl did not have any effect on the sensitivity of the muscles to the three agonists (Figure 5.9) Myo-inositol appears to prevent the effect of LiCl to such an extent that the CRCs are returned towards control levels (Figure 5.10). The responses to NA and PhE in the muscles from the rats which have been treated with LiCl plus myo-inositol are greater than the control, but the increase is not significant. The response induced by ACh is comparable to the control. No standard error bars have been shown for the ACh CRC because only three muscles were examine.

The manner in which these experiments were carried out imposes restrictions on the analysis. That is, these experiments were undertaken as two groups: (1) responses to agonists in tissues which have been harvested from chronically LiCl-treated animals compared with responses to agonists in control tissues and (2) responses to agonists in tissues which have been harvested from chronically LiCl-treated animals compared with responses to agonists in tissues which have been harvested from chronically LiCl plus myo-inositol treated

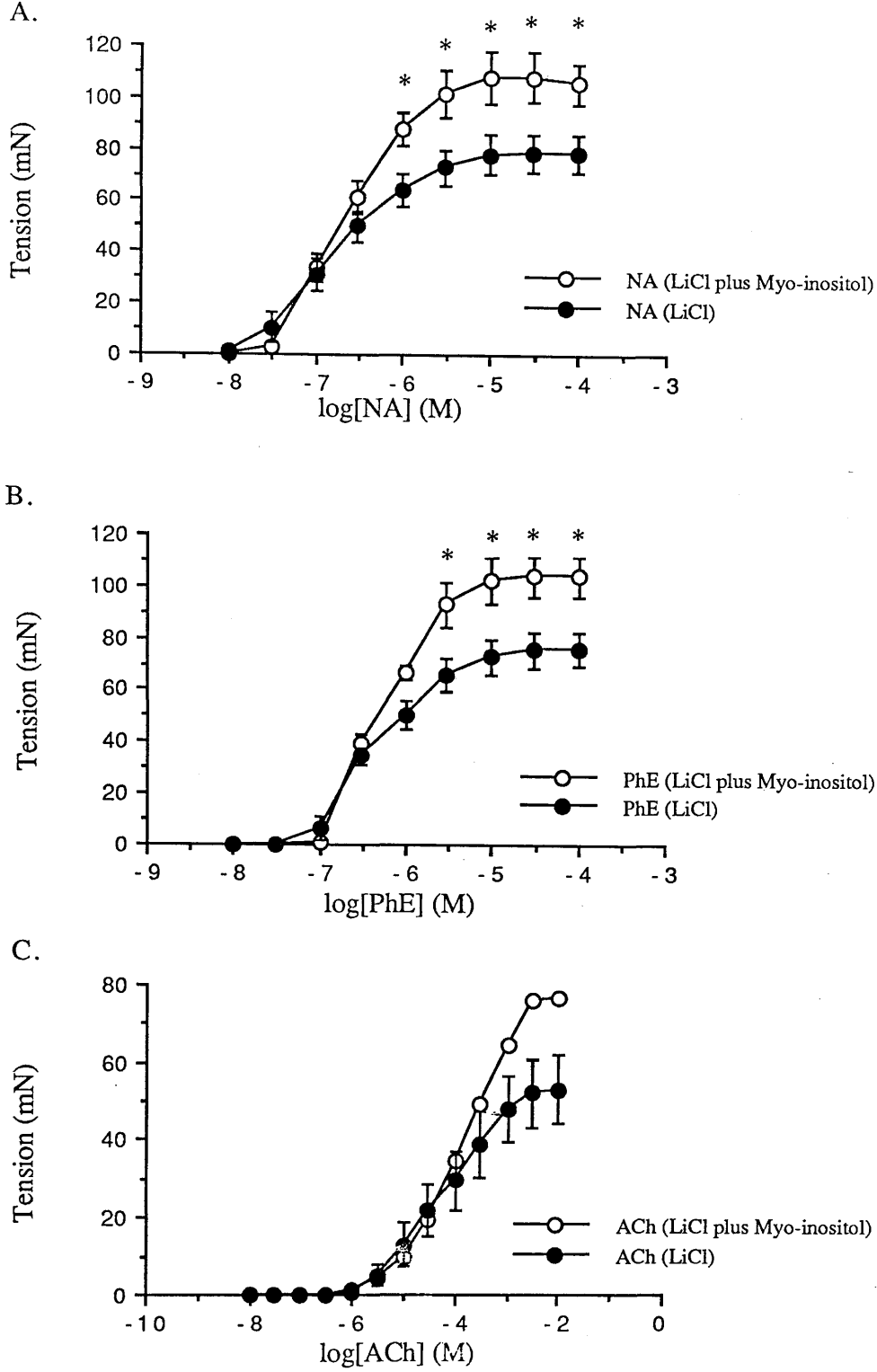
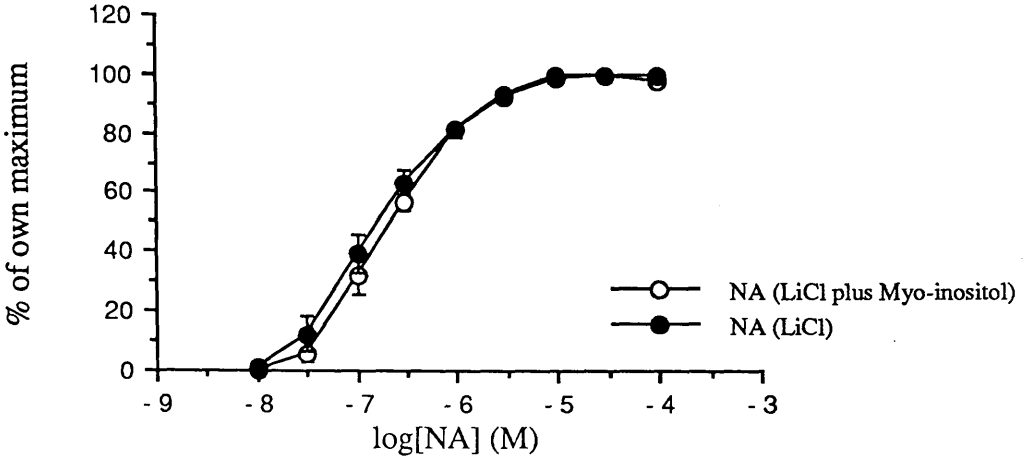


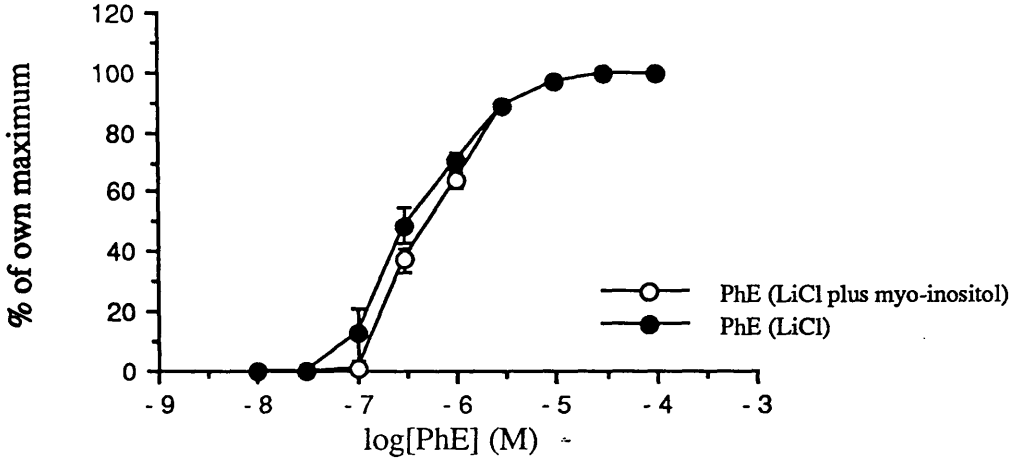
Figure 5.8 shows mean CRC in response to (A) NA, (B) PhE and (C) ACh. The CRCs have been obtained from anococcygeus muscle taken from rats which have been chronically treated with LiCl and myo-inositol (open circles) and in anococcygeus muscle taken from rats which have been chronically treated with LiCl (closed circles). Details of the LiCl and myo-inositol treatment are give in the Materials and Methods of this chapter. (mean±s.e.m., n=3-4, * $p<0.05$)

