Peptide and amine actions on the neurogenic Limulus heart: Biochemical mechanisms of modulation

James Richard Groome

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Peptide and amine actions on the neurogenic Limulus heart: Biochemical mechanisms of modulation

Abstract
The biochemical basis underlying the chronotropic and inotropic effects of peptides and amines on the neurogenic heart of the horseshoe crab, Limulus polyphemus, was investigated. This study focused on the role of cyclic nucleotide and phosphatidylinositol metabolism in specific actions of amines and peptides endogenous to the Limulus nervous system. Biochemical and electrophysiological analyses of amine and peptide actions on specific cellular targets within the neurogenic heart network were performed to characterize the intracellular mechanisms responsible for the excitatory effects of these neuromodulators.

Octopamine and the catecholamines dopamine, norepinephrine and epinephrine utilize the second messenger cyclic AMP at multiple cellular sites to increase the rate and strength of heart contractions. These amines increase burst rate in the cardiac ganglion by a cAMP-dependent mechanism. Amines also increase cardiac muscle contractility and enhance cardiac neuromuscular transmission by a cAMP-dependent process. Cyclic GMP does not appear to be involved in any of the excitatory actions of amines, although it may play a role in cardiac inhibition.

Several proctolin-like and FMRFamide-like peptides are widely distributed in the Limulus CNS and may play a role in the regulation of cardiac output. The peptide proctolin utilizes the phosphatidylinositol second messenger system in its actions on cardiac muscle contractility and excitability. Dopamine may also activate this system, as this amine has several proctolin-like actions on Limulus cardiac muscle. Finally, the FMRFamide-like cardioexcitatory peptide limadrin, like the amines, appears to produce excitation of heart rate by increasing levels of cAMP in the Limulus cardiac ganglion. These results indicate that peptide and amine neuromodulators share several second messenger systems to produce their characteristic responses on the Limulus heartbeat.

Keywords
Biology, Neuroscience, Chemistry, Biochemistry

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Peptide and amine actions on the neurogenic *Limulus* heart: 
Biochemical mechanisms of modulation

Groome, James Richard, Ph.D.

University of New Hampshire, 1988
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PEPTIDE AND AMINE ACTIONS ON THE NEUROGENIC LIMULUS HEART:
BIOCHEMICAL MECHANISMS OF MODULATION

BY

JAMES R. GROOME

B. A. Wake Forest University, 1981

DISSEPTION

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Doctor of Philosophy

in

Zoology

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5/5/88
Date
This dissertation is dedicated to the memory of Tom O'Donohue.
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ABSTRACT

PEPTIDE AND AMINE ACTIONS ON THE NEUROGENIC LIMULUS HEART: BIOCHEMICAL MECHANISMS OF MODULATION

by

James R. Groome

University of New Hampshire, May, 1988

The biochemical basis underlying the chronotropic and inotropic effects of peptides and amines on the neurogenic heart of the horseshoe crab, Limulus polyphemus, was investigated. This study focused on the role of cyclic nucleotide and phosphatidylinositol metabolism in specific actions of amines and peptides endogenous to the Limulus nervous system. Biochemical and electrophysiological analyses of amine and peptide actions on specific cellular targets within the neurogenic heart network were performed to characterize the intracellular mechanisms responsible for the excitatory effects of these neuromodulators.

Octopamine and the catecholamines dopamine, norepinephrine and epinephrine utilize the second messenger cyclic AMP at multiple cellular sites to increase the rate and strength of heart contractions. These amines increase burst rate in the cardiac ganglion by a cAMP-dependent mechanism. Amines also increase cardiac muscle contractility and enhance cardiac neuromuscular transmission by a cAMP-dependent process. Cyclic GMP does not appear to be involved in any of the excitatory actions of amines, although it may play a role in cardiac inhibition.
Several proctolin-like and FMRFamide-like peptides are widely distributed in the *Limulus* CNS and may play a role in the regulation of cardiac output. The peptide proctolin utilizes the phosphatidylinositol second messenger system in its actions on cardiac muscle contractility and excitability. Dopamine may also activate this system, as this amine has several proctolin-like actions on *Limulus* cardiac muscle. Finally, the FMRFamide-like cardioexcitatory peptide limadrin, like the amines, appears to produce excitation of heart rate by increasing levels of cAMP in the *Limulus* cardiac ganglion. These results indicate that peptide and amine neuromodulators share several second messenger systems to produce their characteristic responses on the *Limulus* heartbeat.
INTRODUCTION

i. NEUROMODULATION

Animals possess neuronal circuitries which elicit particular responses in effector tissues under appropriate conditions. However, a nervous system which by necessity employed a separate neural circuit for every conceivable event, be it physiological or behavioral, would be needlessly complex and thus inefficient. Instead, these organisms produce subtle alterations in existing circuitries to cope with a continually changing environment and an equally dynamic set of internal physiological processes.

Modification of the basic properties of neurons is a process termed neuromodulation, and occurs as a consequence of hormonal and synaptic input. These neuromodulatory events are as crucial to the expression of behavior as is the existing circuitry itself (Kaczmarek and Levitan, 1987).

a. Neurohormonal communication

Neurosecretion of peptide and amine hormones is in many ways analogous to synaptic transmission. In both cases, a substance is released from a nerve terminal upon depolarization and binds to a specific receptor on a target cell membrane in order to elicit a particular response. However, neurohormones are released not into the synaptic cleft but into the circulatory system. In this respect, neurosecretory cells act much like endocrine cells. Indeed, the endocrine and nervous systems are closely
related anatomically, functionally and even phylogenetically (reviewed by Roith et al., 1982 and Krieger and Liotta, 1979).

Neurohormones may travel long distances in the circulatory system to act on specific target tissues, or they may act on local targets not readily accessible to the circulation (Branton et al., 1978). Local release and action may prevent inadvertent stimulation of inappropriate tissues. Neurohormones released near their site of action may also facilitate the attainment of appropriate hormonal titer to overcome the effects of enzymes which quickly degrade hormones in the general circulation (Quistad et al., 1984; Previero et al., 1985).

Neurohormones and neurotransmitters influence ionic conductances in cell membranes. Many transmitters open or close ion channels for only the short period of receptor binding. Neurohormones, and some neurotransmitters, induce long-term conductance alteration by initiating intracellular biochemical events (usually enzyme activation and substrate phosphorylation) which persist after the termination of receptor-ligand interaction (reviewed by Greengard, 1976; Cohen, 1982; Levitan et al., 1983a). These neuroregulators provide organisms with a means of communication appropriate for both short- and long-term events.

b. Neuromodulatory actions of amines and peptides

Amines and peptides have powerful influences on physiological and behavioral processes in animals. Investigations into amine and peptide actions in both invertebrate and vertebrate systems have unequivocally demonstrated the importance of these compounds in physiological regulation (Barchas et al., 1979).
Many types of physiological and behavioral phenomena are modulated by peptide and amine neurohormones and neurotransmitters. Peptides and amines may influence an organism's responsiveness to environmental stimuli, or modify efferent processes, by specific actions within the central nervous system and on peripheral targets. In addition, neuronal events crucial in the phenomena of learning and memory are also subject to peptidergic and aminergic influences.

Sensory processes in animals may be influenced by release of a particular neurohormone or transmitter. For example, photosensitivity of the lateral eye in the horseshoe crab, Limulus, is regulated by the activity of a circadian clock in the brain which controls efferent optic nerve activity (Barlow et al., 1980). Octopamine is present in and released from efferent optic nerves (Battelle et al., 1982). These workers have suggested that this amine modulates photoreceptor sensitivity, a critical physiological process in nocturnal behavior.

In many animals, simple behaviors are controlled by central pattern generators (CPG's) whose basic output is independent of sensory information. For example, locomotion in several species of lampreys is controlled by a CPG (Harris-Warrick and Cohen, 1985). The characteristic lamprey swimming program is induced by the classical transmitter glutamate. Serotonin, which has no effect on the swim CPG alone, modulates several parameters of the glutamate-induced motor program.

Investigations into the cellular basis of neuronal plasticity have focused on activity-dependent modulation of synapses. Synaptic efficacy in a variety of invertebrate and vertebrate systems may be modulated by the amount of stimulation a synapse receives (Bliss and Lomo, 1973; Weight and Erulkar, 1976; Hawkins et al., 1983; McNaughton, 1983). Thus, a
given stimulus may produce a strong postsynaptic response after repetitive input, even though the same stimulus was ineffective under conditions of infrequent input. As detailed in the next section, neurohormones have pronounced influences on synaptic efficacy in both vertebrate and invertebrate organisms.

Amines

Biogenic amines are widely distributed in the nervous systems of both vertebrate (Peroutka et al., 1981; Seeman and Grigoriadis, 1987) and invertebrate (Walker and Kerkut, 1978; Laxmyr, 1984; Orchard et al., 1986) animals. Amines serve an important role as neuromodulators of neuronal and effector tissue properties in these organisms (reviewed by Barchas et al., 1979).

Amine enhancement of synaptic efficacy is well documented in vertebrates. In sympathetic ganglia of the bullfrog, long term potentiation (LTP) of synaptic efficacy may be brought about with increased synaptic input (Koyano et al., 1985). However, adrenergic agents also contribute to LTP (Kumamoto and Kuba, 1986). Catecholamines appear to play a similar role in the mammalian hippocampus (Bliss et al., 1983; Hopkins and Johnston, 1984). Release of endogenous amines during repetitive stimulation of hippocampal neurons has been postulated as an important contributor to the development of LTP. Amine-induced enhancement of synaptic responses has been demonstrated for a number of other vertebrate neuronal and neuromuscular systems (Ashe and Libet, 1981; Bergman et al., 1981; Illes, 1986).

Amines have potent influences on the cardiovascular system of
vertebrates. Adrenergic excitation of heart contraction rate and strength has been documented in all major vertebrate groups (Benfey, 1980; Peyraud-Waitzenegger et al., 1980; Earm et al., 1983; Macey et al., 1984). Catecholamines bind with alpha- and beta-adrenergic receptors to produce these chronotropic and inotropic actions (Tsien, 1977; Benfey, 1980). Activation of amine receptors also regulates contraction and relaxation in vertebrate smooth muscle (Jones et al., 1984; Kitazawa et al., 1986).

In invertebrates, amines have been implicated as modulators of well characterized, stereotyped behaviors. For example, in the marine mollusc *Aplysia*, a particular reflex involving the withdrawal of the gill and siphon upon touch is modified by simple non-associative learning (Kandel and Schwartz, 1982). The synaptic events underlying sensitization, or strengthening, of this reflex resemble those observed during LTP in vertebrates. In *Aplysia*, sensitization of gill withdrawal occurs due to increased transmitter released from active siphon sensory neurons (these cells synapse with gill motor neurons in the abdominal ganglion). Modulation of transmitter release is effected by the action of serotonin to close K⁺ channels (and thus broaden presynaptic action potentials) in terminals of the sensory neurons (Kandel and Schwartz, 1982; Hochner et al., 1986). Habituation, or decrement, of this response is inhibited by dopaminergic modulation of gill muscle contractions (Swann et al., 1982; Ruben and Lukowiak, 1983). These amines act to enhance the overall sensitivity of gill withdrawal by modulatory actions on separate elements of the system.

Central pattern generators of behavior in invertebrates appear to receive considerable modulatory input from biogenic amines. Amines have been shown to have significant effects on neural mechanisms underlying
such patterned activities as feeding (Wieland and Gelperin, 1983; Trimble and Barker, 1984) and flight (Bailey et al., 1983; Sombati and Hoyle, 1984; Classen and Kammer, 1986). Serotonin has a profound effect on the pattern generating network for locomotion in the leech *Hirudo* (Willard, 1981). Leeches will swim towards a disturbance in the water when hungry; serotonin enhances both the sensitivity of movement receptors and the probability that the animal will swim (Kristan and Weeks, 1983; Brodfueher and Friesen, 1984). Serotonin also initiates and organizes feeding behavior in the leech (Lent and Dickinson, 1984). Modulation of an existing neuronal circuit by this amine produces several coordinated and appropriate behaviors for this organism under these conditions.

**Peptides**

Like the amines, neuropeptides are important regulators of neuronal and effector cell properties. Many peptides were first discovered in the vertebrate gastrointestinal tract, where they have varied and pronounced effects on gut motility and glandular secretion (Brown and Miller, 1982; Dockray, 1982; Fox et al., 1983; Bartho’ and Holzer, 1985). Many of these peptides (and others) are also present in the vertebrate nervous system (reviewed by Krieger and Liotta, 1979).

Studies on the neuronal regulation of peptide hormone secretion in vertebrates have focused on the hypothalamic-pituitary axis. A variety of approaches such as brain-slice electrophysiology and recombinant DNA techniques have been employed to investigate the influence of peptide release on homeostatic and behavioral regulation (Dudek et al., 1982; Loh et al., 1982; Negro-Vilar, 1982; Tuomisto and Mannisto, 1985).
Many of the peptides found in the pituitary also function within the CNS as neurotransmitters and neurohormones (reviewed by Krieger and Liotta, 1979 and Snyder, 1980). Peptides of the vertebrate CNS are involved in such diverse processes as cardiovascular control (Cowley et al., 1983), water balance (Lind and Johnson, 1982; Woods and Johnston, 1983), arousal and learning (O'Donohue and Dorsa, 1982), sensory perception (Iverson, 1982), hibernation (Muchlinski et al., 1983) and circadian rhythms (Albers et al., 1984).

Behavioral regulation may be influenced by opposing effects of peptides. For example, cholecystokinin promotes ingestive satiety in mammals, while peptides of the opiate family (enkephalins, endorphins and dynorphin) are stimulants to feeding behavior (reviewed by Baile et al., 1986).

Many peptides of the vertebrate gut and CNS are also found in invertebrate animals (reviewed by Haynes, 1980 and Greenberg and Price, 1983). Molluscan and arthropod species have been utilized extensively in isolation and physiological characterization of invertebrate neuropeptides. Peptides have numerous physiological roles in these animals, including modulation of stereotyped behavior patterns.

Several molluscan cardioactive peptides, in addition to their actions on the molluscan heart (Greenberg and Price, 1980; Lloyd, 1980; Painter and Greenberg, 1982; Boyd et al., 1984), have profound effects on non-cardiac muscle and neuronal excitability in a number of molluscs (reviewed by Lloyd, 1982 and Greenberg et al., 1983). For example, FMRFamide or related peptides act as neurohormones on molluscan smooth muscle (Greenberg and Price, 1980) and may act as a transmitter of an identified motor neuron of the cerebral ganglion of Helix (Cottrell et al., 1983).
The molluscan small cardioactive peptides (SCP's) and FMRFamide have opposite actions in some systems. In the prosobranch *Rapana*, SCP$_B$ has a negative chronotropic effect on the heart (Kobayashi and Muneoka, 1986), while FMRFamide excites heart rate. In *Helisoma* buccal ganglion neurons, FMRFamide inhibits (but SCP$_B$ excites) ongoing activity (Coates and Bulloch, 1985).

The arthropod neuropeptide proctolin was first isolated from the cockroach *Periplaneta* (Brown and Starratt, 1975). Proctolin-like peptides have subsequently been found in many species of insects (Brown, 1977; Bishop et al., 1981) and crustaceans (Kingan and Titmus, 1983; Schwarz, 1983). Proctolin has potent cardioexcitatory actions in many arthropod species (Miller, 1979; Benson et al., 1981; Miller and Sullivan, 1981) and modulates neuronal and neuromuscular excitability (Schwarz et al., 1980; Sullivan and Miller, 1984; Watson and Hoshi, 1985; Marder et al., 1986). Proctolin appears to act as a neurotransmitter in some systems (Adams and O'Shea, 1983; Witten and O'Shea, 1985; Bishop et al., 1987).

Peptides, like amines, regulate the expression of well-characterized behaviors in invertebrate animals. For example, ecdysis in certain species of moths is controlled by a CPG (Truman, 1978). The peptide eclosion hormone is present in the insect brain (Copenhauer and Truman, 1986) and is released prior to ecdysis to initiate the motor program for this behavior.

Obviously, amines and peptides have widespread and similar regulatory actions in invertebrate and vertebrate organisms. Not surprisingly, a number of investigations have demonstrated the coexistence of peptides and amines in certain neurons (reviewed by O'Donohue and Dorsa, 1982; Hokfelt et al., 1987). Investigations of dual (peptidergic and aminergic) modulation have addressed the question of coexistence in order
to characterize a particular system (Hoffman et al., 1982; Jacobowitz and Olschowka, 1982; Palmer et al., 1983). Multitransmitter neurons may be the anatomical basis underlying the interaction of peptides and amines in these systems.

Other examples of aminergic and peptidergic modulation of physiological events and behavior in invertebrates are discussed in Sections iii and iv. The relatively simple nervous systems of these animals are amenable to detailed studies of the cellular and biochemical bases for peptidergic and aminergic actions. Investigations of fundamental regulatory events shared by virtually all animals, such as the biochemical events underlying the cellular actions of hormones, have been facilitated by the variety of approaches possible with simple systems.

ii. MOLECULAR MECHANISMS

a. Intracellular signalling

Neurohormones or neurotransmitters elicit specific cellular responses by various biochemical processes, or mechanisms. Many neurotransmitters elicit a brief change in cell membrane potential by producing a conformational change in the ion channel upon transmitter-receptor interaction. More complex mechanisms, such as the intracellular synthesis of second messengers, are required to explain neurohormonal actions, and even some actions of transmitters. For example, in vertebrate sympathetic ganglia, certain transmitters elicit slow hyperpolarizing or depolarizing potentials which may involve the production of cAMP or cGMP (Weight et al., 1974; Briggs et al., 1982; Kobayashi, 1982).
Several basic signalling processes are common to second messenger systems. For example, signal transduction, protein kinase activation and substrate phosphorylation are phenomena generally associated with the actions of peptide and amine neurohormones. These events result in particular cellular responses to external stimulation by the "first messenger".

**Signal transduction**

The target site for peptides and amines is generally a cell-surface receptor whose configuration dictates selective recognition of a particular first messenger. However, multiple receptor types for a given neurohormone or neurotransmitter exist, and these receptors activate different intracellular processes (for example, dopamine D₁ and D₂ receptors, reviewed by Seeman and Grigoriadis, 1987). Furthermore, one ligand may compete for the binding site of a particular receptor with other compounds of similar molecular structure.

In spite of these complexities, it has been possible to determine the sequence of events underlying signal transduction of first messenger activation of a number of receptors. One particularly well-characterized receptor is the beta-adrenergic receptor, which has a high binding affinity for catecholamines. Ligand binding is followed by G protein (guanine nucleotide-binding protein) activation of the membrane-bound enzyme adenylate cyclase (Aurbach, 1982; Cerione et al., 1983; Schramm and Selinger, 1984). The first messenger signal, in this case, is transduced into the second messenger cyclic AMP by the active cyclase moiety.

A number of G protein types have been described, including Gₕ and Gᵢ
(stimulatory and inhibitory regulation of adenylate cyclase; reviewed by Schramm and Selinger, 1984 and Gilman, 1984), transducin (a G protein regulating cGMP phosphodiesterase in the vertebrate eye, Stryer, 1983) and Go (unknown function, Homburger et al., 1987). These proteins consist of several subunits, which in some cases appear to directly couple receptor binding to the conductance state of ion channels without second messenger involvement (Codina et al., 1987; Heschler et al., 1987; Logothetis, 1987).

**Intracellular actions of second messengers**

Increased production of second messenger molecules in response to an external first messenger signal acts as a biochemical signal, initiating a series of enzyme reactions. In general, second messengers bind to the regulatory subunit of a protein kinase, resulting in activation of the catalytic subunit of the kinase (reviewed by Levitan et al., 1983a). In a few cases, second messengers have been shown to have direct, presumably non-kinase mediated effects (reviewed by Huganir, 1987). More typically, the activated protein kinase phosphorylates cellular substrates (e.g. the sarcoplasmic reticulum, ion channels, other regulatory enzymes, etc.), completing the cellular response to external stimulation (Cohen, 1982; Tada and Katz, 1982; Nestler and Greengard, 1983).

Cellular activities involving second messengers, unlike typical synaptic responses of a few milliseconds, are generally longer-lasting phenomena. Not only must ligand-receptor interaction be terminated, but the intracellular processes described above must be reversed before cessation of the cellular response to the original first messenger signal. Substrate phosphorylation may be considered as one biochemical basis for
long-term cellular regulation.

b. Categories of second messengers

The diversity of cellular responses to external stimulation is in large part a consequence of the variety of second messenger systems activated by the interaction of a first messenger with a specific receptor (Fig. 1). In other words, two neurohormones may exert completely different effects because they act via separate second messengers. Cyclic nucleotides, membrane phospholipids, and calcium are three general second messenger systems for which the biochemical events following first messenger stimulation have been worked out in some detail (Huganir, 1987). Protein kinase activation and substrate phosphorylation appear to be the general processes by which these second messengers mediate regulation of cellular activity.

Cyclic AMP

The first demonstration that cyclic nucleotides are involved in the intracellular mechanism underlying hormonal action came from the work of Sutherland and his colleagues on epinephrine-induced glycogenolysis in rat liver (for review see Robison et al., 1971). These investigators found that increased synthesis of cyclic adenosine 3'-5'-monophosphate (cAMP) by the membrane-bound enzyme adenylate cyclase was correlated with the action of epinephrine in this system. They proposed that the effects of epinephrine in rat liver tissue were a result of second messenger (cAMP) production. The discovery of a cAMP-dependent protein kinase (Walsh et
Fig. 1. Cyclic AMP and phosphatidylinositol second messenger systems. In both systems, a hormone or transmitter binds to a membrane-bound receptor. G proteins translate the external signal by activation (or inhibition) of enzymes catalyzing the formation of second messenger molecules. These messengers may have effects of their own, but in general they activate protein kinases. A series of kinase-promoted phosphorylations culminates in an overt cellular response (e.g. the opening or closing of a particular channel in the cell membrane). Other second messenger systems, such as the cGMP and Ca++ / calmodulin systems, also exhibit these basic regulatory phenomena (receptor activation, protein kinase activation, phosphorylation, etc.).

Abbreviations: AC, adenylate cyclase; 5' AMP, adenosine 5' monophosphate; ATP, adenosine triphosphate; cAMP, cyclic 3'-5' monophosphate; DAG, diacylglycerol; G, guanine nucleotide binding protein; H,T (hormone, or transmitter); IC, ion channel; IP₃, inositol 1,4,5 triphosphate; IP₄, inositol 1,3,4,5 tetrakisphosphate; ORG, intracellular organelle (e.g. the sarcoplasmic reticulum); PDE, cyclic nucleotide-dependent phosphodiesterase; PIP₂, phosphatidyl inositol 4,5 bisphosphate; PKA, protein kinase A (cAMP-dependent protein kinase); PKC, protein kinase C (calcium / phospholipid-dependent protein kinase); PLC, phospholipase C; R, cell-surface receptor.
al., 1968) led to the conclusion that this enzyme (protein kinase A), via its phosphorylating actions, was the general effector of cAMP (Kuo and Greengard, 1969; Greengard, 1978).

Since the initial discovery of the cAMP mechanism, many studies have indicated that cAMP is involved in cellular stimulation. A cAMP-dependent mechanism has been demonstrated for many of the actions of amines in the vertebrate brain (Williams and Rodnight, 1977; Kebabian and Calne 1979; Nestler and Greengard, 1983) and periphery (Cerione et al., 1983; Kranias and Solaro, 1983). Many peptides also utilize cAMP as a second messenger in central (Etgen and Browning, 1983) and peripheral (Aurbach, 1982) actions. Cyclic AMP is involved in neuroregulatory actions of both amines and peptides in invertebrate organisms as well. In section iii of this introduction, a general review of cyclic nucleotide involvement in aminergic and peptidergic actions in invertebrates is presented.

Amines and peptides may activate or inhibit the production of cAMP to elicit cellular responses. For example, in many brain regions, dopamine increases cAMP by activating D₁ receptors (Kelly and Nahorski, 1987; Seeman and Grigoriadis, 1987). However, dopamine also activates D₂ receptors, resulting in adenylate cyclase inhibition (Stoof and Kebabian, 1983; Cooper et al., 1986). Catecholamines act on cardiac and smooth muscle beta-adrenergic receptors positively linked to the cyclase enzyme (Cerione et al., 1983), but catecholamines can also inhibit cAMP production in neurons and other cells by activating certain alpha-adrenergic receptors (reviewed by Jakobs et al., 1981). Finally, many of the effects of opioid peptides occur as a result of inhibited cAMP synthesis (reviewed by O'Donohue and Dorsa, 1982).

This widespread involvement of cAMP in aminergic and peptidergic
regulation points out the possibility that amine and peptide interactions may occur via utilization of a common second messenger system. For example, in the rat locus ceruleus, inhibition of adenylate cyclase is observed with the activation of either opiate or \( \alpha_2 \)-adrenergic receptors (Andrade and Aghajanian, 1985). The activity of certain enzymes, such as tyrosine hydroxylase (critical in the synthesis of catecholamines), is regulated in the rat superior cervical ganglion by peptide activation of the cAMP system (Ip et al., 1985). Interestingly, the activity of tyrosine hydroxylase may also be regulated by activation of the cGMP (Roskoski et al., 1987) and phosphatidylinositol (Albert et al., 1984) systems.

