Modulation of Muscle Contraction by a FMRFamide-Related Peptide

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(Submitted in partial fulfilment of the requirements for the degree of Master of Science)

December 1995

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St. Catharines, Ontario, CANADA

Abstract

A FMRFamide-like neuropeptide with the sequence "DRNFLRF-NH_2" was recently isolated from pericardial organs of crayfish (Mercier et al., Peptides, 14, 137-143, 1993). This neuropeptide, referred to as "DF_2", has already been shown to elicit cardioexcitation and to enhance synaptic transmission at neuromuscular junctions.

Possible effects of DF_2 on muscle were investigated using superficial extensor muscles of the abdomen of the crayfish, *Procambarus clarkii.* These muscles are of the tonic type and generate slow contractions that affect posture. DF_2, at concentrations of 10^{-8} M or higher, increased muscle tonus and induced spontaneous, rhythmic contractions. These effects were antagonized by 5 mM Mn^{2+} but not by 10^{-7} M tetrodotoxin (TTX). Thus, they represent direct actions on muscle cells (rather than effects on motor neurons) and are likely to involve calcium influx. In contrast, deep abdominal extensor muscles, responsible for rapid swimming movements, and superficial flexor muscles do not generate contractions in response to the peptide.

Spontaneous contractions were also induced in the superficial extensor muscles by decreasing the temperature to 11-13°C. Such contractions were also TTX-insensitive and they were antagonized by adding calcium channel blockers (Mn^{2+}, Cd^{2+} or Ni^{2+}) or by removing calcium from the bathing solution. This suggests that the spontaneous contractions depend on an influx of calcium from the extracellular solution. N-type and L-type voltage dependent calcium channel blockers did not reduce the effect of the peptide or the spontaneous contractions suggesting that calcium influx is not through N- or L-type calcium channels.

Acknowledgements

First and foremost, I thank Dr. A.J. Mercier for introducing me to invertebrate neurobiology; his enthusiasm for the topic was certainly contagious. I also thank him for allowing me to explore many 'little side projects', critically reading this thesis, and most of all his patience.

Next, I must mention my lab mates Konrad Noronha, Amy Peaire, Simon Kelly, Meena Srivastava, Susanna Knotz, Sal Iodice, Andrea Weston, CarolAnn Bisson and Chris McMullen for making the atmosphere in the lab very enjoyable.

I am very grateful to Dr. H.L. Atwood, Dr. M.K. Worden, and Rainer Friedrich for valuable discussions and suggestions.

The feedback from my committee, Dr. A. Bown and Dr. Carlone, was very useful and much appreciated. I thank them.

Finally, I thank all the technicians in the electronics services who have kept the equipment in good working order and Dr. T.R. Keenan for his help with statistics.

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1. Literature Review

The aim of this thesis is to provide a better understanding of how crustacean muscle contraction is modulated. In particular, it examines the effects of temperature and of a FMRFamide-related peptide on muscle contraction. But, first, some general characteristics of crustacean muscle morphology will be reviewed.

1.1 Striated Muscle Fiber Morphology

Muscles can be grouped into two types according to their morphology and physiology: smooth muscle and striated muscle. Both types contain actin and myosin filaments. However, in smooth muscle the filaments are loosely arranged to form a contractile apparatus, and in striated muscle they are highly ordered (Alberts et al., 1994). Smooth muscle produces contractions that are slow and rhythmic and are often coordinated into peristaltic waves (Davey, 1964). Striated muscles usually produce fast or slow twitch type contractions. All crustacean muscles are striated, each containing multinucleated muscle cells (or fibers) made up of many myofibrils, which are bundles of contractile material surrounded by sarcoplasmic reticulum. The myofibrils are constructed of longitudinally repeated units called sarcomeres (Fig. 1). Each sarcomere is bounded by two Z lines and contains interdigitating thin and thick filaments made of actin and myosin, respectively. The myosin filaments make up the densest part of the sarcomere, the A (ansiotropic) band. Unlike vertebrate skeletal muscle, the A bands of crustaceans are not of constant lengths (Franzini-Armstrong, 1970). In the middle of the A band, where the actin filaments extending from the adjacent Z lines do not meet, is a light region called the H band. The portion of the sarcomere between two A bands is called an I (isotropic) band.

Figure 1. The organization of a segment of a myofibril with three sarcomeres. The thick and thin myofilaments are arranged to form I, A, L and Z lines.

1.2 Muscle Contraction

The most widely accepted theory of how contraction of skeletal muscle occurs is the sliding filament theory proposed by Huxley and Niedergerke (1954). They observed that the lengths of the actin and myosin filaments do not change as the sarcomeres shorten, but the extent of filament overlap does change. In other words, the actin and myosin filaments slide past one another to produce contraction. Tension is produced when cross bridges form between the actin and myosin filaments. This process requires an increase in internal Ca^{2+} to a concentration higher than 10^{-7} M. The possible sources of Ca^{2+} will be discussed later.

Magnesium-dependent myosin ATPase splits the high energy phosphate bond of ATP and causes the attachment of the myosin head to the actin filament (Huxley, 1972). The attached head rotates through an angle of 45° and then separates from the actin. The addition of another ATP aids the separation of the actin and the myosin, and allows the head to rotate back to its original position before undergoing another round of sequential binding further along the actin filament. Several sites on the myosin bind sequentially with sites on the actin filament in an asyncronous manner so that at any instant some of them are attached to the actin while others are not. This allows for sustained contractions as the myosin heads attach and detach (Ruegg, 1992).

1.3 Sources of Ca^{2+}

As in all striated muscles, Ca^{2+} is required for contraction in all crustacean muscles (Atwood, 1982). Direct injection of Ca^{2+} into muscle fibers causes them to contract (Ashley, 1967). The Ca^{2+} concentration must be in between 10^{-7} and 10^{-6} M for contraction to occur (Hagiwara and Najajima, 1966).

Experiments by Ashley and Ridgeway (1970) show that the intracellular Ca^{2+} level rises as

the fiber contracts. They injected the fluorescent Ca^{2+} indicator aequorin into giant barnacle fibers and measured changes in the Ca^{2+} concentration, membrane potential and tension simultaneously. Weak electrical stimulation caused a small membrane depolarization, faint light emission, and no tension, whereas stronger stimulation caused a larger depolarization, larger light emission and considerable tension. This indicates that there is a threshold for mechanical activation. The increase in Ca^{2+} was transient, decayed exponentially and terminated by the time relaxation occurred. Barnacle fibers produce Ca^{2+} action potentials that supply enough Ca^{2+} for contraction. However, these spikes are not produced under normal conditions. This suggests that the Ca^{2+} needed for contraction must come from another source such as the sarcoplasmic reticulum.

1.4 Voltage-Gated Ca^{2+} Channels

Action potentials in crayfish muscle fibers are caused by Ca^{2+} (Fatt and Ginsborg, 1958). Voltage-gated Ca^{2+} channels are found in almost every excitable cell (Hille, 1991) and a variety of different Ca^{2+} channels are found in the same cells (Takahashi and Momiyama, 1993). They regulate a host of Ca^{2+}-dependent intracellular events including specialized functions such as excitability, exocytosis and contraction, and they mediate general functions like metabolism and gene expression. During contraction in crustacean muscle fibers, the initial entry of Ca^{2+} appears to be mediated by voltage-gated channels. These Ca^{2+} influxes are not sufficient to activate the contractile proteins, but they can induce Ca^{2+} release from internal stores which is required for mechanical activity (Mounier and Goblet, 1987).

Voltage-gated Ca^{2+} channels have been classified according to their sensitivity to depolarization: whether they are low voltage activated (LVA) or high voltage activated (HVA).

LVA channels exhibit rapid inactivation following depolarization and, thus, do not remain activated during prolonged depolarizations. HVA channels lack the rapid inactivation. Four types of voltage-gated Ca^{2+} channels have been identified in vertebrates using the following criteria for classification: pharmacological properties, ionic selectivity, metabolic regulation and single-channel conductance. These channels are the L-type, N-type, P-type all three of which are HVA channels and T-type (which is an LVA channel). Ca^{2+} channels may be somewhat different in invertebrates since the agents that block the vertebrate voltage-gated Ca^{2+} channels only partially block *Drosophila* types (Gielow et al., 1995) and crayfish types (Araque et al., 1994).

L-type channels are HVA channels that were first described in heart cells and peripheral neurons by patch clamping (Tsien et al., 1987). Their name comes from their relatively large conductances and long lasting currents which can be elicited with a depolarizing test potential around +10 mV. They provide most of the voltage-gated Ca^{2+} entry involved in the activation of contraction in heart and many smooth muscles, and in the activation of secretion from endocrine cells, sensory neurons and lizard motor nerve terminals (Bean, 1989). L-type channels are sensitive to 1,4-dihydropyridines and phenylalkylamines such as nifedipine, (+) Bay K 8644 and verapamil (Hille, 1992). Interestingly, the 1,4-dihydropyridines are more effective at blocking L-type channels when they are in their inactive state, which occurs after the channel has been opened by depolarization and is closed again and unavailable for reopening (Bean, 1984; Schwart et al., 1985). The peptide ω-Aga-IIIA may be a better blocker of L-type channels at normal resting potentials (Mintz et al., 1991).

N-type channels have only been found in neurons (Tsien et al., 1988). These HVA channels are blocked by the antagonist ω-conotoxin GVIA.

P-type channels were recently identified as HVA channels, and some are known to be

present in crayfish neurons (Araque et al., 1994). They are neither sensitive to 1,4-dihydropyridines nor to ω-conotoxin GVIA. They are blocked by "funnel toxin" FTX (Llinas et al., 1989) and ω-Aga-IVA (Adams et al., 1989; Mintz et al., 1992) both of which are extracted from funnel web spider venom.

T-type channels were originally identified in guinea pig heart (Nilius et al., 1985). These LVA channels have a "tiny" conductance and make a transient current. They can be activated with very small depolarizations from negative resting potentials. This suggests that they support Ca^{2+} entry at negative membrane potentials required during pacemaker activity. Ionic Ca^{2+} channel blockers, such as Ni^{2+} and Cd^{2+}, will antagonize them, but these agents are nonselective. T-type channel blockage has been shown with amiloride (Tang et al., 1988). Unfortunately, amiloride also inhibits a large number of membrane transport processes and enzymes including: Na^+/Ca^{2+} exchange, Na^+/H^+ antiporter, and the Na^+ channel (Kleyman and Cragoe, 1988) at concentrations lower than is necessary to block the Ca^{2+} channels.

There are many subtypes of Ca^{2+} channels which do not fit into one of the four known types of voltage-gated Ca^{2+} channels using the current criteria. Instead, information from molecular cloning will be required to distinguish them from one another (Snutch et al., 1990; Soong et al., 1993) .

1.5 Differences Between Smooth and Striated Muscles

Vertebrate smooth muscle and striated muscle contraction is mediated by different Ca^{2+} binding proteins. Smooth muscle contraction primarily involves the multifunctional Ca^{2+} binding protein calmodulin (Kamm and Stull, 1985). Ca^{2+} combines with calmodulin (CaM) to form a Ca^{2+}/CaM complex. When this complex joins the myosin light chain kinase, the enzyme is

activated. The enzyme then catalyzes the phosphorylation of one of the myosin II light chains which triggers cross bridge cycling between the myosin and actin. The phosphorylation requires ATP to be used up. Contraction is much slower in smooth muscle than in striated muscle.

In contrast, skeletal muscle contraction is initiated by Ca^{2+} binding directly to a protein known as troponin C. The myosin light chains play a modulatory role rather than a primary regulatory role since the light chain has relatively low calcium affinity in skeletal muscle (Ruegg, 1992). In striated muscles the actin helix contains filamentous molecules, called tropomyosin, in its grooves. Troponin is a Ca^{2+} binding protein that attaches to the tropomyosin and inhibits the formation of the crossbridges between the actin and myosin filaments. When calcium is present, it binds to the troponin, and the tropomyosin becomes available for cross bridge formation.

Interestingly, insect visceral muscle, which is striated yet produces slow rhythmic contractions (Nykamp et al., 1994), and *Limulus* muscle (Sellers, 1981 cited in Ruegg, 1992) use calmodulin instead of troponin C to initiate contraction.

1.6 Ca^{2+} Extrusion

In order for Ca^{2+} to be an effective signalling molecule the resting cytosolic Ca^{2+} concentration must be kept very low, less than 10^{-7} M (Carafoli, 1987). This allows minute changes in the Ca^{2+} influx to cause large changes in Ca^{2+} concentration. The Ca^{2+} sequestering mechanisms include: the Ca^{2+} ATPase pump, the Na^+/Ca^{2+} exchange, sequestering organelles and binding proteins (Mills and Kater, 1990). The Ca^{2+} ATPases on the plasma membrane and sarcoplasmic reticulum pump Ca^{2+} out of the cytosol at the expense of ATP. The Na^+/Ca^{2+} exchange at the plasma membrane removes one Ca^{2+} ion for three Na^+ ions allowed in. This process works in conjunction with the Na^+/K^+ ATPase which pumps Na^+ out and K^+ in against

their concentration gradients.

1.7 Excitation-Contraction Coupling and T-tubules

The series of events following synaptic transmission and leading to contraction is called excitation-contraction coupling (Sandow, 1952). Synaptic transmission causes depolarization of the muscle membrane which in turn elicits contraction. In all vertebrate muscle, action potentials precede contraction since the threshold for all or nothing action potentials is lower than the threshold for contraction (Watanabe, 1958, cited in Orkand, 1962). In arthropods the depolarization can produce contraction below the threshold for action potentials. This is why graded potentials elicit contraction in crayfish muscles (Orkand, 1962). The direct influence of depolarization (ie. the increase in Ca^{2+} concentration) is capable of extending less than a micrometer from the inner surface of the plasma membrane within the 2 msec it takes for contraction to occur so that the potential changes across the membrane surface cannot influence most of the myofibrils in a muscle cell 50-100 μm in diameter (Eckert et al., 1988). Thus, the potential change must be conducted deep within the cell interior where it can cause the release of Ca^{2+} from internal stores. This is accomplished via the many clefts in the sarcolemma bringing all portions of the fiber close to the extracellular space. Small invaginations of these clefts, which run along the myofibrils, make up two tubule systems: the transverse or T-tubules and the Z-tubules (Peachey and Huxley, 1964). The T-tubules, which are localized primarily at the A-line in arthropods, form tubule/sarcoplasmic reticulum connections called dyads (Peachey and Huxley, 1964). The Z-tubules which are only located at the Z-lines of tonic fibers of crustacean muscles (Franzini-Armstrong et al., 1978) do not form dyadic contacts. Huxley and Peachey (1964) found that half sarcomere contractions could be induced when the surface membrane was depolarized

with a current-carrying pipette placed over the A-band but not over the Z-lines. This indicates that the tubules opening to these two areas are different. Since the Z-tubules do not appear to be involved in excitation-contraction coupling, as shown by Huxley's and Peachey's experiment, their function is uncertain. It has been suggested the Z-tubules serve to exchange metabolites, provide mechanical support, or aid in supercontraction (Chapple, 1982). The T-tubules, on the other hand, are thought to conduct electrical signals into the fibers and cause Ca^{2+} to be released from the sarcoplasmic reticulum (Hille, 1992). Unfortunately, the mechanism which links sarcoplasmic reticulum Ca^{2+} release with depolarization is still unknown. However, several possible links are being explored, including: the dihydropyridine receptor, the ryanodine receptor, IP_3 and G protein mediated phosphorylation.

1.8 The Dihydropyridine Receptor

The dihydropyridine receptor is also known as the L-type Ca^{2+} channel. It has been suggested (Scheider, 1994) that these channels are opened when the T-tubules are depolarized and cause enough Ca^{2+} influx to induce more Ca^{2+} to be released from the sarcoplasmic reticulum. It is unlikely that dihydropyridine receptors play this role in vertebrate skeletal muscle since their opening kinetics are too slow (Sanchez and Stefani, 1983). However, they may initiate Ca^{2+} release by generating charge movement, which is the proposed signal for the sarcoplasmic reticulum to release its Ca^{2+} stores (Rios and Brum, 1987). In vertebrate skeletal muscle and cardiac muscle, most of the Ca^{2+} needed for contraction comes from the sarcoplasmic reticulum. In cardiac myocytes, however, some Ca^{2+} from the external medium must enter the cell to trigger Ca^{2+} induced Ca^{2+} release (Nabauer et al., 1989; Niggli and Lederer, 1990). Mammalian skeletal muscle E-C coupling is independent of external Ca^{2+} (Franzini-Armstrong and Jorgensen, 1994).

Caffeine, ryanodine and thapsigargin are agents used to induce Ca^{2+} release from the sarcoplasmic reticulum. Thapsigargin and ryanodine both accelerate Ca^{2+} efflux from the sarcoplasmic reticulum, but thapsigargin also blocks the Ca^{2+} pump of the sarcoplasmic reticulum so that Ca^{2+} cannot be resequestered (Janczewski and Lakatta, 1993).

1.9 The Ryanodine Receptor

The ryanodine receptor is the putative intramembrane Ca^{2+} channel in the sarcoplasmic reticulum. This 450 kD receptor has a cytoplasmic domain that spans the cleft between the T-tubules and the sarcoplamic reticulum (Inui et al., 1987; Ma et al., 1988). This cytoplamic domain was first observed by Franzini-Armstrong (1970) who called it the foot structure. It may form a direct mechanical link between these two structures so that a voltage change on the T-tubule membrane could cause a conformational change of the Ca^{2+} channel on the sarcoplasmic reticulum to activate Ca^{2+} release. In mammalian skeletal muscle the ryanodine receptor can be opened by caffeine, Ca^{2+}, and ryanodine. In lobster muscle the ryanodine receptor is opened by millimolar concentrations of Ca^{2+} and not by ATP, caffeine or Mg^{2+}, suggesting that the crustacean isoform is only susceptible to Ca^{2+} induced Ca^{2+} release (Seok et al., 1992).

1.10 Inositol Trisphosphate (IP_3)

Inositol trisphosphate (IP_3) released from the T-tubule by voltage-dependent activation of phospholipase C may cause Ca^{2+} release from the sarcoplasmic reticulum by binding the IP_3 receptor. The following pathway has been proposed to explain the source of Ca^{2+} required for contractions in smooth muscles (Walsh, 1991). A transmitter or hormone binds to a receptor on the plasma membrane, that is associated with a G-protein linked to phospholipase C.

Phospholipase C breaks PIP_2 into membrane-bound diacylglycerol (DG) and IP_3. DG is capable of activating protein kinase C, which can open Ca^{2+} channels on the T-tubule such as L-type channels. IP_3 is free to move through the cytosol where it can bind to the IP_3 receptor on the sarcoplasmic reticulum (Chadwick et al., 1990). The mechanism responsible for raising the intracellular Ca^{2+} concentration may be similar in skeletal muscle and smooth muscle, but the primary release channel on the sarcoplasmic reticulum in skeletal muscle is the ryanodine receptor, whereas in smooth muscle it is the IP_3 receptor (Hille, 1992). There is evidence for an IP_3 receptor mediating Ca^{2+} release in barnacle muscle (Rojas et al., 1987; Lea et al., 1986).

1.11 Comparison of Crustacean Tonic and Phasic Fibers

There are two main types of crustacean muscle: phasic and tonic. The former produce fast contractions that are short-lived, whereas the latter produce slower contractions which generate more force. There are many differences between tonic and phasic muscles, including properties of the cell membrane, the contractile machinery, the internal membrane systems and the innervation. Each of these aspects will be explained in further detail.

Properties of the membrane such as the membrane potential and the input resistance are different in tonic and phasic fibers. In tonic fibers (such as these studied here) the membrane potential is just below the mechanical threshold, allowing small depolarizations to activate the contractile machinery (Atwood, 1973). The membrane potential in phasic muscles on the other hand, is much lower than mechanical threshold, so that the fibers require all-or-nothing Ca^{2+}-dependent action potentials to initiate contraction (Hoyle, 1968). According to Atwood (1973) phasic fibers have a lower input resistance and a much higher probability of generating spikes than do tonic fibers.

The contractile machinery (myosin ATPase and sarcomere length) is also different in the two muscle types. These features have been studied in the dimorphic lobster claws (Govind, 1982). One claw develops all tonic fibers and is used to crush hard shells of food items, and the other claw develops mostly phasic muscles making it ideal for fast cutting actions. More myosin ATPase is present in the phasic closer muscle of the lobster cutter claw than the corresponding tonic muscle from the crusher claw (Govind, 1982). Also, the myosin ATPase has a lower activity in tonic muscles than in phasic ones (Maier et al., 1984). This accounts for the slower formation of crossbridges between the actin and myosin filaments in the tonic muscles than in the phasic muscles.

The most easily noticed difference between the tonic and phasic muscles is the sarcomere length. Tonic fibers have relatively long sarcomeres, generally 6-10 μm, while the phasic fibers have shorter sarcomeres, 2-3 μm long. Such differences have been reported for crayfish abdominal muscles (Jahromi and Atwood, 1967), lobster abdominal muscles (Jahromi and Atwood, 1969) and lobster claws (Jahromi and Atwood, 1971). Thus, a phasic muscle will contract faster than a tonic muscle of the same length because there are more sarcomeres contracting at the same time in series. However, in the long sarcomeres of tonic muscles more force is produced because there are more crossbridges pulling on one actin filament (Atwood, 1973). Regardless of the sarcomere length, when the actin and myosin filaments overlap completely, tension is maximal. One exception is in the case of supercontraction observed in barnacle muscle in which the thick filaments pass through perforations in the Z lines between adjacent sarcomeres (Hoyle et al., 1965). When the fiber is stretched until there is no overlap, stimulation produces no tension beyond the passive elastic tension of the resting state.