Figure 5.9 shows the normalised results from the CRCs obtained with: (A) NA, (B) PhE and (C) ACh. Only curves from muscles which reached a clearly defined maximum were included and each curve was expressed as a percentage of its own maximum. Open circles denote muscles harvested form rats which were chronically treated with LiCl and myo-inositol and the closed circles muscles from LiCl-treated rats. The PD2 values were calculated for the three agonists under each of the conditions and were found not to be significantly different using a Student's unpaired t-test. (mean±s.e.m., n=2-4)

A.


% of own maximum
120
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
-3
log[NA] (M)
NA (LiCl plus Myo-inositol)
NA (LiCl)

B.


% of own maximum
120
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
-3
log[PhE] (M)
PhE (LiCl plus myo-inositol)
PhE (LiCl)

C.

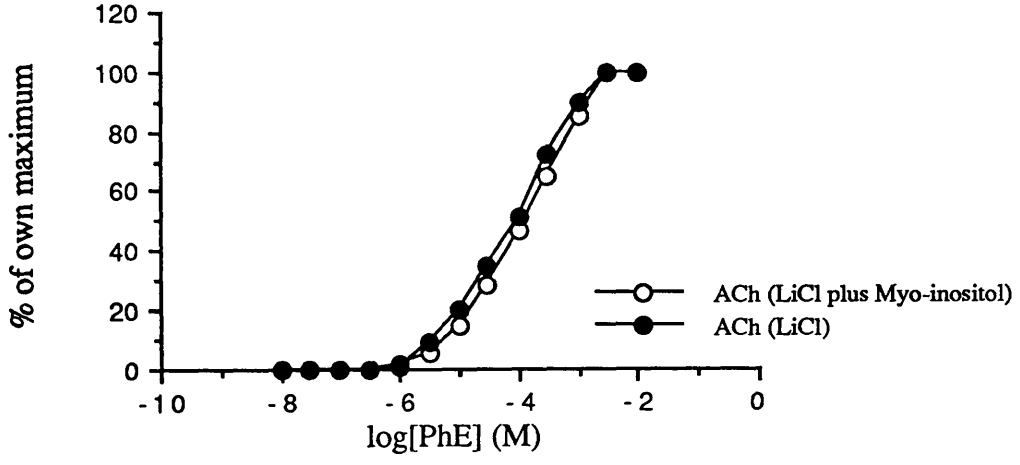
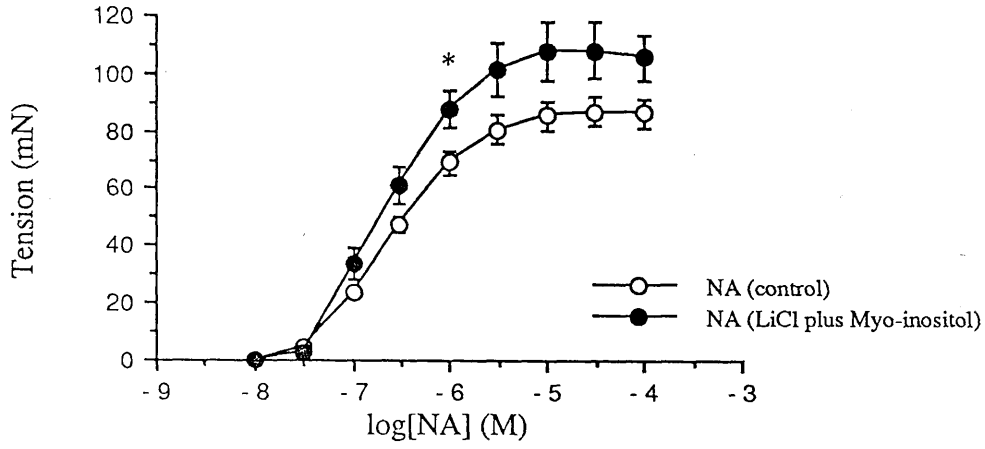
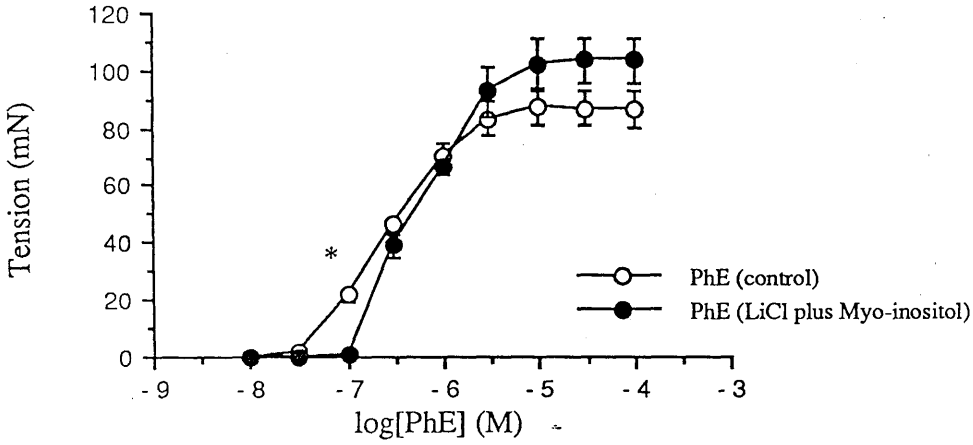

% of own maximum
120
100
80
60
40
20
0
-10
-8
-6
-4
-2
0
log[PhE] (M)
ACh (LiCl plus Myo-inositol)
ACh (LiCl)

Figure 5.10 shows mean CRC in response to (A) NA, (B) PhE and (C) ACh. The CRCs have been undertaken in tissues taken from rats which have (closed circles) and have not (open circles) been chronically treated with LiCl and myo-inositol. Details of the LiCl and myo-inositol treatment are give in the Materials and Methods of this chapter. (mean±s.e.m., n=3-9). Significance was tested using an unpaired Student's t-test. (* $p<0.05$)

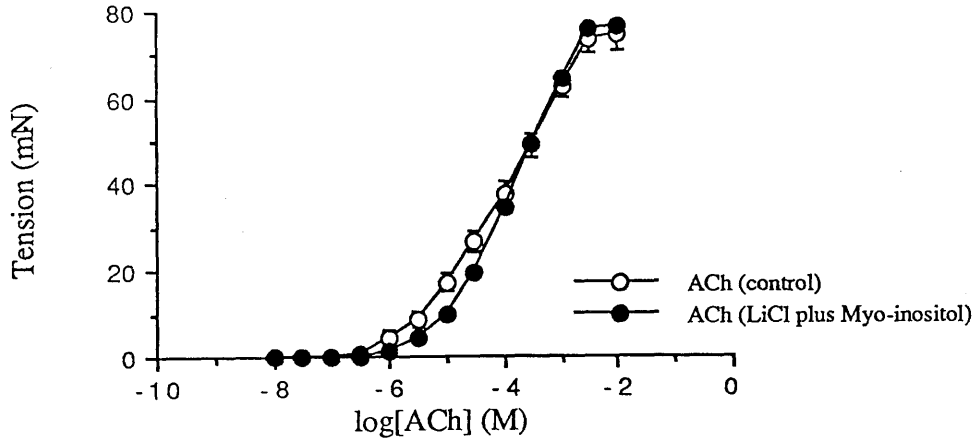
A.