**Cyclic GMP**

Cyclic guanosine 3'-5'-monophosphate (cGMP), like cAMP, also acts as an intracellular second messenger. Cyclic GMP-dependent protein kinase activation and phosphorylation have been demonstrated in nervous and other tissues (Kuo and Greengard, 1970; Nairn and Greengard, 1983; Roskoski et al., 1987). However, several facts have hindered investigations into the messenger role of this molecule. First, cGMP levels within cells are very low. The discovery of cGMP and a reliable assay for its detection were developed only after cAMP had been established as a second messenger (Murad et al., 1979). Second, guanylate cyclase exists in both soluble and particulate forms, and assays or pharmacological activation of this enzyme have proven substantially more difficult than for adenylate cyclase (Garbers and Radany, 1981).

Nevertheless, a number of investigations have indicated that cGMP
does have a second messenger role in at least some systems. For example, there is good evidence indicating that cGMP acts as an intracellular signal in visual transduction (Polans et al., 1981; Zimmerman et al., 1985; Cook et al., 1987). In the vertebrate retina, cGMP appears to bind directly to rod outer segments to close ion channels in response to illumination.

Cyclic GMP may also mediate some actions of neurohormones in bidirectional systems. In the vertebrate myocardium, contractility is decreased by acetylcholine. This action is correlated with both an increase in myocardial cGMP and a decrease in myocardial cAMP (Murad et al., 1979). Agonists of beta-adrenergic receptors, on the other hand, promote increased contractility, decreased myocardial cGMP and increased myocardial cAMP. Similar findings of opposing interactions of cAMP and cGMP in bidirectional systems have led to the proposal that these second messengers are universal antagonists regulating cellular function (Goldberg and Haddox, 1977). However, applicability of this hypothesis to other systems remains uncertain, especially with the relative paucity of studies which have demonstrated a particular function for cGMP as an intracellular signal.

Membrane phospholipids

Many studies have implicated the involvement of membrane phospholipids in central and peripheral actions of peptides and amines (Berridge and Irvine, 1984; Cotechia et al., 1985; Lynch et al., 1985; Cooper et al., 1987). Activation of surface receptors triggers the cleavage of phosphatidylinositol 4,5 bisphosphate (PIP₂) by a membrane-bound phospholipid diesterase (phospholipase C) into inositol 1,4,5 trisphosphate
(IP₃) and diacylglycerol (DAG), two second messengers of the phosphatidylinositol (PI) system (Berridge, 1981, Fig. 1).

Diacylglycerol is an endogenous activator of calcium / phospholipid-dependent protein kinase (Berridge and Irvine, 1984). This enzyme (also termed protein kinase C) apparently is activated in the particulate form (Kraft and Anderson, 1983), a process dependent upon the calcium ion (May et al., 1985; Wolf et al., 1985). Protein kinase C, like the cyclic nucleotide-dependent protein kinases, may modify particular ionic conductances in the plasma membrane (reviewed by Kaczmarek, 1986 and Huganir, 1987). For example, the modulatory action of epinephrine on the voltage-dependent calcium channel in sensory neurons of the dorsal root ganglion appears to be a result of protein kinase C activation (Rane and Dunlap, 1986). Protein kinase C is also involved in the inhibition of calcium and potassium currents in cultured mouse neurons (Grega et al., 1987; Werz and MacDonald, 1987). Protein kinase C phosphorylates a number of substrate proteins and may be involved in neurotransmitter and neurohormone release (Haimann et al., 1987; Nichols et al., 1987; Wang and Friedman, 1987).

Inositol trisphosphate elicits calcium release from internal stores (Streb et al., 1983; Somlyo et al., 1985; Nosek et al., 1986). Additionally, IP₃ may be converted to inositol 1,3,4,5 tetrakisphosphate (IP₄), a second messenger involved in promoting calcium influx (Batty et al., 1985). Finally, the discovery of cyclic phosphoinositides, which apparently act like IP₃ to release internal calcium (Wilson et al., 1985; Agranoff, 1986), has added even further complexity to our understanding of the PI system.

Both DAG and IP₃ appear to be closely linked to calcium mobilization
within cells. Further studies of neuromodulation in which the PI system plays a role are necessary to characterize the general intracellular events associated with PI signalling and its relationship to other second messenger systems.

**Calcium**

The calcium ion is an ubiquitous regulator of intracellular activity. Cells maintain a low concentration of cytosolic calcium (approximately $10^{-7}$ M) so that slight increases in calcium levels may serve as an effective intracellular signal (Kretsinger, 1979). Neurotransmitter and neurohormone release, ion channel conductance, muscular contraction, fertilization, enzyme activities and other cellular processes are regulated by the levels of cytosolic calcium (for reviews see Berridge, 1975, Kretsinger, 1979 and Rasmussen and Barrett, 1984).

Calcium, like other second messengers, effects many of its intracellular actions by activation of specific protein kinases. Calcium binding proteins such as calmodulin are essential for calcium / kinase interaction (Cheung, 1980; Moore and Dedman, 1982). Calcium / calmodulin protein kinase is present in nervous tissues (Kennedy and Greengard, 1981; Nairn et al., 1985) and has been shown to influence pre- and post-synaptic phenomena (Kelley et al., 1984).

**Second Messenger Interactions**

The biochemical basis of neuromodulation does not consist solely of the aforementioned second messenger systems. Consideration must also be
given to interactions between the various second messenger systems in
order to understand the intracellular events ultimately responsible for
cellular responses to external stimulation. The production (or the
degradation) of one second messenger may rely on the relative
concentrations of other messengers. Likewise, intracellular events
responsible for a particular overt cellular response may be shared (or
opposed) by "separate" second messenger systems.

The calcium ion interacts with both the cyclic nucleotide and PI
second messenger systems. Calcium and calmodulin regulate adenylate
cyclase in many systems by stimulating (Amiranoff et al., 1983; Ishikawa
et al., 1985; Wright et al., 1986) or inhibiting (Brostrom et al., 1978;
Schmidt et al., 1982; Oldham et al., 1984) the activity of this enzyme.
Calcium also regulates the activity of guanylate cyclase (Berridge, 1975;
Snider et al., 1984; Ogura et al., 1986). This process also appears to
involve phosphatidylinositides (Berridge and Irvine, 1984; Snider et al.,
1984). Calcium is also an important regulator of cyclic nucleotide
phosphodiesterase and protein kinase activity (Wolff and Brostrom, 1974;
Cheung, 1980; Koletsky et al., 1983).

Like the PI system, cyclic nucleotides may exert their cellular
responses by eliciting intracellular calcium release or by regulation of
calcium channels (Entman et al., 1978; Cachelin et al., 1983; Sawada et al.,
1984; Curtis and Catterall, 1985). Calcium, therefore, may interact with
cyclic nucleotide or PI metabolism at each point in the biochemical
signalling process.

Second messenger interactions may also occur if two or more
separate systems exert a common effect on a particular process, such as
phosphorylation of specific proteins. Cyclic AMP-dependent protein kinase,
for example, has been shown to phosphorylate many of the same proteins in
the nervous system as Ca\(^{2+}\) / calmodulin- or Ca\(^{2+}\) / phospholipid-dependent
Cyclic nucleotides and phosphatidylinositides may co-regulate specific ion
channels. For example, both cAMP, which activates protein kinase A, and
phorbol esters, which activate protein kinase C, reduce the identical
potassium conductance in mouse neurons (Grega et al., 1987). Similarly,
both cGMP and phorbol esters increase a particular calcium current in Helix
neurons (Paupardin-Tritsch et al., 1986).

It is also possible for a single second messenger to have multiple
intracellular effects. Cyclic AMP has recently been shown to regulate the
conductance state of as many as three separate ion channels in Aplysia bag
cells (Strong and Kaczmarek, 1986). In R15 of the Aplysia abdominal
ganglion, serotonin may excite or hyperpolarize the cell, since this amine
may activate specific potassium, calcium and chloride currents by a
cAMP-dependent mechanism (Lotshaw et al., 1986). Additionally, the bag
cell peptide ELH also appears to increase calcium and potassium currents in
this neuron by a cAMP-dependent mechanism (Levitan et al., 1987).
Therefore, second messenger interactions which occur during
neuromodulatory actions of peptides and amines can be quite complex and
intriguing.

iii. INVERTEBRATE MODEL SYSTEMS

a. Invertebrate nervous systems

Many invertebrate organisms possess nervous systems with numerous
advantages to neurobiologists interested in studying the mechanisms underlying modulation of physiological and behavioral processes. In comparison to the vertebrate nervous system, the neural networks of invertebrates are simple, consisting of a relatively small number of neurons. This simplicity of organization has facilitated the determination of the precise neuronal circuitry responsible for the expression of specific behaviors. Furthermore, many of the neurons in invertebrates are large and identifiable. These properties have permitted the detailed investigation of electrophysiological, biochemical and genetic aspects of neural organization and neuromodulation.

Model Systems

Investigations into the neuronal basis of behavior in vertebrate organisms must contend with a highly complex CNS comprised of a vast number of neurons and their projections. Studies of basic neuronal mechanisms require simpler neural networks. For example, some neurobiologists utilize brain slice preparations or discrete ganglia in order to make possible a detailed study of the neuronal circuitry and neurochemistry of one particular system (Dudek et al., 1982; Kobayashi, 1982; Dunwiddie et al, 1983). Other investigators have approached this problem by focusing their efforts on the relatively simple nervous systems of invertebrates. The study of neuroregulation in invertebrate organisms, in addition to its intrinsic value, is often directed towards achieving a greater understanding of similar processes in higher organisms (Maynard, 1972; Kandel, 1976).

Discovery and investigation of the fundamental properties of neural
networks using simple invertebrate systems has contributed greatly in the advancement of neurobiology. For example, the ionic basis of the action potential, one of the most universal properties of excitable cells, was discovered by Hodgkin and Huxley (1952) using the squid giant axon. Likewise, the synapse in the squid stellate ganglion provided a preparation in which pre- and post-synaptic events underlying chemical transmission could be determined in great detail (Bullock and Hagiwara, 1957; Tasaki et al., 1965). The neurosecretory sinus gland found in crustaceans can be considered analogous to the vertebrate neurohypophysis, and therefore has been studied extensively as a model system for neurohaemal release mechanisms (Cooke, 1985; Newcomb et al., 1985; Stuenkel, 1985; Lemos and Nordmann, 1986).

Invertebrates have also served as models for studying the genetic mechanisms underlying neuromodulation of behavior. The large, identifiable neurons of Aplysia are, like those of other invertebrates, particularly well-suited for DNA hybridization, biochemical, electrophysiological and immunohistochemical analyses. These techniques are capable of providing needed insight into the cellular and molecular events underlying specific behaviors.

One behavior in Aplysia amenable to such detailed analyses is egg-laying behavior, a stereotyped sequence of events culminating in egg string deposition (Kandel, 1976; Blankenship et al., 1983). This behavior is largely controlled by peptidergic bag cells of the abdominal ganglion (Pinsker and Dudek, 1977; Cobbs and Pinsker, 1982). Bag cell peptides act at central and peripheral sites in Aplysia to coordinate the behavioral and physiological repertoire of egg-laying (Stuart and Strumwasser, 1980; Rothman et al., 1983a; Ligman and Brownwell, 1985; Rock et al., 1986). The
demonstration of simultaneous release of several peptides to elicit particular and coordinated responses has been explained on the molecular level by examining the genetic machinery of this system. The genes responsible for peptide synthesis specify large precursors, the fragments of which are released together in an all-or-none fashion (McAllister et al., 1983; Rothman et al., 1983b; Scheller et al., 1983). This genetic scheme is quite similar to the pro-opiomelanocorticotropin (POMC) system of vertebrates in which multiple peptide release is guaranteed by the genetic machinery (Loh et al., 1982; O'Donohue and Dorsa, 1982). The bag cells of the Aplysia abdominal ganglion therefore comprise an ideal model system in which to study the genetic control of a stereotyped behavior.

Invertebrates have also been utilized as model systems for the investigation of cellular processes crucial to learning. A form of associative learning is exhibited by the nudibranch Hermissenda: the pairing of light with rotation results in a decrement of this animal's natural positive phototactic response (Farley and Alkon, 1982). In this system, Type B photoreceptors receive excitatory synaptic input from statocyst hair cells during light-rotation pairing, eventually resulting in inhibition of phototactic motor neurons (Crow and Alkon, 1980; West et al., 1982). The well-characterized and long lasting changes in behavior induced by stimulus pairing in Hermissenda may be correlated with second messenger formation and protein phosphorylation (Alkon et al., 1982; Alkon et al., 1983; Neary and Alkon, 1983).

Among the best characterized models of non-associative learning are the gill withdrawal reflex in Aplysia (discussed in section ii) and the escape response of certain arthropods. In the crayfish, escape consists of an extremely rapid tail flick, a behavior for which the complete neuronal
circuitry has been established (Krasne and Wine, 1977). This response, like gill withdrawal in *Aplysia*, is subject to amine modulation (Glanzman and Krasne, 1983), habituation (Wine et al., 1975) and sensitization (Krasne and Glanzman, 1986). These examples point out the value of invertebrate systems as models, especially since the relatively simple neural circuitry of these organisms is amenable to investigations of the biochemical mechanisms underlying neuromodulation of a wide variety of regulatory events.

b. Amine actions in invertebrates: role of cyclic nucleotides

Amines, as discussed in section i, have powerful influences on physiological processes and behavioral expression in invertebrates. Many investigations of the biochemical mechanisms underlying amine actions on identified neurons or their target tissues in these organisms have expanded our general knowledge of the intracellular events associated with neuromodulation. The detection of amine-sensitive adenylate cyclases in nervous and peripheral tissues from a wide distribution of invertebrate groups, especially molluscs (Weiss and Drummond, 1981; Drummond et al., 1985; Deterre et al., 1986) and arthropods (Atkinson et al., 1977; Harmar and Horn, 1977; Morton, 1984; Pratt and Pryor, 1986) is an indication of the widespread role of cAMP in these organisms.

*Amine Modulation of Ionic Currents*

Actions of amines on the conductance state of ion channels in invertebrates have been explained on the molecular level by examination of
the particular second messenger system involved. For example, serotonin, via a cAMP-dependent mechanism, decreases a $K^+$ conductance in sensory neurons of the *Aplysia* abdominal ganglion to promote action potential spike broadening and enhance transmitter release (Castellucci et al., 1980; Kandel and Schwarz, 1982). Serotonin has a similar effect in *Helix* neurons (Deterre et al., 1981). In these neurons, dopamine, acting on separate receptors, also increases cAMP to decrease the same $K^+$ conductance (Deterre et al., 1982).

Other evidence indicates that cGMP may also be involved in amine actions on ion currents. The action of serotonin on certain identified neurons in *Helix* to enhance a calcium current is mimicked by agents which increase levels of cGMP within these cells (Paupardin-Tritsch et al., 1986). Cyclic AMP agents, such as forskolin, do not mimic the action of serotonin.

Perhaps the best characterized second messenger-mediated process is the serotonin-induced hyperpolarization of a large, identifiable neuron, R15, in the *Aplysia* abdominal ganglion. This cell exhibits rhythmic bursting activity consisting of alternating periods of repetitive action potentials and interburst hyperpolarizations. Serotonin enhances the intensity of interburst hyperpolarization, leading to diminution or abolition of bursting activity in this neuron. Cyclic AMP mediates the action of serotonin to increase a $K^+$ conductance in R15 and hyperpolarize the cell (Drummond et al., 1980; Benson and Levitan, 1983). Inhibition of adenylate cyclase (Lemos and Levitan, 1984) or protein kinase (Adams and Levitan, 1982) blocks the serotonin response, indicating that cAMP formation and protein phosphorylation are essential steps in this pathway. Injection of labeled $^{32}$P-ATP into R15 followed by application of serotonin results in an alteration in the labelling pattern of several phosphoproteins (Benson et
al., 1983; Levitan et al., 1983b), one of which might be a regulatory component of the target K⁺ channel.

**Amine Effects on Neuromuscular Transmission and Muscle Contractility**

Amines modulate the contraction strength of invertebrate muscle directly, by affecting excitation-contraction (E-C) coupling, or indirectly, by altering the efficacy of neuromuscular transmission. The role of cAMP in amine actions on either process has been documented in many species.

The existence of amine-sensitive adenylate cyclases in invertebrate muscle is correlated with a messenger role for cAMP in amine actions on muscular contractility. In the gill of *Aplysia*, dopamine and serotonin increase contractility of gill muscles by their actions on separate receptors coupled to adenylate cyclase (Weiss and Drummond, 1981; Weiss et al., 1985). Enhancement of buccal muscle contractility in *Aplysia* by serotonin is also cAMP-dependent (Ram et al., 1983; Weiss et al., 1985).

Cyclic AMP is important in amine enhancement of cardiac output in invertebrates. For example, increased ventricular cAMP is associated with the positive chronotropic and inotropic effects of serotonin on the heart of the clam *Mercenaria* (Higgins, 1974; Paciotti and Higgins, 1985). In *Aplysia*, serotonin-induced enhancement of cardiac muscle contractility is also mediated by cAMP (Weiss et al., 1982; Sawada et al., 1984). In this system, amine-induced elevation of cAMP is greatest in the valve or pacemaker region of the heart, underlining the pronounced increase in heart rate in response to serotonin. In the lobster *Homarus*, octopamine and serotonin increase cardiac muscle cAMP (Sullivan and Barker, 1975; Battelle and Kravitz, 1978), suggesting that the positive inotropic effect
of these amines on the lobster heartbeat may be a consequence of cAMP formation. Thus, amine stimulation of cAMP production in cardiac muscle may be a common mechanism to regulate cardiac output in invertebrate species.

Several studies have suggested that cAMP may also be a common messenger for amine enhancement of neuromuscular transmission. For example, application of a putative phosphodiesterase inhibitor, SQ 20,009, or a cyclic nucleotide analogue, 8-benzylthio cAMP, to the crayfish dactyl opener muscle preparation produces an increase in evoked junction potentials, as does serotonin (Enyeart, 1981). In the locust Schistocerca, octopamine$_2$ receptor activation of adenylate cyclase appears to underly the potentiation of neuromuscular transmission in the extensor tibia muscle (Evans, 1984 b,c).

Other studies have implicated both cAMP and cGMP in amine actions on neuromuscular synapses. In the mollusc Rapana, serotonin enhances neuromuscular transmission in the radula retractor muscle while depressing transmission in the protractor muscle. Both of these effects are mimicked by dibutyryl cAMP, but also (partially) by dibutyryl cGMP (Fujiwara and Kobayashi, 1983). Both cAMP and cGMP may also be associated with serotonergic modulation of neuromuscular transmission in the lobster claw opener muscle (Goy et al., 1984; Beltz and Kravitz, 1986; Goy, 1987). In both of these systems, serotonin has multiple actions on synaptic events, which may explain the difficulty in ascribing a role to cAMP or cGMP for a specific action of this amine. Further work is clearly needed to elucidate the precise second messenger systems underlying specific amine actions at the neuromuscular junction.
Amine Effects on Sensory Processes

Several studies on second messenger mechanisms involved in amine modulation of sensory input in invertebrates stand out as examples of molecular analyses of model systems. These include the serotonin/cAMP modification of sensory neuronal properties in response to tactile stimulation (the *Aplysia* gill withdrawal reflex, discussed in section i), the analogous response of tail sensory neurons of the pleural ganglion in this organism (Ocorr et al., 1985; Scholz and Byrne, 1987), and the alteration of the circadian rhythm of photosensitivity by various amines in the invertebrate eye. The latter phenomenon is extremely intriguing since cyclic nucleotides appear to have a role in primary sensory transduction as well as in mediation of the effects of circulating neurohormones.

In many animals, circadian rhythmicity of behavior is driven by a biological clock in the nervous system. In *Aplysia*, neural output from optic neurons collectively generate a daily rhythm of activity (Olson and Jacklett, 1985). The period of rhythmicity in the *Aplysia* eye is advanced by light pulses (Jacklett, 1974) and by serotonin, a putative entrainment neurohormone released by efferent axons of the optic nerve (Nadakavukaren et al., 1986). This action of serotonin on the optic rhythm is mediated by cAMP (Benson, 1980; Eskin et al., 1982; Eskin and Takahashi, 1983), as is the action of this amine to increase photosensitivity (Eskin and Maresh, 1982). Photic entrainment, on the other hand, may involve a cGMP mechanism, since light selectively increases cGMP in the eye and light-induced phase shifting can be mimicked by cGMP analogues (Eskin et al., 1984). Cyclic GMP involvement in the response to light has also been reported for photoreceptors of the horseshoe crab *Limulus* and the squid.
Loligo (Johnson et al., 1986; Saibil, 1986).

While in Aplysia the eye itself generates circadian rhythmicity, in Limulus the eye is modulated by a biological clock located in the protocerebrum (Barlow et al., 1980). Increased photosensitivity of the lateral eyes appears to be at least partly a response to octopamine released from efferent optic fibers (Battelle et al., 1982). Cyclic AMP is increased in the Limulus lateral eye by octopamine, and pharmacological elevation of cAMP in the eye results in an OCT-like electroretinograph or single photoreceptor response. Thus, in Limulus, Aplysia, and possibly other invertebrates as well, cyclic nucleotides play a crucial role in sensory transduction and in amine modulation of sensory receptors.

c. Peptide actions in invertebrates: role of cyclic nucleotides

Peptides have long-lasting effects on neuronal and peripheral target tissues, as discussed earlier. The involvement of second messengers in cellular responses to neuropeptides is suggested by the typically prolonged expression of peptide actions. As more invertebrate peptides have been isolated and physiologically characterized, evidence has accumulated linking cyclic nucleotide metabolism with their various actions.

Peptide Effects on Invertebrate Hearts

Peptides sharing partial sequence homology, the molluscan cardioexcitatory peptide (FMRFamide) and small cardioexcitatory peptides (SCP's) have, like serotonin, potent effects on the rate and strength of heart contractions in molluscs. Also, like serotonin, these peptides exert
their actions by elevating levels of cardiac muscle cAMP. For example, in
_Aplysia_, SCPB and serotonin both exert positive chronotropic and inotropic
effects and increase myocardial cAMP (Lloyd et al., 1985). Serotonin
appears to influence heart contractions by its long lasting and pronounced
effects on cAMP levels and on contractility in the atrio-ventricular valves.
Small cardioactive peptide, on the other hand, elicits cAMP increases and
contractile effects of shorter duration localized primarily in the ventricle.
These findings indicate separate peptide and amine regulation of the
_Aplysia_ heartbeat, via a common second messenger. In the clam
_Mercenaria_, it is FMRFamide which, like serotonin, utilizes cAMP as a
second messenger to promote increased cardiac muscle contractility
(Higgins et al., 1978; Greenberg and Price, 1979). Apparently, serotonin,
SCPB and FMRFamide augment the rate and strength of heart contractions in
these molluscs by stimulating a myocardial adenylate cyclase.

In the freshwater clam _Lampsilis_, FMRFamide action is inhibitory and
opposite to that of serotonin, although both agents increase cardiac muscle
cAMP (Painter, 1982a; Greenberg et al., 1983). The role of cAMP in peptide
regulation of the molluscan heart, therefore, appears to be more complex
than the original hypothesis that FMRFamide is a general cardioexcitatory /
cAMP-stimulating agent (Greenberg and Price, 1979).

Cyclic AMP may also be involved in peptide modulation of the cardiac
rhythm of neurogenic hearts in arthropods. An unidentified extract,
presumably a peptide, from crustacean pericardial neurohaemal organs
appears to increase (as does serotonin) the cAMP content of the cardiac
ganglion of _Homarus_ (Lemos and Berlind, 1981). Further studies are
necessary to establish the mechanism(s) of peptidergic cardio regulation in
arthropod and molluscan species.
Stereotyped, complex behavior patterns in invertebrates may be initiated by neuropeptide actions on identified neurons. Egg-laying in *Aplysia*, and ecdysis in the moth *Manduca*, are two examples of characterized behaviors in which cyclic nucleotides play an important role in the initiation of a stereotyped action pattern by a peptide.

In *Aplysia*, a sequence of events culminating in egg deposition is elicited by stimulation of the bag cell neurons of the abdominal ganglion, as discussed earlier in this section. These neurons undergo a prolonged period of bursting activity when stimulated electrically (Kupferman and Kandel, 1970) or by peptides released from the reproductive tract (Nagle, et al., 1985; Rock et al., 1986). Bag cell peptides released during bursting activity may also contribute to the afterdischarge in the bag cell clusters (Heller et al., 1980; Rothman et al., 1983b; Rock et al., 1986). During afterdischarge, action potentials of bursting bag cell neurons are progressively broadened, presumably to increase neurosecretory output (and thus insures the necessary circulating peptide titer to trigger egg-laying). Both peptide-induced afterdischarge and spike broadening involve a cAMP-dependent mechanism (Kaczmarek et al., 1980; Kaczmarek and Strumwasser, 1981). At least three potassium conductances in the bag cells are regulated by cAMP (Kaczmarek and Strumwasser, 1984; Strong and Kaczmarek, 1986), and protein phosphorylation occurs during afterdischarge (Jennings et al., 1982; Strumwasser et al., 1982).

