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A STUDY OF THE ELECTRICAL BASIS FOR THE INHIBITION AND EXCITATION IN AUTONOMICALLY INNERVATED SMOOTH MUSCLE

A thesis presented for the degree of
Doctor of Philosophy
in the University of Glasgow

by

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SUMMARY

The aim of this thesis was to investigate the electrical and mechanical responses to inhibitory non-adrenergic non-cholinergic (NANC) nerve stimulation in the bovine retractor penis muscle (BRP) and compare them with those to an inhibitory extract made from this muscle. The extract may contain the NANC inhibitory transmitter of the BRP and possibly of other smooth muscles. Because of species differences in the electrical response to NANC nerves in the rat and rabbit anococcygeus muscles, the effects of the extract on these tissues was also investigated. Prior to the investigation of the extract, both the excitatory and inhibitory responses to field stimulation in the BRP, and the effects of passive membrane potential displacement were studied using conventional intra- or extracellular (sucrose gap) recording techniques.

The majority of cells in the BRP were electrically quiescent independent of the resting tone. The most frequent (in approximately 25% of preparations) form of spontaneous activity, oscillations in membrane potential and tone, may represent a pacemaker activity. The BRP had cable properties; the time constant and space constant indicated a high membrane resistance.

In the absence of tone, field stimulation of the BRP evoked excitatory junction potentials (ejps) in every cell impaled

and contractions, graded with the strength, frequency and number of pulses; spikes were not observed. Guanethidine (1-3 x 10^{-5}M) abolished the ejps and contractions, confirming their adrenergic origin. Noradrenaline added exogenously depolarised and contracted the muscle. These effects were blocked by the α-adrenoceptor antagonists, phentolamine and prazosin. However, phentolamine (2.5 x 10^{-6}M) inhibited the contraction without reducing the ejp significantly. These effects may be independent of adrenoceptor blockade or the ejp may be mediated by a substance other than noradrenaline (e.g. ATP) released from adrenergic nerves. Prazosin (1.4 x 10^{-6}M) failed to block either the ejp or contraction, indicating the possible existence of two types of adrenoceptor in the BRP; one activated by neuronally-released and the other by exogenously-added noradrenaline. ATP, a contaminant in the extract, also depolarised and contracted the BRP.

Physostigmine reduced whilst atropine enhanced the ejps and contractions without similarly affecting the response to exogenous noradrenaline. This confirmed the presence of a cholinergic inhibitory innervation acting on the excitatory adrenergic fibres (Klinge and Sjöstrand, 1977).

TEA (1 x 10^{-4}M) enhanced the ejp and contraction. Higher concentrations (0.5 to 10 x 10^{-3}M) depolarised, increased the tone and evoked electrical and mechanical

oscillations but no spikes. The depolarisation and contraction to exogenous noradrenaline were not enhanced, indicating that TEA acts on the adrenergic nerves. Some post-synaptic effect to block K^+ channels also seems likely.

The relationship between ejp amplitude and membrane potential in the double sucrose gap was linear and indicated a reversal potential more positive than -30mV. Electrotonic pulse amplitude decreased during the ejp, indicating an increased membrane conductance. Ejps and contractions were reduced following the replacement of the NaCl of the Krebs solution with sodium glutamate. This may be due to the effects of glutamate itself (e.g. Ca^{2+} chelation) rather than reduction in the membrane Cl^- gradient.

Tone usually developed spontaneously and was accompanied by membrane depolarisation (from -53 to -45mV) which may open voltage-dependent channels, causing Ca^{2+} entry and/or its release from intracellular binding sites. Field stimulation produced inhibitory potentials (ijps) and relaxations graded with the strength and number of pulses but showing little frequency dependence. Rebound depolarisation and contraction often followed the ijp and relaxation. Tetrodotoxin (3×10^{-6}M), but not adrenergic or cholinergic antagonists, abolished the ijp and relaxation, confirming their non-adrenergic non-cholinergic neurogenic nature.

The extract, prepared and acid-activated as described by Gillespie, Hunter and Martin (1981), hyperpolarised and relaxed the BRP, as did sodium nitroprusside and adenosine triphosphate (ATP). Unlike the activated extract or sodium nitroprusside, desensitisation to ATP occurred rapidly and without any change in the inhibitory electrical or mechanical responses to field stimulation.

The ijp and relaxation in the BRP were insensitive to apamin but abolished by oxyhaemoglobin ($4\text{-}8 \times 10^{-6}$M), as were the responses to extract and sodium nitroprusside. In TEA (10^{-2}M), field stimulation evoked relaxations with no accompanying electrical change. The ijp may be unconnected with or additional to another mechanism producing relaxation.

The relationship between membrane potential and ijp in the BRP was non-linear. Ijp amplitude was initially increased during membrane potential displacement from -45mV to approximately -60mV. Thereafter (-60 to -103mV) the ijp was reduced. Ijps were abolished at -27 and -103mV; reversal was not observed. The hyperpolarisation to extract was also enhanced during passive displacement of the membrane potential to more negative values (-57mV).

Membrane resistance increased during the ijp. The extract produced inconsistent changes in membrane resistance, possibly because of the presence of more than one

active component. K^+ withdrawal failed to enhance the ijp or hyperpolarisation to extract and 20mM K^+ did not abolish the the ijp at membrane potentials exceeding E_K (-49mV). Thus, the ijp or hyperpolarisation to extract are unlikely to be mediated by an increased K^+ conductance.

Reducing the Cl^- abolished the hyperpolarisation to field stimulation and extract. This occurred more quickly than the anticipated reduction in the Cl^- gradient and may be due to Ca^{2+} chelation by the anion substitute (glutamate or benzenesulphonate) or blockade of the resting conductance which is normally inactivated by the transmitter. Ouabain ($1-5 \times 10^{-5}$M), which reduces both the Na^+ and Cl^- gradients, abolished the ijp, implicating either of these ions as the ionic species involved.

In the rat and rabbit anococcygeus, field stimulation and extract each reduced guanethidine-induced tone. This was unaccompanied in the majority of cells in the rat by any significant electrical response. In the remaining cells, inhibition of the membrane potential oscillations occurred. The rabbit anococcygeus differed in that inhibition of the electrical oscillations was observed in every cell exhibiting this behaviour. However, the majority of cells in the rabbit were electrically quiescent and showed only small hyperpolarisations to field stimulation and no electrical response to extract. Apamin (1×10^{-7}M) failed to block the electrical and mechanical response to field stimulation in the rabbit but did inhibit

transiently that to extract. The latter effect may be due to the initial excitatory effects of apamin.

The similarities between the electrical effects of the extract and those of inhibitory nerve stimulation in the BRP, rat and rabbit anococcygeus muscles are generally consistent with their being mediated by the same active component. Moreover, the ijp in the BRP shows properties which have not been reported in other non-adrenergic non-cholinergically innervated smooth muscles.

CHAPTER 1

INTRODUCTION

FOREWORD

The autonomic nervous system comprises, for the purpose of this thesis, a system of nervous pathways with a ganglionic synapse between the effector organ and the central nervous system (Campbell, 1970). Autonomic nerves innervate all smooth muscle, cardiac muscle and secretory cells. Gaskell (1866) first traced the autonomic pathways from the central nervous system, describing the cranial, thoraco-lumbar and sacral outflows. (see Gaskell, 1916) These outflows were grouped together by Langley (1898) under the term autonomic nervous system.

Within the autonomic nervous system, two systems were distinguished: sympathetic (thoraco-lumbar), and parasympathetic (cranial, sacral). Sympathetic fibres, unlike parasympathetic fibres, synapse with ganglia in the paravertebral chains and generally have short pre-ganglionic and long post-ganglionic fibres. The parasympathetic system generally has long pre-ganglionic and short post-ganglionic fibres with ganglia situated in or near the innervated organs. In organs innervated by both the sympathetic and parasympathetic systems, their effects are usually mutually antagonistic. The two systems were thought to differ pharmacologically in that the parasympathetic system was antagonised by atropine, whereas the sympathetic system was not (see Mitchell, 1953: Campbell, 1970).

It was within the framework of the two systems, sympathetic and parasympathetic, that the concept of the chemical transmission of nerve impulses in the autonomic nervous system

was introduced. Similarities between the effects of sympathetic nerve stimulation and those of adrenal extracts, first illustrated by Langley (1901), were confirmed for pure adrenaline (Elliot, 1905). Parasympathetic nerves were mimicked by drugs such as muscarine (Dixon and Brodie, 1903). These observations, together with those of Loewi and Navratil, established that autonomic nerves released specific neurotransmitters which acted on the effector organ (see Gershon, 1970). Accordingly, nerves were classified on the basis of the neurotransmitter released. Dale (1933) used the term "adrenergic" for those nerve fibres which released adrenaline (later found to be noradrenaline; Euler, 1946), and "cholinergic" for those which released acetylcholine. All preganglionic and most post-ganglionic parasympathetic fibres were cholinergic. Sympathetic post-ganglionic fibres were mostly adrenergic. Classically, then, the autonomic nervous system consisted of two systems integrated within the central nervous system.

Deviations from the classical view have often been encountered. That some sympathetic nerve fibres released acetylcholine was demonstrated by Rogwicz (1885) (see Burn, 1971), who found that following degeneration of the facial nerve in the dog, the muscles moving the lips contracted in response to cervical sympathetic nerve stimulation (see Burn , 1971). Euler and Gaddum (1931) concluded that there were nerve fibres releasing acetylcholine in the cervical sympathetic nerve. Other sympathetic fibres also released acetylcholine, e.g.

those innervating the sweat glands and spleen. Burn and Rand (1965) proposed that all adrenergic nerves released acetylcholine which fed back onto the nerve ending to initiate the release of noradrenaline. Thus, sympathetically-mediated cholinergic effects could be due to the overflow of acetylcholine from the nerve endings. A more likely explanation was that some autonomic fibres, although anatomically sympathetic, were functionally cholinergic (Campbell, 1970).

Stimulation of autonomic nerves can produce responses mediated neither by cholinergic nor by adrenergic nerves. Such responses were first recognised in the gastrointestinal tract. Classically, parasympathetic nerve stimulation contracted the gut while sympathetic stimulation produced relaxation (Langley, 1921). However, vagal (parasympathetic) stimulation could produce, in certain circumstances, relaxation of the stomach (Langley, 1898) and small intestine (Bayliss and Starling, 1899). The inhibitory effect of the vagus was facilitated by atropine and therefore was not attributable to cholinergic post-ganglionic fibres (Langley, 1898). The possibility that vagal inhibition was mediated by adrenergic fibres was not fully explored until the advent of the adrenergic neurone blocking drugs. These were ineffective at antagonising the inhibitory responses to vagal or transmural stimulation while blocking those to perivascular sympathetic nerves (see Gillespie, 1982). Electrical recordings demonstrated the presence of inhibitory nerves in the

gut wall which, in response to field stimulation, produced large hyperpolarisations in smooth muscle cells. In contrast, sympathetic nerve stimulation produced little or no electrical change (Bennett, Burnstock and Holman, 1966a,b). The combination of the pharmacological and electrical evidence with that obtained from histochemical and structural studies pointed to the existence of a non-adrenergic, non-cholinergic inhibitory nerve supply to the gut and related smooth muscles (see Burnstock, 1972; Gillespie, 1982).

As implied by the term non-adrenergic non-cholinergic, the transmitter released by these nerves is unidentified. Likely candidates are a purine (purinergic hypothesis), e.g. adenosine triphosphate (ATP), or a peptide (peptidergic nerves), e.g. vasoactive intestinal polypeptide (VIP). The analysis of these two hypotheses has been greatly impeded by the lack of specific antagonists. Neither hypothesis holds for all sites innervated by non-adrenergic non-cholinergic nerves.

Investigations to determine the identity of the non-adrenergic non-cholinergic transmitter have made use of the techniques employed to isolate noradrenaline and acetylcholine. These include attempts to collect the transmitter released by nerve stimulation (Burnstock, Campbell, Satchell and Smythe, 1970), or by extraction from tissues known to possess a non-adrenergic non-cholinergic innervation (Ambache, Killick and Zar, 1975). Thus, an inhibitory extract from the bovine retractor penis muscle, prepared originally by

Ambache, Killick and Zar (1975) and further purified by Gillespie, Hunter and Martin (1981), is being investigated. This extract may contain the inhibitory transmitter of the bovine retractor penis and possibly of other smooth muscles. The isolation and identification of the non-adrenergic non-cholinergic transmitter would considerably aid elucidation of its mechanism of action. Such investigations are presently hampered by the necessity to stimulate nerve fibres in order to release the transmitter. Thus, adrenergic and cholinergic antagonists are required, and changes made to elucidate the mechanism of action of the transmitter, e.g. to the ionic environment, may affect transmitter release. These problems, combined with the possible clinical implications of identifying the transmitter, provide the mandate for continued research in the field.

EVIDENCE FOR THE EXISTENCE OF NON-ADRENERGIC NON-CHOLINERGIC AUTONOMIC NERVES

The first evidence for a third system of autonomic nerves came from studies on the control of gastrointestinal motility. Here, vagal (parasympathetic) stimulation produced two effects: excitation blocked by atropine, and atropine-resistant inhibition (Langley, 1898; Bayliss and Starling, 1899; May, 1904). Thus it was suggested that the response (excitation or inhibition) might be determined by intrinsic neural reflexes in the gut (Carlsson, Boyd and Pearcy, 1922) or the level of resting tone (McSwiney and Wadge, 1928),

neither of which explained why the inhibitory response was atropine resistant. Atropine resistance was attributed to the presence of sympathetic fibres in the vagus (Harrison and McSwiney , 1936), but differences between the inhibitory response to vagal and direct sympathetic (splanchnic) stimulation made this unlikely. The response of the small intestine to sympathetic nerve stimulation was reduced be repetitive stimulation while that to the vagus persisted (Bayliss and Starling, 1899).

Other characteristics of vagal inhibition, notably its shorter latency (Harrison and McSwiney, 1936) and following "rebound" contraction (Brown and Garry, 1932), also distinguished it from those of sympathetic nerve stimulation. Stimulation of the cervical sympathetic trunk produced no change in stomach motility, whereas direct stimulation of the brain evoked relaxations which were abolished by vagotomy (Eliasson, 1952; Semba, Fujii and Kimura, 1964). Inhibitory fibres in the vagus were therefore carried in the cranial outflow and were anatomically parasympathetic. Vagal inhibition was mimicked by nicotine (Ambache, 1951) and reduced or abolished by hexamethonium (Semba et al, 1964; Campbell, 1966), suggesting that it was mediated by post-ganglionic adrenergic fibres with a pre-ganglionic vagal input. The adrenergic antagonists ephedrine (Ambache, 1951), phentolamine (Paton and Vane, 1963) and bretylium (Greeff, Kasperat and Osswald, 1962) were initially shown to reduce or abolish vagal inhibition. However, at the high concentrations employed,

antagonism by these drugs could not be attributed merely to blockade of adrenergic transmission. Phentolamine blocked both the excitatory and inhibitory responses of the stomach to vagal stimulation and drugs (Paton and Vane, 1963), and high concentrations of bretylium blocked ganglionic transmission (Kosterlitz and Lees, 1961; Campbell, 1966). Subsequently, responses to vagal or field stimulation have been observed when sympathetic nerve stimulation was blocked by adrenergic neurone blocking agents (Burnstock, Campbell and Rand, 1966) or a combination of α- and β-adrenoceptor blocking antagonists (Bucknell and Whitney, 1964; Day and Warren, 1967).

Such responses were not confined to the gut. The inhibitory response of other smooth muscles, e.g. the anococcygeous muscles of rat, rabbit and mouse (Gillespie, 1972; Creed, Gillespie and McCaffery, 1977; Gibson and Yu, 1983), rectococcygeous muscle (King and Muir, 1981), blood vessels (Hughes and Vane, 1967), lung (Robinson, McLean and Burnstock, 1971), gall bladder (Davison, Al-Hassini, Crowe and Burnstock, 1978) and accessory smooth muscles of reproduction (Klinge and Sjostrand, 1974) proved equally resistant to blockade by either adrenergic or cholinergic antagonists (see Burnstock, 1972,1977,1979; Furness and Costa, 1973: Gillespie, 1982).

Non-adrenergic non-cholinergic autonomic responses were not restricted to inhibition. The contractile response of the urinary bladder to parasympathetic (classically cholinergic) nerve stimulation was only partially blocked by atropine (Henderson and Roepke, 1934). This was variously

attributed to the inability of atropine to gain access to the receptor sites (Carpenter and Rand, 1965) or its displacement by a high concentration of acetylcholine released in close proximity to the receptors (Hučković, Rand and Vanov, 1965). The former seems incompatible with electron microscopical data showing gaps of 20nm or larger between nerve varicosities and smooth muscle (Caeser, Edwards and Ruska, 1957; Thaemert, 1963). Also, the contractile response to exogenously-added acetylcholine was blocked by atropine (Ambache and Zar, 1970). The amount of acetylcholine required to overcome the blockade exceeded that released during nerve stimulation (Dumsday, 1971), making it unlikely that atropine was displaced from the receptors by neuronally-released acetylcholine. Adrenergic nerves were unlikely to mediate the contractile response because first, it was not blocked by α- or β-adrenoceptor antagonists; secondly, noradrenaline inhibited the urinary bladder _in vitro_ (Edge, 1955; Ambache and Zar, 1970).

Contractile responses resistant to adrenergic and cholinergic antagonists have also been demonstrated in guinea-pig ileum (Ambache and Freeman, 1968), cat colon in response to pelvic nerve stimulation (Hulten and Jodal, 1969), chicken oesophagus in response to vagal stimulation (Hassan, 1969) and chicken rectum in response to stimulation of Remaks nerve (Bartlett and Hassan, 1971),

It has been suggested that the contractile response of the vas-deferens muscle to field stimulation is in part non-adrener-

gic non-cholinergic (Ambache and Zar, 1971). However, recent evidence suggests that the rapid "twitch", which is resistant to adrenergic and cholinergic receptor blocking agents, may be mediated by ATP released as a cotransmitter (Burnstock, 1981) from adrenergic nerves rather than a separate population of non-adrenergic non-cholinergic fibres (Fedan, Hogaboom, O'Donnell, Colby and Westfall, 1981: Sneddon, Westfall and Fedan, 1982).

The development of intracellular and extracellular electrical recording from smooth muscle enabled the sympathetic and intramural inhibitory innervation to be compared at a cellular level. In the taenia coli, perivascular sympathetic nerve stimulation with a single pulse produced no electrical or mechanical response. Trains of pulses at low frequency (below 5Hz) inhibited spike activity and relaxed the muscle without a change in membrane potential. Only at higher stimulation frequencies was the relaxation accompanied by hyperpolarisation which reached a maximum of some 16mV (Bennett, Burnstock and Holman, 1966a). In contrast, field stimulation with a single pulse evoked an inhibitory junction potential (ijp) which could reach 25mV, and relaxation (Bennett and Holman, 1963; Bennett, Burnstock and Holman, 1966b). With trains of pulses, ijps summated to values of up to 30mV. Whereas the inhibitory response to sympathetic nerve stimulation increased with frequencies exceeding 10Hz, that to field stimulation reached a maximum below 10Hz (Bennett et al, 1966a,b). In the colon and

stomach, sympathetic stimulation produced small changes in membrane potential, while field or vagal stimulation produced large hyperpolarisations (compare Gillespie, 1962 with Furness, 1969; Beani, Bianchi and Crema, 1971). Like the relaxations to field or vagal stimulation, ijps were unaffected by atropine or guanethidine and appeared to be mediated by non-adrenergic non-cholinergic nerves (Bennett et al, 1966b: Beani et al, 1971).

The inability of low frequency sympathetic nerve stimulation to produce significant changes in membrane potential is consistent with histochemical studies using specific catecholamine fluorescence (Falck, 1962). With the exceptions of sphincteric muscle and the taenia coli (Gillespie and Maxwell, 1971), adrenergic fibres show little ramification in the smooth muscle layers but terminate mainly in the enteric neural plexuses (Norberg, 1964; Jacobowitz, 1965; Costa, Furness and Gabella, 1971). The distribution of adrenergic fibres is more in keeping with a role as modulators of cholinergic activity than as a direct influence on the smooth muscle of the gut (Norberg and Sjöqvist, 1966).

The inhibitory responses of the gut and related smooth muscles to field stimulation was unaffected by the absence of adrenergic fibres. In the foetal intestine of rabbit or mouse, relaxations to field stimulation resistant to adrenergic and cholinergic antagonists were observed after 16-17 days gestation. The response to sympathetic nerve stimulation was absent until some 30 days after birth (Gershon

and Thompson, 1973). Sympathetic denervation either by physical methods such as storage of (Burnstock et al, 1966) or tissue culture from (Rikimaru, 1971) the taenia coli; freezing the perivascular nerves to the colon (Furness, 1969), or the pharmacological methods of immunosympathectomy in the anococcygeus (Gibson and Gillespie, 1973) and 6-hydroxydopamine in toad lung (Robinson et al, 1971) and anococcygeus (Gibson and Gillespie, 1976), also failed to abolish the inhibitory responses to field stimulation.

The electron microscopical evidence for non-adrenergic non-cholinergic nerves is as yet equivocal. Baumgarten, Holstein and Owman (1970) distinguished three types of nerve profile in Auerbach's plexus. Adrenergic nerves were recognised by the presence of small (40-60nm) or medium-sized (50-80nm) granular vesicles which increased in electron density following treatment with 5-hydroxydopamine. 6-hydroxydopamine caused these profiles to degenerate. Cholinergic profiles were thought to contain mainly small (35-60nm) electron lucent and large (80-110nm) opaque membrane-bound vesicles. The third type of profile allegedly had large granular vesicles (later termed "large opaque vesicles" or "LOVs"; Burnstock, 1972) and small (40-60nm) clear vesicles in equal proportions. Such profiles, designated "p-type" because of their similarity to the peptide secretory neurones of the hypothalamus, have since been reported in other tissues known to exhibit non-adrenergic non-cholinergic responses, e.g. toad lung (Robinson, McLean and Burnstock,

1971), guinea-pig myenteric plexus (Gabella, 1972), avian gizzard (Burnstock, 1972), bovine retractor penis muscle (Eränkö, Klinge and Sjöstrand, 1976) and rat anococcygeus muscle (Gibbins and Haller, 1979). The p-type profile was one of eight types identified in Auerbach's plexus of the guinea-pig by comparing vesicle size, shape and content (Cook and Burnstock, 1976).

However, some inconsistencies have emerged. For example, few p-type profiles were found in the circular muscle of rabbit jejunum, yet atropine and guanethidine-resistant ijps of up to 25mV were recorded in response to a single pulse (Daniel, Taylor, Daniel and Holman, 1977). The authors suggested that the differences between nerve profiled observed previously could arise from the heterogeneous distribution of the vesicles. A detailed comparison of vesicle size and occurrence in nerve profiles from a variety of tissues showed no significant correlation between non-adrenergic non-cholinergic responses and p-type profiles. For instance, there was no significant difference between the number of large granular vesicles present in p-type profiles of the rabbit anococcygeus and hepatic portal vein when compared with cholinergic nerves in rabbit or guinea-pig atria (Gibbins, 1982).

Although the electron microscopical evidence is equivocal, pharmacological, electrical and histochemical studies indicate clearly that there is a third group of autonomic

fibres which release neither acetylcholine nor noradrenaline, their transmitter(s) is as yet unknown.

MECHANISM OF ACTION OF NON-ADRENERGIC NON-CHOLINERGIC AUTONOMIC NERVES AS REVEALED BY ELECTRICAL RECORDING

Inhibition

In many smooth muscles non-adrenergic non-cholinergic nerve-evoked relaxation is accompanied by an inhibitory junction potential (ijp). That of the taenia coli is particularly well characterised. In this muscle, spontaneous tone is maintained by the influx of Ca^{2+} during the spike potential (see Tomita, 1980). Field stimulation with a single pulse evoked ijps of short latency (45-80ms) and sufficiently long duration (1 s) to inhibit spike discharge and relax the muscle. Ijps were graded with stimulus strength and summated at frequencies above 5Hz (Bennett et al, 1966b). Both the ijp and relaxation were abolished by tetrodotoxin (TTX) (Bülbring and Tomita, 1967), confirming their neurogenic origin. Transmitter release appears to be via a Ca^{2+}-dependent process. Ijps in intestinal muscle were reduced by decreasing external Ca^{2+} (Holman and Weinrich, 1975) or by increasing Mg^{2+} (Bauer and Kuriyama, 1982a). Maximum amplitude of the inhibitory potential varies between different tissues. Values of up to 30mV were recorded in the guinea-pig taenia coli (Bennett et al, 1966b), while the maximum in the rat anococcygeus was 6mV (Creed and Gillespie, 1977). The

mechanism by which hyperpolarisation produces relaxation of the anococcygeus has yet to be elucidated. Unlike the taenia , the rat anococcygeus does not exhibit spike potentials (Creed, Gillespie and Muir, 1975).

Hyperpolarisation is not a universal response to inhibitory non-adrenergic non-cholinergic nerve stimulation. The cat trachea relaxed without any apparent electrical change (Ito and Takeda, 1982). This may be because the same transmitter can act via different mechanisms, or different transmitters may be involved (see Burnstock, 1981).

The ionic basis for the non-adrenergic non-cholinergic ijp, in the tissues studied, is a selective increase in K^+ conductance. The equilibrium potential (E_K) for K^+ at approximately -80 to -90mV lies some 30-40mV more negative than the membrane potential of most gut smooth muscle. When E_K was made more negative by reducing or removing the external K^+, the amplitude of the ijp was also increased in the taenia (Bennett, Burnstock and Holman, 1963: Tomita, 1972) and jejunum (Hidaka and Kuriyama, 1969). Raising the external K^+ concentration decreased ijp amplitude, presumably by making E_K more positive. The ijp in the taenia coli (Tomita, 1972), rabbit anococcygeus (Creed and Gillespie, 1977) and guinea-pig ileum (Bauer and Kuriyama, 1982a) were reduced when field stimulation was carried out during passive hyperpolarisation of the membrane potential and abolished at -80 to -90mV when there would have been no

net driving force on K^+. At membrane potentials exceeding -90mV, ijps were reversed to depolarisation. During the ijp there was an increase in membrane conductance as shown by a decrease in the size of electrotonic current pulses (Den Hertog and Jager, 1975; Creed and Gillespie, 1977) and increased rate of $^{42}K^+$ efflux during field stimulation (Den Hertog and Jager, 1975). There is also pharmacological evidence implicating K^+. Apamin, a neurotoxin isolated from bee venom (see Jenkinson, 1981), which blocks Ca^{2+}-stimulated K^+ channels in hepatocytes (Banks, Brown, Burgess, Burnstock, Claret, Cocks and Jenkinson, 1979) also blocks the non-adrenergic non-cholinergic ijp in the taenia (Maas, 1981), stomach (Vladimirova and Shuba, 1978) and small intestine (Bauer and Kuriyama, 1981b). Thus, the increase in K^+ conductance at these sites may be dependent on intracellular Ca^{2+}. Not all non-adrenergic non-cholinergic inhibitory nerve responses are apamin-sensitive. Field stimulation-evoked relaxation of the bovine retractor penis was not blocked by apamin but was abolished by oxyhaemoglobin (Bowman, Gillespie and Pollock, 1982). This suggests that a different mechanism may be involved.

Rebound phenomenon

After the cessation of inhibitory nerve stimulation, a rebound contraction is often observed, particularly if the resting tone level is low (Cocks and Burnstock, 1979). Rebound contraction has been attributed to the secondary release of prostaglandins. The prostaglandin synthesis

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inhibitor, indomethacin, was shown to block rebound contraction in the taenia coli (Burnstock, Cocks, Paddle and Staszewska-Barczak, 1975; Cocks and Burnstock, 1979). However, other studies have failed to confirm the involvement of prostaglandins. Neither indomethacin nor aspirin were effective at blocking rebound contraction of the taenia coli or ileum (Kadlek, Masek and Seferna, 1974) and duodenum (Mitchell and Wood, 1976).

The electrical basis for rebound contraction is a depolarisation, following the ijp, which may reach the threshold for triggering spike activity (Bennett et al, 1966; Furness, 1969) or an oscillation in membrane potential (Creed and Gillespie, 1977). The nature of the rebound response is uncertain. In the taenia coli, an "after-depolarisation" was observed following passive hyperpolarisation of the membrane using externally-applied current (Furness, 1970; Tomita, 1966), implying that it is merely a consequence of the preceding inhibition (Bennett, 1966). However, in guinea-pig intestinal muscle, passive hyperpolarisation failed to produce an after-depolarisation if the time-course of the electrotonic potential was made similar to that of the ijp. A depolarisation was only observed if the current was switched off abruptly (Bywater Holman and Taylor, 1981). Moreover, apamin blocked the ijp without affecting rebound depolarisation in the taenia coli (Maas and Den Hertog, 1979) and guinea-pig intestine (Bywater et al, 1981). This suggests that the rebound response is due to the activity of non-adrenergic non-cholinergic

excitatory nerves.

Excitation

The electrical basis for the motor response to non-adrenergic non-cholinergic nerve stimulation in the urinary bladder (Ursillo, 1961: Creed, Ishikawa and Ito, 1983), chicken rectum (Takewadi and Ohashi, 1977) and guinea-pig ileum (Bywater et al, 1981; Bauer and Kuriyama, 1982a; Bywater and Taylor, 1983) is an excitatory junction potential (ejp). Ejps were graded with stimulus strength and could reach the threshold for generating action potentials. Tetrodotoxin abolished the ejps (Takewaki and Ohashi, 1977; Bauer and Kuriyama, 1982a; Creed et al, 1983), confirming their neurogenic origin. Transmitter release appeared to be via a Ca^{2+}-dependent process because ejps were reduced by increasing the external Mg^{2+} (Bauer and Kuriyama, 1982a). The latency of the ejp varies considerably from 5-15ms in the chicken rectum to 350-900ms in guinea-pig ileum. This may be due to differences in the distance between nerve varicosities and smooth muscle cells, different transmitters or post-synaptic mechanisms.

The ionic basis of the non-adrenergic non-cholinergic ejp remains to be fully elucidated. In the ileum, ejps were enhanced by passive hyperpolarisation of the membrane potential and had a reversal potential of -27mV (Bauer and Kuriyama, 1982a). This may be the equilibrium potential for one ion, e.g. Cl^-, or the net reversal

potential for a number of ions, e.g. Na^+ and K^+.

PUTATIVE NON-ADRENERGIC NON-CHOLINERGIC TRANSMITTERS

Of the various candidates proposed as the transmitter(s) released from non-adrenergic non-cholinergic post-ganglionic nerves, either a purine, e.g. ATP (Burnstock, 1972) or a peptide, e.g. VIP, substance P, somatostatin, neurotensin, enkephalin (Bloom and Polak, 1978: Humphrey and Fischer, 1978) appear the most likely. The possible transmitter role of these candidates is generally assessed according to the Eccles (1964) criteria. These state that the transmitter candidate should mimic nerve stimulation, be present and synthesised in nerve fibres, be released by nerve stimulation, be antagonised by drugs which block the action of the transmitter, be potentiated by drugs which inhibit termination of the transmitter.

Mimicry of nerve stimulation

On the basis that the candidate should mimic nerve stimulation, some of the peptides can be excluded as inhibitory transmitters. Substance P, neurotensin and somatostatin contract the guinea-pig taenia coli. Enkephalin was inactive (Cocks and Burnstock, 1979). VIP in low concentrations produced relaxation but, unlike that to non-adrenergic non-cholinergic nerve stimulation, it was slow in both onset and duration. ATP produced relaxations of similar latency and duration to nerve stimulation. A

rebound contraction also followed the inhibitory response to ATP and inhibitory nerve stimulation but not to VIP (Cocks and Burnstock, 1979). ATP relaxed a number of gut smooth muscles known to possess a non-adrenergic non-cholinergic innervation, including stomach circular muscle, descending colon and ileum of guinea-pigs and rabbits, rat gastric fundus, duodenum and colon, mouse duodenum and colon (Burnstock et al, 1970).

The inhibitory effect of ATP was TTX-insensitive and not therefore due to the production of action potentials in non-adrenergic non-cholinergic nerves (Burnstock et al, 1970). Two types of receptor have been distinguished for ATP and other purines. "P_1 receptors" are most sensitive to adenosine, blocked by methylxanthines and mediate an increase in cAMP levels (Burnstock, 1979 ,1981). "Purinergic P_2 receptors" mediate the response of intestinal muscle to ATP. These receptors are blocked preferentially by quinidine, 2-substituted imidazolines, 2-2'-pyridylisatogen or apamin and evoke prostaglandin synthesis. In the rat anococcygeus, relaxations to ATP were small or absent altogether unless prostaglandin synthesis was inhibited with indomethacin (Burnstock, Cocks and Crowe, 1978). There was no such requirement for the relaxation to field stimulation.

The inhibitory effect of VIP on canine antral muscle was also TTX-insensitive (Morgan, Schultz and Szurszewski,

1978). However, VIP had no direct effect on the longitudinal muscle of guinea-pig ileum but stimulated neurones in the myenteric plexus (Williams and North, 1979; North, 1982). Thus, VIP appears to act both directly and indirectly on smooth muscle preparations.

Electrically, ATP mimics inhibitory nerve stimulation in a number of smooth muscles which exhibit non-adrenergic non-cholinergic ijps, e.g. taenia coli (Axelsson and Holmberg, 1969; Tomita and Watanabe, 1973; Jager and Schievers, 1980), guinea-pig stomach (Vladimirova and Shuba, 1978) and rabbit caecum (Small, 1974). A discrepancy arose when the concentration of ATP required to produce hyperpolarisation was compared with that producing relaxation in the taenia coli. Maximal relaxation was produced at concentrations (approximately 1×10^{-4}M) which, unlike inhibitory nerve stimulation, failed to hyperpolarise. Only at higher concentrations (1×10^{-3}M) was a hyperpolarisation produced (Tomita and Watanabe, 1973). The ionic basis for this hyperpolarisation was the same as that to inhibitory nerve stimulation and involved an increase in conductance to K^+ (Tomita and Watanabe, 1973; Maas, Den Hertog, Ras and Van Den Akker, 1980). Apamin, which blocks the inhibitory potential to field stimulation, abolished the hyperpolarisation to ATP (Vladimirova and Shuba, 1978; Maas and Den Hertog, 1979) and the concomitant increase in ^{42}K efflux (Maas et al, 1980).

VIP also hyperpolarised the taenia coli but the latency of the response (10s or greater) was considerably longer than that to inhibitory nerve stimulation or ATP. Apamin failed to block the hyperpolarisation to VIP (Hills, Collis and Burnstock, 1983), suggesting that the ionic basis of the response is different from that to inhibitory nerve stimulation or ATP. In the cat trachea, low concentrations of VIP (10^{-12} to 10^{-9}M) mimicked inhibitory nerve stimulation producing relaxations with no electrical change. At higher concentrations (10^{-8}M), however, which produced relaxations similar in magnitude to those produced by nerve stimulation, VIP did hyperpolarise and increase membrane conductance (Ito and Takeda, 1982).

In some tissues ATP and VIP produced electrical effects which were opposite to non-adrenergic non-cholinergic nerve stimulation. VIP depolarised opossum oesophageal muscle in which non-adrenergic non-cholinergic nerve stimulation evoked ijps (Daniel, Helmy-Elkoly, Jager and Kannan, 1983). In the smooth muscle of pig stomach (Ohga and Taneike, 1977) and guinea-pig ileum (Bauer and Kuriyama, 1982b), ATP depolarised cells which showed non-adrenergic non-cholinergic ijps and hyperpolarised those showing ejps. Thus, ATP is unlikely to be the transmitter at either site.

Substance P directly stimulates both myenteric neurones

(Katayama and North, 1978; North, 1982; Otsuka and Konishi, 1983) and intestinal muscle (Szeli, Molina, Zappia and Bertaccini, 1977; Yau, 1978) where it may mediate the non-adrenergic non-cholinergic contraction to field stimulation (Franco, Costa and Furness, 1979). Longitudinal and circular muscle cells in the ileum in which field stimulation evoked a non-adrenergic non-cholinergic ejp were depolarised by substance P. Moreover, the ejp was abolished during the depolarisation to substance P. This effect was not reversed by passive hyperpolarisation of the membrane potential and suggests that the ejp and substance P-evoked depolarisation share the same ionic basis (Bauer and Kuriyama, 1982b). Substance P also mimics non-adrenergic non-cholinergic nerve stimulation at other sites including the motor response of the colon and rectum to pelvic nerve stimulation (Anderson, Bloom, Edwards, Järhult and Mellander, 1983), and in the rat parotid gland, where an increase in enzyme secretion, Rb efflux, depolarisation and decreased membrane resistance were observed (Gallacher, 1983).

ATP, VIP and substance P each mimic non-adrenergic non-cholinergic nerve stimulation at some sites. The lack of mimicry observed at others may reflect the existence of yet other autonomic transmitters.

Presence of transmitter candidate in nerves

A number of potential transmitters have been found in

autonomic nerves. Quinicrine, a fluorophore which binds ATP, has been used to demonstrate nerve fibres containing ATP in gastrointestinal smooth muscle (Olson, Alund and Norberg, 1976), urinary bladder (Burnstock, Cocks, Crowe and Kasikov, 1978) and anococcygeus muscle (Burnstock, Cocks and Crowe, 1978). However, these results are consistent with the role of ATP in energy metabolism and may be unrelated to non-adrenergic non-cholinergic transmission. ATP is also known to be present in the synaptic vesicles of adrenergic fibres where it may act as a co-transmitter (Fedan et al, 1981).

Various peptides including VIP, substance P, somatostatin, enkephalin and neurotensin have been detected by immunohistochemical fluorescence in autonomic nerves throughout the gastrointestinal tract (Hokfelt, Johansson, Efendi, Luft and Arimura, 1975; Uddman, Alumets, Edvinsson, Håkanson and Sundler, 1978; Edin, Lund, Lundberg, Ahlman, Dohlström, Fahrenkrug, Hökfelt and Kewenter, 1979; Costa, Furness, Buffa and Said, 1980; Schultzberg, Hökfelt, Nilsson, Terenius, Rothfeld, Brown, Elde, Goldstein and Said, 1980; Jessen, Saffrey, Noorden, Bloom, Polak and Burnstock, 1980: Saffrey, Polak and Burnstock, 1982; Cai, Gu, Huang, McGregor, Guatei, Bloom and Polak, 1983). VIP and substance P immunoreactive fibres were the most abundant, particularly in the myenteric and submucous plexuses. In the colon, VIP was localised in p-type (allegedly non-adrenergic non-cholinergic) fibres in the lamina propria and submucous plexus (Larsson,

1977). The taenia coli was innervated by both VIP and substance P immunoreactive nerves (Jessen et al, 1980), suggesting possible roles in the inhibitory and rebound responses respectively. However, VIP and substance P immunoreactive fibres were sparse or completely absent from intestinal longitudinal muscle (Saffrey et al, 1982; Schultzberg et al, 1980) or gall bladder muscle except in the sphincteric region (Cai et al, 1983), yet both of these show non-adrenergic non-cholinergic responses to field stimulation. This may be because the transmitter reaches the smooth muscle cells by overflow from the ganglionic plexus (see Franco et al, 1979), but the electrophysiological evidence, showing large non-adrenergic non-cholinergic ijps and ejps to a single pulse argues for the direct innervation of the smooth muscle cells (see Gillespie, 1982). Neurotensin immunoreactive fibres were present in the longitudinal muscle of chicken gut but only in the upper tract (Saffrey et al, 1982). The circular muscle layer did possess substance P, VIP and enkephalin immunoreactive fibres (Schultzberg et al, 1980; Costa et al, 1980).

The distribution of peptides in the gut corresponds with a role as neurotransmitters acting either on other neurones, secretory cells, sphincteric and possibly propulsive smooth muscle. With the exceptions of somatostatin and neurotensin, blood vessels also showed a dense innervation, suggesting that the control of intestinal

blood flow may be mediated by peptidergic nerves.

Release of transmitter candidate

First indications that ATP might be released by nerve stimulation came from studies on the perfused stomach of guinea-pig and toad. Vagal stimulation caused a rise in the levels of the ATP metabolites, adenosine and inosine, in the perfusion fluid. A similar effect was observed in response to field stimulation of Auerbach's plexus (Burnstock, Campbell, Satchell and Smythe, 1970). The taenia coli pre-loaded with ^{3}H-adenosine, released ^{3}H-ATP in response to field stimulation (Su, Bevan and Burnstock, 1971). More recent studies have employed the luciferin-luciferase technique to detect directly ATP released by field stimulation of the urinary bladder (Burnstock, Cocks, Crowe and Kasakov, 1978) and rat anococcygeus (Burnstock, Cocks and Crowe, 1978). Adrenergic nerves were excluded as the source of ATP because release was unaffected by 6-hydroxydopamine. There appears some doubt over whether ATP was liberated exclusively from nerves. In the bladder release was TTX-sensitive and Ca^{2+}-dependent, suggesting a neurogenic origin (Burnstock et al, 1978a). However, the release of ^{3}H-ATP from the taenia had both TTX-sensitive and insensitive components (Rutherford and Burnstock, 1978) and was evoked by various exogenously-added inhibitory agents including noradrenaline, papaverine and nitroglycerin (Kuchii, Miyahara and Shibato, 1973).

That such a diverse group of drugs should release ATP from non-adrenergic non-cholinergic nerves seems unlikely. Combined with the partial insensitivity of ATP release to TTX (Rutherford and Burnstock, 1978), the results suggest that some of the ATP released may be of myogenic origin.

VIP release, as measured by an increase in its resting concentrations in blood or lymph, has been demonstrated in response to stimulation of the pelvic (Fahrenkrug, Haglund, Jodal, Lundgren, Olbe and Schaffalitzky de Muckadell, 1978; Anderson, Bloom, Edwards, Järhult and Mellander, 1983) and vagus (Fahrenkrug, Galbo, Holst and Schaffalitzky de Muckadell, 1978; Bloom and Edwards, 1980) nerves. Significantly, VIP was released from the vagus following stimulation of the high threshold fibres responsible for the non-adrenergic non-cholinergic nerve-mediated inhibition of gut motility (Jansson, 1969). Release was also evoked by distension of the oesophagus and small bowel (Fahrenkrug et al, 1978) or proximal stomach (Chayvialle, Miyata, Rayford and Thompson, 1981). This suggests that reflex relaxation of the stomach, and descending inhibition of the intestine (see Abrahamson, 1973) may be mediated by nerve fibres releasing VIP.

Blockade of nerve stimulation and transmitter candidate

Various attempts have been made to compare ATP with non-adrenergic non-cholinergic nerve responses pharmacologically, either by using non-selective blocking agents

or by desensitisation of the tissue to ATP.

Quinidine has been shown to antagonise both ATP and the non-adrenergic non-cholinergic inhibitory nerve response in the taenia but the responses to exogenous noradrenaline and sympathetic nerve stimulation were also affected (Burnstock et al, 1970). Another potential ATP receptor antagonist, 2-2-pyridylisatogen tosylate (PIT), antagonised the motor response of the urinary bladder to field stimulation, ATP, acetylcholine and histamine (Burnstock et al, 1978a). PIT has been used more successfully on the taenia coli. Here the response to ATP was abolished while that to field stimulation and exogenous noradrenaline persisted (Spedding and Weetman, 1975). Phentolamine in a concentration exceeding that required to block α-adrenoceptors also abolished the inhibitory response of the teania to ATP without antagonising field stimulation (Ambache, Daly, Killick and Woodley, 1977). These results suggest either that ATP is not the transmitter or that neuronally-released ATP is less sensitive to blockade by drugs than exogenously-added transmitter.

If ATP was the non-adrenergic non-cholinergic transmitter, desensitisation to its effect should be accompanied by a loss of the response to nerve stimulation. Such experiments have given conflicting results. Desensitisation to ATP or its more stable derivative, αβ-methylene ATP, was accompanied by a reduction in nerve-evoked relaxation of the rabbit ileum (Burnstock et al, 1970), contraction of

the urinary bladder (Burnstock et al, 1972) and colon (Hedlund, Fandriks, Delbro and Fasth, 1983).

In other reports, however, desensitisation to ATP had no effect on the response to non-adrenergic non-cholinergic nerve stimulation, e.g. guinea-pig urinary bladder (Ambache and Zar, 1970) and ileum (Weston, 1973a), rabbit duodenum (Weston, 1973b) and cat trachea (Ito and Takeda, 1982). The contradictory nature of the results may arise from the different methods used to produce desensitisation to ATP.

Pharmacological analysis of VIP has also been hindered by the lack of a selective 'receptor' antagonist. Blockade of the relaxation to both VIP and nerve stimulation by VIP-antiserum was observed in the oesophageal sphincter. The effect appeared to be specific as the relaxation to isoprenaline was not blocked (Goyal, Rattan and Said, 1980). The proteolytic enzyme, α-chymotrypsin, has also been used on the presumption that it would digest neuronally-released VIP. In the taenia coli, α-chymotrypsin blocked the inhibitory response to VIP but not that to field stimulation (MacKenzie and Burnstock,1980). This may either mean that VIP is not the transmitter or that α-chymotrypsin was incapable of breaking down neuronally-released peptide.

Blockade of inhibitory nerve stimulation in the bovine and dog retractor penis muscles by haemolysed blood was unaccompanied by any change in the relaxation to VIP (Bowman, Gillespie and Hunter, 1982). It seems unlikely that haemo-

lysate was preventing transmitter release because the inhibitory responses to sodium nitroprusside and isobultomethylxanthene were also blocked (Bowman and Gillespie, 1981a). Desensitisation to the inhibitory effect of VIP on the cat trachea was accompanied by a marked reduction in response to inhibitory nerve stimulation (Ito and Takeda, 1982), suggesting that VIP may be a transmitter at this site.

Potentiation of nerve stimulation and transmitter candidate

The rapid recovery which followed the excitatory and inhibitory responses to non-adrenergic non-cholinergic nerve stimulation suggested that there was an efficient inactivation mechanism for the transmitter. ATP was also rapidly metabolised to adenosine and inosine (Burnstock et al, 1970). The gut contains 5-nucleotidases, adenosine deaminase and Mg^{2+}-stimulated ATPase enzymes which provide an efficient inactivation mechanism for ATP (Burnstock, 1979). In the taenia coli, there is an uptake system for adenosine which, if present in 'purinergic nerves', would provide fresh supplies of substrate for conversion to the transmitter (Kuchii et al, 1973). The adenosine uptake inhibitors (e.g. dipyridamole) have been shown to potentiate the response of the taenia coli to the inhibitory nerve stimulation and ATP (Satchell, Lynch, Bourke and Burnstock, 1972). This could be explained, within the framework of the purinergic nerve hypothesis, if the increased levels of adenosine caused end-product inhibition of the enzymes responsible for

breaking down ATP. However, the amplitude of the hyperpolarisation to non-adrenergic non-cholinergic nerve stimulation or ATP was not potentiated by dipyridamole, suggesting that the concentration of neither ATP nor the inhibitory transmitter was increased (Jager,1976,1979). The rabbit duodenum (Hulme and Weston, 1974) failed to show any enhancement of the relaxation to non-adrenergic non-cholinergic nerve stimulation in the presence of adenosine uptake inhibitors. Thus, they provide no evidence for purinergic inhibitory nerves in these tissues.

EXTRACTION OF PUTATIVE TRANSMITTERS AND OTHER PHARMACOLOGICALLY ACTIVE SUBSTANCES FROM TISSUES

Extraction from tissues has been an important source of pharmacologically active substances since the early report of Oliver and Schafer (1895a). They demonstrated that extracts of pituitary, thyroid and spleen produced changes in the blood pressure of anaesthetised animals.

The extraction of neurotransmitters is particularly difficult because they are present in small quantities, are often readily broken down by enzymes, or may be chemically unstable. Such extracts may also contain impurities which either mimic or antagonise the desired active component.

In 1895 the peripheral actions of an extract from the adrenal medulla were described (Oliver and Schafer, 1895b). This extract contracted arterioles and the spleen. The

active component in the extract was later isolated and called adrenaline (Takamine, 1901). The peripheral actions of adrenaline on the heart, blood vessels and gastrointestinal motility closely paralleled those of sympathetic nerve stimulation (Elliot, 1904,1905), suggesting that sympathetic nerves might act by releasing adrenaline (Dale, 1935; Cannon and Bacq, 1931). However, Euler (1946), using extracts of lumbar sympathetic chain and splenic arterial nerves, found that their activity correlated more closely with a derivative of adrenaline - noradrenaline. (see Euler, 1951)

Early attempts to extract the transmitter released from parasympathetic nerves met with limited success. Dixon's (1906,1907) method involved prolonged vagal stimulation followed by extraction of the heart in boiling water. The resulting extract weakly mimicked vagally-mediated bradycardia and the active component was termed 'inhibitin'. Inhibitin may have been choline (Dale, 1935). Acetylcholine was not extracted in stable form until 1929 by Dale and Dudley. They mixed ox and horse spleen in cold ethanol to obtain an unstable ester of choline. The active substance was identified as acetylcholine by comparing the pharmacological activity of the two on blood pressure, denervated gastrocnemius and rabbit jejunum, and by physico-chemical tests. Interestingly, the yield of acetylcholine extracted was very much reduced if the tissue was minced before adding the ethanol. This illustrates the influence of extraction conditions on the ability to extract unstable substances.

The pharmacology of histamine, like that of acetylcholine, was known before its identification in tissue extracts. The similarities of the pharmacological properties of histamine, obtained from the decarboxylation of histidine, and of tissue extracts was demonstrated by Dale and Laidlaw (1910). Properties included the ability to produce symptoms similar to those of anaphylactic shock. The active component was crystallised from acid extracts of the mucosal layer of ox intestine (Barger and Dale, 1911), but histamine was not unequivocally identified in body tissues until it was obtained in alcoholic extracts of lung and liver (Best, Dale, Dudley and Sharpe, 1927).

5-Hydroxytryptamine (5-HT) was obtained in the 1930s by Erspamer in extracts of intestinal mucosa (see Douglas, 1975; Erspamer, 1954), which were found to stimulate gastrointestinal motility. The active component was termed enteramine. Independently, Rapport, Green and Page (1948a, b,c) reported the pharmacological and physico-chemical properties of 'serotonin', a crystalline substance obtained from blood serum. Serotonin possessed vasopressor properties, contracted rabbit ileum and was a potent vasoconstrictor in the rabbit ear artery. These pharmacological properties were similar to those of an indolalkylamine - 5-HT (Rapport, 1949). Subsequently, both enteramine and serotonin were identified as 5-HT (Erspamer and Azero, 1952).

A report by Kurzok and Lieb (1930) that human uterine muscle was contracted by seminal fluid prompted Goldblatt

(1935) and Euler (1935) independently to extract the active component(s) in ethanol or acetone. Goldblatt's extract from human seminal fluid decreased blood pressure in anaesthetised cats and rabbits, contracted rabbit ileum and, more interestingly, potentiated the contractile effect of adrenaline in the seminal vesicles. These actions were attributed to the presence of two active components in the extract. Euler (1935) used an extract of monkey vesicular gland. This extract also decreased blood pressure but, unlike extracts of prostate or seminal fluid, it had little effect on intestine or uterus. The active component in the extract was called vesiglandin. Euler (1936) later distinguished pharmacologically between two active substances - vesiglandin and prostaglandin - extracted from the accessory genital glands. Prostaglandin-like activity has also been extracted from other tissues. The active components have been called variously darmstoff, irin, menstrual stimulant and medullin (see Horton, 1972). The activity present in these extracts is now known to be due to a diverse group of structurally-related lipid-soluble unsaturated hydroxyacids called collectively prostaglandins (Bergström, Carlson and Weeks, 1968).

Peptides were also obtained from extracts. Euler and Gaddum (1931) demonstrated that an acid-alcohol extract of horse brain or intestine produced atropine-resistant intestinal contractions of delayed onset and decreased blood pressure. Activity was neither due to histamine nor to

acetylcholine, and was absent from similarly made extracts of intestinal mucosa. The active component, called substance P, was identified in the early 1970s when its composition (Chang and Leeman, 1970) and amino acid sequence (Chang, Leeman and Niall, 1970) were determined.

Vasoactive intestinal polypeptide (VIP) was originally obtained in methanol extracts of pig intestine (Said and Mutt, 1970) which were found to produce cardiovascular changes, hyperglycaemia and respiratory stimulation. Neurotensin, another novel peptide with hypotensive activity, was an active component in an acidified-acetone extract of bovine hypothalami (Carraway and Leeman, 1973). In vitro, neurotensin relaxed rat duodenum and contracted rat uterus and guinea-pig colon.

The existence of an endogenous opioid was demonstrated by the ability of acid extracts of decerebrate brain to antagonise competitively receptor binding of the opiate antagonist dihydromorphine to brain tissue and guinea-pig ileum (Terenius and Wahlström, 1974). The active component in the extract was thought to be a peptide (Terenius and Wahlström, 1975). A similar extract was prepared and further purified by Hughes (1975). The purity was assessed by the concentration of naloxone required to reverse the inhibitory effect of the extract on the twitch response of the vas deferens. Extracts which contained largely "enkephalins" were antagonised by a lower concentration of naloxone than was

required to antagonise less pure extracts. The "enkephalins" where identified as a mixture of two pentapeptides - methionine-enkephalin and leucine-enkephalin - by comparing their mass spectra (Hughes, Smith, Kosterlitz, Fothergill, Morgan and Morris, 1975).