Since phasic muscles have more sarcomeres, they also have more A-I band borders where

the diadic connections between the T-tubules and the sarcoplasmic reticulum are located. There are approximately 4 times as many diads in the lobster phasic extensor than the tonic extensor (Jahromi and Atwood, 1969). The diads are important for Ca^{2+} release, which is necessary for contraction. Thus, in phasic muscles Ca^{2+} may be released more quickly allowing them to contract faster.

Unlike vertebrate fast- and slow-twitch muscles, the amount of sarcoplasmic reticulum in crustacean tonic and phasic muscles is similar (Jahromi and Atwood, 1967, 1969). Differences in speed of contraction in tonic and phasic muscles involve features other than the extent of the sarcoplasmic reticulum, such as the T-tubules which are more extensive in phasic than tonic muscles (Selverston, 1967; Franzini-Armstrong et al., 1978) and ATPase activity.

The arrangement of the actin and myosin filaments is also different. The ratio of thin:thick filaments is 3:1 in phasic fibers of lobster and crayfish; whereas, this ratio is always greater in tonic fibers (approximately 6:1) (Jahromi and Atwood, 1967, 1969). According to Atwood (1982) this feature is not causally related to contraction speed. However, it is thought to be linked to the maximum amount of force generated. Higher thin:thick filament ratios produce greater filament overlap per cross sectional area of muscle.

In crayfish, the abdominal tonic and phasic fibers are grouped in separate muscles supplied with 2-5 motor axons (Kennedy and Takeda, 1965). Tonic muscles are innervated by axons that have higher levels of spontaneous activity and low output synapses that facilitate readily when stimulated repetitively. Conversely, phasic axons have high output synapses that are poorly facilitating, and the level of spontaneous activity in such axons is lower (Parnas and Atwood, 1966; Govind et al., 1981). It is unclear how the neurons and muscles are matched developmentally, yet there is some evidence for neurotrophic factors that suggest the timing of

innervation may be important (Govind and Lang, 1972; Lang et al., 1980).

1.12 Effects of Temperature on Excitation-Contraction Coupling

Temperature has a considerable impact on neuromuscular junctions of crustaceans, influencing the passive electrical properties of the muscle fibers, the contractile apparatus and synaptic transmission (Fischer and Florey,1981). These effects are coordinated in such a way as to allow crustaceans to function at a wide range of temperatures.

The passive electrical properties of muscle fibers, including the resting potential, input resistance, time and length constants and the capacitance, influence muscle excitability and are altered by temperature (Adams, 1987). The excitation-contraction threshold is not significantly altered by changes in temperature (Dudel and Ruedel, 1968), but cooling can cause the resting potential to depolarize (Friedrich et al., 1994), bringing the excitation-contraction threshold and the resting potential closer together. The membrane potential may even surpass the excitation-contraction threshold, increasing muscle tonus as the temperature is decreased (Fischer and Florey, 1981).

A "two-phase" relationship between temperature and membrane potential has been shown in muscles of crab (Stephens and Atwood, 1982) and crayfish (Harri and Florey, 1977). The relationship between membrane potential and temperature in crustacean muscle fibers is biphasic (Harri and Florey, 1977). The membrane potential hyperpolarizes as temperature increases; the increase in hyperpolarization with temperature is greater below the acclimation temperature than above the acclimation temperatures.

Other muscle properties that are temperature-dependent are the input resistance, length constant and time constant which increase as the temperature is lowered, and the membrane

capacitance which decreases with decreasing temperature. Most of these factors contribute to the increased excitability of the muscle fibers at lower temperatures with the exception of the time constant.

Lowering temperature causes a reduction in synaptic efficacy at neuromuscular junctions from phasic axons, but increases synaptic efficacy at neuromuscular junctions from tonic axons (Friedrich et al., 1994). In some neuromuscular junctions transmitter release is greater, albeit more susceptible to fatigue at lower temperatures, such as those in frogs (Pawson and Grinnell, 1989), and in crayfish (Lnenicka and Zhao, 1991). This appears to compensate for the decrease in excitation-contraction coupling at lower temperatures. Thus, the presynaptic effects of low temperature are quite complicated - influencing quantal content, quantal size and the time course of transmitter release.

The changes in membrane excitability counterbalance the accompanying decreased transmitter release under cool conditions. The increased input resistance at low temperatures changes the response of the muscle to synaptic currents such that less current is required to produce the same amount of depolarization. This is important, since low temperature reduces the amount of transmitter released from stimulated nerves (quantal content) at several neuromuscular junctions, as well as decreasing spontaneous transmitter release at some neuromuscular junctions (Fatt and Katz, 1952; White, 1983). Temperature can change other aspects of transmitter release in addition to quantal content. In frog sartorius muscle, only 6% of the fibers failed to produce spikes between 10-30°C but at 5°C, 59% of the fibers did not spike. This effect was caused by a decrease in the temporal dispersion of transmitter release as well as the quantal size, and not by a decrease in the electrical excitability of the postsynaptic membrane (Adams, 1989). The reduced synaptic transmission at low temperature may be caused by the

alteration of the amount of Ca^{2+} entering the nerve terminal (Charlton and Atwood, 1979).

Neurohormones also appear to counteract the reduction in transmitter release at low temperatures. Lowering the temperature decreased EJP amplitudes in the deep extensor muscles of crayfish (Friedrich et al., 1994). The peptide DF_2, which increased EJP amplitudes, was more effective at lower temperatures. The increased effectiveness of the peptide at lower temperatures suggests that one physiological role of the hormone may be to compensate for reduced synaptic efficacy.

1.13 Neurohormones

Neurohormones are transported by the circulatory system to their sites of action. They generally bind to a receptor on the membrane surface and start an intracellular signaling cascade of intracellular messengers. Examples of crustacean neurohormones include the amines, serotonin, octopamine and dopamine, as well as the peptide proctolin (RYLPT). These agents act on a diverse range of tissues, including the heart, muscles, neuromuscular junctions and sensory neurons, as described below.

1.13.1 Proctolin

In insects, proctolin has modulatory effects on locust oviduct (Lange et al., 1984), coxal-depressor motoneuron of cockroach (Adams and O'Shea, 1983), ventral opener muscle of locust (Belanger and Orchard, 1993), and the extensor-tibialis motor neurons of locust (Evans and Myers, 1986; Worden et al., 1985).

Proctolin is thought to act as a neurohormone in crustaceans since it is in very high concentrations in the pericardial organs, which are known to release several neurohormones into

the circulation (Cooke and Sullivan, 1982). However, proctolin is also released as a cotransmitter from some motor neurons in insects and crustacea. Such motor neurons include those innervating the extensor-tibia muscle of locust (O'Shea, 1985; Worden et al., 1985), superficial abdominal flexor muscles of the crayfish (Bishop et al., 1984), and coxal-depressor muscles of cockroach (Adams and O'Shea, 1983).

As a cotransmitter, at crayfish abdominal flexors (Bishop et al., 1984) proctolin does not affect the resting potential or generate tension on its own. However, it does potentiate tension elicited by the depolarizing EJP's of the conventional transmitter (Bishop et al., 1987). Unlike the crayfish abdominal flexors there is no evidence of the localization of proctolin in nerve terminals of crab gill ventilatory muscle yet, proctolin has the same effect on basal tonus and contractions elicited by depolarization in this muscle too (Mercier and Wilkens, 1985). In lobster dactyl opener muscle (Schwarz et al., 1985) and in insect coxal depressor muscle (Adams and O'Shea, 1983) proctolin does induce slow contraction. There is no change in resting potential or input resistance during the contractions in the presence of proctolin in most preparations (Kravitz et al., 1980; Lange et al., 1987; Belanger and Orchard, 1993). One exception is the phasic extensor muscle of *Idotea,* in which the proctolin did cause an increase in the input resistance in half of the fibers and induced action potentials in one quarter of the fibers (Erxleben et al., 1995). An increase in the input resistance would cause an increase in the amplitude of the synaptic junction potentials and their summation properties and, thus, would enhance neurally evoked contractions. In the dactyl opener of lobster proctolin induces Ca^{2+}-mediated action potentials in fibers that do not usually spike without a change in membrane potential (Kravitz et al., 1980).

Proctolin enhances myogenic contractions in several insect muscles. For example, the extensor-tibiae muscle of the locust exhibits myogenic contractions (Voskresenskaya, 1959, cited

in Hoyle, 1978), which are initiated by a small bundle of pacemaker fibers. The contractions are spread electrotonically to the other follower muscle fibers in the bundle (Burns and Usherwood, 1978). This muscle's myogenic rhythm is accelerated or induced in quiescent preparations by proctolin (Evans and Myers, 1986). Proctolin also increases the amplitude and frequency of myogenic contractions in the locust oviduct (Lange et al., 1987).

The modulatory effects of proctolin require extracellular Ca^{2+} in some preparations. The proctolin-induced contractions in the dactyl opener muscle of lobster are voltage-sensitive and are reversibly blocked by the Ca^{2+} channel antagonists Mn^{2+} and Co^{2+} (Kravitz et al., 1980). An increased Ca^{2+} conductance occurs at 20 mV above the resting potential, yet the contractions do not require a change in the membrane potential. This suggests that proctolin may activate some voltage-sensitive Ca^{2+} entry at the resting potential (Kravitz et al., 1980). In the locust oviduct proctolin-induced contractions were not completely blocked by the Ca^{2+}-channel antagonists, nifedipine and verapamil, suggesting a role for receptor-activated Ca^{2+}-channels as well as voltage-gated Ca^{2+}-channels (Lange et al., 1987). Conversely, in the extensor tibiae of locust, changes in the myogenic rhythm caused by proctolin are not dependent on external Ca^{2+} (Worden and O'Shea, 1986). However, the peptide does cause increases in inositol phosphate metabolism which could lead to a release of Ca^{2+} from internal stores.

The effects of proctolin are thought to be mediated by intracellular signaling pathways, such as phosphoinositides (Groome and Watson, 1989; Lange et al., 1989), and/or the cAMP cascade (Bishop et al., 1991; Evans, 1984). There is evidence that these pathways lead to the altering of ionic currents. In *Idotea* the mechanism behind the proctolin-induced increase in tension likely involves a cAMP signal pathway and protein kinase A causing the phosphorylation of a non-voltage dependent K^+ channel (Erxleben et al., 1995). In the tonic abdominal flexor

muscle of the crayfish proctolin causes a cAMP mediated modulation of two types of voltage-gated Ca^{2+} channels (Bishop et al., 1991). Proctolin alone has no effect on the Ca^{2+} channels, but renders them capable of sustained activity following depolarization. The resulting influx of Ca^{2+} is likely responsible for the increase in tension elicited be proctolin following depolarization.

1.14.2 Serotonin and Octopamine

Serotonin and octopamine are found in the pericardial organs of crustaceans (Cooke and Sullivan, 1982). These two aminergic hormones affect many crustacean visceral and skeletal muscles, by both pre- and postsynaptic mechanisms.

Octopamine excites hearts of *Cancer*, *Panulirus*, *Homarus*, *Portunus* (Cooke and Sullivan, 1982) *Astacus*, *Eriphia* (Florey and Rathmayer, 1977) and *Limulus* (Watson et al., 1985). Serotonin also modulates the hearts (Battelle and Kravitz, 1978) and isolated cardiac ganglia (Cooke and Hartline, 1975) of crustaceans. Serotonin increases the basal tonus and myogenic contractions in cockroach oviducts, whereas octopamine decreases them at concentrations greater than 10^{-7} M (Bamji and Orchard, 1995). Octopamine also increases the spontaneous contractions of crayfish hindgut by acting directly on the muscle (Cooke and Sullivan, 1982); whereas, it reduces the amplitude of neurally-evoked contractions of the locust oviduct (Orchard and Lange, 1985). Both these amines seem to exert their effects on visceral muscle through cAMP mediated pathways (Sullivan and Barker, 1975; Battelle and Kravitz, 1978; Lange and Orchard, 1986; Groome and Watson, 1989; Bamji and Orchard, 1995).

Serotonin and octopamine both modulate neuromuscular transmission and muscle contraction in arthropod skeletal muscles. In lobster dactyl opener muscle both amines produce contractures (Florey and Florey, 1954). Octopamine reduces the amplitude of neurally-evoked

contractions of *Schistocerca* extensor tibiae muscle (O'Shea and Evans, 1979), but it increases twitch amplitude, contraction rate and relaxation rate while decreasing basal tonus in several other insect preparations (O'Shea and Evans, 1979; Evans and Myers, 1986; O'Gara and Drewes, 1990). Octopamine reduces the amplitude of EJP's and hyperpolarizes the muscle membrane potential, mediating its effects using cAMP in locust oviduct. Octopamine causes relaxation of the basal tonus and inhibition of myogenic contractions in locust oviduct (Lange et al., 1984) and *Schistocerca* extensor tibia (O'Shea and Evans, 1979) unlike serotonin which enhances the myogenic rhythm of the locust extensor tibiae (Evans and Myers, 1986). Neurally-evoked contractions in the locust oviduct seem to be mediated by raising cAMP levels (Lange and Orchard, 1986), while myogenic contractions appear to use a different mechanism (Evans, 1984).

As is apparent from the preceding paragraghs, that both serotonin and octopamine have a wide range of effects on visceral and skeletal muscles. One explanation would be different types of receptors for these amines. Octopamine is known to have three different types of receptors which mediate physiological changes in the locust extensor tibia which respond to various agonists and antagonists (see Orchard and Lange, 1987). Also, only two of the receptors activate adenylate cyclase. The distribution of these receptor types on the target tissues could account for the inconsistent patterns regarding octopamine's physiological effects.

Both amines have been shown to act postsynaptically. In the walking leg opener muscle of lobster, serotonin and octopamine elicit sustained contractures that are dependent on extracellular Ca^{2+}, through direct effects of the muscles (Kravitz et al., 1980). Serotonin's effect is accompanied by a small depolarization and a slight change in input resistance in lobster dactyl opener muscle (Kravitz et al., 1980). Serotonin also increases an inward Ca^{2+} current through voltage sensitive Ca^{2+} channels in lobster muscle, which leads to the appearance of Ca^{2+} action

potentials (Kravitz et al., 1980).

Serotonin was demonstrated to have a presynaptic action at crustacean neuromuscular junctions by increasing quantal release (Dudel, 1965; Kravitz et al., 1980; Glusman and Kravitz, 1982). In the lobster opener preparation there is evidence that serotonin alters the storage or buffering of internal Ca^{2+} (Glusman and Kravitz, 1982) in part through the cAMP pathway, since the cAMP level increases in this preparation (Battelle and Kravitz, 1978) and in part through a cAMP-independent component (Goy and Kravitz, 1989). In the crayfish opener muscle, the serotonin-induced increase in transmitter released is dependent on phospholipase C and phosphorylation presumably by protein kinase-C (Dixon and Atwood, 1989). It has been suggested that phosphorylation may act by modulating Ca^{2+} channels so that more Ca^{2+} enters the nerve terminals, by modulating the sequestering mechanisms so that the Ca^{2+} transient is prolonged, or by modulating the exocytotic apparatus so that more vesicles are available for release (Swain et al., 1991). Unlike *Aplysia*, in which serotonin causes the cAMP/protein kinase A -mediated phosphorylation of a K^+ channel which broadens the presynaptic action potential to cause increased transmitter release (Shuster et al., 1985; Brada et al., 1993), in crayfish the presynaptic action potentials are not broadened by serotonin as they are in *Aplysia* ruling out K^+ channel inactivation (Dixon and Atwood, 1985). Also in crayfish serotonin requires extracellular Na^+ but not extracellular Ca^{2+} to increase transmitter release (Dixon and Atwood, 1985). It is possible that serotonin's effect in crayfish involves an initial entry of Na^+ into the nerve terminal, with consequent increased availability of Ca^{2+}.

It should be apparent from this review that serotonin and octopamine affect many crustacean motor neurons and muscles. Serotonin, in particular, is known to modulate many different currents through a variety of intracellular signalling pathways.

1.13.3 Dopamine

Dopamine is also found in neurosecretory endings of the pericardial organs and, thus, is thought to act as a neurohormone in crustaceans. It is cardioexcitatory in a number of crustacean hearts, including those of *Libinia*, *Panulirus*, *Homarus*, *Portunus*, *Cancer*, *Astacus*, and *Limulus* (Florey and Rathmayer, 1977; Cooke and Sullivan, 1982; Watson et al., 1985). The thresholds for these responses are between 10^{-9} and 10^{-7} M.

In *Limulus*, dopamine modulates neuromuscular transmission (Watson et al., 1985) and elicits sustained contractures and rhythmic contractions in deganglionated heart muscle. At the cardiac neuromuscular junction, dopamine acts directly on presynaptic terminals to increase spontaneous and evoked transmitter release. Dopamine's effect on contractility involves a direct effect on the muscle and occurs in the absence of changes in membrane potential or input resistance of muscle cells. The latter effect may be a result of changes in excitation-contraction coupling (Watson et al., 1985) mediated by increases in cAMP (Groome and Watson, 1989).

Dopamine increases nerve-evoked contractions, contractures and spontaneous contractions of lobster foregut (Lingle, 1981) and crayfish hindgut (Sullivan, 1980). The effects in the lobster are accompanied by increases in amplitude of excitatory junctional potentials, caused at least in part by increases in muscle fiber membrane resistance. In fibers where dopamine activates spontaneous contractions, the amine also causes spontaneous action potentials (Lingle, 1981).

Dopamine causes the opposite effect (ie. decreased muscle tonus) on *Limulus* midgut (Groome and Lent, 1992) and lobster opener muscle (Kravitz et al., 1980). In *Limulus* it inhibits spontaneous contractions, which counteracts the excitatory effects produced by proctolin and octopamine (Groome and Lent, 1992). Dopamine's effects on the midgut are also mediated by

increased levels of cAMP. Therefore, dopamine produces a variety of different physiological responses in different systems, but appears to act through cAMP in all cases.

1.14 FMRFamide Related Peptides

Another group of potential neurohormones that are localized in the pericardial organs crustaceans along with proctolin, serotonin, octopamine, and dopamine are the FMRFamide-related peptides (Trimmer et al., 1987; Mercier et al., 1993). FMRFamide (named for the one-letter symbols of its amino acid sequence) was originally isolated from the bivalve mollusc *Macrocallista nimbosa* (Price & Greenberg, 1977). Antisera raised against FMRFamide have been used to study FMRFamide-like peptides in many non-molluscan species (eg. Boer et al., 1980). It is now recognized that there is a large family of FMRFamide-related peptides (FaRP's) widely distributed throughout the animal kingdom (Greenberg & Thorndyke, 1983; Price & Greenberg, 1989).

The FaRPs can be characterized into two distinct groups: those isolated from the protostome phyla (Nematoda, Annelida, Mollusca and Arthropoda) and those isolated from the non-protostomes (Coelenterata and Chordata) (Elphick et al., 1989). The protostome FaRP's have the same general C-terminal sequence F(X)RFamide, where X is either methionine, leucine or isoleucine. The non-protostomes share only the C-terminal RFamide sequence.

1.14.1 Arthropod FaRP Sequences

Several FaRP's have been isolated from several types of arthropods, including fruitflies, locusts (Robb and Evans, 1990), cockroaches (Holman et al., 1986), blowflies (Duve et al., 1992), tobacco hornworms (Kingan et al., 1990) and crustaceans (Marder et al., 1987; Trimmer

et al., 1987; Mercier et al., 1991). The arthropod FaRP's have been divided into three groups based on their amino acid sequences (Krajniak, 1991). Group 1, isolated from insects, has the sequence H-*X*-FLRF-amide, where *X* is either a serine or valine residue. Group 2, which has only been found in *Drosophila*, has the sequence DF-*X*-RF-amide, where *X* is either methionine or valine. Group 3 contains peptides found in both insects and crustaceans with the structure NF-*X*-RF-amide, where *X* is either methionine, leucine or isoleucine. This classification scheme however, is inadequate to account for more recently identified FaRP's. For example, Krajniak (1991) has isolated a FaRP from the blue crab whose sequence, GYNRSFLRF-amide, is similar to the structural requirements of both Group 1 and Group 3 but does not fit into either one. Of thirteen FaRPs isolated from the blowfly only two fit into Group 3; however, most have the terminal sequence DFMRF-amide and one has the sequence NMIRF-amide (Duve et al., 1992).

1.14.2 Effects of FaRP's

Physiologically, FaRP's mediate a wide and diverse set of actions on multiple target tissues including: activation of rhythmic bursting in neural circuits (eg. Marder et al., 1987), modulation of neurally evoked contractions of skeletal muscles (eg. Evans and Myers, 1986; Skerrett et al., 1995), modulation of synaptic efficacy at neuromuscular junctions (eg. Baux et al., 1992) and direct excitatory and inhibitory effects on muscle tension (eg. Trimmer et al., 1987; Worden et al., 1995). Specific examples are described in the subsections below.