Recent evidence has indicated that the PI system, in addition to cAMP, is involved in the expression of bag cell afterdischarge. Protein kinase C is
present and active in bag cell neurons (DeRiemer et al., 1985a).
Stimulation of this enzyme by certain phorbol esters (analogues of
diacylglycerol) or by injection of protein kinase C elicits an increase in the
height (not the width, as observed after application of cAMP analogues) of
action potentials, and enhances a calcium current (DeRiemer et al., 1985b).
Increased action potential height is characteristic of bag cell neurons at
the onset of afterdischarge, suggesting that protein kinase C activation
may occur in synergy with protein kinase A (cAMP-dependent) activation in
the normal expression of this event (Kaczmarek, 1986).

It has also been suggested that calcium / phospholipid metabolism
may be responsible for termination of bag cell discharge (Kaczmarek and
Kauer, 1983; Bruehl and Berry, 1985; Berry, 1986). Prolonged activation of
the PI system may prevent subsequent (and inappropriate) bag cell activity,
and also inhibits peptide synthesis. Thus, the Aplysia bag cell system is an
excellent model in which to study the combined intracellular actions of
several second messenger systems to precisely coordinate a stereotyped
behavior.

Ecdysis in insects consists of a complex sequence of behavior
patterns eventually resulting in cuticular shedding. Molting behavior is
triggered by a peptide, eclosion hormone (EH), released into the general
circulation by the insect CNS (Truman and Riddiford, 1970; Reynolds and
Truman, 1980). The pre-eclosion and eclosion behaviors in several species
of moths, including Manduca and Hyalophora, may be expressed by the
isolated ventral nerve cord in response to EH (Truman, 1978). The motor
programs for EH-stimulated behavior occur in association with increases in
CNS levels of cGMP, but not cAMP (Truman et al., 1979; Truman and Levine,
1982). Cyclic GMP analogues and phosphodiesterase inhibitors are capable
of triggering eclosion behavior in intact moths or inducing their underlying motor programs in the isolated nerve cord. Cyclic GMP also appears to be involved in other effects of EH associated with ecdysis (Truman and Levine, 1982; Schwartz and Truman, 1984; Morton and Truman, 1985), making this an attractive system in which to study cGMP-dependent phenomena. Unfortunately, the neurons responsible for the generation of specific ecdysis programs remain undetermined. Identification of these neurons is necessary to permit a more detailed analysis of specific biochemical events which culminate in the expression of this characterized behavior.

iv. THE NEUROGENIC LIMULUS HEART

a. The cardiac rhythm in \textit{Limulus}

In the horseshoe crab, \textit{Limulus polyphemus}, the cardiac rhythm is generated by the cardiac ganglion, a network of approximately two hundred neurons located on the dorsal surface of the heart (Patten and Redenbaugh, 1899; Bursey and Pax, 1970a). The heartbeat is neurogenic in origin, since removal of the cardiac ganglion abolishes heart contractions (Carlson, 1904). The constituents of the neurogenic \textit{Limulus} heart are understood in terms of their relative contributions to the overall activity pattern of the heart (reviewed by Watson and Augustine, 1982). This system has proven useful as a model system to investigate the action of various peptides and amines upon specific cellular elements of a pattern generating network.

\textbf{Cellular Organization of the Limulus Heart}
The cardiac ganglion in *Limulus* consists of two types of neurons which contribute to ganglionic burst formation: small (20-40 μm) pacemaker neurons and larger (60-150 μm) follower, or motor neurons (Patten and Redenbaugh, 1899; Lang, 1971a). This functional arrangement is similar to that found in the neurogenic crustacean heart (Maynard, 1955; Hartline, 1979). The smaller neurons of the *Limulus* cardiac ganglion exhibit slow depolarizing pacemaker potentials leading to overshooting action potentials. These appear to drive the burst activity of the motor neurons, which consists of a non-overshooting depolarization (cumulative synaptic input from several pacemaker neurons) followed by a prolonged plateau potential. During this plateau period, the follower neurons spike repetitively; this activity presumably constitutes motor output to the cardiac muscle fibers. Synchrony of cardiac muscle contractions may be due, at least partially, as a result of electrotonic coupling of the cardiac ganglion neurons (Lang, 1971a; Watson and Augustine, 1982).

Follower cells send projections out of the side branches of the cardiac ganglion and appear to innervate cardiac muscle fibers. The cardiac muscle potentials consist of summated or compound excitatory junction potentials (Abbott et al., 1969; Parnas et al., 1969); the muscle fibers themselves are normally unexcitable.

Under certain conditions deganglionated hearts may exhibit myogenic activity. Stretch, low calcium solutions or the neuropeptide proctolin elicit action potentials in cardiac muscle fibers and regular contractions in the absence of neuronal input from the cardiac ganglion (Lang, 1971b; Rulon et al., 1971; Watson and Hoshi, 1985). The electrical properties of *Limulus* cardiac muscle are thus subject to alteration by physical, chemical and neuromodulatory influences.
Cardioregulation by the CNS

In Limulus, the cardiac ganglion itself is innervated by cardioregulatory nerves which originate in the CNS (Patten and Redenbaugh, 1899; Carlson, 1905). These nerves arise from branches of the dorsal nerves exiting the brain (segmental cardiac nerves 6-8) and ventral nerve cord (segmental cardiac nerves 9-13). Stimulation of these nerves results in cardioinhibitory and cardioexcitatory responses (Pax, 1969; Bursey and Pax, 1970b; Hoegler, 1980). The variety of time courses for the onset and duration of these effects suggests that both synaptic and neurohormonal influences may be involved in cardioregulation (Pax and Sanborn, 1967; Pax, 1969).

Increases in heart rate occur simultaneously with stereotyped ventilatory movements of the book gills in Limulus (Watson and Wyse, 1978). These ventilatory behaviors are controlled by motor programs residing within the ventral nerve cord (Watson, 1980; Wyse et al., 1980). Correlation between these behaviors and alterations of the cardiac rhythm raises the possibility that cardioregulatory neurons of the ventral nerve cord may be associated with the pattern generating network for respiratory behaviors. This system therefore is of considerable interest as a model in which to study neuromodulatory mechanisms underlying physiological processes crucial to the integration of specific behaviors.

b. Amine modulation of the Limulus heart

The biogenic amines serotonin, octopamine, dopamine, epinephrine and
norepinephrine have been identified in various regions of the Limulus CNS and cardiac ganglion (Welsh and Moorehead, 1960; Edwards et al., 1979; O'Connor et al., 1982; Chamberlain et al., 1986). The hypothesis that these amines are important in cardioregulation has been supported by analyses of amine actions on the cardiac rhythm. Serotonin has been proposed as a likely cardioinhibitory substance, since application of this amine to the isolated heart preparation resembles stimulation of cardioinhibitory nerves (Pax and Sanborn, 1967). Octopamine and the catecholamines, on the other hand, all produce cardioexcitation in Limulus (Augustine et al., 1982).

**Amine Actions on the Limulus Heart**

Serotonin has been shown to inhibit the burst activity of the isolated cardiac ganglion, accounting for its negative chronotropic effect (Pax and Sanborn, 1967). However, relatively little attention has been directed towards determination of the cellular sites of cardioinhibitory amine actions on the Limulus heart. By comparison, the cardioexcitatory actions of octopamine and the catecholamines on the Limulus heart network have been analyzed in some detail at the cellular level (Fig. 2). All of these amines excite the isolated cardiac ganglion. In addition, dopamine and norepinephrine transiently inhibit ganglionic output (Augustine et al., 1982). Amines act on both pacemaker and follower neurons within the cardiac ganglion to exert their actions on burst activity (Augustine and Fetterer, 1985). In Chapter One, the intracellular mechanisms underlying amine actions on the Limulus cardiac ganglion are examined.

Octopamine and the catecholamines enhance the strength of heart
Fig. 2. Peptides and amines have long-lasting excitatory effects on the Limulus heartbeat. The pentapeptide proctolin acts directly on cardiac muscle fibers to enhance heart contraction amplitude. Several biogenic amines, including dopamine, act on cellular targets within the cardiac ganglion (pacemaker and follower neurons) as well as on cardiac muscle fibers to produce long-lasting increases in both the rate and strength of heart contractions. Diagram modified from Watson and Augustine, 1982.
contractions in *Limulus* (Augustine et al., 1982) Dopamine and norepinephrine also have transient negative inotropic effects with a time course similar to their initial negative effect on heart rate. Cardiac muscle fibers, as well as the neuromuscular junction, are targeted by these amines as sites of positive inotropic action (Watson and Hoshi, 1981; Watson et al., 1985). In Chapter Two, the role of second messengers in the positive inotropic effects of amines on the *Limulus* heart is investigated.

c. Peptide modulation of the *Limulus* heart

Several cardioactive peptides have been identified in the *Limulus* CNS (reviewed by Watson and Augustine, 1982). *Limulus* chromatophorotropic factor (LCF) may be comprised of several peptides to account for its cardioexcitatory, cardioinhibitory and chromatophorotropic activities (Pezalla et al., 1978; Dores and Herman, 1980; Watson et al., 1981). Several FMRFamide-like peptides are present in the *Limulus* CNS and in the cardiac ganglion: at least one of these *Limulus* peptides appears to have cardioexcitatory and cardioinhibitory effects on the isolated *Limulus* heart (Watson et al., 1984; White and Watson, 1984). Finally, a proctolin-like peptide with a molecular weight of approximately 600-800 daltons has been detected in the *Limulus* cardiac ganglion (Benson et al., 1981; Watson et al., 1983). Like synthetic proctolin, this peptide increases the strength of heart contractions with no apparent effect on the neurons of the cardiac ganglion (Fig. 2). Proctolin also induces myogenicity in the deganglionated *Limulus* heart (Watson and Hoshi, 1985).

For each of these cardioactive peptides, purification efforts and physiological analyses are incomplete. Part Two of this dissertation
describes further characterization of Limulus neuropeptides. In Chapter Three, the distribution and isolation of proctolin-like and FMRFamide-like peptides is investigated. In Chapter Four, the role of cyclic nucleotides and phosphatidylinositides in the action of these peptides on the Limulus cardiac rhythm is examined.

d. Mechanisms of peptide and amine actions in Limulus

As detailed in the previous sections, amines and peptides have pronounced effects on the hearts of a variety of vertebrate and invertebrate species. Cyclic AMP is an important second messenger in the chronotropic and inotropic actions of peptides and amines on the myogenic hearts of vertebrates (reviewed by Tsien, 1977) and many invertebrates, such as molluscs (reviewed by Greenberg et al., 1983).

Amines and peptides also have powerful chronotropic and inotropic effects on the neurogenic hearts of a number of arthropod species (Grega and Sherman, 1975; Florey and Rathmayer, 1978; Miller and Sullivan, 1981; Watson and Augustine, 1982). However, little is known regarding the biochemical basis for neuromodulation of the neurogenic heart network in any species. In some crabs and lobsters, octopamine appears to increase the cAMP content of the cardiac ganglion, as well as in cardiac muscle tissue (Sullivan and Barker, 1975; Battelle and Kravitz, 1978). Also, serotonin and a putative peptide extract in Homarus pericardial organs may increase the cAMP content in the cardiac ganglion of that species (Lemos and Berlind, 1981). However, in none of these studies could a positive correlation be made between the cellular actions of a particular neurohormone and its effect on tissue levels of cAMP. Additionally, the
role of other second messengers, such as cGMP or phosphatidylinositolides, has not been examined in any neurogenic heart.

**Evidence suggesting second messenger involvement in peptide and amine actions on the neurogenic Limulus heart**

Aminergic and peptidergic actions upon a number of cellular sites in the neurogenic *Limulus* heart produce characteristic changes in cardiac output. The biochemical mechanisms underlying these effects have not been determined. Several facts suggest the involvement of second messengers, especially cyclic nucleotides, in the expression of cardioexcitatory responses to these neurohormones. First, an octopamine-sensitive adenylate cyclase has been demonstrated in the *Limulus* CNS (Atkinson et al., 1977). Second, the positive chronotropic and/or inotropic effects of amines and peptides have a slow onset, with effects peaking 10-20 min after exposure of the isolated heart preparation to these compounds (Augustine et al., 1982). Finally, cardioexcitation induced by amines and peptides typically persists for long periods of time (60 min or more) after removal of the agent from the preparation. The actions of peptides and amines in this system, therefore, may be a result of their capacity to influence long-lasting biochemical events within the *Limulus* heart network.

Amines and peptides exhibit partial similarity in terms of overt responses on the cardiac rhythm in *Limulus*. Therefore, it is of interest to determine whether shared, or separate, second messenger systems mediate similar actions of these neuromodulators on the *Limulus* heart. Specifically, do amines and the *Limulus* FMRFamide-like peptide limadrin
both increase heart rate by the same mechanism? Also, do amines and the Limulus proctolin-like peptide both increase contraction amplitude via a shared mechanism?

Approaching these questions from a combined physiological and biochemical perspective is possible using a simple system. The constituents of the neurogenic Limulus heart are amenable to specific electrophysiological and biochemical analyses. These advantages may facilitate the elucidation of the intracellular mechanisms underlying excitation of the cardiac rhythm. This study will examine the roles of cAMP, cGMP and phosphatidylinositides in specific amine and peptide actions on the neurogenic heart network in the horseshoe crab, Limulus polyphemus.
CHAPTER ONE
MECHANISM FOR AMINE ACTIONS ON THE
LIMULUS CARDIAC GANGLION

ABSTRACT

Evidence is presented indicating that biogenic amines produce their characteristic excitatory chronotropic effects on the Limulus heartbeat by increasing cAMP in the cardiac ganglion. Amines, at $10^{-5}$ M, increased cardiac ganglion cAMP with a time course similar to that observed for amine-induced excitation of the contraction rate of the intact heart or the burst rate of the isolated cardiac ganglion. The apparent order of potency, at $10^{-5}$ M amine, for either rate excitation or cAMP elevation, was octopamine > epinephrine > dopamine > norepinephrine. Elevation of cardiac ganglion cAMP by octopamine or dopamine was dose-dependent, with a threshold of $10^{-6}$ M to $3 \times 10^{-6}$ M, at 3 min incubation, for either amine. The phosphodiesterase inhibitor IBMX potentiated amine-induced increases in heart rate and cardiac ganglion cAMP. Cyclic GMP levels in the Limulus cardiac ganglion were unaltered in the presence of any of these amines, at all doses tested.

The adenylate cyclase activator forskolin, 8-substituted cyclic AMP analogues and IBMX had long-lasting positive chronotropic effects on the intact Limulus heart. Like the amines, these agents also increased the burst rate of the isolated cardiac ganglion.

Follower cell burst parameters (interburst interval, burst duration
and number of spikes per burst) were decreased by these cyclic nucleotide agents in an amine-like fashion. Pharmacologically isolated follower neurons were depolarized by octopamine, forskolin and IBMX. These agents, as well as dopamine, also caused burst-like potentials in isolated follower neurons. The combined results indicate that several excitatory effects of these biogenic amines on the Limulus cardiac ganglion are mediated by increases in cardiac ganglion cAMP.
INTRODUCTION

The heartbeat in the horseshoe crab, *Limulus polyphemus*, is neurogenic in origin (Carlson, 1904). Pacemaker and follower neurons of the cardiac ganglion drive rhythmic contractions of the myocardium. The cardiac rhythm is modulated by the biogenic amines octopamine (OCT), dopamine (DA), epinephrine (EPI) and norepinephrine (NE), which are endogenous to the nervous system of the horseshoe crab (O'Connor et al., 1982). These amines have long-lasting excitatory effects on the rate and strength of heart contractions in *Limulus* (Augustine et al., 1982).

The excitatory chronotropic effects of amines on the *Limulus* heartbeat are a result of actions upon cardiac ganglion neurons (Augustine and Fetterer, 1985). Amines increase the burst rate in the *Limulus* cardiac ganglion by increasing the rate of firing of pacemaker neurons. Increased burst rate in the cardiac ganglion is accompanied by multiple changes in follower cell (motor neuron) bursting activity. These include a decrease in the duration of individual bursts and a reduction in the number of action potentials per burst. Amines also depolarize (OCT, EPI) or hyperpolarize (DA, NE) follower neurons isolated pharmacologically from pacemaker neuron input. The cellular sites of action of these amines on neurons in the *Limulus* cardiac ganglion have thus been determined, but the intracellular events underlying amine modulation in this system have not previously been investigated.

Several previous studies have suggested that cyclic nucleotides may be involved in the response of neurogenic hearts to amines. In some species of crabs and lobsters, octopamine increases tissue levels of cAMP in the cardiac ganglion and produces long-lasting excitation of heart rate
(Sullivan and Barker, 1975; Battelle and Kravitz, 1978). In Limulus, amine-induced excitation of heart contraction rate is slow in onset and is long-lasting. For example, peak effect is 10-15 min after amine application, and rate effects typically last 30-60 min following removal of amines from the bath (Augustine et al., 1982). However, there are no studies to date which clearly define the role of cAMP in the response of neurogenic hearts to amines. In this chapter, physiological and biochemical findings are presented which detail the relationship between chronotropic amine actions on the Limulus heart and levels of cardiac ganglion cAMP.
METHODS

Tissue incubations

The incubation, extraction and radioimmunoassay (RIA) protocols for cAMP and cGMP determination in Limulus cardiac ganglia are described in Appendix - General Methodology. The assay for either of these cyclic nucleotides is sensitive enough (0.1 pmol) to determine levels of both cAMP and cGMP in individual cardiac ganglia.

Recording of heart contractions

Limulus hearts were removed as described in Appendix - General Methodology. The isolated hearts were pinned to Sylgard resin in the bottom of small (10 ml) plexiglass chambers. The lateral boundary of each heart was hooked via thread to a force transducer (Grass FT .03 C; Grass Instruments, Co., Quincy, Massachusetts.). Rate and tension measurements were recorded on an oscillograph (Grass Model 79 D). Preparations were perfused continuously (flow rate 5 ml/min) with natural seawater at room temperature. Amine or drug solutions were added via the perfusion reservoir.

Extracellular recording

Isolated cardiac ganglia were pinned to the bottom of small (5 ml) plexiglass chambers and perfused continuously (5 ml/min) with natural seawater at room temperature. The anterior ends of cardiac ganglia were
drawn into the tips of suction electrodes. Extracellular potentials were filtered and amplified (Grass P-15) and then recorded on both an oscillograph and a chart recorder (Brush 220, Gould Inc., Cleveland, Ohio). Noise levels were less than 2 μV peak to peak, so individual cardiac ganglion bursts were clearly distinct.

**Intracellular recording**

Follower neurons within isolated Limulus cardiac ganglia were impaled with 3 M KCl-filled glass microelectrodes (20-40 MΩ). Membrane potentials were recorded with a high impedance electrometer (Dagan 8700, Dagan Corp., Minneapolis, Minnesota or WPI M 701, W-P Instruments, New Haven, Conn.). Potentials were displayed on a storage oscilloscope (Tektronix 5111, Beaverton, Oregon) and recorded on a chart recorder. Apparent input resistances of pharmacologically isolated follower neurons were measured by injecting hyperpolarizing and depolarizing current (0.1 to 0.3 nA internal source).
RESULTS

Amine effects on the Limulus heart and isolated cardiac ganglion

The biogenic amines OCT, DA, EPI and NE, as previously described (Augustine et al, 1982), all increased the contraction rate of the Limulus heart. Most of the experiments in this study focused on the action of two of these amines, OCT and DA. While DA transiently inhibited the heart, both amines produced positive chronotropic responses which were slow in onset and were long-lasting (Fig. 1a).

All four amines tested (OCT, DA, EPI and NE, at 10^-5 M) increased the burst rate of the isolated cardiac ganglion (Table 1). At this concentration, the apparent order of potency was OCT > EPI ~ DA > NE. Like their effect on the intact heart, the excitatory effect of either OCT or DA on the cardiac ganglion was gradual in onset, as burst rate reached a peak increase after 10 min of amine application (Fig. 2). Removal of amine from the bath resulted in a slow decline of the burst rate to control values over the course of 1-2 hr continuous perfusion. Cardiac ganglion burst rate remained elevated if amines were not removed from the recording chamber (data not shown).

Amine effects on levels of cyclic nucleotides in the cardiac ganglion

All four amines, at 10^-5 M, increased cAMP in the Limulus cardiac ganglion (Table 1). The apparent order of potency for cAMP elevation in this tissue was OCT > EPI ~ DA > NE. This result correlated exactly with the apparent order of potency for amine actions on cardiac ganglion burst

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Fig. 1. Amine-induced excitation of the neurogenic Limulus heart (A) is mimicked by cAMP-elevating agents (B). Amines and agents were added at the arrow and continually perfused. Both dopamine and octopamine, at $10^{-6}$ M, produced long-lasting increases in the rate and strength of heart contractions. Forskolin (an activator of adenylate cyclase), at $5 \times 10^{-6}$ M, precisely mimicked the excitatory chronotropic and inotropic actions of these amines. The phosphodiesterase inhibitor IBMX, at $10^{-3}$ M, also produced a long-lasting increase in heart rate, but its positive inotropic action was followed by long-lasting inhibition. Calibration: vertical, 1.5 g; horizontal, 1 min.
### TABLE 1 - Physiological and biochemical effects of amines and pharmacological agents on the *Limulus* cardiac ganglion

<table>
<thead>
<tr>
<th>AGENT</th>
<th>DOSE</th>
<th>% CTL RATE</th>
<th>pmol cAMP</th>
<th>pmol cGMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>100</td>
<td>28.0 ±10.6 (8)</td>
<td>5.8 ± 0.7 (7)</td>
<td></td>
</tr>
</tbody>
</table>

#### Amines

<table>
<thead>
<tr>
<th>AGENT</th>
<th>DOSE</th>
<th>% CTL RATE</th>
<th>pmol cAMP</th>
<th>pmol cGMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCT</td>
<td>$10^{-5}$ M</td>
<td>246.8 ± 15.9 (12)†</td>
<td>117.0 ± 30.6 (8)*</td>
<td>4.1 ± 0.3 (6)</td>
</tr>
<tr>
<td>EPI</td>
<td>$10^{-5}$ M</td>
<td>225.2 ± 48.3 (17)†</td>
<td>81.2 ± 15.6 (8)*</td>
<td>7.3 ± 0.8 (5)</td>
</tr>
<tr>
<td>DA</td>
<td>$10^{-5}$ M</td>
<td>216.8 ± 14.4 (14)†</td>
<td>61.8 ± 7.1 (8)*</td>
<td>5.2 ± 0.7 (6)</td>
</tr>
<tr>
<td>NE</td>
<td>$10^{-5}$ M</td>
<td>166.1 ± 7.2 (20)*</td>
<td>42.6 ± 8.9 (8)</td>
<td>6.6 ± 0.8 (5)</td>
</tr>
</tbody>
</table>

#### Pharmacological agents

<table>
<thead>
<tr>
<th>AGENT</th>
<th>DOSE</th>
<th>% CTL RATE</th>
<th>pmol cAMP</th>
<th>pmol cGMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>FORSK</td>
<td>$5 \times 10^{-6}$ M</td>
<td>225.0 ± 11.3 (12)†</td>
<td>65.8 ± 11.9 (8)*</td>
<td>4.2 ± 0.6 (6)</td>
</tr>
<tr>
<td>IBMX</td>
<td>$10^{-3}$ M</td>
<td>201.4 ± 10.6 (15)†</td>
<td>55.3 ± 10.4 (7)*</td>
<td>73.5 ± 18.3 (6)†</td>
</tr>
<tr>
<td>RO-20-1724</td>
<td>$10^{-3}$ M</td>
<td>118.0 ± 7.6 (8)</td>
<td>21.6 ± 7.8 (9)</td>
<td>---</td>
</tr>
</tbody>
</table>

Table 1. Summary of the effects of various amines and pharmacological agents on burst rate in the isolated *Limulus* cardiac ganglion and on levels of cyclic nucleotides in that tissue. The mean (± S. E.) of n experiments is represented for measurements of peak physiological effect, and for RIA measurements obtained after exposure of cardiac ganglia to these agents for 10 min. Level of significant difference from control was determined by a Student’s t-test.

Level of significance: † (p < .02); * (p < .05).
Fig. 2. Amines produced long-lasting increases in the burst rate of isolated cardiac ganglia. Top: OCT (10^{-5} M) or DA (10^{-5} M) were added to isolated ganglia for a period of 10 min (solid line) and subsequently removed from the bath (note the gradual reduction in frequency towards the control rate). Each point represents the mean increase in rate (± S. E.) recorded in 12 to 14 preparations. Bottom: extracellular recordings of cardiac ganglion output prior to and during amine application. Calibration: vertical, 50 μV; horizontal 2 sec.
The increase in cardiac ganglion cAMP produced by OCT or DA reached a peak with 10 min amine incubation (Fig. 3a). When cardiac ganglia were incubated in $10^{-5}$ M OCT or DA for 10 min and then transferred to amine-free solutions, cAMP levels dropped rapidly (Fig. 4). Within 5 to 10 min after removal of amine, cardiac ganglion cAMP levels had returned to control values. Continued presence of amine in the incubation solution resulted in continued elevation of cardiac ganglion cAMP (data not shown). None of these amines affected the levels of cGMP in the Limulus cardiac ganglion (Table 1, Fig. 3b).

Both OCT and DA increased cardiac ganglion cAMP in a dose-dependent manner (Fig. 5a). The threshold dose for either DA or OCT for cAMP elevation, at 3 min incubation, was between $10^{-6}$ M and $3 \times 10^{-6}$ M. At all doses tested ($10^{-6}$ M to $5 \times 10^{-5}$ M), neither OCT or DA influenced the levels of cardiac ganglion cGMP (Fig 5b).