In 1975, Ambache, Killick and Zar reported the extraction in acid of a substance from the bovine retractor penis which mimicked inhibitory nerve stimulation in this tissue. Extract-evoked relaxation was TTX-insensitive, suggesting a direct effect on the smooth muscle. Antagonists against muscarinic, adrenergic and histamine receptors were ineffective against both the extract and inhibitory nerve stimulation. When the extract was partitioned with ether, inhibitory activity remained in the aqueous phase and was therefore not due to prostaglandins. Neither was the inhibitory activity due to adenine nucleotides, as these could be removed by adsorption onto alumina without loss of extract activity (Bowman, Gillespie and Martin, 1979). Methanol extracts of bovine retractor penis or rat anococcygeus muscles were found to be inactive unless they were exposed briefly (10 min) to acid (pH 2.0) (Gillespie and Martin, 1980; Gillespie, Hunter and Martin, 1981). Thus, the inhibitory material existed in two interconvertible forms. After exposure to acid, the activated form reverted back at a temperature-dependent rate to the inactive form. Extract activity was unaffected by the proteases trypsin, subtillisin or pepsin, suggesting that it was not due to a peptide.

Inhibitory activity was destroyed by periodic acid, which attacks C-C bonds with glycols, and sodium borohydride, which reduces aldehydes, implying that the active component may be a carbohydrate (Gillespie et al, 1981).

The extract also relaxed gut smooth muscle including the taenia, stomach fundal strip, duodenum and colon which possess a non-adrenergic non-cholinergic inhibitory innervation (Crossley and Gillespie, 1983). In common with inhibitory nerve stimulation in the BRP, the effect of the extract was antagonised by a haemolysate from red blood cells (Bowman and Gillespie, 1982), suggesting a common mechanism of action. However, the extract does not appear to contain the non-adrenergic inhibitory transmitter of the taenia coli because, unlike inhibitory nerve stimulation in this tissue, the relaxation to extract was apamin-insensitive (Bowman and Gillespie, 1981b).

The results suggest that the extract contains a novel inhibitor of smooth muscle. The aim of the present study was to investigate the electrical basis for the relaxation to the extract and field stimulation in the bovine retractor penis, and the related rat and rabbit anococcygeus muscles. Combined electrical and mechanical recording using conventional intra- and extracellular (sucrose gap) techniques have been employed. Initially, both the excitatory and inhibitory electrical responses in the bovine retractor penis to field stimulation were characterised and their ionic basis investigated. Pharmacological techniques have been

used to investigate the receptor types and mechanisms involved in the response to field stimulation, extract and drugs and thus elucidate the possible transmitter role of the inhibitory material contained in the extract.

CHAPTER 2

METHODS

TISSUES

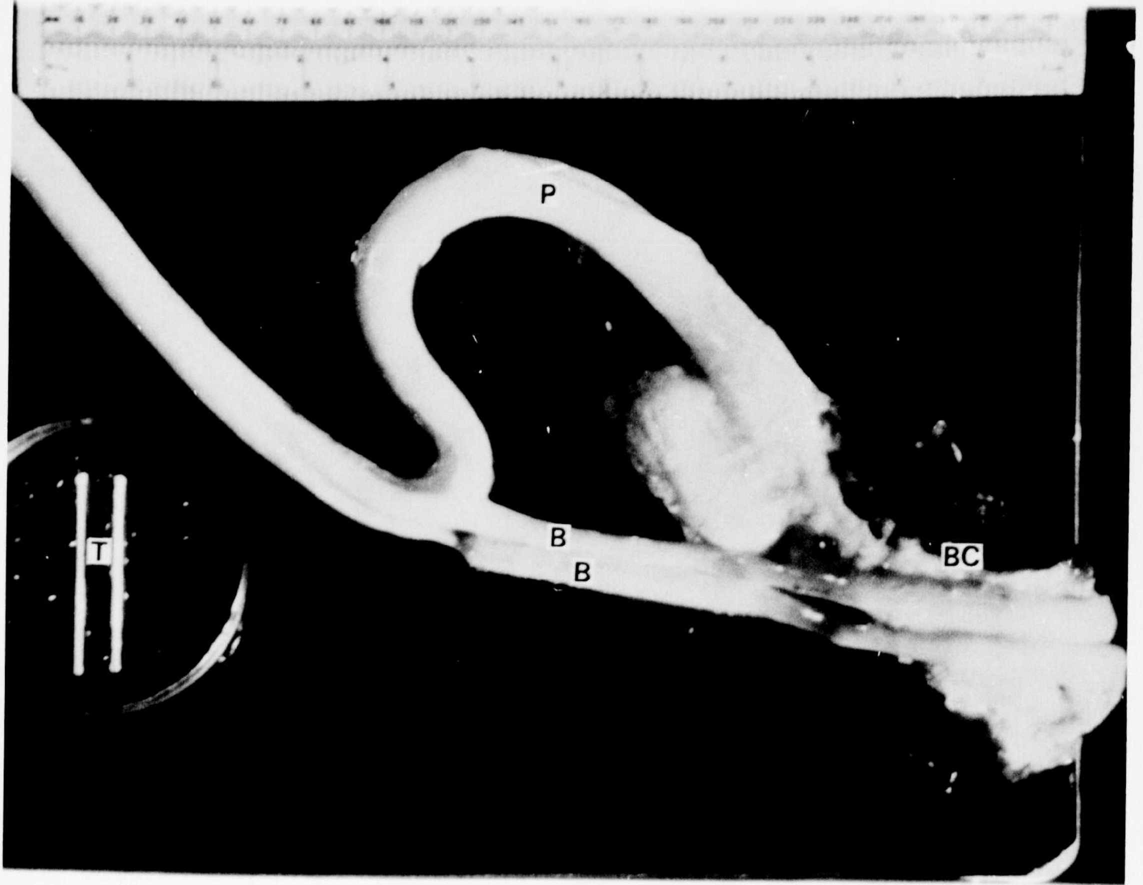
Bovine retractor penis muscle

The bovine retractor penis is a paired smooth muscle originating from the first two coccygeal vertebrae and passing along the ventral surface of the bulbocavernosus muscle to insert into the distal part of the penis. When contracted, the bovine retractor penis muscles keep the penis withdrawn and in a characteristic sigmoid flexure (Fig. 1). Specimens of bovine retractor penis were obtained from the Glasgow abattoir. After slaughter of a bullock, a cut was made through the mid-line of the pelvic region to expose the penis. The penis was severed at the level of the ischiocavernosus and bulcocavernosus muscles and, together with the bovine retractor penis muscles, removed by cutting it free from connective tissue and fat. Specimens were transported to the laboratory in plastic bags. The interval between slaughter and the arrival of the specimens at the laboratory varied from 1-3h.

In the laboratory, the bovine retractor penis muscles were dissected free from the penis and attached connective tissue and stored in Krebs solution at 4°C for up to two days before use. For electrical and mechanical recording, thin strips of muscle (0.1-0.2cm width for sucrose-gap studies or 0.3-0.4cm for intracellular recording) 1.5-2.0cm long were dissected from the middle portion of the bovine retractor penis by cutting along the lines of cleavage separating the smooth muscle bundles.

FIGURE 1

The bovine penis (P) in its characteristic sigmoid flexure and the attached retractor penis muscles (B) extending to the bulbocavernosus (BC). Thin strips of tissue (T), taken from the mid-portion of the bovine retractor penis were used for combined electrical and mechanical recording. The size of the bovine retractor penis is indicated by the 30cm rule in the background.


P
T
B
B
BC

Rat anococcygeus muscle

The rat anococcygeus is a paired smooth muscle originating from the upper coccygeal vertebrae and passing first behind and then to either side of the colon. The muscles join in front of the colon to form a ventral bar approximately 0.1-0.2cm away from the anus.

Male rats weighing 150-250g were killed by stunning and exsanguination and the anococcygeus muscles removed as described by Gillespie (1972). The abdominal contents were exposed by a mid-line incision and deflected to reveal the descending colon which was ligated and cut at the level of the pelvic brim. The pelvis was split and the bladder and urethra removed. The colon was pulled forward and connective tissue behind it removed until the anococcygeus muscles were visible. Both muscles were ligated at their points of attachment to the coccygeal vertebrae and at the ventral bar prior to removal. The isolated muscles were transferred to a petri-dish containing Krebs solution gassed with a 95% O_2/5% CO_2 mixture at room temperature.

Rabbit anococcygeus muscle

The paired anococcygeus muscles in the rabbit originate from the upper coccygeal vertebrae and lie behind the terminal colon some 1cm above the anal margin. The muscles pass on either side of the colon, ending within the longitudinal external muscle. They do not, as in the rat, unite in front of the colon.

Dutch rabbits of either sex (1.5-2.5kg) were killed by stunning and exsanguination, and the anococcygeus muscles removed as described by Creed, Gillespie and McCaffrey (1977). The dissection procedure was similar to that described for the rat. After exposing the abdominal contents, the urinary bladder, and the reproductive organs in the male, were ligated and excised. The anococcygeus muscles were located at their points of attachment to the coccygeal vertebrae, ligated and dissected free up to the colon. The muscles were tied just before their insertion into the colon and transferred to a petri-dish containing Krebs solution gassed with a 95% O_2/5% CO_2 mixture at room temperature. Muscles could be stored without apparent deterioration for up to 24h before use.

SETTING UP MUSCLES FOR ELECTRICAL AND MECHANICAL RECORDING

After removal, the muscles were cleaned of surface connective tissue under a dissection microscope and mounted horizontally in an organ bath for intracellular recording or a sucrose gap. An initial tension of 0.5-1g was applied when investigating the excitatory responses in each muscle. In order to investigate the inhibitory responses of the bovine retractor penis, either the tension was increased in a stepwise fashion up to 3g until tone developed spontaneously, or, as with the rat and rabbit anococcygeous, guanethidine ($1-3 \times 10^{-5}$M) was administered.

TABLE 1 Composition (mM) of normal and modified Krebs solutions

	NaCl	Na Glutamate	Na benzene sulphon-ate	$NaHCO_3$	NaH_2PO_4	KCl	K_2SO_4	$CaCl_2$	$CaSO_4$	$MgCl_2$	$MgSO_4$	Glucose
Normal	111.8	0	0	25	1.13	4.7	0	2.7	0	1.3	0	5.5
K^+-free	116.5	0	0	25	1.13	0	0	2.7	0	1.3	0	5.5
20mM K^+	96.5	0	0	25	1.13	20	0	2.7	0	1.3	0	5.5
Low Cl^-	0	111.8	0	25	1.13	4.7	0	2.7	0	1.3	0	5.5
Zero Cl^-	0	0	111.8	25	1.13	0	2.35	0	2.7	0	1.3	5.5

The ionic composition (mM) of normal and modified Krebs solutions. Solutions were made up in distilled water and gassed with 95% O_2/5% CO_2. The concentration of K^+ was varied without changing the tonicity by making the appropriate changes in Na^+ concentration. Cl^- was reduced by replacement of NaCl with sodium glutamate. Complete removal of Cl^- was by replacement of NaCl with sodium benzenesulphonate, $MgCl_2$ with $MgSO_4$, and $CaCl_2$ with $CaSO_4$.

PHYSIOLOGICAL SOLUTIONS: CHANGES IN IONIC COMPOSITION

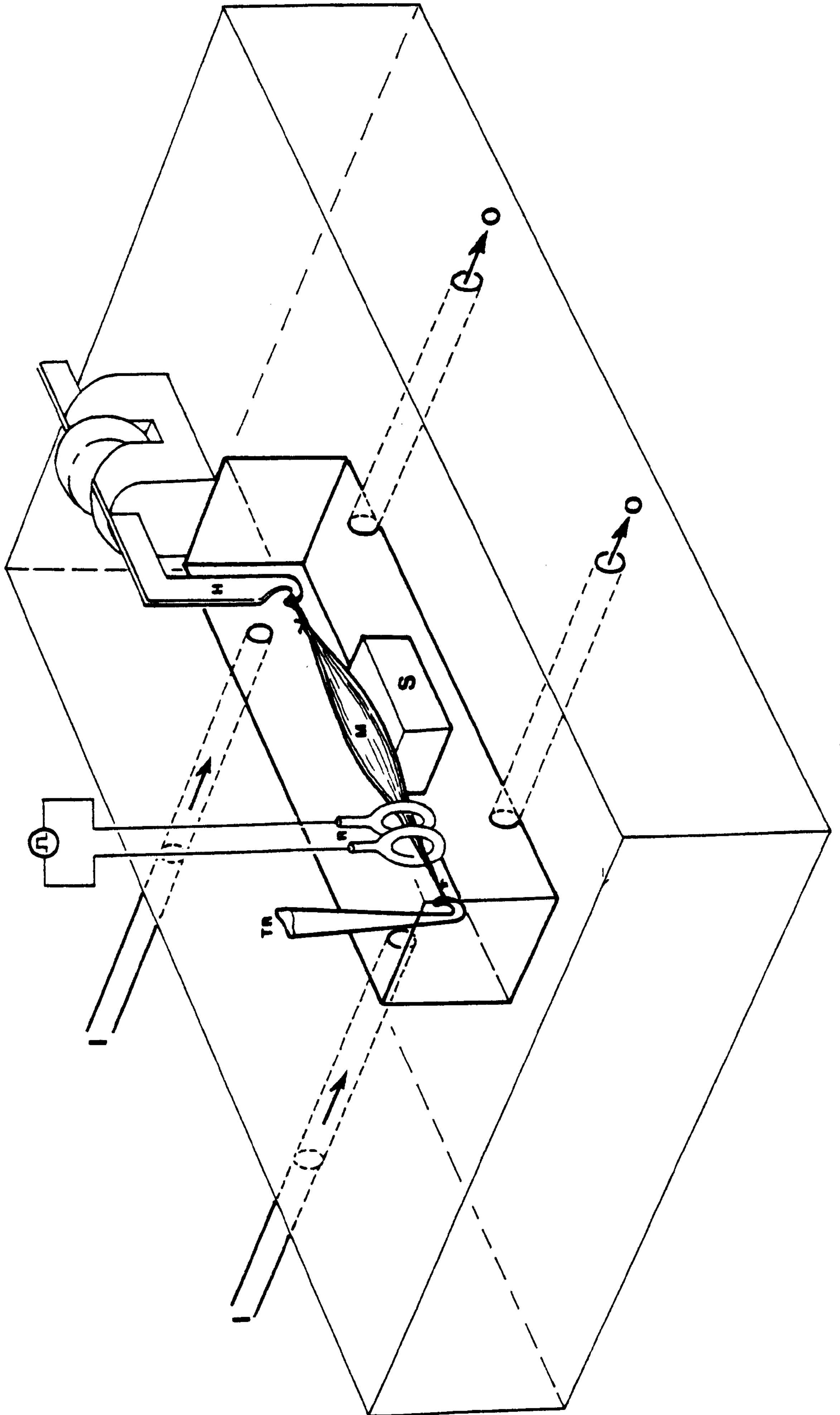
The composition of the Krebs solution used throughout the investigation is shown in Table 1. When the ionic composition of the Krebs solution was modified, isotonicity was maintained by substitution for, or reduction in the concentration of another appropriate ion. In K^+-free Krebs, the total KCl content (4.7mM) was replaced by NaCl. Low Cl^- Krebs solution was obtained by replacing the NaCl content (111.8mM) with Na glutamate. Complete removal of Cl^- was by replacement of NaCl with Na-benzenesulphonate and $CaCl_2$ and $MgCl_2$ by $CaSO_4$ and $MgSO_4$ respectively. Two different impermeable anions, glutamate and benzenesulphonate were used in order to distinguish between the effect of Cl^- withdrawal *per se* and that of the anion substitute itself. In solutions containing an increased concentration of K^+ (20mM), an equivalent reduction in the concentration of NaCl was made. The pH of the Krebs and the modified physiological solution was maintained at 7.4 by gassing with a 95% O_2/5% CO_2 mixture.

DEVELOPMENT OF TECHNIQUES: INTRACELLULAR RECORDING

During the initial investigation of the extract, it became clear that the organ bath in use in the laboratory (Fig. 2) required modification. The heat lability of the extract (half life of inhibitory activity at 37°C, approximately 10-20s; Gillespie, Hunter and Martin, 1981) precluded its addition to the pre-heated perfusion solution. Thus it was necessary to inject the extract at 0-4°C into the bath.

FIGURE 2

Organ bath for combined intracellular electrical and mechanical recording consisting of a central trough (5 x 1 x 1cm) cut from a perspex block (5 x 11 x 2cm) and drilled to accept stainless steel inlet (I) tubes for Krebs solution and outlets (O) for drainage. The muscle (M) was secured at one end by an adjustable stainless steel hook (H) and at the other by an isometric transducer (TR). Field stimulation by an isolated stimulator was effected via Ag/AgCl ring electrodes (R). Intracellular recordings were made from an area of tissue pinned out onto a Sylgard block (S) mounted on perspex. Oxygenated Krebs solution was pumped to the bath by a Watson-Marlow flow inducer at a rate of 6ml/min. The polythene tubing (internal diameter 0.25cm) containing the Krebs solution was surrounded by an outer tube (internal diameter 1.0cm) containing liquid paraffin at 40 ± 0.1°C) pumped from a thermostatically controlled Tempette pump. The temperature of the Krebs solution in the bath was 37 ± 0.5°C.



However, injections (20-200µl) of either warm (36°C) or cold saline were sufficient in themselves to produce relaxations of the muscle which could not be distinguished from those to extract. The cause of this response was a temperature drop (2-3°C over a period of 1-2 min) produced by disturbing the bath fluid.

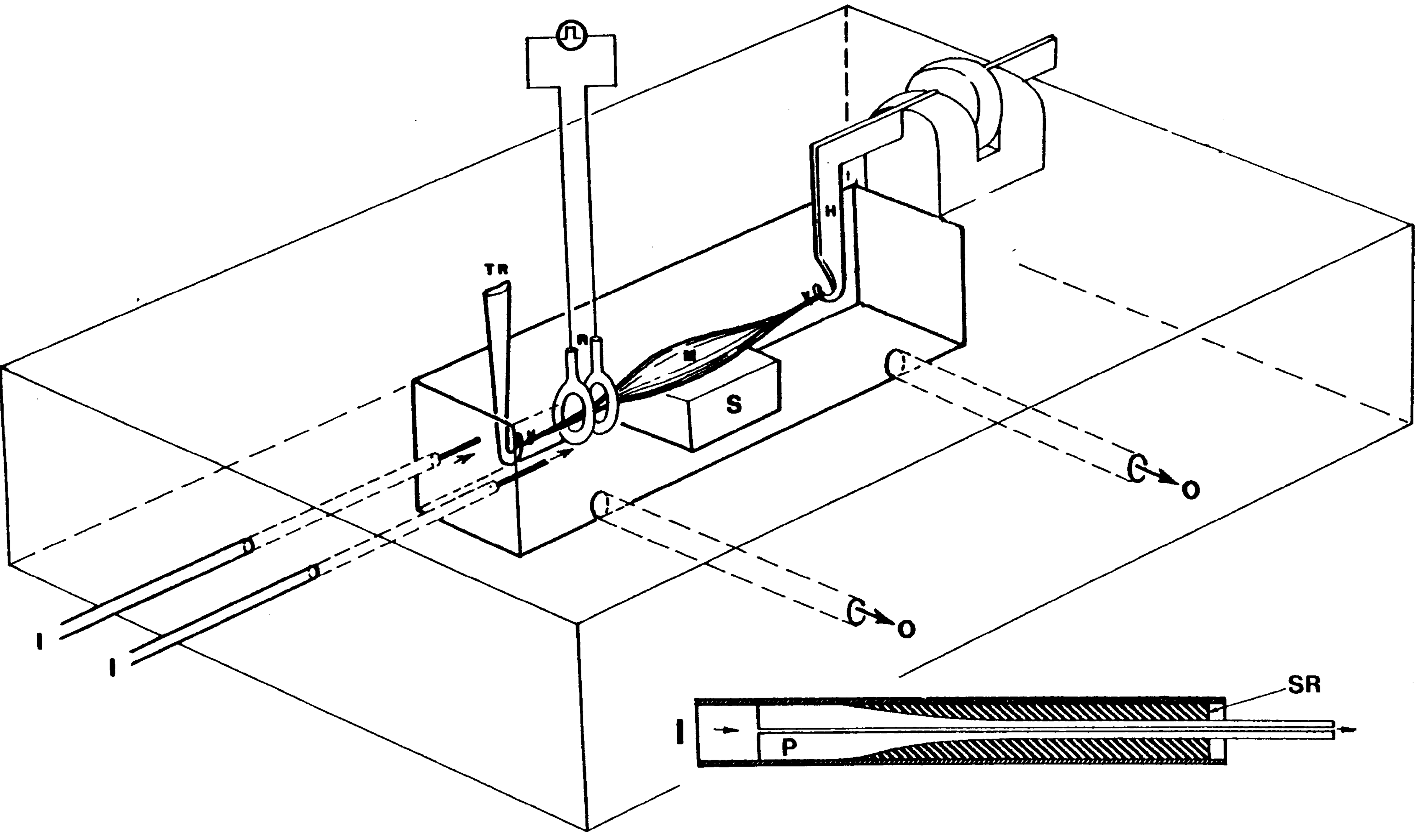
Two approaches to this problem were investigated. First, the injection technique was modified to prevent disturbance of the bath fluid. The fate of added solutions was followed visually after injecting methylene blue (0.01%). Injections made at a slow rate (0.3-1.0ml/min) using a mechanically driven syringe (Palmer slow-injection apparatus) produced a smaller temperature drop but the dye failed to equilibrate quickly. Because of the short active life of the extract, slow equilibration could cause a significant loss of activity before it reached the muscle. Accordingly, a second approach, that of mixing the bath fluid, was adopted. Vigorous gassing with 95% O_2/5% CO_2 at a number of sites failed to mix the bath fluid or to prevent the temperature reduction caused by injection.

The method adopted, and incorporated into the design of the second bath (Fig. 3), was based on the ability of rapid injections to mix the bath fluid. Thus, injections made to occur continuously at a rapid rate would mix the bath contents adequately. This was achieved by reducing the bore of the tubes carrying the Krebs solution to a very small diameter (approximately 0.01cm) by inserting a

FIGURE 3

Organ bath with modified perfusion system. Oxygenated pre-heated Krebs solution was pumped into the bath by a Watson-Marlow flow inducer at a rate of 6ml/min via two inlets (I). In order to mix the bath fluid continuously with injected material, the bore of each inlet was reduced by a drawn-out polythene tube (P in insert, internal diameter approximately 0.01cm) sealed into each inlet with silicon-rubber compound (SR on insert, R.S. Stock No. 555 588). Krebs solution entered the bath as two jets of fluid which ensured that adequate mixing occurred. Other details including method of recording and field stimulation are as described in Fig. 2.

TR = isometric transducer; H = adjustable hook;
R = Ag/AgCl ring electrodes; S = Sylgard block;
M = muscle; O = outlets.


TR
H
R
S
O
O
I
I
I
P
SR

piece of drawn-out polythene tubing (Fig. 3). Krebs solution was forced through each inlet at a rate of 3ml/min using a Watson-Marlow flow inducer. As a result, injections (20-200µl) of cold saline produced small (0.5°C) transient (10-15 sec) reductions in temperature which, unlike equivalent volumes of inhibitory extract, failed to evoke relaxation.

APPARATUS: INTRACELLULAR RECORDING

The organ bath consisted of a trough (5 x 1 x 1cm) cut from a Perspex block (5 x 11 x 2cm). To minimise mechanical vibrations, the bath was bolted to two non-conducting pillars of bakelite which were fastened to a steel plate (200kg) on a table mounted on Mufflite (K-150) anti-vibration dampers. The bath was perfused continuously at a rate of 6ml/min via two inlets with Krebs solution. The polythene tubing containing the Krebs solution was surrounded by an outer tube (internal diameter approximately 1.0cm) containing liquid paraffin at 40 ± 0.1°C, pumped from a Tempette (TET) pump modified to regulate temperature to within 0.1°C limits. (for modifications, see Cunnane, 1979). The temperature of the Krebs solution in the bath was 37 ± 0.5°C.

One end of the muscle was secured to a stainless steel hook and the other passed through bipolar Ag/AgCl ring electrodes (0.1cm width, 1cm apart, mounted in Araldite) and secured to a Grass isometric force-displacement trans-

ducer (FTO3C). Field stimulation was effected by means of a Devices isolated mark III or IV stimulator triggered by a Devices pulse generator (type 2521) and Digitimer. Changes in membrane potential were recorded intracellularly using capillary glass (Clark Electromedical Cat. No. GC150F-10: 1·5mm) microelectrodes (15-40 M Ω) filled with 3M KCl by immersion. Microelectrodes were pulled on a horizontal puller. ([illegible]) Electrical recordings were made from a small area of tissue pinned out onto a Sylgard (Dow Corning) platform (0.6 x 0.6cm) adjacent to the stimulating electrodes. The microelectrode (Shank length 2·2cm) was connected to a unity gain high impedence (10^{10} Ω) DC preamplifier (W.P.I M4A) via an Ag/AgCl half-cell attached to a probe matched and calibrated for the amplifier used. The indifferent electrode was an Ag/AgCl pellet held in solution in the organ bath. Electrical signals passed via the preamplifier, and were displayed on one channel of a Tektronix storage oscilloscope (type 5103N) and digital voltmeter (Fairchild, M53). The other channel of the oscilloscope was used to display electrical signals from the transducer. Electrical and mechanical data were recorded permanently on a Racal instrumentation FM tape recorder (Store 4DE, band width 313-40KHz) and SE UV oscillograph (type 3006 or 6150).

INTRACELLULAR RECORDING FOLLOWING DISPLACEMENT OF THE MEMBRANE POTENTIAL

The passive membrane properties of the bovine retractor penis were investigated by the method of Abe and Tomita (1968). The bath, similar to that described previously,

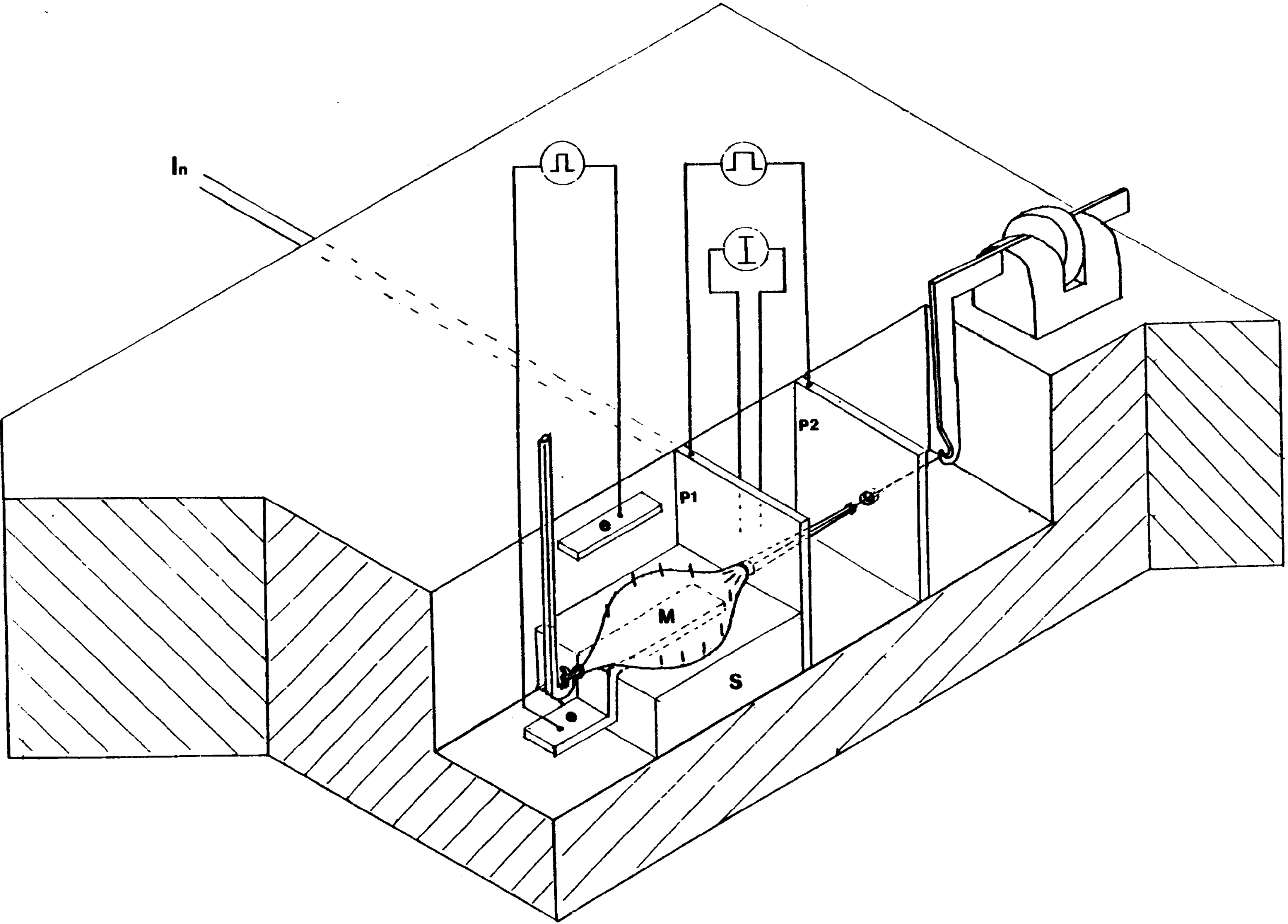
was divided by an Ag/AgCl plate (P1, Fig.4) into two compartments: one for recording and field stimulation, the other for passing current through the muscle. Both compartments were perfused continuously (each at 3ml/min) at 37 ± 0.5°C using a Watson-Marlow flow inducer for the recording and gravity flow for the current-passing compartment.

The current-passing chamber was bounded by two Ag/AgCl plates (250μm thick), 1cm apart and breached with a small hole (diameter 0.15cm) through which the muscle passed. The surface of the plate adjacent to the recording compartment was insulated by a coating of Araldite to electrically isolate the recording and current-passing compartments from each other. The recording compartment contained a Sylgard platform onto which the muscle was pinned, the indifferent electrode, the microelectrode and the electrodes for field stimulation. The electrodes comprised two flattened Ag/AgCl wires (0.15cm wide), one of which lay underneath an area of tissue pinned out onto the Sylgard base; the other was held in the fluid above the surface of the tissue (Fig. 4). The length of tissue extending into the current-passing compartment was 0.8-0.9cm.

In order to investigate the passive membrane properties, current pulses of 1.0-1.5s duration were delivered to the plates by a Devices isolated stimulator triggered by

FIGURE 4

Cut-away view of the organ bath modified to allow displacement of the membrane potential by the technique of Abe and Tomita (1968). The bath was divided by an Ag/AgCl plate (P1) into two compartments. Changes in membrane potential were recorded from the muscle (M) pinned out onto Sylgard (S) in the recording compartment (to the left of P1). Current was applied to the muscle via the Ag/AgCl plates (P1 and P2) which enclosed the current-passing compartment. The relative current intensity (I) was measured via two Ag/AgCl wires which dipped into the current-passing compartment and were connected differentially to one channel of the oscilloscope. The recording compartment contained bipolar Ag/AgCl electrodes (e) for field stimulation. One pole lay underneath the muscle and the other was held in the fluid above. Oxygenated preheated Krebs solution was supplied to the recording compartment by a Watson-Marlow flow inducer as described in Fig. 3. The current-passing compartment was perfused separately with Krebs (37 ± 0.5°C) solution supplied via a separate inlet (In) by gravity flow.



I_n
P2
P1
M
S

a Digitimer. The longer pulse widths (up to 60s) required to investigate the effect of displacing the membrane potential on the response to field stimulation and extract were provided by an isolated constant current source triggered by a Digitimer.

CRITERIA FOR CELL PENETRATION

Satisfactory cell penetration was indicated by an abrupt change in potential from 0 to a steady state value of between -50 and -70mV for muscles with a low level of resting tone and -40 to -50mV after the development of tone. An interval of some 30s was allowed between impalement of a cell and the application of a stimulus. During this period the lack of a significant change in membrane potential was considered to indicate successful penetration.

COLLAGENASE INCUBATION

Considerable difficulty was often experienced in making impalements successfully in the bovine retractor penis, particularly after the muscle had developed tone. The obvious presence of dense layers of connective tissue overlying the muscle appeared to be a possible cause of the problem. As it was impracticable to dissect off all such tissue, collagenase was employed as an alternative method of removal.

A small number of tissues (4) were incubated with collagenase (67-670 units/ml for 30 min) in Krebs solution

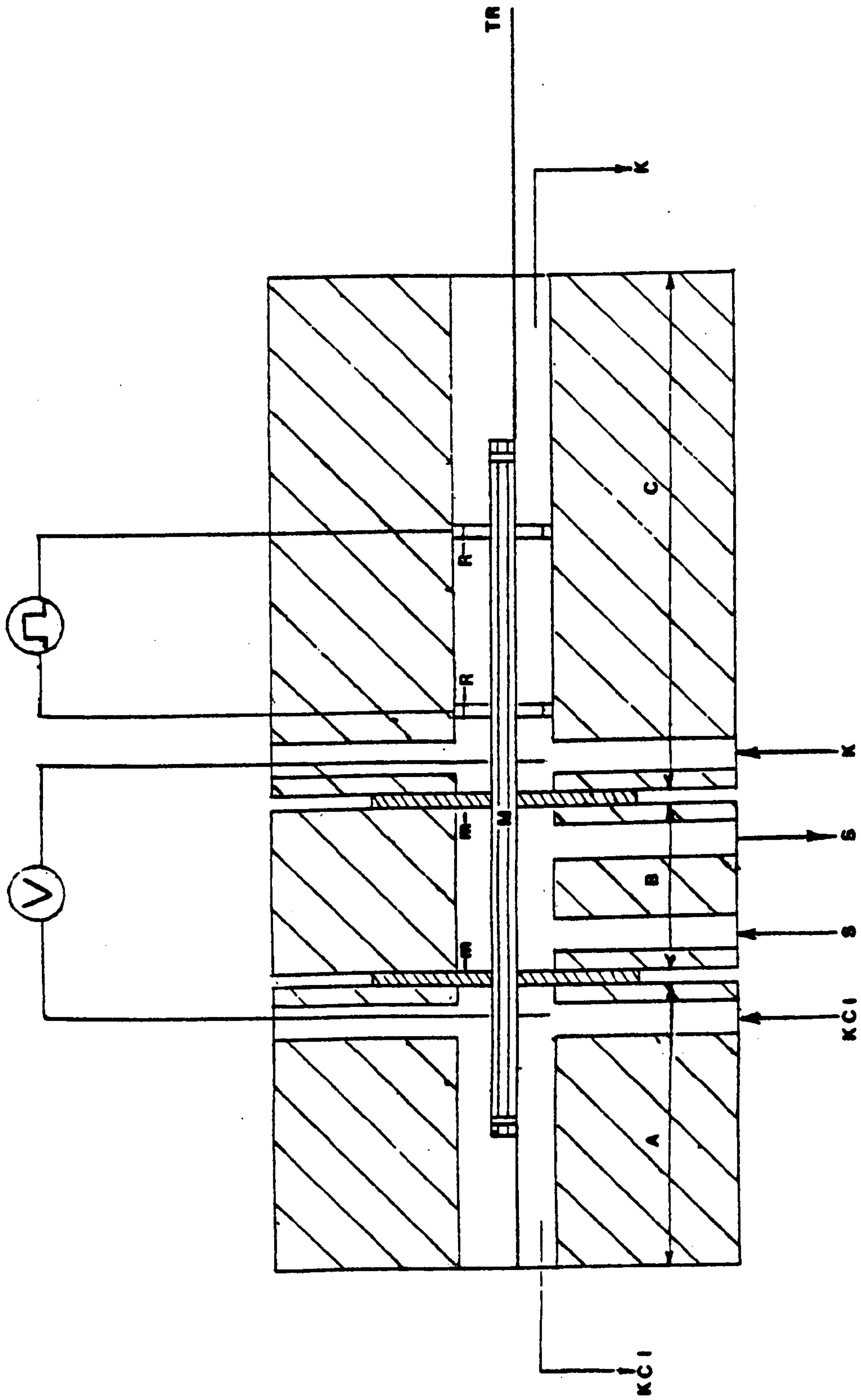
at room temperature. Following incubation, the tissues were washed with Krebs solution before setting up in the organ bath.

EXTRACELLULAR RECORDING BY SUCROSE GAP METHOD.

The use of extracellular electrical recording was prompted by the difficulty in obtaining stable micro-electrode impalements in the bovine retractor penis. This problem is common to other smooth muscles because of the small size of the cells (5-10μm) and has led to the use of the sucrose gap technique (Stämpfli, 1954) for recording from smooth muscle (Burnstock and Straub, 1958). Normally, transmembrane potential changes are effectively short-circuited by the low resistance pathway formed by the external solution. For this reason only a small fraction of the true change in membrane potential can be recorded using external electrodes alone (see Coburn, Ohba and Tomita, 1975), In the sucrose gap, the external resistance is increased by replacement of the normal physiological solution with isotonic sucrose solution of high resistance. Electrical recordings are made from two regions of muscle on either side of a portion bathed in sucrose solution. One of these regions is usually bathed with isotonic KCl to inactivate the muscle and the other is bathed with a physiological (Krebs) solution. The method employed for maintaining a boundary between the different solutions varies (see Bolton, Tomita and Vassort, 1980).

FIGURE 5

Schematic diagram of a cross-sectional view of the membrane-type single sucrose gap. The apparatus is divided by latex rubber contraceptive membranes (m) into three chambers A, B and C made of perspex. Chambers A and B were each perfused with isotonic KCl (150mM) and sucrose (300mM;S) respectively at room temperature. Chamber C was perfused with oxygenated Krebs solution (K). Each solution was supplied via polythene tubing by gravity flow. The polythene tubing (internal diameter 0.25cm) containing the Krebs solution was surrounded by an outer tube (internal diameter 1.0cm) containing liquid paraffin at 40 ± 0.1°C pumped from a thermostatically controlled Tempette pump. The temperature of the Krebs solution at the gap was 37 ± 0.5°C. The muscle (M) passed through small holes (0.03cm) in the membranes. Contact between the different solutions in chambers A, B and C was prevented by the close fit of the membrane around the muscle. Field stimulation (0.1-0.5ms, supramaximal voltage) was effected via Ag/AgCl ring electrodes (R). Changes in potential were recorded via Ag/AgCl wires in contact with the fluid in chambers A and C. Chamber C was earthed. Mechanical responses were measured by an isometric transducer (TR).



TR
K
R
R
C
K
M
m
m
S
B
S
KCl
A
KCl

In the present study, latex contraceptive-rubber membranes were employed. These divided the apparatus into three chambers (Fig. 5), each perfused continuously (0.8-1.2ml/min) with various solutions supplied by gravity flow. Chambers A and B were perfused with isotonic KCl (150mM) and sucrose (300mM) solutions respectively at room temperature. Chamber C, the Krebs chamber, was perfused with oxygenated pre-warmed (37 ± 0.5°C) Krebs solution. The membranes were breached by a small hole (0.03cm diameter) through which the muscle passed. Contact between the different solutions was prevented by the close fit of the membranes around the tissue. Changes in potential were recorded between the KCl and Krebs solution via Ag/AgCl wires. The Krebs side was earthed and the KCl side connected to the input probe of a unity gain pre-amplifier (WPI M4A). Electrical and mechanical responses were recorded as described previously. Field stimulation was carried out via bipolar Ag/AgCl ring electrodes, 1cm apart in the Krebs chamber (Fig. 5) and 0.2cm away from the sucrose chamber, using the apparatus described previously.

APPARATUS: EXTRACELLULAR RECORDING

Two different designs of membrane-type sucrose gaps were used. One was a modification of a prototype given by Dr. Lees (Wallis, Lees and Kosterlitz, 1975), the other was kindly donated by Dr. Mollie Holman. Both designs work on the principle described previously. The apparatus

FIGURE 6

Exploded view of the components of the Holman-type sucrose gap. In single sucrose gap form, the apparatus comprised three main chambers A, B and C. Chambers A and B, the KCl and sucrose chambers respectively, were each made from a single piece of perspex. Chamber C, the Krebs chamber, was made from five individual perspex components including two Ag/AgCl ring electrodes (R) for field stimulation (shown enlarged below their perspex holders) which were mounted in perspex holders. Two latex rubber contraceptive membranes (1cm square), pierced by a small hole (0.03cm) were placed on either side of B to separate the three chambers from each other. The hole in each membrane was aligned with the centre of each chamber. In double sucrose gap form, chamber C was split into a test chamber (T) and a sucrose chamber (D), by inserting another membrane between T and D. Prior to assembly of the apparatus in either single or double sucrose gap form, the mating surfaces of the components were greased (Vaseline + paraffin wax, ratio 1 or 2:1) to prevent leakage. After assembly (b) the components were pressed together by two clamping screws. The muscle was pulled through each chamber and the membranes by a fine thread (8-00 gauge suture), inserted before assembly. Field stimulation in the double gap was carried out via two Ag/AgCl ring electrodes in the test chamber (T). The membrane potential was displaced by passing current via the two Ag/AgCl ring electrodes mounted in chamber D.

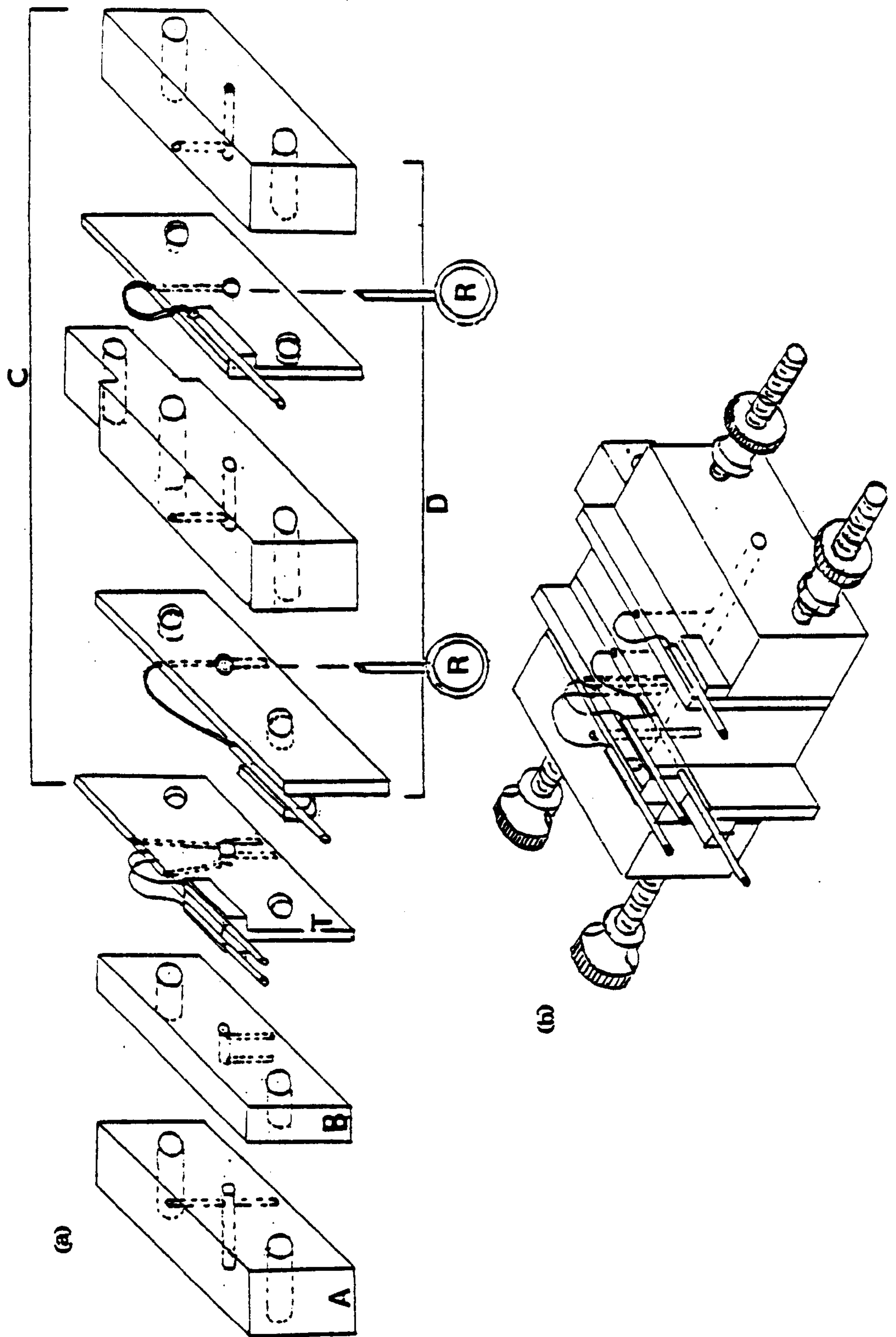
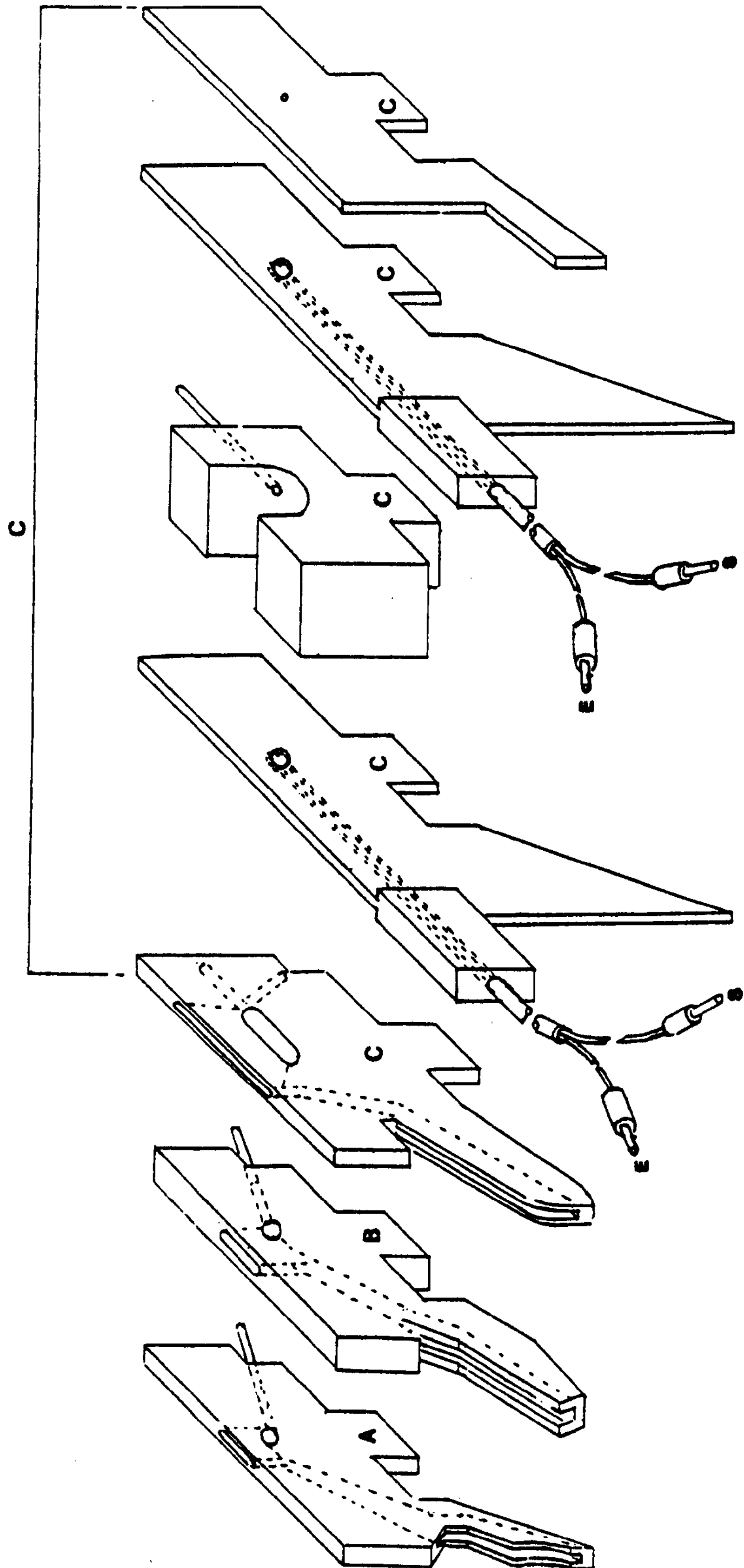

(a)
A
B
T
C
D
R
R
(b)

FIGURE 7

Exploded view of the components of the Wallis, Lees and Kosterlitz-type single sucrose gap. The apparatus comprised three main chambers A, B and C. Chambers A and B, the KCl and sucrose chambers respectively, were each made from a single piece of perspex. Chamber C, the Krebs chamber, was made from five individual perspex components including the Ag/AgCl electrodes for field stimulation, which were mounted in perspex holders. The output from an isolated stimulator was connected to E and the screen (S) to earth. Two latex rubber contraceptive membranes, pierced by a small hole (0.03cm diameter),were placed on either side of B to separate the three chambers from each other. The hole in the membranes was aligned with the centre of each chamber. Prior to assembly, the mating surfaces of the components were greased (Vaseline + paraffin wax, ratio 1 or 2:1) to prevent leakage. After assembly, the components were pressed together firmly by a perspex clamp (Fig. 8). The muscle was pulled through each chamber and the membranes with a fine thread (8-00 gauge suture), inserted before assembly.


C
C
C
C
C
C
B
A
S
E
S
E

comprised three chambers made from perspex and drilled to allow the inflow and outflow of solution (Figs. 6,7). Chambers A and B, the KCl and sucrose chambers respectively, were each made from one piece of perspex. Chamber C, the Krebs chamber, was made up from a number of individual pieces including the ring electrodes for field stimulation which were mounted in perspex. Prior to assembly the mating surfaces of the perspex were coated with grease (Vaseline + paraffin wax, ratio 1 or 2:1) to prevent leakage from the chambers. Also, a fine thread (8-00 gauge suture) was passed through the holes in the membranes and components of the gap. This thread was used after assembly to pull the tissue through the membranes and chambers of the sucrose gap. In the Holman-type gap, the components were clamped together with two screws (Fig. 6b), while the Wallis Lees and Kosterlitz-type employed a separate perspex clamp for this purpose (Fig. 8).

DOUBLE SUCROSE GAP - PRINCIPLE

The double sucrose gap (Fig. 9; Bulbring and Tomita, 1969) allows displacement of the membrane potential by passing current through the muscle membrane. The amplitude of displacement is dependent upon membrane resistance; thus changes in the latter can be measured simultaneously with changes in membrane potential and tension. Current pulses were applied to the muscle via Ag/AgCl ring electrodes mounted in an additional sucrose (300mM) chamber (Fig. 9). Due to the high resistance of the sucrose solution, the

FIGURE 8

Perspex clamp employed to hold together the components of the Wallis, Lees and Kosterlitz-type sucrose gap (Figs. 7,11). Each component slotted firmly into a channel cut into the clamp. The components were pressed together by the block (B) which was pushed by turning the screw (Sc). The whole assembly was held rigid by a brass support bar (S).

