CENTRAL AND PERIPHERAL ACTIONS OF THE NEUROPEPTIDE PROCTOLIN ON A POSTURAL NEUROMUSCULAR MUSCLE SYSTEM IN THE NORWAY LOBSTER, *NEPHROPS NORVEGICUS* (L.)

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Summary

An investigation of the central and peripheral actions of the neuropeptide proctolin in the abdominal superficial flexor muscle (SFM) system of the Norway lobster, *Nephrops norvegicus* has been undertaken. High performance liquid chromatography (HPLC) and a sensitive bioassay have been used to establish the presence of the peptide in various tissues of *Nephrops*, including the SFM system. Proctolin-like bioactivity (PLB) in partially purified tissue extracts co-eluted with synthetic proctolin using these techniques. Quantification of bioassay responses to proctolin revealed that higher concentrations of the peptide are present in the lateral bundle of the SFM compared with the medial bundle of the muscle.

An analysis of the central effects of proctolin on the spontaneous activities of individual SFM motor neurones and on coupling relationships between different motor neurone pairs has been carried out using an isolated abdominal nerve cord preparation. Proctolin can simultaneously enhance and suppress the firing of individual members of the motor neurone pool; the peptide is effective in upregulating the firing of some motor neurones (f1, f4 and f6) and downregulating the firing of others (f2 and f5). These studies have also shown that proctolin can modulate the connectivity between specific superficial flexor motor neurone pairs by evoking or disrupting premotor connections. Thus, proctolin can reconfigure the postural circuitry by specifically modulating the postural flexor motor output pattern.

An analysis of the peripheral actions of proctolin on the two slow fibre phenotypes comprising the SFM has been carried out using both *in vitro* neuromuscular preparations and isolated single fibres. Proctolin alone has no effect on muscle tension, resting membrane potential or EJP amplitude. However, potentiation of SFM tension is produced by proctolin provided that the muscle is partially depolarised prior to exposure of the peptide. Furthermore, the two slow fibre phenotypes of *Nephrops* SFM exhibit a

differential responsiveness to proctolin, lateral fibres being more responsive to proctolin than medial fibres. These results, suggest that the effect of proctolin is mediated via proctolin-sensitive calcium channels and the differential responsiveness of medial and lateral fibres may reflect differences in the distribution and/or proctolin sensitivity of these channels.

Surveys of the pattern of innervation across the SFM have been carried out in an attempt to further investigate the observed correlation between different fibre phenotypes and their pattern of innervation at the level of individual motor neurones. The existence of a 'gross' correlation between fibre phenotypes and pattern of innervation has been confirmed. However, results indicate that the polysynaptic pattern of activity displayed by medial fibres as opposed to the synaptic silence usually observed for lateral fibres is due to the expression of their differential presynaptic properties. Medial fibres show properties of high output synapses and exhibit weak facilitation whereas lateral fibres show properties of low output synapses and facilitate strongly.

Further characterisation of the SFM with regard to fibre heterogeneity by investigating a number of neuromuscular parameters including pattern of innervation, facilitation properties, decay constant and EJP amplitude has suggested that the SFM system is more complex than was previously appreciated.

A preliminary investigation of the distribution and function of proctolin in the SFM system of the crayfish *Pacifastacus leniusculus* has been carried out. Histochemical analysis of the crayfish SFM reveals that it is composed of the same two slow fibre subtypes as *Nephrops* but that in crayfish these fibre subtypes are distributed in a random fashion throughout the SFM. An examination of the concentration of proctolin in the SFM of crayfish reveals that a similar distribution of the peptide exists to that found in the SFM of *Nephrops*; the lateral bundle contains higher concentrations of proctolin than the medial bundle. Given the differences in distribution of the two fibre

subtypes within each SFM, these results suggest that proctolinergic innervation of the SFM in both *Nephrops* and crayfish is position dependent and not correlated with fibre type. Furthermore, fibre type does not appear to be correlated with resting membrane potential or innervation in crayfish SFM. This study has also confirmed that proctolin acts postsynaptically to potentiate neurally induced tension in crayfish SFM.

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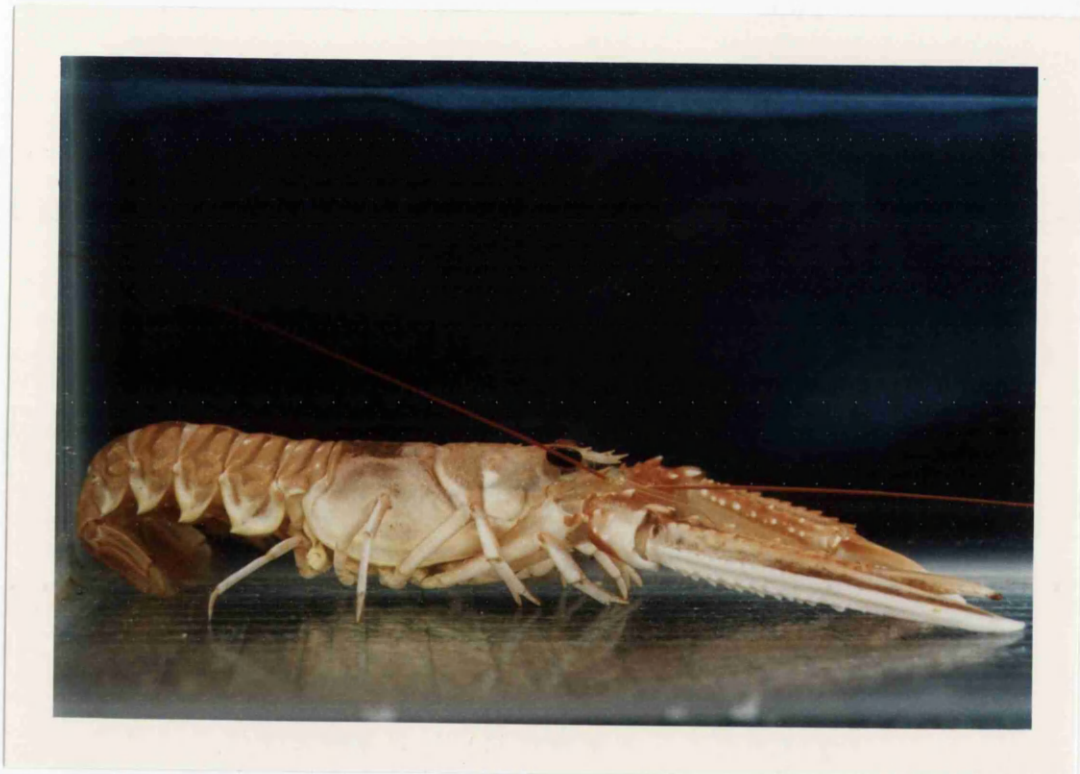
The Norway lobster, *Nephrops norvegicus*.



CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

1.1 Neuropeptides

Our knowledge of neurotransmission has come a long way since Dale (1935) put forward his principle that a single neurone contains a single neurotransmitter. We now know this to be untrue. As well as the 'classical' neurotransmitters, neurones can contain many different neuroactive substances and very often these include neuropeptides (Lundberg & Hockfelt, 1985). Thus, two neuropeptides, substance P and thyrotropin releasing hormone, are present in some mammalian brainstem neurones together with 5-hydroxytryptamine (5-HT) (Hokfelt *et al.*, 1980b).

The first neuropeptides to be characterised were oxytocin and vasopressin, the neurohormones of the hypothalmo-hypophyseal tract (du Vigneaud *et al.*, 1953a). Efforts were then concentrated on finding peptides released from the hypothalamus which in turn controlled the release of anterior pituitary hormones (as reviewed by Dockray, 1990). The first of these was thyrotropin releasing hormone (TRH), shortly followed by luteinizing hormone releasing hormone (LHRH) and the growth hormone inhibitory factor, somatostatin. Originally it was thought that the sole purpose of the releasing factors was to regulate functioning of the anterior pituitary. However, research on the localisation of these peptides and others previously found in the gut (vasoactive intestinal polypeptide and cholecystokinin), using immunohistochemical and radioimmunoassay methods, revealed them to be widely distributed throughout the central and peripheral nervous systems. These findings suggested that peptides may function as neurotransmitters or neuromodulators.

1.2 Neurotransmitter, neuromodulator or neurohormone

Neuroactive substances are classified as neurotransmitters according to whether they fulfil several criteria. These criteria have been constantly reappraised as our knowledge of synaptic function has progressed (McLennan,

1963; Elliot, 1979) and are applied to neurotransmitters in both vertebrate and invertebrate species.

Criteria for a neurotransmitter:

1. The molecule is synthesised and stored within the neurone from which it is released. Therefore it should be possible to visualise the putative transmitter directly using techniques such as immunocytochemistry, or indirectly by marking the enzymes involved in the synthesis of the substance.

2. Presynaptic stimulation should cause the release of the putative transmitter via a calcium-dependent mechanism and at a biologically effective concentration.

3. Exogenous application of the suspected transmitter must produce postsynaptic effects identical to those produced by nerve stimulation.

4. The response to exogenously applied putative transmitter must be blocked by antagonists which block the postsynaptic response to presynaptic stimulation.

5. The postsynaptic response produced by exogenous application of the suspected transmitter must be rapidly terminated. Therefore there should be mechanisms for inactivation or termination of the response, which can include re-uptake of the transmitter into nerve terminals.

Thus, neurotransmitters function by directly affecting ion channels on the subsynaptic membrane and rapidly exciting or inhibiting the postsynaptic cell. The discovery that a neurone could store and release more than one neuroactive substance led to the development of the concept of a neuromodulator, another class of chemical messenger which acts indirectly on ion channels to 'modulate' or 'regulate' the action of neurotransmitters over a longer time scale. The criteria for describing a neuromodulator were put forward by Barchas *et al.* (1978):

1. The substance does not act transynaptically, i.e. it is not released from the presynaptic membrane into the synapse, but acts either (i) to affect the

sensitivity of the subsynaptic membrane to the transmitter or (ii) to influence the release of the transmitter from the nerve terminal.

2. The substance must be present in physiological fluids and have access to the site of potential modulation in physiologically significant concentrations.

3. Pharmacological identity of action, as described above for neurotransmitters. Endogenous concentrations should affect postsynaptic activity in a dose-dependent manner at one or more specific sites of action and be mimicked by direct application of the neuromodulator or pharmacological agonists; these effects being altered in the presence of pharmacological antagonists

4. The time course of inactivating mechanisms should be comparable to that of endogenously induced changes.

Taking these criteria into account, neuromodulators can also be classed as 'local' hormones, released into and transported by body fluids. Their functions may be autocrine (Moed *et al.*, 1987), acting on the cells from which they are themselves released, or paracrine, where their actions are on neighbouring cells. (Buma & Roubos, 1985; Nieuwnehuys, 1985). 'Local hormones' are often contained within discrete sites, for example, the hypothalamo-pituitary system, and are not widespread throughout the brain or body. Some of these peptides e.g. luteinising-releasing hormone (LHRH) and thyrotropin-releasing hormone (TRH), are secreted into the capillary bed of the hypophyseal portal system and travel short distances to another capillary bed where they exert their effects. On the other hand, the peptides oxytocin and vasopressin function as true neurohormones as they are released directly into the general circulation via the capillary bed and are therefore able to reach distant targets over even longer periods of time.

1.3 Peptide families

To date, many novel neuropeptides have been discovered and it has become apparent that the majority can be grouped into families on the basis of

structural similarities, eg. stretches of amino acid sequence homology, or peptide folding. As we presume that peptides are recognised by receptors according to their structure, it seems logical that family members could have similar biological actions. Thus, amongst the secretins, vasoactive intestinal polypeptide (VIP) and secretin can bind to each other's receptors although with much lower affinity than they bind to their own receptors. Yet when secretin is compared with another member of the same family, glucagon, both have strikingly similar structures but different biological actions; secretin being involved in the regulation of pancreatic secretion while glucagon is involved in regulating blood glucose levels. This exemplifies the need to exercise caution when characterising novel neuropeptides since similarity in physiological response does not necessarily mean similarity in structure. On the other hand, the structural similarities of functionally unrelated neuropeptides can cause problems in their immunological identification if the antigenic site for antibody production is a shared region, e.g. the C-terminus region of gastrin and cholecystokinin. In order to achieve specificity the antibody must be directed against the unshared region of the neuropeptides.

1.4 Neuropeptides vs Classical neurotransmitters - Synthesis and metabolism

The modes of synthesis of neuropeptides and classical neurotransmitters are different. The classical neurotransmitters are small, positively charged ions which are synthesised in the cytoplasm by enzymes distributed throughout the neurone. Therefore their synthesis can occur in different regions of the neurone especially the nerve terminals. An example of one such neurotransmitter is the monoamine noradrenaline which is synthesised chiefly in the nerve terminals. However, terminal stores of noradrenaline are topped up by axonal transport of recently synthesised neurotransmitter or by reuptake of noradrenaline released from the nerve terminal.

In comparison, neuropeptides are synthesised from large precursor molecules which are produced only in the cell body (Lundberg, 1981). The

peptide precursors are packaged into granules and travel via axonal transport to the nerve terminals where they are stored prior to release. Processing of the peptide precursors within the granules results in the formation of several peptides with varying degrees of biological activity. There is no apparent method of recycling peptide or of reuptake once release from the nerve terminal has occurred, which would suggest that peptides are utilised in different ways from conventional neurotransmitters. The criteria for release of peptides may be different; they may be released less often, in smaller quantities and may be 'effective' at lower concentrations than the classical neurotransmitters (Harmar, 1987).

1.5 Distribution of Neuropeptides

The distribution of neuropeptides varies widely throughout the mammalian nervous system. Areas such as the hypothalamus, substansia gelatinosa of the spinal cord and nerve networks innervating the gut are rich in a wide variety of neuropeptides, whereas the cerebellum and thalamus have very few neuropeptide-containing neurones. Similarly, some neuropeptides (such as substance P) occur extensively throughout the nervous system whereas others (such as LHRH) are localised to particular areas (Hkfelt *et al.* 1975a,b,c). The widespread distribution of neuropeptides such as substance P and somatostatin is an indication that they may function as neurotransmitters rather than neurohormones.

1.6 Behavioural effects of vertebrate neuropeptides

In rats, oxytocin regulates maternal behaviour (du Vigneaud *et al.*, 1953b), while the expression of lordosis behaviour is promoted by LHRH (Pfaff, 1973). Growth hormone releasing factor (GRF) induces feeding behaviour (Vaccarino, 1990) while it is inhibited by cholecytokinin and bombesin (Antin *et al.*, 1975; Martin *et al.*, 1980). In humans, behavioural effects include the analgesic effect of opioid peptides and the enhancement of memory by vasopressin (See Krieger *et al.*, 1983).

1.7 Advantages of the invertebrate nervous system in studying neuropeptides

The literature concerning the chemical characterisation and localisation of vertebrate neuropeptides is already large and is constantly expanding (Hkfelt *et al.,* 1980a). However, the complexities of the vertebrate nervous system make the investigation of the specific roles of individual peptide-containing neurones, and more importantly, the role of neuropeptides in determining behaviour, extremely difficult. It has been known for a considerable period of time that invertebrates contain biologically active neuropeptides with actions comparable to those of vertebrates (Scharrer, 1967, 1978; Haynes, 1980). In the quest to understand the mechanisms underlying neuropeptide action, it is only relatively recently that the advantages of working with invertebrate preparations have been fully appreciated and exploited.

The main advantages of many invertebrate nervous systems over those of vertebrates are that they contain relatively few neurones (a few thousand rather than millions), and that they contain large peptidergic neurones which are re-identifiable in every individual preparation. Often, the discovery of such neurones leads to the development of 'model systems' in which identified peptidergic neurones innervate specific target organs. These systems not only allow investigation of the properties of peptide-containing neurones, but also permit analysis of the molecular mechanisms involved in regulating the actions of peptides at their target organs. Such studies can be carried out using electrophysiological, biochemical, immunocytochemical and molecular techniques.

1.8 Invertebrate neuropeptides

Many neuropeptides have been discovered as a direct result of invertebrate studies and the following examples represent the great diversity in structure and function of invertebrate neuropeptides.

1.8.1 Insects

1.8.1.1 Adipokinetic Hormone

The peptide named adipokinetic hormone (AKH) was initially isolated from the corpus cardiaca of the locust *Schistocerca gregaria P* (Stone *et al.*. 1976). Further studies on the cockroach corpus cardiaca have demonstrated the presence of the original peptide, now called AKHI and another related peptide AKHII (Carlsen *et al.*, 1979; Seigert *et al.*, 1985). In locusts, adipokinetic hormones cause release of diglycerides from the fat body into the haemolymph during flight (Goldsworthy & Gade 1983; Goldsworthy & Wheeler, 1984) and in terms of behaviour, enhance the speed of flight and tendency to fly (Goldsworthy, 1983). More recently, immunocytochemistry has demonstrated the presence of AKH in neurones of the locust and cockroach central nervous system (Schooneveld *et al.*, 1983; Witten, 1984) and a role for the peptide in skeletal muscle contraction seems likely as it and other AKH-like peptides (MI and MII) produce slow, sustained contracture of locust skeletal muscle (O'Shea *et al.*, 1984).

1.8.1.2 CAP_1 and CAP_2

Two myomodulatory neuropeptides, CAP_1 and CAP_2, have been discovered in the tobacco hawkmoth, *Manduca sexta*. The peptides were originally found to be cardioexcitaory when bioassayed *in vitro* on the heart of *Manduca sexta* (Tublitz & Truman, 1985a,b) but have since been found to have other actions on the hindgut in early stages of development (Broadie *et al.*, 1990). Thus, the peptides have been shown to act specifically during four different stages of the insect's life. In the embryo they are involved in modulating contractility of the hindgut to facilitate yolk ingestion while in the larva the target is again the hindgut, but this time the peptides promote gut emptying, a process which occurs during 'wandering' behaviour. In the newly emergent adult the CAPs accelerate the heart rate and aid wing inflation (Tublitz & Evans, 1986) after which time they continue to regulate the heart

rate during flight throughout the remainder of adult life (Tublitz, 1989). Thus, in the juvenile stages of development the CAPs act as local hormones influencing the hindgut, while in adults they function as blood-borne neurohormones affecting the heart rate during flight. These distinct actions are the result of differences in the distribution of CAP-containing neurones during the four stages of development, as well as changes in the sensitivity of the target organ to the peptides.

O'Shea *et al.,* (1988) have provided an extensive review of the functions of peptidergic and also aminergic skeletal motor neurones in insects.

1.8.2 Crustaceans

Proctolin is undoubtedly the most thoroughly studied peptide in crustaceans but as the actions of the peptide is the main focus of this thesis it will be dealt with in detail elsewhere. The peptide Red Pigment Concentrating Hormone (RPCH) was originally isolated from the prawn *Pandalus borealis* (Fernlund & Josefsson, 1968) and described as a hormone involved in the regulation of pigment migration in chromatophores. Research in the last few years has shown that the peptide has a variety of actions in crustaceans. It is a neuromodulator of the pyloric rhythm in crabs, (Nusbaum & Marder, 1988) the cardiac sac rhythm in lobsters (Dickinson & Marder, 1989) and the swimmeret rhythm in crayfish (Mulloney *et al.*, 1990; Scherff and Mulloney, 1991).

1.8.3 Molluscs

1.8.3.1 Egg-laying hormone

Egg-laying in *Aplysia* is one particularly amenable system in which to study the way peptides coordinate behaviour. During egg-laying the animal expresses a "fixed action pattern" which involves a complex series of behavioural and physiological changes: locomotion and eating are interrupted, respiration and circulation increase and headwaving accompanies the egg-laying process itself. This pattern is mediated by peptides released from 'bag-cells' (Kupfermann, 1967) which are clusters of neurones associated with the

abdominal ganglion (Dudek *et al.*, 1979; Stuart *et al.*, 1980). The best studied of the bag-cell peptides is ELH (Chiu *et al.*, 1979), a peptide which may act both as a circulating hormone and as a neuromodulator in the CNS (Mayeri & Rothman, 1982). The other bag-cell peptides include α-BCP, β-BCP, σ-BCP and acidic peptide (Rothman *et al.*, 1983; Scheller *et al.*, 1983). All of the peptides discovered so far control aspects of the fixed action pattern, but it is likely that other as yet undiscovered peptides are also involved (O'Shea & Schaffer, 1985).

1.8.3.2 Buccalin

The accessory radula closer (ARC) muscle in *Aplysia* and the two motor neurones, B15 and B16, which innervate it offer yet another excellent system in which to study peptides. The ARC muscle is regulated by a variety of modulators, including serotonin (Weiss *et al.*, 1978). In addition, the two motor neurones, B15 and B16, which are cholinergic also contain neuropeptides which can modulate ARC contractions (Cropper *et al.*, 1987). B15 contains three peptides, two of which are homologous cardioactive peptides (SCP_A and SCP_b). These act postsynaptically on the muscle to potentiate contractions produced by the action of the primary neurotransmitter, acetylcholine and increase the muscle relaxation rate (Lloyd *et al.*, 1984). Potentiation of the ARC muscle by the SCP peptides is also linked to an elevation of its cAMP levels. Conversely, the remaining peptide in B15, buccalin, appears to act presynaptically to inhibit acetylcholine release, thereby decreasing the size of contractions of the ARC muscle without affecting the relaxation rate. B16 contains the structurally unrelated peptide, myomodulin (Cropper *et al.*, 1987).

1.8.3.3 FMRFamides

Since the tetrapeptide FMRFamide (Phe-Met-Arg-Phe-NH_2) was first isolated from the clam *Macrocallista nimbosa* (Price & Greenberg, 1977), a tremendous amount of research has been conducted into its distribution and function in a variety of different species. Research has now demonstrated the

existence of other FMRFamide-like peptides (FaRPs) in molluscs which contain a C-terminal sequence of amino acids peculiar to all FMRFamide peptides (Price, 1986). As this sequence seems to be the most active antigenic site of the peptide, immunocytochemistry cannot distinguish between FMRFamide and related peptides. However, different peptides elicit a range of effects, and so far four different receptor types have been identified for this 'family' of peptides (Cottrell, *et al.*, 1987). FaRPs have been isolated from a number of molluscs including *Aplysia* (Austin *et al.*, 1983; Lehman *et al.*, 1982), *Helix aspersa* (Cottrell *et al.*, 1983; Boyd *et al.*, 1984; Price *et al.*, 1985; *Lymnea stagnalis* (Geraerts *et al.*, 1981) and *Octopus vulgaris* (Voight *et al.*, 1983) while FMRFamide-immunoreactivity has been detected throughout the animal kingdom from coelenterates to mammals (see Boer *et al.*, 1980). Recently three anthozoan neuropeptides which may be related to FMRFamide have been found to induce slow contractions on muscles in sea anemones (McFarlane *et al.*, 1991)

FMRFamide peptides have been shown to produce a variety of physiological effects in many species. When originally isolated from *Macrocallista* FMRFamide was found to be cardioexcitatory (Price & Greenberg, 1977) but cardioinhibitory properties have also been reported (Painter, 1982). In *Helix aspersa* the peptide causes contraction of the tentacle retractor muscle (Cottrell *et al.*, 1983) and has actions on central neurones which involve alterations of their membrane permeability to K^+, Na^+ and Ca^{++}. FMRFamide actions in rats include increases in arterial blood pressure (Koo *et al.*, 1982; Barnard & Dockray, 1984), inhibition of somatostatin and insulin secretion from islet cells (Sorenson *et al.*, 1984) and modulation of opiate-induced analgesia (Yang *et al.*, 1985).

Consequently, evidence from various lines of research have suggested a neurotransmitter/neuromodulator function for FMRFamide and related peptides (Price 1986, Cottrell *et al.*, 1988) as well as a neurohormonal role

(Greenberg *et al.*, 1985).

The distribution of FMRFamide-like immunoreactivity (FLI) within crustaceans such as the lobster (Kobierski *et al.*, 1987) and crayfish (Mercier *et al.*, 1991) has also been the subject of intensive investigation. Many similarities were observed between the pattern of immunocytochemical staining in these two animals and in both cases the highest levels of FLI were found in tissues which are known to have a neurosecretory function, especially the pericardial organs (POs). However, FLI was found to be associated with the hindgut and the intestinal nerve innervating it in the crayfish but not the lobster. In contrast, Siwicki & Bishop (1986) have found that proctolin-like immunoreactivity exists in the intestinal nerve of the lobster but not the crayfish. This has led to the suggestion that hindgut motility may be regulated by proctolin in the lobster (see Chapter 2) and FMRFamide-related peptides in the crayfish (Mercier *et al.*, 1991). Thus, an investigation of the functional role of a substance in one animal has its merits but can lead to false generalisations about the same function in other species. Comparative studies represent a more open-minded approach to research, and can lead to a more complete understanding of the way comparable systems work in different species.

1.9 Arthropod preparations as model systems

As we have seen, neuromodulators act at the level of information transfer across the synapse, but their effects are expressed ultimately as modifications of behaviour. Arthropods such as the lobster, crayfish and a variety of insects, provide model systems in which the action of neuromodulators can be studied at all possible levels between these two extremes, and the suitability of such model systems for the study of neuromodulator action can be illustrated by three examples.

1.9.1 The crayfish escape response

A sharp tap on the tail fan of the crayfish produces a characteristic escape behaviour which manifests itself as a tail flip, propelling the animal away

from the site of stimulation (Wine & Krasne, 1972). The neural circuitry involved in this reflex response is well understood (Wine & Krasne, 1982). The tail fan bears sensory receptors which connect to lateral giant fibres both directly and through a group of sensory interneurones. When stimulated, the lateral giant fibres excite the motor neurones innervating the fast flexor muscles of the abdomen. Repeated tactile stimulation of the tail fan leads to a decrease in the number of tail flips produced, i.e. habituation. Synaptic transmission between the afferent fibres and the sensory interneurones is depressed, making it less probable that the lateral giant fibres are activated. On the other hand, strong electric shocks increase the likelihood of producing a tail flip, i.e. sensitization, because the firing threshold of the largest sensory interneurone decreases. The biogenic amines 5-HT and octopamine modulate the synaptic transmission in the sensory circuits and mimic the habituation and sensitization responses (Glanzman and Krasne, 1983). Perfusion of 5-HT through the arterial system decreases the EPSPs in the lateral giant fibres produced by stimulating the sensory neurones, whereas they are increased by perfusion of octopamine. Thus. through the interplay of different modulators, neural circuits can be 'rewired' to orchestrate and tune their outputs to fit the immediate requirements of the animal.

1.9.2 The stomatogastric nervous system

Individual invertebrate ganglia often contain groups of cells which generate patterns of motor activity in the absence of sensory feedback. A well known example of this can be found in the stomatogastic nervous system of lobsters (see Katz & Harris-Warrick, 1990 for review) which consists of four ganglia - the stomatogastric, oesophageal and two commissural. These ganglia contain the central pattern generators (CPGs) which control the movements of three different areas of the animal's foregut - the pylorus, the gastric mill and cardiac sac. The pyloric and gastric mill CPG circuits are located in the single stomatogastric ganglion, and comprise 30 identified cells. The network for the

cardiac sac CPG spans all four ganglia and has not been studied extensively.

The stomatogastric ganglion will continue to generate rhythmic patterns of activity *in vitro* if the entire stomatogastric nervous system is intact, but these patterns cease when the stomatogastric ganglion is isolated from the rest of the system. Bath application of modulators onto the isolated stomatogastric ganglion have identified 13 different substances (including proctolin, see section 1.11.3) capable of initiating or modulating the pyloric and/or gastric mill rhythm. These substances are present in fibres arising from the three remaining ganglia (Hooper & Marder, 1987) and stimulation of these identified neurones can induce modulatory effects on the neural network.

This type of isolated preparation is ideal for studying the effect of neuromodulators on cellular and synaptic properties of CPG circuits, but does not easily allow the simultaneous analysis of the effect of modulators both centrally and on muscles in the system. This type of information is more conveniently gained by using a simpler neuromuscular preparation.

1.9.3 Postural muscle systems

The superficial abdominal flexor muscle system of crayfish and lobsters is an ideal neuromuscular preparation for studying peptidergic transmission. It consists of a nerve containing six identified neurones and its target organ, a thin sheet of postural muscle. Different properties of the superficial abdominal flexor system of crayfish have been studied by a number of workers. Bishop and coworkers have used this preparation extensively to investigate the function of proctolin in crayfish skeletal motor neurones (Bishop *et al.*, 1984; 1987; 1991). Of all the invertebrate peptides known to date, proctolin is perhaps the most thouroughly studied.

1.10 Proctolin

In 1967, Brown reported the biological activity of a "gut factor" which he extracted from both the foregut and hindgut of the cockroach *Periplaneta americana* (Brown, 1967). When applied to the longitudinal muscles of these

tissues the gut factor produced a slow graded contraction similar to that produced both by nerve stimulation and by the addition of 5-HT to the muscle preparation. However, the 5-HT antagonist, bromo-LSD, did not block the effect of the gut factor and the neuroactive compounds ACh, adrenaline, noradrenaline, GABA and glutamate all failed to mimic the pharmacological behaviour of the extract. Brown (1967) also demonstrated the existence of the gut factor in the thoracic peripheral nerves innervating skeletal muscles and in the thoracic central nervous system of the cockroach, but neither tested the gut factor on skeletal muscle nor attempted to elucidate its role in the CNS.

Brown considered that the gut factor fulfilled some of the criteria characterising neuromuscular transmitters and suggested that the substance may act as an excitatory transmitter within insects. He suggested that the active component may be a peptide because its effect on the gut was similar to that produced by two peptides named Factors P_1 and P_2 (Brown, 1975). However, unlike the gut factor, these peptides were biologically inactivated by chymotrypsin.

The name proctolin first appeared in the literature in 1975 when Brown & Starratt (1975) reported on the isolation and primary structure of the active component of the gut factor, a pentapeptide (arginine-tyrosine-leucine-proline-threonine) from extracts of the cockroach proctodeum.

1.11 The distribution of proctolin throughout the animal kingdom

Since Brown's initial report of the existence of the gut factor, much information has accumulated concerning the distribution of proctolin in a wide variety of invertebrates.

1.11.1 Annelids

Proctolin-containing neurones have been identified in the leech *Hirudo medicinalis* by using an antibody against the arthropod neuropeptide (Li & Calabrese, 1983). Although no peptides from the annelids have yet been sequenced, the annelids may provide excellent model systems in the future for

studying peptidergic transmission, as identified neurones have been located in the leech central nervous system using antibodies directed against other peptides such as enkephalin (Zipser, 1980), and AKH (Witten *et al.,* 1984). More recently, immunoreactivity to cholecytokinin, neuropeptide Y, enkephalins, substance P and FMRFamide has been found in the nervous system of the sedentary polychaete, *Sabellastarte magnifica* (DiazMiranda *et al.*, 1991).

1.11.2 Insects

In 1977, Brown surveyed nine representatives from six orders of insects; Orthoptera, Coleoptera, Diptera, Hemiptera, Hymenoptera and Lepidoptera and from his findings suggested that proctolin is a universal constituent of the Insecta. The peptide was not detected in a number of lepidopteran species, although this does not exclude the possibility that closely related peptides do exist as some invertebrate peptides already characterised have been shown to exist in families e.g. the adipokinetic family and the FMRFamide family (as reviewed by Platt and Reynolds, 1988).

Since then, proctolin has been shown to induce tonic contractions of the locust oviduct, extensor tibiae and cockroach foregut and hindgut (May *et al, 1979*; Holman and Cook, 1978, 1982, 1985; Wright and Cook, 1983; Lange and Orchard, 1984a: O'Shea and Adams, 1986). Calcium-dependent release of proctolin from cockroach and locust oviduct has been demonstrated and it is suggested that the role of proctolin in the neural control of visceral muscle is to act as an excitatory co-transmitter with a more conventional neurotransmitter such as glutamate (Holman & Cook, 1985; Lange *et al.*, 1986; Orchard & Lange, 1986). Interestingly, the effect of proctolin on locust oviduct is further regulated by a second peptide related to FMRFamide (Lange *et al., 1991*). Furthermore, a dual innervation of the blowfly hindgut and associated structures by proctolin and FMRFamide has been demonstrated using immunocytochemistry, suggesting that these peptides are involved in ionic and

water regulation as well the regulation of myotropic activities (Cantera and Nassel, 1991). Proctolin has also been shown to increase the strength and rate of heart contractions in cockroaches, yellow mealworms and orb-weaving spiders (Miller, 1979; Bartosz-Bechowski, *et al.*, 1991; Groome, *et al.*, 1991) and thus may act as a cardioregulatory neuromodulator in insects.

Studies of the effect of proctolin on insect skeletal muscle have revealed different actions of the peptide. For example, proctolin is able to produce a sustained contracture in the resting coxal depressor muscle of the cockroach (Washio *et al.*, 1990) but can only induce a tonic contraction in locust mandibular muscle if the muscle is depolarised prior to exposure to proctolin (Baines and Downer, 1991).

1.11.3 Crustacea

Immunocytochemistry, radioimmunoassay and HPLC have all been used to demonstrate the presence of proctolin in the CNS of crustaceans (Schwarz *et al.*, 1984; Bishop *et al.*, 1984; Siwicki & Bishop, 1986; Marder *et al.*, 1986). The discovery that proctolin occurs in the innervation of skeletal muscle in crustaceans as well as in insects (for review see Adams *et al*, 1989) has also resulted in intense research. For example, proctolin has been localised to neuromuscular terminals of the superficial flexor motor neurones in the crayfish *Procambarus clarkii* (Bishop *et al*,. 1984) where it modulates the strength of contraction in the superficial flexor muscle (SFM) (Bishop *et al.*, 1987). Proctolin can also modulate the activity of neurones in the cardiac and the stomatogastric ganglion (Miller and Sullivan, 1981; Hooper and Marder, 1987; Mulloney *et al.*, 1987; Nusbaum and Marder, 1989a,b).

Proctolin-like immunoreactivity has also been reported in the nervous systems of other chelicerate arthropods such as the horseshoe crab *Limulus polyphemus* (Chelicerata) (Benson *et al.*, 1981; Rane *et al.*, 1984; Groome, 1990).

1.11.4 Molluscs

As yet, no reports have appeared in the literature concerning the occurrence of proctolin in the nervous system of molluscs.

1.11.5 Vertebrates

Penzlin *et al.* (1981) demonstrated that proctolin causes contractions of the rat ileum, and proctolin immunoreactivity has since been found in various regions of rat brain: the mesencephalon, third ventricle, paraventricular nucleus of the hypothalamus and Raphe nucleus (Holets *et al.*, 1987). Fone *et al.*, (1988) have shown that proctolin may influence spinal serotonergic function.

1.12 Coexistence of proctolin with other transmitters

Proctolin has been found to coexist in neurones with other conventional neurotransmitters and neurohormones throughout the animal kingdom. Siwicki *et al.*, (1987) have demonstrated that proctolin coexists with 5-HT-, dopamine- and ACh-containing neurones in the lobster *Homarus americanus*. Working on cockroaches, Adams & O'Shea (1983) and Witten & O'Shea (1985) have proposed that proctolin may coexist with glutamate, producing depolarisation of muscle fibres via the slow coxal depressor motor neurone. As has been discussed in section 1.11.3, proctolin is proposed to coexist with a primary transmitter, again possibly glutamate, in crayfish superficial flexor motor neurones (Bishop *et al.*, 1987). The peptide also coexists with thyrotropin releasing hormone, and other neuropeptides including substance P in the ventral and thoracic lateral horns of rat spinal cord (Hokfelt *et al.*, 1980b, Holets *et al.*, 1987).

1.13 Does Proctolin act as a Neurotransmitter, Neuromodulator or Neurohormone?

When Brown published his paper in 1967 there was considerable disagreement over the exact role of the peptide in the cockroach proctodeum. Brown proposed that proctolin acted as an excitatory neuromuscular transmitter released from peptidergic motoneurones. He supported his claim

with various lines of evidence: (1) the similarity of the effect of nerve stimulation and bath application of proctolin, (2) the direct action of proctolin on denervated preparations and (3) the fact that repetitive stimulation released a substance into the perfusate which had similar pharmacological properties to proctolin.

Holman & Cook (1979) compared the effects of proctolin and L-glutamate on the visceral muscle of the cockroach *Leucophaea madaerae*. They concluded that proctolin behaved not as a transmitter but rather as a modulator of glutamate action and a regulator of intrinsic myogenic properties.

Brown's original suggestion that proctolin may function as a transmitter of insect skeletal motoneurones has now been confirmed by various groups working on the locust (Witten *et al.*, 1984; Worden *et al.*, 1985) and the cockroach leg (O'Shea & Bishop, 1982).

In crustaceans, proctolin has been shown to have a variety of neuromodulatory actions on different neurones (Hooper and Marder, 1987; Nusbaum and Marder, 1989ab), muscles (Schwarz *et al.,* 1980; Bishop *et al.,* 1987) and proprioceptors (Pasztor and MacMmillan, 1990) but to date no studies have produced direct evidence that the peptide can act as a primary transmitter. However, Acevedo *et al.* (1993a,b) propose that proctolin is a neurotransmitter in three of the five descending interneuronal 'command elements' that activate and regulate the swimmeret rhythm. Both perfusion of proctolin onto isolated abdominal nerve cord preparations and stimulation of these interneurones can induce and modulate the swimmeret rhythm in crayfish (Mulloney *et al.,* 1987). Proctolin-like immunoreactivity exists in the same region of the nerve cord as these axons (Acevedo *et al.,* 1992a) and a proctolin-like substance is released when the axons are artificially stimulated (Acevedo *et al.,* 1992b).

The pericardial organs in the lobster have been found to contain the greatest amounts of proctolin (Schwarz et al, 1984). This tissue, being a

neurosecretory structure is well placed for releasing substances into the circulation and this has led to the suggestion that proctolin may function as a neurohormone in arthropods. One problem with this proposal is that proctolin is rapidly inactivated in haemolymph, having a half-life of only about six minutes (Schwarz *et al.*, 1984). Another consideration must be the quantity of proctolin which would have to be released to achieve a concentration in blood which would be physiologically effective. If it is assumed that the peptide is uniformly distributed in blood, then most of the proctolin within the pericardial organs would have to be released at once in order to achieve a threshold concentration (Schwarz *et al.*, 1984). This seems highly unlikely, and it is therefore more probable that proctolin acts as a local hormone in crustaceans.

1.14 What motivated this study?

Over the last few years an enormous amount of information has been accumulated concerning the distribution of the neuropeptide proctolin, especially within invertebrate species (Li and Calabrese, 1983; Zipser, 1980; Schwarz *et al.*, 1984; Adams *et al.*, 1989). In many cases studies on the distribution of proctolin have led to the proposal of possible roles for the peptide which have subsequently been proven. The Crustacea, in particular, lobster and crayfish, have provided excellent material for the investigation of the role of peptides thus far.

The neuropeptide proctolin is a recognised neuromodulator of the abdominal swimmeret system in crayfish (Mulloney *et al.,* 1987). Both perfusion of proctolin onto an isolated crayfish ventral nerve cord and stimulation of excitatory command neurones both induce the swimmeret rhythm in silent preparations (Mulloney *et al.*, 1987). Furthermore, it has been proposed that some of these command neurones, originally described by Wiersma and Ikeda (1964), use proctolin as a neurotransmitter (Acevedo *et al.,* 1992a,b).

Very little is known about the central function of proctolin in the abdominal positioning system, but Bishop's group has identified the peptide in

three of five excitatory motor neurones innervating the postural superficial flexor muscle (SFM) in crayfish (Bishop *et al.,* 1984) and have demonstrated that proctolin can act peripherally to modulate tension developed in the SFM in response to the primary transmitter (Bishop *et al.,* 1987).

In the lobster *Homarus*, Kravitz and co-workers have demonstrated both central and peripheral effects of the amine neuromodulators octopamine and serotonin on the SFM neuromuscular system (Kravitz, 1988). However no equivalent information has yet been obtained for proctolin, either in *Homarus* or in other nephropid decapods, including the Norway lobster, *Nephrops norvegicus*. A general aim of the present study was therefore to obtain comparative data from this lobster on the actions of proctolin. In this laboratory, *Nephrops* has been studied extensively in terms of the influence of sensory stimuli on postural motor systems (Priest, 1983; Neil and Miyan, 1986; Newland and Neil, 1987; Knox and Neil, 1987; Goodall et al., 1990). These studies have utilised both intact animals and semi-intact preparations to obtain an understanding of the neuronal mechanisms involved in eliciting particular behavioural responses. An investigation of the proctolin sensitivity of such postural motor systems of *Nephrops* would therefore be expected to give a greater understanding of the extent to which such behaviours can be modulated, and this aim has been pursued in this project.

In addition to the extensive neurobiological data now available for *Nephrops*, a number of recent findings about the organisation of the abdominal SFM system have revealed unexpected features. The SFM in *Nephrops* is a thin muscle sheet which is innervated by six motor neurones, five of which are excitatory and one inhibitory (Knox and Neil, 1991). It was previously assumed that the SFM was composed of a uniform population of slow muscle fibres, but Neil and Fowler (1990) have shown that the muscle in fact comprises two slow fibre subtypes that are more or less segregated into discrete medial and lateral bundles. This discovery prompted a re-examination of the crayfish SFM (Fowler

et al., 1991) which revealed that it is also composed of fibres of the same two slow subtypes, but in this case they are distributed in a mixed fashion throughout the muscle. These findings in *Nephrops* and crayfish suggest that important advances can be made in our understanding of the peripheral actions of proctolin in relation to muscle fibre properties by an in-depth study of their SFM systems, and this has been performed in this project. The segregation of the *Nephrops* SFM into bundles of the two phenotypes is a particularly advantageous feature which has been exploited.

A preliminary survey of the innervation of the SFM in *Nephrops* has revealed that a correlation exists between a particular fibre type and its pattern of synaptic input. Different fibre types (determined by subsequent biochemical analysis) can be recognised by their characteristic pattern of innervation (Neil and Fowler, 1990). This suggests that a detailed analysis of these relationships for each of the six motor axons that innervate SFM may not only throw light on the relationship between innervation and fibre type, but will also make it possible to relate any central effects that proctolin exerts on motor neurones to their innervation properties. For this reason, much attention in this project has been paid to the effect of proctolin on the firing properties and coupling relationships of SFM motor neurones.

The above considerations have determined the experimental programme pursued in this project. As a basis for the more detailed studies, the existence of proctolin in various tissues of *Nephrops*, including the SFM neuromuscular system was first established using extraction procedures (HPLC) and a bioassay (Chapter 2). The central effects of proctolin on the activities of individual SFM motor neurones, and on their coupling relationships were then determined using an *in vitro* preparation of the isolated *Nephrops* abdominal nerve cord (Chapter 3). The peripheral effects of proctolin on muscle fibres of different phenotypes in the SFM of both *Nephrops* and crayfish were then examined using both *in vitro* neuromuscular preparations and

isolated single fibres. (Chapter 4). The results obtained from these investigations have demonstrated that proctolin has differential effects on the firing of SFM motor neurones, modulates their relationships within the motor network and enhances to different extents the tension developed by the two fibre phenotypes of SFM. The significance of these findings, their implications for proctolin action *in vivo* and possible directions for future research are discussed in Chapter 5.

CHAPTER 2.

LOCALISATION AND ESTIMATION OF PROCTOLIN

WITHIN THE SUPERFICIAL FLEXOR MUSCLE SYSTEM.

2.1 INTRODUCTION

In order to explore the function of the peptide proctolin in the superficial flexor muscle (SFM) system of the Norway lobster *Nephrops norvegicus*, it was first necessary to demonstrate its existence within these tissues. A precedent for the existence of proctolin in the motor neurones innervating the visceral muscle in *Nephrops* exists in the work of a number of other groups who have localised proctolin in postural motor neurones of the cockroach (O'Shea and Bishop, 1982; Adams and O'Shea, 1983), the locust (Worden *et al.,* 1985), the grasshopper (Keshishian and O'Shea, 1985), the crayfish (Bishop *et al.,* 1984; 1987) and the lobster *Homarus americanus* (Siwicki *et al.*, 1985). Proctolin has myoactive effects on a number of muscles including the cockroach foregut and hindgut and the locust oviduct and extensor tibiae (May *et al.,* 1979; Holman and Cook, 1982; Wright and Cook, 1983; Lange and Orchard, 1984; O'Shea and Adams, 1986). However, in some other muscles, such as the cockroach coxal depressor muscle, the locust mandibular closer muscle and the crayfish SFM, proctolin acts to enhance neurally evoked contractions (Adams and O'Shea, 1983; Bishop *et al.,* 1984; Baines and Downer, 1991).

The direct myoactive effect of proctolin on some insect tissues has led to the development of a number of bioassay systems for the detection and quantification of the peptide. The first one to be developed was the hindgut bioassay of the cockroach *Periplaneta americana* (Brown, 1967), which is simple to prepare and can be used to produce either neurally evoked contractions or myogenic contractions. This bioassay system is consistent within preparations and is sensitive to low concentrations of proctolin having a threshold concentration for proctolin of around 5×10^{-10} M (this study). Some other cockroach hindgut preparations such as that of *Leucophaea maderae*, offer a slight advantage in sensitivity to proctolin, having a reported threshold excitation of the hindgut of 3×10^{-11} M proctolin (Holman and Cook, 1978) but

this species is less widely available. For convenience, the *Periplaneta* hindgut bioassay system was chosen for use in this study. Another bioassay system based on the ability of proctolin to increase the myogenic leg movements of a locust extensor tibiae muscle has been used by a number of different workers to quantify proctolin (O'Shea and Adams, 1981; Bishop *et al.,* 1984, 1987; Acevedo *et al.,* 1993b). This system offers even greater sensitivity to proctolin, responding with an accelerated rhythm to as little as 0.25 femtomoles and is therefore appropriate for measuring low levels of peptide in individual tissues or proctolin released from stimulated nerves (Lange *et al.*, 1986). However, this bioassay system is more difficult to prepare and to superfuse and although initial trials were performed with the locust leg system, it proved difficult to obtain reliable responses and efforts were concentrated on the cockroach hindgut system instead.

Initially, a general survey of tissues was made to ascertain the distribution of proctolin in *Nephrops* and to confirm the responsiveness and sensitivity of the cockroach hindgut to proctolin. In the lobster *Homarus*, highest concentrations of proctolin exist in the pericardial organs (POs) (Schwarz *et al.*, 1984). Therefore, these same tissues in *Nephrops* were assayed for their proctolin-like bioactivity (PLB) along with other parts of the nervous system including the eyestalks and the ganglia. There is evidence for the existence of proctolinergic endings on the SFM of crayfish (Bishop *et al.,* 1984) and the equivalent tissues in *Nephrops* have also been assayed for proctolin. Since there is a general segregation of muscle fibre types between medial and lateral bundles in *Nephrops* SFM (details in Chapter 4) these two bundles have been assayed separately.

In crayfish, immunocytochemistry has been used to demonstrate the presence of proctolin in three of five excitatory motor neurones innervating the SFMs of the crayfish *Procambarus* (numbered f1, f3 and f4), and proctolin immunocytochemical staining in axon terminals can be visualised across the

whole superficial flexor muscle (Bishop *et al.* 1984). In contrast, only one proctolin immunoreactive axon has been found in *Homarus americanus* and the extent of the arborisations across the superficial flexor muscle (SFM) is unclear (Siwicki *et al.*, 1985). Immunocytochemical mapping of proctolin within the SFM system of *Nephrops* was performed in this study in an attempt to determine the identity of proctolin-containing motor neurones and permit a comparison of the distribution of proctolinergic axon terminals across both medial and lateral bundles. Unfortunately, these experiments were repeated several times without success and the exact localisation of proctolin within the SFM system of *Nephrops* remains to be elucidated.

2.2 MATERIALS AND METHODS

2.2.1 Animals

Cockroaches (*Periplaneta americana*) used in this study were taken from stock colonies maintained at 27°C and fed rat chow pellets and water *ad libidum*. Adult male cockroaches were used for the hindgut bioassay.

Lobsters (*Nephrops norvegicus (L.)*) of carapace length 6 - 9.5 cm, obtained from the University Marine Station, Millport, Isle of Cumbrae, were maintained in tanks supplied with aerated circulating sea water at around 10-12°C and were fed on whitebait.

Crayfish (*Pacifastacus leniusculus L.*) of carapace length 5 - 7.5 cm, were obtained commercially. They were kept in aerated tanks of copper-free tapwater at 14°C and fed on fish meal.

2.2.2 Composition of insect saline

The insect saline used in these experiments was composed of 9.0g NaCl, 0.2g KCl, 0.2g CaCl, 3.96g glucose and 10 ml 0.1 M sodium phosphate buffer, (Pringle, 1938). The pH of the saline (pH 7.0) did not change with oxygenation (Brown, 1965).

2.2.3 Extraction procedure

Tissues from each animal were dissected in physiological saline, placed in a container of known weight, immediately frozen, and then freeze-dried overnight to allow calculation of dry weight. After homogenisation in 100-200 μl of 80% methanol, the resulting tissue suspensions were centrifuged at 13,000 rpm for 10 minutes and the supernatants collected and evaporated to dryness under a stream of nitrogen. Dried samples were then redissolved in 200μl of chromatography solvent for purification by high performance liquid chromatography (HPLC).

2.2.4 HPLC purification

Separations were carried out at ambient temperature using a Gilson 714 Liquid Chromatography system (Fig 2.1) with a variable u.v. absorbance

detector. Proctolin standards and tissue extracts were applied to an Anachem Bondapak C18 column (250x50 mm, 4.6 mm internal diameter) packed with silica (10 μm ODS) by a Hamilton microsyringe. The solvent system eventually employed was ion-pair reverse-phase HPLC using a linear concentration gradient of 5-50% (v/v) acetonitrile containing 0.1% trifluoroacetic acid (TFA) (buffer B) against 0.1% TFA in water (buffer A) at 1 ml/min. The gradient was started 5 minutes after sample injection and 1 ml fractions were collected, evaporated to dryness and redissolved in cockroach saline for bioassay. Of these fractions, only fraction number 19 produced bioactivity (see results) similar to authentic proctolin when applied to the bioassay. For this reason, in further experiments only fractions 19 and 20 were collected. The elution time of authentic proctolin was determined by chromatography of the pure peptide (Sigma) at a detection wavelength of 214 nm (Fig. 2.2). Using this system, proctolin eluted at 19.3 minutes. A Gilson 714 software package was used to analyse the results. The amount of proctolin present in the tissue samples analysed was below the level of detection of the HPLC system (Fig 2.3) and for this reason HPLC could be used only for purification of tissue extracts, but not for quantitative analysis of proctolin. Some of the prepared tissue samples were spiked with pure proctolin in order to check that the retention time remained consistent. During purification of samples, proctolin standards were routinely chromatographed to check for any variability in the retention time of the peptide. HPLC grade reagants (Scotlab) were used throughout.

2.2.5 Musculature of the cockroach proctodeum

The cockroach proctodeum is composed of striated fibres which exhibit properties that are intermediate between smooth and fast skeletal muscle fibres. The proctodeum is divided by a constriction into two regions, the anterior intestine and the posterior intestine (rectum). Although both regions possess circular and longitudinal muscles, the organisation of the fibres in the two regions is considerably different. In the anterior intestine, short flat bundles

of longitudinal muscle are closely associated with the underlying circular muscle fibres. The anterior two thirds of the rectum contains six individual bundles of symmetrically placed longitudinal muscle fibres. These muscle fibre bundles lie over a thin layer of circular muscle that is much thicker at the intestinal constriction and around the anus. In the absence of regulatory motor discharge from the sixth abdominal ganglion via the proctodeal nerve, the muscle fibres of the proctodeum exhibit spontaneous myogenic contractions. The muscle fibres of the proctodeum undergo coordinated contractions that give rise to peristalsis. The activity of the longitudinal muscles during peristalsis can be monitored easily and provides the basis for a pharmacological assay.

2.2.6 Isolation of the proctodeum

Adult male cockroaches were used for dissection. The reproductive system of male cockroaches is more discrete than that of females which makes it easier to dissect the proctodeum. Each insect was immobilised by briefly chilling it on ice before dissection. The head, legs and wings were removed and the specimen was pinned dorsal side up in a dissecting tray. The tray was then flooded with fresh insect saline. The proctodeum was exposed by making a superficial midline incision through the last abdominal sclerite and continuing up to the thorax. The dorsal tegument was then pulled apart and pinned out to reveal the viscera. As it was only intended to use the preparation for pharmacological assay, those nerves innervating the proctodeum and any other restraints were carefully snipped. The intestine was then cut anterior to the insertion of the malphigian tubules and placed on one side. Finally the ventral rectum dilator muscles were severed and the proctodeum lifted free from the abdomen and placed in a petri dish containing fresh saline.

Two lengths of silk thread (5 cm and 1 cm) were prepared with a small noose at one end which would serve to attach the hindgut to the equipment. The smaller length was tied to the rectal end of the proctodeum and the longer tied immediately above the point of insertion of the malphigian tubules.

2.2.7 Hindgut bioassay.

The recording chamber (Fig 2.4), which was made in the Glassblowing Workshop of the Chemistry Department, consisted of an open-ended glass tube with a side arm to provide a simple overflow system which maintained a constant volume of 3.2 ml. The organ bath was mounted vertically with a rubber stopper seated firmly in the bottom. The stopper was penetrated by a pin with a hook at the end, onto which the rectal end of the proctodeum was attached. A gentle stream of oxygen bubbles and fresh saline were are also delivered through fine steel tubes penetrating the stopper. The thread from the anterior end of the proctodeum was attached to a sensitive strain gauge (fabricated from foil strain gauges (RS Ltd)) which was mounted directly above the organ bath. The whole system was mounted in a way such that both the strain gauge and the organ bath could be moved independently. Hindgut contractions were displayed on a oscillograph pen recorder (Washington) or oscilloscope (Gould). Following the initial mounting procedures the tension on the preparation was then increased to approximately 2.5 mN and the preparation was left for 60 - 90 minutes. During this time the longitudinal muscles relaxed to constant length and the preparation became much more sensitive to proctolin. Preparations set up in this way remained viable for at least 12 hours. Experiments were carried out at room temperature. 32-320 μl of various concentrations of proctolin standards were added to the organ bath via a Hamilton microsyringe in order of increasing concentration.

2.3 RESULTS

2.3.1 Bioassay of authentic proctolin

Cockroach hindgut preparations responded to proctolin with three changes in their contractile activity (Fig. 2.5):

1. the amplitude of phasic contractions increased
2. the frequency of phasic contractions increased
3. muscle tone increased, as indicated by a rise in the baseline tension. This feature was dose-dependant and was used as the basis of the pharmacological assay.

The threshold for excitation of the hindgut usually lay between 0.5 - 1.0 x 10^{-9}M proctolin (final concentration in the bath). When hindguts were exposed to low concentrations of proctolin (< 1 x 10^{-9}), only an increase in the contractile activity was seen in the hindgut, without the characteristic tonic contraction produced by larger doses of the peptide. For each experiment the mean maximum tension was measured approximately 1.5 minutes after the addition of a given concentration of proctolin to the bath (Fig. 2.6) and individual dose-response curves were constructed (Fig. 2.7). Figure 2.8 shows a dose response curve constructed from the results of 5 experiments. The response of the hindgut to increasing concentrations of proctolin was graded with the maximum response occurring at approximately 1 x 10^{-7} M. At doses above 1 x 10^{-7} M the effect of proctolin on the hindgut decreased. Repetition of doses of proctolin produced reliably consistent effects on hindguts **within** experiments but the amount of tension generated by hindguts in response to a given concentration of proctolin varied considerably **between** experiments. Due to the differential sensitivity of individual hindguts to standard doses of proctolin, results are expressed as a percentage of the maximum force generated in each experiment. Figure 2.9 shows a variety of responses of hindguts to a standard dose of 1 x 10^{-8} M proctolin. The amount of tension generated in individual hindguts varied from approximately 0.3 mN to 2.5 mN

(Fig. 2.9 b and c respectively). There was also a great diversity in the type of response elicited from individual hindguts. In some preparations (Fig 2.9 a, c and e) the larger tonic response was of more rapid onset than in others (Fig 2.9 b, d and f). There was pronounced variation in the amplitude, frequency and duration of phasic contractions e.g. those of Fig 2.9a are small, irregular and slower than those shown in Fig 2.9f. In most preparations the tonic response to proctolin disappeared after five minutes leaving the phasic contractions which would continue until the washout with saline. In a few preparations, however, the tonic response was very short lived (Fig 2.9e).

2.3.2 Bioassay of tissue extracts

Initially, semi-quantitative experiments were performed to test the responsiveness of the cockroach hindgut bioassay to proctolin in *Nephrops* tissue extracts. In *Homarus* proctolin exists in highest concentrations in the pericardial organs (POs) and high concentrations are also present in the eyestalk (Schwarz *et al.*, 1984). Therefore, these same tissues in *Nephrops* were assayed for their proctolin-like bioactivity (PLB) after HPLC separation. Bioassays of extracts of the 5th thoracic and 1st abdominal ganglia were also performed as these tissues are known to contain proctolinergic cells in crayfish (Siwicki *et al.*, 1985). The observed response of cockroach hindguts to proctolin and tissue extracts was qualitatively very similar and indicated the presence of a proctolin-like substance in all four tissues (Fig. 2.10). As expected, the POs contained high levels of proctolin. Comparison of the hindgut response to an extract of 2 POs (Fig. 2.10a) with that of a standard dose of 1×10^{-8} M proctolin (Fig. 2.10b) yields an estimate of approximately 5×10^{-9} M proctolin per organ. Similarly, the results shown in Figure 2.10c-f, which are from a different experiment, provides estimates for the proctolin content of eyestalk, thoracic and abdominal tissues, and verified that the cockroach hindgut was sufficiently sensitive to detect proctolin in *Nephrops* tissues.

Once it had been established that a proctolin-like substance could be

detected within the nervous system of *Nephrops*, more quantitative experiments were carried out to determine the proctolin content of the SFM system in both *Nephrops* and *Pacifastacus*.

The PLB of HPLC purified tissue samples was tested by applying them (in conjunction with the application of authentic proctolin), to a hindgut bioassay preparation for which a dose response curve had been constructed. PLB in tissue samples containing low concentrations of proctolin was estimated from the mean amplitude of the resultant high frequency contractions. In order to check the efficiency of the extraction and purification procedures employed, authentic proctolin standards were taken through the various steps and then bioassayed. This method indicated that the system used was 90% efficient. The fact that both proctolin standards and tissue extracts underwent identical procedures and produced such similar responses when applied to bioassay, supports the argument that the PLB of the tissue extracts was in fact due to authentic proctolin. Figures 2.11 and 2.12 illustrate the responses obtained when purified lobster and crayfish tissue extracts were applied to hindgut bioassays. The results presented in Figure 2.11 for *Nephrops* tissues are from two different experiments. The slight differences in sensitivity of the two hindguts used is demonstrated by Fig 2.11 (c and e) which show the responses of each hindgut to a standard dose of 3×10^{-9} M proctolin. The results presented in Figure 2.12 and for crayfish tissues are from one experiment. A comparison of the quantitative bioassay results are presented in Table 2.1 which shows the levels of PLB, expressed as proctolin equivalents, in various tissues associated with the superficial flexor system. PLB was detected in all tissues and for all tissues except SFM nerves, crayfish contained greater amounts of proctolin than *Nephrops*.

It is known that peptides are synthesised in the cell bodies of a neurone and are then transported down the axon to the presynaptic terminals where they are stored until required (Harmer, 1987). Bioassay of extracts of the

abdominal ganglia (where the cell bodies are located, see chapter 3), revealed that on a dry weight basis the abdominal ganglia contain the highest amounts of proctolin in the SFM system of both species. Concentrations in *Pacifastacus* were more than double those detected in *Nephrops*. Lower concentrations of proctolin were found in the SFMs of both lobster and crayfish and most likely reflect the content of presynaptic terminals on the muscle, although the possibility that proctolin is inside the muscle fibres cannot be excluded. Interestingly, in both species the lateral superficial flexor muscles contained greater amounts of proctolin than the medial superficial flexor muscles.

2.3.3 Mediation of proctolin-induced contraction in the cockroach hindgut

There are conflicting views of how proctolin-receptor interaction mediates contraction of the visceral musculature in the cockroach but it is clear from the results of this study and those of other workers (Holman and Cook, 1978) that the response of the hindgut to proctolin has both a fast and a slow component. As the two responses are not mutually exclusive it may be postulated that they are produced by different pathways; the fast response may represent an ionotropically mediated response via a traditional neurotransmitter and the slower response may involve a second messenger effect, acting for example via cAMP. To test this hypothesis, some preliminary experiments were performed using the adenylate cyclase activator, forskolin, to investigate the possible involvement of c-AMP in mediating the slower contractile response of the hindgut. The application of forskolin to hindguts resulted in an increase in the frequency and amplitude of contractions but failed to produce the tonic component of contraction characteristic of proctolin (Fig. 2.13). Similarly, the addition of forskolin to a hindgut already exposed to proctolin, significantly increased the amplitude and frequency of contractions. By themselves, these results can only point to a possible role for cAMP in the slow component of the proctolin-induced contraction but not in the fast component.

2.3.4 *Nephrops* hindgut bioassay

During the course of this study, the opportunity was taken to examine the possible role of proctolin in the control of contractility of the crustacean hindgut. A particular aim was to establish the sensitivity of the hindguts of *Nephrops* and *Pacifastacus* to proctolin with a view to using one of them as a convenient alternative for use with crutacean tissue extracts. Initially the hindguts of both *Nephrops* and *Pacifastacus* were set up in the same way as the cockroach hindgut and their response to proctolin tested. The peptide caused an increase in contractility in both hindguts, although the response of the hindgut of *Pacifastacus* was found to be inconsistent and difficult to measure. The application of proctolin to the hindgut of *Nephrops* (Fig. 2.14) produced more consistent responses, and 3 changes in contractile activity could be identified;

1. an increase in the amplitude of phasic contractions,
2. an increase in the frequency of phasic contractions,
3. the onset of a wave of peristaltic-like contraction upon which smaller phasic contractions were superimposed.

However, none of these responses to proctolin were persistent. Within approximately 5 minutes of application the peristaltic wave disappeared, and the levels of contractile activity decreased. Although these results demonstrate that proctolin is undoubtedly involved in the control of the hindgut in *Nephrops*, they also indicate that the preparation does not offer any advantages over the cockroach hindgut as a bioassay for proctolin in terms of sensitivity. The threshold concentration for excitation of *Nephrops* hindgut was approximately 5 x 10^{-9} M as opposed to 5 x 10^{-10} M for cockroach hindgut.

