A PHARMACOLOGICAL STUDY OF SOME RELAXATION PROCESSES IN THE RAT OESOPHAGEAL TUNICA MUSCULARIS MUCOSAE

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A Pharmacological Study Of Some Relaxation Processes In The Rat Oesophageal Tunica Muscularis Mucosae.

by

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ABSTRACT

This thesis deals with pharmacological properties of rat oesophageal smooth muscle, viz. the tunica muscularis mucosae (TMM). Although the TMM contains the bulk of smooth muscle of the rodent oesophagus, its function is not fully understood. However, recent evidence supports an active role for the TMM in oesophageal motility. Present concepts of the processes involved in oesophageal peristalsis have emphasised the importance of neural inhibitory mechanisms in the smooth muscle layer of the oesophagus. The present study was thus chiefly concerned with relaxation responses of the TMM.

The presence of a submucous ganglionated plexus in the isolated TMM was demonstrated by histological examination. The nerve plexus was found to contain acetyl- and butryl-cholinesterases and various peptide immunoreactive nerve fibres such as vasoactive intestinal peptide and calcitonin-gene related peptide. Adrenergic innervation was sparse in the proximal and supradiaphramatic portion of the TMM; serotonin was observed to be localised to mast cells but not to nervous elements.

In the presence of cholinoceptor-induced tone relaxation responses were elicited in the TMM by field-stimulation and by stimulation of the vagus provided excitatory responses to electrical stimulation were blocked. Relaxations were also examined to pharmacological agents.

Field-stimulation of the isolated TMM produced both a tetrodotoxin (TTX)-sensitive and a TTX-insensitive relaxation. The former was sensitive to guanethidine in the distal but not in the proximal segment. The TTX-insensitive response was unaffected by a variety of pharmacological agents and not abolished by cold storage. It was however diminished by cooling and by calcium antagonists. This form of relaxation was characterised as purely myogenic and a consequence of K^+ efflux activated by calcium entry through potential-operated channels following field-stimulation.

Vagally-stimulated relaxations in the whole oesophagus preparation were insensitive to hexamethonium. Relaxations to pharmacological agents such as 5HT and A23187 were found to have a regional gradient with the proximal TMM segments being more responsive than the distal segments.

This study demonstrates that the isolated TMM with its attached submucous plexus is capable of relaxing to both electrical and pharmacological stimuli.

Key Words : oesophagus, tunica muscularis mucosae, smooth muscle relaxations, pharmacology, Ca^{2+} activated K^+ channels, calcium antagonists.

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LIST OF ABBREVIATIONS

TMM : Tunica muscularis mucosae

TMP : Tunica muscularis propria

Ach : Acetylcholie

CD : cis-2-methyl-4-dimetylamino-methyl-1-3 dioxolane methiodide

TTX : Tetrodotoxin

FSR_1: Field-stimulated TTX-sensitive relaxation

FSR_2: Field-stimulated TTX-insensitive relaxation

FSC : Field-stimulated contraction

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CHAPTER 1

INTRODUCTION

1.1 General Introduction

The tunica muscularis mucosae (TMM) extends throughout the length of the alimentary canal as the third and innermost muscle layer beneath the tunica serosa. In all species, the muscularis mucosae consists of smooth muscle, but species differences exist in the composition and arrangement of the surrounding muscle layers particularly in the oesophagus. For example, in humans and marsupials, the outer two muscle layers of the oesophagus (the tunica propria) consist of a striated proximal segment and a distal smooth muscle segment. In rodents, the tunica propria is composed almost entirely of striated musculature.

Little attention has been paid to the TMM with regard to its contribution to the peristaltic activity of the gastrointestinal tract, particularly of the oesophagus. In view of the autonomous capability of the smooth muscle propria to effect oesophageal motility, the TMM of rodents may be conceived of as subserving an analogous function since it is the principal smooth muscle component of the rodent oesophageal body wall. Since the generation of oesophageal peristalsis depends on both efferent and afferent neural connections with the medulla oblongata, it would seem logical to investigate the presence in the TMM of similar neural

arrangements to establish an involvement of the TMM in oesophageal peristalsis. In the case of the smooth muscle propria, inhibitory non-adrenergic non-cholinergic (NANC) intrinsic processes have come to be considered of critical importance in peristalsis. Although some recent studies have suggested the absence of such mechanisms in the oesophageal TMM, the available evidence is neither conclusive nor complete. The present investigation aims to delineate pharmacological characteristics of the inhibitory mechanisms governing relaxations in the rat TMM. There are several reviews in the literature pertaining to oesophageal motility (Ingelfinger, 1958; Christensen, 1975, 1978; Roman, 1982), however, none of these provides more than a cursory account of the muscularis mucosae. The following review emphasises the mechanisms that may be involved in regulating oesophageal smooth muscle tone, and how inhibitory events, reflecting changes in cellular calcium concentration, may contribute to the control of oesophageal peristalsis.

In order to provide a basis for the processes that regulate smooth muscle tone in the oesophagus it is important to have an understanding of the anatomy, the pattern of innervation and control of peristalsis in this organ. An initial emphasis in the introduction is thus directed towards such considerations.

1.2 Structural Organisation of the oesophagus

The oesophagus is a narrow tube extending from the cervical region, where it is continuous with the pharynx, through the thorax, where it pierces the diaphragm at the 'diaphragmatic hiatus' and opens into the stomach at the cardial orifice. The oesophageal wall presents all the layers characteristic of the digestive tube in general. From the external to the internal part of the organ these are: serous membrane, external and internal muscle layers (tunica propria), submucosa, muscularis mucosae, lamina propria and epithelium.

1.2.1 The muscular layer of the oesophagus;

The external and internal layer of the tunica propria consist of muscle fibres that generally lie in a longitudinal and circular direction, respectively. In the rat, however, the two layers lie in a spiral orientation at opposite angles to each other (Gruber, 1968). Based on the differences in the musculature of the tunica externa, animals can be classed into three groups (Ingelfinger, 1958);

a) those in which the external muscle layers are striated throughout the length of the oesophagus (rodents, lagomorphs, pig, dog) (Gruber, 1968; Mann and Shorter, 1969)

b) those in which the upper oesophagus is striated but gradually merges into smooth muscle such that the distal is entirely smooth muscle. (primate, cat, opossum, horse) (Winship, Poindexter, Thayer and Spiro, 1965; Code and

Schlegel, 1968)and

c) those in which the entire tunica propria is smooth muscle (birds, amphibians, reptiles) (Ingelfinger, 1958).

A transition between striated and smooth muscle occurs in all three groups over various lengths and at various levels. In the rat, the distal-most region contains smooth muscle intertwined with striated muscle (Marsh and Bieger, 1987). Similar observations have been reported in the guinea-pig (Thomas and Trounce , 1960). In the oesophagus of the monkey transition from striated to smooth muscle occurs in both layers half-way along the oesophagus (Brown, Gideon, Voelker and Castell, 1978) while in the dog and pig only the circular muscle layer is replaced with smooth muscle in the distal region (Jacobowitz and Nemir, 1969). From an evolutionary viewpoint, all mammals have some features of striated tunica propria, the exception being the duck bill-platypus which has an entirely smooth muscled oesophagus (Ingelfinger, 1958).

1.2.1.1 The tunica muscularis mucosae

The muscularis mucosae contains smooth muscle throughout the length of the oesophagus, although it is reportedly absent in the dog oesophagus in the proximal one-third. The smooth muscle fibres are arranged in a longitudinal direction and increase in thickness towards the distal end (Schofield, 1968; Hughes, 1955). In most species the TMM is separated from the epithelium at the luminal side

by a layer of connective tissue, the lamina propria. This connective tissue is absent in the rat (Schofield, 1968) probably reflecting the absence of subepithelial musocus glands. On the abluminal surface, the muscularis mucosae is separated from the inner muscle layer of the tunica propria by a layer of connective tissue, the submucosa. In the rat, due to the absence of a lamina propria, the epithelium is attached to the muscularis mucosae. In all species, the epithelium consists of stratified nonkeratinizing squamous cells, which changes to simple columnar epithelium at the stomach entrance (Geboes and Desmet, 1978). In humans, at least, there are oesophageal glands which are of the mucous type. These are lodged in the submucous tissue outside the TMM and open into the lumen via ducts which pierce through the muscularis mucosae (Geboes and Desmet, 1978). The submucosal layer contains elastic fibres and allows the mucosa to lie in longitudinal folds (rugae) when the oesophagus is empty.

1.3 Extra-oesophageal TMM

Two main features of the TMM of the stomach, small and large intestine distinguish it from oesophageal TMM. First, there are two layers as opposed to a single layer in the oesophagus. These can be distinguished by differences in the orientation of their fibres and in some areas there is intermingling and decussation of bundles from each layer as observed in the muscularis mucosae of the cat stomach (Schofield, 1968). Second, the TMM protrudes into the villi

and may be drawn into circular folds with the adjacent submucosa in the small intestine or into semilunar folds of the colon (Schofield, 1968).

1.4 Innervation

1.4.1 Extrinsic

Hwang, Grossman and Ivy (1948) using nerve stimulation and X-ray photography demonstrated that motor fibres serving the cervical oesophagus emerge from the vagus either above or below the nodose ganglia and innervate the oesophagus from a branch of the pharyngeal nerve (dog, cat, rabbit) or the superior laryngeal nerve (monkey, guinea-pig, rat). There are also inputs from the glossopharyngeal nerve and sympathetic nerves. The larger intrathoracic and supradiaphragmatic part of the oesophagus receives motor input from the branches of the thoracic vagi.

The presence of a sympathetic innervation to the oesophagus of the cat and rhesus monkey has been demonstrated by Baumgarten and Lange (1969). The course and origin of these fibres has not been identified but nevertheless, it has been suggested that the sympathetic fibres mainly innervate blood vessels (Christensen 1978) or myenteric ganglia (Geboes and Desmet, 1978). Using fluorescense histochemistry for noradrenaline, Baumgarten and Lange (1969) showed that the muscularis mucosae of the cat or rhesus monkey oesophagus was innervated with a well developed adrenergic ground plexus. Nishimura and Takasu (1967) demonstrated that the adrenergic innervation was well developed in the myenteric plexus of the rabbit oesophagus but sparse in the submucous plexus. In the guinea-pig, Kamikawa and Shimo (1979) using the Falck-

Hillarp technique observed very little adrenergic innervation to the TMM. Similarly, Schultzberg, Hokfelt, Terenius, Rehfeld, Brown, Elde, Goldstein and Said (1980) could only detect sparse immunoreactivity to dopamine-b-hydroxylase in the submucous plexus of the guinea-pig and rat oesophagus. Thus species differences and perhaps the nature of the outer tunica propria may account for the differences in noradrenergic innervation.

Pharmacological evidence indicates that in the distal-most region of the primate and marsupial oesophagus, the smooth muscle propria receives both sympathetic and parasympathetic innervation (Langley, 1898; Clark and Vane, 1961; Rattan and Goyal, 1974; Goyal and Rattan, 1975). The cell bodies as observed by horseradish peroxidase tracing are located in the medulla, stellate and other thoracic ganglia and in the coeliac ganglia (Niel, Gonella and Roman, 1980). The parasympathetic fibres activate intramural inhibitory non-adrenergic non-cholinergic (NANC) processes (Gonella, Niel and Roman, 1977; 1979). In the distal-most TMM of the guinea-pig, Okhawa (1980) presented pharmacological evidence to support the presence of excitatory cholinergic and inhibitory adrenergic innervation in the guinea-pig.

1.4.2 Sensory

Vagal sensory pathways have been reported in the rat (Andrew, 1956; 1957), cat (Mei, 1970) and sheep (Falempin, Mei and Rousseau, 1978). These studies have demonstrated

mechanoreceptors in the oesophagus with cell bodies in the nodose ganglia. Vagal sensory fibres from the cervical oesophagus pass through the superior laryngeal nerve, and those innervating the rest of the oesophagus pass through recurrent laryngeal nerves or the oesophageal branches of the rami oesophagi. Sensory endings in the mucosa, submucosa and muscular layers are generally free endings (see Roman, 1982). Intraepithelial nerve endings have also been demonstrated and may be sensory in nature (see Christensen, 1984). Rodrigo, Polak, Fernandez, Ghatei, Mulderry and Bloom (1985) found that calcitonin gene-related peptide (CGRP) immunoreactivity was associated with sensory endings in the subepithelium of the rat, cat and monkey oesophagus. Substance P containing afferent fibres have been demonstrated in the TMM of the opossum (Domoto, Jury, Berezin, Fox and Daniel, 1983).

1.4.3 Intrinsic

The term 'enteric nervous system' was introduced by Langley (1900) to describe the independent nature of the nerve plexuses in the gastro-intestinal tract. These plexuses had been identified by Auerbach (1864) and Meissner (1857) who described an outer myenteric and an inner submucous plexus, respectively. Meissner's plexus is located between the submucosa and the muscularis mucosae whereas Auerbach's plexus or the myenteric plexus is sandwiched between the two muscular layers of the tunica propria,

including those formed by the striated muscle fibres (Schofield, 1968; Gruber, 1958). The plexuses consist of a network of ganglia connected together by nerve fibre strands. The shape of this network varies according to the nature of the plexus (Meissner or Auerbach), to the different areas of the digestive tract and to the species (see Gabella 1979). There are also some differences between the size of the cells of the two plexuses. The plexuses of Meissner and Auerbach are especially well developed in the smooth muscle of the oesophagus (Kuntz 1947). It is now recognised that the axons of the intrinsic enteric neurones make functional contacts with gastro-intestinal smooth muscle (for review see Daniel, 1985). The neurotransmitters are known to vary regionally and may be species-specific. Evidence for non-adrenergic, non-cholinergic transmission is well documented but the nature of the transmitter remains controversial. The presence of 'peptidergic' innervation has been demonstrated in the intramural plexus (Furness and Costa, 1982). Evidence has also been presented for a neurotransmitter role for ATP and hence the concept of 'purinergic' innervation has been proposed (Burnstock, 1972). A more detailed description of the species-dependent distribution and postulated significance of these transmitters is beyond the scope of this thesis, however, the nature of neural and neuro-myal transmission in the oesophageal submucosal plexus is described in this review as it pertains to the control of TMM

motility.

The function of the intra-mural plexus in the striated muscle remains obscure although many histological and pharmacological studies have suggested that the efferent vagal fibres terminate in motor end plates in this region (Gruber 1968, Abe 1959; Floyd 1973, Roman 1982 , Bartlett 1968; Bieger and Triggle 1985; Marsh and Bieger, 1987). It is noteworthy, that CGRP-like immunoreactivity has also been demonstrated in motor end plates in the striated tunica propria of rat, cat and monkey oesophagus (Rodrigo et al., 1985)

In the submucosal plexus, VIP-like immunoreactivity has been demonstrated in the cat and pig oesophagus but is reportedly absent in the rat (Uddman, Almets, Edvinson, Hakanson and Sundler, 1978). Terminal nerve fibres containg VIP were also described in the muscularis mucosae of the opossum (Christensen, Williams, Jew and D'Oriso (1987). A decrease in VIP-like immunoreactivity has been demonstrated in the myenteric plexus of smooth muscle distal oesophagus of patients suffering from achalasia (Aggestrup, Uddman, Sundler, Fahrenkrug, Hakanson, Sorensen and Hambraeus, 1983). Schultzberg et al. (1980) observed the presence of enkephalin-like, and small numbers of cholecystokinin/gastrin-like immunoreactivity in the guinea-pig and rat oesophagus. The absence of somatostatin-like immunoreactivity was also noted by these workers.

1.5 Functional aspects - general remarks

The function of the oesophagus is to convey ingested material from the pharynx to the stomach. The entrance to and the exit from the oesophagus are governed by sphincters at each end. Mechanisms regulating sphincter motility represent a separate topic and will not be considered here except to note that the potential exists for the TMM to exert influence on this region. For instance, Botha (1962) observed thickening of the rabbit distal-most TMM and the inferred that the function may be to control movement of the 'fold' at the cardial orifice.

The motility pattern of primary concern in the oesophageal body is peristalsis, the segmental contraction that moves in an aboral direction (Hirst 1979). In large part the present concept of oesophageal peristalsis (Diamant and El-Sharkawy, 1977) is derived from studies concerned with the organisation of extra-oesophageal regions : however, the intricate mechanisms governing oesophageal motility cannot be understood by simple extrapolation due to the interplay of central and peripheral neural inputs to the various regions of the oesophagus.

Since the late ninteenth century, oesophageal motility has been assessed by a) manometric studies using intra-oesophageal air- or water-filled balloons and b) by barium-contrast radiology. Although contractions can be

observed radiologically, there are inherent technical difficulties in using this technique, such as excess X-ray exposure. Thus most studies have employed manometric recordings. In recent years techniques of radionuclide transit have been used to assess motility patterns. Based on manometric studies, three functional subdivisions of the oesophagus have been established (Ingelfinger,1958, Christensen, 1978). These are the two sphincters, the upper and lower oesophageal sphincters and the oesophageal body. These regions are not clearly anatomically distinct, and the junctions describing the boundaries of the two sphincters have been disputed, although manometric, radiological and pharmacological data seem to justify the existence of these regions.

1.5.1 Neural control of the oesophagus.

Meltzer (1899) divided peristalsis in the oesophagus into primary and secondary types. Primary peristalsis is initiated by swallowing whilst secondary peristalsis is independent of swallowing and initiated by a local stimulus such as distension of the oesophagus. This distinction is based on the observation of both Meltzer (1899) and Mosso (1876) and subsequently confirmed by others (Janssens, DeWever, Vantrappen, Agg, Hellemans and Agg, 1976; see Ingelfinger, 1958), that the progression of a peristaltic wave is not inhibited by transection of the oesophagus once initiated by swallowing. On the other hand, a peristaltic

wave produced by distension of the oesophagus is abolished at the point of transection. These studies suggest that central programming is essential for primary peristalsis. Such a mechanism is suggested to reside in the swallowing center (rhombencephalon) (Doty, 1968; Jean, 1972, Miller, 1986). According to this concept, primary peristalsis initiated by swallowing can run its entire course without input of afferent signals. However, afferent discharges on swallowing have been recorded in the superior laryngeal nerves of the rat by Andrew (1956) and Jean (1972) has suggested the presence of afferent modification of the central programming sequence. The difference in primary and secondary peristalsis appears to be in the method of initiation and not on the apparent contribution of the extrinsic innervation. This has been demonstrated in both striated and smooth muscle tunica propria in the cat (Ueda, Schlegel and Code,1972; Reynolds, El-Sharkawy and Diamant, 1985), monkey (Janssens, De Wever, Vantrappen, Agg, Hellemens and Agg, 1976) and opossum (Janssens, 1978).

Cannon's (1907) earlier observation that peristalsis in the smooth muscle of the cat oesophagus could be elicited by local stimulation after bilateral vagotomy suggested that an intrinsic peripheral mechanism was also present in the smooth muscle. Stimulation of the isolated oesophagus directly or via the vagus also resulted in peristaltic progression along the smooth muscle propria

(Christensen and Lund, 1969). This type of peristalsis was termed as tertiary by Cannon and autonomous by Roman (1982). Cannon (1907) and Jurica (1926) demonstrated that tertiary peristalsis could be observed from a few hours to a few months after bilateral vagotomy. It was suggested that the time lapse could be due to adaptation of intramural neurones on removal of extrinsic connections (Jurica, 1926; see Reynolds, El-Sharkawy and Diamant, 1985). Notwithstanding the nature of peristalsis, it is apparent that the smooth muscle tunica propria has an intrinsic mechanism to generate oesophageal peristalsis. In contrast, bilateral vagotomy induces paralysis in the striated tunica propria.

The inherent capability of the smooth muscle for autonomous peristalsis has been studied both in vivo and in vitro (Christensen,1969; 1970; Roman and Tieffenbach 1971). In vivo, Christensen (1969) observed that in the opossum, distension of the oesophagus produced three distinct responses.

1) Inflation of the balloon produced a single brief contraction - termed the "on" response - above the point of distension which corresponded to an increase in intraluminal pressure.

2) If the balloon distention was maintained for a long period, a "duration' response occurred consisting of active shortening of the longitudinal muscle and the muscularis mucosae.

3) Deflation of the balloon produced a single brief circumferential contraction - the "off" response - which was propagated along the entire oesophagus below the point of stretch.

The 'on', 'duration'and 'off' responses have also been observed in vitro on opossum smooth muscle oesophagus preparations (Christensen and Lund, 1969), in the cat (Christensen, Cocklin and Freeman, 1973; Roman and Tieffenbach 1971) and in the rhesus monkey (Christensen et al. 1973). Stimulation of isolated strips of the opossum distal oesophagus indicate that the "on"-and "off"-responses are localised to the circular muscle layer and the duration response to the longitudinal muscle (Christensen and Lund, 1969). While the "on"-responses may be myogenic (Lund and Christensen, 1969; DeCarle, Templeman and Christensen, 1978) or neurogenic (Diamant and El-Sharkawy, 1975; Crist, Gidda and Goyal, 1984), the mechanism representing peristalsis has been equated with a neurally-mediated "off"-response. However, not all results are in agreement with this inference especially since the "off" response is not always propagated in a peristaltic manner (Roman, 1982). The evidence for the "off"- response being the mechanism for peristalsis comes from:

1) the demonstration by Weisbrodt and Christensen (1972) that the latency of "off" contraction increases in more distal segments. It has been known for some time that the rate of

peristalsis is decreased in the distal oesophagus (Ingelfinger, 1958).

2) Inhibitory junctional potentials (i.j.p.) have been recorded in the circular layer of the smooth muscle tunica propria, following vagal stimulation. These i.j.p.'s are not abolished by cholinergic or adrenergic antagonists and are usually followed by after-depolarisations (Crist, Gidda and Goyal, 1984; Rattan, Gidda and Goyal, 1983; Roman, 1982). The "off" response , on the other hand, is considered a rebound phenomenon following activation of inhibitory NANC nerves. In the isolated strip preparations of the opossum circular muscle, hyperpolarisations insensitive to atropine or adrenergic antagonists have been reported. (Christensen, 1969; Roman, 1982).

3) The circumferential contraction that moves in an aboral direction is limited to the circular muscle. The "off" contraction is also confined to the circular muscle (Sugarbaker, Rattan and Goyal, 1984).

4) Bayliss and Starling (1899) and Cannon (1907) demonstrated relaxations below the point of stretch in the intestine and the oesophagus, respectively. Clinical evidence indicates that repeated swallows do not produce peristalsis until the last swallow (Ingelfinger, 1958). This has been construed as evidence for centrally-mediated deglutitive inhibition (see Miller, 1986). However, peripheral inhibitory responses exist in the intestine (Hirst,1979). It has been suggested

that a phase of descending inhibition occurs prior to excitation . These results are consistent with an inhibitory phase prior to contractions observed in the "off" response.

The studies cited above indicate not only that the "off" response is well correlated with a peristaltic wave, but also that the intramural plexus exerts a profound influence on oesophageal motility. It is relevant to note that the concept of descending inhibition is compatible with the presence of intrinsic inhibitory neurones. Since the demonstration by Langley and Magnus (1905) that distal inhibition was not attributable to extrinsic sympathetic innervation, additional conclusive evidence from pharmacological, electrophysiological and histological studies indicate that intrinsic inhibitory neurones are non-adrenergic (Costa and Furness, 1982 a, b; Daniel, 1984). According to the above studies, the "off' response precludes the involvement of cholinergic inputs. However, El-Sharkawy and Diamant (1976) reported partial sensitivity of the "off"-response to atropine in the cat, and Gidda and Buyniski (1986) demonstrated that the amplitude of the peristaltic wave could be altered by cholinergic agents in the opossum. Furthermore, Bieger (1984) reported that atropine abolished primary peristalsis evoked by electrical stimulation of the brainstem of the rat and Dodds, Dent, Hogan and Arndorfer (1981) reported that low doses of atropine abolished

peristaltic contractions of low amplitude in human subjects. Thus, the influence of cholinergic mechanisms cannot be discounted and is suggestive of mechanisms other than "off"-responses being equally important in peristalsis. Such mechanisms may include the longitudinal muscle and the muscularis mucosae, indeed, depolarisation and hyperpolarisation have been recorded in these muscles by Sugarbaker, Rattan and Goyal (1984).

As noted above, in rodents, the TMM is the only smooth muscled structure of the oesophageal body wall. Therefore, the question poses itself as to whether the TMM is a functional homologue of the smooth muscle propria of other species or, as indicated by its anatomical name, effects localised movements of the mucosa. The following section gives an overview of pharmacological data relevant to this issue.

1.6 Pharmacological responses of the tunica muscularis mucosae of the oesophagus

1.6.1 Cholinergic

The vagal excitatory innervation of the TMM is effected via a pre-ganglionic nicotinic cholinergic synapse and a post-ganglionic muscarinic cholinergic synapse (Bieger and Triggle, 1985). Since hexamethonium blocked the contractile responses of the isolated guinea-pig TMM to nicotine but not to field-stimulation, Kamikawa and Shimo (1979) suggested that the preganglionic synapse lies in the submucous plexus. This finding is in contrast to the reports of Schofield (1960) and Rash and Thomas (1962). These authors using the silver impregnation technique to stain for enteric neurones, failed to demonstrate a submucous plexus in the oesophagus of the rat, mice, guinea-pig and rabbits. On the other hand, at the turn of the century, DeWitt (1900) demonstrated the presence of submucous plexus in the oesophagus of the cat and rabbit. Furthermore, Bieger and Triggle (1985) also demonstrated a cholinesterase positive plexus on the abluminal surface of the rat TMM. Atropine-sensitive excitatory responses to field stimulation of the isolated TMM of the dog, cat and opossum were demonstrated by Christensen and Percy (1984). These results indicate that irrespective of the nature of the tunica propria, the TMM in most species is innervated by excitatory cholinergic neurones.

1.6.2 Adrenergic

Hughes (1955) examined the effects of catecholamines in the TMM of the rat, rabbit (striated TMP) and the cat (mixed TMP). Noradrenaline relaxed the rat TMM but produced contractions of the rabbit and cat TMM. Thus the nature of the tunica propria did not seem to influence the responses to catecholamines. Field-stimulation of the guinea-pig TMM resulted in guanethidine- and propranolol-sensitive relaxations (Kamikawa and Shimo 1979). On the other hand, Kamikawa and Shimo (1983) also demonstrated inhibition of field stimulation-evoked acteylcholine motor responses by noradrenaline. Christensen and Percy (1984) demonstrated that in the dog, cat and opossum, catecholamine responses were species-dependent and regionally differentiated. Based on the inhibition of the field stimulation-evoked cholinergically mediated contraction (FSC), these authors suggested that in non-rodents the sympathetic innervation of the TMM impinges on intrinsic cholinergic neurones. In all three species examined, except for the distal portion of the opossum TMM, noradrenaline inhibited FSC, the inhibition being reversed by yohimbine. Furthermore, clonidine produced responses similar to noradrenaline. Noradrenaline-induced contraction were produced via α_1 adrenoceptors in all three species, except for the proximal region of the opossum oesophagus. Thus in the opossum there are regional

differences with respect to the distribution of adrenoceptors. α-$_1$ are predominant in the distal segment while α-$_2$ are predominant in the proximal. Bieger and Triggle (1985) demonstrated that in the rat noradrenaline-induced relaxation was predominantly via a beta receptor with a minor alpha component.

1.6.3 5-Hydroxytryptamine

Two types of responses were obtained to 5HT in the guinea-pig oesophagus by Bartlet (1968). The sensitivity to blockade of the 5HT-induced contractions was dependent on the presence or absence of the tunica propria. In isolated musclaris mucosae preparations 5HT-induced contractions were 1) methysergide sensitive and 2) methysergide-insensitive and blocked by hyoscine. In the whole oesophagus preparation 5HT responses were blocked by cocaine and hyoscine. 5HT responses were thus suggested to be due to activation of intramural ganglia of the myenteric plexus, to stimulation of intrinsic cholinergic innervation of the muscularis mucosae and due to an effect on the smooth muscle itself. The latter result is in contrast to that reported by Kamikawa and Shimo (1983). These authors demonstrated an inhibition of 5HT-induced contractions by tetrodotoxin and atropine. This suggests that 5HT has no direct effect on the muscle but an indirect effect due to release of acetylcholine from intrinsic neurones. These responses to 5HT were not antagonised by methysergide or ketanserin but were

diminished by cyproheptadine and phenoxybenzamine. The differences in the guinea-pig TMM between the results of Bartlet and Kamikawa and Shimo have been attributed by the latter to be due to differences in dissection. On the other hand, Bieger and Triggle (1985) observed regional differences in inhibitory responses to 5HT in the rat oesophagus. Relaxations were more prominent in the proximal TMM with contractions being evident in the distal segment. The contractile responses were enhanced in whole oesophageal preparations containing the outer tunica propria and were blocked by tetrodotoxin. This exaggerated contractile response was attributed to the presence of the myenteric plexus and therefore represents another site of 5HT-induced response. The contractile responses were ketanserin-sensitive and therefore mediated via $5HT_2$ receptor type. Direct action of 5HT via a methysergide-sensitive receptor was demonstrated in the musclaris mucosae of the chicken (Bartlet and Hassan 1968).

There is substantial evidence that 5HT exists in intramural intrinsic neurones in the intestine (Gershon, 1981). Evidence has also been presented that 5HT may be involved in the peristaltic reflex (Bulbring and Gershon, 1967). A number of studies indirectly indicate that 5HT may act at neuro-neuronal synapses and impinge on excitatory cholinergic nerves in the intestine (Costa and Furness 1979) and on vagal inhibitory neurones in the lower oesophageal sphincter

(Rattan and Goyal 1978)

1.6.4 Substance P responses

The presence of substance P innervation of the muscularis mucosae was demonstrated in the opossum oesophagus by Domoto, Jury Berezin, Fox and Daniel (1983). Substance P and acetylcholine were suggested to be co-transmitted. This interpretation was based on the presence of a phasic and tonic response observed to field-stimulation. The phasic response was blocked by atropine while the tonic response was susceptible to substance P tachyphylaxis and substance P antagonists. Moreover, since atropine enhanced the tonic response, Domoto et al. (1983) suggested that acetylcholine could modulate release of substance P. However, Robotham, Jury and Daniel (1985) reported that substance P was released from afferent nerves and may not be co-transmitted with acetylcholine. Christensen and Percy (1984) failed to observe tonic responses to field-stimulation of isolated muscularis mucosae of the opossum, cat and dog. Although there are no regional differences in responses to this peptide, the opossum is more sensitive than either the cat or dog. In the cat contraction to substance P is associated with spontaneous activity. The dog on the other hand does not develop spontaneous activity and requires greater concentration of substance P to induce contractions (Christensen and Percy, 1984)

1.6.5 Vasoactive Intestinal Polypeptide (VIP)

Although the presence of the peptide has been demonstrated (see section 1.2.2.3), its function remains unclear. Christensen and Percy (1984) failed to observe responses to VIP in the cat, dog or opossum. However, they only examined excitatory effects and did not test VIP on stretch- or agonist- induced tone thus failing to determine whether relaxant effects are present. VIP has been suggested to have relaxant effects in the cat oesophageal smooth muscle propria (Uddman et al.,1978) and in the opossum lower oesophageal sphincter (Rattan, Said and Goyal, 1977; Goyal and Rattan, 1980).

1.6.6 Opiates

Morphine-mediated inhibition of acetylcholine release has been demonstrated in the rat and guinea-pig (Bieger and Triggle, 1985; Kamikawa and Shimo, 1983). Kamikawa and Shimo reported inhibition of cholinergic-mediated excitatory responses to field-stimulation by opioid peptides; the order of potency being dynorphin (1-13) > a-neo-endorphin > b-endorphin > [D-Ala^2]- met-enkephalin >> a-endorphin, met-enkephalin, leu-enkephalin, morphine. It was deduced from the rank order of potency that in the guinea-pig TMM, the inhibition was mediated via a pre-synaptic k opiate receptor. Similar potency for morphine-mediated inhibition was observed in the rat by Bieger and Triggle (1985) suggesting the presence of a pre-synaptic k receptor in this tissue as well. On the other hand, vagally-stimulated

contractions of the rat TMM were also inhibited by morphine with a greater potency than the field-stimulated response. This suggests that opiate receptors also exist on submucosal ganglia which are functionally different from the kappa subtype. In human oesophagus, the met-enkephalin analogue, Hoe 825, inhibits intrinsic inhibitory neurones and increases peristaltic amplitude (Jian, Janssens, Vantrappen and Ceccatelli, 1987). Dowlatshahi, Evander, Walther and Skinner (1985) also demonstrated an increase in peristaltic amplitude with morphine in healthy human subjects.

1.6.7 Cholecystokinin/gastrin responses.

Oesophageal TMM of the opossum, dog and cat were unresponsive to cholecystokinin octapeptide and gastrin (Christensen and Percy, 1984).

1.6.8 Extra-oesophageal TMM

Brucke (1851; cited by Gunn and Underhill,1915) described the existence and distribution of smooth muscle in intestinal villi. Exner (1902; cited by King and Robinson, 1945) observed that puncturing of mucous membrane of the intestine or stroking with a needle produced local contractions. Other studies demonstrated that the movements of villi were effected by the muscularis mucosae (see King and Arnold, 1921). It has therefore been suggested that the function of the TMM could be to prevent potentially hazardous sharp objects in the intestinal lumen from causing mucosal

damage (see King and Robinson, 1945). Hambleton (1914) demonstrated that in the dog intestine, two types of villi movement could be distinguished pharmacologically: a 'lashing' movement, insensitive to atropine, and a retraction and extension of villi which was blocked by atropine and high concentrations of nicotine. Mechanical studies of the cat intestinal TMM revealed that the muscularis mucosae was spontaenously active and responded with contractions to adrenaline (Gunn and Underhill, 1915). The absence of villi in the stomach and colon indicate that the TMM may have functions other than simply to affect villi movement.

In the muscularis mucosae of the canine stomach, field-stimulation evokes TTX-sensitive inhibitory responses. The relaxations may be mediated by VIP as demonstrated by Angel, Go, Schmalz and Szurszewski (1983). VIP-like immunoreactivity in the submucosal plexus and on the TMM in the stomach has been visualised by Jessen, Polak, Van Noorden, Bloom and Burstock (1980), Schultzberg et al. (1980) and Costa, Furness, Bufa and Said (1980). Contractile responses to field-stimulation were not observed in the stomach, but were reported in the colonic muscularis mucosae of the dog. In the latter, Angel, Go and Szurszewski (1984) demonstrated that the contractile responses were due to release of substance P. Inhibitory responses to field-stimulation of the colonic muscularis mucosae, like the

stomach TMM were due to release of VIP. A rich peptidergic neuropile exists in the submucous plexus of the intestine (Costa, Cuello, Furness and Franco 1980). An absence of cholinergic and adrenergic innervation in the stomach and colonic muscularis mucosae of the dog has been suggested by Angel et al. (1983; 1984). These results contrast with those obtained in the oesophageal TMM of the same species where no inhibitory responses to field- stimulation or a role for VIP could be demonstrated (Christensen and Percy, 1984).

In the rabbit and opossum colonic muscularis mucosae, excitatory cholinergic responses and inhibitory NANC responses occur (Gallacher, McKenna and McKirdy 1972; Percy and Christensen, 1985), while the cat colonic TMM relaxes to cholinergic agonists (Onori, Friedmann, Frigo and Tonini, 1971). The responses of the rabbit colonic and oesophageal TMM are similar in that excitatory responses to catecholamines can be obtained in both muscles (Gallacher et al.,1972; Hughes, 1955). In the opossum, however, - adrenoceptor mediated inhibition occurs in the colon while -adrenoceptor mediated excitatory responses predominate in the oesophagus.

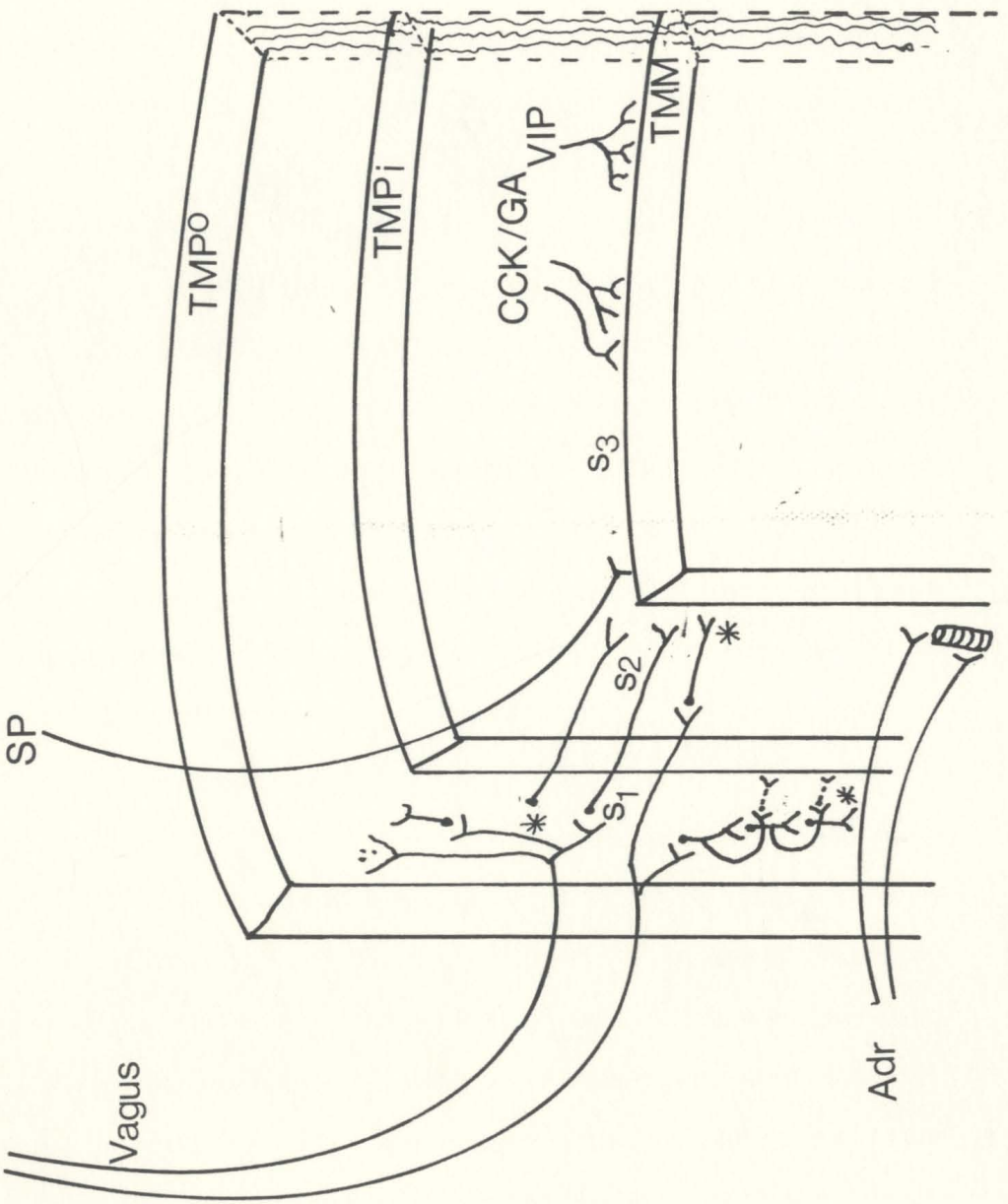
From the above review of the literature it appears that the TMM is responsive to a variety of pharmacological agents and the profile of such responses may change along the length of the gastrointestinal tract. Oesophageal TMM in all species is

innervated via excitatory vagal connections but the organisation of the intramural plexus remains undefined. The presence of peptidergic innervation and the effects of the peptides have not been studied in greater detail and therefore the importance of these agents remains unclear at present.

A schematic diagram of the postulated innervation of the TMM is presented in figure 1. Also illustrated is the neurogenic mechanism postulated by Diamant and El-Sharkawy (1977) to effect peristalsis. Vagal fibres have been suggested to synapse directly with excitatory cholinergic interneurones in the myenteric plexus. These interneurones stimulate NANC inhibitory neurones. The inhibitory nerves at each level are activated by cholinergic nerves from more proximal areas thus allowing for descending inhibition. The NANC inhibitory responses in the smooth muscle propria are manifested as "off"-responses. In the TMM, particularly that of the rodents, however, other workers have been unable to demonstrate either an "off"-responses or neurogenic relaxations. Yet, the presence of a variety of peptides in the TMM provides a basis for such mechanisms to be operative. In the rat TMM, Bieger and Triggle (1985) demonstrated the presence of TTX-insensitive relaxations to field-stimulation. Whether this response represents a form of neurogenic relaxations is the subject investigated in this thesis.

While the foregoing review has focussed on extrinsic mechanisms regulating oesophageal smooth muscle activity, it is well recognised that the cellular mechanism in regulation of motility relates to the alteration in cytoplasmic Ca^{2+}. The next section deals with this aspect.

Figure 1: Innervation of the oesophagus. A general schematic summary is presented with empahsis on the TMM. TMP^{o} and TMP^{i} are the outer and inner layers respectively of the tunica propria. Vagal cholinergic pre- and post-ganglionic fibres are indicated in solid lines, with end-plates in the TMP^{o} and TMP^{i} of striated muscle and interposed intramural ganglia projecting to the smooth muscle. A form of descending inhibiton is presented in the TMP^{o} with inhibitory fibres represented by dashed lines (see Diamant and El-Sharkawy, 1977). 'S' represents regions of 5HT action where '1' is the activation of myenteric ganglia of guinea pig and rat (Bartlet, 1968; Bieger and Triggle, 1985). '2' is the effect of 5HT on intrinsic cholinergic neurones (Kamikawa and Shimo, 1982) and '3' represents smooth muscle effects of 5HT, inhibitory in the rat and excitatory in the guinea-pig (see text). * represents the sites of inhibitory action of the opiates in various species. Adrenergic (Adr) innervation to the TMM and blood vessel is also shown. The origin of VIP/ CCK/Gastrin (GA) is not known. Substance P (SP) and CGRP may be of vagal origin.