Tension (mN)
120
100
80
60
40
20
0
*
-9
-8
-7
-6
-5
-4
-3
log[NA] (M)
NA (control)
NA (LiCl plus Myo-inositol)

B.


Tension (mN)
120
100
80
60
40
20
0
*
-9
-8
-7
-6
-5
-4
-3
log[PhE] (M)
PhE (control)
PhE (LiCl plus Myo-inositol)

C.


Tension (mN)
80
60
40
20
0
-10
-8
-6
-4
-2
0
log[ACh] (M)
ACh (control)
ACh (LiCl plus Myo-inositol)

animals. These two groups of experiments took place separated by several months and so strictly speaking should not be cross interpreted. This means, therefore, that the response from the control tissues should not be compared with the responses from the LiCl plus myo-inositol treated group. It cannot be strictly claimed, therefore, that myo-inositol is preventing LiCl from suppressing the response to the agonists to such an extent that they are no different from the control. It can only be said that myo-inositol is to some extent preventing the effect of LiCl. The other problem is that the design of the experiments does not allow the analysis of the effect of myo-inositol on its own. Myo-inositol is fed to the LiCl treated rats in their drinking water which they take freely because they are natriuretic. It is unknown whether control rats would take the myo-inositol in their drinking water and it is almost certain that, because they would not be natriuretic, probably would not take an equivalent dose of myo-inositol.

In summary, anococcygeus muscle from rats chronically treated with LiCl for four days produces CRCs in response to NA, PhE, ACh and 5-HT which are significantly depressed in comparison to the responses in tissues from control rats. Although the force produced in response to all these agonists is depressed, sensitivity to these agonists is unaffected. This depression in the force produced by these agonists is prevented to some extent by feeding the rats myo-inositol at the same time as they are receiving the injections of LiCl. The extent to which the effect of LiCl is prevented cannot be analysed because two important

controls are missing i.e. CRC's in response to these agonists in tissues from (1) untreated rats and (2) myo-inositol only treated rats.

EFFECTS OF LITHIUM ON THE ITC

Figure 5.11 shows representative ITC contractions in (A) control and (B) muscles from rats chronically treated with LiCl. The effect of chronic LiCl treatment is to suppress the ITC produced by all three agonists. 5-HT does not produce an ITC. Mean observations are shown in Figure 5.12A. LiCl significantly reduces the size of the ITC produced by all three agonists. Surprisingly however, after treatment with myo-inositol plus LiCl the effect on the ITC is not prevented (Figure 5.12B). It is, however slightly increased with regard the effect of LiCl alone. This slight increase is enough to cause the response (ITC in LiCl plus myo-inositol for NA and PhE) to be no longer significantly different from the control ITC (Figure 5.12C). This is most probably due to the manner in which these experiments were undertaken as has been discussed above. The experiments were performed in two sections: (1) controls and LiCl and (2) LiCl and LiCl plus myo-inositol, separated by several months. It would appear that the effect of LiCl in the second set of animals is different from that in the first. This is only a problem when the animals from the two sets are compared. To obtain a better picture of the difference between the LiCl plus myo-inositol treated muscles and control muscles these experiments should ideally have been done at the same time. The other results from within the two sets of experiments are still valid. In summary, LiCl

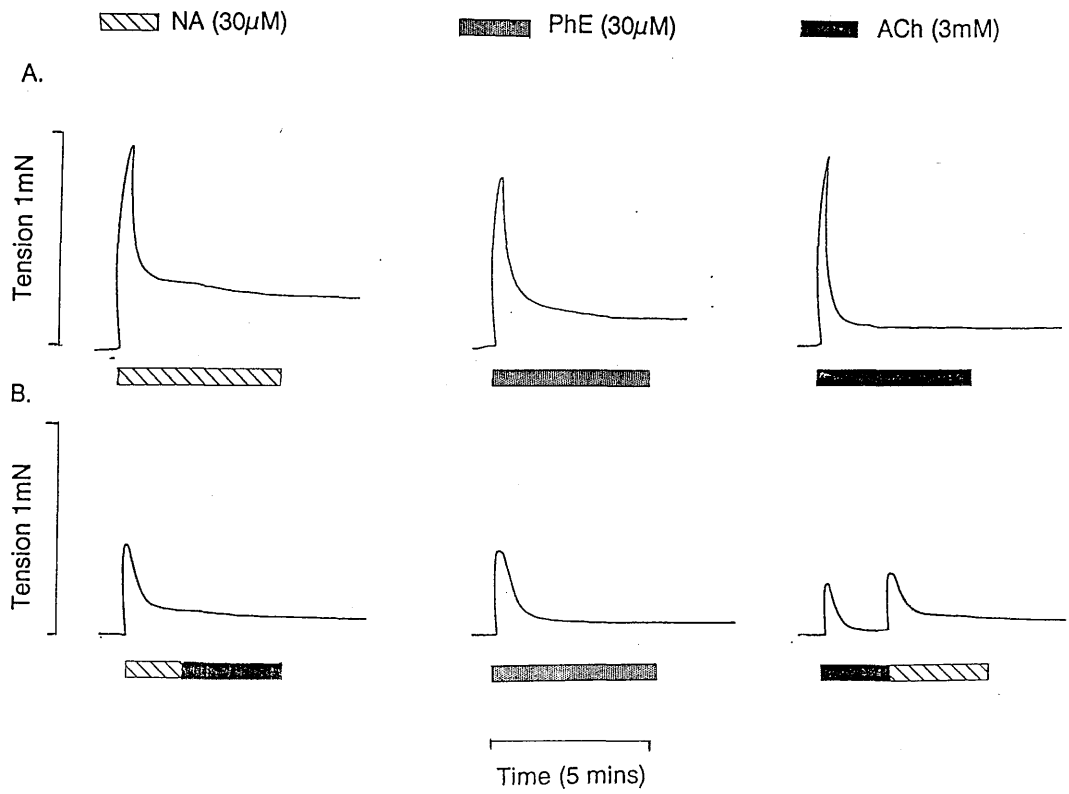
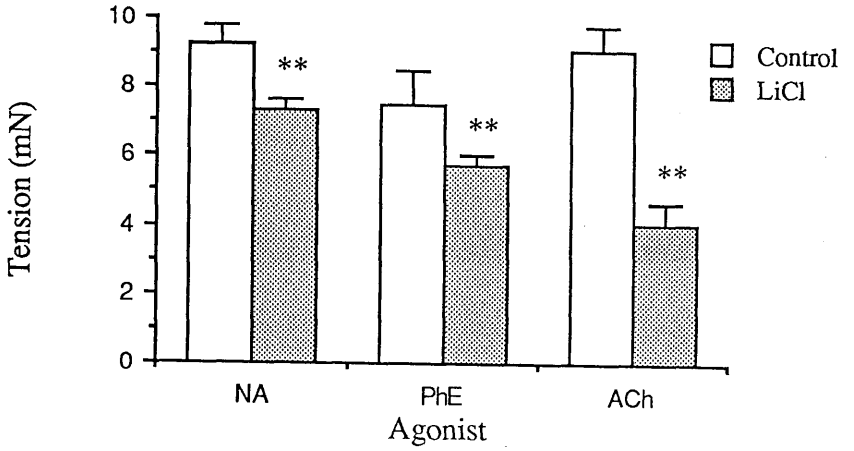