1.14.2.1 Neural circuits

FaRPs have been shown to modulate rhythmically active neural circuits such as the pyloric rhythm in the stomatogastric ganglia of the crab (Hooper and Marder, 1984; Marder et al., 1987)

and shrimp (Meyrand and Marder, 1991), by increasing the frequency of the pyloric cycle. The effects of the FaRP's, F_1 and F_2, on the rhythm are dependent on the pre-application state of the system. Peptide effects were only seen in preparations where the pyloric rhythm was originally inactive or slowly active. The FMRFamide evoked contractions are accompanied by a sequence of rhythmic depolarizations, followed by a state in which the muscle does not spontaneously contract but is close to threshold for the generation of rhythmic activity in pyloric dilator muscle of shrimp (Meyrand and Marder, 1991). The induction of plateau and oscillatory properties in the DG neuron is an important step in the activation of the full gastric rhythm. F_1 and F_2 modify the intrinsic membrane properties of the DG neuron so that it expresses plateau properties and/or becomes a true oscillator (Kliehn and Harris-Warrick, 1992).

1.14.2.2 Neuromuscular Transmission

In contrast to vertebrate muscles, invertebrate muscle fibers do not routinely display regenerative membrane properties, but the amplitude of the muscle contraction is a function of the size of the postjunctional response (Atwood, 1976). Under these conditions movement amplitude can be extremely sensitive to the pattern of activity in the motor neuron. FaRPs are likely to have multiple sites of actions, including presynaptic terminals as well as the muscles themselves (Evans and Myers, 1986; Skerrett et al., 1995).

F_1 and F_2, which were isolated from the lobster pericardial organs (Trimmer et al., 1987), enhance both nerve-evoked and spontaneous transmitter release from neurons innnervating the dactyl opener muscle of lobster (Trimmer et al., 1987; Worden et al., 1995). Both neuropeptides also enhance nerve-evoked contractions and EJP's of the crayfish phasic extensor muscles (Mercier et al., 1990). In the latter neuromuscular system, the peptides were shown to increase

the quantal content, indicating an increase in transmitter release from presynaptic terminals.

In the extensor-tibiae muscle of locust, FaRP's modulate tension generated by stimulation of the slow excitatory motor neuron, but they have no effect on myogenic contractions (Evans and Myers, 1986). FMRFamide increases the frequency, but not the amplitude of spontaneous miniature end-plate potentials in muscle fibers innervated by the slow excitatory motor neuron, indicating a presynaptic action. These actions do not appear to be mediated by increases in cyclic nucleotide levels (Evans and Myers, 1986).

Unlike some modulators such as serotonin and proctolin, F_1 showed no modulatory effect on stretch receptor neurons in the lobster (Pasztor and MacMillan, 1990). So far, there has not been sufficient evidence to extend physiological targets of FaRP's to crustacean sensory neurons.

1.14.2.3 Neurons

The subcellular mechanisms by which FaRP's alter synaptic transmission have been studied in *Aplysia* (Baux et al., 1992) and *Helisoma* (Man-Son-Hing et al., 1989). In a neuro-neuronal synapse of the buccal ganglion of *Aplysia* quantal release is increased by FLRFamide because the voltage sensitivity of an N-type Ca^{2+} channel is increased (Baux et al., 1992). This differs from other reports where FLRFamide decreases neuronal Ca^{2+} currents as in *Helisoma* (Man-Son-Hing et al., 1989).

When FMRFamide was applied to the somatic synapses of cultured *Helisoma* neurons, release of the transmitter, acetylcholine, was depressed (Man-Son-Hing et al., 1989). Using voltage-clamp and calcium-clamp techniques the investigators found the modulation to be caused partly by a decrease in the voltage-dependent Ca^{2+} current and partly by a direct effect on the secretory apparatus. The latter effect was demonstrated when FMRFamide caused a decrease in

transmitter release even when the inside of the presynaptic cell was loaded with Ca^{2+} using a "caged-Ca^{2+}" chemical. This process was shown to require a G-protein when the peptide's effect was mimicked by the injection of the GTP analogue, GTPγS (Haydon et al., 1991).

FMRFamide decreases the duration of the Ca^{2+} current and a cAMP controlled K^+ current in *Helix* neurons (Colombaioni et al., 1985). This contrasts with the effect of FMRFamide in *Aplysia* sensory neurons where the peptide increases the probability of opening of the K^+ channels (Belardetti et al., 1987).

1.14.2.4 Cardiac and Other Visceral Muscles

FaRP's increase both the rate and strength of cardiac contractions in lobster (Trimmer et al., 1987; Worden et al., 1995), locust (Cuthbert and Evans, 1989; Robb and Evans, 1990; Robb and Evans, 1994), crab (Krajniak, 1991) and crayfish (Mercier and Russenes, 1992; Skerrett et al., 1995). The peptides could act on the myocardium, on the nerve terminals innervating the myocardium, and on the rhythm generating neurons on the cardiac ganglion (Cuthbert and Evans, 1989). However, investigations of the effect of FaRP's on isolated cardiac ganglia and isolated myocardium from crustaceans have been lacking. Several FaRP's are cardioexcitatory in the *Limulus* heart (Groome et al., 1994) acting on both the cardiac ganglia and the myocardium itself.

The ionic currents that mediate the effects of FaRP's on arthropod hearts are unknown. There is evidence in leech heart that FMRFamide activates a sustained inward Na^+ current and a K^+ current and modifies Ca^{2+} currents (Thompson and Calabrese, 1991). In locust, FMRFamide's cardioexcitatory effects are not mediated by cyclic nucleotides (Cuthbert and Evans, 1989). The effects of FMRFamide on the locust heart depend on the form of beating of the heart prior to peptide application, sometimes increasing the frequency and amplitude of heart contractions and

sometimes decreasing them (Cuthbert and Evans, 1989). This is also true in leech hearts where FMRFamide can even induce myogenic contractions in quiescent hearts (Li and Calabrese, 1992). Cardioexcitation in the locust likely involves second messengers since the effects last for several minutes after the peptide has been removed unlike the cardioinhibitory effects which cease rapidly following the removal of the peptide (Cuthbert and Evans, 1989).

Some FaRPs are excitatory and some are inhibitory in cardiac and visceral muscles. FMRFamide initiates contractions by itself and potentiates proctolin induced contractions in the locust oviduct (Puiroux et al., 1993). However, SchistoFLRFamide (PDVDHVFLRFamide) and leucomyosuppressin (pQDVDHVFLRFamide) decrease the amplitude and frequency of myogenic contractions and reduce basal tonus in locust oviduct muscle (Lange et al., 1991; Peeff et al., 1993) and inhibit spontaneous contractions of the locust heart (Robb and Evans, 1990; Robb and Evans, 1994). SchistoFLRFamide appears to block Ca^{2+} influx from the cell exterior through voltage-gated or receptor operated Ca^{2+} channels (Wang et al., 1995).

1.14.3 Direct Postsynaptic Effects

There are several reports of direct effects of FaRPs on skeletal muscle. Walther et al. (1991) tested 15 peptides (including several FaRP's and structural analogs) on the locust jumping muscle to determine if their modulatory actions were pre- or postsynaptic in origin. Some postsynaptic effects of FaRP's were demonstrated using the voltage-clamp method. Leucomyosuppressin (pQDVDHVFLRFamide) and YGGFMRFamide decreased the membrane conductance of the muscle cells and shifted the reversal potential towards the equilibrium potential for K^+, suggesting that these peptides may modulate a voltage-independent K^+ channel.

In the pyloric dilator muscle of shrimp, FMRFamide and F_1 evoke short-lived rhythmic

depolarizations and contractions (Meyrand and Marder, 1991). The oscillations in the membrane potential were unaffected by TTX and are suppressed by Co^{2+}, suggesting an inward Ca^{2+} current is necessary for the depolarization. In this preparation FMRFamide and YGGFMRFamide depolarize the membrane by 5-10 mV at $2.5x10^{-7}$ M and cause a 20-30% increase in the input resistance. This is in contrast to the effect of FaRP's on crayfish abdominal muscles where F_1 increased input resistance by only 15%, and F_2 caused no change in input resistance (Mercier et al., 1990).

Other postsynaptic effects of FaRPs include the production of myogenic contractions in *Helix* tentacle retractor muscle (Cottrell et al. 1983) and a sustained increase in tonus in the lobster dactyl opener muscle (Worden et al., 1995).

1.15 Objectives of this Thesis

Two FaRP's isolated from the crayfish pericardial organs (Mercier et al., 1993), have the sequences: NRNFLRF-amide (NF_1) and DRNFLRF-amide (DF_2). Both peptides increase the rate and amplitude of cardiac contractions in a dose-dependent manner. They also increase the size of the excitatory junctional potentials (EJP's) in the phasic extensor muscles of the crayfish abdomen (Mercier et al., 1991). One of the peptides, DF_2, has been shown to be more effective at low temperatures than at high temperatures (Friedrich et al., 1994). This suggests that the peptide may provide a mechanism to compensate for reduced transmitter release at low temperatures.

In general, increases in EJP amplitude can be caused by an increase in the amount of transmitter released, by a change in the sensitivity of the receptor channels to the transmitter, or through an increase in the input resistance. Both peptides increase the number of quanta of

transmitter released from the motor nerve terminals innervating the crayfish phasic extensors. Neither peptide alters quantal size, suggesting no change in the sensitivity of postsynaptic receptors. One peptide, NF_1, causes a slight increase in the muscle fiber input resistance while the other, DF_2 does not (Skerrett et al., 1995). However, other postsynaptic effects such as modulation of evoked contraction or changes in tonus have not been ruled out.

The objective of this study was to examine more closely the possibility that DF_2 elicits direct effects on crayfish muscle. Experiments described here demonstrate such effects and investigate the influence of calcium and temperature on muscle tonus.

2. Methods

2.1 Preparation

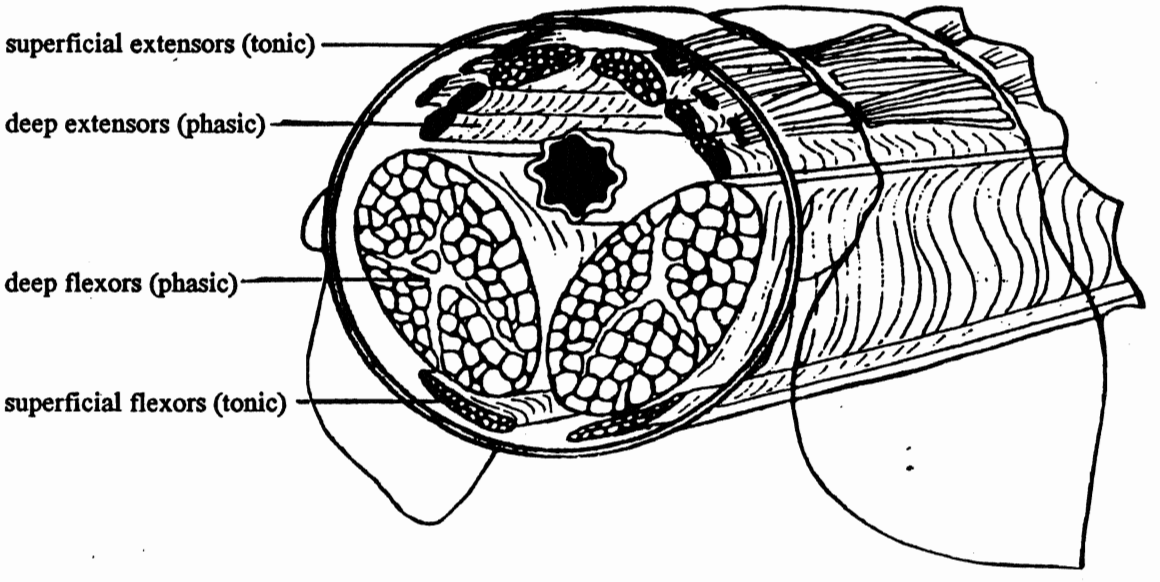
Crayfish (*Procambarus clarkii*), 2-3 inches in length, were obtained from the Atchafalaya Biological Supply Co. and stored in freshwater tanks at 15°C. They were fed a diet of carrots and Tender Vittles cat food. Prior to dissection, the crayfish were anaesthetized by placing them on ice for 30 minutes. The crayfish were euthanised by quick maceration of the brain and the thoracic ganglia. The abdomen was then cut away from the thorax. After making two lateral cuts along the sides of abdominal shell and one connecting cut across the base of the sixth abdominal segment, the dorsal abdominal shell was peeled back exposing the deep extensor muscles. The superficial abdominal extensor muscles were isolated by carefully removing the deep extensors (see Fig. 2). Segments 3 and 4 were left intact, while all the extra segments were removed. The shell, with the superficial extensors, was pinned to the bottom of a recording chamber lined with sylgard. The volume of the recording chamber was 2.0 mL.

2.2 Saline

Preparations were bathed in crayfish physiological saline (Van Harraveld, 1936) with the following constituents: 205 mM NaCl, 5.3 mM KCl, 13.5 mM CaCl, 2.45 mM MgCl and 5 mM HEPES. The pH was adjusted to 7.4 using 10 N NaOH.

The recording chamber was perfused with crayfish saline at a constant rate of 2.5 mL/min using a peristaltic pump at one end of the chamber and suction at the other end. All chemicals were bath applied by changing the solutions delivered to the preparation by the pump.

Figure 2. Cross-section of the abdomen of crayfish, *P. clarkii*, showing all the muscle groups. Note that each hemi-segment contains 3 pairs of superficial extensor muscle the lie right against the dorsal shell. They attach to the shell of one segment at one end and to a membrane lining the shell of the adjacent distal segment.



modified from Govind and Atwood (1982)
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2.3 Temperature Control

The temperature of the saline was carefully controlled with a Forma Scientific refrigerated circulator and monitored with a digital thermometer. Unless otherwise stated all experiments were performed between 7 and 10°C ± 1°C in each experiment.

2.4 Tension Recordings

Isometric contractions were recorded using a Grass Model 7B Polygraph. The muscle was held at a constant length while tension produced was measured with a Grass FT03 tension transducer. The transducer was calibrated by hanging weights of known mass (in mg), and the contractions were measured in units of milliNewtons (mN) using the relationship 102 mg= 1.0 mN. Segments 3 and 4 were cut down the midline with scissors and separated into left and right hemisegments. The ligaments and connective tissue in each preparation were severed, leaving the superficial extensor muscles as the only attachments between the dorsal shells of hemisegments 3 and 4. The shell of hemisegment 3 was them securely pinned to the bottom of the recording chamber, and the shell of hemisegment 4 was attached to the tension transducer using a fine pin, hooked at the end.

2.5 Tension Analysis

All changes in muscle tonus induced by peptide or blocking agents were measured relative to baseline levels that occurred prior to application of such agents. Mean changes in tonus were compared using the Wilcoxon Signed Rank test for matched pairs.

2.6 Intracellular Recordings

Muscle fiber input resistance was examined by passing measured amounts of current into individual fibers through one microelectrode, and measuring induced changes in the membrane potential through a second electrode placed < 50 μm away in the same fiber. Both electrodes were filled with 3M KCl, and each typically had a resistance of 16 MΩ. Current was generated with a Grass S88 Stimulator and was passed through a WPI Cyto 721 electrometer to the cell with one electrode. The corresponding changes in voltage were measured with a Warner Instrument Oocyte clamp (Model OC-725B). Current and voltage measurements were made directly from a Hameg model HM 205-3 digital storage oscilloscope.

Since the V/I recordings revealed active responses which were of interest, the signals were split between the digital storage oscilloscope for viewing and an Axon Instruments TL-1 DMA interface which, in turn, was connected to an IBM 386 compatible computer for computerized data acquisition. The active responses were analysed at a later date using a computer program, called ANACQ, provided by Dr. Milton Charleton (Dept. of Physiology, University of Toronto, Toronto, ON). The active response threshold, was determined by first estimating the start of the active response visually by the change in the slope of the membrane potential. The threshold was estimated as the voltage at the start of the active response relative to 0 mV (Fig. 3C). The amplitude was calculated by taking the difference between the peak of the active response and the threshold (Fig. 3B). An estimate of the duration of the active response was made by determining the time difference between the half-amplitudes on the rising and falling phases (Fig. 3D). The difference between means was determined using the Mann-Whitney U test for unmatched samples or the Wilcoxon signed rank test for matched pairs.

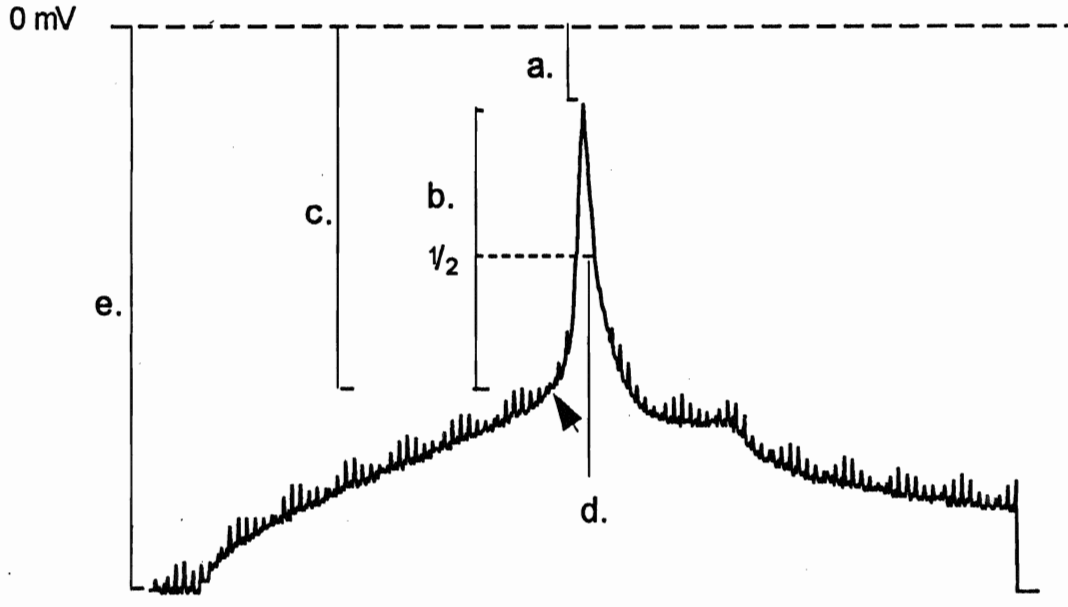
2.7 Concentrations of Toxins and Drugs

All toxins and drugs were made up in stock solutions and were frozen. Strophanthidin (0.1%), and nifedipine (0.1%) were dissolved in 95% EtOH. Both + and - enantiomers of Bay K 8644 (0.1%) and thapsigargin (0.001%) were dissolved in dimethylsulfoxide (DMSO). Manganous chloride, cadmium chloride, nickel chloride, verapamil, tetrodotoxin, ryanodine, ω-conotoxin, and DF_2 were all dissolved in crayfish saline.

2.8 Sources of Chemicals and Toxins

NaCl, $CaCl_2$, $MgCl_2$, KCl, NaOH, $NiCl_2$ and $CdCl_2$ were purchased from BDH Chemical Inc. (Toronto, ON). For some experiments, $MgCl_2$ came from the Fisher Scientific Company (Ottawa, ON). HEPES, DMSO, strophanthidin and tetrodotoxin came from Sigma Chemical Co. (St. Louis, MO). Ryanodine, nifedipine, ω-conotoxin GVIA were purchased from Calbiochem Chemical Co. (La Jolla, CA). Alomone Labs (Jerusalem, Israel) was the supplier of thapsigargin, charybdotoxin, and apamin. Both the positive and negative forms of Bay K 8644, along with the ameliorate and verapamil were purchased from Research Biochemicals International (Natick, MA). DF_2 was synthesized by T.S. Chen of the Biotechnology Service Centre, Banting and Best Institute, Toronto, Canada.

Figure 3. Paramaters for analyzing the active responses. a. Peak voltage relative to zero (ground voltage). b. Active response amplitude is measured from the threshold (indicated by the arrow) to the top of the peak. c. Thresholds are measured relative to zero. d. Duration is measured as the time difference between rising and falling phases at the half the amplitude. e. Resting potential.


0 mV
a.
b.
c.
1/2
d.
e.

3. Results

3.1 Muscle Contraction

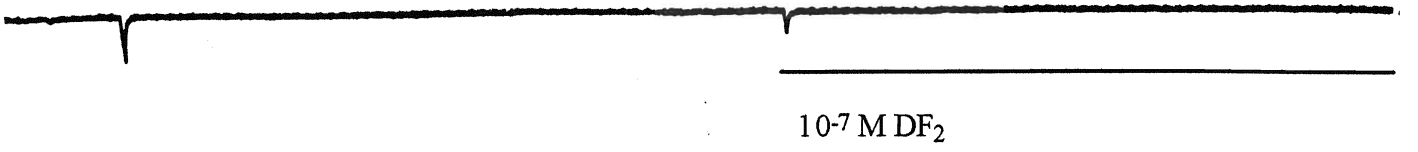
The first step in investigating postsynaptic effects of the neuropeptide was to identify an appropriate muscle that is sensitive to DF_2. Three muscle groups from the crayfish abdomen were surveyed to determine whether or not the peptide would alter muscle tonus. Figure 4 shows the effects of the application of 10^{-7} M DF_2 on the deep extensor muscles, the superficial flexor muscles and the superficial extensor muscles of the crayfish abdomen. The only muscle group affected by the peptide was the superficial extensors, which showed an increase in the tonus in 51 out of 62 preparations. The deep extensors and superficial flexors (N=5 and N=9, respectively) never exhibited alterations in muscle tonus in response to 10^{-7} M DF_2. All subsequent experiments were conducted on the superficial extensor muscles.