2.4 DISCUSSION

The cockroach hindgut bioassay used in this study was sufficiently sensitive to detect proctolin in the tissues and provides a simple and direct means of determining the quantity of proctolin in tissue samples, as synthetic proctolin is available for the calibration of the assay response. Although the cockroach hindgut bioassay is only able to detect greater than 1.6 picomoles of proctolin compared to the locust leg bioassay which can detect 0.25 femtomoles of proctolin, the response of the cockroach hindgut to proctolin standards remains consistent within preparations whereas it has been reported that a variation in response of the locust leg to proctolin standards occurs over time (Acevedo *et al.*, 1993b).

Another potential bioassay system for proctolin which was not investigated in this study is the isolated *Nephrops* heart, which was observed to increase its frequency of spontaneous contractions after addition of 1×10^{-8} M proctolin to the bathing saline. It is perhaps not surprising that this preparation may be sensitive to proctolin since the highest concentrations of proctolin in *Homarus* lobsters have been found within the pericardium (Schwarz *et al.*, 1984). Similarly, extracts of lobster POs have been found to contain high concentrations of several FMRFamide-like immunoreactive peptides (Trimmer *et al.*, 1987) and crayfish heart has been used recently as a sensitive assay of FMRFamide and related peptides (Mercier *et al.*, 1991). The results presented in this chapter have not only confirmed that a proctolin-like substance is present within the superficial flexor system of both lobster and crayfish, but have also indicated a possible role for the peptide in the regulation of hindgut activity within both crustaceans. Siwicki and Bishop (1986), reported the presence of proctolin-like immunoreactive axons in the intestinal nerves of lobsters but not of crayfish, and it has been suggested recently that hindgut motility may be coordinated by release of proctolin in the lobster and by FMRFamide-related peptides in the crayfish (Mercier *et al.*, 1991). In this study, proctolin increased

the strength and frequency of contractions of both *Nephrops* and *Pacifastacus* (data not shown) hindguts, indicating that receptors for proctolin exist within this tissue in the two species.

2.4.1 Distribution of proctolin in the nervous system and SFM system of *Nephrops* and *Pacifastacus*

In conjunction with the sensitive cockroach bioassay, HPLC has been used in this study to demonstrate the presence of a proctolin-like substance in the SFM system of the macruran decapods *Nephrops norvegicus* and *Pacifastacus leniusculus*. PLB was found in all tissues of the SFM system but on a dry weight basis, extracts of the abdominal ganglia of both species contained higher concentrations of PLB than extracts of other tissues within the SFM system. Recently, the PLB of abdominal ganglia in the crayfish *Pacifastacus leniusclus* has also been quantified in another study (Acevedo *et al.*, 1992). These workers found the abdominal ganglia to contain 10 pmoles of PLB compared to 323 pmoles in the present study. These results are not directly comparable, however, as different separation procedures and methods of detection of proctolin were employed: Acevedo and co-workers eluted proctolin from a SEP-Pak cartridge using 80% methanol and detected the peptide with a locust-leg bioassay (O'Shea and Adams, 1981) whereas in this study ion-pair reverse phase HPLC and a cockroach hindgut bioassay were employed (see Methods for more details).

In lobster and crayfish SFMs, lateral fibres were found to contain considerably greater concentrations of proctolin than medial fibres (Table 2.1), indicating that in both species, the majority of proctolin in the SFMs is located in the terminals of the SFM nerve innervating the lateral fibres. Given the differences in distribution of muscle fibre types in the SFMs of lobster and crayfish, these results do not support the theory that proctolinergic axons may be selectively connected to specific fibre types in these muscles as has been demonstrated for a subset of proctolin-containing neurones innervating visceral

and bodywall muscle fibres in *Drosophila* larvae (Anderson *et al.*, 1988). If this were the case, we would expect the levels of proctolin in medial and lateral muscle bundles of *Pacifastacus* to be similar, reflecting the mixed population of fibres in these bundles. On the other hand, because of the segregation of fibre types in the medial and lateral muscle bundles in *Nephrops*, we would expect to see high levels of proctolin in one muscle bundle and little or no proctolin in the other bundle, the relative levels of proctolin in each bundle being dependent on which fibre type was selectively connected to proctolinergic axons.

Clearly this is not the case, and the considerably higher levels of proctolin in the lateral muscles of both *Nephrops* and *Pacifastacus* suggests a similarity in the proctolinergic innervation of these muscles which seems to be related to position across the muscle and not in fact related to muscle fibre type. The significance of these results is discussed in Chapter 4 in relation to data obtained from measures of contractile performance.

In an earlier study (Bishop *et al.*, 1987) proctolin-like immunoreactivity was reported in axon terminals across the whole superficial flexor muscle in the crayfish *Procambarus clarkii* which does not fit with the position-dependence of proctolinergic innervation suggested by the results of this study. One obvious way in which to reconcile these different results is to apply immunocytochemical methods to the lobster SFM system. Attempts were made to do this, both with frozen sections and whole mounts of abdominal nerve cord and SFM, using a sample of polyclonal antibodies kindly donated by Professor B. Mulloney of Davis University, California and successfully used in his laboratory. A number of variations of standard protocols were followed with the proctolin antibodies using both fluorescent and HRP secondary antibodies. In all cases however, it proved impossible to visualise proctolin. Subsequent ELIZA assays which were performed indicated that proctolin was not binding to the antisera, suggesting that the antisera had been degraded, perhaps in transit. Time limitations

prevented the subsequent production of another proctolin antisera.

2.4.2 Involvement of second messengers in proctolin effects

It has been suggested in this study that cAMP may be involved in mediating the slow component of the proctolin-induced contraction in cockroach hindgut to activate calcium channels. Similarly, in the control of vertebrate smooth muscle, slow tonic contractions are maintained by the opening of receptor-operated calcium channels whereas phasic contractions are produced by the opening of fast, voltage-sensitive, calcium channels (Hoyle, 1983).

However, the involvement of cAMP is not straightforward as demonstrated by the results of Wright *et al.* (1986). In their experiments, proctolin exerted a multiphasic effect on *Leucophaea maderae* hindgut adenylate cyclase activity in that adenylate cyclase activity was increased by 50% and 700% above the basal level in the presence of 1×10^{-11} M proctolin and 1×10^{-10} M proctolin respectively. Furthermore, activity fell to 50% in 1×10^{-10} M proctolin only to peak again to 700% above basal activity with 1×10^{-9} M proctolin. Direct antagonism of adenylate cyclase would inhibit cAMP formation and so provide a clearer picture of the possible involvement of cAMP in mediating the effects of proctolin, but, so far, there are no antagonists of adenylate cyclase available. The presence of a specific proctolin receptor in which calcium ions may act as the second messenger has been suggested from studies on locust hindgut but the receptor does not appear to be linked to adenylate cyclase in this tissue and calcium ions have been proposed to be the second messenger mediating proctolins contractile effect (Banner *et al.*, 1987a). More recently, specific proctolin-activated, voltage sensitive calcium channels have been suggested to mediate contraction in locust oviduct (Lange *et al.*, 1987) and crayfish SFM (Bishop *et al.*, 1991). However, whereas proctolin alone is sufficient to produce a contraction in locust oviduct, crayfish SFM requires membrane depolarisation for proctolin to exert its influence. This phenomenon

may be explained by the observation that proctolin can induce a small (3-4 mV) depolarisation of the oviduct muscle membrane (Orchard and Lange, 1986) whereas in crayfish SFM proctolin cannot depolarise the muscle membrane (Bishop *et al.*, 1987). Thus, the ability of proctolin to depolarise muscle membranes seems to be correlated with its ability to evoke contractions.

A possible role for proctolin in the regulation of activity of the lobster hindgut has been suggested in this study. But as of yet, no studies have been carried out on the involvement of other second messengers such as calcium or cAMP in mediating proctolin-induced contractions in the lobster hindgut.

In summary, the ability of proctolin to evoke contractions in a cockroach hindgut preparation has been utilised to establish the presence and quantify the concentration of proctolin in tissues of the SFM system of *Nephrops norvegicus* and *Pacifastacus leniusculus*. PLB is detectable in all tissues in both species but exists in highest concentrations in the abdominal ganglia (on a dry weight basis). The lateral SFM fibres of both species contain considerably greater amounts of proctolin than the medial fibres but this preferential innervation of lateral fibres by proctolinergic neurones is not correlated to fibre type distribution. Instead, proctolinergic innervation appears to be position-dependent.

Table 2.1

The proctolin content of tissues associated with the SFM system of *Nephrops norvegicus* and *Pacifasticus leniusculus* as determined from proctolin-like bioactivity.

a. Results are expressed as picomoles per tissue.

b. Results are expressed as picomoles per mg of freeze dried tissue.

n = number of replicates.

	Nephrops		*Pacifastacus*	
	Proctolin/ tissue (a)	Proctolin/mg dry tissue (b)	Proctolin/ tissue (a)	Proctolin/mg dry tissue (b)
Abdominal ganglion	727 (n=2)	814 (n=2)	323 (n=3)	1697 (n=3)
Sr3 nerve	47 (n=1)	672 (n=1)	47 (n=1)	714 (n=2)
Medial bundle	48 (n=2)	41 (n=2)	74 (n=2)	121 (n=2)
Lateral bundle	209 (n=2)	132 (n=2)	1309 (n=2)	949 (n=2)

Figure 2.1

Diagrammatic representation of a basic high performance liquid chromatography system. After sample injection, sample components are separated on the column as a result of their interaction with the stationary phase (non-polar column packing) and the mobile phase (polar buffers A and B). Separated sample components are detected as they elute from the column according to their absorbance at a specific wavelength and the results are integrated and displayed as a series of peaks. Unknown components in samples of tissue extracts can be identified by comparing their retention time with that of known components analysed under identical conditions, or by the use of an internal standard.

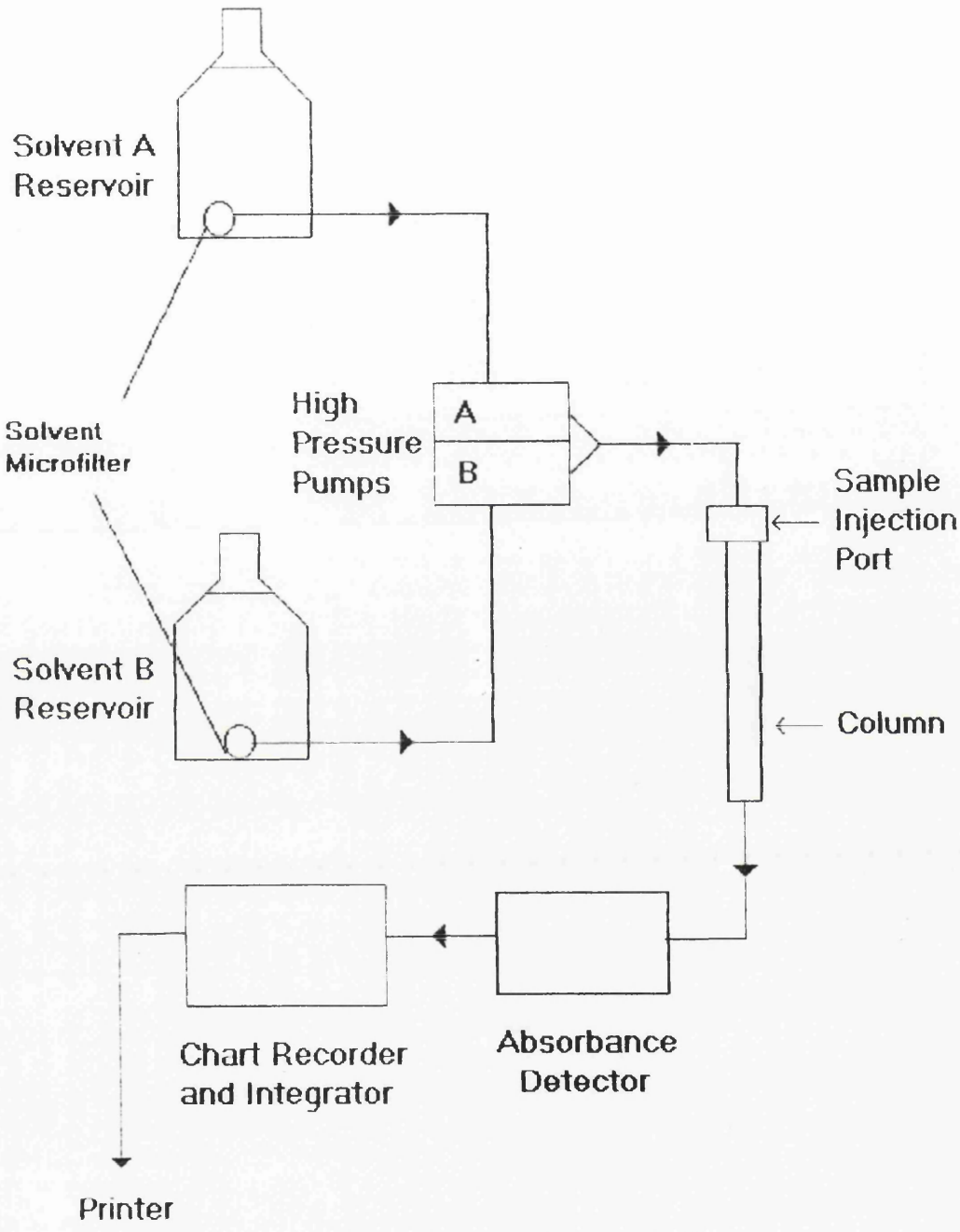

Solvent A
Reservoir
Solvent
Microfilter
High
Pressure
Pumps
A
B
Sample
Injection
Port
Solvent B
Reservoir
Column
Chart Recorder
and Integrator
Absorbance
Detector
Printer

Figure 2.2

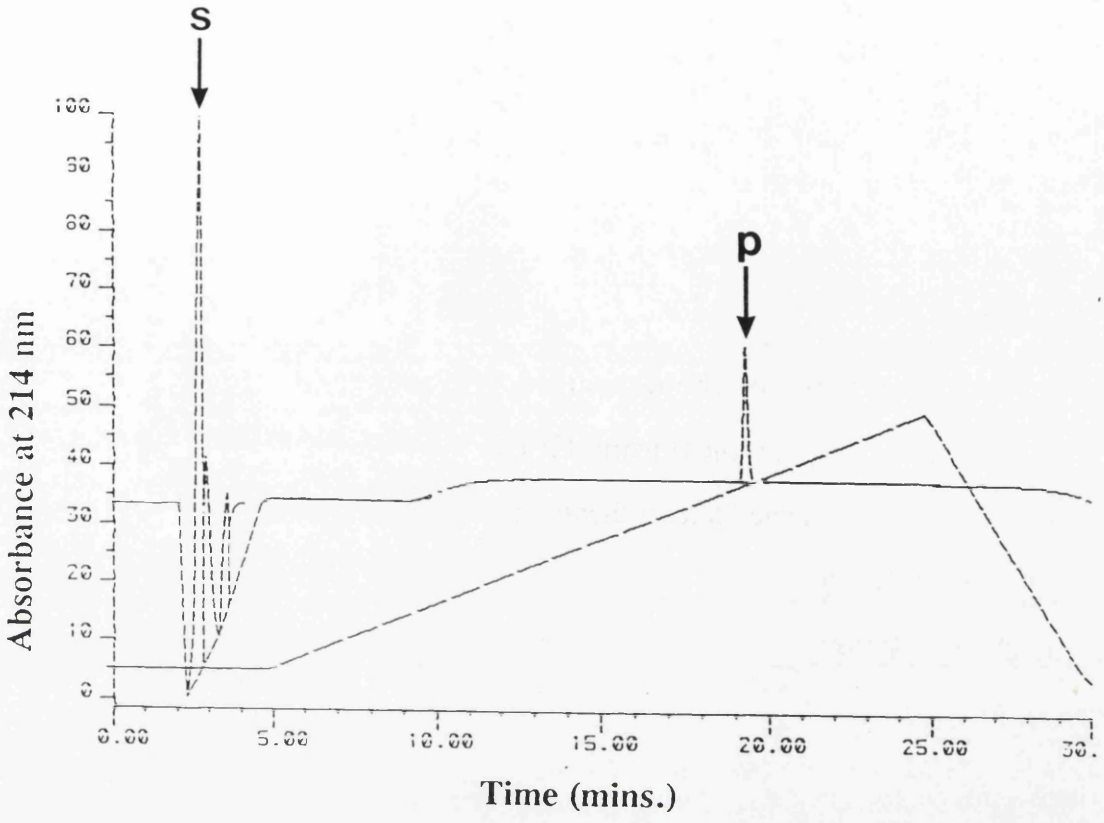
Computerised trace of the detection of a standard of pure synthetic proctolin (equal to 2 nmoles) using HPLC. Proctolin elutes at 19.3 minutes (37% acetonitrile - dashed lines indicate increasing acetonitrile gradient).

s = solvent front

p = proctolin peak

Figure 2.3

HPLC of *Nephrops* medial SFM extract. The amount of proctolin present in the extract is too small to be detected as a peak (p). However, proctolin-like bioactivity in the appropriate eluate fraction can be detected and quantified using a cockroach hindgut bioassay.


S
p
Absorbance at 214 nm
0.00
5.00
10.00
15.00
20.00
25.00
30.
Time (mins.)

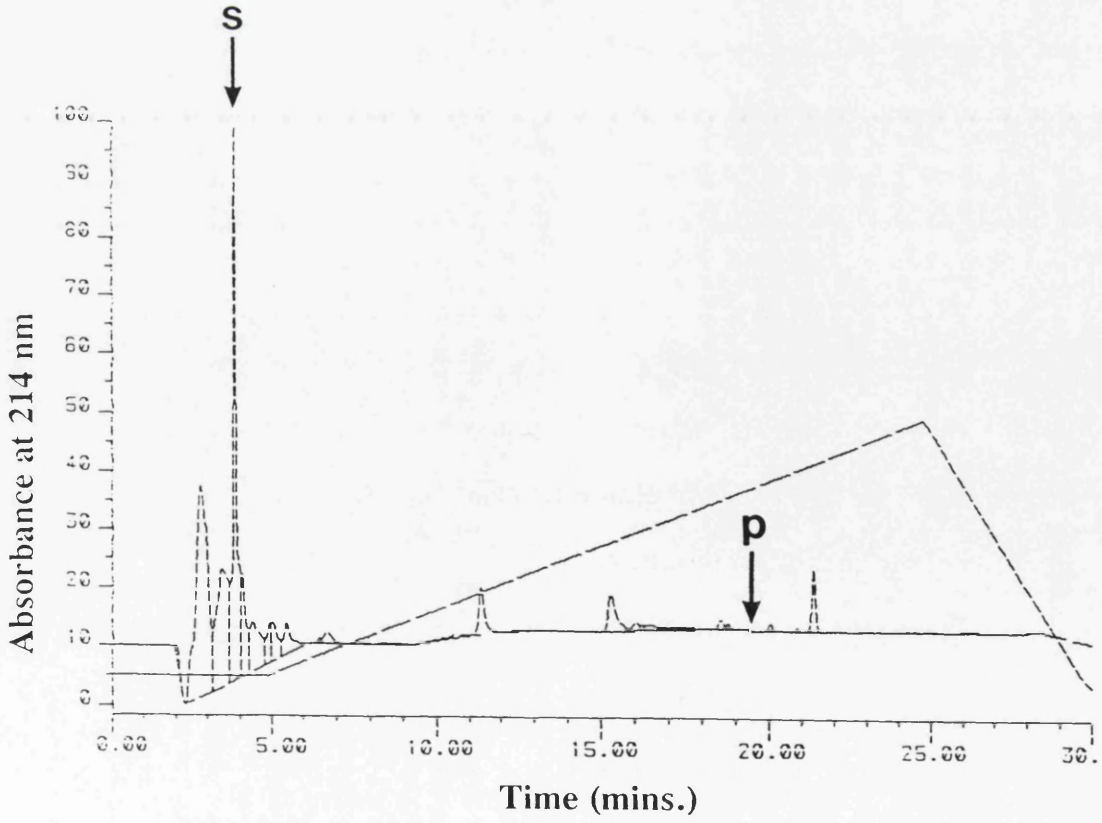

S
p
Absorbance at 214 nm
0.00
5.00
10.00
15.00
20.00
25.00
30.
Time (mins.)

Figure 2.4

Bioassay apparatus used to record cockroach hindgut contractions. A simple overflow system was used in which oxygenated saline was delivered to the bath via a tube embedded in a rubber stopper. Hindgut contractions were measured by a sensitive strain gauge.

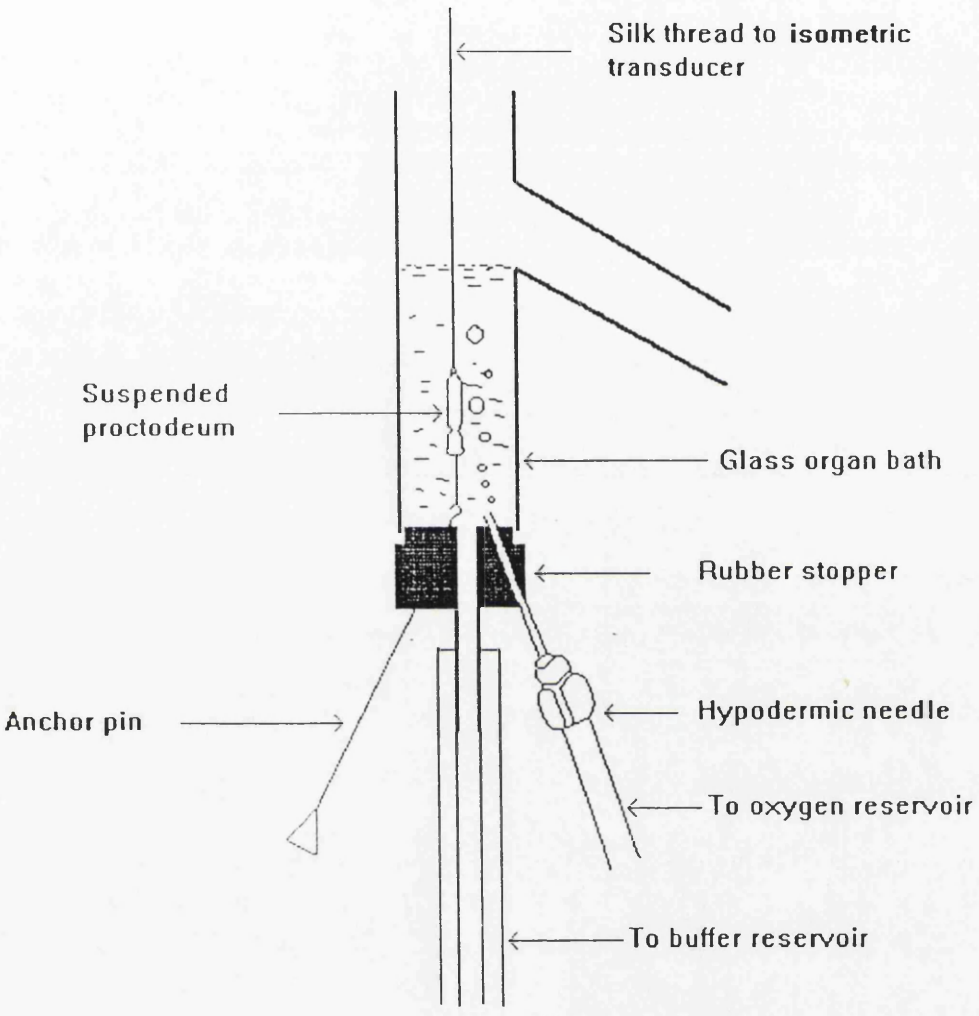

Silk thread to isometric transducer
Suspended proctodeum
Glass organ bath
Rubber stopper
Anchor pin
Hypodermic needle
To oxygen reservoir
To buffer reservoir

Figure 2.5

Dose-dependent effect of proctolin on contractility in the isolated hindgut (filled circle indicates point of addition of peptide).

a. 3×10^{-9} M proctolin.

b. 7×10^{-9} M proctolin.

c. 1×10^{-8} M proctolin.

Scale bar = 1 mN and 1 minute.

(1 mN = 100 mg)

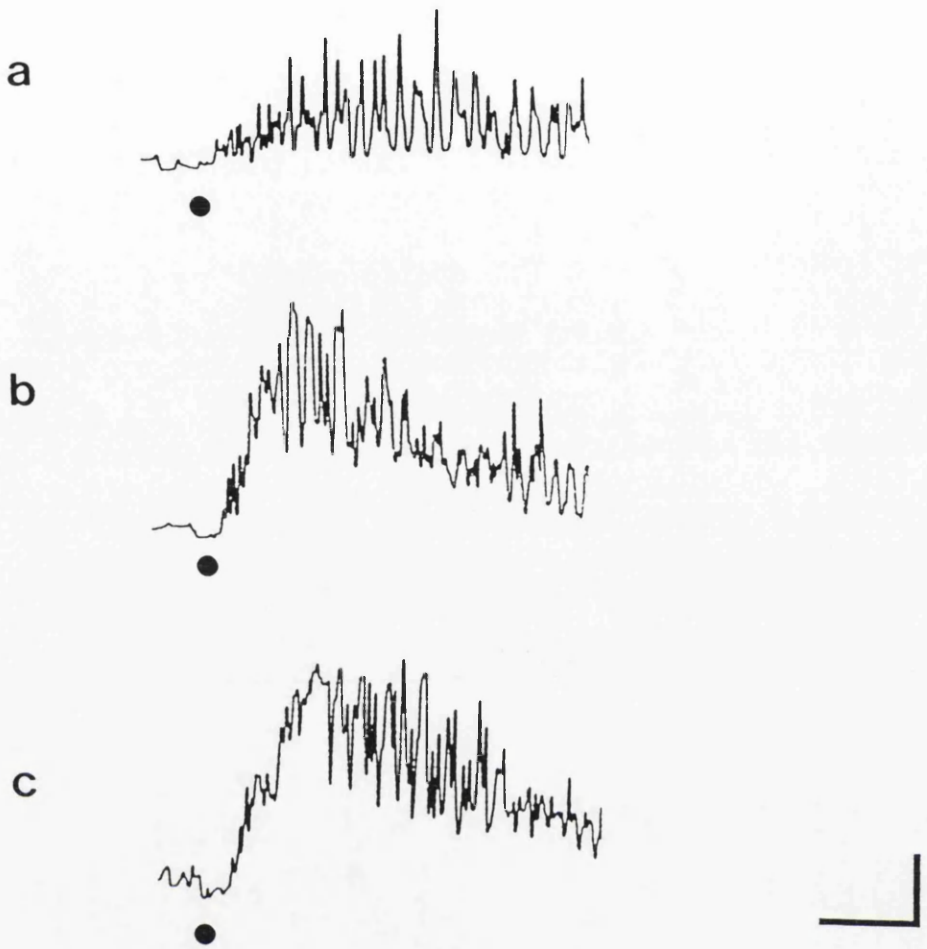
Figure 2.6

Method used to construct proctolin dose response curves.

For each standard dose of proctolin, the mean increase in baseline tension (**a**), was measured (**b**), 1.5 minutes after addition of proctolin to the organ bath (Filled circles - addition of proctolin to the bath, open circles - saline wash).

Scale as in Fig. 2.5.

Dose-Dependent Response Of Cockroach Hindgut To Proctolin.



Construction Of Proctolin Dose-Response Curve.

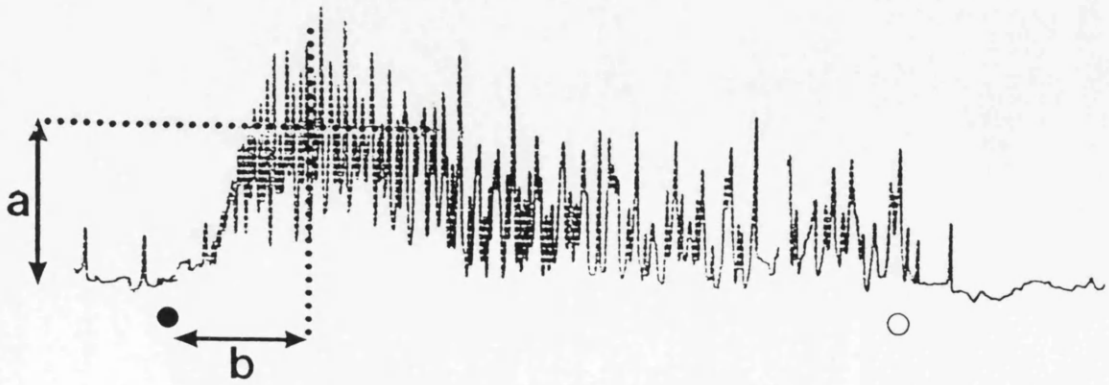
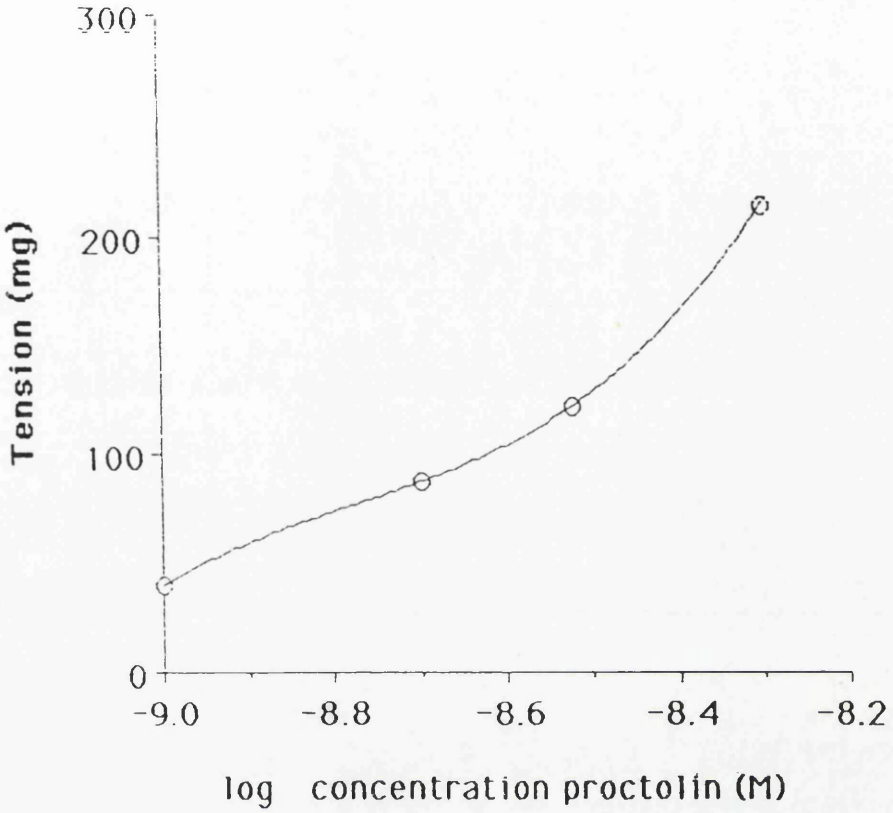


Figure 2.7

Typical dose response curve produced from a single experiment. This curve was used to quantify the proctolin-like bioactivity in tissue extracts shown in Figure 2.11 (a-d).

Figure 2.8

Dose response curve for proctolin-induced contraction of the cockroach hindgut *in vitro*. Each point is the mean of at least 5 replicates (+/- S.E.M).


300
200
100
0
Tension (mg)
-9.0
-8.8
-8.6
-8.4
-8.2
log concentration proctolin (M)

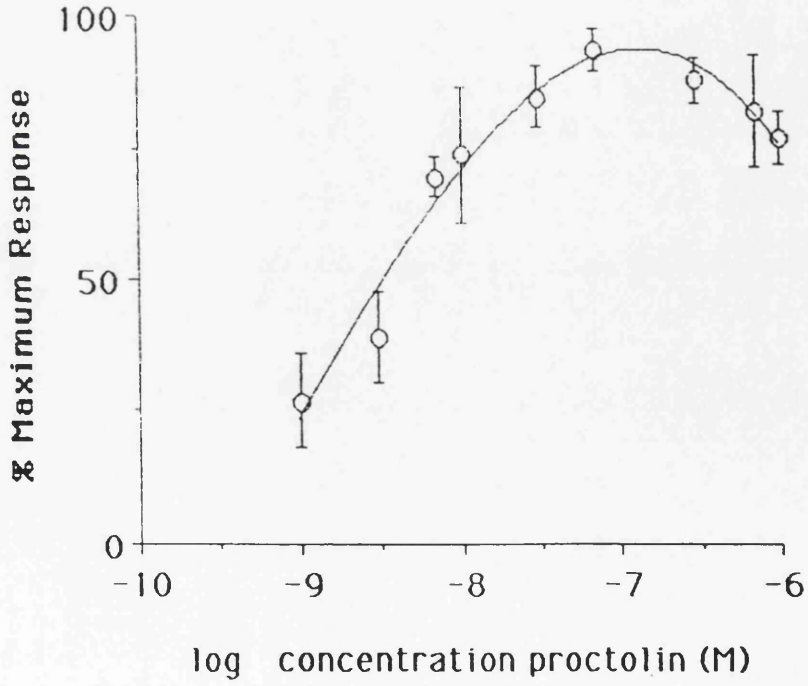

100
50
0
% Maximum Response
-10
-9
-8
-7
-6
log concentration proctolin (M)

Figure 2.9

a-f. The variability in response of a number of individual cockroach hindguts to a standard dose of 10^{-8} M proctolin.

Scale bar = 1 mN, 1 minute. Arrow indicates point of addition of proctolin

Variability Of Cockroach Hindgut Responses To Proctolin.

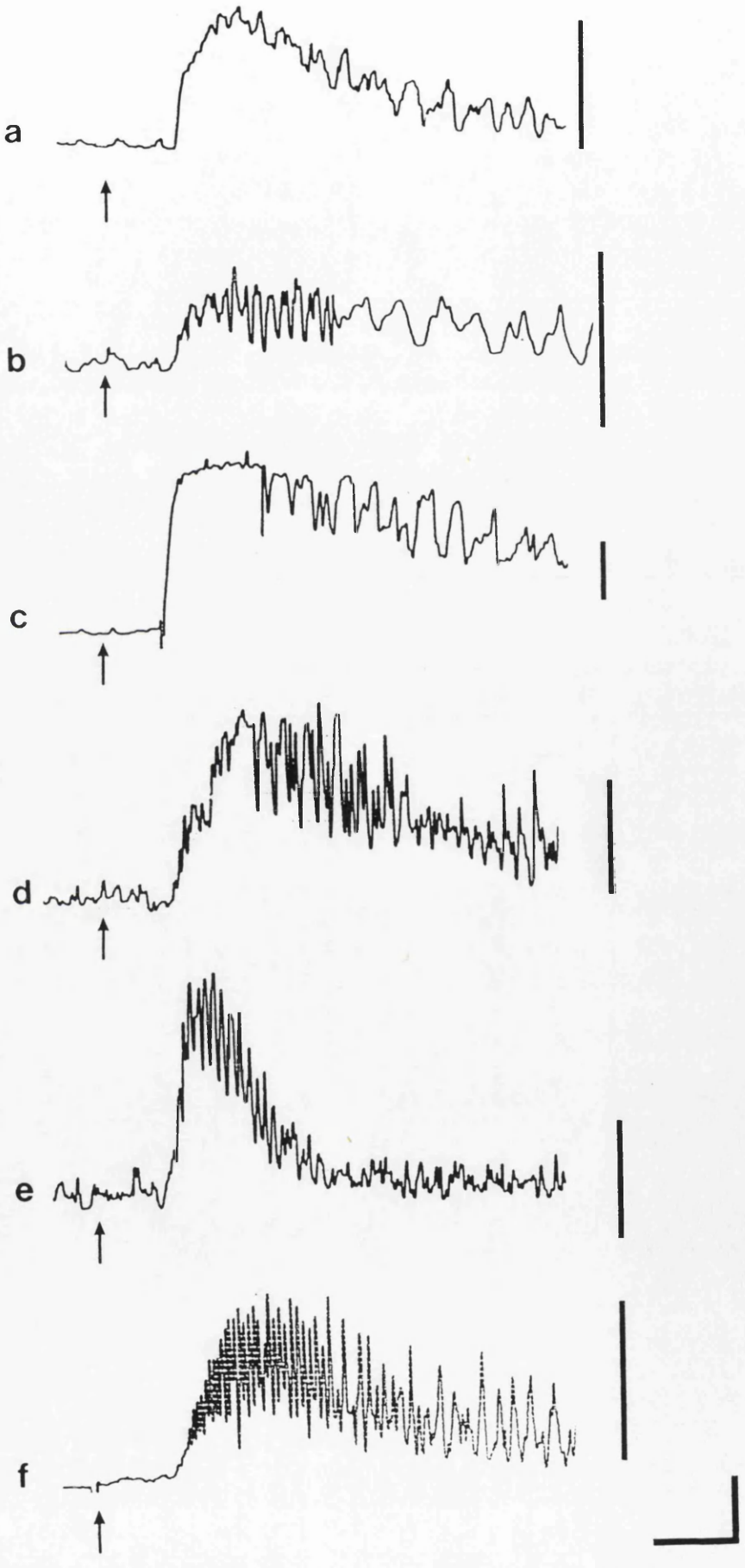


Figure 2.10

Proctolin-like bioactivity contained in a number of *Nephrops* tissue extracts. Traces are of hindgut responses to extracts, and to proctolin standards. Estimated amounts of proctolin derived from the calibration curve constructed for each experiment are indicated in brackets.

a. Pericardial organ (1855 pmoles proctolin/organ).

b. Proctolin standard (1×10^{-8} M proctolin).

c. Proctolin standard (5×10^{-9} M proctolin).

d. Eyestalk (1297 pmoles proctolin/organ).

e. abdominal ganglia (652 pmoles proctolin/organ).

f. thoracic ganglia (675 pmoles proctolin/organ).

a. and **b.** are from one experiment, **c.** to **f.** are from another experiment.

Scale bar = 1 mN, 1 minute. Arrows indicate point of addition of proctolin or purified extract to the bath.

Proctolin Bioactivity Of *Nephrops* Tissue Extracts.

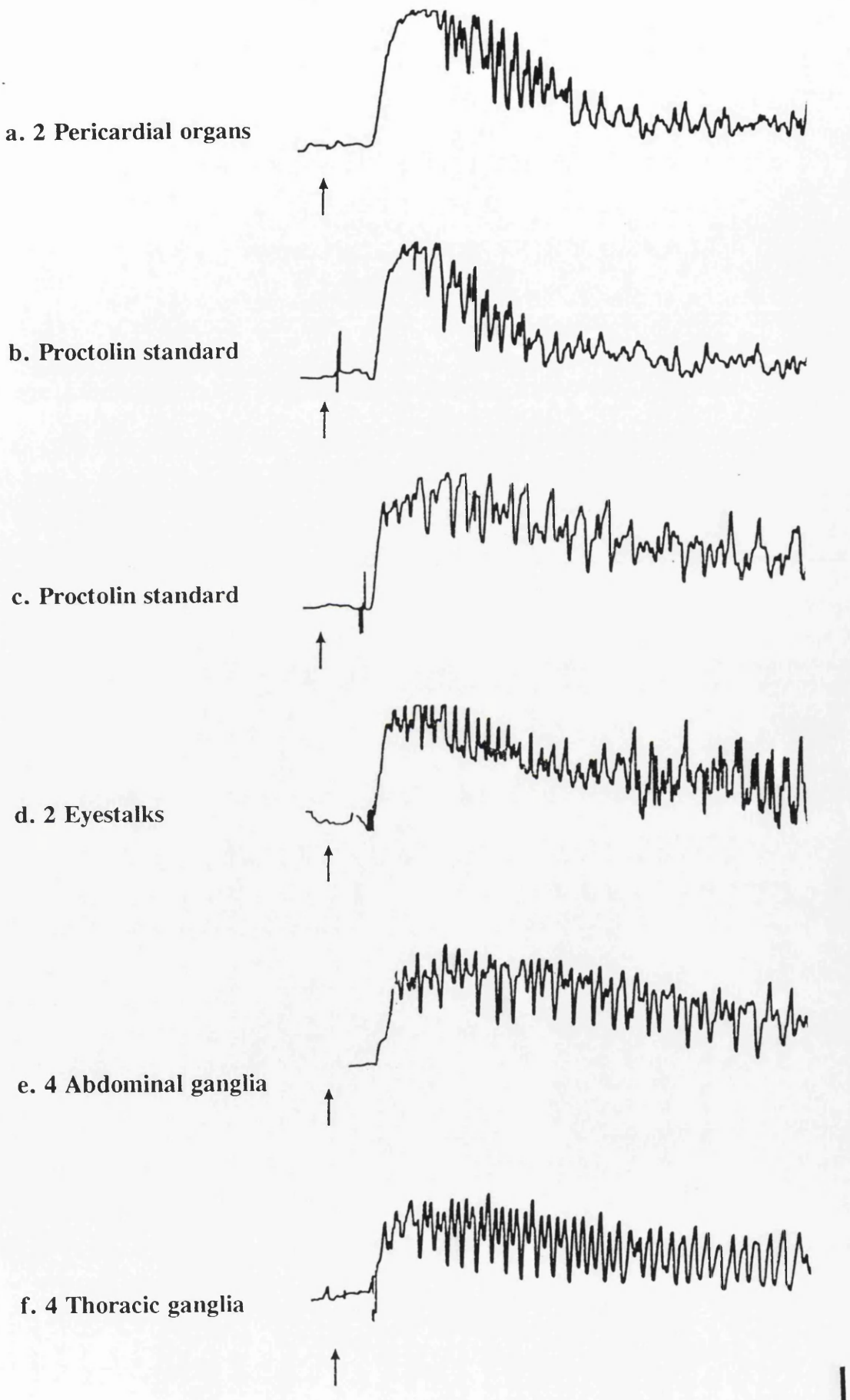


Figure 2.11

Proctolin-like bioactivity of *Nephrops* SFM system tissue extracts. Traces are of cockroach hindgut responses to proctolin standards. Estimated amounts of proctolin derived from the calibration curve constructed for each experiment are indicated in brackets.

a. 7 Sr3 nerves (47 pmoles per tissue).

b. Proctolin standard (3×10^{-10} M proctolin in bath).

c. Proctolin standard (1×10^{-8} M proctolin in bath).

d. 4 Abdominal ganglia (689 pmoles per ganglion).

e. Proctolin standard (3×10^{-9} M proctolin in bath).

f. Lateral fibres of 7 SF muscles (186 pmoles proctolin per bundle).

g. Medial fibres of 7 SF muscles (52 pmoles proctolin per bundle)..

a. to **d.** are from one experiment, **e.** to **g.** are from another experiment. Scale bar = 1 mN, 1 minute. Arrows indicate point of addition of proctolin or purified extract to the bath.

Proctolin Bioactivity Of *Nephrops* SFM System Tissue Extracts.

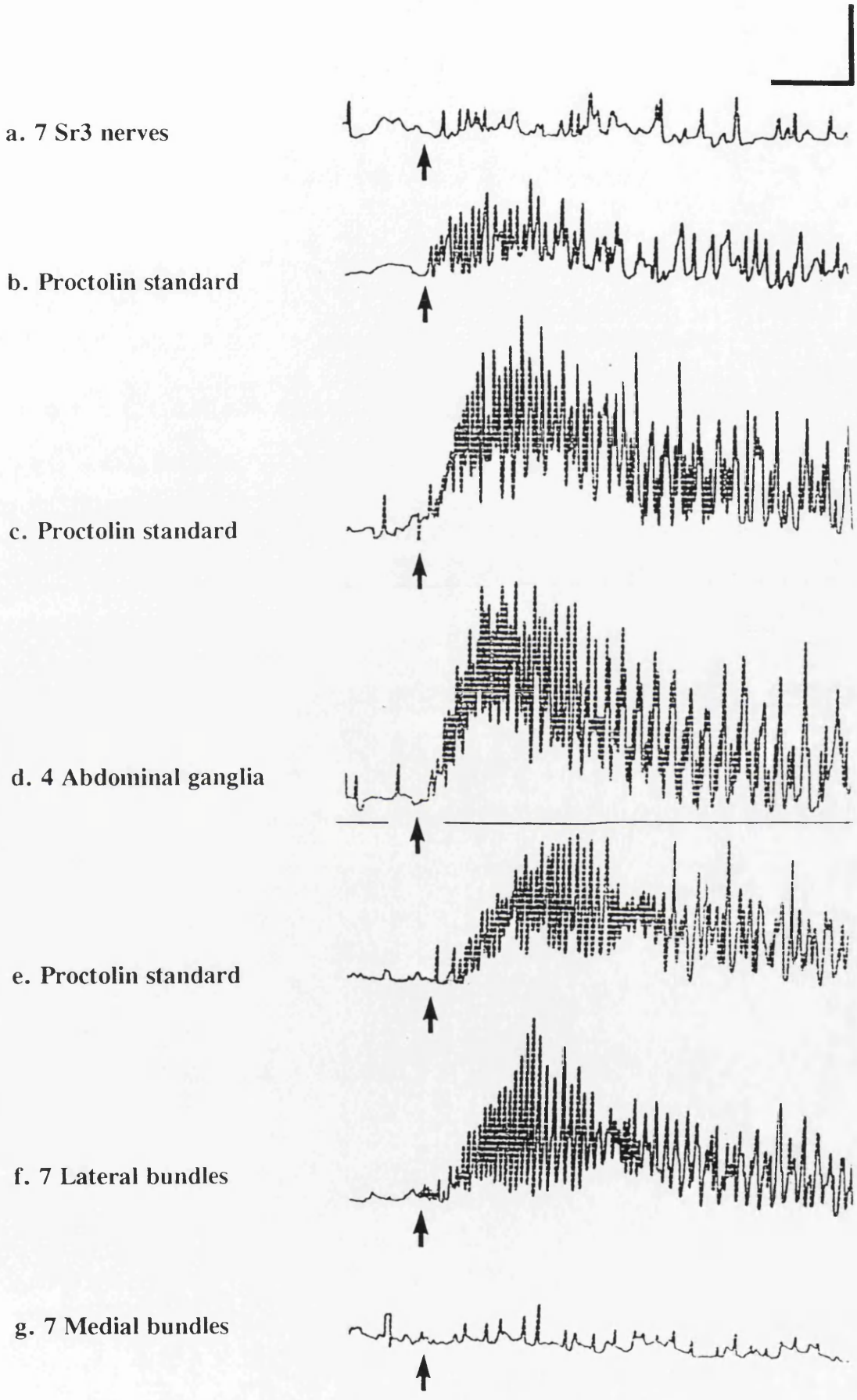


Figure 2.12

Proctolin-like bioactivity in purified *Pacifastacus* SF system tissue extracts. a-f are all from one experiment. Traces are of cockroach hindgut responses to proctolin standards. Estimated amounts of proctolin derived from the calibration curve constructed for each experiment are indicated in brackets.

a. 7 SF nerves (47 pmoles per tissue).

b. Proctolin standard (3×10^{-10} M proctolin in the bath).

c. 4 Abdominal ganglia (346 pmoles proctolin per ganglion).

d. Proctolin standard (4×10^{-8} M proctolin in the bath).

e. Lateral fibres of 7 SF muscles (1182 pmoles per bundle)..

f. Medial fibres of 7 SF muscles (68 pmoles per bundle).

Scale bar = 1 mN, 1 minute.

Proctolin Bioactivity of *Pacifastacus* tissue extracts

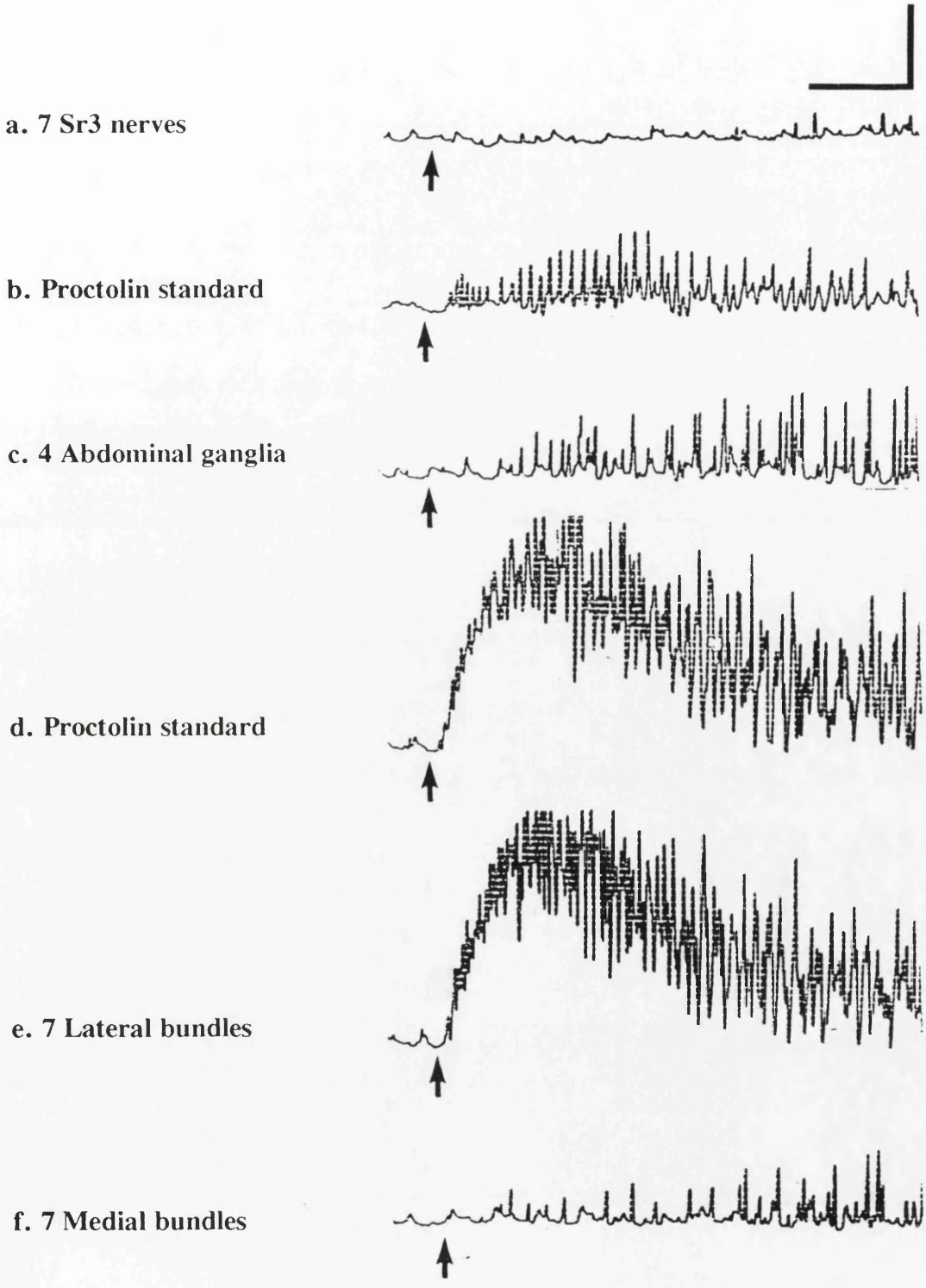


Figure 2.13

a. Addition of 1 x 10^{-5} M forskolin to a cockroach hindgut.

b. Addition of 1 x 10^{-9} M proctolin to the same hindgut (no saline wash between a and b).

c. Gut allowed to relax after saline wash and then exposed to 1 x 10^{-7} M proctolin.

d. Contractile response evoked by 1 x 10^{-4} M forskolin (no wash between c and d).

Scale bars = 1 mN, 1 minute (note change of timescale in d at *).

Effect Of Forskolin On Cockroach Hindgut.

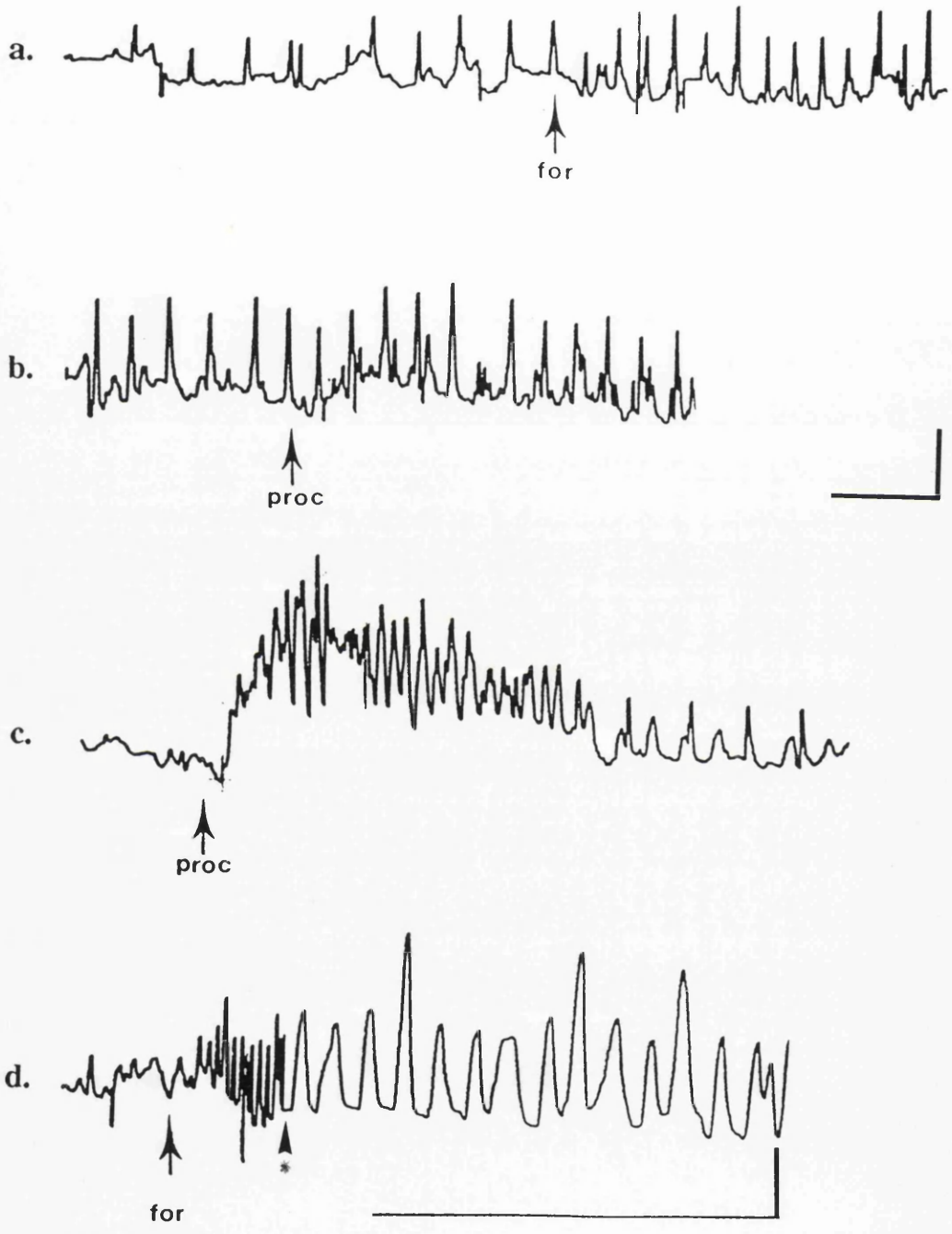


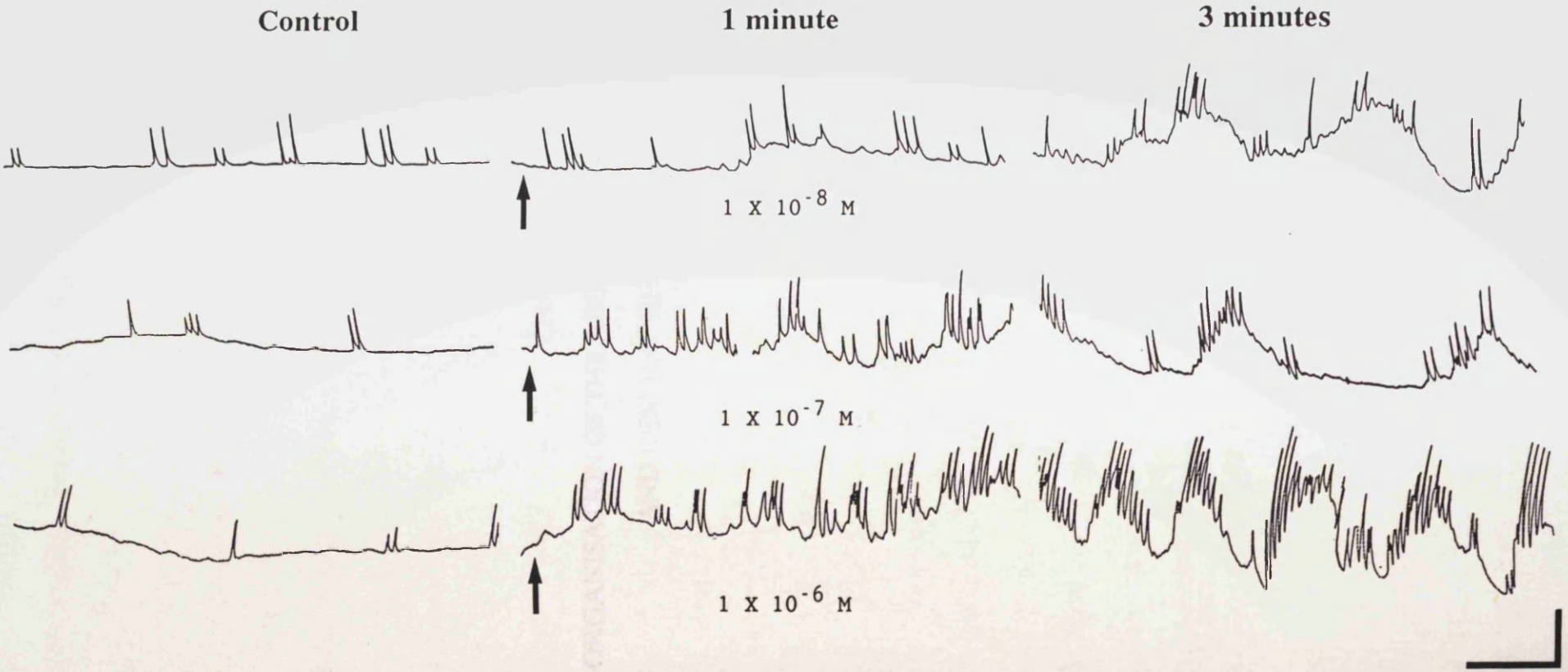
Figure 2.14

Proctolin-induced contraction of the isolated hindgut of the Norway lobster. The three panels show the contractility of the hindgut in the control situation (in normal saline), within one minute of addition of proctolin and within three minutes of addition of proctolin.

Scale bar = 1 mN, 5 secs.

The arrow indicates the point of addition of standard doses of proctolin.

Nephrops Hindgut Bioassay



CHAPTER 3

ORGANISATION OF THE ABDOMINAL POSTURAL SYSTEM AND CENTRAL EFFECTS OF PROCTOLIN

3.1 INTRODUCTION

3.1.1 The abdominal postural system

The abdominal positioning system of decapod crustaceans provides a good model system in which to investigate the neural circuitry involved in the control of a simple motor behaviour.

In both lobsters (*Homarus, Nephrops*) and crayfish, maintenance of posture through coordination of the abdominal segments is fundamental to the proper expression of many complex patterns of behaviour e.g. forward or backward walking, behaviours associated with reproduction such as egg extrusion, egg aeration (in females) and brooding, escape responses, defence and many others. These complex behaviours are the result of the combined execution of a number of basic behaviours which in themselves have been studied extensively. In particular, three motor behaviours associated with the abdomen have been the subject of much research. These are: (i) rhythmic beating of the paired abdominal appendages (the swimmerets), (ii) rapid tailflips that occur during backward swimming and are essential in the escape response and (iii) maintenance of abdominal posture.

The abdomen is organised into six segments (Fig. 3.1A) whose movements are coordinated by the action of four sets of muscles in response to motor patterns generated by each of the six ganglia in the ventral nerve cord. The muscles involved in generating the rapid flexion (powerstroke) and extension (returnstroke) of the abdomen seen during backward swimming and escape tail flips are the fast extensor (FE) and fast flexor (FF) muscles (Kennedy and Takeda, 1965a; Wine and Krasne, 1982). These are phasic, deep muscles which occupy most of the central core of the abdomen (Fig. 3.1B). The superficial slow extensor and flexor muscles (SEM's and SFM's) (Fig. 3.1B) are thin, dorsal and ventral sheets of muscle which span the first five abdominal segments and are active in the control of posture (Pilgrim and Wiersma, 1963; Kennedy and Takeda, 1965).

3.1.2 The neuronal control of abdominal posture

Within decapod crustaceans, the motor patterns controlling abdominal postural movements represent the coordinated and integrated activity of sensory, interneuronal and motor neuronal elements within the postural system.

Sensory Elements. Reflex activation of the postural system occurs as a result of activating different sensory systems: the visual system (Wiersma, 1966), the vestibular system (Knox and Neil, 1991), mechanosensory hairs over the body (Goodall *et al.*, 1990) and proprioceptors (Sukhdeo and Page, 1992). The mechanisms underlying proprioceptive control have been studied most extensively (for review see Fields, 1976; Bush and Laverack, 1982).

The proprioceptors which monitor abdominal position by responding to abdominal flexion are the muscle receptor organs (MROs). Each MRO consists of a pair of specialised receptor muscles (lying parallel to the abdominal extensor muscles) and innervated by separate sensory neurones. The lateral tonic MRO responds to extension of the body, and initiates reflex activation of the SFM's (Wiersma, 1953; Fields, 1976).

Recently, Sukhdeo and Page (1992) have demonstrated in lobsters that this MRO reflex exhibits a state-dependent 'reflex reversal' in which stretch activation can elicit reflex extension, accompanied by inhibition of the SFM, rather than reflex flexion. This switching occurs in such a way that a centrally generated motor pattern is accompanied by an assistance reflex from the MRO, rather than the resistance reflex which occurs normally. This illustrates how sensory inputs may be gated or modified by the activity of central networks.