TMP^o
TMPi
CCK/GA
VIP
TMM
S_3
S_2
S_1
SP
Vagus
Adr

1.7 Calcium and smooth muscle contractility

According to the generally accepted view, contraction of smooth muscle results from an increase in intracellular Ca^{2+} (Ca^{2+}_i) which leads to activation of contractile proteins. Correspondingly, relaxations occur by reduction in free cytoplasmic Ca^{2+} levels (see Fleckenstein, 1983). This addmittedly simplified concept may, however, need to be revised in light of recent studies on the role of Ca^{2+} in excitation-contraction coupling including *inter alia* the decrease in Ca^{2+} levels (Morgan and Morgan, 1984) and myosin phosphorylation (Dillion, Aksoy, Driska and Murphy, 1983) observed during maintained tonic contractions of certain smooth muscles. Moreover, Ca^{2+}-induced relaxation of the uterus (Sakai, Yamagishi, Nakamura and Uchida, 1984) is inconsistent with the above hypothesis.

1.7.1 Excitation-contraction coupling.

Calcium is required to initiate phosphorylation of myosin resulting in myosin-actin interaction, splitting of ATP and shortening or tension generation. Calcium first binds to calmodulin, the resulting complex activates myosin light chain kinase (MLCK) which in turn phosphorylates the two P-light chains (mol. wt. 20,000 each) of myosin. Phosphorylated myosin forms cross-bridges with actin and sliding of actin over myosin filament by cross bridge cycling occurs concomitantly with splitting of ATP (for more details see Hartshorne 1980; Hartshorne and Gorecka, 1980).

The Ca^{2+} utilised for excitation-contraction coupling can either be released from intracellular stores e.g. endoplasmic reticulum (ER) (Raeymakers and Casteels, 1981), or enter through the plasma membrane from the extracellular space. At least three Ca^{2+} entry routes have been proposed (Bolton, 1979; Aaronson and van Breemen, 1979). These are Ca^{2+} influx through 1) the potential operated channel (POC) 2) the receptor operated channel (ROC) and 3) the leak pathway. In addition, Winquist and Bevan (1981) and Bevan, Hwa, Laher and Wen (1985) have described a 'stretch' channel which mediates development of myogenic tone in certain blood vessels such as the rabbit facial vein, when the tissue is subjected to stretch.

Evidence for the existence of separate entry routes for Ca^{2+} has been presented (Cauvin and van Breemen, 1985; Karaki, Satake and Shibata, 1986; Karaki, Nakagawa and Urakawa, 1986). Furthermore, the pharmacological characterisation of these channels has been assisted by the use of selective Ca^{2+} channel antagonists in the smooth muscle, particularly of the 1-4 dihyropyridine type. It has been demonstrated that Ca^{2+} entry through POC is more sensitive to antagonism than Ca^{2+} entry through ROC (see Schwartz and Triggle, 1984).

Calcium channel opening on depolarisation has also been demonstrated in single smooth muscle cells by patch clamp recordings of single channels (Hess, Fox, Lansman,

Nilius, Nowycyk and Tsien, 1984). Patch clamp studies indicate that there are at least two types of channels in smooth muscle (Sturek and Hermsmeyer, 1986):
1) the 'L' channel which is activated rapidly and requires strong depolarisations. This type of channel also rapidly deactivates upon repolarisations, and 2) the 'T' channel which has a transient time course due to a fairly rapid inactivation and is activated at relatively small depolarisations.

These two types of POC have been suggested to display different permeation properties, gating kinetics and drug sensitivities (Tsien, 1983; Hess et al. 1984). A receptor modulation hypothesis has been proposed for the interaction of Ca^{2+} antagonists (CATs) with POC (Fox et al. 1986; Chin, 1986). At higher membrane depolarisations, the channel is in an inactivated form and displays greatest affinity for the 1-4 dihydropyridines. Thus, the L channel is more sensitive to CATs.

Patch clamp studies have not identified ROC per se. Receptor-stimulated Ca^{2+} entry is generally regarded as insensitive to CAT (Bolton, 1986). However, Cauvin and van Breemen (1985) reported that in some vascular smooth muscles, Ca^{2+} entry via ROC could be antagonised by CATs which was not dependent on membrane depolarisation. These authors inferred that CATs were effective under conditions when receptor activation did not lead to release of Ca^{2+}_{i}.

1.7.2 Regulation of contraction by calcium.

It has been demonstrated that myosin phosphorylation and stress or tone maintenance are not co-incidental (Dillion, Aksoy, Driska, and Murphy, 1982; Chaterjee and Murphy, 1983). Thus on stimulation of the swine carotid artery by various agonists it was observed that there was an initial surge in myosin phosphorylation and in cross-bridge cycling rate which fell to lower levels while tonus was maintained. As a result of such studies it has been suggested that tension maintenance in the absence of phosphorylation results from the development of a 'latch' state. The decreased phosphorylation and cross-bridge cycling rate has been demonstrated in the swine carotid artery (Dillion et al., 1982; Moreland, Moreland and Singer, 1986; Aksoy, Murphy and Kamm, 1982), tracheal smooth muscle (Silver and Stull, 1984; Gerthoffer, 1986) and feline oesophageal smooth muscle (Weisbrodt and Murphy, 1985).

Measurements of intracellular Ca^{2+} levels using the aequorin luminescence or the fluorescent dyes, quin2 and fura2, indicate that transients similar to those observed for myosin phosphorylation also occur with Ca^{2+}. Such transients are not observed with K^{+}-depolarisation. A causal relationship between Ca^{2+} and myosin phosphorylation can be postulated. For instance, Silver and Stull (1984) demonstrated that in bovine tracheal smooth muscle decreases

in myosin phosphorylation and hence, the onset of the latch state were more immediate for carbachol-induced responses than for K^+-depolarisation. Moreover, contractions induced by phorbol esters are slow in onset, not accompanied by transient increases in Ca^{2+} and myosin phosphorylation (Morgan, 1987; Chaterjee and Tajeda, 1986). Studies by Moreland and Moreland (1987) indicate that the initial rapid phase of tone development resulting from depolarisation may be associated with Ca^{2+} entry through a dihydropyridine-insensitive Ca^{2+} channel with a rapid rise in myosin phosphorylation and cross-bridge cycling rate. The sustained tonic response initiated by high K^+ and mimicked by the Ca^{2+} channel activator, Bay K 8644, is associated with Ca^{2+} entry through a dihydropyridine-sensitive channel, lower levels of myosin phosphorylation and slowly cycling cross-bridges. These results are in agreement with the presence of 'T' and 'L' type channels, the former being the main initial route for Ca^{2+} entry and insensitive to dihydropyridines (Bean, Sturek, Puga and Hermsmeyer, 1986).

The above studies suggest that while the role of Ca^{2+} in excitation-contraction coupling is obligatory, questions pertaining to its precise mechanism of action remain unanswered. For instance, Morgan and Morgan (1984) observed that the vasodilators, isoprenaline, papaverine and forskolin caused relaxation of ferret vascular smooth muscle while Ca^{2+} levels measured by the aequorin luminescence

slightly increased or did not change. On the other hand, sodium nitroprusside decreased both tonus and intracellular Ca^{2+} levels. These results indicate that relaxations can occur without lowering of intracellular Ca^{2+} levels.

1.8 Relaxation of Smooth Muscle.

Relaxation of smooth muscle is achieved by reduction of free cytoplasmic Ca^{2+} levels (Ca^{2+}_i) (but see above) or by reducing the rate of myosin phosphorylation in the presence of Ca^{2+}-calmodulin through phosphorylation of MLCK by a cyclic -AMP dependent phosphokinase (Godfraind and Miller, 1984). Ca^{2+}_i can be reduced by inhibiting influx of Ca^{2+}_e or the release of Ca^{2+}_i, or by enhancing the activity of Ca^{2+} removing mechanisms. A further possibility is that the catalytic ability of MLCK might be reduced by agents that interfere with the binding of Ca^{2+} to calmodulin or by phosphorylation by a cAMP dependent mechanism. Relaxations can also be achieved by opening of K^+ channels that are activated by an increase in Ca^{2+}_i (see Godfraind and Miller, 1984; Fleckenstein, 1983; Meech, 1978).

Thus drug-induced relaxations can occur via interaction with any of these mechanisms. Alternatively, release of other factors such as the endothelium-derived relaxing factor,(EDRF), which has recently been suggested as being or leading to the production of nitric oxide (Palmer, Ferrige and Moncada, 1987), may contribute to an agents' ability to relax smooth muscle.

1.8.1 Calcium extrusion and sequestration

Two main mechanisms have been suggested to be responsible for extruding Ca^{2+} from the smooth muscle cell - the Ca^{2+}, Mg^{2+} ATPase (the Ca^{2+} pump) and a Na^{+}-Ca^{2+} exchange mechanism. The ATP-dependent active transport of Ca^{2+} is mediated by a calmodulin-regulated Ca^{2+}-ATPase (Morel, Wibo and Godfraind, 1981,; Grover, Kwan, Crankshaw, Crankshaw, Garfield and Daniel, 1980). The Ca^{2+}-ATPase is thought to be the major contributor to Ca^{2+} extrusion. There is an extensive literature on the Ca^{2+} accumulating properties of microsomes from vascular and visceral smooth muscles (see Daniel, Grover and Kwan, 1982;1983). While the Ca^{2+} pump has been well characterised, less is known about the properties of Na^{+}/Ca^{2+} exchange. The existence of a form of Ca^{2+} extrusion driven by the Na^{+} gradient was proposed for smooth muscle by Reuter, Blaustein and Haeusler (1973). Experiments since have indicated that Na/Ca exchange probably has a quantitatively unimportant role in extruding Ca^{2+} except perhaps under special conditions (see Bolton, 1986). Undoubtedly, the low activity of the Na^{+}/Ca^{2+} exchange compared with that of the Ca^{2+}-pump present problems in its characterisation (Daniel et al. 1982).

Ca^{2+} removal from the cytoplasm may also be accompanied by sequestration by the ER and the mitochondria. Calcium accumulation by the ER is an energy-dependent process supported by ATP (Raeymakers and Casteels, 1981). The ER may

be the intracellular store which releases Ca^{2+} upon stimulation by agonists and may be involved in pharmacomechanical coupling. Additional sites for sequestration are the mitochondria, where the affinity for Ca^{2+} is low but the capacity for binding is high. Their role in regulation of Ca^{2+} is not entirely clear but it is unlikely that they play a role in the regulation of physiological levels of Ca^{2+}_i.

1.8.2 Cyclic Nucleotides

1.8.2.1 Cyclic AMP

There is extensive evidence to support the idea that many cell processes are regulated by cAMP-dependent phosphorylation of proteins and that cellular levels of cAMP can be influenced by the interaction of various agents with membranal receptors (Greengard, 1978; Hardman, 1981;). However various studies have indicated an apparent lack of correlation between the effect of different agonists on cellular cAMP levels in smooth muscle and the corresponding state of contraction of the tissue (Diamond 1977; Diamond, 1978). These discrepancies possibly reflect compartmentation of cAMP in the cell (Rapoport, Drazin and Murad, 1982; Diamond, 1984).

The enzyme adenylate cyclase, located in plasma membranes, catalyses the production of cAMP from ATP. An agonist-sensitive adenylate cyclase system is composed of at least three separable components; the agonist-receptor,

adenylate cyclase and a guanine-nucleotide binding (G) protein that regulates the rate of enzyme hydrolysis of ATP to cAMP. Two types of G proteins that when activated exert a stimulatory (G_s) or inhibitory (G_i) effect on adenylate cyclase (reviewed by Godfraind and Miller, 1985). Each regulatory unit in its basal, non activated state binds GDP and the associated adenylate cyclase converts ATP to cAMP at some basal rate. G_s, in the non-activated state, exists as a heterotrimer of , and subunits with GDP bound on the a-subunit. The interaction of agonist, receptor and G-GDP accelerates the exchange of GTP for bound GDP. The a-GTP dissociates from the agonist-receptor complex and from the bg-subunit via mechanism not yet identified. The G_i subunit exerts an inhibitory effect on adenylate cyclase and in addition may also be involved in the control of activity of other second-messenger systems such as phosphotidyl ionisitol pathway. Other G proteins have also been demonstrated that may regulate Ca^{2+} channels (see Dunlap, Holz and Rane, 1987 for review).

1.8.2.2 cGMP

Some agonists increase cGMP levels in various tissues (Goldberg and Haddox 1977). The increase in cGMP levels may be Ca^{2+}-dependent (Schultz and Hardman 1975) and/or Ca^{2+}-independent (Bohme, Graf and Schultz, 1978; Murad, Mittal, Arnold and Braighler, 1978). Organic nitrates such as sodium nitroprusside increase cGMP levels, however,

in non-vascular smooth muscle a dissociation between relaxations and increases in cGMP was demonstrated (Diamond and Janis, 1978) indicating a lack of cause and effect relationship.

In the lower oesophageal sphincter of the opossum, Torphy, Fine, Burman, Barnette and Ormsbee (1986) demonstrated tetrodotoxin-sensitive increases in cGMP levels during field-stimulation evoked relaxations. On the other hand, the putative NANC transmitter VIP (Rattan et al. 1977) increased cAMP levels accompaning relaxations.

1.8.3 Endothelium/Epithelium-mediated relaxations

It is now well established that acetylcholine (Ach)-induced relaxation of vascular smooth muscle is dependent upon the presence of an intact endothelium (Furchgott and Zadawski, 1980; see Peach, Loeb, Singer and Saye, 1985 for review). Relaxation seems to be mediated via an endothelium-dependent relaxing factor (EDRF) with the ultimate mediator being nitric oxide (Palmer et al., 1987). An analogous factor in tracheal smooth muscle, which contains epithelium, has been demonstrated by Flavahan, Aarhus, Rimele and Vanhoutte (1985). These authors showed increased responsiveness of Ach, 5-hydroxytryptamine and histamine in canine bronchial smooth muscle after epithelium removal and inferred that a relaxant factor was released from the epithelium. Conversely, the relaxations induced by isoprenaline were enhanced in unrubbed tissues. Increased

responsiveness to histamine was also obtained in guinea-pig tracheal smooth muscle by Goldie, Papadimitriou, Paterson, Rigby, Self, and Spina (1986) and to methacholine in human isolated tracheal muscle by Raeburn, Hay, Farmer and Fedau (1986). These studies demonstrate the presence of an epithelium-derived relaxing factor at least in tracheal smooth muscle. A 'sandwich protocol', similar to that employed by Furchott and Zawadzki (1980) to demonstrate the presence of EDRF in VSM, suggest that an epithelial factor is indeed released (Tschirhart and Landry, 1986). This study negates the conclusions of Holroyde (1986) who suggested that increased responsiveness to certain agonists could be due to easier access of agonists to the smooth muscle in epithelium denuded preparations. The nature of this factor and its presence in other non-vascular smooth muscle is still obscure.

1.8.4 Calcium-activated potassium channels

Ca^{2+} activated K^{+} channels have been identified in a number of excitable tissues including smooth muscle (see Meech, 1978 for review). The outward K^{+} current has been suggested to be responsible for the repolarising phase of the action potential triggered upon depolarisation. In some cells, such as pacemaker cells and aplysia neurones that display rhythmic discharges of action potentials, it has been demonstrated that Ca^{2+} ions entering the cell during the rising phase of the action potential activate the following

outward current. This leads to a silent period in between the bursts of action potentials. The presence of such currents in smooth muscle was first demonstrated in the guinea-pig taenia coli. Bulbring (1957) reported that adrenaline-induced relaxation was associated with abolition of spontaneous spike discharge and hyperpolarisation in this smooth muscle. An increase in K^+ permeability was suggested to account for the hyperpolarisation and relaxation (Jenkinson and Morton, 1967; Bulbring and Tomita, 1969). The absolute requirement for Ca^{2+} in the adrenaline-mediated relaxation led to the suggestion of the presence of Ca^{2+}-activated K^+ channels in the guinea- pig taenia coli (Bulbring and Tomita, 1977; den Hertog, 1981). Ca^{2+} activated K^+ currents have been implicated for ATP- and field stimulation-evoked relaxations in the taenia coli (den Hertog and Mass, 1979; Mass, 1981) on the basis of the ability of the bee venom, apamin to block such channels (Banks et al. 1979). It has also been reported that neurotensin-induced relaxations in the rat duodenum and ileum are dependent on extracellular Ca^{2+}, blocked by the Ca^{2+} channel antagonist, nifedipine and enhanced by the Ca^{2+} channel activator Bay K 8644. Apamin blocked these relaxations and it was therefore suggested that neurotensin-induced relaxation was due to activation of Ca^{2+} activated K^+ channels (Kullak,Donoso and Huidobro-Toro, 1987).

The presence of Ca^{2+}-activated K^+ channels have also been demonstrated by the patch-clamp technique. Single channel

recordings indicate that such channels exist in large numbers in the rabbit jejunum and guinea-pig mesenteric artery (Benham, Bolton, Lang and Takewaki, 1986). Benham et al. (1986) also demonstrated large conductances of these channels and a high sensitivity to Ca^{2+}.

The precise mechanism of the translation from hyperpolarisation to mechanical relaxation may be due to closure of Ca^{2+} channels at lower membrane potentials, and hence, a decrease in Ca^{2+}_i. On the other hand, in the case where POC are not involved other messenger systems such as cGMP (Ignarro and Kadowitz, 1985) and phosphorylation of Ca^{2+} channels by cAMP-dependent protein kinase (Levitan, Lemos and Novak-Hofer, 1983) may be responsible.

OBJECTIVES

Bieger and Triggle (1985) considered the possibility of the rat TMM asisting in oesophageal peristalsis. This conclusion was based on the presence of an aboral gradient in electrical and pharmacological excitability and its ability to generate intraluminal pressure. As proposed by Roman (1982) inhibitory mechanisms governing peristalsis arise in neural structures located in the end organ. While relaxations to long pulse field-stimulation were reported by Bieger and Triggle (1985), these were found to persist in the presence of TTX. Since lack of sensitivity to TTX may indicate either a non-neural mechanism or activation of nerves not utilising fast sodium channels, it seemed pertinent to investigate the basis of the field-stimulated relaxation in greater detail. Thus the questions specifically addressed in this study were:

1) Is the field-stimulated relaxation of isolated TMM neurally-mediated?

To answer this question the experimental approach needed means of inducing denervation and the use of pharmacological agents capable of releasing substances from nerve endings. It was also necessary to demonstrate the presence of a nerve plexus.

2) What is the pharmacological basis of TMM relaxations?

In this regard, antagonists of known receptor types

could be used to elucidate the nature of transmitter(s) involved. It was also necessary to determine the presence of and the pharmacological effects of putative inhibitory neurotransmitters in the rat TMM.

3) Is there any inhibitory vagal input to the rat TMM?

To address this question it was necessary to determine whether the stimulation of the vagus would lead to the excitation of inhibitory nerves in the TMM and if pharmacological manipulation would modify such responses.

4) What is the role of calcium in supporting both excitatory and inhibitory events in the TMM?

The role of calcium in the excitation-contraction coupling and uncoupling was also examined. Since clinical evidence supports the use of calcium antagonists in the treatment of certain oesophageal disorders, such as diffuse oesophageal spasm, the effects of these agents on the TMM appeared of particular interest.

CHAPTER 2

METHODS AND MATERIALS

2.1 Animals

Male Sprague-Dawley rats, 250-350g, were purchased from Canada Hybrid Farms, Halifax, Nova Scotia. Male Hartley guinea-pigs were obtained from Charles River Inc., Montreal, Quebec. The animals were housed, four to a cage, in the animal quarters of the Faculty of Medicine, Memorial University of Newfoundland, under minimal disease conditions. Rats and guinea-pigs were kept in separate rooms and had a 12hr light/12hr dark light-cycle with controlled humidity and temperature. The animals had access to food and water ad libitum. Purina Rat chow and Supersweet rabbit chow were fed to rats and guinea-pigs, respectively.

In experiments where canine TMM was used (section 3.11), the oesophagi was obtained from 11 dogs that had undergone surgery at the Surgical Research Unit of the Faculty of Medicine, Memorial University. The oesophagus was transected from the supradiaphragmatic region of dogs that were maintained under nembutal anaesthesia. The oesophagus was immediately placed in ice-cold Tyrode solution and brought to the lab within 10 min for experimentation.

2.2 Preparation of tissues for in vitro mechanical studies

2.2.1 Vagus nerve-oesophagus preparation

Rats were killed by a blow to the head and exanguinated through the jugular veins. The thorax was exposed with a midline incision, the rib cage cut open and the oesophagus dissected out from the pharynx to the stomach. A metal rod was slipped through the lumen and the oesophagus placed in a Sylgard-coated Petri dish containing Tyrode solution aerated with 95%O_2/5%CO_2. The right and left vagus nerves from the middle supradiaphragmatic region were gently dissected free for a length of 1.5 - 2.0 cm up to the distal 1/3 of the oesophagus. This portion of the oesophagus was used for recording of vagally-evoked responses. At both ends, 0.3-0.5 cm of the outer striated tunica propria was removed and the underlying muscularis mucosae was tied with 5-0 silk thread (Ethicon). The distal end was attached by a loop to a tissue holder while the upper end was tied to a force transducer (Grass FT03C). The vagus nerves were draped over bipolar silver wire electrodes. The preparation was maintained in a 25ml jacketed organ bath thermostatically regulated at 37^{o}C and containing Tyrode solution aerated with 95%O_2/5%CO_2.

2.2.2 Isolated TMM of rat and guinea-pig

The oesophagus was dissected and placed in a Petri dish as described above. Care was taken to maintain the length of the oesophagus during dissection equivalent to its original length. The tunica propria was split lengthwise and

dissected away leaving the smooth muscle tube, viz. the tunica muscularis mucosae (TMM). The TMM was divided into three or four segments depending on the length of the oesophagus dissected from the rat and guinea-pig. The segments corresponded to proximal (cervical), one or two middle (supradiaphragmatic) and distal (infradiaphragmatic) portions, each of 1 1/2 - 2cm in length. Each segment was secured at one end via a loop of silk thread (5-0 silk, Ethicon) to a tissue holder and pulled through two concentric platinum ring electrodes spaced 10 mm apart. The other end was tied with the silk thread to a Grass FT03C transducer. The preparation was placed in a 25 ml jacketed organ bath containing Tyrode solution aerated with 95%O_2/5%CO_2 and maintained at 37^{o}C.

2.2.3 Guinea-pig taenia caeci.

The white smooth muscle band overlying the caecum of the guinea-pig viz. the taenia, was dissected free, care being taken to avoid contamination with caecal contents. 1 1/2 cm long strips were set up in similar fashion to the TMM .

2.2.3 Canine TMM strips

The tunica propria from the canine oesophagus was stripped off and the underlying muscularis mucosae was cut into strips measuring 0.5 cm in width to 1.5 cm in length. The strips were set up as described above for the rat TMM.

2.3 Mechanical Recording

Isometric longitudinal tension was recorded via the Grass FT03C transducers on a Grass 7D polygraph or Beckman R-611 dynograph. The transducers were mounted on adjustable clamps permitting fine adjustments of resting muscle tension without overstretching the tissues. All tissues were set up at a preload of 0.3 g except for guinea-pig taenia caeci which was set up at 1.0 g preload.

2.4 Electrical Stimulation

Field-stimulation of the isolated TMM and stimulation of the vagus nerve were delivered via a Grass S88 stimulator with rectangular pulses. Standard stimulation protocols employed pulse trains of 10 s durations delivered every 1.5 min. Voltages did not exceed 10 V for stimulation of the vagus nerve and 40 V, for field stimulation of the isolated TMM. Pulse width and frequencies were varied depending on the type of response examined.

2.5 Cooling

The bath temperature was controlled using a Lauda K2/R thermostat-regulated circulator pump equipped with a refrigerator which permitted cooling within a short-time (5-8 min).

2.6 Cold Storage.

The TMM was dissected and stored unaerated in the refrigerator for 1-7 days in Tyrode buffer. 1% bovine serum albumin (BSA) was routinely added to the buffer. In the absence of BSA, most tissues failed to respond to agonists after 2-3 days. The cold-stored tissues were removed from the refrigerator after varying periods of time and set up for tension recording in fresh Tyrode solution at a preload of 0.3 g and maintained at 37°C for 2 h prior to experimental tests.

2.7 Reserpine and PCPA treatment

Four rats were injected intraperitoneally with reserpine (5 mg/kg) and four with PCPA (300 mg/kg, on the 1st day and 100 mg/kg on the day of the experiment (2nd day)). The rats were sacrificed and TMM set up as usual.

2.8 Experimental Protocol

Tissues were allowed to equilibrate in Tyrode solution for 1 hr with continuous aeration. They were intermittently washed with fresh warm Tyrode (37°C) during this interval. Concentration-response curves for spasmogenic agents were obtained by cumulative additions to the organ bath. For relaxant agents, the cumulative concentration-response relationship was examined in the presence of steady-state tonus achieved by a fixed concentration of the spasmogen. In the case where drugs were examined for their ability to inhibit field stimulation-

evoked relaxations, tissues were contracted with an agonist and allowed to achieve a steady-state tonus. The antagonists were then applied to the bath and changes in field-stimulation evoked relaxations (FSR) observed. Some drugs produced relaxations and in such cases tonus was titrated back to its orignal tension with additional spasmogen.

2.9 Calcium antagonists.

The calcium antagonists were either applied during steady state tonus or for 10 min prior to addition of contractile agent. Field stimulation-evoked relaxations were examined under both conditions. Calcium concentration-response curves were carried out in fresh TMM segemnts which had been set up in the organ bath and equilibrated for 1 hr. The tissues were subsequently incubated in calcium-free buffer and after 45 min challenged with the spasmogens. Calcium (0.1-1.8 mM) was added to the bath and the ensuing tension measured.

2.10 Histochemical studies.

2.10.1 Fixation methods.

The rat oesophagi were fixed either in situ or post mortem. In situ fixation was achieved by retrograde perfusion (under nembutal anaesthesia) through the abdominal aorta with ice-cold saline (50 mls) containing 1% $MgCl_2$ followed by 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.2)

for 20 min at a pressure of 120-140 mmHg. The oesophagus was then excised, tied off at both ends after distension with sufficent fixative, and left for post-fixation for 12-14 h at 4 ^{o}C. The outer tunica propria was then peeled away and the TMM thoroughly washed in phosphate buffered saline (PBS).

Post mortem fixation was carried out after the oesophagus was dissected from the rat and the outer tunica propria peeled away. The TMM was then split longitudinally, pinned flat on a Sylgard-coated Petri dish and immersed in Zamboni's fixative for 24 h at 4 ^{o}C. The TMM was then washed sequentially for 10 min in 80% ethanol, 95% ethanol, 100% ethanol, xylene, 90% ethanol, 70% ethanol, 50% ethanol and PBS.

2.10.2 Cholinesterase staining

The TMM was reacted with acetylthiocholine medium by the method of Shute and Lewis (1967). Dissection of the TMM was carried out in Tyrode and fixation for 5-10 min in a glutaraldehyde/formaldehyde (1%:4%) mixture with subsequent washing in distilled water. The muscle was then inflated with the Shute and Lewis medium in the presence or absence of the butyryl cholinesterase inhibitor iso-OMPA ($1x10^{-5}$ M), and left immersed in this medium for 12-16 h at 4^{o}C. Staining was developed with 10% K^{+} ferricyanide, washed in distilled water, split longitudinally and mounted on chromalum coated slides and observed under a light

microscope.

2.10.3 Immunohistochemistry

Segments of TMM were processed according to the peroxidase-antiperoxidase method of Sternberger (1979). Whole mounts were incubated for 4-5 min at room temperature in 10% normal goat serum in PBS containing 0.3% H_2O_2 and 0.2% Triton X-100 to preblock nonspecific immunoreactivity and inactivate endogenous peroxidases. After washing in PBS, the segments were incubated at 4 ^{o}C for 24 h with an optimum dilution of primary antiserum raised in rabbits. The optimum dilution corresponded to staining revealing faint background but suficent staining of intramural structures. The dilutions were generally cross-checked in sections of brain stem and spinal cord in a seperate study by Vyas, Bieger and White (1985). Antisera and dilutions used were serotonin (1:2000), VIP (1:333), tyrosine hydroxylase (1:100) and calcitonin-gene-related peptide (1:20,000).

After primary antibody treatment, the segments were washed in PBS for 1 h and incubated sequentially in 1:150 dilution of goat antirabbit-antisera at room temperature for 1 1/2 h and 1:300 dilution of rabbit peroxidase antiperoxidase complex for 2 hr at room temperature with repeated washings in PBS in between incubations.

Staining was developed in 3,3,diaminobenzidine HCl (0.5 mg/ml), glucose oxidase (3.8 U/ml) and D-glucose (2

mg/ml) made up in 0.1 M phosphate buffer, pH 7.2. The sections were mounted on chromalum coated slides, air-dried, dehydrated in ethanol, cleared in xylene and coverslipped with a mounting medium, glycerine, or after air drying mounted with Farrants medium (BDH Chemicals).
Controls were run with either primary antibody preabsorbed with excess antigen (100 mg antigen/ml diluted antisera) or in pre-immune rabbit serum.

2.10.4 Tryptophan/nialamide treatment

To enhance serotonin immunoreactivity, rats (n=5) were pretreated with L-tryptophan (300 mg/kg i.p.) and 90 min later with nialamide (100 mg.kg i.p). The rats were sacrificed one hour later.

2.10.4 Toluidine blue staining.

Toluidine blue staining was carried out by the Electron microscopy unit by the method of Hyam (1981). The tissues were fixed in Karnovsky's fixative (pH 7.4) at room temperature for 20 min, embedded in Epon 812, sectioned on a Cambridge-Huxley Ultramicrotome and stained with 1% toluidine blue.

2.11 Buffers

2.11.1 Tyrode

The composition of the Tyrode solution was (in mM):

NaCl 137.0, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.88, NaH_2PO_4 0.36, $NaHCO_3$ 12.0, and glucose 5.5. Stock solutions were generally prepared and stored in the refrigerator at 4°C. Prior to use the buffer was aerated with 95%O_2/5%CO_2 and the pH adjusted to 7.4 with 1N HCl or 1N NaOH as necessary. Depolarising isotonic K^+ solutions were prepared by equimolar substitution of NaCl with KCl.

2.11.2 Phosphate-buffered saline (PBS)

The buffer contained 13.4g $Na_2HPO_4.7H_2O$ and 8g NaCl in 1 l of distilled water. The pH was adjusted to 7.2 with 2N HCl.

2.11.3 0.2M Phosphate buffer.

The phosphate buffer contained KH_2PO_4 (8.06 g/l) and $Na_2HPO_4.7H_2O$ (37.75 g/l). The pH was adjusted to 7.2.

2.11.4 4% Paraformaldehyde buffer.

Paraformaldehyde powder (40 g) was dissolved in 500 ml distilled water by heating to 55 °C. The resulting cloudy solution was cleared with 2 N NaOH and diluted with 500 ml of 0.2 M phosphate buffer.

2.11.5 Zamboni's fixative

This fixative contained 2%(w/v) paraformaldehyde in 0.1M phosphate buffer and 15% saturated picric acid.

2.11.6 Thiocholine medium

100 mg of acetylthiocholine was dissolved in 4 ml of distilled water. To this 7 ml of 0.1 M copper sulphate was added and centrifuged at 2 K for 15-20min; to 10 ml of supernatant was added 62 mg glycine. The pH was adjusted to 5.5 with 1.0 M sodium acetate and a final volume of 20 ml was made up with distilled water.

2.12 Chemicals

The drugs used in this study and their sources were: Sigma, USA: (+) propranolol HCL, isoprenaline, indomethacin, ATP (disodium salt), α-chymotrypsin (bovine pancreatic), histamine dihydrochloride, nordihydroguaretic acid, vasoactive intestinal polypeptide, apamin (bee venom), scorpion (Lierieus quinquestriatus) venom, serotonin oxalate, A23187 (calimycin), GABA, tetrodotoxin, picrotoxin, trypsin, veratridine sulphate, α-β-methylene ATP, metiamide, ouabain, 3,3'-diaminobenzidine HCl, glucose oxidase (Aspergillus niger Type V)

Calbiochem:

methscopolamine, tetrodotoxin, (+) tubocurare HCl, (+) muscarine hydrochloride,

RBI chemicals: (+) cis-2-metyl-4-dimetylamino-methyl-1-3-dioxolane methiodide (CD).

The following drugs were generously donated:

(+) etidocaine (Duranest, Astra Pharmaceuticals, USA)

Phenoxybenzamine HCL (Smith, Kline and French, Canada)

Verapamil (Isoptin-Hyrochlorid, Knoll A.G. Germany)

Nifedipine (Bayer, Germany)

CD (Dr D.J. Triggle, S.U.N.Y. at Buffalo, USA)

Trazodone (Bristol Myers Co., USA)

ATX II (Dr O. Wassermann, Kiel, Germany)

Cromoglycic acid (Dr D. Biggs, University of Alberta, Canada)

Fenfluramine (A.H. Robbins, Richmond, Virginia, U.S.A.)

ETYA (5,8,11,14 Eicosatetraynoic acid) (Hoffman La Roche, USA)

BRL 34915 ((+) 6-cyano-3,4 -dihydro- 2,2 dimethyl-trans-4-(2-oxo-1-pyrollidyl) -2H -benzo[b]pyran -3-ol) (Beecham Pharmaceuticals, U.K.

Trifluroperazine HCl (Smith, Kine and French, USA)

Bay K 8644 (methyl-1, 4-dihyro-2, 6-dimethyl -3-nitro -4- (2-trifluromethylphenylpyridine -5 -carboxylate) (Miles Laboratory, USA)

PN-200-110 (isopropyl -4 -(2,1,3 -benzoxadiazol -4-yl) -1,4, -dihydro 2,6 -dimethyl -5- methoxy-carbonylpyridine -3-carboxylate) (Dr Hof, Sandoz Ltd., Switzerland)

The antisera were purchased from:

Tyrosine Hydroxylase (Eugene Tech. Int., Allendale, N.J. USA.

Serotonin, Substance P and VIP (Immuno Nuclear Corp., Stillwter, MN., USA)

CGRP (Amersham, Oakville, Ont. Canada)

Goat anti-rabbit antiserum (Boehringer Mannheim Biochemicals, Dorval, Quebec, Canada)

Rabbit peroxidase-antiperoxidase (Sternberger Meyer Immunocytochemicals, Jarrettsville, MD, USA)

2.13 Statistics

The means and standard error of means for each concentration in the concentration-response curve were calculated and are represented in figures as points with error bars. The 'n' reported in tables and figures refers to number of tissues unless indicated otherwise.

In some cases the concentration-response curves were plotted according to the method of Carpenter (1986). Cumulative concentration-response curve were normalised and log concentrations interpolated for each predetermined response. Curve normalisation involved expressing each response as a percentage of the maximal response; log concentrations were then averaged and the mean maximal response was expressed as a fraction of the maximal attainable response. This method was applied to preserve the shape of the curve particularly in cases where regional differences were apparent. This method was not utilised in the case where variability in responses between segments was not apparent.

Relaxations to drugs were measured as percentages of

the steady state tonus before application of the relaxant according to the equation (A-B)/A x100% where A represents the tension due to muscarine and B the tension after relaxant. Either student's t distribution or Wilcoxon rank sign test was used to test for significance of differences between two means. Differences with p values of less than 0.05 as determined by a two tailed test were considered significant. Calculations were performed by the use of statistical package of Tallarida and Murray (1981) on an Apple IIe computer or the Epistat program on the Tandy 1200.

CHAPTER 3

RESULTS

3.1 Anatomical observations

In rats of 250-300 g the oesophagus, as measured between the cricoid cartilage to the cardia was about 10 cm in length. Both the right and left vagus were observed to course alongside the striated outer tunica externa (TME) of the caudal half of the oesophagus . The striated muscle could be distinguished from the inner smooth muscle visually. The TME was reddish brown and could be stripped away revealing the white smooth tunica muscularis mucosae (TMM) underneath. The lower oesophageal sphincter could be identified as the region resisting passage on slipping a rod through the lumen. Furthermore, the region was thicker and consisted of both striated and smooth muscle (Marsh and Bieger, 1987). This region corresponds to the 'loop of Willis' (c.f. Ingelfinger, 1958).

3.2 Histochemical observations

The myenteric plexus, as visualised by the thiocholine technique, lies between the two layers of striated muscle (see Introduction) and the submucosal plexus between the striated tunica propria and smooth muscle tunica muscularis . Axons of the submucosal plexus were identified in close proximity to the smooth muscle cells by electron microscopy and the plexus in its entirety was observed by

cholinesterase staining.

3.2.1 Cholinesterase staining

When reacted with acetylthiocholine, in the presence of iso-OMPA ($1x10^{-5}$M), the TMM revealed a cholinesterase positive nerve plexus on its abluminal surface with little staining of the smooth muscle. In the absence of iso-OMPA staining was evident in the smooth muscle in addition to the nerve plexus thus providing a dark background for the latter (Fig 2). Cell bodies (ganglia) in some cases could be identified along the inter-nodal strands. No set pattern for the fibre projections were noted. Furthermore, no marked difference between the proximal and distal segments was observed.

3.2.2 VIP-like immunoreactivity (VIP-IR)

Nerve fibres demonstrating VIP-IR were observed throughout the length of the TMM (Fig 3). The nerve fibres lay alongside the muscle bundles and tended to be in a longitudinal direction. Ganglionic cell bodies were not apparent and there was no regional difference in the pattern of innervation. Oval-shaped cells of dimensions smaller than 5HT-immunoreactive cells (see below) were observed in close proximity to the smooth muscle. These cells characteristically displayed VIP-like immunoreactivity in the circumference and nuclei with the major part of the cytoplasm devoid of immunoreactivity. There was no apparent relationship between these cells and nerve fibres.

Fig 2: Acetylthiocholine-induced staining of the rat TMM. A photomicrograph of whole mount of the TMM shows the submucosal nerve plexus as visualised by the thiocholine technique.

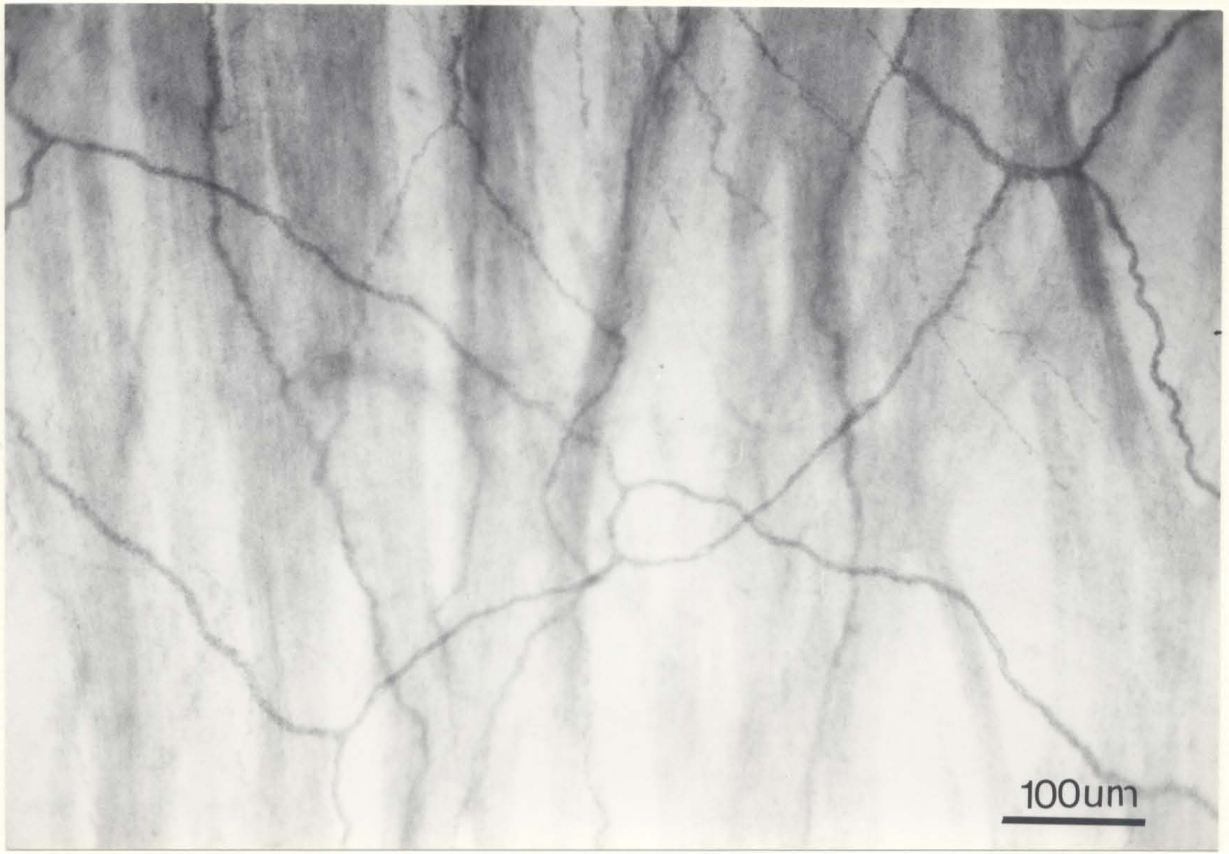
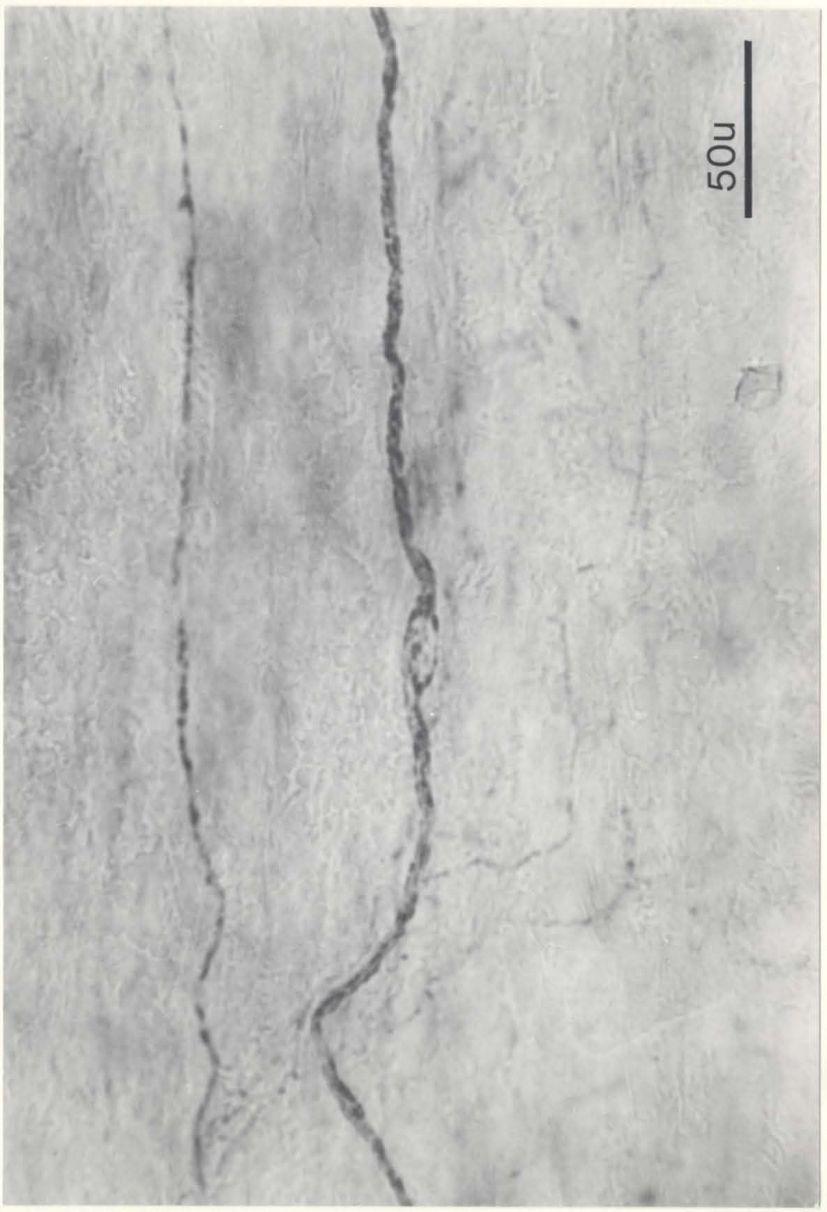

100um

Fig 3: VIP-like immunoreactivity of the isolated TMM. Figure is a photo- micrograph of whole mount TMM showing nerve fibres and some cells (arrow) lying close to the fibres.