Effects of forskolin and IBMX on the Limulus heart and isolated cardiac ganglion

Forskolin, which increases intracellular levels of cAMP by stimulating adenylate cyclase (Seaman et al., 1981), and IBMX, which elevates cAMP and/or cGMP by inhibiting cyclic nucleotide-dependent phosphodiesterases (Chasin and Harris, 1976; Levitan and Norman, 1980), elicited amine-like increases in the rate of Limulus heart contractions (Fig. 1b). The excitatory effects of both compounds were dose-dependent (Fig. 6a), but forskolin was approximately 100 x more potent than IBMX in its ability to increase heart rate.
Fig. 3  Octopamine (10⁻⁵ M) and dopamine (10⁻⁵ M) increased cAMP, but not cGMP, in Limulus cardiac ganglia. A. Amines produced increases in cardiac ganglion cAMP with a time course and potency similar to their effect on burst rate. Each point represents the mean content of cardiac ganglion cAMP (± S. E.) compared to control levels in 6 to 8 experiments. B. The same concentration of OCT or DA had no effect on cGMP levels in the cardiac ganglion in these experiments.
Fig. 4. Onset and washoff kinetics of amine-induced changes in cardiac ganglion cAMP content. Cardiac ganglia were immersed in $10^{-5}$ M OCT or $10^{-5}$ M DA for 30 sec to 10 min (solid line). Ganglia were then processed for determination of cAMP content, or washed in amine-free seawater for 5 to 80 min and then processed. Cyclic AMP levels were increased by either amine, and dropped back to control values within 10 min after amines were removed from the incubation solution. Each point represents the mean content of cardiac ganglion cAMP ($\pm$ S. E.) compared to control levels in 6 to 8 experiments.
Fig. 5. Octopamine and dopamine produced dose-dependent increases in cAMP (A), but not cGMP (B) in Limulus cardiac ganglia. Each point represents the mean ± S. E. of 5 experiments in which individual cardiac ganglia were exposed to a particular dose of amine for 3 min. The threshold dose for either amine was between $10^{-6}$ M and $3 \times 10^{-6}$ M.
Fig. 6. Forskolin and IBMX, which increase cAMP in Limulus cardiac ganglia, also increased heart contraction rate. A. Both forskolin and IBMX produced dose-dependent increases in heartbeat frequency. Each point represents the mean increase in heart rate (± S. E.) of 10 to 16 experiments. B. Onset and washoff kinetics of equipotent doses of forskolin (5 x 10^-6 M, n=14) and IBMX (10^-3 M, n=16). Agents were added to the isolated Limulus heart as indicated by the solid line. Both agents, like the amines, produced long-lasting increases in heart rate.
In order to compare the time course of the responses of the *Limulus* heart to these drugs, doses of each compound which produced comparable increases in heart rate were applied (5 x 10^{-6} M forskolin, 90.8 ± 10.0% S.E.M. increase, n=17 and 10^{-3} M IBMX, 89.3 ± 7.5% increase, n=16). The effects of either agent, like those of the amines, were slow in onset and long-lasting (Fig. 6b). The IBMX-elicited increase in the rate of *Limulus* heart contractions was slightly more rapid in onset, and in decline, than the effect of forskolin.

Forskolin (5 x 10^{-6} M) or IBMX (10^{-3} M) increased the burst rate in isolated *Limulus* cardiac ganglia (Table 1, Fig. 7). The excitatory effect of forskolin or IBMX was similar in time course and magnitude to their effects on the contraction rate of the intact *Limulus* heart.

Forskolin and IBMX both increased cAMP in the *Limulus* cardiac ganglion (Table 1). IBMX-, but not forskolin-induced increases in cardiac ganglion cAMP were observed as early as 3 min incubation. At this time point, IBMX increased cardiac ganglion cAMP levels to 162.5 ± 11.4% control (n=6), while forskolin did not increase cAMP levels (84.5 ± 16.7% control, n=6). This finding may explain the more rapid onset of the positive chronotropic effect of IBMX on the intact heart or isolated cardiac ganglion. Forskolin had no effect on levels of cGMP in the cardiac ganglion (Table 1). IBMX, on the other hand, produced a dramatic increase in cardiac ganglion cGMP content after 3 min (1531.5 ± 313.6 % control) or 10 min (1270 ± 328.4 % control) incubation.

The effects of forskolin, IBMX and the amines suggested a correlation between the relative magnitude of increased cAMP and of increased burst frequency in the cardiac ganglion (Fig. 8). Therefore, the potency and kinetics of the effect of these agents and of the amines on burst frequency...
Fig. 7. Forskolin (5 x 10^{-6} M) and IBMX (10^{-3} M) increased burst frequency in the isolated Limulus cardiac ganglion. Drugs were added to the preparation for 10 min, as indicated by the solid line (top). The time course and magnitude of the effect of either drug was similar to their effect on heart rate. Each point represents the mean increase in cardiac ganglion burst rate (± S. E.) in 12 to 16 experiments. Bottom: extracellular recordings of cardiac ganglion output before addition of agent to the preparation and during the peak response. Calibration: vertical, 30 μV; horizontal, 2 sec.
Fig. 8. Correlation between the extent to which amines (10^{-5} M), forskolin (5 \times 10^{-6} M) and IBMX (10^{-3} M) produced increases in the burst rate of the isolated Limulus cardiac ganglion and their capacity to increase cardiac ganglion cAMP. For any agent, the y co-ordinate represents the mean increase in cAMP (± S. E.), after 10 min incubation in 7 to 8 experiments. The x co-ordinate represents the mean increase in burst rate after 10 min in 12 to 20 experiments. Simple regression of these data points indicates a good correlation (R = 0.94) between levels of cAMP and burst rate in Limulus cardiac ganglia.
was consistent with the kinetics of their effect on cardiac ganglion cAMP.

Effects of cyclic nucleotide analogues and phosphodiesterase inhibitors

The cAMP analogue 8-para-chlorophenylthio cAMP (10\(^{-3}\) M) produced a 34.0 ± 9.9% increase in the contraction rate of the intact Limulus heart in 7 preparations. Dibutyryl cAMP, dibutyryl cGMP and 8-bromo cGMP (each at 10\(^{-3}\) M) had no effect on the cardiac rhythm (n=5).

In contrast to the excitatory chronotropic actions of IBMX, several phosphodiesterase inhibitors elicited weak effects when applied to the isolated heart preparation. Papaverine (10\(^{-3}\) M) elicited a 10.5 ± 4.3% increase in heart rate (n=13). RO-20-1724 (10\(^{-3}\) M), in 12 preparations, increased contraction rate only slightly (4.9 ± 2.4%). Theophylline (10\(^{-3}\) M) had no effect (n=6). Finally, SQ 20,009 (10\(^{-4}\) M) had no effect on heart rate (n=14). At higher doses SQ 20,009 caused the cardiac rhythm to become irregular.

Cyclic nucleotide analogues, like forskolin and IBMX, mimicked the action of amines on the isolated Limulus cardiac ganglion (Fig. 13). The cAMP analogues 8-benzylthio cAMP and 8-parachloro-phenylthio cAMP, at 10\(^{-3}\) M, produced increases of 49.7 ± 9.7% (n=9) and 33.9 ± 7.7% (n=7), respectively, in the burst rate of cardiac ganglia exposed to these agents for 30 min. The phosphodiesterase inhibitor RO-20-1724, at 10\(^{-3}\) M, had only a mild stimulatory effect on cardiac ganglion burst rate (18.0 ± 8.4% increase, n=8). The finding that RO-20-1724 did not increase cardiac ganglion cAMP (Table 1) was consistent with its weak effect on cardiac ganglion burst rate.
Potentiation of amine responses by IBMX

Octopamine, dopamine or IBMX were topically applied to the continually perfused Limulus heart preparation at doses that produced only slight increases in contraction rate. After the contraction rate returned to its original value, the same dose of amine was then added with IBMX present. Dopamine produced a 21.4 ± 5.7% (n=7) greater increase on heart rate when IBMX was present, and octopamine elicited a 13.5 ± 2.7% (n=7) greater response with IBMX (Fig. 9a). At this low dose (1 ml, 10^{-5} M), IBMX itself had a minimal effect on heart rate (2.6 ± 1.7% increase, n=14).

IBMX also potentiated amine-induced elevation of cAMP. At 10^{-4} M, IBMX had no effect on levels of cardiac ganglion cAMP after 3 min incubation. However, when IBMX was combined with a threshold dose (10^{-6} M) of either OCT or DA, significant increases in cAMP were produced after 3 min (Fig. 9b). These results suggest that in the cardiac ganglion, IBMX suppressed cAMP-dependent phosphodiesterase activity sufficiently to permit additional cAMP accumulation with exposure to low doses of amine. Therefore, inhibition of phosphodiesterase activity in the Limulus cardiac ganglion was associated with potentiation of the physiological and biochemical actions of amines.

Suppression of cAMP accumulation by phentolamine

The alpha-adrenergic receptor antagonist phentolamine (10^{-4} M) inhibited the elevation of cAMP induced by 10^{-5} M OCT or 10^{-5} M DA in the Limulus cardiac ganglion (Fig. 10). Suppression of amine-induced elevation of cAMP levels by phentolamine was more effective for OCT than
Fig. 9. Potentiation of physiological and biochemical effects of amines in the Limulus cardiac ganglion. A. The phosphodiesterase inhibitor IBMX enhanced the chronotropic effects of either DA (10^{-6} M in 1 ml) or OCT (3 x 10^{-7} M in 1 ml). B. In separate experiments, IBMX also enhanced the capacity of DA (10^{-6} M) or OCT (10^{-6} M) to increase cardiac ganglion cAMP. IBMX (10^{-4} M) was added to isolated cardiac ganglia in the presence or absence of amine for 3 min after which cardiac ganglion cAMP levels were measured by RIA. In either type of experiment, level of significance was determined using a paired Student's t-test comparing the effects of a particular amine + IBMX to the sum of the effects of that amine and IBMX added individually.

Level of significance: ** (p ≤ .03); * (p ≤ .05).
Fig. 10. The alpha-adrenergic antagonist phentolamine blocked amine-induced increases in cardiac ganglion cAMP. Cardiac ganglia were treated with $10^{-5}$ M DA or OCT for 3 min in the presence or absence of $10^{-4}$ M phentolamine and then processed for cAMP content. Numbers positioned over the areas representing cAMP accumulation in ganglia exposed to amine + phentolamine signify the percent inhibition by phentolamine of the response to a particular amine in comparison to control cAMP levels.
for DA in this tissue. Earlier findings indicated that phentolamine effectively blocks amine induced excitation of heart rate in Limulus (Augustine et al., 1982). Together, these findings indicate that phentolamine blocks the excitatory chronotropic action of amines by inhibiting amine-induced synthesis of cAMP in the cardiac ganglion.

Pharmacological agents: effects on follower neuron bursting activity

Forskolin (5 x 10^{-6} M) and IBMX (10^{-3} M) produced multiple amine-like effects on the bursting activity of individual follower neurons in the Limulus cardiac ganglion (Figs. 11, 12). First, interburst interval was decreased by these agents as burst frequency increased. Second, the duration of each burst was shortened. Finally, the number of action potentials fired per burst decreased as the plateau component of each burst was compressed. These changes in burst parameters are quite similar to those elicited by amines (Augustine and Fetterer, 1985).

Cyclic nucleotide agents also had amine-like effects on follower neuron bursting activity (Fig. 13). Both 8-benzylthio cAMP and 8-parachloro-phenylthio cAMP, at 10^{-3} M, produced alterations in follower neuron burst characteristics similar to those observed during the application of amines, forskolin or IBMX.

Direct effects of forskolin and IBMX on follower neurons

Follower neurons were pharmacologically isolated from pacemaker neuron input with the introduction of 30 mM Mn^{++} to the bath. At this concentration, manganese reversibly eliminated most spontaneous activity
Fig. 11. Amines (10\(^{-5}\) M), forskolin (5 x 10\(^{-6}\) M) and IBMX (10\(^{-3}\) M) alter several characteristics of follower cell activity in the Limulus cardiac ganglion. Agents were perfused over the ganglion for 10 min and then removed from the bathing solution. Left: intracellular recordings of follower cells 1 min before addition of a particular agent. Right: recordings of the same cells during the peak rate response of the cardiac ganglion to a particular amine or drug. Follower neuron bursts were more frequent, shorter in duration and contained fewer spikes. Calibration: vertical, 20 mV; horizontal, 2 sec.
A. Interburst Interval

B. Burst Duration

C. Spikes / Burst

Fig. 12. Time course of alteration in follower cell characteristics in cardiac ganglia exposed to $5 \times 10^{-6}$ M forskolin or $10^{-3}$ M IBMX for 10 min. Interburst interval (A), burst plateau duration (B) and number of action potentials recorded per burst (C) all declined during application of these agents. These effects were long-lasting and only gradually returned towards control values as the burst rate in the cardiac ganglion declined. Each point represents the mean value ± S. E. of 10 to 14 experiments.
Fig. 13. Cyclic nucleotide analogs produced amine-like effects on the Limulus cardiac ganglion. At $10^{-3}$ M, 8-benzylthio cAMP and 8-parachlorophenylthio cAMP increased cardiac ganglion burst rate (top record in each trace). These agents, like the amines, also decreased follower neuron interburst interval, burst duration and the number of spikes per burst (bottom record in each trace). Calibration: vertical, 50 µV (top); 10 mv (bottom); horizontal, 2 sec.
in the cardiac ganglion. Follower neurons were depolarized by 10-15 mV and became quiescent, with the exception of infrequent (< 1 per 10 min) and small (1-2 mV) changes in resting potential.

In cobalt-treated cardiac ganglia, DA decreases the apparent input resistance of and hyperpolarizes these cells, while OCT depolarizes follower neurons without altering apparent input resistance (Augustine and Fetterer, 1985). In the present study, similar effects of these amines, at $10^{-5}$ M, were observed in follower cells of cardiac ganglia treated with manganese (Fig. 14). Forskolin, at $5 \times 10^{-6}$ M, depolarized these cells by $7.4 \pm 0.7$ mV (n=8) and IBMX, at $10^{-3}$ M, produced a $6.0 \pm 1.1$ mV depolarization (n=9) over the course of 15 min drug application. Neither forskolin or IBMX altered the apparent input resistance of follower neurons.

In these experiments, amines, forskolin and IBMX all increased the frequency and magnitude of changes in follower neuron resting potential (Fig 14). These changes in resting potential resembled follower neuron bursts to some extent, since they consisted of a sharp rise in potential followed by a slow depolarizing plateau. Like the depolarizing effect of octopamine and the pharmacological agents, this effect persisted well into the wash period.
Fig. 14. Effects of DA ($10^{-5}$ M), OCT ($10^{-5}$ M), forskolin ($5 \times 10^{-6}$ M) and IBMX ($10^{-3}$ M) on follower neurons in Limulus cardiac ganglia pretreated with 30 mM manganese to isolate these cells from synaptic input. While DA hyperpolarized follower neurons, OCT, forskolin and IBMX all gradually depolarized these cells by several mV. Input resistance was decreased slightly during exposure to DA, as evidenced by a lesser response to current injection and a reduction in the amplitude of membrane "noise". Other agents did not influence apparent input resistance. All these agents elicited burst-like potential changes in follower cells after several minutes. Calibration: vertical, 15 mV; horizontal, 1 min.
DISCUSSION

The findings of this study indicate that the excitatory chronotropic actions of OCT, EPI, DA and NE on the neurogenic Limulus heart result from amine-induced increases in levels of cAMP in the cardiac ganglion. The magnitude of the excitatory effect of these amines on burst frequency in the cardiac ganglion was precisely correlated with their relative effects on cAMP metabolism within this tissue. Further, pharmacological agents which also increased cardiac ganglion cAMP had amine-like effects on heart rate and on burst rate in the isolated cardiac ganglion. These agents also mimicked several actions of the amines on follower cell activity. Finally, cGMP was not involved in any actions of the amines. Octopamine and the catecholamines apparently utilize a common second messenger, cAMP, to produce their characteristic excitation of heart rate in Limulus.

Amines, especially octopamine, have excitatory chronotropic actions on other neurogenic hearts in a variety of crustacean species (Grega and Sherman, 1975; Battelle and Kravitz, 1978; Florey and Rathmayer, 1978; Miller and Sullivan, 1981). Neurons of the cardiac ganglion are the cellular site of chronotropic amine actions on the neurogenic heart of crustaceans (reviewed by Beltz and Kravitz, 1986) and Limulus (Augustine and Fetterrer, 1985). Further, OCT has been reported to increase the cAMP content of cardiac ganglia in crabs and lobsters (Sullivan and Barker, 1975). However, this is the first study to demonstrate a cAMP-dependent mechanism underlying chronotropic amine actions on any neurogenic heart.

Role of cAMP in chronotropic amine actions
In the *Limulus* cardiac ganglion, the small (20-40 μm) bipolar or multipolar cells control the heart rate (Lang, 1971a). These neurons exhibit a slowly decaying pacemaker potential which leads to a single overshooting action potential during each heartbeat. The pacemaker neurons apparently synapse with larger (60-150 μm) follower neurons and initiate the burst activity in these cells.

Octopamine and the catecholamines DA, EPI and NE all increase burst frequency in the *Limulus* cardiac ganglion by increasing the frequency of pacemaker neuron action potentials (Augustine and Fetterer, 1985). These amines increase the rate of pacemaker potential depolarization between action potentials and increase the rate of pacemaker repolarization. A consideration of the biochemical and pharmacological data outlined in this chapter suggests that the excitatory action of these amines on the *Limulus* cardiac ganglion is probably the result of elevated cAMP in the pacemaker neurons. Amines, forskolin and IBMX had identical effects on heart contraction rate, ganglionic burst rate or follower cell activity. All of these agents increased cardiac ganglion cAMP to a degree matched precisely with their capacity to increase burst rate in the isolated cardiac ganglion. Potentiation or suppression of chronotropic amine action was associated with enhanced or inhibited synthesis, respectively, of cAMP in the cardiac ganglion. Therefore, cAMP clearly is the second messenger underlying the positive chronotropic actions of amines on the *Limulus* cardiac ganglion, and these actions most likely involve increased cAMP synthesis in the pacemaker neurons.

**Role of cAMP in amine actions on follower neurons**
Excitation of burst rate in the Limulus cardiac ganglion by amines, or by other agents which also increased cAMP, was associated with an alteration of activity in the follower, or motor neurons. Cyclic AMP appears to be involved in some direct amine actions on follower cells. Both OCT and EPI gradually depolarize follower neurons without affecting membrane conductance (Augustine and Fetterer, 1985). The depolarizing action of OCT on pharmacologically isolated follower cells was mimicked by adenylate cyclase activation or phosphodiesterase inhibition. Therefore, OCT and EPI probably depolarize follower neurons by increasing cAMP in these cells.

The hyperpolarizing effect of DA and NE on follower neurons, on the other hand, involves a rapid conductance increase (Augustine and Fetterer, 1985). Apparently, neither of these effects of DA and NE involves cyclic nucleotides, since they are not mimicked by forskolin or IBMX.

Many of the effects of amines on follower cell activity (decreased interburst interval, burst duration, or number of action potentials per burst) appear to be an indirect consequence of increased pacemaker firing frequency (Augustine and Fetterer, 1985). Local application of DA onto pacemaker neurons produces these characteristic follower cell responses, suggesting that amine-induced alteration in pacemaker input to these cells underlies the observed changes in follower burst activity. Further, it is possible to entrain the bursts of isolated cardiac ganglia with rhythmic electrical stimulation and gradually increase burst rate in the absence of amines (Watson and Groome, unpublished results). In these experiments, artificially increased burst rate resulted in an alteration of follower neuron burst activity similar to that elicited by amines or forskolin. These findings suggest that cAMP may be only indirectly responsible (by
increasing burst rate) for observed changes in follower neuron burst activity during amine application.

**Possible involvement of cAMP in pacemaker-follower synaptic efficacy**

The observation that follower cells exposed to 30 mM Mn^{++} exhibited (albeit very infrequently) small changes in resting potential indicates that Mn^{++} was not quite as effective as other cations (e.g. cobalt, Augustine and Feterrer, 1985 and personal observations) in blocking calcium-dependent synaptic transmission in the *Limulus* cardiac ganglion. Therefore, pacemaker neurons may release transmitter during Mn^{++} blockage which at times is sufficient to elicit partial activity in the follower cells.

The results of the experiments performed with Mn^{++} suggest a possible action of amines in this system that has not previously been described. Marked increases in the size, duration and frequency of these potential changes occurred during exposure to OCT, DA, forskolin and IBMX. These potential changes resembled, to some extent, the burst activity in follower cells receiving normal pacemaker input. In cobalt-treated cardiac ganglia, application of amines does not result in the appearance of burst-like potentials (Augustine and Feterrer, 1985 and unpublished observations).

The appearance of these burst-like potential changes in Mn^{++} treated follower neurons may be the expression of a cAMP-mediated action of amines to enhance synaptic efficacy between pacemaker and follower neurons. Amines may increase cAMP in pacemaker neuron terminals, leading to an increase in the amount of transmitter released per
pacemaker action potential (the pacemaker-follower synapse appears to be chemical, Augustine and McCulloh, unpublished).

It is possible that these burst-like potential changes represent the appearance of slow driver potentials in *Limulus* cardiac ganglion neurons. Calcium-dependent driver potentials have been demonstrated in the cardiac ganglion of several crustacean species (Tazaki and Cooke, 1979; Berlind, 1982). In lobsters, the small pacemaker neurons exhibit oscillating driver potentials while the driver potential of the larger motorneurons is triggered by synaptic input from the pacemaker cells (Hartline, 1979; Tazaki and Cooke, 1983). Interestingly, driver potentials of the pacemaker neurons in the isolated *Homarus* cardiac ganglion are particularly resistant to manganese in the bathing saline (Berlind, 1985). In *Limulus*, amines may utilize cAMP to enhance (e.g. via increased transmitter released from pacemaker neurons) a driver potential present in follower neurons. Possible amine/cAMP influences on driver potentials in follower neurons can only be thoroughly investigated, however, if such potentials can be demonstrated in these cells.

Enhancement of the plateau component of the follower neuron burst may be of considerable importance in coordinating the effects of amines on heart contraction rate and strength. Several facts outline the relationship between follower cell activity, ganglionic burst rate and heart contraction amplitude. First, the follower cell burst is initiated by a rapid depolarization consisting of summated EPSP's (Palese et al., 1970; Lang, 1971a). Second, burst plateau duration is associated with the relative number of action potentials delivered to the cardiac muscle fibers per burst (Augustine and Fetterer, 1985; Watson et al., 1985). Finally, increases in cardiac ganglion burst rate are associated with decreased
plateau duration and, presumably, decreased motor output to cardiac muscle fibers (Watson et al., 1985; Watson and Groome, unpublished). The amines may act, via cAMP, on pacemaker terminals to enhance transmitter release, increasing the size of the compound EPSP in follower neurons. Enhancement of follower cell depolarization could be important to lengthen the plateau portion of the burst. Therefore, the ultimate consequence of this possible action of amines might be directed at enhancing heart contraction strength, or at least offsetting the negative effects of increased rate on heart contraction amplitude.

Amine receptors and adenylate cyclase in the cardiac ganglion

Octopaminergic and catecholaminergic excitation of heart rate or burst rate in the isolated Limulus cardiac ganglion is blocked by the alpha-adrenergic antagonist phentolamine (Augustine et al., 1982; Augustine and Fetterer, 1985). These results pointed out the similarity of amine action on the Limulus heart to OCT responses in other invertebrate preparations (Batta et al., 1979; Orchard, 1982; Classen and Kammer, 1986; Lange and Orchard, 1986; Evans, 1987). Octopamine receptors have been extensively characterized in insects (reviewed by Evans, 1980). Of these, OCT2 receptors are specifically linked to adenylate cyclase (Nathanson, 1985; Lange and Orchard, 1986; Pannabecker and Orchard, 1986). These receptor types have been described in insect nervous tissue (Nathanson, 1979; Gole et al., 1983, Morton, 1984), smooth muscle (Lange and Orchard, 1986; Orchard and Lange, 1986) and skeletal muscle (Evans, 1984 b,c; Evans, 1987).

An octopamine-sensitive adenylate cyclase has also been described
in the *Limulus* protocerebrum and circumesophageal ganglia (Atkinson et al., 1977). The similarity of octopamine actions on adenylate cyclase activity in the *Limulus* brain and cardiac ganglion suggests the presence of OCT2 receptors in these tissues. Dopamine inhibits the ability of octopamine to activate adenylate cyclase in the *Limulus* brain, even though DA does appear to activate this enzyme in the cardiac ganglion.

The pharmacological profile of adenylate cyclase activity in the *Limulus* cardiac ganglion suggests the presence of separate OCT and DA receptors positively linked to adenylate cyclase. Phentolamine did inhibit the ability of both amines to increase cardiac ganglion cAMP, but its effect was markedly greater for OCT-induced than for DA-induced cAMP elevation. However, OCT itself was more potent than DA in its action to stimulate cAMP production. These data suggest that, in the *Limulus* cardiac ganglion, DA acts on a receptor distinct from the OCT receptor.