Central elements. To date, it is not known which factors are responsible for switching motor patterns from one type to another, however, interneurones known as 'command elements' are thought to be involved. Direct stimulation of these cells can generate specific patterns of activity in the postural efferents and therefore cause the animal to assume specific postural stances (Evoy and Kennedy, 1967; Kennedy *et al.*, 1967; Bowerman and Larimer, 1974a). These

evoked patterns of activity are similar to those recorded from intact animals when subjected to sensory stimulation (Larimer and Eggleston, 1971; Sokolove, 1973; Page 1975a). Dye-filling and microelectrode stimulation of these interneurones has revealed that they can be grouped according to whether they are flexion-producing, extension-producing or inhibitory cells (Larimer 1988). However, the current interpretation of Larimer's extensive experiments is that a 'command system' exists in the form of a network of intersegmentally connected interneurones. The motor patterns underlying different abdominal positions are therefore achieved by activation of different subsets of the interneurone pool (Murphy *et al.*,, 1989). Heitler (1986) has shown that different central pathways may be activated depending on how these motor patterns are initiated (either by electrical stimulation of command interneurones or by spontaneous central activity). This may explain why some interneurones which are capable of producing complete motor patterns when stimulated are not activated during spontaneous motor neurone activity (Murphy *et al.*,, 1989).

The motor system. In crayfish and lobster, both sets of postural muscles in each segment are innervated by six motor neurones (f1-f6); five are excitatory, the other is an inhibitor (Kennedy and Takeda, 1965b; Wine *et al.*, 1974; Thompson and Page, 1982). The motor neurones innervating the SFM's have been numbered sequentially on the basis of axon diameter and spike amplitude in motor roots, the smallest being f1 and the largest f6 (Kennedy and Takeda, 1965b; Wine *et al.*, 1974; Thompson and Page, 1982). The inhibitor is numbered f5. The motor neurones can also be distinguished according to their levels of spontaneous activity (Kennedy *et al.*,, 1965; Sokolove and Tatton, 1975).

3.1.3 The neuromodulatory control of abdominal posture

It is known that a variety of amines and peptides can influence and modulate the output of neural networks within many species of crustacea (Marder, 1984; Hooper and Marder, 1987; Heinzel and Selverston, 1985; Mulloney *et al.*, 1987) including the one which controls abdominal posture.

The opposing modulatory effects of the biogenic amines, octopamine and serotonin on posture have been well documented (Harris-Warrick and Kravitz, 1984; Kravitz *et al.*, 1985). Injection of the amines into the circulation of freely moving lobsters causes them to assume particular stances; serotonin causes the animals to assume a rigid fully flexed, aggressive position while octopamine results in extended, submissive-type positions. The cellular mechanisms involved in the modulation of posture by serotonin and octopamine have been investigated by these workers who were able to show differences in the responses of the smaller and larger units to perfusion of these amines. However, their analysis was not sufficiently detailed to permit them to discriminate between (i) the tonic motor neurones f1 and f2, and (ii) the tonic-phasic motor neurones f3 and f4 (Harris-Warrick and Kravitz, 1984).

Several lines of evidence suggest that the neuropeptide proctolin can modulate the output of individual neurones or entire networks through the synaptic connections of these neurones. Proctolin has been found in many central neurones within lobster and crayfish (Siwicki and Bishop, 1986). For example, the existence of the peptide in fibres leading onto the stomatogastric ganglion (but not in the ganglion itself) is in accordance with the ability of proctolin to initiate strong pyloric and gastric rhythms from the stomatogastric ganglions of lobsters and crabs (Marder and Hooper, 1985, Marder *et al.*, 1986). Our understanding of the action of proctolin on the pyloric rhythm has been extended by establishing that proctolin acts directly on only two of the neurones in the network, the rest being unresponsive (Hooper and Marder, 1987). Thus, it is the synaptic connectivity between proctolin-sensitive and proctolin-insensitive neurones that determines the network's overall response to proctolin.

3.1.4 Proctolin within the abdominal superficial flexor system

In crayfish, proctolin coexists with L-glutamate in three of the six identified motor neurones of the abdominal superficial flexor system (Bishop *et*

al., 1984). This system is a particularly appropriate one in which to examine the effects of this neuromodulator at both its potential sites of action: at the level of pre-motor inputs onto individual motor neurones and at the level of the neuromuscular synapses. However, in crayfish most work has focussed only on the latter (Bishop *et al.,*, 1984; 1987; 1990; 1991).

Previous work has indicated the presence of proctolin within the lobster SFM system (Schwarz, 1984) and this has been confirmed in this study by the use of a sensitive bioassay (Chapter 2).

Very little is known about the actions of proctolin at a central level. The work presented in this chapter has attempted to measure the effect of proctolin on central premotor modulation by comparing the spontaneous firing patterns and the coupling relationships of individual slow flexor motor neurones before and after application of the peptide.

The neuroanatomy of crayfish abdominal SFM motor neurones is already known (Wine *et al.,*, 1974). However, in order to make meaningful comparisons of the effect of proctolin on firing patterns of superficial flexor motor neurones in crayfish and lobster it has been necessary to study the neuroanatomy of these motor neurones in *Nephrops*. These results are also reported in this chapter.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Lobsters (*Nephrops norvegicus* L.) of carapace length 6-9.5 cm, obtained from the University Marine Station, Millport, Isle of Cumbrae, were maintained in tanks supplied with aerated circulating sea water at around 10-12° C and were fed on whitebait.

Crayfish (*Pacifastacus leniusculus* L.) of carapace length 5 - 7.5 cm, were obtained commercially. They were kept in aerated tanks of copper-free tapwater 14° C and fed on fish meal.

The majority of electrophysiological studies were carried out on male animals.

3.2.2 Physiological saline

The recipe for lobster and crayfish saline is given in Table 3.1.

The saline bathing the nerve cord was introduced into the dish via plastic tubing by a peristaltic pump (Autoclyde). Under normal conditions the flow rate was set at 1.3 ml/min. The set up also permitted the replacement of saline by test solutions of proctolin or other drugs.

3.2.3 The isolated nerve cord preparation

This preparation was used to investigate the central effects of proctolin. Animals were killed by decapitation and the abdomen separated at its junction with the thorax. Lateral cuts were made down each side of the abdomen and the dorsal carapace dissected free and discarded, revealing two large deep muscle bundles. These muscles were separated at their point of fusion, directly above the nerve cord and then carefully dissected in turn to prevent damage to the underlying nerve cord.

Figure 3.2A shows a diagramatic representation of the dissected preparation at this stage. The first and second roots respectively, were snipped at the points indicated by the arrows and eased free. Once this had been done for each hemi-segment, the nerve cord was grasped by forceps at the anterior

end (above the 1st ganglion) and lifted clear of the underlying membrane. Each superficial flexor nerve (Sr3) was then dissected in turn along with a small patch of SFM as indicated by broken lines on the muscle of the top left segment (Fig. 3.2A). These small pieces of flexor muscle were found to be useful in preventing damage to the Sr3s during pinning out of the preparation. Preparations were mounted dorsal side up on a Sylgard-lined petri dish and perfused with fresh saline maintained at a temperature of approximately 14°C by a peltier-effect cooling system.

3.2.4 Extracellular recordings

In order to monitor spontaneous extracellular activity in the isolated abdominal nerve cord, platinum wires were placed in contact with the nerves and insulated from the saline in the bath with Vaseline. The recording apparatus (Fig. 3.3) included a switch box which enabled differential recording or stimulation of up to 8 nerves at any one time. The preamplified signals were fed to a Textronix oscilloscope to monitor activity, a loudspeaker to produce an audio output, a Racal reel to reel FM tape recorder in order to obtain permanent records of activity and also to a Gould digital storage oscilloscope which could be directly connected to a HP compatible plotter (Roland 980) to provide hard copy of recorded activity either during or after an experiment.

3.2.5 Analysis of frequency of firing of individual motor neurones

The frequency of spontaneous activity of individual motor neurones (f1-f6) before and after the application of proctolin and other neuroactive substances was investigated. Sequences of extracellular spontaneous activity recorded from one or more Sr3 nerves were played back from FM tape to a multi-channel analogue to digital converter (Cambridge Electronic Design (C.E.D) 1401). Various analyses were performed using different software packages (e.g. Spike2, Sigavg).

From each data file collected by the AD conversion, event channels were created for each category of spike, either by conventional methods of 'window

discrimination', or by using an equivalent iterative process (Fig. 3.4). An event was created whenever the rising part of a spike waveform crossed a preset threshold amplitude, illustrated by the position of the cursor over the waveform data. These events were then put into a separate channel. By increasing the threshold amplitude (indicated by level of cursor) a number of different channels could be produced which corresponded to different subsets of the total spike population. These event channels were then successively subtracted from each other by a small program written for the purpose to produce event channels which corresponded to the firing pattern of individual motor neurones. Another small program was written to analyse the different event channels for two variables: mean frequency of firing and mean interspike interval.

3.2.6 Cross-correlation analyses

The aim of these studies was to investigate whether proctolin was able to influence spontaneously induced activity of the six motor neurones innervating each SFM in the Norway lobster, and if so, to investigate whether any of these motor neurones were able to influence others by performing cross-correlation analyses between different pairs of motor neurones, both before and after perfusion of proctolin. Cross correlation analyses were performed by comparing one event channel (the stimulus channel) with another event channel (the response channel). Initially, it was intended to present data on the association between two individual nerve cells as standard cross-correlation histograms as shown in Figure 3.5. However, it was felt that presentation of data in this way was inadequate as it did not allow for any kind of probablistic interpretation of the data. The application of stochastic point process analysis to data was achieved through collaboration with Dr. David Halliday of the Physiology Department, Glasgow University. This yielded estimates of the cross intensity function of a bivariate stationary point process (spike output of two nerve cells) (Brillinger, 1976). The cross intensity function is estimated from the cross-correlation histogram and corrects for the effects of differences in record length

and bin width used to generate different cross-correlation histograms. A square root transformation of the cross intensity function results in estimates which have a known and constant sampling variability (Brillinger, 1976). This allows a confidence interval to be constructed to determine whether values of the estimated cross-intensity function are significantly different from that expected for two independent processes. Values lying outside the 95% confidence band can be taken to indicate a significant correlation.

3.2.7. Template matching.

In some cases, the amplitude of spike sizes of some motor neurones, usually f1 and f2, were too similar to allow identification by amplitude alone. In these instances, a template matching programme was employed which was designed and created by Mark Browning in this laboratory. This system detects individual spikes on the basis of their shape. Sections of analogue data were transferred into Spike 2 and then imported into a database. Variable envelope templates for the different spikes were generated and the spike database was searched for template matches. Result views of the data could be any combination of one or more templates. Figure 3.6 shows one result view. Figure 3.6**A** contains the raw data (both f1 and f2) and Figure 3.6**B** contains one population extracted by template matching.

3.2.8 Cobalt backfilling

The central projections of the six motor neurones contained within the superficial flexor root were visualised by a technique originally developed by Pitman *et al.*,, (1972). Nerve cords were dissected as described in 3.2.3. A vaseline well was constructed next to the superficial nerve to be filled in such a way that the nerve passed through the wall of the well. The well was filled with 0.2M cobalt chloride ($CoCl_2$) and the nerve cut in the $CoCl_2$ solution. The well was then covered with Vaseline to prevent evaporation of the $CoCl_2$. The preparation was placed in a refrigerator (4°C) and left for 36-48 hrs to allow the cobalt to diffuse into the cut end of the nerve. After this period the nerve cord

was rinsed thoroughly in saline and then placed in a solution of ammonium sulphide (8 drops of ammonium sulphide for every 10 mls of saline) for 10 minutes. The ammonium sulphide causes the cobalt to be precipitated out as cobalt sulphide which is a dark brown salt. The cord was then taken through a series of dehydration steps and cleared with methyl salicylate. After this step, cell bodies and associated dendrites were clearly visible and camera lucida drawings were made. Cords were then mounted in Canada Balsam or Histomount and photographed.

3.2.9 GABA Immunocytochemistry

The abdominal nerve cords of Norway lobsters were dissected in saline. The 2nd and 3rd ganglia and their connectives were then fixed with 2% gluteraldehyde in 0.1% sodium cacodylate buffer (pH 7.4) for three hours.This was followed by a period of five hour fixation in Bouin. Post fixation, the tissue was rinsed in 0.05M Tris buffer (pH 7.6) for 15 minutes and then dehydrated in an ascending alcohol series (10 minutes for each step). The tissue was then embedded in paraffin and 10 μM sections cut and dried onto slides. The sections were rehydrated in a descending alcohol series and the wax removed with xylene.

Sections were incubated overnight with rabbit anti-GABA antibodies (Professor Geffard) diluted 1:1000 in PBS containing 1% BSA. After rinsing in PBS the antiserum was visualised by using the peroxidase-antiperoxidase method with diaminobenzidine as chromogen. Finally, sections were washed, dehydrated, cleared and mounted in DPX for viewing.

GABA sections were kindly prepared and stained for me by Dr I. Cournil, C.N.R.S., Archacon.

3.3 RESULTS

3.3.1 Neuroanatomy of superficial flexor motor neurones

Cobalt backfills were performed on the Sr3s of abdominal nerve cords of the lobster *Nephrops norvegicus* and of the crayfish *Pacifastacus leniusculus*. Similar results were obtained for both species. Cobalt backfilling of a single Sr3 (Figs. 3.7, 3.8) reveals six motor neurones located ventrally within the ganglion. Five of the cell bodies are resident in the ganglion anterior to the Sr3: two of the five motor neurones are located contralaterally to the filled root, the other three are ipsilateral (Figs. 3.9A and 3.9B). The remaining motor neurone is always found on the contralateral side of the ganglion posterior to the filled root. Figure 3.9 (C and D) traces the axon as it ascends through the connective from the posterior ganglion. It was found to be impossible to trace the axonal path of any one motor neurone from the Sr3 to the ganglion as the axons became very closely associated within the ganglion and at this point the axon diameter of each motor neurone had tapered to such an extent that association of a single axon with a its cell body was impossible.

The somata of superficial slow flexor motor neurones in lobster (Figs. 3.7, 3.8) are approximately twice the diameter of the homologous neurones in the crayfish (Thompson and Page, 1982). Camera lucida drawings of two crayfish preparations are shown in Figure 3.10. Cobalt backfilling reveals that each soma gives off a thin neurite which ascends up towards the dorsal portion of the ganglion where it gives off dendritic branches. The process or axon then continues posteriorly through the connective and exits via the Sr3.

3.3.2 Identification of superficial flexor motor neurones from their anatomy

In order to study the functional organisation of the flexor motor system it was necessary to identify individual motor neurones in terms of both their anatomical organisation and physiological activity. This has been done by using a number of indirect methods, some of which exploit the pharmacology and anatomy of the system. Attempts have also been made to correlate the

anatomical and physiological properties directly.

The position of the six superficial flexor motor neurones within the ganglia of *Nephrops* and *Pacifastacus* agrees with previous studies (Wine *et al.*,, 1974; Miall and Larimer, 1982; Stretton and Kravitz, 1973; Thompson and Page, 1982). A cross section through a superficial flexor nerve reveals six axons of different diameter (Fig. 3.11). Although previous studies assumed a direct correspondance between soma size and axon diameter, in no case was it directly demonstrated. In this study, direct evidence relating to these properties was obtained for one cell, the peripheral inhibitor, by employing immunostaining for its neurotransmitter, GABA.

3.3.3 Immunocytochemistry of the peripheral inhibitor - f5

A survey of the distribution of GABA-immunoreactivity in the motor roots and connectives of the abdominal nervous system was carried out by staining serial sections taken from the area of the ventral nerve cord lying just posterior to the third root and just anterior to the ganglion. Within the ganglion the following main regions can be recognised (dorsal to ventral): giant fibres (Fig. 3.12 shows medial and lateral giant fibres), four layers of axonal tracts interspersed with three layers of commissures, four different types of neuropil and a ventral layer of somata (Skinner, 1985a,b; Leise *et al.*,, 1986). Anti-GABA antibodies visualised immunoreactive fibres (which are indicated by arrows in Fig. 3.12A) in the neuropil of the 2nd and 3rd ganglia.

The specificity of the antibody was tested by absorption controls in which the antibody was preincubated with the antigen resulting in a subsequent loss of immunological reaction to the nervous tissue. A small amount of the staining present in the ganglionic and connective profiles will be due to cross-reactivity with molecules other than glutamate, aspartate, glycine or taurine none of which cross-react with this anti-GABA antiserum (Seguela *et al.*,, 1984). An examination of the motor roots reveals more specific information about the identity of GABA stained neurones. The second largest of the six axons

contained within the Sr3 (Fig. 3.12B) shows a positive reaction to the GABA antibody. This axon is identified as belonging to the peripheral inhibitor, f5. The main branch of the third root which innervates the fast flexor muscles (FFM) , and is also known to contain one inhibitory motor neurone (Mulloney and Hall, 1990), also shows a single stained profile (Fig. 3.12C).

As previously shown in Figure 3.9, C and D, the axons of the superficial flexor motor neurones travel as a tight group through the connective until just posterior to the midline of the ganglion at which point they disperse laterally before descending ventrally to terminate as cell bodies. Serial sections allowed the position of GABA stained axons (and also the position of the other axons around them) to be followed from the root, along the connective to the ganglion. Figure 3.13 shows the position of GABA-stained axons in the Sr3 (black arrows), and in the branch of the third root which innervates the FFM (white arrows), at the point where the Sr3 root merges with the connective. It can be seen that the position of the stained axons and their coherent group of fibres changes relative to the medial and lateral giant fibres as they travel through the connective (Fig. 3.13 A-E). Within the ganglion, the Sr3 axons lie in approximately the same horizontal plane as the fast flexor (FF) axons, whereas they lie ventral to the FF axons throughout the connective. The ganglionic position of the soma of the Sr3 GABA-stained axon corresponds to the position of the soma designated f5 according to soma size (Fig. 3.9). Thus, in the Sr3, a direct correspondance between soma size and axon diameter seems to exist as the position of the soma of the second largest axon, which stains positively for GABA, corresponds to the position of the second largest cell body visualised from cobalt backfill studies.

3.3.4 Identification of superficial flexor motor neurones from their physiology

Extracellular recordings allowed the individual SFM motor neurones to be identified in isolated nerve-muscle preparations. In this study the use of platinum wire instead of suction electrodes for extracellular recordings (see

Methods 3.2.6) gave a high signal to noise ratio, produced very clean recordings and thus allowed most spikes to be uniquely identified according to spike amplitude. Six classes of spikes could be identified (Figs. 3.14 A and B) and these were labelled f1-f6; f1 being the smallest, f6 the largest, as in other studies (Kennedy and Takeda, 1965b; Wine et al., 1974; Thompson and Page, 1982; Miall and Larimer, 1982). The relative spike heights were found to be consistent from preparation to preparation and this also greatly facilitated identification.

Template matching. In the past, many workers have been unable to discriminate between f1/f2 and f3/f4 using the parameter of spike height alone (Harris-Warrick and Kravitz, 1984). This difficulty was sometimes encountered in this study when attempting to discriminate between f1 and f2, even though good records were obtained. In such cases where spikes were similar in height they nevertheless differed in their shape. In order to exploit this fact a system of template matching was applied (see section 3.2.7 for details). The upper trace in Figure 3.14C shows a typical data set in which f1 and f2 could not be separated by conventional methods. The lower trace shows an expanded view of the waveforms of f1 and f2 and the differences in shape between the spikes can be clearly seen. The waveform data shown in Figure 3.14D on the other hand shows a rare case where the similarity in shape of the two motor neurone spikes goes beyond the resolution of even the template matching system.

Intracellular recording of motor neurones. An attempt was made to conclusively identify the superficial flexor motor neurones f2-f6 by matching intracellular recordings from the cell bodies with extracellular activity recorded from the Sr3 followed by intracellular dye filling of the motor neurone with Lucifer Yellow or some other suitable dye. As the majority of somata in crayfish (Miall and Larimer, 1982) and lobster (Thompson and Page, 1982) are silent, however, individual motor neurones can only be identified by depolarisation/hyperpolarisation of penetrated cell bodies (Fig. 3.15). However, this method proved to be extremely time-consuming with very little return for

effort and was subsequently abandoned.

Identification of f1 and f5. The organisation of the flexor system described in **3.3.1** allowed two of the motor neurones to be identified with greater certainty. Firstly, it was possible to identify the smallest flexor motor neurone, f1 (whose soma lies in the next posterior ganglion), by cutting the connective posterior to the third root and hence selectively eliminating its activity from the extracellular record (Fig. 3.16). Secondly, on some occasions it was possible to identify the flexor inhibitor, f5, by matching its extracellular spikes with inhibitory junctional potentials (IJP's) in the SFM fibres (Fig. 3.17). However, since the IJPs produced by f5 were often recorded as depolarising as opposed to hyperpolarising IJPs, they could not always be distinguished in this way.

The remaining motor neurones, f2, f3, f4 and f6 were identified in extracellular records according to both spike height and rate of spontaneous activity.

3.3.5 Rates of spontaneous activity of different SFM motor neurones in lobster

Different SFM motor neurones can be further identified according to their rate of spontaneous discharge in the isolated preparations. As this discharge rate is dependent on the 'intactness' of the ventral nerve cord (see section 3.3.6), these experiments were carried out under standardised conditions. Spontaneous activity was recorded from the Sr3 of the second or third ganglia, one hour after dissection. Only ventral nerve cords consisting of all six abdominal ganglia were used and these were perfused with saline and maintained at 14°C by a cooling system.

The rates of spontaneous discharge do not include data from preparations for axons which were silent and therefore do not reflect the proportion of experiments in which the different axons were spontaneously active, i.e. f1 and f2 were spontaneously active in approximately 95% of preparations, f3 in 60%, f4 in 40 %, f5 in 15%, and f6 in 5% of preparations.

The following results are summarized in Table 3.2.

f1 and f2. The two smallest tonically active motor neurones, f1 and f2, exhibited the highest rates of impulse discharge. f1 fired at a mean frequency of 11.79 Hz (S.D. +/-6.40) within a range of 3.3 - 26 Hz (n=15). The mean frequency of firing of f2 was 10.96 Hz (S.D. +/-6.85) within a range of 1 - 26 Hz (n=18).

f3 and f4. The discharge rates of the middle sized 'tonic-phasic' motor neurones, f3 and f4, were much more variable than those of the small tonic flexors. The mean frequency of firing of f3 was 4.31 Hz (S.D. +/-3.13) within a range of <1.00 - 10 Hz (n=17). The mean frequency of firing of f4 was 2.19 Hz (S.D. +/-2.31) within a range of <1.00 - 5.36 Hz (n=11). f3 often tended to fire with a steady rate whereas the firing pattern of f4 was often erratic.

The inhibitor, f5, was often silent but when spontaneously active, tended to fire at a regular rate. The mean frequency of firing of f5 was 1.91 Hz (S.D. +/-1.6) within a range of <1.00 - 4.20 Hz (n=6).

f6. The largest, most 'phasically active' motor neurone f6, was normally silent. During the experiments when it was spontaneously active the mean frequency of firing of f6 was 1.39 Hz (S.D. +/-2.56) within a range of <1.00 - 7.36 Hz (n=6). f6 activity was often observed as single, infrequent spikes.

3.3.6 Dependence of motor neurone firing pattern on the integrity of the ventral nerve cord

Abdominal intrasegmental connections in the lobster ventral nerve cord were demonstrated by cutting the abdominal ventral nerve cord connective at different positions and observing the change in the pattern of spontaneous activity recorded from the Sr3 (Fig. 3.18). This figure represents an experiment where the connective was systematically cut in five different places (A - E respectively). Each cut was made anterior or posterior to the exit of the Sr3 as indicated by the arrow positions (Fig. 3.18). Each record consists of two sections: (i) the immediate effect of cutting on the spontaneous activity

recorded from the Sr3 and (ii) the effect on spontaneous activity in the Sr3, thirty seconds after the cut was made. All cuts were associated with immediate transient firing of the largest motor neurone, f6.

A. The first cut was made between the 5th and 6th abdominal ganglia. This produced an increase in the rate of spontaneous activity of two of the three flexor motor neurones which were previously active: f1 and f4. The rate of firing of f3 was decreased.

B. The second cut between the 1st and the 2nd ganglia had the opposite effect to the first cut. After a brief period of firing of f6 followed by high frequency firing of f3, the rate of activity in all three motor neurones decreased to levels lower than before.

C. The third cut was made between the 4th and 5th ganglia. This produced a 2 second period of firing of f6 after which the rate of firing of both f1 and f3 was enhanced.

D. The fourth cut was made between the 2nd and 3rd ganglia. This cut was much less effective in activating f6, and only a few f6 impulses are seen. The immediate effect of cutting was to evoke high frequency activation of f3 and f4. Thirty seconds after firing the rate of firing of f1, f3 and f4 was slightly elevated above levels prior to cutting.

E. The final cut was made between the 3rd and 4th ganglion posterior to the RSr3 of G3. This stimulated high intensity firing of f6, followed by high frequency firing of f3 which stopped altogether for a period of time before returning. Note that f1 is no longer firing as its posterior axon was severed by the cut.

3.3.7 Effect of proctolin on the spontaneous activity of lobster Sr3

Extracellularly recorded spontaneous activity from the Sr3s of complete abdominal nerve cords was analysed before and after the application of proctolin (see 3.2.5 for methods). In all cases extracellular recordings were made from the Sr3s of either the second or third abdominal ganglia and no

significant differences in proctolin effects were noticed between these ganglia. In some experiments, the effects of other neuromodulators on the spontaneous activity of Sr3s were also investigated (see 3.3.9). In these cases, the data for the various analyses have all been plotted on the same graph to facilitate comparison. Each figure (Figs. 3.19, 3.20, 3.21, 3.22) represents the results from a different preparation, although the left and right Sr3 from the second ganglion in one preparation were analysed separately and results are presented in Figs. 3.20A and 3.20B.

The mean frequency of spontaneous firing and the mean interspike interval of each motor neurone were determined after a 30 minute period of exposure to proctolin.

The effects of proctolin on the experiments analysed are summarised in Table 3.3. Bath application of proctolin consistently increased the rate of firing of the excitatory postural flexors f1, f4 and f6. In contrast, the mean firing frequency of the small excitatory tonic flexor, f2, invariably decreased. Both an increase and a decrease in the rate of firing of the remaining excitatory tonic flexor, f3, was observed after perfusion of proctolin. In both experiments where the inhibitor, f5, was spontaneously active, proctolin decreased its rate of firing. In most cases bath application of proctolin did not initiate firing in any of the Sr3 motor neurones which were previously silent. Only those units which were already spontaneously active were affected.

The central effect of proctolin on frequency of firing of active superficial flexor motor neurones was dose-dependent (Figs. 3.19A, B). The results presented in Figure 3.19B show that proctolin at a concentration of 10^{-8} M was more effective in enhancing the rate of firing of f1, f3, f4 and f6 and decreasing the rate of firing of f2 than it was at 10^{-10} M. The standard deviation of the mean interspike interval (Fig. 3.19C) provides an estimate of the effect of proctolin on the regularity of the firing pattern of individual motor neurones, small standard deviations indicating a very regular pattern of activity. Proctolin

increased the regularity of firing of the flexor excitors whose rate of firing was enhanced. Conversely, the impulse discharge of f2 became less regular in the presence of proctolin as indicated by the larger standard deviation of the mean interspike interval.

These results suggest that proctolin can modulate tonic abdominal flexion in very specific ways by increasing the level of firing of the majority of the spontaneously active excitatory motor neurones while decreasing the rate of firing in one excitatory axon, f2, as well as in the inhibitor, f5.

3.3.8 Effect of other neuromodulators on the spontaneous activity of the Sr3

The effects of other known neuromodulators on spontaneous activity of the superficial flexor motor neurones were analysed and compared to the effects of proctolin in an attempt to relate the strength of the effect of proctolin to other neuromodulators known to function within the abdominal postural system.

Octopamine and serotonin were chosen for comparison because of their known actions in inducing opposing postural stances in lobsters (Harris-Warrick and Kravitz, 1984). In addition, the effects of oxotremorine were investigated. This muscarinic, cholinergic agonist has recently been found to induce two types of interaction between the abdominal postural and swimmeret systems (Chrachri and Neil, in press).

In agreement with the findings of Kravitz and co-workers (Harris-Warrick and Kravitz, 1984) application of 10^{-6} M octopamine and serotonin to the isolated nerve cord, most often resulted in the production of motor output patterns consistent with activation (serotonin) and inhibition (octopamine) of the superficial flexor muscles (Fig. 3.21A).

Octopamine. The typical pattern of spontaneous activity prior to octopamine application was a high frequency of firing of f1 and f2 and less frequent but regular firing of either f3 or f4. Both f5 and f6 were usually silent. Compared to control levels of spontaneous activity, octopamine increased the rate of firing of

the superficial flexor inhibitor, f5 and frequently increased the rate of firing of the superficial excitors, f1 and f4. This was accompanied by a marked decrease in the firing rate of the other small tonic unit, f2 (3.21B, 3.22A). The effects of octopamine on the remaining flexor excitors f3 and f6 were found to be variable. Often the rate of firing of f6 was unaltered but could increase or decrease in the presence of octopamine.

Serotonin. The motor output pattern recorded from the Sr3 following bath application of serotonin was completely different to the pattern observed after application of octopamine (Fig. 3.21A). Serotonin enhanced the firing of the postural flexors f2, f3 and f4 to well above control levels (Fig. 3.21B). The frequency of firing of f1 and f6 often remained at around control levels, but f5 was usually completely inhibited.

Oxotremorine. Bath application of oxotremorine produced a pattern of activity that was quantitatively very similar to the pattern produced by proctolin in the experiment shown (Fig. 3.22B). Firing rates of f1, f2 and f4 were nearly identical to rates during perfusion of proctolin. Firing of the inhibitor was greater in oxotremorine than in proctolin but was lower than control levels.

3.3.9 Analysis of connectivity between postural flexor motor neurones

In order to be able to interpret properly the effect of proctolin on postural flexor motor neurone activity, an investigation of the functional organisation of the central circuitry of this motor system was undertaken. Tables 3.4 and 3.5 present a summary of all correlations found between SFM motor neurones before and after proctolin.

The time course and extent of coordinated firing of postural flexor motor neurones within one or both Sr3s of the third ganglion was investigated by constructing estimates of the cross intensity function derived from cross-correlation histograms for their spontaneous activity (see section 3.2.6 for details of methods). Within a single experiment, it was usually not possible to do this for the full complement of postural flexor motor neurones as some were

often silent. This was especially true of the larger phasic motor neurones, f5 and f6 (see section 3.3.5). However, by collating results from a number of experiments, it was possible to obtain data on most possible relationships. The functional classification of Tatton and Sokolove (1974) has been adopted and revised for these purposes to facilitate description of the results of analysis. Thus, the following subgroups of the flexor motor pool are recognised: (1) Intragroup synergists - postural flexor motor neurones which produce excitation in the muscle which they commonly innervate (2) Intragroup antagonists - postural flexor motor neurones which have opposing effects on the muscle which they commonly innervate.

3.3.10 Correlations between Intragroup Synergists

3.3.10.1 Positive correlation

Figure 3.23 presents cross intensity estimates of analyses of the postural flexor synergists, f3 and f4. The general picture which emerges from this analysis is the existence of strong positive correlations between both ipsilateral f3 and f4 pairs (e.g. Rf4 and Rf3) and contralateral f3 and f4 pairs (e.g. Rf4 and Lf3). These correlations lie well above the 95% confidence limits calculated for the activity of each pair of motor neurones, hence they are statistically significant. Estimate A (Fig. 3.23) shows two prominant features present in a number of analyses; (i) a well defined central peak and (ii) symmetrical periodic peaks either side of the origin representing the times of occurence of subadjacent spikes in the test train. Figure 3.23A also shows a statistically significant inhibition of the contralateral pair (Rf4 and Lf3) for approximately 200 ms before and after they fire. The presence of troughs either side of the central peak is typical when analysing periodic data. The ipsilateral pair (Ci) show a narrower peak structure than the contralateral pair (A and B) as emphasised by estimate (Cii) which shows an expanded view of the central peak. The width of the peak is approximately 10 ms and this indicates that neither the coordination of the ipsilateral pair nor the coordination of the contralteral pair can be

attributed to central cross connections. According to Tatton and Sokolove, (1975), coordination via central connections is indicated by central peaks of 5 ms or less, i.e. monosynaptic latency plus conduction delays. Hence, the data suggests that these motor neurones are coordinated by polysynaptic connections at the premotor neurone level.

The periodicity often seen in analyses is particularly evident in histogram D which shows a typical strong positive correlation of f6 with its contralateral synergist, f4. Similarly, f6 was positively correlated with its contralateral synergist, f3 (histogram E) but to a lesser extent. As well as being statistically positively correlated, Figure 3.23D shows a strong periodic inhibition of the contralateral pair, f4 and f6 after firing. With the existence of such strong connections between one of the pairs of the contralateral flexors (Lf6 and Rf4), it is perhaps significant to note that the other contralateral pair (Rf6 and Lf4) were both silent in this experiment. Again, the width of the central peak in these estimates indicates that premotor neurone connections may account for the coordination of these motor neurones. In this and other experiments, cross intensity estimates also demonstrated weaker, positive coordination between between f6 and the ipsilateral motor neurones f3 and f4 (data not shown).

3.3.10.2 No correlation

In all of the experiments analysed, no evidence was obtained for any coordination of firing between f1 and f2 recorded from a single Sr3. Similarly, no correlations between f1 and f2 were found when extracellular recordings from both left and right Sr3s of the same ganglia were compared. In fact, no correlations were found between f1 and any of the other flexors (f2 - f6) recorded from the same or opposite Sr3. Examples of estimates of the cross intensity function applied to some of these motor neurone pairs are shown in Figure 3.24 (A-D). The lack of correlation of f1 and these other superfical flexor motor neurones is, perhaps, not surprising when it is considered that the cell body of f1 is located in the ganglion posterior to that which contains the

other flexor neurones (see section 3.3.1) of the same superficial flexor root. The tonic units, f1 and f2, fire with extreme regularity but no evidence was found which would indicate any connections between them. For this reason, the possibility of coordination between f1 and the other motor neurones lying in the same ganglion was investigated (i.e. between f1 of segment 2 and f2-f6 of segment 3). No correlations were found between f1 and any other motor neurones (ipsilateral or contralateral) in the same ganglion (data not shown). In the same way, no correlations were found between the contralateral homologues of the intragroup synergists (Rf3 and Lf3) and (Rf4 and Lf4) (Figs. 3.24, E and F).

3.3.11 Correlations between Intragroup Antagonists

3.3.11.1 No correlation

In the SFM the peripheral inhibitor, f5, acts to antagonise the other excitatory motor neurones innervating the same muscle. In crayfish, using antidromic stimulation of the inhibitor, Tatton and Sokolove (1975) showed negative correlations between the ipsilateral antagonists f5, and f3 and f4, but not between f5 and f6. In this study, using spontaneous activity, no negative correlations were found between the intragroup antagonists, i.e. f5 with reference to f1, f2, f3, f4, and f6. This was possibly due to the low level of spontaneous activity of f5 in these preparations.

3.3.12 Correlations between Intragroup Synergists in the presence of proctolin.

In most experiments, proctolin was effective in coordinating the firing of the smaller tonic flexors. Conversely, proctolin often disrupted the correlations between the more phasic motor neurones which existed in the control situation.

3.3.12.1 Proctolin-induced negative correlation

Figure 3.25 shows the results of cross-correlation analysis of two of the ipsilateral flexor motor neurones, f2 and f3 of the experiment shown in Figure 3.19. No correlation existed in the control situation (Fig. 3.25A) but the firing of the two tonic flexor motor neurones became increasingly negatively correlated

as the concentrations of proctolin increased. However, this negative correlation is only statistically significant at 10^{-8} M Proctolin as can be seen more clearly from Figure 3.25F which shows an expanded view of the analysis between f2 and f3.

The pattern of correlation shown in Figure 3.25 was frequently encountered after application of proctolin. More examples of other effects are shown in Figure 3.26. Estimates A and B show the results of a cross intensity function applied to the contralateral motor neurones Lf6 and Rf3, before and after the application of proctolin. Proctolin abolished the positive correlation which was previously present, and may have induced a small, but detectable negative correlation, as represented by the central depression, flanked on either side by depressions at approximately 100ms intervals.

A more pronounced effect of this kind is shown in Figure 3.26C, D. In this case, proctolin caused two strongly positively correlated cells, Rf3 and Rf4, to become negatively correlated.

3.3.12.2 Proctolin-induced positive correlation

The effect of proctolin in coordinating the firing of the bilateral pairs of f2 motor neurones or f3 motor neurones is shown in Figure 3.27. In the control situation the nature of any possible correlation between motor neurones is obscure (Figs. 3.27A and 3.27C). However, in the presence of proctolin the firing of these cells became coordinated (estimates B and D).

3.3.12.3 Correlations unaffected by proctolin

Not all correlations existing in the control situation were affected by proctolin. The coordinated activity of f6 and f4 remained very similar both before and after exposure to proctolin (Fig. 3.28) although the mean intensity of spike firing (dashed line between 95% confidence bands) increased in the presence of proctolin.

3.4 DISCUSSION

3.4.1 Evolutionary impact on neurones within the postural system

The neuroanatomy of the abdominal superficial flexor motor neurones in the lobster *Nephrops norvegicus* has been investigated. Apart from the fact that the superficial flexor motor neurones in *Nephrops* are about twice the size of homologous neurones in the crayfish *Pacifastacus*, no obvious differences in number, morphology or location within the ganglia were observed. This agrees with previous studies of animals within the orders Astacidea (*Procambarus*: Wine and Hagiwara, 1977); Nephropidae (*Homarus*: Thompson and Page, 1982) and Caridea (*Crangon*: Bothe, 1989). The SFM in each of these species are innervated by six motor neurones whose position within the ganglion are characteristically stable (see 3.3.1).

However, in contrast to this stability, differences in the branching patterns of homologous nerves exist. For example *Homarus* differs from *Nephrops* in the number of axons which run in the Sr3s of the first five abdominal ganglia. In *Nephrops* the axons of the six flexor motor neurones only run in the superficial branch of the third root. However, in *Homarus*, each Sr3 contains an extra three axons which ascend to innervate the phasic extensor muscles after passing laterally over the SFM (Thompson and Page, 1982).

In other decapod species, further modifications of the abdominal postural system are evident, including differences in: (1) physiological properties and organisation of the six flexor motor neurones and (2) structure of the SFM. One of the six flexor motor neurones (f1 or f2) of the mud shrimp *Callianassa* and *Upogebia* (Thalassinidea) projects its axon through the next anterior Sr3 and only two motor neurones exhibit spontaneous activity (f1 and f2 in *Callianassa*; f1 and f3 in *Upogebia*). The anatomical differences in the SFMs of these species are discussed in Chapter 4.

Thus, these neuroanatomical observations reinforce the idea that the ganglionic position of the six motor neurones which innervate the SFMs in the

decapods has been relatively conserved throughout evolution. However, differences in behavioural requirements have resulted in changes in physiological properties and branching patterns of nerves and adaptations in the superficial flexor muscle (see Chapter 4, Discussion).

3.4.2 Relation of effect of proctolin to the 'intactness' of preparation

The effect of proctolin on patterns of motor output was investigated by analysing its effects on (i) spontaneous firing of motor neurones and (ii) coordination of firing between subsets of the total superficial flexor motor neurone pool.

Any study of the effect of proctolin on a single set of superficial flexor motor neurones requires a much reduced preparation consisting of at least two ganglia. This is due to the neuroanatomy of the smallest motor neurone, f1, which has its cell body resident in the ganglion posterior to the SFM it innervates. However, intersegmental connections between the two ganglia would remain intact in such a preparation and could only be eliminated by sacrificing f1. Thus a study of the effect of application of proctolin onto a single isolated ganglion would represent a more controlled experimental situation but would only include activity of five of the six superficial flexor motor neurones (f2 to f6).

In contrast, the experiments presented in this chapter were carried out using an isolated abdominal nerve cord preparation consisting of all six abdominal ganglia. Variations in the level of 'intactness' of the abdominal nerve cord (i.e. number of ganglia in chain) were found to affect the levels of spontaneous firing of SFM motor neurones, presumably by removal of inhibitory and excitatory influences of intersegmental connections (Miall and Larimer, 1982b; Larimer and Jellies, 1983). In a similar way, the mean period of the crayfish swimmeret rhythm is longer in abdominal preparations than in thoraco-abdominal preparations (Paul and Mulloney, 1985; Barthe *et al.,* 1991).

It is important to realise that a different set of results relating to the

effect of proctolin on the output of the SFM motor neurones could possibly have been obtained if a more or less 'intact' preparation had been employed. The motor pattern expressed by the isolated nervous system can also vary with the preparation. This can be illustrated by considering some recent work on crayfish. Whereas Barthe *et al.,* (1991), using a crayfish thoraco-abdominal preparation, recorded rhythmic activity in both extensor and flexor nerves, related to the swimmeret activity rhythm, Mulloney *et al.* (1987) found that no corresponding rhythmic activity was evoked in the abdominal positioning system in the reduced abdominal nerve cord preparation. Furthermore, perfusion of proctolin through the isolated abdominal nervous system causes excitation and rhythmic beating of the swimmeret system. Therefore, the different results obtained in these two preparations reflect different levels of activity that exist, the influence of central mechanisms of coordination of different motor activities, and the different apparent effects of proctolin on these systems.

The pattern of motor activity recorded in the Sr3 nerves in the present study was always tonic and it would be interesting to study the activity patterns of this system, and the influence upon them of proctolin in the more intact thoraco-abdominal preparation.

3.4.3 Effect of proctolin on motor output of superficial flexor motor neurones in *Nephrops*

Perfusion of proctolin onto isolated preparations invariably enhanced the firing frequency of f1, f4 and f6 when spontaneously active, whereas the rate of firing of f2 was consistently decreased. In experiments where the inhibitor, f5, was spontaneously active, proctolin was also effective in decreasing its rate of firing. The only motor neurone to display both an increase and a decrease in its firing frequency when exposed to proctolin was f3. The variability in responsiveness of f3 may be real, but it may also be due to a number of other factors such as differential penetration of bath-applied proctolin to active sites in the neuropil. Since proctolin was added to

preparations by bath application, there is always the possibility of activation of proctolin-sensitive neurones not normally involved in the generation of posture affecting the activity of those which are usually involved in the generation of posture.

The relative effects of serotonin and octopamine on the patterns of motor neurone activity in the Sr3 have been investigated (Harris-Warrick and Kravitz, 1984). However, the methods of analyses used by these workers were not sufficiently detailed to permit discrimination between (i) the tonic motor neurones f1 and f2, and (ii) the tonic-phasic motor neurones, f3 and f4. Reliable identification of different classes of motor neurones in this study has demonstrated the specific action of neuromodulators on individual motor neurones. For example, the excitation of firing of the f1/f2 class of motor neurones by serotonin, described by Harris-Warrick and co-workers (1984), can be attributed solely to an increase in the firing of f2 (Fig. 3.21A).

The opposing effect of proctolin on the rate of discharge of the smallest excitatory flexors, f1 and f2, was found in all five of the experiments analysed. This is an intriguing find, since in the control condition, these two motor neurones exhibit high levels of spontaneous activity appropriate to the maintenance of postural tone. It could be argued that a general neuromodulatory enhancement of postural flexion would be expected to increase the firing both of f2 and f1. The differential effects observed suggest that modulatory bias of activity occurs in an extremely precise manner, at the level of individual motor neurones.

3.4.4 Effects of proctolin on abdominal posture

In the crayfish, application of proctolin onto isolated crayfish nerve cords evokes the readout of a pattern of activity which is biased towards flexion (this study, data not shown). However, in considering the combined effect of proctolin on the pattern of motor neurone activity controlling the SFMs of *Nephrops*, it is unclear what postural changes would be evoked. It cannot be

predicted whether proctolin would produce either a more flexed or a more extended posture since this peptide is able to simultaneously increase and decrease the firing of different subsets of the total population of slow flexors. This expectation is borne out by the recent study of Ma *et al.* (1992) who find no effect on postural regulation after injection of proctolin into freely moving lobsters. This is in contrast to the defined actions of serotonin and octopamine on the firing patterns of postural motor neurones. Serotonin activates a coordinated firing pattern of postural flexion whereas the firing pattern activated by octopamine results in postural extension (Harris-Warrick, 1985; this study).

Bath application of proctolin onto *Nephrops* isolated nerve cords activates a specific pattern of motor output which is assumed to reflect premotor modulation of postural motor neurone(s). However, the source of this premotor modulation (both in the crayfish and in *Nephrops*) is not known. Proctolin has also been shown to coexist with serotonin in two pairs of identified interneurones in the 5th thoracic and 1st abdominal ganglia of the lobster *Homarus americanus* (Beltz and Kravitz, 1983; 1987). The modulatory role of serotonin in these cells is known, and a similar modulatory role for proctolin in these cells is possible. Stimulation of these cells does not activate the readout of specific motor programs but rather acts as a 'gain-setter', modulating the interaction between command inputs and motor neurone outputs (Ma *et al.*, 1992). For a variety of reasons, this gain-setting is biased towards a facilitation of postural flexion. Thus, the production of a flexion motor program by bath application of serotonin can be explained in terms of an amplification of this bias. Similarly, proctolin could function as a 'gain-setter', and the simultaneous increase of some tonic flexor motor neurones and decrease of others could represent a simultaneous enhancement and suppression in the responsiveness of these motor neurones to normal physiological activity. Proctolin and other circulating neurohormones, have already been described as 'gain-setters' in that

they can influence the output of peripheral proprioceptors in lobsters (Pasztor and MacMillan, 1990). The major source of circulating proctolin in lobsters seems to be the pericardial organ, a well known neurosecretory structure (Sullivan, 1979; Schwarz *et al.,* 1984). Proctolin is also able to act peripherally in insects and crustacea to increase tension in muscle fibres. However, whereas depolarisation of some types of muscle is a requirement for proctolin's ability to potentiate muscle tension (crayfish SFM: Bishop *et al.,* 1987; lobster SFM: this study, Chapter 4), proctolin can act directly on other types of muscle to induce tension without depolarisation (cockroach coxal depressor muscle: Adams and O'Shea, 1983; O'Shea *et al.,* 1985; lobster leg muscle: Schwarz *et al.,* 1980). These peripheral effects are discussed in more detail in Chapter 4.

3.4.5 Coordination of firing of superficial flexor motor neurones in *Nephrops*

The motor patterns which represent a particular behaviour are produced by neuronal networks, which may incorporate central pattern generators (CPGs). Three networks involved in the control of locomotion have been described in Crustacea. One of these networks is found in the thorax and controls leg movements. The other two networks are located within the abdominal nervous system and control rhythmic swimmeret beating and abdominal positioning.

The superficial flexor motor neurones form part of the CPG controlling abdominal positioning. The connectivity of these postural flexor motor neurones in crayfish has been studied (Sokolove and Tatton, 1975; Tatton and Sokolove, 1975), revealing that small tonic and larger tonic-phasic motor neurones are controlled entirely by premotor connections, whereas connections at the motor neurone level control the output of the larger phasic motor neurones (see also Kirk and Glanz, 1981; Thompson and Page, 1982). This study has used similar methods of analyses as those used by Tatton and Sokolove in their innovative set of experiments. An attempt been made to analyse the organisation of the central circuitry of the postural flexors by cross-

correlation analysis of spontaneous activity in the Sr3. This allows an investigation of the probability that firing of a single spike in one cell will produce a spike in a second cell.

Cross-correlation analyses of spontaneous activity from *Nephrops* Sr3 under control conditions have revealed the existence of premotor connections between the following subsets of the motor neurone pool:

(i) contralateral pairs of f3 and f4 (i.e. between Rf3 and Lf4)

(ii) ipsilateral pairs of f3 and f4 (i.e. between Rf3 and Rf4)

(iii) contralateral pairs of f3 and f6 (i.e. Lf6 and Rf3)

(iv) contralateral pairs of f4 and f6 (i.e. between Lf6 and Rf4).

No evidence was found for any other connections between motor neurones, but this should not be interpreted as proof that other such connections do not exist as levels of spontaneous activity observed in the isolated VNC may be too low to show the existence of some connections. For example, high frequency stimulation of f6 was used by Evoy *et al.* (1967) to demonstrate an excitatory cross connection from f6 to e5, which is the fifth largest postural extensor, but the existence of this connection could not be confirmed at lower firing rates (60/s) (Tatton and Sokolove, 1975). Thus, there is a strong argument for investigating the existence of cross connections at levels of activity which are within natural firing rates. However, a number of methods were used by Tatton and Sokolove (1975) to stimulate postural motor neurones in a semi-intact crayfish preparation, including telson extension and flexion, stimulation of flexion and extension command elements and stimulation of distant peripheral abdominal axons. In this way, they were able to enhance the activity of postural motor neurones but remain within the limits of naturally occurring firing rates. This may explain why the presence of connections between larger motor neurones (i.e. excitatory connections between contralateral f6 motor neurones or inhibitory connections between f6 and the inhibitor) which are found in crayfish (Tatton and Sokolove, 1975; Kirk and

Glanz, 1982) were not reflected in the analysis of *Nephrops* postural flexor circuitry (see Table 3.6). Studies using semi-intact preparations, or isolated preparations in which sensory inputs (e.g. from MRO) are retained, would obviously be instructive.

The extent to which longitudinal, intersegmental connections influence the connections found in this study is not known. One possible means of eliminating this difficulty in the future would be to investigate coupling between motor neurones in a single isolated ganglion preparation. Once the basic ganglionic connections had been established, further work could then investigate the possibility of hierarchical connectivity as suggested by the work of Larimer and coworkers (Larimer, 1988; Murphy *et al.*, 1989).

3.4.6 Effect of proctolin on coordination of firing of superficial flexor motor neurones in *Nephrops*

CPGs are influenced by inputs which are capable of 'rewiring' the network, and so altering their output. Networks can be influenced by both 'intrinsic' inputs (sensory reflexes) and 'extrinsic' inputs (other synaptic and neuromodulatory influences). Application of proctolin to isolated nerve cords in an attempt to mimic such 'extrinsic' inputs was effective in 'rewiring' the postural flexor circuitry in that some connections which were present in the control situation disappeared in the presence of proctolin. Furthermore, proctolin induced coordination between motor neurones where there had previously been none. Proctolin was effective in disrupting coordination between phasic and tonic motor neurones which existed in the control situation. The proctolin-induced negative correlations found in this study all involved either one, or other, or both of the motor neurones, f2 and f3. Interestingly, these motor neurones were the only two whose firing rate decreased in the presence of proctolin; the firing of f2 was invariably decreased and the firing of f3 decreased in three of five experiments. Proctolin-induced negative connections were found between the following motor neurones:

(i) From f3 to f2 (ipsilateral pairs).

(ii) From f6 to f3 (contralateral pairs).

(iii) From f4 to f3 (ipsilateral pairs).

These correlations may help to explain the specific opposing action of proctolin on the firing of f2 and f3, and other SFM motor neurones. Proctolin was also effective in coordinating the firing of the following smaller tonic flexors:

(i) Contralateral pairs of f2 motor neurones.

(ii) Contralateral pairs of f3 motor neurones.

Given the inhibitory effect of proctolin on these motor neurones, this coordination points to a mechanism for ensuring that the inhibition of firing in both sets of motor neurones is balanced. This should help to ensure equal tension in opposing muscles although the mechanisms through which proctolin could achieve this action are not known.

It has been known for some time that neuromodulators, such as peptides and monoamines, can reconfigure networks of neurones to form a number of different functional circuits (softwiring). Softwiring is equivalent to cells being temporarily incorporated or removed from a network due to the presence of a specific neuromodulator. Much of our understanding of the effect of these neuromodulators on cellular and synaptic properties of CPG circuits has come from work on the stomatogastric ganglion (STG) (Marder, 1984, 1987; Flamm and Harris-Warrick, 1987; Heinzel and Selverston, 1988; Nusbaum and Marder, 1989a,b). This ganglion contains networks that generate several different rhythmic motor patterns (Selverston and Moulins, 1987). Proctolin is found in two identified cells whose axons project onto the STG and bath application of the peptide can exert a state-dependent modulation of the pyloric rhythm (Nusbaum and Marder, 1989a,b). It has been shown recently that proctolin achieves this modulation by gating an inward current that increases the excitability of neurones within the STG network (Golowasch and Marder, 1992). This action may represent a universal excitatory effect of proctolin on

target cells within networks and may explain the excitatory effect of proctolin on some of the proctolin-sensitive neurones within the abdominal positioning network. However, this does not explain the ability of proctolin to simultaneous enhance the activity of one motor neurone while suppressing the activity of another. This could be achieved through the selective depression or sensitisation of synaptic transmission, as occurs between tactile input neurones and sensory interneurones of the crayfish tailflip circuitry on application of serotonin and octopamine, respectively (Wine and Krasne, 1982). However, this example involves two different neuromodulators.

It may be possible to explain the different proctolin effects by demonstrating differences in ion-channel receptor properties such as single ion channels with opposite ligand-gating properties, or multi-ion channels carrying currents in opposite directions, with proctolin receptors.

Similar arguments have been put forward for the peripheral actions of proctolin on muscle membranes, and a general discussion of these possibilities is therefore deferred to Chapter 4.

Table 3.1

Recipe for saline used in experiments on *Nephrops norvegicus* and *Pacifastacus leniusculus*. pH of saline was adjusted to 7.45 and 7.4 respectively.

Salt	*Nephrops* (mM)	*Pacifastacus* (mM)
NaCl	478.95	195.07
KCl	12.74	5.36
$CaCl_2$	13.70	13.60
$MgSO_4$	20.48	-
$NaSO_4$	3.9	-
Hepes	5.0	-
$MgCl_2$	-	2.61
Tris	-	9.91

Table 3.2

Mean frequency of spontaneous firing of individual motor neurones (expressed in Hz) in the isolated abdominal nerve cord preparation of *Nephrops*.

Table 3.3

Summary of the effect of bath applied proctolin on the mean frequency of spontaneous firing recorded extracellulary from five Sr3s of four different *Nephrops* preparations (Figs. 3.20A and 3.20B show the effect of proctolin on mean firing levels on the left and right Sr3s of a single ganglion). An increase or decrease in firing is indicated by or and - indicates that an axon was not spontaneously active.

	Mean frequency of firing	± S. D.	Range	n
f1	11.8	6.4	3.3 - 26.0	15
f2	11.0	6.9	1.0 - 26.0	18
f3	4.3	3.1	1.0 - 10.0	17
f4	2.2	2.3	1.0 - 5.4	11
f5	1.9	1.6	1.0 - 4.2	6
f6	1.4	2.6	1.0 - 7.4	6

	f1	f2	f3	f4	f5	f6
Fig. 3.19	↑	↓	↑	↑	–	↑
Fig. 3.20A	↑	↓	↓	–	–	↑
Fig. 3.20B	↑	↓	↓	↑	↓	–
Fig.3. 21	↑	↓	↑	–	–	–
Fig. 3.22	↑	↓	↓	↑	↓	–

Table 3.4

Summary of the connections found between motor neurones innervating the SFM of a single hemisegment (ipsilateral connections between motor neurones innervating either the left or the right SFM) in the control situation (C) and in the presence of proctolin (P).

Table 3.5

Summary of the connections found between motor neurones innervating the SFMs of opposite hemisegments (contralateral connections between motor neurones innervating the right and left SFM) in the control situation (C) and in the presence of proctolin (P).

C P	C P	C P	C P	C P	C P	
f1	f2	f3	f4	f5	f6	
	0 0	0 0	0 0	0 0	0 0	f1
		0 +	0 +	0 0	0 0	f2
			+ -	0 0	+ -	f3
				0 0	+ +	f4
					0 0	f5
						f6

	C P	C P	C P	C P	C P	C P
	Rf1	Rf2	Rf3	Rf4	Rf5	Rf6
Lf1	0 0	0 0	0 0	0 0	0 0	0 0
Lf2	0 0	0 +	0 +	0 0	0 0	0 0
Lf3	0 0	0 +	0 +	+ 0	0 0	+ -
Lf4	0 0	0 0	+ 0	0 0	0 0	+ +
Lf5	0 0	0 0	0 0	0 0	0 0	0 0
Lf6	0 0	0 0	+ -	+ +	0 0	0 0

Table 3.6

Evidence for coupling was found between several pairs of contralateral flexors in the semi-intact crayfish preparation used by Tatton and Sokolove (1975; also Sokolove and Tatton, 1975) in their study of connectivity in the postural circuitry of *Procambarus clarkii*. No evidence was found for coupling between these motor neurones in *Nephrops* using an isolated abdominal nerve cord preparation.

Contralateral Homologues	Nephrops norvegicus (Isolated preparation)	Procambarus clarkii (Semi-intact preparation)
Rf1 —> Lf1	0	0
Rf2 —> Lf2	0	0
Rf3 —> Lf3	0	+
Rf4 —> Lf4	0	+
Rf5 —> Lf5	0	++
Rf6 —> Lf6	0	++

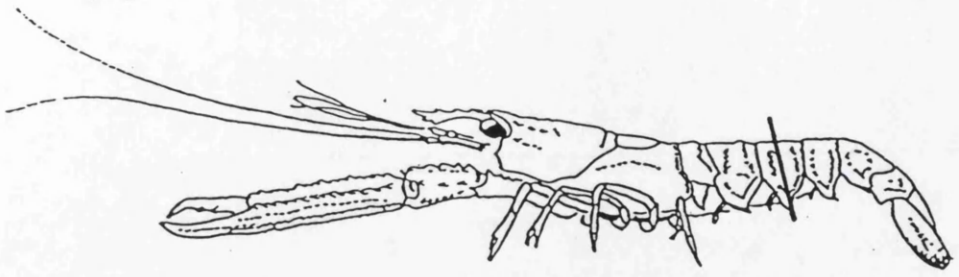
Figure 3.1

A. Drawing of the Norway lobster *Nephrops norvegicus* . The line through the third abdominal segment indicates the plane of the section used to draw diagram (**B**)

B. Diagrammatic representation of the nerves and muscles within an abdominal segment of a lobster or crayfish. The nerves (roots 1, 2 and 3) and nerve cord have been enlarged for clarity. The general direction of nerve 2 has been indicated with dashed lines as it passes out of the plane of this section. Sensory axons of nerves 1 and 2 have been omitted. (modified from Leise, *et al.*, 1986).

Scale bar applies to both figures. A = 2.5 cm, B = 0.5 cm.

A



B

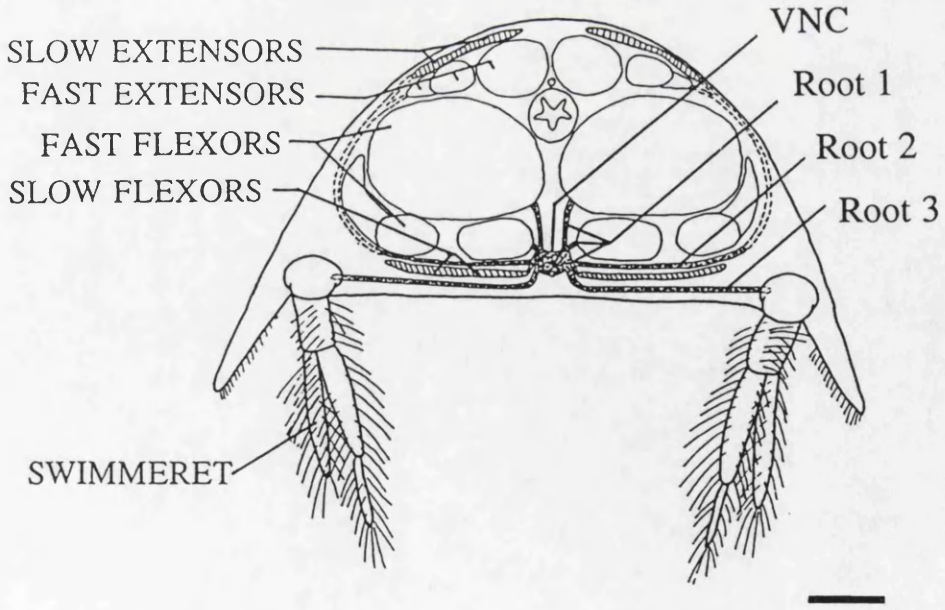

SLOW EXTENSORS
FAST EXTENSORS
FAST FLEXORS
SLOW FLEXORS
SWIMMERET
VNC
Root 1
Root 2
Root 3

Figure 3.2A

Diagrammatic representation of a dorsal view of the ventral nerve cord and superficial flexor muscles of a Norway lobster.

VNC = ventral nerve cord.

r1 = the first ganglionic root which innervates the swimmerets and contains motor and sensory axons.

r2 = the second ganglionic root which innervates the slow and fast extensor muscles, stretch receptors and sensory hairs of the body wall.

Sr3 = the superficial branch of the third ganglionic root which innervates the superficial flexor muscles.

SFM = superficial flexor muscles.

G = ganglion.

Figure 3.2B

Different preparations were used depending on the type of experiment to be carried out.

The **isolated ventral nerve cord** was dissected by first snipping roots 1 and 2 of each segment, (as indicated by the arrows at **a** and **b**) and easing them free. Rather than damaging the fine superficial flexor nerves themselves, a patch of superficial flexor muscle (broken lines) was dissected for each hemisegment which served as a convenient handle when manipulating the fine superficial flexor nerve (Sr3).

The **isolated nerve-muscle preparation** was dissected in the same way with the exception that one of the superficial flexor muscles and its superficial flexor root was left intact. The entire hemisegment was isolated by making two parallel cuts through the superficial flexor muscles and underlying exoskeleton of the two adjacent hemisegments (e-f and c-d) and another perpendicular cut (e-c), taking care not to cut through the fine Sr3.

The **modified nerve-muscle preparation** was used to measure tension produced by an individual superficial flexor muscle. Instead of cutting through the anterior segment (e-f), a cut was made from g-h through the soft membrane onto which the superficial flexor muscles insert.