50u

3.2.3 CGRP-like immunoreactivity

CGRP-like immunoreactivity was observed on the abluminal surface of the rat TMM. Nerve fibres were generally aligned in the longitudinal direction (Fig 4). As with VIP-IR, cell bodies could not be identified in this region. No apparent regional differences were observed.

3.2.4 Tyrosine Hydroxylase immunoreactivity (TH-IR)

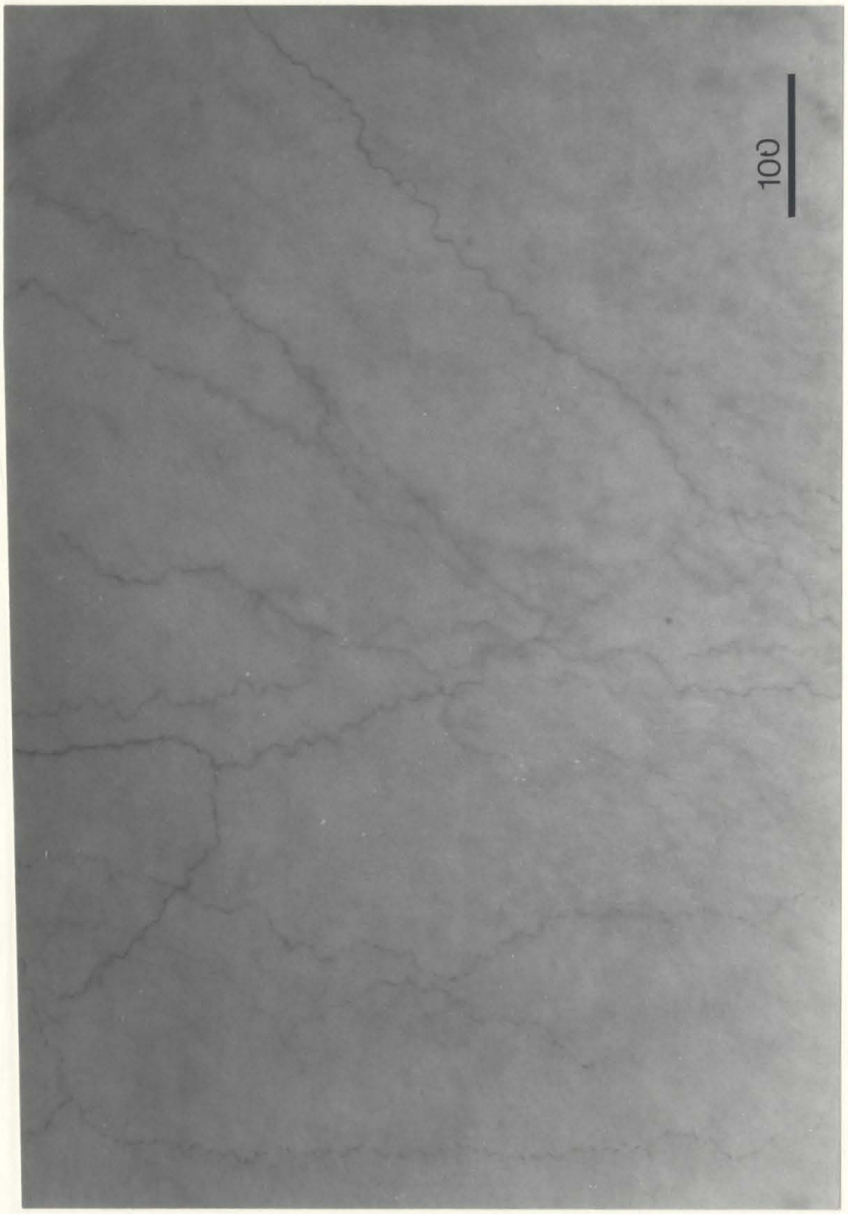
TH-IR was sparse and localised mainly to the blood vessels. Nerve fibres generally ran in a circular direction around the blood vessel. Varicosities could be distinguished along the fibres.

3.2.5 5-Hydroxytryptamine-like immunoreactivity (5HT-IR).

Neither 5HT-IR nerve fibres nor cell bodies could be detected in the TMM. However, 5HT-IR was observed in cells that were scattered throughout the smooth muscle layer of the TMM (Fig 5). These cells were generally oval in shape and measured approximately 40 um in the longitudinal axis and 10 um in width. Some cells displayed short apiculate appendages (Fig 6). No preferential localisation to the vasculature or the epithelium could be detected. Immunoreactivity was markedly enhanced in animals pretreated with a tryptophan/nialamide regimen. There were no detectable regional differences in size, shape or number of these cells. Cells similar to those stained for 5HT could also be stained

Fig 4: CGRP-like immunoreactivity in the rat TMM.

Photomicrograph of whole mount of the rat TMM reacted for CGRP (see methods). Nerve fibres are generally aligned in a longitudinal direction. Note lack of cell bodies.


100

metachromatically with toluidine blue (Fig 7) and were found to contain granular material. These cells were identified as mast cells. Control experiments in which antibodies were treated with excess 5HT failed to demonstrate 5HT-IR structures.

Fig 5: 5HT immunoreactivity in the rat TMM.

Oval shaped cells were observed under light microscope scattered along the smooth muscle. 5HT immunoreactivity was observed after in vivo treatment with tryptophan and nialamide.

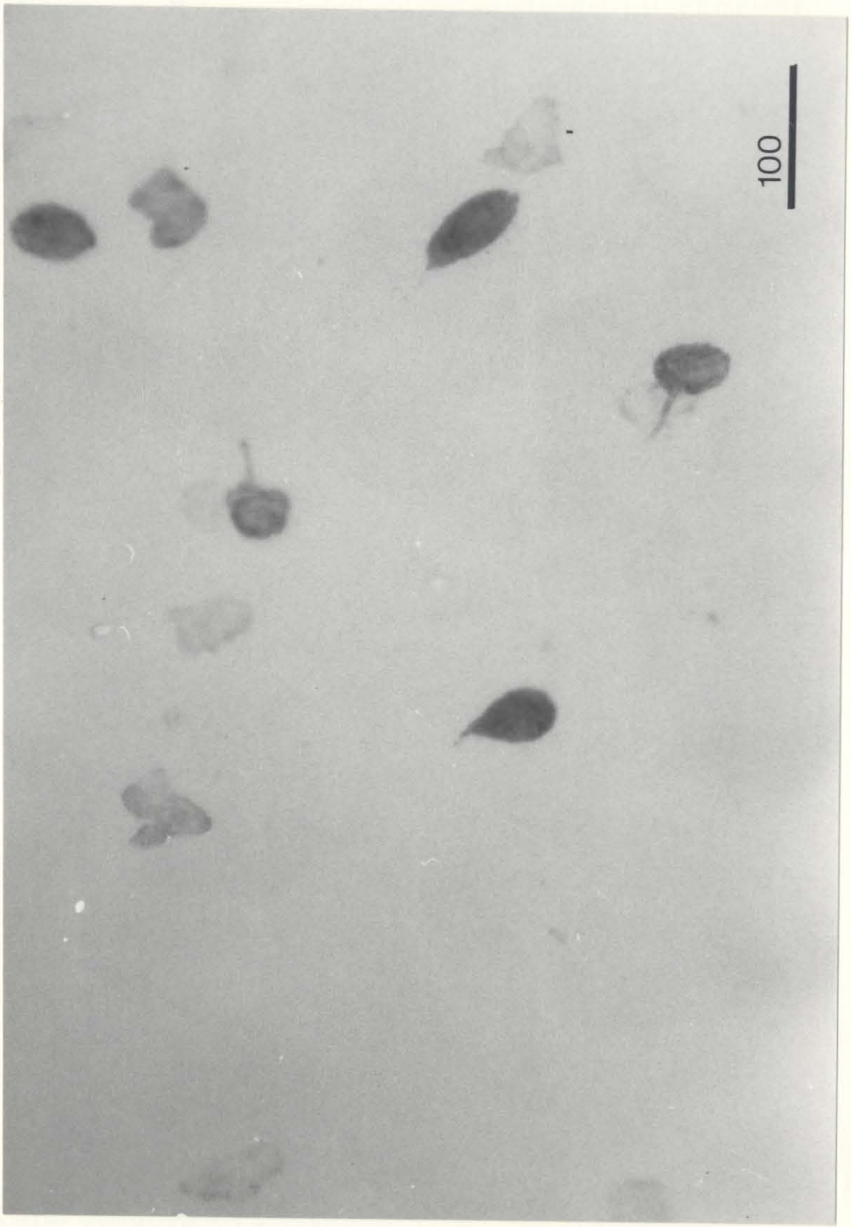

100

Fig 6: A higher magnification of a 5HT immunoreactive cell. Note the presence of short process (arrow).


50u

Fig 7: Toluidine blue staining in a cross section of rat oesophagus smooth muscle.

sm, smooth muscle, viz. tunica muscularis mucosae. Note granular structure of mast cell (arrow).


50u
sm

3.3 Agonist-induced motor responses of the TMM

3.3.1. Contractile

3.3.1.1 Cholinoceptor agonists

Acetylcholine, (+) muscarine, (+) CD and carbachol ($1x10^{-8}$M -$1x10^{-5}$M) produced graded contractions of the TMM. In general, the contractions by these agents could be separated into phasic and tonic components. Methscopolamine and atropine inhibited the contractions. Although EC_{50} values revealed no regional differences (Table 1) the maximal amplitude of contraction of the distal segment was greater than that of the proximal. However, after correction for wet wt of tissue, the differences were not significant suggesting that differences in muscle mass may account for the increased amplitude of contraction. The amount of tonus induced in the middle segments was similar to that induced in the proximal.

3.3.1.2 Potassium

Contractions to K^+ could be elicited by isotonic depolarising solutions (Fig 8) or by cumulative additions of K^+ (Fig 9). In the former case, contractions consisted of a phasic and a tonic component which could be distinguished at concentrations > 10 mM. The contractions under both conditions of K^+ application were diminished by methscopolamine and hemicholinium. The depression of the response to isotonic K^+ (50 mM) by methscopolamine ($1x10^{-8}$M) was 64 + 19% (n=3). A non-competitive inhibition was revealed

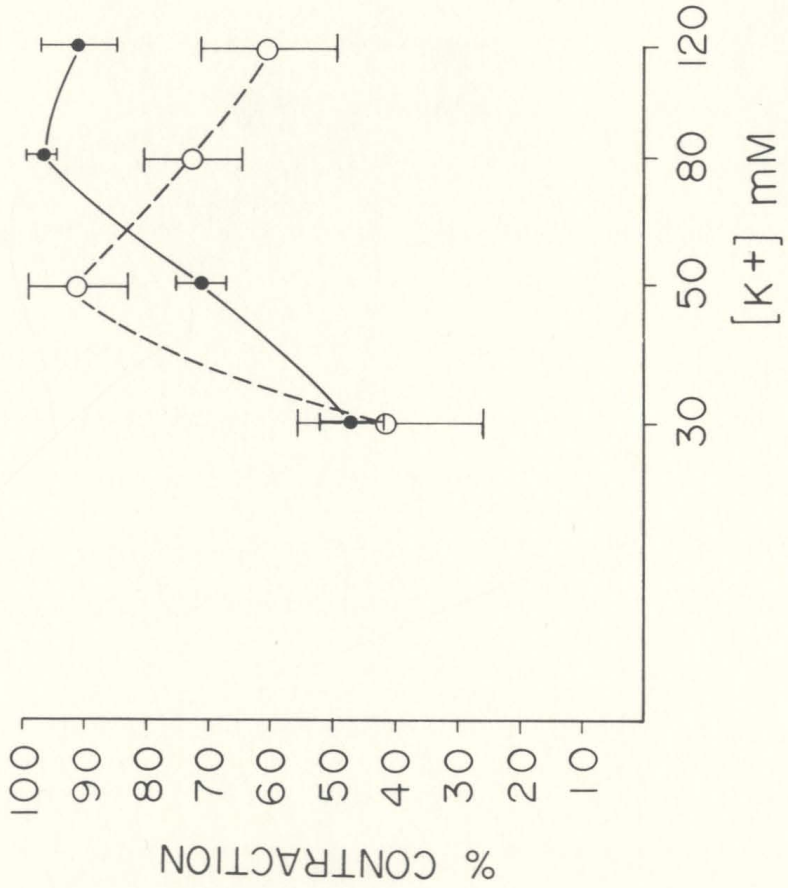
TABLE 1

A comparison of proximal and distal segments of the rat isolated TMM with respect to maximal tension and EC_{50} of muscarine.

	Proximal	**Distal**
Max. tension muscarine (g)	1.66 ± .14	2.27 ± .18*
EC_{50} muscarine	1.1×10^{-7}M	1.0×10^{-7}M
mg tension/wet wt(mg)	95 ± 28 (P=0.217)	120 ± 63

* significant difference (Wilcoxon rank test, two-tailed)

Fig 8: Concentration-response curves for potassium-induced phasic (●) and tonic (○) contractions of isolated TMM. Tissues were pretreated with 1 x 10^{-8}M methscopolamine. Vertical bars represent S.E.M.(n=6).


% CONTRACTION
100
90
80
70
60
50
40
30
20
10
30
50
80
120
[K+] mM

to cumulative additions of K^+ (Fig 9). The distal segment developed significantly more tension than the proximal (Table 2). In the presence of 50 mM isotonic K^+, CD ($1x10^{-6}M$) induced further contractions (n=4) indicating the separate pathways for Ca^{2+} influx utilised by these two agents.

3.3.1.3 Histamine and Substance P.

Contractions to histamine ($1x10^{-6}M$) and substance P ($1x10^{-7}M$) were transient in nature. The contractions to these agents were not sensitive to methscopolamine, hemicholinium or TTX.

3.3.2 Relaxations

3.3.2.1 5-Hydroxytryptamine

5HT produced relaxations of the TMM in a concentration-dependent manner. The relaxations were evident in tissues precontracted with either muscarinic agonists or K^+. The relaxations were regionally differentiated with complete relaxations occurring in the proximal but not the distal segment (Fig 10). The middle segment responded to 5HT like the proximal segment. While the distal segment did not relax maximally to 5HT, isoprenaline ($1x10^{-6}M$) and papaverine ($1x10^{-4}M$) were effective in returning tone to baseline levels. 5HT relaxations were dependent on the level of tone with rightward shifts in the concentration-response curve occurring in the presence of maximal tonus (Fig 11).

3.3.2.2 VIP

Fig 9: Concentration response curve to cumulative addition of potassium in the rat TMM in the presence of methscopolamine and hemicholinium.

Potassium chloride was added cumulatively to the Tyrode solution bathing the TMM. Responses are expressed as percentage of maximal response. (○) control (n=15), (△) methscopolamine treated (1×10^{-8}M) (n=4), (□) hemicholinium treated (1×10^{-5}M) (n=3). Vertical bars represent S.E.M.

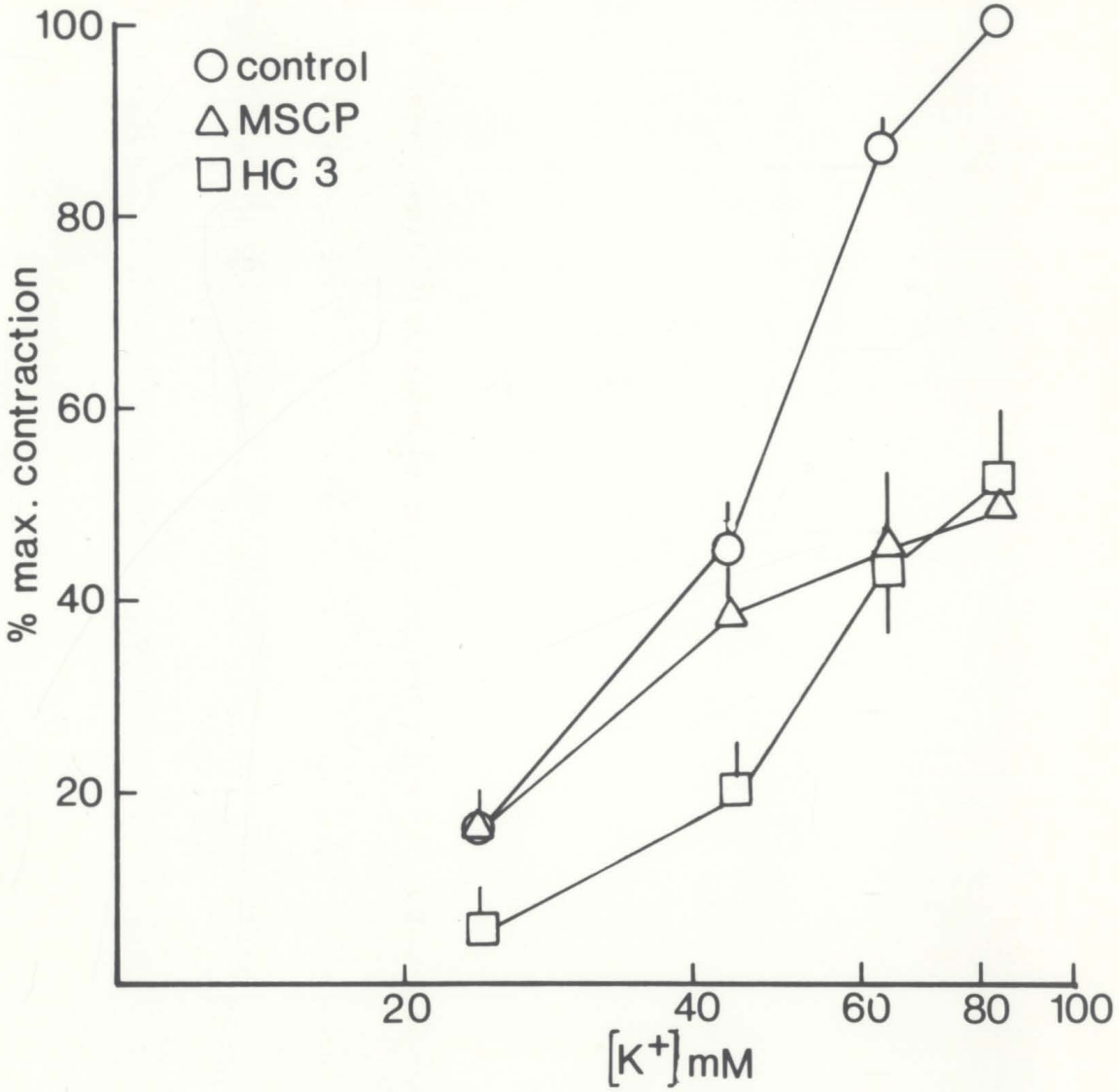

100
80
60
40
20
% max. contraction
control
MSCP
HC 3
20
40
60
80
100
[K+]mM

TABLE 2

Tension due to isotonic depolarising K^+ and cumulative addition of K^+ in the rat isolated TMM.

Isotonic depolarising K^+

Segment	mg tension/ wet wt (mg).	% of max. contr.
Distal	66 + 35 (n=10)*	55
Proximal	44 + 13 (n=10) (P= 0.05)	46

Cumulative K^+

	EC_{50}	Max. tension (g)	
Distal	43 mM	68 + 36	57
Proximal	46 mM	42 + 12* (P=0.01)	44

(*) significant difference (Wilcoxon rank test, two tailed)

Fig 10: 5HT-induced relaxation in proximal (A) and distal (B) segments of the rat isolated TMM. Maximum relaxations were achieved in the proximal but not distal segment. Bottom tracing marks field-stimulation delivered in 10s trains at a pulse-width of 2 ms, frequency of 4 Hz and supramaximal voltage. Tissues were treated with 1×10^{-7}M TTX and tone was induced with 3×10^{-7}M muscarine. Note proximodistal difference in concentration of 5HT required to produce relaxation of equal amplitude.

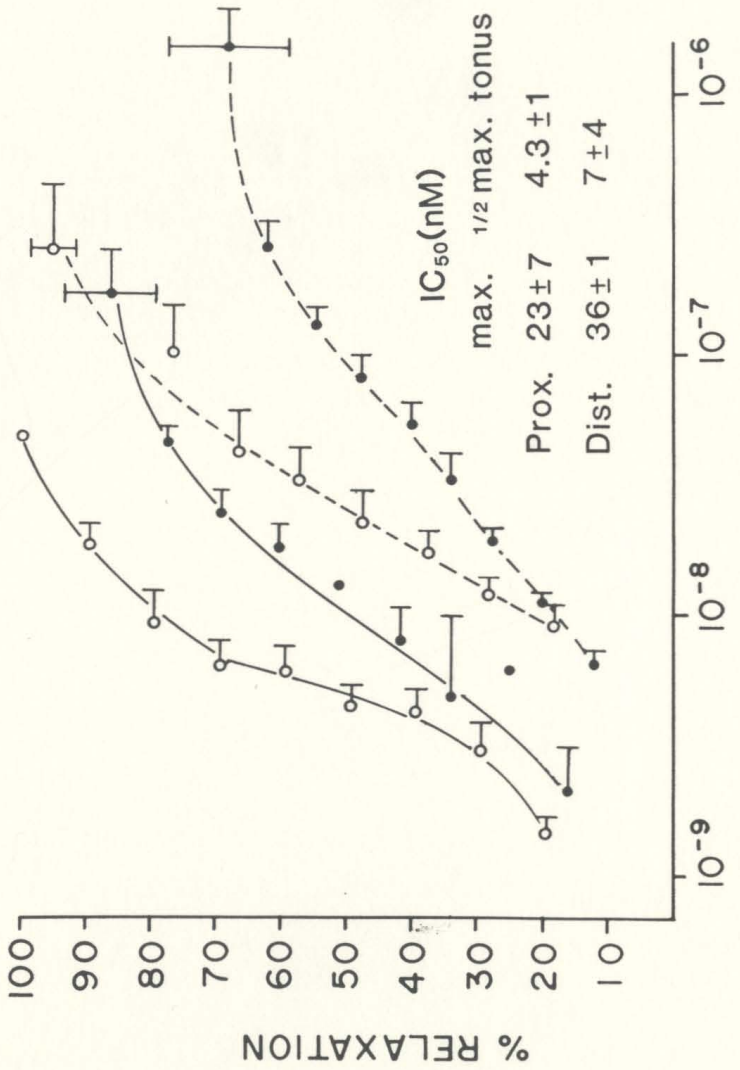
PROXIMAL

TTX 10^{-7}M

Musc. $3x10^{-7}$M

DISTAL

Fig 11: Concentration-response curves for inhibition of muscarine-induced tonus by 5HT in the rat isolated TMM. Inhibition was measured at maximal (broken line) and half-maximal tonus (solid line). Curves were plotted according to the method of Carpenter (1986). Horizontal bars indicate S.E.M. (n=5). IC_{50} values for proximal (O) and distal segments (●) are presented at bottom right hand corner of graph (mean ± SEM, n=5 for each curve).

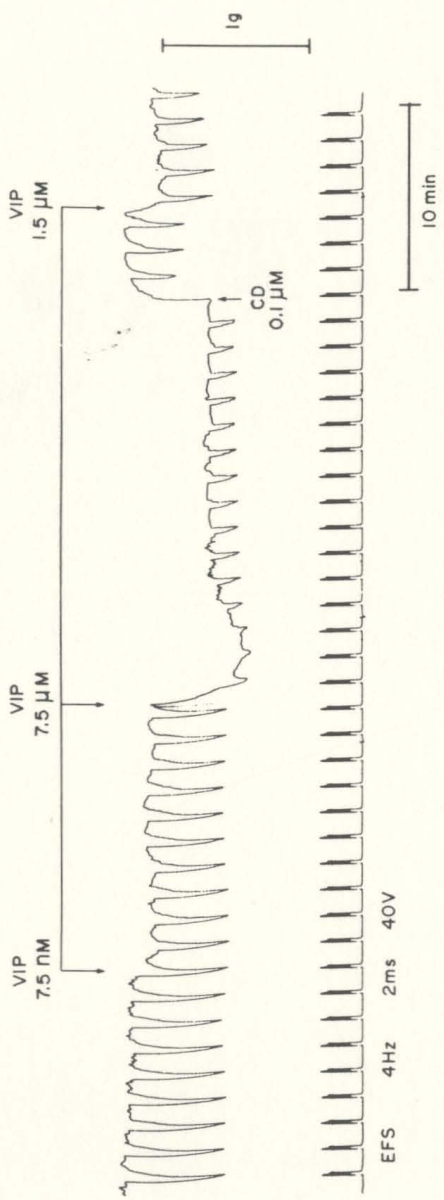

% RELAXATION
100
90
80
70
60
50
40
30
20
10
10^{-9}
10^{-8}
10^{-7}
10^{-6}
5HT M
IC_{50}(nM)
max. 1/2 max. tonus
Prox. 23±7 4.3±1
Dist. 36±1 7±4

VIP (7.5×10^{-8}M - 7.5×10^{-7}M) relaxed all segments of the TMM precontracted with muscarine or CD (Fig 12). These relaxations were not affected by tetrodotoxin (1×10^{-7}M), hemicholinium (1×10^{-5}M) or methscopolamine (1×10^{-8}M) but were blocked by α-chymotrypsin (0.1 U/ml to 10 U/ml).

3.3.2.3 Noradrenaline

Noradrenaline and isoprenaline produced relaxations of the TMM precontracted with muscarinic agonists. The relaxations could be partially inhibited by propranolol (1×10^{-6}M).

Fig 12: Relaxant effect of VIP on the rat isolated TMM. The tissue was pre-contracted with CD in the presence of 1×10^{-7}M TTX and subjected to field-stimulation as indicated below the stimulus monitoring trace (bottom). VIP produced concentration related relaxation without affecting FSR_2. Although FSR_2 is decreased after 7.5×10^{-6}M VIP, this is probably due to decreased tonus since raising tone with CD shows FSR_2 of similar amplitude can be restored.


VIP
7.5 nM
VIP
7.5 µM
VIP
1.5 µM
CD
0.1 µM
1g
10 min
EFS 4Hz 2ms 40V

3.4 Vagally-evoked motor responses of the TMM

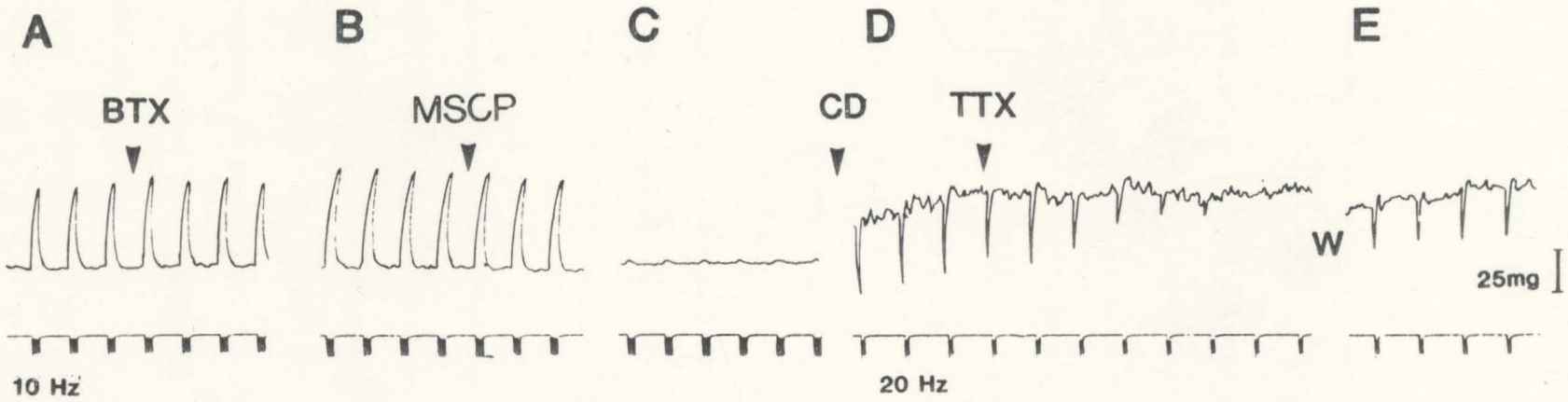
3.4.1 Contractile

Stimulation of the right and left vagus produced an initial fast twitch followed by slow contractile phasic response. The fast twitch response was blocked by α-bungarotoxin ($1x10^{-7}M$) or d-tubocurare ($5x10^{-7}M$). In the presence of these agents, the slow contractile response was enhanced. The latter was abolished by methscopolamine ($1x10^{-8}M$).

3.4.2 Relaxations

On blocking both contractile responses with d-tubocurare and methscopolamine, high concentrations of CD ($1x10^{-6}M$ - $1x10^{-5}M$) produced a tonic contraction. Vagal stimulation under these conditions produced relaxations (Fig 13) which were not blocked by hexamethonium ($1x10^{-5}M$), or propranolol ($1x10^{-6}M$) alone or in combination with phenoxybenzamine ($1x10^{-6}M$). Tetrodotoxin ($1x10^{-7}M$) completely abolished the relaxations. a-chymotrypsin (10U/ml) produced relaxations of the whole oesophagus preparation but did not block vagally-evoked relaxations. Cooling the bath to $28^{o}C$ resulted in depression of the vagally-evoked relaxation which was concomitant with the depression of active tonus.

Fig 13: Vagally-elicited inhibitory responses in the rat oesophageal TMM in situ. Relaxation responses to vagal stimulation were not in evidence until excitatory responses were abolished by succesive administration of 1×10^{-7}M β-bungarotoxin (BTX) and 2×10^{-8}M methscopolamine (MSCP) (traces A, B and C respectively). Trace C shows subtotal blockade of contractile response 120 min after BTX and 30 min after HMBr. Subsequent to inducing active tonus in the TMM with 4×10^{-6}M CD, vagal stimulation produced a TTX-sensitive relaxation response (D). After washing out (W) the TTX from the bath for 10 min and reintroducing tonus with CD, vagally elicited relaxation reappears.


A
B
C
D
E
BTX
MSCP
CD
TTX
W
25mg
10 Hz
20 Hz

3.5 Field stimulation-evoked motor responses in the TMM

3.5.1 Contractile

3.5.1.1 TTX-sensitive

Isolated TMM preparations set up in the organ bath at 37^{o}C and maintained at a preload of 0.3 g were mechanically quiescent. Field-stimulation either applied as single pulses at 0.5 ms pulse widths or as trains every 1.5 min for 10 sec durations resulted in contractions. As reported by Bieger and Triggle (1985) there was an initial rapid rise in tension followed by a plateau within 3 sec. Cessation of stimulation resulted in rapid decay of tension. Furthermore, regional differences existed between proximal and distal segments not only in frequency and pulse width dependency (Bieger and Triggle, 1985) but also in longitudinal tension (Table 3). Thus at 0.5 ms pulse width, 8 Hz frequency and supramaximal voltage (40 V), the amplitude of tension developed in the distal segment was greater than that developed in the proximal segment.

Single pulse stimulation resulted in small contractions which were generally more evident in the distal segment indicating the greater responsiveness of this segment. Maximal tension was observed after 7-8 stimulation periods and remained constant thereafter. It was possible to obtain contractions of the same amplitude for the duration of the experiment (usually 7-8 h).

The field stimulation-evoked contraction (FSC)

TABLE 3

Amplitude of tension developed in proximal and distal segments to field stimulation.

Segment	mg tension /wet wt (mg)	N
Proximal	30 + 9	38
Distal	45 + 23	43
	(P= 0.06)	

Field-stimulation parameters: 0.5 ms pulse-width, 8 Hz frequency, 40 V amplitude, 10 s trains.

could be blocked by TTX ($1x10^{-7}$M), methscopolamine ($5x10^{-9}$M), atropine ($1x10^{-8}$M), hemicholinium ($1x10^{-5}$) and 48 h cold storage (see section 3.6.2). Hemicholinium pretreatment required 15-20 mins with continued field stimulation to achieve block of FSC. Hexamethonium ($1x10^{-5}$-$1x10^{-4}$M) and nifedipine ($1x10^{-6}$M) failed to abolish FSC. On the other hand the nicotinic agonist DMPP depressed FSC while the mixed muscarinic/nicotinic agonist isoarecolone ($1x10^{-7}$M) slightly increased the amplitude of FSC and at concentrations greater than $5x10^{-7}$M produced a tonic contraction.

The above results suggest that field-stimulation-evoked contractions are a consequence of activation of post-ganglionic cholinergic fibres.

3.5.1.2 TTX-insensitive

In the presence of TTX, single contractions followed by after-contractions were produced by field-stimulation when TEA, a non selective K channel blocker was applied to the bath. Direct muscle stimulation was indicated by the lack of effect of methscopolamine. Furthermore, nifedipine ($1x10^{-6}$M) abolished these responses.

3.5.2 Relaxant responses.

3.5.2.1 TTX-sensitive (FSR_1)

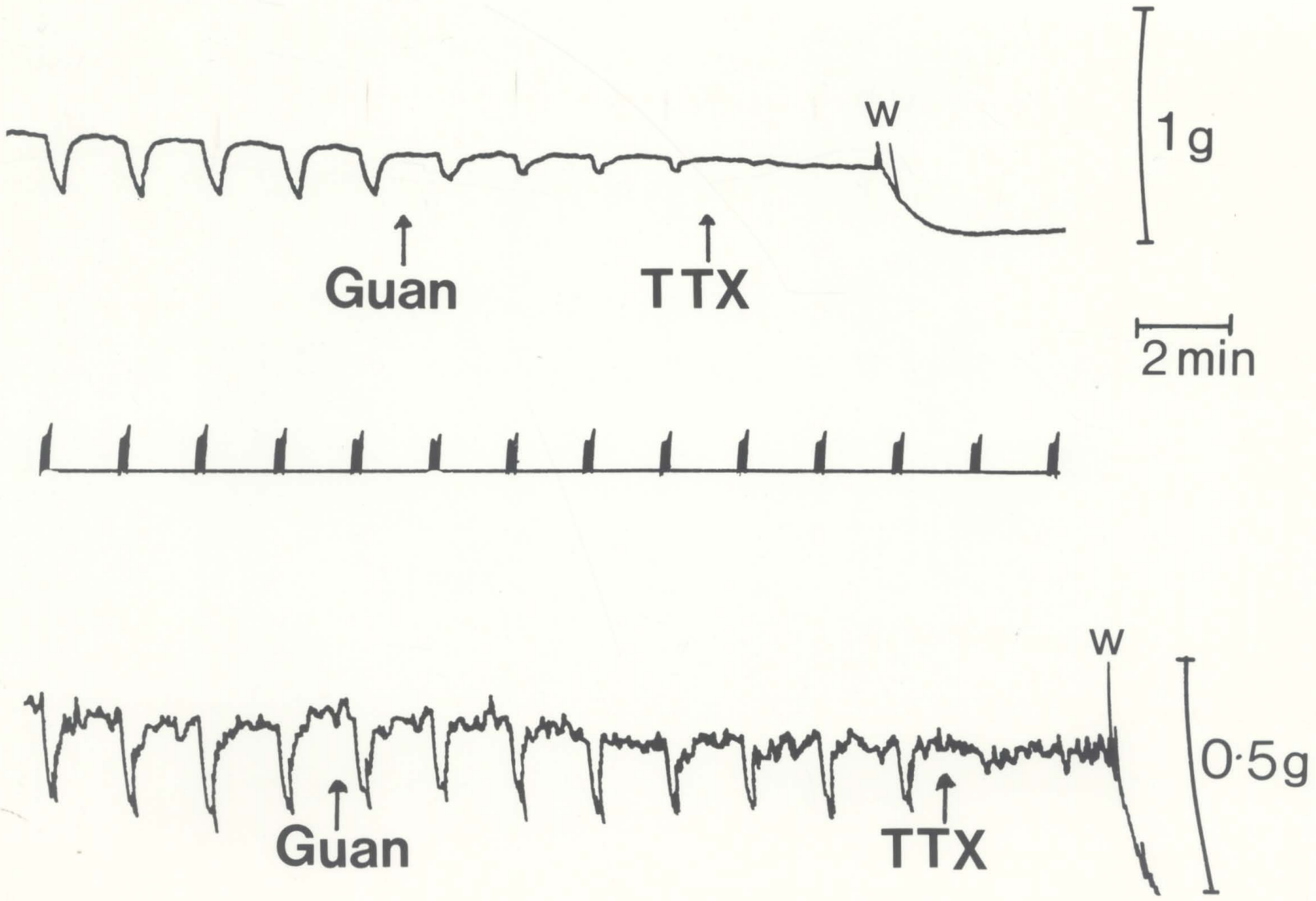
Relaxations to field stimulation could be revealed in the presence of tone induced by muscarinic agonists provided FSC were blocked by methscopolamine ($5x10^{-9}$M). The

concentration of the agonist required to initiate tension was approximately 100 fold greater in the presence of this concentration of methscopolamine. Relaxations observed under these conditions could be produced at pulse-widths of 0.5 ms and 8 Hz and characteristically were of slow onset and offset (Fig 14). The relaxations were insensitive to hexamethonium ($1x10^{-5}M$), phenoxybenzamine ($1x10^{-6}M$), trypsin (1U/ml) or a-chymoptrypsin (10U/ml). They were blocked by TTX ($1x10^{-7}M$) and enhanced by veratridine ($5x10^{-7}M$). Regional differences were observed in sensitivity to propranolol and guanethidine. The distal segment produced relaxations at short pulse widths (0.5ms) which were diminished and modified in their relaxation characteristics by guanethidine ($1x10^{-6}M$). In some tissues FSR_1 was totally abolished by guanethidine. The proximal segment remained unresponsive to the adrenergic neurone blocker. Furthermore, while TTX totally abolished relaxations at short pulse widths in the proximal segment, a residual component was present in the distal after $1x10^{-6}M$ TTX. Relaxations in the middle segments at 0.5ms pulse widths were generally weak. The frequency characteristics of this relaxation revealed maximal responses around 50-60 Hz.

3.5.2.2 TTX-insensitive (FSR_2)

Relaxation to field-stimulation (FSR) were also observed when FSC was blocked by TTX and tonus induced with muscarinic agonists. During transient tonus development with

Fig 14: TTX-sensitive field-stimulated relaxations in the rat TMM. TTX sensitive relaxations were obtained in the distal (A) and proximal (B) segments of the TMM after contractile responses were blocked by methscopolamine (1 x 10^{-8}M) and contraction induced with CD (5 x 10^{-6}M) 10 min prior to start of trace. Middle tracing is signal marker of field-stimulation delivered at 0.5 ms pulse width and 8 Hz frequency. Guanethidine (Guan, 1 x 10^{-6}M) diminished relaxations in the distal but not proximal segment. The relaxations were completely abolished by TTX (1 x 10^{-7}M).


W
1g
Guan
TTX
2 min
W
0·5g
Guan
TTX

histamine or substance P, FSR_2 could also be demonstrated. In the presence of high K^+, FSR_2 was absent except in the case of two out of six tissues which exhibited small relaxations when depolarisation was induced with 30mM isotonic K. Relaxations were immediate in onset and reached a plateau within 3-4 sec. When stimulations were delivered in 10 sec trains the plateau of relaxation was maintained for 4-5 sec after which they tended to return towards the active tension levels. On cessation of stimulation, tension generally returned immediately to its pre-stimulation level. Pulse-width and frequency characteristics were markedly different from those observed in the absence of TTX. Longer pulse-widths and lower frequencies were required to elicit maximal relaxations (Fig 15; Fig 16).

The amplitude of FSR_2, unlike that of FSC, did not show any regional differences (table 4). A bell-shaped relationship was observed between FSR amplitude and the level of active tension (Fig 17). Relaxation amplitude increased to a maximum which roughly coincided with the half maximal tension response to muscarine and then declined again to zero as tension approached its maximum. This indicated a window effect on FSR which seemed to be dependent on the level of tonus and hence the magnitude of cellular response initiated by the cholinoceptor agonist.