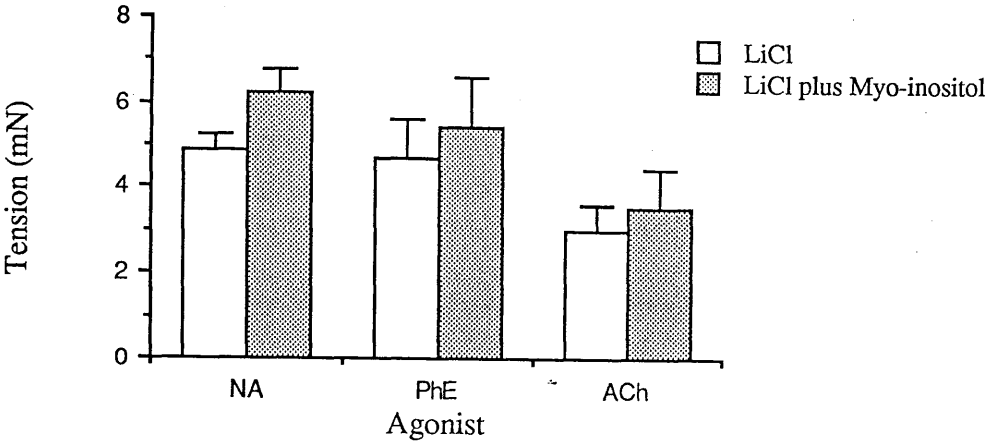
Figure 5.11 shows typical contractions evoked by NA (30μM), PhE (30μM) and ACh (3mM) in low calcium Krebs (0.1μM) in: (A) anococcygeus from control rats and (B) anococcygeus from rats which have been chronically LiCl treated.

Figure 5.12 shows the mean ITC for NA (30μM), PhE (30μM) and ACh (3mM). In (A) the open bars represent the mean ITC of anococcygeus muscles from control rats and the filled bar the mean ITC of anococcygeus muscles from rats chronically treated with LiCl. (mean±s.e.m., n=6-12, **$p<0.05$) (B) compares the mean ITC from anococcygeus muscles from rats chronically LiCl treated (open bars) and rats chronically LiCl and myo-inositol treated (filled bar). (mean±s.e.m., n=4) (C) compares the mean ITC from anococcygeus muscles from control rats (open bars) and rats chronically LiCl and myo-inositol treated (filled bar). (mean±s.e.m., n=4-6, **<0.5)

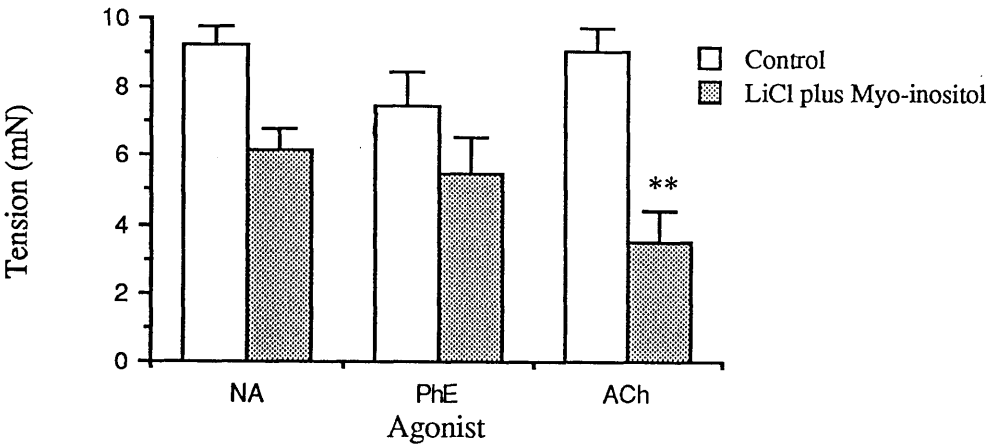
A.


Tension (mN)
10
8
6
4
2
0
**
**
**
NA
PhE
ACh
Agonist
Control
LiCl

B.


Tension (mN)
8
6
4
2
0
NA
PhE
ACh
Agonist
LiCl
LiCl plus Myo-inositol

C.


Tension (mN)
10
8
6
4
2
0
**
NA
PhE
ACh
Agonist
Control
LiCl plus Myo-inositol

treatment reduces the ITC to NA, PhE, and ACh and this effect could not be shown to be prevented by myo-inositol. However, LiCl reduces the maintained response to these agonists and this effect is prevented to some extent by myo-inositol.

THE EFFECTS OF LITHIUM ON ALPHA-TOXIN PERMEABILIZED MUSCLE

One explanation of the effect of LiCl alone on the ITC and CRC might be that the intracellular store has been depleted or is not loading to the same extent. To examine this idea responses evoked by caffeine were examined in muscle which had been permeabilized with alpha-toxin. Caffeine releases calcium from the sarcoplasmic reticulum (Weber & Herz, 1968) as does Ins(1,4,5)P_3 (Streb *et al*, 1983). It has been shown in the previous chapter that if caffeine is applied at regular intervals its addition causes contractions of equal size and this might be used as an indication of the amount of calcium available for release in the sarcoplasmic reticulum. To examine the effect of LiCl on the size of the releasable pool of calcium, four repeat contractions 10 minutes apart were induced in normal '0.2 Relaxing solution' (two are shown in the diagram), four were induced in '0.2 Relaxing' with 10mM NaCl (to examine the effect of increased ionic strength) and four were induced in '0.2 Relaxing' solution with 10mM added LiCl. NaCl (10mM) inhibits the contractions evoked by caffeine, but not immediately (Figure 5.13). LiCl or NaCl were added at the same time as the caffeine to try to discern whether the reduction in the size of the