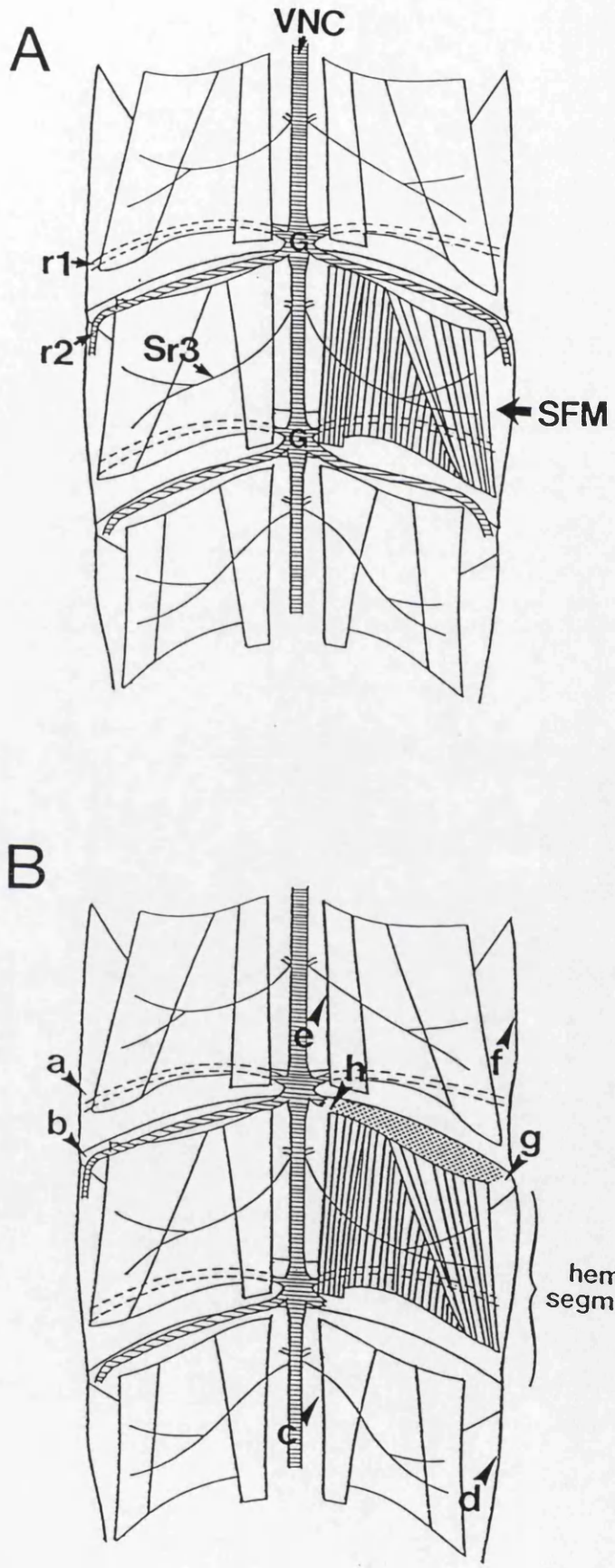

A
VNC
r1
r2
Sr3
SFM
G
G
B
a
b
e
h
f
g
hemi-
segment
c
d

Figure 3.3

Diagram of the experimental layout used to make extracellular recordings from superficial flexor nerves of the isolated abdominal nerve cord of lobster.

VNC = ventral nerve cord

AMP = amplifier

STIM = stimulator

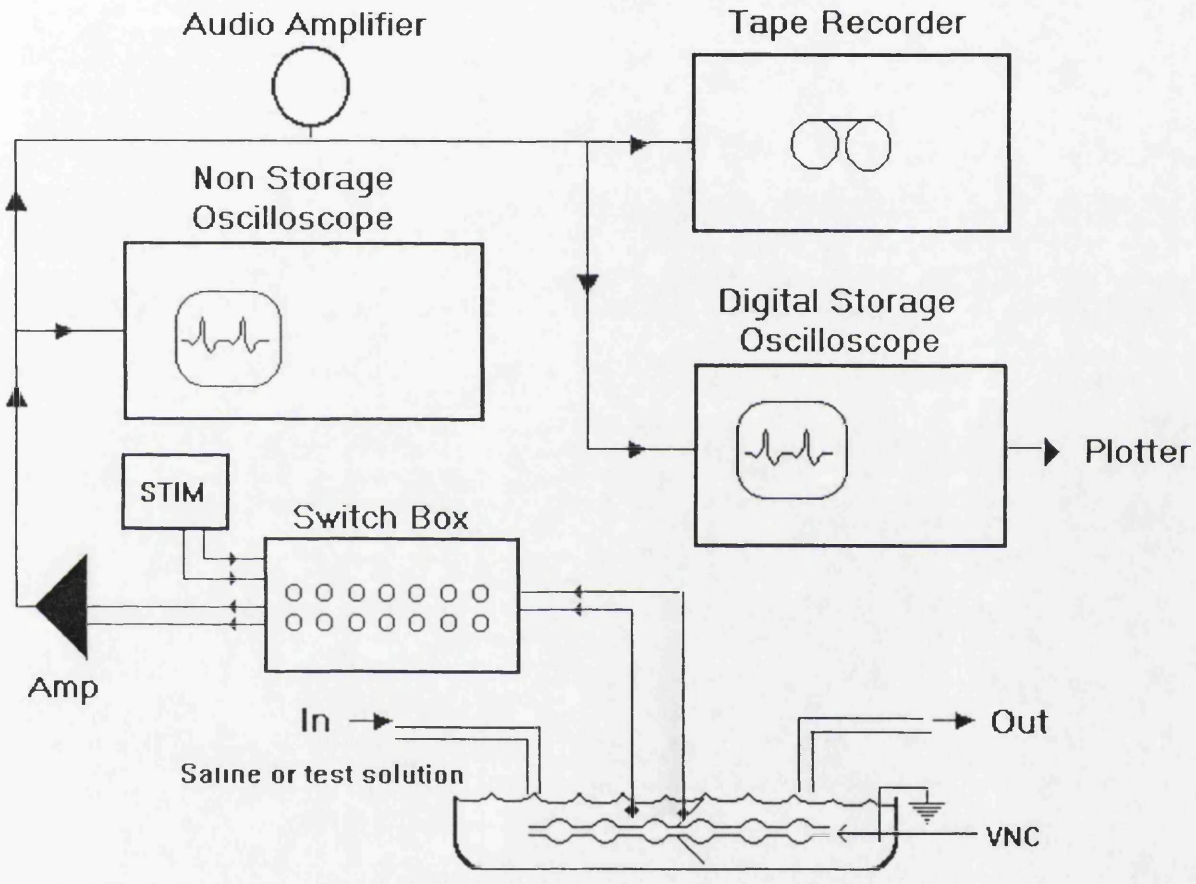

Audio Amplifier
Tape Recorder
Non Storage
Oscilloscope
Digital Storage
Oscilloscope
Plotter
STIM
Switch Box
Amp
In
Out
Saline or test solution
VNC

Figure 3.4

Computerised production of event channels from waveform data.

A. Channel 1 represents the original waveform data.

B. An event is produced for every spike as their peaks all lie above the value of the cursor. These events are stored as channel 2.

C. Only the larger spike has an event associated with it. The peak of the smaller spike lies below the value of the cursor. Therefore each event in channel 3 represents the firing of the large spike.

D. In order to produce an event channel which represents only the small spikes it is necessary to subtract channel 3 (large spikes) from channel 2 (all spikes). A small programme was written for this purpose. The result of running this programme for this data is channel 4 (events corresponding to small spikes).

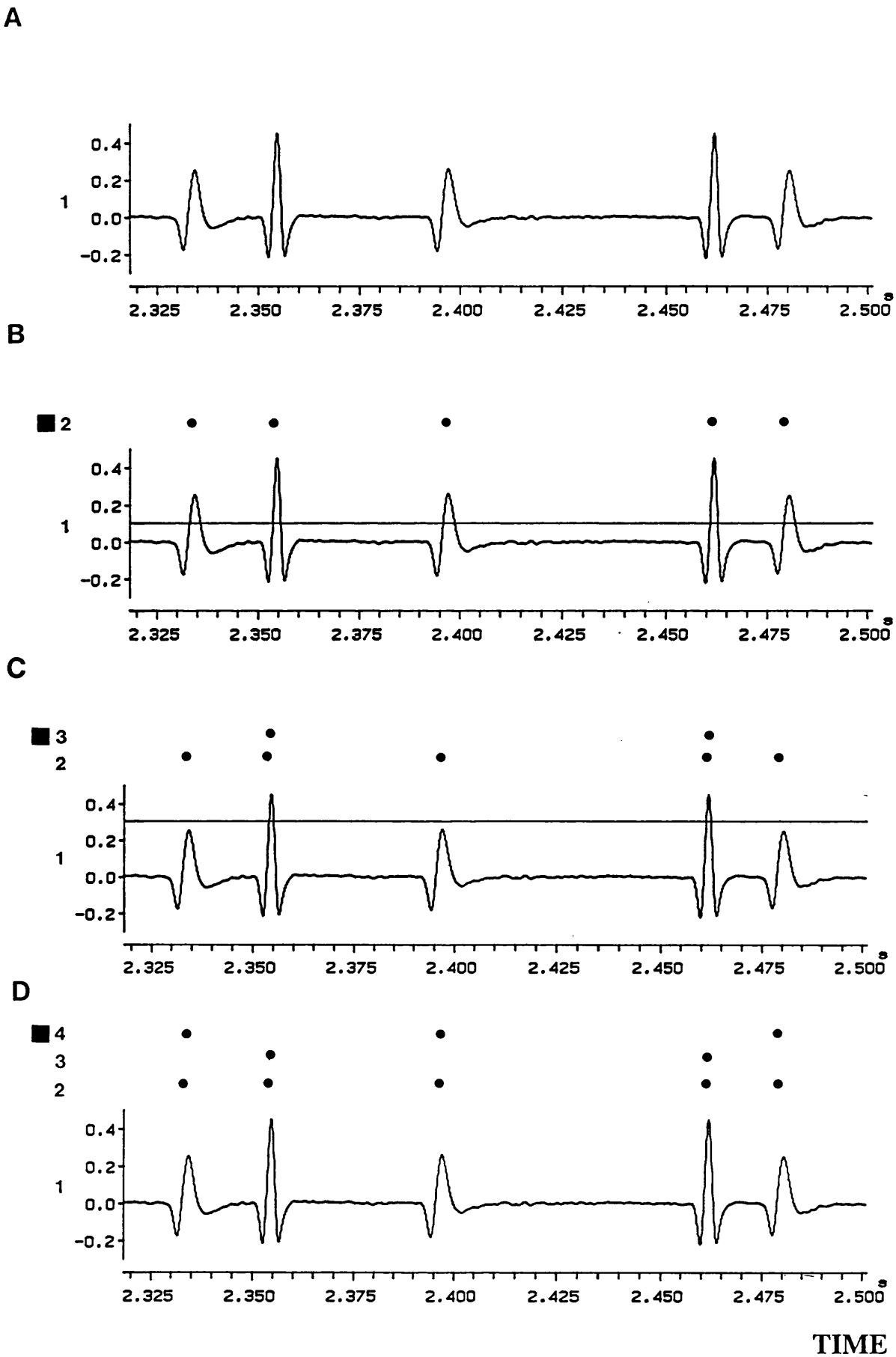

A
1
0.4
0.2
0.0
-0.2
2.325
2.350
2.375
2.400
2.425
2.450
2.475
2.500
s
B
2
1
C
3
2
D
4
3
TIME

Figure 3.5

In the analysis of relationships between motor neurones, cross-correlation histograms were initially produced by comparing one event channel (the stimulus channel) with another event channel (the response channel). Event channels were derived from waveform channels as explained in Figure 3.4.

A represents a typical cross-correlation histogram showing the association between two motor neurones. However, this method of data analysis does not allow for any probablistic interpretation of the association, i.e. whether it is statistically significant or not. This was achieved by the application of a staochastic point process analysis (see section 3.2.6 for details) which gives an estimate of the mean intensity of spikes (dashed line) and applies 95% confidence limits. (For normal distributions, 95% of values lie within +/-2 standard deviations). **B** shows the result of application of the process to the same data as used in **A**.

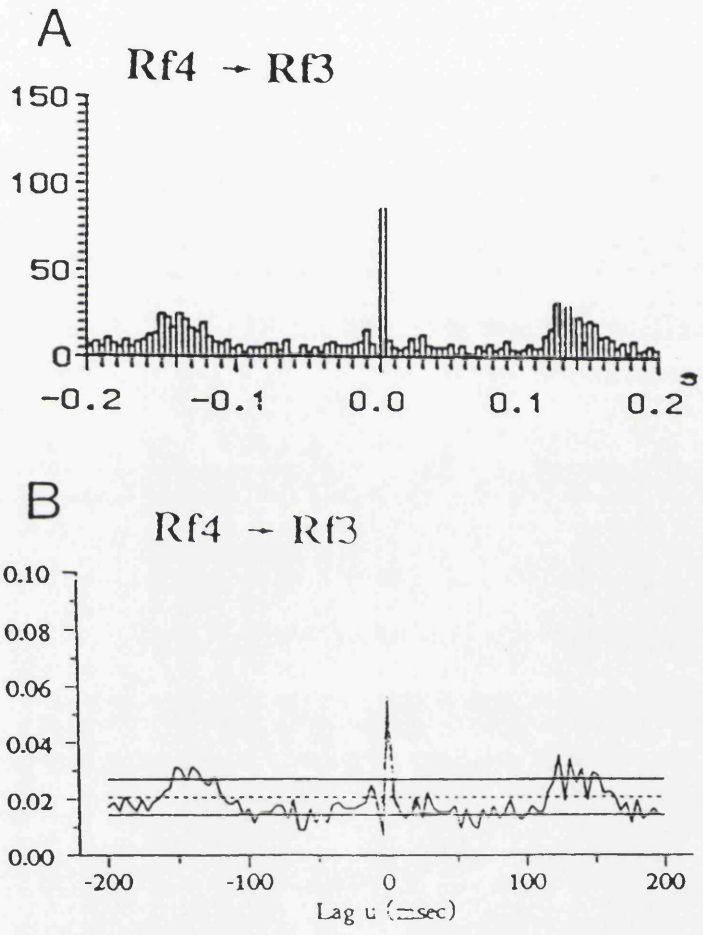

A
Rf4 → Rf3
150
100
50
0
−0.2
−0.1
0.0
0.1
0.2
s
B
Rf4 → Rf3
0.10
0.08
0.06
0.04
0.02
0.00
-200
-100
0
100
200
Lag u (msec)

Figure 3.6

Template matching.

Channel A contains raw data.

Channel B contains only one of the tonic motor neurones after the file has been template matched. Note the regularity of firing of the motor neurone.

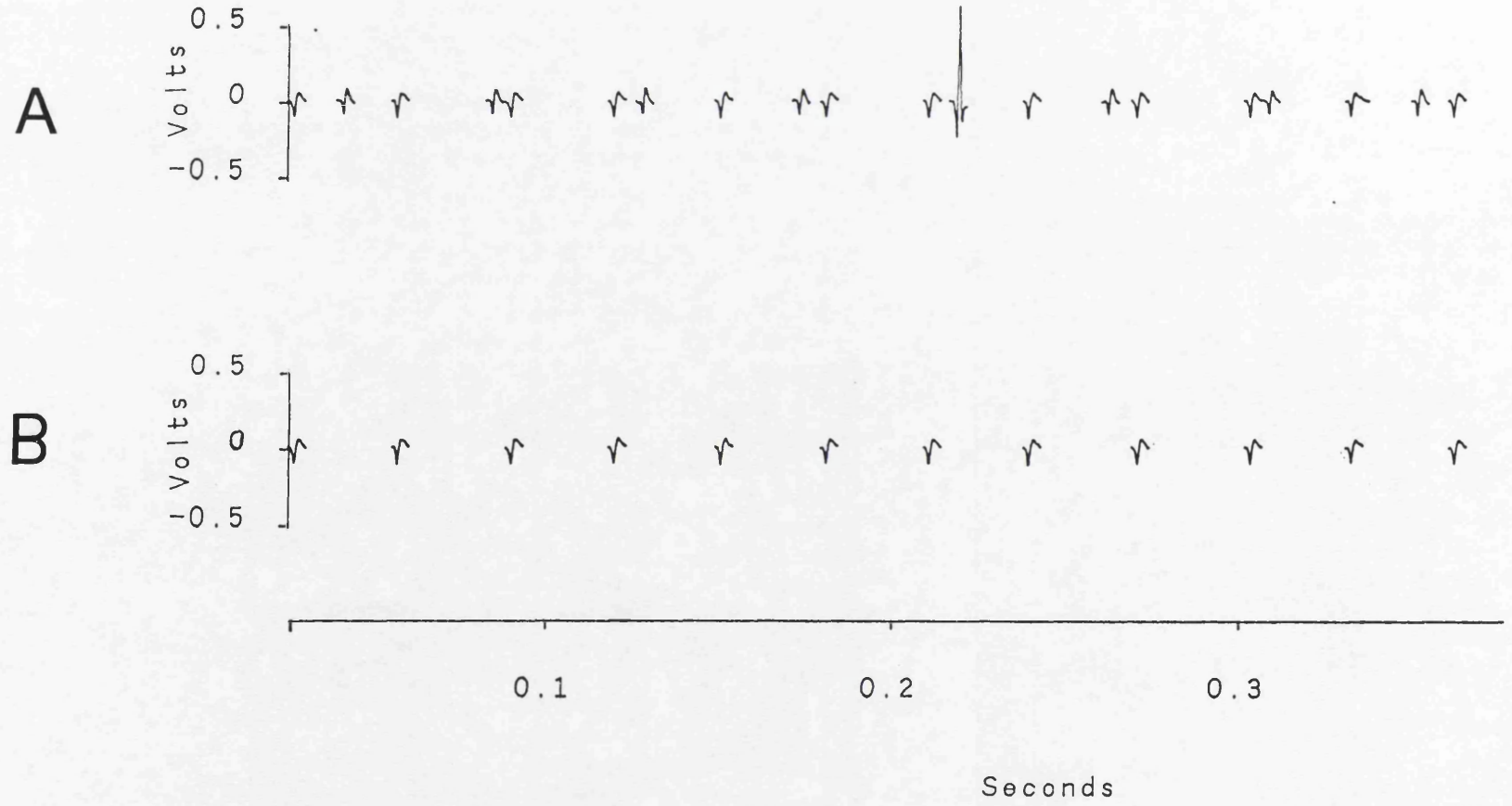

A
B
0.5
0
-0.5
Volts
0.1
0.2
0.3
Seconds

Figure 3.7

Camera lucida drawing of a cobalt backfill of one superficial flexor root. The backfill shows staining in six motor neurones, labelled according to size of soma. Five of these are located in the ganglion anterior to the Sr3; three lie ipsilaterally within the ganglion (f2, f4 and f6), the other two are contralateral (f3 and f5). The remaining motor neurone, f1, has its cell body located contralaterally within the posterior ganglion. Mag. = x10

G2, G3 = abdominal ganglion 2 and 3

Scale bar = 200 μm

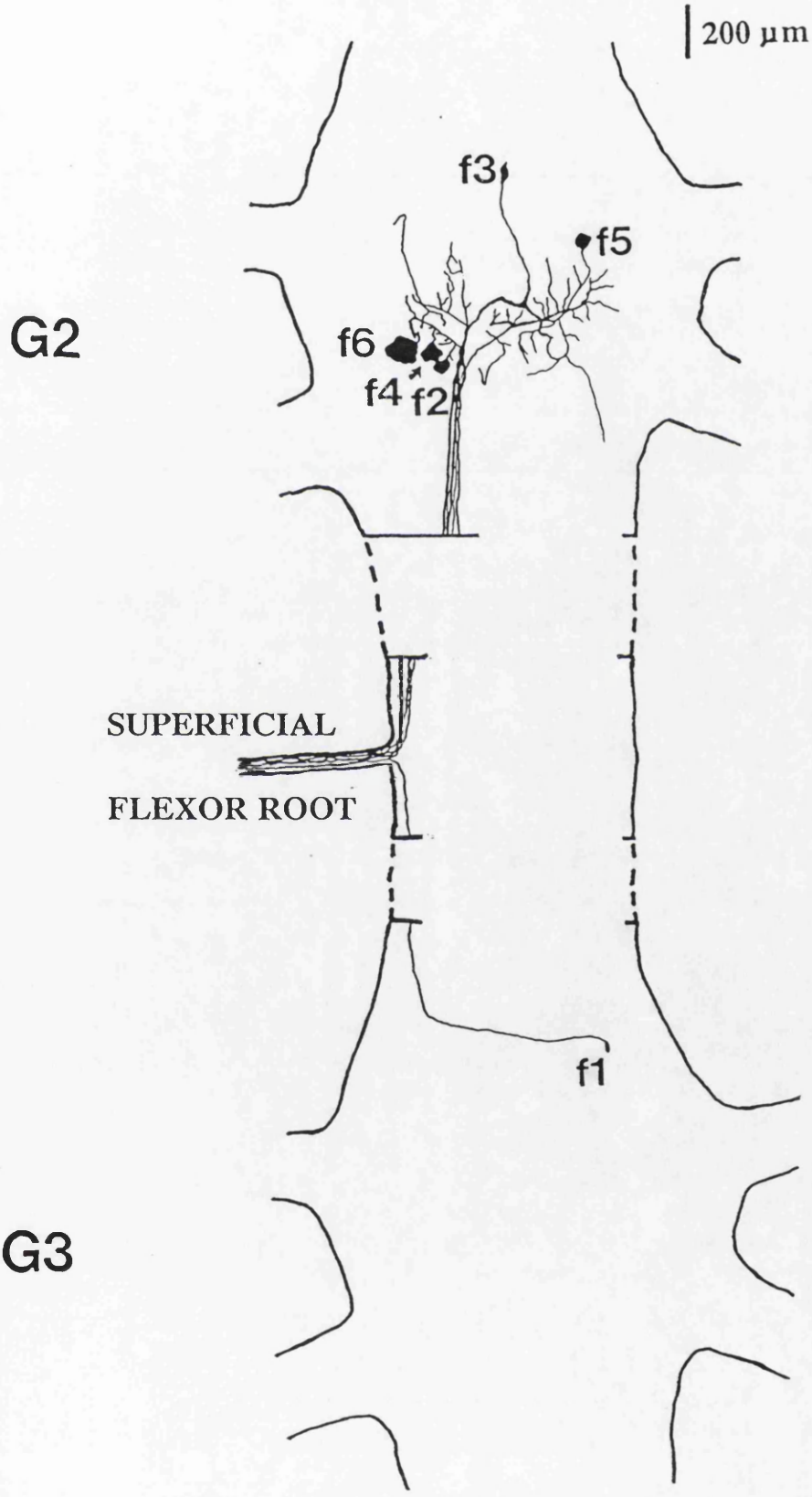

200 µm
G2
f3
f5
f6
f4
f2
SUPERFICIAL
FLEXOR ROOT
f1
G3

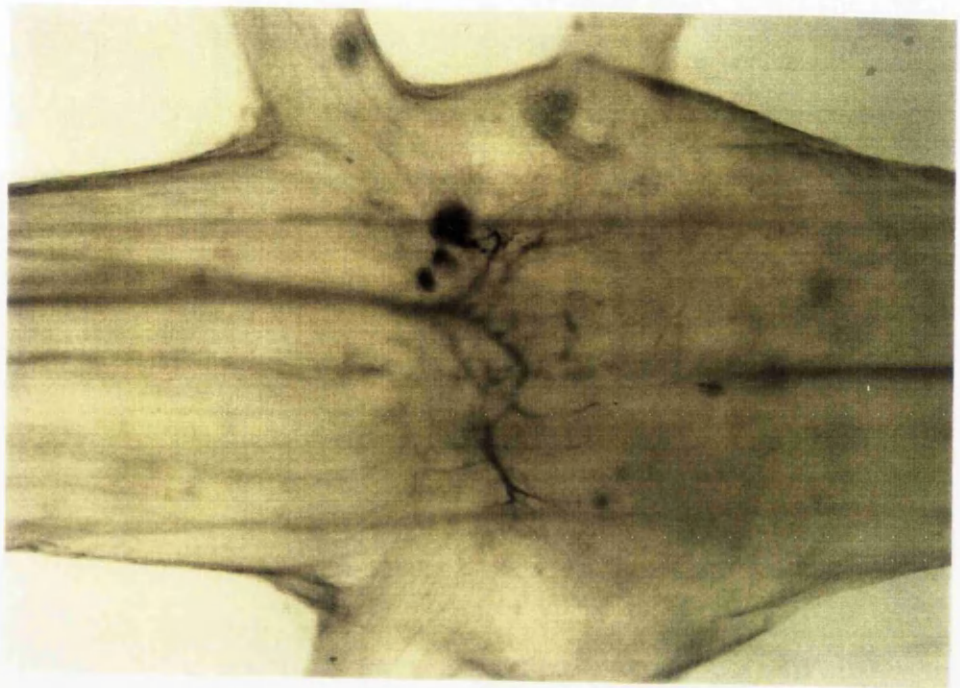
Figure 3.8

Comparison of photograph of cobalt backfill of a single Sr3 (A) with camera lucida drawing (B). The ability to focus through different planes of the ganglion while constructing camera lucida drawings allowed the discrimination of fine detail which was not visible in photographs taken at one focal depth.

Mag. = x10

Scale bar = 200 μm

A



B

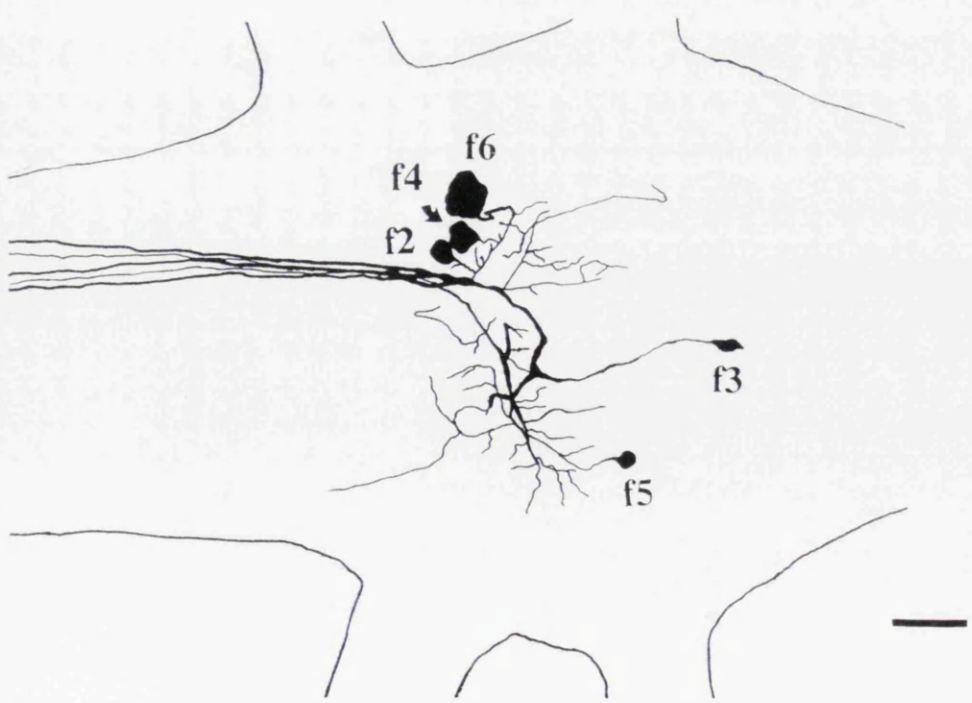


Figure 3.9

Fine detail of cobalt backfilled structures.

B. Magnified view of soma of three motor neurones f2, f4 and f6, seen in **A**. Plane of photograph is focussed on f6 which has a diameter of approximately 75 μm.

D. Magnified view of backfilled axons of a Sr3 as they leave the connective and travel together in the Sr3. The solitary axon of f1 ascending from the posterior ganglion is clearly seen.

Scale bar **A, C** = 200 μm. Mag. = x10

Scale bar **B, D** = 50 μm. Mag. = x40

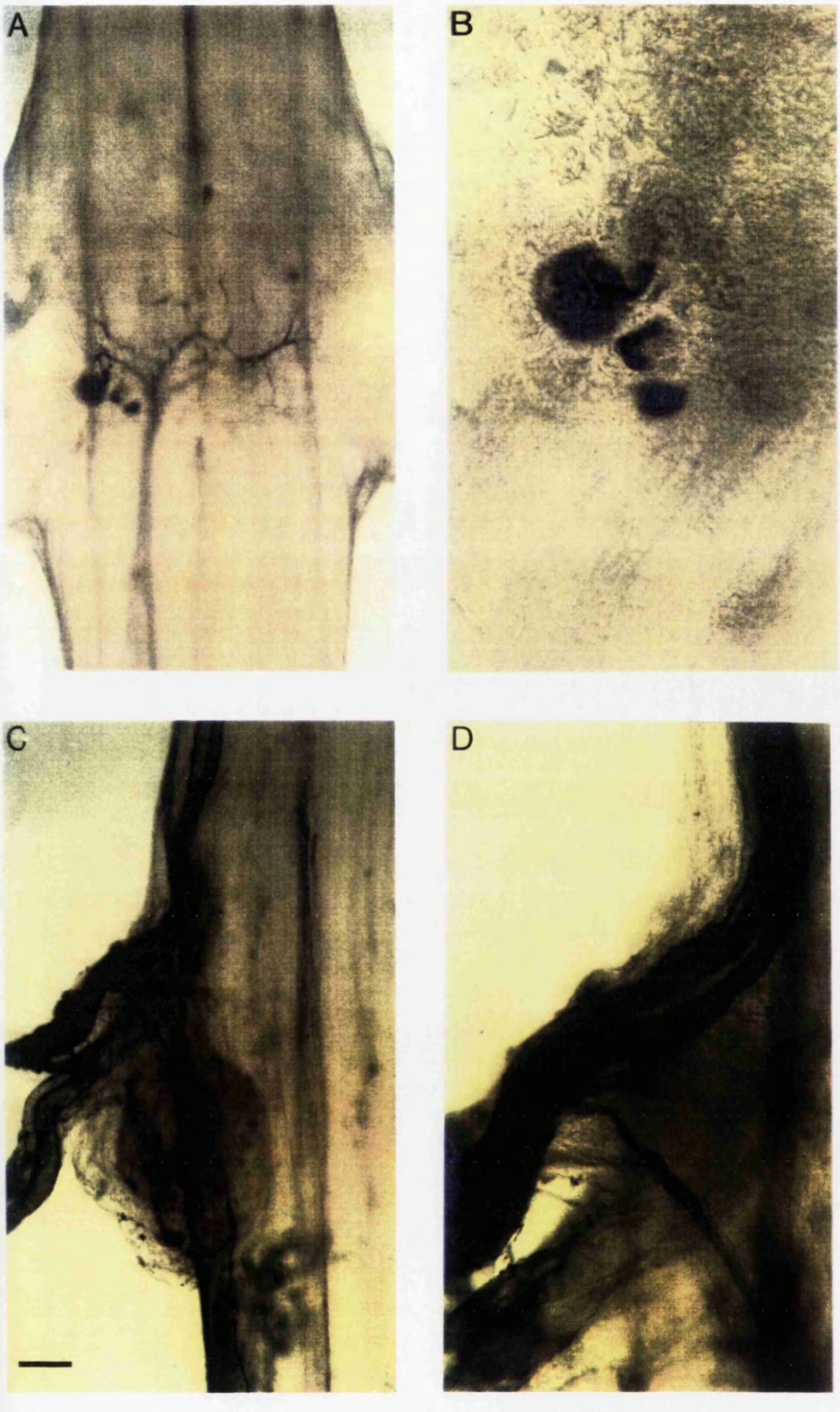

A
B
C
D

Figure 3.10

A. Camera lucida drawing of a backfill of both superficial roots of the 2nd abdominal ganglion of a crayfish. All ten Sr3 flexor motor neurones were filled (both f1s not seen as they are in the ganglion below). For ease of comparison, neurones with axons in the right Sr3 have been darkened whereas only the soma outline of neurones with axons in the left Sr3 has been drawn.

B. Camera lucida drawing of another crayfish cobalt backfill in which both Sr3s anterior and posterior to abdominal ganglion 2 were filled. This enabled a soma map of all twelve superficial flexor motor neurones in one ganglion to be constructed. Identification of left or right motor neurones as explained in **3.10A.**

Scale bar = 100 μm for both photographs

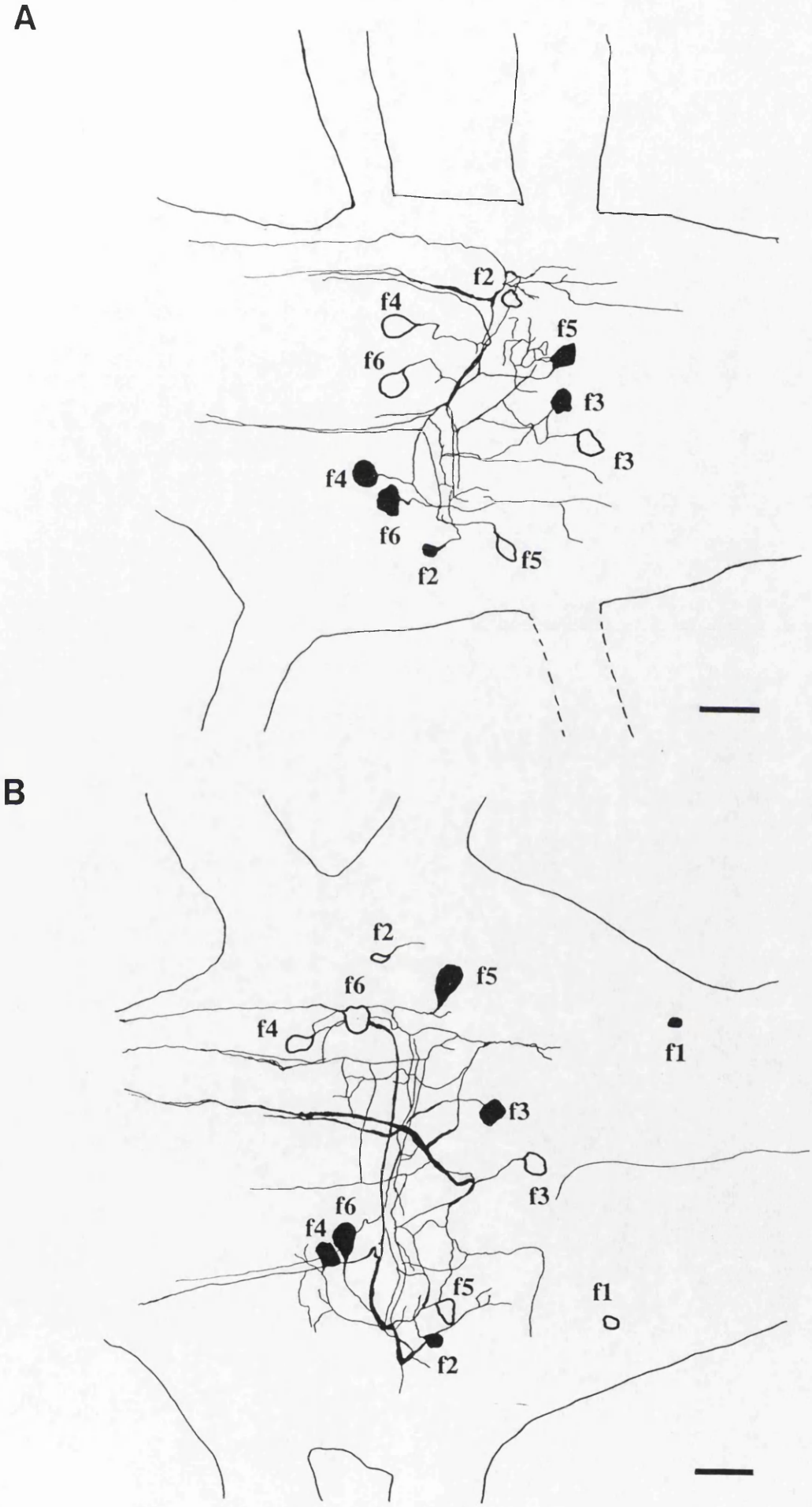
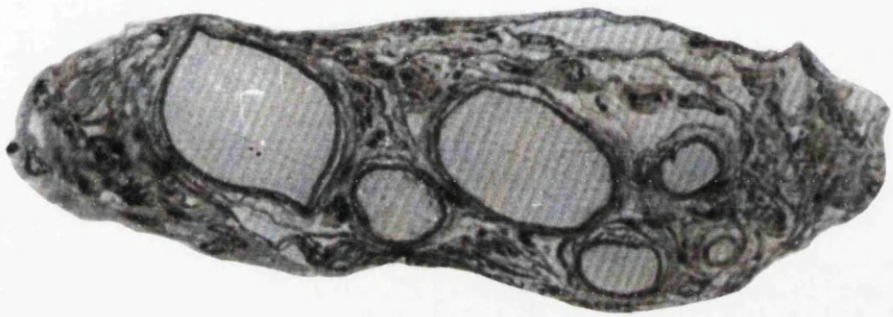

A
f2
f4
f5
f6
f3
f3
f4
f6
f5
f2
B
f2
f6
f5
f4
f1
f3
f3
f6
f4
f5
f1
f2

Figure 3.11

Cross section of Sr3 of 2nd abdominal ganglion. Six axons can be seen which have been labelled 1 - 6 according to increasing axon diameter.

Scale bar = 10 μm



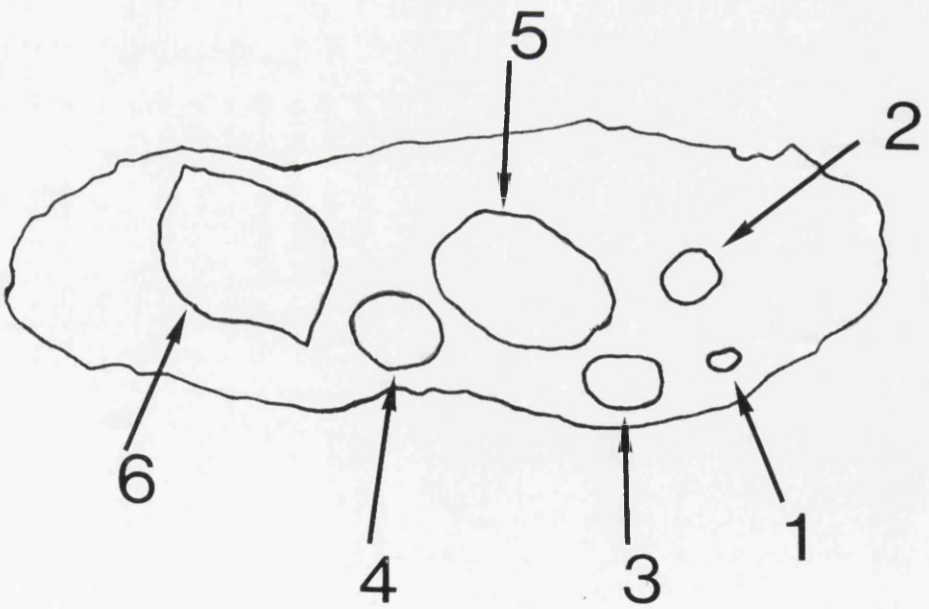

5
2
6
4
3
1

Figure 3.12

A. GABA-stained cross section of the 2nd abdominal ganglion of a Norway lobster. MGF-medial giant fibres, LGF-lateral giant fibres. Scale bar = 100μm

B. GABA-stained cross section of a Sr3. The second largest axon profile is stained.

C. GABA-stained cross section of the branch of the third root which innervates the fast flexor muscles. One GABA-stained profile is visible here too.

Bottom scale bar (25 μm) applies to both **B** and **C**.


MGF
A
LGF
B
C

Figure 3.13

By cutting serial sections, the path of the GABA-stained axon, f5, within the Sr3 can be traced as it travels through the connective towards the ganglion. The paths of both the superficial flexor inhibitor, f5, (black arrows) and the fast flexor inhibitor (white arrows) are shown in this figure.

Distance between sections shown in different figures is as follows:

A - B = 300 μm

B - C = 70 μm

C - D = 180 μm

D - E = 30 μm

Scale bar = 100μm

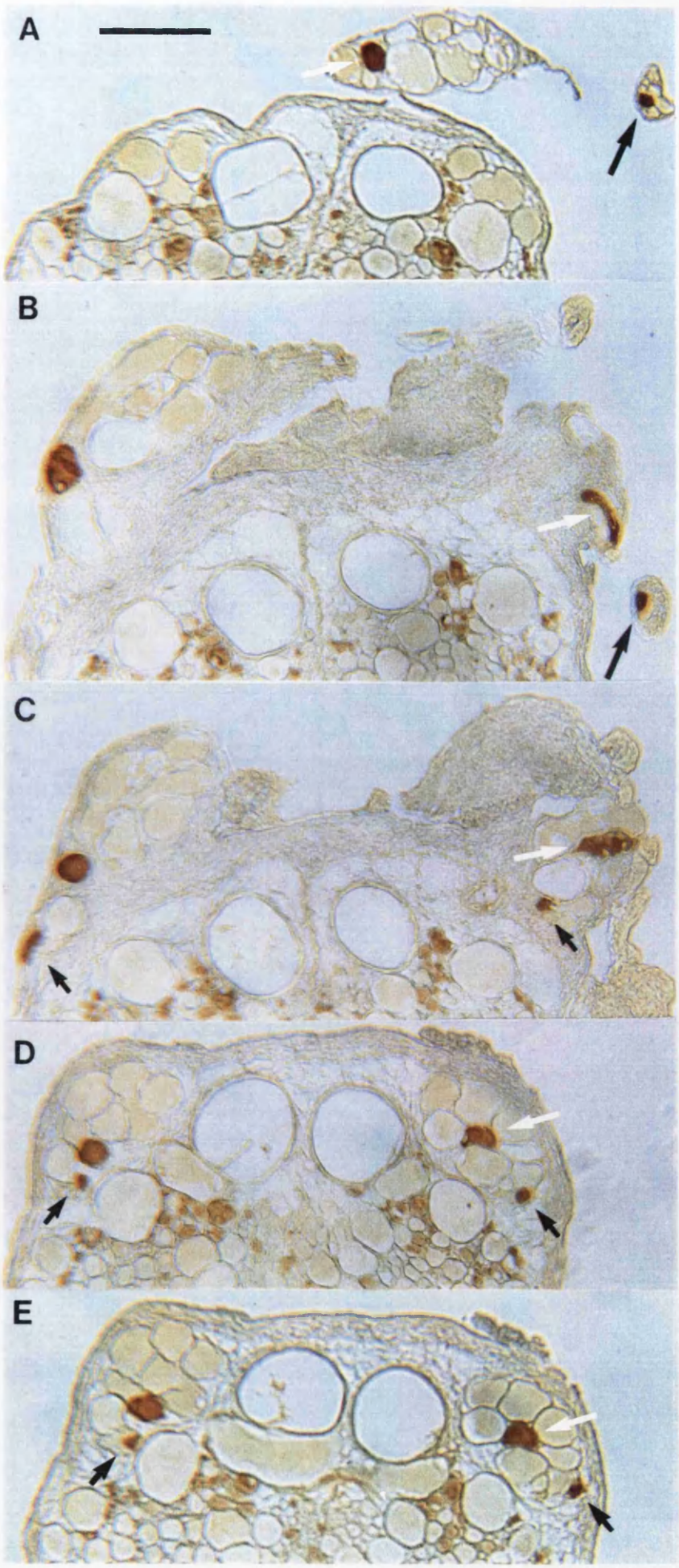

A
B
C
D
E

Figure 3.14

Identification of six classes of motor neurone according to the amplitude of extracellularly recorded spikes.

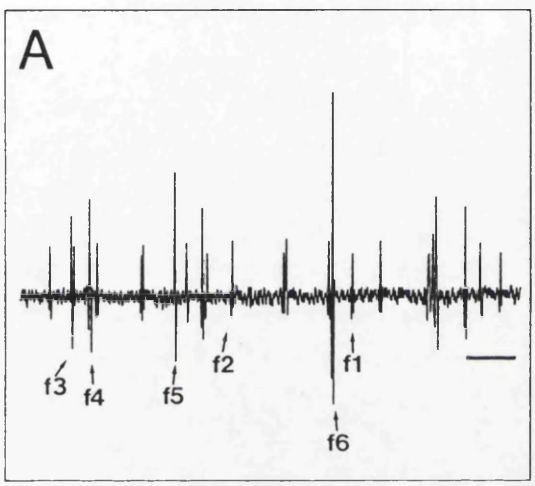
A. All six axons are firing.

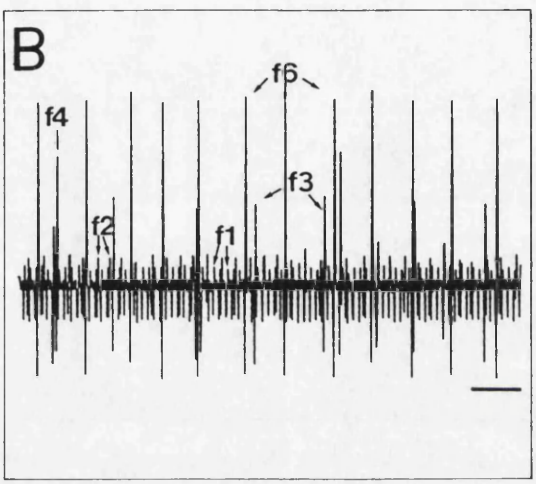
B. All axons are firing except f5. The frequency of firing of f6 is unusually high in this example.

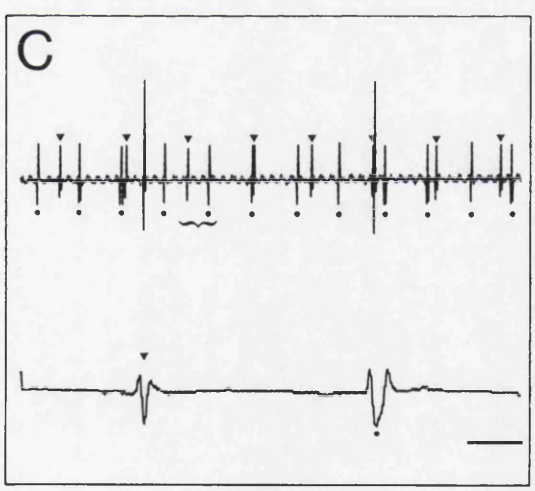
C. An example of an extracellular record (upper trace) in which f1 (triangle) and f2 (dot) could not be successfully separated using window discrimination or C.E.D software. Template matching is able to discriminate between the different spikes on the basis of spike shape (lower trace).

D. The extracellular recording (upper trace) shown in this example goes beyond the limits of separation of even the template matching system as the motor neurones, f1 and f2 are so similar in both size and shape (lower trace).

Scale bar = A. - 50 ms, B. - 250 ms, C. and D. upper trace - 100 ms, lower trace - 5 ms.


A
f3
f4
f5
f2
f1
f6


B
f6
f4
f3
f2
f1


C

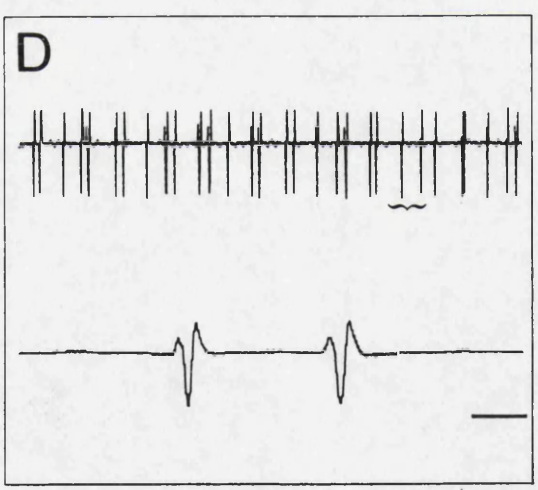

D

Figure 3.15

In the opposite figure the lower record is an expanded view of part of the upper record. Simultaneous extracellular recordings from the Sr3 supplying the superficial flexor muscle of the first abdominal segment of the lobster (middle trace) and intracellular recordings from a single motor neurone, f6, (upper trace) and a medial fibre in the muscle sheet (lower trace).

Scale bars for intracellular records; cell - 10 mV, muscle fibre - 5 mV, upper record - 1 second, lower record - 10 ms.

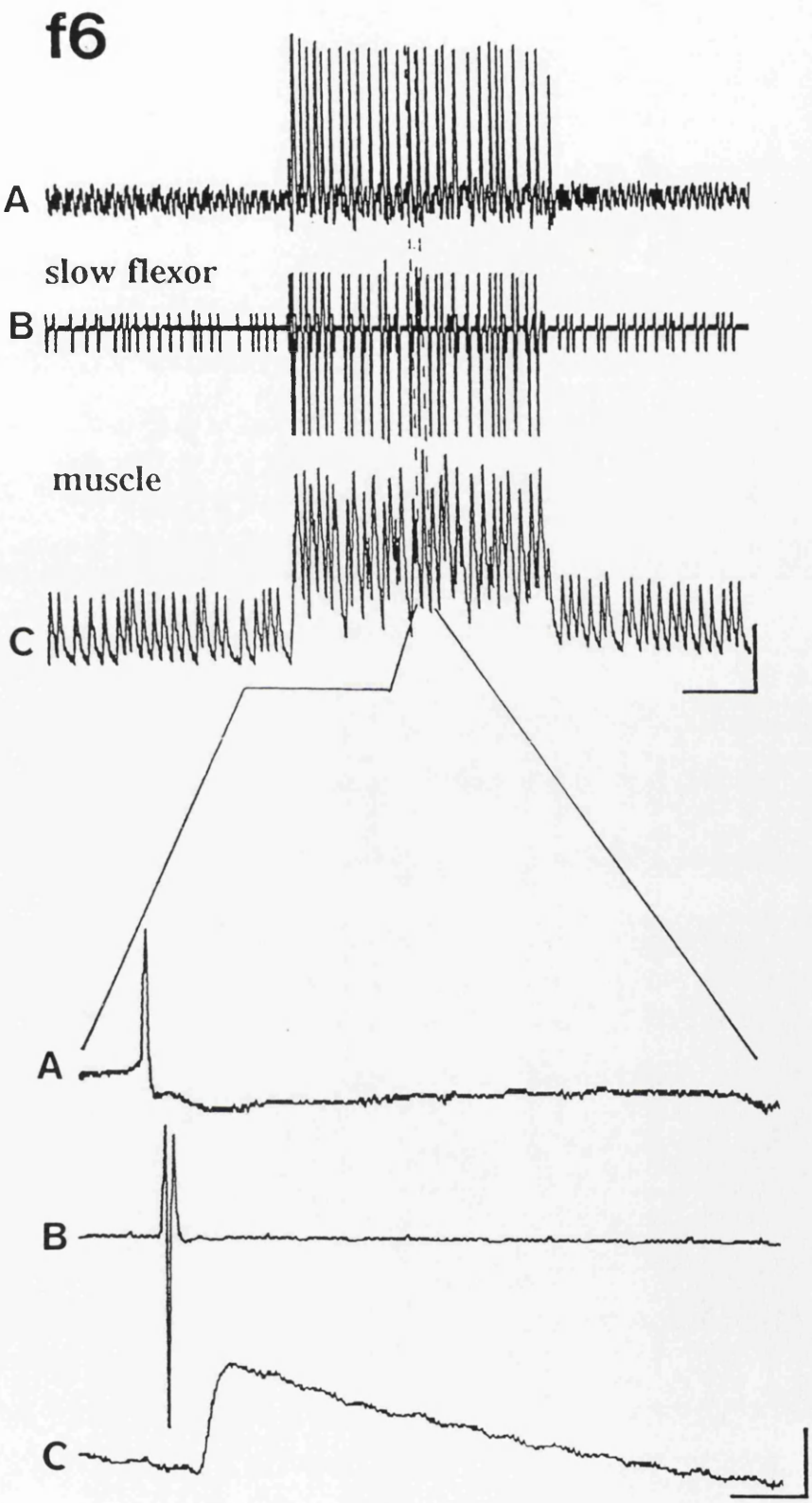

f6
A
slow flexor
B
muscle
C
A
B
C

Figure 3.16

Knowledge of the anatomical organisation of the central circuitry of the superficial flexor system is useful when identifying the smallest motor neurone, f1. This cell has its soma in the ganglion posterior to the Sr3. At the end of an experiment, it was possible to identify f1 by cutting the nerve cord connective below the Sr3 whose extracellular activity was being recorded.

The spontaneous activity of the smallest spike and its corresponding EJP in the record shown ceases after the connective has been cut (the other spontaneously active spike is f4).

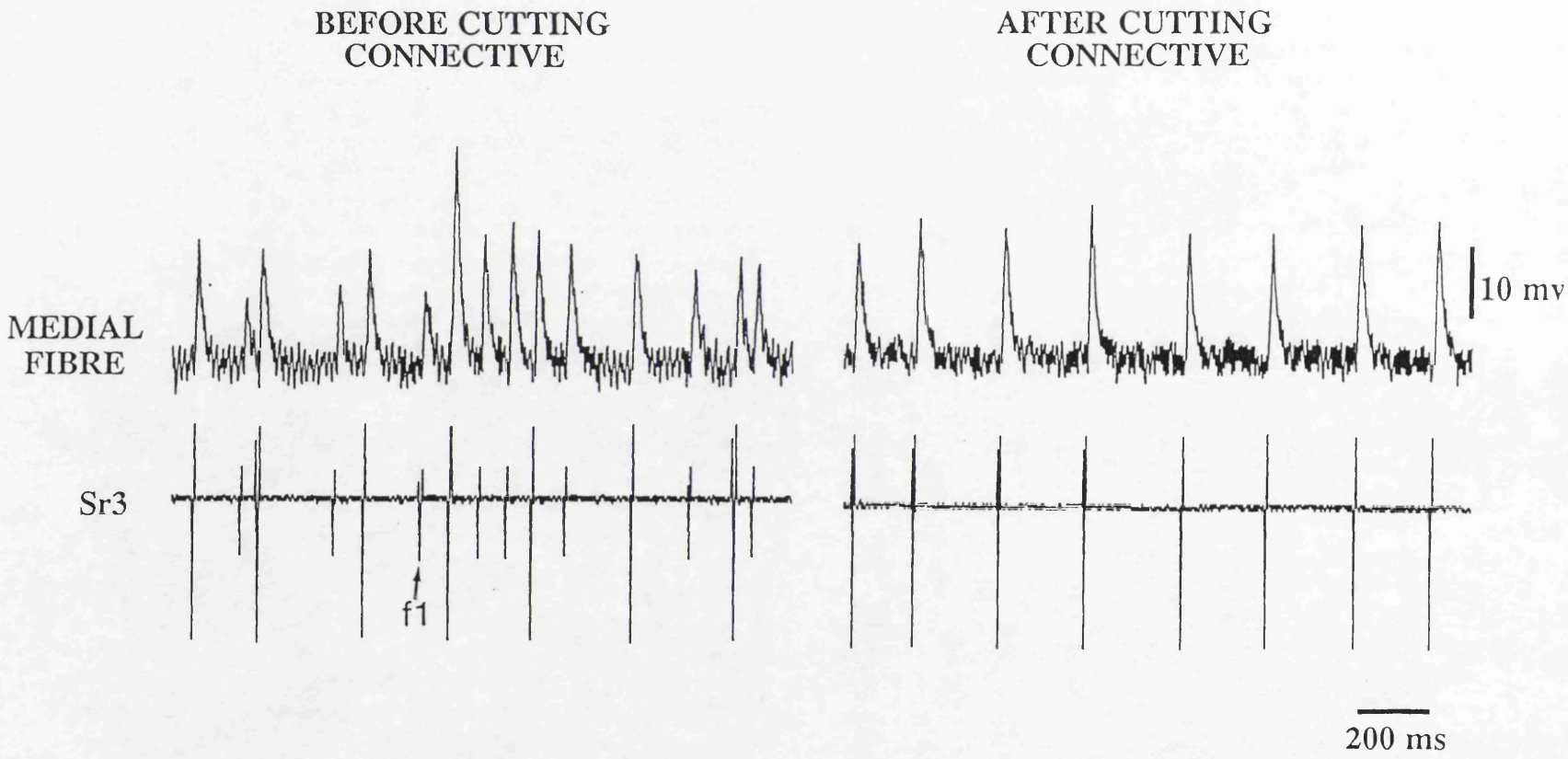

BEFORE CUTTING
CONNECTIVE
AFTER CUTTING
CONNECTIVE
MEDIAL
FIBRE
Sr3
f1
10 mv
200 ms

Figure 3.17

The SF inhibitor, f5, can be identified by matching its extracellular spike with an inhibitory junction potential (IJP) in the intracellular muscle record. A and B show records from one experiment in which the inhibitor was firing.

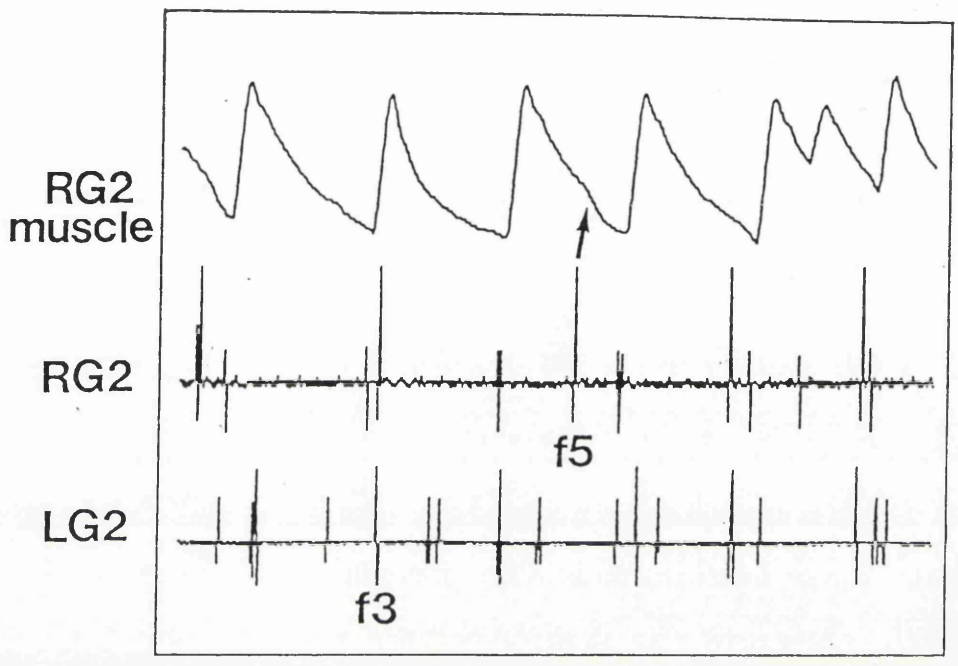
RG2 muscle = Intracellular recording from the right superficial flexor of the second segment.

RG2 = Extracellular recording from the right superficial flexor nerve of the second ganglion.

LG2 = Extracellular recording from the left superficial flexor nerve of the second ganglion.

Scale bar = 5 mV, 200 ms for upper trace; 10 mV, 500 ms for lower trace.

A



B

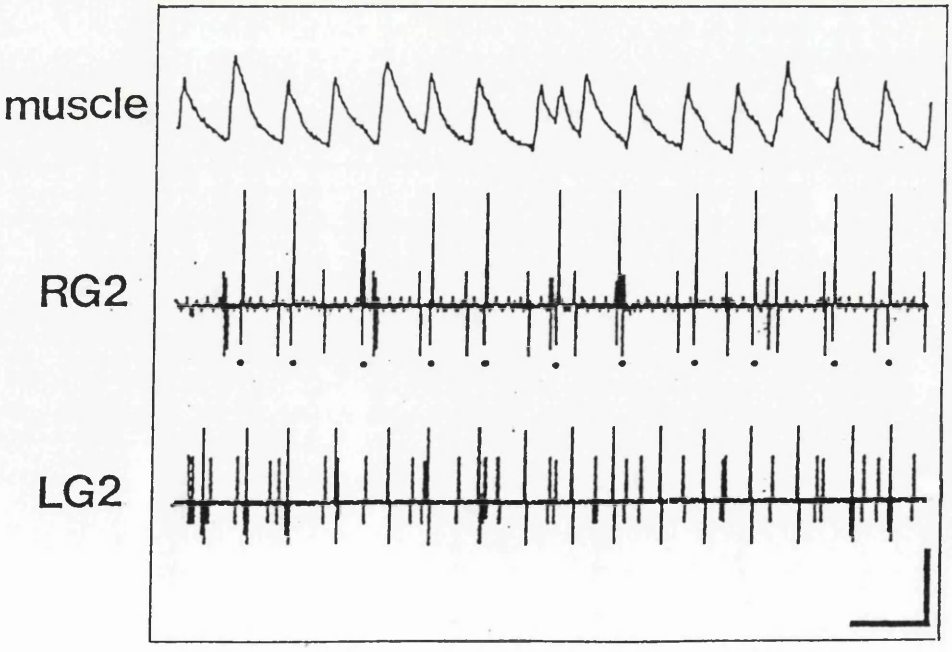


Figure 3.18

Demonstration of abdominal intrasegmental connections in the lobster ventral nerve cord.

Effect of cutting the abdominal ventral nerve cord connective at different places on the extracellular activity recorded from the right Sr3 of the third ganglion (black spot represents platinum extracellular electrode). The connective was cut at five different places in turn; A - E, respectively. Each record consists of two sections: (i) the immediate effect of cutting on the spontaneous activity recorded from the Sr3 and (ii) the effect on spontaneous activity in the Sr3 30 seconds after the cut was made.

All cuts were associated with immediate transient firing of the largest motor neurone, f6

A. The first cut was made between the 5th and 6th abdominal ganglia. This produced an increase in the rate of spontaneous activity of two of the three flexor motor neurones which were previously active: f1 and f4. The rate of firing of f3 was decreased.

B. The second cut between the 1st and the 2nd ganglia had the opposite effect to the first cut. After a brief period of firing of f6 followed by high frequency firing of f3, the rate of activity in all three motor neurones decreased to levels lower than before.

C. The third cut was made between the 4th and 5th ganglia. This produced a 2 second period of firing of f6 after which the rate of firing of both f1 and f3 was enhanced.

D. The fourth cut was made between the 2nd and third ganglia. This cut was much less effective in activating f6, only a few f6 impulses are seen. The immediate effect of cutting was to evoke high frequency activation of f3 and f4. 30 seconds after firing the rate of firing of f1. f3 and f4 was slightly elevated above levels prior to cutting.