Fig 15: Amplitude of relaxation of rat oesophageal muscularis mucosae as a function of the pulse frequency in Hz. Tissues were stimulated in the presence of 1×10^{-7}M TTX and 1×10^{-7}M muscarine. Vertical bars represent S.E.M. (n=8).

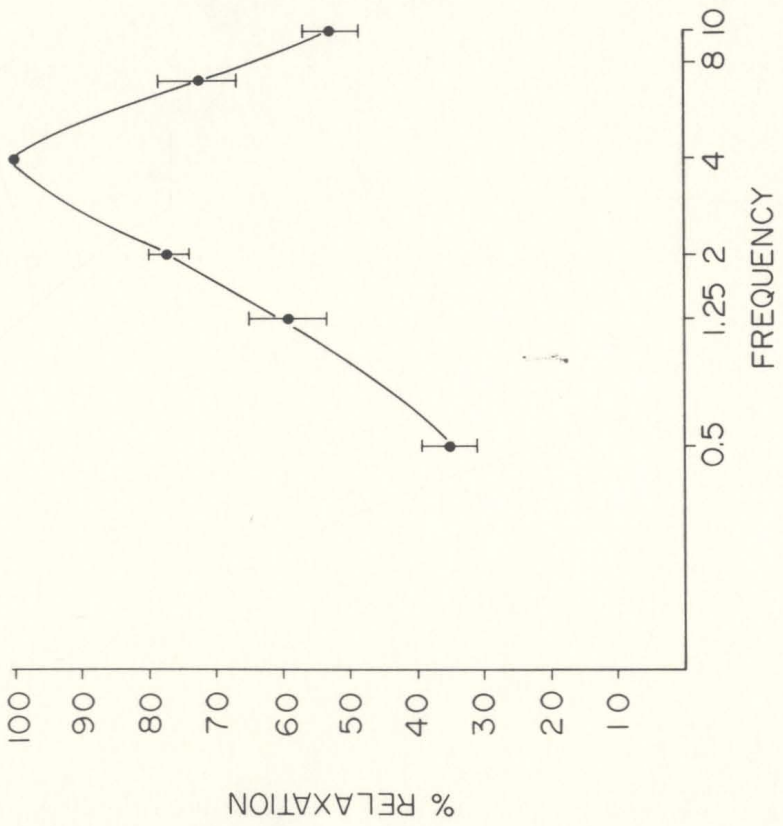

% RELAXATION
100
90
80
70
60
50
40
30
20
10
0.5
1.25
2
4
8
10
FREQUENCY

Fig 16: Amplitude of relaxation of rat isolated TMM as a function of the pulse-width in ms. Tissues were pretreated with TTX and muscarine as in Fig 15. Vertical bars represent S.E.M. (n=8).

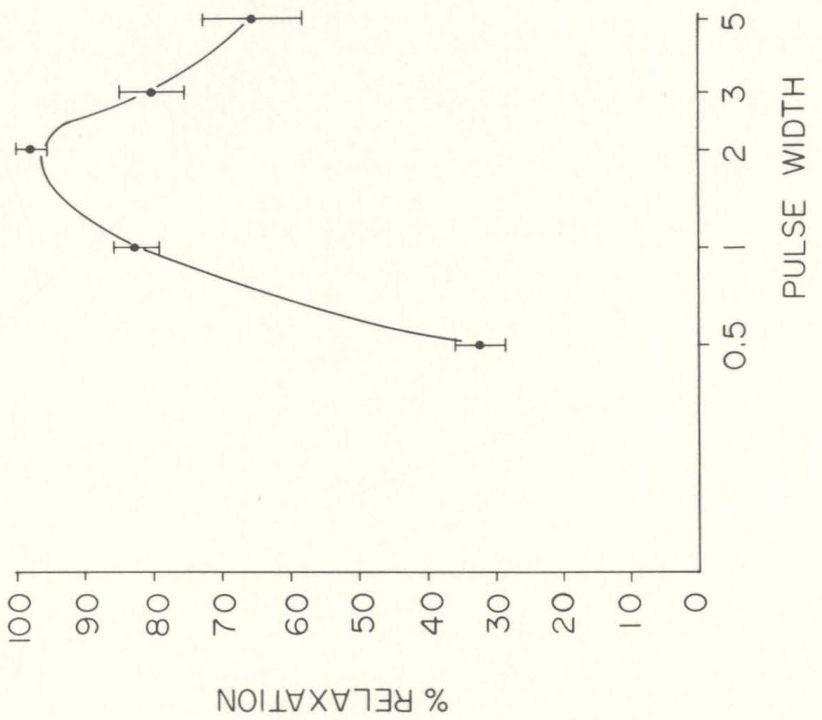

% RELAXATION
100
90
80
70
60
50
40
30
20
10
0
0.5
1
2
3
5
PULSE WIDTH

Fig 17: Plot of muscarine concentration vs TMM contraction (●,top) and vs amplitude of relaxation elicited by field-stimulation (□ ,bottom). Tissues were pretreated with 1×10^{-7} M TTX; field-stimulation parameters were pulse rate 4 Hz and pulse width 2 ms delivered in 10 s trains. Vertical bars represent S.E. of mean (n=8).

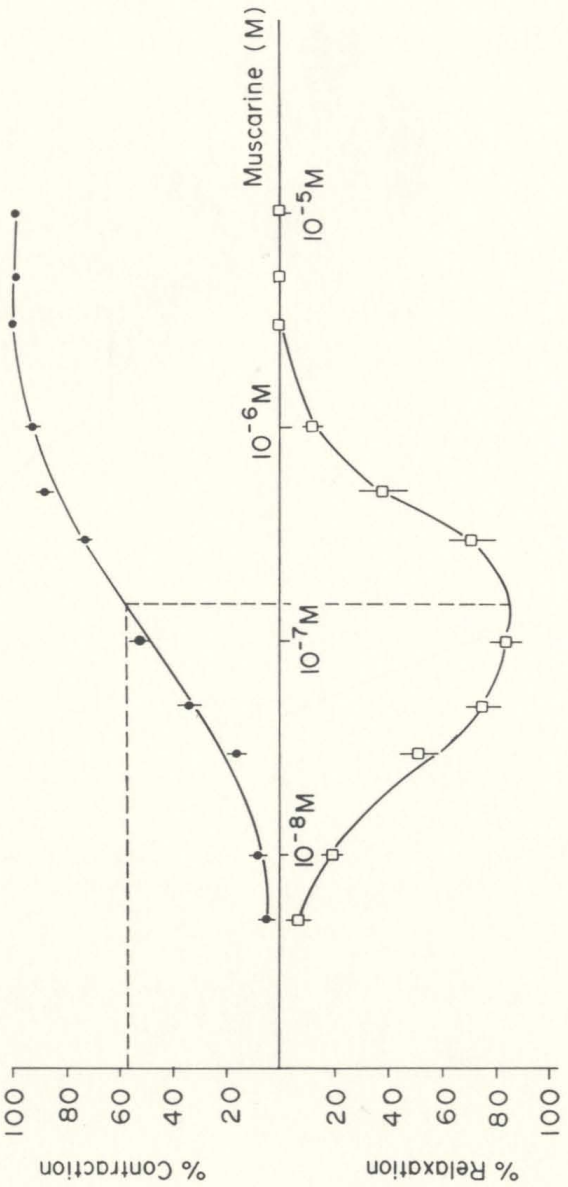

Muscarine (M)
10^{-8}M
10^{-7}M
10^{-6}M
10^{-5}M
% Contraction
% Relaxation
100
80
60
40
20
20
40
60
80
100

TABLE 4

Maximal amplitude of field stimulation-evoked relaxations in the TMM.

Segment	Tension (g)	N
Distal	0.34 + 0.033	17
Middle (distal)	0.27 + 0.023	12
Middle (proximal)	0.27 + 0.021	15
Proximal	0.33 + 0.047	16

Field stimulation parameters: 2 ms pulse-width, 4 Hz pulse-freqency, 40 V amplitude and 10 s train duration. Maximal amplitude of relaxations were measured at EC_{50} of muscarine concentration.

3.6 Evidence supporting myogenic relaxations.

The evidence presented above supports the hypothesis that two different inhibitory mechanisms exist in the TMM i.e TTX-sensitive and TTX-insensitive, hereinafter refered to as FSR_1 and FSR_2, respectively. The persistence of relaxations at longer pulse-widths in the presence of TTX posed the question of whether a relaxation process entirely of myogenic origin may be operative in the TMM. Thus TTX-insensitive relaxations (FSR_2) were further examined under conditions where these could be separated from relaxations of neurogenic origin.

3.6.1 Temperature dependence

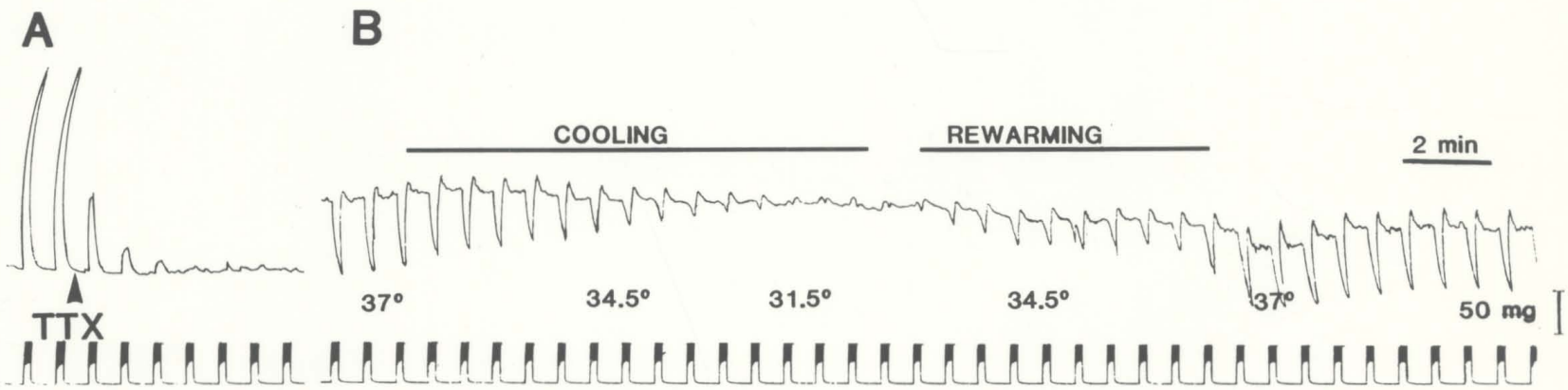
TTX-sensitive FSC and FSR_1 were generally not altered by cooling from 37 ^{o}C to 28 ^{o}C (Fig 18) except in some distal segments where FSR_1 was slightly prolonged. Lowering of bath temperature either increased or decreased muscarinic agonist-induced tone. Changes in baseline tension were unaffected by ouabain (5×10^{-5}M) whereas indomethacin (1×10^{-5}M) abolished relaxation of agonist-induced tone during cooling. Indomethacin produced transient relaxations which returned to baseline tension within 5 min.

The effect of cooling on FSR_2 is illustrated in Fig 19. Lowering the bath temperature had a pronounced effect on FSR_2. Cooling from 37 ^{o}C to 28 ^{o}C diminished FSR_2 with a threshold between 33 and 34 ^{o}C. At 28 ^{o}C FSR_2 was totally abolished and replaced by small contractions. Rewarming

Fig 18: Effect of cooling on FSR_1 in the proximal segment of rat TMM. Relaxations were unmasked after tissues were treated with methscopolamine 5×10^{-9}M and tonus induced with 1×10^{-6}M CD. The bath temperature was lowered to 28 ^{o}C. Bottom tracing shows field-stimulation at 0.5 ms pulse widths and 4 Hz, 2 ms and 8 Hz, and 0.5 ms and 8 Hz. Unlike the effects on FSR_2, cooling did not alter FSR_1. The relaxations were abolished by TTX (not shown).

34°C
30°C
28°C
0·5
0·5 ms
2 ms
0·5ms

Fig 19: Inhibitory effect of cooling on relaxation induced by field-stimulation in isolated TMM of the rat. A relaxation response (B) was unmasked after contractile responses to electrical stimulation were blocked by 1 x 10^{-7}M TTX (A) and active tonus was induced by addition of the muscarinic agonist CD (3 x 10^{-7}M; added to the bath 10 min before start of trace shown in B). Trace at bottom marks field-stimulation trains (train length 10 s, pulse frequency 5Hz, pulse-width 1 ms) Note decrease in amplitude of relaxation response in the absence of change in CD-induced basal tonus.


A
B
COOLING
REWARMING
2 min
TTX
37°
34.5°
31.5°
34.5°
37°
50 mg

restored the relaxation to its original amplitude. FSR_2 was neither altered by indomethacin prior to cooling nor was its sensitivity to cooling affected.

3.6.2 Cold storage.

Isolated oesophageal TMM, cold-stored at 4°C and subsequently allowed to equilibrate for 2 hr at 37°C in the organ bath at a preload of 0.3 g, responded to field stimulation and pharmacological agents in a 'time-dependent' manner. TTX-sensitive FSC was absent after 48hr of cold storage (n=12). Instead, field-stimulation evoked relaxations at baseline tension were usually observed in the distal segment. In those tissues, stimulation at lower pulse widths elicited relaxations (Fig 20) which were TTX-resistant, indicating the presence of FSR_2 at these lower pulse widths. Muscarinic agonists contracted 48 h cold-stored tissues; the EC_{50} values were not different from those of fresh tissues and not regionally differentiated. However, the maximal tension to muscarine was greater in the distal than in the proximal segment as observed for fresh tissues. The amplitude of contraction decreased with increasing duration of cold-storage (Fig 21). Similarly, FSR_2 also decreased with increasing duration of cold storage. Decreases in FSR_2 paralleled those to muscarine.

Muscarine-induced tone was generally not inducible in tissues cold stored for longer than 5 days. In such cases, field-stimulation failed to evoke relaxations. When BSA was

not included in the Tyrode solution during cold storage, the tissues generally failed to respond to muscarinic activation which suggests that albumin has a membrane protective role as observed in cardiac and red blood cell preparations (Neufield, Lederman, Choy and Man, 1985).

3.6.3 Local anaesthetics

Etidocaine, $1x10^{-4}$M, partially inhibited FSR_2. The muscarine induced steady state tonus was however simultaneously decreased in two out of four preparations. FSR was inhibited by 66 + 10% (n=4). Cocaine, $5x10^{-5}$M, did not alter FSR_2.

3.6.4 Effects of some pharmacological agents on FSR_2.

A variety of agents were examined as potential inhibitors of FSR_2 (Table 5). These included 5HT uptake blockers, inhibitors of prostaglandin synthesis, inhibiton of intracellular Ca^{2+} release, chloride channel inhibitor and catecholamine receptor antagonists. None of these interventions blocked FSR_2. Some agonists were also ineffective in either mimicking FSR_2 or blocking it.

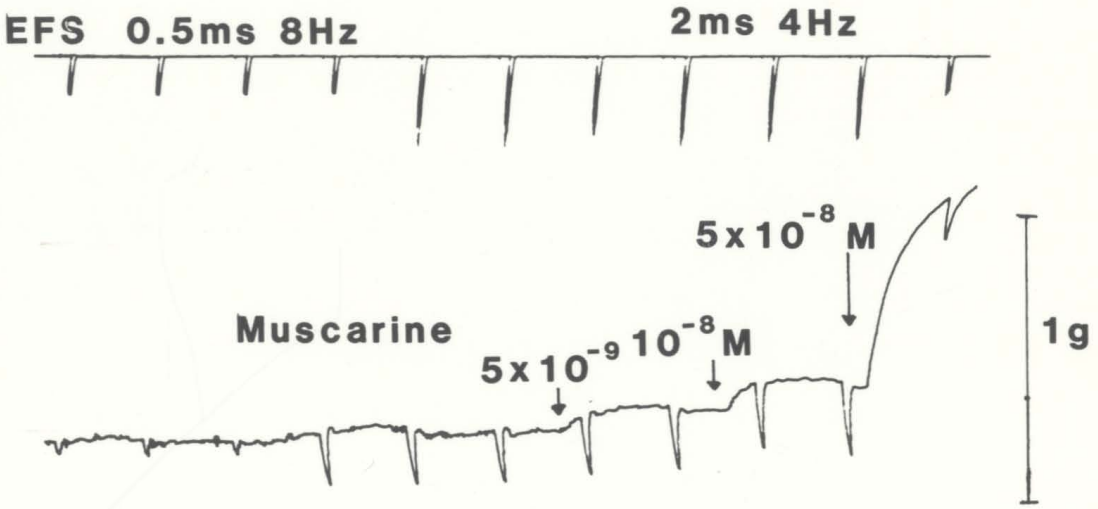
3.6.5 Releasing agents.

Since most commonly used antagonists failed to abolish FSR_2, it seemed warranted to examine agents capable of releasing endogenous mediators.

3.6.5.1 LQV

Fig 20: Effect of cold storage on FSR_2 on 2 d and 5 d cold-stored TMM. Field-stimulation evoked relaxations were present at basal tonus while excitatory responses were abolished. Responses were diminished to both field-stimulation and muscarine after 5 d cold storage (bottom). Tracing of electrical field stimulation (EFS) is given at top.

2d C.S.


EFS 0.5ms 8Hz
2ms 4Hz
5x10^{-8} M
Muscarine
5x10^{-9} 10^{-8}M
1g

5d C.S.

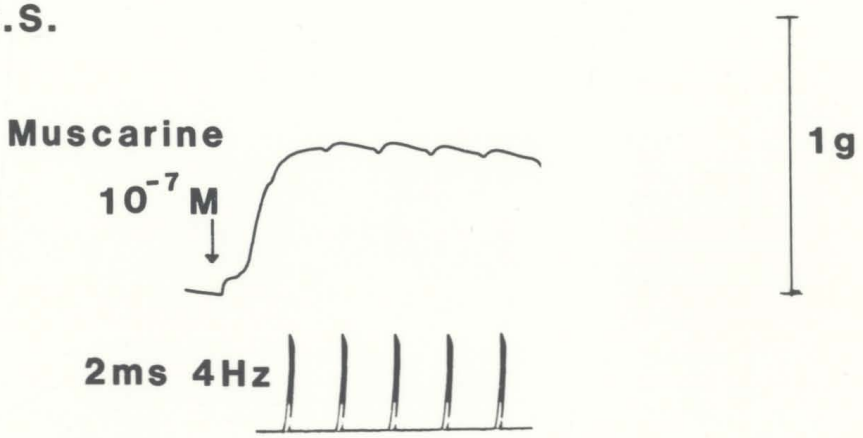

Muscarine
10^{-7} M
1g
2ms 4Hz

Fig 21: Comparison of maximal tension induced by muscarine (A) and maximal relaxation by field-stimulation (B) in fresh, 2-day and 5-day cold-stored isolated TMM. * denotes significant difference ($P < 0.05$, group t-test, two tailed) between fresh and cold-stored preparations. A significant difference in muscarine-induced tension was also observed between proximal and distal segments in freshly excised and 2-day cold stored tissues. Number of tissues given in parentheses. Vertical bars represent SEM.

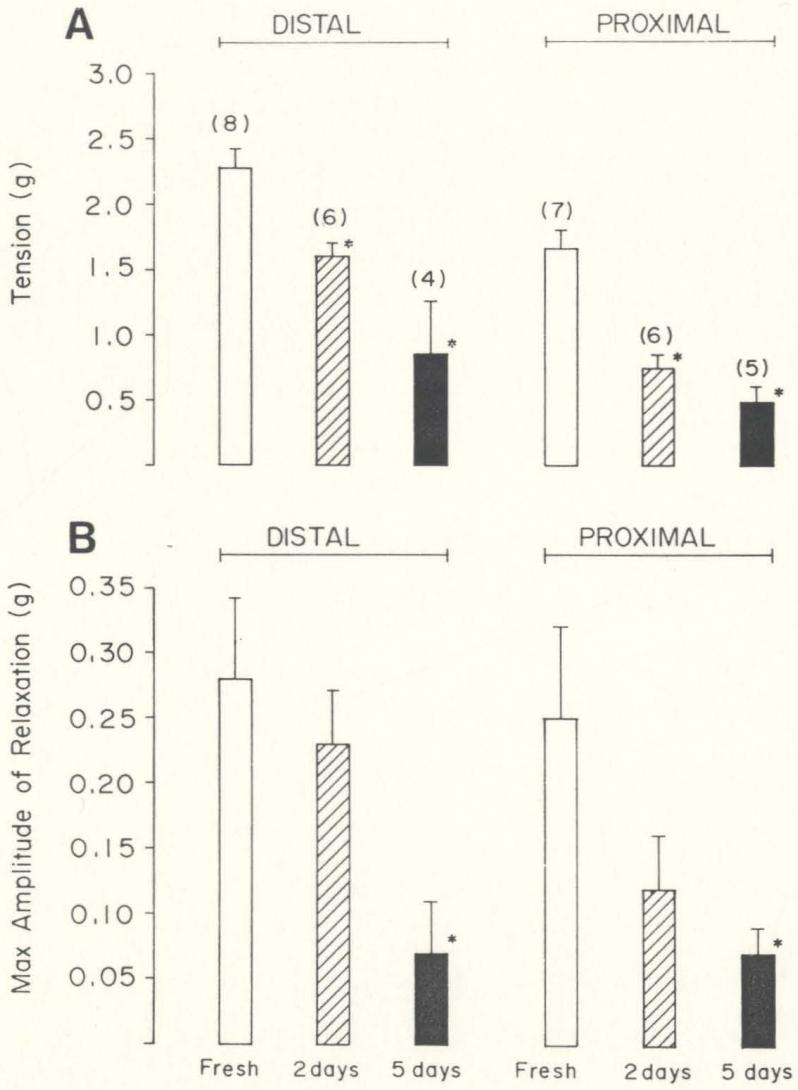

A
DISTAL
PROXIMAL
Tension (g)
3.0
2.5
2.0
1.5
1.0
0.5
(8)
(6)
(4)
(7)
(6)
(5)
B
DISTAL
PROXIMAL
Max Amplitude of Relaxation (g)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
Fresh
2 days
5 days
Fresh
2 days
5 days

TABLE 5

Effect of a variety of pharmacological agents on FSR_2.

Agent	Pharmacological profile	max.conc.	N
ATX II	nerve toxin (prolongs inactivation of Na^+ channel)	10 ug/ml	4
Cromoglycic acid	mast cell stabiliser	1 ug/ml	3
Phenoxybenz.	alpha antagonist	0.1 uM	6
Propranolol	beta antagonist	10 uM	6
Timolol	beta antagonist	20 uM	3
Fenfluramine	neuronal 5HT releasing agent	5 uM	3
GABA	excitatory amino acid	10 uM	3
Indomethacin	cyclo-oxyg. inhibitor	5 uM	5
Nordihydro-guaretic acid	lipox. inhibitor	10 uM	3
Ouabain	Na/K ATPase inh.	10 uM	10
Trypsin	cleaves peptides	1 ug/ml	3
TMB-8	blocks release of int. calcium	0.1 uM	3
Veratridine	prolongs inactivation of Na^+ channels	0.5 uM	3
α - β methylene ATP	ATP analogue	10 uM	3
Metiamide	H_2 antagonist	1 uM	3

Venom from the scorpion Leiurus quinquestriatus has been reported to induce and enhance release of transmitters from the nerve endings by delaying inactivation of Na channels (Koppenhofer and Schmidt, 1968). LQV (10 ug/ml) produced contractions (n=5) of the TMM. The contractions could be blocked by methscopolamine and atropine ($5x10^{-8}$M). Relaxations to LQV were produced in the presence of TTX or MSCP provided tonus had been induced with muscarinic agonist. These relaxations were transient and self-limiting (Fig 22) and FSR_2 remained unaltered in the presence or after LQV treatment. LQV-induced relaxations persisted at 28°C.

3.6.5.2 A23187

The Ca^{2+} ionophore, A23187, produced concentration-dependent relaxations in tissues pretreated with TTX or methscopolamine in the presence of cholinoceptor agonists (Fig 23). Regional differences in relaxation were apparent with complete relaxations occurring in the proximal segment but not the distal segment which exhibited a concentration-dependence curve situated to the right of that determined for the proximal segment (Fig 24). The middle segments were as sensitive as the proximal segment with maximal relaxations occurring at concentrations of $1x10^{-7}$M. Contractions to A23187 were not observed in either of the segments in the presence or absence of the muscarinic agonists.

Spontaneous activity was usually observed after washout of the ionophore. FSR_2 was not affected by A23187. Relaxations produced by this agent were not repeatable for at

Fig 22: Relaxant effect of LQV on rat isolated TMM. Contractile responses to field stimulation were blocked by 1 x 10^{-7}M TTX and tension was induced by muscarine (to a total of 3 x 10^{-7}M). (●) indicates addition of LQV (10 ug/ml). Although LQV caused a decrease in tonus, its effect was self-limiting and was not accompanied by decrease in FSR_2. Bottom trace marks field stimulation trains (small ▮, 0.5 ms 8 Hz; large ▮ 2 ms and 4 Hz).

TTX
1x10-7M
Muscarine
1x10-7M
3x10-7M

Fig 23: Relaxant effect of A23187 on the rat TMM. The tissue was pretreated with 3 x 10^{-7}M TTX and tonus induced with 2 x 10^{-7}M CD. Note appearance of spontaneous activity.

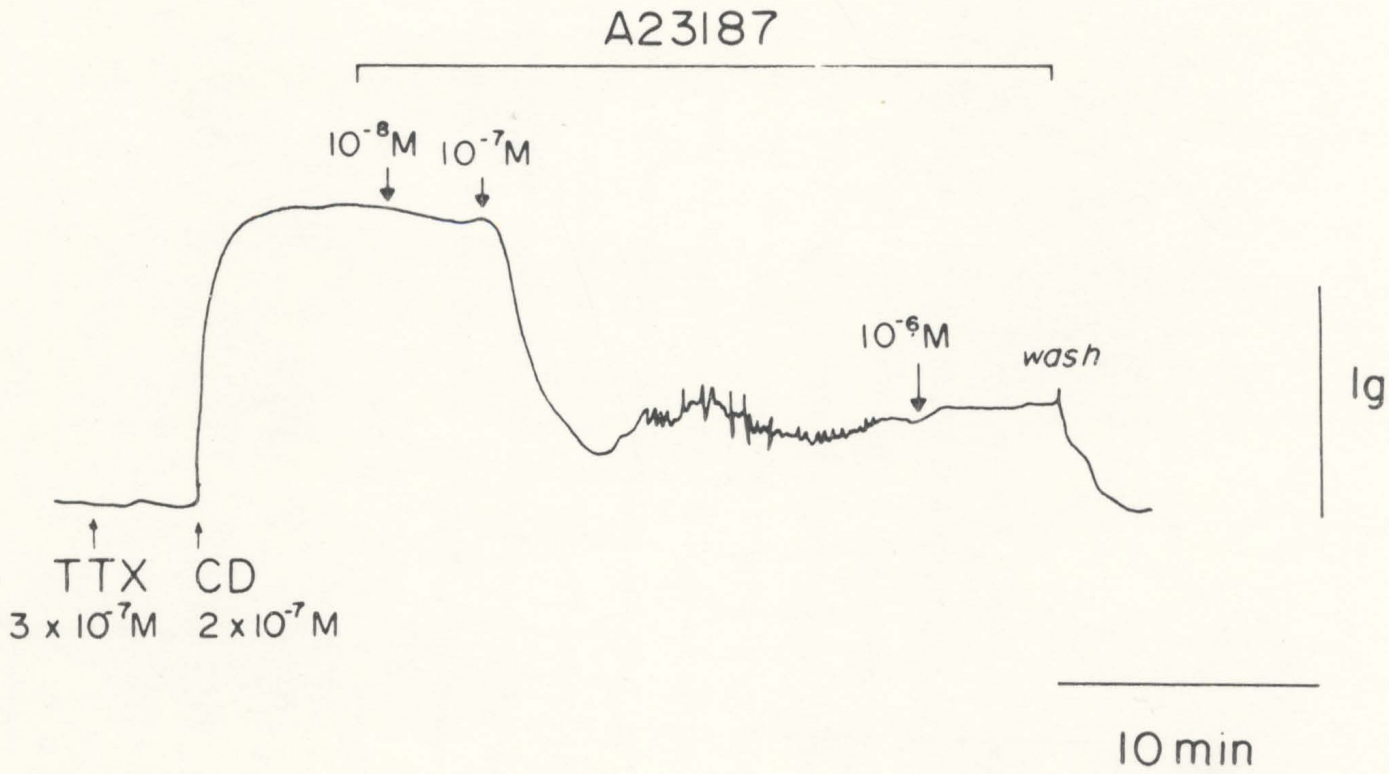
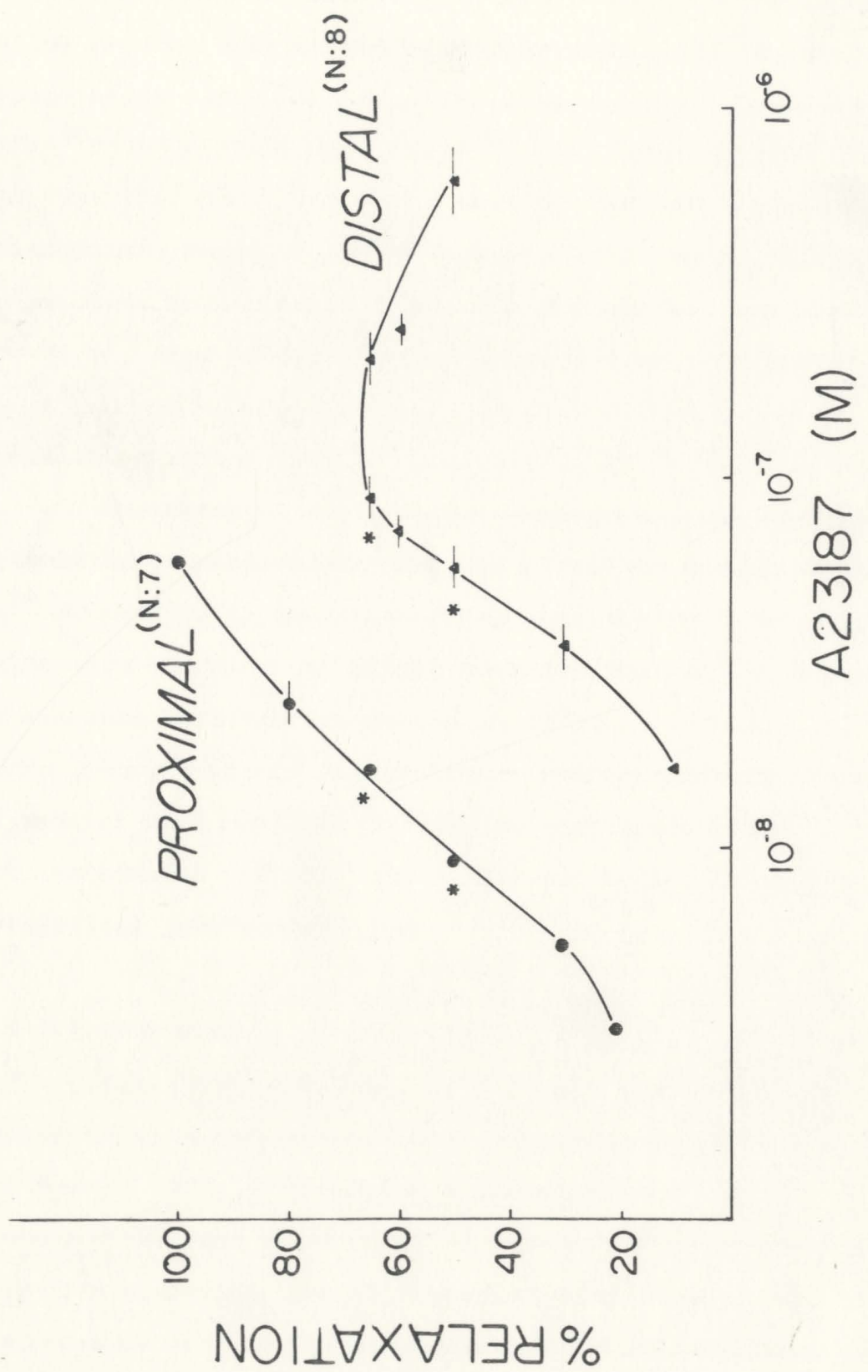

A23187
10⁻⁸M
10⁻⁷M
10⁻⁶M
wash
TTX
3 x 10⁻⁷M
CD
2 x 10⁻⁷M
1g
10 min

Fig 24: Cumulative concentration-effect curves for relaxation induced by A23187 in the distal (▲) and proximal (●) segments of the rat TMM. Maximal relaxation was obtained in the proximal but not the distal segment. Horizontal bars indicate S.E. of mean and were calculated by plotting curves for each tissue and determining the concentration of A23187 required to produce a given degree of relaxation. Proximo-distal differences are significant ($P < 0.05$, Students t-test, n=7) at all points indicated by asteriks. Points with no S.E. bars indicate that the variability remained within the symbol.


% RELAXATION
100
80
60
40
20
PROXIMAL (N:7)
DISTAL (N:8)
10^{-8}
10^{-7}
10^{-6}
A23187 (M)

least 1 hr of repeated washings in Tyrode. The relaxations by A23187 were not antagonised by methysergide ($1x10^{-7}$M; n=6), indomethacin ($5x10^{-5}$M; n=4), LQV (10ug/ml; n=4), trazodone ($1x10^{-5}$M; n=4), ketanserin ($1x10^{-6}$M; n=6), apamin ($1x10^{-5}$M; n=3) or TEA ($1x10^{-6}$M; n=2). Cooling did not attenuate relaxations, however, in the presence of 50 mM K^+, A23187 relaxations were blocked (n=6). The ionophores, ionomycin and X537A, also produced relaxations similar to A23187.

3.6.5.3 Magnesium

Excitatory cholinergic responses to field-stimulation were blocked by 20 mM Mg^{2+}. This concentration of Mg^{2+} relaxed TMM segments as well as abolished FSR_2 (Fig 25) which were replaced by slight contractions. Mg^{2+}-induced relaxations were not attenuated at 28^oC.

In the presence of Mg^{2+} significantly greater concentrations of agonist were required to initiate contraction. Mg^{2+} did not antagonise A23187, VIP, LQV or forskolin-induced relaxations (see section 3.8)

3.6.5.4 Potassium

Low concentrations of K^+ have previously been demonstrated to release mediators from nerve endings (Gibson and James, 1977). Increasing extracellular K^+ by 2-4 mM produced transient relaxations of the rat TMM precontracted with CD or muscarine (Fig 26). The relaxations were evident in the presence of TTX or methscopolamine. The relaxations were

Fig 25: Effect of magnesium on field-stimulated relaxations. In the presence of 20 mM Mg^{2+}, an approximate 10 fold higher concentration of CD was required to produce tone of equal amplitude as in the absence of Mg^{2+}. Note loss of FSR_2 in the presence of Mg^{2+} and its return in the absence of the ion. The figure has been hand traced from original record.

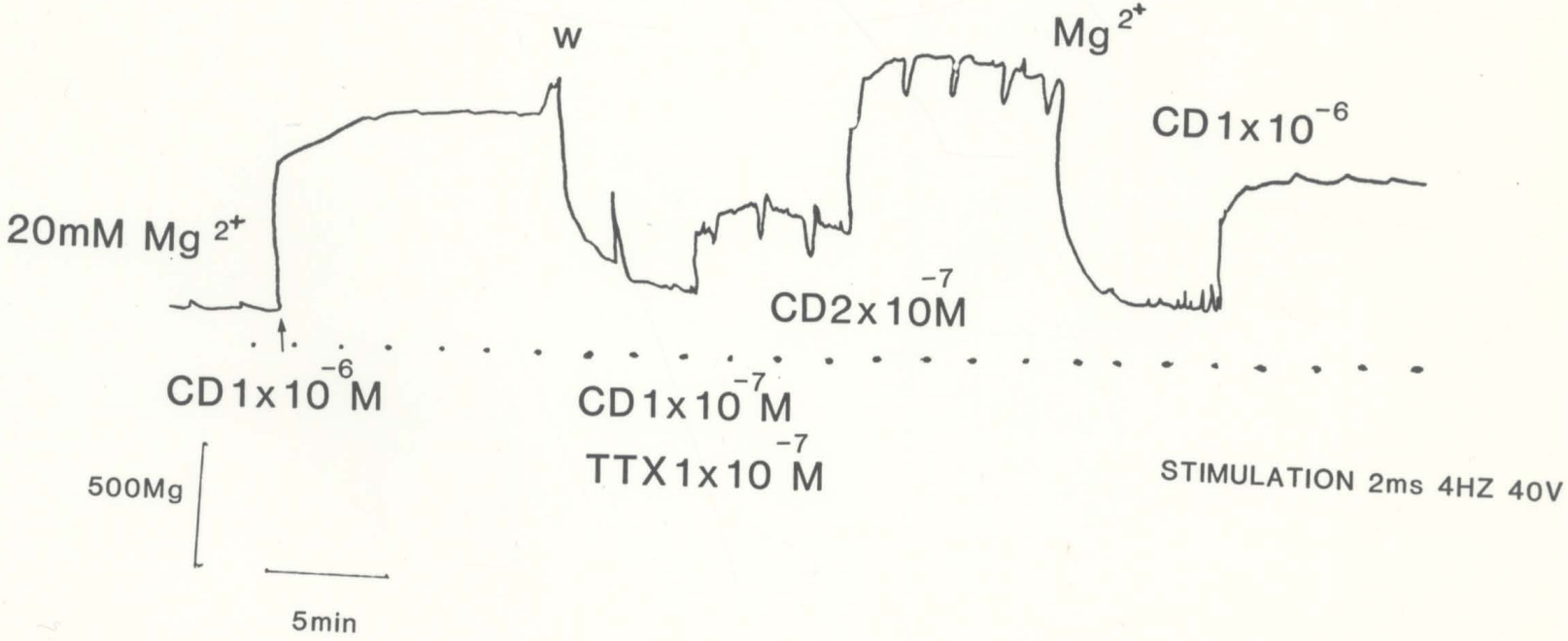
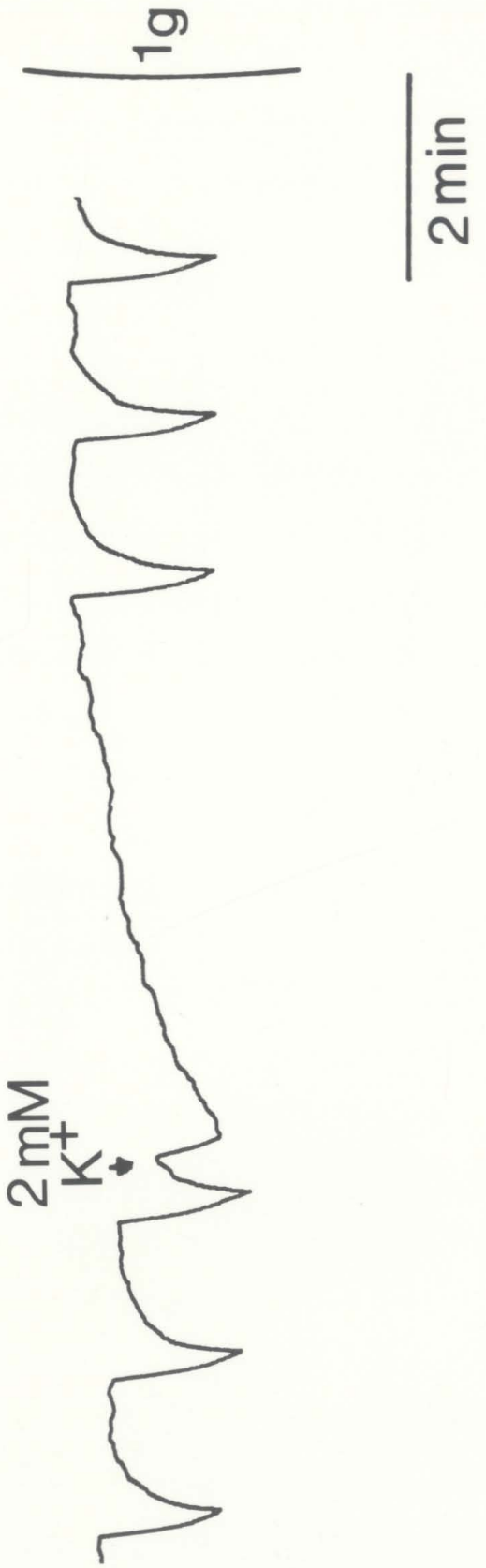

20mM Mg 2+
W
Mg 2+
CD1x10-6
CD2x10M-7
CD1x10-6M
CD1x10-7M
TTX1x10-7M
STIMULATION 2ms 4HZ 40V
500Mg
5min

Fig 26: K^+-induced relaxation in the rat TMM. Isolated TMM was pretreated with methscopolamine (1×10^{-8}M) and CD (3×10^{-5}M) prior to begining of trace. Field-stimulated relaxations were produced at 2 ms pulse-width and 4 Hz frequency. KCl (2 mM) was added to the bath.


1g
2min
2mM
K+

generally of an amplitude equal to that of FSR_2 and were followed by a delayed increase in tonus beyond the orignal tension level. In some cases, the amplitude of FSR_2 also increased following K^+. The relaxations persisted in the presence of ouabain, $1x10^{-5}M$, but were abolished at 28^oC and by nifedipine ($5x10^{-8}M$) (n=4).

3.6.6 K^+ channel blockers.

TTX-insensitive relaxations produced by field-stimulation in the presence of muscarinic agonists were not antagonised by TEA (0.1-5mM). At these concentrations, TEA relaxed all segments of the TMM. This relaxation was also reflected in the ability of the blocker to depress FSC. As described in section 3.5.1.2, TEA, at concentration > 5 mM unmasked contractile responses which were TTX insensitive. The depressant effects of TEA on field stimulation-evoked contractions at lower concentrations were tachyphylactic since repeated exposures failed to produce relaxations (n=4). Further application of CD in the presence of TEA produced spontaneous activity superimposed on tonus. FSR was observed in the presence of 1 mM TEA but due to spontaneous activity and the presence of TTX-insensitive contractions, FSR_2 could not be demonstrated.