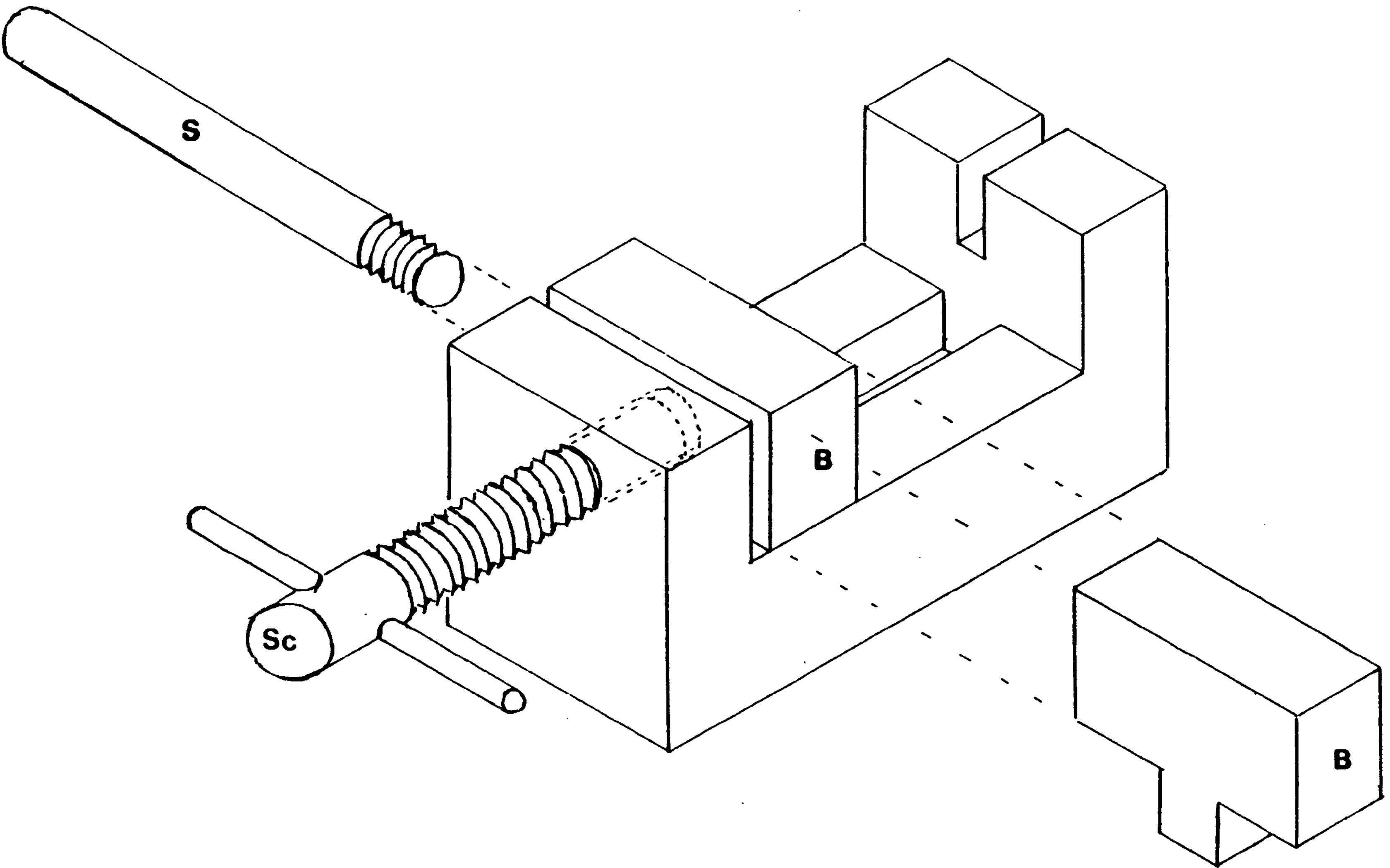
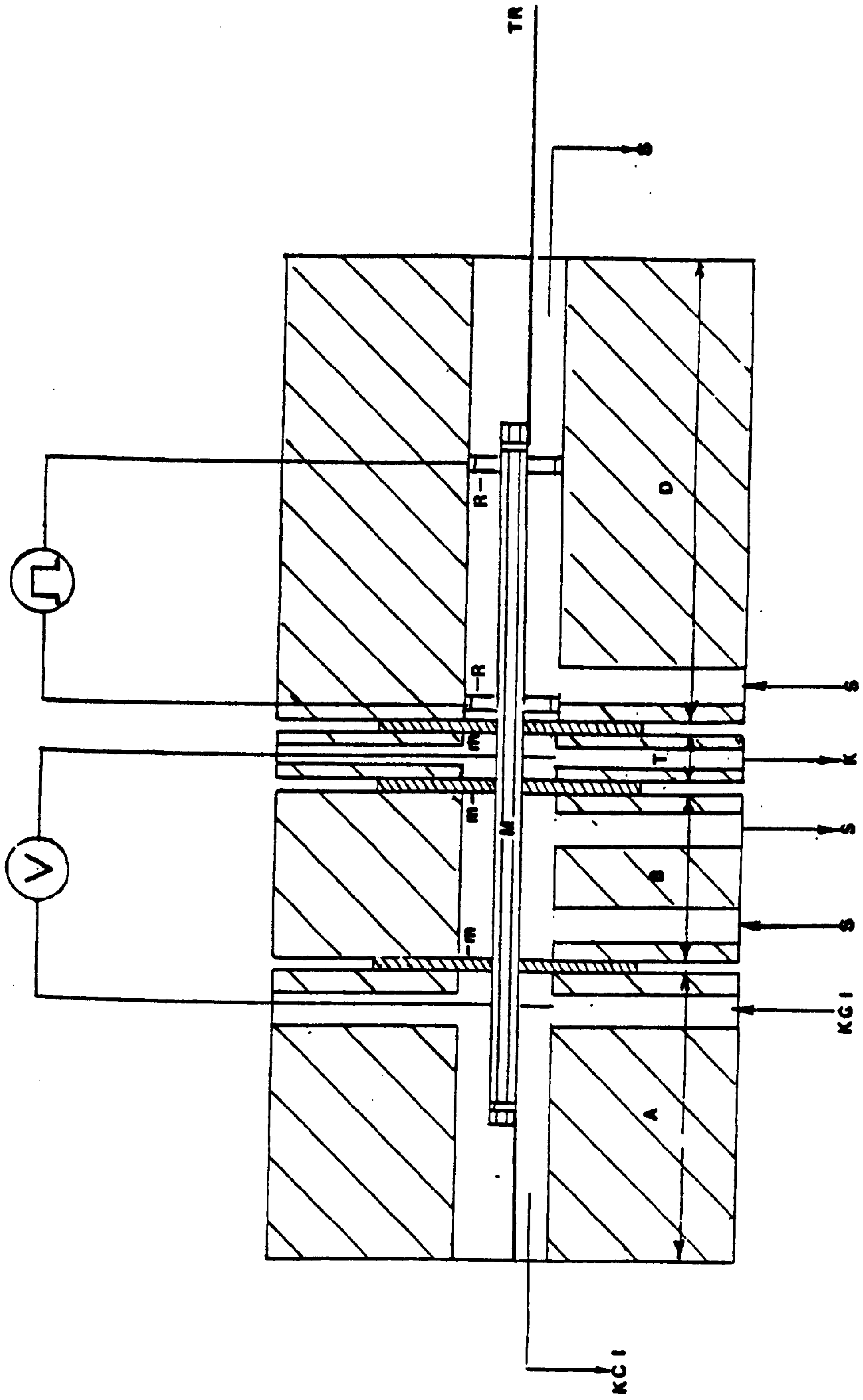

S
B
Sc
B

FIGURE 9

Schematic cross-sectional view of the membrane-type double sucrose gap. The apparatus is divided into four chambers A, B, T and D, separated by latex rubber contraceptive membranes (m). Chambers A and B were perfused with isotonic KCl (150mM) and sucrose (300mM; S) solutions respectively at room temperature. Chamber T was perfused with oxygenated Krebs solution (K) pre-warmed to 37 ± 0.5°C by the liquid paraffin heating jacket, described previously (see legend, Fig. 2). Chamber D was perfused with isotonic sucrose (300mM) solution at room temperature. Each solution was supplied by gravity flow. The muscle (M) passed through small holes (0.03cm diameter) in the membranes. Contact between the different solutions in chambers A, B, T and D was prevented by the close fit of the membrane around the tissue. Only a small portion of muscle (0.15cm length) was exposed to Krebs solution. Field stimulation (0.1-0.5ms, supramaximal voltage) was effected via bipolar Ag/AgCl electrodes (not shown on diagram) in chamber T. The membrane potential was displaced by applying current to the muscle via two Ag/AgCl ring electrodes (R) in Chamber D. Changes in potential were recorded via Ag/AgCl wires in contact with the fluid in chambers A and T. Chamber T was earthed. Mechanical responses were measured by an isometric transducer (TR).


TR
S
R
D
K
T
M
m
B
KCl
A
KCl

current passes mainly through the tissue (see Coburn et al, 1975; Szurszewski, 1974). A biphasic converter (Fig. 10) was used to provide pulses of alternating polarity. Only a small portion of tissue (0.15cm) was exposed to Krebs solution (at 37 ± 0.5°C) in the test chamber (T). Contact between the solutions in chambers T and D was prevented by a latex rubber membrane. Changes in potential were recorded between the KCl and Krebs solution via Ag/AgCl wires, as described previously for the single sucrose gap. Field stimulation was effected by means of bipolar Ag/AgCl electrodes in chamber T.

APPARATUS: DOUBLE SUCROSE GAP

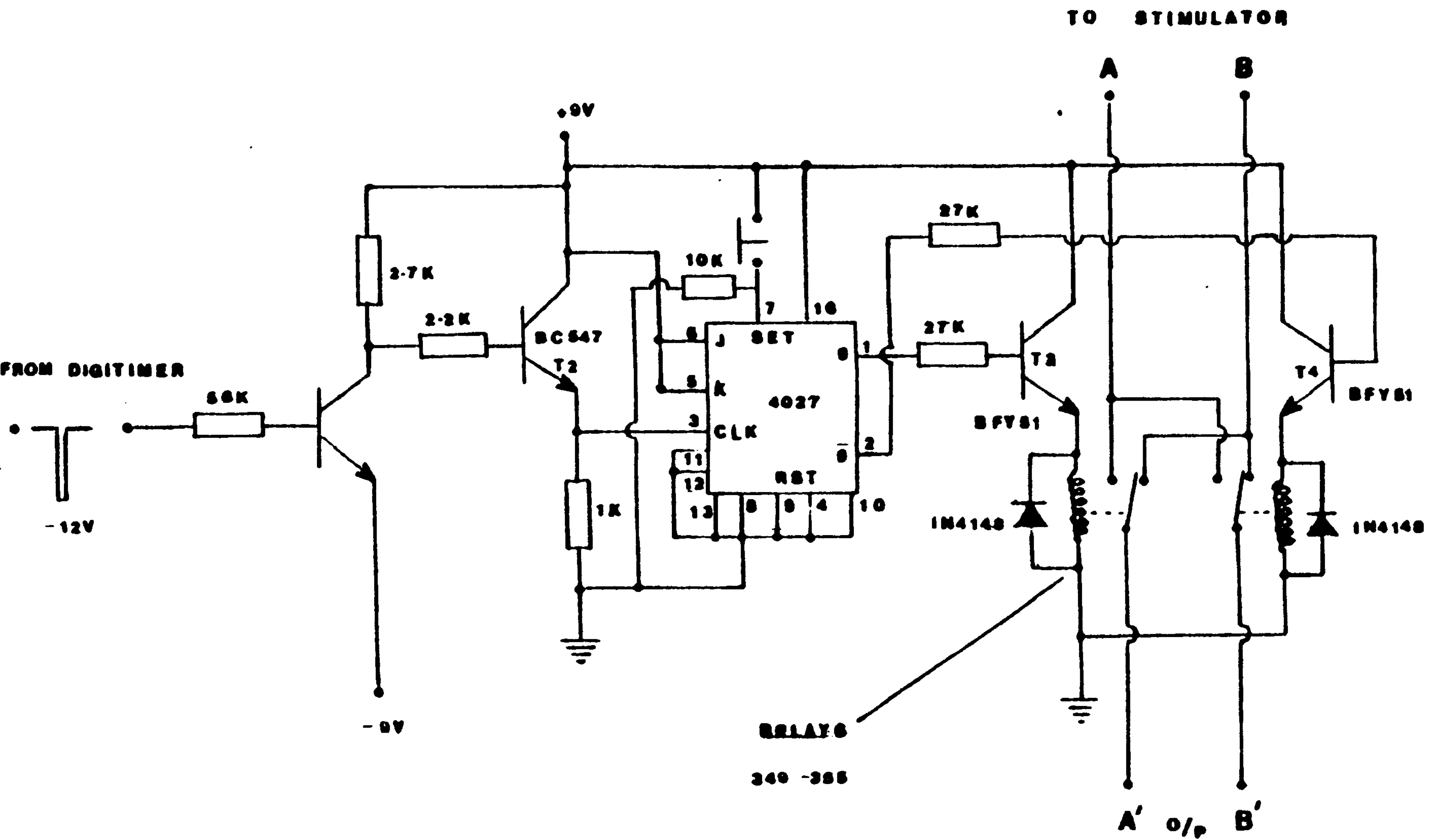
The Holman-type double sucrose gap employed the same components as the single gap version (Fig. 6), except that chamber C was divided by a membrane to give two chambers, T and D. Field stimulation was effected via two Ag/AgCl ring electrodes 0.15cm apart at each end of the test chamber.

The Wallis, Lees and Kosterlitz-type double sucrose gap (Fig. 11) employed different components to the single gap version. The Krebs inlet and outlet of the test chamber consisted of grooves cut into the perspex. Field stimulation was effected by means of two flattened Ag/AgCl wires mounted on either side of the muscle in the test chamber.

FIGURE 10

Circuit diagram of the biphasic converter employed for alternately changing the polarity of the current pulses applied in the double sucrose gap. The output of an isolated stimulator was connected to AB. Both the stimulator and biphasic converter were triggered simultaneously at the required frequency (1 pulse every 6-20s) by a Digitimer. The output A'B' was connected to Ag/AgCl ring electrodes in the sucrose chamber of the double sucrose-gap.

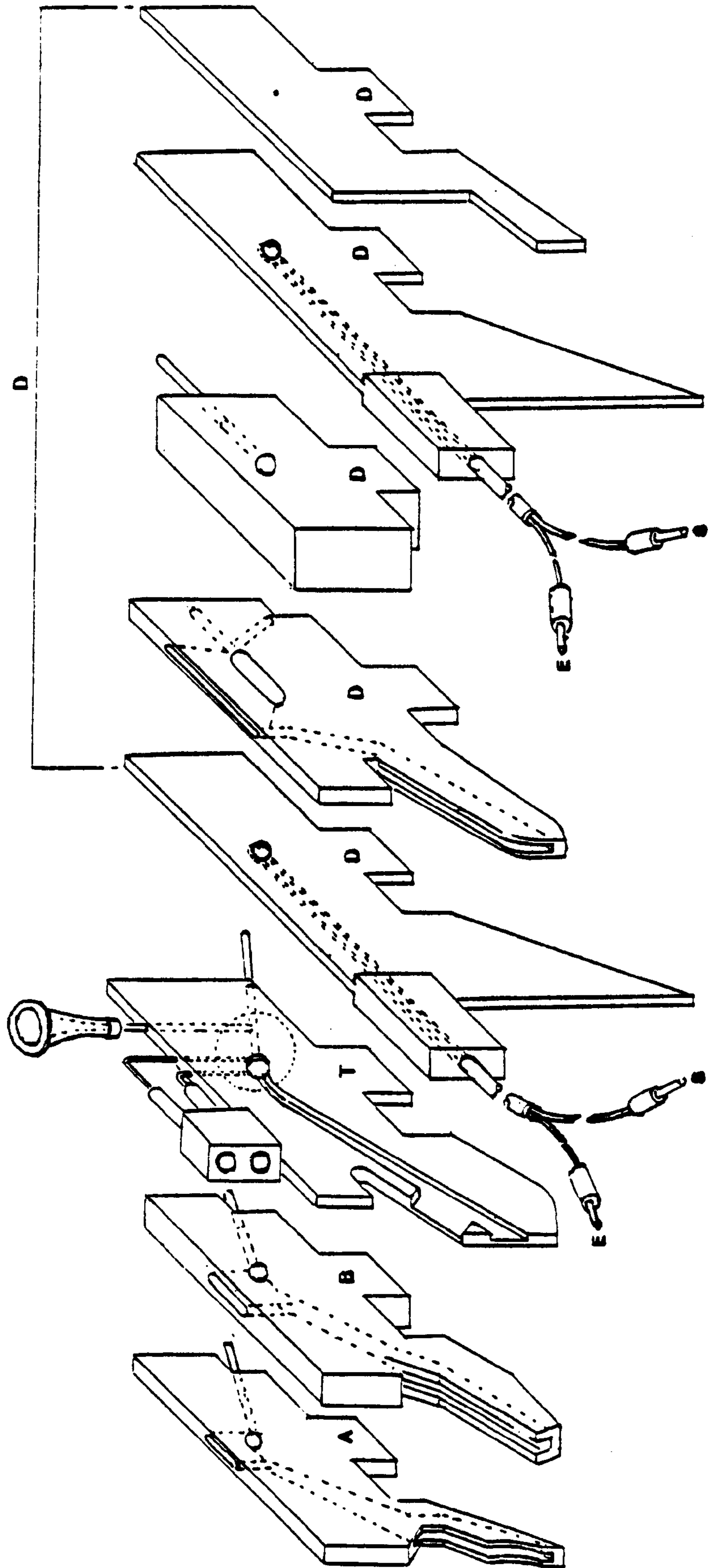
K = Kilo-ohms, T = transistor, ⧓ = diode



70

FIGURE 11

The components of the Wallis, Lees and Kosterlitz-type double sucrose gap. The apparatus comprised four main chambers A, B, T and D. Chambers A and B, the KCl and sucrose chambers respectively, were each made from a single piece of perspex and were also part of the single sucrose gap (Fig. 7). The test chamber (T) was also made from a single piece of perspex in which inlet and outlet grooves had been cut in order to allow perfusion with Krebs solution. Chamber D, the second sucrose chamber, was made of five individual components. These included two Ag/AgCl ring electrodes mounted in perspex through which current was applied to displace the membrane potential. Three latex rubber contraceptive membranes, each pierced by a small hole (0.03cm diameter), were placed on either side of B and between T and D to separate the four chambers from each other. Prior to assembly, the mating surfaces of the components were greased (Vaseline + paraffin wax, ratio 1 or 2:1) to prevent leakage. After assembly the components were pressed together firmly with a perspex clamp (Fig. 8). The muscle was pulled through each chamber and the membranes with a fine thread (8-00 gauge suture), inserted before assembly. Field stimulation was carried out via two flattened Ag/AgCl wires in the test chamber (T).


D
D
D
D
D
D
E
T
E
B
A

PREPARATION OF MEMBRANES FOR SUCROSE GAP RECORDING

Unrolled condoms were washed in propan-2-ol and rinsed in water to remove lubrication. After drying with paper tissues, each contraceptive rubber was coated with chalk to aid handling. With the Wallis, Lees and Kosterlitz sucrose gap, a sheet of membrane (1cm width) was cut and used to cover the mating surfaces of each chamber. The Holman-type sucrose gap used a smaller piece of membrane (1cm square) between each chamber. Holes were punched in line with the centre of each chamber using a punch made from a 27 gauge needle. To facilitate the making of holes, the punch was applied to the membrane through a holder (Fig. 12), which held the membrane flat against a piece of card. The holes were inspected under a microscope (x 40) to ensure that they were uniform and free from ragged edges which might interfere with the seal between the tissue and the membrane. A fine stiff wire passed through the punch removed any adhering material.

MAKING THE MEMBRANE PUNCH

Punches were made from stainless steel tubing 1-2cm long. Various gauges of tubing (36-27) were tested, the most suitable for the bovine retractor penis being 27 gauge. The end of the tube was ground flat using an oil-stone. To ensure that the flattened end was perpendicular to the wall of the tube, a clamp (designed by T. Clark, Fig. 13a) was used to hold it in contact with the oil-

FIGURE 12

Apparatus used to cut holes in contraceptive rubber membranes for sucrose gap recording. The membrane (M) was held flat by pressing it between two pieces of perspex, the upper of which was drilled to allow access of the punch (P). The punch, held in a pin-chuck, was applied lightly to the membrane (M) and rotated to produce a clean hole. A piece of card placed underneath the membrane prevented blunting of the punch.

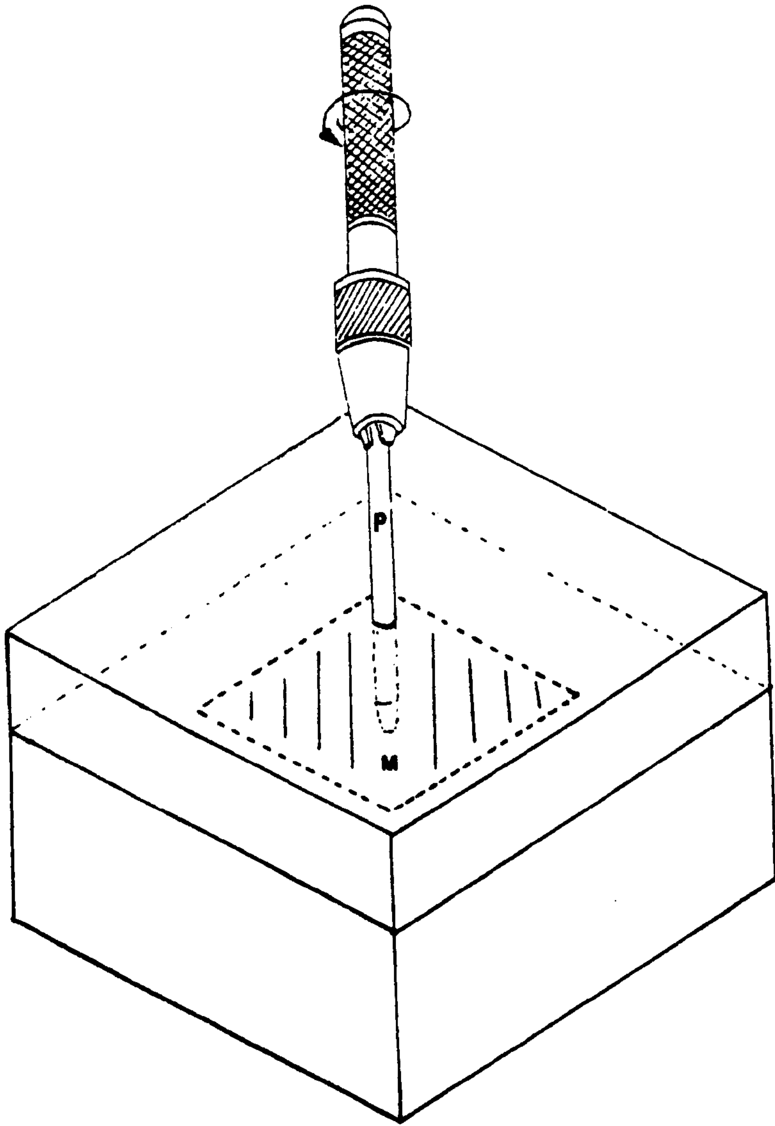
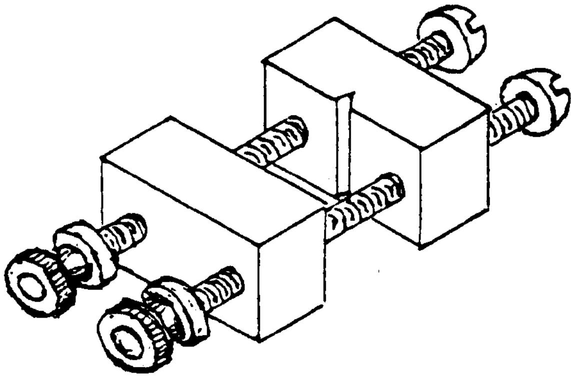
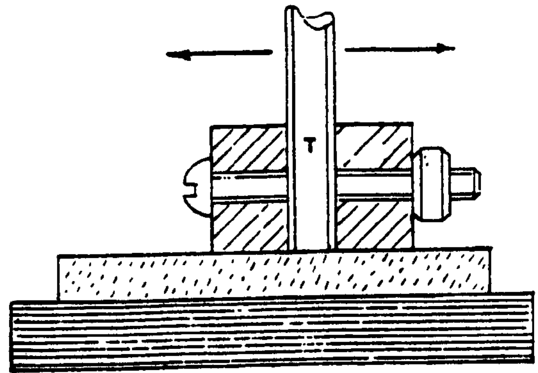

P
M

FIGURE 13

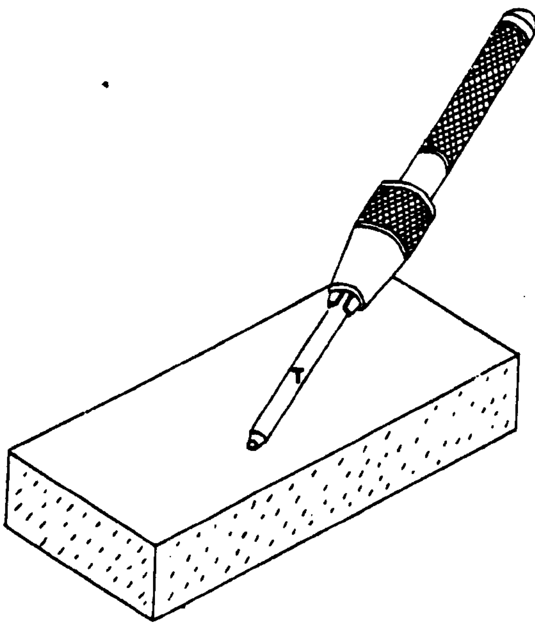
Apparatus used to make membrane punches. A piece of stainless steel tubing (T, 27 gauge, internal diameter 0.025cm), gripped in a purpose-made clamp (a), was ground flat at one end using an oil-stone (b). The outer edge of the flattened end was bevelled by drawing the tubing, held in a pin-chuck, lightly across an oil-stone. At the same time, the tubing was rotated (c). Finally, the inner edge of the tubing was sharpened (d) using a watchmakers' broach (B) to produce the finished punch, shown enlarged in cross-section (e).



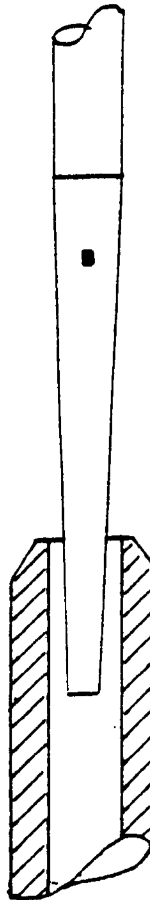
a



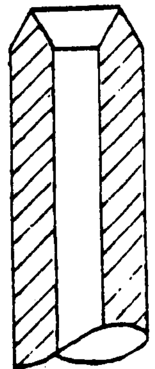
b



c



d



e

stone (Fig. 13b). A sharp cutting edge was formed by bevelling the internal and external walls of the tube such that they met to form a point. The outer cutting edge was bevelled by drawing it lightly across an oil-stone, the tube being held in a pin-chuck (Fig. 13c) and rotated at the same time. The internal diameter was then increased by sharpening it with a watchmakers' broach (Fig. 13d). These two procedures were repeated until a sharp, uniform cutting edge of the correct diameter (approximately 0.03cm) was produced (Fig. 13e).

PREPARATION OF THE INHIBITORY EXTRACT OF THE BOVINE RETRACTOR PENIS MUSCLE

The inhibitory extract was prepared in advance by staff in Professor Gillespie's laboratory. Since the method of preparation has been modified on several occasions, that used during the present investigation is described.

Bovine retractor penis muscles, free of connective tissue, were weighed, minced (Moulinex domestic mincer) and extracted in ice-cold (0-4°C) methanol (5ml/g wet weight of tissue) for a period of 30 min. At the beginning of the extraction and thereafter at 15-min intervals, the mixture was stirred vigorously (MSE stirrer) for 2-5 min and then filtered (Whatman No. 1). The filtrate was treated by one of the following methods.

Method I

The filtrate was pumped (2-3ml/min) through an anion exchange resin (Bio-Rad AG1 x 8 formate form, 200-400 mesh) at 0-4°C in a cold room by a Watson-Marlow flow inducer. The effluent was discarded and the column washed with distilled water (10ml). The column was then eluted with 500mM NaCl (25ml), the first 5ml of effluent being discarded and the rest (containing the inhibitory extract) divided equally among 10 glass ampoules in which it was freeze-dried overnight. A small amount of silica gel in the neck of each ampoule kept the extract dry. Each tube was sealed and stored at -30°C.

Method II

The filtrate was put into centrifuge tubes, chilled in liquid N_2 and spun for 6 min at 4000 rpm. The supernatant was filtered through a Millipore filter (0.45µm). The rest of the procedure was exactly as described in method I.

Reconstitution of the extract

Freeze-dried extracts were reconstituted in distilled water on ice at 0-4°C. For sucrose gap studies, the contents of two or three ampoules (usually some 12g) were dissolved in 2.5-3.0ml distilled water. For intracellular studies, in which the maximum addition volume was greater, the extract was made up to a concentration equivalent to

2g wet weight tissue/ml. In both cases the resulting solution was adjusted to pH 9.0 by adding NaOH (1N), and passed through an alumina column at 0-4°C to remove adenine nucleotides (Bowman, Gillespie and Martin, 1979). This procedure is referred to as 'cleaning' and the effluent as 'cleaned extract'. The concentration of adenine nucleotides present before and after cleaning was determined spectrophotometrically at 260nm by reference to a standard curve.

The cleaned extract, which contained the inhibitory factor in unactivated form (Gillespie et al, 1981), was divided into two parts. One part was adjusted to pH 7.0-7.4 by adding HCl (1N) and this comprised cleaned unactivated extract (CUE). The other part was adjusted to pH 2.0 by adding HCl (1N) and kept for 10 min on ice (0-4°C). This process was termed 'activation' and the product 'cleaned activated extract' (CAE). After activation the pH was readjusted to 7.0-7.4 by the addition of NaOH (1N). Both forms of the extract (CUE and CAE) were kept at 0-4°C on ice before use. The CUE served as a control for the activated form. On some occasions, the electrical and mechanical activity of uncleaned extract was investigated.

DRUGS

The following drugs were used: Apamin (Uniscience), adenosine triphosphate (ATP, Boehringer), atropine sulphate

(BDH), collagenase (Sigma), guanethidine sulphate (Ciba), indomethacin (Sigma), noradrenaline bitartrate (Koch-Light), oxyhaemoglobin (prepared from human red blood cells by Dr. D. Pollock containing glucose 2% dry weight to act as stabiliser), phentolamine mesylate (Ciba), physostignine salicylate (BDH), prazosin hydrochloride (kindly donated by Pfizer), propranolol hydrochloride (ICI), 2-o-propoxyphenyl-8-azapurin-6-one (M and B 22948, kindly donated by Dr. A. Drummond), sodium nitroprusside (BDH), tetraethylammonium bromide (Koch-Light) and tetrodotoxin (Sankyo).

With the following exceptions, drugs were dissolved in saline (0.9%) prior to their addition to the organ bath or sucrose gap. Collagenase was dissolved in Krebs solution, indomethacin in sodium carbonate solution (0.09M), M and B 22948 in sodium hydroxide (approximately 0.01M) noradrenaline in saline with ascorbic acid (2×10^{-4}M) and ethylene diamine tetra-acetic acid (3×10^{-5}M) to prevent oxidation of the catecholamine. Oxyhaemoglobin and prazosin were each dissolved in distilled water.

ADDITION OF DRUGS AND INHIBITORY EXTRACT

Antagonist drugs were added to the Krebs solution perfusing the organ bath or sucrose gap unless otherwise stated. Inhibitory extract and agonist drugs, which included adenosine triphosphate, noradrenaline and sodium nitroprusside, were added by injection into the organ bath

or perfusion flow to the sucrose gap. The maximum volume added, some 200μl in intracellular studies or 20μl in sucrose gap studies, was limited by the sensitivity of the tissue to the injection of cold solutions. In the sucrose gap, the injection volume was further limited by artefactual electrical changes (usually an apparent depolarisation) caused by injecting volumes in excess of 20μl into the perfusion flow. The cause of this effect was the change in the perfusion rate produced by the injection.

EXPRESSION OF INTRA- AND EXTRACELLULAR RESULTS

The results obtained with intra- and extracellular recording were qualitatively similar. The terms 'excitatory' and 'inhibitory' potentials refer to responses to field stimulation using either method of recording. Excitatory (ejps) and inhibitory (ijps) junction potentials refer respectively to intracellularly-recorded events. The values of resting membrane potential, amplitude, latency, rise time and time to decay to half-amplitude were obtained from intracellular recordings. Results are quoted as means ± standard deviation. Statistical significance was evaluated using the Student's t-test or a paired t-test. P values of <0.05 were considered to be significant.

CHAPTER 3

RESULTS

BOVINE RETRACTOR PENIS: RESTING ACTIVITY AND EXCITATION

RESTING ACTIVITY IN THE BOVINE RETRACTOR PENIS

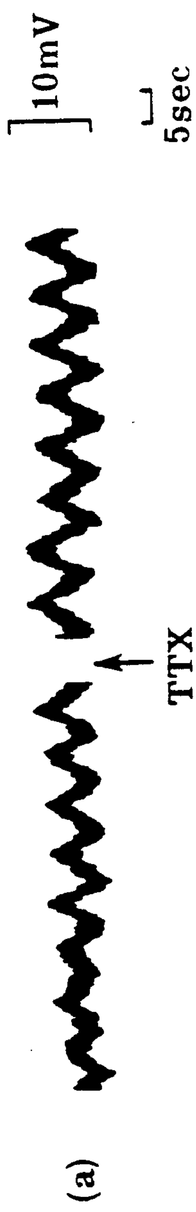
When first set up the muscle had no resting tone. Under these conditions the resting membrane potential was -53 ± 7mV (n=184, range -42 to -70mV). Tone developed spontaneously and was accompanied by a significant ($p < 0.001$) depolarisation of the cells to a membrane potential of -45 ± 5mV (n=92, range -37 to -56mV).

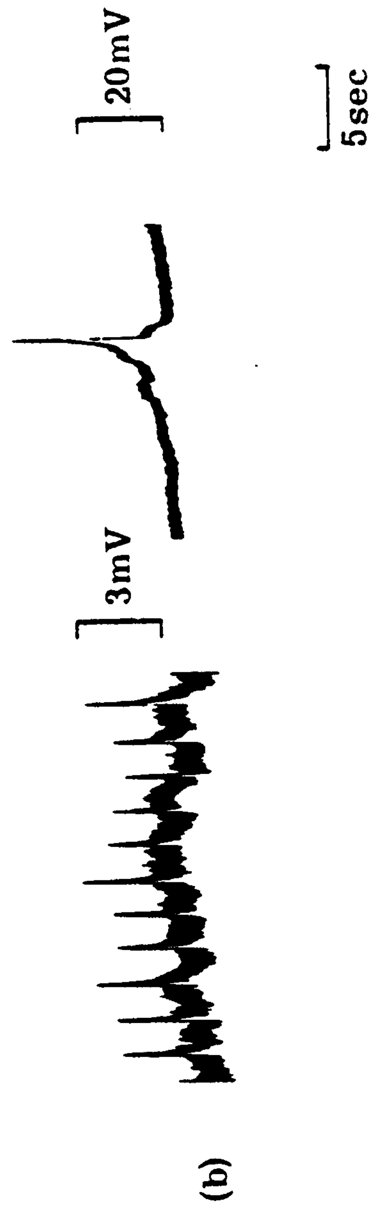
Most of the preparations were electrically quiescent, independent of the amount of resting tone. However, in about 25% of preparations, spontaneous electrical activity was observed. This took various forms; the most common, recorded both intra- and extracellularly, was an intermittent oscillation in membrane potential of 2-15mV and a periodicity of 10-15s unaccompanied by action potentials but often associated with corresponding fluctuations in tone. Oscillations were unaffected by tetrodotoxin (TTX, 3×10^{-6}M), indicating their myogenic origin (Fig. 14a).

Spontaneous ejps (Fig. 14b) were seen infrequently (less than 1% cells impaled). Their amplitude ranged from 1-2.5mV (mean 2.0 ± 0.4mV), the time to peak was 119 ± 25ms, and the time to decay to half amplitude 121 ± 25ms (n=23 in each case). On one occasion, a spike potential of some 40mV was seen (Fig. 14b).

FIGURE 14

Spontaneous electrical activity in the bovine retractor penis recorded intracellularly. (a) Tetrodotoxin (↑TTX, 3×10^{-6}M for 7 min)-resistant rhythmic oscillations in membrane potential (from the same cell). (b) Excitatory junction potentials and spike (different cells). (c) Inhibitory junction potentials at different times in same cell; time between panels 3 min. (a), (b) and (c) were recorded from different preparations.


(a)
10mV
5sec
TTX


(b)
20mV
3mV
5sec

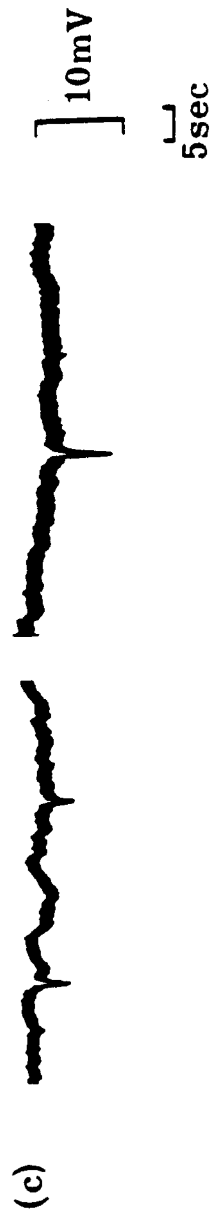
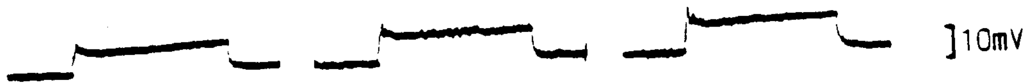
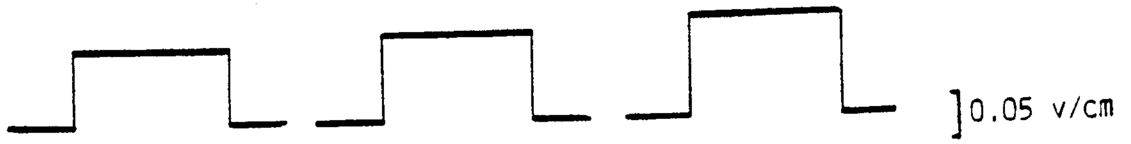

(c)
10mV
5sec

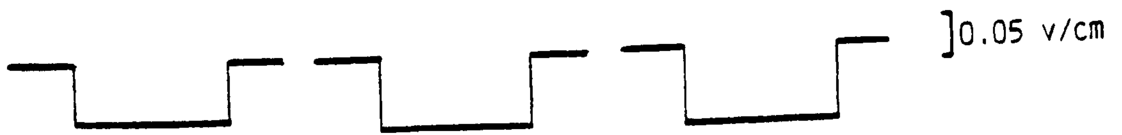
FIGURE 15

Electrotonic potentials (upper trace) recorded from one cell in the bovine retractor penis in response to outward (upper panel) and inward (lower panel) current pulses (duration 1s) of different intensities (V/cm, lower trace) using the method of Abe and Tomita (1968). The amplitude of the membrane potential change recorded comprised the electrotonic potential together with the voltage change caused by the current spread from the current-passing compartment. Accordingly, the true electrotonic potential was represented by the difference between these values (Bywater and Taylor, 1979). Spikes were not observed even in response to large outward current pulses.





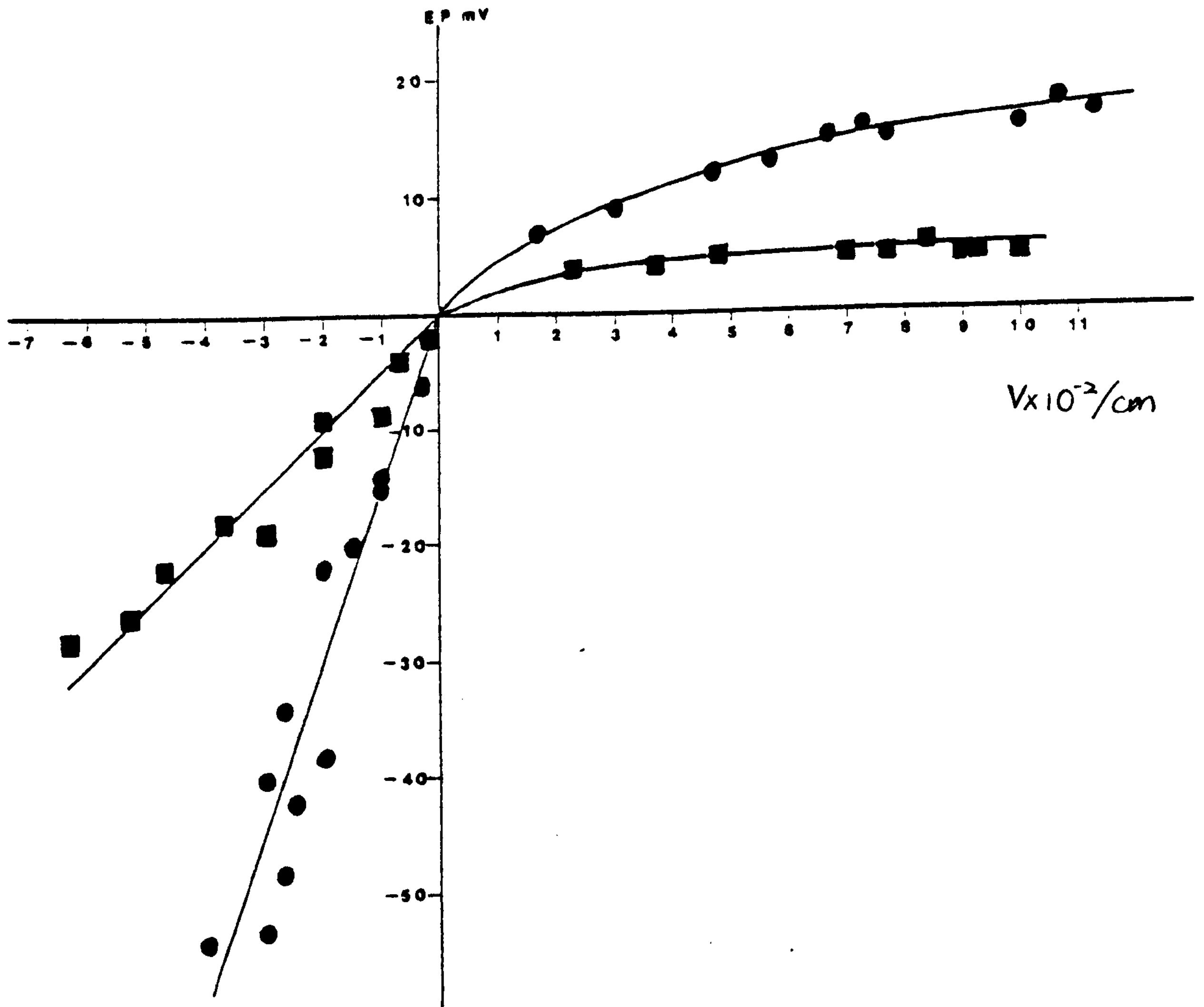
0.2 sec



0.2 sec

FIGURE 16

Current (V/cm) voltage (E.P. mV) relationships recorded by the method of Abe and Tomita (1968) from two cells in the bovine retractor penis preparation at different distances from the stimulating plate (●——●, 0.3mm; ■——■, 0.5mm) in response to inward and outward current pulses (duration 1s). Rectification was apparent with outward current. In response to inward current, the membrane behaved as an ohmic resistor.


EP mV
20
10
-10
-20
-30
-40
-50
-7
-6
-5
-4
-3
-2
-1
1
2
3
4
5
6
7
8
9
10
11
Vx10^-2/cm

Spontaneous ijps were also infrequent (less than 1% cells impaled). These could reach 8mV (mean 3.0 ± 2.0mV, n=26), their time to peak was 333 ± 219ms, and the time to decay to half amplitude 410 ± 160ms (n=13, Fig. 14c).

PASSIVE MEMBRANE PROPERTIES OF THE BOVINE RETRACTOR PENIS

Recording of electrotonic potential

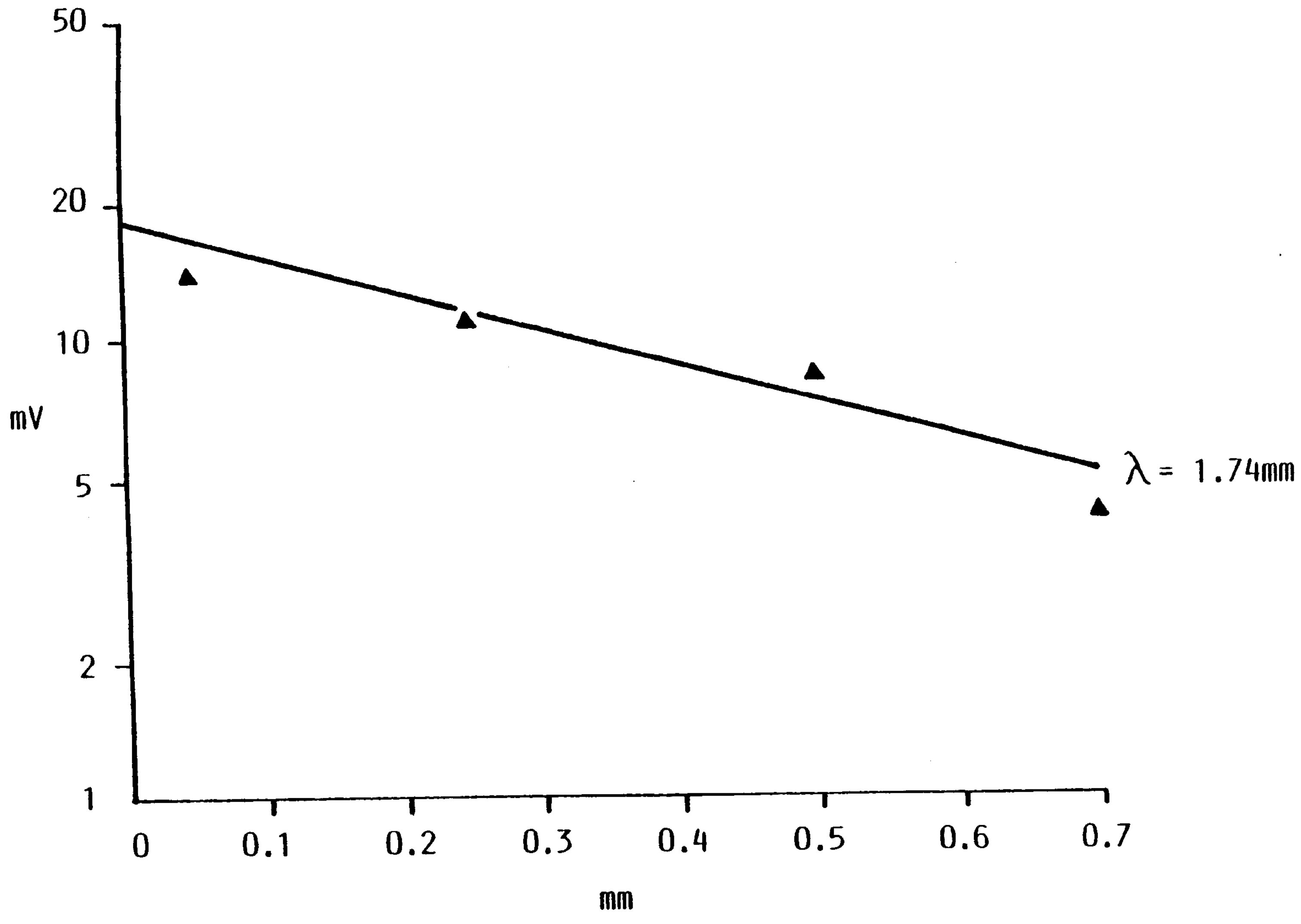
The electrical changes recorded intracellularly in response to inward (hyperpolarising) or outward (depolarising) current pulses (1-2s duration) comprised the electrotonic potential together with the voltage change caused by the spread of current from the current-passing compartment (Bywater and Taylor, 1980). The amplitude of the electrotonic potential was obtained by subtracting the potential change recorded outside from that recorded inside the cell.

Voltage current relationship

The steady-state electrotonic potentials evoked by outward and inward current pulses of different intensities are shown in Figure 15. Spikes were not observed in response to outward current pulses, even when large currents were used. In response to inward current pulses, the membrane behaved as an ohmic resistor, there being no evidence of non-linearity (Fig. 16). Rectification was apparent with outward current pulses (Fig. 16).

FIGURE 17

Decay of the electrotonic potential (mV) with distance (mm) from the stimulating plate recorded by the method of Abe and Tomita (1968) from four cells in the bovine retractor penis at different distances from the stimulating plate. The current intensity was constant throughout. The amplitude of the electrotonic potential decayed exponentially with distance from the plate. The space constant (λ = 1.74mm) was obtained from the slope of the semi-log plot.


50
20
10
mV
5
2
1
λ = 1.74mm
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
mm

Space constant (λ)

Electrotonic potentials in response to inward current pulses were recorded at distances of up to 4mm from the stimulating plate, indicating that the muscle possesses cable properties. The amplitude of the electrotonic potential decayed exponentially with distance from the plate(Fig. 17). The space constant (λ) was obtained from the slope of the semi-log plot of amplitude of electrotonic potential against distance from the plate (Fig. 17). λ ranged from 1.3-2.1mm with a mean of 1.7 ± 0.3mm (n=7).

Time constant (τ)

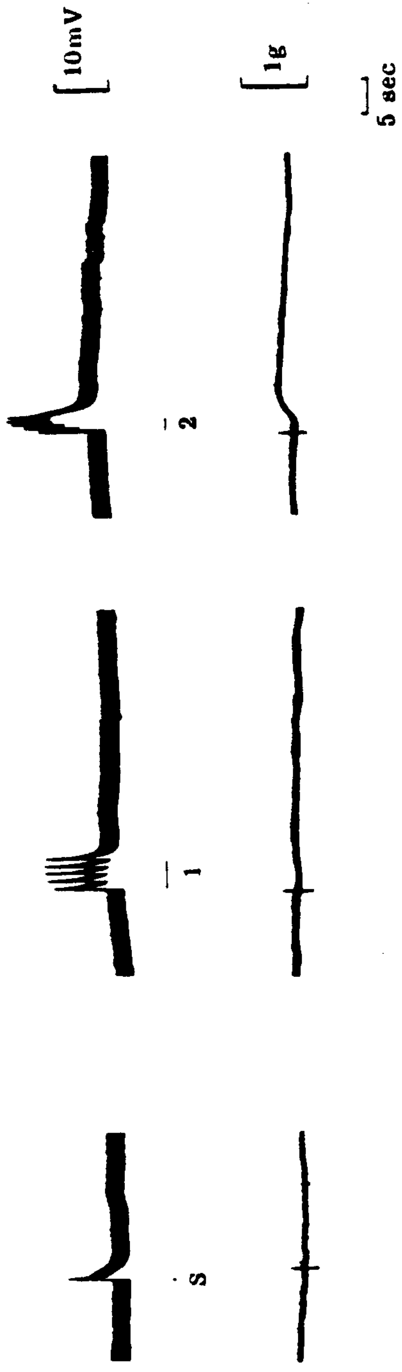
The membrane time constant was obtained from a plot of time for the electrotonic potential to reach 50% steady-state against distance from the stimulating plate. The slope of this plot approaches $\tau m/2\lambda$ when the distance increases and when the length of tissue between the plates is greater than 3λ (Bywater and Taylor, 1980). Thus, τm (slope x 2λ) was obtained by substitution of the value of λ. Values for τm showed a marked variation from 160-450ms with a mean of 296 ± 113ms (n=6).

EXCITATORY RESPONSES TO FIELD STIMULATION OF THE BOVINE RETRACTOR PENIS

In the absence of tone, field stimulation (0.1-0.5ms supramaximal voltage) produced excitatory potentials and contractions. Single stimuli evoked excitatory potentials

FIGURE 18

Excitatory potentials (upper trace) and contractions (lower trace) to field stimulation (single pulse at S and 5 pulses at 1 to 20Hz as indicated below bars) of the bovine retractor penis. Excitatory potentials summated at frequencies of 2Hz or above. Optimal frequency was about 10Hz. Spikes were not observed in response to either single or trains of stimuli. Sucrose gap recording: supramaximal voltage, 0.5ms pulse width.


10mV
1g
5 sec
S
1
2

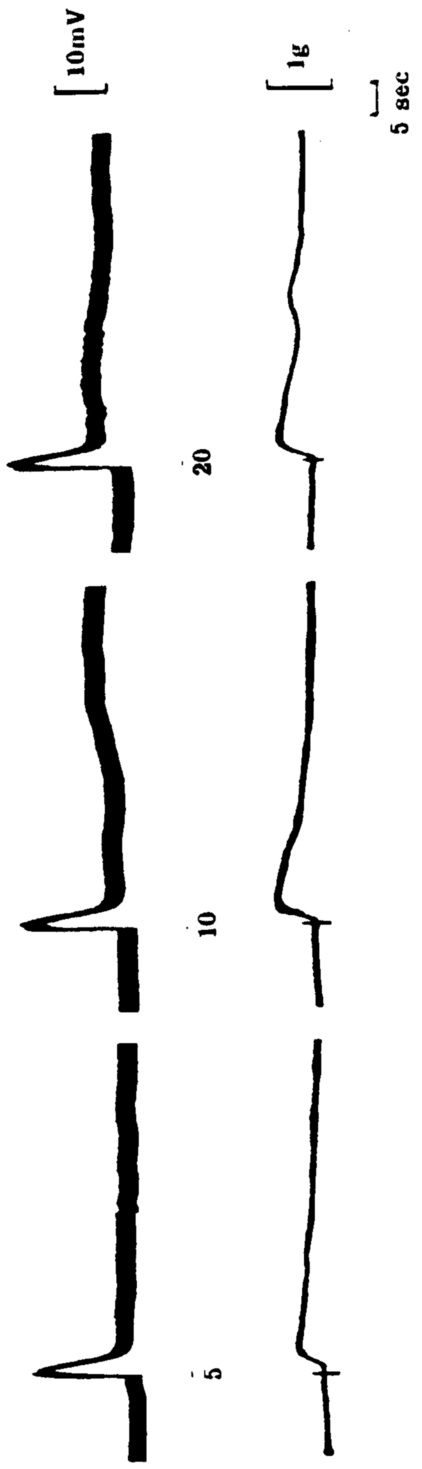
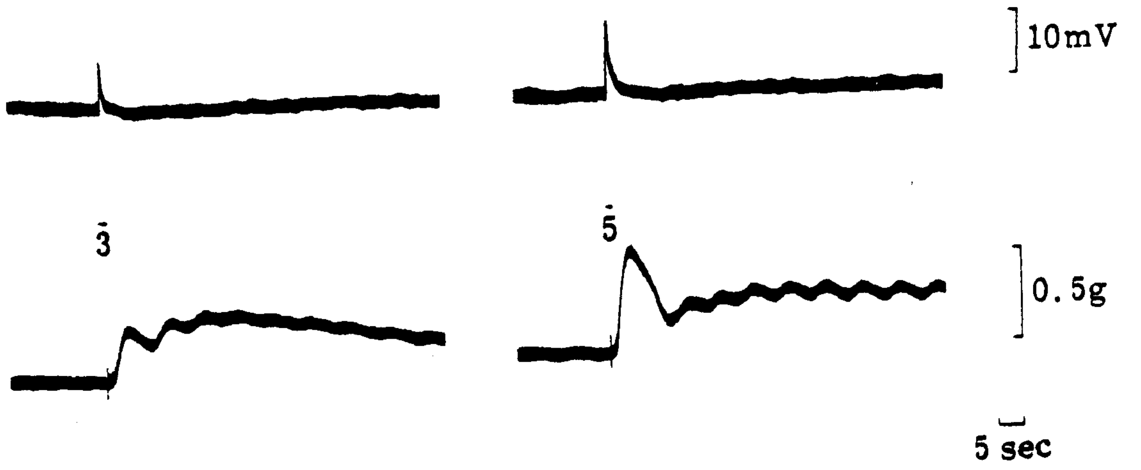

10mV
1g
5 sec
5
10
20

FIGURE 19

Excitatory potentials (upper trace) recorded from a single cell and contractions (lower trace) of the bovine retractor penis to field stimulation (supramaximal voltage; 0.5ms pulse width; 3 to 10 pulses, 10Hz as indicated below bars). Excitatory potentials and contractions were graded with increasing numbers of pulses. Spikes were not observed. Loss of impalement is shown by the sharp vertical deflection in the lower right panel.