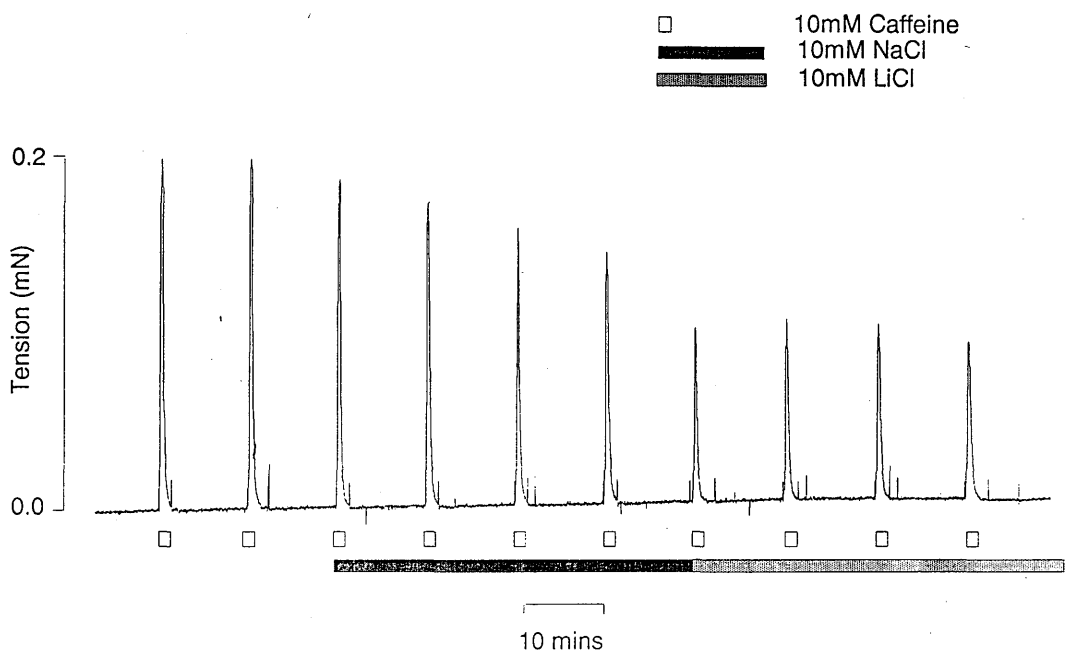
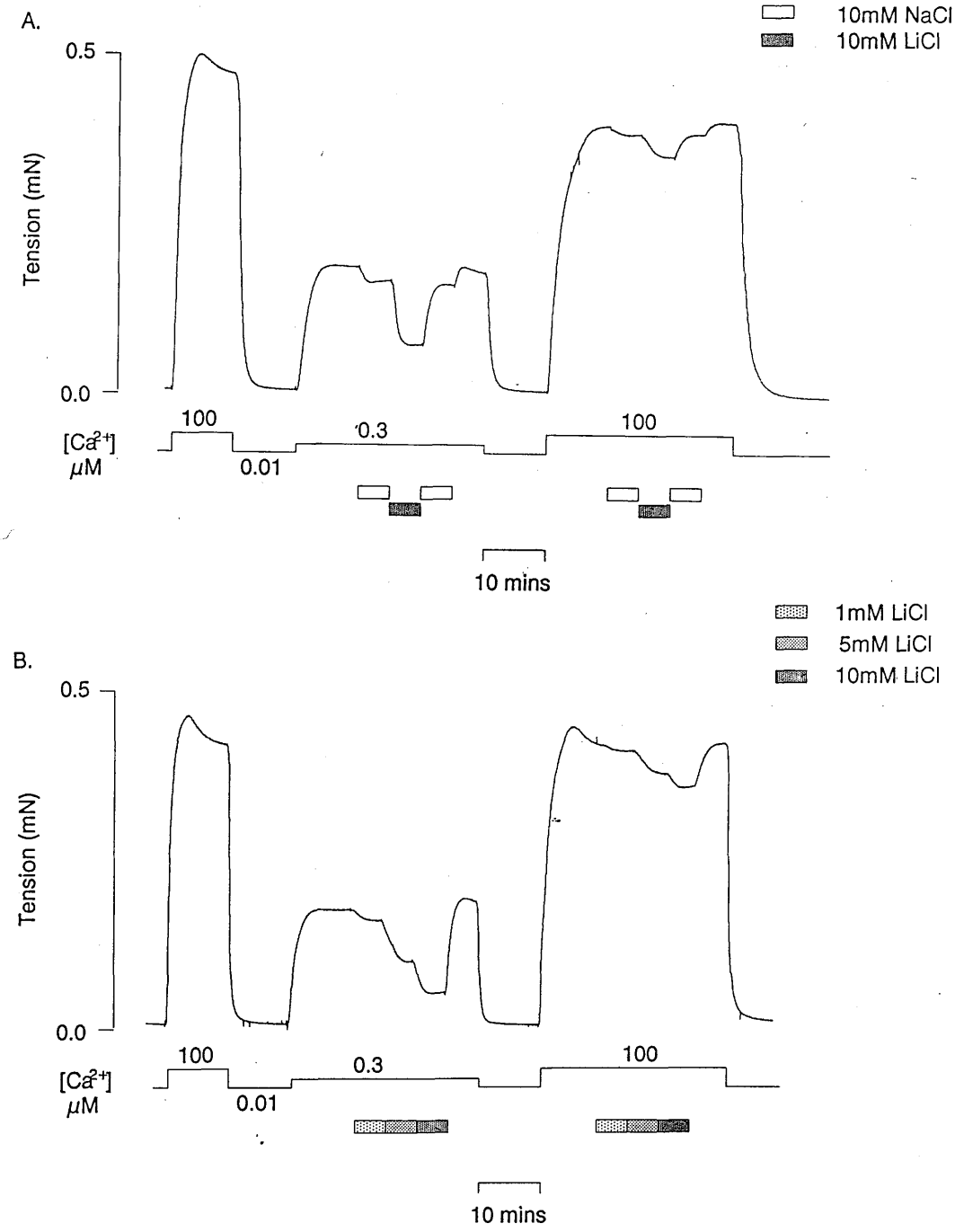


Figure 5.13 shows a typical experimental trace of contractions evoked by caffeine (10mM) in alpha-toxin permeabilized rat anococcygeus muscle. The open bars below the trace denote exposure time to caffeine. 10mM NaCl was applied to the solution at the time indicated by the solid bar. This was exchanged for 10mM LiCl at the time indicated by the stippled bar.

contraction in response to caffeine was due to a depression of calcium sensitivity or due to a reduction in the amount of calcium within the store. If, when the compound is added at the same time as caffeine, there is a reduction in the size of the contraction then this is most likely to be due to a decrease in sensitivity. If, however, the effect does not occur until the next contraction then this indicates that the loading of the store has been affected. Since NaCl did not immediately inhibit the contraction in response to caffeine then it is probable that it is having its effect on the ability of the store to load with calcium. LiCl produced a further reduction in size of the contraction in response to caffeine. This inhibition is immediate and so is most likely to be due to a decrease in the calcium sensitivity of the contractile proteins. The size of the contraction does increase slightly indicating that the store is able to load more in the presence of LiCl. This possible effect on calcium-sensitivity was examined in half maximally and maximally calcium-activated muscle. The results in figure 5.14A show that NaCl depresses the contraction produced at 0.3μM and 100μM [Ca^{2+}]. LiCl (10mM) causes a further depression in calcium-activated force. The depressive effect is greater at 0.3μM calcium than 100μM calcium which indicates that 10mM LiCl is depressing calcium sensitivity of the myofilaments. This effect of LiCl is concentration dependent (Figure 5.14B) and is fully reversible.

As has been discussed in Chapter 3, one of the advantages of the alpha-toxin-treated preparation is that it retains functional membrane-bound

Figure 5.14A shows the effect of 10mM NaCl (open bar) and 10mM LiCl (stippled bar) on calcium-activated force in alpha toxin permeabilized rat anococcygeus muscle. The muscle was exposed to the $[Ca^{2+}]$ indicated by the 'stepped' line. Panel B shows the effect of increasing concentrations of LiCl on calcium activated force. The $[Ca^{2+}]$ that the muscle was exposed to is indicated by the lines under the trace and the LiCl concentration is indicated by the bars. The solutions used in these experiments comprised ratios of '10 Activating' and '10 Relaxing' (their composition is detailed in Chapter 2, Table 2.3).