Some muscle preparations produced small contractions that ranged in amplitude from 0.02 mN to 0.30 mN. The spontaneous contractions were usually smaller than the nerve-evoked contractions produced when the nerve was stimulated for 2 seconds at 10 Hz with 2 volts (Fig. 6). The spontaneous contractions were not present in every preparation, but the contractions could usually be obtained if the temperature was brought below 13°C (Fig.5A). As the temperature was lowered the spontaneous contractions became more noticeable. Even though the amplitude of the contractions increased as the temperature was lowered (Fig.5C), the frequency of the contractions decreased (Fig.5B). A typical time course of the spontaneous contractions recorded at approximately 8°C consisted of a 200 msec rise time followed by an 800 msec falling phase. These contractions were sometimes rhythmic and sometimes very irregular.

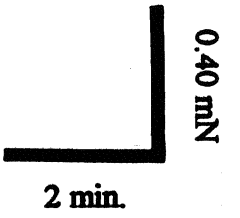
10^{-7} M DF_2 appeared to enhance the spontaneous contractions in some preparations as shown in Fig. 4. In 40 out of 47 preparations the peptide did not elicit such contractions and in

Figure 4. Survey of the responsiveness of three abdominal muscles to 10^{-7} M DF_2. Muscle contraction was monitored by recording isometric tension. The peptide was applied during times indicated by the solid black bars.

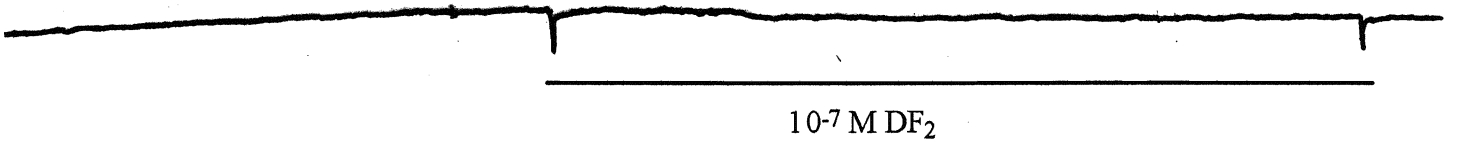
Deep Extensors



10^{-7} M DF_2

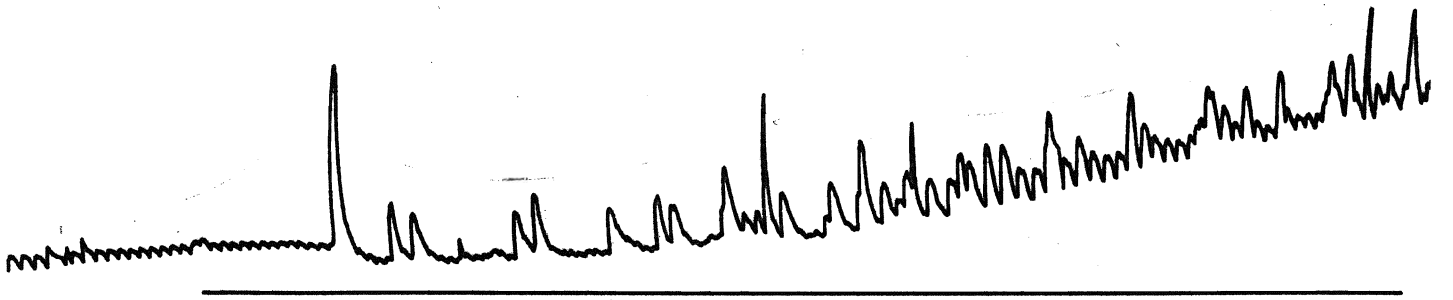


Superficial Flexors



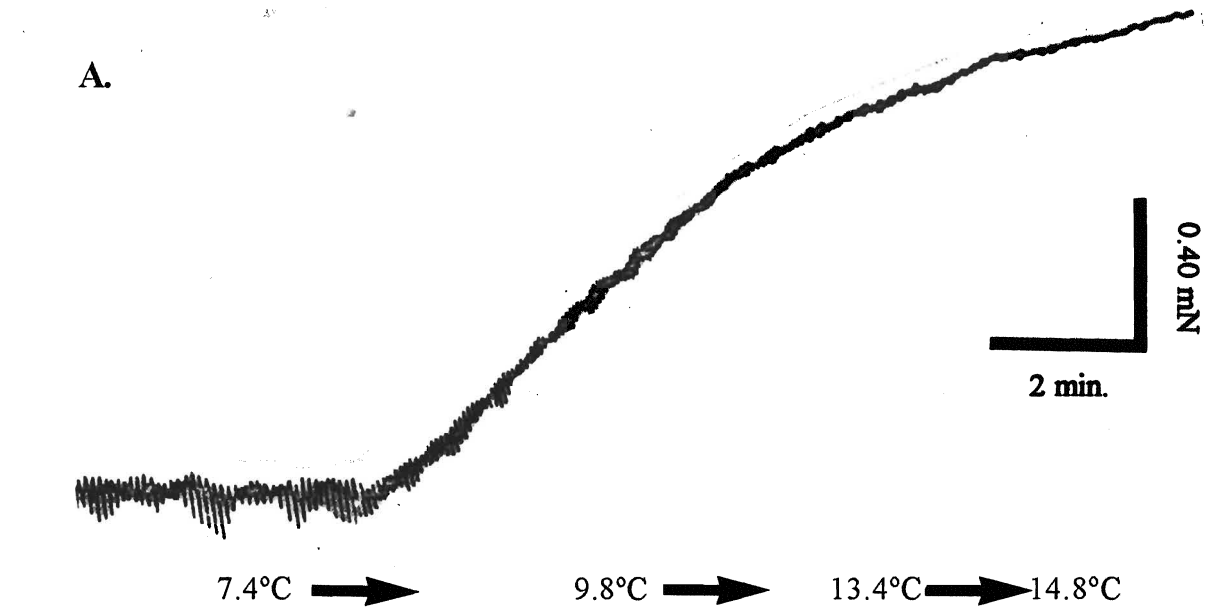
10^{-7} M DF_2

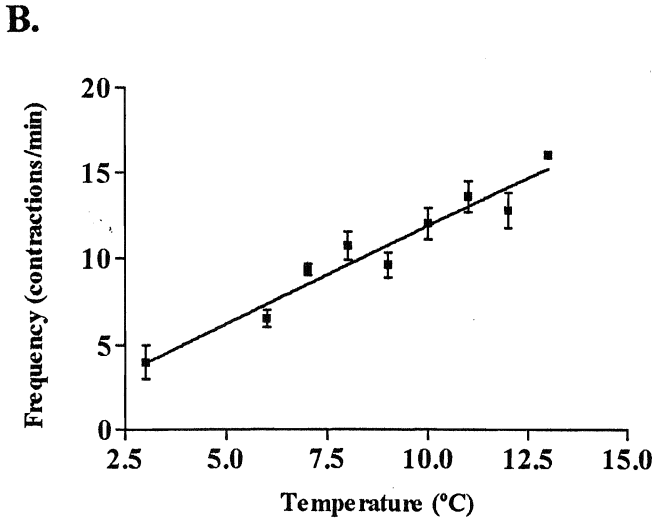
Superficial Extensors

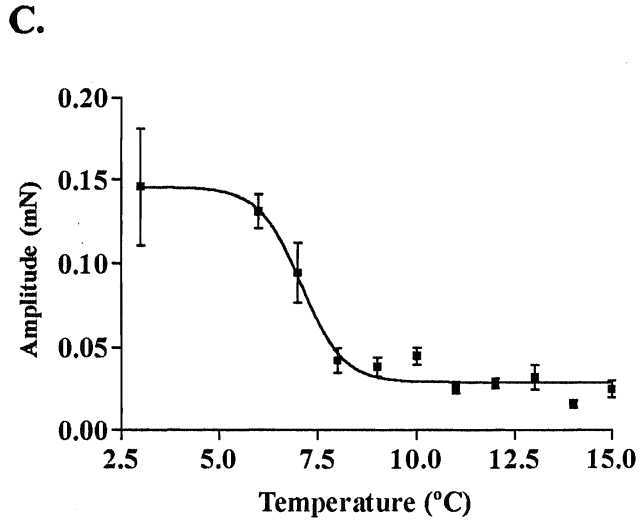


10^{-7} M DF_2

Figure 5. The superficial extensor muscles produce rhythmic contractions that are temperature-dependent. A. Sample tension recording from the superficial extensor muscle as the temperature is raised from 7°C to 15°C. B. The frequency of the contractions increases as the temperature increases. Above 9.8° C the contractions become indistinguishable and impossible to count. C. The amplitude of individual contractions decreases as the temperature increases. (n=17).


A.
0.40 mN
2 min.
7.4°C
9.8°C
13.4°C
14.8°C


B.
Frequency (contractions/min)
20
15
10
5
0
2.5
5.0
7.5
10.0
12.5
15.0
Temperature (°C)


C.
Amplitude (mN)
0.20
0.15
0.10
0.05
0.00
2.5
5.0
7.5
10.0
12.5
15.0
Temperature (°C)

27 out of 47 preparations the peptide did not increase the amplitude of such contractions. The effect on tonus was more consistently observed than the effect on the rhythmic and irregular contractions.

In two preliminary experiments where tension was evoked by stimulating the nerve, the peptide enhanced the amplitude of the contractions (Fig. 6 A and B (lower traces)) suggesting it may enhance neuromuscular transmission as in the deep extensor muscle of the crayfish abdomen (Skerrett et al., 1995).

It was not expected that the spontaneous muscle contractions would be caused by neural activity because the nerve containing all the motor axons had been cut. Nonetheless, experiments were performed to examine the unlikely possibility that the distal stumps of the severed axons might be spontaneously active. Recordings from the nerve through a suction electrode detected some activity associated with the sensory neurons of the muscle receptor organs (MROs), whose cell bodies are located next to the muscle (Alexandrowicz, 1951). This activity was abolished with the application of 10^{-7} M TTX (in 10 out of 10 preparations) as indicated in figure 8. However, the rhythmic contractions persisted. Thus, the spontaneous contractions are not caused by neural activity, but are myogenic in nature. The effect of DF_2 was also unaffected by TTX (Fig. 8).

In an attempt to understand the temperature-dependent nature of the spontaneous contractions, the effects of the Na^+/K^+ pump inhibitor, strophanthidin, were examined. If the Na^+/K^+ exchange pump were inhibited at low temperatures, one would predict a corresponding increase in the intracellular Na^+ concentration. This, in turn, could inhibit the Na^+/Ca^{2+} exchange which would increase the concentration of intracellular Ca^{2+} (Ortiz and Orkand, 1979) and might increase muscle tonus. Figure 7 shows two tension recordings from the same preparation. At 11°C the rhythmic contractions were present but at 18° C they were no longer visible.

Figure 6. Nerve-evoked contractions from two preparations stimulated every minute for 2 seconds at 10 Hz (arrows). Black lines indicate the application of 10^{-7} M DF_2. A. Upper and lower traces are from the same preparation. Note the spontaneous contraction (*) in the upper trace. B. (Upper trace) The first two contractions were cut off by the chart recorder (**). Subsequent contractions were elicited by a lower voltage.

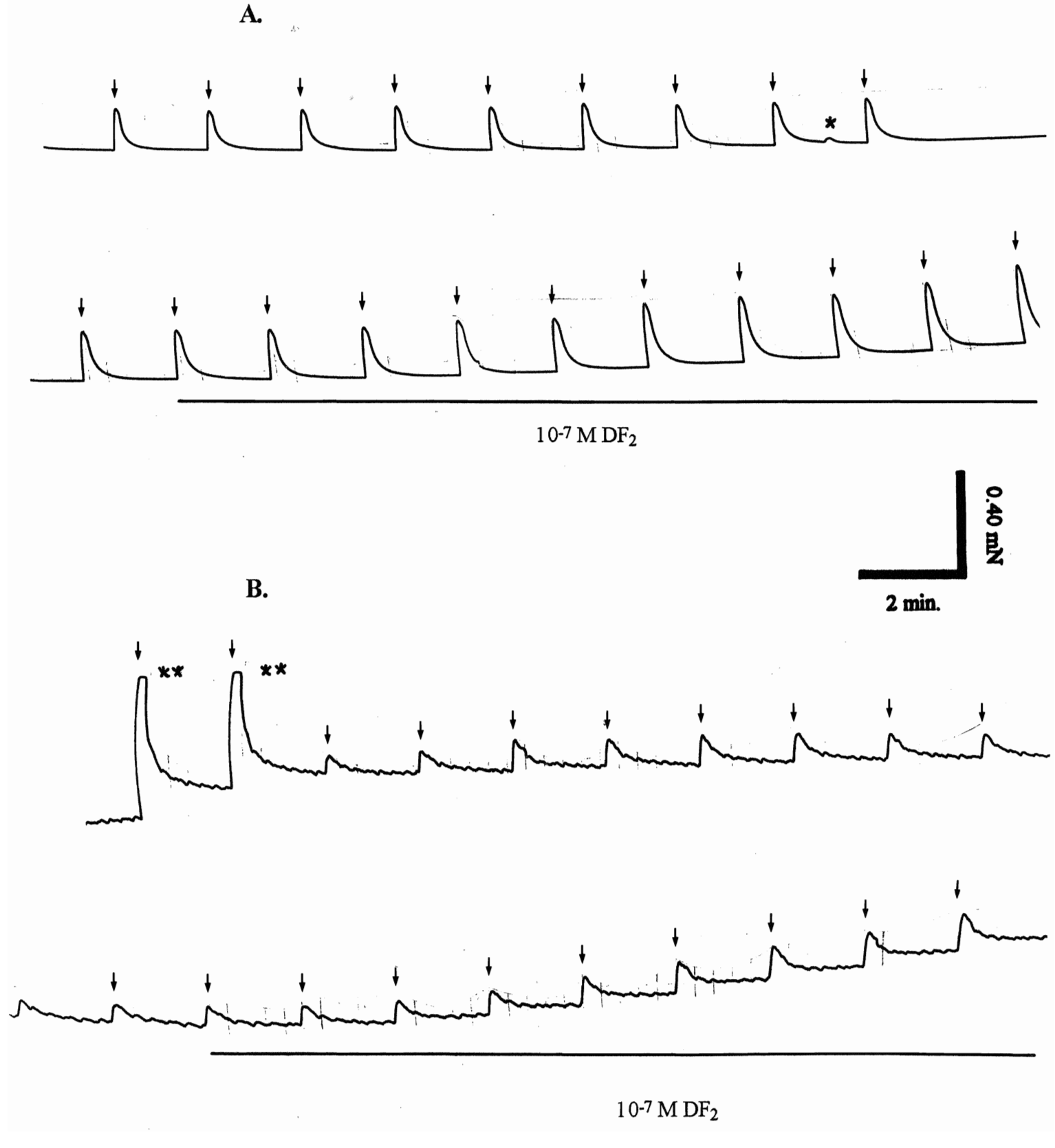

A.
*
10-7 M DF2
0.40 mN
2 min.
B.
**
**
10-7 M DF2

Figure 7. Tension recordings from a single preparation showing the rhythmic contractions at 11° C (top trace) and the effect of the Na^+/K^+ exchange inhibitor, strophanthidin, at 18° C (bottom trace). The preparation was superfused with saline containing 0.1% EtOH throughout all the recordings. The black line indicates the application of strophanthidin.

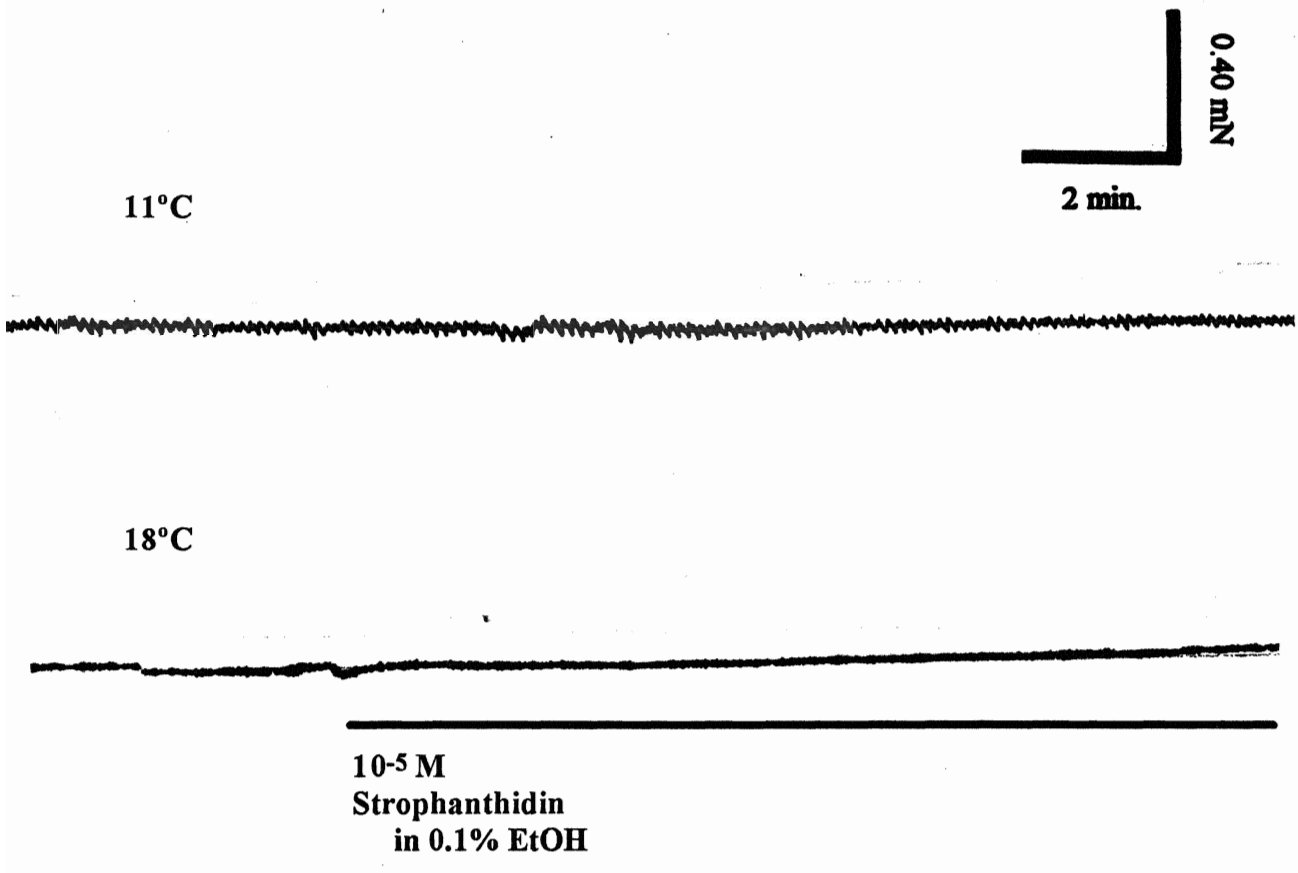
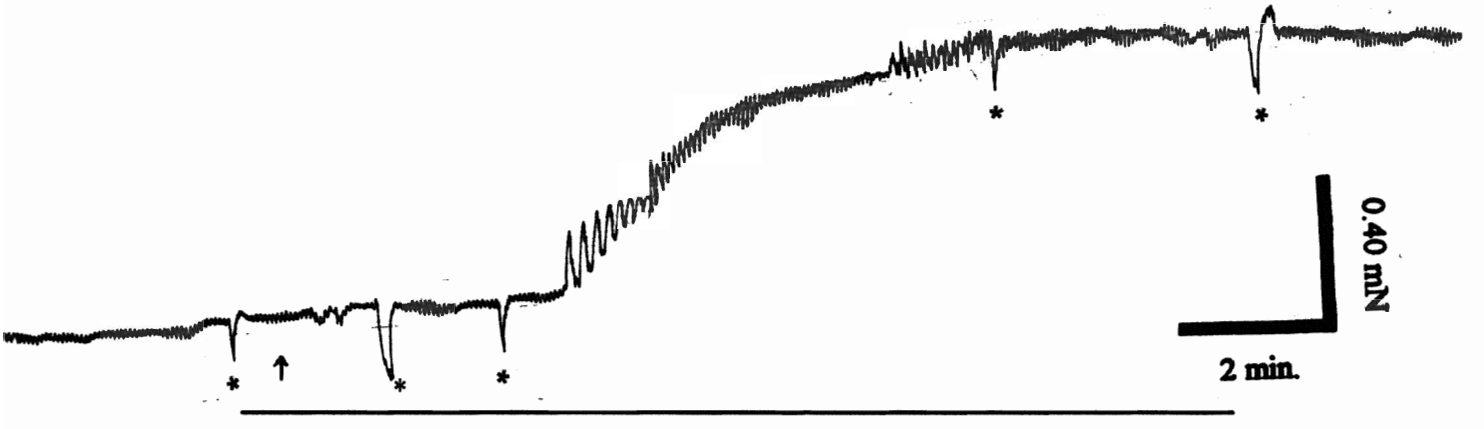

0.40 mN
2 min.
11°C
18°C
10-5 M
Strophanthidin
in 0.1% EtOH

Figure 8. The rhythmic contractions are TTX-resistant. 10^{-7} M TTX was applied during the time indicated by the solid black bar, and 10^{-7} M DF_2 was applied when indicated by the dotted line. Shortly after application of TTX (arrow) nerve activity was abolished. (* Artifact caused by entry of air bubble into the recording chamber).