10mV
3
5
0.5g
5 sec

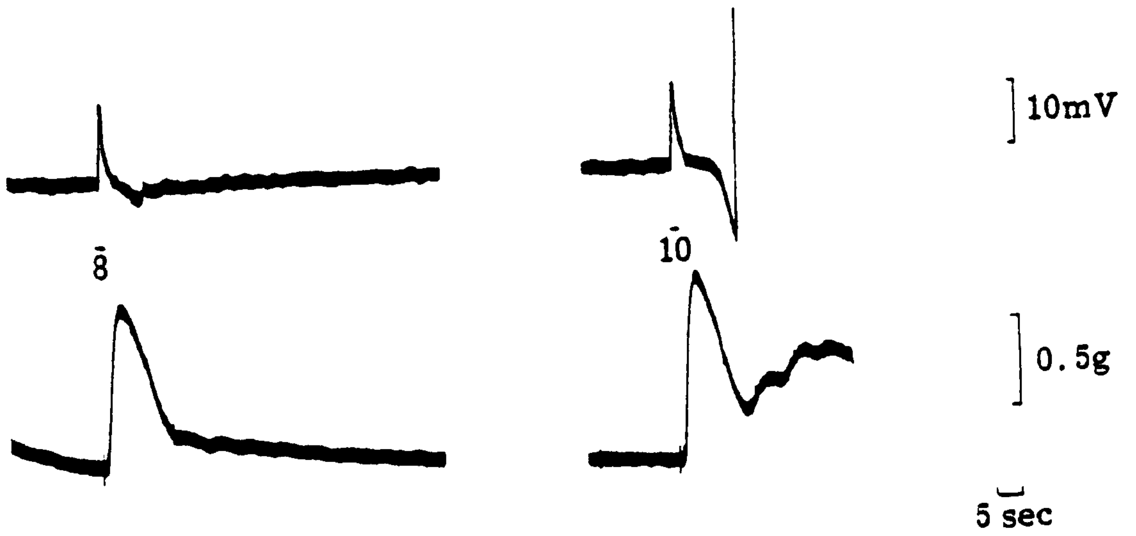
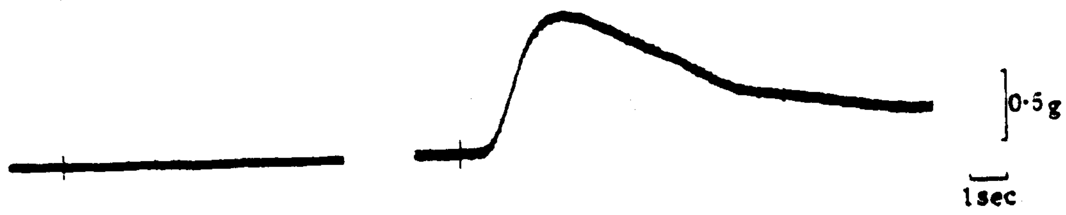
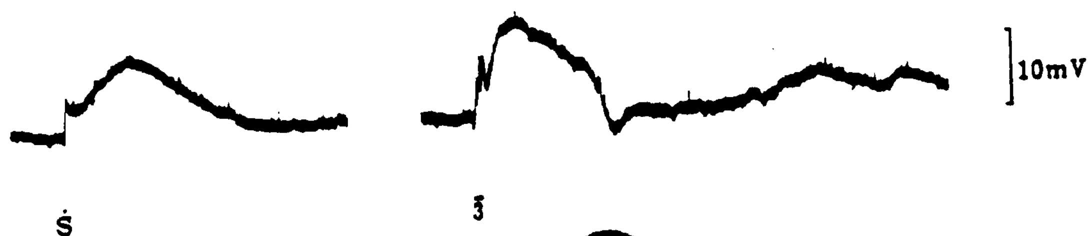

10mV
8
10
0.5g
5 sec

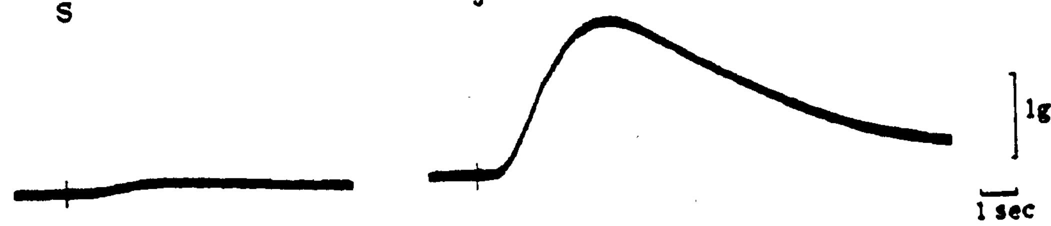
FIGURE 20

Variation in the duration of the excitatory potential (upper trace) to field stimulation (single pulse at S and three at 10Hz) and the accompanying contraction (lower trace) recorded from two different bovine retractor penis preparations (upper and lower panels). The duration of the excitatory potential varied from a brief depolarisation which reached a maximum during field stimulation (upper panels), to a prolonged depolarisation reaching a peak after the cessation of field stimulation. Intracellular electrical recording: supramaximal voltage, 0.5ms pulse width.

10mV
S
3


0·5g
1sec


10mV
S
3


1g
1 sec

which could reach 7mV (mean 3.5 ± 2mV, n=16) and contractions. The latency of the excitatory potential to single stimuli was 47 ± 5ms, the time to peak 111 ± 44ms, and time to decay to half amplitude 268 ± 135ms (n=16 in each case). Trains of stimuli (1-50Hz) produced excitatory potentials and contractions graded with frequency (optimal 10Hz, Fig.18) and, at constant frequency, with increasing numbers of pulses (Fig.19). The excitatory potentials did not facilitate but summated at frequencies of 2Hz or above. The time to peak of the excitatory potentials (3-12 pulses, 10Hz) varied between 80 and 1500ms (mean 499 ± 331ms, n=54). The duration of the excitatory potentials in response to the same stimulus also varied. On some occasions, peak depolarisation was reached during field stimulation (Fig.20) and decayed rapidly. On others, the initial peak was followed by a slower depolarisation, often of greater amplitude (Fig.20). These differences may arise from the varying activity of the inhibitory transmitter which may act to truncate the excitatory potential.

The latency of the contraction (3-12 pulses, 10Hz) was 400-1200 ms (mean 647 ± 197ms, n=47). Spikes were not observed to either single or trains of stimuli. Excitatory potentials and contractions were abolished by guanethidine ($1\text{-}3 \times 10^{-5}$M, Fig.21), indicating that they were mediated by adrenergic fibres.

FIGURE 21

The effect of guanethidine (3×10^{-5}M at bar for the times indicated) on the excitatory potential (upper trace) and contraction of the bovine retractor penis in response to field stimulation (20 pulses at 20Hz; 0.5ms pulse width, supramaximal voltage). The excitatory potentials and contractions were abolished, leaving a small hyperpolarisation (last panel). Sucrose gap recording.

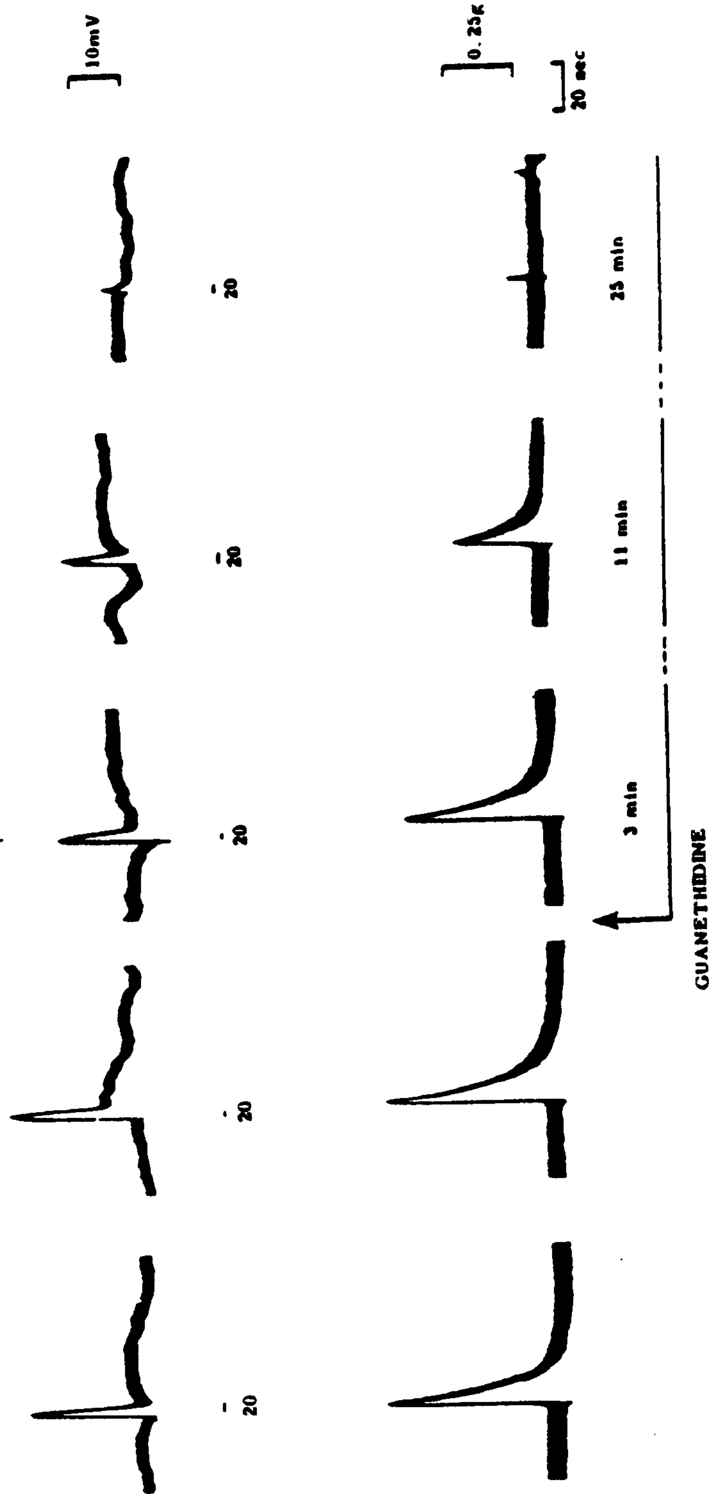
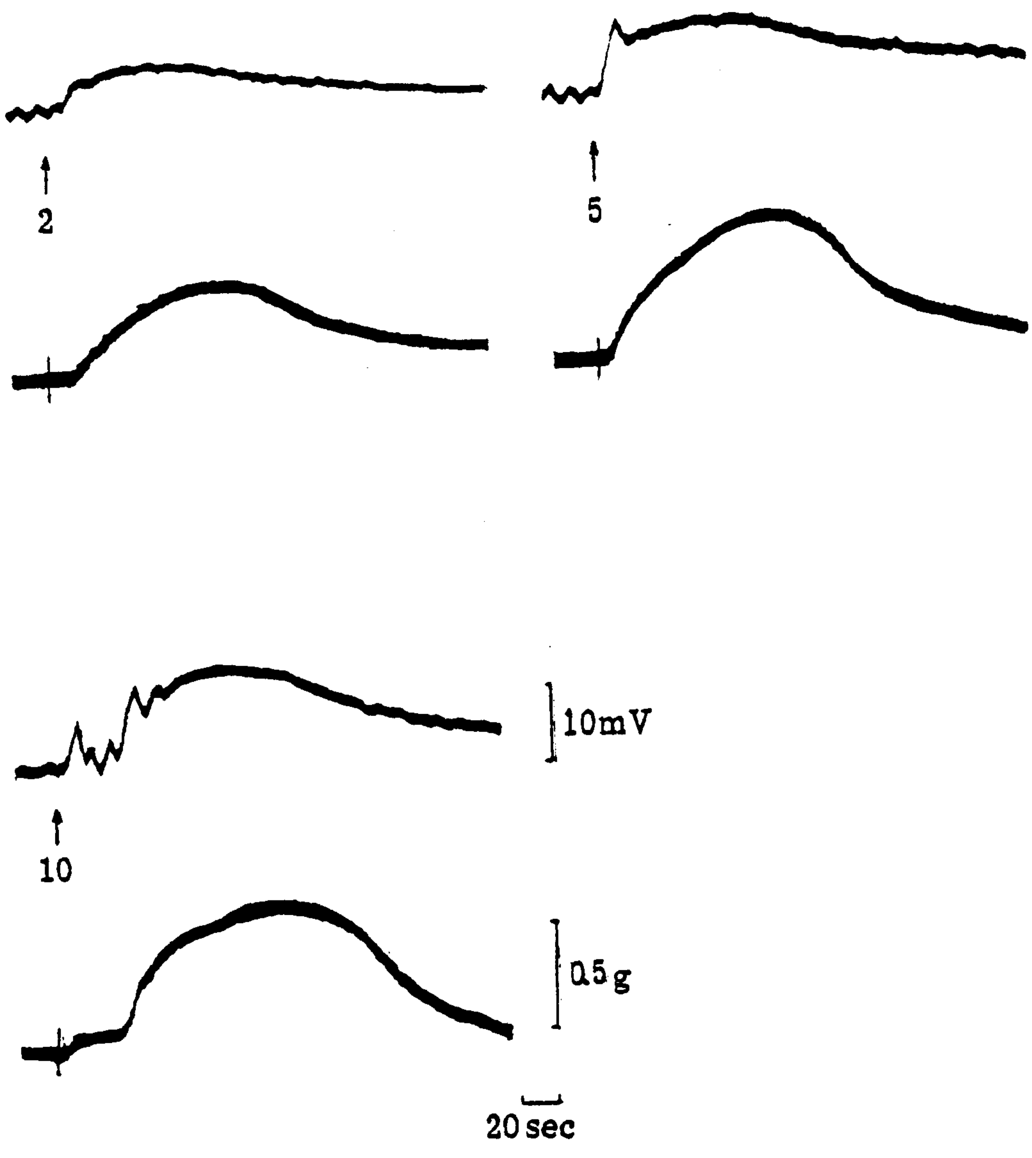

10mV
0.25g
20 sec
25 min
11 min
3 min
GUANETHIDINE

FIGURE 22

Dose-dependent depolarisation (upper trace) and contraction (lower trace) of the bovine retractor penis in response to exogenously added noradrenaline ($2\text{-}10 \times 10^{-10}$ moles). The depolsarisation and contraction was sometimes biphasic (response to 5 and 10×10^{-10} moles), consisting of an initial rapid component, sometimes associated with an oscillation in membrane potential, followed by a slower, more prolonged response. Sucrose gap recording.


2
5
10
10mV
0.5g
20 sec

EFFECTS OF NORADRENALINE ON THE BOVINE RETRACTOR PENIS

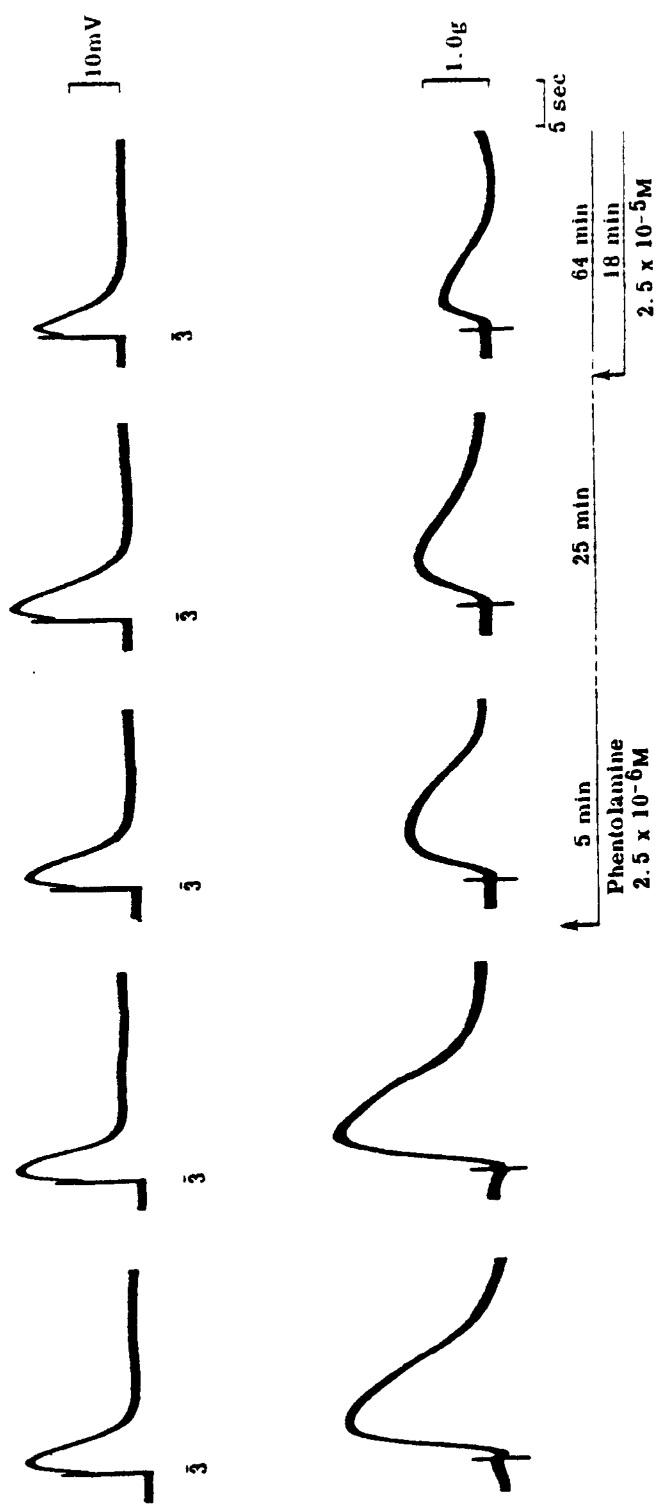
Noradrenaline (NA, $0.02\text{-}10 \times 10^{-9}$ moles) produced a dose-dependent depolarisation (1-20mV) and contraction which sometimes appeared biphasic, comprising an initial rapid component followed by a slower, more prolonged response (Fig. 22). The sensitivity of different specimens of bovine retractor penis to noradrenaline varied considerably. Threshold dose ranged from $0.02\text{-}0.5 \times 10^{-9}$ moles. This may have been due to the use of specimens taken from animals of different age and breed.

EFFECT OF α-ADRENOCEPTOR ANTAGONISTS ON THE EXCITATORY RESPONSES TO FIELD STIMULATION AND EXOGENOUSLY ADDED NORADRENALINE

The excitatory response to field stimulation (10Hz, 0.5ms) of intramural nerves was noticeably resistant to α-antagonists, the contraction being more sensitive than the excitatory potential. Thus, while phentolamine (2.5×10^{-5}M for some 60 min) reduced the mechanical contraction in response to field stimulation, usually by some 60-80%, the accompanying excitatory potential was much less (20-30% reduction) affected (Fig. 23). When the preparation was perfused with very large concentrations (2.5×10^{-4}M) of the drug, a further small (10%) reduction in both electrical and mechanical responses to field stimulation occurred, but neither component was abolished. Prazosin ($0.05\text{-}1.4 \times 10^{-6}$M) was even less effective. In its presence, the excitatory

FIGURE 23

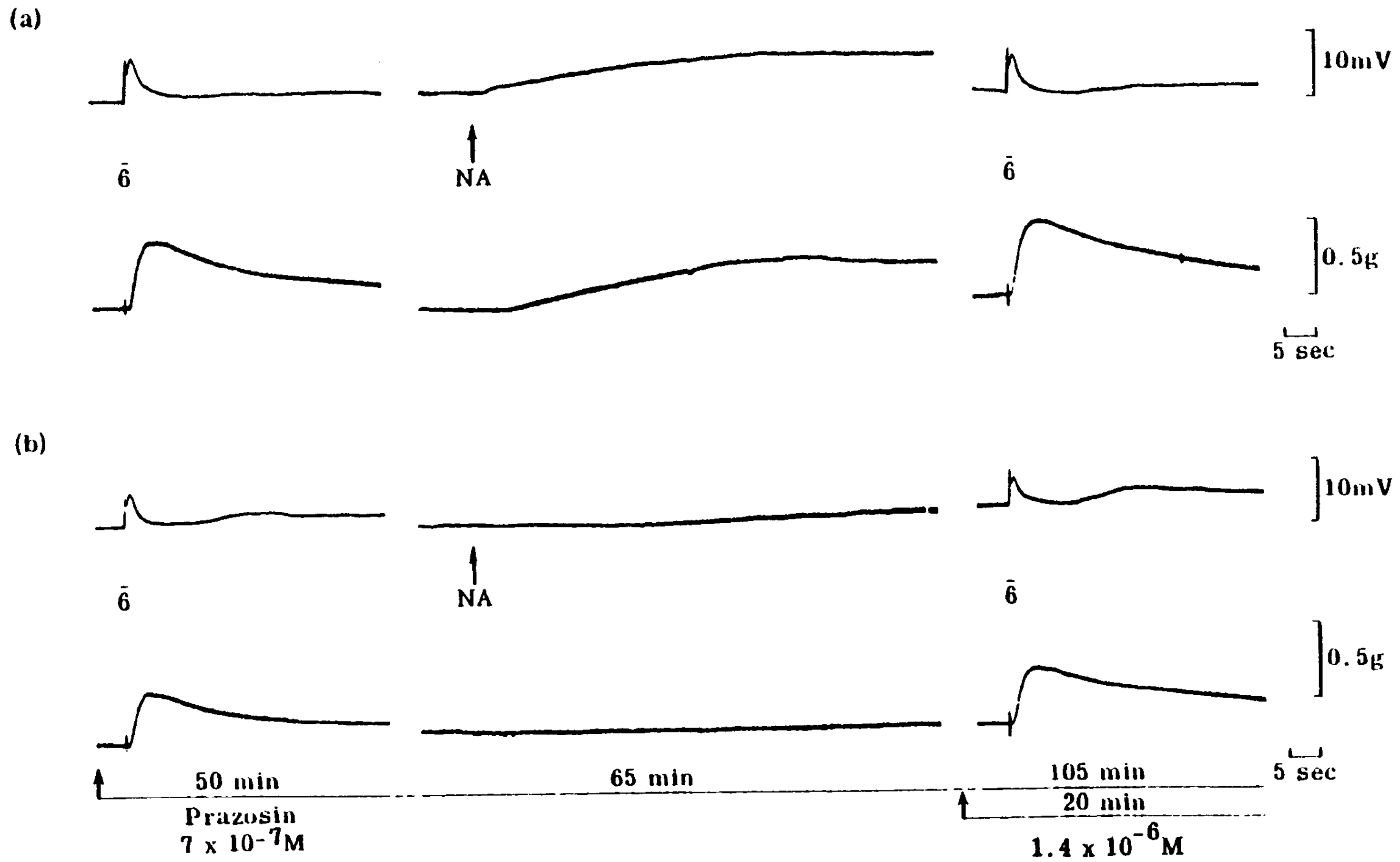
The effect of phentolamine (2.5 x 10^{-6}M and 2.5 x 10^{-5}M at arrows for the times indicated) on the excitatory potential (upper trace) and contraction of the bovine retractor penis to field stimulation (3 pulses, 10Hz; 0.5ms pulse width, supramaximal voltage). In the presence of phentolamine (2.5 x 10^{-6}M) the contraction was reduced at a time (5 to 25 min) when the excitatory potential was not affected significantly. The excitatory potential was reduced noticeably only after the application of higher (2.5 x 10^{-5}M) concentrations of drug for prolonged (18 min) periods. Sucrose gap recording.



10mV
1.0g
5 sec
64 min
18 min
2.5 x 10-5M
25 min
5 min
Phentolamine
2.5 x 10-6M

FIGURE 24

The effect of prazosin (7×10^{-7} and 1.4×10^{-6}M in (b) at bar for the times indicated) on the excitatory potential (upper trace) and contraction (lower trace) of the bovine retractor penis to field stimulation (6 pulses, 10Hz; 0.5ms pulse width, supramaximal voltage) and noradrenaline (↑NA, 1×10^{-8} moles). Compared with controls in (a), the excitatory response to noradrenaline was eliminated while that to field stimulation was only slightly reduced. Sucrose gap recording.



(a)
10mV
0.5g
5 sec
NA
(b)
10mV
0.5g
5 sec
NA
50 min
Prazosin
7 x 10-7M
65 min
105 min
20 min
1.4 x 10-6M

potential and contraction were at best only slightly reduced (10-15%; Fig. 24). Prazosin frequently depolarised the membrane and raised tone. When this occurred, both the electrical and mechanical responses to field stimulation were reduced. In contrast, both phentolamine (5×10^{-6}M) and prazosin (7×10^{-7}M) inhibited (Fig. 24) the depolarisation and contraction produced by exogenous noradrenaline ($0.02-2 \times 10^{-8}$ moles).

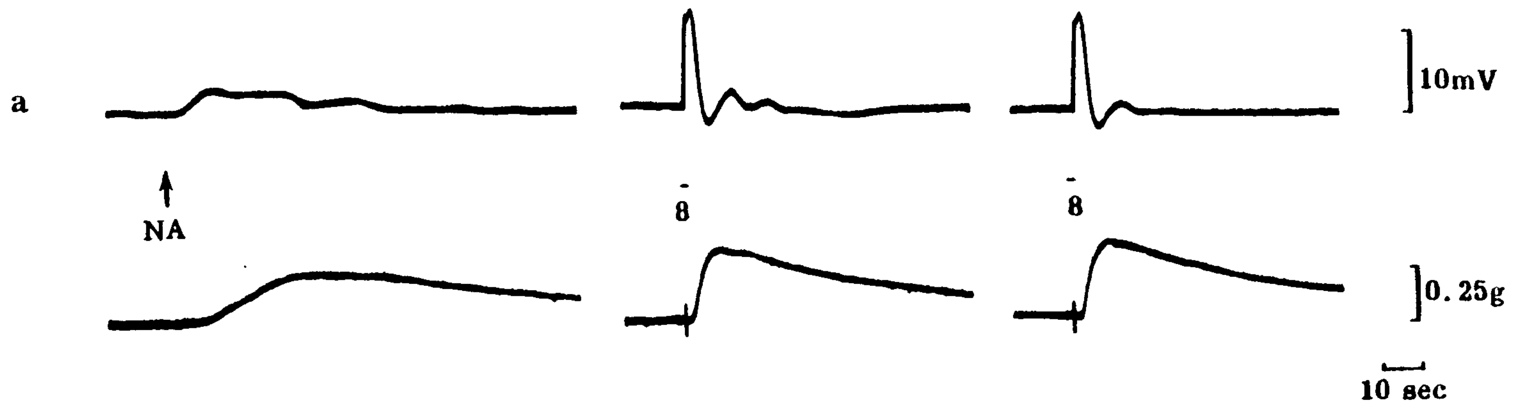
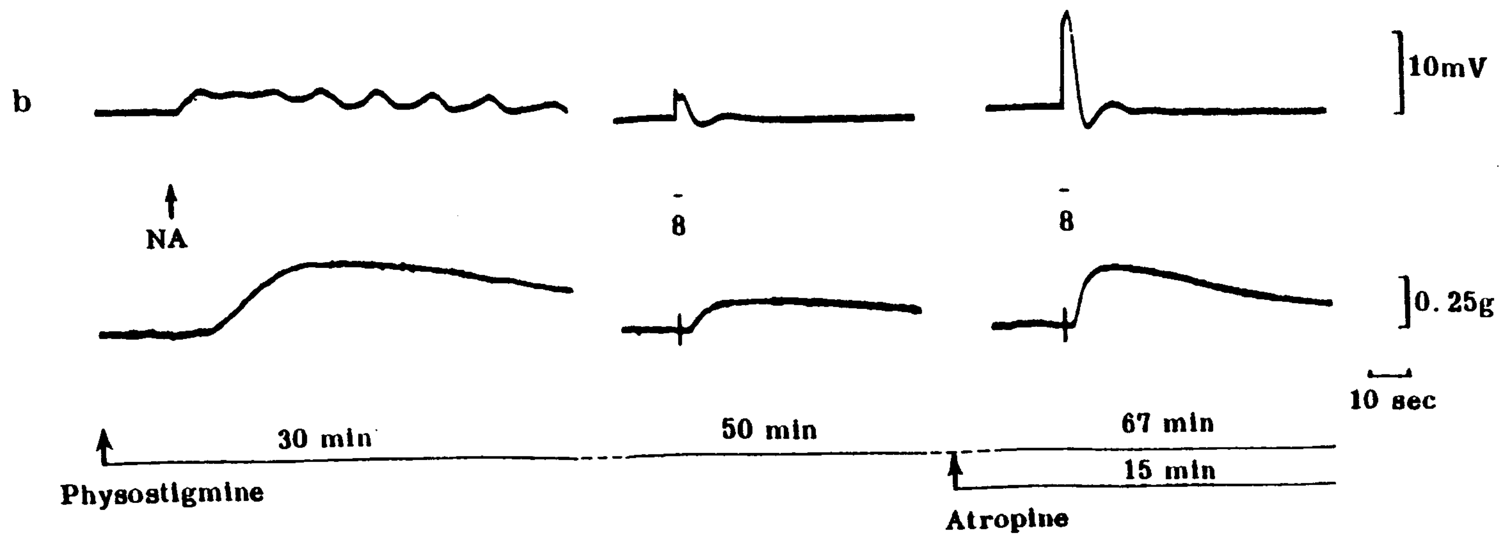
CHOLINERGIC INFLUENCE ON THE EXCITATORY ELECTRICAL AND MECHANICAL RESPONSES TO FIELD STIMULATION

Recent evidence (Klinge and Sjöstrand, 1977) for a cholinergic component which regulates the release of the excitatory transmitter in the bovine retractor penis prompted the investigation of the effects of atropine and physostigmine on the excitatory potentials and contraction in response to field stimulation (5-10 pulses, 10Hz every 5 min) with the sucrose gap technique.

Atropine (0.5×10^{-6}M) initially hyperpolarised the membrane by 2-3mV over a 10-15 min period. As a result, the amplitude of the excitatory potential was slightly increased, while that of the accompanying contraction was either not affected significantly or slightly reduced. After some 45 min in the presence of atropine, during which there was no further membrane hyperpolarisation, both the excitatory potential and contraction were increased by up to 50 and 75% respectively. In contrast, the electrical

FIGURE 25

The effects of physostigmine (b, 5×10^{-6}M at upper arrow for the times indicated) alone and in combination with atropine (1×10^{-6}M at lower arrow for the time indicated) on the depolarisations (upper trace) and contractions (lower trace) of the bovine retractor penis to noradrenaline (↑NA, 5×10^{-10} moles) and to field stimulation (8 pulses, 10Hz; 0.5ms pulse width, supramaximal voltage). Compared with controls (a), the depolarisations and contractions to field stimulation were depressed by physostigmine; those to exogenous noradrenaline were not. Atropine reversed the effects of physostignine. Sucrose gap recording.



a
NA
8
8
10mV
0. 25g
10 sec
b
NA
8
8
10mV
0. 25g
10 sec
30 min
50 min
67 min
15 min
Physostigmine
Atropine

and mechanical responses to exogenous noradrenaline (20-50 $\times 10^{-12}$ moles) were reduced.

Physostigmine ($1-5 \times 10^{-6}$M) initially (10-15 min contact) enhanced the excitatory potential and contraction in response to field stimulation by up to 20 and 50% respectively. Following this, both the excitatory potential and the contraction were inhibited by a maximum of 60-80% after 45 min contact time. The response to exogenous noradrenaline was either not significantly affected or slightly enhanced (Fig. 25). Atropine (1×10^{-6}M) restored the excitatory potential and contraction to pre-physostigmine levels (Fig. 25).

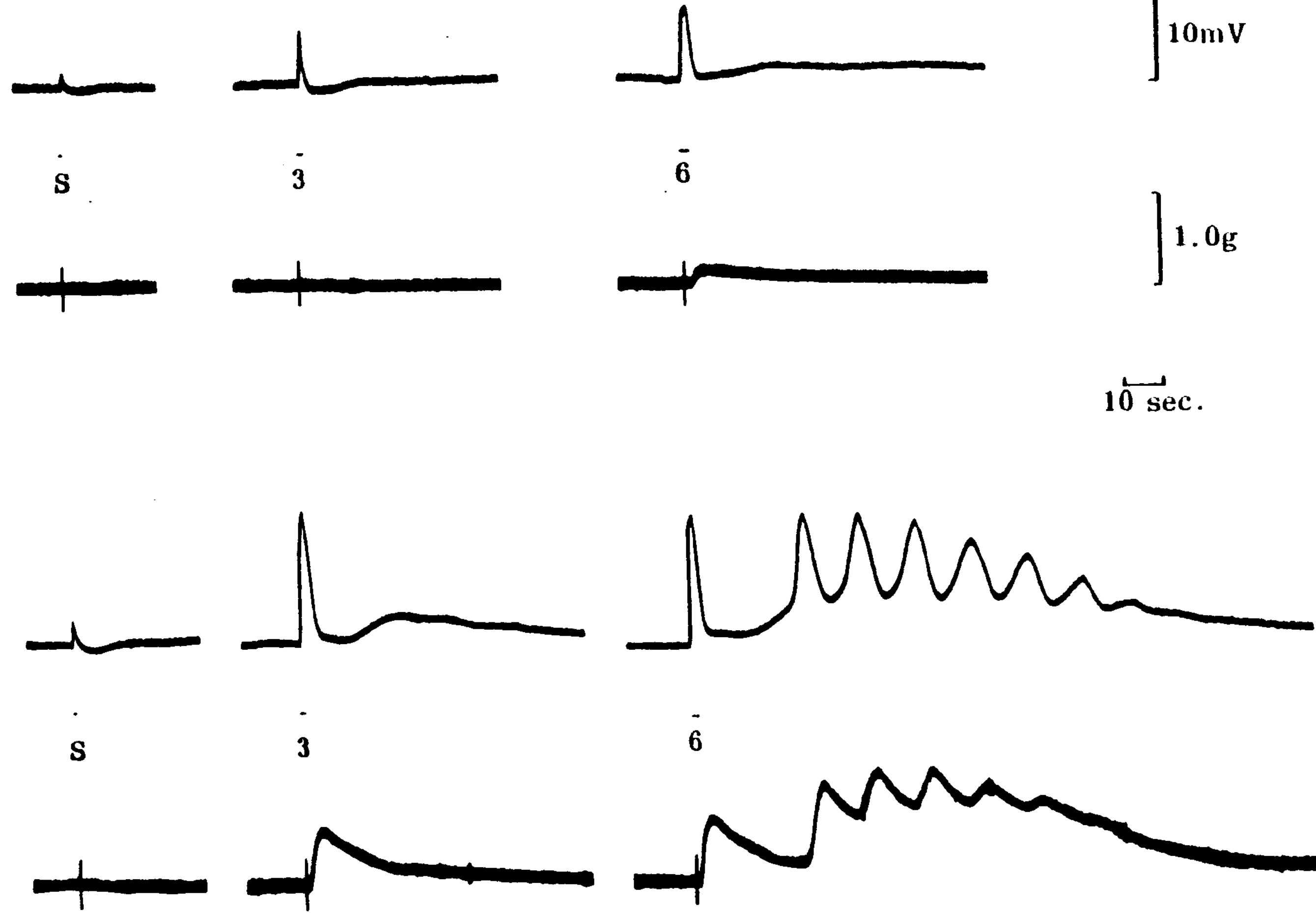
EFFECTS OF TETRAETHYLAMMONIUM (TEA) ON THE EXCITATORY ELECTRICAL AND MECHANICAL RESPONSES TO FIELD STIMULATION AND EXOGENOUSLY APPLIED NORADRENALINE

The effects of TEA ($0.1-10 \times 10^{-3}$M) were studied in the single sucrose gap. TEA (0.1×10^{-3}M) increased the amplitude of the excitatory potential in response to single pulses or trains of stimuli (3-6 pulses, 10Hz) by up to 200% (Fig. 26). The accompanying contraction was also enhanced by between 400 and 1100%. In the presence of TEA, the excitatory potential to short trains was followed by an oscillation in membrane potential and tone.

Higher concentrations of TEA ($0.5-10 \times 10^{-3}$M) initially depolarised the membrane potential (10-20mV) and increased the resting tone level. After some 15-20 min, the membrane

FIGURE 26

The effect of tetraethylammonium (b, 1×10^{-4}M for 15 min) on the excitatory potential (upper trace) and contraction (lower trace) of the bovine retractor penis in response to field stimulation (single pulse at S, 3 and 6 pulses, 10Hz: 0.5ms pulse width, supramaximal voltage). Compared with controls (a), the responses to field stimulation were enhanced in the presence of TEA and electrical and mechanical oscillations developed. Sucrose gap recording.


a
S
3
6
10mV
1.0g
10 sec.
b
S
3
6

potential and tone recovered partially and fluctuations in both were observed. The amplitude of the excitatory potential and contraction were subsequently reduced during periods of depolarisation and high tone, or enhanced during phases of repolarisation and low tone. The rate of rise of the excitatory potential was increased but spikes were not observed even in response to trains of pulses at high frequency (50Hz).

In contrast to field stimulation, the depolarisation and contraction in response to exogenously added noradrenaline ($0.1-2 \times 10^{-9}$ moles) was not enhanced by TEA ($0.5-5 \times 10^{-3}$M).

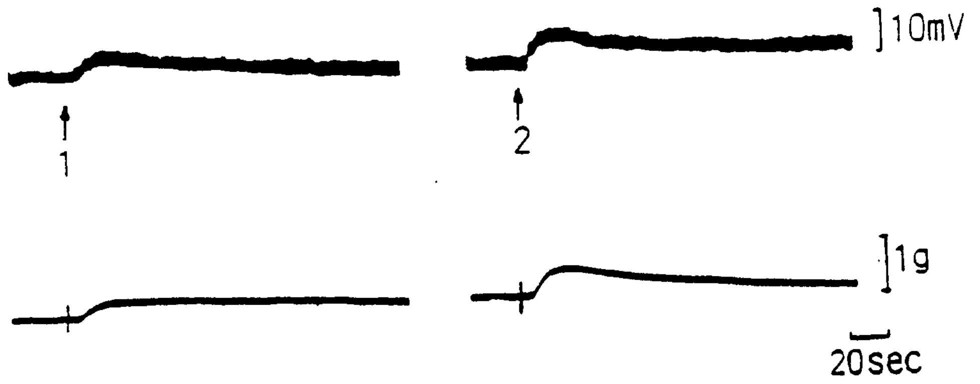
THE EFFECT OF INHIBITORY EXTRACT AND ADENOSINE TRIPHOSPHATE (ATP) IN THE ABSENCE OF TONE

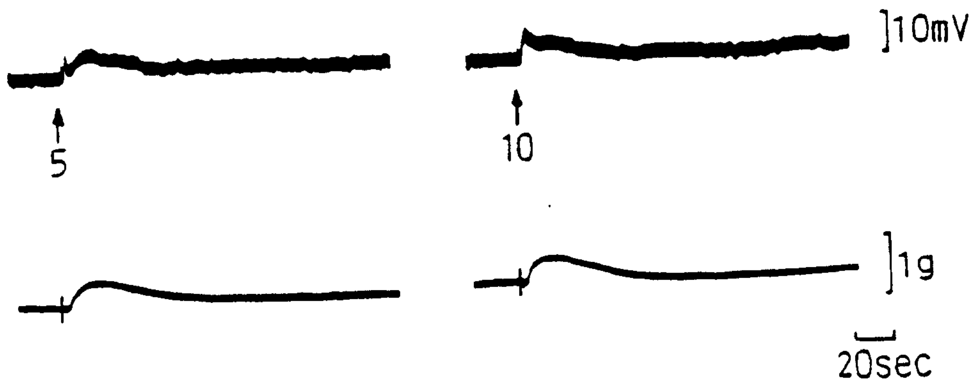
In the absence of tone, both the cleaned activated and cleaned unactivated forms of the extract (10-50μl) produced a depolarisation (up to 8mV), but without any significant mechanical response. Because the cleaned extract contained ATP (approximately 10^{-4}M), the possibility that this was responsible for the depolarisation was investigated.

ATP ($0.1-20 \times 10^{-9}$ moles) produced a depolarisation (up to 18mV) which, unlike that produced by the extract, was accompanied by a contraction (Fig. 27). These effects lacked dose-dependency and the maximum response was often small by comparison with that produced by field stimulation or exogenously added noradrenaline. This may be due to

FIGURE 27

Depolarisations (upper trace) and contractions (lower trace) of the bovine retractor penis in response to adenosine triphosphate (ATP, 1-10 x 10^{-9} moles as indicated below bars). There was no increase in the amplitude of the electrical or mechanical response when the dose of ATP was increased from 2 to 5 or 10 x 10^{-9} moles. This may be because of desensitisation to ATP, which was evident when dose intervals of up to 10 min were used. Compared with the depolarisations and contractions to noradrenaline (Fig.9), those to ATP were brief and unaccompanied by oscillations in membrane potential. Sucrose gap recording; time between panels approximately 8 min.


10mV
1
2
1g
20sec


10mV
5
10
1g
20sec

desensitisation to ATP, which was observed when dose intervals of up to 10 min were used, or the onset of the inhibitory effects of ATP.

The ability of 'pure ATP', but not equivalent doses of ATP present in the cleaned unactivated extract, to contract the bovine retractor penis suggested that the extract contained a non-acid activated component which inhibited the contraction to ATP. To investigate this, cleaned unactivated extract (containing approximately 10^{-4}M ATP) and ATP (10^{-4} or 2×10^{-4}M) were mixed (0.5ml of each). Additions made from this solution depolarised the muscle without contracting it. This suggests that ATP may be responsible for the depolarisation produced by the extract and that the contractile effect might have been inhibited by other substances present, e.g. sodium chloride (500mM approximately) or formate, but not the acid-activated inhibitory substance.

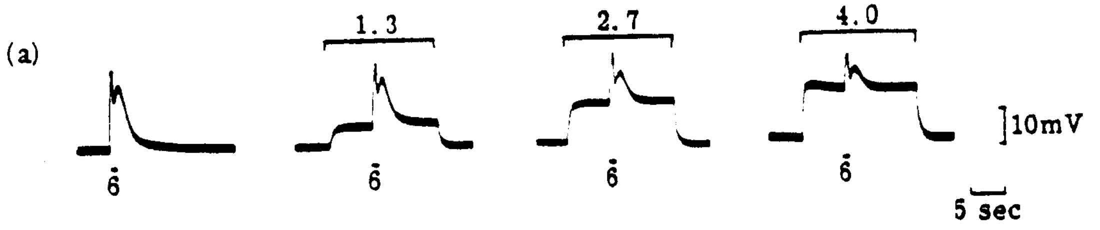
RELATIONSHIP BETWEEN THE AMPLITUDE OF THE EXCITATORY POTENTIAL AND THE MEMBRANE POTENTIAL

Field stimulation (6-10 pulses, 5-10Hz: 0.5ms pulse width, supramaximal voltage) was applied during displacement of the membrane potential in the depolarising or hyperpolarising directions (10s pulses, $0.2\text{-}6 \times 10^{-6}$A) in the double sucrose gap. Conditioning depolarising currents reduced, while hyperpolarising currents enhanced the amplitude of the excitatory potential (Fig. 28). It was difficult

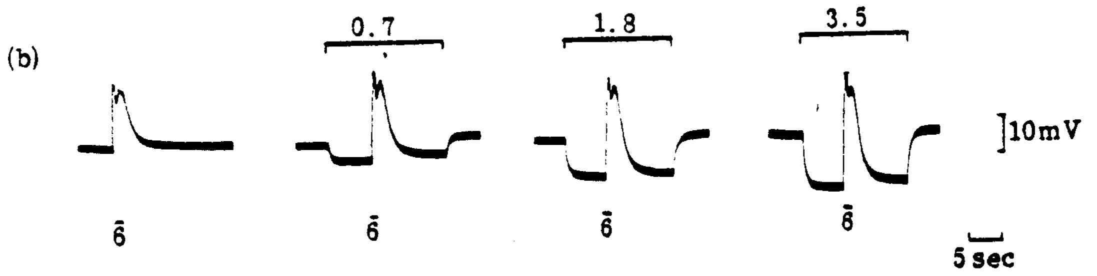
FIGURE 28

The effect of displacement of the membrane potential by (a) depolarising and (b) hyperpolarising currents on the second phase of the excitatory potentials to field stimulation (6 pulses, 10Hz) in the bovine retractor penis. Current values (x 10^{-6}A) are indicated above the traces: double sucrose gap recording. The amplitude of the excitatory potential was reduced when field stimulation was carried out during conditioning depolarisation of the membrane potential. During the conditioning hyperpolarisation, the excitatory potential was enhanced. The relationship (c) between amplitude of the excitatory potential (EP) and membrane potential displacement (ΔMP) obtained from the results shown in (a) and (b) was linear (correlation coefficient 0.883, degrees of freedom 5, $p < 0.001$). The value of membrane polarisation at which the excitatory potential = o was obtained by extrapolation.

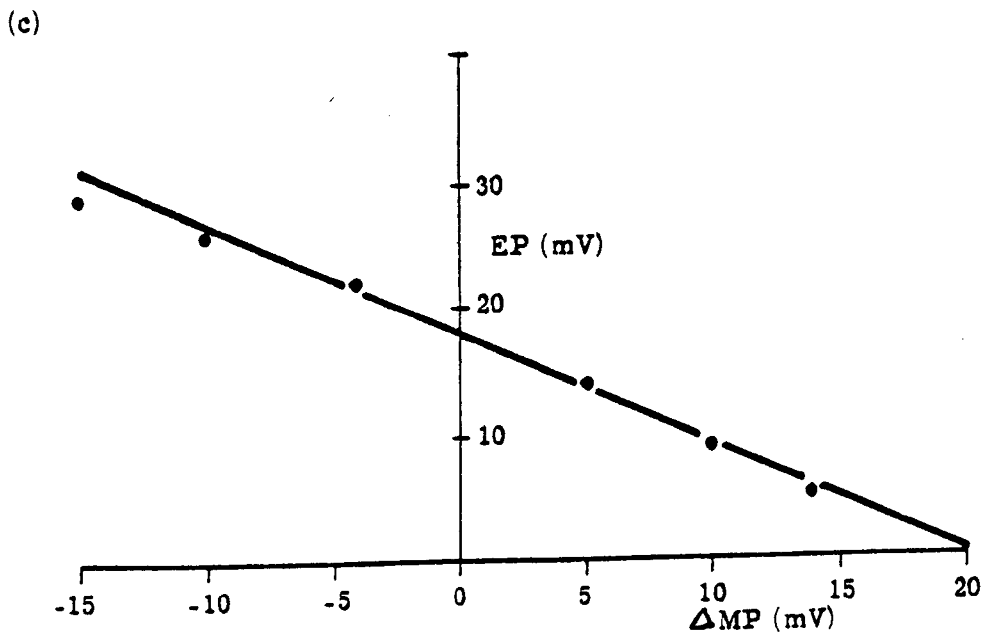
(a)



(b)



(c)



to depolarise the membrane sufficiently to abolish the excitatory potential, but extrapolation of the linear plots (correlation coefficient 0.99 ± 0.01, n=3) of membrane potential against excitatory potential revealed the latter to be abolished by a displacement of 20-25mV (mean 23 ± 2.0 mV, n=3). Since the resting membrane potential of the cells in low tone is -53mV (intracellular recording), this gives an apparent reversal potential for the excitatory potential of approximately -30mV. The true value is presumably more positive than -30mV, as membrane potential changes recorded in the sucrose gap are less than those recorded intracellularly (Bennett and Burnstock, 1966). Because of the spontaneous development of tone and difficulty in maintaining impalements, the reversal potential was not determined by intracellular methods.

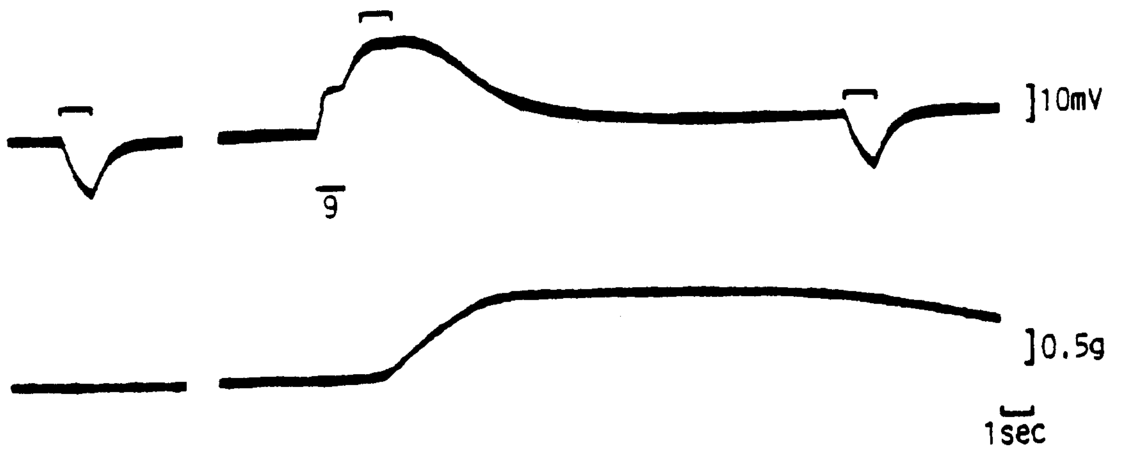
Interestingly, there was no emergence of inhibitory potentials to field stimulation during displacement of the membrane potential by depolarising current pulses.

CONDUCTANCE CHANGE DURING THE EXCITATORY POTENTIAL

Brief (duration 1s) electrotonic pulses were reduced or abolished during the excitatory potential (Fig. 29). This indicates that the transmitter increases the permeability of the membrane to one or more ions with a net equilibrium potential more positive than the resting membrane potential.

FIGURE 29

Change in membrane resistance during the excitatory potential (upper trace) and contraction (lower trace) to field stimulation (9 pulses, 10Hz) in the bovine retractor penis. Brief (duration 1s) electrotonic pulses (applied at bars shown above the upper trace) were abolished during the excitatory potential, indicating that the excitatory transmitter decreased the membrane resistance. Double sucrose gap recording.



10mV
g
0.5g
1sec

EFFECT OF REDUCING THE EXTERNAL CONCENTRATION OF CHLORIDE ON THE EXCITATORY ELECTRICAL AND MECHANICAL RESPONSES TO FIELD STIMULATION AND EXOGENOUS NORADRENALINE

The possible role of chloride in the excitatory potential and the depolarisation to exogenous noradrenaline was investigated in the single sucrose gap. Replacement of the sodium chloride content (111.8×10^{-3}M) of the normal Krebs solution with sodium glutamate depolarised the membrane potential (6-8mV) and slightly increased the resting tone level. The excitatory potential (single pulse to 7 pulses at 10Hz) was initially unchanged. After 25-30 min, both the excitatory potential and contraction were reduced to below control values (Fig. 30). Maximum reduction of the excitatory potential (25-68%) was observed after some 35 min at which time the accompanying contraction was reduced by 30-60%. These effects were reversed partially by restoring the chloride content of the Krebs solution.

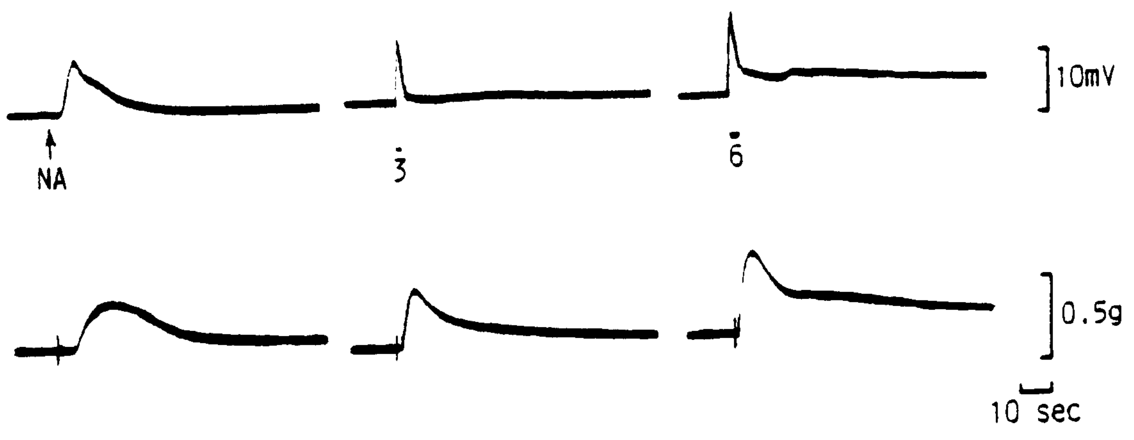
In the presence of low chloride (40-60 min), the depolarisation response to exogenously added noradrenaline ($1\text{-}2 \times 10^{-10}$ moles) was reduced by 27-70% and the contraction by 30-80% (Fig. 30).