Therefore, in *Limulus*, as in several other arthropods (cockroach - Harmar and Horn, 1977; moth - Bodnaryk, 1979; mosquito - Pratt and Pryor, 1986), separate OCT and DA receptors may be linked to a common second messenger system.

**Cyclic AMP, cyclic GMP and phosphodiesterase activity in the *Limulus* cardiac ganglion**

Cyclic GMP does not appear to be involved in excitatory actions of amines on the *Limulus* cardiac ganglion. None of the amines, or forskolin, influenced levels of cardiac ganglion cGMP. However, IBMX was extremely potent in its capacity to increase cGMP in this tissue. The relative increases in cAMP and cGMP in the cardiac ganglion after exposure to $10^{-3}$
M IBMX indicate that while this agent supressed both cAMP- and
cGMP-dependent phosphodiesterases, its action was greatest on the
cGMP-dependent phosphodiesterase.

Although IBMX significantly increased cardiac ganglion cGMP, this
effect was not correlated with an observed physiological response.
Forskolin, OCT and the catecholamines, on the other hand, increased
cardiac ganglion cAMP in proportion with their ability to increase burst
rate, but had no effect on cardiac ganglion cGMP. Therefore, while
increases in cardiac ganglion cGMP may bear some physiological
significance, cGMP does not appear to play a role in chronotropic amine
modulation.

The kinetics of amine actions on cAMP levels within the Limulus
cardiac ganglion indicate the presence of a strong cAMP-dependent
phosphodiesterase. Removal of amine from cardiac ganglion receptors
resulted in a rapid drop in cardiac ganglion cAMP. Cardiac ganglion burst
rate declined during the wash period at a slower rate, presumably due to a
gradual reversal of cAMP stimulation of protein kinase A activity and of
protein phosphorylation.

In the intact heart, negative inotropic actions of amines may occur
during periods of intense chronotropic excitation. The physiological
significance of strong phosphodiesterase activity in the Limulus cardiac
ganglion may be that possible negative inotropic actions of amines are
offset by limiting the duration of chronotropic amine actions. Relative
phosphodiesterase activities in the cardiac ganglion and in cardiac muscle
may coordinate the biochemical effects of amines in these tissues as one
means to maximize cardiac output.
In summary, cyclic AMP is a general effector of amine action in the *Limulus* cardiac ganglion. Octopamine and the catecholamines DA, EPI and NE exert their positive chronotropic actions via a cAMP-, but not cGMP-dependent mechanism most likely occurring within pacemaker neurons. Octopamine, but not DA, utilizes cAMP to depolarize follower cells. Finally, both OCT and DA may enhance synaptic efficacy within the cardiac ganglion. Apparently, amines utilize a common second messenger, cAMP, to elicit a number of excitatory effects on cardiac ganglion neurons of the *Limulus* heart.
CHAPTER TWO
MECHANISM FOR AMINE ACTIONS ON CARDIAC MUSCLE
CONTRACTILITY AND ON NEUROMUSCULAR TRANSMISSION IN THE LIMULUS HEART

ABSTRACT

The mechanism by which octopamine, dopamine, norepinephrine and epinephrine increase heart contraction strength in Limulus was investigated. Amines produced a long-lasting increase in the amplitude of spontaneous (neurally evoked) or electrically evoked heart contractions. At 10^{-5} M, the apparent order of potency for amine-induced increases in evoked contraction amplitude was dopamine ~ octopamine > norepinephrine ~ epinephrine. Amines also elevated cardiac muscle cAMP (octopamine > dopamine ~ norepinephrine ~ epinephrine) in a dose-dependent manner. None of the amines influenced levels of cGMP in Limulus cardiac muscle.

Forskolin, an activator of adenylate cyclase, enhanced cardiac muscle contractility and increased levels of cAMP, but not cGMP, in cardiac muscle tissue. The phosphodiesterase inhibitor IBMX occasionally increased cardiac muscle contractility for a short period, but typically had long-lasting negative effects on contractility. This agent increased levels of both cAMP and cGMP in Limulus cardiac muscle.

Forskolin, and to a lesser extent IBMX, had amine-like effects on cardiac neuromuscular transmission. These agents elicited a prolonged increase in the size of evoked, unitary excitatory junction potentials.
(EJP's) in cardiac muscle fibers without affecting the apparent input resistance of these cells. The results of these studies indicate that cAMP acts as a second messenger of excitatory inotropic amine actions on multiple cellular targets in the Limulus heart.
INTRODUCTION

The neurogenic heart of the horseshoe crab, *Limulus polyphemus*, is modulated by a number of biogenic amines endogenous to the CNS and cardiac ganglion (Augustine et al., 1982). These amines, which include octopamine (OCT), dopamine (DA), norepinephrine (NE) and epinephrine (EPI) act on multiple cellular targets within this system to produce increases in both the strength and rate of heart contractions (reviewed by Watson and Augustine, 1982).

The excitatory inotropic effects of these amines on the *Limulus* heartbeat are the result of enhancement of both cardiac neuromuscular transmission and cardiac muscle contractility (Watson and Hoshi, 1981; Watson et al., 1985). Amines appear to act presynaptically to increase individual quanta of transmitter released by follower neurons onto cardiac muscle fibers. They also act postsynaptically on the muscle fibers to enhance contractility, apparently by altering the process of excitation-contraction coupling.

While the cellular sites of inotropic amine actions in this system have been determined, the biochemical mechanisms by which aminergic modulation of heart contraction amplitude is effected have not been investigated previously. In this chapter, the role of cyclic nucleotides in amine actions on cardiac neuromuscular transmission and on cardiac muscle contractility is examined.
METHODS

Recordings of cardiac muscle contractions

Recordings from intact Limulus hearts were carried out as described in Appendix - General Methodology. To examine the direct effect of amines and drugs on cardiac muscle contractility, cardiac muscle rings (1-2 cm) were excised from deganglionated hearts. Muscle contractions were evoked by electrical stimulation using a modification of the technique employed by Watson et al. (1985). A lateral edge of each muscle ring was pinned to the bottom of a 20 ml stimulation chamber. A steel pin was inserted through the lumen of the ring and attached via a threaded hook to a force transducer (Grass FT. 03C). Contractions were evoked by passing current (1-2 volts, 80 msec at 0.1-0.2 Hz) between vertical stainless steel plate electrodes located 2 mm to either side of the cardiac muscle ring. Two stimulators (Grass Model SD 9) were synchronized (100 msec delay) to send equal and opposing current pulses to prevent electrode plate polarization. Contraction force was recorded with an oscillograph (Grass Model 79D). The preparation was perfused continuously at 5 ml/min with seawater at room temperature. Test solutions were added via the perfusion reservoir.

Neuromuscular transmission

Small sections (2-3 cm) of deganglionated Limulus hearts were opened with a mid-ventral incision and pinned, dorsal side up, to the bottom of a 5 ml perfusion chamber. Flow rate was adjusted to approximately 3 ml/min. The membrane potential of cardiac muscle fibers was recorded (Dagan 8700) after impaling the cells with 10-25 MΩ glass
microelectrodes filled with 3 M KCL. Potentials were displayed and monitored on an oscilloscope (Tektronix 5111). A suction electrode was used to stimulate residual motor nerves in this region of the preparation, thereby eliciting excitatory junction potentials (EJP's) in these muscle fibers. Stimulation parameters were adjusted (0.1-0.4 V, 2-6 msec at 0.5 pps) to obtain the smallest, or unitary, EJP's. Successive EJP's were averaged (30 sweeps) and stored at 5 min intervals using a Nicolet digital oscilloscope (Nicolet Biomedical Instr., Madison, Wisconsin). Test solutions were added to the preparation via the perfusion reservoir.
RESULTS

Amine effects on naturally occurring or evoked heart contractions

As has been reported earlier (Augustine et al., 1982; Watson et al., 1985), dopamine and octopamine typically produced long-lasting increases in contraction amplitude when applied to the intact Limulus heart preparation (Fig. 1a, Chapter One). The positive inotropic effect of DA was preceded by a transient inhibition of contraction amplitude. Norepinephrine and epinephrine also increased heart contraction strength, but to a lesser extent.

In order to test the effect of amines on cardiac muscle contractility, deganglionated myocardial rings were prepared and contractions were evoked by electrical stimulation. All of the amines tested, at 10^{-5} M, produced a gradual increase in the amplitude of evoked heart contractions (Table 2, Fig. 1). Both DA and OCT had larger effects than either NE or EPI at this dose. The enhancement of evoked heart contraction amplitude by DA and OCT was particularly long-lasting (Fig. 2). Contractions typically continued to increase in size for at least 15 min after removal of amine from the bath and only gradually declined to control height with 1-2 hr continuous washing.

Amine effects on levels of cyclic nucleotides in cardiac muscle

Levels of cyclic AMP in Limulus cardiac muscle rings were elevated by 10^{-5} M amine (Table 2, Fig. 3a). Octopamine had a significantly greater effect on cardiac muscle cAMP than any of the other amines tested. The apparent order of potency, at 10^{-5} M, was OCT > DA ~ NE ~ EPI. Both DA and
<table>
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<th>% CTL AMPL.</th>
<th>pmol cAMP</th>
<th>pmol cGMP</th>
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<td>CONTROL</td>
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<td>1.3 ± 0.2 (9)</td>
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<td>OCT</td>
<td>10^-5 M</td>
<td>659.6 ± 135.4 (14)†</td>
<td>54.6 ± 9.6 (8)†</td>
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<td>DA</td>
<td>10^-5 M</td>
<td>680.7 ± 163.0 (18)†</td>
<td>16.4 ± 2.6 (9)*</td>
<td>0.8 ± 0.1 (6)</td>
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<td>NE</td>
<td>10^-5 M</td>
<td>227.7 ± 53.7 (12)†</td>
<td>14.9 ± 1.3 (8)*</td>
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<td>EPI</td>
<td>10^-5 M</td>
<td>217.6 ± 99.9 (12)*</td>
<td>14.4 ± 0.5 (8)*</td>
<td>1.1 ± 0.3 (6)</td>
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<td>FORSK</td>
<td>5 X 10^-6 M</td>
<td>341.1 ± 100.4 (15)*</td>
<td>11.8 ± 0.8 (8)*</td>
<td>1.6 ± 0.2 (6)</td>
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<td>IBMX</td>
<td>10^-3 M</td>
<td>109.1 ± 5.8 (14)</td>
<td>10.6 ± 0.5 (7)*</td>
<td>5.1 ± 1.3 (6)†</td>
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<td>SQ 20,009</td>
<td>10^-4 M</td>
<td>191.3 ± 24.7 (6)*</td>
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<td>RO-20-1724</td>
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<td>110.8 ± 6.4 (4)</td>
<td>7.7 ± 1.0</td>
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Table 2. Summary of the effects of various amines and agents on evoked contractions of deganglionated cardiac muscle and on levels of cyclic nucleotides in that tissue. The mean (± S. E.) of n experiments (shown in parentheses) is represented for measurements of peak physiological effect, and for RIA measurements after 10 min incubation. Level of significant difference from control was determined by a Student's t-test.

Level of significance: † (p ≤ .02); * (p ≤ .05).
Fig. 1. Amines, at $10^{-5}$ M, increased the amplitude of contractions of deganglionated cardiac muscle rings stimulated by electrical current pulses. Amines were added at the arrow and continually perfused over the preparation. Responses to DA and to OCT were more rapid and larger in magnitude than to either NE or EPI. Calibration: vertical: 2 g; horizontal, 1 min.
Fig. 2. Dopamine ($10^{-5}$ M) and octopamine ($10^{-5}$ M) produced similar long-lasting increases in the size of electrically evoked contractions of deganglionated cardiac muscle. Amines were added for 10 min, as indicated by the solid line, and contractions continued to increase for approximately 20 min after removal of amine from the perfusion solution. Each point represents the mean increase in contraction amplitude ± S. E. from 14 to 18 experiments.
Fig. 3. A. Both octopamine (10⁻⁵ M) and dopamine (10⁻⁵ M) increased cardiac muscle cAMP levels with a time course similar to their effect on evoked contraction amplitude. However, the relative magnitude of the cAMP increase for OCT is much greater than that for DA. Each point represents the mean content of cardiac muscle cAMP (± S. E.) in 8 to 9 experiments. B. Neither OCT or DA, at 10⁻⁵ M, increased the cGMP content of Limulus cardiac muscle.
OCT, at $10^{-5}$ M, produced long-lasting increases in cardiac muscle cAMP (Fig. 4). Amine-induced increases in cardiac muscle cAMP persisted for at least 20 min after removal of amine from the incubation solution. None of the amines increased cGMP in Limulus cardiac muscle (Table 2, Fig. 3b).

Amine-induced increases in cardiac muscle cAMP levels were dose-dependent (Fig. 5a), with a threshold of approximately $10^{-6}$ M at 3 min incubation for either amine. Octopamine increased levels of cardiac muscle cAMP to a larger extent than dopamine at all doses tested ($10^{-6}$ M to $5 \times 10^{-5}$ M). Neither OCT or DA affected cardiac muscle cGMP content at any dose tested (Fig 5b).

**Effects of forskolin and IBMX on naturally occurring or evoked heart contractions**

The adenylate cyclase activator forskolin and the phosphodiesterase inhibitor 3-isobutyl 1-methyl xanthine (IBMX) increased the strength of contractions when applied to the intact Limulus heart (Fig. 1b, Chapter One). The excitatory inotropic effects of these compounds were dose-dependent (Fig. 6a), but forskolin was at least 100 X more potent than IBMX in its capacity to increase heart contraction strength.

The time course of the inotropic responses of the Limulus heart to forskolin and IBMX were quite different. Forskolin, at $5 \times 10^{-6}$ M, in most preparations, elicited a gradual and prolonged increase in heart contraction amplitude (Fig. 1b (Chapter One), Fig. 6b). IBMX ($10^{-3}$ M), on the other hand, consistently produced enhancement of heart contractions for only a short period of time, followed by a prolonged decrease in contraction amplitude. Such a biphasic inotropic response was also occasionally observed upon forskolin, OCT or DA application to the intact heart.
Fig. 4. Time course of the onset and washoff of amine-induced cAMP increases in Limulus cardiac muscle. Octopamine (10⁻⁵ M) or dopamine (10⁻⁵ M) were added (solid line) to cardiac muscle rings for up to 10 min, or for 10 min followed by removal of amine. Each point represents the mean content of cardiac muscle cAMP (± S. E.) in 8 to 9 experiments. Amine-induced increases in cAMP declined only gradually after removal of amine from the incubation solution.
A. Cyclic AMP

![Graph showing the effect of amine on cAMP levels](image)

B. Cyclic GMP

![Graph showing the effect of amine on cGMP levels](image)

Fig. 5. Dose-response curve for amine effects on levels of cyclic nucleotides in *Limulus* cardiac muscle. Tests were performed by incubating cardiac muscle rings in various doses of OCT or DA for 3 min. A. Octopamine produced greater increases in cardiac muscle cAMP than DA at all doses above threshold (10⁻⁶ M). B. Neither OCT or DA, at any dose, influenced cardiac muscle cGMP levels in these experiments.
Fig. 6. Forskolin and IBMX, agents which elevate cAMP levels in Limulus cardiac muscle, produced increases in the contraction amplitude of the intact heart. A. Both agents produced dose-dependent increases in heart contraction amplitude. Each point represents the mean increase ± S. E. in 10 to 16 experiments. B. Forskolin (5 x 10⁻⁶ M, n=14) produced a long-lasting increase in contraction amplitude. IBMX (10⁻⁵ M, n=14) had a short-lived positive inotropic effect, followed by long-lasting inhibition of contraction amplitude.
Forskolin, at $5 \times 10^{-6}$ M, induced a gradual and long-lasting increase in the size of evoked heart contractions (Table 2, Fig. 7). Enhancement of contraction amplitude was not blocked by pre-treatment of the preparation with $3 \times 10^{-7}$ M tetrodotoxin (TTX). Since TTX appears to block residual neuronal activity in this preparation (Watson et al., 1985), this result suggests a direct effect of forskolin upon cardiac muscle fibers to increase contractility.

In contrast to the positive inotropic effect of forskolin, IBMX ($10^{-3}$ M) consistently decreased the amplitude of evoked contractions (Table 2, Fig. 7). The effect of IBMX was long-lasting and TTX-resistant. However, in 6 of 14 experiments, IBMX elicited a transient increase in contraction amplitude prior to the onset of inhibition (not shown).

**Effects of forskolin and IBMX on levels of cyclic nucleotides in cardiac muscle**

Forskolin, at $5 \times 10^{-6}$ M, and IBMX, at $10^{-3}$ M, significantly elevated cardiac muscle cAMP (Table 2). Levels of cAMP in cardiac muscle were increased to $164.1 \pm 6.6\%$ control ($n=8$) after 10 min exposure to forskolin, while IBMX increased cardiac muscle cAMP to $172.8 \pm 8.0\%$ control ($n=7$) at this time point. IBMX, but not forskolin, also increased levels of cardiac muscle cGMP (Table 2). The percentage increase in cardiac muscle cGMP elicited by IBMX ($401.3 \pm 102.9\%$ control, $n=6$) at 10 min was greater than the corresponding cAMP increase in this tissue.

A comparison of the physiological and biochemical effects of amines, forskolin and IBMX suggested a rather weak correlation between effects on levels of cardiac muscle cAMP and enhancement of evoked contractions by these agents (Fig. 8). However, a much better correlation was observed if
Fig. 7. Effect of forskolin (5 x 10^{-6} M) and IBMX (10^{-3} M) on electrically evoked contractions in *Limulus* cardiac muscle rings. A. Records of contractions before and 30 min after initial addition of agent to the preparation. Calibration: vertical 1.5 g; horizontal, 1 min. B. The time course of the effect of forskolin and IBMX (added for 15 min; solid line) on evoked contractions was similar to that on the intact heart. Each point represents the mean change in amplitude ± S. E. in 14 to 15 experiments.
Fig. 8. Relationship between capacity of amines (10^{-5} M), forskolin (5 \times 10^{-6} M) and IBMX (10^{-3} M) to increase cardiac muscle cAMP and to alter cardiac muscle contractility. For each point, the y co-ordinate represents the mean increase in cardiac muscle cAMP ± S. E. in 7 to 9 experiments after 10 min incubation. The x co-ordinate represents the mean increase in amplitude of evoked heart contractions in 12 to 18 experiments after 10 min exposure to a particular agent. Using all data points, a simple regression indicated a poor correlation between the effects of these agents on cardiac muscle cAMP and on contractility (r = 0.60). Using the four data points indicated in black, a better correlation was observed (r = 0.98). The agents which do not fit into this line (dopamine and IBMX) may affect other second messenger systems in Limulus cardiac muscle (see discussion).
only OCT, NE, EPI and forskolin were considered. These findings suggest that DA and IBMX may influence cardiac muscle contractility in a way which is not solely dependent on their capacity to increase cardiac muscle cAMP.

**Effect of IBMX on DA- and OCT-induced inotropic responses and cAMP elevation in cardiac muscle**

The phosphodiesterase inhibitor IBMX was ineffective in potentiating the effects of threshold doses of either DA or OCT to 1) increase the contraction strength of the intact *Limulus* heart (Fig 9a) or 2) elevate levels of cardiac muscle cAMP (Fig. 9b). Pharmacological and biochemical tests were performed in the same manner as described in Chapter One.

**Suppression of amine-induced increases in cardiac muscle cAMP by phentolamine**

The alpha-adrenergic antagonist phentolamine, at $10^{-4}$ M, blocked the elevation of cAMP in cardiac muscle rings exposed to either $10^{-5}$ M DA or to $10^{-5}$ M OCT for a period of 3 min (Fig. 10). As was observed in tests of the cardiac ganglion, phentolamine was more effective in inhibiting the effect of OCT on cardiac muscle cAMP levels than it was in suppressing the corresponding effect of DA. Phentolamine itself had a negligible effect on levels of cAMP in *Limulus* cardiac muscle.

**Neuromuscular transmission**

Unitary excitatory junction potentials (EJP's) were increased in size
Fig. 9. The phosphodiesterase inhibitor IBMX did not potentiate amine-induced physiological or biochemical actions in Limulus cardiac muscle. A. IBMX, at doses ranging from $10^{-5}$ M to $10^{-4}$ M in 1 ml, had a slight effect on heart contraction amplitude. IBMX did not enhance the inotropic action of either DA ($10^{-6}$ M in 1 ml) or OCT ($3 \times 10^{-7}$ M in 1 ml) on the intact Limulus heart. B. IBMX, at $10^{-4}$ M, had no effect on the capacity of DA ($10^{-6}$ M) or OCT ($10^{-6}$ M) to increase cardiac muscle cAMP with 3 min of incubation.
Fig. 10. The alpha-adrenergic antagonist phentolamine blocked amine-induced increases in cardiac muscle cAMP. Cardiac muscle rings were treated with $10^{-5}$ M DA or OCT for 3 min in the presence or absence of $10^{-4}$ M phentolamine. Numbers positioned over the areas representing cAMP accumulation in cardiac muscle exposed to amine + phentolamine signify the percent inhibition by phentolamine of the response to a particular amine in comparison to control cAMP levels.
by the application of $5 \times 10^{-6}$ M forskolin or by $10^{-3}$ M IBMX (Fig. 11). This effect was slow in onset and long-lasting, similar to the reported effect of DA on this preparation (Watson et al., 1985). Application of forskolin caused a larger and more prolonged increase in EJP size ($180.2 \pm 5.9\%$ control, $n=11$) than that produced by IBMX ($131.2 \pm 7.0\%$ control, $n=14$). Neither agent altered the apparent input resistance or resting membrane potential of cardiac muscle fibers in these experiments.
Fig. 11. Forskolin and IBMX enhance cardiac neuromuscular transmission. A. Record of averaged (30 sweeps) excitatory junction potentials (EJP's) in cardiac muscle fibers before and during peak response to 5 x 10^-6 M forskolin. Calibration: vertical, 2 mV; horizontal, 80 ms. B. Time course of the increase in EJP size after addition (solid line) of forskolin (5 x 10^-6 M) or IBMX (10^-3 M). Apparent input resistance was unaltered by either forskolin or IBMX, as indicated by no change in the response of cardiac muscle fibers to a hyperpolarizing current pulse prior to elicitation of the EJP.
DISCUSSION

Amines regulate the strength of heart contractions in Limulus by their actions on cellular targets in both the cardiac ganglion and in the myocardium. Specifically, cAMP appears to be an important second messenger of amine actions on both cardiac muscle contractility and on neuromuscular transmission. While cAMP may be the sole second messenger underlying the actions of OCT, NE and EPI to enhance cardiac muscle contractility, DA appears to use some other mechanism, in addition to cAMP, to elicit a similar effect.

The importance of cAMP as a second messenger in the positive inotropic actions of amines has been documented for a wide variety of vertebrate species (Reuter, 1974; Tsien, 1977; Benfey, 1980; Kranias and Solaro, 1982; Earm et al., 1983; Macey et al., 1984). Similarly, a cAMP-dependent mechanism appears to underly the excitatory actions of amines on the myogenic hearts of a number of molluscan species (Higgins, 1974; Weiss et al., 1982; Sawada et al., 1984; Paciotti and Higgins, 1985).

In contrast to these studies correlating cAMP with inotropic amine modulation of myogenic hearts, very little is known of the intracellular mechanisms by which amines enhance contraction strength in arthropod neurogenic hearts. Octopamine has been shown to increase the cAMP content and contraction strength of the lobster heart (Battelle and Kravitz, 1978). However, in that study, physiological and biochemical tests were performed on the intact neurogenic heart, so the site of inotropic OCT action was not determined. Further, that study did not examine the role of cAMP in amine actions on cardiac neuromuscular transmission. The results from the present study of the Limulus heart represent the first demonstration of a cAMP-dependent mechanism underlying specific and
localized actions of amines to enhance the contraction strength of a neurogenic heart.

**Role of cAMP in amine actions on cardiac muscle contractility**

Octopamine, NE, EPI and forskolin all increased the amplitude of electrically evoked contractions of *Limulus* cardiac muscle with a potency and time course consistent with their relative capacity to increase cardiac muscle cAMP. Since these effects are not affected by TTX, it appears that amines influence the process of excitation-contraction coupling in the *Limulus* heart by increasing cardiac muscle cAMP. Amines, most notably 5-HT, also utilize cAMP to enhance E-C coupling in the hearts of *Aplysia* (Sawada et al., 1984; Drummond et al., 1985) and *Mercenaria* (Higgins, 1974, Paciotti and Higgins, 1985). Cyclic AMP has been implicated in a variety of direct actions of amines on other invertebrate muscles as well (Weiss et al., 1979; Ram et al., 1983; Evans, 1984 b,c; Weiss et al, 1985; Gies, 1986). These and similar studies in vertebrates (reviewed by Cerione et al., 1983) suggest a general messenger role for cAMP in amine modulation of muscle contractility.

The subcellular sites of cAMP action in the *Limulus* heart are presently unknown. In the vertebrate heart, cAMP-dependent phosphorylation is important in regulating a number of processes influencing the strength of heart contractions (reviewed by Tsien, 1977; Katz, 1979; Winegrad et al., 1983). A likely site of action for cAMP in inotropic modulation in the *Limulus* heart is promotion of internal calcium release (e.g. phosphorylation of sarcoplasmic reticulum proteins, reviewed by Tada and Katz, 1982 and Kranias and Solaro, 1983). In invertebrates, cyclic AMP-induced release of internal calcium has been proposed as the
means by which serotonin increases cardiac muscle contractility in Aplysia (Sawada et al., 1984).