10^{-7} M TTX

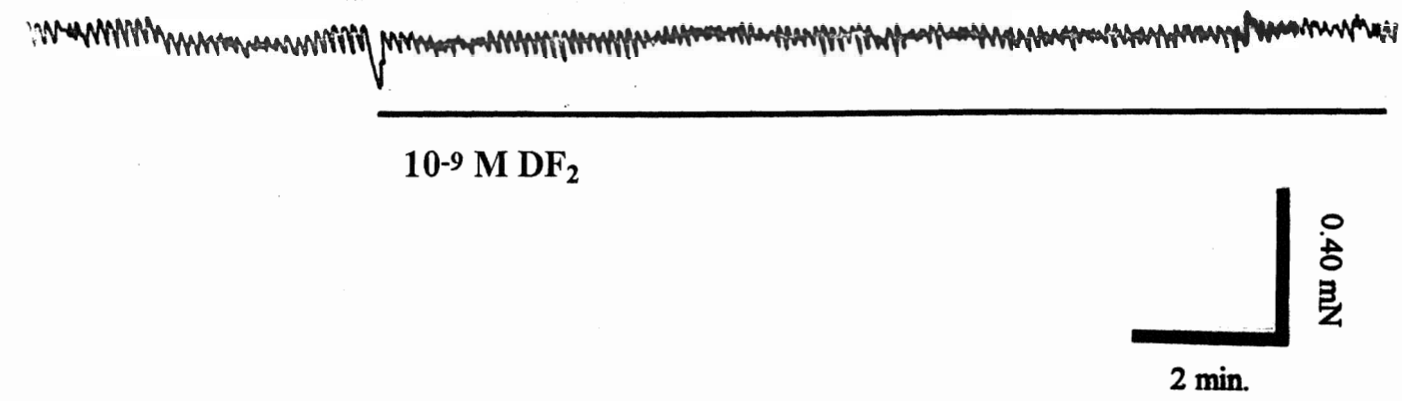
10^{-7} M DF_2

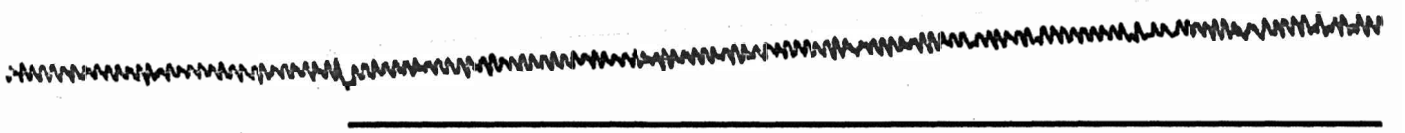
The application of 10^{-5} M strophanthidin did not induce any contractions at the higher temperature in 8 preparations (all of which displayed oscillatory contractions at low temperatures). This suggests that the Na^+/K^+ pump is not involved in the temperature-dependent induction of the rhythmic contractions.

DF_2 was applied to preparations in increasing concentrations, with wash periods of approximately 20 minutes between successive applications. The tonus of the muscle increased with each successive application of the peptide in a dose-dependent manner. The threshold for the effect on tonus was between 10^{-9} and 10^{-8} M DF_2 (Fig.9). The dose-response curve generated from 5 preparations is shown in figure 10(A). Each hemi-segment of the crayfish abdomen consists of 3 superficial extensor muscles, referred to here as the medial, middle and lateral muscles. When isolated and treated with DF_2, each muscle showed a similar dose-dependent increase in tonus (Fig.10B).

To test the dependence of the peptide's effect on external calcium, several divalent cations which are known to block Ca^{2+} channels were used. Tension recordings from two different preparations exposed to Mn^{2+} and $Cd^{2+}$are shown in figure 11(A). Both of these blockers decreased the tonus and the amplitude of rhythmic contractions, in the presence and absence of DF_2. In the top trace, DF_2 increased the tonus and subsequent addition of 10 mM Mn^{2+} decreased both the tonus and the amplitude of the rhythmic contractions. In the second trace, the rhythmic contractions were present even in the absence of DF_2. The addition of Cd^{2+} reduced the tonus, suppressed the rhythmic contractions and minimized DF_2's effect. Nickel also inhibited the effect of the peptide, as shown in figure 11(B). In each of these sets of experiments the blocker was applied before DF_2 (Fig. 11B). In Ca^{2+}free saline, in which Ca^{2+} is replaced by Mg^{2+} without EGTA, the effect of DF_2 on muscle tonus is blocked (Fig. 12). In contrast, the sodium channel

Figure 9. DF_2 increases the basal tonus of the superficial extensors in a dose-dependent manner. Sample tension recordings from a single preparation. Increasing concentrations of DF_2 were applied for approximately 10 minutes with a 20 minute wash period between each application.

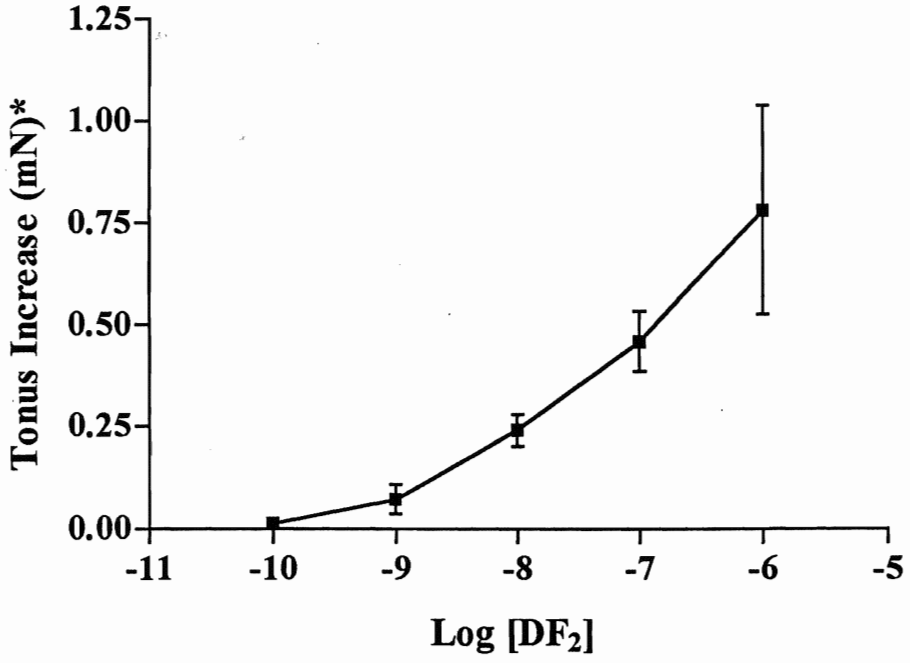

10-9 M DF2
0.40 mN
2 min.


10-8 M DF2

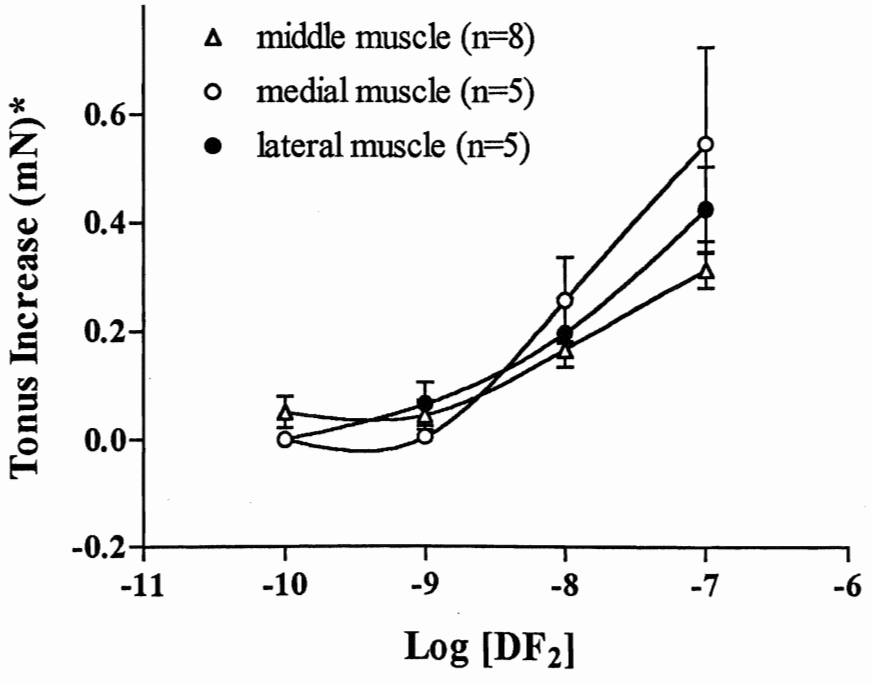

10-7 M DF2

Figure 10. A. The dose-response curve for the effect of DF_2 on tonus for all 3 superficial extensor groups in the third hemi-segment (n=5). B. The dose-response curves for each of the 3 muscles.

A.



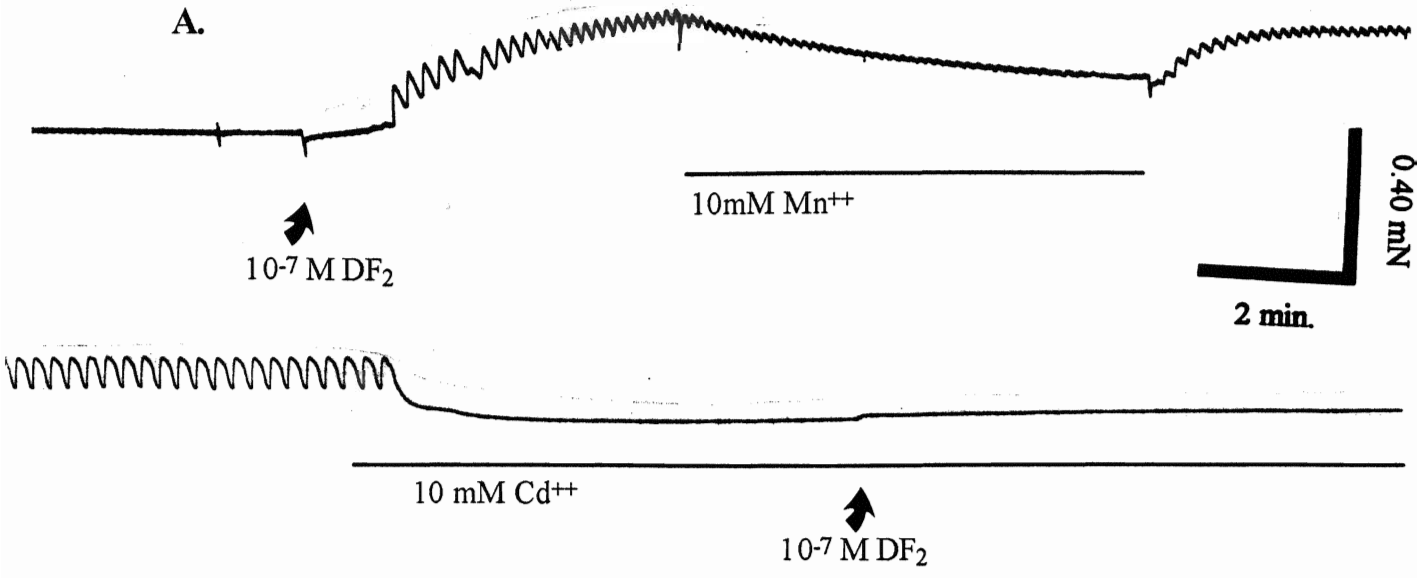
B.



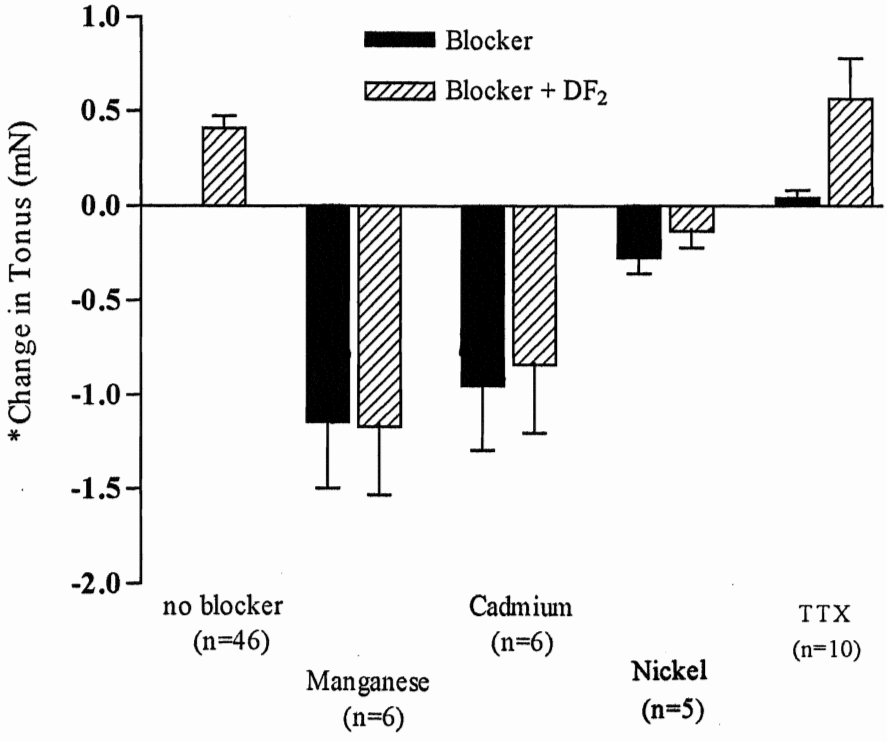
*Relative to baseline tonus in saline

Figure 11. Muscle tonus and the rhythmic contractions are dependent on external Ca^{2+}. A. Sample tension recordings showing the effects of the application of the ionic Ca^{2+} channel blockers Mn^{2+} and Cd^{2+} on two different preparations. DF_2 was applied continuously starting at each of the arrows. The black lines denote the Ca^{2+} channel blockers were present in the bathing solution. B. Bar graph summarizing the effects of 3 Ca^{2+} channel blockers and the Na^{+} channel blocker, TTX on muscle tonus. Data are shown as the mean ± SEM. The concentration of Mn^{2+}, Cd^{2+} and Ni^{2+} was 10 mM and the concentrations of DF_2 and TTX were 10^{-7} M. In each set of experiments the blocker was applied first, followed by DF_2.

A.

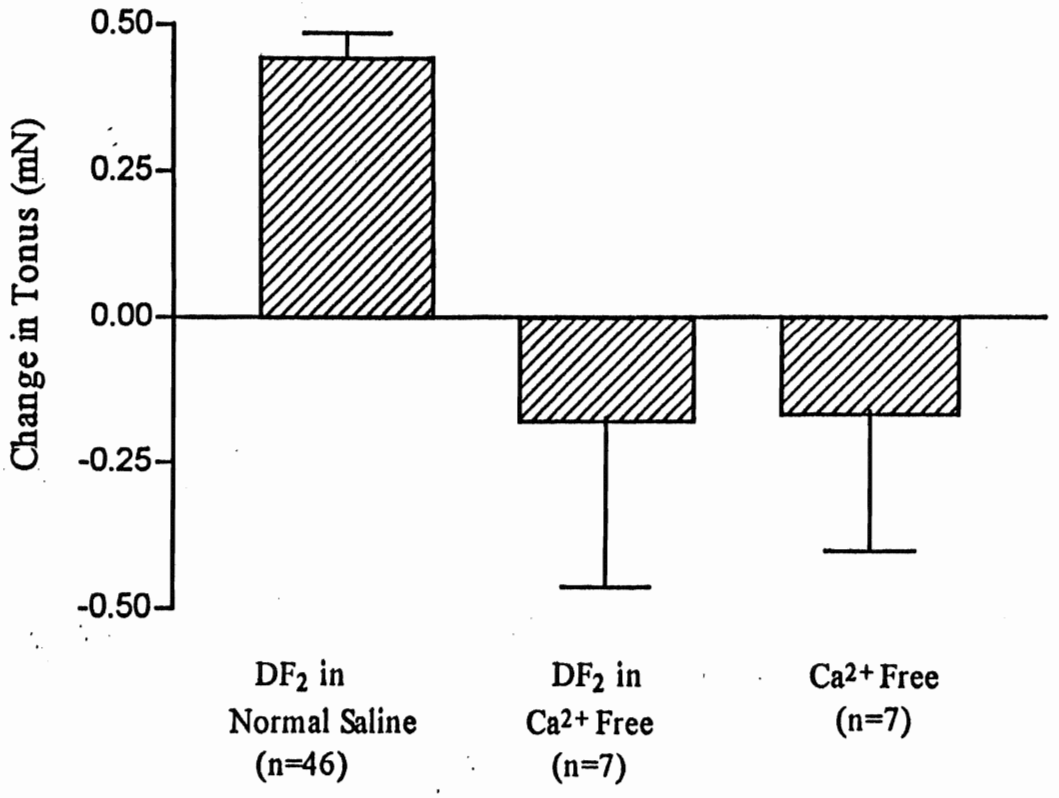


B.



* Relative to baseline tonus prior to the application of blocker.

Figure 12. The effects of DF_2 on muscle tonus are blocked in Ca^{2+}-free saline (Ca^{2+} was replaced by Mg^{2+}). The effect of DF_2 in normal saline is shown for comparison. Data are shown as means ± SEM.


0.50
0.25
0.00
-0.25
-0.50
Change in Tonus (mN)
DF2 in Normal Saline (n=46)
DF2 in Ca2+ Free (n=7)
Ca2+ Free (n=7)

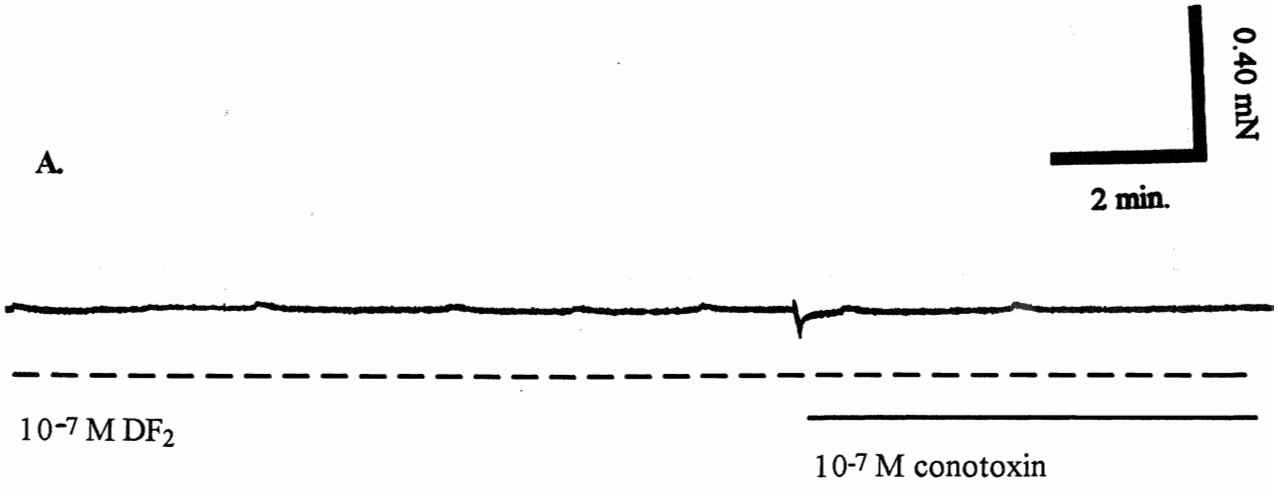
blocker, TTX, did not alter muscle tonus or the peptide's effect on tonus (Fig. 11B). This suggests that external calcium is required for both the rhythmic contractions and the peptide's effect but, sodium is not.

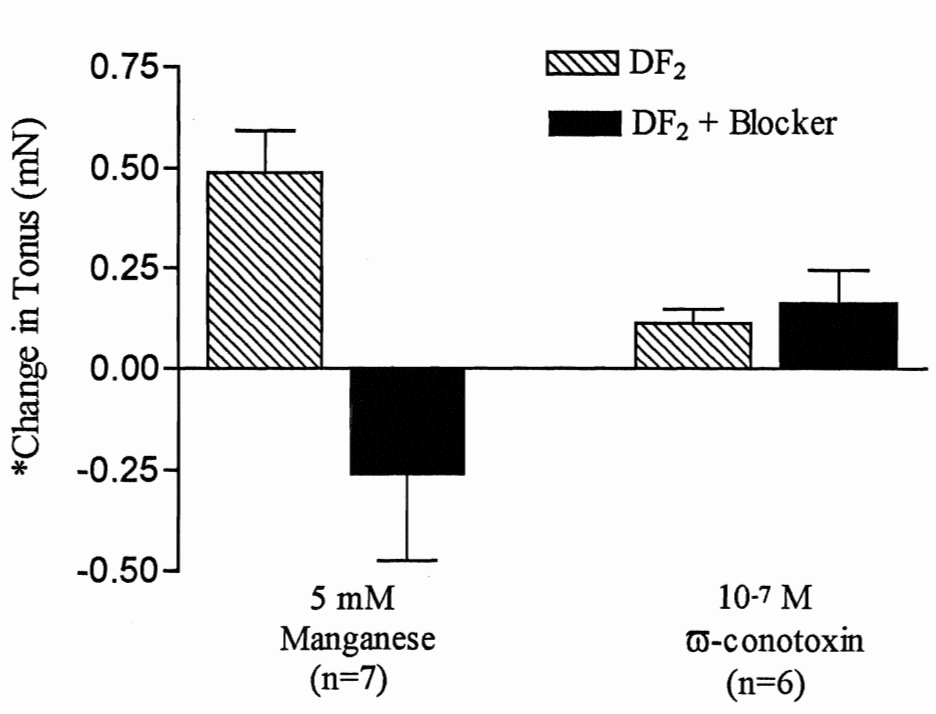
To examine which types of Ca^{2+} channels are involved in the peptide's effect, blockers specific to N-type and L-type voltage-gated Ca^{2+} channels were used. The N-type Ca^{2+} channel blocker ω-conotoxin (Takahashi and Momiyama, 1993) did not inhibit the peptide's effect (Fig. 13). DF_2 alone increased muscle tonus, and the tonus did not change with the subsequent application of 10^{-7} M ω-conotoxin. This was particularly obvious when compared to the effect of 5 mM Mn^{2+} (Fig. 13) which decreased the tonus following the application of DF_2 much the same as did 10 mM Mn^{2+} (Fig.11B).