Recent studies (Aickin and Brading, 1983; see Discussion, this thesis) suggest that inhibition of the depolarisation and contraction in response to field stimulation or exogenous noradrenaline following the partial withdrawal of Cl^- may not be due to a reduction in the Cl^- gradient across the smooth muscle cell membrane. Another effect of Cl^- withdrawal

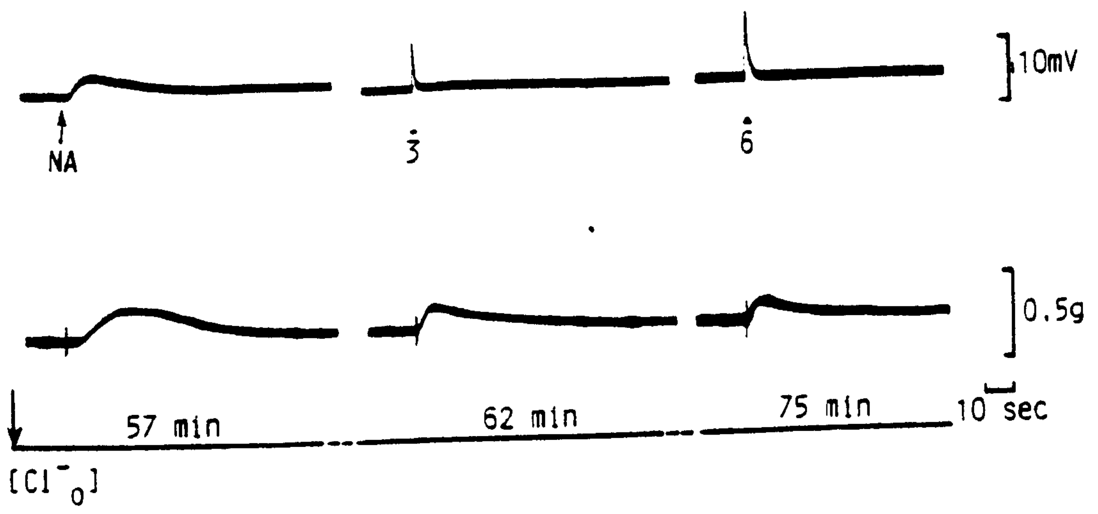
FIGURE 30

The effect of reducing the external chloride levels from 111.8×10^{-3}M in (a) to 12.7×10^{-3}M in (b) (by substitution of sodium chloride with sodium glutamate) for the times indicated on the excitatory electrical (upper trace) and mechanical (lower trace) responses to field stimulation (3 and 6 pulses, 10Hz; 0.5ms pulse width, supramaximal voltage) and exogenously added noradrenaline (1×10^{-10} moles) in the bovine retractor penis. The depolarisations and contractions to both stimuli were reduced by prolonged chloride withdrawal. Single sucrose gap recording.

(a)



(b)



such as a change in intracellular pH or some action of glutamate, e.g. chelation of Ca^{2+}, may account for the results obtained.

BOVINE RETRACTOR PENIS: INHIBITION

INTRACELLULAR ELECTRICAL RECORDING FROM THE BOVINE RETRACTOR PENIS AFTER THE DEVELOPMENT OF TONE

After the development of tone (up to 15g), impalements were particularly difficult to make. Strips of bovine retractor penis were incubated with collagenase (67-670 units/ml) to investigate the contribution of dense connective tissue to the difficulty of cell impalement.

Collagenase-treated tissue was fragile, the smooth muscle bundles tending to separate as the preparation was pinned out in the organ bath. There was no significant difference between the ability to make impalements in collagenase-treated and untreated preparations. Moreover, it was difficult to obtain prolonged impalements in collagenase-treated preparations, presumably because of the reduced ability to pin-out the tissue securely. Thus, no electrical recordings were made from collagenase-treated preparations.

INHIBITORY RESPONSE TO FIELD STIMULATION

The spontaneous development of tone in the bovine retractor penis was accompanied by depolarisation of the membrane potential, reduction in or abolition of the excitatory potential, and the emergence of inhibitory electrical and mechanical responses to field stimulation (Fig. 31).

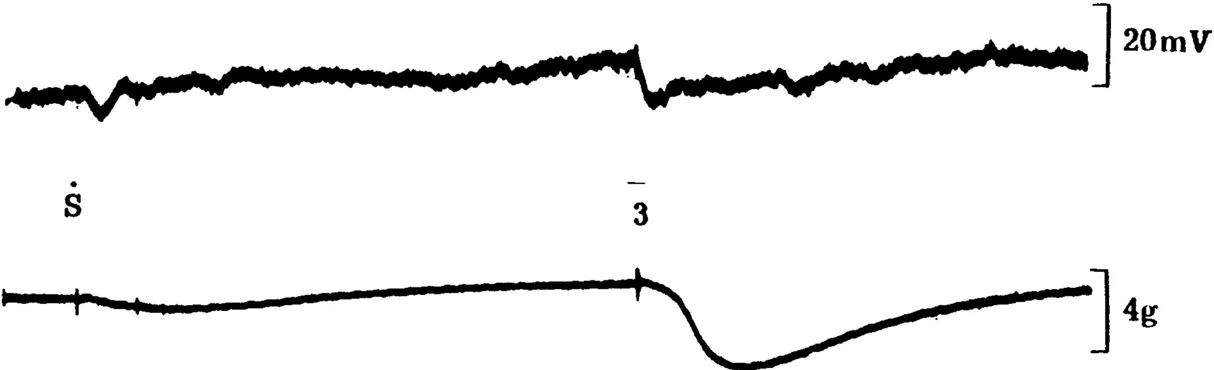
FIGURE 31

Change in the response to field stimulation (5 pulses at 1 and 5Hz, 0.5ms pulse width, supramaximal voltage) after the spontaneous development of tone in the bovine retractor penis. Before the development of tone, field stimulation produced an excitatory potential, poorly maintained in response to 1Hz, and contraction. There followed a period of spontaneous depolarisation and contraction during which field stimulation evoked an inhibitory potential and relaxation. Continuous sucrose gap recording.

10mV
5
1
5
1g
5sec

FIGURE 32

Inhibitory potentials (upper trace) from a single cell, and relaxations (lower trace) of the bovine retractor penis in response to field stimulation (1 at S to 9 pulses, 5Hz; 0.5ms pulse width as indicated below bars, supramaximal voltage) after the development of tone. The amplitude of the inhibitory potential and relaxation was graded with increasing numbers of pulses. A rebound depolarisation, as in the lower right-hand panel, and rebound contraction sometimes followed the inhibitory response.


20mV
Ṡ
3
4g
5 sec.

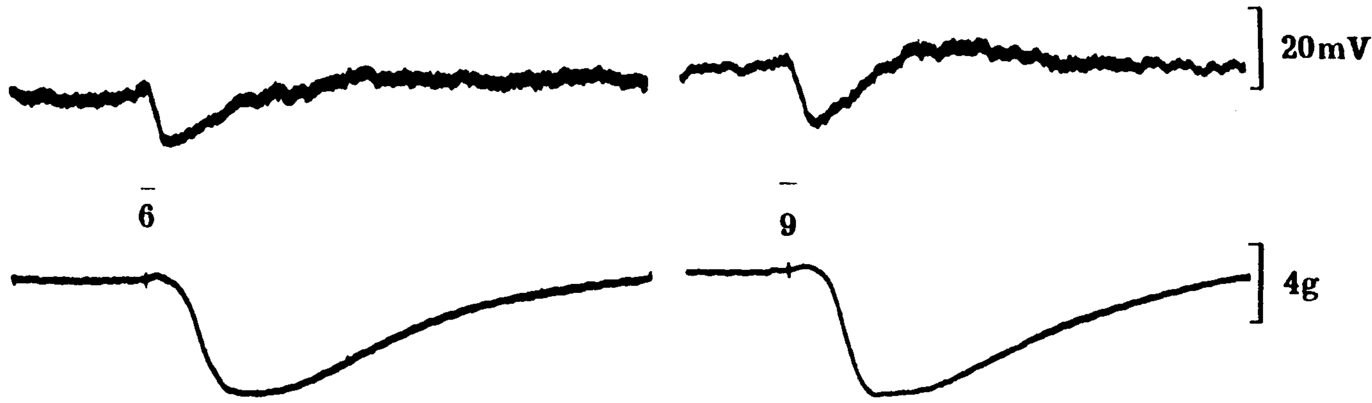
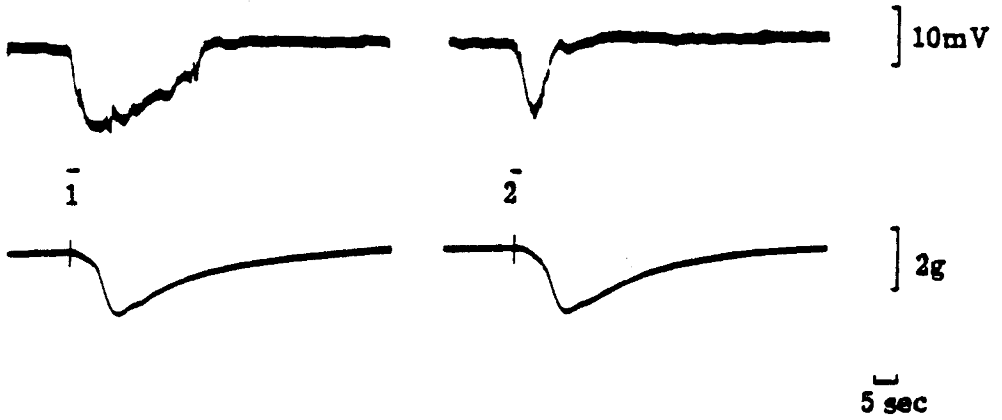
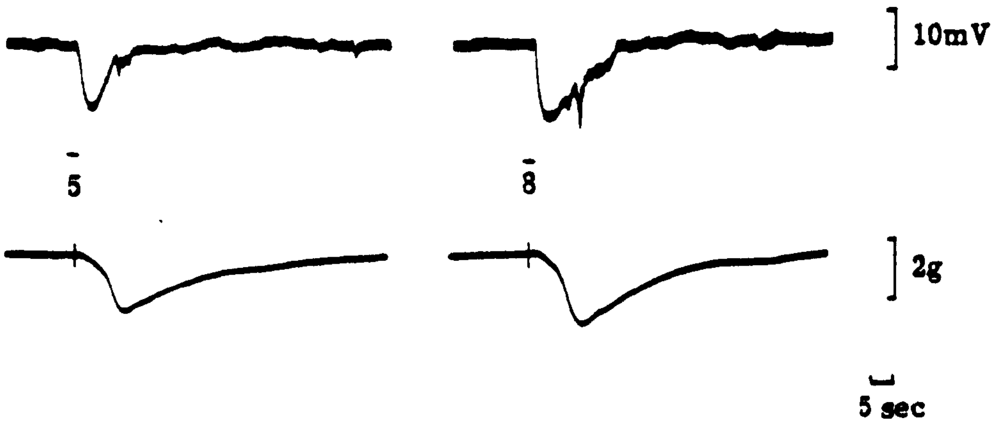

20mV
6
9
4g

FIGURE 33

Inhibitory potentials (upper trace) from a single cell and relaxations of the bovine retractor penis in response to field stimulation (3 pulses, 1-20Hz; 0.5ms pulse width, supramaximal voltage as indicated below bars) after the development of tone. The amplitude of the inhibitory potential and relaxation showed little variation with stimulus frequency.


10mV
1
2
2g
5 sec


10mV
5
8
2g
5 sec

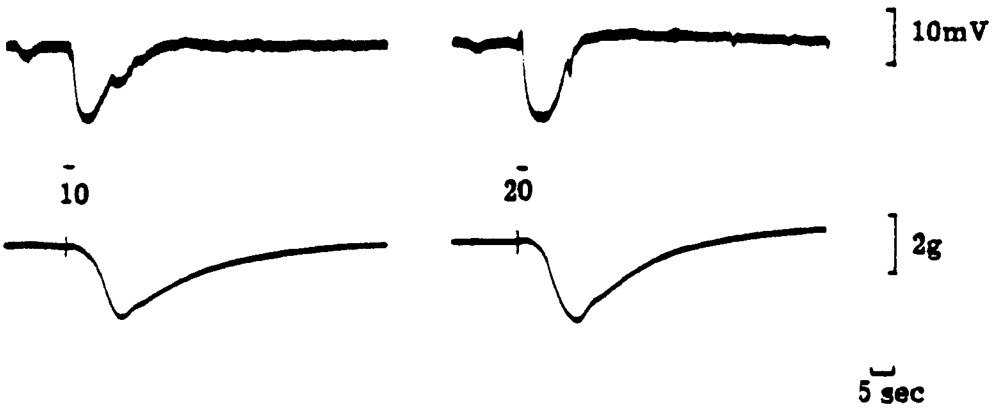
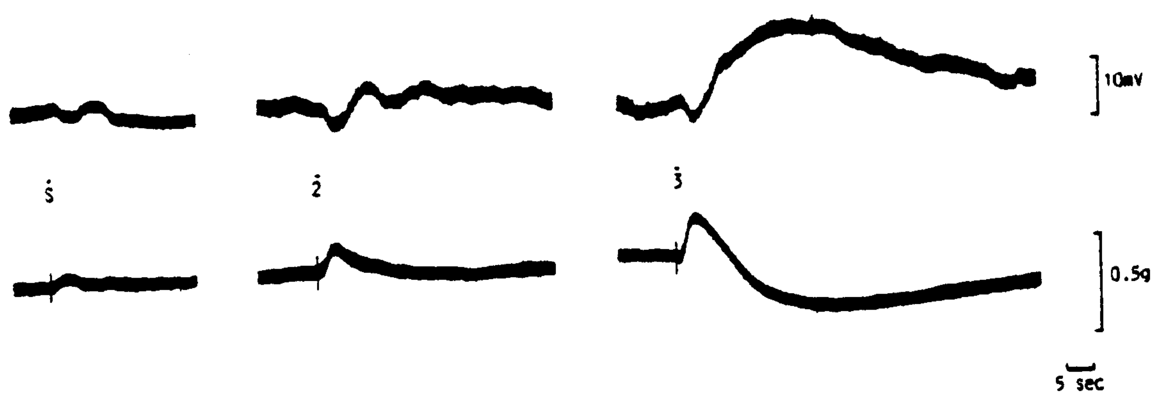
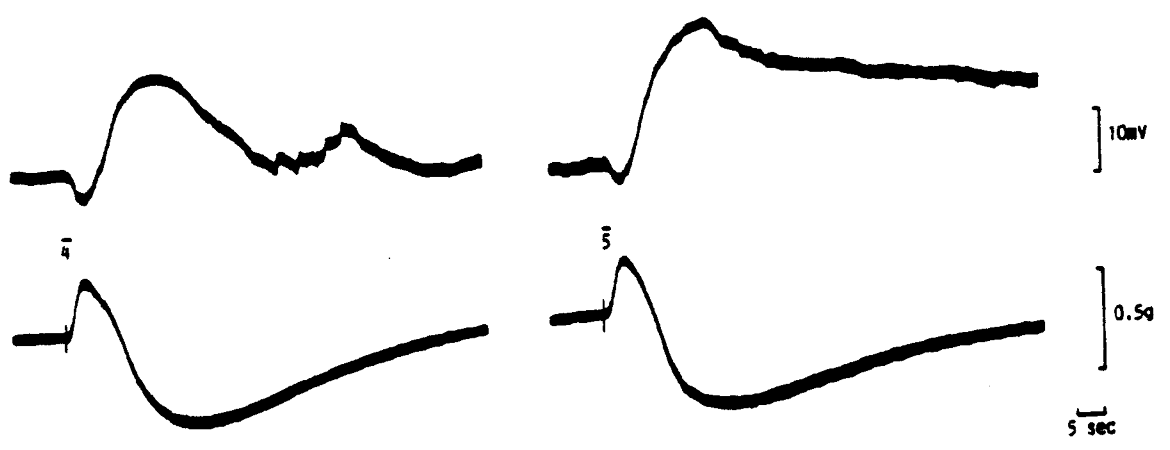

10mV
10
20
2g
5 sec

FIGURE 34

Inhibitory potentials, rebound depolarisations (upper traces) from a single cell and accompanying mechanical responses to field stimulation (single pulse at S and 2-5 pulses, 5Hz; 0.5ms pulse width as indicated below bars, supramaximal voltage) in the bovine retractor penis. The amplitude of the rebound depolarisation was graded with increasing numbers of pulses and appeared independent of the amplitude of the preceding inhibitory potential.


10mV
S
2
3
0.5g
5 sec

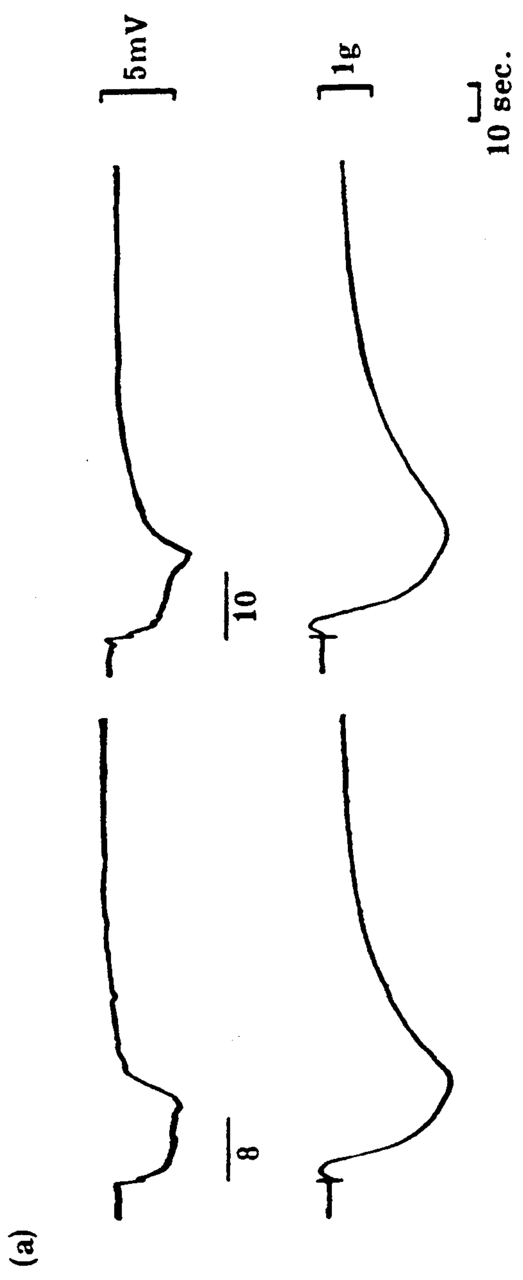

10mV
4
5
0.5g
5 sec

When tone had developed fully, single stimuli produced inhibitory potentials which could reach an amplitude of 8mV (mean 3 ± 1mV, n=18); the time to peak was 222 ± 94ms (n=18) and time to decay to half amplitude 2082 ± 1140ms (n=17). Latency measurements were made difficult by the slow rate of rise of the inhibitory potential and its relatively small amplitude. The values ranged from 100ms to 2500ms (mean 1081 ± 782ms, n=18). The amplitude of inhibitory potentials (up to 20mV) and relaxations to trains (3-20 pulses, 5-50Hz) of stimuli were graded with the strength and number of stimuli (Fig. 32), but showed little variation with stimulus frequency (1-20Hz; Fig. 33). The time course of the inhibitory potential following trains of pulses was prolonged, the time to peak being 2750 ± 640ms (n=48). A rebound depolarisation, graded with increasing numbers of pulses (Fig. 34), and contraction often followed inhibitory responses to single or trains of stimuli.

Both the inhibitory potential and relaxation were well maintained during prolonged (20-40s) periods of field stimulation at 1-10Hz (Fig. 35a). If during the inhibitory potential, field stimulation at low frequencies (1-2Hz) was continued, small excitatory potentials were often observed to be superimposed upon the existing hyperpolarisation (Fig. 35b). This demonstrates the discrete effects of the excitatory and inhibitory transmitters. Facilitation of the inhibitory potentials occurred at low (0.2Hz)

FIGURE 35

Characteristics of the electrical (upper trace) and mechanical responses of the bovine retractor penis to field stimulation (0.2ms pulse width, supramaximal voltage) following the development of tone. (a) The inhibitory potential and relaxation were well maintained during prolonged (20s) periods of field stimulation (8 and 10Hz). (b) During the inhibitory potential elicited by field stimulation (10 pulses, 1Hz), small excitatory potentials emerged. (c) Facilitation of inhibitory potentials occurred in response to single stimuli (0.2Hz at dots) before loss of impalement. (a) and (b) sucrose gap, (c) intracellular recordings.


(a)
5mV
1g
10 sec.
8
10

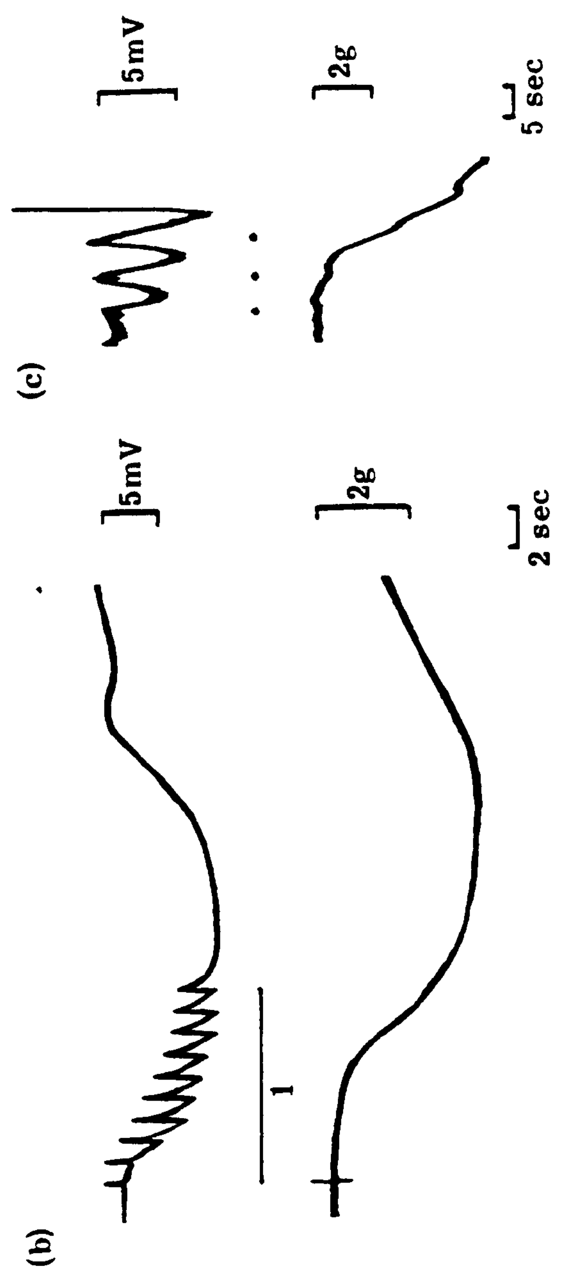
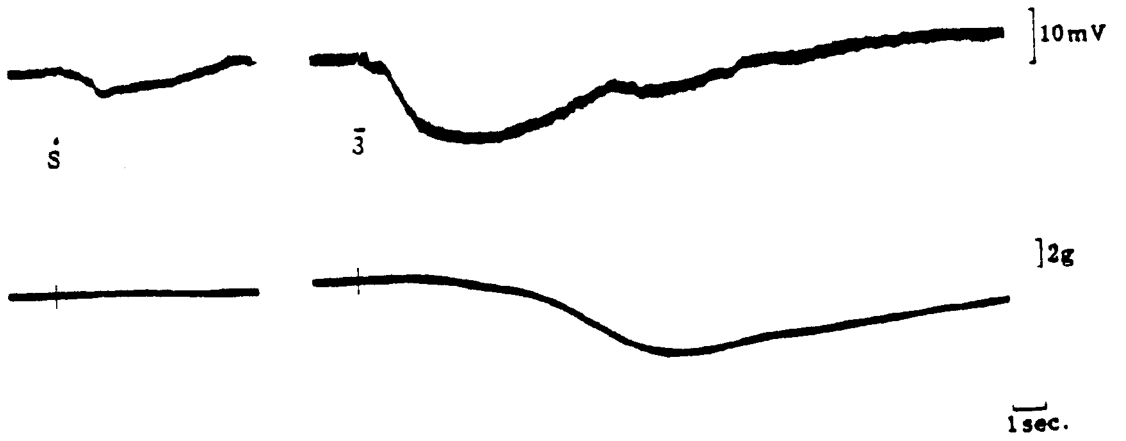

(b)
5mV
2g
2 sec
1
(c)
5mV
2g
5 sec

FIGURE 36

Variations in the nature of the inhibitory potential (upper trace) to field stimulation (single pulse at S or 3 at 10Hz, 0.5ms pulse width, supramaximal voltage) and the accompanying relaxation (lower trace) in the bovine retractor penis recorded at high chart speed. The majority of inhibitory potentials were continuous (upper panels) but others were interrupted after the cessation of field stimulation by a brief repolarisation to the base line membrane potential (lower panels). Intracellular electrical recordings from two different preparations (upper and lower panels).


10mV
S
3
2g
1sec.

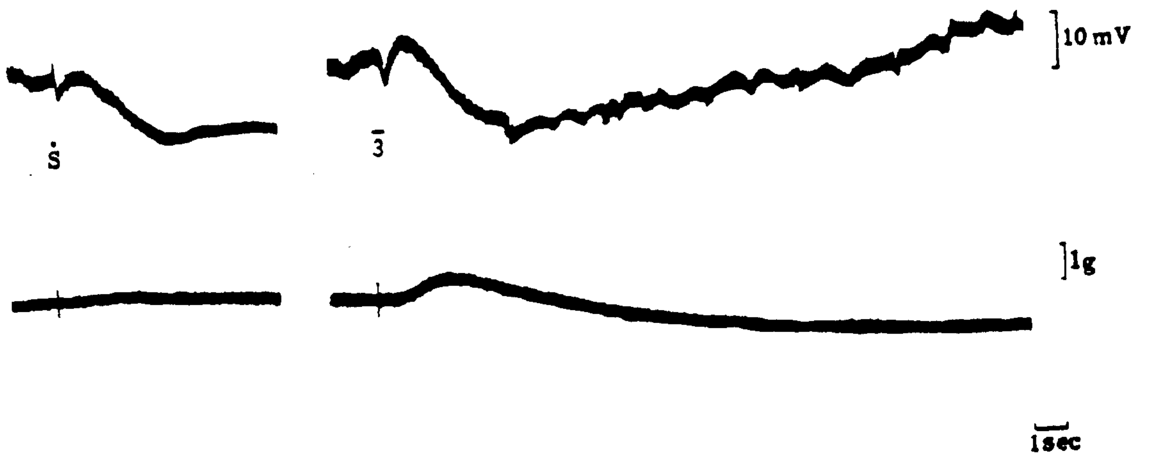
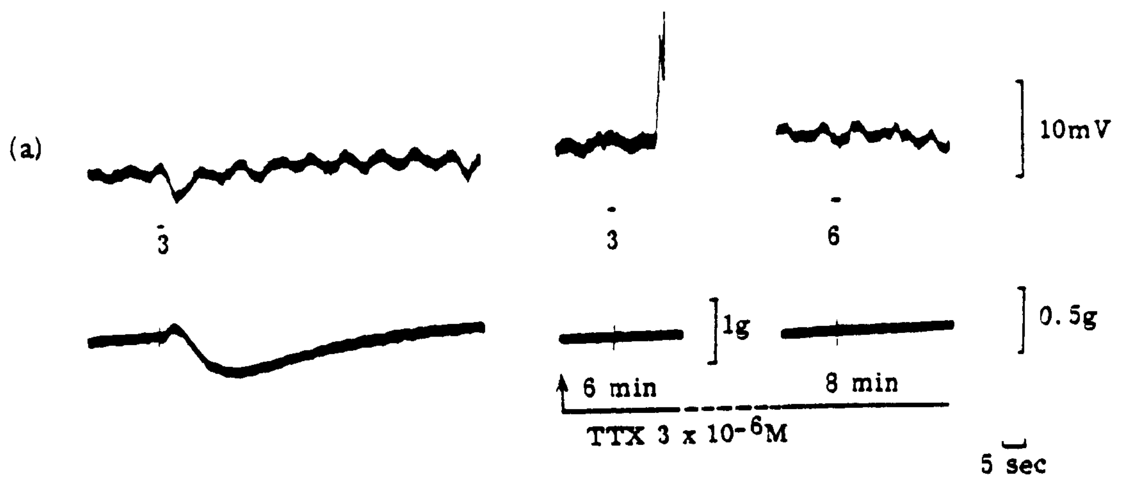
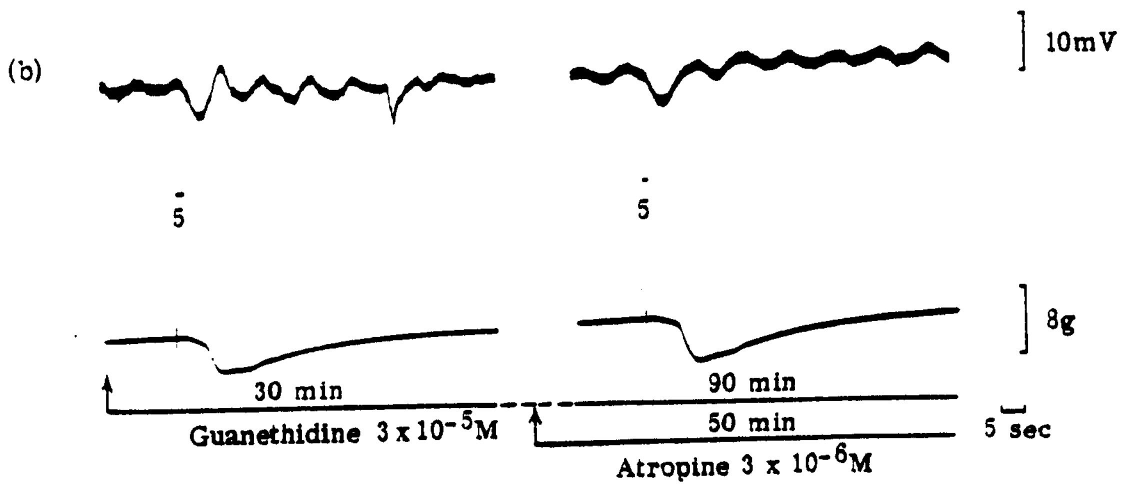

10mV
S
3
1g
1sec

FIGURE 37

The effects of tetrodotoxin, guanethidine and atropine on the inhibitory potential (upper trace) and relaxation of the bovine retractor penis in response to field stimulation (5Hz; 0.5ms pulse width, supramaximal voltage). (a) Abolition of the inhibitory potential (3 pulses) by tertodotoxin (TTX, 3×10^{-6}M for the times indicated) was followed by loss of impalement. In another cell in the same preparation (third panel in (a)), 6 pulses failed to evoke an electrical or mechanical response in the presence of TTX. In (b), recordings from another preparation, inhibitory potentials and relaxations (5 pulses) were evident after prolonged contact with guanethidine (times as indicated) and, from a different cell, a mixture of guanethidine (90 min contact) and atropine (50 min contact). Intracellular electrical recordings.


(a)
3
3
6
10mV
1g
0.5g
6 min
8 min
TTX 3 x 10-6M
5 sec


(b)
5
5
10mV
8g
30 min
90 min
50 min
Guanethidine 3 x 10-5M
Atropine 3 x 10-6M
5 sec

119

frequencies of stimulation (Fig. 35c) and summation at 1Hz or above. After the cessation of field stimulation, some inhibitory potentials were briefly interrupted by a repolarisation back to the resting membrane potential, following which hyperpolarisation continued (Fig. 36). Such responses were sometimes associated with a small contraction, which preceded relaxation, suggesting that repolarisation during the inhibitory potential may have been due to the activity of an excitatory transmitter.

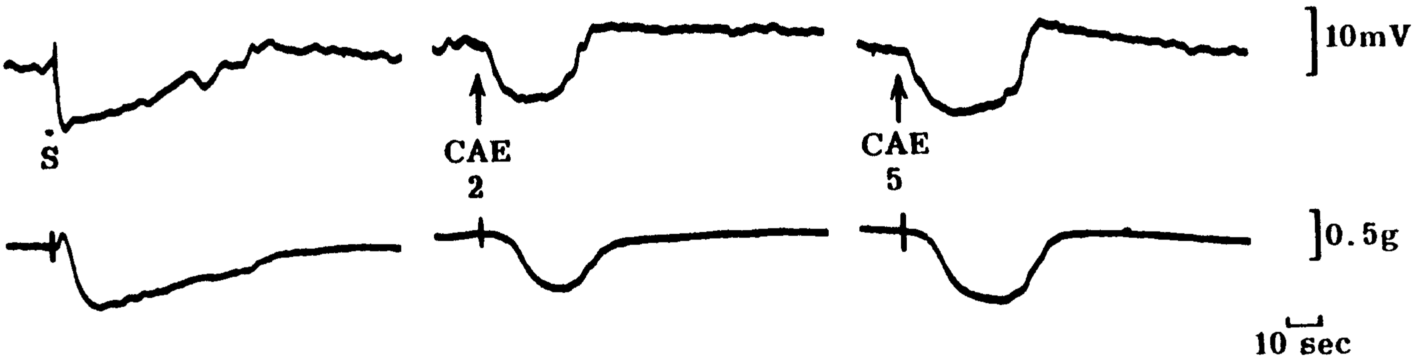
The inhibitory potential and relaxation were abolished by tetrodotoxin (3×10^{-6}M) but unaffected by phentolamine (5×10^{-6}M), propranolol (4×10^{-6}M) or a mixture of guanethidine (3×10^{-5}M) and atropine (3×10^{-6}M), confirming that they were mediated by non-adrenergic non-cholinergic nerves (Fig. 37).

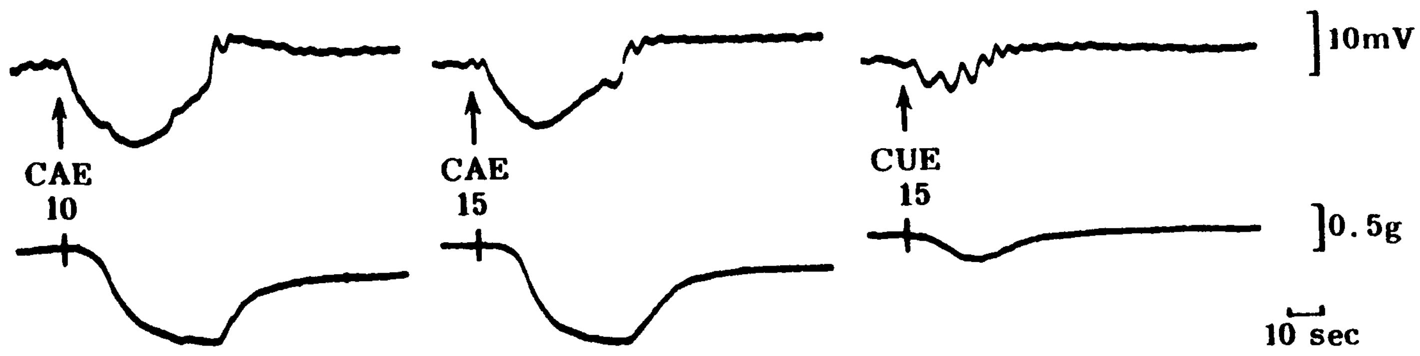
EFFECT OF THE INHIBITORY EXTRACT ON MEMBRANE POTENTIAL AND TONE

The acid-activated form of the extract, either cleaned or uncleaned, produced a dose-dependent hyperpolarisation and relaxation of the bovine retractor penis (Fig. 38). These effects were not mimicked by equivalent volumes of cleaned unactivated extract. The time of onset of the hyperpolarisation response to extract was about 2s and its duration ranged from 40 to 150s. The amplitude of the hyperpolarisation to activated extract or inhibitory nerve stimulation recorded intracellularly in the

FIGURE 38

The effect of inhibitory extract on the membrane potential (upper trace) and tone of the bovine retractor penis. Cleaned activated extract (CAE, 2-15μl as indicated below arrows) produced a dose-dependent hyperpolarisation and relaxation, thus mimicking the effect of field stimulation (single pulse at S). The hyperpolarisation to both field stimulation and extract was followed by a rebound depolarisation. In comparison, the cleaned un-activated extract (CUE, 15μl) produced only a slight electrical and mechanical inhibition. Sucrose gap recording.


10mV
S
CAE
2
CAE
5
0.5g
10 sec


10mV
CAE
10
CAE
15
CUE
15
0.5g
10 sec

same tissues (n=4) ranged from 2 to 15mV. The inhibitory response to the extract was often followed by a rebound depolarisation and contraction which further confirmed the similarity between the extract and inhibitory nerve stimulation. Occasionally, the hyperpolarisation to activated extract was preceded by a brief depolarisation. Depolarisations were also observed in response to cleaned unactivated extract and were probably due to the presence of impurities, e.g. ATP.

EFFECT OF SODIUM NITROPRUSSIDE ON MEMBRANE POTENTIAL AND TONE

Previous reports (Bowman and Gillespie, 1981; Bowman and Drummond, 1984) that the inhibitory extract and sodium nitroprusside might relax the bovine retractor penis via a similar mechanism prompted the investigation of the electrophysiological effects of sodium nitroprusside.

Sodium nitroprusside (10^{-10}-10^{-8} moles) produced a dose-dependent hyperpolarisation ranging from 2-10mV in amplitude, 25-150s duration, and accompanied by a relaxation (Fig. 39). When the level of spontaneously-developed tone was low, the inhibitory response to sodium nitroprusside was followed by a prolonged rebound depolarisation and contraction, sometimes accompanied by electrical and mechanical oscillations (Fig. 39).

FIGURE 39

The effect of sodium nitroprusside (NP, 1-5 x 10^{-9} moles) on the membrane potential (upper trace) and tone (lower trace) of the bovine retractor penis. A dose-dependent hyperpolarisation was followed by a prolonged rebound depolarisation. Sucrose gap recording.

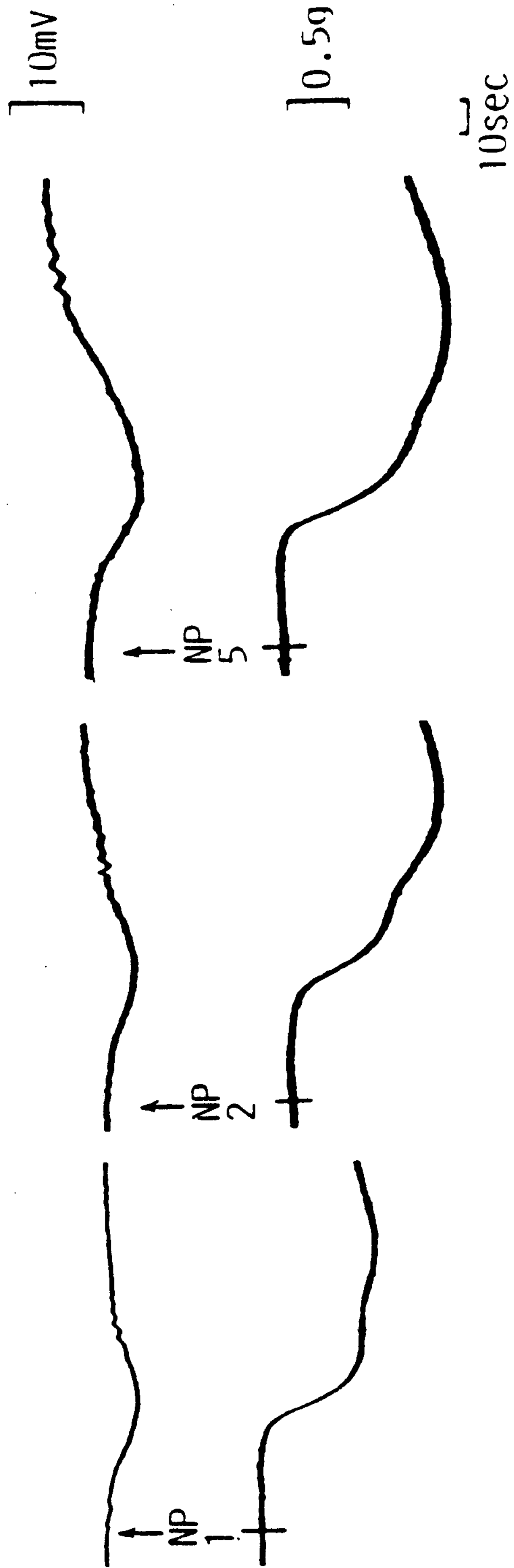
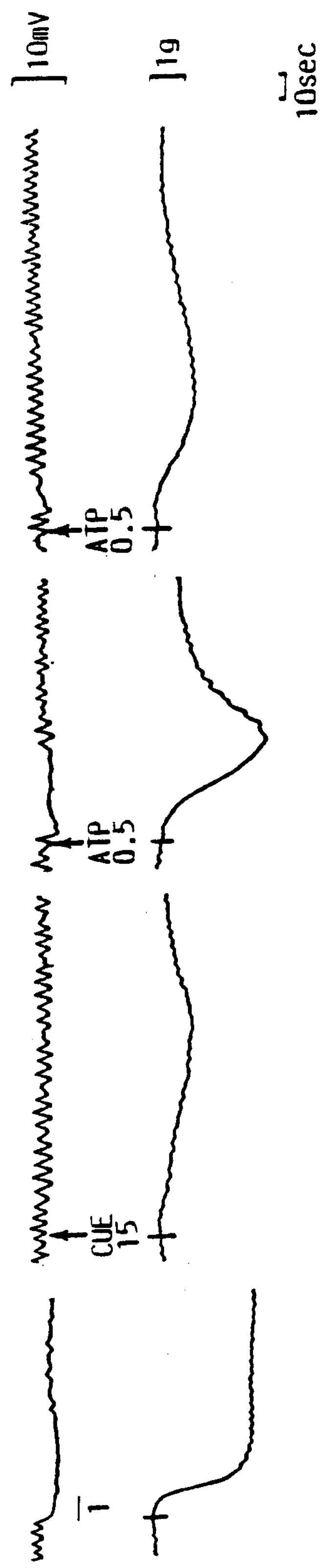
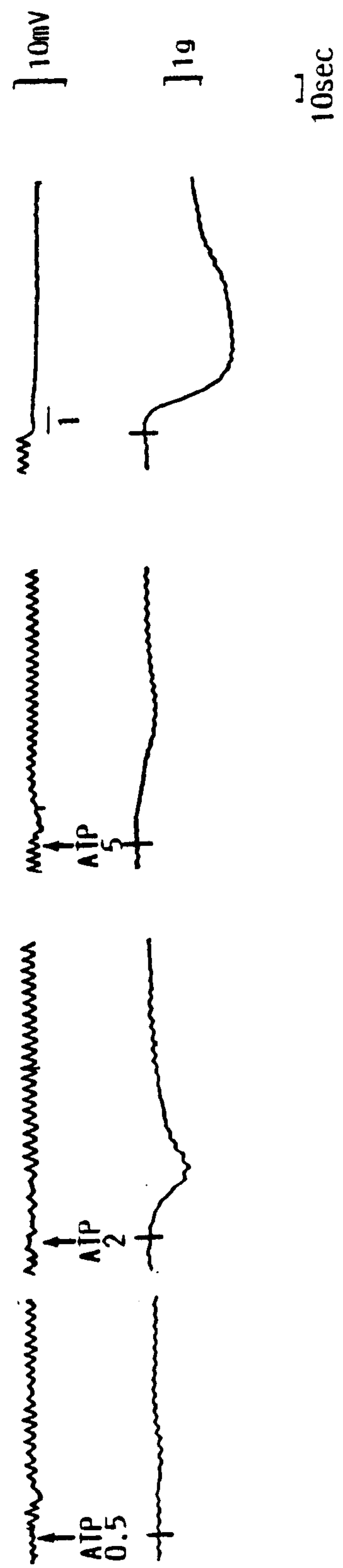

10mV
0.5g
10sec
NP 5
NP 2
NP 1

FIGURE 40

The effects of field stimulation (10 pulses, 1Hz; 0.5ms pulse width, supramaximal voltage), adenosine triphosphate (ATP, $0.5-5 \times 10^{-9}$ moles as indicated below arrows) and cleaned unactivated extract (CUE, 15µl containing approximately 1.5×10^{-9} moles ATP) on the membrane potential (upper trace) and tone of the bovine retractor penis. Although added before the ATP, CUE produced no significant electrical or mechanical inhibition. ATP (1st dose) hyperpolarised and relaxed the muscle in similar manner to field stimulation. Desensitisation to ATP was apparent with the second dose, added 5 min after the first. Successive doses of larger amounts failed to produce a response of comparable magnitude to the first. The response to field stimulation was unaffected. Sucrose gap recording.


10mV
1g
10sec
CUE
15
ATP
0.5
ATP
0.5


10mV
1g
10sec
ATP
0.5
ATP
2
ATP
5

EFFECT OF ATP ON THE BOVINE RETRACTOR PENIS AFTER THE DEVELOPMENT OF TONE

ATP (0.2-5 x 10^{-9} moles) produced initially a hyperpolarisation which was rapid in onset, and a relaxation (Fig. 40). However, the muscle rapidly desensitised to ATP. Higher doses (1-5 x 10^{-8}moles) then produced a biphasic electrical and mechanical response consisting of an initial rapid depolarisation and contraction followed by a small prolonged hyperpolarisation and relaxation. Interestingly, although the cleaned extract contained ATP (approximately 10^{-4}M), there was no hyperpolarisation in response to cleaned unactivated extract (up to 20µl - equivalent to 2 x 10^{-9} moles ATP). Neither was the muscle desensitised to ATP by frequent doses of extract. This suggests that ATP present in the extract may be inhibited by another component.

Desensitisation to ATP was unaccompanied by any change in the electrical or mechanical response to inhibitory nerve stimulation (Fig. 40), suggesting that the transmitter and ATP acted via different mechanisms.

EFFECTS OF NORADRENALINE ON MEMBRANE POTENTIAL AND TONE

On one occasion, in the presence of prazosin (1.4 x 10^{-6}M), following depolarisation of the membrane potential and the development of tone, noradrenaline (0.1-2 x 10^{-9} moles) produced small hyperpolarisations (1-3mV) and relaxations (Fig. 41a). Propranolol (4 x 10^{-6}M) blocked the inhibitory response to noradrenaline, confirming its medi-

FIGURE 41

The effect of noradrenaline (NA, 0.5×10^{-9} moles) on the membrane potential (upper trace) and tone of the bovine retractor penis. (a) In the presence of prazosin (1.4×10^{-6}M), following depolarisation and an increase in resting tone, noradrenaline produced a small hyperpolarisation and relaxation. (b) After 30 min propranolol (4×10^{-6}M, prazosin still present), the inhibitory electrical and mechanical responses to noradrenaline were abolished.

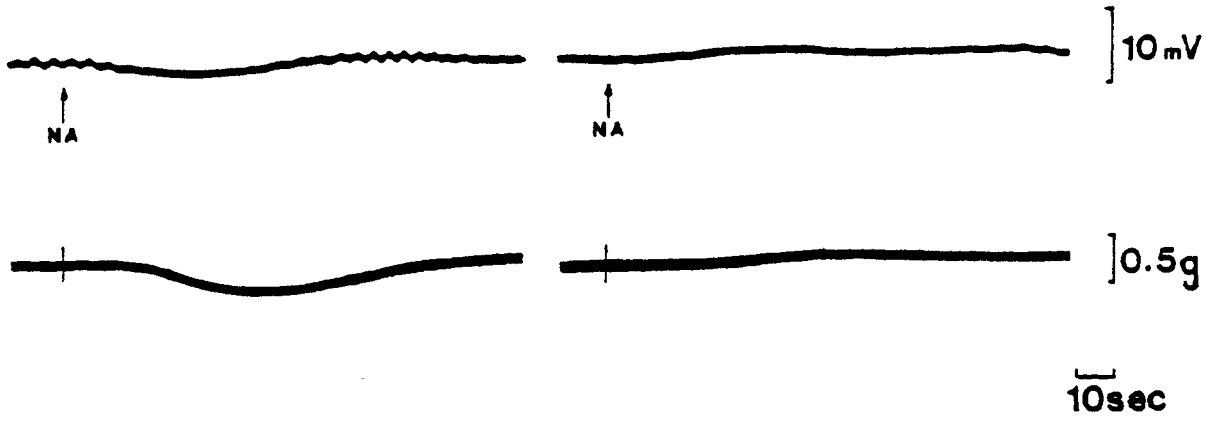

(a)
(b)
NA
NA
10 mV
0.5g
10sec

FIGURE 42

The effect of apamin (5×10^{-7}M at bar for the times indicated) on the inhibitory potential (upper trace) and relaxation (lower trace) of the bovine retractor penis in response to field stimulation (10 pulses, 1-10Hz, supramaximal voltage, 0.2ms pulse width as indicated below bars). The inhibitory potential was slightly reduced without any decrease in the amplitude of the relaxation. Sucrose gap recording.

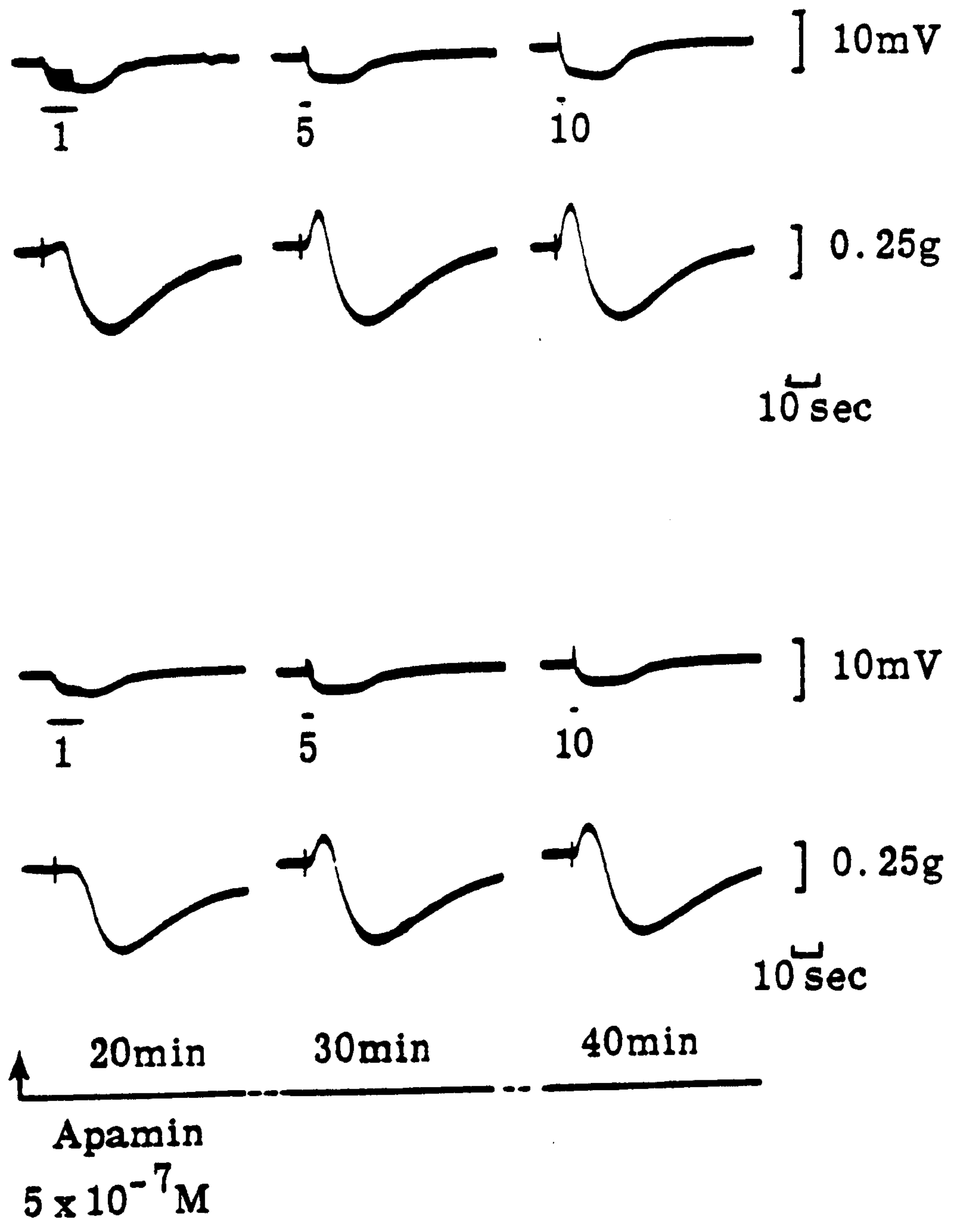
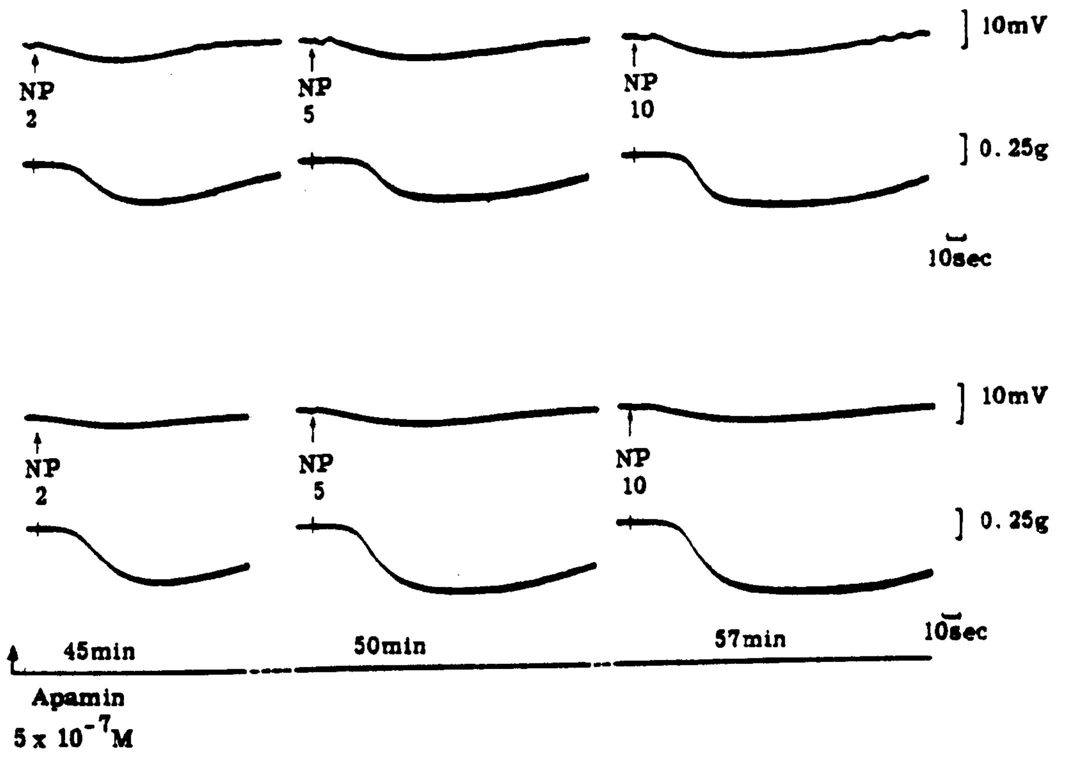

10mV
1
5
10
0.25g
10 sec
10mV
1
5
10
0.25g
10 sec
20min
30min
40min
Apamin
5 x 10^-7 M

FIGURE 43

The effect of apamin (5×10^{-7}M at bar for the times indicated) on the hyperpolarisation (upper trace) and relaxation (lower trace) of the bovine retractor penis in response to sodium nitroprusside (NP, $2-10 \times 10^{-12}$ moles). The hyperpolarisation to sodium nitroprusside was slightly reduced without any decrease in amplitude of the relaxation. Sucrose gap recording.