Apamin, $1x10^{-5}M$, a blocker of Ca^{2+}-activated K^+ channel (Banks et.al. 1979) produced no effect on either FSC or FSR (n>10). On the other hand, 4-aminopyridine relaxed TMM segments without altering FSR_2 ($1x10^{-4}M$; n=6).

3.6.7 Epithelium removal

To investigate the possibility of the epithelium as a source of a relaxing factor to field-stimulation and its interaction with A23187 (analogous to the effects of endothelium in the vasculature), TMM segments denuded of the epithelium were examined. The squamous epithelium layer was peeled off with forceps. In segments which were inverted such that the epithelium faced outwards and in direct contact with the drugs, field-stimulation produced contractions which were not inhibited by TTX or methscopolamine suggesting the epithelium acted as a barrier. Furthermore, CD $1x10^{-4}$M, did not induce any contractions. In inverted preparations where the epithelium had been removed FSC could be blocked by TTX or atropine, tone induced with CD and FSR_2 was unmasked (Fig 27). Smooth muscle damage was indicated by the increase in CD concentration required for contraction. The threshold for such contraction was $1x10^{-7}$M CD. Nevertheless, FSR could still be observed although the amplitude of FSR in these tissues was significantly less (0.044 ± 0.004g) than in tissues with epithelium (0.33 ± 0.047g). In the presence of moderate tonus, A23187 relaxed in a similar manner as observed in fresh tissues whose epithelia were present (Fig 28). CD-induced contractile responses were not affected by preparing TMM segments denuded of epithelium (Fig 29).

In TMM segments set up in the normal manner with the smooth muscle layer facing outward, ascorbic acid,

superoxide dismutase (86 U/ml) and catalase (775.5U/ml) did not alter FSR_2.

Fig 27: Relaxations in the epithelium-denuded rat TMM strip. Contractile responses were blocked by TTX and tension induced with CD. Field-stimulation (horizontal bars at bottom) of 2 ms pulse-width and 4 Hz frequency produced relaxations. Note low amplitude of both contractile and relaxation responses.

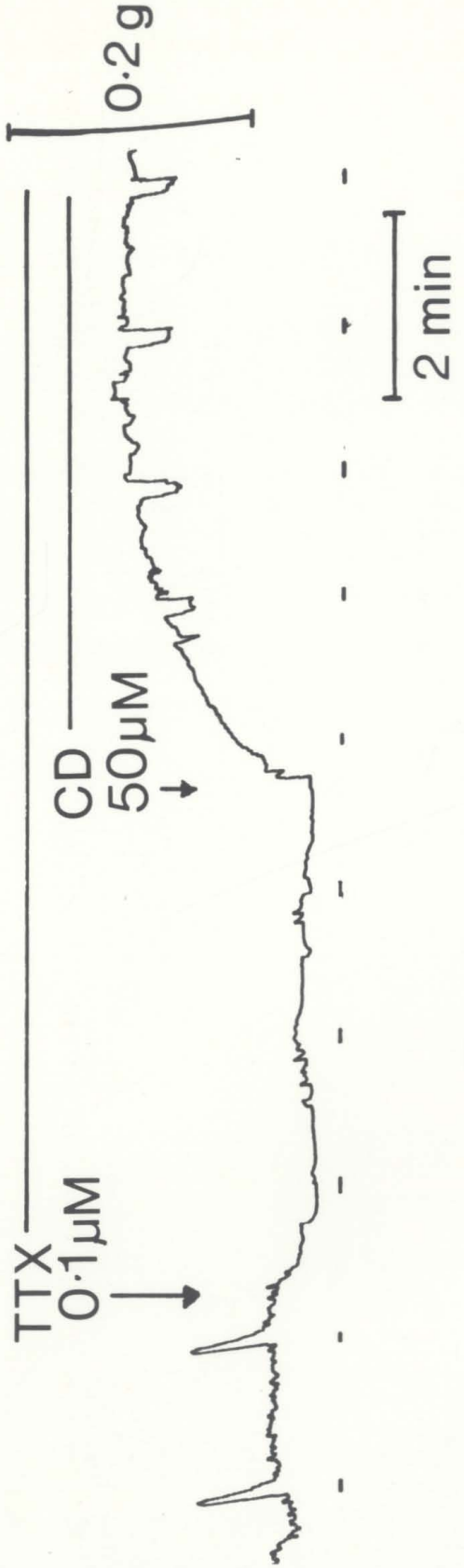

TTX
0·1μM
CD
50μM
0·2 g
2 min

Fig 28: A23187-induced relaxation in epithelium denuded strip. Tissue was pretreated with TTX and CD as in previous figure. Note that concentration required to produce relaxation is similar to that in isolated segments. (w) denotes wash out with Tyrode solution.

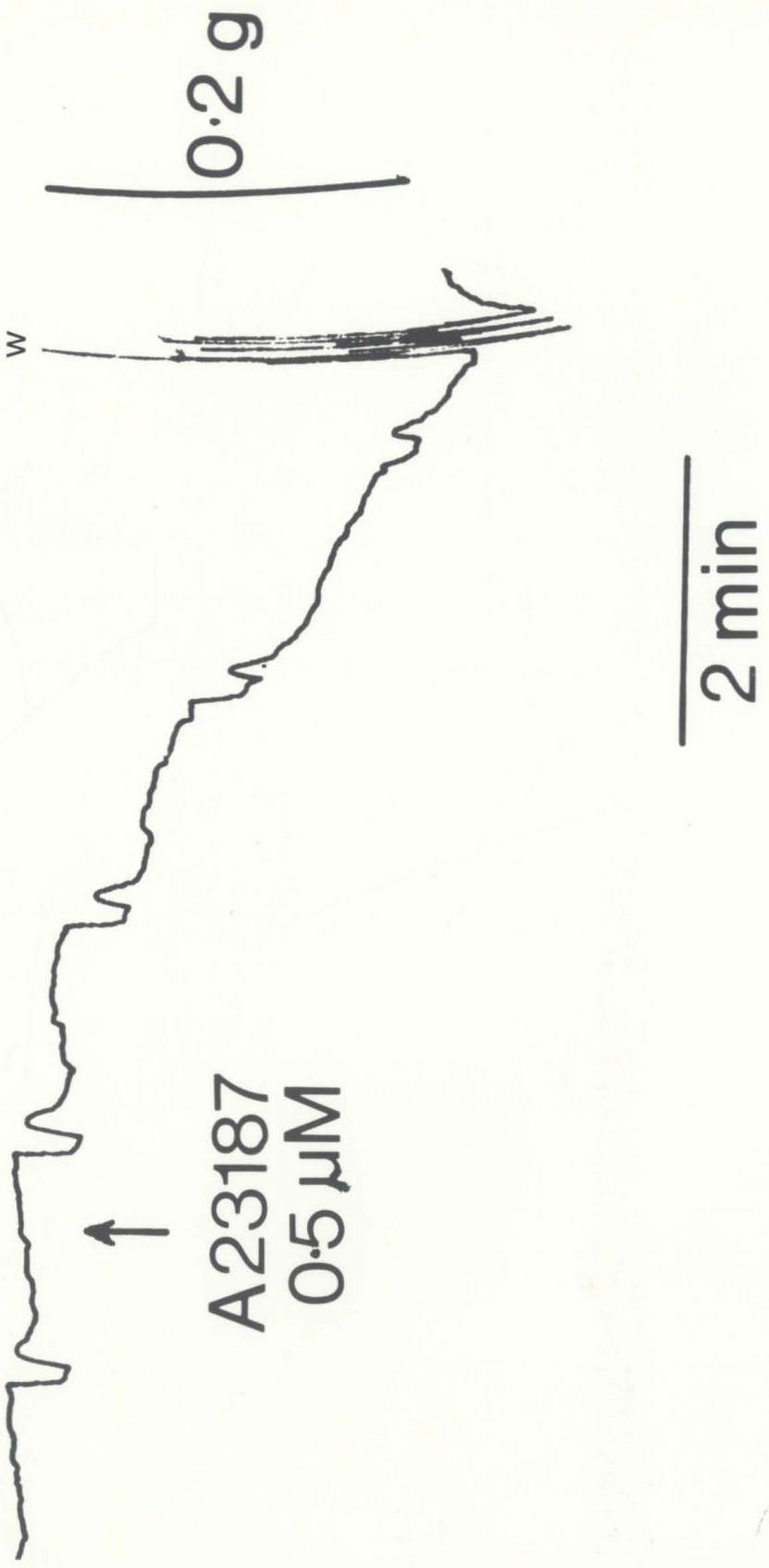
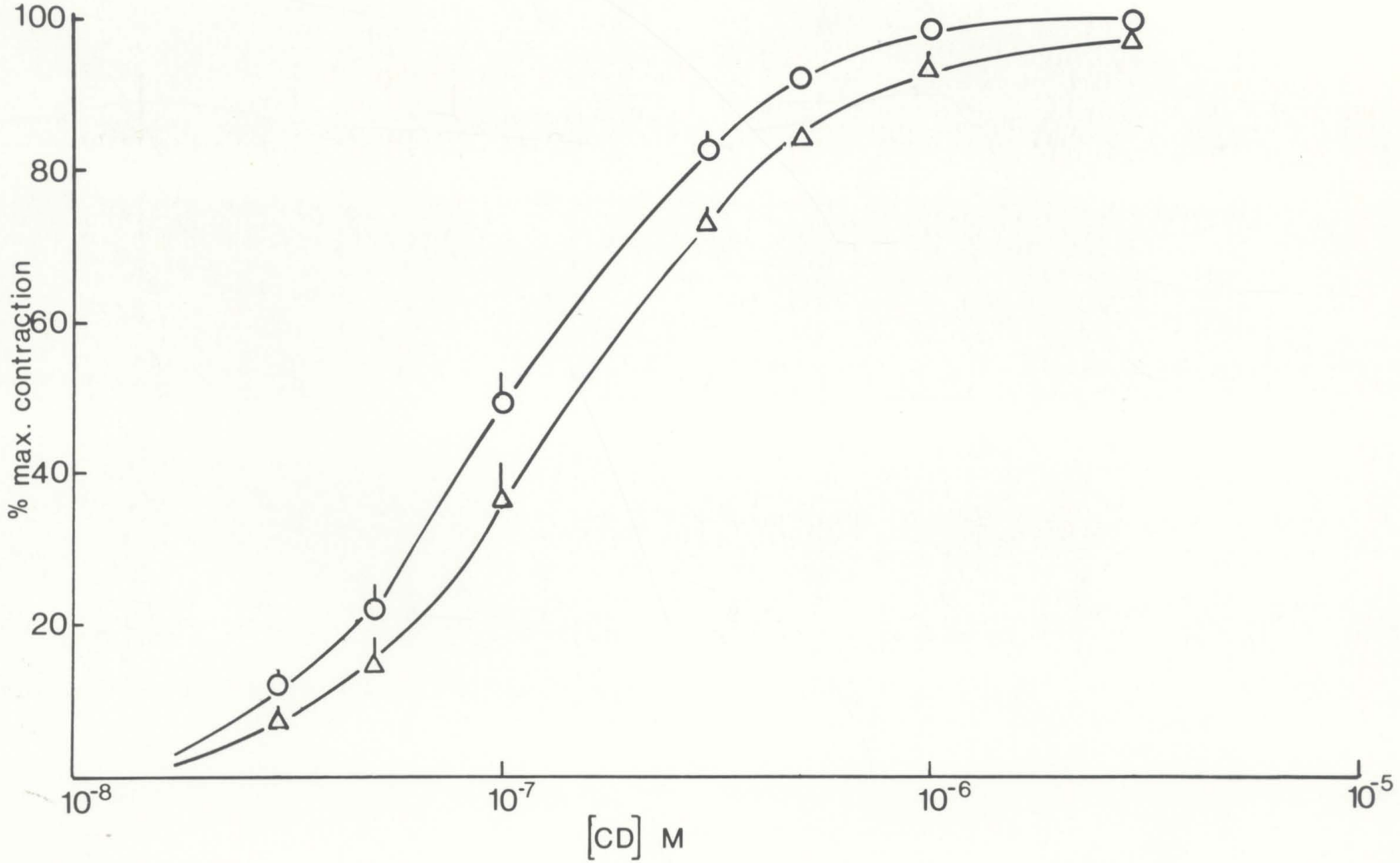

0·2 g
W
A23187
0·5 µM
2 min

Fig 29: Concentration-response curve to CD in the absence and presence of epithelium in rat isolated TMM. (Δ) + epithelium (n = 20) and (o) - epithelium (n= 5). Results are pooled data from proximal and distal segments of TMM. Vertical bars represent S.E.M.


100
80
60
40
20
% max. contraction
10^{-8}
10^{-7}
10^{-6}
10^{-5}
[CD] M

3.7 Calcium and FSR inhibition

3.7.1 Calcium

In Tyrode buffer from which Ca^{2+} was omitted, field-stimulation evoked contractions (FSC) were abolished within 2 min of replacing with Ca^{2+}-free buffer. CD also failed to initiate contractions in the proximal and middle segments. The distal segment, however, contracted to CD at concentrations greater than $3x10^{-7}$M. These responses were about 10% of maximal contractions in 1.8mM Ca^{2+} (n=6). FSR of very low amplitude could be demonstrated under these conditions in the distal segment. The CD-induced contractions were markedly depressed in the presence of 2 mM EGTA. Ca^{2+}-induced contractions (0.1mM-1.8mM) in the presence of $1x10^{-7}$M CD were reproducible twice but diminished maximal responses were produced on the third application (Fig 30). The pD_2 values for Ca^{2+} in the presence of CD $1x10^{-7}$M and isotonic K^+, 50 mM, were 0.74±0.03 mM (n=20) and 1.31 ± 0.06 mM (n=6) respectively. At lower Ca^{2+} concentrations, FSR_2 could be demonstrated in the presence of CD but not K^+. However, in the case of the muscarinic agonist, tone was generally not maintained.

3.7.2 Calcium channel antagonists

3.7.2.1 FSR_1

In normal 1.8 mM Ca^{2+} containing Tyrode buffer, FSR_1 was not blocked by the Ca^{2+} channel antagonists, PN-200-110 (1 x

10^{-7}M) or nifedipine (1 x 10^{-7}M).

3.7.2.2 FSR_2

FSR_2 was blocked by the Ca^{2+} channel antagonists, nifedipine, Pn-200-110, nimodipine, verapamil and diltiazem. The antagonists relaxed tissues precontracted with muscarinic agonists; however, tonus could be titrated back to the original tension by small increaments in the concentration of the muscarinic agonists (Fig 31; Fig 32) thus remaining within the concentration range that allowed FSR_2 to be observed in control tissues. Pretreatment with the antagonist produced similar effects on FSR_2.

FSR_2 was stereoselectively inhibited by the isomers of PN-200-110. The (+) isomer was considerably more potent than its optical antipode. The IC_{50} for inhibition of FSR was 0.2 nM and 200 nM for the (+) and (-) isomers respectively. The effects of (+)- and (-)- PN were also examined against CD-induced and K^+-induced contractions. In the case of K^+ stimulation inhibition was equal to that observed for FSR_2 (Fig 33) with similar selectivity. The CD-induced contraction was also inhibited in a stereoselective manner, however, maximal inhibition was not observed (Fig 34) at these concentrations.

Similar inhibition of CD, K^+ and FSR_2 was observed for nifedipine, verapamil and diltiazem. As with PN, FSR_2 and K^+ were antagonised to a greater extent than CD.

Correlation of IC_{50} values of the Ca^{2+} channel

Fig 30: Three consecutive concentration-response curves to calcium in the rat isolated TMM. Tissues were incubated in 0 calcium buffer for 20 min before cumulative addition of the ion. First concentration-response curve (●) was determined and followed 20 min later after continuous washing in 0 calcium by a second test (□). The third curve (△) was obtained 20 min later. Note decrease in maximal response after two consecutive cumulative applications of calcium.

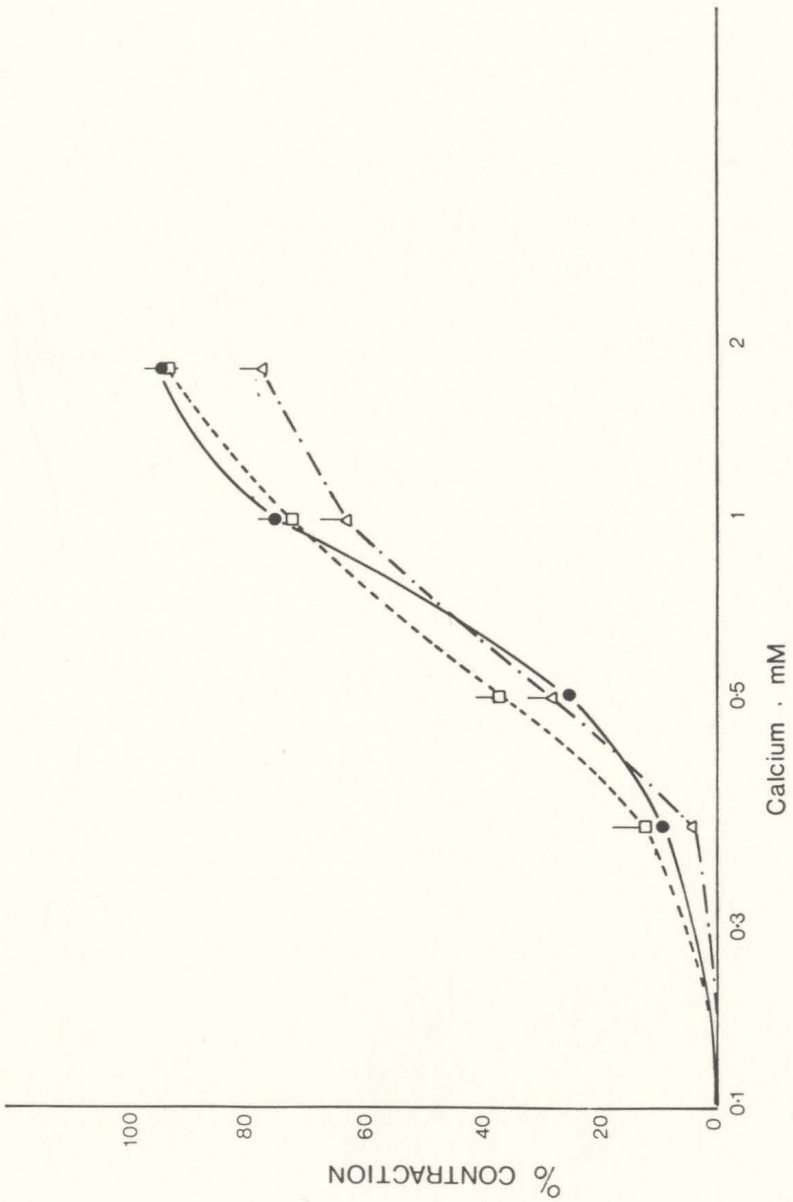

% CONTRACTION
100
80
60
40
20
0
0·1
0·3
0·5
1
2
Calcium . mM

Fig 31: Stereoselective inhibition of field-stimulated relaxation (FSR_2) in rat TMM by the two enantiomers of PN-200-110. Isometric tension records from two isolated TMM segments showing effects of (+) PN (A,B) and (-) PN (C,D). Control FSR_2 (A,C) were observed after excitatory responses to field-stimulation were blocked with 0.15 uM TTX and tension induced with 0.1 uM CD. After washout with Tyrode (w), the tissues were pretreated with 1 nM (+) PN (B) or 5 nM (-) PN (D) for 10 min and re-challenged with 0.15 uM TTX and 0.1 uM CD. Note loss of FSR_2 in B and peristence in D.

Dots at the bottom of each recording indicate 10 sec train of field-stimulation delivered every 1.5 min at 2 ms pulse width, 4 Hz pulse rate and 40 V amplitude.

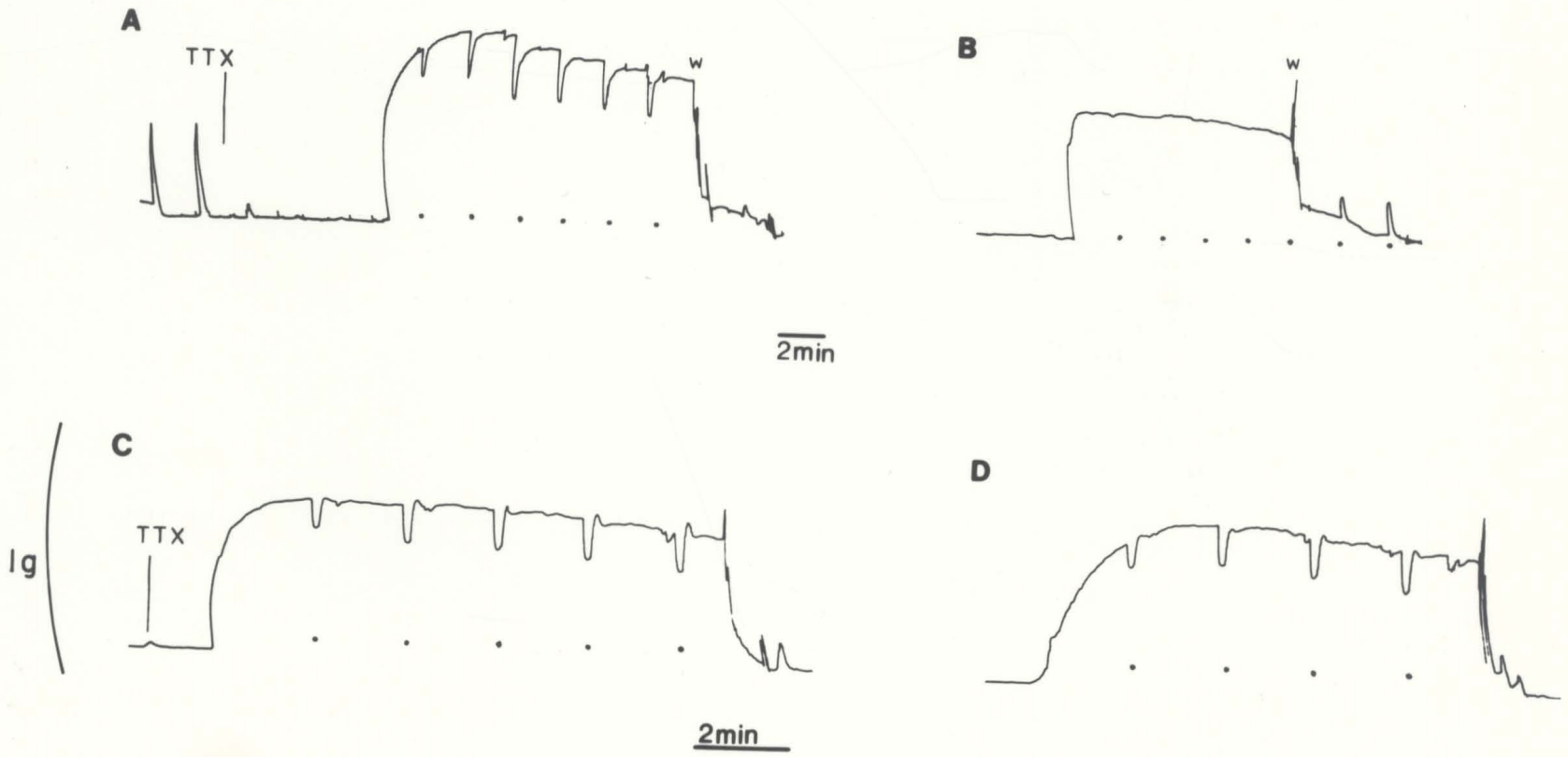

A
TTX
W
B
W
2min
C
Ig
TTX
2min
D

Fig 32: Inhibitory effect of verapamil on FSR_2 and muscarine. Tissue was pretreated with 1 x 10^{-7}M TTX and 1 x 10^{-7}M muscarine before the start of trace. Verapamil inhibition of tonus was surmounted by restoring tone by the addition of 2 x 10^{-7}M muscarine (↑) and the subsequent addition of 1 x 10^{-7}M muscarine (o) revealing an inhibition of FSR_2. Stimulation parameters : small bars 2 ms 4 Hz and large bars 2 ms 8 Hz.

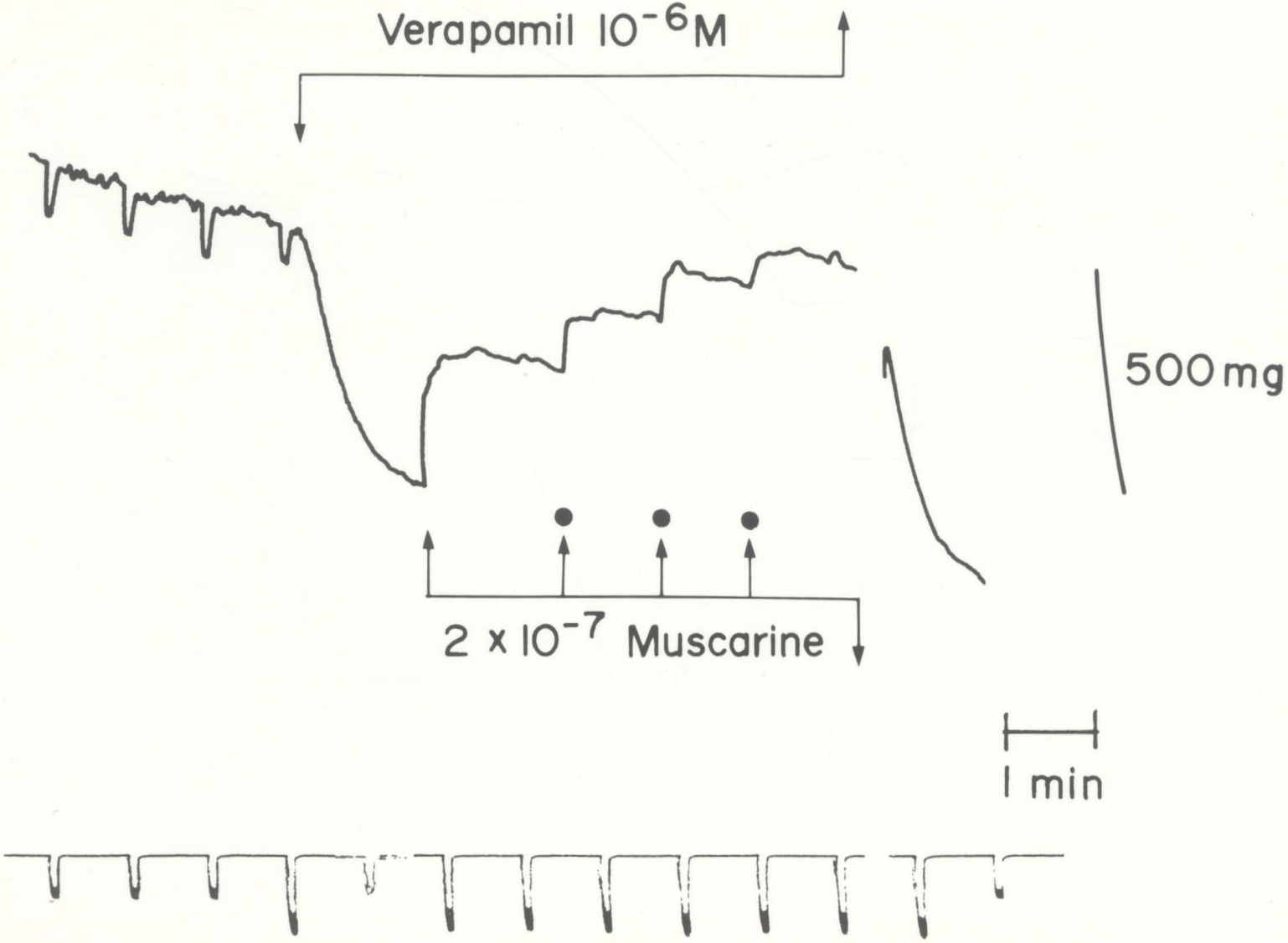

Verapamil 10^{-6}M
500 mg
2×10^{-7} Muscarine
1 min

Fig 33: Inhibition of FSR_2 and K^+ contractions by enantiomers of PN-200-110. Percent inhibition of FSR was calculated with respect to control responses obtained before pretreatment with PN-200-110. K^+ contractions were produced by isotonic depolarising 50 mM KCl buffer. Percent inhibition was determined for the tonic component of the contraction after pretreatment with PN-200-110 for 10 min. Cross-hatched columns represent inhibition of FSR and open columns inhibition of K^+. Concentration of PN (nM) is indicated below each set of columns. (+) and (-) indicate the enantiomers of PN-200-110. Number of tissues are indicated within columns.

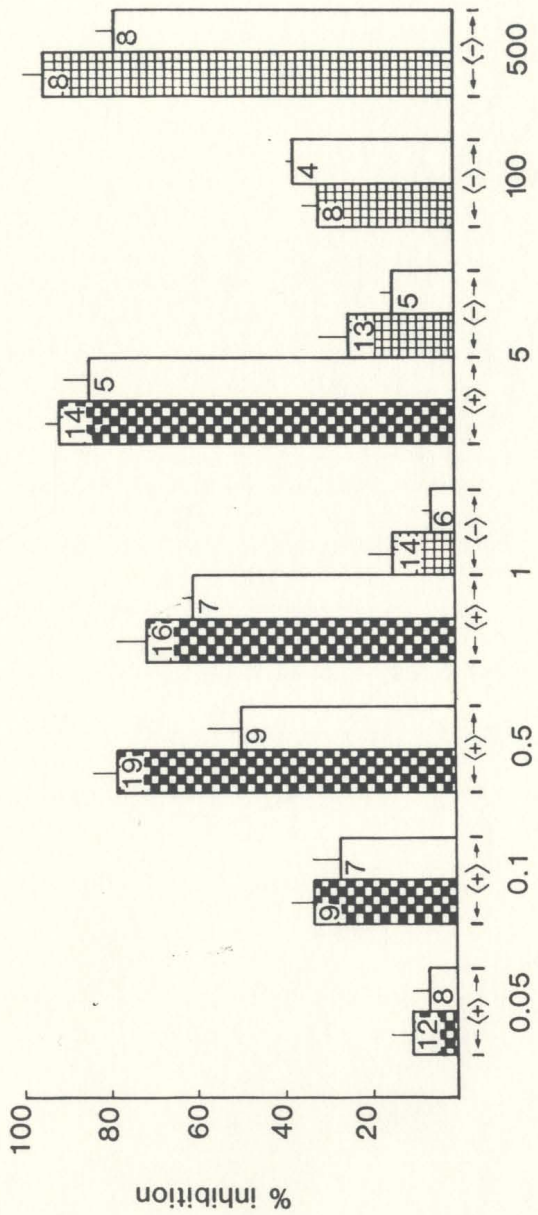

% inhibition
100
80
60
40
20
0.05
0.1
0.5
1
5
100
500
12
8
9
7
19
9
16
7
14
6
14
5
13
5
4
8
8
8

Fig 34: Inhibition of CD-induced tone by enantiomers of PN-200-110. Percent inhibition of 0.1 uM CD-induced tone was calculated with respect to control responses. PN-200-110 (nM) concentrations are indicated below columns, % inhibitions on the ordinate. Open columns represent (+) PN and hatched columns (-) PN. Number of tissues are given within the columns. Vertical bars represent standard errors. (*) indicate significant difference ($p > 0.05$).

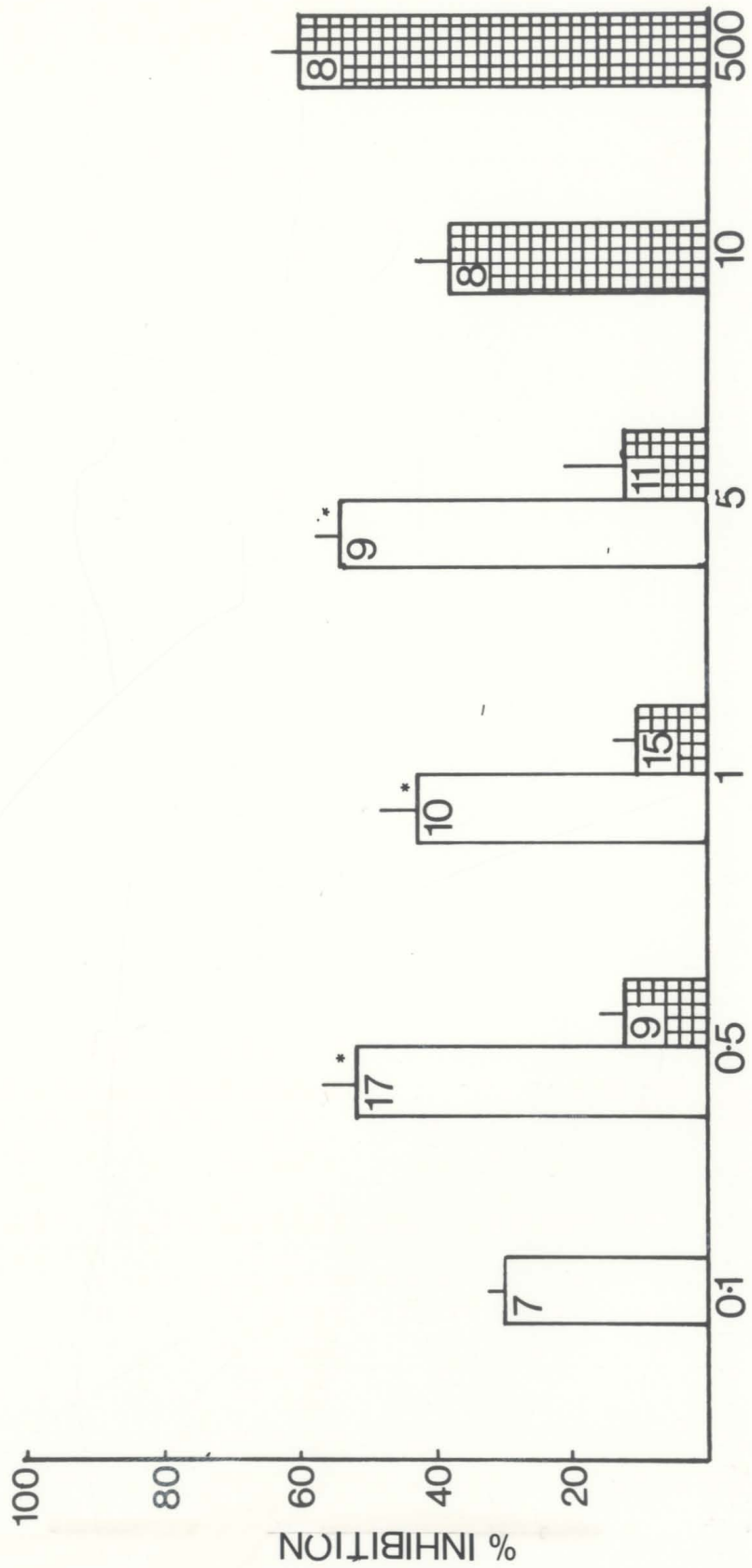
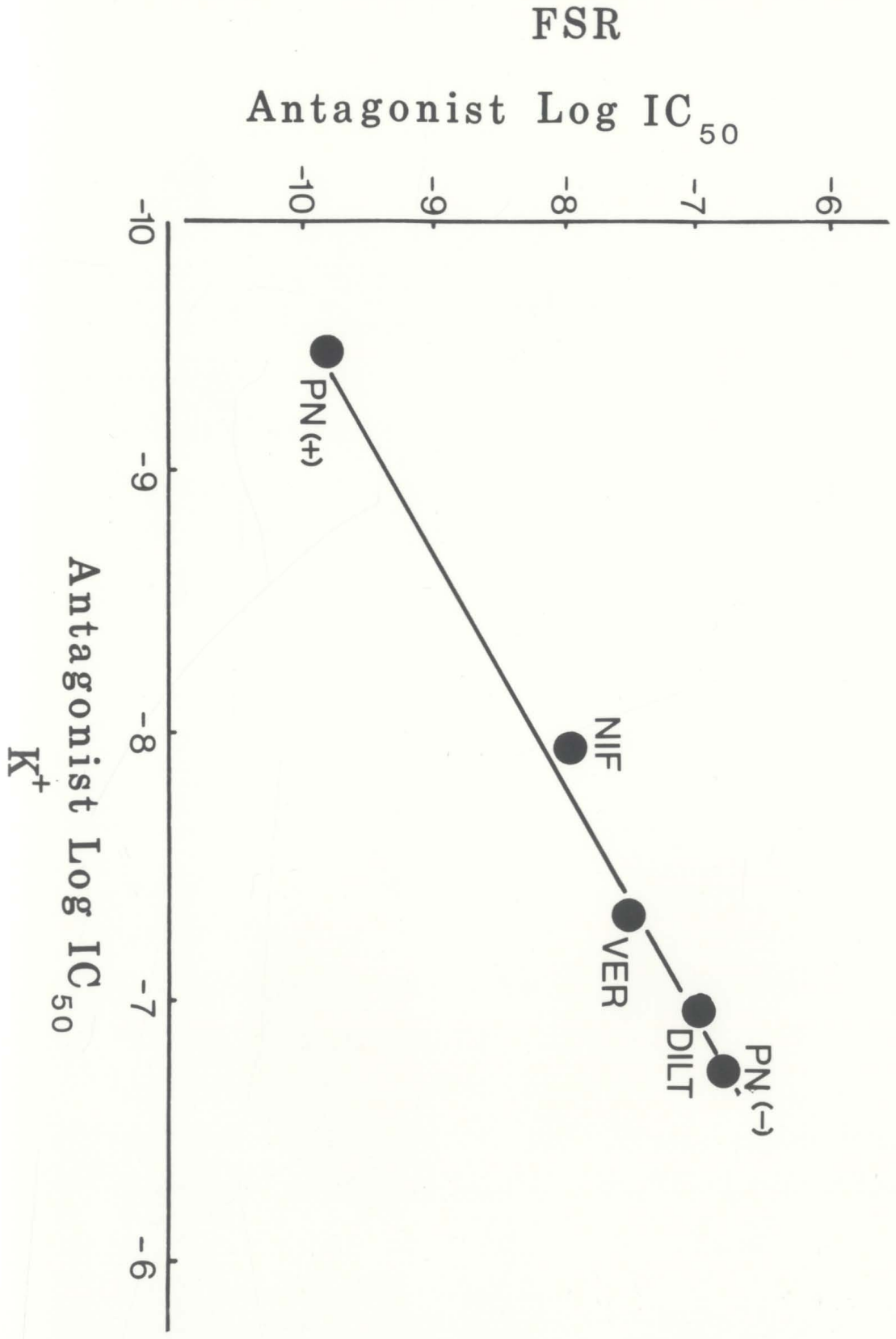

% INHIBITION
100
80
60
40
20
0·1
0·5
1
5
10
500
[PN] nM
7
17
9
10
15
9
11
8
8
*
*
*

Fig 35: Correlation between inhibition of FSR_2 and K^+. IC_{50} values are plotted for inhibition of FSR_2 and K^+-induced tone.


FSR
Antagonist Log IC50
-10
-9
-8
-7
-6
PN (+)
NIF
VER
DILT
PN (-)
Antagonist Log IC50
K+
-10
-9
-8
-7
-6

antagonists against FSR_2 and K^+ produced a slope of 1.11 ± 0.19 (Fig 35) and the rank order of potency was PN(+) > nifedipine > verapamil > diltiazem > PN(-).

3.7.3 Sensitivity of K^+-induced contraction to Ca^{2+} channel antagonists in the presence of methscopolamine.

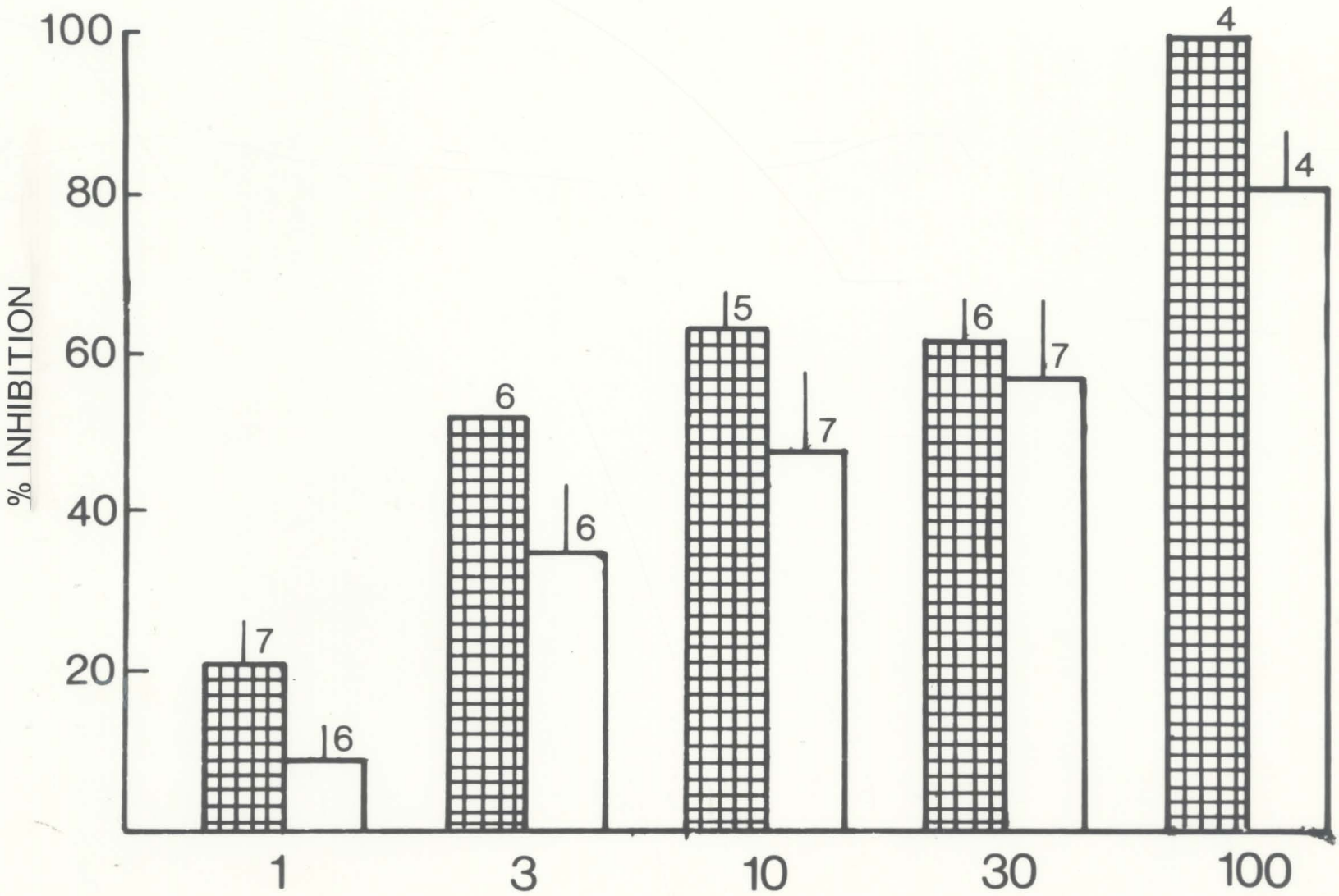
Since a large component of the K^+ response was dependent on the release of acetylcholine, it seemed warranted to examine the effects of the Ca^{2+} antagonists on the depolarisation in the presence of methscopolamine. As shown in Fig 36, the sensitivity of the antagonists did not vary between methscopolamine treated and untreated preparations. This may reflect the partial sensitivity of cholinoceptor response to the Ca^{2+} channel antagonists.