E. The final cut was made between the 3rd and 4th ganglion posterior to the RSr3 of G3. This stimulated high intensity firing of f6, followed by high frequency firing of f3 which stopped altogether for a period of time before returning. Note that f1 is no longer firing as its posterior axon was severed by the cut.

Scale bar = 1 sec.

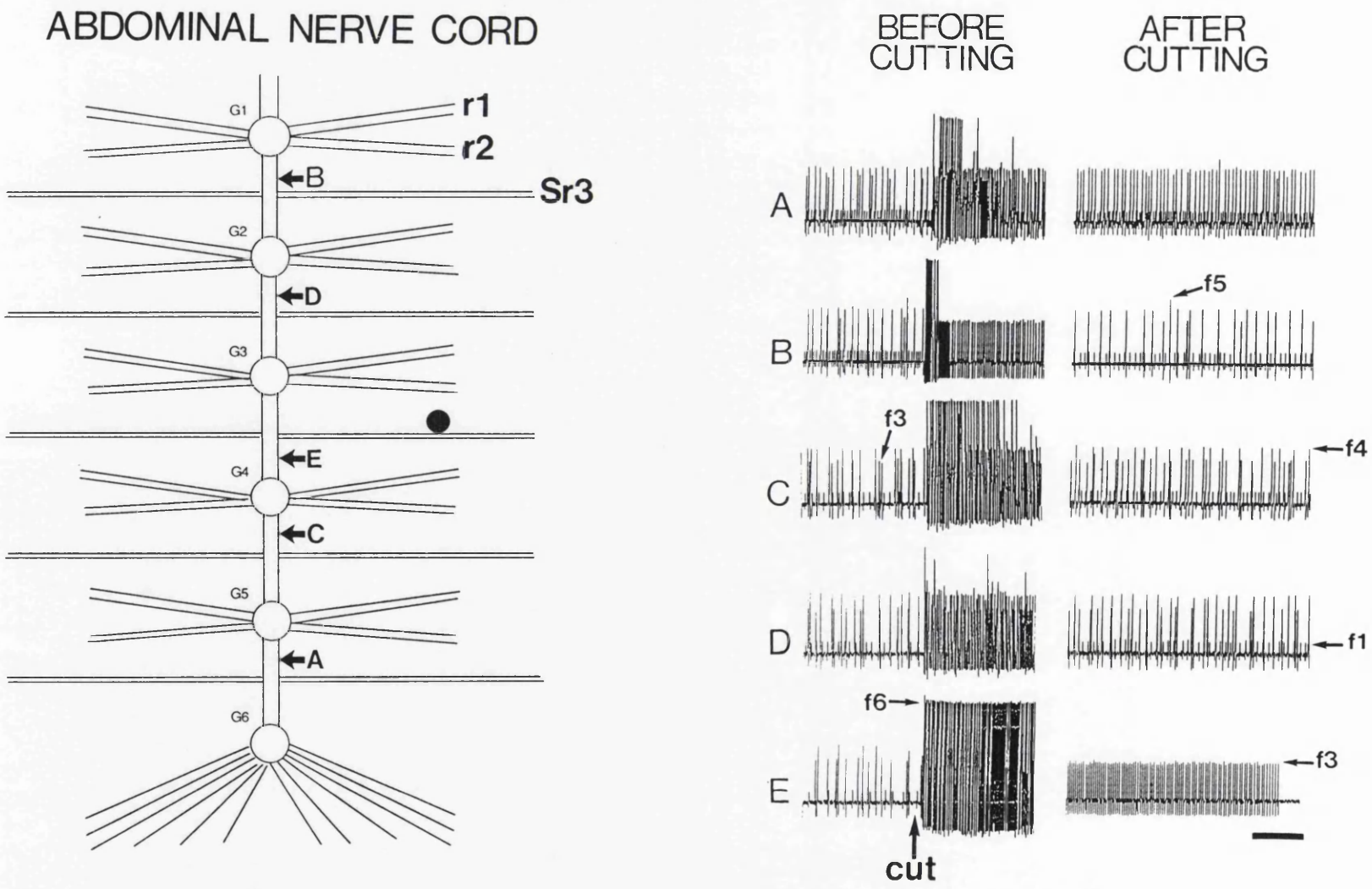

ABDOMINAL NERVE CORD
G1
G2
G3
G4
G5
G6
r1
r2
Sr3
B
D
E
C
A
BEFORE CUTTING
AFTER CUTTING
A
B
C
D
E
f5
f3
f4
f6
f1
f3
cut

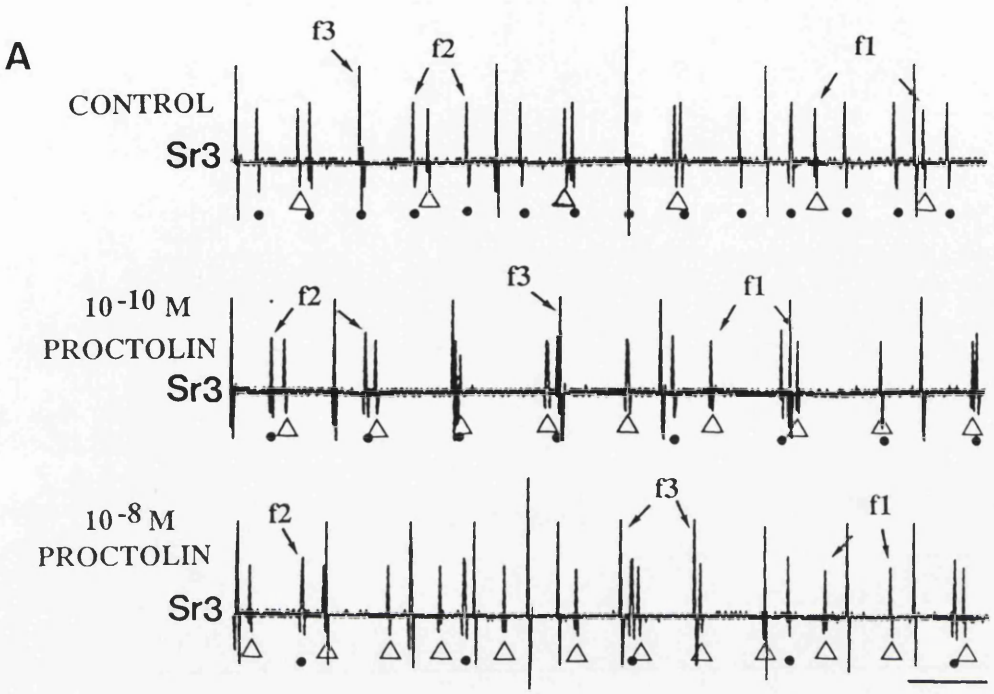
Figure 3.19

Dose-dependent effect of proctolin on the mean frequency of spontaneous firing of individual Sr3 motor neurones.

A. Extracellular record of the left Sr3 of the second abdominal segment showing the effect of proctolin on the individual motor neurones before and after proctolin (10^{-10} and 10^{-8} M). Firing of the two motor neurones, f1 (empty triangles) and f3 increased linearly with increasing concentrations of proctolin. The rate of firing of f2 (black dots), however, was significantly decreased by proctolin.

Scale bar = 500 ms.

B. Mean frequency data from above experiment.


A
CONTROL
Sr3
f3
f2
f1
10-10 M
PROCTOLIN
Sr3
f2
f3
f1
10-8 M
PROCTOLIN
Sr3
f2
f3
f1

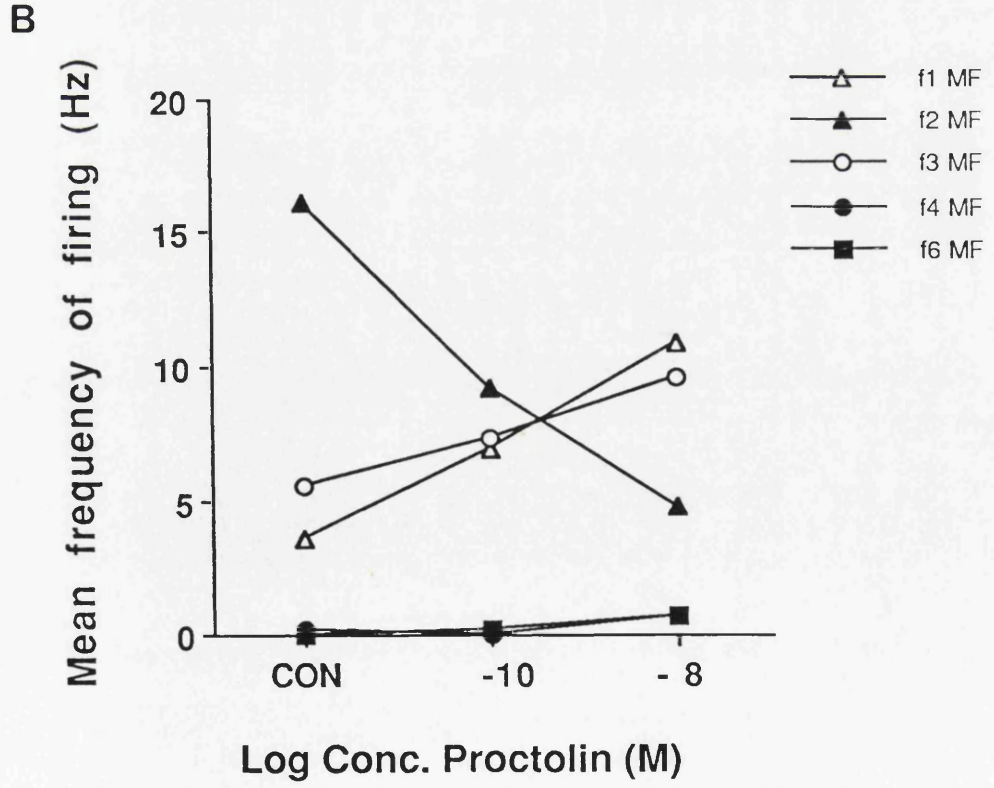
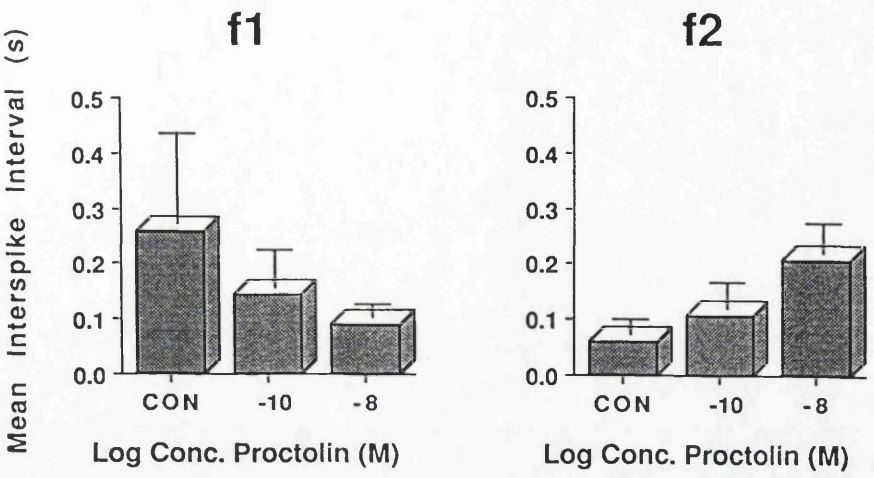

B
Mean frequency of firing (Hz)
20
15
10
5
0
CON
-10
- 8
Log Conc. Proctolin (M)
f1 MF
f2 MF
f3 MF
f4 MF
f6 MF

Figure 3.19C

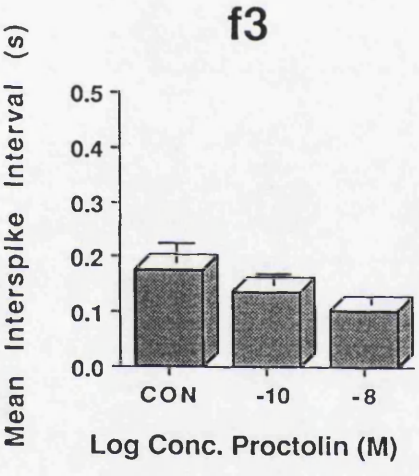
The graphs opposite have been constructed using the same data as **Figure 3.19A.** The mean interspike interval for the spontaneous activity of each motor neurone has been plotted.

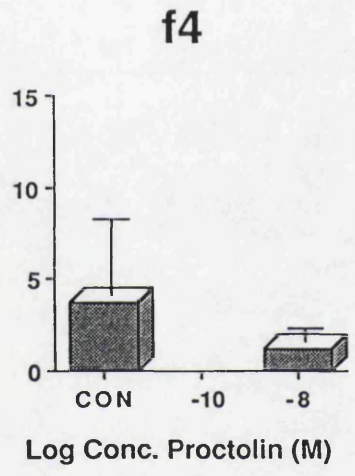
An increase in frequency of firing of a motor neurone is associated with a reciprocal decrease in the interspike interval of that motor neurone. The pattern of firing of the motor neurone is reflected by the standard deviation of the mean interspike interval. A small standard deviation reflects a regular firing pattern, whereas a large standard deviation reflects an irregular firing pattern.

Proctolin decreased the mean interspike interval for f1, f3, f4 and f6 (increased the mean frequency of firing) and also caused the pattern of firing to become more regular in these motor neurones. Conversely, the frequency of firing of f2 was decreased and this was associated with a less regular pattern of firing.


f1
Mean Interspike Interval (s)
0.5
0.4
0.3
0.2
0.1
0.0
CON
-10
-8
Log Conc. Proctolin (M)

f2
0.5
0.4
0.3
0.2
0.1
0.0
CON
-10
-8
Log Conc. Proctolin (M)


f3
Mean Interspike Interval (s)
0.5
0.4
0.3
0.2
0.1
0.0
CON
-10
-8
Log Conc. Proctolin (M)


f4
15
10
5
0
CON
-10
-8
Log Conc. Proctolin (M)

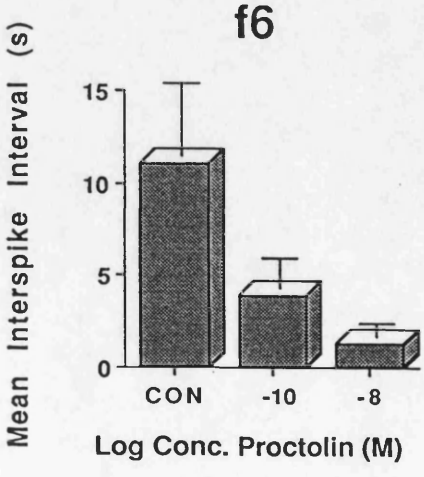

f6
Mean Interspike Interval (s)
15
10
5
0
CON
-10
-8
Log Conc. Proctolin (M)

Figure 3.20A

The data presented in Figures 3.20A and 3.20B was derived from extracellular recordings of both Sr3s from the second ganglion of one preparation. 3.20A shows the effect of proctolin on mean frequency of firing of individual motor neurones recorded extracellularly from the left Sr3.

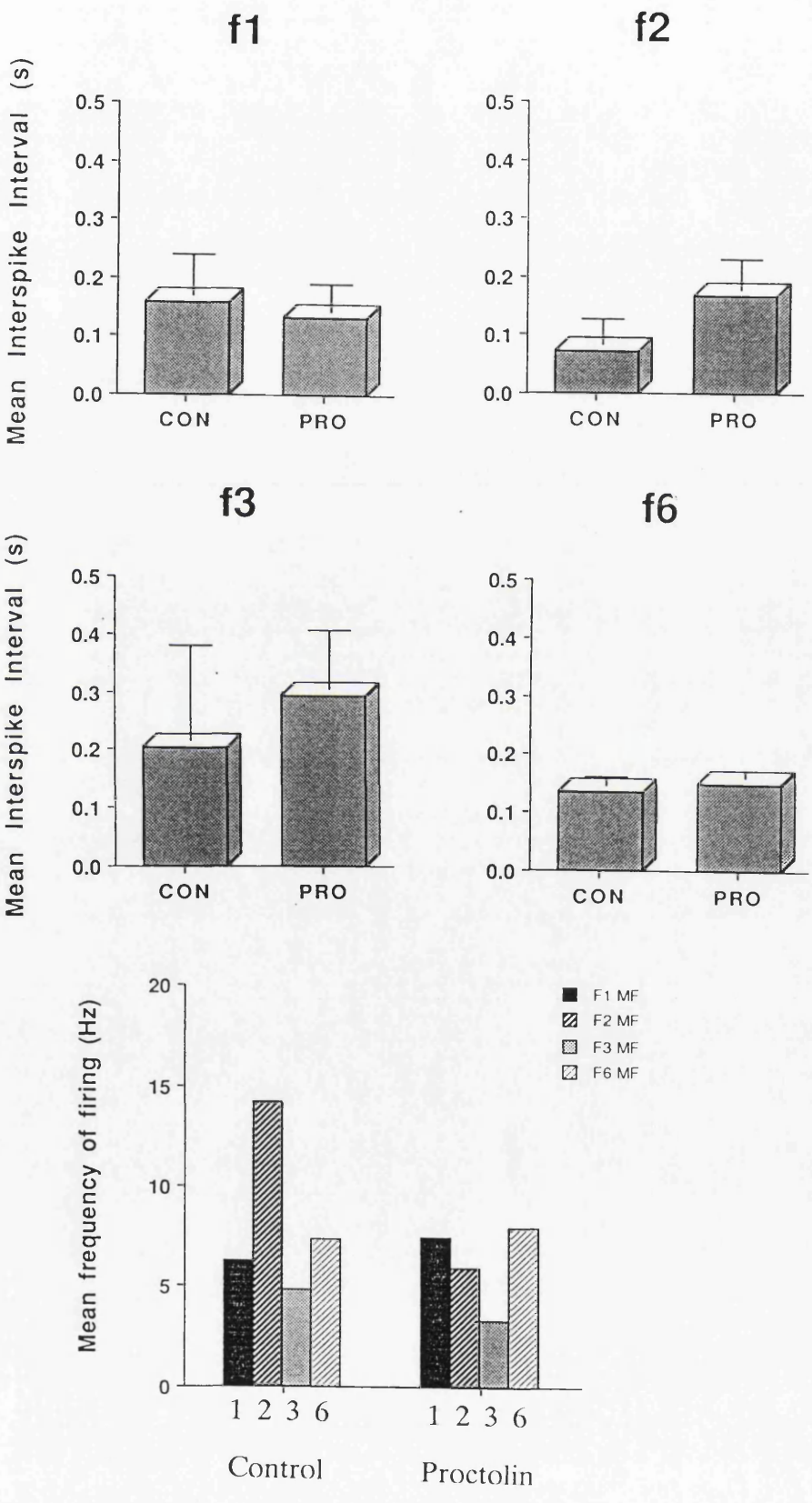

f1
f2
f3
f6
Mean Interspike Interval (s)
0.5
0.4
0.3
0.2
0.1
0.0
CON
PRO
Mean frequency of firing (Hz)
20
15
10
5
0
F1 MF
F2 MF
F3 MF
F6 MF
1 2 3 6
Control
Proctolin

Figure 3.20B.

Figure 3.20B shows the effect of proctolin on mean frequency of firing of individual motor neurones recorded extracellularly from the right Sr3.

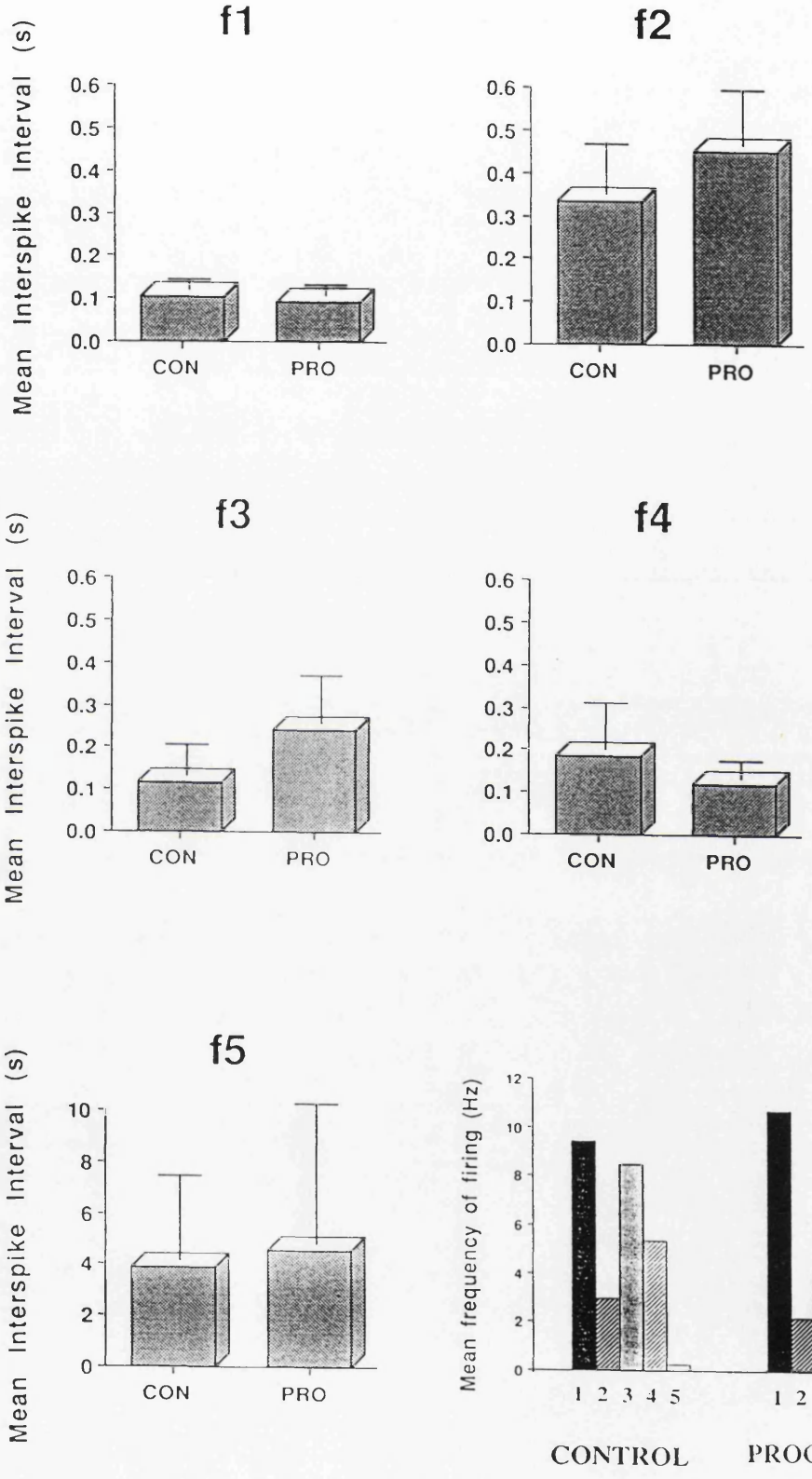

f1
f2
f3
f4
f5
Mean Interspike Interval (s)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
CON
PRO
10
8
6
4
2
0
Mean frequency of firing (Hz)
12
1 2 3 4 5
CONTROL
PROCTOLIN

Figure 3.21A

Effects of different neuromodulators on spike frequency in isolated ventral nerve cord preparations. Each trace shows spontaneous activity recorded extracellularly from the left/right Sr3 of the second ganglion.

CONTROL In the control situation, f1, f2 and f3 were spontaneously active.

PROCTOLIN Bath application of 10^{-6} M proctolin increased the rate of spontaneous firing of f1 and f3 and decreased the rate of spontaneous firing of f2.

OCTOPAMINE 10^{-6} M octopamine produced an entirely different pattern of firing to that observed in proctolin or in the control situation. Octopamine activated the inhibitor, f5 which was previously silent but totally inhibited the firing of f2. The firing of f1 and f3 were both increased.

SEROTONIN Perfusion of 10^{-6} M serotonin increased the rate of firing of the flexors f1, f2, f3 and f4 to above control levels. The inhibitor was silent in the presence of serotonin.

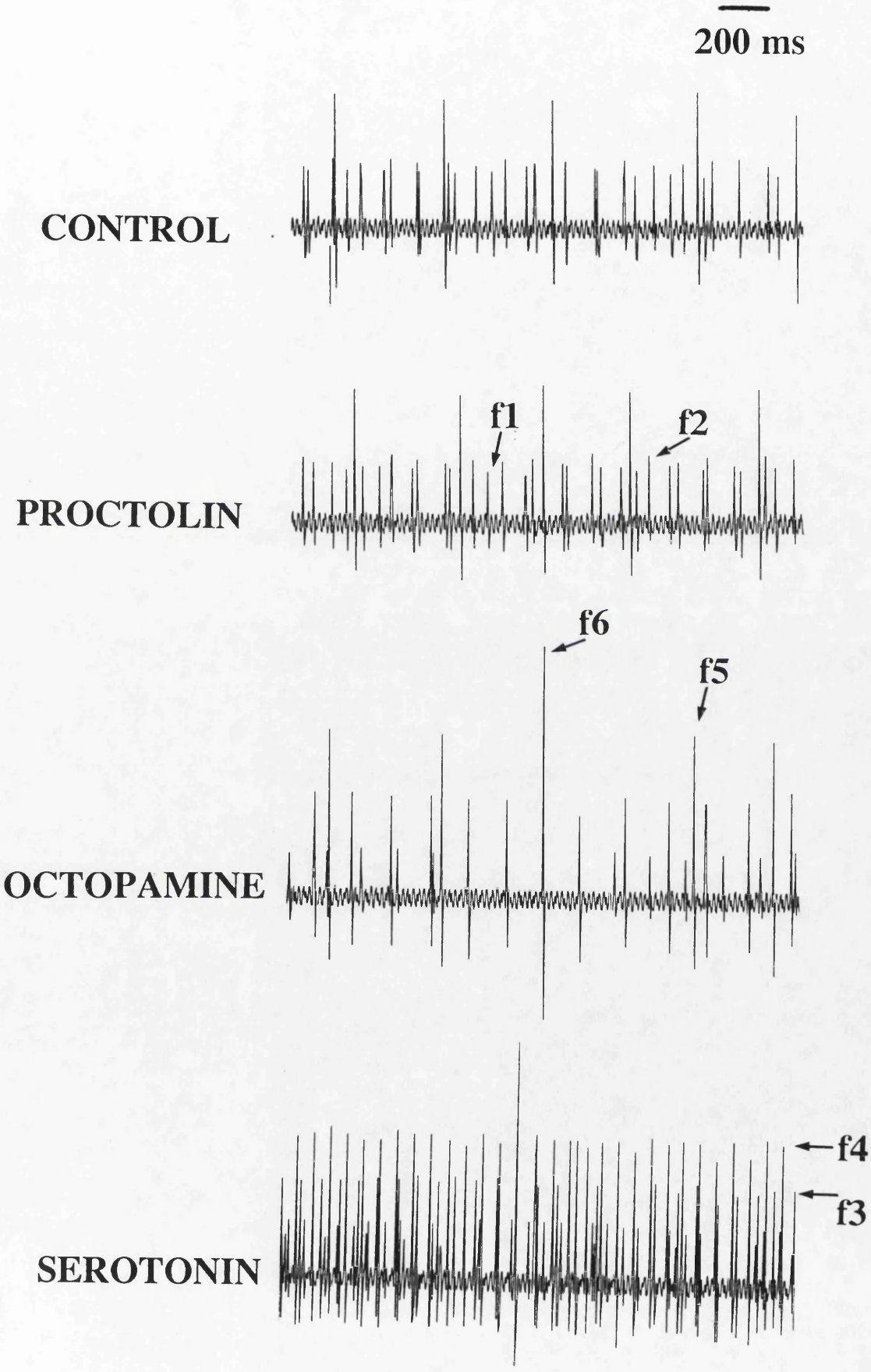

200 ms
CONTROL
f1
f2
PROCTOLIN
f6
f5
OCTOPAMINE
f4
f3
SEROTONIN

Figure 3.21B

Cumulative graph showing the effect of different neuromodulators on the mean frequency of spontaneous activity of individual motor neurones. Same data as shown in **Figure 3.21A**.

CON - control

PRO - proctolin

OCT - octopamine

5HT - serotonin

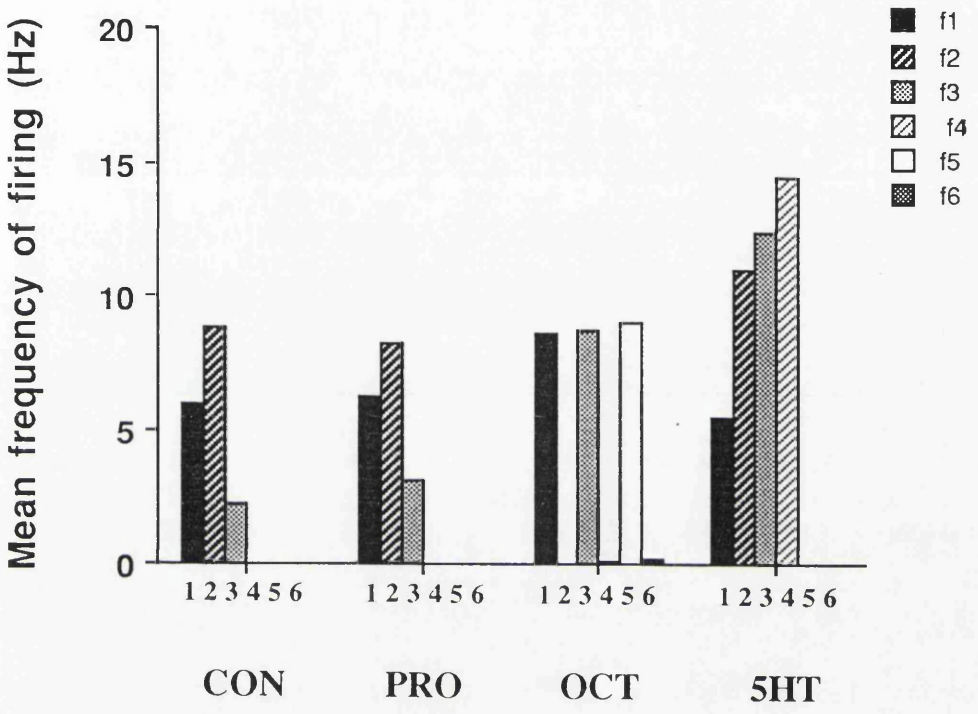

Mean frequency of firing (Hz)
20
15
10
5
0
f1
f2
f3
f4
f5
f6
1 2 3 4 5 6
1 2 3 4 5 6
1 2 3 4 5 6
1 2 3 4 5 6
CON
PRO
OCT
5HT

Figure 3.21C

Analysis of the effect of different neuromodulators on the mean interspike interval of individual motor neurones. Size of standard deviation bars is a measure of the regularity of firing of individual motor neurones.

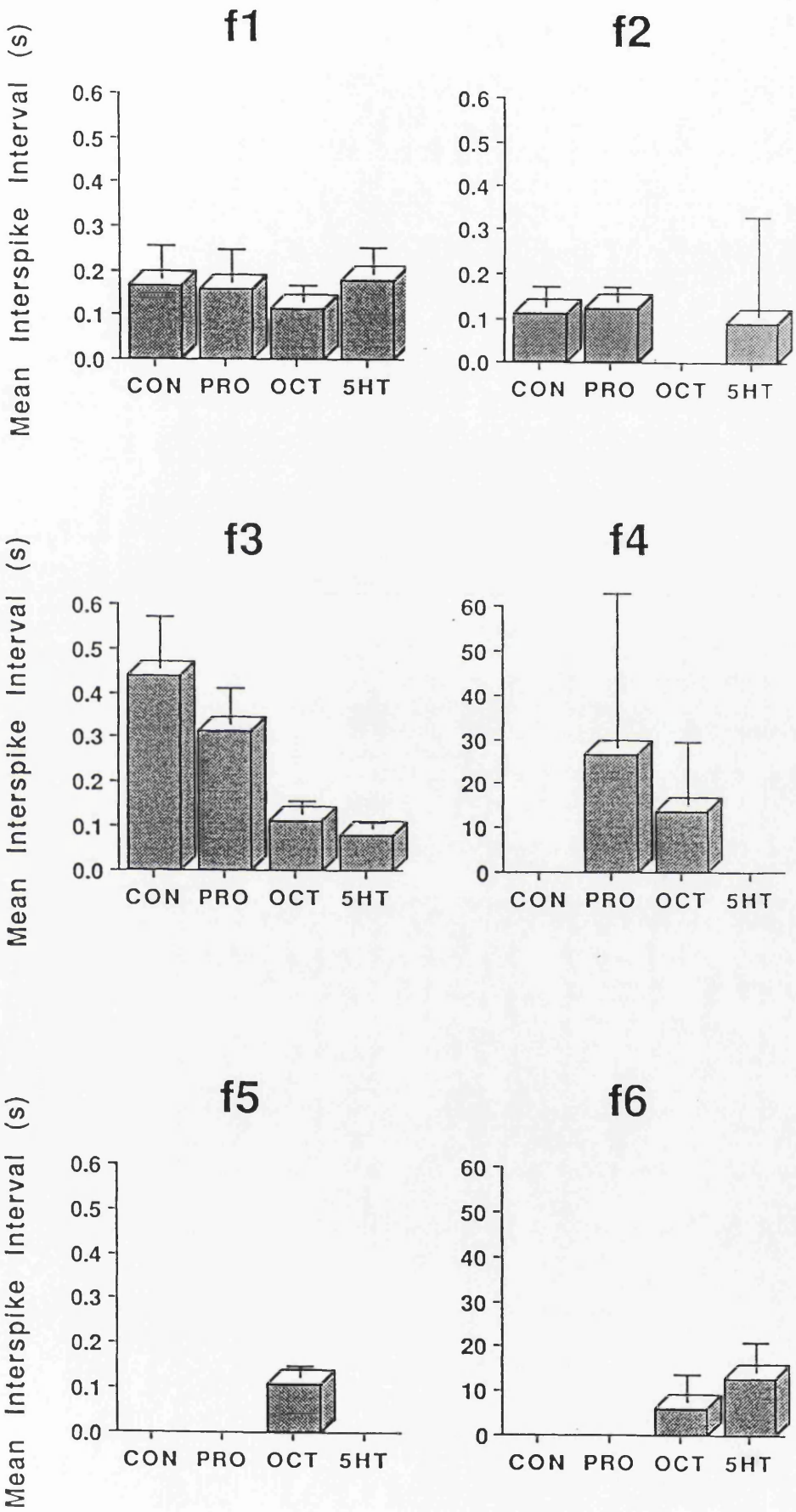

f1
f2
f3
f4
f5
f6
Mean Interspike Interval (s)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
10
20
30
40
50
60
CON
PRO
OCT
5HT

Figure 3.22A

Effect of proctolin, octopamine and oxotremorine on the mean frequency of spontaneous firing of individual motor neurones in the isolated abdomianl nerve cord preparation.

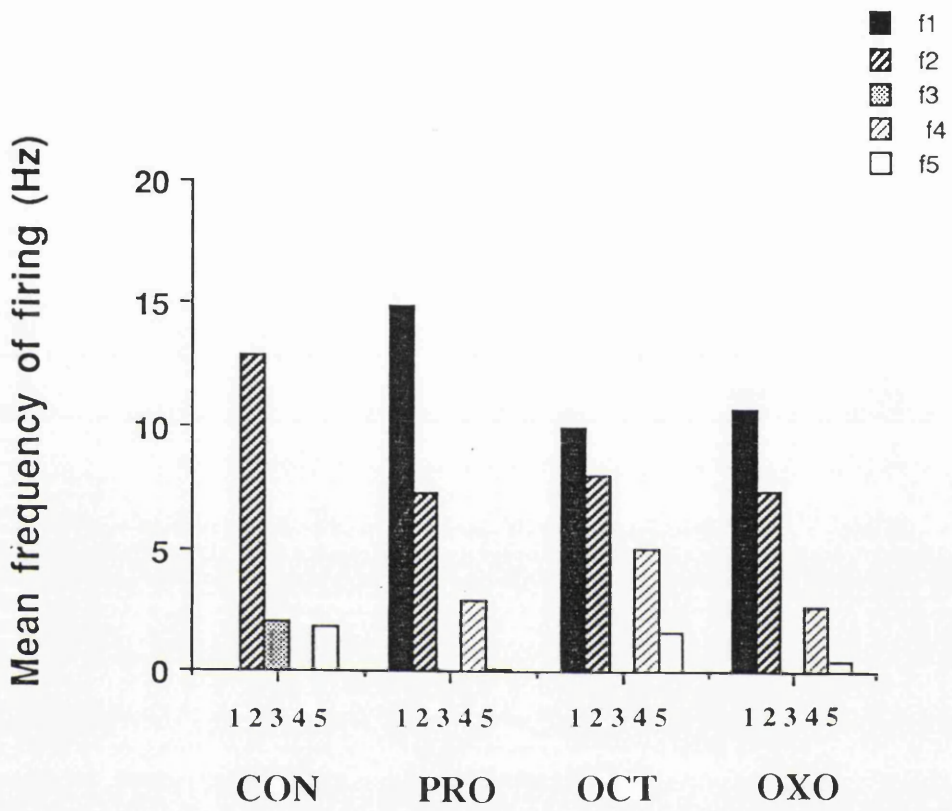

f1
f2
f3
f4
f5
Mean frequency of firing (Hz)
20
15
10
5
0
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
CON
PRO
OCT
OXO

Figure 3.22B

Effect of proctolin, octopamine and oxotremorine on the mean interspike interval of individual motor neurones. Size of standard deviation bars is a measure of the regularity of firing of individual motor neurones.

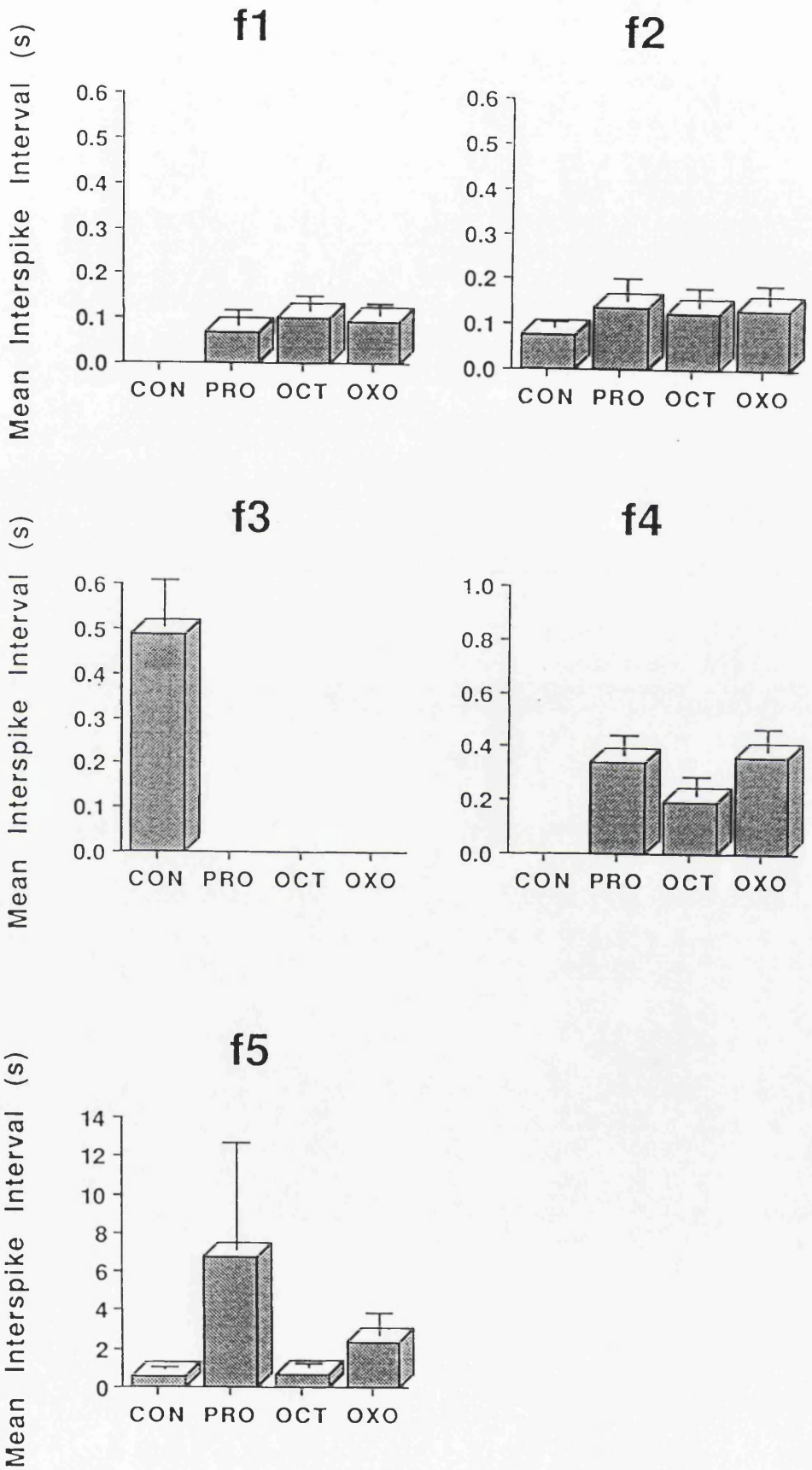

f1
f2
f3
f4
f5
Mean Interspike Interval (s)
Mean Interspike Interval (s)
Mean Interspike Interval (s)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1.0
0.8
14
12
10
8
6
4
2
0
CON PRO OCT OXO

Figure 3.23

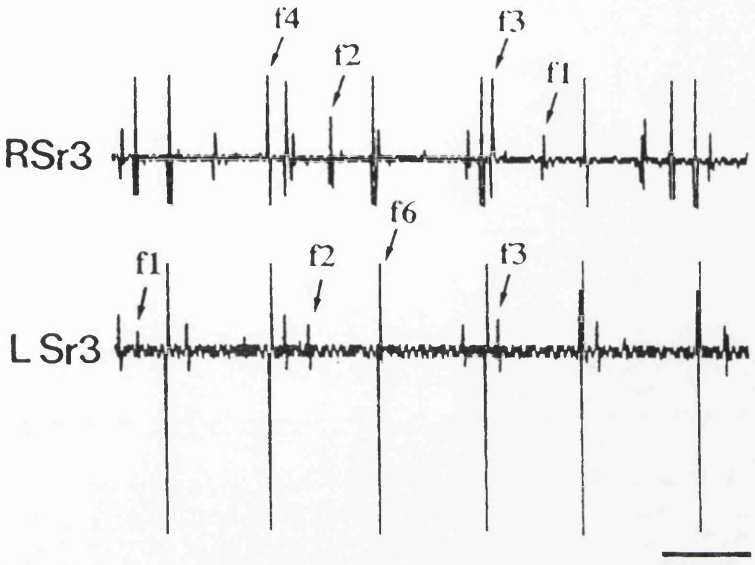
Positive correlation.

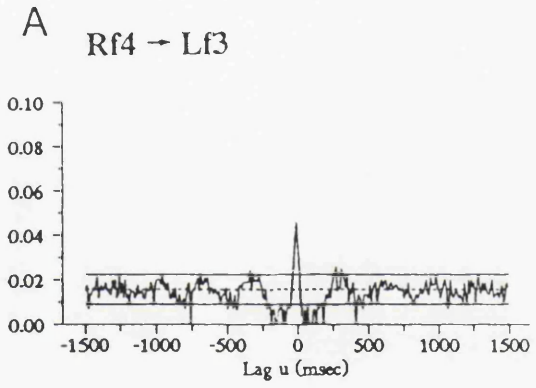
Cross-correlation analysis of the tonic-phasic flexor motor neurones and the phasic flexor motor neurones. The spike activity recorded from the right and left Sr3 of the third abdominal ganglion (experiment c2) was used to construct the analyses shown in **Ci, Cii, D** and **E**. Cross intensity estimates **A** and **B** were constructed using the data from experiment c1 (shown in **Figure 3.20**).

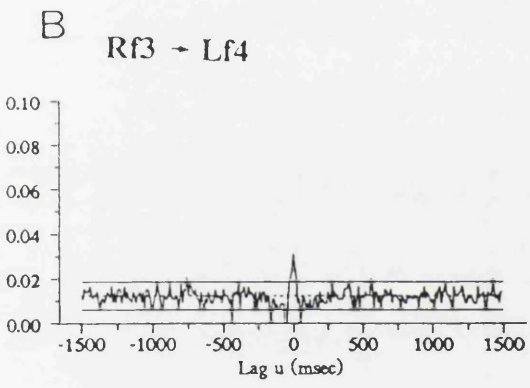
In these and the following cross intensity estimates, the stimulus is initiated at time 0 on the abscissa and the ~~number~~ of ~~events~~ is indicated by the ordinate.

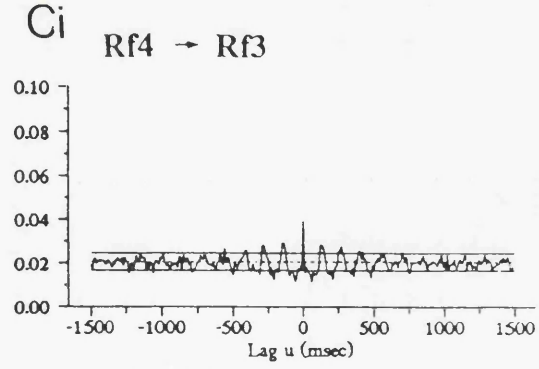
Rf4 is motor neurone, f4, whose axon is contained in the Sr3 innervating the SFM of the right hand side hemisegment (from a dorsal view). Similarly, the axon of Lf4 travels in the Sr3 which innervates the SFM of the left hemisegment. This nomenclature will be used from here on.

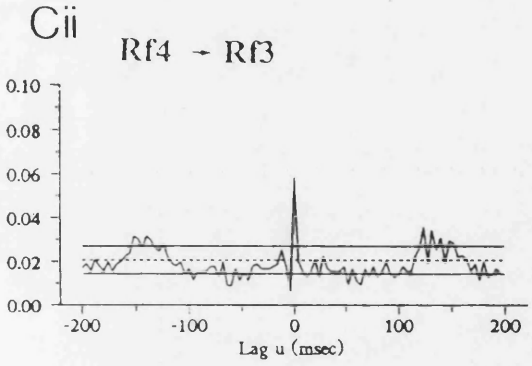
Scale bar = 200 ms.

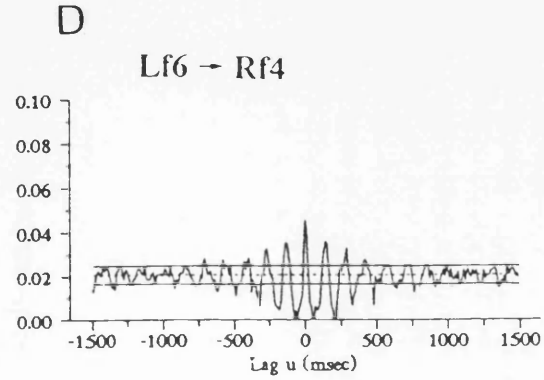

f4
f2
f3
f1
RSr3
f6
f1
f2
f3
L Sr3


A
Rf4 → Lf3
0.10
0.08
0.06
0.04
0.02
0.00
-1500 -1000 -500 0 500 1000 1500
Lag u (msec)


B
Rf3 → Lf4
0.10
0.08
0.06
0.04
0.02
0.00
-1500 -1000 -500 0 500 1000 1500
Lag u (msec)


Ci
Rf4 → Rf3
0.10
0.08
0.06
0.04
0.02
0.00
-1500 -1000 -500 0 500 1000 1500
Lag u (msec)


Cii
Rf4 → Rf3
0.10
0.08
0.06
0.04
0.02
0.00
-200 -100 0 100 200
Lag u (msec)


D
Lf6 → Rf4
0.10
0.08
0.06
0.04
0.02
0.00
-1500 -1000 -500 0 500 1000 1500
Lag u (msec)

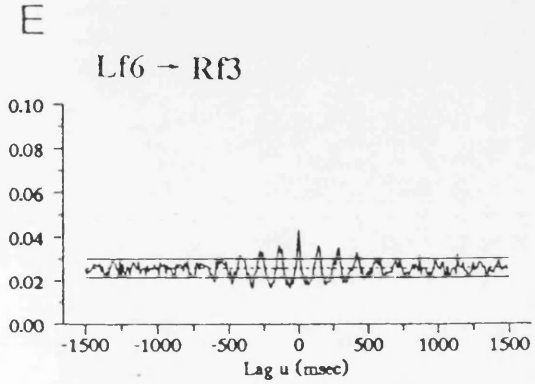

E
Lf6 → Rf3
0.10
0.08
0.06
0.04
0.02
0.00
-1500 -1000 -500 0 500 1000 1500
Lag u (msec)

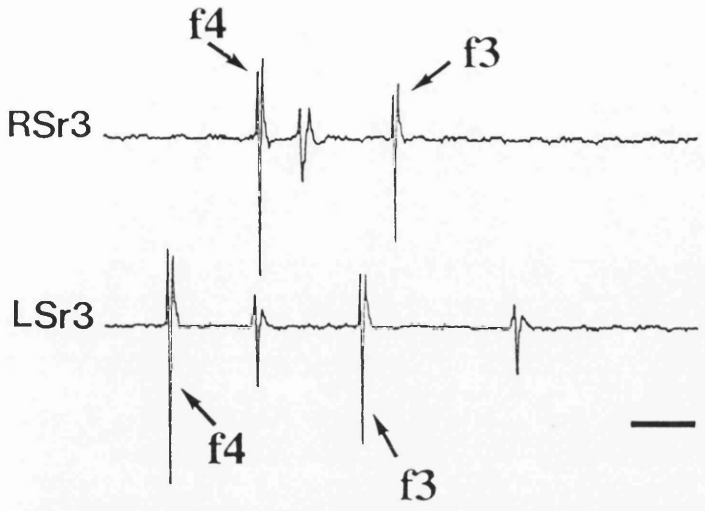
Figure 3.24

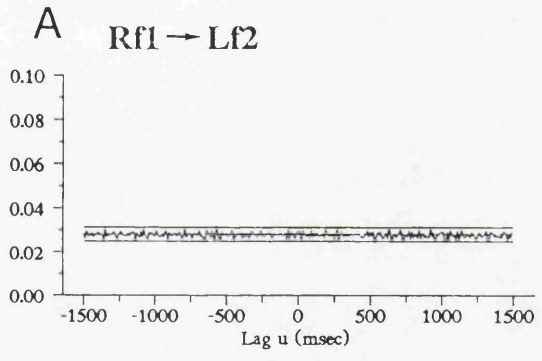
No correlation.

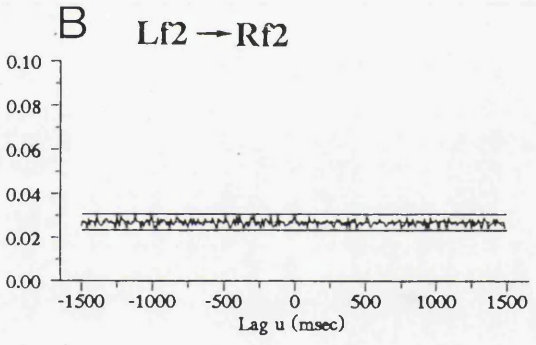
Cross-correlation analyses of the activity of the tonic flexor motor neurones and the tonic-phasic flexor motor neurones. Correlations were constructed using the data from experiment (c1) shown in the top two traces. These traces show only a short section of the spontaneous activity recorded extracellularly from the right and left Sr3s of the third abdominal ganglion (unfortunately the raw data stored on tape was accidentally erased).

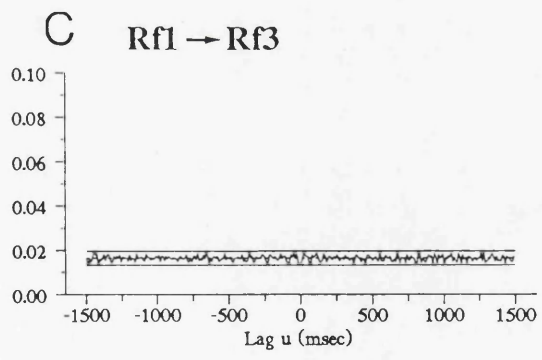
All correlations were constructed using bin widths of 10 ms.

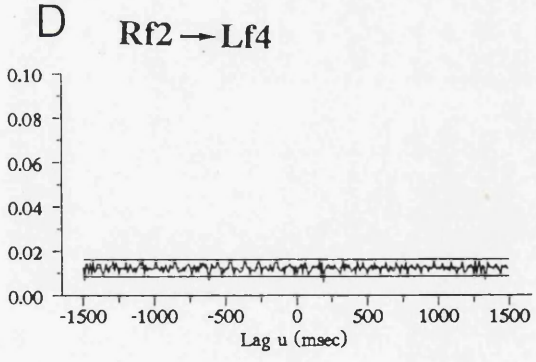
Scale bar = 10 ms.

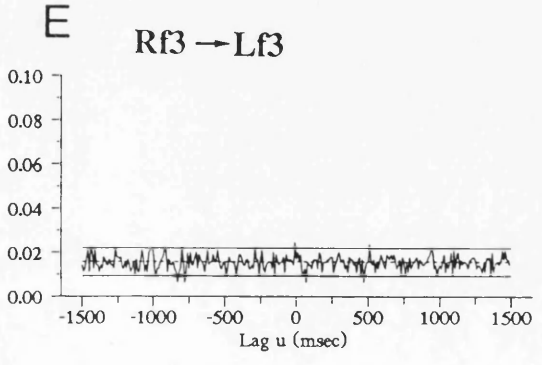

f4
f3
RSr3
LSr3
f4
f3


A
Rf1 → Lf2
0.10
0.08
0.06
0.04
0.02
0.00
-1500 -1000 -500 0 500 1000 1500
Lag u (msec)


B
Lf2 → Rf2
0.10
0.08
0.06
0.04
0.02
0.00
-1500 -1000 -500 0 500 1000 1500
Lag u (msec)


C
Rf1 → Rf3
0.10
0.08
0.06
0.04
0.02
0.00
-1500 -1000 -500 0 500 1000 1500
Lag u (msec)


D
Rf2 → Lf4
0.10
0.08
0.06
0.04
0.02
0.00
-1500 -1000 -500 0 500 1000 1500
Lag u (msec)


E
Rf3 → Lf3
0.10
0.08
0.06
0.04
0.02
0.00
-1500 -1000 -500 0 500 1000 1500
Lag u (msec)

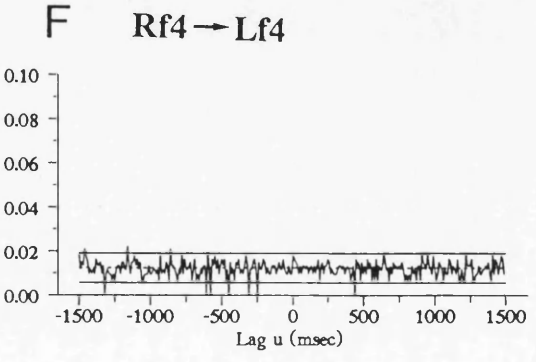
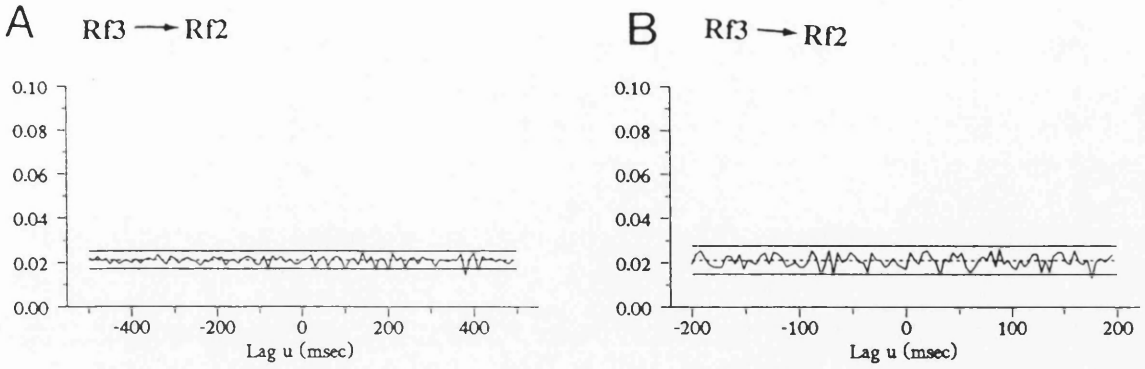

F
Rf4 → Lf4
0.10
0.08
0.06
0.04
0.02
0.00
-1500 -1000 -500 0 500 1000 1500
Lag u (msec)

Figure 3.25

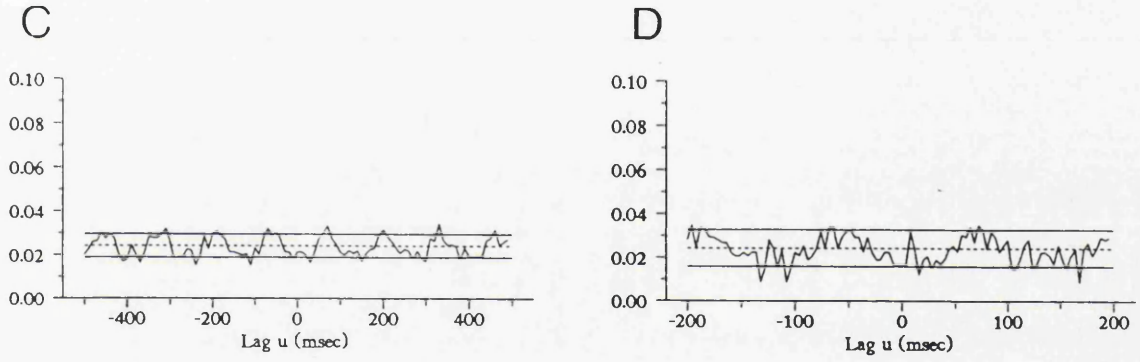
Proctolin-induced negative correlation.

Cross intensity estimates of the activity of the ipsilateral flexor motor neurones f2 and f3 before and after the perfusion of proctolin to the preparation. **A**, **C** and **E** were constructed with a 10 ms bin-width using the spike data shown which was recorded from the right Sr3 of the 2nd abdominal ganglion (experiment c3). **B**, **D** and **E** show cross intensity estimates constructed for the same data as **A**, **C** and **E** respectively but using a bin-width of 4 ms.

CONTROL



10^{-10} M PROCTOLIN



10^{-8} M PROCTOLIN

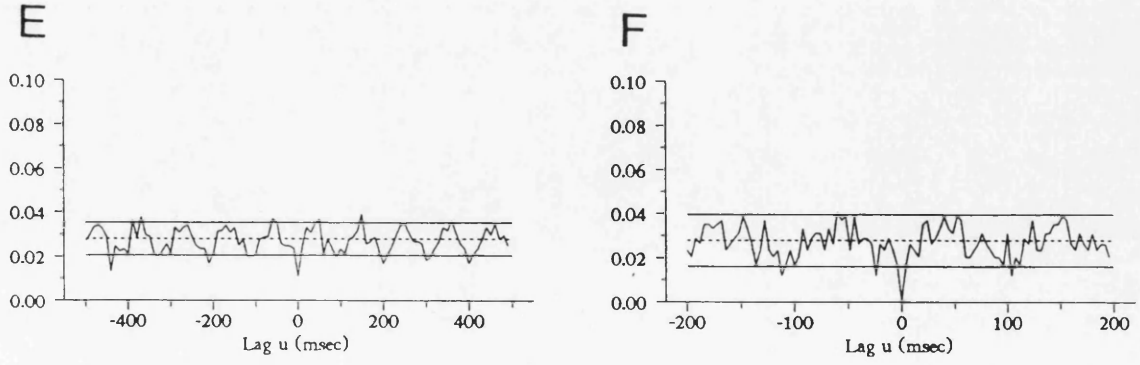


Figure 3.26

Proctolin-induced negative correlations.

Estimates of cross-correlation analyses of the effect of proctolin on the coordination of firing of the tonic-phasic flexor motor neurone with:

A and **B** its contralateral phasic flexor motor neurone, f6, and

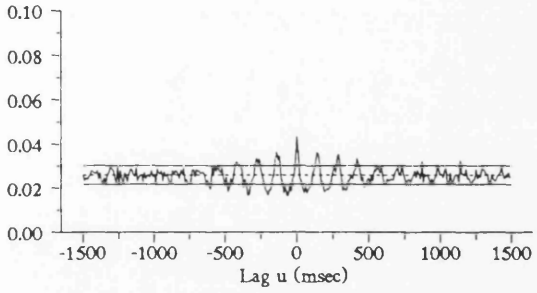
C and **D** its ipsilateral tonic-phasic flexor motor neurone, f4.

Estimates were constructed using the spike data from experiment c2 (**Figure 3.22**), bin-width 4 ms.

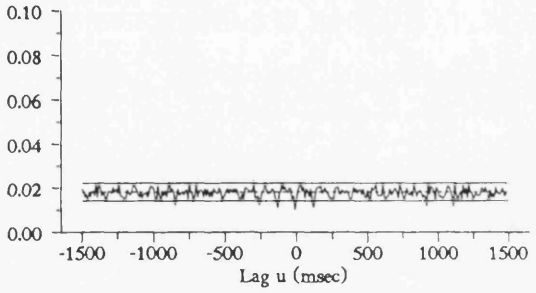
CONTROL

PROCTOLIN

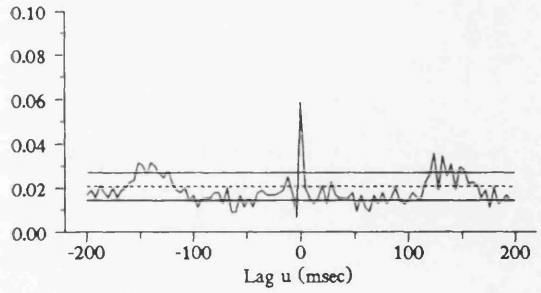
A Lf6 → Rf3



B Lf6 → Rf3



C Rf4 → Rf3



D Rf4 → Rf3

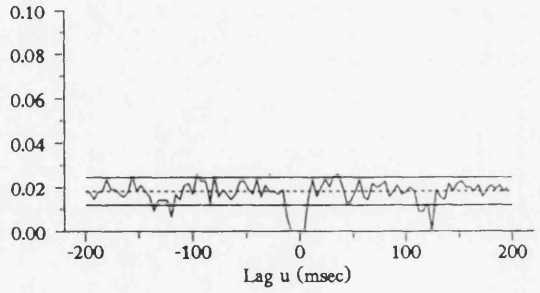


Figure 3.27

Proctolin-induced positive correlations.