A.
10mM NaCl
10mM LiCl
0.5
Tension (mN)
0.0
100
0.3
100
[Ca2+]
μM
0.01
10 mins
B.
1mM LiCl
5mM LiCl
10mM LiCl
0.5
Tension (mN)
0.0
100
0.3
100
[Ca2+]
μM
0.01
10 mins

receptors. However, some of the disadvantages of this technique for use in this preparation have also been discussed in Chapter 4. The size of the response to repeated NA challenges declines and this cannot be readily restored. The response to ACh is also lost in these muscles. Therefore, only the response to NA was examined in alpha-toxin permeabilized muscle. Since the amplitude of the response to NA declines with time in alpha-toxin permeabilized muscle the effect of 10mM LiCl on the response to NA has had to be compared with the response to NA in an untreated preparation. The effect of LiCl on the contraction induced by NA is shown in Figure 5.15. It can be seen that the response is very small and prolonged, very different from the response to NA that was described in Chapter 4. Of the five tissues which were examined in this manner two of them did not respond to NA at all. These contractions (in presence of 10mM LiCl) induced by NA are only 22.5%±27.4% (x±S.D, n=5) of the previous contraction to caffeine (also in LiCl), whereas the other contractions (no LiCl) to NA are 73.7%±21.0% (x±S.D., n=13) of the previous response to caffeine. These results would indicate that the response to NA is significantly reduced in the presence of LiCl and that this reduction cannot be completely accounted for by a reduction in calcium-activated force. The results, therefore, imply that LiCl interferes with the process linking NA to calcium release.

The next step was to examine whether myo-inositol could reverse the effect of LiCl in the alpha-toxin permeabilized muscle as it does in the intact

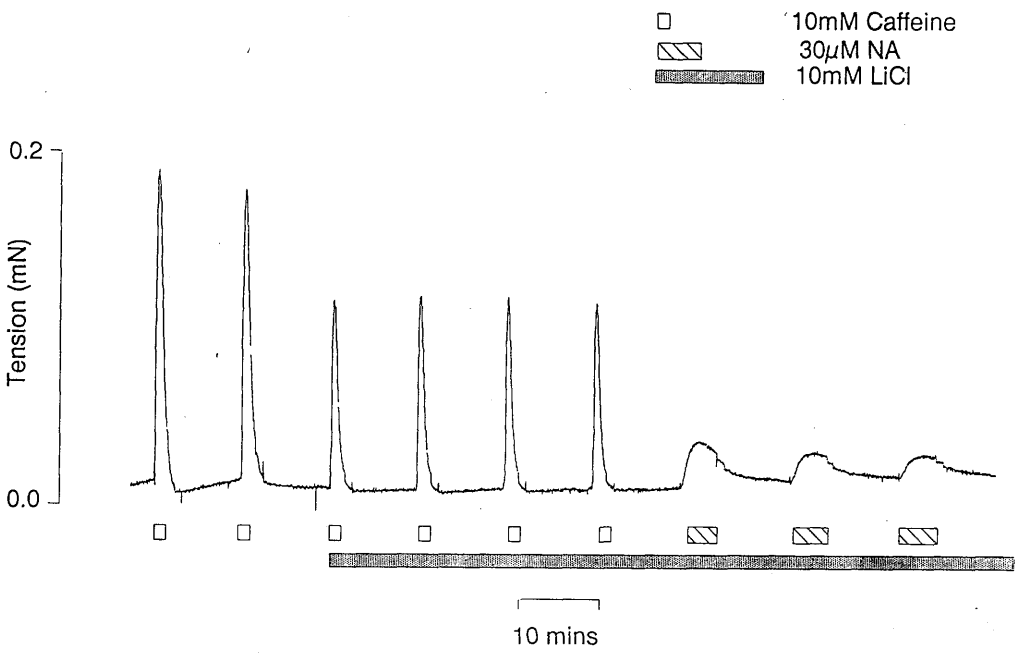


Figure 5.15. shows the effect of 10mM LiCl (stippled bar) in the '0.2 relaxing' solution on contractions evoked by caffeine (open bar) and noradrenaline (hatched bar).

muscle. Figure 5.16 shows the effect of 10mM NaCl plus 10mM sucrose (used to examine the effect of increased ionic strength and osmolarity) and 10mM LiCl plus 10mM myo-inositol on a half maximally and maximally calcium-activated muscle. NaCl plus sucrose depresses calcium activation at both $[Ca^{2+}]$'s (0.3μM and 100μM) and these are further depressed by LiCl and myo-inositol. These effects were also fully reversible. Thus myo-inositol did not reverse the effect of LiCl on calcium activated force. Myo-inositol also did not reverse the effect of LiCl on the contractions induced by caffeine or noradrenaline on the two occasions that this experiment was tried (results not shown).

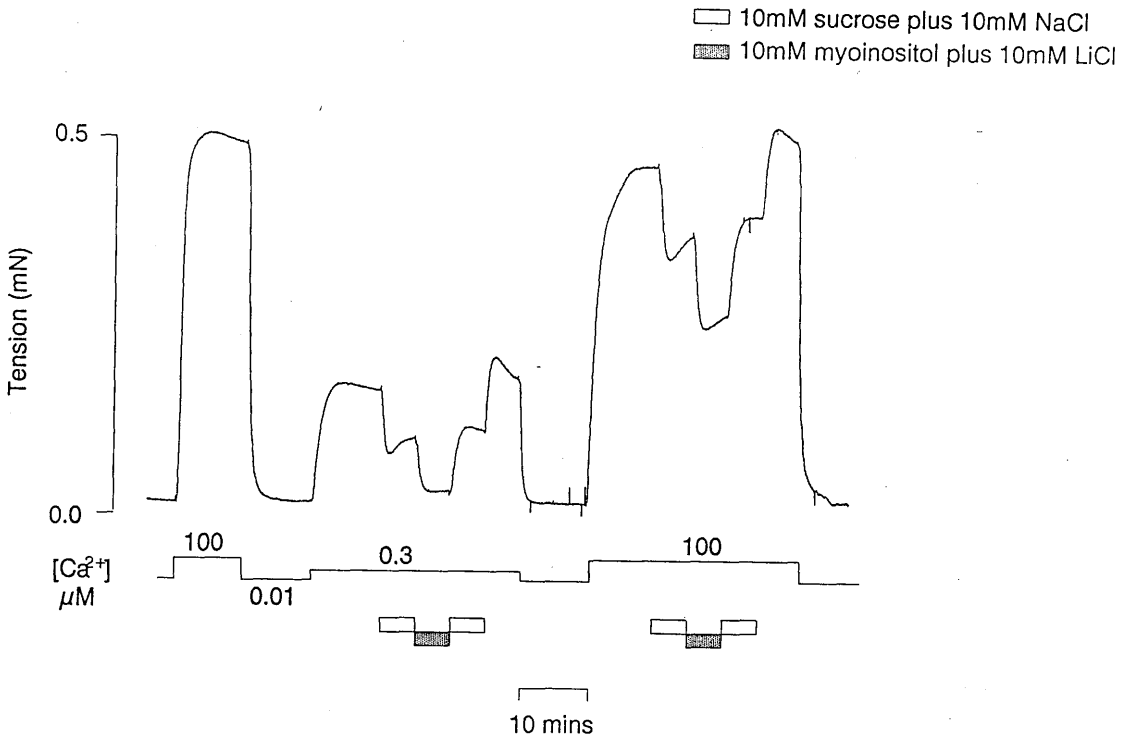


Figure 5.16 shows the effect of 10mM NaCl plus 10mM sucrose (open bar) and 10mM LiCl plus 10mM myo-inositol (stippled bar) on calcium-activated force. The $[Ca^{2+}]$ in the solutions was changed as indicated by the stepped lines below the trace.