Figure 14 shows the effects of the L-type Ca^{2+} channel antagonists, 10^{-5} M nifedipine (Rios and Brum, 1987), 2.5 x 10^{-4} M (+)Bay K 8644 (Mintz et al., 1991), and 10^{-4} M verapamil (Gielow and Singh, 1995), on muscle tonus following the application of DF_2. None of these putative antagonists inhibited the peptide's effect on tonus, nor did they inhibit the rhythmic contractions (Fig.14). 2.5 x 10^{-4} M (-)Bay K 8644 , which is reported to be an L-type Ca^{2+} channel agonist (Mintz et al., 1991), did not alter the tonus. This suggests that L-type Ca^{2+} channels are not involved in the peptide's effect. 10^{-4} M verapamil increased the muscle tonus in the presence of DF_2 in 6 out of 6 preparations (Fig.15). This was found to be significant using a Wilcoxon signed-rank test for matched pairs ($p<0.005$).

Neither the N-type Ca^{2+} channel blocker, ω-conotoxin, nor the toxins specific to L-type calcium channels (nifedipine, (+)Bay K 8644, and (-)Bay K 8644) significantly changed the muscle tonus when they were applied alone (Fig.16). On the other hand, 10^{-4} M verapamil increased the tonus substantially when applied in normal saline (Fig.16A). Verapamil induced

Figure 13. Neither the tonus nor the rhythmic contractions are modulated by the N-type Ca^{2+} channel blocker, ω-conotoxin. A. Sample tension recording superficial extensor preparation. Dashed line indicates the presence of DF_2 and the solid line indicates the presence of the ω-conotoxin. B. Bar graph comparing the changes (means ± SEM) in tonus caused by Mn^{2+} and ω-conotoxin following the application of DF_2.





*Relative to baseline prior to the application of DF_2.

Figure 14. Sample tension recordings showing the effects of several L-type Ca^{2+} channel blockers in the presence of DF_2. The peptide was present throughout the times indicated by the dashed lines. The solid lines indicate the presence of each specified blocker. Note: nifedipine was applied in the presence of 0.1% EtOH and (+) Bay K 8644 was applied in 0.1% DMSO. Neither EtOH nor DMSO altered the muscle tonus at these concentrations.

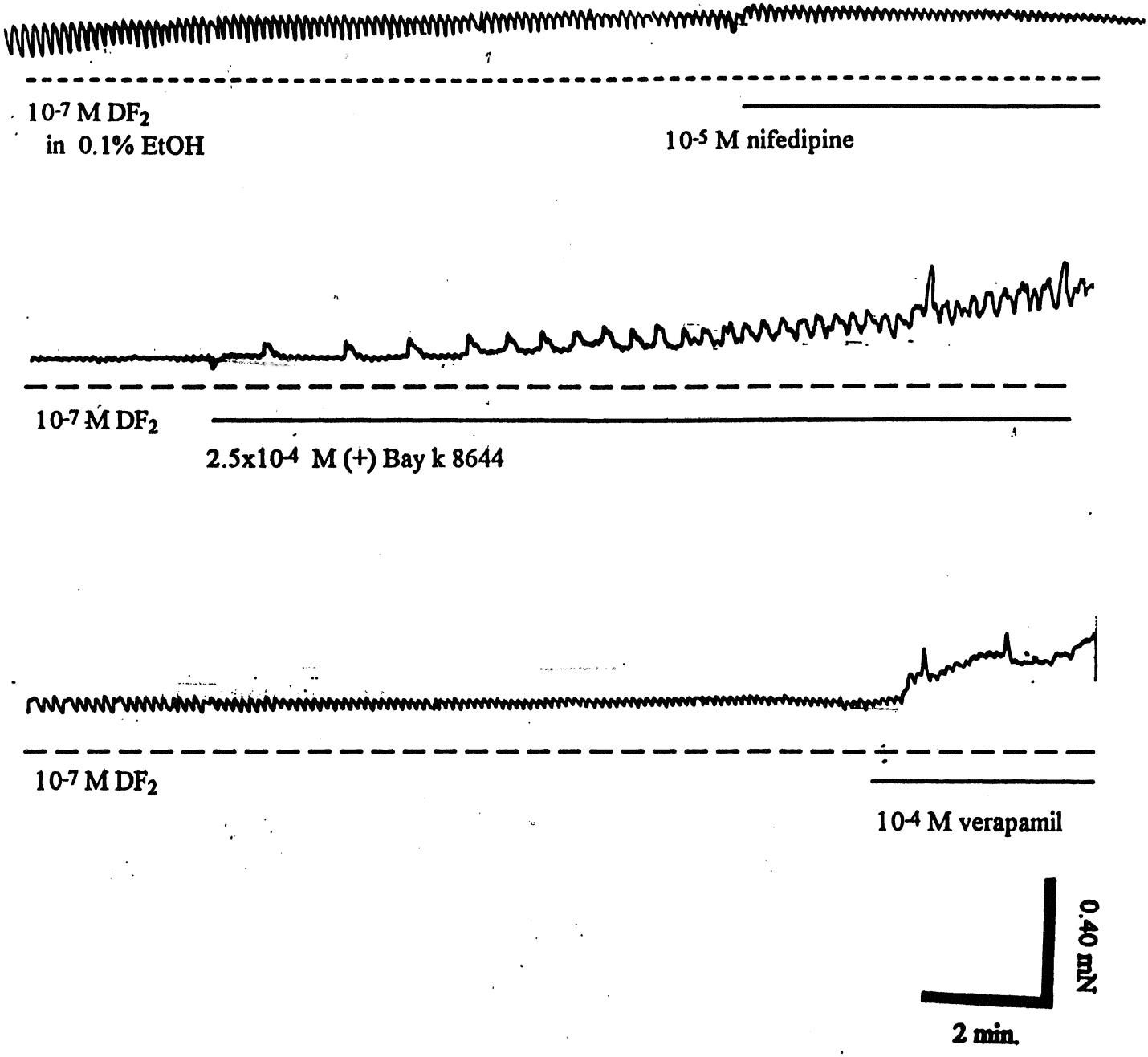
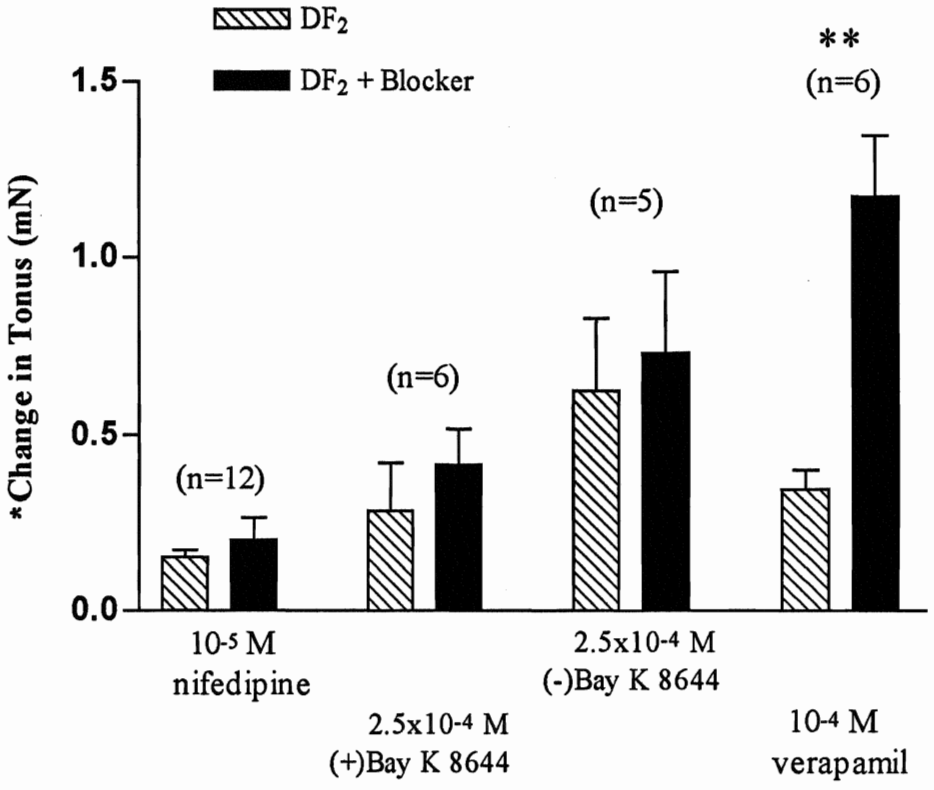

10-7 M DF2
in 0.1% EtOH
10-5 M nifedipine
10-7 M DF2
2.5x10-4 M (+) Bay k 8644
10-7 M DF2
10-4 M verapamil
0.40 mN
2 min.

Figure 15. Bar graph summarizing the effects of several L-type Ca^{2+} channel blockers on tonus of the superficial extensor muscles in the presence of DF_2. The nifedipine experiments were run in the presence of 0.1% EtOH and both the (+) and (-) Bay K 8644 experiments were run in 0.1% DMSO. Neither EtOH nor DMSO altered the muscle tonus at these concentrations. Data are shown as means ± SEM. and the significant increase in tonus ($p<0.005$) in the presence of verapamil is indicated by the double asterisks (**).

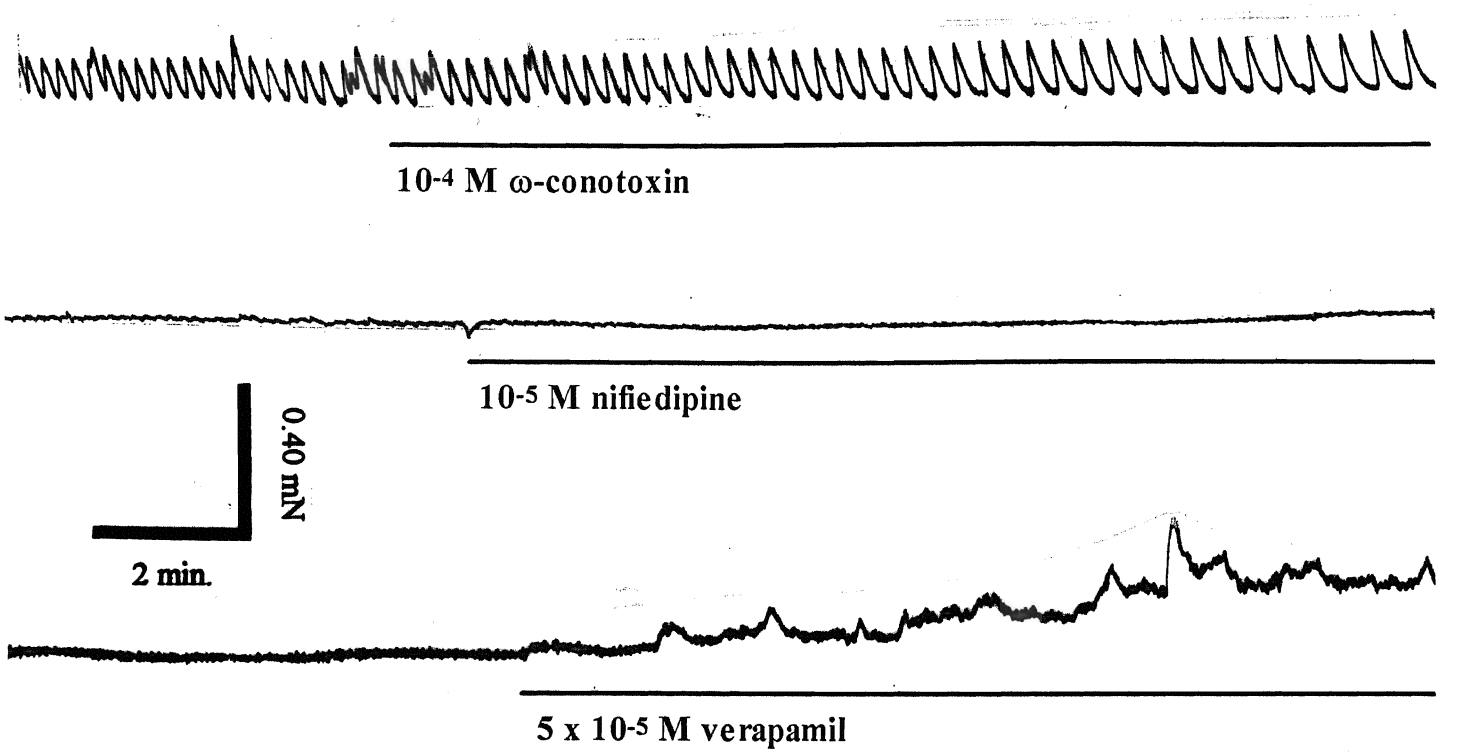


*Relative to baseline prior to the application of DF_2.

**Significantly different ($p<0.005$)

Figure 16. A. Tension recordings showing the effect of an N-type Ca^{2+} channel blocker, ω-conotoxin, and the L-type Ca^{2+} channel blockers, nifedipine and verapamil, on muscle contraction without DF_2 present. Blocker application is indicated by the solid black bars. B. Bar graph summarizing the tonus changes (means ± SEM) caused by ω-conotoxin and several L-type Ca^{2+} channel blockers.

A.


10-4 M ω-conotoxin
10-5 M nifiedipine
0.40 mN
2 min.
5 x 10-5 M verapamil

B.

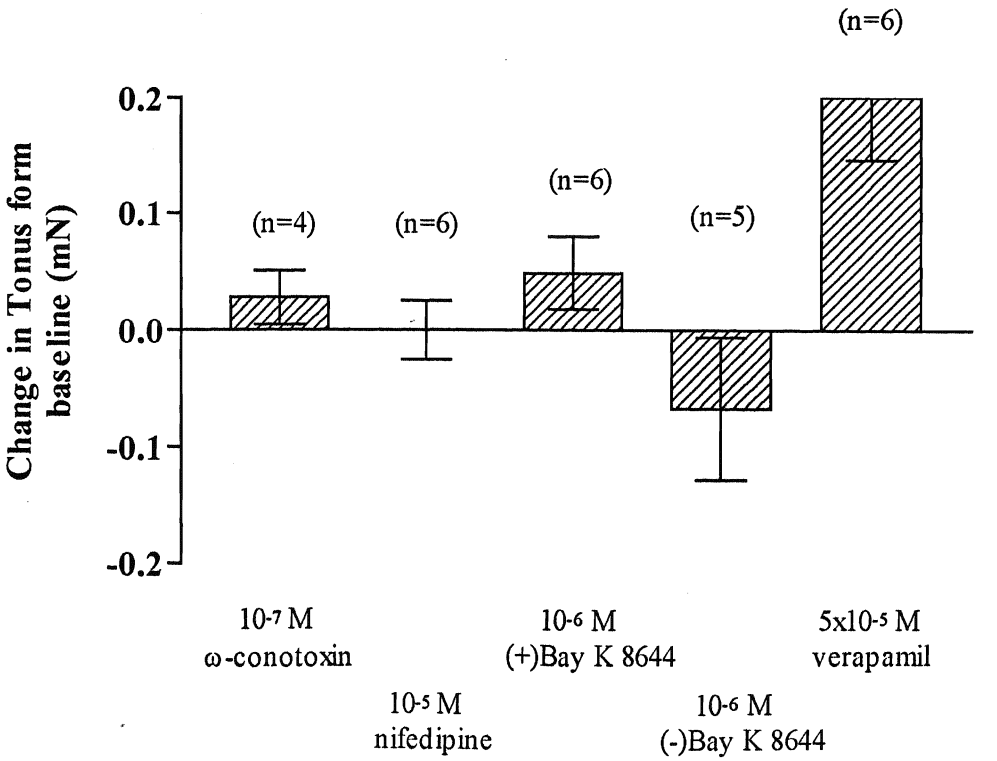
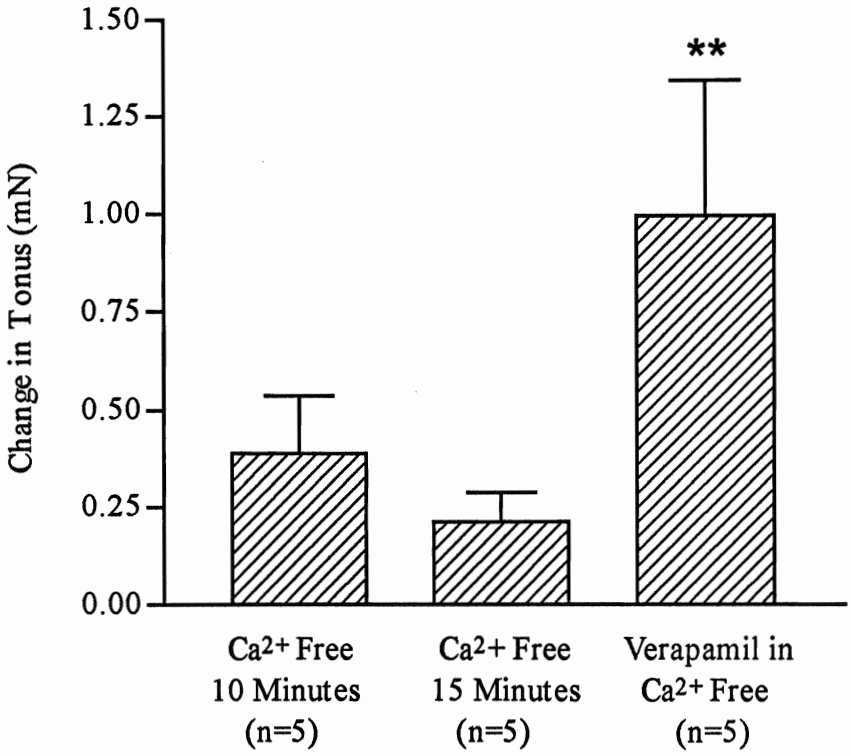

Change in Tonus form baseline (mN)
0.2
0.1
0.0
-0.1
-0.2
(n=4)
(n=6)
(n=6)
(n=5)
(n=6)
10-7 M ω-conotoxin
10-5 M nifedipine
10-6 M (+)Bay K 8644
10-6 M (-)Bay K 8644
5x10-5 M verapamil

Figure 17. Bar gragh summarizing the effects of verapmil in Ca^{2+}-free saline buffered with EGTA on tonus of the superficial extensor muscles. The first two bars indicate the changes in tonus after 10 and 15 minutes in Ca^{2+}-free saline, respectively. The significant increase in tonus ($p<0.005$) in the presence of verapamil is indicated by the double asterisks (**). Data are shown as means ± SEM.


1.50
1.25
1.00
0.75
0.50
0.25
0.00
Change in Tonus (mN)
**
Ca2+ Free
10 Minutes
(n=5)
Ca2+ Free
15 Minutes
(n=5)
Verapamil in
Ca2+ Free
(n=5)

some spontaneous contractions (although not rhythmic ones) along with the increase in tonus. Even in Ca^{2+}-free saline buffered with 5 mM EGTA, so that pCa^{2+} is greater than 9, verapamil significantly changed the tonus suggesting that verapamil's effect does not require the influx of external Ca^{2+} (Fig. 17; Wilcoxon signed-rank test for matched pairs, $p<0.005$). When the saline bathing the preparation was changed to Ca^{2+}-free saline buffered with EGTA, there was a large increase in tonus followed by a slow decrease which took longer than 15 minutes to return to baseline. Figure 17 shows the effect on tonus after 10 minutes and 15 minutes in Ca^{2+}-free saline. After 15 minutes in Ca^{2+}-free saline the tonus was closer to the tonus level in normal saline. The initial increase in tonus may be caused by the release of Ca^{2+} from internal stores.

3.2 Electrical Properties of Muscle Cells

To determine if the increased tonus caused by DF_2 was accompanied by a change in resting potential, the resting potential was monitored using standard intracellular recording techniques. There was no significant difference (Mann-Whitney U test) between the distributions of the resting potential of 5 muscle fibers bathed in normal crayfish saline (-67.3 ± 5.7 mV) for 10 minutes and 6 different muscle fibers bathed in 10^{-7} M DF_2 (-66.1 ± 6.3 mV) for the same length of time (Table 1). There was also no significant difference (Wilcoxon signed-rank test for matched pairs) between the distributions of the resting potentials of 5 muscle fibers measured in saline (-65.7 ± 6.4 mV) and the same fibers after bathing in 10^{-7} M DF_2 for 10 minutes (-66.1 ± 6.3 mV).

Initially the input resistance was measured by injecting a series of hyperpolarizing current pulses followed by a series of depolarizing current pulses into a single fiber and recording the corresponding change in voltage. However, the occurrence of active responses by the muscle

Table 1. Comparison of means ± SEM of membrane properties of fibers bathed in saline and peptide. V_m indicates the resting potential; R_{input} indicates the input resistance; Amplitude refers to the amplitude of the active response; Threshold refers to the threshold of the active response. (** indicates a significant difference at the $p<0.001$ level).