**Dopamine effects on contractility and cardiac muscle cAMP**

Dopamine, like the other amines tested, significantly increased cAMP in cardiac muscle with a time course similar to its effect on cardiac muscle contractility. However, the magnitude of the DA-induced cAMP increase, relative to its inotropic effect, was much less than that produced by OCT, NE or EPI. While DA appears to utilize cAMP to enhance cardiac muscle contractility, this amine probably employs some other system, in addition to cAMP, to produce its positive inotropic effect.

The peptide proctolin, like the amines, enhances cardiac muscle contractility (Benson et al., 1981; Watson et al., 1983). Dopamine has been shown to have a proctolin-like action on deganglionated Limulus heart muscle during glutamate-induced contractions (Watson et al., 1985). These authors speculated that dopamine and proctolin may act via similar mechanisms to produce their similar inotropic effects. In Chapter Four, the biochemical mechanisms underlying the similarity of dopamine and proctolin actions on Limulus cardiac muscle are investigated.

**Role of cAMP in amine actions on neuromuscular transmission**

Amines enhance cardiac neuromuscular transmission, and this effect contributes to overall inotropic modulation of the Limulus heart (Watson and Hoshi, 1981; Watson et al., 1985). The finding that, like the amines, forskolin and IBMX increased the size of EJP's in Limulus cardiac muscle fibers supports the hypothesis that amines utilize a cAMP-dependent
mechanism in their excitatory action at the cardiac neuromuscular junction.

Increases in EJP size brought about by amines, forskolin or IBMX may be a result of cAMP elevation in follower cell terminals. Presynaptic cAMP-dependent protein kinase activity appears to be associated with the phosphorylation state of synaptic vesicles (Ueda and Greengard, 1977; Browning et al., 1987) and voltage-sensitive calcium channels (Curtis and Catterall, 1985; Armstrong and Eckert, 1987) in the vertebrate brain. Cyclic AMP-dependent protein kinase has also been shown to be present and active in arthropod synaptosomes (Kelly, 1983; Haro et al., 1987). These findings suggest that cAMP may play an important role in the regulation of synaptic (e.g. neuromuscular) efficacy via modulation of transmitter release.

Other work indicates that Ca++/calmodulin protein kinase (Kelly, 1983; Llinas et al., 1985; Robinson et al., 1987) and calcium/phospholipid protein kinase (Yandrasitz et al., 1986; Nichols et al., 1987; Haimann et al., 1987; Ozawa et al., 1987) also are important in the regulation of transmitter release. These systems may act independently or in concert with cAMP to modulate synaptic efficacy.

In Limulus, octopamine, the catecholamines and forskolin all produce a similar excitatory effect on evoked transmitter release at the neuromuscular junction. While cAMP appears to be important in this process, the contribution of other second messenger systems for the action of particular amines remains unknown. For example, dopamine may have a biochemical effect at the neuromuscular junction similar to its action in cardiac muscle, where the activity of multiple second messenger systems appear to be involved.
Cyclic AMP and cyclic GMP as opposing regulators of heart contraction strength

Cyclic AMP appears to play a major role in the excitatory pre- and post synaptic inotropic effects of amines on the Limulus heart. Cyclic GMP is not involved in excitatory amine actions in this system. It is possible, however, that cGMP opposes the excitatory influences of cAMP on heart contraction strength.

The phosphodiesterase inhibitor IBMX increased levels of both cAMP and cGMP in the Limulus cardiac ganglion (Table 1, Chapter One). Increased cGMP in the cardiac ganglion might explain the relatively weak effect of IBMX on evoked EJP's. While forskolin (5 x 10^{-6} M) and IBMX (10^{-3} M) increased cAMP to a similar extent in the cardiac ganglion, forskolin produced much greater and longer-lasting increases in EJP size than did IBMX. The magnitude of the effect of IBMX on evoked EJP's could possibly have been partially masked by concomitant increases of both cAMP and cGMP in follower neuron terminals. In the Mytilus anterior byssus retractor muscle, IBMX and 8-bromo cGMP relax muscle tension, apparently via actions in presynaptic terminals (Matsuura, 1984). However, cAMP has also been proposed as the messenger for relaxation in this system (Painter, 1982b; Castellani and Cohen, 1987). Similarly, both cAMP and cGMP analogues mimic the inhibitory action of serotonin on neuromuscular transmission in the radula protractor muscle of Rapana (Fujiwara and Kobayashi, 1983). The nature of neuromuscular inhibition in these systems, and in Limulus, clearly requires a further investigation of the relative roles of cAMP and cGMP as possible intracellular messengers.

It is possible that the biphasic inotropic response of the Limulus heart to IBMX is a consequence of the biochemical effect of IBMX on both
cAMP and cGMP content in cardiac muscle. IBMX clearly inhibited cardiac muscle contractility, even though it did increase cardiac muscle cAMP. IBMX was capable of enhancing the strength of naturally occurring or evoked contractions, but only transiently. The negative inotropic effects of this compound may be a result of increased cGMP in Limulus cardiac muscle. In the locust Schistocerca, millimolar concentrations of IBMX increase levels of both cAMP and cGMP in the extensor tibiae muscle (Evans, 1984a). Increased muscle cGMP may oppose the stimulatory effects of cAMP on the myogenic rhythm in this preparation. In vertebrates, cGMP mediates contractile inhibition in vascular muscle in opposition to cAMP (Winquist et al., 1984; Macleod et al., 1987).

The discovery that cAMP and cGMP have opposing actions in many bidirectional systems has led to the hypothesis that these messengers are universal antagonists of intracellular regulation (Goldberg and Haddox, 1977). The results of the present study suggest that while this relationship may be applicable in the Limulus heart, further work is clearly necessary to clarify the role of cGMP in this system.

**Contraction amplitude, heart rate and IBMX**

A negative inotropic effect is sometimes observed during bath application of amines to the isolated, intact Limulus heart (Watson et al., 1985). However, this effect appears to be a consequence of increased heart rate, and not a direct, negative effect of amines on cardiac muscle contractility. Further support for this hypothesis is suggested by a comparison of the effects of forskolin or IBMX on neurally evoked (rate effects influence heart contraction amplitude) versus evoked heart contractions (rate effects eliminated). Forskolin elicited a much greater
effect on contraction size when applied to electrically stimulated cardiac muscle in comparison to its effect on the intact heart. Additionally, IBMX decreased the size of evoked heart contractions to a lesser extent than it did the size of spontaneous contractions. Therefore, amines, forskolin and IBMX are all capable of reducing heart contraction amplitude due to excessive rate increases. However, IBMX also has a direct inhibitory action on cardiac muscle fibers, and this action may involve a cGMP-dependent mechanism.

Amine receptors and adenylate cyclase

A number of amine receptors may be present in Limulus cardiac muscle. The effects of phentolamine on OCT- and DA-induced elevation of cAMP in cardiac muscle suggest that separate OCT and DA receptors may be coupled to adenylate cyclase in this tissue. Multiple amine-sensitive adenylate cyclases have been described in Aplysia cardiac muscle (Kebabian et al., 1979; Drummond et al., 1985). In this system, 5-HT is approximately 10 x more potent than DA in its ability to increase cardiac muscle cAMP, but the two amines are virtually equipotent with regard to inotropic actions on the Aplysia heart (Wernham and Lukowiak, 1983; Drummond et al., 1985). A similar discrepancy exists between OCT and DA in Limulus cardiac muscle; the relative magnitude of amine effects on adenylate cyclase activity and on inotropy are dissimilar. These findings suggest that in the myogenic Aplysia heart, and in the neurogenic Limulus heart, DA acts on several receptors to elicit its overall effect on cardiac muscle contractility.

Cardiac muscle phosphodiesterase
Several facts indicate that the cAMP-dependent phosphodiesterase in cardiac muscle exhibits weak enzymatic activity in comparison to the phosphodiesterase present in the cardiac ganglion. First, cyclic AMP produced in cardiac muscle transiently exposed to amine was only slowly degraded to the inactive 5'-AMP metabolite. Second, IBMX was ineffective in potentiating subthreshold doses of DA or OCT on heart contraction amplitude or cardiac muscle cAMP. Finally, various phosphodiesterase inhibitors had little or no effect on cardiac muscle cAMP.

The relative activities of cardiac ganglion and cardiac muscle cAMP phosphodiesterases may be important in the fine tuning of simultaneous modulation of heart contraction rate and strength by amines. As discussed in Chapter One, rate increases by amine-stimulated cAMP production in the cardiac ganglion may be limited by a highly active cAMP-dependent phosphodiesterase. Likewise, cAMP elevation (and contractility increases) may be maximized in Limulus cardiac muscle by a weak cAMP-dependent phosphodiesterase. A balance between the excitatory effects of amines on cellular targets in this system might be achieved by the relative activities of cardiac ganglion and cardiac muscle phosphodiesterases.

In summary, cAMP is an important second messenger of excitatory inotropic amine actions on the neurogenic Limulus heart. Amines utilize a cAMP-dependent mechanism to enhance cardiac neuromuscular transmission. Additionally, amines increase cardiac muscle contractility by increasing levels of cardiac muscle cAMP. Cyclic GMP may have a cAMP-opposing, inhibitory action on cardiac muscle contractility. These findings, taken together with the results presented in Chapter One, indicate that amine modulation of the Limulus heartbeat is effected, in general, by
simultaneous increases in cAMP at specific cellular targets in the cardiac ganglion and in cardiac muscle.
CHAPTER THREE

DISTRIBUTION AND PURIFICATION OF PROCTOLIN-LIKE AND

FMRFAMIDE-LIKE PEPTIDES IN THE *LIMULUS* NERVOUS SYSTEM

ABSTRACT

The distribution of proctolin-like and FMRFamide-like peptides in *Limulus* was determined by radioimmunoassay. Immunoreactive (iR-) proctolin was found in greatest concentrations in the cardiac ganglion (185.8 ± 24.8 pg / mg). The brain (97.7 ± 9.7 pg / mg), ventral nerve cord (74.1 ± 4.5 pg / mg) and cardioregulatory nerves (53.8 ± 7.5 pg / mg) also contained substantial amounts of iR-proctolin. Peripherally, high levels of iR-proctolin were detected in the *Limulus* hindgut (69.5 ± 11.3 pg / mg).

Proctolin-like peptides were isolated from acetic acid extracts of *Limulus* brain, cardiac ganglion, ventral nerve cord and hindgut. Two factors exhibiting proctolin-like immunoreactivity and bioactivity were separated by gel filtration. One of these factors (proctolin-like peak one, PP1) co-eluted with synthetic proctolin on Sephadex G-15. The PP1 factor also co-eluted with synthetic proctolin on ion exchange and reverse phase high pressure liquid chromatography (HPLC) columns. The other proctolin-like factor (proctolin-like peak two, PP2) eluted late on Sephadex G-15, but co-eluted with synthetic proctolin and with PP1 on HPLC. Both factors had proctolin-like actions on cockroach hindgut, *Limulus* hindgut and on the *Limulus* heart.

FMRFamide-like peptides were also found to be widely distributed in
**Limulus.** The ventral nerve cord (12.6 ± 3.3 pg / mg) and brain (11.9 ± 1.7 pg / mg) contained the highest quantities of iR-FMRFamide. Immunoreactive FMRFamide was also detected in the branchial nerves (11.2 ± 2.6 pg / mg) and in the cardiac ganglion (4.6 ± 2.4 pg / mg).

**Limulus** brain, cardiac ganglion and ventral nerve cord extracts were used to purify FMRFamide-like peptides. A high molecular weight factor (FMRFamide-like peak one, F1) was detected on Sephadex G-15. This factor did not co-elute with synthetic (clam) FMRFamide on this column, but did co-elute with FMRFamide using ion exchange chromatography. The F1 peak was separated into two iR-FMRFamide peaks by HPLC. One of these factors co-eluted with clam FMRFamide and the other factor co-eluted with the *Helix* FMRFamide-like peptide pQDPFLRFamide. These findings describe the presence and partial purification of several proctolin-like and FMRFamide-like peptides in *Limulus.*
INTRODUCTION

Several cardioexcitatory peptides are present in the Limulus nervous system. These include a proctolin-like peptide or peptides and several FMRFamide-like peptides (Watson et al., 1983; Watson et al., 1984). Proctolin or proctolin-like peptides are present in many arthropods, including insects (Brown, 1977; Bishop et al., 1981; Kingan and Titmus, 1983), crustaceans (Sullivan, 1979; Marder et al., 1986; Siwicki and Bishop, 1986) and arachnids (Groome et al., in preparation). FMRFamide and FMRFamide-like peptides have primarily been described in molluscan species (reviewed by Price et al., 1987). However, other members of the FMRFamide-like family of peptides are present in coelenterates (Grimmelikhuijzen and Spencer, 1984), flatworms (Jennings et al., 1987), annelids (Kuhlman et al., 1985), insects (Boer et al., 1980; White et al., 1986) and crustaceans (Callaway et al., 1987; Marder et al., 1987).

A proctolin-like peptide has been identified in the cardiac ganglion of Limulus (Benson et al., 1981; Watson et al., 1983). This peptide is similar to synthetic proctolin in terms of apparent molecular weight and susceptibility to enzymatic degradation. Both synthetic proctolin and the Limulus cardiac ganglion proctolin-like peptide have positive inotropic effects on the Limulus heart. However, purification and physiological characterization of this peptide or other proctolin-like peptides in Limulus is incomplete at present.

Several FMRFamide-like peptides have also been partially purified from various regions of the Limulus nervous system (Watson et al., 1984). One of these has tentatively been named limadrin (Limulus adrenalin) because of its pronounced positive chronotropic effect on the isolated
Limulus heart (White and Watson, 1984). Limadrin appears to be a relatively high molecular weight peptide which elicits prolonged contracture of the FMRFamide-sensitive radula protractor muscle of the left-handed whelk, Busycon contrarium. Isolation and purification of this cardioexcitatory peptide, and other FMRFamide-like peptides in Limulus, is necessary in order to understand their physiological actions.

The actions of limadrin and proctolin on the neurogenic Limulus heart are quite distinct. The positive chronotropic action of limadrin is similar to that produced by biogenic amines, while the positive inotropic action of proctolin resembles the effect of amines to enhance cardiac muscle contractility. Purification of cardioexcitatory peptides in Limulus will facilitate investigation of the comparative cellular and molecular actions of amines and peptides on the Limulus cardiac rhythm.
METHODS

Extractions and assays

These procedures are detailed in Appendix - General Methodology. Peptide distribution measurements were obtained from acetic acid extracts of Limulus tissues using radioimmunoassay (RIA).

Four chromatographic procedures were used to purify peptides from extracts of Limulus tissues. Proctolin-like and FMRFamde-like fractions from each chromatographic purification step were detected using coincidental RIA and bioassay. Peptide levels were calculated by linear regression of the standard curve and expressed as immunoreactive (iR-) or bioactive pg peptide per milligram of original wet tissue weight.

1. Gel filtration chromatography

Samples were reconstituted in 2 ml column buffer (0.1 N acetic acid with .01% thiodiglycol) and applied to a 65 x 1.6 cm Sephadex G-15-120 molecular sieve column. Fractionation was accomplished at a flow rate of 0.25 ml/min and 3 ml fractions were collected (Buchler LC-100; Buchler Instr., Fort Lee, New Jersey). Fractions were assayed for optical density at 280 nm, dried by vacuum centrifugation and stored at -20 °C until RIA and bioassay. The column was calibrated with the following compounds (the molecular weight and assay for these is in parentheses): blue dextran (void volume, visible band), vitamin B$_{12}$ (1355, visible), proctolin (648, cockroach hindgut), FMRFamide (599, RIA), dopamine (190, O.D.$_{280}$) and serotonin (176, O.D.$_{280}$).
2. **Sep-Pak elution**

Sep-Pak C₁₈ cartridges (Waters Associates, Milford, Massachusetts) were rinsed with 5 ml 95% ethanol, followed by 10 ml of distilled water. Samples were reconstituted in 5 ml distilled water and loaded into the cartridges. Elution was carried out with successive 5 ml rinses of 0%, 10% and 80% acetonitrile containing 0.1% trifluoroacetic acid (TFA). Immunoreactive peptides were concentrated in the 80% elution. All samples were dried and stored at -20 °C.

3. **Ion Exchange chromatography**

Pooled proctolin-like or FMRFamide-like activities from Sephadex G-15 (brain extracts only) were reconstituted in 2 ml 50 mM ammonium acetate buffer (pH 6.0) and applied to a 10 x 1 cm CM Sephadex C-25-120 ion exchange column. The material was eluted with a rinse series of 20 ml each of the following: 50 mM ammonium acetate (pH 6.0), 50 mM ammonium acetate (pH 8.0) and 500 mM ammonium acetate (pH 8.0). Flow rate was set at 0.35 ml/min and 3.3 ml fractions were collected (LKB 2212 Helirac, Bromma, Sweden). Samples were assayed for both O.D₂₈₀ (Beckman DB-GT spectrophotometer) and osmolality (Wescor 5100 C vapor pressure osmometer), dried, and stored at -20 °C until assayed for peptide content.

4. **High pressure liquid chromatography (HPLC)**

Samples and standards were reconstituted in 140 μL 0.1% TFA (Buffer
A). Buffer B consisted of 90% acetonitrile in 0.1% TFA. Samples and standards were then injected onto a 5 μm reverse phase C_{18} HPLC column (RP-18 Spheri-5, Brownlee Labs, Santa Clara, California). Flow rate was set at 1 ml/min at 1300-1400 psi and 1-2 ml fractions were collected (LKB 2212 Helirac). Isocratic and linear acetonitrile gradients were established by mixing Buffer A and Buffer B (Perkin Elmer Series 410 BIO LC pump and controller). All fractions were measured for absorbance (204 -285 nm; LKB 2140 Rapid Spectral Detector), assayed by RIA, and the active fractions pooled and dried for further HPLC purification if necessary. The column was rinsed for 30 min with 100% acetonitrile and 30 min with Buffer A before injection of the next sample.
RESULTS

Distribution of iR-proctolin in Limulus

Immunoreactive proctolin was detected in all regions of the Limulus nervous system, and peripherally in the hindgut (Table 3). The distribution of iR-proctolin using RIA corresponded to immunohistochemical observations (Watson and Detschaschell, personal communication; Wyse, personal communication).

The cardiac ganglion contained the greatest amount (185.8 ± 24.8 pg) of iR-proctolin per mg tissue. This finding is in agreement with earlier results using bioassay to detect cockroach hindgut-stimulating factors in the Limulus nervous system (Watson et al., 1983).

In the Limulus brain (prosomal CNS), iR-proctolin was distributed equally between the protocerebrum (103.2 ± 4.4 pg / mg) and the circumesophageal ganglia (91.7 ± 9.4 pg / mg). The first abdominal ganglion (127.0 ± 35.7 pg / mg) contained more iR-proctolin than the remaining ganglia of the ventral nerve cord, while the second ganglion (44.8 ± 6.7 ± pg / mg) contained the least amount. Segmental cardiac nerves 9 and 10, which originate from the first and second ganglia of the ventral nerve cord, respectively, showed a similar but less dramatic distribution of iR-proctolin (SCN 9, 63.1 ± 12.3 pg / mg, n=8; SCN 10, 47.1 ± 7.8 pg / mg, n=11).

The Limulus hindgut contained high levels of iR-proctolin (69.5 ± 11.3 pg / mg), distributed equally in the four regions tested. In contrast, the foregut and esophagus contained almost no iR-proctolin.

The gill nerves (the proximal portions of the medial, external and
<table>
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<tr>
<th>Tissue</th>
<th>pg proctolin / mg</th>
<th>ng proctolin / tissue</th>
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<tr>
<td>Cardiac Ganglion</td>
<td>185.8 ± 24.8</td>
<td>3.3 ± 0.4</td>
<td>(15)</td>
</tr>
<tr>
<td>Cardiac Muscle</td>
<td>3.1 ± 0.9</td>
<td>6.9 ± 2.0</td>
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</tr>
<tr>
<td>Prosomal CNS</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>a. Protocerebrum</td>
<td>97.7 ± 9.7</td>
<td>30.1 ± 1.2</td>
<td>(15)</td>
</tr>
<tr>
<td>b. C. E. Ganglia</td>
<td>91.7 ± 9.4</td>
<td>19.7 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>Ventral Nerve Cord</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>a. Abd. Ganglion 1</td>
<td>74.1 ± 4.5</td>
<td>12.0 ± 0.8</td>
<td>(15)</td>
</tr>
<tr>
<td>b. Abd. Ganglion 2</td>
<td>127.0 ± 35.7</td>
<td>4.4 ± 1.2</td>
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<td>c. Abd. Ganglion 3</td>
<td>44.8 ± 6.7</td>
<td>1.6 ± 0.2</td>
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<td>d. Abd. Ganglia 4,5</td>
<td>60.8 ± 17.8</td>
<td>1.8 ± 0.5</td>
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<td>Ventral Nerve Cord</td>
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<td>a. Abd. Ganglion 1</td>
<td>63.6 ± 13.4</td>
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<td>Optic Nerve</td>
<td>10.0 ± 3.0</td>
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<td>Lateral Eye</td>
<td>2.2 ± 0.6</td>
<td>0.7 ± 0.1</td>
<td>(8)</td>
</tr>
<tr>
<td>Leg Nerve</td>
<td>12.1 ± 2.6</td>
<td>2.2 ± 0.5</td>
<td>(10)</td>
</tr>
<tr>
<td>Leg Muscle</td>
<td>4.4 ± 0.6</td>
<td>0.8 ± 0.1</td>
<td>(13)</td>
</tr>
<tr>
<td>Gill Nerve</td>
<td>27.4 ± 2.5</td>
<td>0.8 ± 0.1</td>
<td>(12)</td>
</tr>
<tr>
<td>Gill Muscle</td>
<td>7.3 ± 1.2</td>
<td>0.5 ± 0.1</td>
<td>(10)</td>
</tr>
<tr>
<td>Esophagus</td>
<td>4.9 ± 1.0</td>
<td>2.7 ± 0.5</td>
<td>(13)</td>
</tr>
<tr>
<td>Foregut</td>
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<td>26.1 ± 4.3</td>
<td>(15)</td>
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<tr>
<td>Hindgut</td>
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<td>272.3 ± 44.3</td>
<td>(17)</td>
</tr>
<tr>
<td>a. Region 1</td>
<td>59.2 ± 10.2</td>
<td>68.1 ± 9.8</td>
<td></td>
</tr>
<tr>
<td>b. Region 2</td>
<td>71.9 ± 10.6</td>
<td>72.3 ± 10.1</td>
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</tr>
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<td>c. Region 3</td>
<td>78.6 ± 14.7</td>
<td>56.6 ± 12.3</td>
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</tr>
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<td>d. Region 4</td>
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<tr>
<td>Ostial Tissue</td>
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<td>(10)</td>
</tr>
<tr>
<td>a. Region 1</td>
<td>8.0 ± 3.1</td>
<td>0.3 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>b. Region 2</td>
<td>8.2 ± 2.6</td>
<td>0.3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>c. Region 3</td>
<td>6.6 ± 3.7</td>
<td>0.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Hepatopancreas</td>
<td>1.7 ± 0.8</td>
<td>********</td>
<td>(11)</td>
</tr>
<tr>
<td>Blood</td>
<td>2.0 ± 0.3 x 10^-10 M</td>
<td>********</td>
<td>(35)</td>
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Table 3. RIA for proctolin content of Limulus tissues. Levels of proctolin are expressed in terms of pg / mg tissue weight ± S. E. and ng / region ± S. E. For certain tissues (e. g. ventral nerve cord), the sum of several ganglia or tissue regions is also calculated.
internal branchial nerves) contained a relatively high concentration (27.4 ± 2.5 pg / mg) of iR-proctolin. In contrast, the optic nerves and leg nerves had lower levels of iR-proctolin. Several tissues (lateral eye, tibia flexor muscle of the second or third walking leg, cardiac muscle and gill muscle 48 of the second or third gill appendage) contained less iR-proctolin than the nerves associated with these tissues. Ostial connective tissues were assayed (7.7 ± 3.1 pg / mg) in a preliminary investigation of putative pericardial organs in *Limulus*. Finally, blood levels of iR-proctolin were detectable (2 x 10^{-10} M) in cardiac puncture samples.

**Purification of proctolin-like peptides from *Limulus* tissues**

Several *Limulus* tissues (brain, cardiac ganglion, ventral nerve cord and hindgut) were used to isolate and purify proctolin-like peptides. Extracts of these tissues were subjected to several chromatographic steps and purified using reverse phase HPLC.