Estimates of cross-correlation analyses of the effect of proctolin on the coordination of firing of:

A and **B** the contralateral tonic flexor f2 motor neurones,

C and **D** the contralateral tonic-phasic flexor f3 motor neurones.

Figure 3.28

Correlations unaffected by proctolin.

Estimates of cross-correlation analyses of the coordination of firing of the contralateral flexor motor neurones Rf4 and Lf6 in the control situation (**A**) and in the presence of proctolin (**B**).

Proctolin did not affect the coordination of firing of the motor neurones. The bin count in histogram **B** reflects an increase in the mean frequency of firing in both f4 and f6 induced by proctolin.

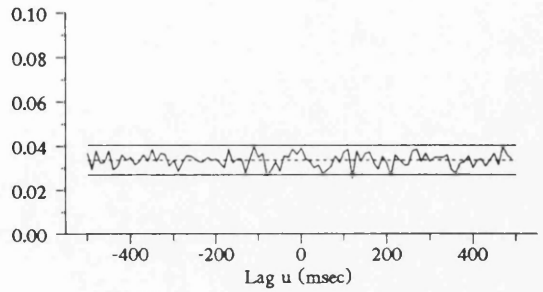
Estimates were constructed using data from experiment c2 (**Figure 3.22**) using bin-widths of 4 ms.

CONTROL

PROCTOLIN

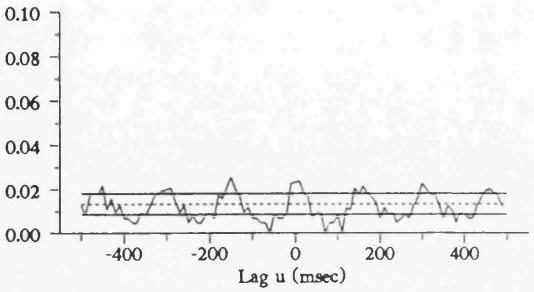
A

Lf2 → Rf2



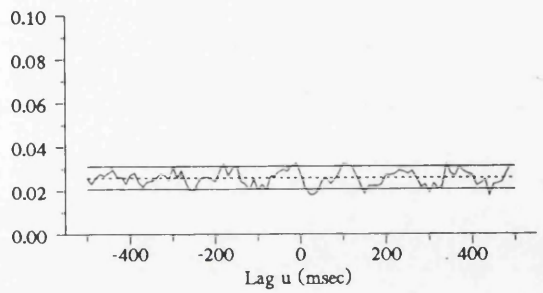
B

Lf2 → Rf2



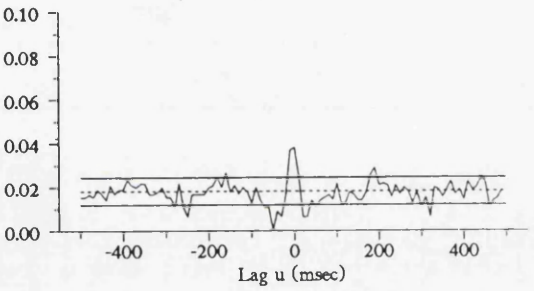
C

Lf3 → Rf3



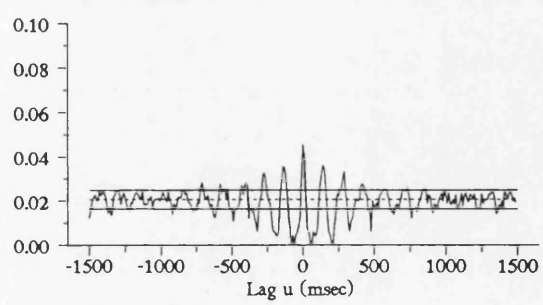
D

Lf3 → Rf3



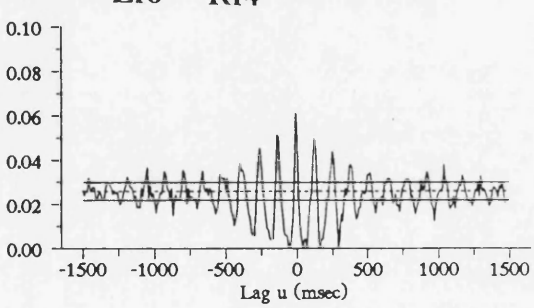
A

Lf6 → Rf4



B

Lf6 → Rf4



CHAPTER 4

CHARACTERISATION OF AND PERIPHERAL EFFECTS OF PROCTOLIN ON *NEPHROPS* SFM.

4.1 INTRODUCTION

The superficial flexor muscles (SFMs) of decapod crustaceans which are involved in the control of fine postural adjustments, are composed of 'slow' fibres, compared with the 'fast' fibres of the deep flexors which are involved in escape responses (Kennedy and Takeda, 1965). SFMs, which consist of a medial and a lateral bundle, were previously thought to be comprised of a homogeneous group of slow fibres. However, using a number of biochemical and histochemical techniques, Neil and Fowler (1990) have demonstrated a heterogeneity in the fibre composition of the SFM of *Nephrops norvegicus*. Significantly, these different procedures reveal the same two subpopulations of fibres.

Biochemical characterisation using electrophoretic separation of myofibrillar protein assemblages (Mykles, 1985a) identifies two slow fibre phenotypes in *Nephrops* SFM (Neil and Fowler, 1990), designated S1 and S2 in accordance with the criteria of Mykles, (1985b).

Histochemical characterisation of the SFM has involved the use of the following tests: (1) total myofibrillar ATPase activity, indicative of speed of contraction of fibres (Ogonowski *et al.*, 1980; Govind *et al.*, 1981; Maier *et al.*, 1984), (2) succinate dehydrogenase (SDH) activity, (Fig. 4.1) indicative of the oxidative capacity of fibres (Mabuchi and Streter, 1980) and (3) the presence of pH-sensitive isoforms of mATPase (Silverman and Charlton, 1980; Maier *et al.*, 1984; Li and Mykles, 1990). All three tests have confirmed the presence of two distinct populations of fibre types within the SFM, distributed in a non-uniform pattern. Medial fibres, which express the S2 phenotype exclusively, show intense staining for SDH activity, low levels of staining for total myofibrillar ATPase activity and contain a stable isoform of mATPase. Lateral fibres, however, are predominantly of the S1 phenotype, interspersed with a few fibres expressing the S2. The majority of lateral fibres show weak staining for SDH activity and high levels of staining for total myofibrillar ATPase activity. Thus, fibres in the

medial SFM bundle of *Nephrops* are more fatigue resistant and contract at a slower rate than the majority of fibres in the lateral SFM bundle.

In the crayfish *Pacifastacus leniusculus* the SFM also contains two slow fibre subtypes according to histochemical tests, but only expresses one phenotype, S1, according to the assemblages of its myofibrillar proteins (Fowler et al., 1990).

The many reports of 'matching' between physiological properties of muscle fibres and the motor neurones which innervate them (crustaceans: Costello and Govind, 1983; Maier et al., 1986; Rathmayer and Maier, 1987; Wiens *et al.*, 1991, insects: Anderson *et al.,* 1988) have prompted a more in-depth investigation of the innervation of the SFM of *Nephrops*. Although previous work suggests that a correlation exists between *Nephrops* SFM fibre subtypes and their pattern of innervation (Neil and Fowler, 1990), the basis of this innervation at the level of individual motor neurones is not known. Given the segregation of fibre subtypes, it was necessary to determine whether medial and lateral bundles of the SFM are innervated by different subsets of motor neurones. This was investigated in a number of SFMs by surveying their dorsal surfaces with an intracellular microelectrode and testing for synaptic connectivity in response to spontaneous motor neurone activity in isolated nerve-muscle preparations. Attempts to further characterise the muscle with regard to fibre heterogeneity were made by an investigation of other neuromuscular parameters such as their facilitation properties, excitatory junction potential (EJP) amplitude and time constant. In crab and crayfish muscle evidence already exists for matching between these presynaptic and postsynaptic properties. Fibres containing synapses which generate EJPs with high quantal content (high output synapses) exhibit low facilitation whereas fibres containing synapses which generate EJPs with low quantal content (low output synapses) exhibit high facilitation (Sherman and Atwood, 1972; Parnas and Dudel, 1982c; Rathmayer and Hammelsbeck, 1985).

Since it has been established for *Nephrops* SFM that there is an exact correspondence between the two histochemical fibre types and the two phenotypes, S1 and S2, according to the myofibrillar proteins expressed (Neil and Fowler, 1990), the latter method could be used to establish the identity of muscle fibres following innervation studies. As the latter technique can be performed more conveniently on single fibres, it was chosen for this purpose.

Since a detailed investigation of the histochemical properties of the SFM in *Nephrops* had already been performed in this laboratory (Fowler and Neil, 1989, 1992; Neil and Fowler, 1990; Fowler, 1990), it was thought unnecessary to repeat this work in a detailed way. However, the opposite arguments hold in the case of crayfish. Since all fibres have been found to express the S1 phenotype but segregate into two populations according to histochemical tests (Fowler *et al.*, 1990) recourse had to be made to histochemistry to distinguish the fibre types following innervation studies. This technique is less precise, as individual fibres are not identified.

The previous two chapters have demonstrated the existence of the neuropeptide proctolin within the SFM system of *Nephrops* (Chapter 2) and shown its central action on spontaneously induced activity in the SFM postural motor neurones (Chapter 3). The peripheral targets of proctolin released from the Sr3 axonal terminals are the SFMs. Thus, this segregation of fibre types in the SFM of the Norway lobster provides an appropriate system in which to investigate the role of proctolin in the generation of mechanical tension on two, different, identified, fibre phenotypes. If each muscle bundle is considered, at a first approximation, to consist of a single fibre type, the mechanical properties of each fibre type can be studied by making simultaneous tension measurements on each muscle bundle. This is certainly true for the S2 fibres of the medial bundle but is only an approximation for the lateral bundle which contains both fibre types (75% are S1, 25% are S2, on average).

Unlike the segregation in *Nephrops*, the two histochemical fibre types in

crayfish are mixed throughout the SFM. Thus, it is not possible in crayfish to compare the effect of proctolin on the two fibre types by measuring the gross tension in medial and lateral bundles before and after application of the peptide, as it is possible in *Nephrops*.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Norway lobsters (*Nephrops norvegicus* L.) of carapace length 9.5 cm, obtained from the University Marine Station, Millport, Isle of Cumbrae, were maintained in tanks supplied with aerated circulating sea water at 10-12° C and were fed on whitebait.

Crayfish (*Pacifastacus leniusculus* L.) of carapace length 5.0 - 7.5 cm, were obtained commercially. They were kept in aerated tanks of copper-free tapwater at 14° and fed on fish meal.

The majority of electrophysiological studies were carried out on male animals.

4.2.2 Neuromuscular preparations

The electrophysiological experiments described in this chapter fall into two main categories, those investigating: (1) the pattern of innervation across the superficial flexor muscle and (2) the effect of proctolin on tension development in muscle fibre bundles in response to evoked stimulation. The neuromuscular preparations which were used for each purpose were slightly different. All experiments were performed on the muscles of the right second or third segments and were maintained at a temperature of approximately 14°C by a Peltier cooling device.

4.2.3 The pattern of innervation across the SFM

An isolated neuromuscular preparation was used to study the pattern of innervation across the superficial flexor muscle. Each muscle was exposed via a dorsal approach (as explained in section 3.2.3) and was dissected by making two incisions (Fig. 3.2B **c** - **d** and **e** - **f**) into the cuticle on either side of the segment in question parallel to the exoskeletal ribs. Another cut was then made through the soft ventral exoskeleton along the axis of the nerve cord (**e** -**c**) and below the 3rd root, enabling the whole preparation (muscle and associated Sr3 with attached abdominal ventral nerve cord) to be lifted free and pinned dorsal side

up in a Sylgard-lined 10 ml bath.

4.2.4 The effect of proctolin on tension development.

A modified neuromuscular preparation was used to determine the effect of proctolin on the development of tension in the fibre bundles of the superficial flexor muscle. The dissection of the preparation was carried out in a similar fashion to that for the nerve-muscle preparation described above, with the following modifications:

1. The connectives anterior and posterior to the superficial flexor nerve were severed to eliminate tonic activity in the motor neurones and the influence of intersegmental connections.

2. The thin sheet of membrane onto which the superficial flexor muscle fibres insert (stippled area in Fig. 3.2B) was cut from **h** to **g** as close to the anterior rib as possible. The nerve cord and muscle were removed and the muscle was then securely pinned along the remaining exoskeletal rib. Mechanical tension in the medial and lateral fibre bundles of the superficial flexor muscle was recorded using two strain gauge transducers (Fig. 4.2). These were made from foil strain gauges, mounted back-to-back on a cellophane sheet (and connected in a Wheatstone bridge). The needle attached to the transducer was thrust into the sheet of membrane close to the insertion point of the muscle fibres. As a standard procedure 100 mg tension was applied to both muscle bundles to take up any slack. The muscle bundles were then left for approximately 30 minutes to rest. This level of resting tension was taken as the baseline value.

Proctolin. A stock solution of 10^{-3} M proctolin was used to make up appropriate concentrations for experiments. The stock solution was stored frozen (-20°C) in Eppendorf tubes in volumes of 1 ml. In experiments where the role of proctolin in tension development was studied, the preparation was continuously perfused with 100 ml of proctolin solution at a rate of 3.15 ml/minute.

A number of experiments were carried out to determine the effect of proctolin on muscle tension produced by smaller muscle bundles, containing only a few fibres. These were totally isolated from medial or lateral parts of the muscle and induced to contract in high potassium saline (1.2 - 4.7 x normal strength). A sensitive force transducer (Scientific Instruments GMBH, model KG3) was used to measure the increases in tension produced by these small bundles.

4.2.5 Electrophysiological recordings

The flexor motor neurones were stimulated and recorded with platinum electrodes as described in Chapter 3 (see section 3.2.6). A conventional bridge circuit was used for intracellular recording and passing current pulses with a single microelectrode. Glass microelectrodes filled with 3M KCl with tip resistances of 10-30 mΩ were used. The layout of the apparatus used in shown in Figure 4.3.

4.2.6 Innervation Survey.

A survey of the pattern of innervation across the dorsal surface of the SFM was carried out. Intracellular recordings were made from each muscle fibre in turn whilst simultaneously recording extracellular activity from the associated Sr3. Data were then analysed by capturing sweeps (50 sweeps, each of 100ms) of each spontaneously active motor neurone and its corresponding EJP. Each sweep was automatically triggered by the peak of the spike falling in between the preset boundaries of a window discriminator. The sweeps were then averaged and the peak of the EJP measured. The amplitude of EJP produced by each spontaneously active SFM motor neurone was plotted for each SFM fibre.

4.2.7 Measurement of neuromuscular parameters

The membrane time constant τ was measured as the time taken for an EJP to decay to 37% of the peak amplitude. Facilitation of f6-induced EJPs was measured by stimulation of the excitatory axon by trains of 10 pulses at a

frequency of 20Hz. Facilitation was calculated by a comparison of the amplitude of the tenth EJP in the train with the first EJP in the train according to the formula $f_n=(a_n/a_1)-1$, where a_n is the amplitude of the nth EJP.

4.2.8 Histochemistry

Adult species of *Pacifastacus leniusculus* were used for histochemical tests. Either whole abdomens or dissected SFMs were held at resting length and fast frozen in liquid nitrogen. The specimen was then mounted on the cryostat chuck and left for half an hour to equilibrate to the cutting temperature (-25°C). Serial sections (15-20 μm) were cut using a cryostat (BRIGHT Starlet model 1202), air-dried and stained for the following:

1. Total myofibrillar ATPase activity
2. pH-sensitivity of mATPase isoforms
3. Succinate dehydrogenase (SDH)

4.2.8.1 Total myofibrillar ATPase activity

In muscle, membrane-bound enzymes, ATPases, hydrolyse ATP to ADP and inorganic phosphate thereby providing chemical fuel for contraction. Consequently, high levels of total myofibrillar ATPase activity in muscle are associated with fast contraction of the fibres. The method used to test for total myofibrillar ATPase activity was modified from Ogonowski *et al.,* (1980).

Stock Solutions:

Solution 1. Buffer - 0.05M N glycylglycine (pH 8.0, 500ml).

Solution 2. 1M $CaCl_2$ (250ml).

Solution 3. 0.1M $MgCl_2$ (250ml).

On the day of use the stock solutions were mixed together as follows to produce the reaction medium: 19 ml solution 1 + 0.36 ml solution 2 + 0.25 ml solution 3 and to this 0.1806 g of ATP and 0.60 ml of distilled H_2O were added. The pH of the solution was adjusted to 9.4.

Procedure:

Sections were placed in Columbia jars and incubated with the Reaction

Medium for 30 minutes at 4°C. They were then rinsed in distilled H_2O three times and incubated in 1% $CaCl_2$ for 3 minutes at 4°C. After a further rinse with distilled H_2O (5 times) sections were incubated in 1% $(NaH_4)2S$ for one minute or less. Finally, sections were rinsed in distilled H_2O (3 times), dehydrated in an alcohol series, cleared and mounted onto slides.

4.2.8.2 pH-sensitivity of mATPase isoforms

Different muscle fibre types can be distinguished according to the myofibrillar ATPase isoforms which they contain. The fact that different mATPase isoforms vary in their pH stability across the muscle can be exploited by conducting histochemical tests in which muscle sections are preincubated at different pHs before the incubation procedure. Fibres which contain a mATPase isoform which is stable at the pH of the preincubation solution will still be reactive (with ATP) in the incubation medium and so produce a dark reaction. Conversely, fibres which contain a mATPase isoform which is labile at the preincubation pH will no longer be reactive with the ATP in the incubation medium and consequently will not stain. The method used was modified from Mabuchi and Streter (1980).

Stock solutions:

Preincubation Medium

2.72g sodium acetate + 1.49g potassium hydroxide in 160 ml distilled water. Solution was adjusted to pH 5.0 using 100% acetic acid, made up to 200ml and stored at 4°C. On the day of use the preincubation medium was split into four volumes of 50 mls and the pH adjusted accordingly.

Incubation Medium

1.90g glycine (0.05M) + 1.45g NaCl (0.05M) + 2.20g $CaCl_2.2H_2O$ (0.03M) added to 500 ml distilled water. The solution was stored at 4°C until required. On the day of use 0.09g of ATP was added to 50 ml of incubation medium. This was then pH'd to 9.4 with 10M NaOH.

Procedure:

Sections were placed in Columbia jars each containing preincubation medium at a different pH and left for 20 minutes at 4°C. After a rinse with distilled H_2O (three times), sections were incubated in the incubation medium for 30 minutes at 4°C. The procedure for total mATPase was then followed from step B.

4.2.8.3 Succinate dehydrogenase (SDH)

Levels of this respiratory enzyme give an indication of the oxidative capacity and hence fatigue resistance of a muscle.

Stock solutions:

1. 1M sodium succinate
2. 0.1M sodium phosphate (containing 1 mg/ml nitro blue tetrazolium)

10 mls of each solution were made up at a time. The pH of both solutions was adjusted to 7.5 and they were stored at 4°C.

On the day of use solutions (1) and (2) were mixed in the proportions 1:9 respectively to produce a yellow operating solution.

Procedure:

Sections were incubated with a few drops of this solution at around 30-35°C until the reaction was complete. Sections were then dehydrated in an alcohol series, cleared and mounted onto slides.

Staining of histochemical sections was carried out for me by Dr. D. Gunzel, University of Konstanz, Germany.

4.2.9 Photography

Histochemically stained sections were photographed with a WILD M37 Type-S photomicrograph and WILD MPS45 photoautomod (both by Heerburgg) using Kodak Panatomic 32 film (FX135).

4.2.9 Biochemistry

SDS-PAGE discontinuous gel electrophoresis according to the method of Laemmli (1970) was used to analyse the protein content of different muscle fibres of the superficial flexor muscle. This system offers high resolution of dilute protein samples. The principle of the technique is as follows: application

of an electric field across a polyacrylamide gel results in the differential migration of charged proteins towards one of the electrodes. Migration of individual proteins is also affected by pore size of the gel (dependent upon the percentage of acrylamide content of the gel), the size of the proteins to be separated and to a lesser extent, the pH of the buffer system used.

With the SDS-discontinuous buffer system, the protein sample to be analysed is loaded onto a large-pore 'stacking' gel polymerized on top of a small-pore 'resolving' gel. The system used to produce the gels was either a Biorad II System, or a Hoefer 'Might-small' mini-gel system.

Gels containing a 12.5% acrylamide stacking gel and a 10% separating gel were prepared from a 30% (w/v) acrylamide and 0.8% (w/v) N,N'-methylene bisacrylamide stock solution.

Pieces of muscle were put into cold glycerination buffer containing 20mM Tris-acetate (pH 7.5), 50% glycerol, 0.1M KCl, 1mM EDTA, 0.1% Triton X-100 for 2-3 hours. This process facilitated separation of individual muscle fibres, which were then separated and transferred to 100ul of SDS sample buffer (62.5mM Tris-HCl (pH 6.8), 12.5% glycerol, 1.25% SDS, 1.25% B-mercaptoethanol). Samples were immediately boiled for 3 minutes, then stored at -20°C until required.

Samples, and standards of known molecular weight (Sigma Dalton Mark VII-L), were applied to the wells in the stacking gel. The gels were then mounted in a chamber containing a reservoir buffer (0.25M Tris, 1.92M glycine and 1% SDS (pH 8.3)) and run with applied currents of up to 40mA per gel.

Gels were removed from the system and fixed in 10% (w/v) trichloroacetic acid, stained in 0.2% (w/v) Coomassie Blue in 45% (v/v) methanol and 10% (v/v) acetic acid for up to 8 hours, and subsequently destained in a acetic acid/methanol/distilled water mixture in a ratio of 1:33:90. In some cases gels were further stained with silver; a technique which can visualise small protein bands not apparent after staining in Coomassie Blue.

4.3 RESULTS

4.3.1 Innervation of *Nephrops* SFM

The SFM muscle is innervated by five excitatory neurones and one inhibitory neurone. In an initial series of experiments, intracellular recordings were taken randomly from fibres across the dorsal surface of the SFM. This revealed differences in the pattern of activity of medial and lateral fibres in response to spontaneous activity of the six axons in the Sr3 innervating the muscle. Medial fibres consistently showed a characteristic pattern of activity from several axons, each inducing relatively large EJPs (Fig. 4.4A, B). However, the majority of lateral fibres encountered in most preparations were synaptically silent, only producing small EJPs in response to normally infrequent impulses of f6. Due to their lack of response to normal levels of spontaneous activity in the Sr3, these fibres are referred to as 'silent' lateral fibres. In a few preparations tiny EJPs were observed in these 'silent' lateral fibres in response to firing of either f3 or f4, but never simultaneously to both units. Figure 4.4B shows an example of the innervation pattern seen in the remaining lateral fibres. These fibres displayed patterns of synaptic activity normally associated with medial fibres, i.e. highly active and polyneuronal, and are referred to as 'active' lateral fibres. Thus, two characteristic patterns of innervation were displayed by SFM fibres. All medial and a few lateral fibres displayed a polyneuronal pattern of innervation while the remainder of lateral fibres displayed a silent or monosynaptic pattern of innervation. This distribution of different fibres based on their pattern of innervation suggested a possible correlation between, the innervation properties of individual muscle fibres, and their biochemical/histochemical phenotype. In order to establish the existence of such correlations with more certainty, a protocol was developed which enabled both the innervation and biochemical properties to be determined for individual muscle fibres.

4.3.2 Biochemical characterisation of *Nephrops* SFM

Slow fibre phenotypes of SFM are distinguishable from fast fibre phenotypes of deep flexor muscle according to variations in the profiles of their myofibrillar protein assemblages on SDS-PAGE gels. Electrophoretic separation of these muscles allows the discrimination of one fast phenotype and two slow phenotypes. All three phenotypes commonly express the same isoforms of the myosin heavy chain, actin and tropomyosin, but differ in their expression of other myofibrillar proteins. Profiles of both slow subtypes lack the presence of two specific bands which are always present in fast fibres profiles (Mykles, 1985b; 1988). These bands correspond to a P_1 variant of paramyosin (Mr = 110,000) and a 75kD protein (Fig. 4.5A) (Mykles, 1988; Neil and Fowler, 1992). The different slow fibre phenotypes can themselves be discriminated as S1 or S2 according to the presence or absence of other isoforms in their biochemical profiles, in particular those of troponin T (Mykles, 1985b). Fibres which do not contain the troponin T1 isoform (Mr = 55kDa) are identified as S1; fibres in which the troponin T1 isoform is present are identified as S2 fibres (Mykles, 1985b, Neil and Fowler, 1990). Another variant of troponin T, T3, (Mr = 47 kDa), can most notably be discerned in fast fibres, but occurs in all three phenotypes, along with T2 (Mr = 49 kDa), another troponin T variant.

4.3.3 Correlation of Physiological and Biochemical Properties In Single Identified *Nephrops SFMs.*

Single, identified SFM fibres whose intracellular properties were known were dissected and run on an SDS-PAGE gel, and produced one of the two slow fibre phenotypic subtypes as described in section 4.3.2. In this way it was found that fibres which exhibited the highly active pattern of innervation characteristic of medial and 'active' lateral fibres always expressed the S2 phenotype (Fig. 4.5B), while fibres which were 'silent' or active in response to only the phasic motor neurones (depending on the levels of spontaneous activity of these units) always expressed the S1 phenotype (Fig. 4.4B). These findings provide

confirmatory evidence for a consistent relationship between the biochemical phenotype of a fibre and the physiology of its synaptic input, measured in terms of the general level of polysynaptic activity.

4.3.4 Mapping of innervation across *Nephrops* SFM

The above correlation which appears to link different fibre phenotypes with characteristic patterns of intracellular activity, prompted a detailed investigation of the pattern of innervation of each of the six SFM axons, in order to determine whether different fibre types received preferential innervation from certain axons.

As well as attempting to stimulate individual motor neurones selectively, advantage was taken of the spontaneous activity of motor neurones in isolated preparations, which permitted identification of synaptic connections between identified axons and different SFM fibres.

The results represent the analysis of data from six *Nephrops* SFM preparations in which a survey was made of every fibre across the dorsal surface of the muscle.

Each fibre in turn across the entire dorsal surface of the SFM was sampled for its pattern of spontaneous activity while simultaneous recordings were made from the Sr3 innervating the muscle. Spikes of identified motor neurones were matched with EJPs in each of the SFM fibres to produce maps of the innervation of each motor neurone across the SFM. The EJP amplitude for each axon in every fibre was measured by averaging up to 50 events. This procedure was necessary because high levels of tonic activity often resulted in summation of EJPs, which hindered the accurate measurement of EJP amplitude. Natural variation in the levels of spontaneous activity of individual motor neurones (see section 3.3.5) meant that little data were available concerning the innervation of SFM by the phasic motor neurones, f5 and f6. For this reason and because inhibitory junctional potential (IJP) amplitude varied with the condition of the preparation, the inhibitor, f5, was not included in this

analysis. In all of the SFMs of *Nephrops* analysed, the majority of lateral fibres produced no EJPs in response to normal patterns of spontaneous activity in the Sr3. EJPs were generated in many lateral fibres in response to the most phasic motor neurone, f6, but these were rarely seen due to the infrequent nature of firing of f6.

Of the six complete muscle surveys carried out (s1-s6) (Fig. 4.6 - 4.11), one (s6) showed a pattern of innervation which differed substantially from the other five and will be discussed later. In the remaining 5 (of 6) innervation surveys, EJPs were measured in the majority of all medial fibres in response to at least two motor neurones while the great majority of lateral fibres were silent i.e. they displayed no measurable EJP in response to activity in any of the spontaneously active motor neurones. Table 4.1 summarises the percentage of medial and lateral fibres in each survey which were synaptically connected to spontaneously active axons. The mean values of these figures are presented in Table 4.2.

f1 - Medial. No consistent pattern or gradient of f1 innervation across the medial SFM bundle was evident from the survey. In three experiments, a high percentage of fibres responded to f1 with small EJPs (s1, s3, s5). However, in two experiments (s2, s4), none of the medial fibres displayed a response to f1 and although the possibillity of damage to the f1 axon distal to the point of recording is unlikely, it cannot be ruled out.

f1 - Lateral. In the five preparations s1-s5, none of the lateral fibres showed any EJPs in response to spontaneous firing of f1.

f2 - Medial. In every preparation analysed, f2 innervated the majority of medial fibres. The pattern of innervation of f2 was often mirrored by the pattern of innervation of f1, except that the EJPs produced by f2 were greater in amplitude.

f2 - Lateral. In three preparations (s2,s3,s4) a minority of lateral fibres were innervated by f2 (s2, s3, s4) but no lateral fibres were innervated by f2 in

two experiments (s1, s5).

f3 - Medial. In four preparations where f3 was spontaneously active a high percentage of medial fibres produced EJPs (s1, s2, s4, s5). One preparation showed fewer f3-innervated fibres tightly grouped together (s4).

f3 - Lateral. The SFM motor neurone, f3, was spontaneously active in four of the preparations analysed but did not produce EJPs in three of them (s1, s2, s5). In the remaining preparation, (s4), very small EJPs were produced in a quarter of the lateral fibres sampled.

f4 - Medial. The medial fibres of one preparation (s2) were extensively innervated by f4. In this muscle, the pattern of distribution of f4-innervated fibres and size of EJPs was very similar to that of f3-innervated fibres.

f4 - Lateral. f4 was spontaneously active in two of the preparations analysed (s2, s5), both of which showed a tiny f4-induced EJPs in a single lateral fibre.

f6. The largest and most phasic motor neurone, f6, was rarely spontaneously active in the experimental neuromuscular preparations used and was therefore, the most difficult to analyse. A complete survey of the innervation of f6 across the SFM was obtained for one experiment (s4) in which f6 was highly active.

f6 - Medial. 16 of 17 medial fibres were innervated by f6 in survey (c4). The average size of EJPs was 3 mV although EJPs recorded in two fibres (the 1st and the 8th fibres) were much larger (9.8 mV and 7.6 mV respectively). A distinct trend in EJP size can be seen from one edge of the medial muscle to the other which does not relate to resting potential.

f6 - Lateral. 35% of lateral fibres were innervated by f6 in this muscle. Rather than being distributed across the muscle, the f6-innervated fibres were grouped in this case. From survey s4 it is apparent that f2 and f3 also contibute to the innervation of fibres in this area. A correlation between both the medial and 'active' lateral fibres and the S2 phenotype and between 'silent' lateral

fibres and the S1 phenotype has already been established. Thus, it is possible that this group of fibres represents a cluster of S2 type fibres.

In the sixth survey (s6), analysis of the innervation showed a very different pattern to that found in the other five muscles. Clear segregation of the medial and lateral muscle bundles into 'active' and 'silent' regions was not as apparent as in surveys s1-s5 although 48% of lateral fibres showed no obvious synaptic connectivity with the five axons analysed, f1, f2, f3, f4 and f6. It is unlikely that these fibres do not receive input from any of the excitatory axons.

The data from the above muscle surveys were also used in conjunction with data from subsequent experiments utilising spontaneous activity to investigate EJP amplitude and other neuromuscular parameters in medial and lateral fibres (see table 4.3). No significant difference was found between the mean resting membrane potential of medial (S2) fibres and lateral (S2) or (S1) fibres ($p > 0.05$, unpaired t-test). With the exception of f1, the mean EJP amplitude for all motor neurones was greater in medial (S2) fibres than in lateral (S2) fibres (see table 4.3). However, only f4 and f6 produced EJPs whose amplitudes differed significantly between the two fibres ($p < 0.05$, Students t-test). There was no significant difference between f6-induced EJPs in lateral (S2) and lateral (S1) fibres ($p > 0.05$, Students t-test). For all axons except f6, the mean decay constant τ was larger in lateral S2 fibres than medial fibres but this was only statistically significant for f3-induced EJPs ($p < 0.01$, Students t-test). The mean decay constant of f6-induced EJPs medial (S2), lateral (S1) and lateral (S2) fibres did not differ significantly ($p > 0.05$, unpaired t-test).

4.3.5 Facilitation Properties

4.3.5.1 Facilitation in response to supramaximal stimulation of the Sr3

Synaptic facilitation is a characteristic feature of many nerve terminals which involves an increase in the quantal content of transmitter release with maintained stimulation in a frequency-dependent way. Several crustacean muscles display matching of presynaptic facilitation with postsynaptic properties

(Sherman and Atwood, 1972; Wiens *et al.*, 1991) hence, the facilitation properties of electrophysiologically identified SFM fibres were investigated in an attempt to (1) further differentiate muscle fibres and (2) permit a correlation of these presynaptic characteristics with known postsynaptic properties.

This approach resulted in the classification of SFM fibres into three groups based on their facilitation properties in response to supramaximal stimulation of the Sr3 (Fig. 4.12). In general, facilitation was inversely related to EJP amplitude, as has been reported for other muscles (Atwood and Bittner, 1971). Fibres in each group were found to exhibit differential facilitation properties in response to stimulation, which became more pronounced when the frequency of the stimulus trains was increased. These observed differences in facilitation were consistent from preparation to preparation and were usually only observed at frequencies of 10 Hz or greater. In most experiments, facilitation properties were tested routinely at the following frequencies; 1, 5, 10, 20 and 40 Hz. Typical responses in each category of fibre to stimulation of the Sr3 (muscle still spontaneously innervated) at the first four of these frequencies are presented in Figure 4.12. Medial (S2 type) fibres displayed low facilitation in response to supramaximal stimulation of the Sr3. At frequencies of 10 Hz and above, after an initial short period of growth, a plateau of facilitation was reached i.e. the amplitude of EJPs did not increase any further, and this situation continued for the stimulus duration. In 'silent' lateral (S1 type) fibres, however, both the rate and amount of facilitation increased with frequency. There was always an increase in facilitation with the duration of stimulation for all frequencies tested (for trains up to 4 seconds long). In no case was a decline of facilitation observed during continued stimulation. Lateral 'active' fibres tended to show facilitatory properties which were intermediate between those of medial and lateral 'silent' fibres. Further examples of the different facilitatory properties of medial and lateral 'silent' fibres (muscle isolated from Sr3) are shown in Figure 4.13.

4.3.5.2 Facilitation in response to selective stimulation of f6

Although supramaximal stimulation of the Sr3 producing synchronous stimulation of all 6 SFM axons, was able to show consistently that different SFM fibre types have different facilitatory properties, selective stimulation of a single axon was attempted to permit a more direct comparison of the facilitation of different fibre types. The axon chosen for selective stimulation was f6 as it has the lowest excitation threshold. Dorsal SFM fibres were sampled for the innervation pattern produced in response to spontaneous activity. After sampling, the Sr3 was sectioned and f6 stimulated at 1, 10 and 20 Hz in each identified fibre to investigate their facilitatory properties. The relative position and resting membrane potential of each fibre before and after Sr3 section was also noted in order to aid in identification of fibres. Data comparing type with pattern of innervation, EJP amplitude, decay constant and facilitation are presented in Table 4.4.

In agreement with spontaneously derived data, synaptic connections were found from f6 to every medial fibre in two muscles surveyed but not to all lateral fibres. In comparison to the medial fibres where an f6 impulse always produced an EJP, the low output type of synapse made by f6 with some lateral fibres meant that EJPs were visible only during periods of high frequency stimulation (>10 Hz). A large amplitude EJP was produced by f6 impulses at low frequency in the remaining lateral connected fibres (see table 4.4). No gradient was observed for amplitude of EJP or time of EJP decay in response to f6 stimulation over the dorsal surface of the muscle or across regions of the muscle where fibre types were randomly mixed, the fibres received similar innervation from spontaneously active axons. A variety of EJP amplitudes were observed among the different fibres innervated by f6; these ranged from 0.48 - 6.4 mV.

Selective stimulation of f6 in the Sr3 revealed at least four distinct fibre types (I-IV) which can be classified according to their time constant for decay

of the f6 elicited EJP (defined as the time for the EJP amplitude to fall to 0.37 of its peak amplitude (Sherman and Atwood, 1972) (Fig. 4.14). Types I and II had short time constants compared to types III and IV which had larger time constants. Fibre type I was further subdivided into Ia, Ib and Ic types although the latter two types were only found in lateral fibres. Type Ic fibres (not shown) apparently did not receive any innervation from f6 as no EJPs were produced at any combination of stimulus frequency or amplitude in these fibres.

The facilitation properties of each type differed. At stimulation frequencies of 20 Hz, the greatest facilitation was observed in type Ia fibres (0.48 - 3.58, as derived from the formula shown in section 4.2.6). Type III fibres also displayed significant facilitation (1.48) but less than observed in type Ia fibres. Type II and IV fibres both did not facilitate significantly and the amplitude of their EJPs declined with repetition during a stimulus train. This latter property was most apparent in type IV fibres which have the longest time constants and is probably due to voltage-dependent postsynaptic effects, such as rectification (Sherman and Atwood, 1972). Type IV fibres were only found in medial fibres. Type Ib fibres were more difficult to characterise by stimulation at 1, 10 and 20 Hz because of the tiny amplitude of their EJPs. In these fibre types significant facilitation was only achieved at frequencies of 20 Hz and above over longer time periods. Type Ic fibres were not innervated by f6 and hence their facilitatory properties are unknown.

4.3.5.3 Evidence for innervation derived from spontaneous bursts of activity

The highly facilitating nature of f6 in 'silent' lateral fibres in response to high frequency selective stimulation of this axon suggested the possibility that these fibres could make similar highly facilitating 'low output' type synaptic connections with the remaining SFM motor axons. Theoretically, such connections could produce very tiny EJPs which would only become visible during high frequency bursts of activity due to facilitation. This would explain the apparent silence of these fibres during spontaneous tonic levels of SFM activity.

Attempts to induce high frequency bursts of activity in axons other than f6 proved impossible, as increasing the level of stimulation for smaller axons also recruited larger axons of lower threshold stimulation. However, it was possible to obtain evidence of highly facilitating synaptic connectivity for individual axons from naturally occurring bursts of spontaneous activity.

Figure 4.15 shows a recording obtained during a burst of spontaneous activity. This type of burst occurred infrequently and only in some preparations. Prior to the burst, f4 was spontaneously active and producing EJPs in the medial fibre only; the lateral fibre was silent. However, during the burst, activation of f3 at a frequency of approximately 30 Hz occurred, resulting in the production of highly facilitating EJPs in the lateral fibre. Closer examination of the facilitation revealed that the f3-induced EJPs were not visible above baseline noise until the third f3 impulse. Moreover, single f3 impulses occurring minutes after the burst did not produce any discernible EJPs in the lateral fibre, but were still visible in the medial fibre.

4.3.6 Mechanical Properties

The correlation observed at the gross level between innervation pattern of fibres and their histochemical, biochemical and physiological properties prompted an investigation of a possible further correlation between these features and the relative contribution to overall tension made by the medial and lateral bundle of each SFM. The isolated neuromuscular system provides a convenient preparation in which to investigate this, and to examine the physiological action and ultimately, the functional significance of the peptide proctolin in the two muscle bundles.

4.3.6.1 Neurally induced tension in medial and lateral bundles of SFM

Simultaneous tension measurements were made from the medial and lateral bundles of the SFM, *in situ*, using relatively simple apparatus (Fig. 4.2). At a first approximation these represent bundles of S1 and S2 fibres respectively.

The Sr3 innervating the SFM was stimulated supramaximally at 40Hz to ensure full facilitation of both medial and lateral fibres. For a given level of stimulation at 40 Hz, a significantly greater magnitude of tension was produced by the lateral bundle than by the medial bundle (Fig. 4.16). This is perhaps not surprising as the lateral bundle consists of approximately twice the number of fibres than the medial bundle, however, no conclusion is possible from this result alone about the forces produced by the different fibre types.

4.3.6.2 Potentiation of neurally induced tension by proctolin

The effect of proctolin on neurally induced tension in both fibre types of the Norway lobster was assessed in relation to the tension produced by a level of stimulation sufficient to excite all six motor neurones simultaneously. Some of these motor neurones are assumed to be proctolinergic (see Chapter 2).

Proctolin was effective in enhancing tension produced in *Nephrops* SFM as a result of supramaximal stimulation of the Sr3. Figure 4.17A shows a typical experiment in which the Sr3 was stimulated with a regime which consisted of a 2 second burst of 50Hz at 60 second intervals. In the control condition, each burst produced a peak tension of 0.14 mN in medial fibres and 0.27 mN in lateral fibres. After a 30 minute perfusion of 10^{-8} M proctolin, neurally induced tension was potentiated in both medial and lateral bundles. However, when expressed as a percentage of the tension produced in the control situation, lateral tension was increased to a greater extent (100% increase over control) than was medial tension (40% increase) in the presence of proctolin.

In both muscle bundles, both the rate of production of tension and the rate of tension release were increased in the presence of proctolin. As a result the duration over which tension was maintained was not altered significantly by the presence of proctolin but the amount of tension produced over this time was greater.

Repeated attempts were made to investigate the dose-responsiveness of medial and lateral SFM bundles and hence construct a dose-response curve.

However, this was found to be impossible due to the following phenomenon. In each experiment, the initial perfusion of a concentration of proctolin between 10^{-10} and 10^{-6} M produced potentiation of neurally induced tension in both medial and lateral bundles. However, even after extensive periods of washing with normal saline, the level of tension produced with identical stimulation was much lower and not comparable to control levels. This appears to represent some from of desensitisation of the proctolin effect. In contrast, the crayfish SFM did not display the same type of desensitisation effect in that it was possible to measure the dose-dependancy of its tension response (section 4.3.7.4).

A comparison of intracellular recordings of SFM fibres before and after perfusion of proctolin revealed that the summated EJPs associated with generation of tension were identical under the two conditions (Fig. 4.17B). Similarly, proctolin had no effect on the amplitude or shape of single unfacilitated EJPs, the resting potential of muscle fibres or the resting muscle tension. These results clearly imply a postsynaptic site of action for proctolin.

4.3.6.3 Postsynaptic effect of proctolin on tension produced by isolated, denervated fibres

It is possible that proctolin acts by binding to its own receptor on the muscle and thereby allowing a greater influx of Ca^{2+} ions into the muscle in response to depolarisation. Such proctolin-dependant calcium channels have been found recently in crayfish SFM (Bishop *et al.*, 1990; 1991) and the differential effects of proctolin may represent variations in the total number of proctolin-sensitive channels in the two fibre types. In order to investigate this experimentally a series of tension measurements were performed on isolated muscle bundles consisting of a few fibres. For this purpose, a highly sensitive strain gauge system designed to measure the mechanical properties of single skinned fibres was utilised. In an attempt to provide more detailed and more comparable information on the effect of proctolin upon the mechanical properties of the two slow fibre phenotypes in *Nephrops*, measurements were

made from isolated bundles of approximately equal numbers of intact fresh fibres. Small bundles of two or three identified fibres were mounted in the strain gauge system in such a way that they could be quickly and easily moved in and out of a number of different solutions while continuously measuring changes in tension.

Perfusion of proctolin in normal saline onto the isolated intact fibres did not affect the resting tension of medial or lateral fibre bundles at any concentration. However, when the fibres were depolarised with high-KCl saline, a depolarisation-induced tension ('contracture') was produced and the effect of proctolin on this response was studied.

Initially, fibres were depolarised with a solution containing 300 mM KCl to produce large amplitude contractures but, at this concentration, proctolin was unable to produce any further amplification of tension, indicating that contractile saturation had occurred (Hodgkin and Horrowitz, 1960). The depolarising solution eventually employed contained KCl at a concentration 60 mM which generated about one third of the amount of tension generated by 300mM KCl saline. Thus, by lowering the KCl concentration in the saline it became possible to observe the effect of proctolin on tension development.

Dealing with such small numbers of fibres, it proved possible to make initial electrophysiological recordings while the muscle was still innervated. Thus it was established that all 3 fibres in the lateral bundle were of the 'silent' type (corresponding to the S1 phenotype) and indeed that (as expected) all 3 fibres of the medial bundle were of the 'active' type (corresponding to the S2 phenotype). Fig. 4.18 shows the results obtained from a medial and lateral bundle, each containing three fibres, obtained from the same SFM. The fibres were taken from the extreme medial and lateral edges of the muscle. The tension profiles of the different fibres, when depolarised with KCl showed very different rates of tension development: in the medial fibres tension reached a plateau in 20 seconds whereas in lateral fibres tension was still increasing after

66 seconds.

10^{-10} M proctolin in 60 mM KCl produced an 18% increase in maximum medial tension; i.e. in the control situation, 0.45 mN of tension was generated after 20 seconds of depolarisation whereas in the presence of proctolin, 0.48 mN of tension was generated in the same time period. The situation in lateral fibres was as follows: lateral fibres were depolarised for 60 seconds during which time they developed 0.40 mN of tension. In the presence of proctolin, an equivalent amount of tension was generated in lateral fibres in only 10 seconds. Therefore, proctolin increased the amount of tension generated in the first 10 seconds from 0.025 mN to 0.40 mN, (an increase of 1,600%!). Not only was proctolin able to potentiate the rate of development of tension but, it was also able to increase the rate of decay of tension so that the resting value was achieved faster than in the control situation.

In a preliminary further series of tests, carried out on only one lateral fibre bundle, the potential of this experimental approach for establishing a dose-response curve for proctolin was demonstrated. The concentration of the depolarising solution was lowered from 60 mM to a value which represented the threshold for tension development, in this case 15 mM. Figure 4.19 demonstrates the dose dependent effect of proctolin on lateral fibres depolarised to a very low level with 15 mM KCl saline. In this case no observable tension was produced in the presence of 15 mM KCl alone, but with the addition of 10^{-10} M proctolin, a tension of 0.025 mN was produced. Repetition of the experiment, substituting 10^{-8} M proctolin for 10^{-10} M proctolin resulted in the production of less than 0.01 mN of tension. Both doses were repeated twice to ensure that the dose dependency of the muscle was not artefactual. It is of particular interest that 10^{-8} M proctolin produces less tension than 10^{-10} M proctolin in the muscle bundle. Although this represents the results of a single experiment, it may represent the expression of the saturation effect described in 4.3.10.

4.3.7 Crayfish

Unlike the situation in *Nephrops* SFM, biochemical analysis of *Pacifastacus* SFM does not correlate with fibre heterogeneity of the muscle (Fowler *et al.*, 1990); both histochemical fibre types exhibit the same biochemical profile when run on an SDS-PAGE gel. Thus, further characterisation of the muscle was attempted by histochemical analysis of crayfish SFM which had previously been surveyed for innervation pattern. This approach also facilitated direct comparison of the innervation of the SFM in crayfish with the innervation of the muscle in *Nephrops*.

4.3.7.1 Muscle Histochemistry

Serial sections of whole abdomens of crayfish (Fig. 4.20a) were stained for total myofibrillar ATPase to determine their fibre composition. The intensity of staining in the deep fast flexor muscles was markedly greater than the levels of staining in both the superficial slow extensor (Fig. 4.21a) and flexor muscles (Fig. 4.22a). Differences in staining intensities reflect the contractile properties of the fibres in the different muscles. The deep flexors are fast contracting muscles and have higher levels of total myofibrillar ATPase activity than the superficial extensors and flexors which contract much less rapidly. This test also shows that within the slow SEMs and SFMs there is a heterogeneity in the staining reaction for mATPase, indicating that both of these muscles contain two populations of fibres with different properties. Both the SFMs and SEMs muscles in crayfish show a random pattern of distribution of the two fibre types. This contrasts with *Nephrops*, in which the different fibre types are almost completely segregated into medial and lateral bundles (Fowler and Neil, 1992).

When an alkali pre-incubation step was included in the procedure, a reversal of the pattern of staining occurred in both the SFMs and the SEMs (and the swimmeret). Fibres with higher levels of myofibrillar ATPase activity contained an isoform of myosin ATPase which was labile after alkali pre-incubation, while fibres with lower levels of myofibrillar ATPase contained an

alkali-stable myosin ATPase isoform (Figs. 4.20b - 4.22b).

4.3.7.2 Innervation

Crayfish SFM did not contain 'silent' lateral fibres commmonly found in *Nephrops* SFM. EJPs from at least one excitatory axon were seen in all lateral and medial crayfish fibres. Figures 4.23 and 4.24 show one partial data set (D1) and one complete data set (D2) for two crayfish SFM muscles which were surveyed extensively for their pattern of motor innervation. A comparison of both innervation maps reveals some close similarities in pattern of innervation. The innervation profile for the complete survey (D2) suggests a preferential innervation of medial fibres by the smallest axon, f1 and of lateral fibres by the tonic-phasic axon, f3. This is also reflected by the innervation pattern of these axons in survey D1. A high percentage of medial fibres were innervated by f1 whereas lateral fibres apparently received no innervation from this motor neurone. On the contrary, medial fibres were not innervated by f3 whereas all lateral fibres were, some producing very large EJPs in response to this motor neurone (up to 20 mV). The largest excitatory axon, f6, was only spontaneously active in survey D1 and sampling of the fibres across the muscle indicated that a high proportion of medial fibres but none of the lateral fibres were innervated by f6 suggesting a preferential innervation of medial fibres by f6. The flexor excitors, f2 and f4, innervate both medial and lateral fibres, although f2 produces smaller amplitude EJPs than f4. A higher proportion of medial fibres than lateral fibres received innervation from f2 and this axon produced low amplitude EJPs (approx. 1mV). The majority of medial fibres were innervated by f4 in the muscle shown in Fig. 4.24 and every fibre sampled was innervated by f4 in the other muscle analysed (Fig. 4.23). In both muscles the majority of lateral fibres were not innervated by f4.

The values for resting membrane potential were not significantly different for medial and lateral fibres ($p > 0.05$, Students t-test).

4.3.7.3 Correlation of histochemical and innervation data for crayfish muscle

Following characterisation with regard to innervation pattern (Fig. 4.24), muscle D2 was analysed histochemically. Figure 4.25 shows the muscle stained for total mATPase activity, revealing the presence of (at least) two histochemical fibre types. In this particular muscle, mixing of the two fibre types was less pronounced than was usually observed (see Fig. 4.21). Histochemically stained fibres were numbered to correspond with the results of the innervation survey and their histochemical and innervation properties were compared directly.

Results of these crayfish experiments indicate that the distribution of innervation does not appear to be correlated with fibre type. Also, there was no apparent correlation between resting membrane potential and fibre type in this data set e.g. the 4th medial fibre (M4) and the 4th lateral fibre (L4) had similarly low resting potentials (44mV and 51mV, respectively) but were of different fibre types. Furthermore, there was no obvious evidence for the existence of any gradient of innervation from one side of the SFM to the other as suggested by Velez and Wyman (1978).

4.3.7.4 Effect of proctolin on neurally induced tension in crayfish SFM

It is known that proctolin potentiates neurally induced tension in crayfish SFM and that the peptide is co-localised with a conventional transmitter in three of the five excitatory motor neurones which innervate the muscle: f1, f3 and f4 (Bishop *et al.*, 1894; 1987). The same experimental regime as the one applied to *Nephrops* was used except that, in light of the mixed distibution of fibre types, simultaneous intracellular recordings and tension measurements were made for the muscle as a whole and not for both medial and lateral bundles. From a practical point of view, separation of the bundles was much more difficult as the medial bundle is much reduced in *Pacifastacus* consisting of a much smaller number of fibres.

Proctolin was effective in enhancing tension produced in the crayfish

SFM as a result of supramaximal stimulation of the Sr3. This enhancment of tension was dose-dependent. Maximum tension was produced in 10^{-8} M proctolin, and the response was lower at higher concentrations (Fig. 4.26). The rate of production of tension was increased in the presence of proctolin but so was the time to return to baseline after cessation of stimulation; therefore the duration of tension was not altered by the presence of proctolin. Resting muscle tension was not affected by the perfusion in the presence of proctolin.

A comparison of intracellular recordings of SFM fibres before and after perfusion of proctolin revealed that the summated EJPs associated with generation of tension were identical. Similarly, proctolin had no effect on the amplitude or shape of single unfacilitated EJPs.

4.4 DISCUSSION

The aims of the experiments presented in this chapter were (i) to characterise the SFM of the Norway lobster *Nephrops norvegicus* with regard to fibre heterogeneity using data from biochemical, electrophysiological and mechanical studies and (ii) to investigate the role of the neuropeptide proctolin in this neuromuscular system.

The results obtained have revealed complexities in the pattern of innervation relative to fibre type which were not previously appreciated, and have established the differential responsiveness of the two slow fibre subtypes to proctolin.

4.4.1 Correlation of fibre subtype with innervation

A number of studies have proposed a correlation between the different subtypes in crustacean muscles and their pattern of motor innervation (Costello and Govind, 1983; Rathmayer and Maier, 1987; Wiens *et al.*, 1991). Lang *et al.*, (1980) provide convincing evidence for such a correlation in the claw closer muscles of the lobster *Homarus americanus* based on the oxidative capacity of fibres and their excitatory innervation. In *Nephrops*, it has been suggested by Neil and Fowler (1990) that S2 fibres in both the medial and lateral bundles are preferentially innervated by the smaller axons f2 and f3 whereas lateral S1 fibres are preferentially innervated by a larger unit, f4. The present study has confirmed such a correlation at a 'gross' level between fibre subtypes and their pattern of motor innervation. Medial and lateral fibres of the S2 phenotype characteristically displayed a pattern of high postsynaptic activity as a result of synaptic inputs from the majority of the five excitatory axons in the Sr3 innervating the SFM. The lateral S1 type fibres, on the other hand, most often showed no signs of synaptic input in response to normal levels of spontaneous activity of the axons in the isolated preparations used. In these preparations, an approximate inverse relationship was noted between the frequency of firing of axons and their spike amplitude (see Chapter 3, section 3.3.5); the smallest

units, f1 and f2, showed the highest levels of spontaneous activity whereas the largest unit, f6, was normally silent. Hence, the ability of f6 to produce an EJP in the majority of lateral fibres was not often apparent due to the infrequent nature of its firing. In some cases, small EJPs were also evoked in these fibres by spontaneous activity of either f3 or f4, supporting the notion that there is a correlation between type S1 lateral fibres and innervation by larger excitatory axons.

However, attempts to disentangle these 'gross' correlations by examining the underlying innervation of each of the excitatory axons across the SFM revealed a more complex picture. Data from the innervation surveys and from subsequent sampling during later experiments revealed that fibres in the medial bundle of the SFM are innervated by a varying combination of the five excitatory axons. Since all these medial fibres are of the same phenotype, it cannot be said that there is a precise correlation of a particular innervation pattern with fibre type. These results are consistent with those of Maier *et al.* (1986) who were also unable to correlate the pattern of innervation with the metabolic profile of fibres in the crab closer muscle. Thus, it seems that a certain degree of variability ('noise') occurs in the matching of induced axons to muscle fibres within the medial S2 populations. The detailed pattern of innervation obtained from medial SFM fibres could not be ascertained in lateral fibres due to their synaptic silence.

This observed variability in matching of innervation with muscle properties in *Nephrops* SFM is in contrast to the precise matching of muscle properties and motor neurone firing patterns which occurs during the early stages of development (Vrbova *et al.,* 1985) and continues into adulthood (Eisenberg, 1985) in vertebrate muscle. Recently, a similar responsiveness has been demonstrated in adult crustacean muscle by imposing a 'tonic' pattern of activity on phasic neurones innervating fast muscles in the crayfish claw closer muscles (Atwood and Nguyen, 1991). This leads to an adaptive alteration of the

fast muscles, both physiologically and morphologically, to a more tonic phenotype. It is possible that the observed differentiation of *Nephrops* SFM fibres into two subtypes (S1 and S2) is the result of exposure of medial and lateral fibres to different patterns of motor activity which would each place different metabolic demands on the fibres. Since the lateral fibres are histochemically 'faster' than medial fibres this implies that they receive a more phasic pattern of activity than medial fibres. *Nephrops* medial fibres are exposed to a tonic pattern of activity of at least 30 Hz, but as of yet the mean firing rate of axons to which lateral fibres are exposed cannot be defined. The reason is that it is still not clear whether (i) lateral fibres are synaptically connected to all six Sr3 motor neurones but the depolarising effects of the majority are masked by the presynaptic properties of these fibres during normal levels of spontaneous activity or (ii) they are only innervated by a subset of the total Sr3 population. Methylene blue staining of *Nephrops* SFM nerve-muscle preparations supports the latter theory as only three stained axons have been observed arborising across the lateral muscle fibres whereas all six axons can be counted arborising across medial fibres (unpublished observation, this study). Furthermore, in crayfish, the most lateral fibres of the SFM receive no input from f2 and even fewer lateral fibres are innervated by f1 (Clement *et al.*, 1983). In further studies, aside from selective stimulation of individual axons, it may be possible to position fine suction electrodes onto the branches of the Sr3 arborising across the lateral fibres to investigate which axons are branching across these fibres and hence innervating them.

In crayfish SFM, selective stimulation of individual axons has led to the proposal that some sort of gradient exists which controls the development of neuronal connectivity, resulting in either an increase or decrease in the strength of innervation for each axon across the muscle (Velez and Wyman, 1978a,b). In the two crayfish SFM surveys carried out in this study, it could be argued that the EJP amplitude of axons f1 f2, f4 and f6 decreased from medial to lateral

across the muscle, especially if the value of EJP size of groups of three fibres were averaged as in the study of Velez and Wyman (1978). In the same way, there seems to be an opposite trend of increasing EJP size from medial to lateral, for f3. However, the gradients in EJP amplitude for f3, f4 and f6 which exist in this study oppose those seen for the same axons in the study of Velez and Wyman (1978). Furthermore, these workers predicted that differentiation of muscle fibres in the crayfish SFM would follow the axonal gradients across the muscle. In fact, crayfish SFM tend to display a mixed distribution of two fibre types (Fowler and Neil, 1989) which show no obvious correlation with axonal supply or EJP amplitude.

In *Nephrops* SFM, the evidence for the existence of a gradient guiding innervation is even less convincing. Only the SFM analysed in survey s6 contained a reasonably high number of lateral fibres which were synaptically active. In this survey it could be argued that the strength of innervation of all five axons decreased from medial to lateral across the muscle. In the remaining five surveys, a spatially graded strength of innervation in the medial fibres was only suggested in survey s5 for the axons f1, f2 and f3 but the gradients of innervation of these axons oppose those seen in survey s6. Rather than being directed by a gradient, the pattern of innervation, at the gross level at least, seems to be directed to the two discrete regions which can be distinguished by their individual histochemical, biochemical and electrophysiological parameters.

4.4.2 Correlation of fibre subtype with presynaptic facilitation

Compared to medial fibres, lateral fibres exhibit low levels of postsynaptic activity. However, the apparent lack of synaptic input which this suggests may simply reflect presynaptic properties of the lateral fibres themselves. Lateral S1 fibres show high levels of presynaptic facilitation, a feature which is now generally accepted to be associated with 'low output' synapses (Sherman and Atwood, 1972; Parnas *et al.*, 1982c; Rathmayer and Hammelsbeck, 1985). In their comparative study of presynaptic and

postsynaptic matching in the singly innervated crab stretcher muscle preparation, Sherman and Atwood, (1972) found some fibres exhibited high levels of facilitation and released smaller amounts of transmitter ('low output' synapses) while others exhibited low levels of facilitation and released higher amounts of transmitter ('high output' synapses). Thus, it seems likely that lateral fibres will only be depolarised during periods of high frequency activity due to high facilitation of EJPs, whereas unfacilitated EJPs may be too small to be seen during normal levels of activity. Some evidence for this has been obtained in isolated preparations. In contrast, medial and lateral S2 fibres, exhibit properties of 'high output' synapses: they produce large amplitude EJPs in response to normal levels of activity and facilitate to a small extent. Thus, increasing levels of neuronal activity should result in the progressive recruitment of first the medial and then the lateral fibres within the muscle.

4.4.3 Correlation of presynaptic facilitation with contractile properties

The 'high output' synapses associated with low levels of facilitation are commonly found in muscles which are capable of generating fast 'twitch-like' contractions; on the other hand, 'low output' synapses associated with high levels of facilitation are usually found in muscles which generate slow, sustained contractions (Atwood, 1963; Rathmayer and Erxleben, 1983; Rathmayer and Maier, 1987). However, the opposite relationship seems to exist in *Nephrops* SFM between facilitation properties of medial and lateral fibres and their contractile properties, as reflected in their histochemical staining. The fibres with the higher contractile capacity (S1) have the higher levels of facilitation, while those with the lower contractile capacity (S2) have low facilitation rates. Lateral fibres show higher total mATPase activity, and lower levels of SDH activity than do medial fibres, indicating that they are faster contracting fibres and are less resistant to fatigue (Neil and Fowler, 1992). In considering such relationships between different properties of muscle fibres, it is important to realise that they do not always follow a predictable pattern. Thus, the expected

correlations between enzymatic and contractile properties do not always occur. For instance, Costello and Govind (1983) found a number of anomalies in their study of the contractile properties of the lobster claw closer muscle. Three contractile types were identified in the study but only two histochemical types were apparent.

In a similar way, in many crustacean muscles, including the SFM (this study and Galler and Neil, in press) a correlation has been found to exist between mean sarcomere length and contractile speed; (slow contracting fibres have long sarcomeres while fast contracting fibres have short sarcomeres) (Atwood, 1973), although this is not universally found. Thus, no strict correlation is evident in lobster claw closer muscles between contractile type and sarcomere length. Similarly, in the 'deep pink' fibres of the basal swimming muscles of the blue crab, fibres with short sarcomeres have slow contracting properties (Tse, *et al.,* 1983).

4.4.4 Selective stimulation of f6

The use of selective stimulation of the most phasic axon, f6, has suggested that further subclasses of fibre type may exist in *Nephrops* SFM. Although only two slow subtypes are obvious from SDS-PAGE gel electrophoresis, the existence of additional categories has been indicated by non-uniform levels of histochemical staining in some muscle regions (Neil *et al.,* unpublished observations). This could be investigated in future studies by lowering the pH at which histochemical tests are carried out. Such an approach has been used successfully by other groups to visualise differences in total mATPase activity which were not evident at higher values of pH (Tse *et al.,* 1983; Rossi-Durant and Pagni, 1986).