	V_m (mV)	R_{input} (Ω)	Active Responses Amplitude (mV)	Active Responses Threshold (mV)	Preparations with Active Responses (%)
Saline	**-67.3** ±5.7 (n=11)	**300** ±222 (n=11)	**18.4** ±2.1 (n=6)	**-35.0** ±2.5 (n=6) **	**54.5** (n=11)
DF_2	**-66.1** ±6.3 (n=8)	**304** ±194 (n=11)	**19.2** ±8.0 (n=6)	**-53.2** ±5.1 (n=5)	**72.7** (n=11)

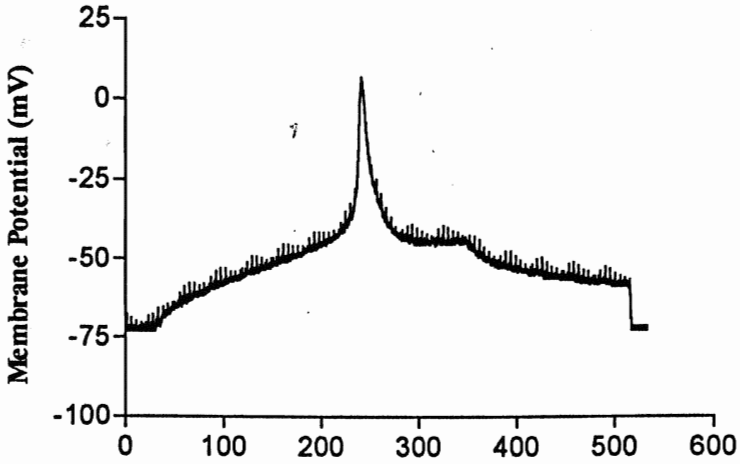
fibers when depolarized led to the exclusion of depolarizing currents in the input resistance calculations. The mean input resistance was determined from 11 preparations in saline (300 ± 222 Ω) and then after 10 minutes in 10^{-7} M DF_2 (304 ± 194 Ω) from the voltage/current relationships shown in Appendix 1. Application of 10^{-7} M DF_2 did not cause a significant change (Wilcoxon signed-rank test for matched pairs) in muscle cell input resistance (Table 1).

The amplitudes and thresholds of active responses in the muscle cells were also estimated in saline and 10^{-7} M DF_2 (Table 1). There was no significant difference between the distributions of the amplitudes of the active responses recorded from 6 preparations in saline and 6 different preparations in 10^{-7} M DF_2. However, in 6 muscle fibers that were first bathed in saline and subsequently bathed in 10^{-7} M DF_2 for 10 minutes, the distributions of the amplitudes of the active response did change significantly ($p<0.0277$, Wilcoxon signed-rank test for matched pairs). The distributions of the threshold voltages from 4 muscle fibers was significantly more negative (Mann Whitney U, $p<0.1429$) in the presence of 10^{-7} M DF_2 than from 5 different muscle fibers bathed in saline. These data suggest that the peptide increases the excitability of the muscle fibers, even though there was no significant difference (Chi squared test) in the percentage of the 11 preparations that produced active responses when bathed in saline (54.5%) compared with DF_2 (72.7%) (Table 1).

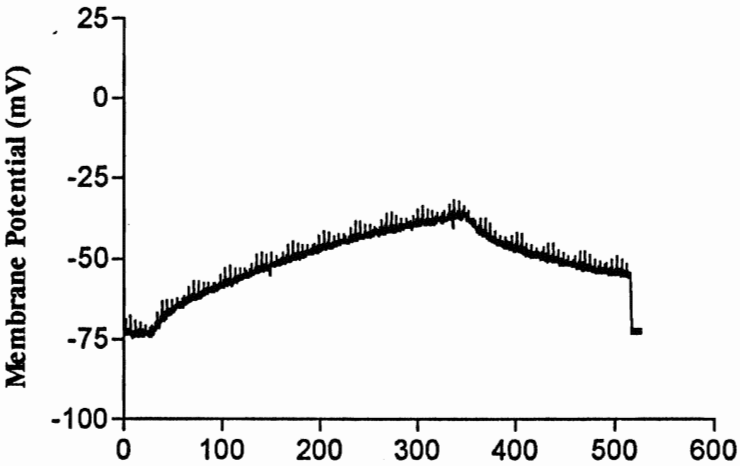
The active responses appeared to be TTX-resistant but depended at least in part on extracellular Ca^{2+}. Figure 18 shows active responses recorded from a single muscle fiber. The addition of 10 mM $MnCl_2$ completely abolished the active response (Fig.18B) in 1 out of 10 trials. In 6 out of the 10 trials the active response were reduced by 10 mM $MnCl_2$. In one trial 50 mM $MnCl_2$ was applied, and it completely blocked the active response that had remained in the presence of 10 mM $MnCl_2$. The blocking effect of Mn^{2+}, when it occurred, was reversible

Figure 18. Voltage recordings from a single muscle fiber injected with depolarizing current. A. Preparation bathed in 10^{-7} M TTX for 10 minutes. B. Recording following 10 minute exposure to 10 mM Mn^{2+}. C. Washout in normal saline.

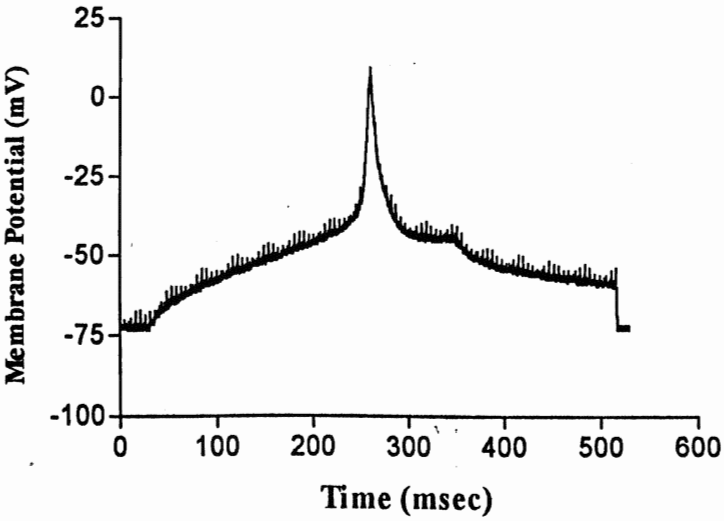
A. Saline and 10^{-7} M TTX



B. 10 mM Manganese



C. Saline



(Fig.18C). In the last two trials there was no change in the active response even in the presence of Mn^{2+}. This suggests that the active responses require external Ca^{2+} and another cation (possibly Na^{+}).

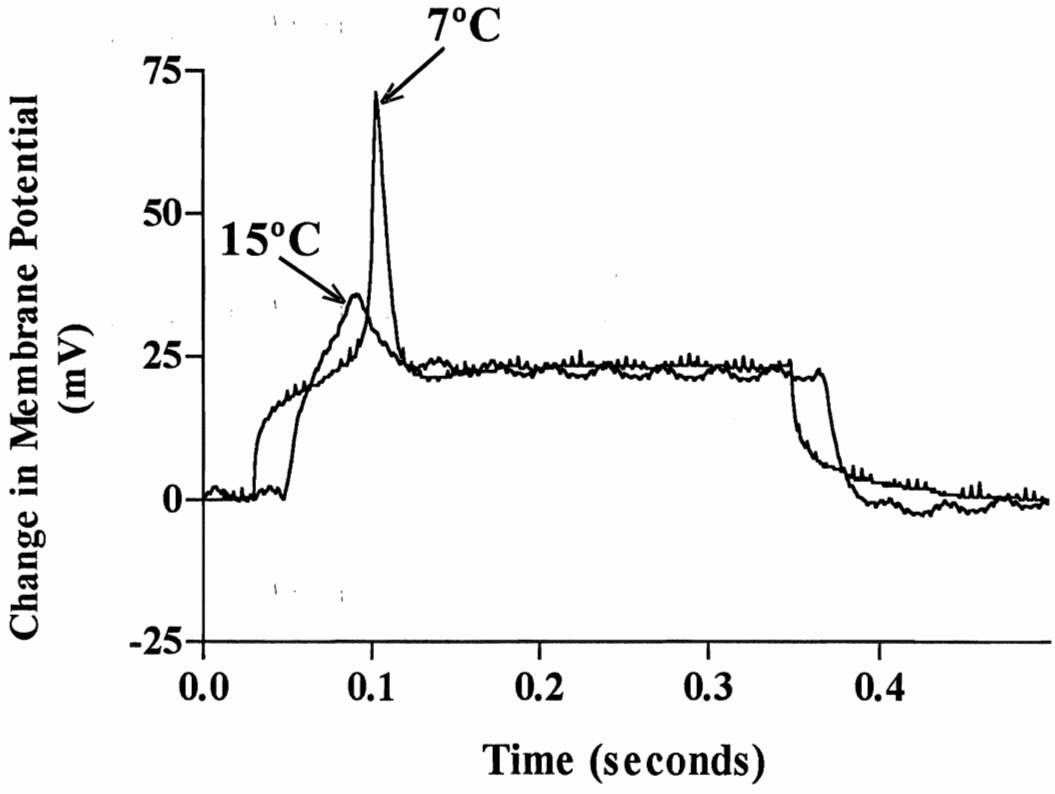
The active responses appeared to become larger in amplitude over long recording periods. Individual fibers were penetrated, and the initial responses were compared with responses obtained after the electrodes had been in the cell for 45 minutes. The distribution of the amplitudes increased from time 0 (22.6 ± 17.3 mV) to time 45 (38.7 ± 18.6 mV). This was found to be a significant difference using a Wilcoxon signed-rank test for matched pairs $p<0.005$. To determine whether the increases in amplitude were due to an effect of temperature (rather than other factors, such as cell damage), active responses recorded at 7°C were compared with active responses obtained at 15°C. The distributions of the amplitudes of the active responses was significantly higher at 7°C (-28.3 ± 5.0 mV) than at 15°C (5.1 ± 1.3 mV; Mann-Whitney U, $p<0.01$) (Table 2). The active responses also appeared to be of shorter duration (Fig. 19) at 7°C (12.4 ± 7.3 msec) than at 15°C (35.0 ± 28.6 msec) but, there was no significant difference between the distributions of the durations taken at half-amplitude. There were also no significant differences in the distributions of the resting membrane potentials, input resistances or the active response thresholds at 7°C and 15°C, respectively (Table 2).

Of 11 preparations studied at 7°C, 90.9% produced active responses. This is significantly greater than the 37.5% of 8 preparations that produced active responses at 15°C (Chi squared, $p<0.01$; Table 2). This indicates that the membrane is more excitable at the lower temperature, even though the membrane potential, input resistances and threshold voltage of the active responses are not significantly different at the two temperatures.

Table 2. Properties of muscle fibers at 7° C and 15° C. Values are means ± SEM. The double asterisks indicate the properties that are significantly different ($p<0.01$) at the 2 temperatures.

	V_m (mV)	R_{input} (Ω)	Active Responses Amplitude (mV)	Active Responses Threshold (mV)	Preparations with Active Responses (%)
7°C	**-69.4** ±7.4 (n=12)	**289** ±225 (n=11)	**28.3** ±5.0 (n=10) **	**-38.8** ±4.6 (n=8)	**90.9** (n=11) **
15°C	**-72.6** ±7.0 (n=8)	**282** ±127 (n=8)	**5.1** ±1.3 (n=3)	**-39.5** ±6.5 (n=3)	**37.5** (n=8)

Figure 19. Voltage recordings from muscle fibers injected with depolarizing current at two different temperatures, as indicated by the arrows.


7°C
15°C
75
50
25
0
-25
0.0
0.1
0.2
0.3
0.4
Change in Membrane Potential (mV)
Time (seconds)

4. Discussion

4.1 DF_2 as a Modulator of Muscle Contraction

DF_2, which was isolated from crayfish pericardial organs, has been shown to increase transmitter release from neurons innervating the deep extensor muscles (Skerrett et al., 1995). In order to examine possible postsynaptic effects of DF_2, an appropriate preparation was required that could be tested without any neural stimulation and that would not contract vigorously when the fibers are stimulated. Three groups of muscles from the crayfish abdomen were tested to determine whether or not DF_2 would increase muscle tonus without any neural stimulation: the superficial flexors, deep extensors and superficial extensors. Of these, the superficial extensors were the only muscle group to respond to the peptide by showing an increase in tonus. This increase was dose-dependent, and all three of the superficial extensor muscles in each hemi-segment responded similarly. All showed an increase in tonus at the same threshold and over the same concentration range. The amount by which tonus increased was also similar in all three muscles. This indicates that at least some of the fibers from all three superficial extensor muscles possess receptors for the peptide. The absence of such tonus changes in the deep extensors and superficial flexors does not mean that such muscles lack FaRP receptors. Modulatory effects other than tonus changes are possible. For example, lobster peptides F_1 and F_2 are reported to enhance nerve-evoked contractions of crayfish deep extensor muscles (Mercier et al., 1990), but it is not clear whether this effect reflects modulation of EJP amplitude or of excitation-contraction coupling.

If an increase in spontaneous transmitter release had occurred when the peptide was applied, a depolarization and a decrease in input resistance would have been expected. Since there was no change in either the membrane potential or the input resistance, the effects on

contraction cannot be attributed to an increase in spontaneous transmitter release. The peptide's effect is not blocked by TTX. Therefore it is not associated with any spontaneous electrical activity in the severed axons. Instead, the peptide's effects are postsynaptic.

FaRPs have been shown to modulate synaptic transmission and contraction in neuromuscular systems of locusts (Evans and Myers, 1986), leech (Li and Calabrese, 1992), crayfish (Mercier et al., 1990; Skerrett et al., 1995), and lobster (Worden et al., 1995). In several cases, postsynaptic effects of FaRPs have been clearly demonstrated, and the present experiments with the superficial extensor muscles from the crayfish abdomen are another example of this. FaRPs produce a slow contracture in other invertebrate muscles including the locust extensor tibiae (Evans and Myers, 1986), *Helix* tentacle retractor muscle (Cottrell et al., 1983), leech hearts (Li and Calabrese, 1992), lobster dactyl opener muscle (Worden et al., 1995) and the anterior byssus retractor muscle bivalve mollusc *Mytilus edulis* (Muneoka and Matsuura, 1985).

Some neuromodulators have been reported to cause a change in membrane potential or input resistance while inducing tonus. These include serotonin in lobster opener muscle (Kravitz et al., 1980), and F_1 in shrimp pyloric dilator muscle (Meyrand and Marder, 1991). In other cases, neuromodulators induce contracture in invertebrate muscles with little or no change in membrane potential or input resistance. Examples include the effects of octopamine and proctolin on lobster opener muscle (Kravitz et al., 1980) and crayfish opener muscle (Fischer and Florey, 1983), the effect of proctolin on the superficial flexor muscles of the crayfish abdomen and the effect of dopamine on *Limulus* cardiac muscle (Watson et al., 1985). The mechanisms for such effects on contraction are unknown, but others have suggested the direct modulation of the excitation-contraction coupling process (Fischer and Florey, 1983), or inhibition of the Na^+/Ca^{2+} exchanger (Khananshvili et al., 1993).

In the crayfish opener muscle, serotonin and octopamine do not induce contracture but, both amines do increase the amplitude of EJP's evoked by nerve stimulation and nerve-evoked contractions (Fischer and Florey, 1983). Contractions induced by direct stimulation of muscle fibers with depolarizing current could be modulated by serotonin and octopamine. The amines increased the amount of contraction induced by a given depolarization, indicating a direct effect on excitation-contraction coupling (Fischer and Florey, 1983). The cotransmitter proctolin also potentiates tension induced by direct depolarization in crayfish superficial flexor muscle without affecting tension or membrane potential on its own (Bishop et al., 1987). Instead proctolin affects excitation-contraction coupling by sustaining the activity of two voltage-dependent Ca^{2+} channels following depolarization generated by the conventional transmitter (Bishop et al., 1991).

A DF_2-induced shift in the excitation-contraction coupling threshold towards the resting potential might account for the increases in tonus observed in the present study. The excitation-contraction coupling threshold in crayfish opener muscle did not change in serotonin or octopamine (Fischer and Florey, 1983).

4.2 Dependence on External Calcium

To examine the mechanisms underlying the peptide's ability to increase muscle tonus, several Ca^{2+}-channel blockers were employed. The response to DF_2 is very similar to responses elicited by other neurohormones, where the peptide-induced tonus increase was inhibited by 5 mM Mn^{2+} or Co^{2+} (Kravitz et al., 1980). Octopamine, serotonin and proctolin induce sustained contracture in fibers of the lobster opener muscle (Kravitz et al., 1980) and proctolin has the same effect on locust oviduct (Lange et al., 1987). Since the effects of these neuromodulators are antagonized by Ca^{2+}-channel blockers it has been suggested that they require the influx of

extracellular Ca^{2+} (Kravitz et al., 1980; Lange et al., 1987). The involvement of calcium influx in the present work is also suggested by the observation that the effect of DF_2 on tonus is antagonized by reducing the extracellular calcium concentration (ie. by using "calcium free" saline with or without EGTA).

The role played by extracellular Ca^{2+} is not clear. One possibility is that the influx of Ca^{2+} is sufficient to induce contractions. Alternatively, Ca^{2+} influx may trigger Ca^{2+}-induced-Ca^{2+}-release from internal stores, similar to the Ca^{2+} waves produced after fertilization of sea urchin eggs (Galione et al., 1993).

4.3 Voltage-Gated Ca^{2+} Channels

It has been suggested that serotonin, octopamine and proctolin activate voltage-sensitive Ca^{2+} entry at the resting level of membrane potential and thereby induce contracture with little or no change in membrane potential (Kravitz et al., 1980). The contractures observed might be accounted for by a very small entry of Ca^{2+} through a hormone-induced change in a population of voltage-sensitive channels that are ordinarily closed at rest.

In an attempt to further characterize the type of Ca^{2+} channels required for the peptide's effect, several voltage-gated Ca^{2+} channel blockers were used. It was thought that DF_2 may increase tonus by increasing the influx of Ca^{2+} through certain voltage-gated channels. There is evidence that FLRFamide shifts the voltage-dependence of N-type Ca^{2+} channels in neurons towards lower voltages, thereby making it easier for such channels to open (Baux et al., 1992). L-type Ca^{2+} channels are known to be present in crayfish muscles and can be inhibited by 10^{-6} M nifedipine (Araque et al., 1994). Neither the N-type blocker (ω-conotoxin) nor L-type Ca^{2+} channel blockers (nifedipine, (+) Bay K 8644, and verapamil) reduced the peptide's effect on

tonus. These results suggest that these voltage-gated Ca^{2+} channels are not employed by DF_2 to increase muscle tonus. However, it is possible that the N-type blocker, ω-conotoxin, which has been shown to work in other organisms, is less effective in arthropods due to differences in the Ca^{2+} channels (Gielow et al., 1995; Hille, 1992). In addition, the 1,4-dihydropyridines block channels in their inactive state, after they have been opened by depolarization (Hille, 1992). Since DF_2 does not cause depolarization, these blockers may be ineffective at inhibiting such channels.

One particularly interesting yet unexpected result occurred with the L-type Ca^{2+} channel blocker, verapamil. This blocker caused a large increase in tonus when applied alone or in the presence of the peptide. Even though 10^{-4} M verapamil has been shown to block Ca^{2+} channels in *Drosophila* (Gielow et al., 1995) and proctolin-induced contractions in locust oviduct (Lange et al., 1987), it may have other non-specific effects in crustacean muscle fibers. The increase in tonus in the superficial extensors bathed in 10^{-4} M verapamil with Ca^{2+}-free saline buffered with EGTA indicate that verapamil is not causing an influx of external Ca^{2+}, but it may release Ca^{2+} from internal stores.

The mechanism through which DF_2 increases tonus does not depend on depolarization. If Ca^{2+} influx does occur, as suggested by the dependence on external Ca^{2+}, it is likely that the influx would involve a non-voltage activated channel such as a receptor operated channel. If DF_2 increases tonus by opening Ca^{2+} channels at the plasma membrane, one might expect the peptide to increase membrane conductance and, thus, decrease input resistance. No such change in input resistance was observed. It is possible that Ca^{2+} channels were being opened by DF_2 but the effect was masked by the opening of other channels (ie. K^+ channels) at the same time, or that the increase in Ca^{2+} conductance is small compared to the resting conductance.

One technical difficulty in these experiments was movement of muscle fibers during

intracellular recording. Worden et al. (1995) reported the same problem when recording from the lobster opener muscle in the presence of lobster peptide F_1. If muscle fibers were damaged by such movement, such damage would be expected to produce depolarization and a drop in input resistance. The inability of the peptide to depolarize the cells or to lower input resistance, therefore, cannot be attributed to muscle damage.

As an alternative to Ca^{2+} channel opening, it is also possible that the peptide may act by inhibiting the Na^{+}/Ca^{2+} exchange at the sarcolemma. FaRPs (including FLRFamide, FIRFamide and FNRFamide) have been shown to inhibit Na^{+}/Ca^{2+} exchange in sarcolemmal vesicles from vertebrate cardiac muscle (Khananshvili et al., 1993). Should this pump be inhibited in the crayfish skeletal muscles, an intracellular build up of Ca^{2+} would occur which would lead to an increase in contraction without changing the membrane potential.

4.4 Intracellular Signalling

The long latency of DF_2's effect on the superficial extensors (usually several minutes) and the persistence of the effect suggest the involvement of a second messenger system. It is not known yet which second messengers system function in this case. The proctolin-enhanced myogenic rhythm in the locust extensor tibiae and dopamine's effect on *Limulus* cardiac muscle are mediated by cAMP (Evans, 1984; Groome and Watson, 1989), as is proctolin's modulation of Ca^{2+} channels in the crayfish superficial flexor muscle which leads to an increase in tension (Bishop et al., 1991).