Molecular weight separation (Sephadex G-15) of TCA extracts of these tissues yielded several peaks of proctolin-like activity as determined by the cockroach hindgut bioassay and by RIA (Figs. 1,2). In general, four peaks of bioactivity were observed in each tissue. Two of these peaks, Peak B (PP1, proctolin-like peak one) and Peak C (PP2, proctolin-like peak two) were also detected by RIA. The two bioactive peaks which did not display immunoreactivity were not considered further in the purification sequence. Synthetic proctolin co-eluted with PP1, while PP2 eluted further on this column. Activities from PP1 and from PP2 were pooled separately and prepared for ion exchange or HPLC chromatography by passing the sample through Sep-Pak C_{18} cartridges and collecting the 80%
Fig. 1. Sephadex G-15 gel filtration profile of proctolin-like activity in Limulus brain extracts. A. Four peaks of cockroach hindgut-stimulating activity are observed. Two of these, Peak B (PP1) and Peak C (PP2), also are detected by the proctolin radioimmunoassay (B). Elution position of standards are indicated by arrows in the top graph. Peak B (PP1) co-elutes with synthetic proctolin, while Peak C (PP2) is of an apparently lower molecular weight.
Fig. 2. Sephadex G-15 gel filtration profile of proctolin-like activity in extracts of Limulus ventral nerve cord, cardiac ganglion and hindgut. Like the Limulus brain, these tissues contain two peaks (PP1, PP2) of coincidental bioactivity and immunoreactivity.
elution.

In a separate Sephadex G-15 run of 20 Limulus brains, active fractions from either PP1 or PP2 were pooled and passed through Sep-Pak cartridges. The two peaks were dried, reconstituted in 2 ml seawater, adjusted to pH 7.6 with .05 M NaOH and applied directly to an intact Limulus heart in a 5 ml recording chamber. The effect of either extract was similar to that of synthetic proctolin (Fig. 3). Heart contraction amplitude was increased by PP1 (80.0%) and by PP2 (63.7%). Rate increases produced by PP1 (16.7%) and PP2 (23.8%) were less pronounced.

Proctolin-like peak one was of considerable interest at this time since it appeared to resemble the proctolin originally isolated from the cockroach Periplaneta (Brown and Starrat, 1975). Most of the material collected for further analysis was restricted to PP1 using Limulus brain extracts.

Ion exchange chromatography (Limulus brain PP1) yielded one peak of activity as revealed by either bioassay or RIA (Fig. 4). This material eluted with an increase in osmolarity in the ammonium acetate buffer. Measurements of O.D._280 of these fractions revealed an absorbance peak in the first four fractions, indicating that the pH 6, 50 mM ammonium acetate stage separated contaminating material from the proctolin-like activity which eluted later.

Limulus brain PP1 was run on three separate gradients on reverse phase HPLC. At each step, active fractions were detected by RIA and pooled. The final HPLC protocol consisted of a 10 min isocratic step (18.0% acetonitrile in 0.1% TFA) followed by a 50 min linear gradient to 31.5% acetonitrile. Proctolin-like peak one co-eluted with synthetic proctolin on this and the other gradients employed. Synthetic proctolin eluted at 10.2
Fig. 3. Partially purified *Limulus* proctolin-like peptides excite the neurogenic *Limulus* heart. Proctolin-like activities (PP1 and PP2) from Sephadex columns were concentrated and passed through Sep-Pak C18 cartridges to eliminate hydrophilic contaminants. Synthetic proctolin (30 ng), PP1 (20 brain equiv.) or PP2 (20 brain equiv.) was reconstituted in 2 ml seawater, adjusted to pH 7.5 and added directly to the continually perfused intact heart preparation as indicated by the arrow. Proctolin, PP1 and PP2 all caused a long-lasting increase in the amplitude of heart contractions. Effects on heart rate were lesser in magnitude and duration. Calibration: vertical, 2 g; horizontal, 1 min.
Fig. 4. Ion exchange chromatography of Limulus brain extract PP1. This peak was pooled and passed through Sep-Pak C18 cartridges. The 80% acetonitrile elution was dried, reconstituted in 50 mM, pH 6.0 ammonium acetate buffer and applied to a 10 x 1 cm CM Sephadex column. Bioactive (A) and immunoreactive (B) proctolin-like activity was concentrated in two fractions with the onset of increased buffer osmolarity. Inset (A): response of the cockroach hindgut to a 3 brain equiv. aliquot of fraction 14.
min (fraction 11) and iR-proctolin in PP1 was detected in fractions 10-12 (Fig. 5a). Cockroach hindgut-stimulating activity was observed in fractions containing iR-proctolin (not shown).

The PP1 factor from the ventral nerve cord, cardiac ganglion and hindgut had similar an elution pattern to synthetic proctolin or Limulus brain PP1 run on this gradient (Figs. 5b, 6a,c). Differences in elution times between iR-proctolin in these extracts and synthetic proctolin were generally 1 min or less.

The cardiac ganglion and hindgut extracts already processed through G-15 provided an excellent source of material to compare and purify PP1 and PP2 by HPLC. Interestingly, both proctolin-like factors from Limulus cardiac ganglia and hindgut extracts co-eluted with synthetic proctolin on HPLC (Fig. 6). Also, cockroach hindgut-stimulating activity was noted for cardiac ganglion PP1 and PP2 after HPLC in the same proportion as that observed for these factors after molecular weight separation (Fig. 7a). These data indicate that some portion of the proctolin-like factor in PP1 is structurally very similar to the factor in PP2.

Aliquots of hindgut PP1 and PP2 purified by HPLC were applied to the Limulus hindgut (Fig 7b). Like the effect of synthetic proctolin, both PP1 and PP2 produced rhythmic contractions imposed upon a sustained contracture of the hindgut. Purified PP1 from the ventral nerve cord also produced a proctolin-like response on the Limulus gut (Fig. 7c).

Distribution of iR-FMRFamide in Limulus

Immunoreactive FMRFamide was detected in all regions of the Limulus nervous system (Table 4). Levels of iR-FMRFamide were highest in the
Fig. 5. HPLC profiles of PP1 from Limulus brain (A) or ventral nerve cord (B) extracts. Samples were reconstituted in 20% buffer B (90% acetonitrile containing 0.1% TFA), injected onto a reverse phase HPLC column and run through the gradient shown by the solid line. For either extract, proctolin-like immunoreactivity eluted within 1 min of the elution time of synthetic proctolin.
Fig. 6. HPLC profiles of *Limulus* cardiac ganglion and hindgut PP1 (A,C) and PP2 (B,D). Samples were run through Sephadex G-15 columns and Sep-Pak C$_{18}$ cartridges to concentrate proctolin-like activities. The HPLC profiles of PP1 or PP2 were very similar, and both activities eluted closely to the elution time of synthetic proctolin.
Fig. 7. HPLC purified Limulus PP1 and PP2 elicited proctolin-like responses on the hindgut of Periplaneta and Limulus. A. Synthetic proctolin (5.1 ng in 1 ml), cardiac ganglion PP1 (4.3 ng iR-proctolin in 1 ml) and PP2 (5.6 ng iR-proctolin in 1 ml) all produced similar responses on the cockroach hindgut. B. Synthetic proctolin (10.0 ng in 2 ml), hindgut PP1 (4.1 ng in 2 ml) and PP2 (15.2 ng in 2 ml) produced contracture and contractions when applied to the Limulus hindgut. C. An aliquot of iR-proctolin from an extract of ventral nerve cord PP1 (1.2 ng in 2 ml) produced a similar response to that produced by synthetic proctolin (5.1 ng in 2 ml) in a more sensitive Limulus hindgut preparation. Calibration: vertical, 250 mg (A), 400 mg (B,C); horizontal, 2 min.
TABLE 4 - Distribution of immunoreactive FMRFamide in *Limulus*

<table>
<thead>
<tr>
<th>Tissue</th>
<th>pg FMRFamide / mg</th>
<th>pg FMRFamide / tissue</th>
<th>(n)</th>
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<tbody>
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<td>Cardiac Ganglion</td>
<td>4.6 ± 2.4</td>
<td>81.0 ± 42.2</td>
<td>(11)</td>
</tr>
<tr>
<td>Cardiac Muscle</td>
<td>0.5 ± 0.2</td>
<td>817.5 ± 327.0</td>
<td>(13)</td>
</tr>
<tr>
<td><strong>Prosomal CNS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Protocerebrum</td>
<td>11.9 ± 1.7</td>
<td>3650.7 ± 262.4</td>
<td>(18)</td>
</tr>
<tr>
<td>b. C. E. Ganglia</td>
<td>11.1 ± 1.6</td>
<td>2387.5 ± 344.2</td>
<td></td>
</tr>
<tr>
<td><strong>Ventral Nerve Cord</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Abd. Ganglion 1</td>
<td>12.6 ± 1.8</td>
<td>1263.2 ± 180.5</td>
<td>(13)</td>
</tr>
<tr>
<td>b. Abd. Ganglion 2</td>
<td>9.8 ± 2.3</td>
<td>341.0 ± 80.0</td>
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</tr>
<tr>
<td>c. Abd. Ganglion 3</td>
<td>16.2 ± 2.0</td>
<td>466.6 ± 57.6</td>
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<tr>
<td>d. Abd. Ganglia 4,5</td>
<td>11.3 ± 2.0</td>
<td>836.2 ± 148.0</td>
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<td>Cardiac Nerves 9,10</td>
<td>4.0 ± 0.9</td>
<td>17.9 ± 4.0</td>
<td>(9)</td>
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<td>Optic Nerve</td>
<td>2.9 ± 0.6</td>
<td>207.7 ± 43.0</td>
<td>(8)</td>
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<tr>
<td>Lateral Eye</td>
<td>1.8 ± 0.6</td>
<td>136.4 ± 45.5</td>
<td>(11)</td>
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<tr>
<td>Leg Nerve</td>
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<td>177.9 ± 35.6</td>
<td>(8)</td>
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<tr>
<td>Leg Muscle</td>
<td>0.3 ± 0.1</td>
<td>51.5 ± 17.2</td>
<td>(10)</td>
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<tr>
<td>Gill Nerve</td>
<td>11.2 ± 2.6</td>
<td>331.5 ± 77.0</td>
<td>(11)</td>
</tr>
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<td>Gill Muscle</td>
<td>0.4 ± 0.1</td>
<td>27.6 ± 6.9</td>
<td>(11)</td>
</tr>
<tr>
<td>Esophagus</td>
<td>0.3 ± 0.1</td>
<td>240.0 ± 78.5</td>
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<tr>
<td>Foregut</td>
<td>0.2 ± 0.1</td>
<td>987.7 ± 456.3</td>
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<td>Hindgut</td>
<td>0.8 ± 0.2</td>
<td>2328.3 ± 582.0</td>
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<tr>
<td>a. Region 1</td>
<td>1.4 ± 0.4</td>
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<tr>
<td>b. Region 2</td>
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<td>614.2 ± 164.2</td>
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<td>c. Region 3</td>
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<td>d. Region 4</td>
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<tr>
<td>Ostial Tissue</td>
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<td>(9)</td>
</tr>
<tr>
<td>a. Region 1</td>
<td>1.8 ± 0.3</td>
<td>68.9 ± 11.5</td>
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</tr>
<tr>
<td>b. Region 2</td>
<td>1.9 ± 0.6</td>
<td>64.0 ± 19.3</td>
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</tr>
<tr>
<td>c. Region 3</td>
<td>0.7 ± 0.2</td>
<td>23.9 ± 6.2</td>
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</tr>
<tr>
<td>Hepatopancreas</td>
<td>0.1 ± 0.02</td>
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<td>(8)</td>
</tr>
<tr>
<td>Blood</td>
<td>1.6 ± 0.5 x 10^{-10} M</td>
<td></td>
<td>(40)</td>
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Table 4. RIA for FMRFamide in *Limulus* tissues. Levels of FMRFamide are expressed in terms of pg / mg tissue weight ± S. E. and pg / region ± S. E. For certain tissues (e. g. prosomal CNS), the sum of several ganglia or tissue regions is also calculated.
ventral nerve cord (12.6 ± 3.3 pg / mg) and brain (11.9 ± 1.7 pg / mg). The cardiac ganglion contained lesser concentrations of iR-FMRFamide (4.6 ± 2.4 pg / mg). These findings are consistent with earlier RIA results (Watson et al., 1984), although tissue levels of iR-FMRFamide reported here are approximately twice those reported in the earlier study. The Limulus brain, ventral nerve cord and cardiac ganglion all stain positively for FMRFamide using immunohistochemistry (Watson et al., 1984).

The protocerebrum (12.6 ± 1.8 pg / mg) and circumesophageal ganglia (11.1 ± 1.6 pg / mg) contained essentially equal concentrations of iR-FMRFamide. Likewise, in the ventral nerve cord, all ganglia tested had comparable levels of iR-FMRFamide. There may be a slight difference between iR-FMRFamide levels of the first (13.1 ± 4.9 pg / mg) and the second abdominal ganglion (9.8 ± 2.3 pg / mg). This trend was reflected in levels of iR-FMRFamide detected in segmental cardiac nerves 9 (5.0 ± 2.5 pg / mg, n=5) and 10 (2.7 ± 1.4 pg / mg, n=4). These nerves originate separately from the dorsal nerves of the first two abdominal ganglia.

Other tissues in Limulus contained considerably lesser levels of iR-FMRFamide, with the notable exception of the branchial gill nerves (11.2 ± 2.6 pg / mg). No region of the digestive tract contained a high concentration of iR-FMRFamide. However, iR-FMRFamide in the hindgut may be regionally distributed, as levels in the anterior sections of the hindgut (1.4 ± 0.4 pg / mg in region 1) were much greater than those observed in posterior hindgut sections (0.2 ± 0.1 pg / mg in region 4). Ostial tissue contained low levels of iR-FMRFamide (1.6 ± 0.3 pg / mg). Immunoreactive FMRFamide was detected in Limulus blood at a concentration of 1.6 ± 0.5 x 10^{-10} M.
Purification of FMRFamide-like peptides from the Limulus nervous system

Several nervous tissues (brain, ventral nerve cord and cardiac ganglion) were used to purify *Limulus* FMRFamide-like peptides. Acetic acid and TCA extracts were prepared as described in Appendix - General Methodology for application to Sephadex G-15 molecular weight columns.

For each of these tissues, Sephadex G-15 filtration revealed three areas of FMRFamide-like activity (Figs. 8,9). Two of these peaks, Peak A (FMRFamide-like peak one, F1) and Peak C (FMRFamide-like peak two, F2), exhibited coincident immunoreactivity and bioactivity. The F2 region appeared to be comprised of two overlapping areas of activity: peak bioactivity consistently eluted slightly earlier than the main peak of immunoreactivity.

Several pieces of evidence indicated that Peak B was acetylcholine (Ach) rather than a *Limulus* FMRFamide-like peptide. First, this factor was not immunoreactive. Second, Ach is present in the *Limulus* nervous system (Sukumar et al., 1983). Third, Ach causes a FMRFamide-like contracture of the Busycon RPM at $10^{-7}$ M (Nagle and Greenberg, 1982). Fourth, bioactive aliquots from Peak B, like Ach, eluted from the 0% and 10% acetonitrile washes when loaded onto Sep-Pak cartridges (synthetic FMRFamide and F1 elute with the 80% wash). Finally, Peak B contained a substance which produced an Ach-like increase in the frequency of contractions of the isolated *Lumbricus* gizzard (Fig. 10), a preparation in which Ach is a putative transmitter (Wu, 1939). For these reasons, Peak B was not considered further in purification efforts.

The FMRFamide-like activity of F1 was of the most interest due to earlier findings associating *Limulus* cardioexcitatory activity with a
Fig. 8. Sephadex G-15 gel filtration profile of FMRFamide-like activity in Limulus brain extracts. Three peaks of activity were observed. Peak A (F1) and Peak C (F2) exhibited coincidental bioactivity (A) and immunoreactivity (B), while Peak B may be an Ach-like factor. Elution positions of standards are indicated by the arrows: note that synthetic FMRFamide did not co-elute with any of the brain FMRFamide-like peaks on this column.
Fig. 9. Sephadex G-15 gel filtration profiles of FMRFamide-like activity in *Limulus* cardiac ganglion and ventral nerve cord extracts. These tissues displayed a similar G-15 FMRFamide-like profile in comparison to *Limulus* brain extracts.
Fig. 10. Response of the isolated *Lumbricus* gizzard to synthetic Ach ($10^{-5}$ M) and to fraction 20 (10 brain equiv.) from Peak B, Sephadex G-15. Both Ach and fraction 20 produced an increase in the size and frequency of contractions of the gizzard. This finding, and the fact that Ach is active on the *Busycin* RPM, suggests that the effect of this Peak B fraction on the RPM is due to the presence of Ach rather than to a FMRFamide-like peptide. Calibration: vertical, 0.5 g, horizontal, 2 min.
factor eluting near the void volume on Sephadex G-25 columns which produced a FMRFamide-like contracture of the Busycon radula protractor muscle (White and Watson, 1984). The F1 peak fractions were pooled for further purification, loaded onto Sep-Pak cartridges and eluted with 80% acetonitrile after removing hydrophilic compounds with successive 0% and 10% washes.

The latter peak(s) of FMRFamide-like activity (F2) also overlapped with PP2 (proctolin-like activity), and most of the material eluting in this area was used in the purification of PP2. A limited amount of F2 (from hindgut extracts also run through Sephadex G-15) was available for further analysis. Immunoreactive material was eluted with the 80% acetonitrile wash from Sep-Pak cartridges, dried and stored at -20 °C until injected onto the HPLC column.

Ion exchange chromatography was performed on F1 activity in the Limulus brain. The major peak of FMRFamide-like bioactivity in the brain extract eluted with the pH 8, 500 mM ammonium acetate buffer (Fig. 11a). This peak was also immunoreactive, and co-eluted with synthetic FMRFamide (Fig. 11b). A small amount of bioactivity was also observed in fraction 2 before the increase in buffer osmolarity. This fraction was within the peak in optical density (280 nm) and was not immunoreactive. The coincidental bioactive and immunoreactive fractions (13,14) were pooled for HPLC.

FMRFamide-like activity from Limulus brain, cardiac ganglion, abdominal ganglion or hindgut extracts subjected to Sephadex, Sep-Pak and ion exchange (brain only) chromatography were then purified by 2 to 3 passes through a reverse phase HPLC column. The majority of these experiments focused on F1. For each tissue examined, two peaks of
Fig. 11. Ion exchange chromatography of Limulus brain extract F1. This peak was concentrated, passed through a Sep-Pak C\textsubscript{18} cartridge and eluted with 80% acetonitrile. This material was reconstituted in the 50 mM, pH 6.0 ammonium acetate buffer and applied to a 10 x 1 cm CM Sepahdex column. Bioactivity (A) was noted early (fraction 2) and with an increase in buffer osmolarity (fractions 13 and 14). The latter fractions were also immunoreactive (B). Inset: responses of the Busycon RPM to fractions 2 and 14.
iR-FMRFamide were detected by HPLC when F1 was put through a sequence of 10 min isocratic 18.0% acetonitrile, followed by a linear gradient to 29.7% acetonitrile in 50 min. The two peaks were run again on a similar HPLC gradient (to 31.5% acetonitrile this time) for comparison with known peptides (Fig. 12). The first HPLC peak of iR-FMRFamide from brain and cardiac ganglion F1 had a similar elution time to clam FMRFamide, while the first HPLC peak of iR-FMRFamide from abdominal ganglion F1 eluted approximately 3 min earlier. This peak had eluted earlier than clam FMRFamide by approximately 3 min in the first HPLC run as well. For each of these tissues, the second HPLC peak of iR-FMRFamide from F1 eluted very closely with the Helix FMRFamide-like peptide pQDPFLRFamide.

Hindgut F1 and F2 from Sephadex G-15 were also run on this HPLC gradient to compare the elution times of these compounds. The first run indicated that F2, as well as F1, contained two FMRFamide-like compounds (data not shown). At this stage of the purification process, the material was not purified sufficiently to compare elution times between the two peaks of F2 and known FMRFamide-like peptides. A second passage of the two F2 peaks through HPLC yielded insufficient material for assay.
Fig. 12. HPLC profiles of *Limulus* brain (A), cardiac ganglion (B) and ventral nerve cord (C) F1. Samples were reconstituted in 20% buffer B (90% acetonitrile containing 0.1% TFA) and run on the gradient shown (solid line). Two peaks of iR-FMRFamide were observed for each F1 extract and their elution times are compared to those of known FMRFamide-like peptides.
DISCUSSION

Proctolin-like and FMRFamide-like peptides are widely distributed in the *Limulus* CNS and may act as neurotransmitters, or be released into the hemolymph (or locally) as neurohormones to modulate peripheral function. These peptides, especially iR-proctolin, are also found in the cardiac ganglion and in selected peripheral nerves or tissues. The distribution of proctolin-like and FMRFamide-like peptides in *Limulus* agrees with other work using immunohistochemical techniques to localize these peptides in *Limulus* (Watson et al., 1984; Watson and Detschaschell, personal communication; Wyse, personal communication). These studies and the present work support physiological studies on the actions of peptides in *Limulus* and lay the groundwork for further investigations of peptide actions on the heart and other systems in this animal.

Distribution of proctolin-like peptides in *Limulus*

The *Limulus* cardiac ganglion contained the highest concentrations of iR- and bioactive proctolin of any tissue examined. The high levels of iR-proctolin in this tissue, in cardioregulatory nerves and in the CNS is supportive of the proposed role for this peptide as a regulator of cardiac output (for review see Watson and Augustine, 1982).

Synthetic proctolin and a proctolin-like cardiac ganglion peptide have a pronounced positive inotropic effect on the *Limulus* heart (Watson et al., 1983). In *Limulus*, proctolin acts directly on cardiac muscle fibers and not on either the neuromuscular junction or on the cardiac ganglion itself. However, in the lobster *Homarus*, proctolin excites cardiac ganglion
neurons to produce increases in heart rate while having little or no effect on cardiac muscle (Sullivan and Miller, 1984). Limulus and Homarus also differ in their respective levels of cardiac ganglion iR-proctolin. While the Limulus cardiac ganglion contains 10.0 pmol iR-proctolin / mg protein, the Homarus cardiac ganglion contains almost no iR-proctolin (0.4 pmol / mg protein, Schwarz, 1983).

The difference in cardiac ganglion proctolin levels in these two species might be paralleled by different release sites for this peptide. The lobster cardiac ganglion may be acted upon by proctolin released into the hemolymph from the pericardial organs (Berlind and Cooke, 1970; Sullivan, 1979). In Limulus, proctolin may be released from some other site, such as the follower (motor) neurons of the cardiac ganglion, in order to produce its inotropic actions on cardiac muscle. In the crayfish Procamparbus, proctolin acts as a co-transmitter in abdominal postural motoneurons of the ventral nerve cord (Bishop et al., 1987). Similarly, in the cockroach Periplaneta, proctolin is released as a co-transmitter from motoneurons onto skeletal muscle (O'Shea and Bishop, 1982; Adams and O'Shea, 1983). Immunohistochemical observations in Limulus have shown that iR-proctolin is present within the cardiac ganglion as well as in branches leading into the myocardium (Wyse, personal communication). The proctolin detected by RIA in Limulus cardiac muscle might possibly be attributed to motor roots of the cardiac ganglion within this tissue.

Alternatively, proctolin may be released into the bloodstream by cardioregulatory neurons of the Limulus brain or ventral nerve cord in order to reach the heart or other tissues. Levels of iR-proctolin in the bloodstream ($2 \times 10^{-10}$ M) were quite close to the threshold concentration ($3 \times 10^{-10}$ M) for the inotropic effect of this peptide on the Limulus heart.
Proctolin also acts to augment evoked contractions of skeletal muscle in the Limulus leg at a threshold dose of $3 \times 10^{-9}$ M (Rane et al., 1984). These findings suggest that proctolin might be released hormonally to reach cardiac or skeletal muscle fibers. Further studies are clearly necessary in order to determine the site(s) of release of proctolin in *Limulus*.

The existence of a pericardial organ system for release of peptides, like that described in crustaceans (reviewed by Cooke and Sullivan, 1982) may exist in *Limulus*, and peptidergic terminals of pericardial organs may be located in connective tissue located over the ostia of the heart. Evidence in favor of this hypothesis comes from the observation that cardioregulatory nerves send branches into ostial tissue (unpublished observations), and that ostial tissue does contain iR-proctolin. Ostial connective tissue in *Limulus* contained approximately 20-30 pmol iR-proctolin/mg protein. It should be noted that it is not known to what extent ostial tissue in *Limulus* is comprised of cardioregulatory nerves, connective tissue, or nerve terminals. Further studies on the anatomy and function of the cardioregulatory nerves and putative pericardial organs in *Limulus* are needed in order to answer this question.

The abdominal ganglia of the *Limulus* CNS are involved in a variety of physiological processes such as cardioregulation and respiration (Fourtner et al., 1971; Watson and Wyse, 1978; Wyse et al., 1980). Immunoreactive proctolin was detected in the ventral nerve cord and in cardioregulatory nerves 9 and 10 of the first two abdominal ganglia, suggesting that part of the cardioregulatory function of the abdominal ganglia may involve the release of this peptide. Immunohistochemical observations (Watson and Detschaschell, personal communication; Wyse, personal communication)
have indicated the presence of proctolin-positive somata and fibers in the ventral nerve cord and immunoreactive fibers in the cardioregulatory nerves to support this hypothesis.