3.7.4 Calmodulin antagonists

Trifluroperazine and W7 abolished CD-induced contraction, K^+-induced contraction and FSR to the same degree. W7 at $1x10^{-7}M$ did not abolish either FSR_2 or CD contraction but at $1x10^{-4}M$ was effective in totally abolishing CD-induced responses. With the low amplitude of tension that could be generated FSR_2 was blocked. Trifluroperazine $3x10^{-5}M$ antagonised CD-induced responses by 71+14% and FSR by 93+7% (n=4). The calmodulin antagonists therefore exhibited a lack of selectivity for FSR_2.

3.7.4 Non-competitive blockade

Fig 36: Inhibition of K^+-induced contractions by nifedipine in the absence and presence of methscopolamine. Percent inhibition of K^+ 50 mM was calculated with respect to control responses in the absence of nifedipine pretreatment. Inhibition of K^+ contraction by nifedipine was examined in the presence (hatched column) and absence (open column) of methscopolamine (1×10^{-7}M). Concentration of nifedipine (nM) is indicated below each set of columns. % inhibition is given on the ordinate. Number of tissues are given above each column. Wilcoxon rank sign test showed no significant differences between methscopolamine treated and untreated tissues. Probability values were 1 nM (0.137), 3 nM (0.39), 10 nM (0.343), 30 nM (>0.5), 100 nM (>0.5).


100
80
60
40
20
% INHIBITION
7
6
6
6
5
7
6
7
4
4
1
3
10
30
100
NIFEDIPINE nM

In order to determine if the effects of PN(+) on CD and K^+ reflected a competitive antagonism of extracellular Ca^{2+}, the effects of the antagonists on the concentration-response relationship to Ca^{2+} in tissues preincubated with CD $1x10^{-7}$M and K^+ 50mM were examined. As shown in Fig 37, non-competitive antagonism was observed with both CD and K^+. Furthermore PN(+) was more potent in antagonising K^+ than in antagonising CD. An about equal depression of the maxima occurred at 0.1 nM and 1 nM PN(+) for K^+ and CD respectively suggesting a 10 fold increased selectivity for membrane depolarising events.

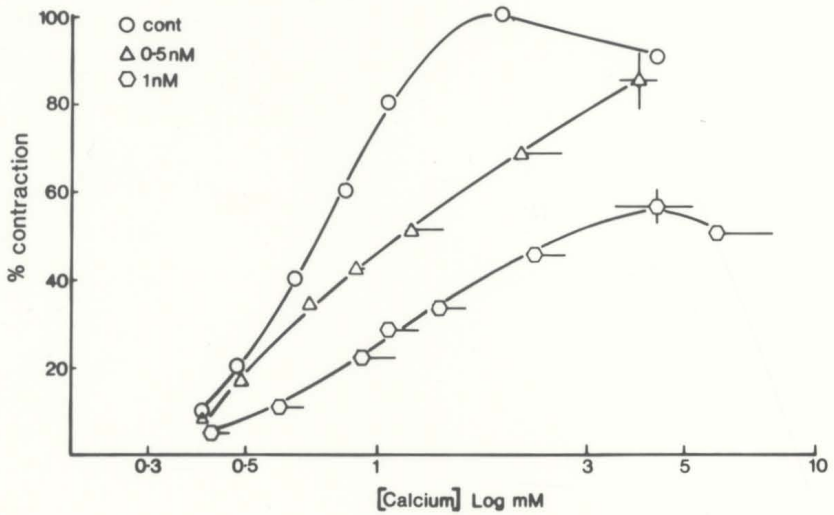
In the presence of PN(+), FSR_2 inhibition exhibited 'time or use'dependent phenomena as illustrated in fig 38.

3.7.5 Calcium channel agonists

The effects of BayK 8644, a Ca^{2+} channel agonist, were examined on tissues precontracted with CD $1x10^{-7}$M and in the presence of TTX $1x10^{-7}$M. Under these conditions the racemic mixture of Bay K produced further contractions (fig 39). At the same time, spontaneous activity was revealed. In the absence of CD, TTX-insensitive FSC was revealed on application of Bay K8644 (Fig 40). These TTX-resistant contractions were blocked by concentrations of nifedipine ($1x10^{-6}$M; Fig 41) which greatly exceeded those required to abolish FSR_2. Furthermore, pretreatment with the antagonist ($1x10^{-7}$M) did not prevent induction of TTX-resistant contractions. Similar effects were observed with the (-)

isomer of Bay K 8644. On the other hand, the (+) isomer behaved as an antagonist as evidenced by its ability to relax CD-induced tonus and blockade of FSR. TTX-resistant contractions to field-stimulation were also observed with CGP ($5x10^{-7}$M - $1x10^{-6}$M).

Fig 37: Effects of (+) PN on the Ca^{2+} concentration-response curves in the presence of 0.1 uM CD (A) and 50 mM K^{+} (B). Curves were plotted according to the method of Carpenter (1986). Horizontal lines represent standard errors. Note approximately equal depression of maxima by 1 nM PN in (A) and 0.1 nM PN in (B). The responses are expressed as a % of maximal contraction.


100
80
60
40
20
% contraction
cont
0·5nM
1nM
0·3
0·5
1
3
5
10
[Calcium] Log mM

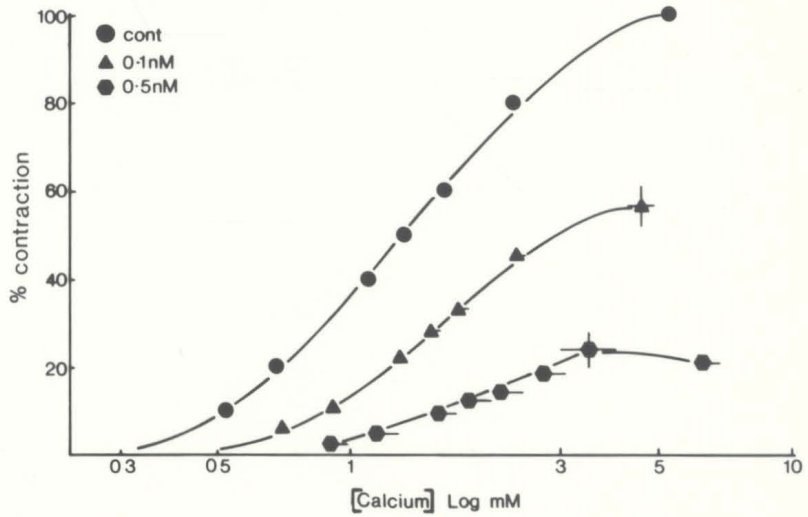

100
80
60
40
20
% contraction
cont
0·1nM
0·5nM
0 3
0 5
1
3
5
10
[Calcium] Log mM

Fig 38: Time or use-dependent inhibition of FSR_2 by PN-200-110. Tissue was incubated in Ca^{2+} free buffer for 30 min and in 1 nM (+) PN and 0.1 uM CD for 10 min prior to begining of trace. $CaCl_2$ was added cumulatively. Note increase in isometric tone and appearance of FSR with increasing Ca^{2+} concentration. Small horizontal bars at the bottom of tracing indicate field-stimulation applied at pulse width of 2 ms and frequency of 4 Hz.

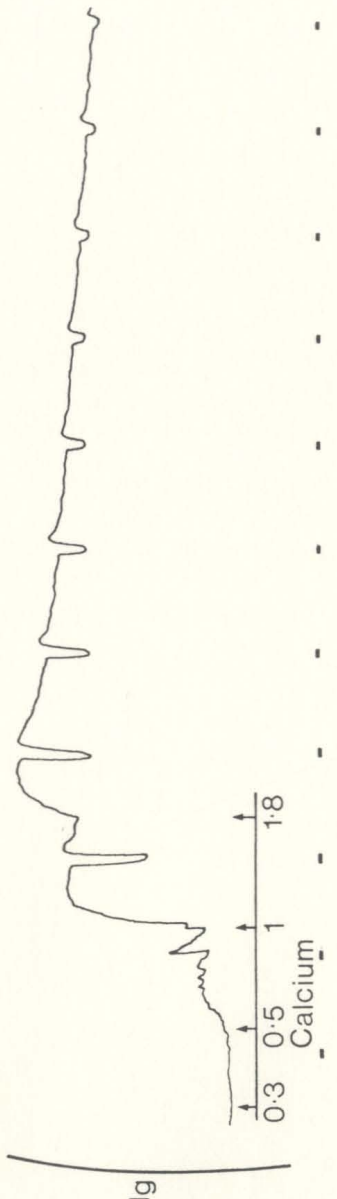

1g
0·3
0·5
1
1·8
Calcium
2 min

Fig 39: Bay K 8644 (-)-induced contraction of rat TMM. Tissue was pretreated with TTX (1×10^{-7}M) and CD (5×10^{-8}M) prior to start of trace. Bay K (-) was added as shown. Note the presence of field-stimulated relaxations after 50 nM Bay K. Field-stimulation parameters were 2 ms pulse width and 4 Hz frequency.

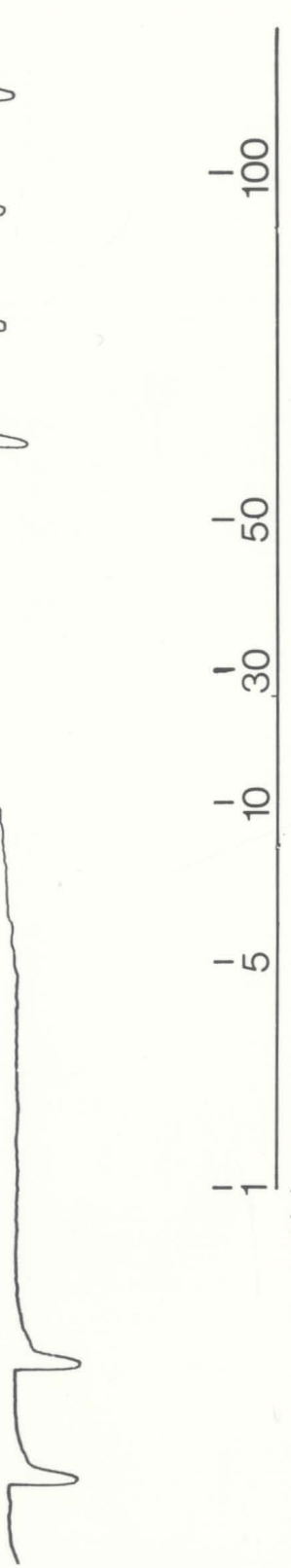
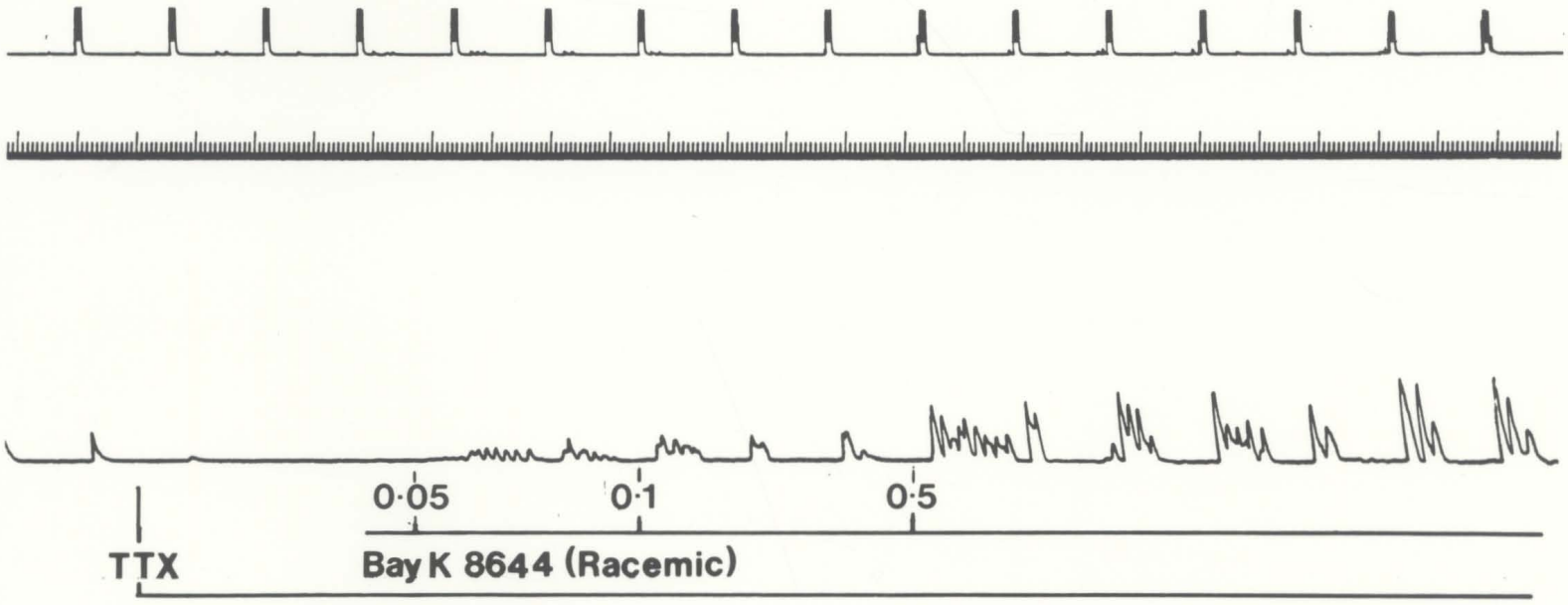
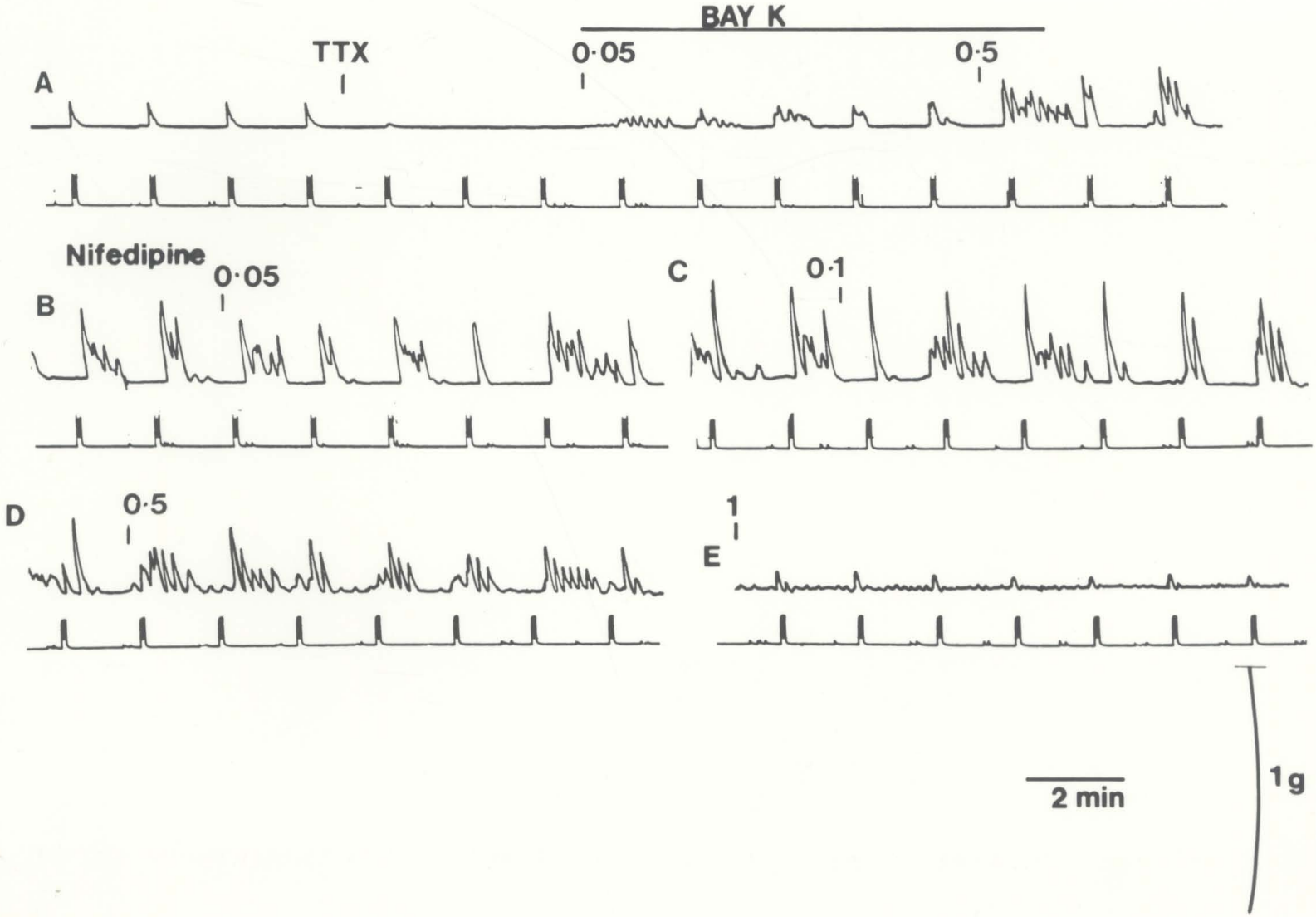

1g
2 min
1
5
10
30
50
100
BayK(-) nM

Fig 40: Bay K 8644-induced contractions to field-stimulation in the presence of TTX in the rat isolated TMM. Tissue was treated with TTX (1×10^{-7}M) to block excitatory responses to field-stimulation. Racemic mixture of BayK 8644 (uM) was applied in the continued presence of TTX and field-stimulation. Top tracing marks field-stimulation signals of 0.5 ms pulse-width and 8 Hz pulse-frequency. Middle tracing denotes time signal of 1 min interval between each maximal deflections.



g

Fig 41: The effect of nifedipine on BayK 8644-induced contractions. BayK (racemic) induced TTX insensitive contractions (A) which was not blocked by nifedipine 0.05 uM (B), 0.1 uM (C), 0.5 uM (D). BayK effect was abolished by 1 uM nifedipine (E). Bottom tracing under each figure marks field-stimulation at 0.5 ms pulse-width and 8 Hz pulse-frequency.


BAY K
TTX
0·05
0·5
A
Nifedipine
0·05
B
C
0·1
D
0·5
1
E
2 min
1g

3.8 Drug-evoked relaxations : mode of action and comparision with FSR_2

3.8.1 5-Hydroxytryptamine

As reported above (section 3.4) 5HT produced regionally differentiated relaxations. The 5HT-induced relaxation was not antagonised by trazodone ($1x10^{-6}M$; n=6), methysergide ($1x1)^{-6}M$; n=8), ketanserin ($1x10^{-5}M$; n=8), cyproheptadine ($1x10^{-5}M$; n=2), picrotoxin ($1x10^{-6}M$; n=2), phenoxybenzamine ($1x10^{-6}M$; n=3), timolol ($2x10^{-5}M$; n=2), or lithium chloride ($1x10^{-4}M$; n=4). Thus in the presence of these agents, 5HT ($5x10^{-8}M$ - $1x10^{-7}M$) produced complete relaxations in the proximal and middle segments. Ketanserin ($1x10^{-6}M$) pretreatment did not enhance 5HT-induced relaxations in the distal segment. Analogues of 5HT produced relaxations in a concentration-dependent manner. These fell into two groups based on their ability to produce relaxations. 5HT and 5-methoxytryptamine were clearly more potent than 5-methoxy-N,N, dimethyltryptamine, tryptamine, MK212, 8-hydroxy DPAT and 5-fluromethyltryptamine (Fig 42). Thus 5HT and 5-methoxytryptamine were approximately 100 fold more potent agonists.

These analogues of 5HT were also examined for their ability to antagonise 5HT-induced relaxations. Neither 5HT nor FSR_2 were antagonised by these agents. Cooling did not depress 5HT-induced relaxations.

5HT induced relaxations were also observed in

tissues FSR_2 was also evident with amplitudes of 0.26 ± 0.04 g and 0.25 ± 0.03 g in PCPA and reserpinised animals respectively.

The mast cell depletor, compound 48/80 (20ug/ml) produced contractions of approximately 10% of maximal CD-induced contractions. These contractions were blocked by $5x10^{-9}$M methscopolamine. In hemicholinium-treated tissues (n=3) and in 3day cold stored segments, compound 48/80 did not elicit any contractions. In precontracted TMM, 1 ug/ml 48/80 produced relaxations which were self-limiting. FSR was not altered by 48/80 treatment.

Regional differences in 5HT responses were exaggerated after 48hr cold storage. Proximal and middle segments relaxed to 5HT with an IC_{50} of 7nM , however, contractions were apparent in the distal segement at concentrations greater than $1x10^{-7}$M (Fig 43). Contractions to 5HT could be observed after 5 d of cold storage. These responses were not abolished by methscopolamine ($1x10^{-6}$M) or tetrodotoxin ($1x10^{-7}$M). When applied in the absence of tone, 5HT-induced contractions were observed at $1x10^{-7}$ - $1x10^{-4}$M. The contractions were blocked by ketanserin ($1x10^{-6}$M). 5HT induced contractions were not observed in segments left for 3 days at room temperature.

3.8.1.1 Effects of cold storage on A23187

As described in section 3.6.5.2, A23187-induced relaxations were also regionally selective and in the same

Fig 42: % Relaxation of CD-induced tone by 5HT analogues. % Relaxation (ordinate) was plotted against concentration of 5HT analogues (abssisa). In all cases relaxations were carried out at half maximal tonus induced by CD. (●) 5HT, (□) 5-methoxytryptamine, (○) 5-methoxy N, N, dimethyltryptamine, (■) tryptamine, (△) MK 212, (⬡) 8 OHDPAT, (▲) 5 fluro- methyl tryptamine.

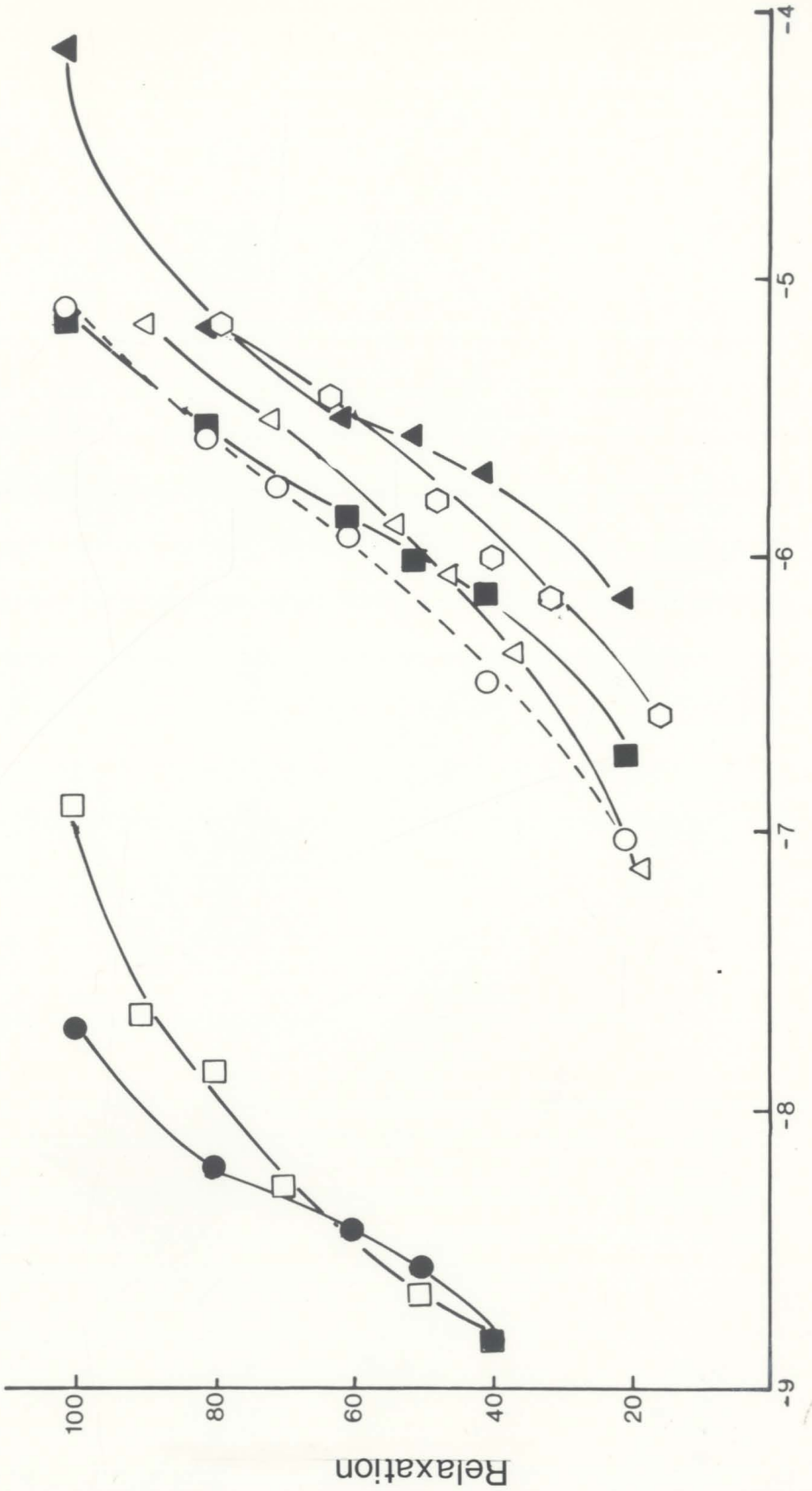

Relaxation
100
80
60
40
20
-9
-8
-7
-6
-5
-4
5HT Analogues

Fig 43: Relaxation induced by field-stimulation and 5HT in 2 day cold-stored proximal and distal segments. Tension was induced with 1 x 10^{-7}M muscarine applied at upward arrows. Complete relaxation was obtained in the proximal segment with contractions being evident in the distal segment. Contractions in the distal segment persisted in 3 d cold-stored distal segment (bottom). Field-stimulation periods (2 ms pulse width, 4 Hz pulse rate) are indicated below each trace.

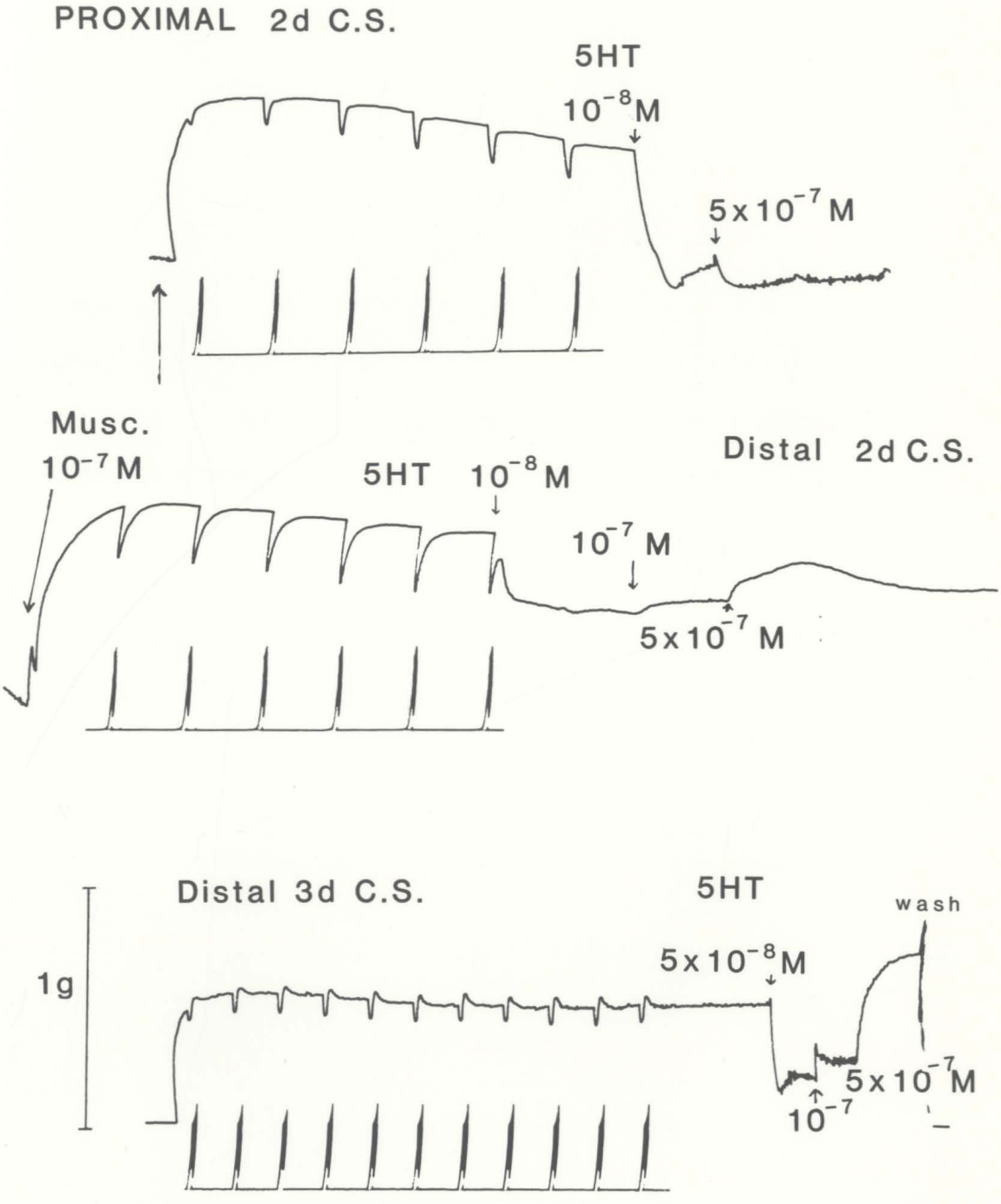
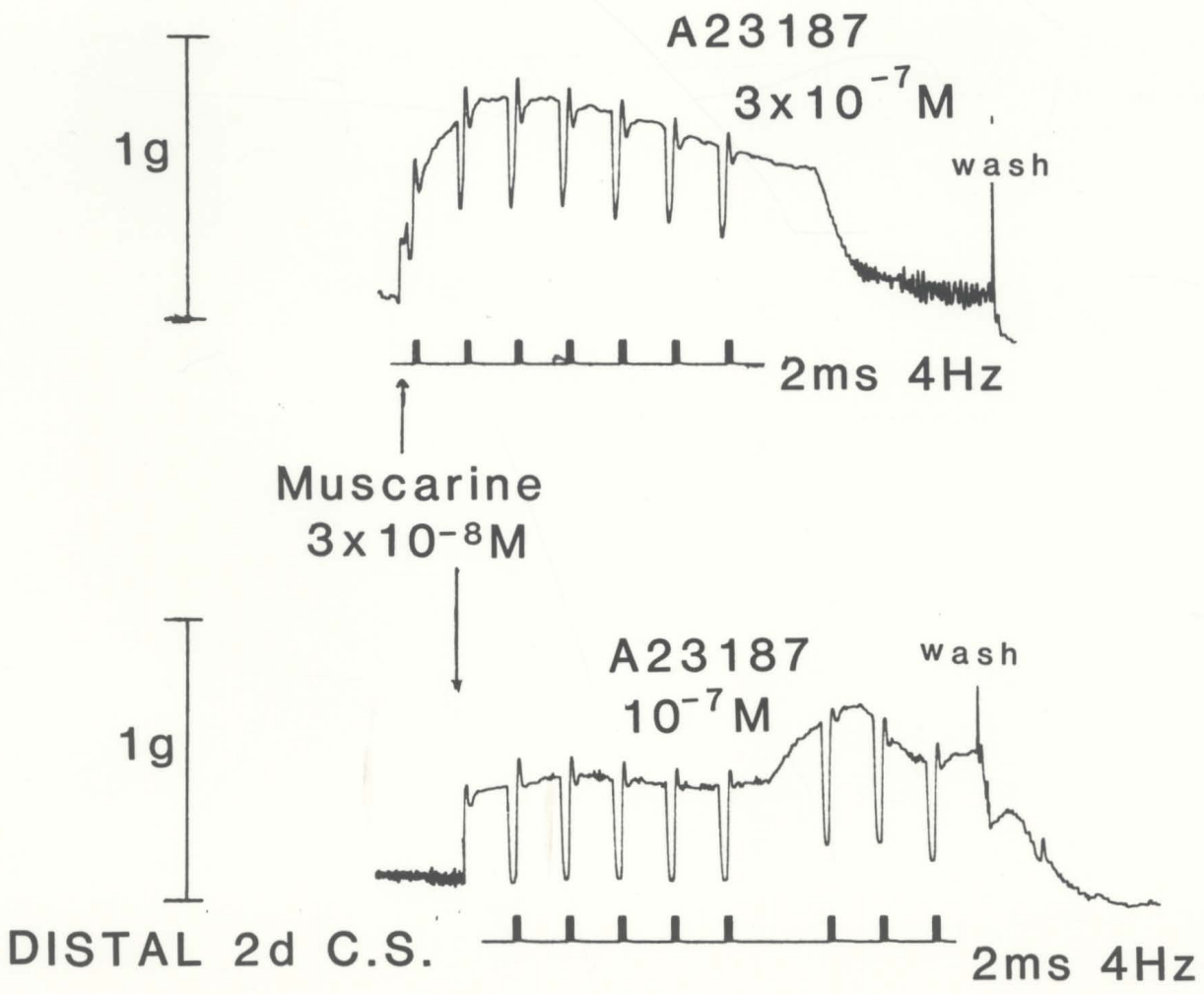

PROXIMAL 2d C.S.
5HT
10⁻⁸M
5x10⁻⁷M
Musc.
10⁻⁷M
5HT 10⁻⁸M
Distal 2d C.S.
10⁻⁷M
5x10⁻⁷M
1g
Distal 3d C.S.
5HT
wash
5x10⁻⁸M
10⁻⁷
5x10⁻⁷M

Fig 44: A23187-induced responses in 2 day cold-stored proximal (A) and distal (B) segments of rat TMM precontracted with 3 x 10^{-8}M muscarine. As for 5HT, the proximal segment relaxes, whereas contraction is seen in the distal segment.


PROXIMAL 2d C.S.
A23187
3x10⁻⁷M
wash
1g
2ms 4Hz
Muscarine
3x10⁻⁸M
A23187
10⁻⁷M
wash
1g
DISTAL 2d C.S.
2ms 4Hz

direction as 5HT (Fig 44). Cold storage exaggerated this regional selectivity of A23187 in a manner similar to that observed for 5HT. Thus 48 h cold-stored proximal segments produced relaxations to A23187 while contractions were observed in the distal. Unlike 5HT, A23187 did not produce relaxations at lower concentrations in the distal segments. Moreover, A23187-induced contractions were not antagonised by ketanserin, $1x10^{-6}$M. These effects of A23187 persisted upto 5 days of cold storage.

3.8.2 Vasoactive Intestinal Polypeptide (VIP)

VIP, ($7.5x10^{-8}$M -$7.5x10^{-7}$M) relaxed all segements of the TMM precontracted with muscarine or CD as indicated in section 3.3.2.2. These relaxations were not affected by tetrodotoxin ($1x10^{-7}$M), hemicholinium ($1x10^{-5}$M) or methscopolamine ($1x10^{-8}$M). α-chymotyrpysin, 0.1 U/ml to 10 U/ml, also produced relaxations, however, pretreatment with the peptidase (1U/ml) abolished VIP-induced relaxations. Neither VIP nor α-chymotrypsin abolished FSR_2. VIP-induced relaxations were not antagonised by cooling.

3.8.3 ATP and related agents.

ATP transiently relaxed TMM at 0.1mM with biphasic effects being apparent at 1mM. The biphasic effect consisted of an initial contraction followed by a relaxation. In the presence of ATP, FSR_2 remained unchanged. The ATP induced relaxations were not blocked by apamin ($1x10^{-5}$M) or α-β-

methylene ATP ($1x10^{-5}$M). ATP-induced relaxations persisted undiminished at 28°C. α- β-methylene ATP produced contractions of the TMM (n=3). The guinea-pig taenia caeci was relaxed by ATP (3.8 uM) and field-stimulation evoked relaxations were abolished by apamin ($1x10^{-7}$M) as was ATP-induced relaxation

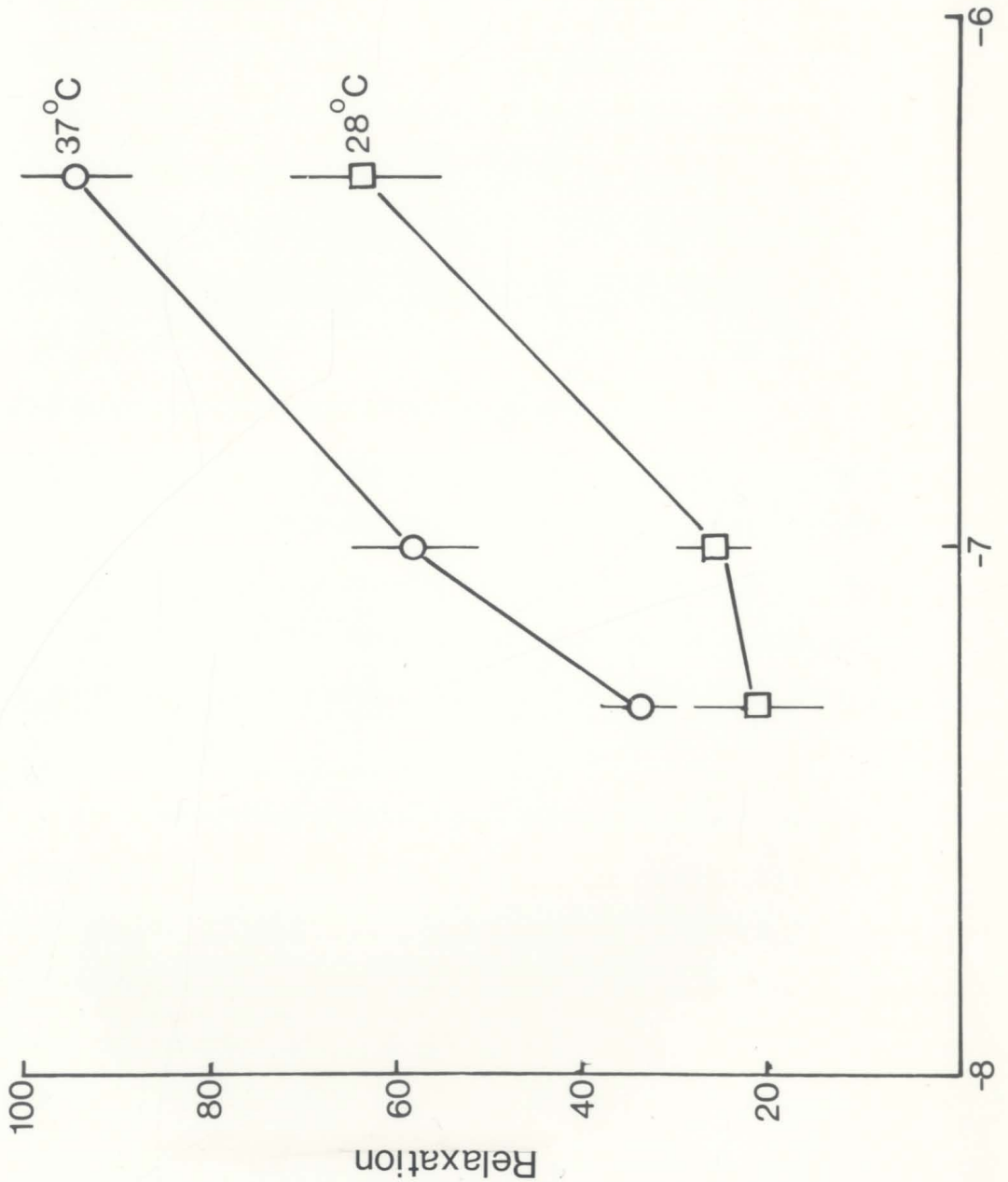
Dibutryl cyclic AMP ($5x10^{-5}$M) also relaxed TMM segments precontracted with CD in the presence of TTX ($1x10^{-7}$M). Relaxations were also evident with isobutyl methyl xanthine ($5x10^{-6}$M). None of these agents altered FSR_2. The adenylate cyclase activator, forskolin, relaxed tissues precontracted with either muscarine or CD. While not altering FSR_2, forskolin-induced relaxations were attenuated on cooling (Fig 45). Relaxations were slower in onset at 28°C. Myrecitin ($3x10^{-6}$M) or quercitin ($1x10^{-5}$M) did not block FSR and furthermore the former more potent putative forskolin antagonist did not block forskolin-induced relaxation.