The preliminary investigations presented here on the effects of an individual axon (f6) on different *Nephrops* SFM fibres emphasise that the ultimate expression of stimulation measured at the intracellular fibre level is dependant upon both the synaptic properties of the axon and the cable

properties of the muscle fibres. Thus, it is not possible to classify f6 as a fast or slow excitor as it seems to show properties of both in different fibres. Fast axons are usually associated with high-output synapses which are able to generate rapid twitch-like contractions, whereas slow axons usually make low-output, strongly facilitating synapses enabling their target fibres to develop slow tension on repeated firing of the slow axons. Thus, the synaptic properties of f6 appear to be 'fast' both for type II and type IV fibres and 'slow' for type I and type III fibres. The facilitation properties of type II and type IV fibres in response to f6 stimulation resemble those exhibited by lobster limb flexor muscle fibres upon selective stimulation of the 'fast' α axon (Wiens *et al.*, 1992), whereas selective stimulation of the 'slow' p axon in the same fibres results in similar synaptic properties to those induced in the present study in type I and type III fibres by stimulation of f6. It is unclear from Wiens *et al.* (1991) whether selective stimulation of an individual axon always resulted in the production of one type of synaptic property i.e. high or low output, or whether the synaptic properties represented those seen in the majority of fibres and was taken as being representative. However, the approach taken by these workers to identify different muscle fibre types in response to stimulation of a single, identified axon may only reveal part of the picture as stimulation of an individual axon may produce a variety of responses in different fibres.

4.4.5. Mechanism of action of proctolin

The results presented in this chapter indicate that proctolin can act directly on both medial and lateral muscle fibres to enhance depolarisation-induced tension of the conventional transmitter. Proctolin amplification of tension occurs without observable depolarisation of the muscle or alteration of the EJPs. This suggests that it is acting at sites away from the synapses, perhaps to modulate the activity of one or more populations of proctolin-sensitive channels in the plasma membrane of the SFM. The presence of two such types of proctolin-modulated, calcium channels has been reported in crayfish SFM

(Bishop *et al.*, 1991). The channels are individually responsive to proctolin. Both proctolin and depolarisation are required to activate the opening of the larger channel, whereas the smaller channel is still partially open in the absence of proctolin. The consequential influx of extracellular calcium through these open channels results in an increase in the levels of free cytosolic calcium and hence increased tension generation for a given level of depolarisation. This suggests that co-release of proctolin with the conventional transmitter serves to make the SFM system more efficient as it provides a means of producing tension with less neural activity.

Alternatively, or additionally, proctolin's role in the SFM system may be to provide additional calcium required for muscular contraction. According to classical studies on vertebrate skeletal muscle, extracellular calcium is not involved directly in the production of contraction. Instead, depolarisation spreads through the muscle via the transverse tubules causing the release of calcium from the sarcoplasmic reticulum (SR). Binding of calcium to troponin then initiates contraction. As relaxation occurs through the uptake of calcium back into the SR, all of the calcium which is required for contraction is internally recycled. Thus the only need for extracellular calcium is to bolster internal stores (Hoyle, 1983). However, in cardiac muscle and frog skeletal muscle, there is evidence for calcium-induced calcium release (CICR) from the SR, resulting from the entry of extracellular calcium. In some crustacean muscles (Gainer, 1968; Atwater *et al.*, 1981) and some molluscan muscles (Huddart and Hill, 1988) the inward current on depolarisation is carried mainly by calcium, and there is good evidence that CICR is an important mechanism for muscle activation. It has been proposed that this difference in mechanism of contraction between vertebrate skeletal muscle and crustacean muscle results in an enhanced role for the sarcolemma and tubular systems in the latter muscles. Recently, lobster abdominal fast flexor muscle has been shown to contain a deeply invaginating transverse tubular system (T system) which is comprised of

50% more surface area than the SR (Crowe and Baskin, 1981). This greater area would be expected to carry a larger number of calcium channels. It has been proposed that the developed T system can more effectively produce fast contractions by mediating a more rapid influx of extracellular calcium directly to the contractile machinery, with CICR from the SR playing a secondary role (Crowe and Baskin, 1981). It is possible that this influx of extracellular calcium is susceptible to upregulation by proctolin in *Nephrops* SFM as has been shown for crayfish SFM (Bishop *et al.*, 1991). EM evidence suggests that the T system in *Nephrops* SFM is more extensively developed in lateral fibres than it is in medial fibres (Fowler and Neil, 1992). Lateral fibres also have faster contractile properties. Thus of the two fibre types, lateral fibres may be expected to show the greatest responsiveness to proctolin to facilitate the entry of extracellular calcium needed to mediate faster contractions. This may help to explain the increased quantities of proctolin found in extracts of SFM lateral fibres compared to medial fibre extracts (Chapter 2) and the ability of proctolin to potentiate neurally induced tension in lateral fibres to a much greater degree than it is able to in medial fibres (this Chapter). The enhanced action of proctolin on tension generation in *Nephrops* lateral fibres compared to medial fibres could represent the activity of differentially-sensitive populations of proctolin channels.

4.4.6 Functional interpretation of proctolin effect

Studies of the action of proctolin, both in whole muscle bundles and isolated bundles of a few fibres showed that it is more effective in potentiating tension in the lateral fibres than in the medial fibres. An understanding of the functional significance of this relies upon knowledge both of the mechanisms of the action of muscle bundles across the intersegmental joint, and of the recruitment of these fibre bundles in various motor behaviours. One possible functional interpretation of the enhanced effect of proctolin on the lateral fibres is that medial fibres have a greater mechanical advantage than lateral fibres

across the segmental joint. Figure 3.2 shows that medial fibres lie parallel to the ventral nerve cord whereas lateral fibres are inserted at an angle of as much as 45° at their most medial edge. Thus, the mechanical force generated by lateral fibres may have to be greater than medial fibres to contract the muscle to the same extent. The orientation of the medial and lateral fibre bundles suggests that they may have functionally different roles. Tension generated in the medial fibres acts along the axis of symmetry. Tension generated in the lateral fibres would impart a tortional force on each side of the segment. These forces could effectively brace the abdomen, locking it more firmly into the hemisegment in front at the intersegmental joint.

Table 4.1

Percentage of synaptically active medial and lateral fibres in each muscle survey. The absence of spontaneous activity of superficial flexor motor neurones in individual experiments is represented by (-) whereas (0) denotes spontaneous activity of motor neurones where no corresponding synaptic input onto muscle fibres was evident.

Table 4.2

Total percentage of synaptically active medial and lateral fibres.

	Medial fibres						Lateral fibres					
	s1	s2	s3	s4	s5	s6	s1	s2	s3	s4	s5	s6
f1	86	0	87	0	91	92	0	0	0	0	0	12.5
f2	95	90	87	59	65	96	0	10	7	25	0	58
f3	90	100	-	82	52	38	0	0	-	25	0	37.5
f4	-	100	-	-	0	96	-	10	-	-	7	54
f6	-	-	-	94	-	50	-	-	-	35	-	29

	No. of synaptic-ally active medial fibres	Total no. of medial fibres	% of synaptic-ally active medial fibres	No. of synaptic-ally active lateral fibres	Total no. of lateral fibres	% of synaptic-ally active lateral fibres
f1	83	120	69	3	94	3
f2	99	120	67	21	94	22
f3	65	97	67	14	80	17.5
f4	35	59	59	15	48	31
f6	29	43	67	14	44	32

Table 4.3

Properties of muscle fibres comprising SFM in *Nephrops*. Means +/- S.D. are given in all cases except for facilitation where f_{10} denotes facilitation of the 10th EJP in a train of 20/s stimulation. The number of fibres (x) and of muscles (y) used is indicated as n=x/y. Data labelled * are derived from Fowler and Neil (1992).

Position in muscle	Medial					Lateral					Lateral
Biochemical fibre type	S2					S2					S1
mATPase*	low					low					high
isoform of mATPase at preincubation pH 9.6	stable					stable					labile
SDH activity*	high					high					low
Membrane potential (mV)	-57.1 ± 8.1 n=119/6					-55.9 ± 8.7 n=29/5					-55.5 ± 9.0 n=68/6
Facilitation	-0.7 to 1.7 n=19/6					-0.2 to 1.8 n= 11/5					-0.2 to 3.0 n= 15/6
Innervation	f1	f2	f3	f4	f6	f1	f2	f3	f4	f6	f6
EJP (mV)	1.2 ± 1.0 (0.1 to 5.1) n=50/3	5.2 ± 5.0 (0.2 to 18) n=96/6	5.5 ± 5.0 (0.3 to 22) n=54/5	2.7 ± 2.0 (2.9 to 7.3) n=50/3	7.3 ±6.2 (1.3 to 2.1) n=29/2	2.2 ±1.8 (0.5 to 3.8) n=20/3	4.4 ± 4.8 (0.2 to 16) n=20/3	3.8 ±3.7 (0.2 to 13.7) n=13/2	1.6 ± 1.4 (0.1 to 4.3) n=15/3	3.3 ± 1.0 (0.1 to 5.1) n=50/2	3.1 ± 2.0 (0.8 to 6.4) n=10/2
τ (ms)	16 ± 10 (9 to 45) n=12	22 ± 14 (11 to 64) n= 14	25 ± 15 (12 to 72) n=18	24 ± 4 (16 to 29) n=8	47 ± 16 (22 to 80) n=14	23 ± 8 (9 to 39) n=8	28 ± 9 (10 to 46) n= 10	32 ± 10 (11 to 41) n=8	36 ± 6 (25 to 44) n=9	36 ±16 (22 to 66) n=7	36 ± 6 (30 to 44) n=10

Table 4.4

Comparison of neuromuscular properties of a single *Nephrops* SFM including membrane time constant (τ), range of amplitude of single EJPs, facilitation of the 10th EJP in a train of 20 Hz stimulation (f_{10}) and pattern of innervation of spontaneously active axons. All four axons, f2, f3, f4 and f6 were spontaneously active. Fibres were sampled successively from the medial to the lateral edge of the SFM.

Fibres were classified according to:

(a) position in muscle

(b) pattern of innervation of spontaneously active axons (from which the biochemical subtype of fibres was predicted)

(c) facilitation and decay properties.

Evidence of synaptic connectivity in the form of an observable EJP is displayed in the table as + or - for each axon.

Fibre type			Neuromuscular properties			Spontaneously active axons			
a	b	c	τ (ms)	EJP (mV)	f10	f2	f3	f4	f6
M1	S2	Ia	42	3.00	1.2	+	+	+	+
M2	S2	Ia	41	3.30	1.3	+	+	+	+
M3	S2	Ia	36	3.00	1.7	+	+	+	+
M4	S2	Ia	55	3.58	1.1	+	+	+	+
M5	S2	Ia	40	3.00	1.6	+	+	+	+
M6	S2	Ia	30	1.90	1.2	+	+	+	+
M7	S2	III	80	1.48	1.2	+	+	+	+
M8	S2	IV	200	1.20	-0.2	+	+	+	+
M9	S2	IV	116	2.00	-0.6	+	+	+	+
M10	S2	IV	100	1.68	-0.4	+	+	+	+
M11	S2	II	40	1.92	-0.2	+	-	+	+
M12	S2	II	20	1.36	-0.7	+	-	+	+
M13	S2	Ia	30	1.60	1.3	+	-	+	+
M14	S2	Ia	40	0.48	1.2	+	-	+	+
M15	S2	Ia	30	1.50	1.0	+	-	+	+
M16	S2	Ia	40	1.30	1.3	+	+	+	+
M17	S2	Ia	30	1.40	1.6	+	+	+	+
M18	S2	IV	160	1.30	-0.6	+	+	+	+
M19	S2	Ia	40	1.40	1.3	+	-	-	+
M20	S2	II	55	1.30	-0.1	+	-	-	+
M21	S2	II	50	3.40	-0.2	+	-	-	+
L1	S1	Ib	?	?	high	-	-	-	-
L2	S1	Ib	?	?	high	-	-	-	-
L3	S1	Ib	?	?	high	-	-	-	-
L4	S1	Ib	?	?	high	-	-	-	-
L5	S2	Ia	50	1.04	1.1	+	-	-	+
L6	S2	Ia	40	0.90	1.3	+	-	-	+
L7	S1	II	44	6.40	-0.2	-	-	-	+
L8	S1	Ia	35	3.60	1.5	-	-	-	+
L9	S1	Ia	40	3.80	1.5	-	-	-	+
L10	S1	Ia	30	0.80	1.1	-	-	-	+
L11	S1	Ia	30	1.00	1.3	-	-	-	+
L12	S1	Ic	-	-	-	-	-	-	-
L13	S1	Ic	-	-	-	-	-	-	-
L14	S1	Ic	-	-	-	-	-	-	-

Figure 4.1

Typical pattern of histochemical staining observed in *Nephrops* SFM (from Neil and Fowler, 1991). Muscle was stained for SDH activity. The majority of fibres in the lateral bundle stain lightly for SDH, indicative of low oxidative capacity. The majority of medial fibres show intense staining for SDH, indicating that they are more fatigue resistant than lateral fibres.

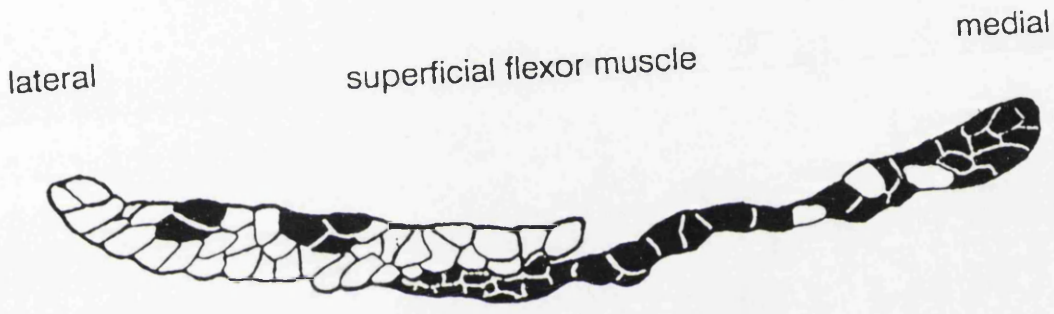
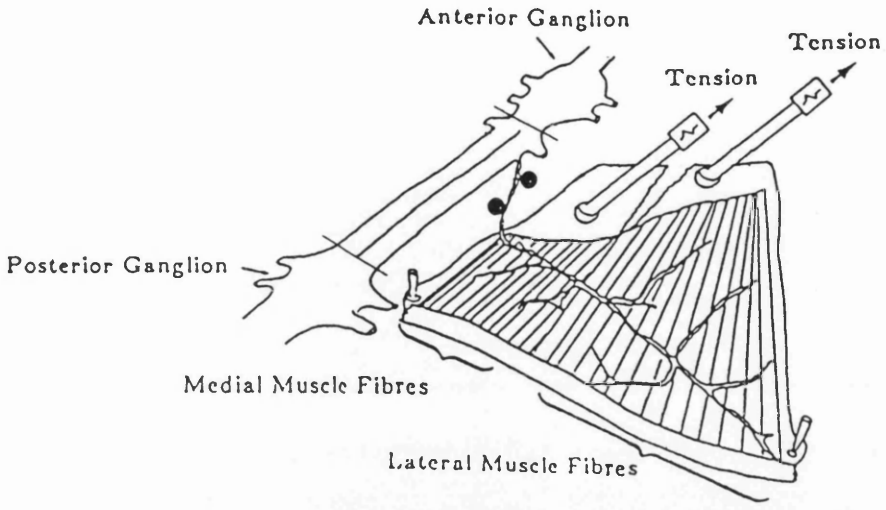

lateral
superficial flexor muscle
medial

Figure 4.2

Modified nerve-muscle preparation used to record mechanical tension in the medial and lateral fibre bundles of the SFM. Tension in each bundle was monitored independantly by the use of identical strain gauges. Platinum electrodes (black dots) were placed against the Sr3 in order to stimulate and record from the nerve and intracellular activity in medial and lateral fibres was sampled with glass microelectrodes (not shown).

Figure 4.3

Diagram of the experimental layout used to make intracellular recordings fron SFM fibres. A description of the equipment and procedure used for recording of extracellular activity can be found in Chapter 3 (section 3.2.4).


Anterior Ganglion
Tension
Tension
Posterior Ganglion
Medial Muscle Fibres
Lateral Muscle Fibres

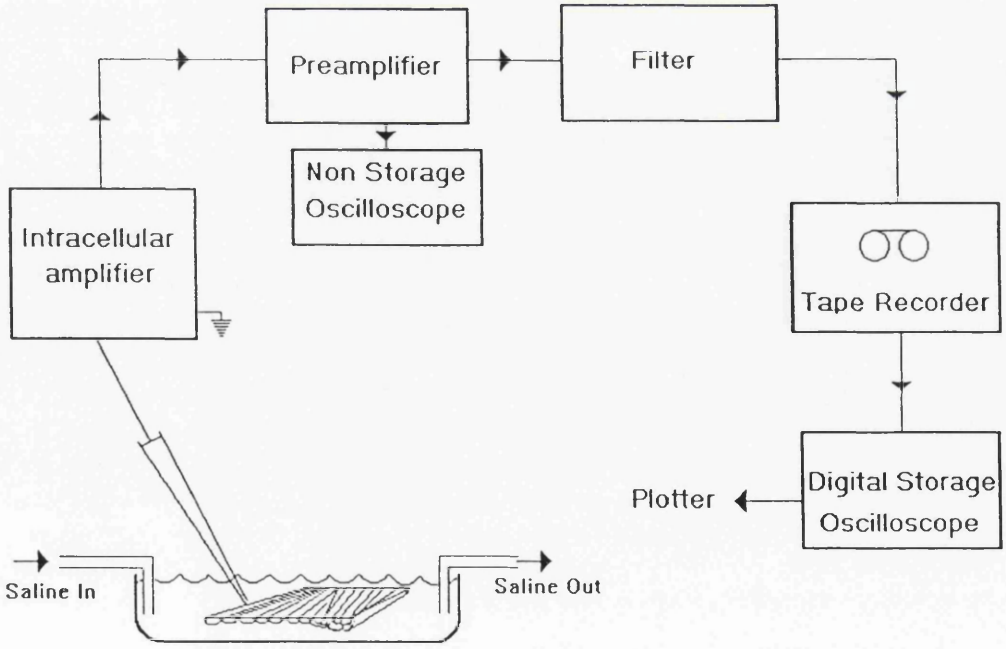

Preamplifier
Filter
Non Storage
Oscilloscope
Intracellular
amplifier
Tape Recorder
Digital Storage
Oscilloscope
Plotter
Saline In
Saline Out

Figure 4.4

Typical patterns of activity observed in medial and lateral fibres in response to normal levels of spontaneous activity in the isolated nerve-muscle preparation.

A. Simultaneous intracellular and extracellular recordings showing the different patterns of innervation observed in a medial fibre and a 'silent' lateral fibre in response to spontaneous motor activity in the Sr3.

B. Simultaneous intracellular and extracellular recordings showing the similarity in pattern of innervation of medial and 'active' lateral fibres in response to spontaneous motor activity in the Sr3.

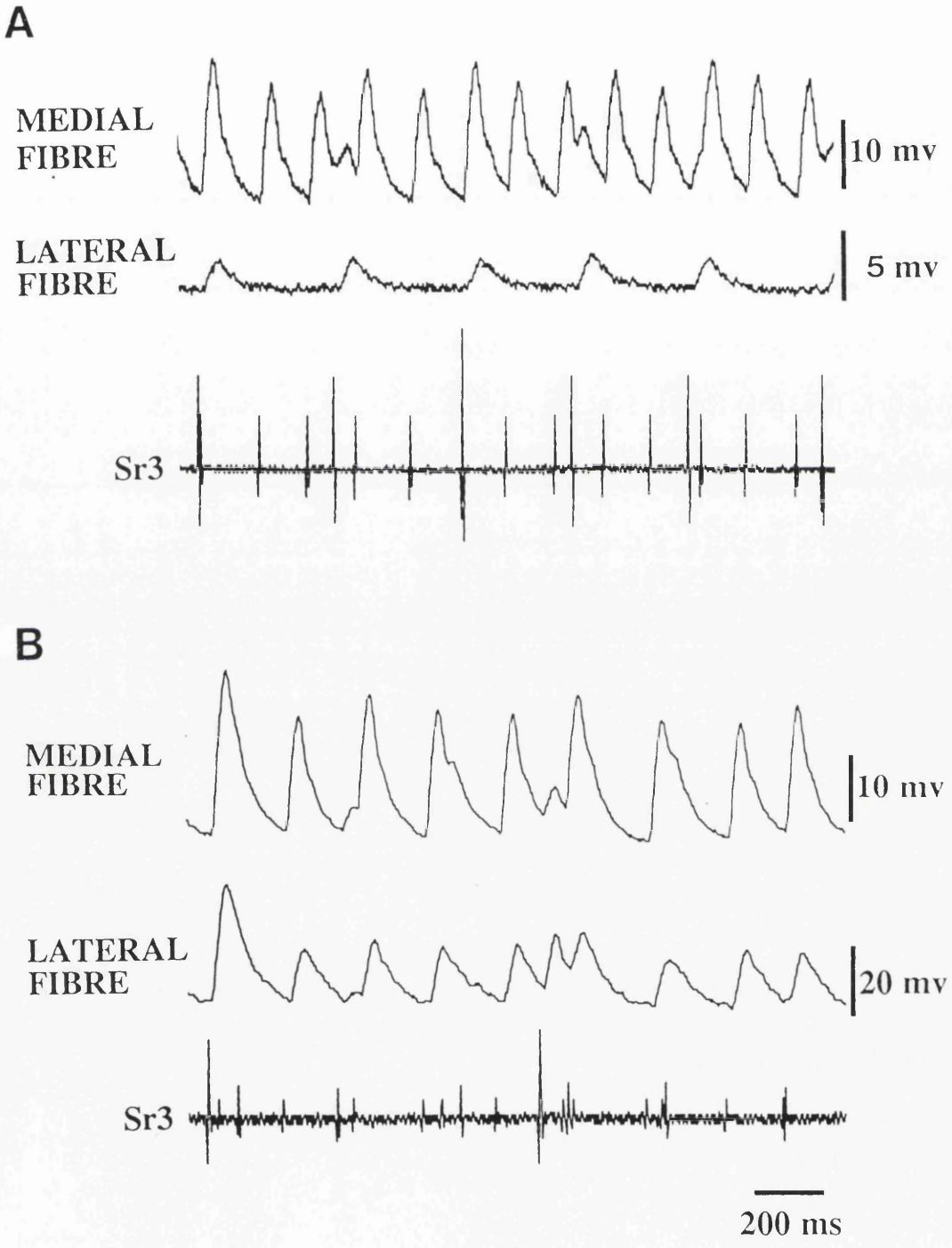

A
MEDIAL FIBRE
10 mv
LATERAL FIBRE
5 mv
Sr3
B
MEDIAL FIBRE
10 mv
LATERAL FIBRE
20 mv
Sr3
200 ms

Figure 4.5

A. Typical example of a good SDS-PAGE gel run showing the electrophoretic separation of myofibrillar protein assemblages in **Lane a.** SFM S_2 fibre **Lane b.** SFM S_1 fibre and **Lane c.** fast flexor fibre.

MHC - myosin heavy chain.

P_1 - paramyosin.

T_1 - troponin T_1 isoform.

A - actin.

TM - tropomyosin.

B. Correlation of biochemical profile with pattern of innervation in SFM fibres. The figure shows simultaneous recordings of a 'silent' lateral fibre, a medial fibre and spontaneous motor activity in the Sr3. Subsequent biochemical analysis of these electrophysiologically identified fibres revealed that lateral fibres (lat) which exhibited the 'silent' pattern of activity were identified as S1 type fibres according to the criteria of Mykles (1985b). In the same way, both medial fibres (med) and 'active' lateral fibres (not shown) which were innervated by the majority of flexor excitors, were identified as S2 type fibres.

Scale bar = 10 mV, 200 msecs.

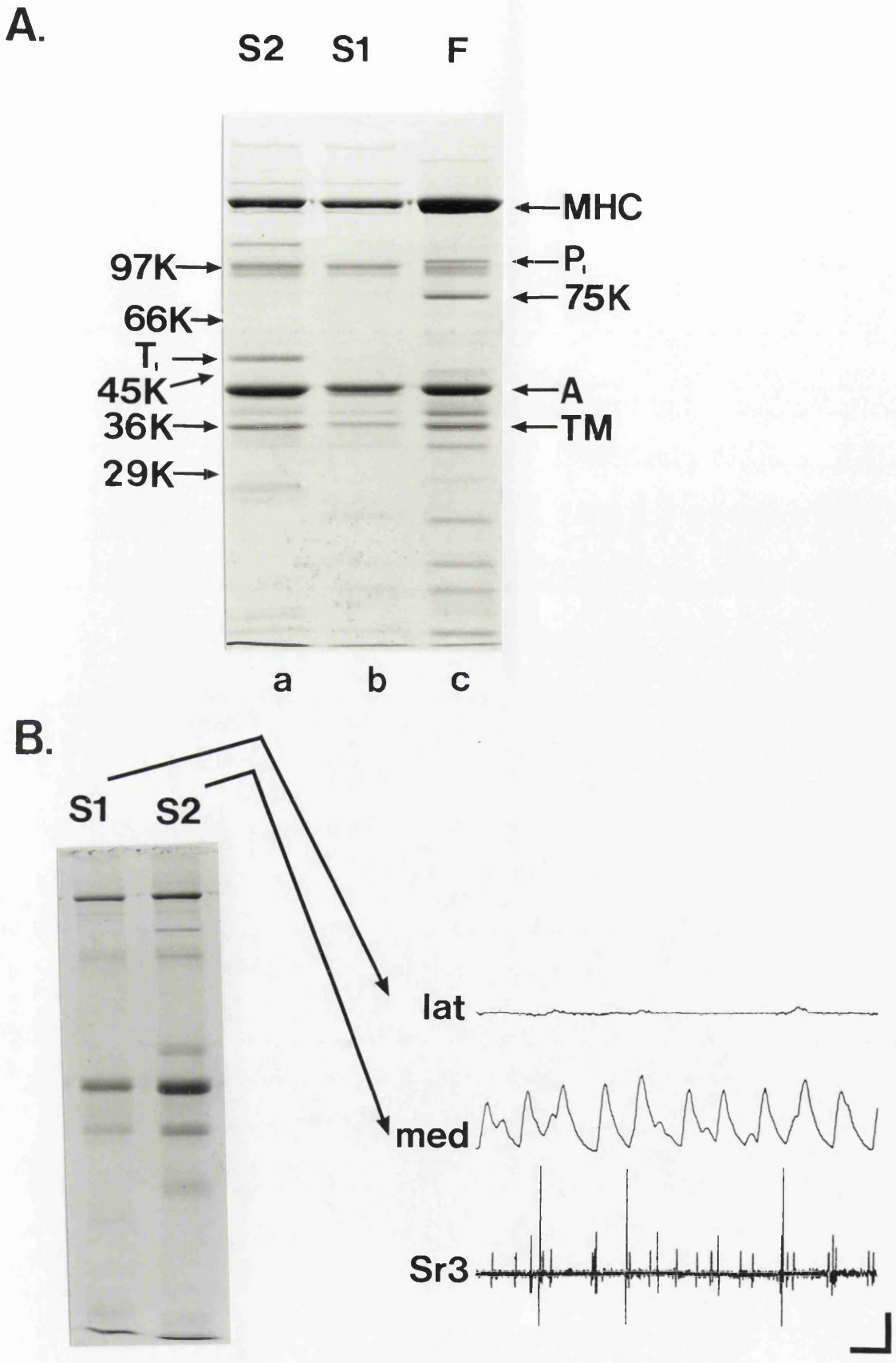
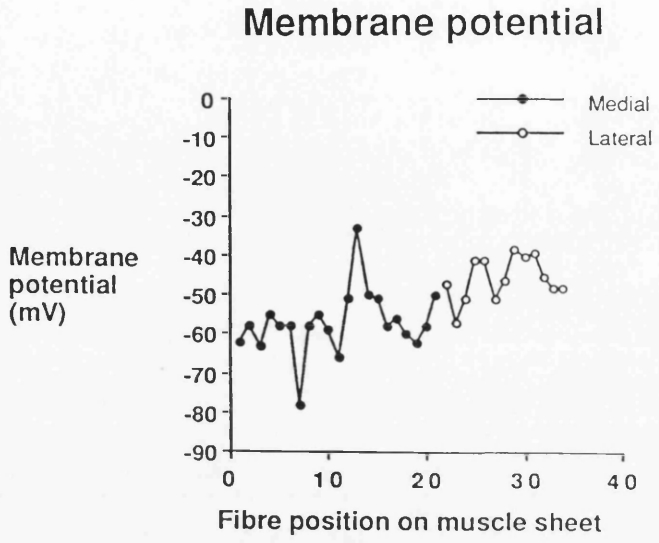

A.
S2
S1
F
MHC
97K
P_i
75K
66K
T_i
45K
A
36K
TM
29K
a
b
c
B.
S1
S2
lat
med
Sr3

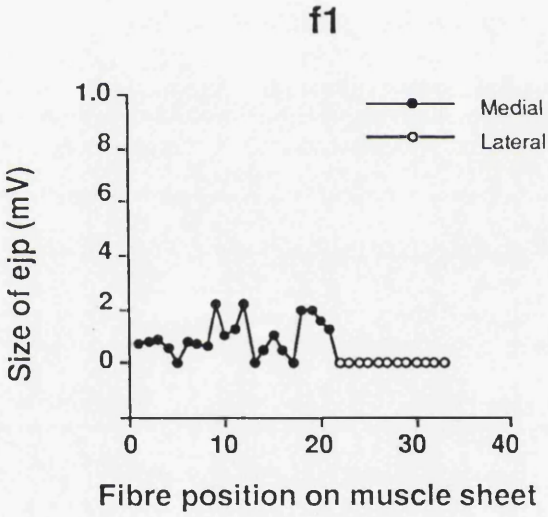
Figure 4.6

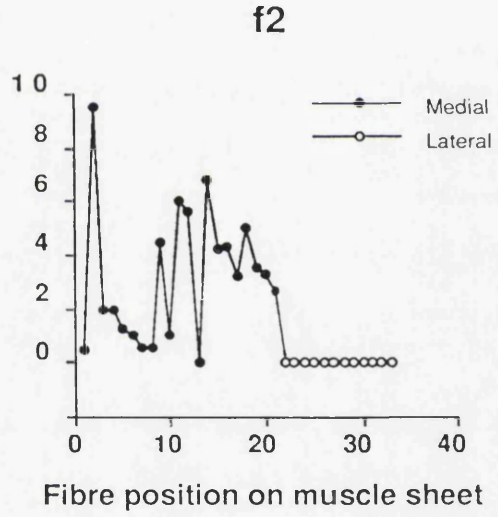
Survey (S1) of membrane potential and pattern of innervation of single fibres sampled successively from medial to lateral edge of *Nephrops* SFM.

Filled circles represent medial fibres, open circles represent lateral fibres.

Innervation of S.F. muscle - Lobster







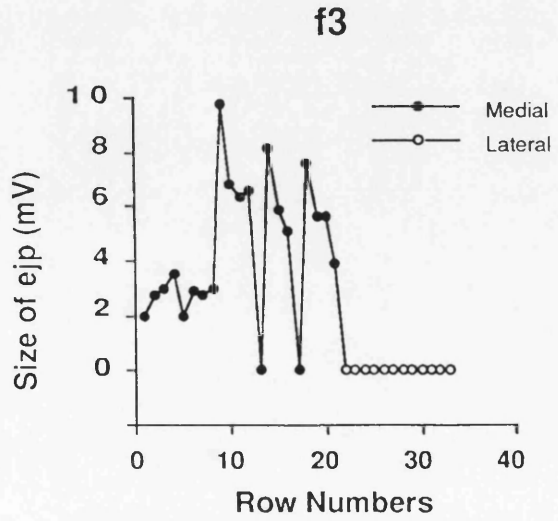
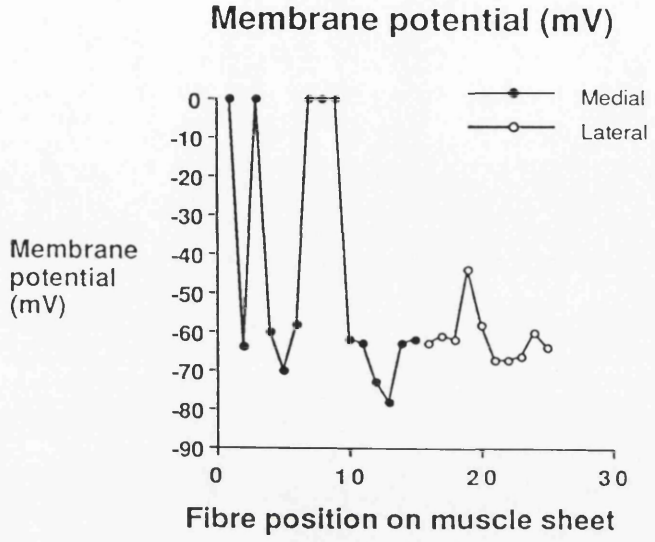


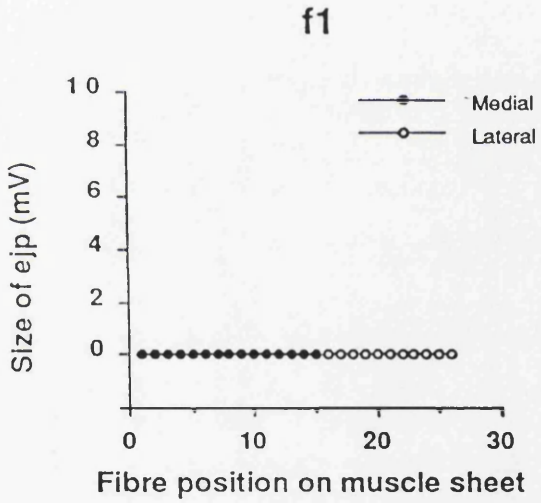
Figure 4.7

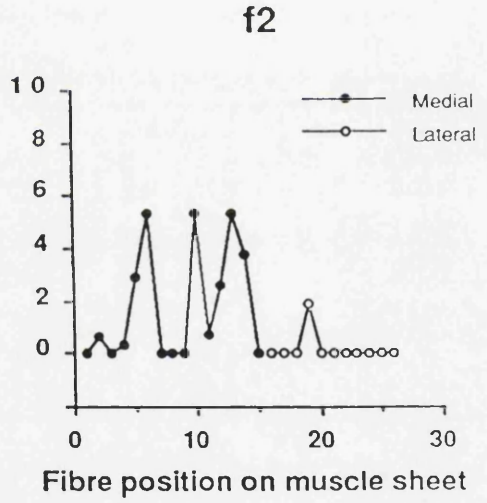
Survey (S2) of membrane potential and pattern of innervation of single fibres sampled successively from medial to lateral edge of *Nephrops* SFM.

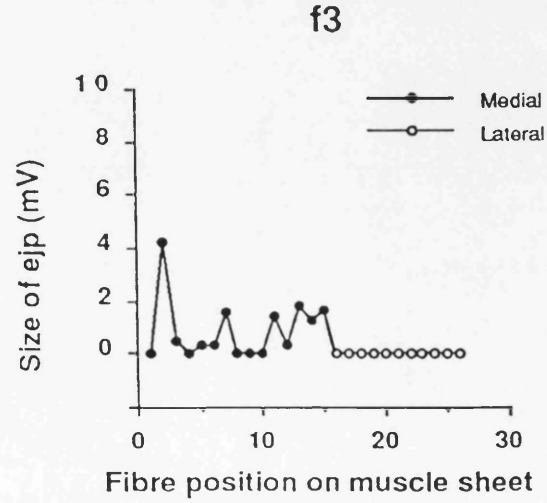
Filled circles represent medial fibres, open circles represent lateral fibres.

Innervation of S.F. muscle - Lobster









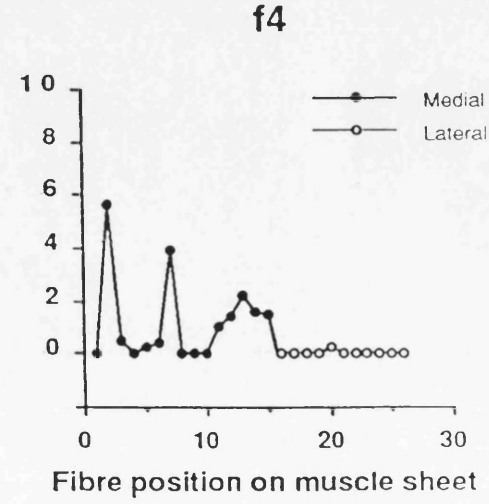
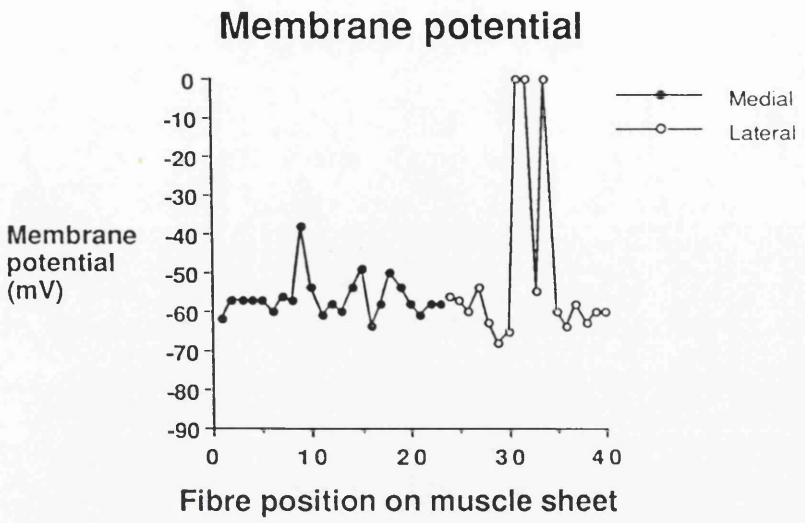


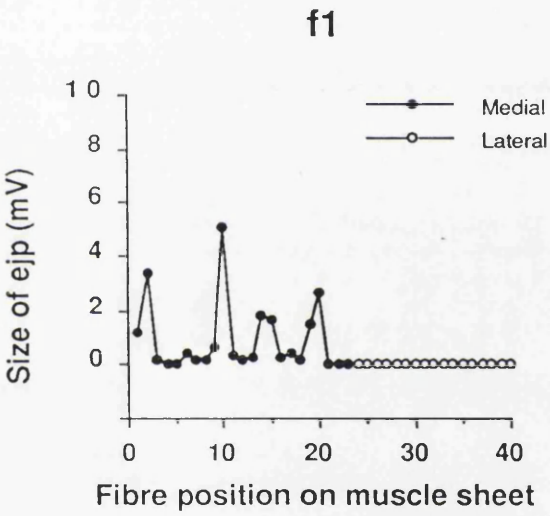
Figure 4.8

Survey (S3) of membrane potential and pattern of innervation of single fibres sampled successively from medial to lateral edge of *Nephrops* SFM.

Filled circles represent medial fibres, open circles represent lateral fibres.

Innervation of S.F. muscle - Lobster





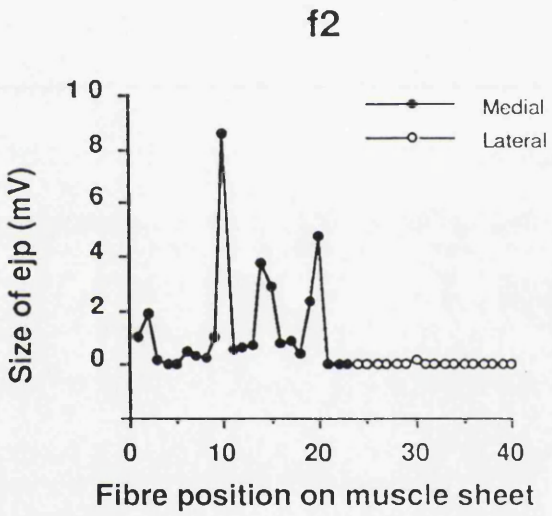
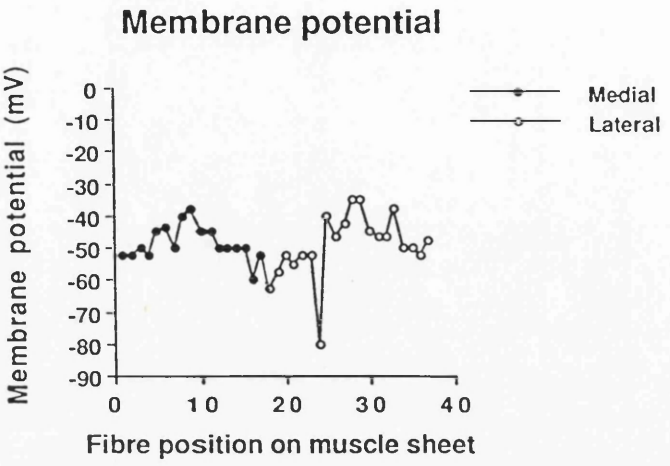


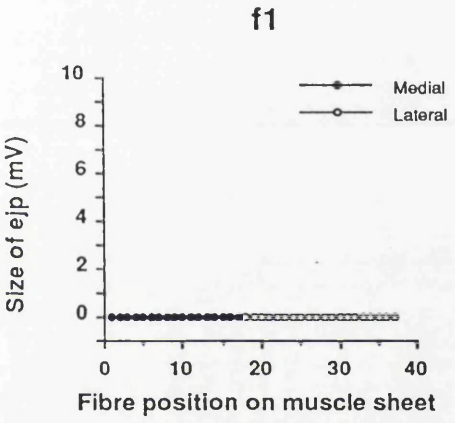
Figure 4.9

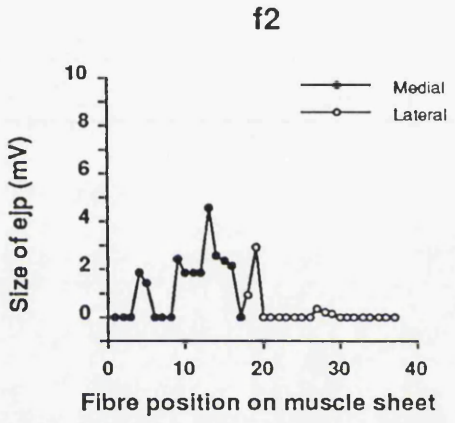
Survey (S4) of membrane potential and pattern of innervation of single fibres sampled successively from medial to lateral edge of *Nephrops* SFM.

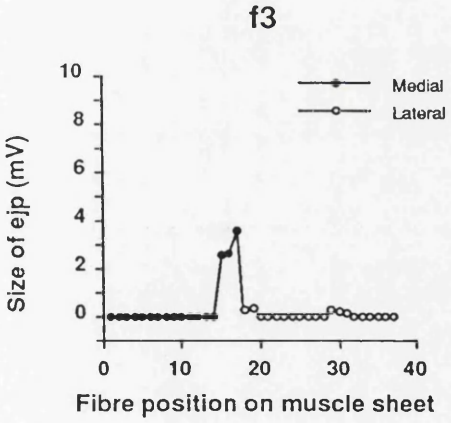
Filled circles represent medial fibres, open circles represent lateral fibres.

Innervation of S.F. muscle - Lobster









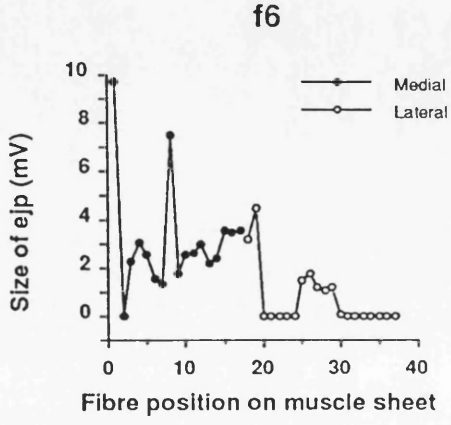
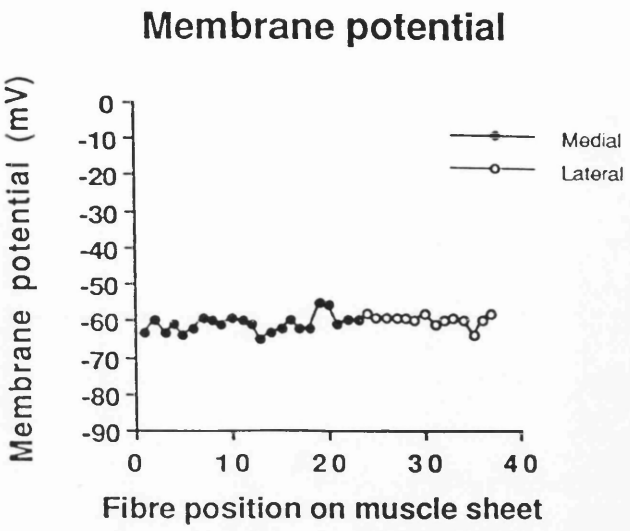


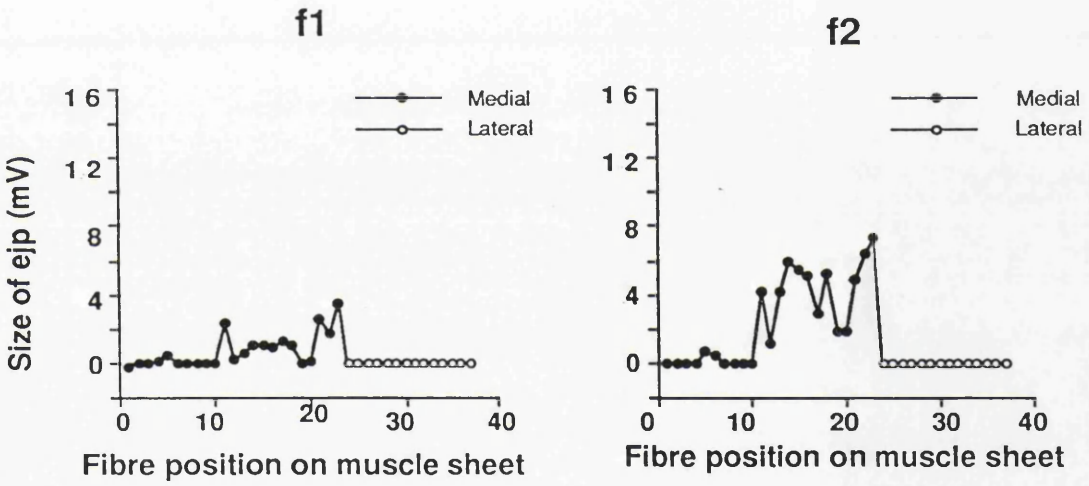
Figure 4.10

Survey (S5) of membrane potential and pattern of innervation of single fibres sampled successively from medial to lateral edge of *Nephrops* SFM.

Filled circles represent medial fibres, open circles represent lateral fibres.

Innervation of S.F. muscle - Lobster





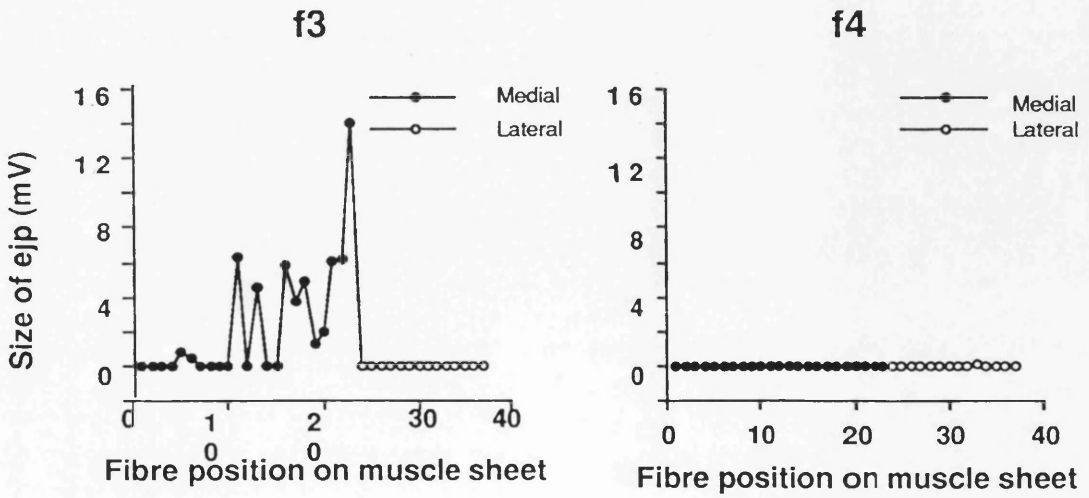
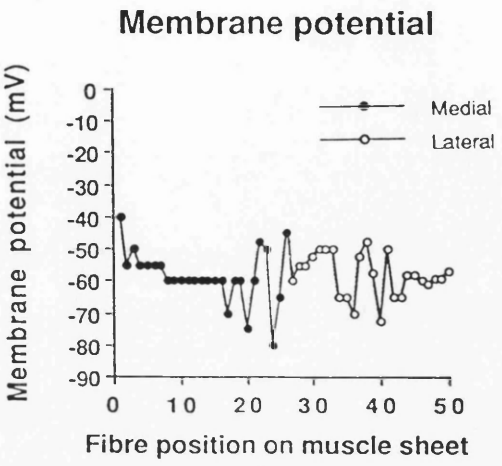


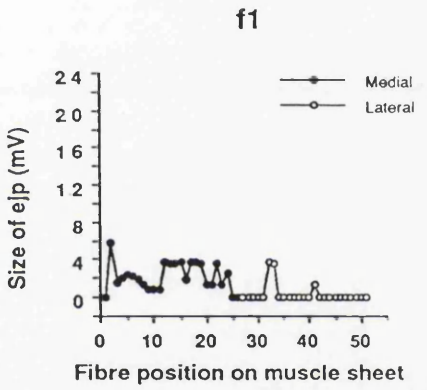
Figure 4.11

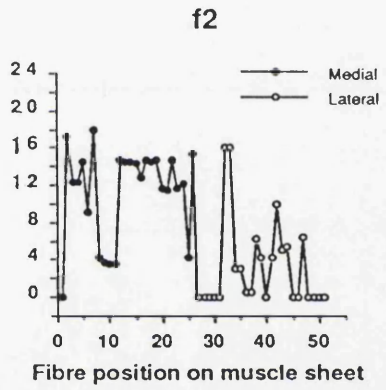
Survey (S6) of membrane potential and pattern of innervation of single fibres sampled successively from medial to lateral edge of *Nephrops* SFM.

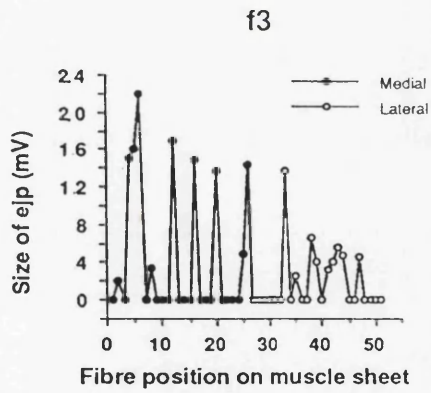
Filled circles represent medial fibres, open circles represent lateral fibres.

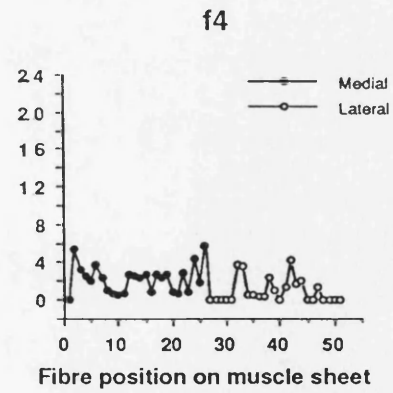
Innervation of S.F muscle - Lobster











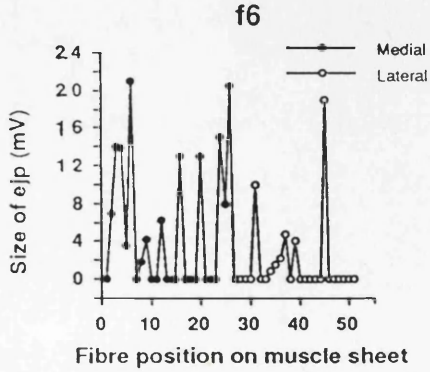


Figure 4.12

Recording of EJPs from *Nephrops* SFM fibres upon supramaximal stimulation of the Sr3 at a frequency of 1, 5, 10 and 20Hz. This preparation was still spontaneously innervated to emphasise the lack of activity in 'silent' lateral fibres as opposed to medial or 'active' lateral fibres. Thus, the baseline in these fibres represents spontaneous activity and not bad signal to noise ratio.

All recordings are from one preparation.

LS - 'silent' lateral fibre.

La - 'active' lateral fibre.

M - medial fibre.

Scale bar = 20 mV for medial and 'active' lateral fibres, 1 mV for 'silent' lateral fibres; 500 msecs.

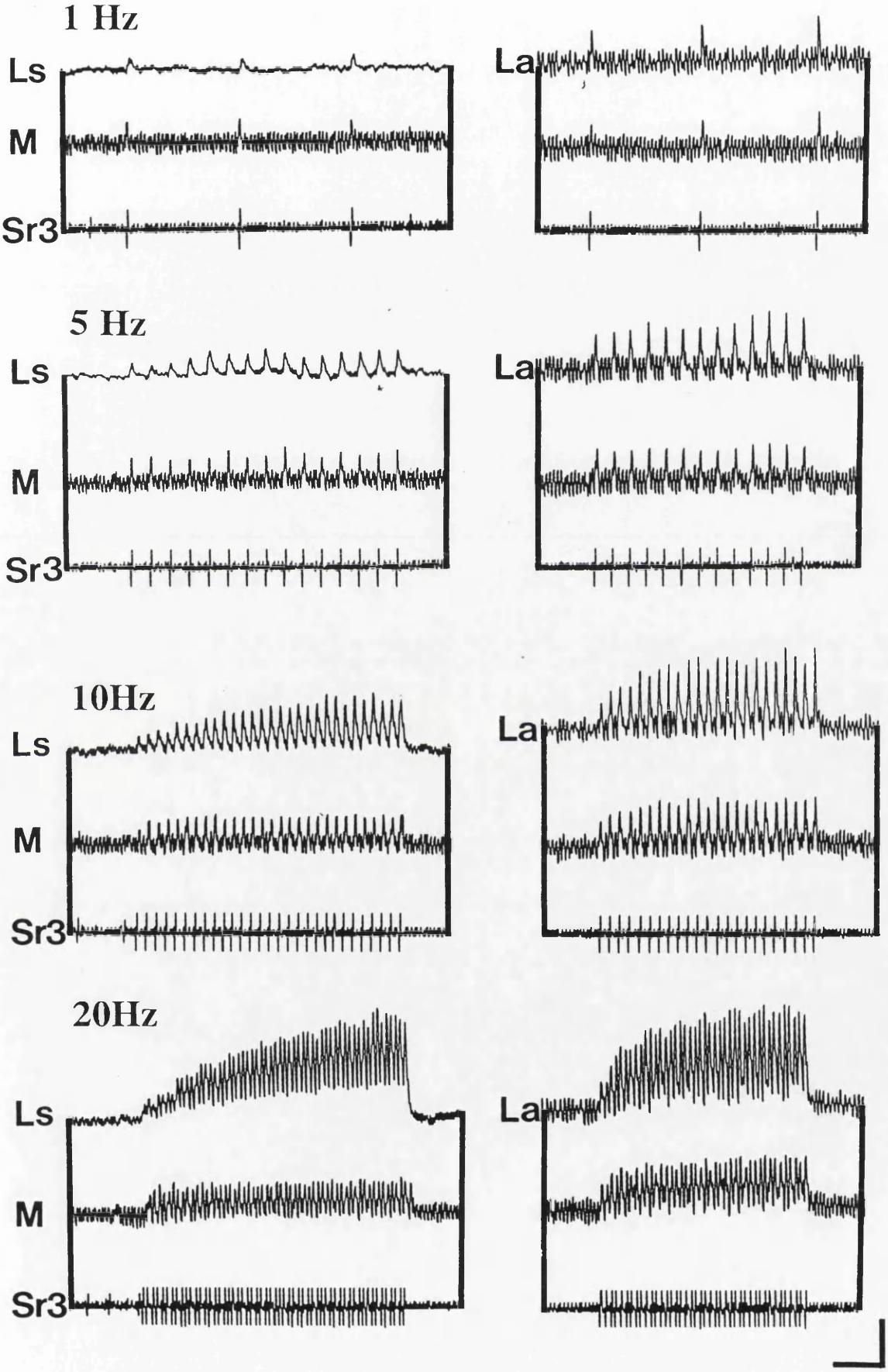

1 Hz
Ls
M
Sr3
La
5 Hz
Ls
M
Sr3
La
10Hz
Ls
M
Sr3
La
20Hz
Ls
M
Sr3
La

Figure 4.13

Recording of EJPs from a medial and a lateral fibre upon stimulation of the Sr3 with a 2 second pulse at a frequency of 5, 10 and 20 Hz. In this preparation the fibres were identified electrophysiologically before the Sr3 was cut. Both fibres were recorded at the same gain to demonstrate the difference in size of EJPs generated in the different fibre types. Many 'silent' lateral fibres displayed a dramatic rise in baseline when stimulated at 20 Hz or above. In some cases, this rise in baseilne was not accompanied by any observable EJPs.

Scale bar = medial - 10 mV, lateral - 1 mV; 500 msec.

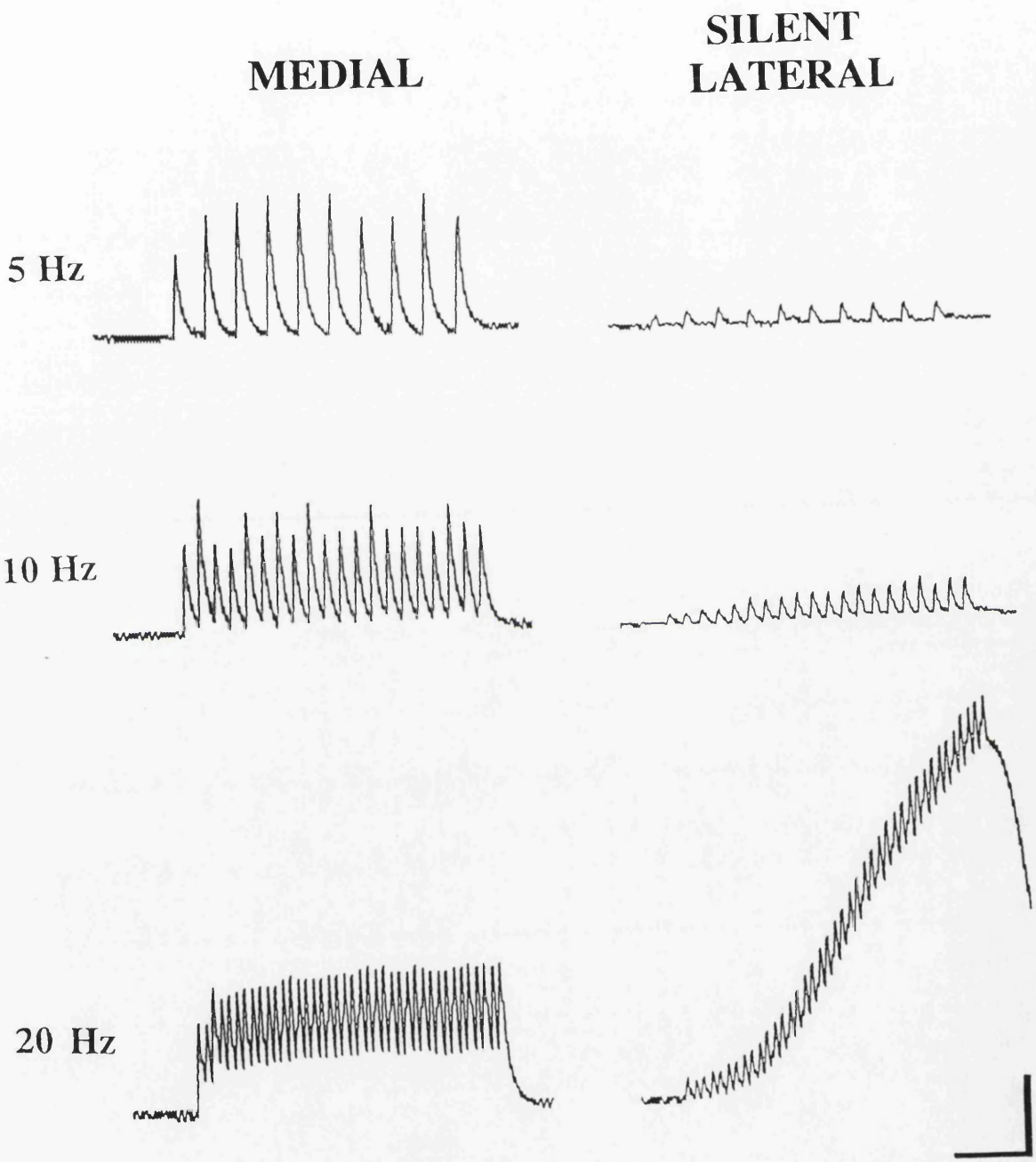

MEDIAL
SILENT
LATERAL
5 Hz
10 Hz
20 Hz

Figure 4.14

Typical synaptic responses of EJPs from both medial and lateral SFM fibres to selective stimulation of f6 with 1Hz, 10 Hz (5 pulses) and 20z (10 pulses). All recordings are from one preparation.

Scale bars = Ia, III, IV - 2 mV, II - 1 mV, Ib - 0.1 mV; 200 msecs.