DISCUSSION

This study has shown that LiCl has wide ranging effects on intact and alpha-toxin permeabilized rat anococcygeus muscle. A number of explanations are possible and some of these are discussed below.

POSSIBLE EFFECT OF LiCl ON RECEPTOR NUMBER

LiCl depressed the size of the contractions to NA, PhE, ACh and 5-HT, but it did not appreciably affect the 'sensitivity' of the muscles to these agonists. A possible factor which could reduce maximum activated force, but not sensitivity, would be a non-competitive reduction in the number of receptors in the surface membrane. Maximum force would be depressed because there would be a reduced number of channels that could be opened by this reduced population of receptors. Prestronk and Drachman (1987) have already reported that 24-48 hours incubation of cultured cells in 1.5mM LiCl reduces the synthesis and, therefore, insertion of acetylcholine receptors into the surface membrane. This effect can be mimicked by raising the intracellular $[Ca^{2+}]$ and the effect is blocked if the cultured cells are bathed in inositol with LiCl. LiCl, therefore, might be having its effect directly on the synthesis of the receptors or it might be blocking the PtdIns cycle which might raise intracellular $[Ca^{2+}]$ and this might affect receptor synthesis. Intracellular $[Ca^{2+}]$ might be raised if the factors postulated to cause calcium release and entry are elevated, for example $Ins(1,4,5)P_3$, DG and $Ins(1,3,4,5)P_4$. This possibility could be examined to some

extent by measuring intracellular [Ca^{2+}] in the presence of LiCl and comparing this with the [Ca^{2+}] in LiCl plus myo-inositol treated cells.

POSSIBLE EFFECT OF LiCl ON CALCIUM-ACTIVATED FORCE

Another possible explanation of the reduced maximum response, but not reduced sensitivity, in tissues from chronically LiCl treated rats would be if the contractile proteins had become less responsive to calcium. There is some evidence from this study that, in permeabilized muscle, LiCl did reduce calcium-activated force. This has also be reported by Hori *et al* (1989). In both studies LiCl's effect was reversible. The measurements made in the intact muscles were made after the animals had been LiCl treated for 4 days. When the measurements were taken the intact muscle was not in contact with LiCl. The effect of LiCl on calcium sensitivity in the permeabilized muscle is reversible. However, it is not known whether the effects of long term exposure to LiCl are reversible. These experiments could not be carried out because the integrity of the muscle cannot be retained for the length of time needed. Gow and Ellis (1990) have reported that extracellularly applied LiCl takes hours to accumulate intracellularly and that the concentration which is achieved is greater than the extracellular concentration. LiCl might not be having a direct effect on calcium sensitivity, the effect might be supplemented by a reduced Ins(1,4,5)P_3 and DG production. Indeed the response to NA in the permeabilized muscle was reduced by LiCl proportionately more than the effect on calcium sensitivity. DG has been reported to raise calcium sensitivity (Itoh

et al, 1986; 1988; Fujiwara *et al*, 1989) through its ability to activate PKC. If the breakdown pathway for $Ins(1,4,5)P_3$ is blocked by LiCl then the amount of inositol within the cell will decrease and so will the amount of $PtdIns(4,5)P_2$ in the membrane. This will mean that, when the cycle is stimulated, a reduced amount of $Ins(1,4,5)P_3$ and DG will be produced. This could mean that DG will have a lesser effect on raising calcium sensitivity which will manifest itself as a decrease from control in calcium sensitivity. One way of examining this might be to add a phorbol ester or 1-oleoyl-2-acetylglycerol (OAG) at the height of the contraction to try and further activate PKC. OAG is a synthetic analogue of DG and has been shown to work in a similar manner to DG (Nishizuka, 1984). Other compounds which might be depressing calcium sensitivity are the breakdown products of $Ins(1,4,5)P_3$ which will be accumulating within the cell. The effect of these compounds on calcium sensitivity has not been tested most probably because they are expensive and have a very short half life. Their effect on calcium sensitivity will be easier to examine once stable analogues have been produced as has been done for $Ins(1,4,5)P_3$ (Taylor *et al*, 1989). This effect of LiCl on calcium sensitivity assumes that the muscle is apparently maximally activated. This could be easily checked by (1) raising the extracellular $[Ca^{2+}]$ when the muscle is maximally contracted to examine if the muscle contracts more or (2) by measuring the $[Ca^{2+}]$ within the cells. If the $[Ca^{2+}]$ rises to the same extent in the LiCl treated cells as the control cells then this would indicate, since the LiCl treated muscle is producing less tension, that calcium sensitivity is reduced.

POSSIBLE EFFECT OF LiCl ON CALCIUM ENTRY AND EFFLUX

Calcium influx is less likely to have been affected because maximum tension has been reduced and sensitivity of the muscle to the agonists has not been affected. If influx had been the limiting factor then it would mean that eventually the response would have reached the original maximum. This again relies on the assumption that the LiCl treated muscles are maximally activated. On the other hand if calcium efflux has been increased this would reduce the $[Ca^{2+}]$ which was allowed to accumulate within the muscle cells and so the size of the contractions would be reduced. A mechanism linked to PtdIns to explain this is hard to imagine. DG is normally thought to stimulate calcium efflux (Drummond, 1985). However, if it is reduced by LiCl then this should decrease calcium efflux.

EFFECT OF LiCl AND MYO-INOSITOL ON THE INTRACELLULAR STORE AND FACTORS RELEASING THIS STORE

LiCl reduced the ITCs and CRCs induced by the agonists examined. It also reduced the contraction induced by NA in the permeabilized muscle and the contraction in response to caffeine. Part of this blockade has been shown to be an effect on sensitivity in the permeabilized muscle. LiCl per se does not seem to affect the size of the intracellular store, although the calcium which can be released is affected by increased ionic strength.

The surprising result came when myo-inositol was administered to the rats at the same time as they were being LiCl treated. This prevented to some extent the effect of LiCl on the CRCs in response to the agonists, but did not

prevent the effect of LiCl on the ITC in response to the same agonists. Myo-inositol also did not prevent the effect of LiCl on calcium sensitivity in the permeabilized muscle nor did it reduce the effect of LiCl on the contraction in response to caffeine or NA. This might suggest an imbalance between the two breakdown products of PtdIns(4,5)P_2 breakdown. If it is assumed that the ITC is a measure of Ins(1,4,5)P_3 activity and the CRC a measure of Ins(1,4,5)P_3 and DG activity then since the CRC to some extent is restored, but the ITC is not, in tissues from rats which have been chronically LiCl and myo-inositol treated, it might suggest either that not enough Ins(1,4,5)P_3 is being produced to cause release of intracellular calcium or that the rate of Ins(1,4,5)P_3 production has been reduced to a level where the breakdown rate of Ins(1,4,5)P_3 is approximately the same as the production rate. The latter of these two is the more likely considering that: (a) enough DG appears to be produced to cause calcium-entry and (b) the two breakdown products are produced at the same rate and in the same amounts. The rate of production of DG could be reduced without it causing a problem since it has a slower rate of breakdown than Ins(1,4,5)P_3. On the other hand a slower rate of production of Ins(1,4,5)P_3 could mean that its concentration never reaches a level at which it can have an effect because Ins(1,4,5)P_3 is broken down so rapidly. Measuring Ins(1,4,5)P_3 and DG production would be a means for examining this hypothesis.