3.8.4 cGMP and related agents

Concentration-dependent relaxations of CD-induced tone were produced by 8-bromo cyclic GMP ($1x10^{-5}$ - $1x10^{-4}$M; n=4). The guanylate cyclase inhibitor methylene blue, also relaxed TMM at concentrations > 10 uM. Neither of these agents altered FSR. In the presence of methylene blue 5HT-induced relaxations persisted. Relaxations by sodium nitroprusside were self-limiting in that 80±3% (n=3) relaxation was achieved in the first exposure at $5x10^{-6}$M whereas higher

concentrations up to $2x10^{-5}M$ on the second application produced relaxations of only 24% (n=2). Pretreatment with methylene blue, $1x10^{-5}M$, did not prevent sodium nitroprusside-induced relaxations.

Fig 45: Forkolin-induced relaxation of rat TMM at 37 ^{o}C and 28 ^{o}C. Relaxation of half maximal CD-induced tone by forskolin was measured at the two different temperatures. % Relaxation is plotted on the ordinate and Log concentration of forskolin on the abssica. Each point is a mean of 6-8 tissues.


37°C
28°C
100
80
60
40
20
Relaxation
-8
-7
-6
Forskolin

3.9 Comparision of drug-induced relaxation with FSR_1

As described above, FSR_1 was not blocked by hexamethonium, propranolol or phenoxybenzamine. On the other hand, guanethidine blocked FSR_1 in the distal segment but not the proximal segments. The effects of ATP were examined on FSR_1. Regional selectivity was apparent in that FSR_1 in the distal segment was not affected but was modified by ATP in the proximal segment to much slower relaxations.

3.10 Drug-evoked relaxations mimicking FSR_2

3.10.1 BRL 34915

3.10.1.1 As a K^+ channel blocker

BRL 34915 exerted antispasmogenic effects on TMM which was manifested as an inhibition of 1) contraction of K^+ depolarised tissues, 2) contractions evoked by field-stimulation in TTX treated tissues in the presence of TEA and 3) tonic contractions due to muscarinic cholinoceptor stimulation.

BRL34915 ($1x10^{-5}M$), displaced the foot of the K^+ concentration-dependent curve to the right while the maximum effect of K^+ was unchanged (Fig 46). In the presence of methscopolamine ($1x10^{-8}M$), the depression of the K^+ response remained unaltered.

Direct stimulation of the muscle induced by TEA was abolished by BRL 34915 (Fig 47). BRL34915 slightly depressed FSC which was reflected in its ability to relax TMM in a concentration-dependent manner (Fig 48). Relaxations were

also observed in the guinea-pig taenia caeci in agreement with other reports (Weir and Weston 1986).

3.10.1.2 Similarity with FSR_2

BRL 34915-induced relaxation was absent in tissues contracted with high K^+ (64mM). Under these conditions FSR was not observed (section 3.4.2). Lowering the bath temperature to 28^oC also antagonised BRL 34915-induced relaxation as did pretreatment with nifedipine (Fig 49; Fig 50). These conditions also abolished FSR_2.

3.10.1.3 Interaction with FSR_2

At concentrations greater than 10 uM, BRL 34915 blocked FSR_2 which were replaced with slight contractions as shown in Fig 51. Pretreatment with BRL 34915, resulted in a similar blockade, however, a 'use' dependent phenomenon was observed as 4-5 stimulation periods were required to demonstrate complete antagonism.

3.10.2 Interaction with FSR_1.

BRL, 1×10^{-5}M, did not alter FSR_1 in either the proximal or distal segments.

The contractions due to Bay K 8644 were also abolished by BRL suggesting that K^+ channel blockade may represent another putative action of the Ca^{2+} channel agonist (Fig 52).

Fig 46: Effect of BRL 34915 on concentration-response curve to KCl in the rat oesophageal TMM. Control responses (●); Responses in the presence of BRL 34915, 10 uM (□). Each point is a mean derived from 8 experiments; vertical bar shows S.E.M. (*) denotes significant difference (Student's t test; $p > 0.05$).

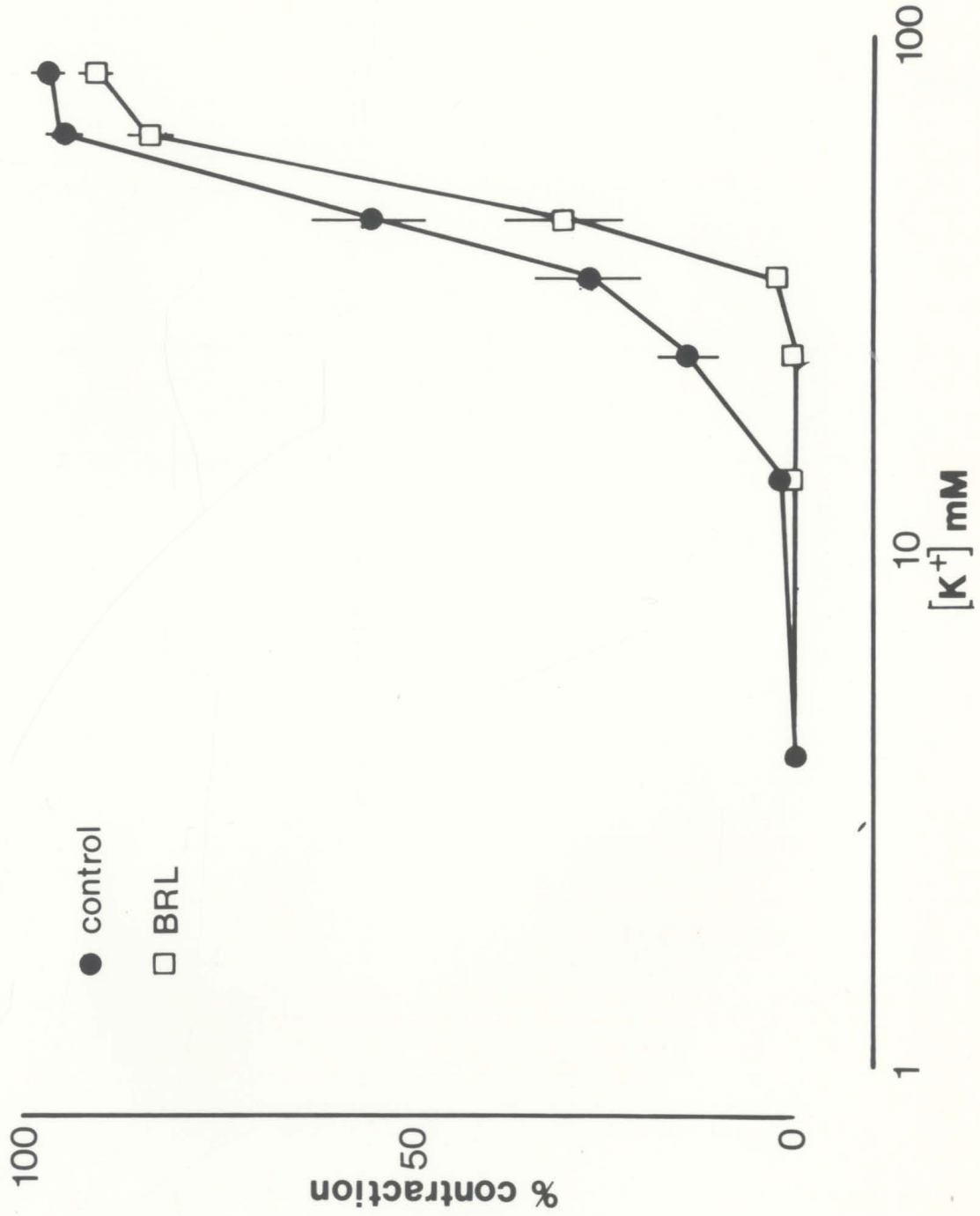

100
50
0
% contraction
● control
□ BRL
1
10
100
[K+] mM

Fig 47: BRL 34915 inhibition of TEA-dependent TMM direct muscle contraction. The top trace is a continuous record with a 10 min gap indicated by two dots. Initial contractions evoked by field-stimulation (indicated in bottom trace) were blocked by TTX (0.1 uM). Contractile responses reappeared after additional application of TEA (5 mM) which were blocked by BRL 341915 (1 uM). Field stimulation parameters: train length 10 sec, pulse frequency 8 Hz, pulse width 0.5 ms.

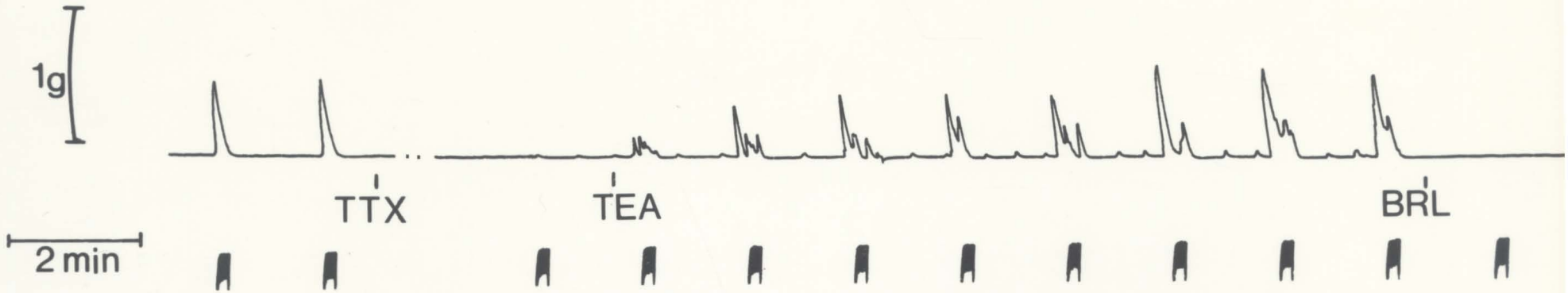

1g
TTX
TEA
BRL
2 min

Fig 48: Concentration-response relationships of relaxant effects of BRL 34915 in the rat TMM (●) and guinea pig taenia caeci (Δ). Relaxations are expressed as % of maximal relaxation in the presence of tone induced by CD (0.1 uM) for both tissues. Vertical bars show S.E.M.; rat TMM n=8; guinea pig taenia caeci n=4.

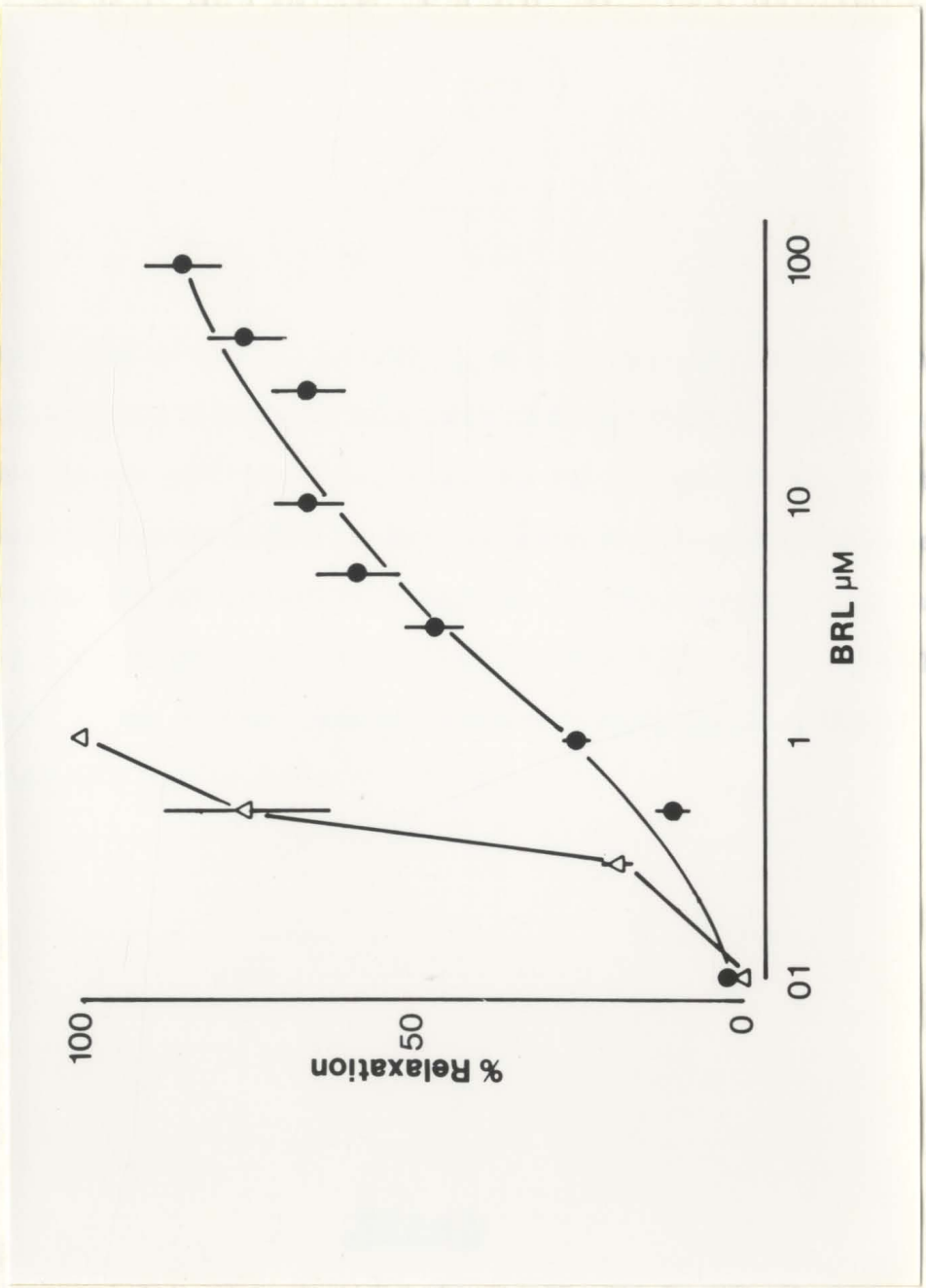

100
50
0
% Relaxation
01
1
10
100
BRL μM

Fig 49: The effects of cooling on FSR_2 and BRL 34915-induced relaxations. Muscle tension was induced by CD (0.1 uM) in the presence of TTX (0.1 uM) applied 10 min prior to start of record. Note decrease in FSR_2 as bath temperature is lowered and absence of relaxant response to BRL 34915 (5-100 uM). Stimulation parameters: 10 sec train length, 2 ms pulse-width; 4 Hz pulse rate). Vertical calibration 500mg and horizontal bar 1 min.

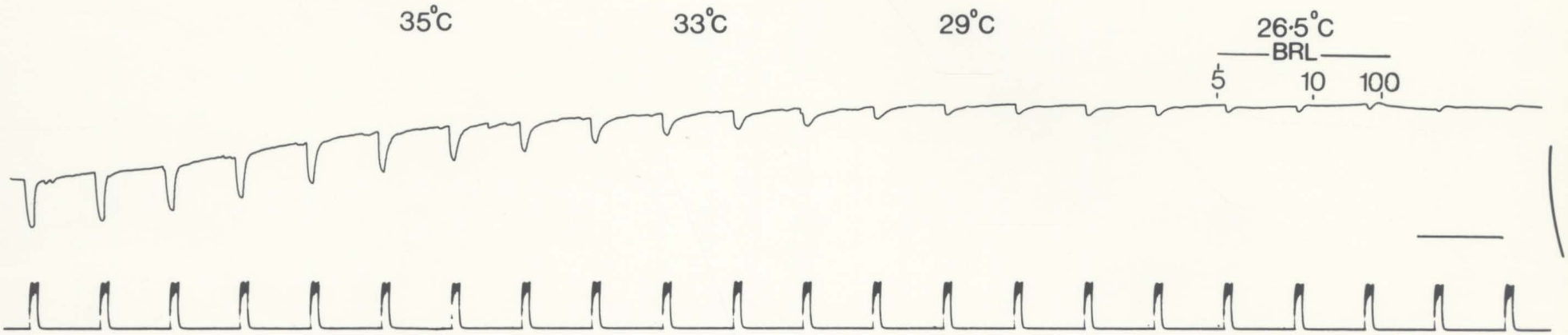

35°C
33°C
29°C
26·5°C
BRL
5
10
100

Fig 50: Block of FSR_2 and antagonism of BRL 34915 by nifedipine. Isometric tension recording from rat TMM. Following pretreatment with TTX (0.1 uM) and induction of active tonus with CD (0.1 uM) the tissue displayed FSR_2 during application of field-stimulation (bottom trace). The decrease in active tone but not of FSR_2 amplitude after nifedipine (NIF 0.05 uM) was overcome by addition of CD (0.05 uM). Note failure of BRL 34915 (1 - 10 uM) to induce a relaxation. Vertical bar indicates 1 g and horizontal bar 2 min. Stimulation parameters same as in previous figure.

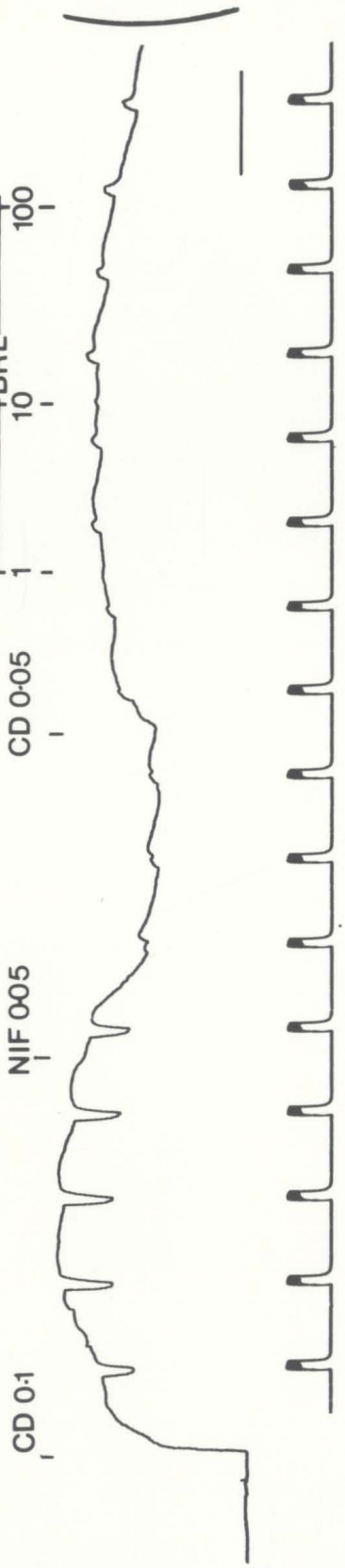

CD 0·1
NIF 0·05
CD 0·05
1
BRL
10
100

Fig 51: Relaxation induced by BRL 34915 (BRL) in the rat oesophageal TMM. Tissue was pretreated with TTX (0.1 uM) and tonus induced by CD (0.1 uM). Note conversion of FSR_2 to contractions. Dashes indicate field-stimulation as in previous figures. Calibration : Vertical bar 1 g.

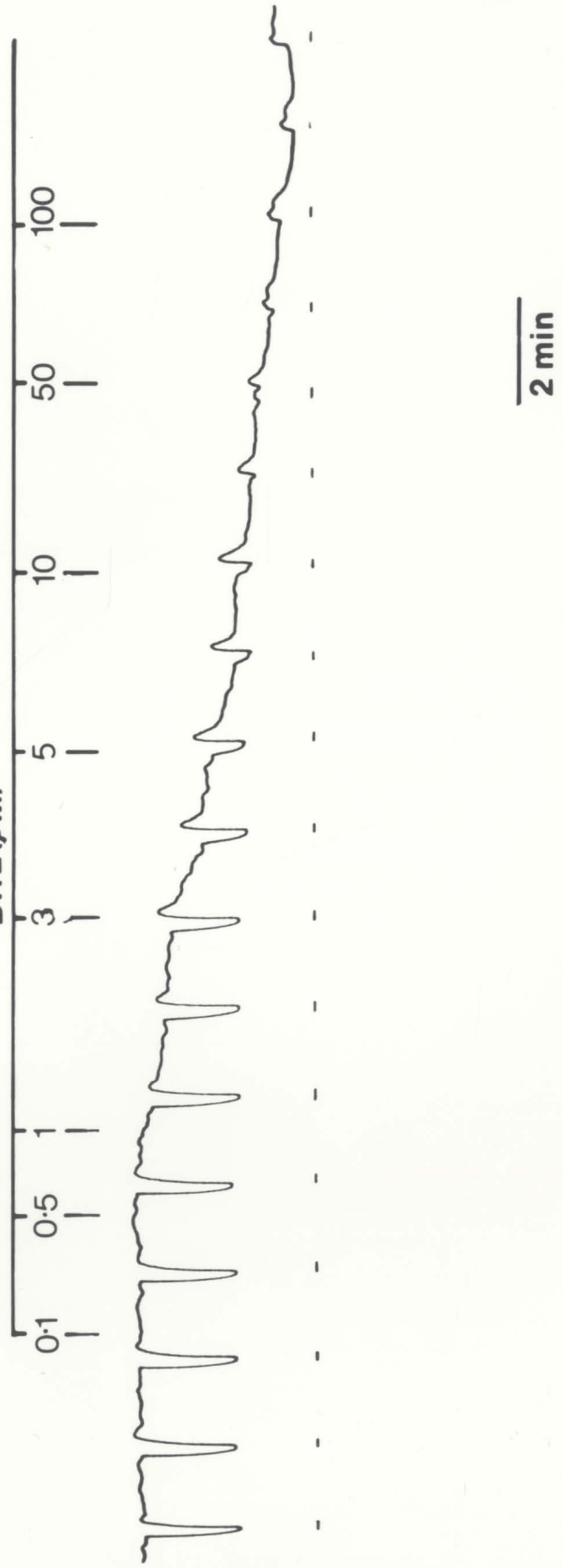
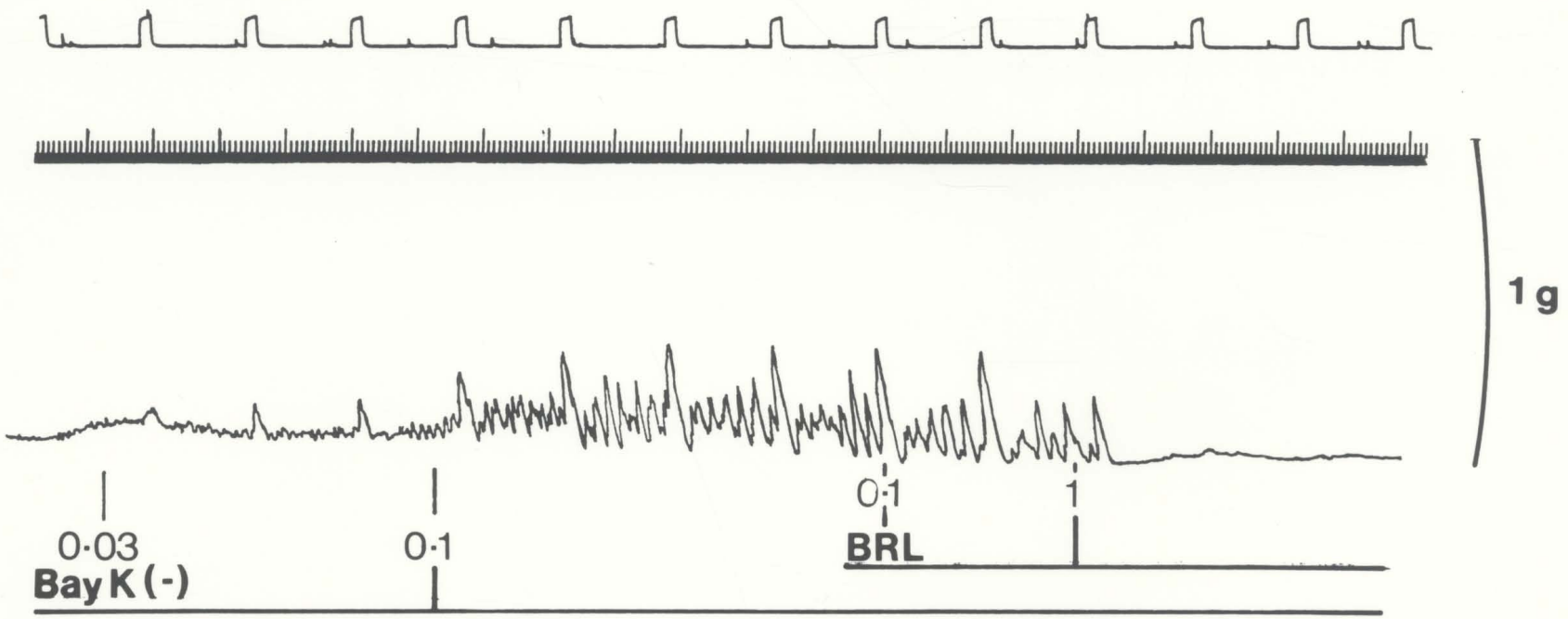

BRL(μM)
0·1
0·5
1
3
5
10
50
100
2 min

Fig 52: Block of Bay K 8644-induced direct muscle contraction by BRL 34915. The tissue was pretreated with TTX (0.1 uM) 5 min prior to start of record. Bay K (-) isomer dependent field-stimulated contractions were abolished by 1 uM BRL 34915. Top tracing marks field-stimulation at 0.5 ms pulse width and 8 Hz frequency. Middle tracing denotes 1 min interval between maximal deflections.


1 g
0·03
Bay K (-)
0·1
0·1
BRL
1

3.10 Canine TMM

Strips of canine TMM from the supradiphagramatic region responded to field stimulation with contractions at 0.5 ms pulse-widths and 8 Hz frequency. These contractions were blocked by methscopolamine ($1x10^{-8}$ -$1x10^{-7}$M) or TTX ($1x10^{-7}$M). Sustained contractions could be induced with histamine ($1x10^{-6}$M - $1x10^{-5}$M). Field-stimulation under these conditions revealed relaxations (Fig 53) which were observed in 19 out of 36 strips from 11 dogs. Relaxations were observed at 2 ms pulse-widths and 4 Hz and were insensitive to TTX except in the case of two out of the 19 strips in which they were sensitive. The relaxations in the canine TMM resembled those in the rat TMM in that the TTX-insensitive relaxations were not blocked by TEA (1mM), apamin ($1x10^{-5}$M), hexamethonium ($1x10^{-5}$M), propranolol ($1x10^{-6}$M) or phenoxybenzamine ($1x10^{-6}$M). Furthermore relaxations in the canine TMM were sensitive to cooling (Fig 54) but were not blocked by the calcium channel antagonists, nifedipine, PN-200-110 or verapamil. The relaxations were also blocked in high K^+ solution (Fig 55). Table 6 illustrates the comparative effects of some agents in the rat and canine TMM. In order to eliminate the possibility of strip preparations affecting FSR_2, strips of 2-4 mm wide and 1.5 cm in length from rats were also examined for FSR_2. Such strips continued to demonstrate FSR_2.

Fig 53: Field-stimulation evoked relaxations in the canine isolated TMM. The strip of TMM was treated with methscopolamine (MSCP) (1×10^{-6M}) and tone induced with histamine (HIST) (1×10^{-6}M). Bottom tracing marks field stimulation at parameters indicated below.

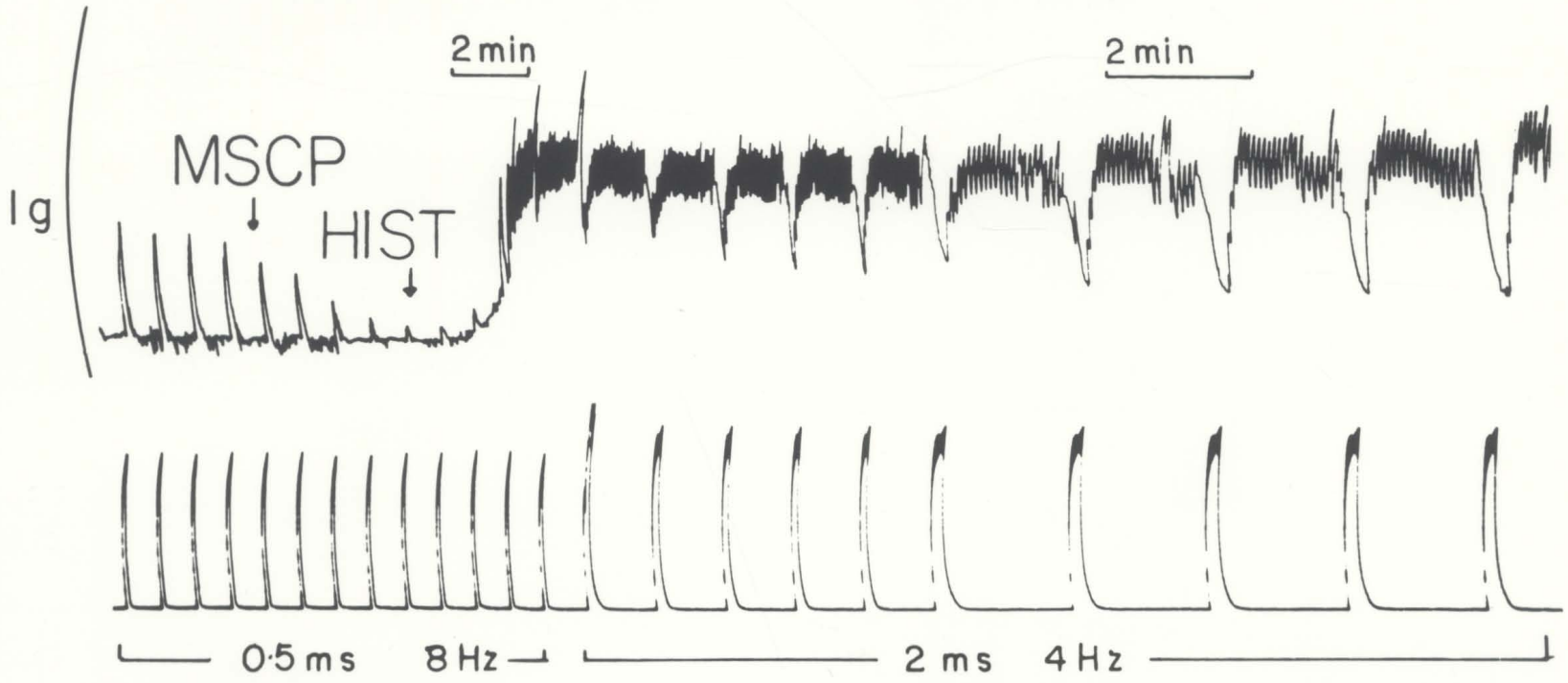

2 min
2 min
MSCP
HIST
1g
0·5 ms
8 Hz
2 ms
4 Hz

Fig 54: Effect of cooling on relaxation in the canine TMM. The tissue was pretreated and stimulated as in previous figure. Cooling the bath to 31 ^{o}C abolished FSR, rewarming restored it (not shown). Note slight increase in basal tone on cooling loss of spontaneous activity.

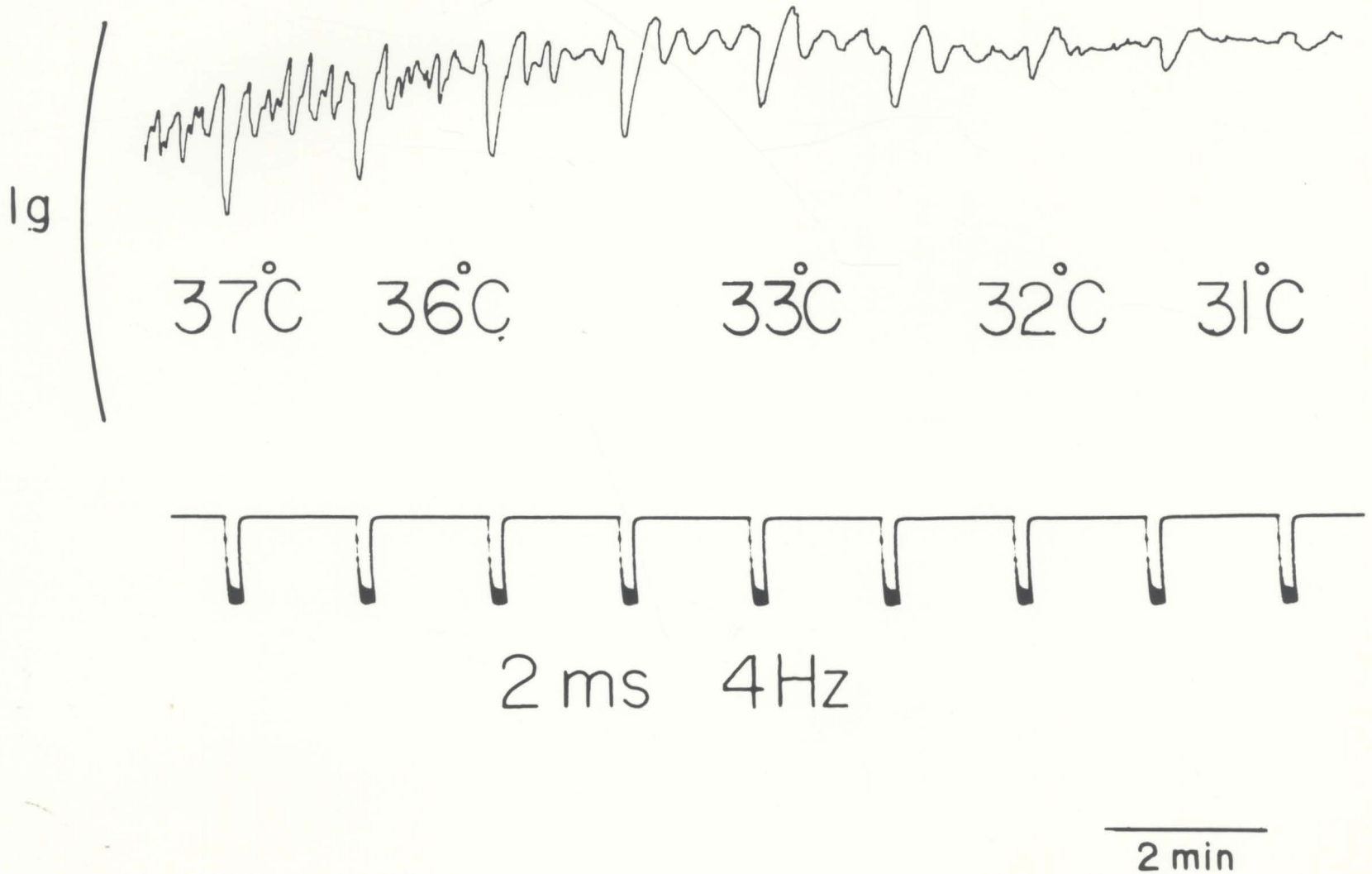

Ig
37°C
36°C
33°C
32°C
31°C
2 ms 4Hz
2 min

Fig 55: Loss of field-stimulated relaxation in high K^+ in the canine TMM.

Relaxations were revealed in the strip of TMM treated with 1 x 10^{-7}M TTX and tension induced with 1 x 10^{-6}M histamine. Note increase in tone and loss of relaxation to field-stimulation on application of 40 mM K^+.

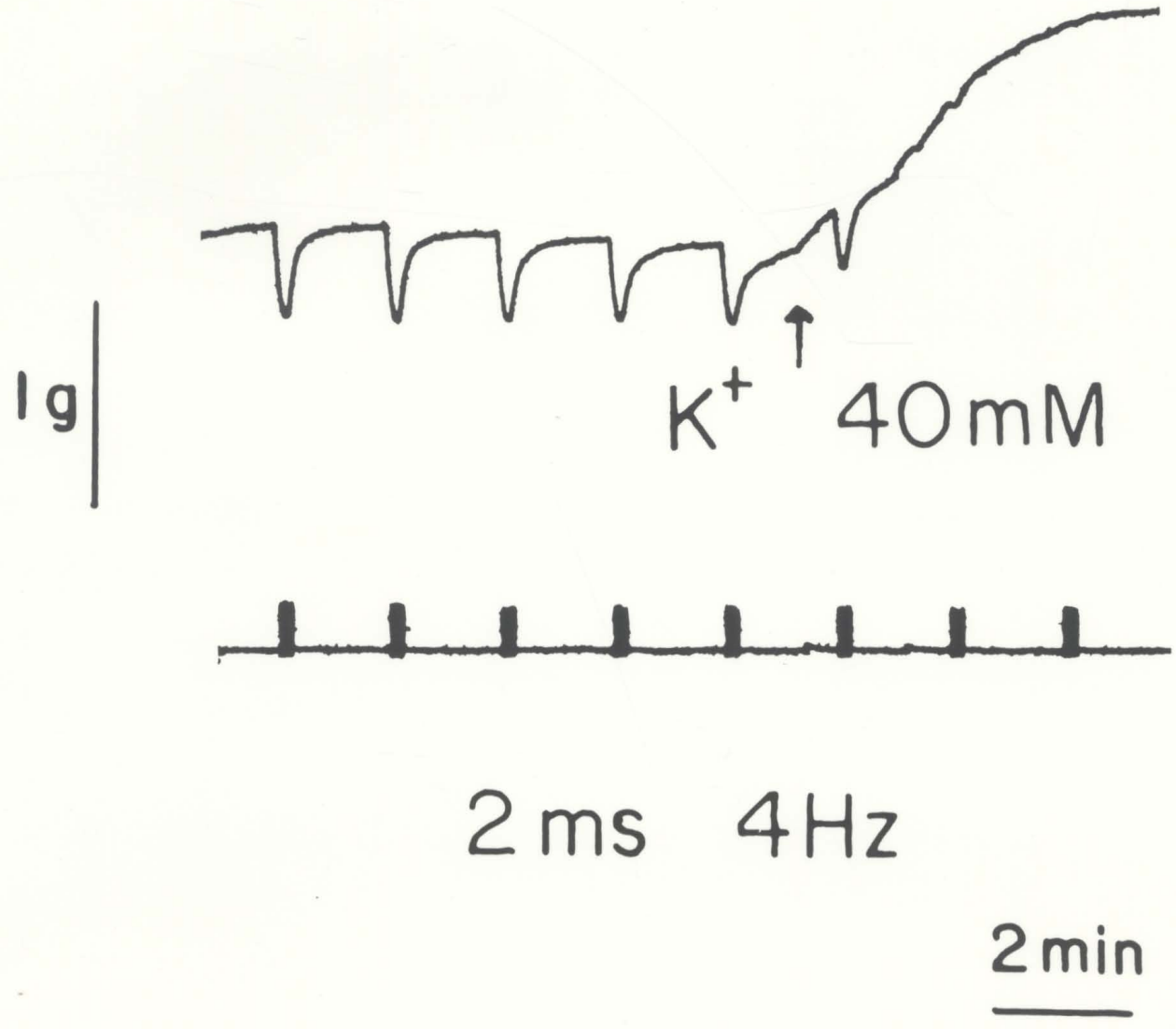

1g
K+ 40mM
2ms 4Hz
2min

TABLE 6

Comparison of field stimulation- and drug-evoked relaxation between rat and canine TMM

Pharmacological agent	Rat TMM	Canine TMM
TTX	N.E.	N.E.
TEA	N.E.	N.E.
Apamin	N.E.	N.E.
Hexamethonium	N.E.	N.E.
Propranolol	N.E.	N.E.
Phenoxybenzamine	N.E.	N.E.
Cooling	Blocks FSR	Blocks FSR
K	Blocks FSR	Blocks FSR
Mg	Blocks FSR	N.E.
Verapamil, Nifedip.	Blocks FSR	N.E.
5HT	Relaxation	N.E.
A23187	Relaxation	N.E.
LQV	Relaxation	N.E.

N.E. = No Effect.

CHAPTER 4

DISCUSSION

4.1 General considerations

The present study was mainly concerned with mechanical activity of the smooth muscle as a result of pharmacological and electrical stimuli. Tension development represents the final physiological output of the smooth muscle and is only an indirect measure of earlier events such as an increase in membrane conductance, depolarisation and hyperpolarisation and attending transmembrane ionic movements. In addition what is recorded is a result of the interaction of a change of an active state with a system displaying complex viscous-elastic properties which may produce considerable delay or modification of the time course. Thus, changes in tension in the TMM can be considered as a result of various membrane events, the output of which may be distorted in amplitude and time course by the isometric recording system.

The primary objective of this study was to delineate neural and non-neural responses in the smooth muscle. Smooth muscle and their nerve networks in particular, can be vulnerable to rough handling. Exposure to cold during preparation can radically alter ionic content of smooth muscle (Goodford and Freeman-Narrod, 1962). Therefore the first concern in isolated organ bath experiments involved examining contractions evoked by field-stimulation, the

reponse providing an index of the viability of the preparation in terms of both its neural and muscular elements. Transmural field-stimulation was used to excite intramural nerves. According to Paton (1975) four types of evidence need to be sought to support the conclusion that a response is mediated by nervous elements selectively excited by transmural stimulation. These are : a) single short electrical shocks should be effective, b) that there is some drug, known not to effect the excitability of smooth muscle, which inhibits the response studied; c) that the response is sensitive to TTX; and d) that the response is abolished by denervation. It is the convergence of these types of evidence which lead to acceptance of intramural nerve stimulation. The research strategy employed in this investigation considered each of these criteria. Differences in stimulus strength, as reflected by pulse-width, for the activation of nerve and muscle were apparent as discussed in the following section. With regard to sensitivity to pharmacological agents, contraction and relaxation responses were differentially affected thus posing the question of the role of neuronal mediation in these responses. To this end, denervation experiments were also carried out in order to meet with the criteria outlined above. The utility of the above criteria in making secure conclusions about the nature of the responses obtained in this study may be questioned particularly in the case of cold storage as a means of denervation (see section

4.4.1).

A potential drawback in the use of isolated tissues is that the preparations may behave in a pathological manner owing to their artificial environment. In this context, it was thus necessary to evaluate the functional integrity of the oesophageal TMM. Both histochemical and pharmacological data presented in this thesis indicate that the rat isolated TMM contains a functional submucosal plexus although it may not be completely intact. This is supported by responses obtained by field-stimulation and by vagal stimulation. Although, in vitro effects of vagal stimulation may not be totally representative of its motor control of oesophageal motility, the pharmacological basis of the responses due to vagal stimulation is supportive of the presence of functional neuronal network.