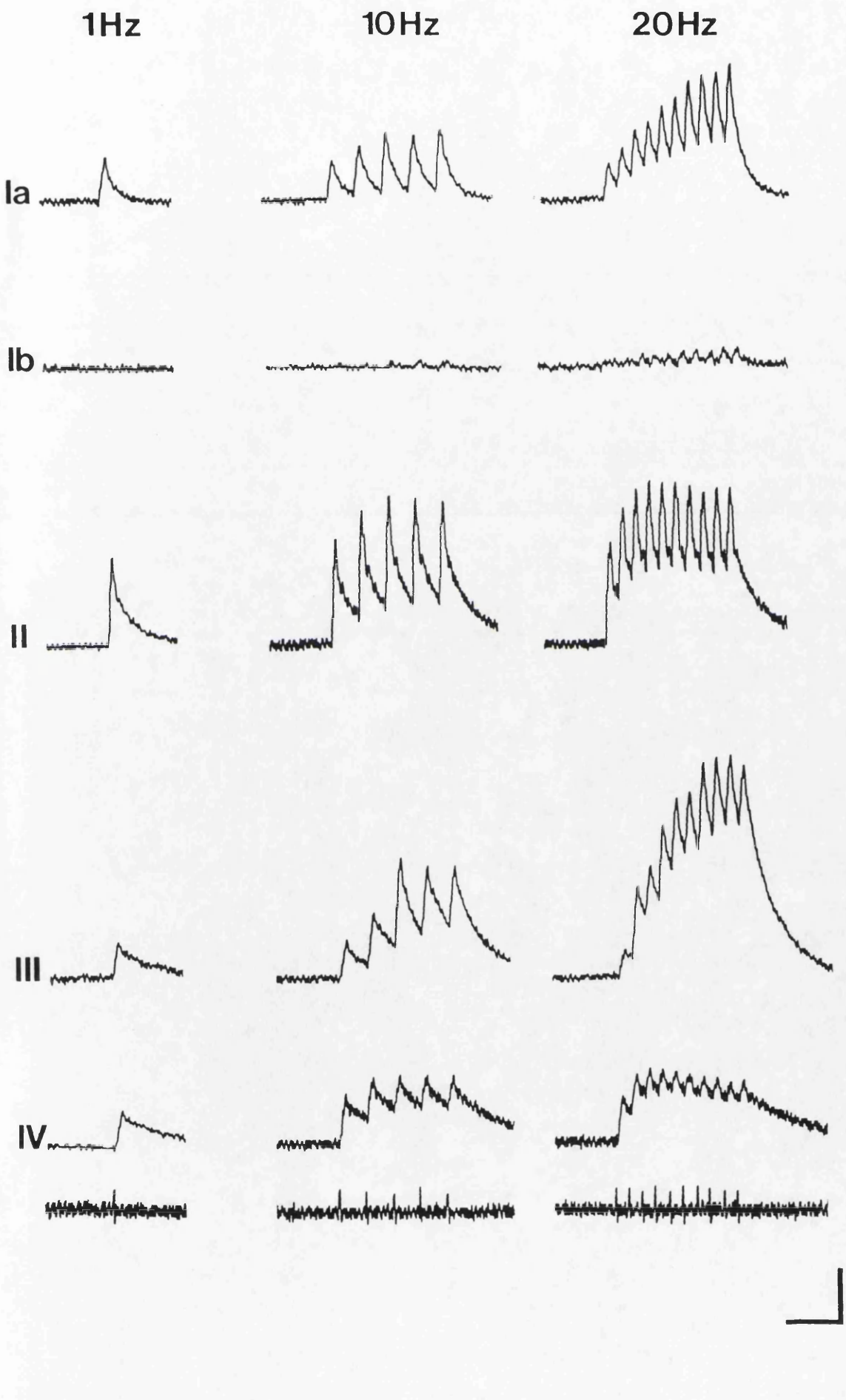

1Hz
10Hz
20Hz
Ia
Ib
II
III
IV

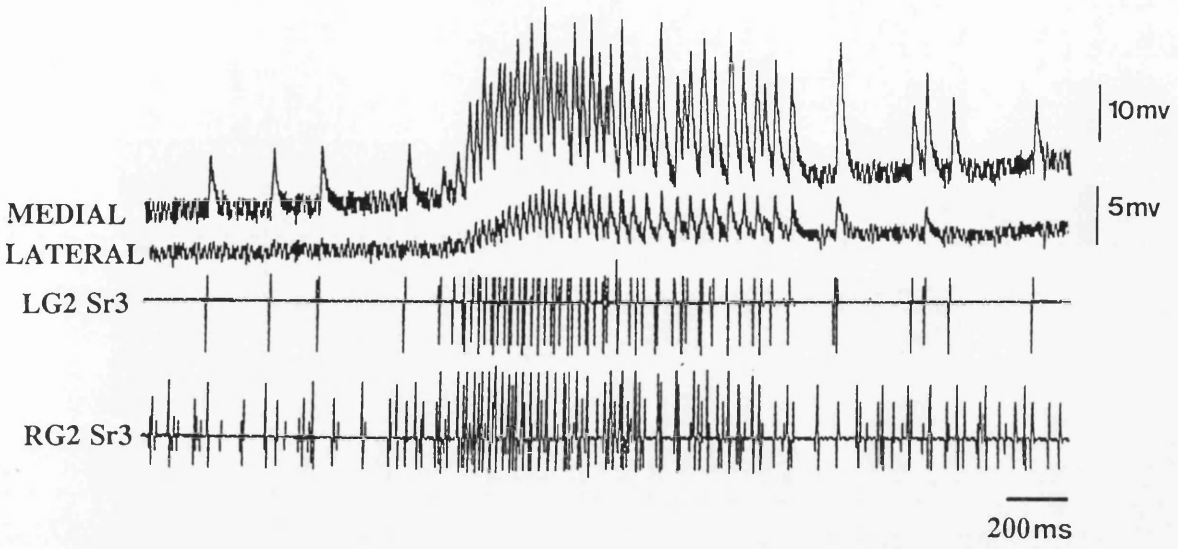
Figure 4.15

Spontaneous bursting in the isolated nerve-muscle preparation.

A shows simultaneous intracellular and extracellular activity recorded from the left SFM of the second segment in an isolated nerve-muscle preparation during the course of a burst of spontaneous activity. The highly facilitating EJPs generated in the lateral fibre are due to high frequency firing of f3 which was previously silent. Upper trace - medial fibre; second trace - 'silent' lateral fibre; third trace - Sr3 innervating the muscle; lower trace - Sr3 innervating the muscle of the opposite hemisegment.

B. Activity in the same preparation as shown above 30 seconds after the end of the spontaneous burst. A single spike of f3 is now unable to generate an observable EJP in the lateral fibre but can still produce a EJP of fairly large amplitude in the medial fibre.

A


10mv
5mv
MEDIAL
LATERAL
LG2 Sr3
RG2 Sr3
200ms

B

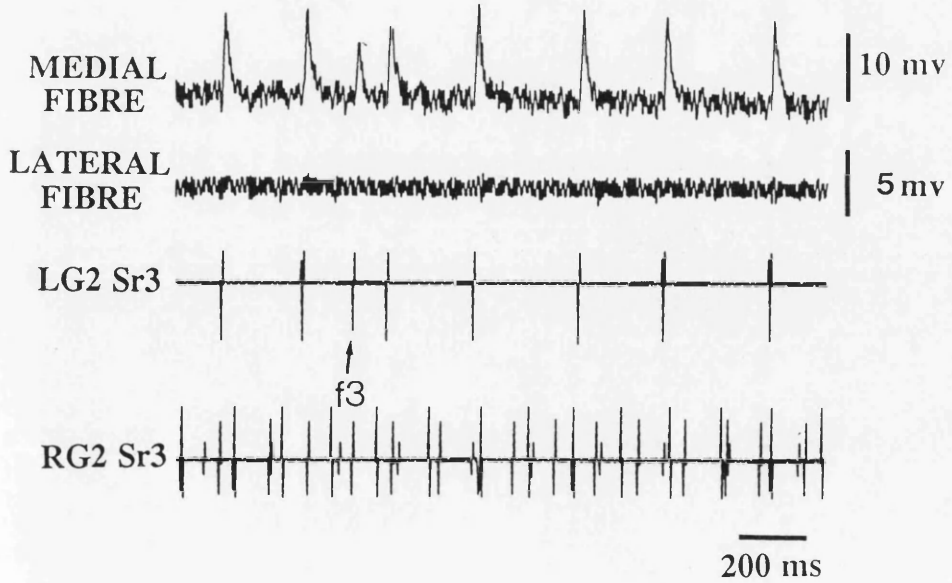

MEDIAL FIBRE
10 mv
LATERAL FIBRE
5 mv
LG2 Sr3
f3
RG2 Sr3
200 ms

Figure 4.16

Simultaneous tension measurements of the medial and lateral bundles of *Nephrops* SFM made by supramaximally stimulating the Sr3 (root) with a 4 sec. pulse at 50 Hz. Scale bar = 5 secs.; 0.4 mN (medial tension), 0.78 mN (lateral tension).

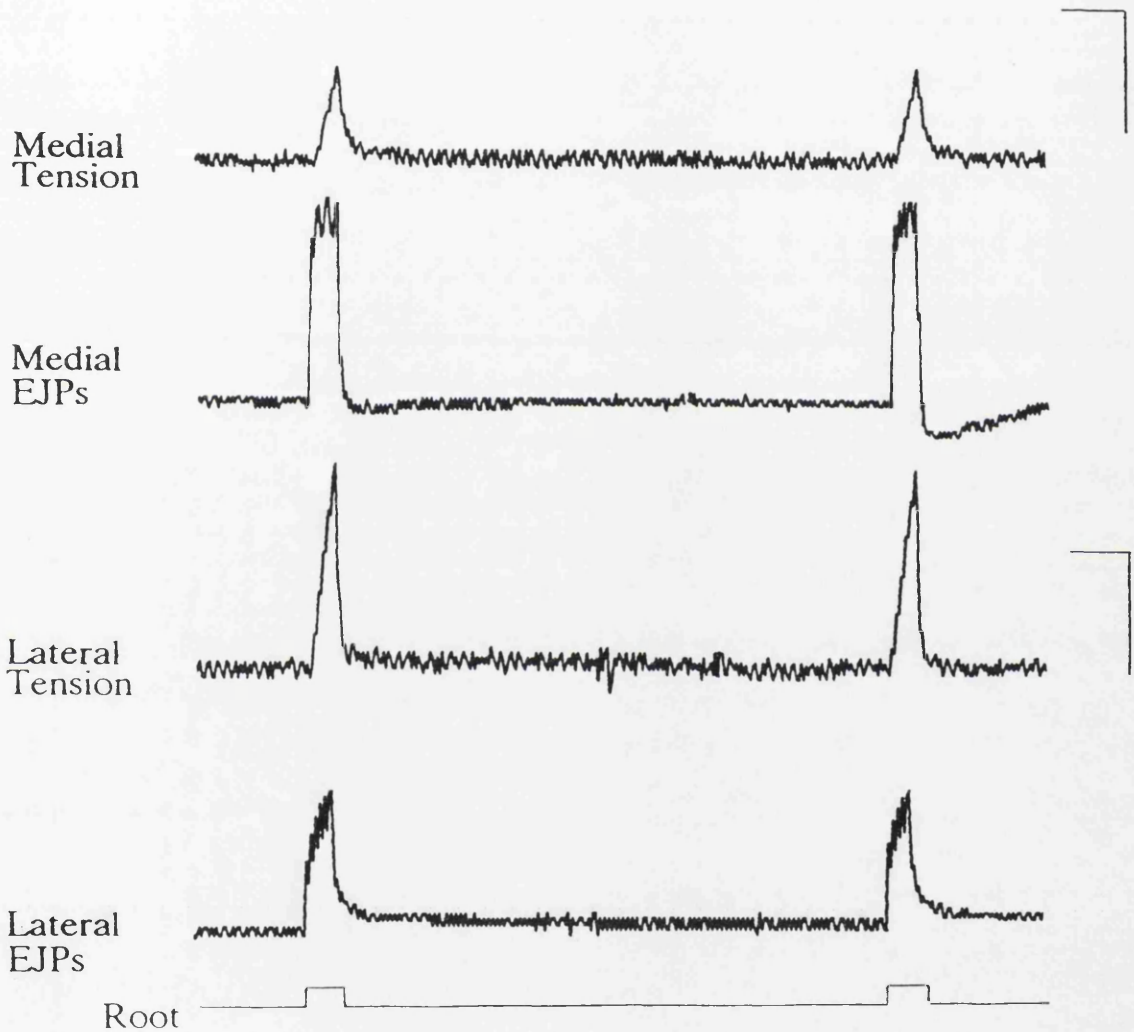

Medial
Tension
Medial
EJPs
Lateral
Tension
Lateral
EJPs
Root

Figure 4.17

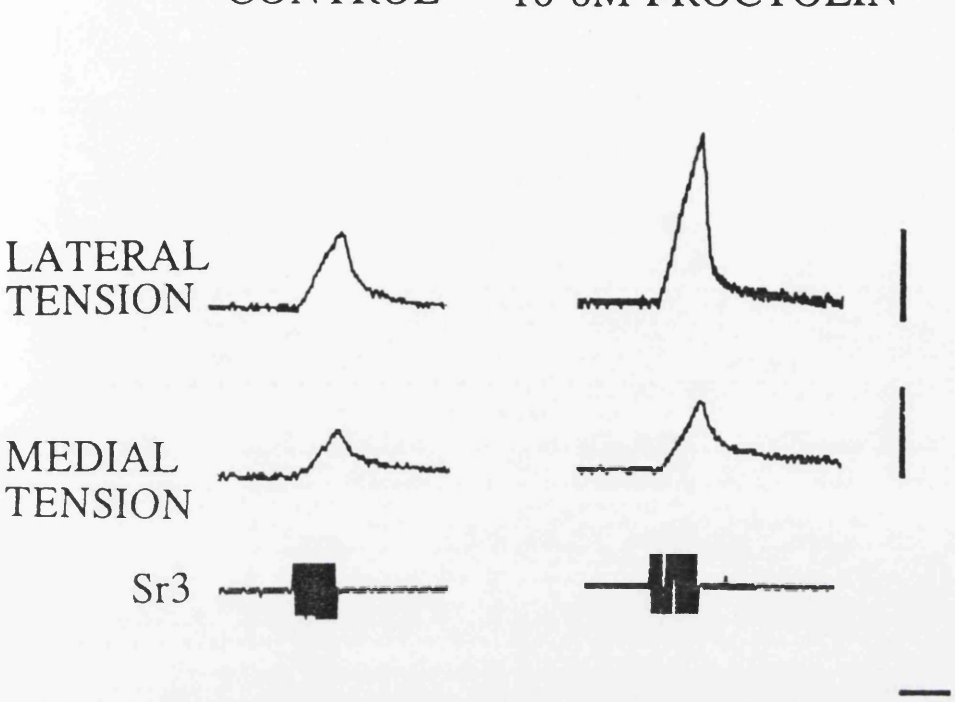
A. Effect of bath application of proctolin on neurally induced tension in medial and lateral fibres. Proctolin produced a 100% increase in lateral tension in response to a 2 sec, 50z train of supramaximal stimulation whereas the tension increase in medial fibres was only 40%.

Scale bar = 2 secs.; 0.33 mN (lateral tension), 0.25 mN (medial tension).

B. Effect of bath application of proctolin on tension produced by supramaximal stimulation of the Sr3, (2 secs, 50 Hz, top trace) and size of summated EJPs. EJP size remained unchanged even though tension doubled.

Scale bar = 2 secs.; 0.33 mN (lateral tension); 10 mV (lateral EJPs).

A


CONTROL
10-8M PROCTOLIN
LATERAL TENSION
MEDIAL TENSION
Sr3

B

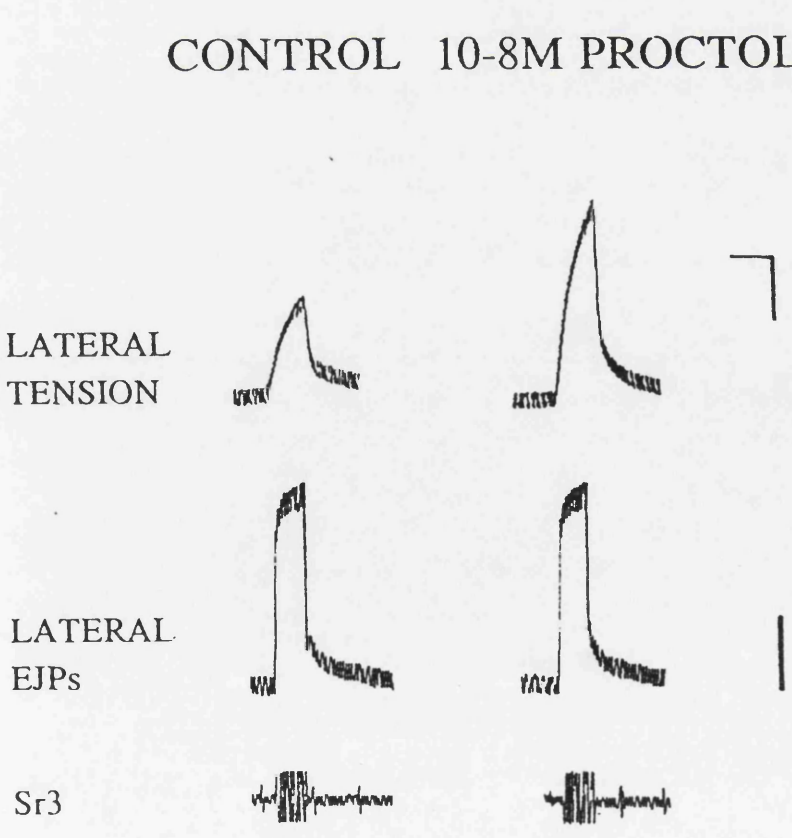

CONTROL
10-8M PROCTOLIN
LATERAL TENSION
LATERAL EJPs
Sr3

Figure 4.18

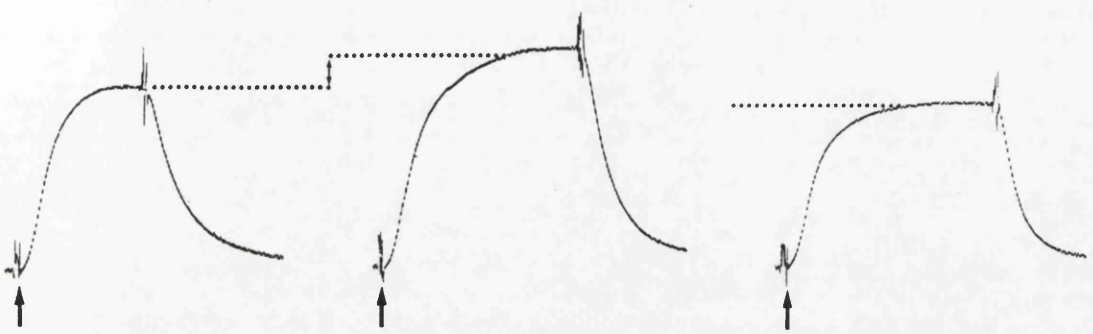
Postsynaptic effect of bath-applied proctolin.

A. Tension reponse of isolated, denervated medial fibres to bath-applied proctolin.

B. Tension reponse of isolated, denervated lateral fibres to bath-applied proctolin. Fibres were sequentially depolarised with high-KCl saline (4.7 x normal), high KCl-saline in the presence of proctolin and high KCl-saline again.

Scale bar = medial fibres - 18 mg, lateral fibres - 10 mg; 15 secs.

A. MEDIAL FIBRES



B. LATERAL FIBRES

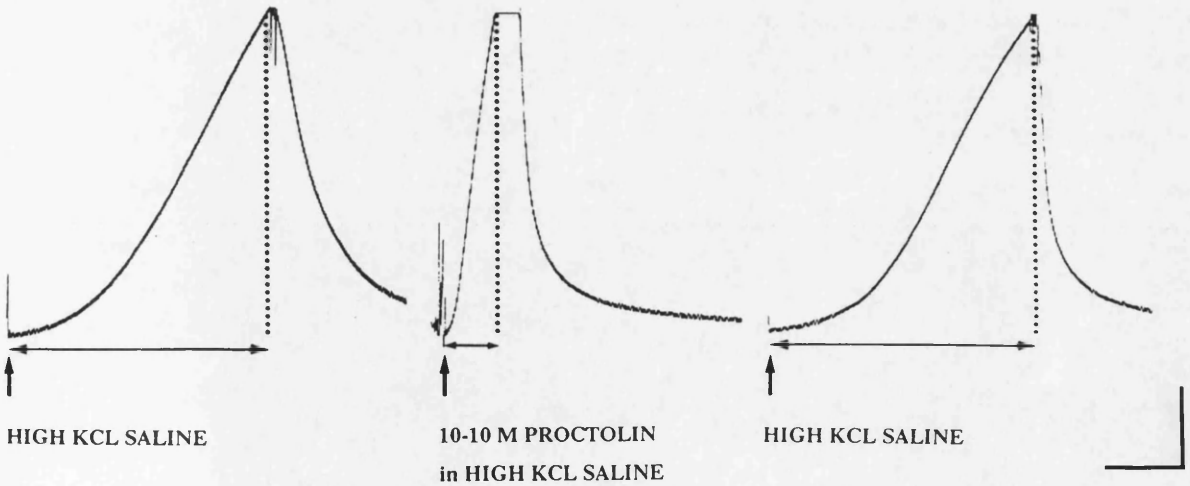


Figure 4.19

Desensitising postsynaptic effect of increasing concentrations of bath-applied proctolin. Tension response of denervated SFM lateral bundle to bath-applied proctolin. Fibres were sequentially depolarised with high-KCl saline (1.2 x normal), high KCl-saline in the presence of proctolin, high KCl-saline, and normal saline. This process was carried out four times in succession, each time alternating the dose of proctolin. A desensitisation of the tension response was observed for the higher dose of proctolin.

Scale bar = 4 mN; 20 secs.

LATERAL FIBRES

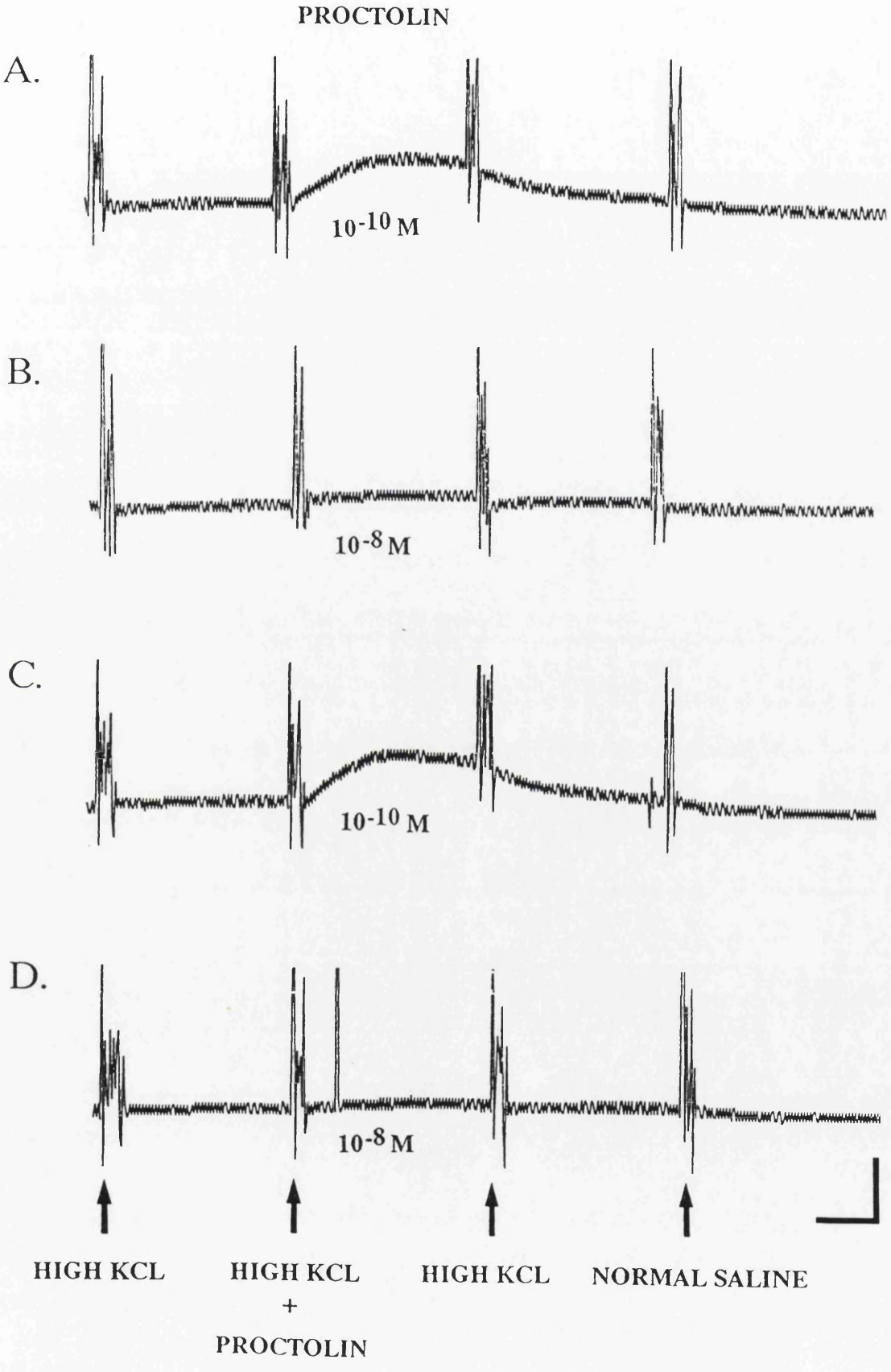


Figure 4.20

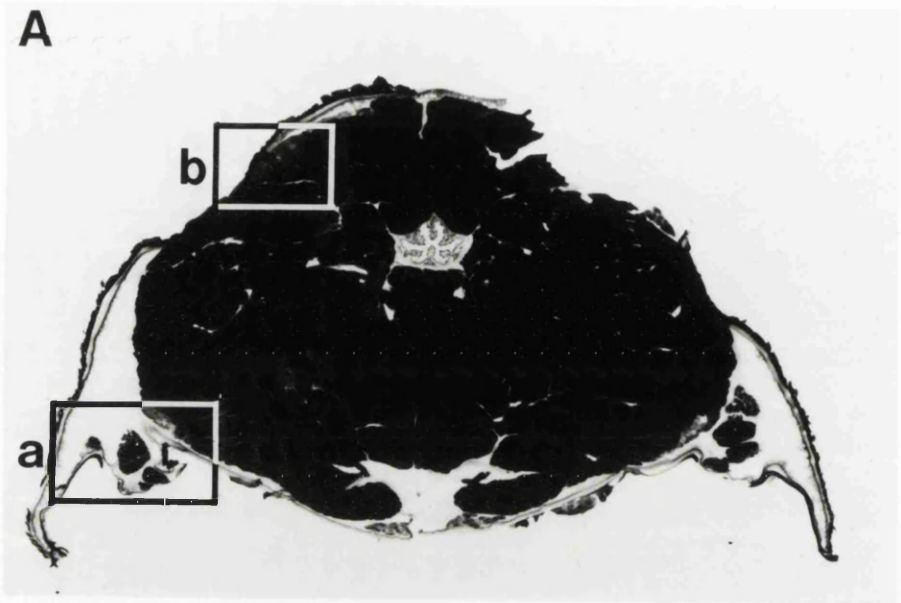
A. Whole mount section through 2nd abdominal segment of *Pacifastacus*, stained for total mATPase activity at pH 9.4.

B. Whole mount section through 2nd abdominal segment of *Pacifastacus*, stained for pH stability of the mATPase after preincubation at pH 10.05.

Boxes labelled (**a**) and (**b**) in both sections refer to the areas detailed in the next two consecutive figures:

Area (**a**) shows superficial flexor muscle and swimmeret muscles (**Figure 4.21**). Area (**b**) shows superficial extensor muscle (**Figure 4.22**).

Scale bar = 0.5 cm.


A
b
a

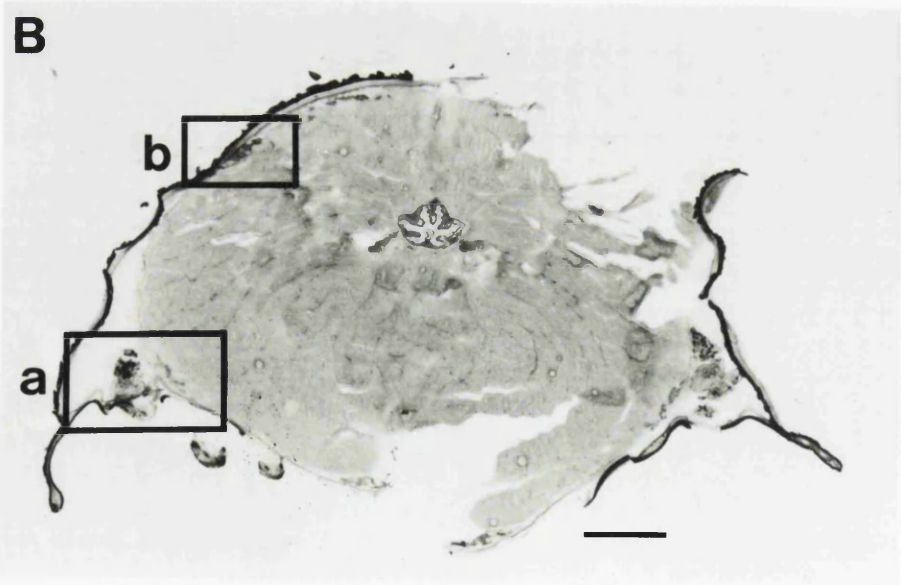

B
b
a

Figure 4.21

A. Section through superficial flexor muscle of *Pasifasticus* (box labelled (**a**) in **Figure 4.20**) stained for total mATPase activity at pH 9.4.

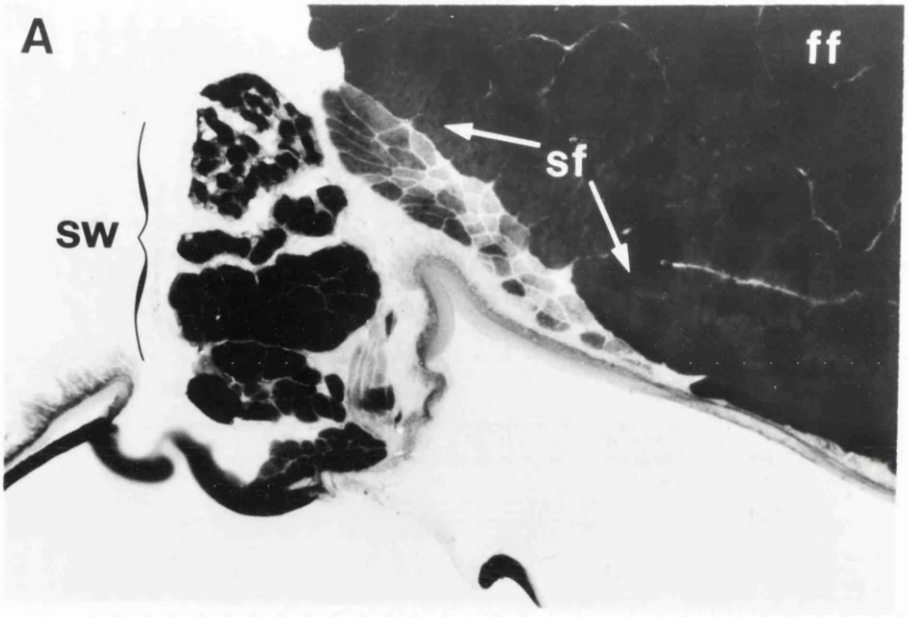
B. Section through superficial flexor muscle of *Pasifasticus* (box labelled (**a**) in **Figure 4.20**) stained for pH stability of the mATPase after preincubation at pH 10.05.

sw - swimmeret muscles

sf - superfical flexor muscle

ff - fast flexor (deep muscle)

Scale bar = 0.8 mm.


A
ff
sf
sw

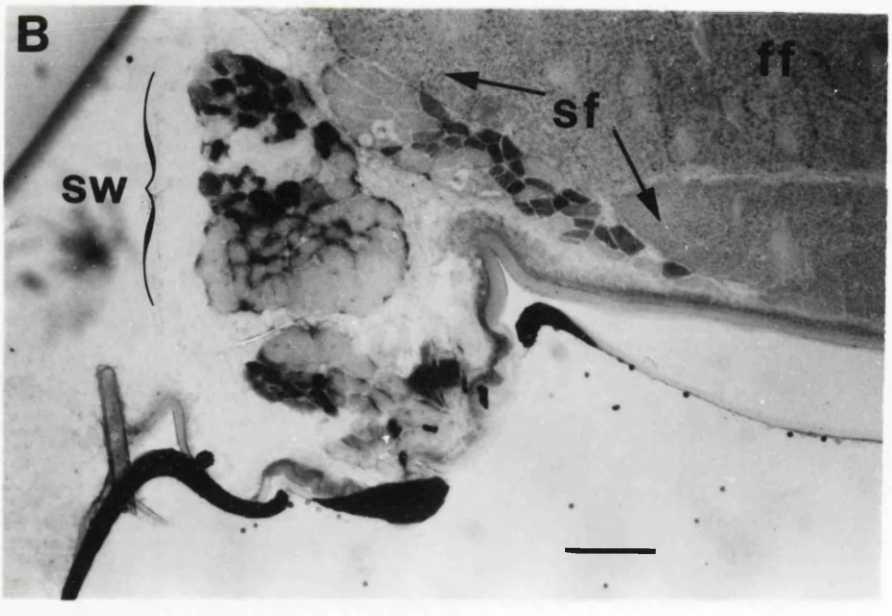

B
ff
sf
sw

Figure 4.22

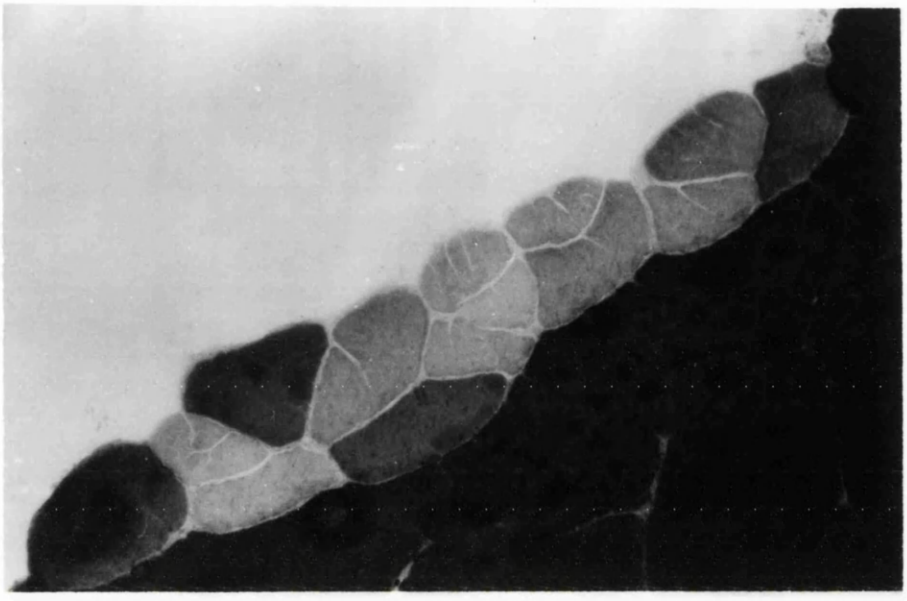
A. Section through superficial extensor muscle of *Pasifasticus* (box labelled (**b**) in **Figure 4.20**) stained for total mATPase activity at pH 9.4.

B. Section through superficial extensor muscle of *Pasifasticus* (box labelled (**b**) in **Figure 4.20**) stained for pH stability of the mATPase after preincubation at pH 10.05.

se - superfical extensor muscle

fe - fast extensor (deep muscle)

Scale bar = 0.2 mm.



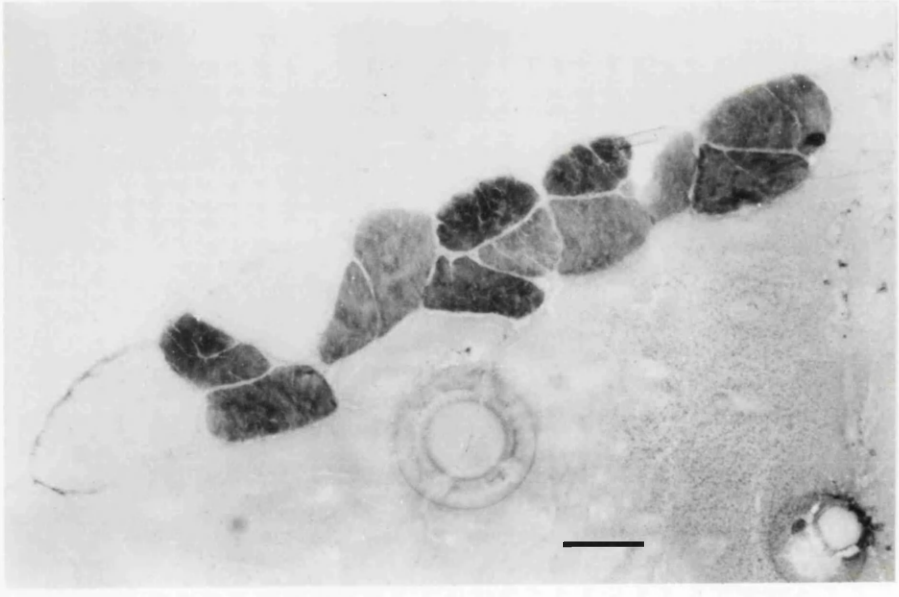
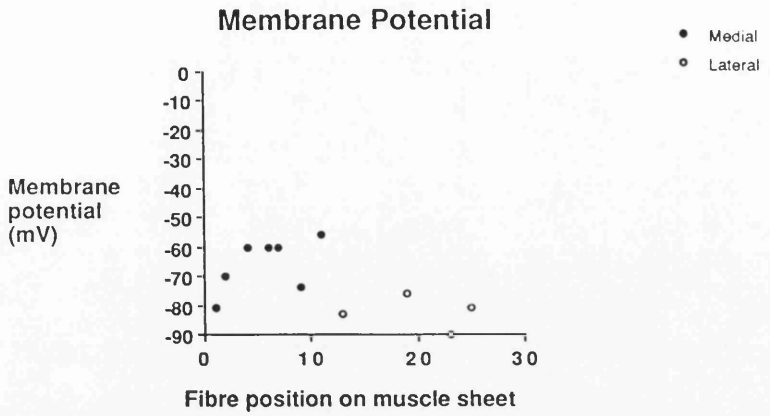
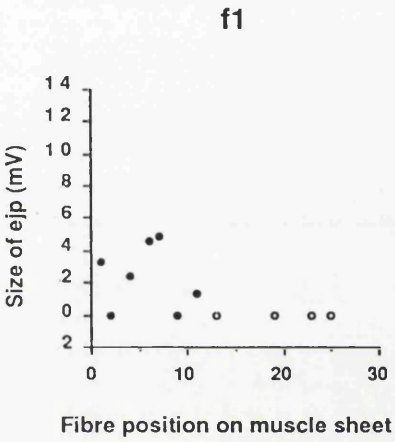


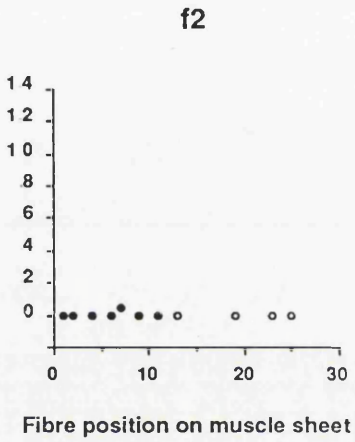
Figure 4.23

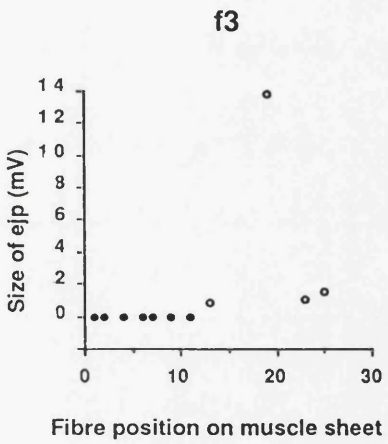
Partial survey (D1) of membrane potential and pattern of innervation of single fibres sampled successively from medial to lateral edge of *Pacifastacus* SFM. Filled circles represent medial fibres, open circles represent lateral fibres.

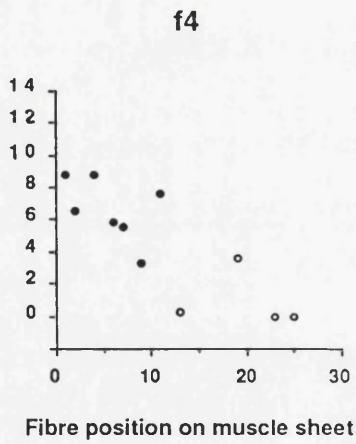
Innervation of S.F. muscle - Crayfish











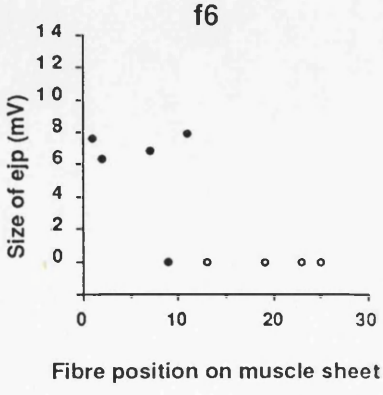
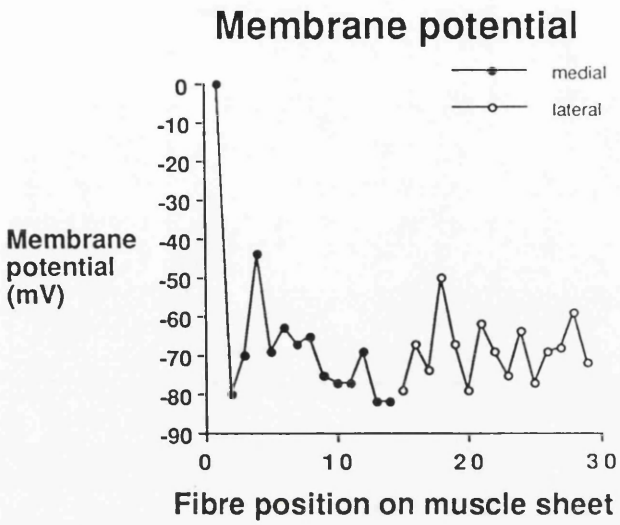


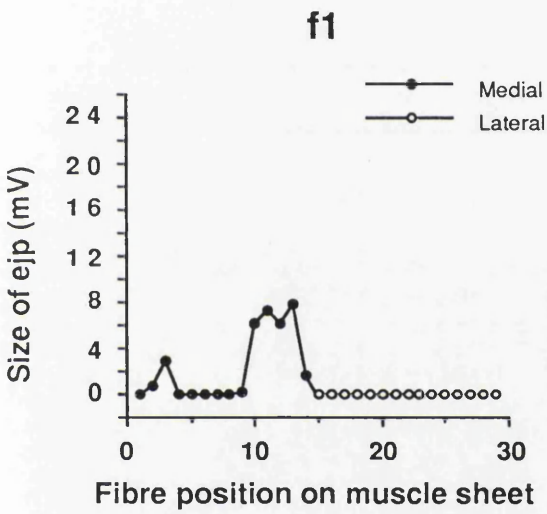
Figure 4.24

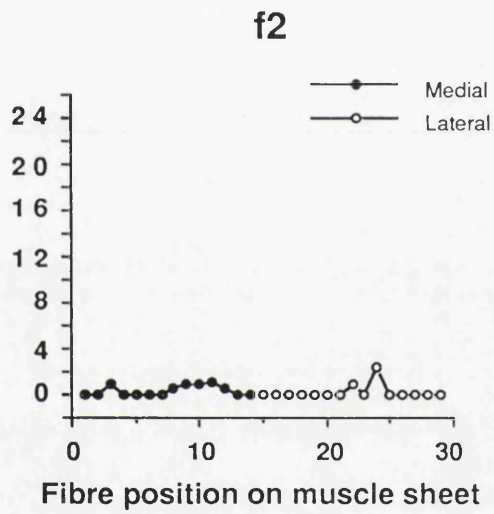
Complete survey (D2) of membrane potential and pattern of innervation of single fibres sampled successively from medial to lateral edge of *Pacifastacus* SFM.

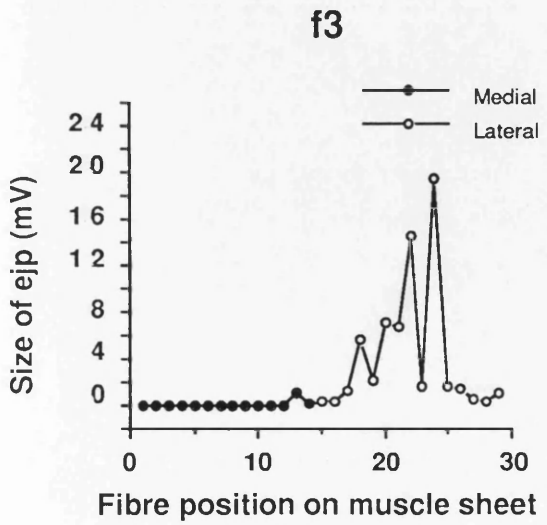
Filled circles represent medial fibres, open circles represent lateral fibres.

Innervation of S.F. muscle - Crayfish









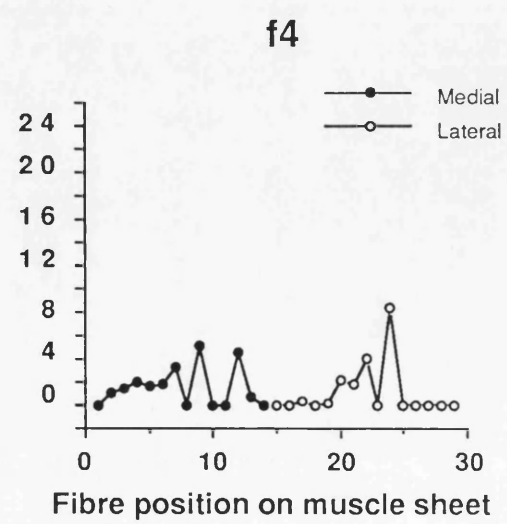


Figure 4.25

Histochemical profile of *Pacifastacus* SFM used to produce innervation survey D2. Muscle was stained for total mATPase activity at pH 9.4. Fibres are numbered in accordance with innervation survey.

Scale bar = 250μm.

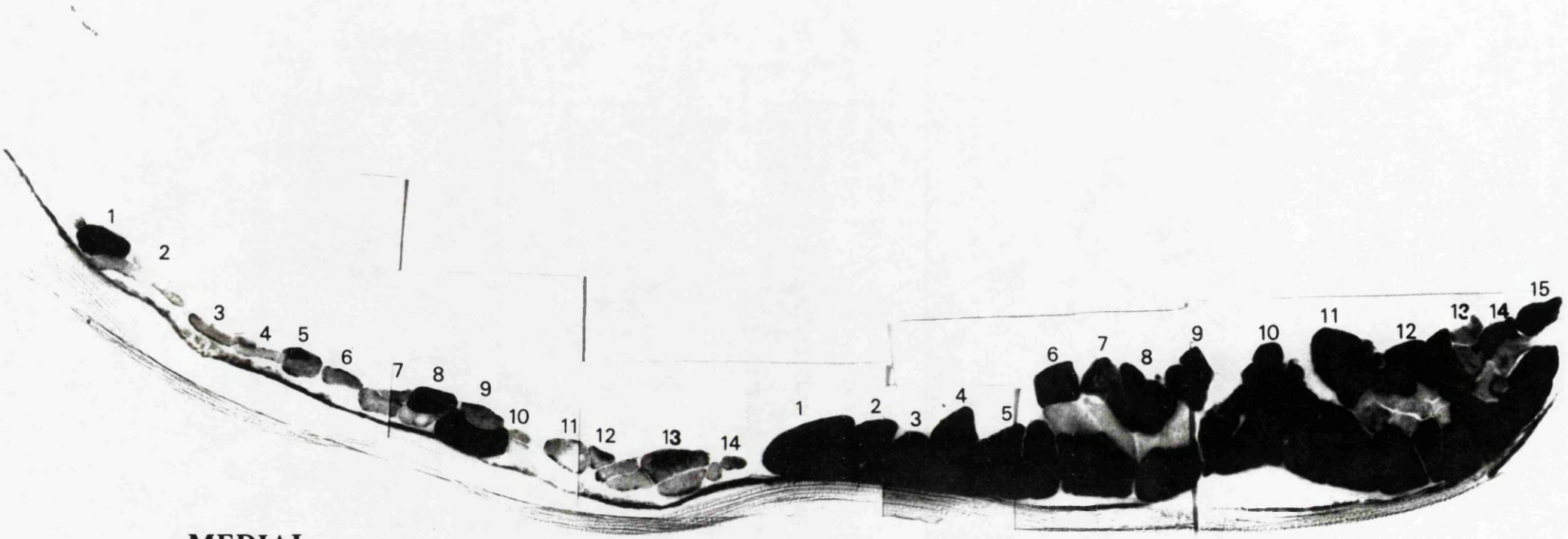
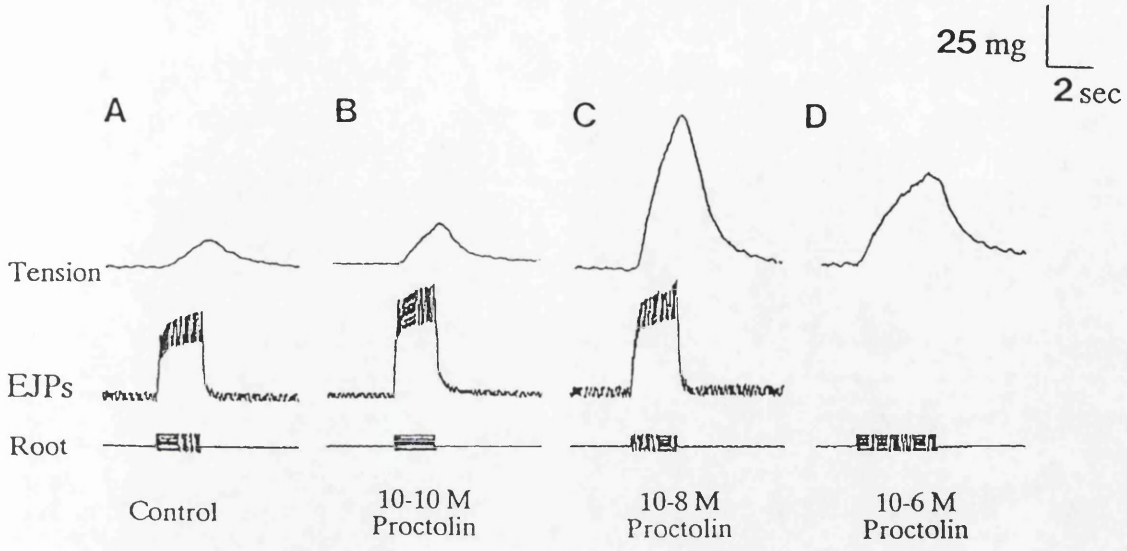

1
2
3
4
5
6
7
8
9
10
11
12
13
14
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
MEDIAL
LATERAL

Figure 4.26

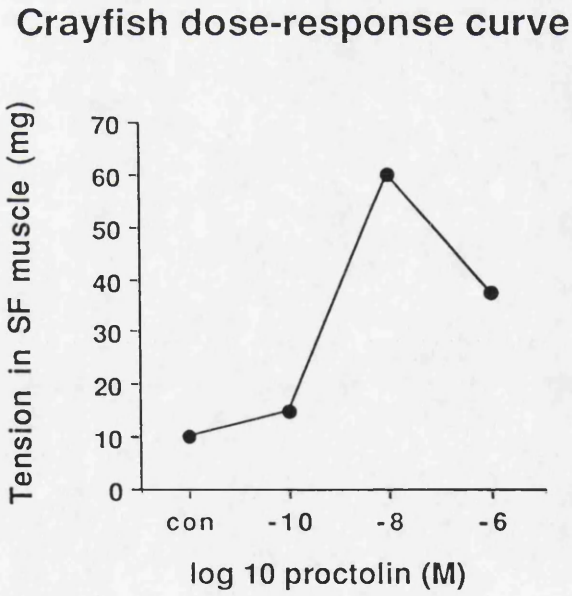
A. Effect of bath application of proctolin on neurally induced tension in *Pacifastacus* SFM fibres. Proctolin dose-dependantly increased tension in response to a 2 sec, 50Hz train of supramaximal stimulation. Desensitisation of response was observed at 10^{-6} M proctolin. Proctolin had no effect on size of summated EJPs in crayfish fibres.

B. Dose response curve of data shown in **A.**

A


25 mg
2 sec
A
B
C
D
Tension
EJPs
Root
Control
10-10 M
Proctolin
10-8 M
Proctolin
10-6 M
Proctolin

B


Crayfish dose-response curve
Tension in SF muscle (mg)
70
60
50
40
30
20
10
0
con
-10
-8
-6
log 10 proctolin (M)

GENERAL DISCUSSION AND FUTURE STUDIES

5.1 GENERAL DISCUSSION AND FUTURE STUDIES

This study has established that endogenous proctolin is present in the tissues of the SFM system and in other tissues of the nervous system of *Nephrops* and has confirmed that the peptide exists in the SFM system of crayfish (Chapter 2). The presence of higher concentrations of proctolin in extracts of lateral muscles compared to medial muscles in the SFMs of both species suggests a preferential innervation of lateral fibres by proctolinergic motor neurones, although this remains to be more fully established by direct methods, such as immunocytochemistry. Evidence of specific proctolinergic connections has already been found in other arthropod neuromuscular systems. In a study of the innervation of *Drosophila* muscle, abdominal body wall muscle fibres were found to be targets of proctolin-expressing efferents (Anderson *et al.*, 1988). The presence of proctolin in small, specialised subsets of neurones innervating specific muscles has also been shown in other insects including the grasshopper (Keshishian and O'Shea, 1985) and the cockroach (Bishop and O'Shea, 1982a). An immunocytochemical study of proctolin in *Nephrops* SFM system is required to localise the peptide to one or more specific motor neurones and to demonstrate the extent of arborisation of proctolinergic motor neurones across the medial and lateral SFM bundles. This should be one of the primary aims of a further investigation.

This study has, for the first time, demonstrated the specific modulatory action of the neuropeptide proctolin on the firing patterns of individual superficial flexor motor neurones (Chapter 3). Proctolin is able to upregulate some motor neurones (f1, f4 and f6) while simultaneously downregulating others (f2 and f5). These modulatory effects can in part be explained by the ability of proctolin to selectively induce or disrupt premotor connections between motor neurones. It is assumed that proctolin is released from nerve terminals in the central ganglia in high enough local concentrations to be able to activate sensitive target neurones, and so evoke the readout of a particular

pattern of motor output. The identity of the proctolin-containing neurones involved in the mediation of these effects is not known, but the presence and activity-dependent release of proctolin from previously identified command neurones which can activate the swimmeret system (Wiersma and Ikeda, 1964), has recently been demonstrated (Acevedo *et al.,* 1992a,b). It is possible that descending interneurones with similar properties may be involved in regulating the abdominal positioning system. It may even be possible that the same interneurones are involved in the activation of the two systems since interneurones which can synaptically activate the motor outputs of both the swimmeret and postural motor systems have already been demonstrated (Murchison and Larimer, 1990, Chrachri and Neil, 1993). Alternatively, proctolin may indirectly modulate the interaction between central command inputs and motor neurone outputs of the motor output of superfical flexor motor neurones by functioning as a 'gain setter' in a similar manner to that proposed for serotonin (Ma *et al,* 1992).

In considering the central effects exerted by proctolin on motor neurones the question arises: is it possible to relate these specific effects to recruitment of SFM fibres which are the targets of innervation by these motor neurones? In order to answer this question a knowledge of the pattern of innervation across the SFM is required. It has been shown in this study that the different patterns of postsynaptic activity exhibited by medial and lateral fibres are in part due to differences in their presynaptic properties (Chapter 4). Lateral fibres are often synaptically 'silent' on account of their 'low output' synapses and high facilitation properties. During normal levels of synaptic activity, only the largest SFM excitor, f6, is able to depolarise lateral fibres sufficiently to produce an observable EJP. In contrast, medial fibres exhibit properties of high output synapses and low facilitation properties and hence display a polysynaptic pattern of innervation in response to normal levels of spontaneous activity of the superficial flexor motor neurones. At this gross level

of consideration, it seems that the expression of fibre phenotype relates to the level of synaptic activity impinging on the fibre. Although this does not prove the causality of this relationship, it is known that phenotypic changes can be induced by imposing altered levels of synaptic activity (Atwood and Nguyen, 1991).

At a more detailed level, individual medial fibres receive innervation from different subsets of the total motor neurone pool, and there is no evidence for matching of innervation with fibre type as all of the fibres in the medial bundle express the same phenotype. Modulation of tonic activity in the superficial flexor motor neurones by proctolin results in an increase in the firing of f1, f4 and f6 and a decrease in the firing of f2 and f5; proctolin exerts variable effects on the firing of f3. However, the peripheral expression of this central proctolin modulation will mainly be translated to postsynaptic activity in medial fibres. Postsynaptic activity in lateral fibres will be affected to a much lesser extent. The reason for this is that in most cases, depolarisation of lateral fibres by all postural motor neurones except f6, requires the type of high frequency motor neurone activity (i.e. > 30 Hz) which is normally associated with reflex bursting behaviour. The observed enhancement of firing of specific postural motor neurones by proctolin was much more subtle than this. The maximum firing rate of any motor neurone after exposure to proctolin was 15 Hz and this figure represents the firing rate of the smallest motor neurone, f1. All other motor neurones which were upregulated, still displayed lower rates of firing. Furthermore, even high frequency activity of f1 may not affect *Nephrops* lateral fibres as these fibres do not appear to receive much input from f1 as has already been demonstrated for crayfish lateral fibres (Clement *et al.,* 1983). It is difficult to interpret the differential effects of proctolin in terms of a general enhancement or reduction of medial flexion since proctolin exerts opposite effects on motor neurones which act synergistically to produce excitation in the muscles they commonly innervate. For example, the ability of proctolin to

selectively depress firing of f2 may result in an overall reduction in the depolarisation of medial fibres as f2 produces large amplitude EJPs in comparison to its highly tonic counterpart, f1.

This study has focussed on only one part of the abdominal postural system, the nerves and muscles involved in controlling slow flexion movements. These muscles work antagonistically with another set of slow extensor muscles and an obvious extension of this work would be to investigate the actions of proctolin on the co-ordinated action of both the extensor and flexor systems as has been done for octopamine and serotonin (Kravitz *et al.,* 1988).

In addition to an investigation of the central role of proctolin in the SFM system of the Norway lobster, analysis of the peripheral actions of the peptide on the two slow fibre phenotypes comprising the SFM was carried out. This revealed that the two slow fibre phenotypes of *Nephrops* SFM are differentially responsive to proctolin, both in *in vitro* neuromuscular preparations and in isolated single fibres, but only if the fibres are partially depolarised prior to exposure of proctolin. It is possible that proctolin is acting to enhance contractions in the SFM fibres through the activation of various calcium channels, similar to those of crayfish (Bishop *et al.,* 1991), which require both an activated receptor and a membrane voltage. The differential enhancement of tension in each of the fibre types may be due to differences in the distribution of populations of calcium channels displaying differential sensitivity to proctolin. The application of patch clamp techniques to each of the slow fibre phenotypes of *Nephrops* SFM would allow an investigation of the kinetics and proctolin-sensitivity of their calcium channels and would also permit a direct comparison of the distribution of different calcium channel populations in each fibre type. This is an obvious direction for future research.

An attempt was also made in this study to characterise the SFM with regard to fibre heterogeneity by utilising the feature of spontaneous activity of the axons innervating this muscle. One advantage in using spontaneous activity

is that the intracellular activity recorded in the muscle fibre represents a true picture of the output of the synapses onto that fibre. One disadvantage lies in the fact that only the innervation of those motor neurones which are spontaneously active are represented. It may be possible to use neuromodulators such as octopamine and serotonin to increase pharmacologically the frequency of firing of individual motor neurones and then study their innervation of the SFM to overcome the problems of low output synapses. Furthermore, bearing in mind that the isolated preparation used in this study has no sensory feedback, future studies of the innervation of this muscle would benefit from use of a more intact preparation i.e. one containing some sort of sensory receptors (such as the MRO) which could be activated to induce reflex activation. Since such reflex effects are known to involve particular members of the Sr3 motor neurone pool (Fields, 1966; Page, 1982), this approach might be expected to reveal specific information about the involvement of particular motor neurones. This should enhance our knowledge of the innervation of lateral fibres and hence their contribution to the development of tension in this muscle. Further studies utilising selective stimulation of each of the six axons should prove invaluable in gaining a much clearer understanding of the contribution of each axon to the output of the SFM, especially with regard to type S1 lateral fibres whose innervation is still uncertain due to the presynaptic properties of these fibres. Such studies would permit a much greater analysis of the synaptic properties and innervation of each axon, the extent of inhibition of the inhibitor, the contribution of each axon to tension and also the effect of intrinsic neuromodulators such as proctolin on levels of tension induced by individual axons.

In summary, this work has shown that proctolin can act at two levels within the SFM system of the Norway lobster. At the central level, the peptide can differentially affect the firing of specific members of the motor neurone pool and can 'rewire' the motor network by selectively coordinating or

disrupting the relationships between neurones within the motor network. Thus proctolin can act centrally in the ventral nerve cord to specifically modulate the motor output pattern being fed to the SFM. At the peripheral level, proctolin can differentially modulate the level of tension produced by each of the two slow fibre phenotypes comprising the *Nephrops* SFM. The exact mechanisms involved in the expression of proctolin's effects both centrally and peripherally are as yet, far from understood. However, the neuromuscular abdominal flexor preparation offers a ideal model system in which to further investigate the actions of this peptide. In addition to demonstrating the different actions of proctolin in the SFM system of *Nephrops*, this study has demonstrated an even greater complexity of the muscle than was previously appreciated. Fowler and Neil, (1992) have shown that the muscle is composed of two populations of fibre type but the presence of further subtypes based on presynaptic (facilitation) and postsynaptic (time constant) properties is suggested by the results of experiments involving selective stimulation. Undoubtedly, the way forward is to concentrate efforts on single fibre studies which would allow an investigation of the innervation and electrophysiological properties of identified fibres, their contractile properties and responsiveness to proctolin followed by biochemical analysis to confirm fibre type.

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