10mV
NP 2
NP 5
NP 10
0. 25g
10sec
10mV
NP 2
NP 5
NP 10
0. 25g
45min
50min
57min
10sec
Apamin
5 x 10⁻⁷M

ation by inhibitory β-adrenoceptors (Fig.41b; Klinge and Sjöstrand, 1974).

THE EFFECTS OF POTENTIAL BLOCKING AGENTS ON THE INHIBITORY RESPONSE TO FIELD STIMULATION, EXTRACT AND DRUGS

Apamin

Apamin ($1-5 \times 10^{-7}$M) depolarised the membrane potential (by some 2-3mV, sucrose gap recording) and slightly increased the tone.

The amplitude of the inhibitory potential (5-10 pulses, 1-10Hz; Fig.42) and hyperpolarisation to sodium nitroprusside ($0.5-5 \times 10^{-12}$ moles; Fig.43) were slightly reduced (some 20%) by apamin (5×10^{-7}M) without any reduction in the accompanying relaxation. Thus, in comparison with the inhibitory potential and relaxation in the taenia coli which are abolished by 5×10^{-7}M apamin (Maas and Hertog, 1979; Maas, 1981; Hills et al, 1983), the bovine retractor penis was insensitive to the drug.

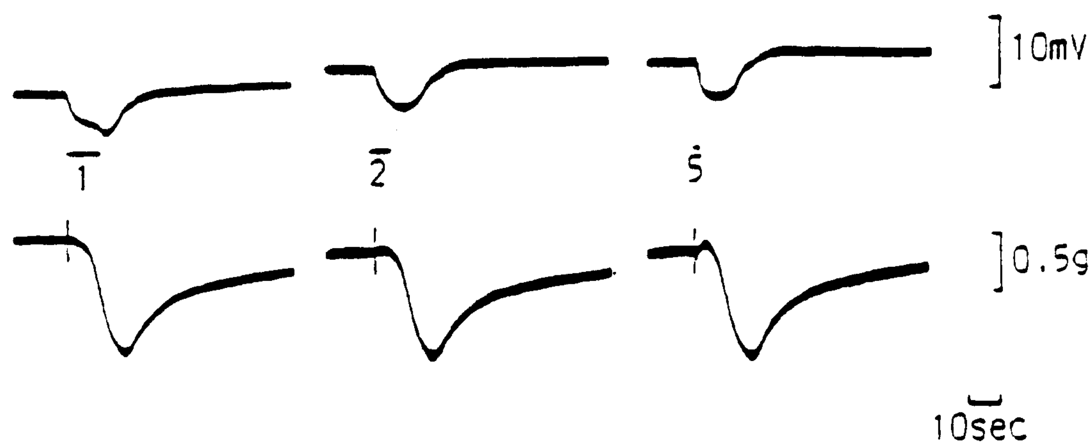
Oxyhaemoglobin

Initially, oxyhaemoglobin ($4-8 \times 10^{-6}$M) depolarised (by up to 8mV) the membrane potential and increased the tone. Repolarisation to the original membrane potential (within 4-6 min) was followed by a slow hyperpolarisation and decrease in resting tone.

The inhibitory potential and the accompanying relaxation to field stimulation (1-10 pulses, 1-10Hz) were abolished

FIGURE 44

The effects of oxyhaemoglobin (Oxy Hb, 8×10^{-6}M for the times indicated) on the inhibitory potential (upper trace) and relaxation (lower trace) to field stimulation (10 pulses, 1-5Hz, supramaximal voltage, 0.5ms pulse width) of the bovine retractor penis. The inhibitory potential and the relaxation were blocked by oxyhaemoglobin. Sucrose gap recording; phentolamine (5×10^{-6}M) present throughout.


10mV
1
2
5
0.5g
10sec

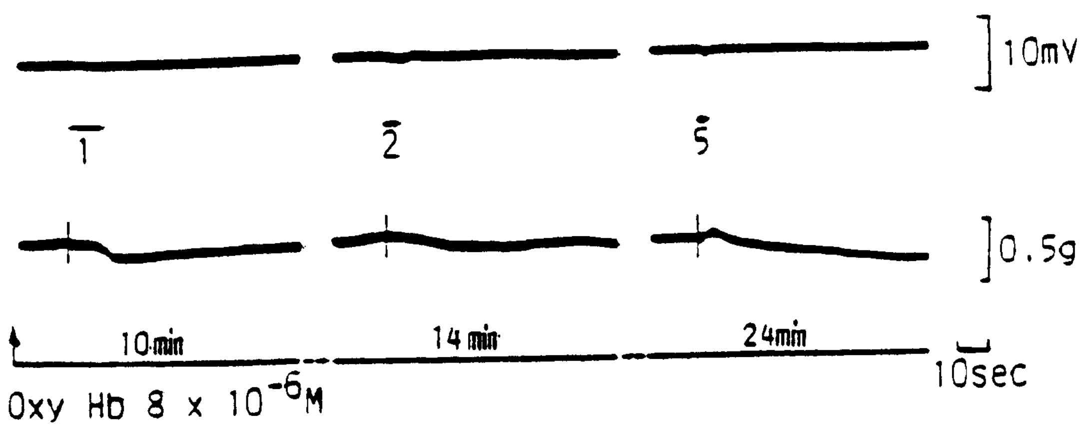

10mV
1
2
5
0.5g
10min
14min
24min
10sec
Oxy Hb 8 x 10-6M

FIGURE 45

The effects of oxyhaemoglobin (Oxy Hb, 4×10^{-6}M for the times indicated) on the hyperpolarisation (upper trace) and relaxation (lower trace) of the bovine retractor penis to cleaned activated extract (CAE, 5-10µl as indicated below arrows) and field stimulation (single pulse at S and 5 pulses at 1Hz, supramaximal voltage, 0.5ms pulse width as indicated below bars) compared in the same tissue. Blockade of the responses to the extract (CAE) and field stimulation was accompanied by the emergence of a contraction to the latter which was unassociated with any significant electrical change. The tone level in (b) was greater than that in (a). Sucrose gap recording.

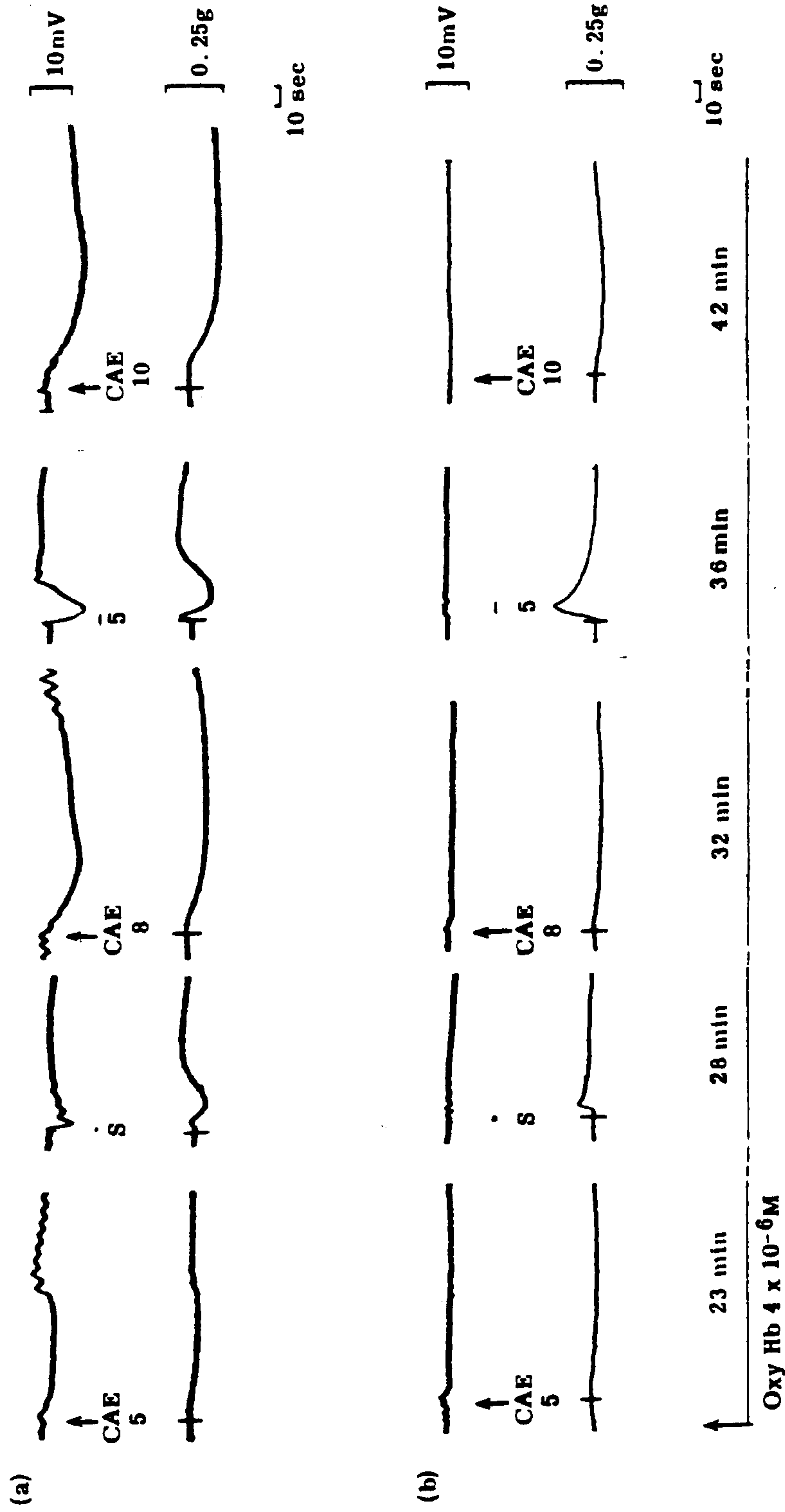
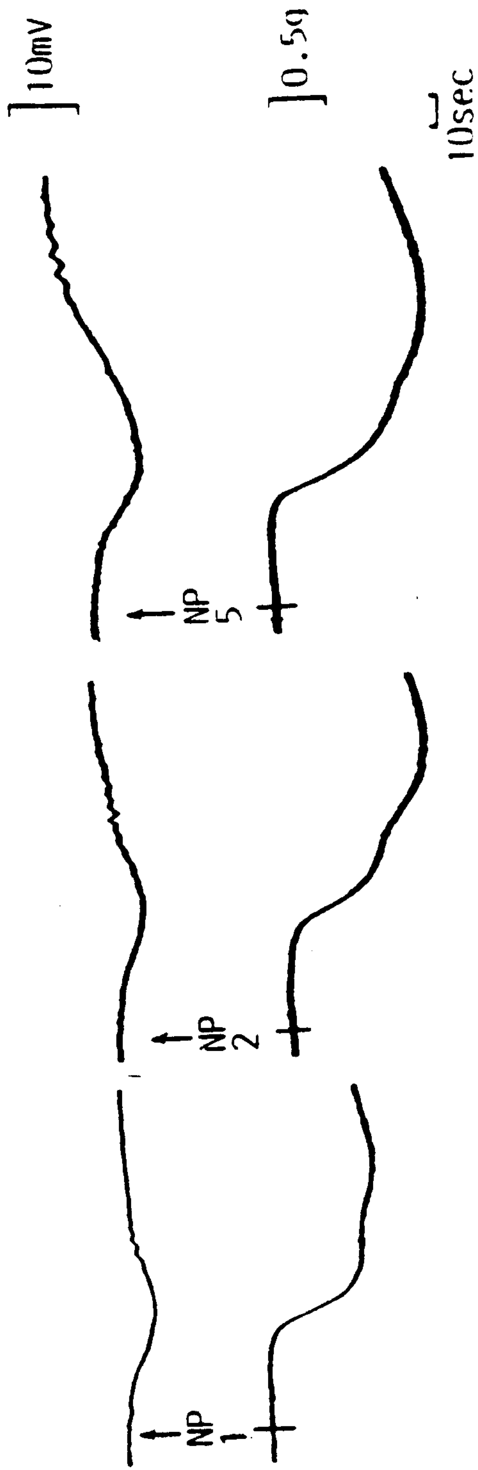
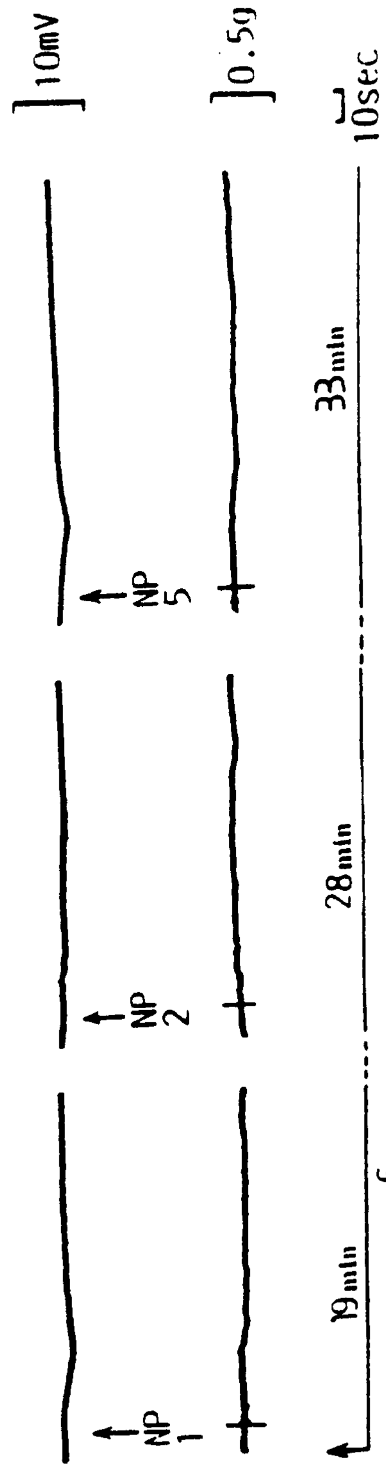

(a)
(b)
CAE 5
S
CAE 8
5
CAE 10
10mV
0.25g
10 sec
23 min
28 min
32 min
36 min
42 min
Oxy Hb 4 x 10-6M

FIGURE 46

The effects of oxyhaemoglobin (Oxy Hb, 8×10^{-6}M for the times indicated) on the hyperpolarisation (upper trace) and relaxation (lower trace) of the bovine retractor penis to sodium nitroprusside (NP, $1-5 \times 10^{-12}$ moles as indicated below arrows). The inhibitory electrical and mechanical responses together with the rebound depolarisation to sodium nitroprusside were blocked by oxyhaemoglobin. The tone level was increased in the presence of oxyhaemoglobin. Sucrose gap recording.


10mV
0.5g
10sec
NP 1
NP 2
NP 5


10mV
0.5g
10sec
NP 1
NP 2
NP 5
19min
28min
33min
Oxy Hb 8 x 10-6M.

by oxyhaemoglobin ($4-8 \times 10^{-6}$M) after some 5-10 min (Fig. 44), sometimes revealing a contraction which was unassociated with any electrical change (Fig.45). Blockade of the inhibitory potential and relaxation was not overcome by increasing the number of pulses applied (from 10 to 20).

The hyperpolarisations and relaxations to the inhibitory extract (1-20µl; Fig.45) and sodium nitroprusside ($1-50 \times 10^{-12}$ moles; Fig.46) were also blocked by oxyhaemoglobin ($4-8 \times 10^{-6}$M). Blockade of the inhibitory electrical responses to the extract, sodium nitroprusside and field stimulation occurred during periods both of depolarisation and hyperpolarisation by oxyhaemoglobin, suggesting that it was not due to a change in resting membrane potential.

Tetraethylammonium (TEA)

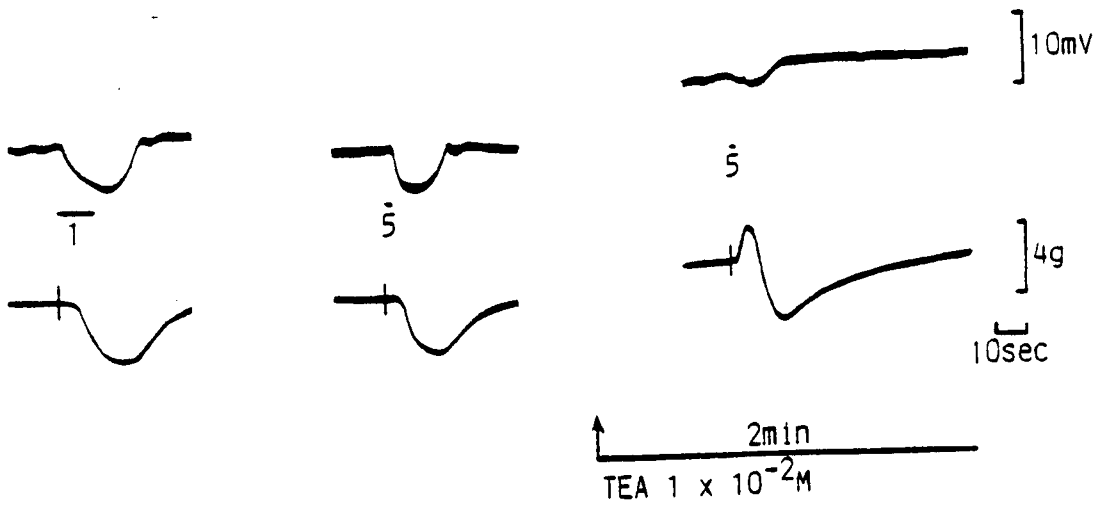
Neither the inhibitory potential nor the relaxation to field stimulation (1-10 pulses, 1-10Hz) was enhanced by TEA (1×10^{-4}M). Higher concentrations ($0.5-10 \times 10^{-3}$M) produced a concentration-dependent depolarisation (up to 40mV, sucrose gap recording) and reduction in the amplitude of the inhibitory potential. The accompanying relaxation to field stimulation was not abolished (Fig.47) and thus occurred without any apparent change in membrane potential.

Ouabain

Ouabain ($1-5 \times 10^{-5}$M) initially depolarised (2-4mV) the membrane potential and increased the resting tone. The inhibitory potential (1-10 pulses, 1-10Hz) was reduced (by

FIGURE 47

The effect of tetraethylammonium (TEA, 10^{-2}M for the times indicated) on the inhibitory potential (upper trace) and relaxation of the bovine retractor penis to field stimulation (10 pulses, 1 and 5Hz, supramaximal voltage, 0.5ms pulse width). The membrane potential was depolarised by TEA and the tone level increased. The inhibitory potential but not the relaxation to field stimulation was abolished. The effect of TEA was reversed following its removal (↓). Sucrose gap recording.


10mV
1
5
5
4g
10sec
2min
TEA 1 x 10-2M

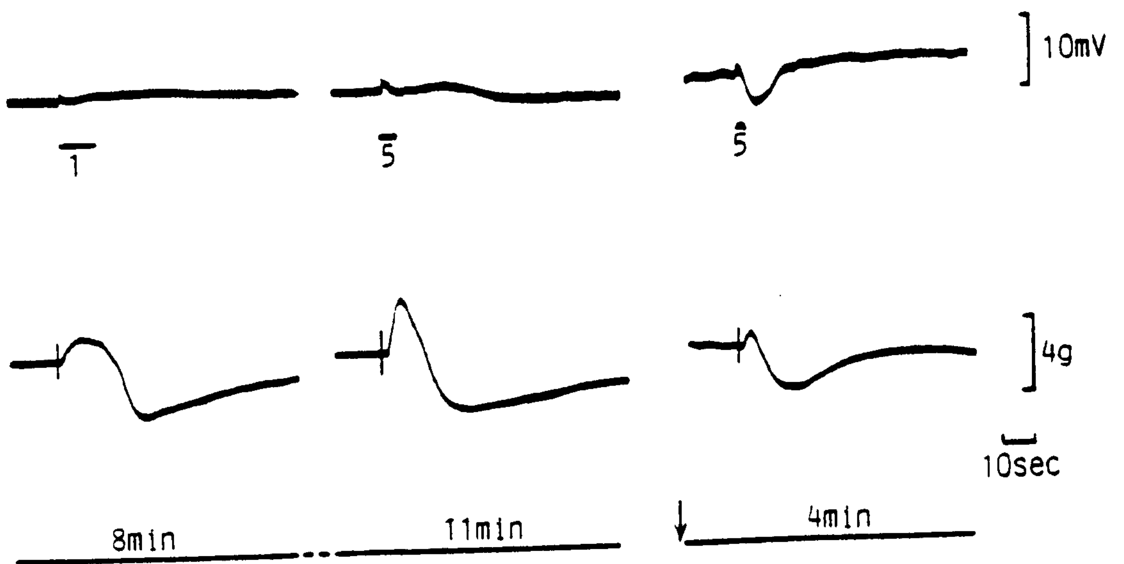
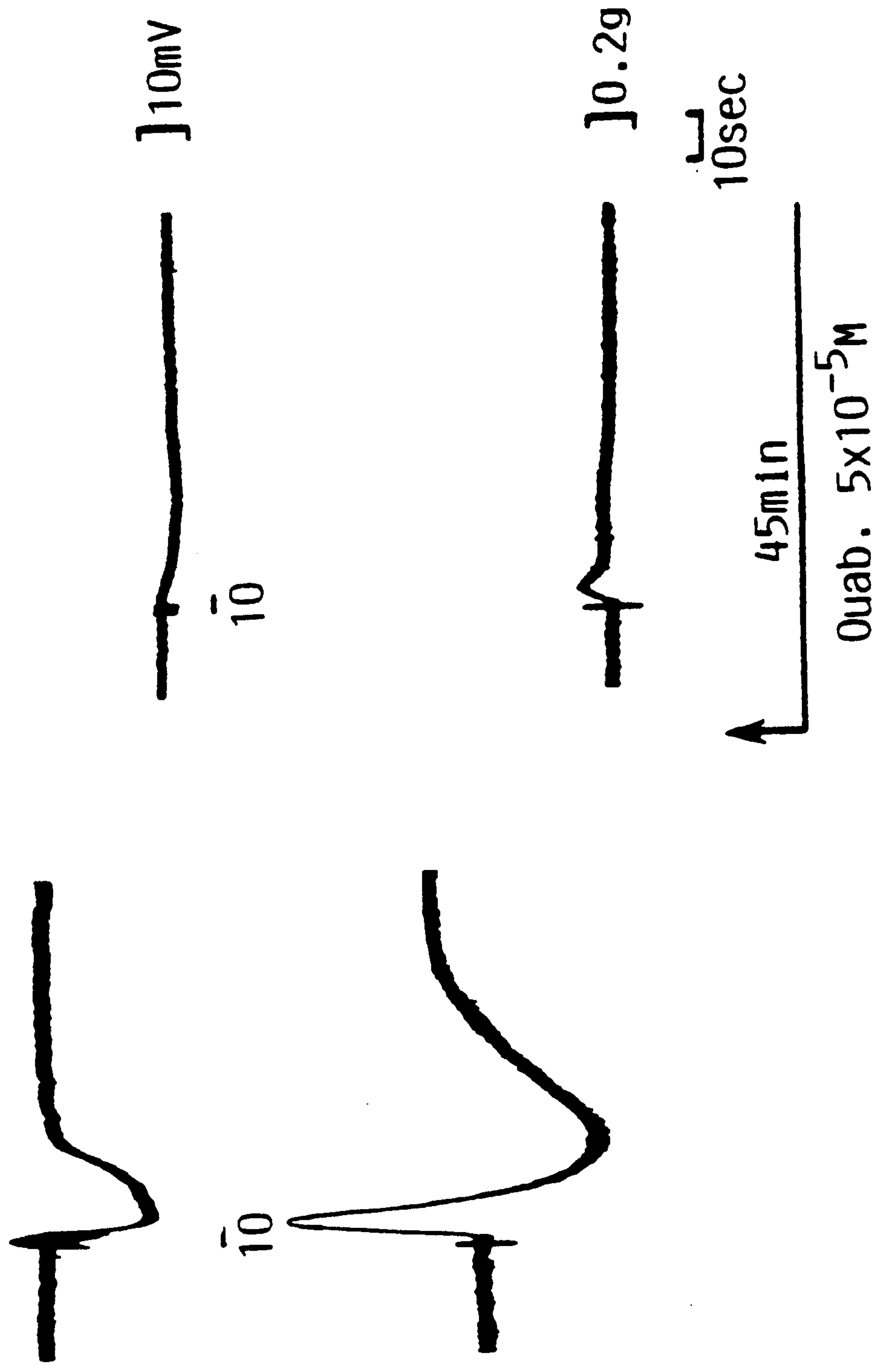

10mV
1
5
5
4g
10sec
8min
11min
4min

FIGURE 48

The effect of ouabain (Ouab, 5×10^{-5}M, 45 min contact) on the inhibitory potential (upper trace) and relaxation of the bovine retractor penis to field stimulation (10 pulses, 10Hz, supramaximal voltage, 0.2ms pulse width). The inhibitory potential was almost abolished after prolonged contact with ouabain during which time the membrane hyperpolarised and tone declined. In this preparation, which exhibited biphasic electrical and mechanical responses to field stimulation, a small excitatory potential and contraction were also reduced by ouabain. Sucrose gap recording.


10mV
0.2g
10sec
10
10
45min
Ouab. 5x10^{-5}M

30-40%) during this period with either no effect or apparent enhancement of the mechanical response caused presumably by increased tone. After 6-10 min there was a slow hyperpolarisation of the membrane and a progressive loss of resting tone. During this time the inhibitory potential and relaxation were reduced further or, after prolonged contact (45-60 min), abolished (Fig.48). The prolonged contact time required for this effect indicated that antagonism of the inhibitory potential was not due to a reduction in Na^{+}-K^{+} ATPase activity *per se*.

Removal of ouabain produced a transient hyperpolarisation (some 3mV). There was no recovery of the membrane potential, spontaneous tone or inhibitory electrical and mechanical responses to field stimulation during a 15-20 min period.

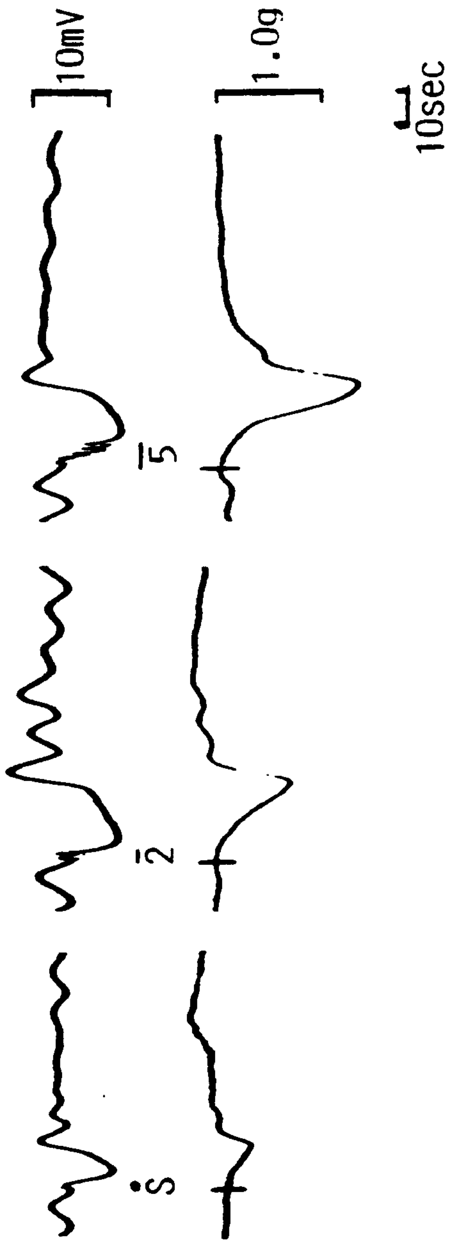
THE EFFECT OF M and B 22948 ON THE INHIBITORY ELECTRICAL AND MECHANICAL RESPONSES TO FIELD STIMULATION AND INHIBITORY EXTRACT

M and B 22948 (2-o-propoxyphenyl-8-azapurin-6-one), which inhibits cGMP-specific phosphodiesterase (Bergstrand, Lungquist and Schurmann, 1978) potentiated the relaxation of the bovine retractor penis to field stimulation (Bowman and Drummond, 1984). The effects of M and B 29948 on the inhibitory potential was therefore examined using the sucrose gap.

Initially, M and B 22948 ($3-9 \times 10^{-6}$M) produced a transient (approximately 5 min) hyperpolarisation (5-8mV) and

FIGURE 49

The effect of the cGMP-specific phosphodiesterase inhibitor, M & B 22948 (2-O-propoxyphenol-8-azapurin-6-one, 9×10^{-6}M for the times indicated) on the inhibitory potential (upper trace) and relaxation (lower trace) of the bovine retractor penis to field stimulation (single pulse at S, 2 and 5 pulses at 1Hz, supramaximal voltage, 0.2ms pulse width, as indicated below bars). Both the amplitude and duration of the relaxation were increased by M & B 22948. The amplitude of the inhibitory potential was not enhanced, although its duration was slightly increased. Sucrose gap recording.


10mV
1.0g
10sec
S
2
5

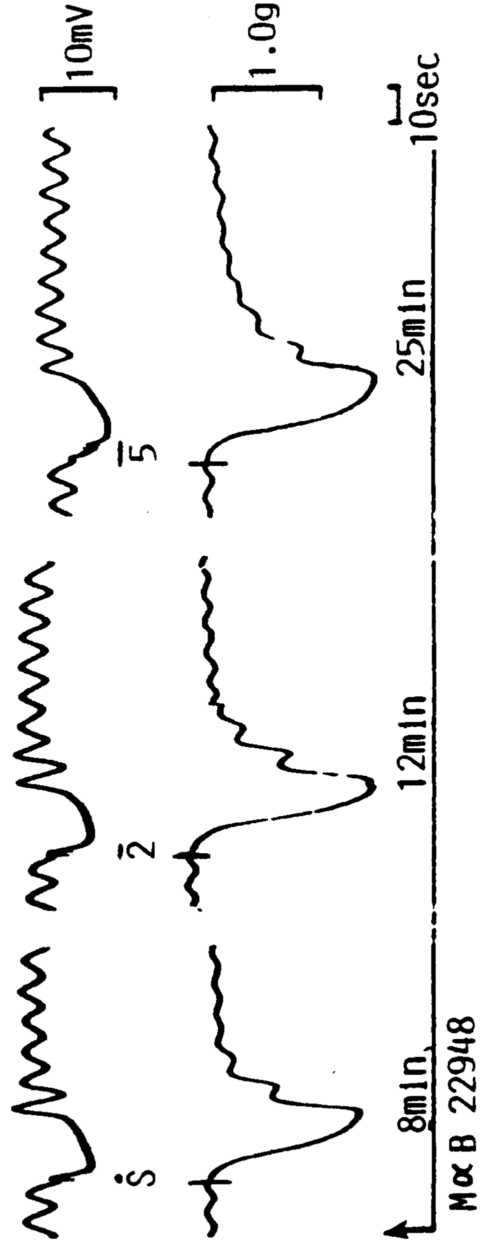
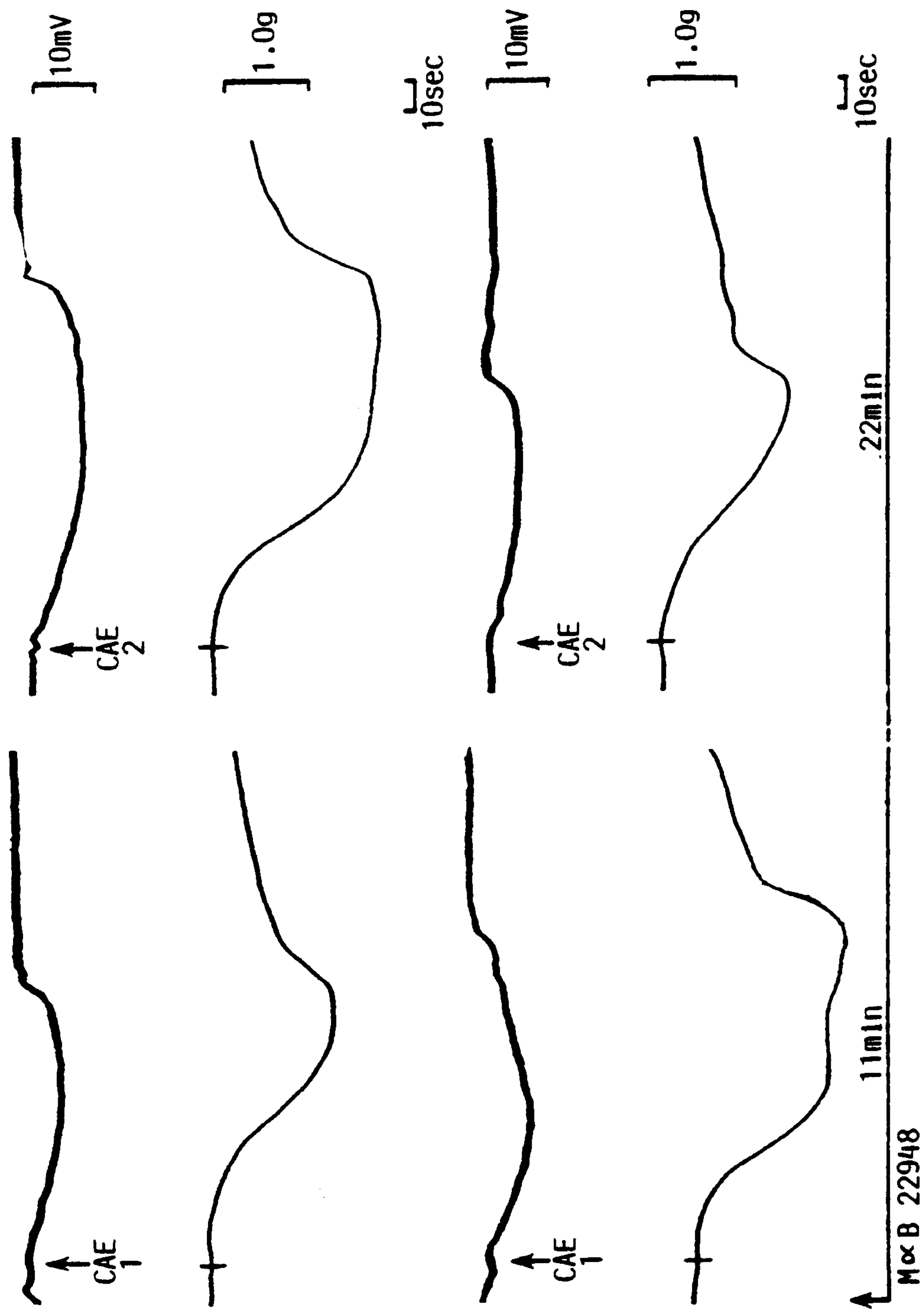

10mV
1.0g
10sec
S
2
5
8min
12min
25min
MαB 22948

FIGURE 50

The effect of the cGMP-specific phosphodiesterase inhibitor, M & B 22948 (9×10^{-6}M for the time indicated) on the hyperpolarisation (upper trace) and relaxation (lower trace) of the bovine retractor penis to cleaned activated extract (CAE, 1-2μl). In the presence of M & B 22948 the relaxation to inhibitory extract was initially enhanced in amplitude and duration without any apparent change in the accompanying hyperpolarisation. However, after some 20 min, both the hyperpolarisation and relaxation to inhibitory extract was reduced. Sucrose gap recording.


10mV
1.0g
10sec
10mV
1.0g
10sec
CAE 1
CAE 2
CAE 1
CAE 2
11min
22min
MαB 22948

relaxation, after which the membrane potential and tone recovered. The relaxation to field stimulation (1-10 pulses, 1Hz) was increased in amplitude, by up to 800% (single pulse), and in duration in the presence of M and B 22948. The accompanying inhibitory potential was unchanged in amplitude, though its duration was sometimes increased (Fig.49).

The amplitude of the relaxation to inhibitory extract was also increased by some 50% in the presence of M and B 22948 (9×10^{-6}M), without any enhancement of the accompanying hyperpolarisation (Fig.50). However, in contrast with the inhibitory response to field stimulation, continued exposure to M and B 22948 (20-25 min contact) inhibited the hyperpolarisation and relaxation in response to extract (Fig.50).

RELATIONSHIP BETWEEN THE INHIBITORY POTENTIAL AMPLITUDE AND MEMBRANE POTENTIAL

In these studies, the membrane potential was displaced by passing current through the muscle and field stimulation (1-15 pulses, 1-10Hz, supramaximal voltage, 0.5ms pulse width) carried out. The results obtained intracellularly using the technique of Abe and Tomita (1968) differed from those obtained with the double sucrose gap, and are considered to be correct. The sets of results will be considered separately.

Intracellular results

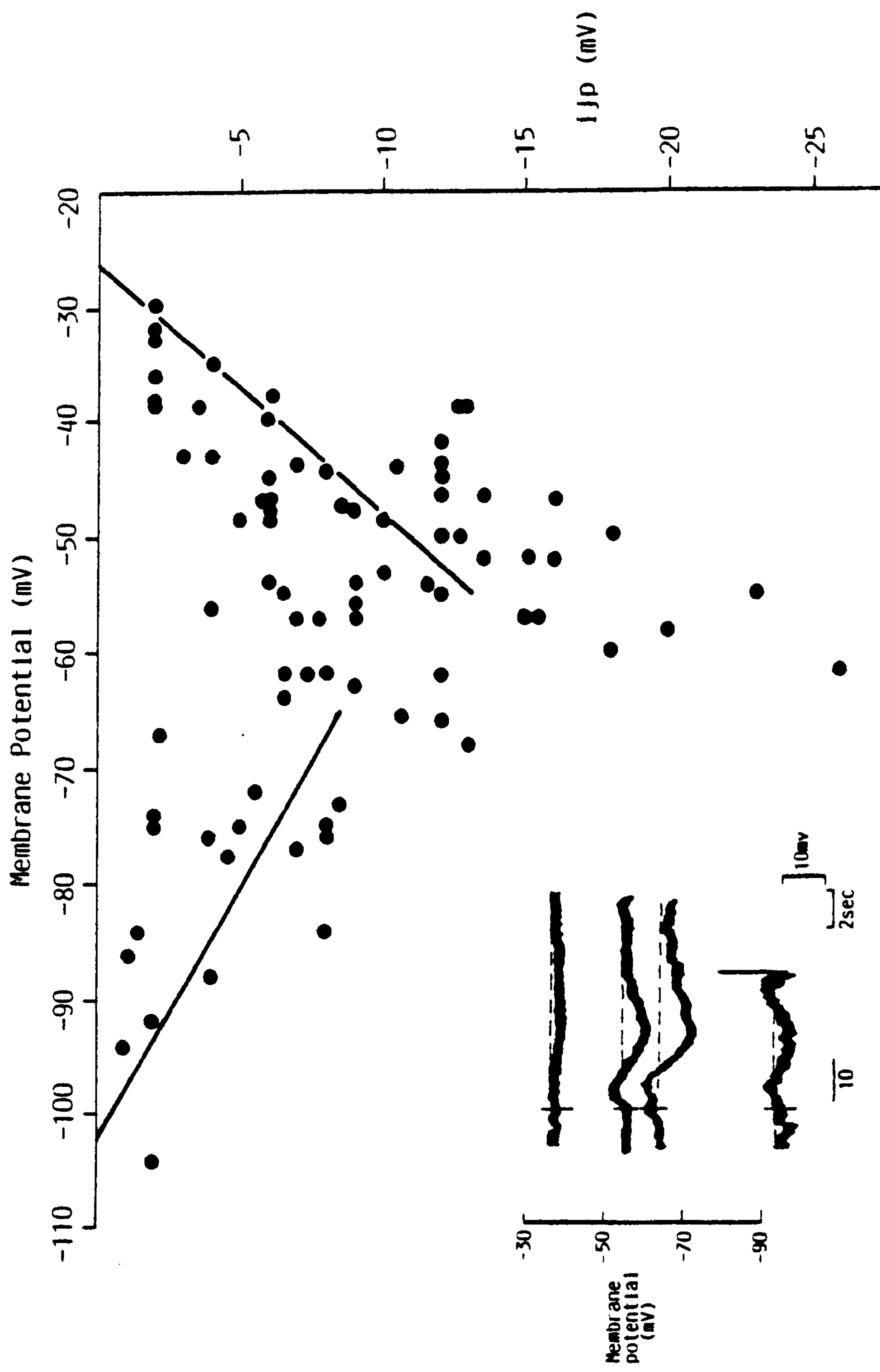
The amplitude of the inhibitory potential was reduced

or abolished when field stimulation was carried out during displacement of the membrane potential from rest (some -40 - -45mV) to more positive values using outward (depolarising) current pulses. When inward (hyperpolarising) current pulses were applied, there were two distinct changes in the inhibitory potential. First, over the range -45 to approximately -60mV, the amplitude of the inhibitory potential was increased (Fig.51). Secondly, with further passive hyperpolarisation to membrane potentials exceeding -60mV, the inhibitory potential was reduced in proportion to the change in membrane potential (Fig. 51). In the absence of guanethidine, inhibitory potentials at values of membrane potential more negative than rest were preceded by excitatory potentials which increased in amplitude with further passive hyperpolarisation. For this reason, guanethidine (0.2×10^{-5}M) was usually present to abolish the excitatory potential.

In order to obtain the average values for the membrane potential at which the inhibitory potential was abolished, the results obtained from nine tissues were combined and regression analysis carried out (Fig.51). Data obtained over the range of membrane potentials -55 - -65mV and values of membrane potential at which the inhibitory potential was zero were excluded from the analysis. The two linear plots obtained had opposite slopes of different magnitude. Although this method of analysis is limited, it does suggest that over the membrane potential range -27mV to -55mV the inhibitory potential showed proportionately larger changes

FIGURE 51

Relationship between inhibitory potential amplitude (ijp mV) in response to field stimulation (10 pulses, 5Hz, supramaximal voltage, 0.5ms pulse width) and membrane potential recorded from the bovine retractor penis (9 different preparations). Displacement of the membrane potential from rest (some -40 to -45mV) to more positive values using outward current pulses reduced the amplitude of the inhibitory potential. Displacement to more negative values produced two distinct changes. As shown in the insert (recorded from one cell), over the range -40 to -60mV the amplitude of the inhibitory potential was enhanced. With further displacement to membrane potentials exceeding -60mV, the amplitude of the inhibitory potential was reduced in proportion with the change in membrane potential. The two lines shown were obtained by regression analysis. Data obtained over the range of membrane potentials -55 to -65mV and values at which the inhibitory potential was abolished were excluded from the analysis. (Respective correlation coefficients and degrees of freedom 0.598, 43 for membrane potentials -55 or less; 0.613, 21 for membrane potentials -65 or greater.) The values for membrane potential at which the inhibitory potential was abolished obtained by extrapolation of the plot were -103mV and -27mV. No reversal of the inhibitory potential (to give a depolarisation) was observed. Intracellular recordings using the method of Abe and Tomita (1968).


Membrane Potential (mV)
IJP (mV)
-20
-30
-40
-50
-60
-70
-80
-90
-100
-110
-5
-10
-15
-20
-25
Membrane potential (mV)
-30
-50
-70
-90
10mv
2sec
10

in amplitude than over the range -65mV to -103mV. The values of membrane potential at which the inhibitory potential was abolished, obtained by extrapolation of the plots (Fig.51), were -103mV and -27mV. There was no reversal of the inhibitory potential when the membrane was either depolarised to values of -20mV or hyperpolarised to some -120mV.

Double sucrose gap results

Inhibitory potentials were recorded only with difficulty in the double sucrose gap. The maximum amplitude was about 5mV. The cause of this difficulty appeared to be the presence of a second sucrose chamber (current-passing chamber, see Fig. 9, Methods section). Figure 52 shows the abolition of the inhibitory potential following the admission of sucrose to the current-passing chamber. Inhibitory potentials were only recovered by depolarising the tissue with high concentrations ($3\text{-}5 \times 10^{-5}$M) of guanethidine.

The resulting inhibitory potentials were unaffected by displacement of the membrane potential in either the hyperpolarising (up to -20mV) or depolarising (up to +15mV) direction. This discrepancy with the intracellular results may be due to the presence of an additional sucrose (current-passing) chamber. The tissue exposed to sucrose undergoes electrical changes which may spread electrotonically to the region in the Krebs chamber (see Coburn et al, 1975).

FIGURE 52

Double sucrose gap recording from the bovine retractor penis showing (a) inhibitory potential (upper trace) and relaxation (lower trace) in response to field stimulation (10 pulses, 5Hz supramaximal voltage; 0.5ms pulse width as indicated below bar) when sucrose was present on the recording side of the gap only. The current-passing chamber contained Krebs solution at room temperature. In (b), 15 min after the admission of sucrose to the current-passing chamber, the inhibitory potential and relaxation were abolished.

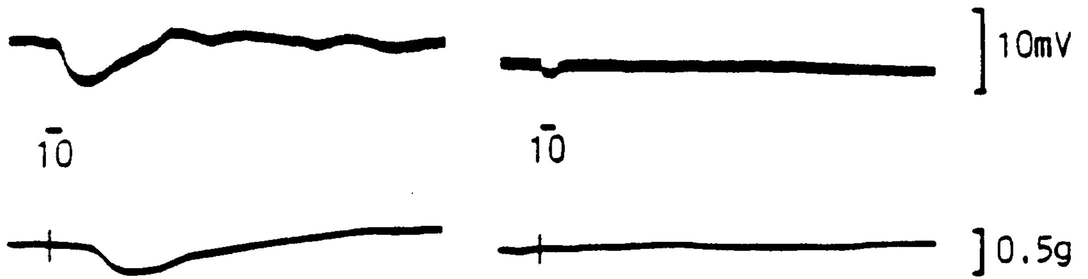
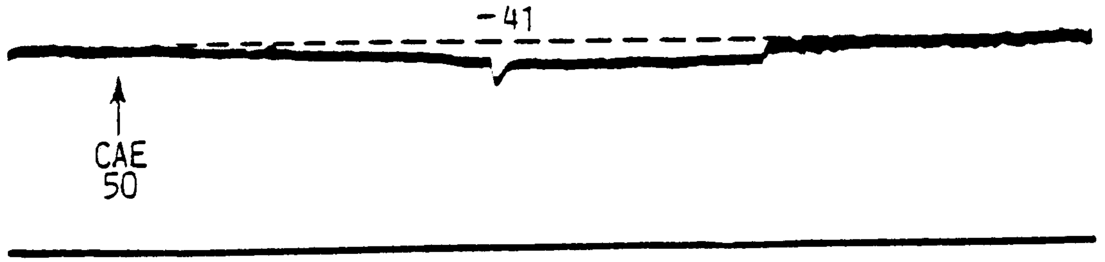
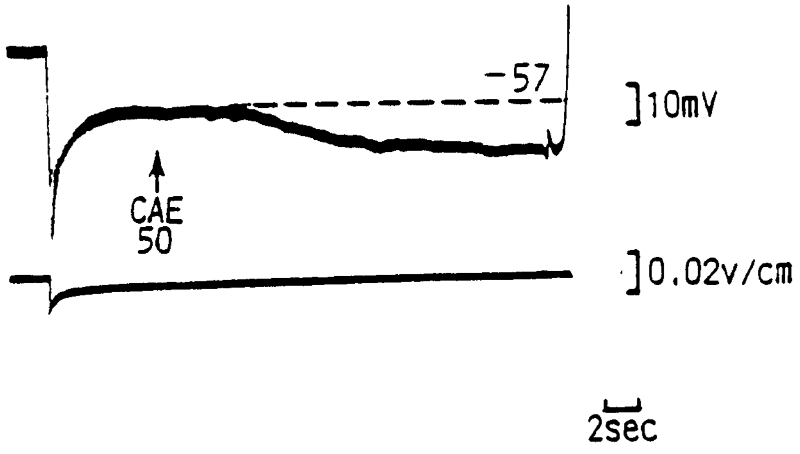

(a)
(b)
10
10
10mV
0.5g
5sec

FIGURE 53

The effect of displacement of the membrane potential by an inward (hyperpolarising) current pulse (Relative intensity V/cm, lower trace) on the hyperpolarisation (upper trace) in response to cleaned activated extract (CAE, 50µl) in a single cell of the bovine retractor penis. The amplitude of the hyperpolarisation to extract was increased during displacement of the membrane potential from rest (-41mV) to a more negative value (-57mV). Intracellular recording using the method of Abe and Tomita (1968).


-41
CAE
50


-57
10mV
CAE
50
0.02v/cm
2sec

RELATIONSHIP BETWEEN AMPLITUDE OF HYPERPOLARISATION TO INHIBITORY EXTRACT AND MEMBRANE POTENTIAL

This was investigated by the method of Abe and Tomita (1968). The membrane potential was displaced passively for prolonged periods (30-45s), during which the extract (20-100µl) was added. Because of the difficulty of maintaining impalements throughout this period, the relationship was investigated over a limited range of membrane potentials (-45mV to -57mV). A further restraint was that prolonged displacement with large currents caused a loss of electrical response to extract and field stimulation.

The amplitude of the hyperpolarisation to cleaned activated extract (CAE) was increased and, on occasion, preceded by a slight depolaristion, following displacement of the membrane potential from rest (approximately -45mV) to more negative values (up to -57mV) by inward current pulses (Fig.53).

Cleaned unactivated extract (CUE) was either ineffective or depolarised slightly following passive hyperpolarisation of the membrane potential.

CHANGE IN MEMBRANE RESISTANCE DURING THE INHIBITORY POTENTIAL

The effect of the inhibitory transmitter on membrane resistance was determined intracellularly by the application of inward or outward current pulses (duration 1s) via external electrodes (Abe and Tomita, 1968) at the peak of the

FIGURE 54

Change in membrane resistance during the inhibitory potential recorded from a single cell in the bovine retractor penis using the method of Abe and Tomita (1968). An electrotonic current pulse (duration 1s; current intensity V/cm shown in lower trace) was applied before and during the inhibitory potential to field stimulation (10 pulses, 5Hz; supramaximal voltage, 0.5ms pulse width as indicated below bar). The amplitude of the electrotonic pulse was increased during the inhibitory potential indicating an increase in membrane resistance. Guanethidine (2×10^{-6}M) present throughout.