According to Baux et al. (1992), in most preparations neurotransmitter-induced modulation of Ca^{2+} currents involves the transducing role of G-proteins. Their studies have led to a model for modulation of transmitter release in molluscan neurons by FMRFamide. In that case,

a receptor to FMRFamide is thought to be associated with a G-protein, which stimulates the hydrolysis of membrane phospholipids via phospholipase C to produce diacylglycerol and IP_3. IP_3 liberates Ca^{2+} from internal stores and, along with diacylglycerol, activates protein kinase C which, in turn, increases influx through Ca^{2+} channels (Baux et al., 1992).

4.5 Spontaneous Contractions

The superficial extensors exhibited spontaneous contractions similar to those observed in locust extensor-tibia muscle and locust oviduct (Hoyle, 1978; Lange et al., 1984). Sometimes these contractions were rhythmic and at other times they were irregular. The TTX-insensitivity of the contractions indicates that they do not depend on presynaptic action potentials and that they do not require a TTX-sensitive Na^+ current. Hoyle (1978) surveyed several tonic skeletal muscles from the locusts, *Schistocerca* and *Locusta*, and found that only the extensor tibia produced such contractions. In the present study the superficial flexor muscles of the crayfish abdomen, which are tonic muscles, did not exhibit spontaneous contractions. Since only three muscles were examined in the present study, it is not known whether other tonic muscles exhibit similar contractions. There is no obvious function for such spontaneous contractions in tonic muscles. It has been suggested that they may increase the flow of haemolymph (Usherwood cited in Hoyle, 1978).

A variety of experimental conditions have been reported to stimulate myogenic contractions in arthropod skeletal muscle. Spontaneous contractions in the locust extensor tibiae muscle could be reliably obtained by adding acetylcholine and eserine to insect physiological saline, by bathing the muscle in haemolymph or by chronic denervation (Hoyle, 1978). 'Fibrillations' in tension were induced in Hawaiian ghost crab closer muscles when the muscle was

rapidly cooled below 18°C (Florey and Hoyle, 1976). The myogenic contractions in the crayfish superficial extensors were reliably obtained at temperatures below 13°C. Thus, temperature is only one of several factors that can promote the appearance of myogenic contractions.

In the extensor tibiae of locusts, depolarizations corresponding to the oscillations in tension were not found in every fiber of the muscles (Hoyle, 1978). Only a relatively small number of fibers located near the end of the muscle were involved in generating the tension and showed depolarizing waves varying in amplitude from the barely detectable to 24 mV. The large waves were presumably recorded from pacemaker fibers, and the smaller waves were from fibers weakly electrically coupled to them. It is possible that tonic muscles produce spontaneous, rhythmic, myogenic contractions that are partially synchronized by electrical coupling between some of the fibers. This is an unlikely explanation for the contractions in the superficial extensors since they are not electrically coupled (Parnas and Atwood, 1966). Since recordings were not made systematically from all regions of the superficial extensor muscle, it is possible that fibers showing oscillations in membrane potential could have been missed. It is also possible that rhythmic contractions might have been restricted to a subset of fibers, but this was not examined.

The present study examined the possibility that low temperature induces myogenic contractions by inhibition of the Na^+/K^+ pump. If this pump's activity is reduced, a build up of intracellular Na^+ would be predicted. This, in turn, would inhibit the Na^+/Ca^{2+} exchange (Atwood et al., 1983; Pannabecker and Orchard, 1988) and might increase intracellular Ca^{2+} to a level which could cause oscillatory release of Ca^{2+} from internal stores and rhythmic contractions. However, addition of the Na^+/K^+ pump inhibitor strophanthidin did not induce rhythmic contractions at high temperatures in preparations which had exhibited such contractions at lower temperatures. This indicates that the rhythmic contractions are not caused by the temperature-

dependent inhibition of the Na^+ pump.

4.6 Effects of DF_2 on Myogenic Contractions

Some muscles display conditional regenerative properties because modulatory substances such as amines and peptides can elicit myogenic activity (Lingle, 1981; Meyrand and Marder, 1991). FaRP's can induce myogenic activity in *Helix* tentacle retractor muscle (Cottrell et al., 1983) and leech hearts (Li and Calabrese, 1992), but have not been shown to do so in locust skeletal muscle (Evans and Myers, 1986). In shrimp pyloric dilator muscle FaRP's evoke rhythmic depolarizations and contractions, followed by a state in which the muscle does not spontaneously contract but is close to threshold for the generation of rhythmic activity (Meyrand and Marder, 1991). DF_2 only occasionally induced contractions in the crayfish superficial extensor muscles (7/47 preparations). This might reflect the fact that the superficial extensor muscles are tonically active and do not normally display rhythmic contractions.

4.7 Effects of Temperature on Membrane Properties

In other crustacean muscles (Hawaiian ghost crab and crayfish closer muscles), the input resistance increases and the membrane potential depolarizes with decreasing temperature (Florey and Hoyle, 1976; Harri and Florey, 1977; Adams, 1987). Based on such reports, one would expect that in the present study input resistance would be higher at the lower temperature. Since the muscles were contracting at low temperature, it is possible that fiber damage could have reduced input resistance. The muscle cells typically appeared white and damaged by the end of the experiments. Thus, the input resistance may be underestimated.

4.8 Active Responses

Tonic muscle fibers of crustaceans generate graded electrical responses but rarely produce active responses (Wiersma, 1961). Active responses have been found in some fibers of the extensor muscles of the crayfish carpopodite (Ozeki, 1969), crab stretcher muscle fibers (Atwood et al., 1965), and tonic flexor fibers of the crayfish abdomen (Lehouelleur et al., 1983). In the present study, active responses were elicited in the superficial extensor muscle of the crayfish abdomen when the fibers were injected with depolarizing current. These active responses are largely Ca^{2+}-dependent but may also have a minor contribution from Na^{+} channels.

Different forms of electrical responsiveness to direct stimulation are found in different fibers of the crab stretcher muscle fibers recorded at 12°C including: all-or-nothing responses, graded responses and passive responses (Atwood et al., 1965). All-or-nothing responses are distinguished by a sharp threshold for the production of large spikes. Graded responses, which occur most frequently, can be anything from small oscillatory responses to large, variable spike-like responses. Passive responses show a rapid, brief depolarization followed by marked delayed rectification. All these response types were observed in the crayfish superficial extensor muscles, but most of the active responses analysed were all-or-nothing responses.

The appearance of Ca^{2+}-dependent active responses so reliably in the superficial extensor muscles contrasts with early work by Parnas and Atwood (1966), who reported only graded responses in these muscles in *Procambarus* and *Panulirus*. Small responses resembling graded responses were observed in the present study in muscles held at 15°C. The ability to induce large Ca^{2+} action potentials appears to be a direct result of decreasing the temperature to 7°C. Thus, Ca^{2+} action potentials, thought to be rare in crustacean tonic muscles can be induced reliably by lowering temperature. The effect of decreasing temperature is to increase the size of the voltage

response, rather than to decrease the threshold.

Lehouelleur et al. (1983) reported the presence of Ca^{2+} spikes in tonic flexor fibers from the crayfish *P. clarkii*. After chronic axonotomy (several weeks) half the fibers produced action potentials caused by Na^{+} channels, suggesting that these channels were somehow activated by the procedure. The muscles used in this experiment did not undergo chronic axonotomy yet produced responses that could not be completely blocked by Mn^{2+}. The appearance of Na^{+} active responses might depend on the location of the muscle fibers or on the part of the fiber from which recordings were made. These factors were not kept constant in the present experiments.

In other crustacean muscle the input resistance increases and the membrane potential depolarizes with decreasing temperature (Adams, 1987). This makes the muscle more excitable as the temperature is decreased so that it requires smaller depolarizations to reach threshold for an active response. As the input resistance increases, the depolarization in response to a stimulus will increase as well (Adams, 1987). Normally, at neuromuscular junctions the increased electrical excitability of the muscle compensates for the reduced amount of transmitter released at lower temperatures (Florey and Hoyle, 1976; Harri and Florey, 1977; Stephens, 1990). As temperature decreases, the time constant (τ) increases (Hagiwara et al., 1968). Since τ is equal to the product of resistance and capacitance, the lengthening of τ with decreasing temperature must be entirely due to the increase in resistance, because capacitance is either constant or decreases with decreasing temperature; decreasing capacitance would decrease, not increase τ (Adams, 1987).

In the present experiments a greater percentage of the fibers produced active responses at lower temperatures indicating that the membrane excitability has increased. Strangely, there was no change in membrane potential, input resistance or threshold of the active responses. The

amplitude of the active responses is also greater at low temperature. It has been suggested that the range of electrical responses encountered in crustaceans can be accounted for by the variable activity of a voltage-dependent K^+ conductance which shunts to different degrees the inward Ca^{2+} current (Moody, 1978). It is possible that a voltage-dependent K^+ channel is inhibited at low temperature increasing the amplitude of the active response. Alternatively, more Ca^{2+} channels could be opening in response to depolarization at the low temperature.

Octopamine, serotonin and proctolin have been reported to induce voltage-dependent active responses in lobster opener muscle (Kravitz et al., 1980) and proctolin has a similar effect in *Idotea* abdominal extensor muscle (Erxleben et al., 1995). DF_2 caused the threshold for the active response to be lowered, indicating that the peptide increases the muscle fiber excitability. The size of the response was unaltered. The changes in excitability are not accompanied by any change in fiber resting potential or input resistance.

The lowered threshold for active response suggests that DF_2 lowers the threshold for activating voltage-gated Ca^{2+} channels. It is unlikely that the active responses contribute to the effects of DF_2 on tonus or spontaneous contractions since the active responses were only seen when the fibers were depolarized by approximately 13 mV. Also DF_2 did not affect the passive electrical properties of the muscle fibers; neither membrane potential nor input resistance was significantly altered by DF_2.

5. Conclusions

1. The superficial extensors exhibit myogenic contractions which are temperature-dependent.

2. Neuropeptide DF_2 increases tonus in the superficial extensor muscles but not in the deep extensors or the superficial flexors.

3. The spontaneous contractions, tonus and the effect of DF_2 are blocked by Ca^{2+}-channel blockers. These effects might depend on Ca^{2+} influx through Ca^{2+} channels located in the plasma membrane. However, the type of Ca^{2+} channel has yet to be determined.

4. Decreasing temperature causes the appearance of large voltage-activated Ca^{2+}-dependent active responses in 90.9% of muscle fibers. This effect involves both an increase in percentage of cells which generate membrane responses and an increase in amplitude of the membrane responses.

5. At 7°C, DF_2 lowers the threshold for active responses.

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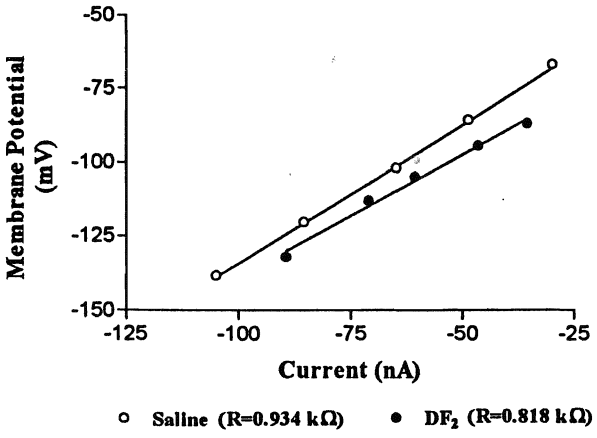
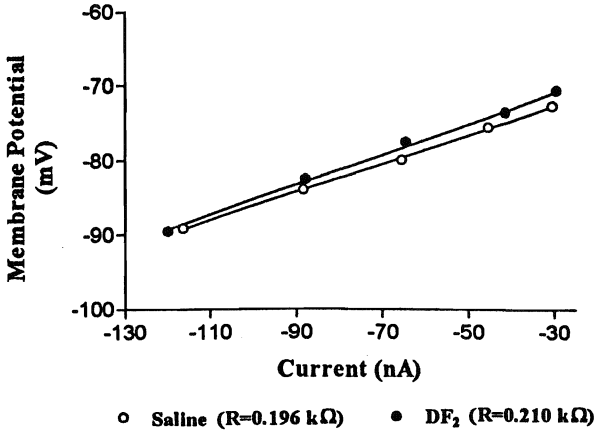
#2



○ Saline (R=0.934 kΩ) ● DF_2 (R=0.818 kΩ)

#3



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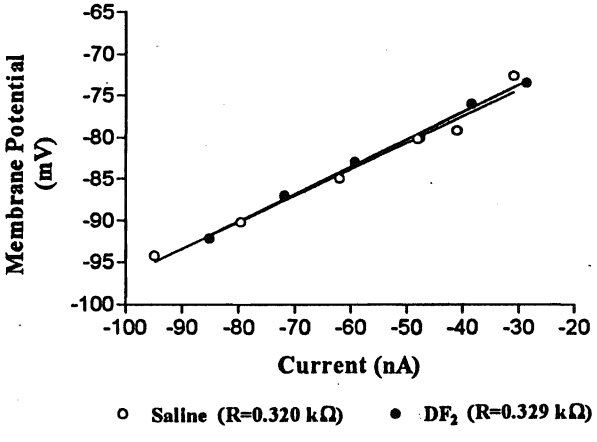

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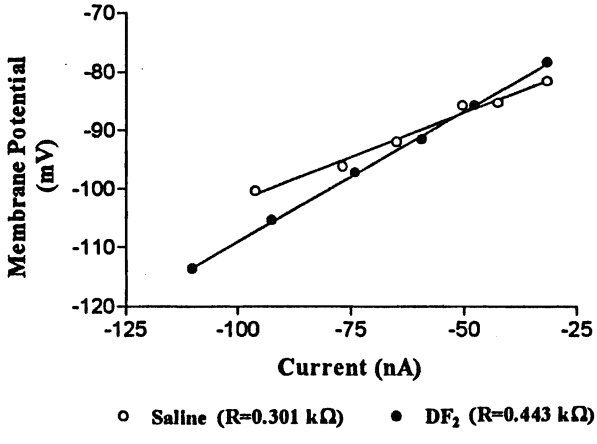
#5



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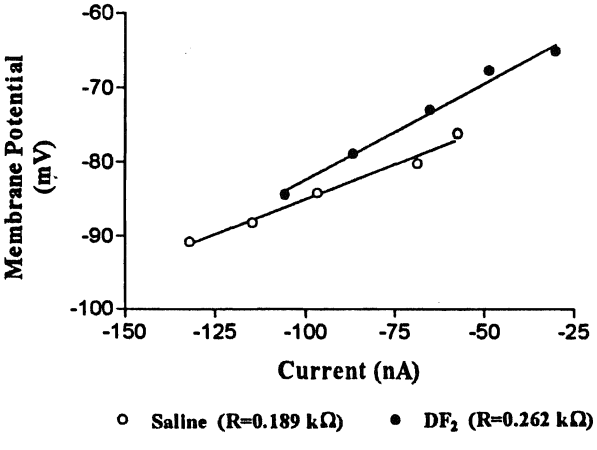
#6



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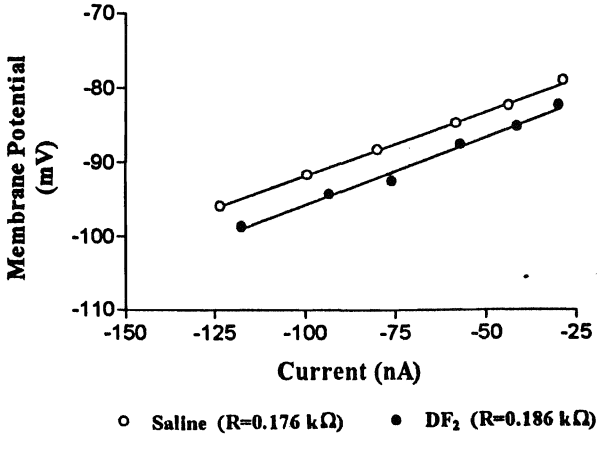
#7



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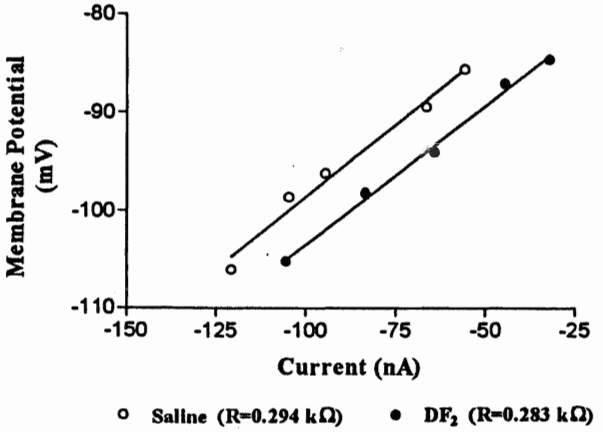
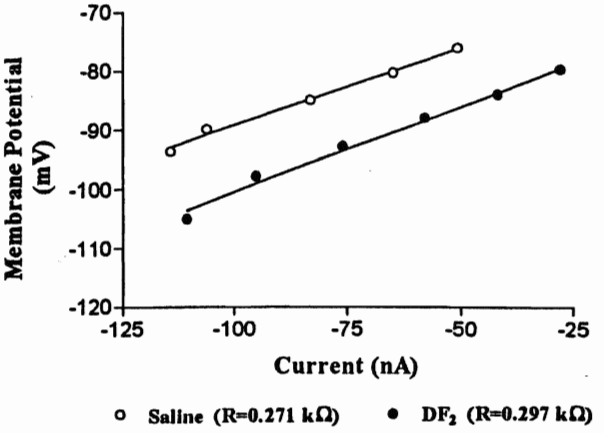
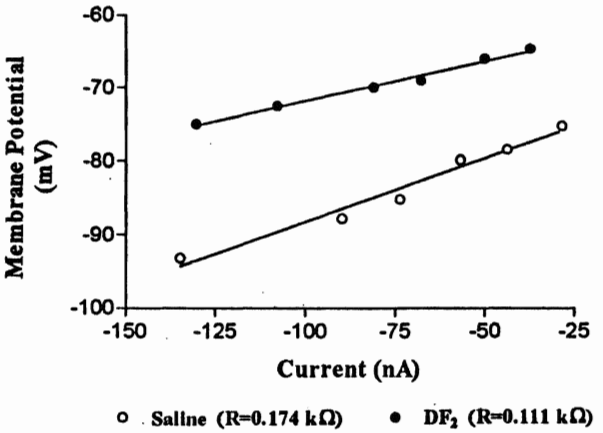
#8


Membrane Potential (mV)
-80
-90
-100
-110
-150
-125
-100
-75
-50
-25
Current (nA)
Saline (R=0.294 kΩ)
DF2 (R=0.283 kΩ)

#9


Membrane Potential (mV)
-70
-80
-90
-100
-110
-120
-125
-100
-75
-50
-25
Current (nA)
Saline (R=0.271 kΩ)
DF2 (R=0.297 kΩ)

#10


Membrane Potential (mV)
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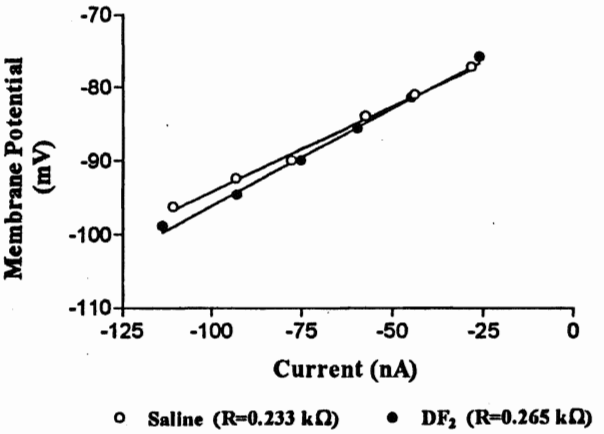
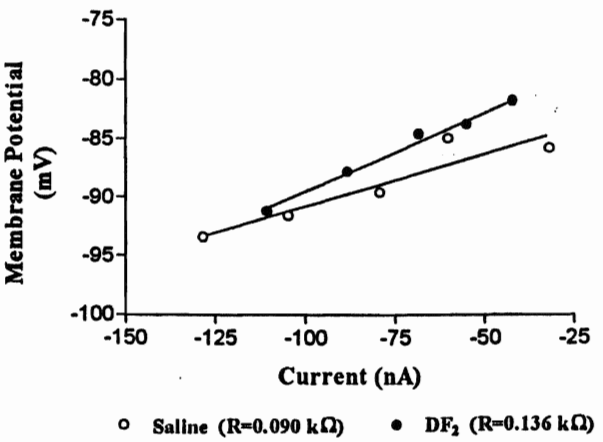
-70
-80
-90
-100
-150
-125
-100
-75
-50
-25
Current (nA)
Saline (R=0.174 kΩ)
DF2 (R=0.111 kΩ)

#11


Membrane Potential (mV)
-70
-80
-90
-100
-110
-125
-100
-75
-50
-25
0
Current (nA)
Saline (R=0.233 kΩ)
DF2 (R=0.265 kΩ)

#12


Membrane Potential (mV)
-75
-80
-85
-90
-95
-100
-150
-125
-100
-75
-50
-25
Current (nA)
Saline (R=0.090 kΩ)
DF2 (R=0.136 kΩ)