The effect of myo-inositol might also be a consequence of how these experiments were carried out. The missing substrate (inositol) was restored, but the breakdown processes of the PtdIns cycle were still blocked. Perhaps a

better way to examine whether LiCl's effect was due to the PtdIns cycle would have been to replace the enzymes which are supposed to have been blocked. The intracellular effect of NA could have been to examined more effectively in alpha-toxin permeabilized muscle from a rat which had been chronically treated with LiCl.

In conclusion, LiCl has a wide range of effects including those of directly and indirectly decreasing calcium sensitivity. This affects the responses to NA, PhE, ACh and 5-HT. However, only in the case of NA is there evidence that LiCl is affecting something more than simply a reduced response of the myofilaments to calcium to produce its effect. There is no direct evidence, however, that this has anything to do with the PtdIns cycle as the effect could not be prevented using myo-inositol.

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GENERAL CONCLUSIONS

The general aim of this thesis was to obtain a better understanding of the transduction mechanism linking membrane bound receptors to tension production in smooth muscle. Initial studies were carried out to investigate the viability of three different permeabilizing techniques. The experiments undertaken provide evidence that all three techniques (saponin, EGTA and α-toxin from *Staphylococcus aureus*) permeabilize the smooth muscle of rat anococcygeus. However, each treatment produced preparations with different properties and suitability for further experimentation. The basis of the different properties may be because each technique forms different sized 'pores' in the surface membrane (Thelestam & Mollby, 1979). Saponin-treatment appears to produce the largest pores, and as a result of this the agonist-induced responses are absent and there is a rapid decline in the amplitude of maximum calcium-activated tension. The reduction in maximal calcium-activated force can only be partially prevented by including the protein calmodulin to the bathing solutions and using low ionic strength to favour its affinity for calcium. Alpha-toxin-treatment produces the next largest 'pores'. Muscles permeabilized by this means retain their ability to respond to agonist stimulation and low ionic strength is sufficient to prevent the decline in the amplitude of maximum calcium-activated force. EGTA-treatment of muscle appears to produce the smallest 'pores' and retains the ability to respond to agonist stimulation, showing little decline in the amplitude of maximum calcium-activated force. However, EGTA-treated muscle only produces a third of the tension produced by

equivalent saponin- or α-toxin-treated muscles. Overall, α-toxin-treated muscle was considered to be the most suitable permeabilization technique and was used throughout the rest of the study.

The advantages of α-toxin permeabilization, that is that membrane bound receptors were left functional and the muscle internal environment could be changed by changing the external bathing solution of the muscle, allowed the study of the involvement of G-proteins in the receptor transduction mechanism. The effect of GTP and other related compounds on the response induced by noradrenaline and calcium activated force were investigated in two different smooth muscle types rat anococcygeus, a visceral smooth muscle, and guinea pig portal vein, a vascular smooth muscle. These two tissues responded very differently to GTP. This difference includes their response to GTP with respect to both calcium release and calcium-activated force. In guinea pig portal vein GTP does not itself release calcium, but does potentiate noradrenaline-activated calcium release. In this preparation, GTP has only a small effect on calcium-activated force in comparison with the effect of noradrenaline on calcium activated force. The results obtained with guinea pig portal vein are consistent with current understanding of the involvement of G-proteins in the agonist transduction system in smooth muscle (Kitazawa *et al*, 1989). Rat anococcygeus, on the other hand, reacted in a novel manner to GTP with respect to both calcium release and calcium-activated force. GTP itself caused a contraction and only transiently potentiated noradrenaline activated force. It also had a greater effect on calcium-activated force than noradrenaline. Both muscle types could be made to react in the same manner by using GTP-γ-S instead of GTP;

that is, both contracted in response to GTP-γ-S itself and GTP-γ-S had a greater effect on calcium-activated force than noradrenaline. This response is similar to the way GTP affects calcium release and calcium-activated force in rat anococcygeus. This would indicate that permeabilized rat anococcygeus muscle has a population of chronically activated G-proteins which effects its response to GTP.

It is now widely accepted that α-adrenoreceptors have their intracellular effect via the phosphatidylinositol cycle (Fain & Garcia-Sainz, 1980). A method to manipulate this cycle is to block the phosphatases which normally breakdown Ins(1,4,5)P_3 to inositol using LiCl (Berridge *et al*, 1983). The effect of LiCl was examined in both intact and α-toxin permeabilized rat anococcygeus muscle. LiCl has a wide range of effects including directly and indirectly decreasing calcium sensitivity. This affects the responses to NA, PhE, ACh and 5-HT. However, only in the case of NA is there evidence that LiCl is affecting something more than simply a reduced sensitivity of the myofilaments to calcium to produce its effect. The evidence, suggests that this effect is via the PtdIns cycle, however, the effect could not be prevented by supplying the muscle with myo-inositol.

The majority of this experimental work could be further developed by measuring calcium as well as tension. For example it would be interesting to see if GTP alone in rat anococcygeus was indeed releasing calcium and if the decline in the response to noradrenaline was actually due to decreased release of calcium. Another interesting situation in which to measure calcium would be in the presence of LiCl when the muscle is stimulated by noradrenaline.

ERRATUM

Page 4, line 10 "via of low resistance" should read "via low resistance".

Page 10, line 3 "Inoue and Isenber, 1990" should read "Inoue and Isenberg, 1990".

Page 16, line 8 "mitochondiria sequesters" should read "mitochondria sequester".

Page 16, line 9 "taht" should read "that".

Page 22 "MLCK" and "MLCP" should be inserted after "myosin light chain kinase" and "myosin light chain phosphatase" in the figure subscript.

Page 26, line 9 "one" should read "either".

Page 26, line 19 insert "piece" after "Hence the".

Page 39, line 12 insert "(Smith, 1985)" after the equation for ionic strength.

Page 52, line 21 "Figure 4.8" should read "Figure 4.14".

Page 54, line 17 "suppress" should read "depress"

Page 56, line 7 insert after "depolarised muscle." "The transient fall in force in the intact depolarised muscle is a movement artefact."

Page 58, line 8 "6 or 10 times" should read "6 to 11 times".

Page 65, figure legend "ionic strength 0.1M" should read "ionic strength 0.07M"

Page 66, figure legend "ionic strength 0.1M" should read "ionic strength 0.07M"

Page 68, figure legend "ionic strength 0.1M" should read "ionic strength 0.07M"

Page 77, line 2 "Rojas and Birnbaumer, 1985" should read "Rojas and Birnbauer, 1985".

Page 86 "0.8μM" should read "0.08μM".

Page 93, line 1 insert "after the muscle had contracted in response to

noradrenaline three times" after "response to noradrenaline".

Page 117, line 16 insert "Arylazido Aminopropionyl Adenosine Triphosphate" after "$ANAPP_3$".

Page 119 Insert "than Guinea Pig Portal Vein" after "Rat Anococcygeus" in the title.

Page 122, line 21 insert "12-0-tetraecanoylphorbol-13-acetate" before "TPA".

Page 143 "1M nifedipine" should read "1μM nifedipine".

Page 164, line 12 "Prestronk and Drachman" should read "Pestronk and Drachman".

Page 171, Bers, 1980 reference date should be 1982.

Page 176, Gilkey *et al*, 1987 reference "oryzias latipes" should read "Oryzias latipes".

Page 181, Miller, 1975 reference should read "Miller D.J. (1985)".