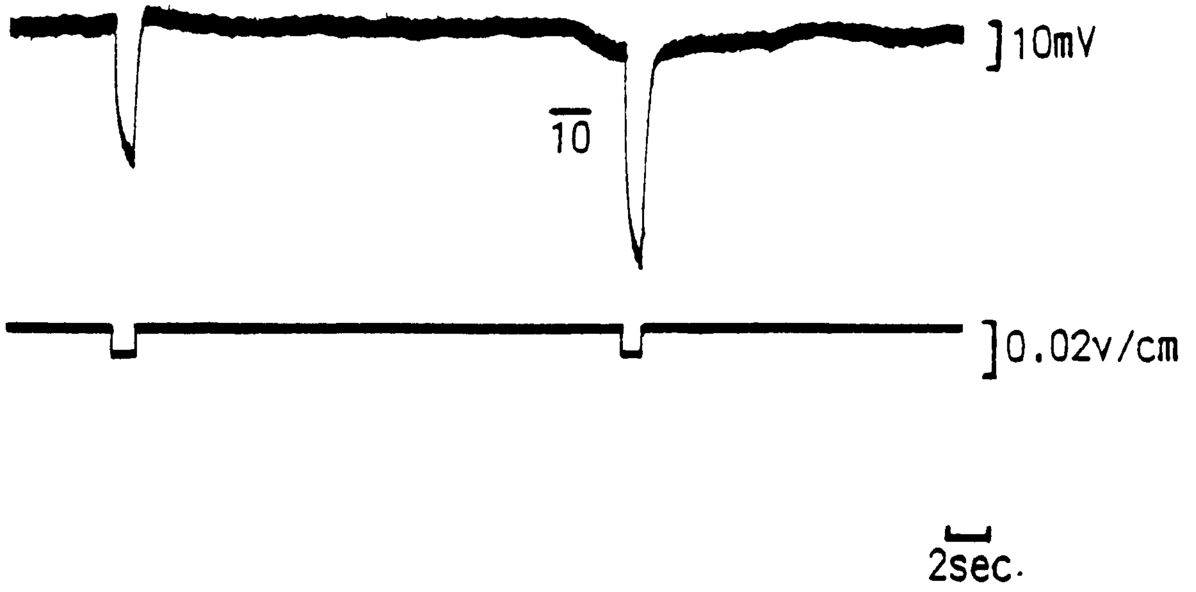
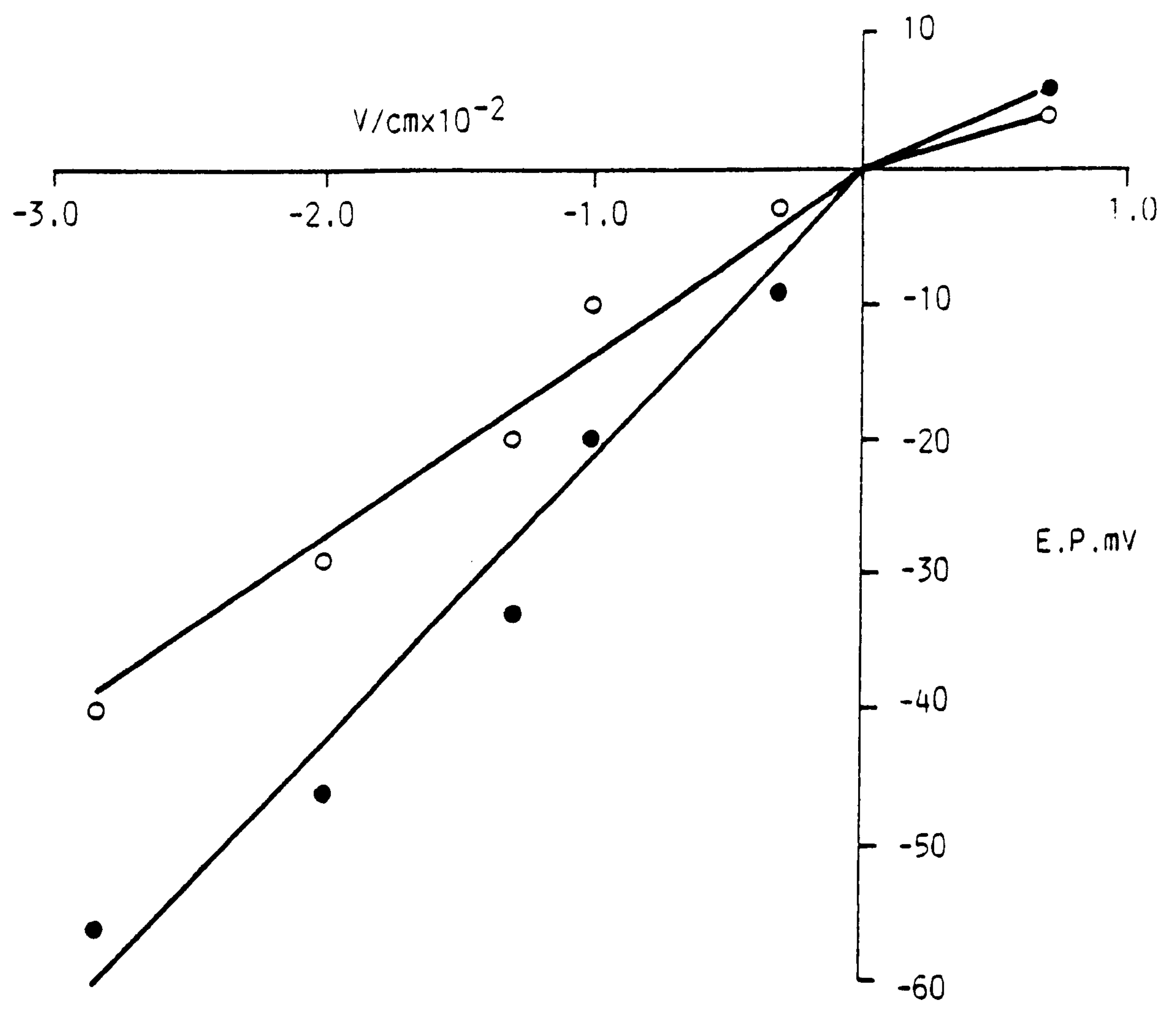

10mV
10
0.02v/cm
2sec.

FIGURE 55

Current (V/cm)-voltage (E.P.mV) relationship recorded from a single cell in the bovine retractor penis in response to inward and outward pulses (duration 1s) in the absence (open circles) and during (filled circles) the peak amplitude of the inhibitory potential (10 pulses, 5Hz; supramaximal voltage, 0.5ms pulse width). There was a significant ($p < 0.0025$, paired t-test) shift in the V/I plot to the right during the inhibitory potential indicating a decrease in membrane conductance.



V/cmx10^{-2}
-3.0
-2.0
-1.0
1.0
10
-10
-20
-30
-40
-50
-60
E.P.mV

inhibitory potential (usually some 2s after the stimulus).

The amplitude of electrotonic pulses was increased by up to 125% during the inhibitory potential (Fig.54), causing a shift in the V/I curve to the right (Fig.55). Electrotonic potentials were increased by an average of 74 ± 22% (n=14) during inhibitory potentials of 4mV amplitude. These results indicate that the basis for the inhibitory potential is a decrease in membrane ionic conductance.

THE EFFECT OF THE INHIBITORY EXTRACT ON MEMBRANE RESISTANCE

The effect of the extract on the amplitude of electrotonic current pulses (duration 1s, every 6-8s) was investigated intracellularly using the method of Abe and Tomita (1968). CAE produced inconsistent changes in the amplitude of the electrotonic pulses. These were either not affected significantly (n=26), increased (n=26; Fig.56) or, on some occasions (n=9) reduced. These inconsistencies may arise from the presence of impurities in the extract. CUE produced no significant change in the amplitude of electrotonic pulses (Fig.56).

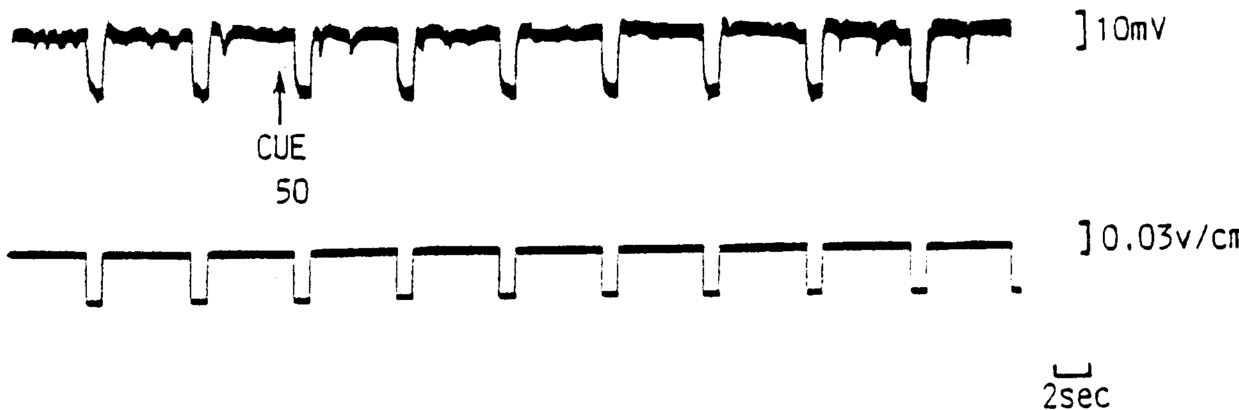
EFFECTS OF CHANGES TO THE IONIC ENVIRONMENT ON THE INHIBITORY RESPONSES TO FIELD STIMULATION AND INHIBITORY EXTRACT

Removal of K^+

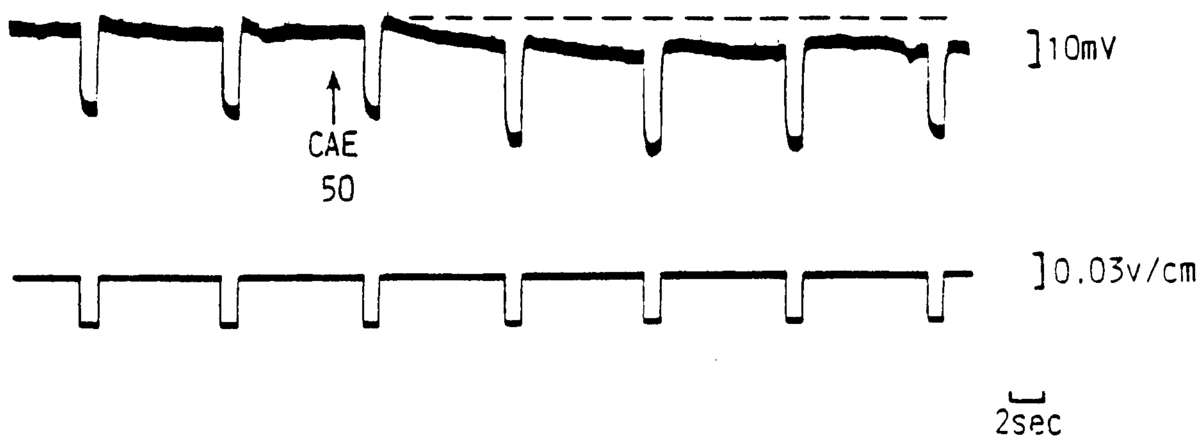
After some 3-6 min, the membrane potential hyperpolarised (3-10mV) and resting tone declined. The inhibitory potential

FIGURE 56

Changes in membrane resistance produced by the inhibitory extract as indicated by the amplitude of electrotonic current pulses (duration 1s, relative current intensity V/cm shown in lower trace) recorded from two cells (upper and lower panels) in the same bovine retractor penis preparation. Cleaned unactivated extract (CUE) produced no change in membrane potential or resistance. Cleaned activated extract (CAE, 50µl) hyperpolarised the membrane and increased the amplitude of the electrotonic pulses. Intracellular recording using the technique of Abe and Tomita (1968).



]10mV
CUE
50
]0.03v/cm
2sec



]10mV
CAE
50
]0.03v/cm
2sec

FIGURE 57

The effect of removing the external potassium ($\downarrow K^+_o$ for the times indicated) on the inhibitory potential (upper trace) and relaxation of the bovine retractor penis in response to field stimulation (10 pulses, 1-10Hz; supramaximal voltage, 0.5ms pulse width as indicated below bars). There was no initial enhancement of the inhibitory potential during the K^+-withdrawal. After prolonged contact (25-33 min) with K^+-free Krebs, the membrane potential was hyperpolarised, and both the resting tone and the inhibitory potential reduced. Readmission of K^+ ($\uparrow K^+_o$) produced initially a further hyperpolarisation and a reduction in tone. The inhibitory electrical and mechanical responses recovered in the continued presence of normal Krebs solution. Sucrose gap recording.

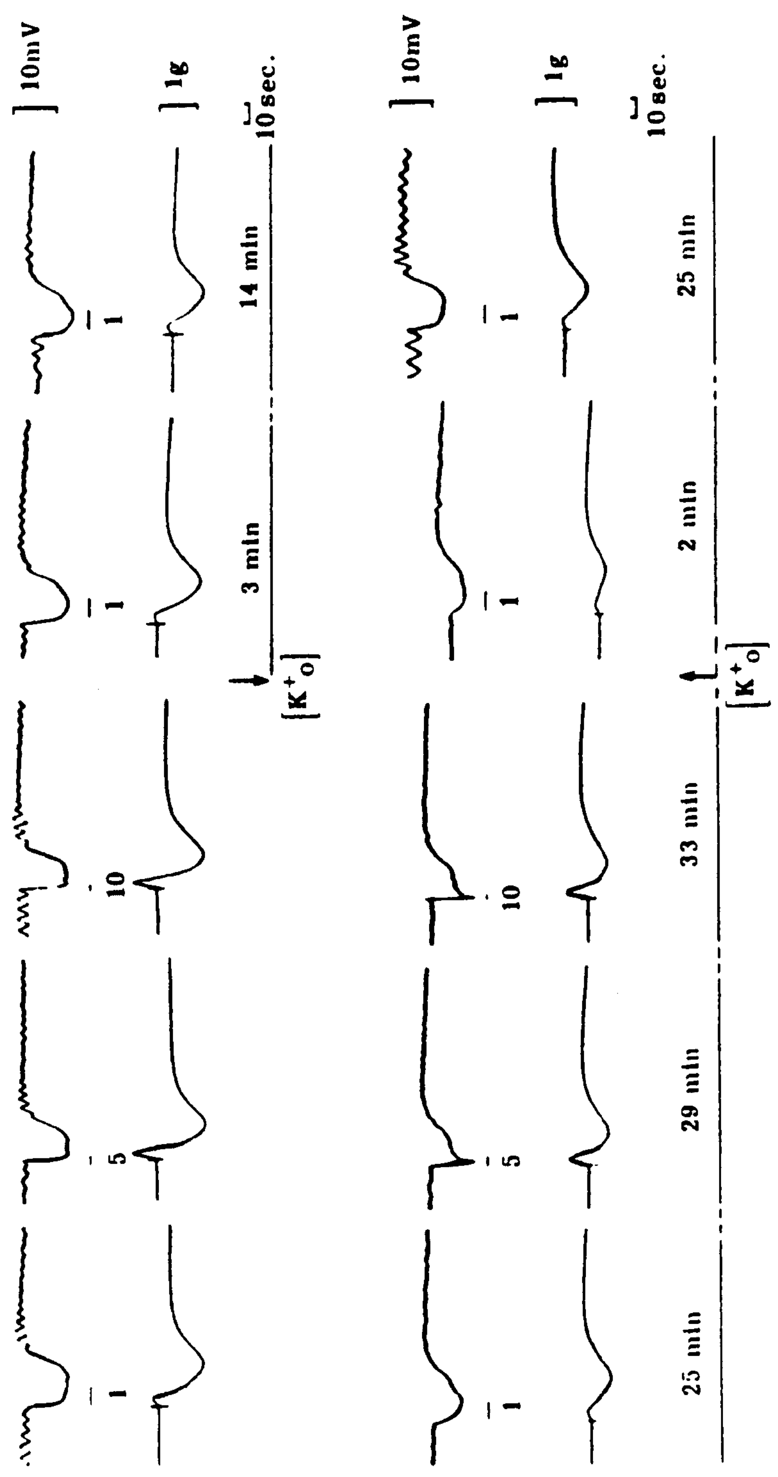
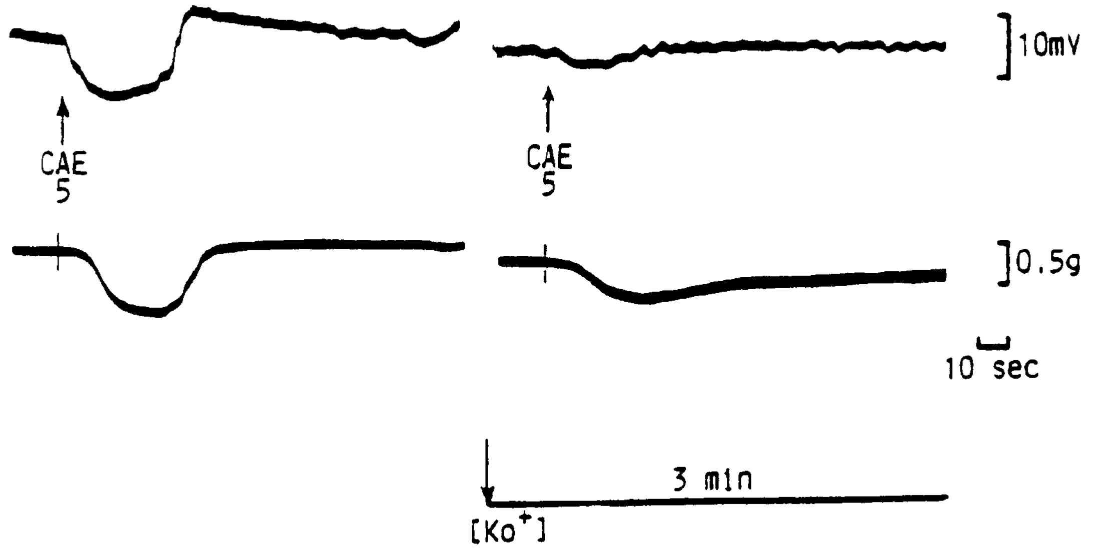
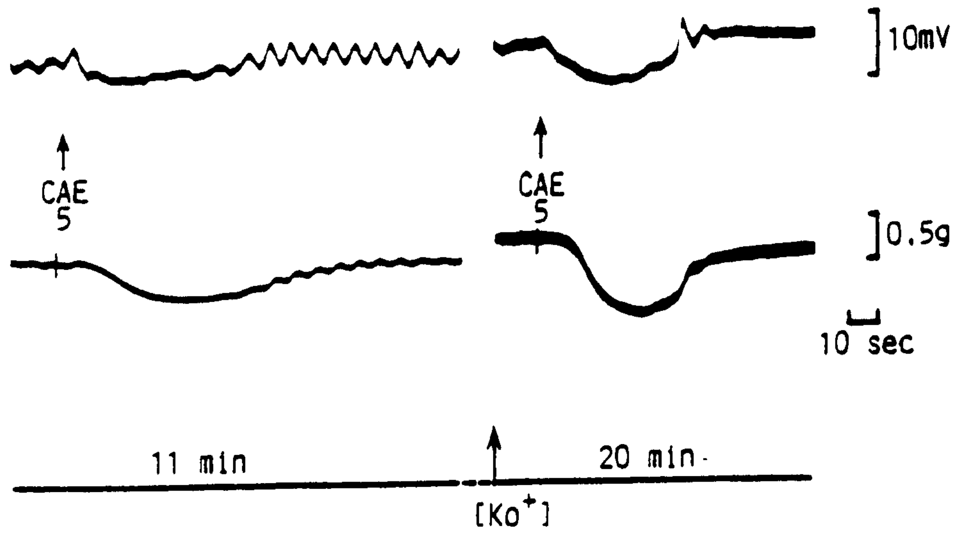

10mV
1g
10 sec.
1
5
10
1
1
14 min
3 min
[K+o]
10mV
1g
10 sec.
1
5
10
1
1
25 min
29 min
33 min
[K+o]
2 min
25 min

FIGURE 58

The effect of removing the external potassium ($\downarrow K^+_o$ for the times indicated) on the hyperpolarisation (upper trace) and relaxation of the bovine retractor penis in response to cleaned activated extract (CAE, 5µl). The hyperpolarisation to CAE was inhibited within some 3 min in the absence of K^+. Continued exposure to K^+-free solution hyperpolarised the membrane and reduced both the resting tone and the inhibitory electrical and mechanical responses to extract. These effects were reversed by readmitting K^+ to the Krebs solution ($\uparrow K^+_o$). Sucrose gap recording.



10mV
CAE
5
CAE
5
0.5g
10 sec
3 min
[Ko+]



10mV
CAE
5
CAE
5
0.5g
10 sec
11 min
20 min
[Ko+]

in response to field stimulation (1-10 pulses, 1-10Hz) was reduced by up to 50% after some 20 min (sucrose gap recording). The accompanying relaxation was also reduced, possibly because of the decline in resting tone caused by the removal of K^+ (Fig.57). In an intracellular study, inhibitory potentials, which were reduced in the absence of potassium, recovered when field stimulation was carried out during passive hyperpolarisation of the membrane to values of up to -60mV. Thus, the inhibitory potential was not reduced as a result of the hyperpolarisation caused by K^+ withdrawal. Neither was the inhibitory potential abolished because of a decrease in membrane resistance, as the V/I plot for the cell was unchanged.

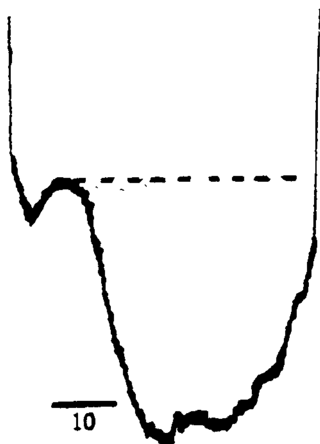
The hyperpolarisation in response to inhibitory extract (2-20µl: sucrose gap recording) was reduced within 5 min of K^+ withdrawal. The maximum reduction (between 60 and 75%) occurred usually after some 20 min (Fig.58). The accompanying relaxation was reduced, again possibly because of the loss of resting tone caused by K^+ withdrawal.

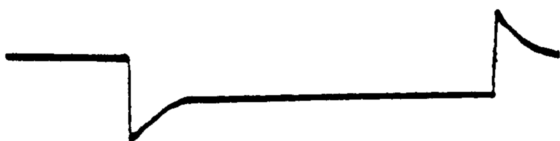
Readmission of K^+ transiently hyperpolarised the membrane potential (5-7mV) and decreased resting tone. During this period, the inhibitory potential and relaxation in response to field stimulation were further reduced. Repolarisation of the membrane and restoration of spontaneous tone was accompanied by recovery of the inhibitory electrical

FIGURE 59

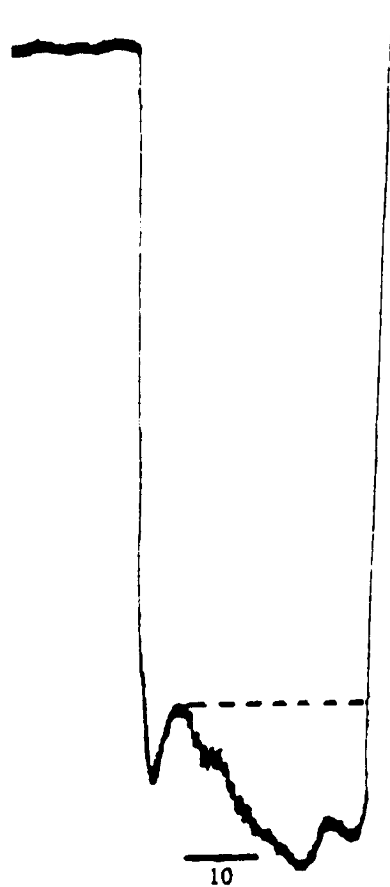
The effects of increasing the concentration of potassium from normal (4.7×10^{-3}M in (a)) to 20×10^{-3}M in (b) on the inhibitory potential in response to field stimulation (10 pulses, 5Hz; 0.5ms pulse width, supramaximal voltage) recorded from two cells in the same bovine retractor penis preparation. In (a), the inhibitory potential recorded in normal Krebs solution (resting membrane potential -42mV) was enhanced during passive displacement of the membrane potential to -73mV (shown by broken line). (b) After some 20 min in Krebs solution containing 20mM K^+ (resting membrane potential -33mV) in a different cell to (a), an inhibitory potential was recorded at a membrane potential of -83mV (shown by broken line), i.e. 34mV more negative than the estimated potassium equilibrium potential. In both (a) and (b), the value of the displaced membrane potentials were obtained following subtraction of the voltage transient recorded outside the cell during the current pulse. Intracellular electrical recording using the method of Abe and Tomita (1968). Guanethidine (2×10^{-6}M) present throughout.

(a)





(b)



0.02v/cm [ ] 10mV

2sec

and mechanical responses to field stimulation and to inhibitory extract.

The lack of enhancement of the hyperpolarisation to extract or field stimulation during K^+ withdrawal suggested that the response was not mediated by an increase in K^+ conductance.

Increasing potassium

Krebs solution containing 20mM K^+ (NaCl content reduced accordingly, see Table 1) depolarised the cells significantly from -38 ± 6 to -30 ± 5mV ($p < 0.001$, n=28 and 29 respectively). The inhibitory potential was abolished but recovered during passive hyperpolarisation of the membrane (Abe and Tomita, 1968) by electrotonic current pulses to values of up to -83mV (Fig.59). If the internal concentration of K^+ ($[K^+]_I$) in the bovine retractor penis is similar to that in the rat anococcygeus (127mM; Creed and Pollock, unpublished), E_K in 20mM K^+ ($[K^+]_0$) would be some -49mV (from $E_K = 2.3RT/ZF \log [K^+]_0/[K^+]_I$). Since inhibitory potentials were obtained at membrane potentials exceeding -49mV, this suggests that the response is not mediated by an increase in K^+ conductance.

Reducing or removing chloride

The effects of reducing the external chloride chloride on the inhibitory potential were similar regardless of whether

glutamate or benzenesulphonate/sulphate were used as substitutes. The inhibitory potential was reduced within 5 min contact with the modified Krebs solution (Fig.60). This effect appeared independent of any change in membrane potential, as it occurred during periods of depolarisation caused by substitution with glutamate, and hyperpolarisation produced by benzenesulphonate/sulphate. The accompanying relaxation in response to field stimulation was enhanced in accordance with the increase in tone produced by glutamate or reduced in the presence of benzenesulphonate/sulphate.

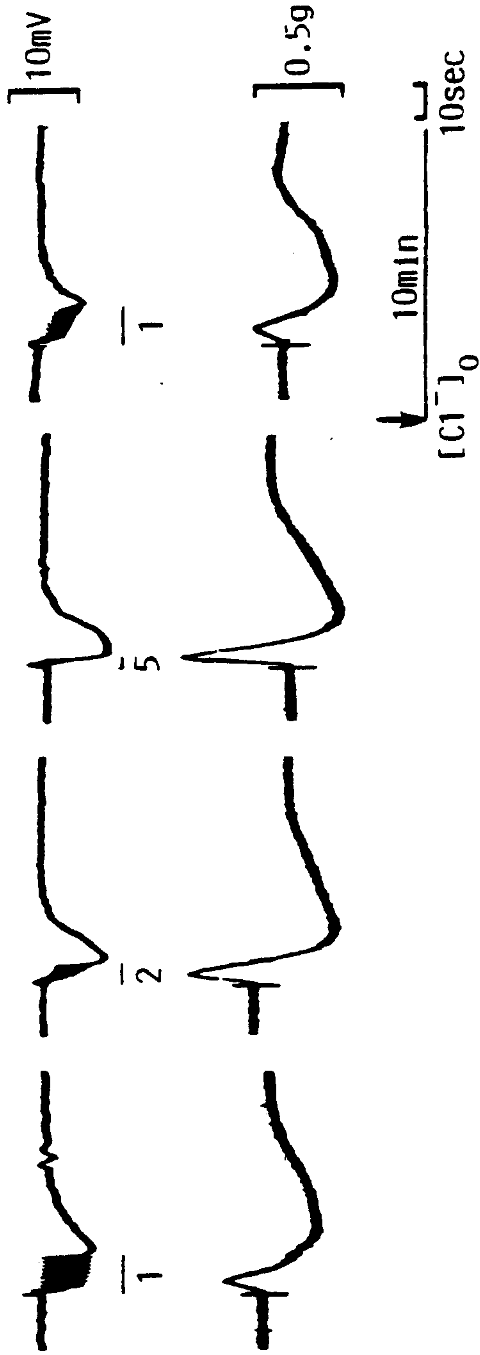
Continued exposure to low Cl^- (glutamate) or Cl^--free (benzenesulphonate/sulphate) solution caused a slow hyperpolarisation and loss of tone. The inhibitory potential and relaxation was further reduced or abolished after 25-30 min (Fig.60). The inhibitory potential was not restored during passive displacement of the membrane potential over a range from -26mV to -104mV by externally applied current using the method of Abe and Tomita (1968).

The hyperpolarisation and relaxation to inhibitory extract (2-20μl) was reduced or abolished within 6 min after replacement of the external chloride with benzenesulphonate/sulphate (Fig.61). Reduction of the relaxation appeared greater than could be accounted for merely by the decreased level of resting tone.

Readmission of Cl^- restored (after some 30-60 min) the membrane potential, tone and inhibitory electrical and mech-

FIGURE 60

The effect of reducing the external concentration of chloride from 111.8mM to 12.7mM ($\downarrow Cl^{-}_{o}$; replacement anion glutamate for the time indicated) on the inhibitory potential (upper trace) and relaxation of the bovine retractor penis in response to field stimulation (10 pulses, 1-5Hz; supramaximal voltage, 0.5ms pulse width as indicated below bars). The inhibitory potential was reduced during the initial depolarisation produced by glutamate (10 min) and thereafter (25-35 min) when the membrane was hyperpolarised and resting tone abolished. Small excitatory potentials and contractions were observed in this preparation after prolonged Cl^{-}-withdrawal. Readmission of Cl^{-} ($\uparrow Cl^{-}_{o}$) restored the membrane potential, tone and inhibitory electrical and mechanical responses to field stimulation. Sucrose gap recording.


10mV
0.5g
10sec
10min
[Cl⁻]o
1
5
2
1

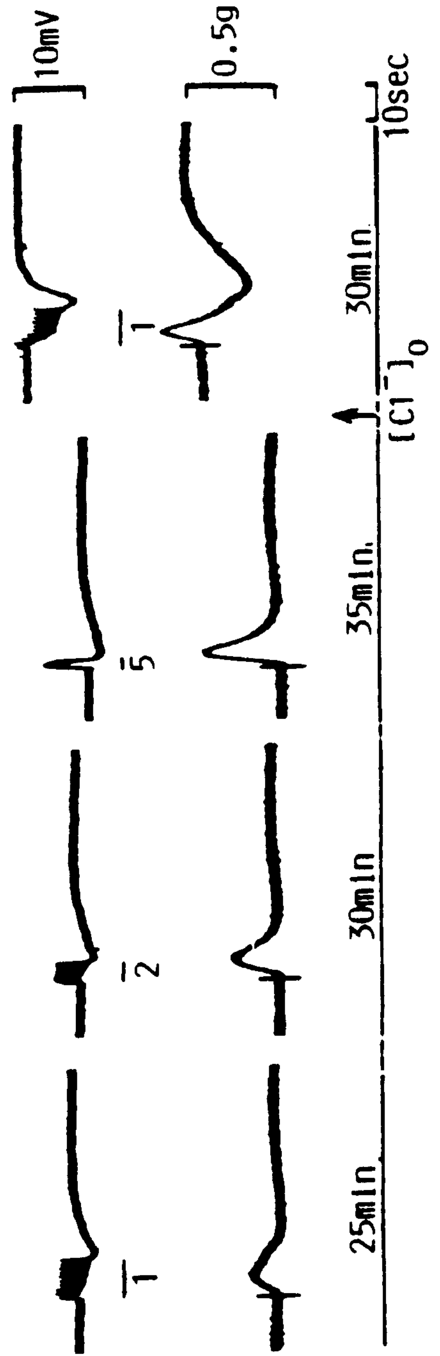
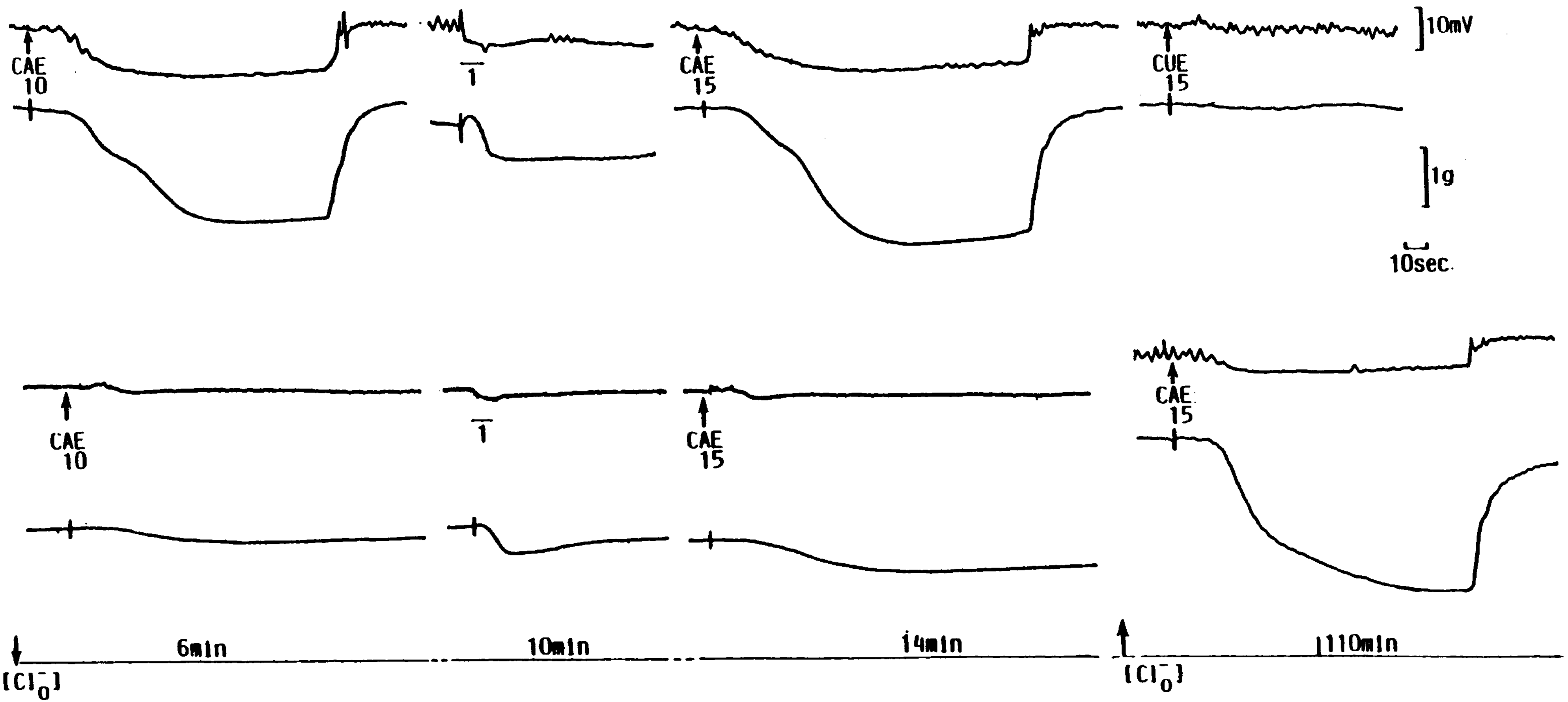

10mV
0.5g
10sec
30min
[Cl⁻]o
35min
30min
25min
1
5
2
1

FIGURE 61

The effect of removing the external chloride ($\downarrow Cl^-_o$; replacement anions sulphate and benzene-sulphonate for the times indicated) on the hyper-polarisation (upper trace) and relaxation of the bovine retractor penis in response to cleaned activated extract (CAE, 10-15µl) and field stimulation (10 pulses, 1Hz; supramaximal voltage, 0.5ms pulse width). In normal Krebs, CAE and field stimulation, but not CUE (15µl) produced hyperpolarisation reduction in electrical oscillation and relaxation. The hyperpolarisation to CAE was reduced or abolished with 6 min of Cl^--withdrawal. The resting tone level was also reduced and the membrane potential hyperpolarised. Readmission of Cl^- ($\uparrow Cl^-_o$) restored the membrane potential, tone and inhibitory electrical and mechanical responses to extract. Sucrose gap recording.


10mV
1g
10sec.
CAE 10
1
CAE 15
CUE 15
CAE 10
1
CAE 15
CAE 15
[Cl$_o^-$]
6min
10min
14min
[Cl$_o^-$]
110min

anical responses to field stimulation (Fig.60) and inhibitory extract (Fig.61).

RABBIT AND RAT ANOCOCCYGEUS

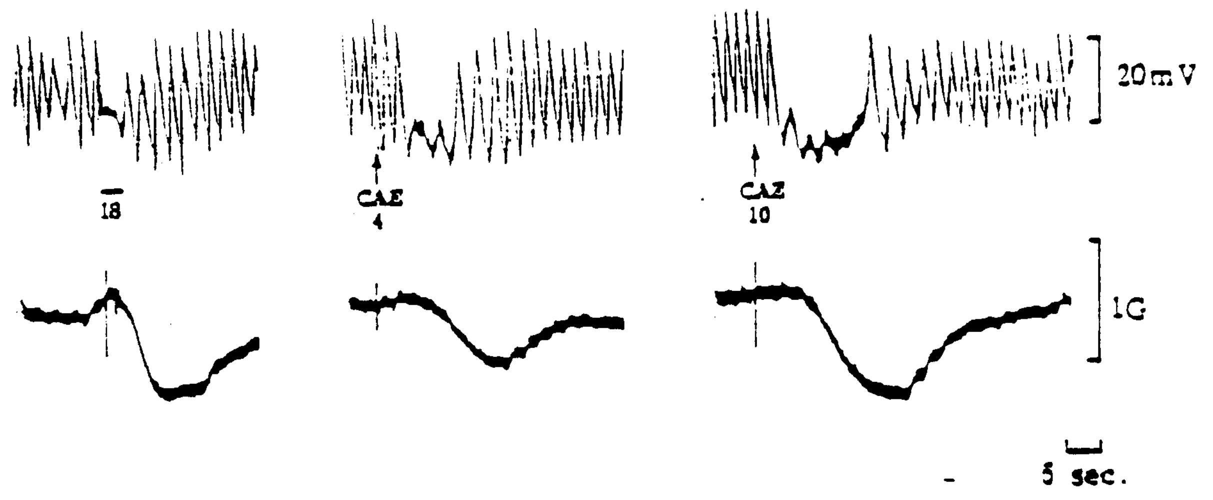
THE EFFECTS OF INHIBITORY NERVE STIMULATION AND EXTRACT ON THE RABBIT ANOCOCCYGEUS

The response to field stimulation and extract was studied by intracellular recording from preparations with either spontaneously-developed or guanethidine ($1-3 \times 10^{-5}$M)-induced tone. The development of tone was accompanied by a significant ($p < 0.01$) depolarisation of the cells from -55 ± 7mV (n=68) to either a stable membrane potential of -52 ± 8mV (n=102) in the majority (70%) of cells, or oscillations in membrane potential (periodicity of oscillations 1.5-4.5s, amplitude 5-36mV). The average peak membrane potential in these cells (-46 ± 6mV, n=78) was significantly more depolarised ($p < 0.001$) than in electrically quiescent cells.

Both field stimulation (5-20 pulses, 5-10Hz) and the cleaned activated extract (CAE), but not the cleaned unactivated extract (CUE), relaxed the muscle. This was accompanied by an inhibition of the oscillations in membrane potential, and additionally with the extract, a dose-dependent hyperpolarisation (Fig.62). In the absence of electrical oscillations, field stimulation evoked a small (2-5mV) inhibitory potential and CAE produced no significant electrical change (Fig.63).

FIGURE 62

The effects of field stimulation (18 pulses, 5Hz; supramaximal voltage, 0.5ms pulse width at bar) and cleaned activated extract (CAE, 4-20μl as indicated at arrows) on the membrane potential (upper trace) and tone (lower trace) of the rabbit anococcygeus. The relaxation to either stimulus was accompanied by inhibition of the oscillations in membrane potential, and additionally with the extract, a dose-dependent hyperpolarisation. Cleaned unactivated extract (CUE, 100μl) was relatively ineffective. Intracellular electrical recording. Guanethidine (1×10^{-5}M) present throughout.


20mV
18
CAE
4
CAZ
10
1G
5 sec.

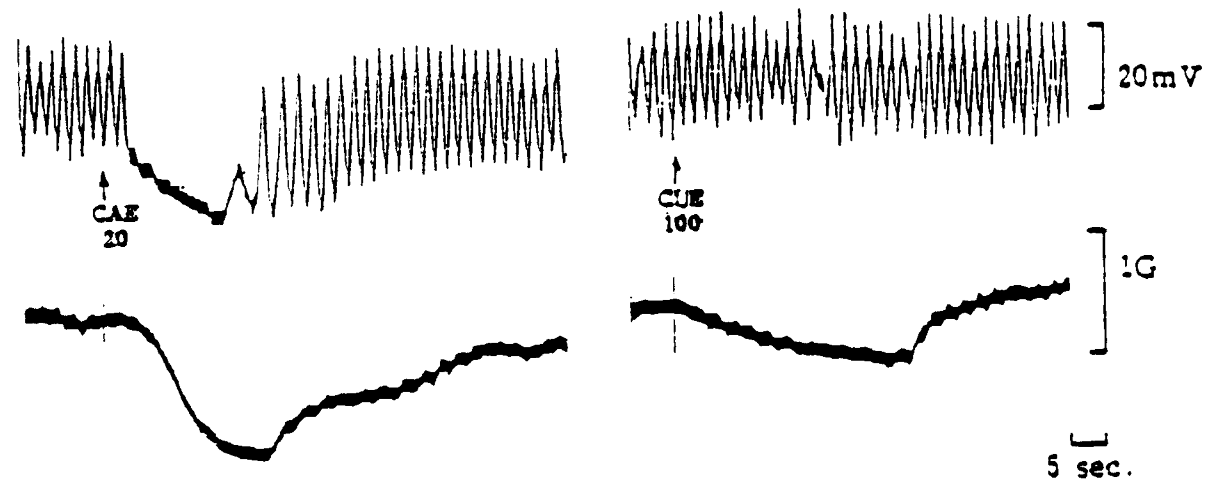
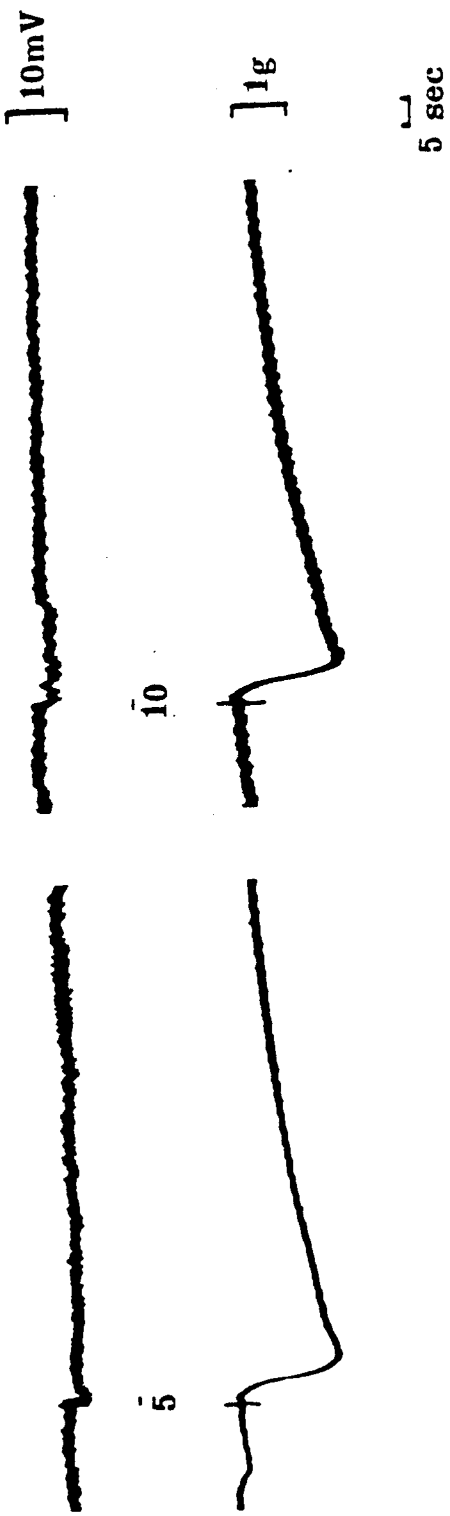

20mV
CAE
20
CUE
100
1G
5 sec.

FIGURE 63

The effects of field stimulation (5 pulses at 5 and 10Hz; supramaximal voltage, 0.5ms pulse width as indicated below bars) and cleaned activated extract (CAE, 100µl) on membrane potential (upper trace) and tone (lower trace) of the rabbit anococcygeus. The relaxation in response to inhibitory nerve stimulation was accompanied by a small hyperpolarisation (inhibitory potential) while that to the extract was not. These electrical responses were characteristic of those cells which did not exhibit oscillations in membrane potential. Intracellular recording. Guanethidine (1×10^{-5}M) present throughout.


10mV
1g
5 sec
10
5

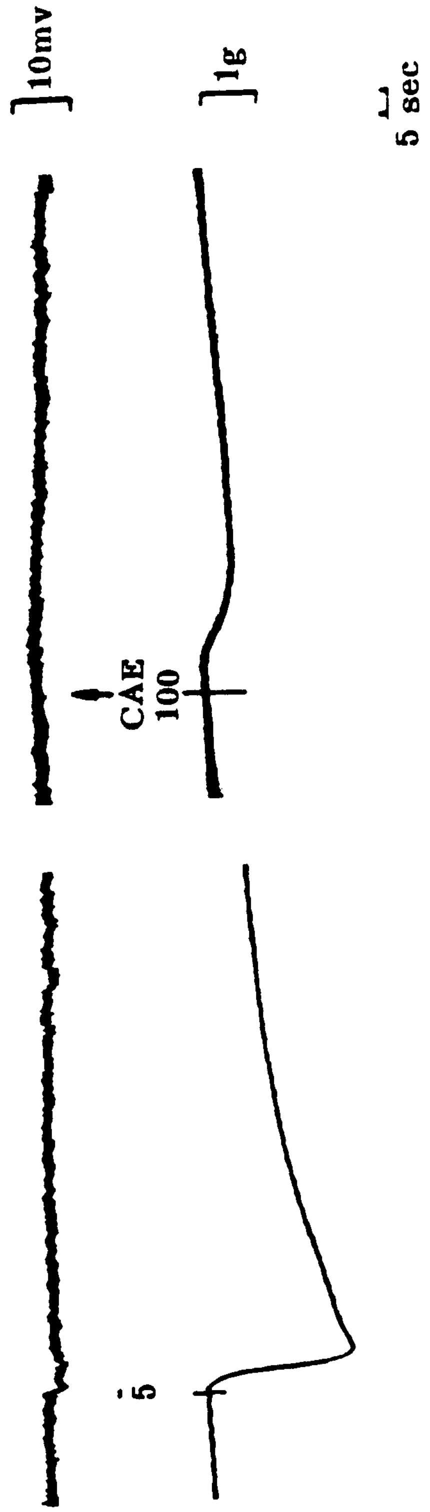
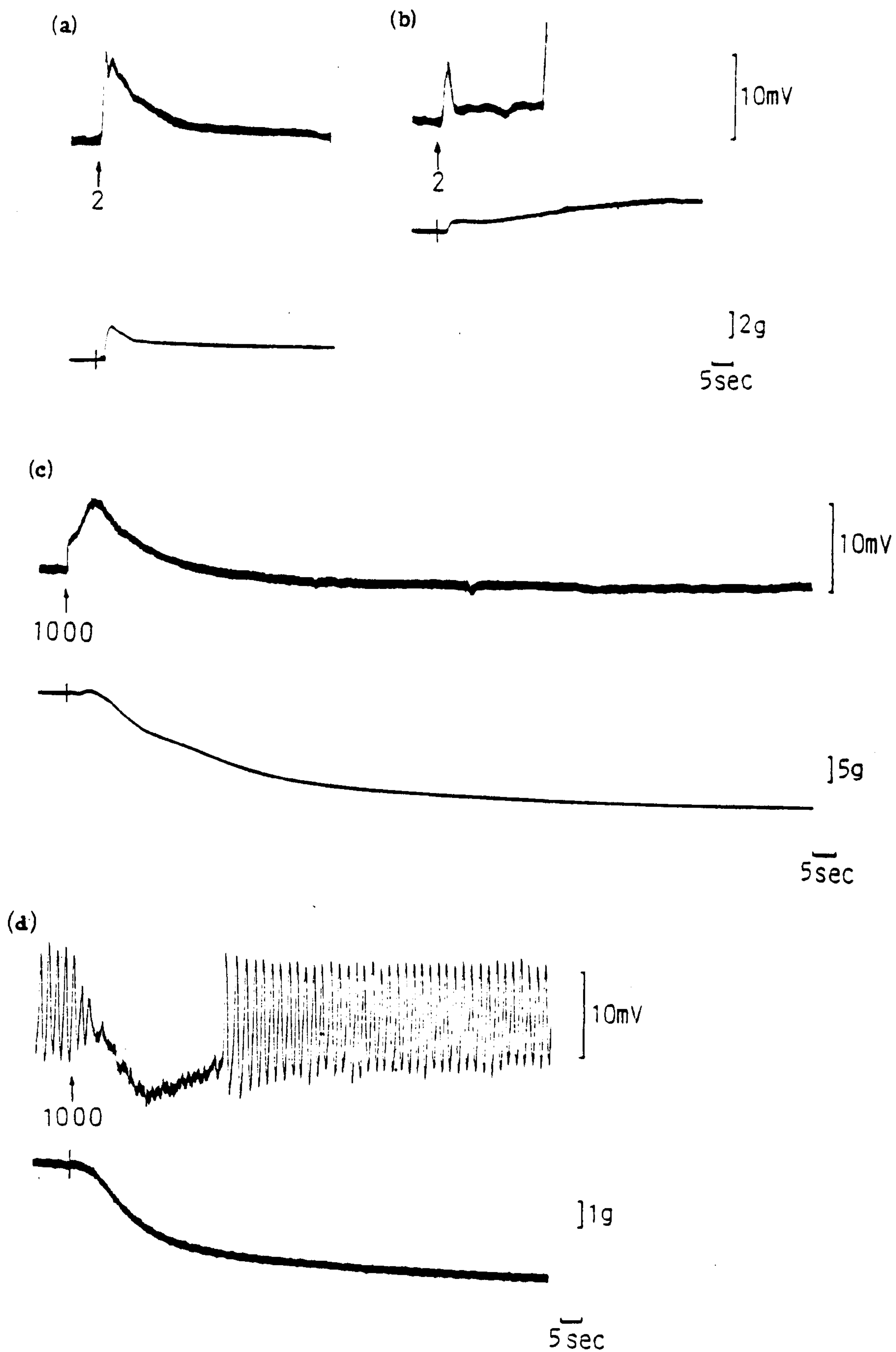

10mv
1g
5 sec
CAE
100
5

FIGURE 64

The effects of adenosine triphosphate (ATP; 2-1000 x 10^{-9} moles, as indicated below arrow) on the membrane potential (upper trace), recorded from four different cells, and tension in the rabbit anococcygeus. In the absence (a) and presence (b) of tone, low doses of ATP produced depolarisation and contraction. Higher doses (c) produced, in the same preparation, depolarisation followed by a small, prolonged hyperpolarisation. The accompanying relaxation preceded the hyperpolarisation. In (d), recordings from a different preparation: when present the electrical oscillations were inhibited and the membrane hyperpolarised by large doses of ATP. Intracellular electrical recording. Guanethidine (1×10^{-5}M) present during (b), (c) and (d).


(a)
(b)
2
2
10mV
2g
5sec
(c)
1000
10mV
5g
5sec
(d)
1000
10mV
1g
5sec

THE EFFECTS OF ADENOSINE TRIPHOSPHATE (ATP) ON MEMBRANE POTENTIAL AND TONE IN THE RABBIT ANOCOCCYGEUS

In the absence or presence of tone, ATP ($1-20 \times 10^{-9}$ moles) evoked a rapid depolarisation (up to 12mV) and contraction (Fig.64, a and b). Higher doses of ATP ($1-20 \times 10^{-6}$mol) relaxed the tone. The accompanying electrical response was either a depolarisation sometimes followed by a small hyperpolarisation (Fig.64c), or an inhibition of the electrical oscillations and hyperpolarisation (Fig.64c). This effect was produced by doses of ATP which exceeded those present in volumes of the CAE which produced an equivalent electrical and mechanical inhibition (50µl CAE contained approximately 5×10^{-9}mol).

EFFECT OF APAMIN ON THE INHIBITORY RESPONSES OF THE RABBIT ANOCOCCYGEUS TO FIELD STIMULATION AND INHIBITORY EXTRACT

Apamin (10^{-7}M for up to 90 min) failed to block the inhibitory potential and relaxation in response to field stimulation (5-10 pulses, 2-10Hz; Fig.65).

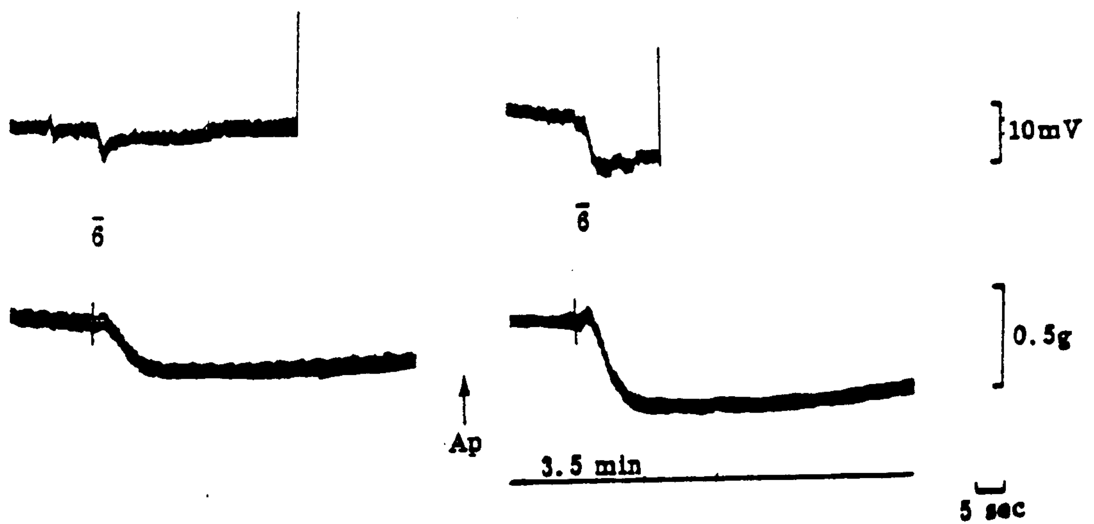
The electrical and mechanical responses to CAE were transiently blocked (for some 10 min) by apamin (10^{-7}M). The blockade became ineffective in the continued presence of the drug (Fig.66).

THE EFFECTS OF FIELD STIMULATION AND INHIBITORY EXTRACT ON THE RAT ANOCOCCYGEUS AFTER THE DEVELOPMENT OF TONE

Tone was induced by guanethidine ($1-3 \times 10^{-5}$M) which

FIGURE 65

The effects of apamin (Ap, 1×10^{-7}M for the times indicated) on the inhibitory potential (upper trace) and relaxation (lower trace) in response to field stimulation (6 pulses, 5Hz: supramaximal voltage, 0.5ms pulse width as inducated below bars) in the rabbit anococcygeus. Apamin depolarised the cells and increased the tone. The inhibitory potential and relaxation were enhanced. Intracellular electrical recordings from four different cells in the same preparation. Loss of impalement is shown by the sharp vertical deflections (upper trace). Guanethidine (3×10^{-5}M) present throughout.