The main focus of this study was to elucidate the mechanisms underlying relaxations of the rat TMM. The ability of the smooth muscle to generate an energy-dependent active tonus during exposure to cholinoceptor agonists enabled relaxations to be demonstrated in a reproducible and convenient manner. These responses could therefore be compared with field-stimulation evoked contractions, drug-induced contractions and relaxations. Moreover, the dependency of responses on the state of the smooth muscle was also conveniently explored by inducing contractions by depolarisation. This effect was achieved by high K^+

solutions. High K^+ solutions may be either hypertonic or isotonic. Examination under both procedures were required since apart from the effects of tonicity on the smooth muscle response, contractions due to isotonic K^+ may reflect changes in the Na^+ content rather than elevation of the K^+.

To summarise, this study deals with mechanisms governing oesophageal smooth muscle contractility. The interpretation of the observed effects are contingent on the viability of the isolated preparation and the functional response of the neuronal plexus in an artificial environment. The method of pursuing this problem entails comparing responses of electrical and pharmacological origin.

4.2 MORPHOLOGIC FINDINGS

The isolated TMM preparation contains the submucous plexus (Bieger and Triggle, 1985). It is reasonable to assume that both TTX-sensitive contraction and relaxation to field-stimulation may arise from this plexus. The sensitivity of the FSC to methscopolamine, coupled with the presence of a cholinesterase-rich plexus indicates the likely presence of acetylcholine. Apart from the dense cholinesterase staining, the submucous plexus also contains VIP-like fibres, calcitonin-gene related fibres and some noradrenergic fibres. The sparse adrenergic innervation present in proximal and supradiapharagmatic TMM is consistent with that reported in the guinea-pig (Kamikawa and Shimo, 1979) and the rat (Schultzberg et al., 1980). Cell bodies of the peptidergic fibres were not observed in the submucous plexus. These fibres could arise from myenteric plexus ganglionic perikarya or may have their cell bodies in sensory ganglia. Thus neither a motor nor sensory function can be attributed to these peptides at present. Although peptides could be co-localised with acetylcholine, the blockade of field-stimulated contractions (FSC) by methscopolamine would negate a co-transmitter function. On the other hand, a modulatory role of the peptides cannot be discounted.

Interestingly, VIP containing cells were also observed in the rat TMM. No obvious neuronal connections

could be observed. Similar cells have been reported by Christensen et al. (1987) in the opossum TMM but their function remains undefined.

5HT containing cells were identified as mast cells on the basis of their resemblance to cells stained with toluidine blue. One of the possibilities considered in this study was the release of 5HT from these cells (see below).

Overall, the rat isolated TMM contains a rich submucosal plexus which indicates its potential for responding to a variety of neurally-mediated stimuli. In the present study, immunohistochemistry was limited to the whole mount preparation of the TMM. Thus spatial details of fibre distribution vis a vis muscle, epithelium etc., may not be revealed. However, results from the whole mount preparations were suitable for evaluating the presence of a peptidergic innervation.

The relationship between these different immunoreactive structures and their role in TMM motility presents an interesting area for future investigation.

4.3 CONTRACTILE RESPONSES OF THE TMM

4.3.1 Field-stimulation

The TTX-sensitivity of field stimulation-evoked contractile responses implies the presence of a nerve-mediated excitatory process in the TMM. This event was predominant over the TTX-insensitive contractions, which were of much smaller amplitude, being elicited at larger pulse widths (>5ms) and also in the presence of TEA (at pulse widths < 1ms) suggesting direct muscle stimulation. Electrically inexcitable smooth muscles usually show outward rectification (Mekata, 1971; Kannan, Jager, Daniel and Garfield, 1983). In the rabbit aorta (Mekata, 1981) and in the monkey carotid artery (Mekata, 1986), there is close coupling between electrical and mechanical output. Thus, in the monkey carotid artery, tension changes were only observed on prolonged currents at membrane potential more positive than -40 mV. Current application at resting membrane potentials (more negative than -40 mV) were ineffective in inducing tension changes (Mekata, 1981; 1986). Although electrical measurements were not carried out in this study, a close coupling between electrical and mechanical output can also be assumed in the rat TMM. This assumption is based on the inability of the TMM to contract when treated with TTX reflecting a lack of direct muscle stimulation. Furthermore, direct muscle contraction could only be observed in the

presence of TEA. TEA may be regarded as decreasing the membrane potential by blocking rectifying K^+ channels (Kirkpatrick, 1981). Indeed, a decrease in resting membrane potential by 10-12 mM depolarising solutions of K^+ also produced similar responses. These lower concentrations which required the application of current to induce contractions can be assumed to be insufficient to evoke opening of Ca^{2+} channels .

With regard to nerve-mediated contractions, the results are consistent with those reported by Bieger and Triggle (1985). In the isolated TMM, the sensitivity to methscopolamine but not to hexamethonium suggests field-stimulated contraction results from activation of intrinsic postganglionic cholinergic nerve terminals. In the whole vagus-oesophagus preparation, the sensitivity to hexamethonium indicates that the motor control of the TMM is effected by at least a 2-neurone pathway composed of a preganglionic nicotinic synapse and a postganglionic muscarinic synapse.

4.3.2 Drug-induced contractions.

As with other smooth muscle preparations, the phasic and tonic components of cholinoceptor agonists and K^+ depolarisation were differentially affected by Ca^{2+} channel antagonists and their requirements for metabolic activity. Extensive studies (Chang and Triggle, 1973; Triggle, Swamy and Triggle, 1979; Rosenberger, Ticku and Triggle, 1979)

support the contention that the Ca^{2+} pool supporting the tonic component of the agonist response is derived from an extracellular source and presumably reflects free extracellular Ca^{2+}, whereas the Ca^{2+} pool that supports the phasic component is superficially bound pool. Others have postulated (Hurwitz, McGuffee, Little and Blumberg, 1980) that extracellular Ca^{2+} associated with the phasic component enters the cell via Ca^{2+} channels which possess different characteristics from those supporting tonic components. Rangachari and Triggle (1986) reported, that at least in the guinea-pig ileal smooth muscle, agonist-induced contractions may utilise three different pools of Ca^{2+}. In the rat TMM, both phasic and tonic components of the muscarinic agonist and K^{+} depolarisation were dependent on extracellular Ca^{2+}. The comparative insensitivity of the phasic response to organic Ca^{2+} channel antagonists is suggestive of different Ca^{2+} pools utilised in the two phases of the contractions. The tonic phase initiated by high K^{+} was inhibited to a greater extent than the response resulting from cholinoceptor activation, suggesting that the source of Ca^{2+}, or the Ca^{2+} entry routes are different for these two activation processes. In keeping with findings reported by other workers (see Cauvin et al. 1983 for review), the greater sensitivity to the Ca^{2+} channel antagonists of K^{+} mediated tonic response suggests activation of potential operated Ca^{2+} channels. In contrast, the cholinoceptor-mediated

responses, being only partially sensitive to Ca^{2+} channel antagonists may be considered as activating receptor operated Ca^{2+} channels. On the other hand, Ca^{2+} levels may be sufficiently decreased during tonic responses conferring partial sensitivity to the Ca^{2+} antagonists. This simply means that similar channels may be utilised for both forms of contraction, but in the case of muscarinic stimulation, a Ca^{2+} transient, responsible for the initial phase of contraction occurs (Morgan and Morgan, 1984).

It has been reported that the energy storage in the form of ATP, of vascular smooth muscle is limited and can only be maintained by continuous production (Paul, 1980;1983). The inability of the tonic phase to be maintained in the absence of glucose, indicates a similar dependency in the TMM. Moreover, Ashoori, Takai, Tokuno and Tomita (1984) reported that Ca^{2+} channels may remain inactivated during decreased intracellular ATP levels in the guinea pig taenia caeci thus inhibiting tonic contractions. The differences between the continued presence of phasic responses and the absence of tonic responses upon glucose deprivation may be due to increased demand and consumption in the latter state for ATP than is available. This may suggest that the contractile machinery retains the ability to produce tension but the limitation may be due to availability of ATP. Moreover, under relaxed conditions the tissue content of high

energy phosphates may recover at least sufficient to produce phasic responses (Ashoori et al. 1984).

Since a large part of the tonic component of K^+ was inhibited by methscopolamine it may be surmised that K^+ evokes release of acetylcholine from nerve terminals. Depolarisation of nerve terminals can induce TTX-resistant, Ca^{2+} dependent release (Katz, 1969; reviewed by Augustine, Charlton and Smith, 1987).

4.3.3 Regional Differences

A consistent feature of TMM contractility was the proximo-distal gradient in the amplitude of tone to field-stimulation, drug-induced contraction and K^+ induced contraction. Contractions in the distal segment were considerably greater than in either the proximal or middle segments particularly in the case of K^+ depolarisation and field-stimulated contractions. Regional gradients in the oesophagus can be attributed to either neurogenic (Gidda and Goyal, 1985), myogenic (Schulze, Conklin and Christensen, 1977) factors or simply to decreased smooth muscle mass in the proximal segment. Although no significant difference was observed in the muscle mass this could be compensated by other tissue elements such as the epithelium. Inhibition in the distal segment due to vagal stimulation in the opossum oesophageal smooth muscle was prolonged compared

to more proximal regions; the inference being that gradation in neurally-mediated responsiveness occurs (Gidda and Goyal, 1985). A decline in intracellular K^+ concentration of the opossum oesophageal smooth muscle from proximal to distal segment was reported by Schulze et al. (1977). However, the decline in $[K^+]$ was not correlated with changes in membrane potential. Crist, Surprenant and Goyal (1987) observed no regional differences in active or passive electrical membrane properties of the opossum oesophageal circular smooth muscle. The question as to whether neurogenic mechanisms account for regional differences remains to be fully answered. The possibility of better coupling between smooth muscle cells in the distal compared to the proximal may also account for differences in contractility. Thus, a greater number of gap junctions have been observed in the opossum oesophageal body smooth muscle as compared to the lower oesophageal sphincter of this species (Daniel and Posey-Daniel, 1985). Differences in field-stimulation evoked contractions may indicate a closer proximity of nerve terminals to smooth muscle in the distal than the proximal segment.

It can be concluded that the rat TMM is an electrically inexcitable smooth muscle, possibly reflecting a large resting outward rectification. It is nevertheless responsive to cholinoceptor agonists producing both phasic

and tonic responses. The motor control of the TMM via the vagus and the effects of field-stimulation of the isolated tissue indicate that the submucosal plexus represents an important site for control of intrinsic cholinergic neurones.

4.4 RELAXATION RESPONSES IN THE TMM

The present sudy has been mainly concerned with inhibitory mechanisms operating in the TMM. The results demonstrate that in the rat oesophageal TMM, relaxations can be produced by pharmacological stimulation on the one hand, and by electrical stimulation applied either transmurally or via the vagus nerve on the other. The main issue dealt with in this study concerns the pharmacological basis of field-stimulated TTX-insensitive relaxation. The experimental approach was based on delineating the source of this relaxation and the congruity with other forms of relaxations. This included the relaxations evoked by stimulation of a small pulse-width, vagal stimulation and pharmacological agents. Notwithstanding the insensitivity to TTX, in each case the interrelationships between these forms of relaxations were examined under conditions that affected them. For instance, the sensitivity to cooling and to pharmacological agents formed a simple basis to separate each response.

Two types of relaxations produced by field-stimulation were identified as evidenced by their sensitivity to TTX. For convienience, these have been termed as FSR_1 and FSR_2, denoting the TTX-sensitive and TTX-insensitive relaxations, respectively. In smooth muscle pharmacology, TTX has generally been used as a tool to distinguish between nerve- and muscle-induced tissue

response. However, TTX insensitivity per se does not rule out nerve-mediated responses since action potentials in nerves can be generated either by Ca^{2+} spikes or through slow Na^{+} channels (Blaschke and Uvnas, 1981; Hirst and Spence, 1973; McAfee and Varowski, 1979). These relaxations could be regarded as non-cholinergic, non-adrenergic since adrenoceptor antagonists were ineffective, except in the distal segment where FSR_1 was abolished by the adrenergic neurone blocking agent, guanethidine. However, since phenoxybenzamine, phentolamine and propranolol were all ineffective in inhibiting FSR_1, one must assume that either a nonconventional adrenoceptor mediates the relaxation or that guanethidine has effects other than those of an adrenergic neurone blocker. Among the latter effects, local anaesthetic-like properties have particularly been noted (Haesler and Haefley, 1979; Brock and Cunnane, 1987). Hirst, Neild and Silverberg, (1982) described the presence of - receptors in the guinea-pig arterioles. This novel adrenoceptor is insensitive to and antagonists. Whether FSR_1 is mediated via such a receptor remains undefined at present. It is noteworthy, that relaxations did not usually accompany guanethidine treatment suggesting that noradrenaline may not be released in sufficient quantity.

4.4.1 Relationship between FSR_1 and FSR_2

At first glance, FSR_1 and FSR_2 seem to be of the same origin. Both types of relaxations could be evoked by field-

stimulation in the presence of active tonus induced by cholinoceptor agonists but not K^+. Although FSR_2 was insensitive to TTX, this could be attributed to stimulation with sufficient current to overcome Na^+ channel blockade. However, other distinguishing features such as frequency response-relationship, insensitivity of the FSR_1 to cooling and the effects of various pharmacological agents render it unlikely that the two forms of relaxation originate from the same source. The source of FSR_2, as determined by cold storage and pharmacological agents known to induce release from nerve terminals, may be regarded as non-neuronal. On the other hand, a neuronal basis for FSR_1 is clearly indicated not only by the effects of TTX but also by cold storage and releasing agents.

Cold storage is known to induce denervation of intramural nerves (Guimares, Oswald, Cardoso and Branco, 1971; Lee, Hume, Su and Bevan, 1978; Shibata, Hattori, Sakurai, Mori and Fujiwara, 1977). FSR_2 could be elicited for up to 5 days of cold storage while excitatory responses to field stimulation were abolished after 2 days. The persistence of relaxation paralleled smooth muscle responsitivity. This evidence could be construed to mean either that a) FSR_2 arises from inhibitory nerves resistant to cold storage or b) FSR_2 is due to activation of a non-neuronal structure.

Burnstock, Campbell and Rand (1966) observed inhibitory responses to transmural field-stimulation in the guinea pig taenia coli after 4 days of cold storage. The authors suggested that NANC inhibitory nerves activated by field-stimulation were resistant to cold storage. However, no evidence regarding the sensitivity to TTX was presented thus leaving open the question of a TTX-insensitive mechanism operating in this muscle. Moreover, Hattori, Kurahashi, Mori and Shibata (1972) could not induce relaxations in the guinea pig taenia coli after 3 days of cold storage which is in contrast to the results of Burnstock et al. (1966). King and Robinson (1945) also demonstrated inhibitory responses to nicotine in the muscularis mucosae of the canine small intestine after 38 hrs of cold storage, suggesting stimulation of inhibitory ganglia. Again, the presence of neurally-mediated relaxation was not clearly established. Thus, unless a resistance to cold storage of inhibitory nerves is postulated for the TMM, FSR_2 would appear to arise from a non-neuronal structure closely associated with the muscle or as a result of muscle stimulation. Further evidence that one of these alternatives represents the likely process involved is presented by the effects of cooling and scorpion venom.

4.4.1.1 Cooling

The remarkable sensitivity of FSR_2 to cooling also distinguished it from FSR_1. Both neurally-mediated

contractions and relaxations were unaffected by cooling. De Carle, Szabo and Christensen (1977) have also noted that cooling affects the 'off' response of the in vitro opossum oesophageal smooth muscle preparation. In that study a decrease in amplitude and an increase in latency was observed for the rebound 'off' response. Relaxations in the lower oesophageal sphincter were however reduced at temperatures of 14-20 ^{o}C. The temperature dependency of FSR_2 observed in this study could be due to either altered transmitter release and dispersion (Jager and den Hertog, 1974) or as speculated by de Carle et al. (1977) due to changes in membrane conductance. It is noteworthy, that cooling can induce an increase in sodium content and depolarisation (Freeman-Narrod and Goodford, 1962). Since cooling did not alter FSR_1 or FSC in the temperature range examined, it may be surmised that it is smooth muscle rather than neural tissue that is affected. This provides further support for the view that FSR_2 represents a non-neuronal response.

4.4.1.2 Scorpion venom (Leiurus quinquestriatus, LQV)

By delaying inactivation of fast Na^+ channels, LQV should enhance mechanisms involving nerve mediated release (Koppenhoffer and Schmidt, 1968). However, FSR_2 remained unaltered after LQV treatment, lending further support for a non-neuronal mechanism. Relaxation produced by LQV itself cannot be ascribed to delayed inactivation of Na^+ channels since this effect was not antagonised by TTX. The crude

preparation obtained from Sigma may contain serotonin (Adam and Weiss, 1956) which may be responsible for relaxations. On the other hand, veratridine, enhanced FSR_1 but not FSR_2, indicating the neuronal basis of the former.

To summarise, the results support the conclusion that FSR_2 may be effected via a mechanism different from that underlying FSR_1. The contention that FSR_2 originates from a non-neuronal source is most strongly supported by the effects of cold storage. It is tempting to speculate on the possible inter-relationship between FSR_1 and FSR_2. Thus while the former may be involved in whole TMM motility as represented by peristalsis, the latter could be involved in affecting local inhibition of the muscle.

Of the non-neuronal sources, mast cells seemed worth investigating for three reasons. 1) Histochemical data indicated the close apposition of the mast cells to the smooth muscle; 2) due to the presence of 5HT in these cells. 5HT could be postulated as a likely candidate of FSR_2 since this agent was a potent relaxing agent and has been previously demonstrated to serve an inhibitory neurotransmitter function in G.I. motility (Bulbring and Gershon, 1967) and 3) A23187-induced relaxations may reflect mediator relase from mast cells (Foreman, 1981).

Since compound 48/80, a mast cell releasing agent,

did not abolish FSR_2, it may be surmised that these cells belong to the class of atypical mast cells i.e. mucosal mast cells (Pearce, 1986; Jarret and Haig, 1984). The presence of projections from some of these cells raised the possibility that these could be paraneurones (Fujita and Kobayshi, 1979). However, several lines of evidence suggest that mast cells may not be the source of the putative mediator of FSR_2: 1) 5HT seems unlikely to be the mediator of FSR_2 since contractions were induced by 5HT in the distal segment after cold storage and under such conditions FSR_2 could still be observed and 2) A23187 may not relax TMM by releasing 5HT since the contractile responses to the ionophore after cold storage, unlike those to 5HT itself, were not abolished by ketanserin.

The effects of cold storage on 5HT-induced responses were remarkable in that $5HT_2$ receptors were unmasked only in the distal segment. Selective effects of cold storage on receptors have been previously reported in the rabbit jejunum (Lum, Kermani and Heilman, 1966) and guinea pig taenia caeci (Honda, Katsuki, Miyahara and Shibata, 1977). The process(es) by which excitatory effects to 5HT appear in the cold stored TMM is not known and was not pursued. However, the possibility exists that changes in second messenger systems reflecting a change in coupling mechanism may occur. Honda et al. (1977) demonstrated a decrease in intracellular cAMP during cold storage of the guinea pig taenia caeci. Alternatively, inhibitory receptors may be internalised

during cold storage thus allowing for excitatory $5HT_2$ receptors to be expressed. Nevertheless, the persistence of FSR_2 in the distal segment argues against the possibility of 5HT as the candidate of this relaxation. However, it should be emphasised that there may be differences between exogenous application of the drug and endogenous release during field stimulation.

The relaxant effects of the ionophore A23187 resembled those of 5HT in that they showed a regional gradient with the proximal segment being more reponsive. It is unlikely, for reasons already noted, that A23187 induces relaxation by releasing 5HT from mast cells. It is noteworthy that A23187-induced relaxation, unlike that induced by 5HT was abolished in high K^+. The possible basis for this difference is discussed in a later section.

It may thus be concluded that mast cells are unlikely to be the source of a putative mediator of FSR_2 and that 5HT is unlikely to be this mediator. The question therefore presents itself as to the presence of other non-neuronal sources. Interstitial cells of Cajal could be a likely alternative since they have been hypothesised to mediate TTX-insensitive relaxations of the lower oesophageal smooth muscle of the opossum (Daniel, Crankshaw and Sarna, 1983). Daniel and Posey-Daniel (1985) also demonstrated close apposition of these cells to nerves and to smooth muscle indicating that

these may be involved in transmission from nerve to muscle. The interstitial cells were not identified in this study but if they were to function in the rat TMM as in the opossum oesophagus, species and tissue differences aside, they are unlikely to be the sources of the putative mediator of FSR_2 for two reasons: 1) the electrical inexcitability of the TMM is incompatible with the postulated pacemaker role of the cells of Cajal (Thunenberg, 1982) and 2) unlike the smooth muscle of the opossum (Daniel et al. 1983), prostaglandin involvement in the generation of FSR_2 is not indicated due to the lack of effect of indomethacin. The epithelium may also be considered a potential source for a relaxing factor since there was a decreased FSR_2 response in epithelium-denuded preparations. However, the accompanying decrease of muscarinic-induced response may account for a loss in the amplitude of FSR_2. Consideration to the role of free-radical formation in generating FSR_2 must also be given since the enzymes used to intervene with the free-radical system may not penetrate sufficiently to achieve an effect in the TMM. However, a role for free-radicals in FSR_2 is unlikely since free radical-induced relaxations have a slow onset of action, at least in vascular smooth muscle (Lamb and Clinton Webb, 1984), compared to the fast on-off response observed for FSR_2. Barring a non-free radical epithelial derived factor being the mediator of FSR_2, the most likely basis of FSR_2 is therefore, the direct activation of the smooth muscle itself.

4.4.2 Relationship of field-stimulated and drug-induced relaxations.

A comparison of TTX-insensitive relaxation to field-stimulation and 5HT revealed differences in the sensitivity to cold storage, cooling and high K^+. In high K^+ and on cooling, FSR_2 was abolished whereas 5HT mediated relaxations were not affected. Similarly, one could also discount 5HT as the mediator of FSR_1 since under high K^+ the relaxation to field-stimulation was abolished. Nevertheless, it is difficult to entirely rule out 5HT as the mediator due to the possibility that field-stimulation may release small amounts of the mediator whose actions may be antagonised by the above interventions. Moreover, high K^+ may block release of 5HT. One possible approach to clearly rule out 5HT as the mediator would be to antagonise 5HT-induced relaxations and cross-examine FSR. However, the inability of known 5HT antagonists to abolish either forms of relaxations leaves open the possibility of a 5HT-mediated field-stimulated relaxation. Since relaxations were observed in the presence of tone due to muscarinic agonists, the question of an anti-muscarinic action needs to be ruled out. Anti-muscarinics usually have a slower onset of action whereas the effects of 5HT were immediate and therefore suggests a physiological antagonism. Moreover, as the IC_{50} values for 5HT-mediated inhibition were significantly lower at half-maximal than at maximal tonus, the observed interaction with muscarinic receptor

agonists satisfies a criterion for functional (physiological) antagonism (van den Brink, 1973). The regionally selective effects of 5HT were not antagonised by antagonists of $5HT_1$ or $5HT_2$ types. For the former, both trazodone (Kendall, Taylor and Enna 1983) and methysergide were ineffective thus ruling out the $5HT_1$ response. Moreover, the low potency of 8-OH DPAT indicates that $5HT_{1a}$ subtype are unlikely to mediate 5HT-induced relaxation. $5HT_2$ recptors except for the cold-stored distal segment, do not exert a profound influence as indicated by the lack of effect of ketanserin. The present data do not allow for a specific receptor subtype to be implicated for the 5HT response. Of the three types of receptors currently postulated for 5HT, it remains to be determined whether those of the $5HT_3$ type are involved in FSR. This latter receptor type has been suggested to be insensitive to ketanserin and methysergide but antagonised by such agents as MDL 72222 and ICS 205 930 (Richardson and Engel, 1986).

VIP would also seem to be an unlikely candidate as the mediator of FSR_2 since -chymotrypsin did not affect FSR_2 although it abolished VIP-induced relaxation. In the canine colonic muscularis mucosae, Angel et al. (1984) demonstrated that field-stimulation evoked relaxation were blocked reversibly by chymotrypsin and trypsin. Hills, Meldrum, Klarskov and Burnstock (1984) have also reported inhibition

of field stimulated relaxations by -chymotrypsin in the pig bladder. The lack of effect of -chymotrypsin in the rat TMM is thus unlikely to be due to its inability to reach synaptic sites. Moreover, VIP-induced relaxations were not attenuated by cooling or calcium antagonists. In as far as the relationship of VIP-induced relaxation and FSR_1 is concerned, the inability of -chymotrypsin to abolish the relaxation suggests that VIP may not be the mediator. However, a more vigorous examination may be required to completely rule out this possibility especially since both the presence and the effects of VIP would suggest that it may be an inhibitory neurotransmitter in the TMM.

As regards K^+-induced relaxations, these could be due to release of an inhibitory transmitter (Gibson and James, 1977) or stimulation of Na^+/K^+ ATPase (Shibata, Fukuda and Kurahashi 1973). However, 1×10^{-5} M ouabain failed to block both K^+-induced relaxation and FSR_2 negating the involvement of the ATPase. Although, the rat Na^+ pump is relatively insensitive to the cardiac glycoside (Allen and Schwartz, 1969; Toda, 1974), 10^{-5} M was sufficient to block K^+-induced relaxations in the rat tail artery (Webb and Bohr, 1974). In the TMM, K^+-induced relaxations resembled FSR_2 in that they were abolished by cooling and Ca^{2+} channel antagonists. It would thus seem that K^+-induced relaxations may involve Ca^{2+} entry and represent the same mechanism as

that responsible for FSR_2. Relaxations to K^+ have been observed in the rabbit coronary artery which were dependent on the concentration gradient for K^+ and in this study (Norton and Detar, 1972) exposure to low K^+ (1.8-5.88 mM) produced relaxations. It was thus suggested that alterations in the transmembrane K^+ gradients, within a certain range, can lead to muscle relaxation. Other possibilities that were not examined by Norton and Detar (1972) include the effects of increased K^+ on the stimulation of the Na^+/K^+ ATPase and alternatively, the release of an endothelium-derived relaxing factor.

In the rat TMM, an analogous epithelium dependent relaxing factor is not indicated at least with respect to A23187-induced relaxation and FSR_2.

The calcium ionophore, A23187, may produce relaxations of the TMM by either releasing an unknown mediator or by transducing Ca^{2+} in the smooth muscle which may trigger relaxation. The former effect is unlikely at least in as far as release from mast cell is concerned. Moreover, a release from nerve terminal is also not indicated since the calcium ionophore did not produce any contractions that could be attributed to the release of acetylcholine. Thus, it may be assumed that Ca^{2+} entry leads to a relaxation of the smooth muscle. This seemingly paradoxical effect has also been demonstrated by Morgan and Morgan (1984) who showed that in the ferret vascular smooth muscle, relaxations due to vasodilators

could still occur without concommitant decreases in Ca^{2+} levels. It is noteworthy that A23187-induced relaxations were abolished in high K^+. If it is assumed that free intracellular Ca^{2+} concentrations remain sufficiently high during K^+-depolarisation (Morgan and Morgan, 1984), then a further entry of Ca^{2+} by A23187 may be restricted. Further support that relaxations may be initiated by Ca^{2+} entry is indicated by the high sensitivity of FSR_2 to the inhibitory effects of the organic Ca^{2+} channel antagonists.

4.4.3 Calcium and FSR_2

Since calcium antagonists exhibited equal potency in inhibiting FSR_2 and K^+-induced contractions, it may be postulated that Ca^{2+} entry via potential operated channels mediates FSR_2. Furthermore, the Ca^{2+} channel antagonist PN-200-110 was stereoselective, with the (+) isomer being more potent than its optical antipode. A similar stereoselectivity for PN-200-110 in inhibiting vascular smooth muscle contraction has been reported by Hof et al. (1984). Cholinoceptor-induced contractions, on the other hand, were less sensitive to calcium antagonists. This difference permitted the selective inhibitory effect of these agents on FSR_2 to be clearly demonstrated. In keeping with findings reported by other workers (Cauvin et al., 1983) contractile events mediated by activation of potential-operated channels, such as resulting from high K^+ contractions were observed to be considerably more sensitive

to inhibition than receptor mediated contractions.

Four possible explanations may be considered to account for the paradoxical effects of the Ca^{2+} channel antagonists in inhibiting relaxations. First, Ca^{2+} channel antagonists may act to inhibit release of a mediator from nerve endings. Second, they may act on an unidentified interstitial cell type, or, third directly on the smooth muscle itself. Fourth, Ca^{2+} channel antagonists may have effects unrelated to their actions as Ca^{2+} entry blockers. As regards the first explanation, the inability of the Ca^{2+} channel antagonists to inhibit FSR_1 or FSC would rule out the effects on nerve endings. Besides, Ca^{2+} channel antagonists may be ineffective at the nerve terminal (Miller and Freedman, 1984). The possibility of unrelated effects can be discounted in view of the low concentration required for inhibition. The order of potency for the Ca^{2+} channel antagonists in inhibiting FSR_2 and K^+ correlate well with those reported in the literature (Hof et al., 1984). The most attractive hypothesis therefore remains an effect on smooth muscle or some other cell type. As described earlier, mast cells and interstitial cells of Cajal are unlikely sources, at least in the rat TMM, for a possible mediator.

That FSR_2 is critically dependent on Ca^{2+} is also indicated by the inhibitory action of Mg^{2+}. Although Mg^{2+} abolishes synaptic transmission (Mordes and Wacker, 1978), its effects as a calcium antagonist have also been described.

To summarise, the two types of relaxations evoked by field-stimulation, FSR_1 and FSR_2, represent distinct events. The neurally-mediated FSR_1 is unlikely to be due to VIP or 5HT, although further investigations are required. FSR_2 would seem to have a purely myogenic basis and clearly depends on a Ca^{2+} entry process. The paradox, however, that presents itself concerns how Ca^{2+} entry into smooth muscle can lead to relaxation. Conceivably, Ca^{2+} entry may lead to the activation of K^+ channel (see Introduction).

4.4.4 Is FSR_2 due to activation of Ca^{2+}-dependent K^+ channel?

The inability of TEA, 4-aminopyridine and apamin to abolish FSR_2 would seem to negate the involvement of K^+ channels. However, K^+ channels mediating inhibitory junction potentials in opossum oesophageal smooth muscle were not blocked by TEA, 4-aminopyridine or apamin (Jury et al. 1985). Similar channels may thus be postulated to exist in the rat TMM. Apamin-insensitive Ca^{2+} activated K^+ channels have been shown to occur in red blood cells (Burgess, Claret and Jenkinson, 1981) and in the fundus circular muscle, proximal colon longitudinal muscle and distal colon of the guinea pig (Costa, Furness and Humphreys, 1986).

In order to further investigate the role of K^+ channels in FSR_2, the effects of BRL 34915, a putative K^+ channel activator in a number of smooth muscle preparations

including guinea-pig taenia caeci (Weir and Weston, 1986a), rabbit aorta (Cook et al. 1987), rat uterus (Edwards et al. 1987) and rat portal vein (Weir and Weston, 1986b), were studied in the rat TMM. BRL 34915 shifted the lower end of the K^+ concentration response curve to the right and antagonised TEA-inducued contractions in the rat TMM. Such actions are consistent with the putative action of this agent as a K^+ channel activator. The depression by BRL 34915 of the base of K^+ concentration-response curve has been demonstrated in the rat portal vein, rat uterus, guinea pig taenia caeci and trachealis. This effect of BRL 34915 has been attributed to the opening of K^+ channels at equilibrium potentials more negative than the transmembrane potential. A similar effect of BRL 34915 is thus indicated in the rat oesophageal smooth muscle. The increase in smooth muscle excitability by TEA and the block by BRL 34915 suggests the involvement of K^+ channels. Relaxations produced by BRL 34915 resembled FSR_2 in that both were blocked in high K^+ solutions, by cooling and by nifedipine. Furthermore, like FSR_2 BRL-induced relaxations were TTX-insensitive. These results therefore support the hypotheses that 1) FSR_2 may be a consequence of K^+ channel activation and 2) both forms of relaxations are dependent on Ca^{2+} influx through potential-operated channels.

The inability to demonstrate FSR_2 in the presence of high concentrations of BRL would appear to be inconsistent with the hypothesis outlined above. However, maximal

activation of K^+ channels by BRL may preclude further relaxations by field-stimulation. In any case, an interaction between BRL 34915 and FSR_2 is indicated.

The effects of BRL 34915 on the responses by Ca^{2+} channel agonists merits seperate comment. Both BayK 8644 and CGP 28,392 unmasked TTX-insensitive contractions similar to the effects of TEA. This effect was stereoselective with the (-)-isomer of BayK 8644 inducing contractions while the (+) isomer blocked FSR_2. The opposite effects of the isomers on Ca^{2+} channel functions have been previously described (Franckowiak, Bechem, Schramm and Thomas, 1985) The contractile responses in the presence of the Ca^{2+} channel agonists were blocked by BRL 34915 indicating the involvement of K^+ channels in the resposes of the putative Ca^{2+} channel agonists. Since BRL 34915 did not alter the responses to high K^+ an effect on potential-operated Ca^{2+} channels may be disregarded. The results therefore suggest that Bay K 8644 may induce a depolarisation of smooth muscle in part by a process that involves blockade of outward rectifying K^+ channels. Supportive of such an action is the fact that Ca^{2+} channel antagonists were less effective on the contractile responses due to Bay K 8644 than on FSR_2.

4.4.5 Proposed mechanism for FSR_2.

The results presented in this thesis indicate that FSR_2

1) may have a purely myogenic basis, and

2) could be mediated by Ca^{2+}-activated K^+ channels.

The myogenic basis for FSR_2 is suggested by cold storage experiments and the inability of releasing agents to alter FSR_2. As well this concept is reinforced by the high sensitivity to Ca^{2+} channel antagonists.

The question presents itself as to the Ca^{2+} dependent mechanism of relaxation produced directly by stimulation of smooth muscle. Since the muscle is generally quiescent and does not generate contractions at pulse widths of 2 ms in the presence of TTX, it has to be assumed that excitability is induced in the presence of cholinoceptor activation, which allows direct stimulation of the muscle to elicit an inhibitory response. An increase in muscle excitability could result if muscarinic activation leads to a block of outward rectifying currents. An indication that this may arise is provided by data, obtained by Dr Ohia in this laboratory and presented at the I.U.P.H.A.R. meeting in Sydney, Australia (1987) that CD does not affect ^{86}Rb efflux. Moreover, other studies have reported muscarinic cholinoceptor-induced block of K^+ conductance (North, 1986; Cassel and McLachlan, 1987). Clearly the depolarisation that may accompany this effect of muscarine is not sufficient to block FSR_2 or to activate potential-operated channels. It is noteworthy, that TEA alone does not produce contractions, which may suggest that blocking of resting K^+ channels does

not necessarily induce sufficient depolarisation for contraction. It could also mean that there are many different types of K^+ conductances which offset TEA-induced depolarisation. Nevertheless, maximal activation of muscarinic receptors may induce a depolarisation sufficent to inhibit FSR_2. In a similar manner high K^+ depolarisation also inhibits FSR_2. Field-stimulation in the presence of increased excitability of the muscle resulting from muscarinic receptor activation would depolarise the membrane sufficiently to open potential-operated Ca^{2+} channels. The transient increase in Ca^{2+} would then lead to K^+ channel activation and an efflux of K^+. The mechanism whereby K^+ efflux produces relaxation is not clearly understood but may either involve hyperpolarisation and concommitant changes or other as yet undefined mechanisms. Voltage-independent relaxations, however, have been described in other smooth muscles (reviewed by Bolton and Large, 1986).

4.4.6 Vagally-evoked relaxations

The failure of hexamethonium to inhibit vagally evoked relaxations would seem to preclude involvement of a classical ganglionic pathway. Thus relaxations could be mediated via antidromic stimulation of afferent fibres or via non-nicotinic ganglionic synapse. Vagally stimulated relaxations resembled FSR_1 in that both were TTX-sensitive. On the other hand, vagally evoked relaxations were also

sensitive to cooling and resembled FSR_2 in that respect. It could be argued that TTX may block nerve conduction along the exposed vagus and therefore bear no relationship to FSR_1. On the other hand, cooling can affect transmission along the nerve.

Regarding sensory fibres, calcitonin-gene related peptide seems to qualify as a potential candidate in view of its presence in the rat TMM and its demonstrated efficacy as a smooth muscle relaxant (Brain, Williams, Tippins, Morris and MacIntyre, 1985). Again, further studies are required in order to elucidate the processes that contribute to the relaxations induced by vagal stimulation. In any case the demonstration of a vagally-evoked relaxation of the TMM indicates the functional integrity of this preparation with regard to both contractile and relaxant effects.

4.5 TTX-insensitive relaxations in other smooth muscle.

TTX-insensitive relaxations have been attributed to histamine release from mast cells in the rat tail artery (Ebeigbe, Gantzos and Webb, 1983) and canine coronary artery (Rooke, Cohen, Verbeuren and Vanhoutte, 1982), to stimulation of interstitial cells of Cajal in the opossum oesophageal sphincter (Daniel et al. 1979), or stimulation of intramural nerves not utilising fast Na^+ channels in the canine saphenous vein (Senaratne and Kappagoda, 1984). It is noteworthy that relaxations in the canine saphenous vein but not in the coronary artery were abolished by cold storage.

Cole and Marquis (1985) also reported TTX-insensitive relaxations in the bat ileum.

The inability of cimetidine to block FSR_2 would negate the role of histamine acting on H_2 receptors and since indomethacin did not block FSR_2, prostaglandin involvement, unlike in the opossum oesophageal sphincter, is not indicated. Although Senaratne and Kappagoda (1986) reported a block of TTX-insensitive relaxations by lignocaine in the canine saphenous vein , the post synaptic effects of the anaesthetic cannot be ruled out.

This thesis also describes TTX-insensitive relaxations that were observed in canine TMM. These relaxations were not expressed in every tissue and could therefore be attributed to loss of structures during strip preparations. In contrast strip preparations of the rat TMM continued to demonstrate FSR_2. It is possible that structurual differences between the rat and canine TMM may be the basis for the lack of reproducibility in the appearance of FSR_2. Alternatively, close coupling in the rat allows one to observe FSR_2 in strips but not always so in the dog. Although the relaxations in the canine and the rat TMM were similar in their sensitivity to cooling, relaxations in the canine TMM were not blocked by Ca^{2+} channel antagonists suggesting a difference in the Ca^{2+} dependency of relaxations from canine tissues.

4.6 Clinical considerations.

Some aspects of the present study correlate with clinical findings. For instance, ingestion of cold beverages can cause decrease in swallow-induced peristaltic amplitude in normal volunteers (Meyer and Castell, 1981) and precipitate or worsen dysphagia in patients suffering from diffuse oesophageal spasm (Winship, Viegas de Andrade and Zboralske, 1970). Such a condition could arise if relaxation mechanisms were affected. It should also be noted that although clinical data suggest Ca^{2+} channel antagonists to be pure oesophageal spasmolytics (Bortolotti and Labo, 1981; Richter, Sinar, Cordova and Castell, 1982; Morales-Olivas, Cortijo, Esplugues, Rubio and Esplugues, 1985), the decreased potency of these agents towards non-vascular smooth muscle contractility (Sorkin, Clissold and Brogden, 1985) may in part reflect inhibition of relaxations in these tissues.

In conclusion, this thesis demonstrates that the rat TMM is capable of relaxations to both field-stimulation and pharmacological agents. While this property alone may not be compelling evidence to indicate that the TMM assists in peristalsis, regional differences in both contractile and relaxation processes however, does indicate that the TMM resembles the tunica propria smooth muscle of other species. This study also demonstrates that relaxations which have been denied by others are present in the rat and leads to the

suggestion that the tunica muscularis mucosae may be a misnomer in as far as its functions are not restricted to localised movements of the mucosae.

4.7 Future experiments.

The results and the hypotheses presented in this thesis raise several issues for future consideration.

1) In view of the postulated mechanism for FSR_2, the TMM clearly lends itself to electrophysiological analysis. In particular the relationship between the various forms of relaxations and the electrical response of the tissue needs to be correlated.

2) Whether vagally-evoked relaxations result from antidromic stimulation of sensory fibres or intramural ganglia, or from the orthodromic stimulation of vagal noncholinergic efferents requires further investigation.

3) Although this study provides pharmacological evidence for the presence of both contractions and relaxations in the TMM, the role of this muscle in oesophageal peristalsis still needs to be further explored.

4) An important consideration that has not been fully resolved in this thesis is the nature of the NANC inhibitory transmitter mediating FSR_1. Clearly, pharmacological, histochemical and electrophysiological techniques should help elucidate the nature of the putative inhibitory transmitter(s).

5) The present study has been limited to the oesophageal body. Comparative data from TMM of extra-oesophageal origins may provide further understanding of the functions of the TMM.
6) With regard to regional differences in the rat TMM to both spasmogenic and spasmolytic agents, diversity in receptor types and intracellular messenger systems within the same organ provides an opportunity to elucidate mechanisms governing excitation-contraction coupling and uncoupling processes.

4.8 Summary

From the data obtained in the present study, several points should be emphasised.

1) The isolated TMM contains a functional neuronal plexus which can be excited by field-stimulation thus leading to contractions. These contractions can be attributed to the stimulation of intrinsic cholinergic nerves.
2) Pharmacological studies of the rat TMM indicate the existence of at least three forms of relaxations; a neurally-mediated, drug-induced and a field-stimulated direct activation of the muscle.
3) The neurally mediated relaxation would seem to arise from the submucous plexus which was shown to contain VIP-like, CGRP-like immunoreactivity as well as a sparse adrenergic innervation.
4) The potency of 5HT in initiating relaxations and its presence in mast cells suggests a possible role for this

indoleamine in regulation of TMM motility. The receptor that mediates the 5HT-induced relaxation remains to be identified.

5) From analysis of the inhibitory potency of Ca^{2+} channel antagonists towards non-neuronal relaxation (FSR_2), it is postulated that Ca^{2+} entry coupled to K^+ efflux leads to a relaxation.

6) An interesting property of the relaxations mediated by these atypical Ca^{2+}-activated K^+ channels is their high sensitivity to temperature.

5) The effects of pharmacological agents on the rat TMM indicate that this muscle is capable of assisting in oesophageal motility and further investigations are required to assess its role in primary oesophageal peristalsis.

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