10mV
6̄
6̄
0.5g
Ap
3.5 min
5 sec

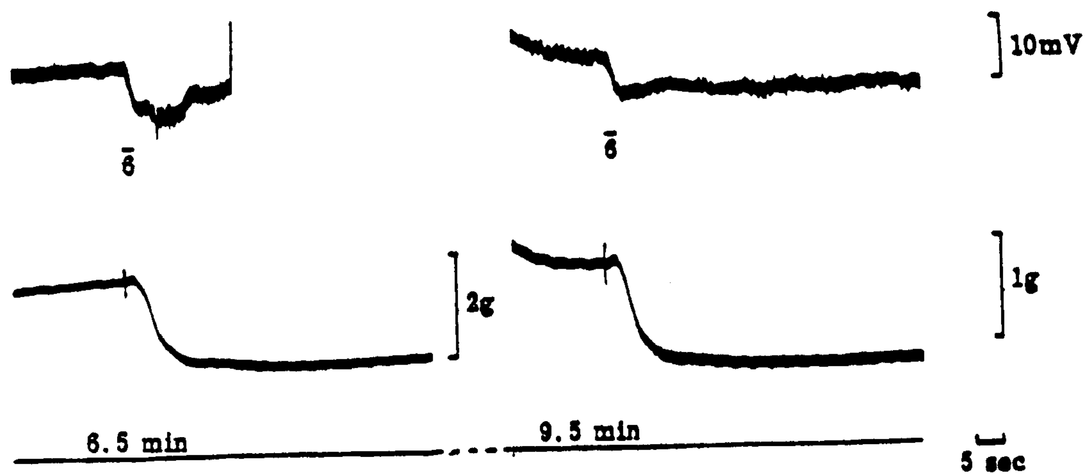
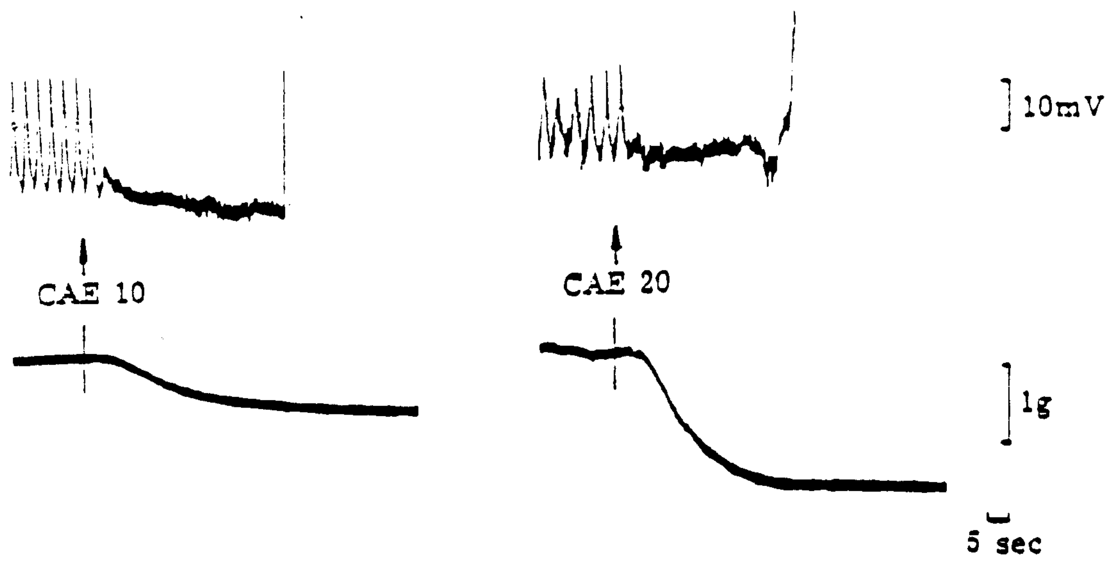
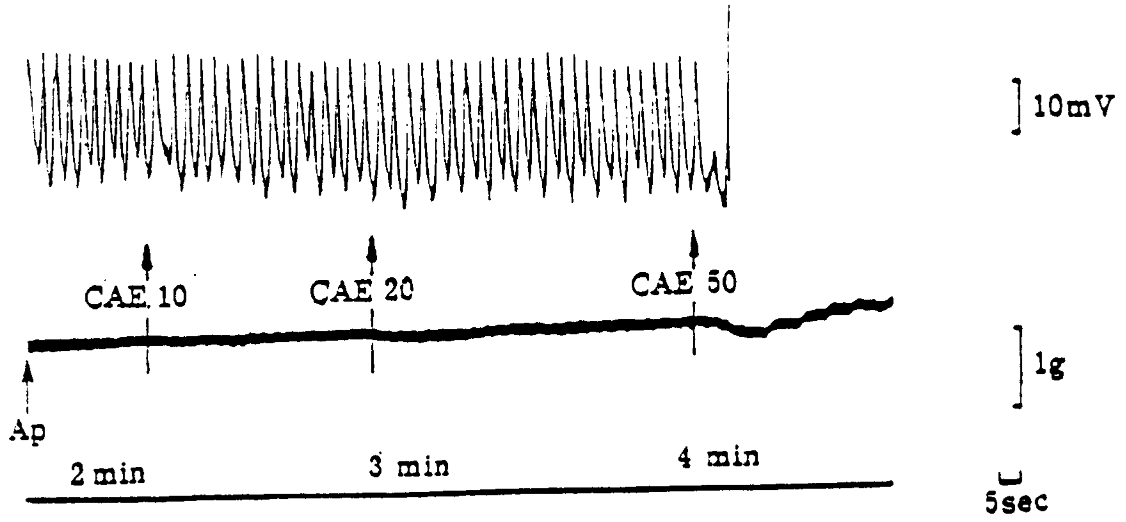

10mV
6̄
6̄
2g
1g
6.5 min
9.5 min
5 sec

FIGURE 66

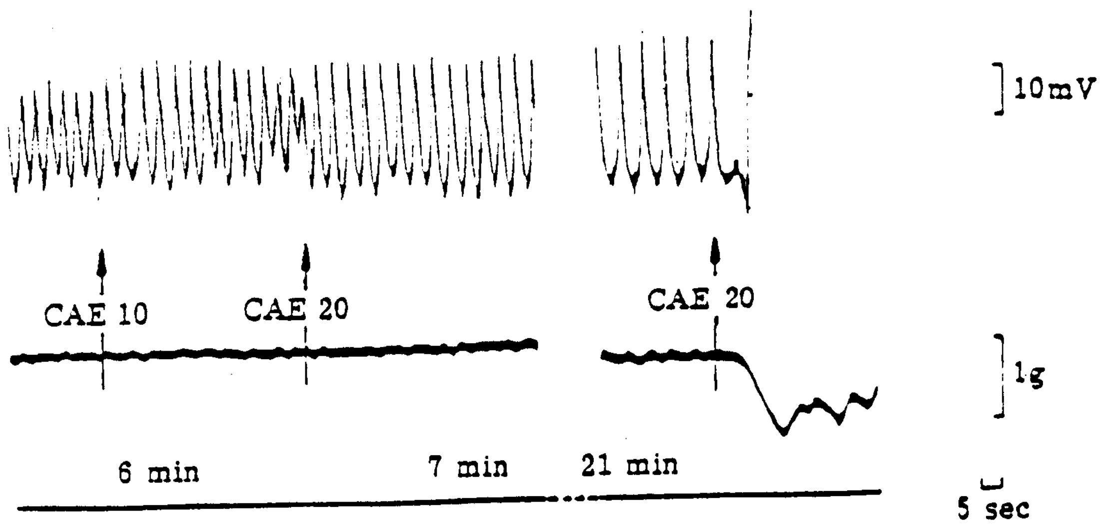
The effect of apamin (Ap, 1×10^{-7}M for the times indicated) on the electrical (upper trace) and mechanical (lower trace) responses to cleaned activated extract (CAE, 10-50μl as indicated) in the rabbit anococcygeus. The inhibitory effects of the extract on the oscillations in membrane potential and tone (upper panels) were blocked transiently by apamin. Blockade was overcome by increasing the dose of extract to 50μl or during the continued presence of apamin (21 min). Intracellular electrical recording. Guanethidine (1×10^{-5}M) present throughout.



10mV
CAE 10
CAE 20
1g
5 sec



10mV
CAE 10
CAE 20
CAE 50
1g
Ap
2 min
3 min
4 min
5sec



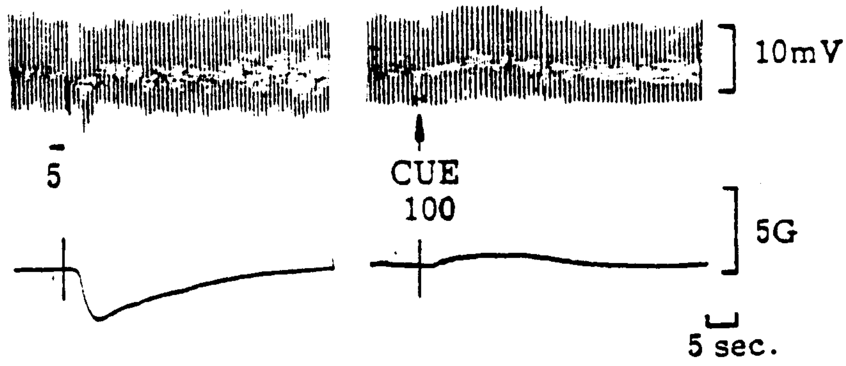
10mV
CAE 10
CAE 20
CAE 20
1g
6 min
7 min
21 min
5 sec

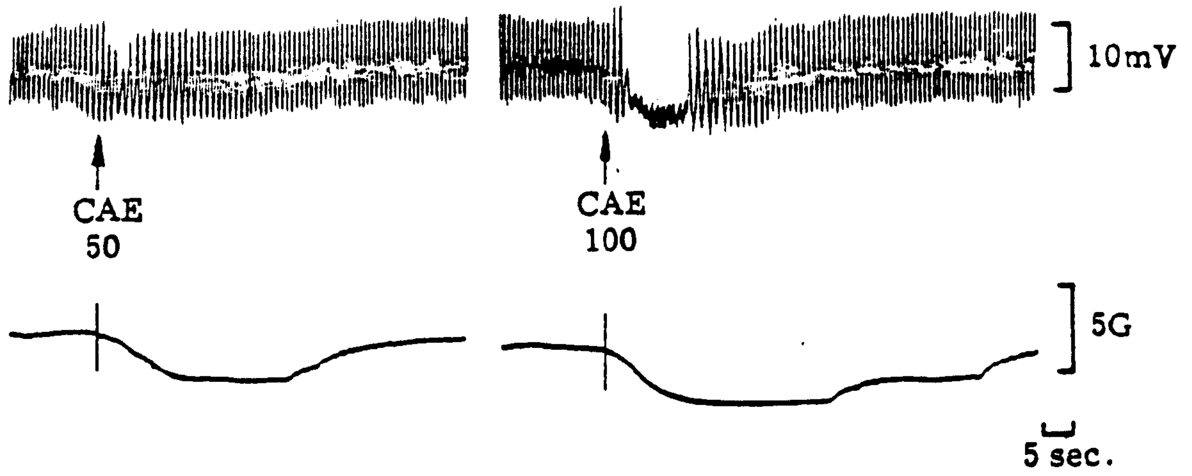
also significantly ($p < 0.001$) depolarised the cells (from -64 ± 9mV, n=8 to -43 ± 7mV, n=26) and caused oscillations in membrane potential (periodicity 3-4.2s).

Both field stimulation (5-20 pulses, 1-10Hz) and CAE (20-100μl) relaxed the tone. The accompanying electrical response was, in the majority of cells, insignificant. In some 25% of cells, relaxation was preceded by inhibition of the oscillations in membrane potential (Fig.67). CUE produced no significant electrical or mechanical responses.

FIGURE 67

The effects of field stimulation (5 pulses, 5Hz; supramaximal voltage, 0.5ms pulse width) and cleaned activated extract (CAE, 50-100μl) on the membrane potential (upper trace) and tone (lower trace) in the rat anococcygeus. The relaxation to field stimulation or CAE was accompanied by an inhibition of the oscillations in membrane potential. Cleaned unactivated extract (CUE, 100μl) was relatively ineffective. Intracellular electrical recording. Guanethidine (1×10^{-5}M) present throughout.


10mV
5
CUE
100
5G
5 sec.


10mV
CAE
50
CAE
100
5G
5 sec.

CHAPTER 4

DISCUSSION

THE BOVINE RETRACTOR PENIS

INTRACELLULAR RECORDING FROM THE BOVINE RETRACTOR PENIS

The bovine retractor penis comprises smooth muscle bundles separated by wide bands of connective tissue. Collagen fibrils surround the individual cells (Eränkö et al, 1976). The presence of such connective tissue may explain the difficulty, particularly after the development of tone, in making microelectrode impalements. Collagenase caused separation of the smooth muscle bundles but failed to facilitate cell impalement, possibly because collagen fibrils surrounding the cells remained intact.

RESTING ACTIVITY AND PASSIVE MEMBRANE PROPERTIES

The resting electrical activity of the bovine retractor penis displayed features common to other smooth muscles. Spontaneous junction potentials were rare, confirming the presence of large (some 200nm) junctions between the nerve varicosities and smooth muscle cells (Crossley, 1981, unpublished). The most common form of spontaneous electrical activity was oscillations in membrane potential which had a myogenic basis and were similar to those recorded in the dog retractor penis (Orlov, 1962), rabbit anococcygeus (Creed and Gillespie, 1977), retococcygeus (Blakely, Cunnane and Muir, 1978) and, recently, the bovine retractor penis (Samuelson, Sjöstrand and Klinge, 1983). Oscillations were not due to membrane depolarisation *per se* since they were not evoked by passive displacement of the membrane potential. Like the bovine trachea (Kirkpatrick, 1980), oscillations

were unassociated with action potentials. This is in contrast to the sucrose gap recordings from the bovine retractor penis by Samuelson, Sjöstrand and Klinge (1983) in which small (1-2mV) spikes were recorded. Electrical oscillations were often accompanied by small fluctuations in tone and may be due to the activity of pacemaker regions.

The bovine retractor penis, like other smooth muscles (see Creed, 1979) had cable-like properties. The space constant (1.7mm) is similar to that in other smooth muscles, e.g. pulmonary artery (1.5mm), taenia coli (1.5mm), and urinary bladder (1.7mm) (see Creed, 1979), but higher than that in the rat anococcygeus (2.7mm; Creed, 1975). This suggests a relatively low value of membrane resistance (Rm) or high internal resistance (Ri; since $\lambda^2 = Rm/Ri$). The former view is favoured by the relatively high value of τm (290ms) which exceeds that of the rat anococcygeus (178ms; Creed, 1975) but is similar to the value obtained for the bovine trachea (305ms) which has a relatively low resting membrane potential (-47mV) and also exhibits spontaneous electrical oscillations (see Kirkpatrick, 1975,1980).

EXCITATORY ELECTRICAL AND MECHANICAL RESPONSES TO FIELD STIMULATION AND EXOGENOUS NORADRENALINE

The excitatory electrical and mechanical responses of the bovine retractor penis to field stimulation resembled those of the rat anococcygeus (Creed et al, 1975) rather than the rabbit anococcygeus (Creed and Gillespie, 1977) or dog retractor penis (Orlov, 1962), both of which show spike activity. Spikes in response to field stimulation

of the bovine retractor penis have been reported (Samuelson et al, 1983), but the prolonged duration (some 1s) and small amplitude (approximately 2mV) of these responses suggest that they may represent excitatory junction potentials rather than spikes.

In the absence of tone, excitatory potentials, though often small, were recorded from every cell impaled, indicating a wide distribution of excitatory nerves. A number of factors may account for the relatively small size of the excitatory potentials. (a) The transmitter may reach the cells in low concentrations, a view consistent with the presence of large (200nm) junctions between nerve varicosities and smooth muscle cells (Crossley, 1981, unpublished). (b) The membrane potential (-53mV) is lower and therefore closer to the reversal potential for the excitatory potential than in the rat anococcygeus. (c) The inhibitory transmitter, which is released during field stimulation, may reduce the size and duration of the excitatory potential and contraction (Klinge and Sjostrand, 1974). The view that the small excitatory potentials arise because only a small proportion of key cells are innervated and from which current spreads electrotonically is unlikely. A prerequisite for this view, the presence of large excitatory potentials in certain key cells, was not observed. Moreover, the latency of the ejp (47ms) was small and showed very little variation among different cells, further indicating that each cell receives an excitatory innervation.

The latency of the excitatory potential in the bovine retractor penis was shorter than that of the rat anococcygeus (165ms; Creed et al, 1975) and more in keeping with transmission in the vas deferens and arteries where the values range from 10-50ms (see Holman, 1970; Suprenant, 1980). Such differences are not accounted for by diffusion. The average distance between nerve varicosities and smooth muscle cells in the anococcygeus (260nm; Gillespie and Lüllman-Rauch, 1974) is similar to that in the bovine retractor penis (some 200nm; Crossley, unpublished). Both of these values are larger than the minimum separation in the vas deferens.

Potential antagonists of excitatory electrical and mechanical responses to field stimulation and exogenous noradrenaline

The excitatory potential in the bovine retractor penis displayed certain pharmacological characteristics similar to those of the vas deferens and blood vessels. Both the excitatory potential and contraction were abolished by guanethidine, confirming their mediation by adrenergic nerves (Klinge and Sjöstrand, 1974). However, phentolamine in concentrations which depressed the contraction to field stimulation had little effect on the excitatory potential. Phentolamine may inhibit contraction independently of the receptors for neurally released noradrenaline as proposed for blood vessels (Holman and Suprenant, 1980). This is supported by the ineffectiveness of the α_1-adrenoceptor antagonist, prazosin, against both the excitatory potential

and contraction. Alternatively, the excitatory potential may not be mediated by noradrenaline but by a co-transmitter (e.g. ATP) released from the adrenergic fibres as proposed for the vas deferens (Sneddon and Westfall, 1984). If so, the noradrenergically-mediated component of the contraction would be produced via a non-electrical mechanism. Interestingly, the excitatory potential in the rat anococcygeus has a phentolamine-resistant component which is mimicked by ATP (Byrne and Large, unpublished).

Both phentolamine and prazosin were effective inhibitors of the depolarisation and contraction in response to exogenous noradrenaline. Differences in the effectiveness of α-adrenoceptor antagonists against neurally released and exogenous noradrenaline are well known (Nickerson, 1949) and may reflect differences in the susceptibility to or sensitivity of post-synaptic α-adrenoceptors. In view of the relatively large distances between nerve varicosities and smooth muscle cells (200nm), access to the receptors for neurally-released noradrenaline should be unhindered. A more likely alternative is that originally proposed by Hotta (1969) for the vas deferens, and more recently by Holman and Suprenant (1980) for vascular smooth muscle, that there may be 'junctional' or γ- (Hirst and Neild, 1980; Hirst, Neild and Silverberg, 1982) adrenoceptors which are activated by neurally-released noradrenaline but which are insensitive to α-adrenoceptor antagonists, and 'extrajunctional' receptors activated by exogenous

noradrenaline and sensitive to α-antagonists. There is also some pharmacological evidence for two subpopulations of α-adrenoceptor in the rat anococcygeus (Coats, Jahn and Weetman, 1982; McGrath, 1982) which may be due to the presence of junctional and extrajunctional types. The same may be true of the bovine retractor penis.

Effects of drugs potentiating or antagonising cholinergic transmission

The effects of physostigmine and atropine on the excitatory electrical and mechanical responses to field stimulation and noradrenaline are consistent with the presence of a cholinergic innervation acting on adrenergic nerves to inhibit transmitter release (Klinge and Sjöstrand, 1977; Sjöstrand, 1980). Electron microscopical studies have shown allegedly cholinergic nerve profiles in close contact with the adrenergic nerve endings in the bovine retractor penis (Eränkö et al, 1976). However, it may be impossible to distinguish microscopically between cholinergic and non-adrenergic non-cholinergic nerve profiles (Gibbins, 1982). *In vivo* the tone of the muscle may be due in part to sympathetic nerve activity, in which case cholinergic fibres, by inhibiting noradrenaline release, would cause relaxation.

Physostigmine initially enhanced the excitatory potential and contraction. In the rat anococcygeus also, physostigmine enhances the contraction in response to field stimulation without changing noradrenaline overflow (Smith

and Spriggs, 1983). The bovine retractor penis is contracted by high concentrations of cholinergic agonists (Klinge and Sjostrand, 1974), suggesting that enhancement of the response to field stimulation by physostigmine may be due to acetylcholine released from cholinergic fibres acting on the smooth muscle directly.

Effects of tetraethylammonium (TEA) on resting activity and excitation

TEA in low concentrations enhanced the amplitude of the excitatory potential and contraction in response to field stimulation. This was unaccompanied by membrane depolarisation and was presumably due to the prolongation of the action potential in the adrenergic nerve fibres, and consequently an increase in transmitter release (Tasaki and Hagiwara, 1957; Thoenen, Hafely and Stachelin, 1967). The antimuscarinic activity of TEA (Gillespie and Tilmisany, 1976) is unlikely to account for the potentiation observed because of the relatively low concentration required (10^{-4}M) and the rapid onset of effect (some 15 min). Potentiation of the excitatory response to field stimulation by atropine took some 30-45 min to occur. The depolarisation produced by higher concentrations ($0.5 - 10 \times 10^{-3}$M) of TEA may have been due either to a direct action on the smooth muscle cell membrane or to the release of noradrenaline from adrenergic nerves. The latter is supported by the lack of any enhancement of the depolarisation and contraction in response to exogenously-added noradrenaline. In other

smooth muscle preparations, e.g. rabbit ear artery (Droogmans, Raeymaekers and Casteels, 1977; Droogmans and Casteels, 1977) and cat spleen (Thoenen et al, 1967), TEA ($1.2 - 10 \times 10^{-3}$M) enhanced the depolarisation and contraction in response to exogenous noradrenaline. However, in the rat anococcygeus, although the response to noradrenaline was not enhanced by TEA, the contraction in response to TEA ($5 - 20 \times 10^{-3}$M) had phentolamine-sensitive and insensitive components, suggesting both the liberation of transmitter and a direct action on the smooth muscle cell membrane (Gillespie and Tilmisany, 1976). The action of TEA on nerves and muscle is presumably to block K^+ channels. This increases the amplitude of action potentials in nerves and smooth muscle by reducing the rate of repolarisation (Tasaki and Hagiwara, 1957; Ito, Kuriyama and Sakamoto, 1970). Blockade of the resting K^+ conductance could also explain the depolarising action of TEA.

The absence of action potentials in the presence of TEA was surprising, particularly in view of the report by Samuelson et al (1983) who recorded spontaneous spike activity from the bovine retractor penis using the sucrose gap technique. In other smooth muscle preparations, e.g. carotid artery (Mekata, 1971), rat anococcygeus (Creed et al, 1975), tracheal smooth muscle (Kirkpatrick, 1975) and ear artery (Droogmans and Casteels, 1977), which do not normally exhibit spike activity, TEA destabilised the membrane and evoked action potentials. The inability to

record spike activity in the present study may have been due to their very small size in the sucrose gap (approximately 1mV: Samuelson et al, 1983) or the presence of rubber membranes which may, by constricting the tissue, have prevented the propagation of spike activity to cells at the Krebs-sucrose interface.

<u>Change in membrane conductance during the excitatory potential</u>

There was an increase in membrane conductance during the excitatory potential and a linear relationship between its amplitude and the membrane potential. An apparent reversal potential of -30mV was obtained for the excitatory potential in the double sucrose gap. Since the potential changes recorded in the sucrose gap are less than those measured intracellularly (Bennett and Burnstock, 1966), the true value for the reversal potential is probably more positive than -30mV and may be closer to the value of -20mV obtained by Creed (1975) for the rat anococcygeus. A reversal potential of -20 to -30mV is more negative than the anticipated equilibrium potentials for Cl^-, Na^+ (-13mV and +13mV respectively in the rat anococcygeus; Creed and Pollock, unpublished), but may reflect the net equilibrium potential for more than one ion, e.g. Na^+ and K^+ or Cl^- and K^+.

<u>Effect of replacing sodium chloride with sodium glutamate on the excitatory potential and contraction</u>

Replacement of the sodium chloride in the Krebs solution

with sodium glutamate reduced the amplitude of the depolarisation and contraction in response to field stimulation and exogenous noradrenaline. The basis for this effect was unlikely to be a reduction in the chloride gradient across the smooth muscle membrane as has been proposed for the myometrium (Bulbring and Szurszewski, 1974). Although the intracellular chloride is rapidly depleted (Casteels, 1971), recent studies on the vas deferens using chloride-sensitive microelectrodes (Aicken and Brading, 1982,1983) indicate that in the presence of low chloride solutions the chloride gradient was increased by active uptake. The reduction in amplitude of the excitatory responses in the bovine retractor penis may have been due to a change in intracellular pH (Aicken and Brading, 1984) or the effect of glutamate. In common with other anion substitutes (Vaughan-Jones, 1979), glutamate chelates calcium. This could have been overcome by increasing the concentration of Ca^{2+} present in the modified Krebs solution. Glutamate may inhibit transmitter release by field stimulation, but this would not account for the reduction in response to exogenous noradrenaline.

EXCITATORY RESPONSES TO ATP AND EXTRACT

ATP and extract each depolarised (by up to 18 and 8mV respectively) the bovine retractor penis. ATP also depolarises other smooth muscles (Ohga and Taneika, 1977; Bauer and Kuriyama, 1982b). The presence of ATP as a

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contaminant could account for the depolarisation of the bovine retractor penis by the extract. However, while the depolarisation to ATP was accompanied by a small contraction, equivalent depolarisations produced by the extract were unaccompanied by a mechanical response. The lack of a mechanical response was common to both activated and unactivated cleaned forms of the extract and was therefore not due to the activity of the acid-activated component. The extract has a high tonicity (approximately 500mM NaCl), which together with other impurities (e.g. formate) may inhibit the contractile response.

SPONTANEOUS DEVELOPMENT OF TONE

The spontaneous development of tone was accompanied in the bovine retractor penis by a maintained membrane depolarisation, sometimes interrupted by oscillations in membrane potential but not spikes. Spontaneous tone was unaffected by TTX or phentolamine, suggesting a myogenic mechanism. That an inhibitory substance released from the tissue during dissection was subsequently destroyed after setting up seems unlikely. The interval required for the development of tone (0.5-3h) varied considerably whereas the effect of any postulated inhibitory substance would have been expected to wear off rapidly in a tissue which was being continuously perfused. Membrane depolarisation presumably opens voltage-dependent Ca^{2+} and possibly Na^{+} channels, causing an influx or intracellular release of Ca^{2+} with subsequent activation of the contractile proteins (see Bolton, 1979).

The initial stimulus for membrane depolarisation may be stretch, causing the whole muscle to depolarise, or spontaneous oscillations in potential, possibly arising from pacemaker cells. Physiologically, the spontaneous development of tone is probably an important property of the muscle which *in vivo* is usually contracted.

INHIBITORY RESPONSE TO FIELD STIMULATION

Following spontaneous membrane depolarisation and the development of tone, the excitatory potential in response to field stimulation was reduced or abolished. This is not wholly accounted for by either membrane depolarisation or the concomitant release of inhibitory transmitter. Relaxation of the bovine retractor penis to field stimulation was accompanied by an inhibitory potential and often followed by a rebound depolarisation and contraction. These responses are similar to those reported in other smooth muscles innervated by non-adrenergic non-cholinergic inhibitory nerves, e.g. taenia coli (Bennett et al., 1966a,b), rabbit anococcygeus (Creed and Gillespie, 1977) and rectococcygeus (Blakeley et al., 1978). A single pulse was sufficient to evoke an inhibitory potential and relaxation in the bovine retractor penis, indicating the presence of dense inhibitory innervation. The inhibitory potential and relaxation were blocked by TTX but not by adrenergic or cholinergic antagonists, confirming their non-adrenergic non-cholinergic neurogenic origin.

The latency of the inhibitory potential was unusually

long (1087ms) in comparison with other smooth muscles (e.g. rabbit anococcygeus 185ms; Creed and Gillespie, 1977; rabbit rectococcygeus 200-340ms; Blakeley et al, 1978). This is not due to slow electrotonic conduction of the response because (a) the latency was not significantly decreased when electrical recordings were made from tissue regions in close proximity to the stimulating electrodes (see Figure 4, Methods section), (b) the conduction velocity in the taenia coli (6-10cm s^{-1}; Bülbring and Tomita, 1967; Tomita, 1970) is fast. One factor which may have contributed to the large latency was the difficulty in making measurements from inhibitory potentials which were often small and with a slow rate of rise. The latency was not reduced in the presence of guanethidine and was not therefore due to the opposing activity of the adrenergic nerves. The inhibitory transmitter may be stored in the nerve terminals in an inactive form and converted to the active form on arrival of the nerve impulse (Bowman, Gillespie and McGrath, 1983). Thus, the long latency could be due to the time required for the conversion of the transmitter from inactive to active form. Diffusion of transmitter to the muscle cells is an unlikely cause because of its rapidity in other tissues (within 50µs at the neuromuscular junction; Katz and Miledi, 1965) and the rapid onset of the excitatory potential (less than 50ms) in the bovine retractor penis.

Other examples of long latency inhibitory potentials

have been reported following the iontophoretic application of muscarinic agonists in both nerves (Koketsu, Nishi and Soeda, 1968; Hartzell, Kuffler, Stickgold and Yoshikami, 1977) and smooth muscle (Purves, 1974; Bolton, 1976). The rate of agonist-receptor binding is a possible rate-limiting step, though this occurs within 0.3ms in response to acetylcholine at the neuromuscular junction (Fatt and Katz, 1952). The latency may be determined by the rate of activation of ionic channels (Hartzell Kuffler, Stickgold & Yoshikami, 1977). Delayed activation may result from the involvement of a biochemical mechanism in the response. Such a mechanism may exist in some ganglia (Greengard, 1976) where the slow synaptic potential may be mediated by an acetylcholine-activated guanylate cyclase. A similar mechanism in the bovine retractor penis could also explain the considerable variation in latency (standard deviation 782ms) which may have been due to the different levels of substrates or enzymes in the postulated biochemical step. Differences in the distances between stimulation and recording electrodes may also have contributed to the variability in latency.

Relationship between the inhibitory potential and relaxation

The inhibitory potential preceded the relaxation to field stimulation. However, the relationship between membrane hyperpolarisation and relaxation is unclear. In smooth muscles which admit Na^+ or Ca^{2+} during spontaneous action potentials, e.g. taenia coli or uterus, hyperpolaris-

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ation causes the cessation of firing and thus inhibits Ca^{2+} entry or intracellular release. The development of tone in the bovine retractor penis was accompanied not by action potentials but by a maintained depolarisation of the membrane. Presumably the intracellular calcium is maintained at a constantly elevated level. By closing voltage-dependent Ca^{2+} and Na^{+} channels, membrane hyperpolarisation would decrease Ca^{2+} entry and its release from intracellular binding sites. This implies that both Ca^{2+} and Na^{+} are continuously cycling across the membrane, i.e. are pumped out of the cell and re-enter via voltage-dependent channels. Such a mechanism could exist, but the small size of the inhibitory potential in some cells (1-2mV, 10 pulses at 5Hz) appears insufficient to close voltage-dependent Ca^{2+} channels unless there is very tight coupling between the two. The inhibitory transmitter may reduce intracellular Ca^{2+} by activating its sequestration to intracellular binding sites, e.g. cell membrane, sarcoplasmic reticulum or mitochondria, or extruding Ca^{2+} into the extracellular space via a pump. These changes may be triggered by the inhibitory potential.

The inhibitory potential did not appear to be an essential step to producing a relaxation in response to field stimulation (see Samuelson, 1983). This was illustrated by the action of TEA which, in high concentrations, abolished the inhibitory potential but not the relaxation to field stimulation. Ouabain also caused a reduction in

the amplitude of the inhibitory potential without reducing the mechanical response, though the latter effect may have been masked by the increase in tone level in the presence of the drug.

INHIBITORY RESPONSES TO EXTRACT, ATP, SODIUM NITROPRUSSIDE AND NORADRENALINE

The acid-activated inhibitory extract, as previously reported (Gillespie et al, 1981), relaxed the bovine retractor penis while the unactivated extract did not. The relaxation was accompanied by a membrane hyperpolarisation and, in this respect, the extract mimicked the inhibitory transmitter. The duration of the hyperpolarisation to extract was usually longer than that to field stimulation. Such differences may arise from the method of administration of the extract.

ATP and sodium nitroprusside each produced a hyperpolarisation and relaxation of the bovine retractor penis. The response to ATP was of particular interest because of its possible transmitter role in other tissues (Burnstock, 1979) and presence as a contaminant in the extract. ATP hyperpolarises a number of smooth muscles innervated by non-adrenergic non-cholinergic inhibitory nerves, e.g. taenia coli (Tomita and Watanabe, 1973) and guinea-pig ileum (Bauer and Kuriyama, 1982b). The bovine retractor penis desensitised to ATP so rapidly that it was impossible to obtain a dose-response curve. For this reason, and because it did not require acid activation, ATP is unlikely

to be the active component in the inhibitory extract. Neither is ATP a candidate for the inhibitory transmitter because first, ATP also produced excitation, and secondly desensitisation developed to ATP without loss of the response to inhibitory nerve stimulation.

The effects of 'pure' ATP and ATP present in the extract showed distinct differences. Although the unactivated extract contained sufficient ATP to relax the muscle and produce desensitisation to ATP, neither was observed. This was not because ATP in the extract was inactivated by one of the other components, because the unactivated extract depolarised the muscle. The lack of inhibitory effect of ATP present in the extract may have been due to the presence of other impurities, e.g. sodium chloride, formate.

Sodium nitroprusside was a very potent inhibitor of tone in the bovine retractor penis, producing hyperpolarisation and relaxation in doses as low as 1×10^{-13} moles. The onset to (1-2s) and duration of inhibition, and the following rebound excitation to sodium nitroprusside, were similar to that to inhibitory extract, suggesting that the two may act via a similar mechanism.

In one preparation, following prolonged treatment with α-adrenoceptor antagonist prazosin, noradrenaline produced a small hyperpolarisation and relaxation mediated by inhibitory β-adrenoceptors. These do not appear to contribute

to the inhibitory response to field stimulation because of the ineffectiveness of guanethidine and propranolol as antagonists and the dominant excitatory effect of exogenous noradrenaline (Klinge and Sjöstrand, 1974).

Rebound response to field stimulation, extract, sodium nitroprusside and ATP

A rebound depolarisation and contraction often followed the relaxation of the bovine retractor penis to field stimulation, particularly in muscles with a low level of resting tone. The amplitude of the rebound response appeared to be independent of the preceding inhibition but was dependent on the number of pulses applied. This suggests that the rebound response may be mediated by non-adrenergic non-cholinergic excitatory nerves, as suggested for the guinea-pig ileum (Bywater et al, 1981) and taenia coli (Maas, 1981), in which apamin blocked the inhibitory potential and relaxation without affecting the rebound depolarisation. In the bovine retractor penis, however, the inhibitory extract, ATP and sodium nitroprusside also produced rebound depolarisation and contraction. Although the extract could contain a non-adrenergic non-cholinergic excitatory transmitter and ATP could be that transmitter, the rebound response to sodium nitroprusside is difficult to explain on this basis unless it released the transmitter. Moreover, blockade of the inhibitory response to field stimulation with oxyhaemoglobin did not reveal a delayed depolarisation and contraction.

Sites of action of extract, sodium nitroprusside and ATP

The short onset time required for the effects of the activated extract, ATP and sodium nitroprusside, and the non-tachyphylactic nature of the extract and sodium nitroprusside, suggest a direct action on the smooth muscle cell membrane rather than the release of transmitter from the inhibitory nerves. The absence of a technique to eliminate the non-adrenergic non-cholinergic innervation meant that an indirect action of the agonists could not be excluded.

POTENTIAL ANTAGONISTS OF THE INHIBITORY RESPONSE TO FIELD STIMULATION, EXTRACT AND SODIUM NITROPRUSSIDE

A pharmacological comparison between the inhibitory responses to field stimulation, extract and sodium nitroprusside was made using oxyhaemoglobin. Oxyhaemoglobin blocked the relaxation to each, confirming recent reports (Bowman, Gillespie and Pollock, 1982). The accompanying hyperpolarisation to each stimulus was also blocked. The large size of the oxyhaemoglobin molecule (molecular weight approximately 60,000 daltons) suggests that it acts outside the cell, possibly by binding the transmitter, the active component in the extract or sodium nitroprusside. preventing their actions on the post-synaptic membrane (Bowman and Drummond, 1984). Blockade of receptors could explain the antagonism of the responses to inhibitory nerve stimulation and extract but not sodium nitroprusside which is thought to act directly on the membrane to cause hyperpolarisation (Häusler and Thorens, 1976; Ito, Suzuki and

Kuriyama, 1978) or stimulate guanylate cyclase directly (Hardman, 1980).

POSSIBLE ROLE OF cGMP IN THE INHIBITORY RESPONSES TO FIELD STIMULATION AND EXTRACT

The similarities between the effects of inhibitory nerve stimulation, extract and sodium nitroprusside implied a common mechanism of action. This mechanism might involve cGMP. Sodium nitroprusside is known to increase cGMP levels in smooth muscle by increasing the activity of guanylate cyclase (Schultz, Schultz and Schultz, 1977; Diamond, 1977; Hardman, 1980). Recently, field stimulation, extract and sodium nitroprusside have been shown to increase cGMP levels in the bovine retractor penis, an effect which was blocked by oxyhaemoglobin (Bowman and Drummond, 1984). Moreover, the compound M and B 22948, which inhibits cGMP-specific phosphodiesterase, potentiated the relaxation in response to field stimulation. This was confirmed in the present study, though M and B 22948 failed to enhance the amplitude of the inhibitory potential significantly. This was not because the inhibitory potential was already maximal, since increasing the number of pulses applied further increased its amplitude. The duration of the inhibitory potential was sometimes increased by M abd B 22948, but the inconcistency of this effect makes it unlikely to account for the potentiation of the relaxation. Similarly, the relaxation to inhibitory extract was initially increased by M and B 22948, though to a lesser extent that field stimulation, without any apparent increase in the amplitude or duration

of the hyperpolarisation. Thus, although cGMP does appear to be involved in the relaxation to field stimulation and extract, it does not appear to be involved in the accompanying hyperpolarisation. The effects of dibromo-cGMP, which relaxes the bovine retractor penis (Bowman and Drummond, 1984), on membrane potential should be investigated to confirm this.

Although the initial effects of M and B 22948 on field stimulation and the extract were similar, after prolonged contact with the drug both the hyperpolarisation and relaxation to extract were inhibited, while field stimulation continued to be enhanced. Inhibition of the response to extract usually occurred after the same period of exposure to the M and B compound (approximately 20 min). Thus, it was unlikely to be due to loss of inhibitory activity in the extract caused by its reconversion to the unactivated form, because the time between extract activation and its addition to the preparation varied.

CHANGE IN MEMBRANE CONDUCTANCE DURING THE INHIBITORY POTENTIAL

The inhibitory potential in the bovine retractor penis was accompanied by a decrease in membrane conductance. This is in contrast to the taenia coli (Jager and Scheivers, 1980) and rabbit anococcygeus (Creed and Gillespie, 1977), in which an increase in conductance was observed. Decreases in membrane conductance have been recorded during inhibitory (Engberg and Marshall, 1971; Weight and Padjen, 1973a,b)

and excitatory (Dudel and Kuffler, 1960; Shuba, 1977) potentials in both nerves and muscle. In crayfish muscle fibres the increase in membrane resistance during the excitatory post-synaptic potential (EPSP) was attributed to the non-linear voltage-current relationship of the membrane. The EPSP, though produced by an increased conductance, itself caused a voltage-dependent decrease in membrane conductance (Rueben and Gainer, 1962; Dudel, 1974). Such a mechanism does not account for the increased membrane resistance during the inhibitory potential in the bovine retractor penis because (a) the V/I relationship was linear for inward currents, (b) small (2mV) inhibitory potentials which were unlikely to cause voltage-dependent changes in membrane characteristics were accompanied by significant decreases in membrane conductance.

IONIC BASIS FOR INHIBITORY POTENTIAL

The ionic species responsible for evoked changes in membrane potential is usually indicated by the reversal potential of the response. In the bovine retractor penis, however, the relationship between the inhibitory potential and the membrane potential was non-linear and gave two apparent values for the reversal potential. This contrasts with other smooth muscles innervated by non-adrenergic non-cholinergic inhibitory nerves, e.g. taenia coli (Tomita, 1972), rat anococcygeus (Creed and Gillespie, 1977) and guinea-pig anal sphincter (Lim and Muir, 1983), in which the relationship was linear and had a reversal potential

at approximately that of the potassium equilibrium potential (some -80 to -90mV) and shows a greater similarity to the slow inhibitory post-synaptic potential (ipsp) of certain ganglia (Hartzell et al, 1977; Hartzell, 1981; Horn and Dodd, 1981).

Potassium

The inhibitory potential in the bovine retractor penis was abolished at -103mV, which is higher than the anticipated equilibrium potential for potassium (-87mV, assuming that the internal concentration of potassium is the same as measured in the rat anococcygeus, 0.127M; Creed and Pollock, unpublished). Unlike the taenia coli (Tomita, 1972) and bullfrog sympathetic ganglia (Smith and Weight, 1977), the amplitude of the inhibitory potential in the bovine retractor penis was not increased when E_{K^+} was made more negative by removing K^+ from the Krebs solution. Increasing the external K^+ concentration to 20mM would be expected to change E_K to -49mV. Inhibitory potentials were recorded at membrane potentials exceeding this value. In normal Krebs solution, reversal of the inhibitory potential was not observed at membrane potentials more negative than -103mV. Reversal would have been expected if the action of the transmitter was to cause the membrane potential to shift towards E_{K^+} via an increase in K^+ permeability (Tomita, 1972; Horn and Dodd, 1981).

Together with the increase in membrane resistance during the inhibitory potential, the results suggest that the basis of the response in the bovine retractor penis is

not an increase in K^+ conductance. This may also explain why apamin, which blocks K^+ channels activated by the inhibitory transmitter in other tissues (Maas, 1981; Bauer and Kuriyama, 1982b), was relatively ineffective against inhibitory responses to field stimulation in the bovine retractor penis.

Chloride

The reduction in or removal of chloride from the Krebs solution depressed or abolished the inhibitory potential suggesting that it may be due to a change in permeability to chloride. The inhibitory transmitter could either stimulate Cl^- uptake into the cell or decrease its loss. The former explanation requires that Cl^- be pumped into the cell against its concentration gradient via an active mechanism stimulated by the transmitter. However, the mechanism responsible for Cl^- uptake, though uncertain, does not appear to involve a distinct pump. Cl^- uptake appears to be via a carrier-operated mechanism (Aickin and Brading, 1983) which may be driven by the sodium gradient (see Brading, 1979, 1980). Selective enhancement of chloride uptake also fails to explain why an increase in membrane resistance occurred during the inhibitory potential. The latter effect could be due to a decrease in Cl^- conductance. Recent studies (Aickin and Brading, 1982,1983) suggest that the resting Cl^- conductance in smooth muscle cells may be a small proportion (some 4%) of the total membrane conductance. This may increase after the development of

tone in the bovine retractor penis, although the inactivation of an already low Cl^- conductance by the inhibitory transmitter could explain the small size of the inhibitory potential.

The reduction in amplitude of the inhibitory potential at membrane potentials ranging from -60 to -27mV may be due to the combined effects of a decrease in potential difference between E_{Cl^-} and the membrane potential and reduced membrane resistance. The latter effect may prevent reversal of the inhibitory potential at membrane potentials more positive than -27mV.

The inhibitory potential was reduced or abolished after prolonged contact with ouabain or K^+-free Krebs solution, both of which reduce the Cl^- gradient across the membrane indirectly by inhibiting the Na^+-K^+ ATPase and hence reducing the Na^+ gradient (Brading, 1980). That a Na^+-K^+ ATPase is present in the bovine retractor penis was suggested by the depolarising action of ouabain and the hyperpolarisation which occurred following the readmission of K^+ after prolonged K^+ withdrawal (see Widdicombe, 1980). The prolonged contact time required for the abolition of the inhibitory potential by ouabain suggests that the inhibitory transmitter does not stimulate the Na^+-K^+ ATPase itself.

Interestingly, the inhibitory potential was depressed rapidly (within some 6 min) following K^+ withdrawal or the replacement of Cl^- with glutamate or benzene sulphonate.

This cannot be attributed to a reduction in the Cl^- gradient, and consequently the abolition of the resting Cl^- conductance. Intracellular Cl^- activity in the vas deferens dropped by only some one-seventh six minutes after complete withdrawal of chloride. Moreover, after prolonged periods in low Cl^- (10% normal) solutions the chloride gradient was larger than that in normal Krebs (Aicking and Brading, 1982). Unless Cl^- regulation in the bovine retractor penis differs considerably from that in the vas deferens, these observations suggest that the reduction in amplitude of the inhibitory potential in low (12.7×10^{-3}M) and thus possibly zero chloride solution may not be due to the abolition of the Cl^- gradient.

Both of the anion substitutes employed, glutamate and benzene sulphonate, have Ca^{2+}-chelating properties. The resulting decrease in the external Ca^{2+} concentration may interfere with drug-receptor combination or receptor activation. A decrease in membrane resistance caused by benzene sulphonate or glutamate appears unlikely, because in other smooth muscles, e.g. taenia coli (Ohashi, 1970), uterus (Bulbring and Szursweski, 1974), longitudinal muscle of the ileum (Benham and Bolton, 1983) and rat anococcygeus (Byrne and Large, unpublished) replacement of part or all of the external chloride with impermeant anions (including benzene sulphonate) either did not affect or increased membrane resistance.

Blockade of non-adrenergic non-cholinergic 'receptors'

also appears an unlikely mechanism of action for the anion substitutes. Although some muscarinic receptor blocking activity has been reported for benzene sulphonate (Benham and Bolton, 1983), its effects on the inhibitory potential in the bovine retractor penis were qualitatively similar to those of glutamate which has a different chemical structure.

Blockade of a resting conductance normally inactivated by the action of the inhibitory transmitter is another possibility. Trinitrobenzene sulphonate reduced K^+ channel-closing rates in frog myelinated nerves (Cahalan and Pappone, 1984), though this effect was irreversible. Replacement of chloride with glutamate or benzene sulphonate hyperpolarised the membrane after some 5 min, suggesting that the anion substitutes may block ionic channels. That membrane hyperpolarisation was not due to changes in liquid junction potentials (Coburn et al, 1975) is supported by the accompanying decrease in muscle tone.

The effect of the low Cl^- solutions may have been to inhibit transmitter release by field stimulation. This, again, seems unlikely in view of the persistence of non-adrenergic non-cholinergic inhibitory potentials in the taenia coli (Bennett, Burnstock and Holman, 1963; Tomita, 1972) and jejunum (Hidaka and Kuriyama, 1969) in low chloride solutions.

Sodium

The effect of ouabain on the inhibitory potential is compatible with the transmitter inactivating a Na^+ conductance. Reduction in the Na^+ gradient, caused by prolonged treatment with ouabain, may inactivate the Na^+ channels involved and thus reduce the effect of the inhibitory transmitter. Some information on the role of Na^+ may have been gained by reducing its concentration in the Krebs solution. However, the results would have been difficult to interpret. Complete removal of sodium would presumably abolish nerve activity whereas partial withdrawal may be insufficient to affect the sodium conductance. A further problem is that if Cl^- uptake into smooth muscle cells is driven by the Na^+ gradient (Brading, 1979,1980). any reduction in the latter would invariably reduce the Cl^- gradient.

Calcium

A further possibility is that the inhibitory transmitter reduced membrane Ca^{2+} conductance. After the development of tone in the bovine retractor penis, Ca^{2+} may be continuously entering the cell and be pumped out in a cycle, the net effect being a stable level of increased intracellular free Ca^{2+}. Thus, if the inhibitory transmitter decreased Ca^{2+} entry it would cause both hyperpolarisation and relaxation of the muscle. The effect of replacing Cl^- with benzene sulphonate or glutamate may have been due to their Ca^{2+}-chelating properties which

would reduce the levels of external Ca^{2+} and hence the Ca^{2+} gradient across the membrane. That this effect did occur is suggested by the slow hyperpolarisation and reduction in tone observed after prolonged contact with the low chloride solutions. However, the inhibitory potential always preceded and was of shorter duration than the relaxation to field stimulation, suggesting that the basis for the two effects was different.

VOLTAGE DEPENDENCE OF THE INHIBITORY POTENTIAL

The voltage dependence of the inhibitory potential over the membrane potential range -60 to -103mV was compatible with the response being due to a decrease in Cl^-, Na^+ or Ca^{2+} conductance. Anderson and Stevens (1973) proposed that the voltage dependence of transmitter-evoked conductance changes arose from the voltage sensitivity of channel 'open time'. In their model, channel open time was increased at high membrane potentials because the energy barrier for a conformational change from open to closed was increased. Such a model, if applicable to the bovine retractor penis, would predict that as the membrane potential was increased (beyond -60mV), the channel inactivated by the inhibitory transmitter would spend progressively less time in the closed conformation. Hence, the amplitude of the inhibitory potential would be reduced.

The voltage dependence of the inhibitory potential raised an interesting paradox. At a membrane potential of

some -60mV the inhibitory potential was maximal, yet at this membrane potential the tone level would normally be zero and the muscle unable to relax. In order to observe the inhibitory potential it was necessary for the muscle to depolarise (Fig.31), yet after this had occurred, passive hyperpolarisation of the membrane then enhanced the inhibitory potential. Moreover, passive depolarisation of the membrane potential in the sucrose gap failed to unmask an inhibitory potential to field stimulation. The difference between passive and spontaneous depolarisation may be that in the latter, the conductance which is inactivated by the inhibitory transmitter is switched on and may even be the basis for spontaneous depolarisation.

IONIC BASIS FOR THE HYPERPOLARISATION TO INHIBITORY EXTRACT

If the extract contained the inhibitory transmitter, then it should share the same mechanism of action. The membrane resistance change during the hyperpolarisation to inhibitory extract (cleaned activated) was inconsistent. Most frequently, either no significant change or an increase in membrane resistance occurred. The absence of any change in some cells was not due to the hyperpolarisation being small, since inhibitory potentials of only 1-2mV produced large increases. The lack of a consistent increase to extract may have been due to contaminants, e.g. ATP, which may have decreased membrane resistance, thus masking the effect of the acid-activated component. The amplitude of the hyperpolarisation to extract was

increased during membrane potential displacement from rest (-45mV) to more negative values (-57mV). This indicates a similar voltage dependence to the inhibitory potential.

In common with the inhibitory potential, the hyperpolarisation to cleaned activated extract was reduced or abolished following the withdrawal of K^+ or Cl^- from the Krebs solution. However, the effects of Cl^- withdrawal occurred more rapidly than could be accounted for by a reduction in the Cl^- gradient alone and suggest that the responses were inhibited directly by benzene sulphonate.

CONCLUSIONS

The inhibitory potential in the bovine retractor penis showed some similarities to those recorded in other smooth muscles possessing a non-adrenergic non-cholinergic innervation. Inhibitory potentials were evoked by a single pulse, preceded relaxation and were TTX-sensitive but insensitive to adrenergic or cholinergic antagonists. Particularly interesting characteristics of the response in the bovine retractor penis included the accompanying increase in membrane resistance, lack of enhancement by removing the external K^+, sensitivity to Cl^- replacement by other anions and its voltage dependency. These characteristics were largely shared by the hyperpolarisation to inhibitory extract and were compatible with the view that the extract may contain the inhibitory transmitter in the bovine retractor penis.

Also of particular interest in this muscle was the ability of the inhibitory transmitter to produce relaxation independently of any change in membrane potential in the presence of TEA (0.5 to 1×10^{-2}M). This indicates two possible mechanisms, electrical and non-electrical, by which relaxation may be produced.

RAT AND RABBIT ANOCOCCYGEUS

The electrical and mechanical effects of the cleaned activated extract and inhibitory nerve stimulation in the rat anococcygeus were consistent with their mediation by the same active component. The same proportion of cells (some 25%) exhibited an electrical response to the extract or field stimulation comprising inhibition of the oscillations in membrane potential.

Differences between the amplitude of the electrical response to inhibitory nerve stimulation in the rat and rabbit anococcygeus reported previously (Creed and Gillespie, 1977) were less apparent. Unlike the rat, however, in the rabbit the cleaned activated extract and inhibitory nerve stimulation inhibited the oscillations in membrane potential in every cell exhibiting this behaviour. Thus, the extract did appear to distinguish between the two species in a similar manner to inhibitory nerve stimulation. Relaxation to ATP was also accompanied by inhibition of the oscillations in membrane potential, though the dose of ATP required exceeded that present in volumes of activated extract which produced equivalent electrical and mechanical effects by approximately 1000-fold.

In the majority of cells which failed to exhibit electrical oscillations in the rabbit, neither inhibitory nerve stimulation nor the activated extract produced large electrical changes, although both relaxed the muscle. The

absence of electrical response was particularly apparent with the extract, possibly due to the presence of ATP which depolarised the tissue. Differences between inhibitory nerve stimulation and the extract also emerged when the effect of apamin was investigated on the rabbit anococcygeus. Apamin failed to block the inhibitory potential and relaxation to field stimulation, suggesting that the K^+ channels mediating the response differ from those in the taenia coli. The effects of the extract were inhibited transiently by apamin, possibly because of the initial excitatory effects of the drug.

In both the rat and, to a lesser extent, the rabbit anococcygeus the activated extract did mimic inhibitory nerve stimulation, suggesting that it may contain the non-adrenergic non-cholinergic transmitter in these tissues and thus, that this transmitter may be the same as that of the bovine retractor penis.

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