Stereotyped patterns of ventilation and cleaning of the book gills in *Limulus* are controlled by central pattern generators within the ventral nerve cord (Wyse et al., 1980). These programs consist of coordinated, rhythmic outputs in recordings from the medial, external and internal branches of the ventral nerves. The finding that the ventral nerve cord and gill nerves contained high levels of iR-proctolin suggests that abdominal ganglion motorneurons may utilize proctolin to initiate or augment particular gill movements in the normal expression of these behaviors. Proctolin is located in lobster ventilatory motor neurons (Siwiki and Bishop, 1986), increases ventilatory output in the crab *Carcinus* (Mercier and Wilkens, 1985) and modulates the swimmeret program of the crayfish (Mulloney et al., 1987). In *Limulus*, proctolin may act centrally or peripherally to modify specific respiratory movements of the gill appendages. This peptide might also be important in the maintenance of cardiac output during coordinated heart and respiratory activity (Watson and Wyse, 1978).

Immunoreactive proctolin was highly concentrated in the *Limulus* hindgut, while other portions of the digestive tract contained little or no iR-proctolin. Proctolin-like peptides have been detected in the hindgut and other visceral muscles of a number of arthropod species (Holman and Cook, 1985; Eckert et al., 1981; Lange et al., 1986). Proctolin has several actions on the *Limulus* hindgut (Fig. 7 b,c and unpublished results) which are similar to the action of this peptide on the hindgut and visceral muscles of other arthropods (Brown, 1975; Holman and Cook, 1985). In contrast, the
Limulus foregut is responsive only to high concentrations of proctolin (10^{-6} \text{ M or greater, unpublished results}), a finding in agreement with studies in Leucophaea (Cook and Holman, 1978) in which the effect of proctolin is 1000 x greater on hindgut muscles than on foregut muscles.

Proctolin-like immunoreactivity has been observed in nerve fibers of the Limulus gut (Watson and Detschaschell, personal communication; Wyse, personal communication). A proctolin-like peptide is present in hindgut motorneurons in the lobster (Siwicki and Bishop, 1986) and has been shown to be released by high potassium from visceral muscle innervated by proctolinergic motorneurons of the terminal abdominal ganglion in the cockroach Periplaneta (Lange et al., 1986; Orchard and Lange, 1987).

Proctolin might reach the Limulus hindgut from neurons in the abdominal ganglia which send projections to the gut via the intestinal nerve (Patten and Redenbaugh, 1899), or via the bloodstream. Interestingly, the isolated Limulus hindgut shows a considerable amount of spontaneous activity for many hours, which at times resembles the response to application of synthetic proctolin (unpublished observations).

Multiple proctolin-like peptides in Limulus

There are at least two proctolin-like peptides (PP1 and PP2) in the Limulus CNS, cardiac ganglion and hindgut. The presence of two separate iR-proctolin peptides has not previously been described in other arthropods.

Sep-Pak C_{18} and HPLC purification of PP1 and PP2 did not alter their activity on any of the preparations used in this study. Additionally, these peptides co-eluted with synthetic proctolin on reverse phase HPLC, suggesting that both compounds are similar in structure and hydrophobicity.
to each other and to cockroach proctolin. Several possibilities exist to explain the presence of two proctolin-like peptides in *Limulus*.

Proctolin-like peak one (PP1) may be identical to or a close analog of synthetic proctolin (H-Arg-Tyr-Leu-Pro-Thr-OH). The second proctolin-like peptide (PP2), which is of an apparent lower molecular weight, could be a metabolic breakdown product. In the cockroach *Periplaneta*, proctolin degradation in the hemolymph, nervous and gut tissues can proceed in two directions (Quistad et al., 1984). The primary degradation pathway involves cleavage of the Tyr-Leu bond to yield Arg-Tyr and Leu-Pro-Thr, but some of the proctolin is cleaved at the Arg-Tyr bond to yield Arg and Tyr-Leu-Pro-Thr. A structure-function analysis of proctolin fragments and analogues has been performed for the cockroach hindgut receptor (Sullivan and Newcomb, 1982). None of the proctolin breakdown products proposed by Quistad et al. (1984) are active on this preparation. However, as mentioned earlier, both PP1 and PP2 cardiac ganglion extracts purified by HPLC were equally active on the cockroach hindgut. These findings suggest that PP2 is an independent proctolin-like peptide in *Limulus*, rather than a metabolite.

Both of these peptides are active on the *Limulus* heart and hindgut, suggesting that one or both of these compounds are active in vivo. Interestingly, several if not all of the leukokinins (octapeptides isolated from head extracts of *Leucophaea*) also are active on both the heart and gut in that species of cockroach (Holman et al., 1986). The presence of multiple peptides with apparently similar actions in *Limulus* and other arthropods presents an interesting challenge in understanding peptidergic regulation of particular physiological events in vivo.
Distribution of iR-FMRFamide in Limulus

The brain and ventral nerve cord of the Limulus CNS contained the highest quantities of iR-FMRFamide. The cardiac ganglion and cardioregulatory nerves contained 2-3 x less iR-FMRFamide per mg tissue than the CNS. The relative distribution of iR-FMRFamide in the CNS and cardiac ganglion reported here is in agreement with an earlier study (Watson et al., 1984). One exception is the distribution of iR-FMRFamide within the brain (prosomal CNS). Earlier results suggested a 3-fold greater concentration of iR-FMRFamide in the protocerebrum than in the circumesophageal ganglia, while in the present study essentially equivalent concentrations were detected.

FMRFamide may function as a transmitter or neurohormone in the regulation of cardiac function in Limulus. Immunocytochemical observations have indicated the presence of iR-FMRFamide in somata of the Limulus brain and ventral nerve cord, nerve fibers in the cardiac ganglion and cardiac muscle, and in the arterial sheath surrounding the CNS (Watson et al., 1984). Release of Limulus FMRFamide-like peptides into the hemolymph could occur within the CNS arterial sheath, or from putative pericardial organs within the ostial connective tissues of the heart. Release of FMRFamide from neuroheamal organs has been demonstrated in molluscs (Voight et al., 1983) and in arthropods (Walther and Schiebe, 1987).

It is possible that other FMRFamide-like peptides play a role as a neurohormone or neurotransmitter in the regulation of the heart in Limulus. While synthetic FMRFamide has weak effects on the Limulus heart (Watson et al., 1984), a high molecular weight FMRFamide-like factor present in the
Limulus brain (termed limadrin, or Limulus adrenalin) has potent cardioexcitatory actions (White and Watson, 1984). The endogenous FMRFamide-like peptide or peptides which exert these actions in Limulus may differ considerably in structure (and thus action) from clam FMRFamide. In molluscs, for example, an entire family of FMRFamide-related peptides sharing partial sequence homology has been described (reviewed by Price et al., 1987). Further, many of these FMRFamide-like peptides have pronounced chronotropic and inotropic actions on the myogenic hearts of a number of molluscan species (Greenberg and Price, 1980; Painter and Greenberg, 1982; Boyd et al., 1984; Kobayashi and Muneoka, 1986; Smith, 1987). Therefore, purification, sequence analysis and pharmacological characterization of FMRFamide-like peptides in Limulus is necessary in order to clarify the role of these peptides in the regulation of cardiac output.

FMRFamide-like peptides in the Limulus CNS may have central actions, but little is known regarding central neural circuits in the horseshoe crab. However, the motor output of the ventral nerves of the abdominal ganglia have been characterized in some detail (Watson, 1980; Wyse et al., 1980). Synthetic FMRFamide has no effect on the motor programs exhibited by the isolated ventral nerve cord at concentrations up to $10^{-5}$ M (Watson et al., 1984). However, the FMRFamide-like limadrin peptide present in the Limulus brain initiates patterned ventilatory output in the isolated ventral nerve cord (Watson, personal communication).

FMRFamide and related peptides have been shown to have central actions on the crustacean stomatogastric ganglion (Hooper and Marder, 1984) and on certain identified neurons in molluscs (Murphy et al., 1985; Ruben et al., 1986; Boyd and Walker, 1987).
The branchial gill nerves contained a substantial concentration of FMRFamide-like material. The iR-FMRFamide somata of the Limulus abdominal ganglia (Watson et al., 1984) may be the origin of iR-FMRFamide in the gill nerves. These cells might release FMRFamide-like peptides onto gill muscles to modulate contractions during specific respiratory behaviors. However, peripheral regulation of the book gills in Limulus has not yet been investigated.

Levels of iR-FMRFamide in the hindgut were extremely low, especially in comparison to the high concentration of iR-proctolin located there. However, FMRFamide immunoreactive fibers are present in the Limulus gut (Watson, personal communication). Synthetic FMRFamide relaxes the Limulus gut (unpublished results), but only at high concentrations. In Mercenaria, FMRFamide has a contractile effect on hindgut muscles (Doble and Greenberg, 1982), while in Aplysia FMRFamide has inhibitory actions on contractions of the stomach and gizzard (Austin et al., 1983). FMRFamide-like peptides may act on some portion of the Limulus digestive system, but at present the significance of iR-FMRFamide in the Limulus digestive tract and of its apparent regional distribution in the hindgut is uncertain.

**FMRFamide-like peptides in Limulus**

Immunoreactive FMRFamide in Limulus appears to consist of several FMRFamide-like peptides. In crustaceans, the FMRFamide-like peptides present in the stomatogastric nervous system are more hydrophobic than clam FMRFamide (Marder et al., 1986). HPLC analysis in the present study indicates that one of the FMRFamide-like peptides in Limulus nervous
tissues is also more hydrophobic than clam FMRFamide, but that the other
FMRFamide-like peptide may be identical to or very similar to clam
FMRFamide. However, while comparison of elution times of Limulus
FMRFamide-like peptides to known peptides is a useful tool in their
purification and characterization, amino acid analysis of the two
FMRFamide-like peptides in the Limulus brain purified by HPLC is necessary
to reveal whether or not these peptides are novel or identical to previously
described FMRFamide-like species.

In summary, proctolin-like and FMRFamide-like peptides are widely
distributed in the Limulus nervous system. The presence of proctolin-like
peptides in specific nervous and peripheral tissues support previous
reports of the inotropic action of synthetic proctolin and a Limulus
proctolin-like peptide on the neurogenic Limulus heart, and the present
finding that synthetic proctolin and Limulus proctolin-like peptides have
pronounced effects on hindgut motility. The presence of iR-FMRFamide in
the Limulus CNS and cardiac ganglion suggest a role for Limulus
FMRFamide-like peptides in cardioregulation and possibly for modulation of
respiratory movements of the book gills.

Limulus may contain authentic proctolin and FMRFamide in addition to
other proctolin-like and FMRFamide-like peptides. The precise regulatory
roles played by these peptides in Limulus will be greatly facilitated by
determination of their amino acid structure and sequence.
CHAPTER FOUR

MECHANISMS UNDERLYING ACTIONS OF PROCTOLIN-LIKE AND FMRFAMIDE-LIKE PEPTIDES ON THE LIMULUS HEART

ABSTRACT

The involvement of cyclic nucleotides and protein kinase C in peptide actions on the Limulus heart was investigated. Partially purified limadrin, a Limulus FMRFamide-like peptide, had a long-lasting positive chronotropic effect and biphasic inotropic actions on the isolated heart. These actions were mimicked by the phosphodiesterase inhibitor IBMX. Both limadrin and IBMX increased levels of cAMP and cGMP in the cardiac ganglion and in cardiac muscle. The positive chronotropic effect of limadrin, like that of the amines, appears to involve a cAMP-dependent mechanism. The biphasic inotropic action of this compound may involve opposing actions of cAMP and cGMP in Limulus cardiac muscle.

Synthetic FMRFamide (10^-5 M) or the Helix FMRFamide-like peptide pQDPFLRFamide (10^-5 M) had only slight effects on the Limulus heart and did not increase cAMP levels in the cardiac ganglion or in cardiac muscle. Experiments using different extraction procedures revealed that cardioexcitation associated with particular fractions from Sephadex G-15 containing partially purified limadrin was not directly proportional to the level of immunoreactive FMRFamide in those fractions.

Proctolin, at 10^-6 M, had no effect on cyclic nucleotide levels in Limulus cardiac muscle. However, the protein kinase C activator phorbol
12,13 dibutyrate (3 x 10^{-7} M) mimicked the positive inotropic action of 10^{-7} M proctolin on the intact heart or electrically stimulated myocardium. This agent, like proctolin, also elicited contracture and rhythmic contractions of deganglionated Limulus cardiac muscle rings. Protein kinase C activation, therefore, may be an essential process in the modulatory actions of proctolin in this system.

The contractile and myogenic actions of proctolin or phorbol 12,13 dibutyrate on deganglionated Limulus cardiac muscle were also mimicked by 10^{-5} M dopamine. Octopamine (10^{-5} M) and forskolin (10^{-5} M) had only slight effects on this preparation. These results suggest that dopamine activates protein kinase C, in addition to protein kinase A, to enhance cardiac muscle contractility. Apparently, peptides and amines share several second messenger systems to produce similar chronotropic and inotropic effects on the Limulus heart.
INTRODUCTION

The neurogenic heart of the horseshoe crab, *Limulus polyphemus*, is modulated by a number of amines and peptides present in the nervous system of this animal (for review see Watson and Augustine, 1982). Amines have long-lasting positive chronotropic and inotropic actions on the *Limulus* heartbeat. These effects of amines involve activation of cAMP metabolism in this system (Groome and Watson, 1983; Groome and Watson, 1987).

Several cardioactive peptides found in the *Limulus* nervous system have amine-like actions on the *Limulus* heartbeat. Synthetic proctolin, and a cardiac ganglion proctolin-like peptide, have been shown to have direct actions on cardiac muscle fibers to enhance heart contraction strength (Benson et al., 1981; Watson et al., 1983). Synthetic proctolin also induces rhythmic contractions in the deganglionated *Limulus* heart (Watson and Hoshi, 1985).

A FMRFamide-like cardioexcitatory compound (limadrin) present in the *Limulus* brain, on the other hand, appears to act on the *Limulus* cardiac ganglion to increase heart rate (White and Watson, 1984). In addition to its positive chronotropic action, limadrin has a biphasic effect on heart contraction amplitude resulting in prolonged inhibition. These effects are shared only partially by synthetic FMRFamide (Watson et al., 1984).

The positive inotropic action of proctolin, and the positive chronotropic action of limadrin are overtly similar to the excitatory actions of amines on the *Limulus* heartbeat. Additionally, the prolonged time course of peptide actions on the *Limulus* heart suggests that second messengers may be involved. This system, therefore, presents the
opportunity to study the biochemical basis for similar actions of amines and peptides on particular cellular targets of a pattern generating network.
METHODS

Biochemistry

Tissue incubations and cyclic nucleotide assays were performed as described in Appendix - General Methodology. Synthetic proctolin, FMRFamide (clam FMRFamide) or pQPDLRFamide (Helix FMRFamide-like peptide) were used in experiments on peptide effects on cyclic nucleotide levels in particular tissues.

The effects of partially purified limadrin from the Limulus brain on cardiac ganglion or cardiac muscle biochemistry were examined in three separate experiments. In general, Limulus brains were extracted as described in Appendix - General Methodology. However, in the first set of experiments TCA was not employed in the procedure, and extracts were applied directly (e.g. without employing Sep-Pak purification) to a Sephadex G-25 column. In the second experiment, both TCA and Sep-Pak were used prior to molecular weight separation of the brain extract. In the third experiment, TCA was used for one half of the extract only.

Aliquots of 3-10 brain equivalents from the first peak of FMRFamide-like (Busycon RPM and RIA) activity were utilized in tests of Limulus heart cardioexcitation and for effects on tissue levels of cyclic nucleotides.

Pharmacology

Limulus hearts or cardiac muscle rings were prepared as described in Appendix - General Methodology. Recordings were accomplished as described in Chapters One and Two. The intact Limulus heart and
deganglioniated myocardium provided two preparations in which to investigate the excitatory actions of proctolin, amines and various pharmacological agents. Cardiac muscle rings were prepared for electrical stimulation as described, or pinned out in 5 ml perfusion chambers for tension recordings in the absence of neuronal or electrical stimulation.

The intact *Limulus* heart was employed to compare the chronotropic and inotropic actions of limadrin, amines and pharmacological agents. This preparation was also used to compare several modifications in the basic extraction procedure for limadrin. All experiments were carried out at room temperature. Natural seawater was used in most experiments. In other experiments, artificial *Limulus* saline was employed. Normal saline was of the following composition: 445 mM NaCl, 12 mM KCl, 10 mM CaCl₂, 46 mM MgCl₂, with 10 mM Hepes buffer at pH 7.5. Zero calcium saline was made by omitting CaCl₂ and adding 2 mM ethylene glycol bis-tetraacetic acid (EGTA).
RESULTS

Effects of proctolin on cyclic nucleotide levels in cardiac muscle

Proctolin, at concentrations up to 10^{-5} M, had no effect on the levels of either cAMP or cGMP in *Limulus* cardiac muscle. A 10 min incubation of cardiac muscle rings in 10^{-7} M, 10^{-6} M or 10^{-5} M proctolin did not significantly alter levels of either cyclic nucleotide from control values (n=6). When cardiac muscle rings were incubated in 10^{-6} M proctolin solutions for periods of time ranging from 30 sec to 10 min, no transient increase in levels of cAMP or cGMP was observed (Fig. 1).

Proctolin and phorbol ester actions on neurally and electrically evoked heart contractions

The protein kinase C activator phorbol 12,13 dibutyrate (Ph dB), at 3 x 10^{-7} M, mimicked the positive inotropic action of 10^{-7} M proctolin on the isolated *Limulus* heart (Fig. 2). Both Ph dB and proctolin produced a comparable increase in contraction amplitude, but the effect of the phorbol ester was much slower in onset and in decay. In most experiments the effects of proctolin and Ph dB were exclusively inotropic, although in a few experiments these agents did produce slight increases in the contraction rate of the intact heart.

The inotropic effect of proctolin and Ph dB was the result of an alteration in the properties of cardiac muscle fibers, since these agents also increased the amplitude of electrically evoked contractions of deganglionated myocardial rings (Fig. 3). The effect of either agent was
Fig. 1. Proctolin, at $10^{-6}$ M, has no effect on levels of cAMP or cGMP in *Limulus* cardiac muscle. Cardiac muscle rings were incubated in $10^{-6}$ M proctolin for 30 sec to 10 min and processed for cAMP and cGMP content. Each point represents the mean cyclic nucleotide content ± S. E. in 3 to 6 experiments.
Fig. 2. Proctolin and the protein kinase C activator phorbol 12, 13 dibutyrate elicit positive inotropic, but not chronotropic, responses when applied to the intact Limulus heart. A. Record of heart contractions before and during peak response to $10^{-7}$ M proctolin or $3 \times 10^{-7}$ M phorbol ester. Calibration: vertical, 1.5 g; horizontal, 1 min. B. Time course of the inotropic effect of proctolin or phorbol ester (added to the preparation as indicated by the solid line).
Fig. 3. The positive inotropic effect of proctolin and phorbol ester was the result of their direct actions on cardiac muscle fibers. Cardiac muscle rings were induced to contract by applying pulses of electrical current. A. Record of evoked contractions before and during peak response to $10^{-7}$ M proctolin or $3 \times 10^{-7}$ M phorbol 12,13 dibutyrate. Calibration: vertical, 2 g; horizontal, 1 min. B. Time course of the increase in evoked contraction amplitude in response to proctolin or phorbol ester (solid line indicates application).
not altered by pretreatment of the preparation with $3 \times 10^{-7}$ M TTX (not shown). The phorbol ester produced an increase in contraction amplitude which declined only after two or more hours of perfusion with phorbol-free seawater. The positive inotropic response to proctolin was also long-lasting, but to a much lesser extent than observed with phorbol-treated preparations.

Like proctolin, Ph dB ($3 \times 10^{-7}$ M) had no effect on levels of cAMP in cardiac muscle rings incubated in this drug for 10 min ($90.2 \pm 6.6\%$ control, n=5) or 20 min ($86.9 \pm 3.4\%$ control, n=5). Phorbol 12-myristate 13-acetate and 1-oleyl 2-acetyl sn-glycerol, at $10^{-6}$ M, had negligible effects on the intact heart or electrically stimulated myocardium. At higher concentrations, these agents occasionally produced positive inotropic effects as well.

Comparison of the effects of proctolin, amines and pharmacological agents on Limulus cardiac muscle

Contractures of cardiac muscle rings were consistently observed during proctolin ($10^{-6}$ M) application to preparations stimulated by electrical current pulses. In several experiments dopamine ($10^{-5}$ M) produced a similar effect (Fig. 4). In several preparations, proctolin and dopamine caused the gradual appearance of smaller contractions occurring between electrical stimulations.

Proctolin, at a concentration of $10^{-6}$ M, has been shown to produce both a contracture and rhythmic contractions when applied to deganglionated Limulus hearts (Watson and Hoshi, 1985). In this study, these effects were observed with the application of $10^{-6}$ M proctolin to
Fig. 4. Proctolin ($10^{-7}$ M) or dopamine ($10^{-5}$ M) occasionally caused contracture when applied (continuously at arrow) to cardiac muscle rings stimulated to contract by regular current pulses. The contractile effect of proctolin was observed more often and was more pronounced than that of dopamine. Both agents occasionally produced contractions following evoked contractions (bottom arrow points to small after-contraction observed during dopamine application). Calibration: vertical, 2 g, horizontal, 1 min.
deganglionated 2 cm cardiac muscle rings (Table 5, Fig. 5).

Dopamine (10^{-5} M), like proctolin, consistently elicited contractures and rhythmic contractions in deganglionated cardiac muscle rings (Table 5, Fig. 5). In contrast, OCT (10^{-5} M) produced a modest contracture in only one of 15 preparations. Octopamine elicited rhythmic contractions in a few experiments, but these contractions were of much lesser amplitude and frequency than dopamine-elicited contractions.

The phorbol ester Ph dB, at 3 x 10^{-7} M, also produced a proctolin-like effect in this preparation (Table 5, Fig. 5). While the degree of contracture produced by this agent was less than that elicited by either proctolin or DA, Ph dB more consistently produced rhythmic contractions than any agent tested. Forskolin (10^{-5} M), on the other hand, was much less potent in either of these effects. Forskolin-induced contracture was observed in only one instance, and the effects of this agent on rhythmicity were infrequent and slight.

Rhythmic contractions produced by these agents persisted for 1-2 hr after their removal from the bath medium. However, contractions were abolished with the introduction of the calcium channel blocker Mn^{++} (20 mM) to the bath (not shown). Five to ten minutes after removal of Mn^{++}, rhythmic contractions were again apparent. A similar dependency on external calcium has been demonstrated for the action of proctolin on the deganglionated Limulus heart (Watson and Hoshi, 1985). When cardiac muscle rings were perfused with saline containing 0 Ca^{++} / 2mM EGTA, application of proctolin, phorbol ester or DA was without effect (not shown). Subsequent washing of the preparation with normal saline resulted in contracture and long-lasting rhythmic contractions.
**Table 5** - Effects of proctolin, amines and pharmacological agents on deganglionated cardiac muscle

<table>
<thead>
<tr>
<th>AGENT</th>
<th>DOSE</th>
<th>(n)</th>
<th>Contracture tension (g)</th>
<th>Rhythmic contractions (% of preparations tested)</th>
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<tbody>
<tr>
<td>Proctolin</td>
<td>$10^{-6}$ M</td>
<td>12</td>
<td>$0.92 \pm 0.26$</td>
<td>83.3</td>
</tr>
<tr>
<td>DA</td>
<td>$10^{-5}$ M</td>
<td>30</td>
<td>$0.34 \pm 0.10$</td>
<td>63.3</td>
</tr>
<tr>
<td>Phorbol</td>
<td>$12,13 \text{ dB}$</td>
<td>$3 \times 10^{-7}$ M</td>
<td>22</td>
<td>$0.13 \pm 0.04$</td>
</tr>
<tr>
<td>Forskolin</td>
<td>$10^{-5}$ M</td>
<td>15</td>
<td>$0.02 \pm 0.02$</td>
<td>23.3</td>
</tr>
<tr>
<td>Octanol</td>
<td>$10^{-5}$ M</td>
<td>15</td>
<td>$0.01 \pm 0.01$</td>
<td>23.3</td>
</tr>
</tbody>
</table>

Table 5. Summary of the effects of various agents to promote contracture of, or initiate rhythmic contractions in, deganglionated cardiac muscle rings. Agents were applied to the preparation for 10 min and then removed from the perfusion solution. Contracture tension was measured as the difference from resting tension and is expressed as the mean tension $\pm$ S.E. Appearance or absence of rhythmic contractions in response to a given agent was noted and the overall percentage of preparations which responded positively is represented. The frequency and magnitude of contractions in response to particular agents were variable, but most pronounced for proctolin, dopamine and phorbol ester.
Fig. 5. Effect of various agents on deganglionated Limulus cardiac muscle. Agents were applied to the preparation at the arrow and washed off after 10 min exposure. Proctolin (10^{-6} M), dopamine (10^{-5} M) and phorbol 12,13 dibutyrate (3 \times 10^{-7} M) all produced rhythmic contractions of cardiac muscle. These agents often produced contracture of the myocardium prior to the initiation of contractions. Octopamine (10^{-5} M) and forskolin (10^{-5} M) occasionally produced small contractions. Interruptions in the lower 3 traces are equal to a 10 min time period. Calibration: vertical, 2 g; horizontal, 1 min.
Limadrin

Partially purified limadrin from experiment one produced several effects when applied to the Limulus heart preparation (Table 6, Fig. 6). A single dose of limadrin (3 brain equiv. of fraction 23, Sephadex G-25) applied directly to the Limulus heart by pipette caused a 90.0% increase in heart rate. The positive chronotropic effect was long-lasting, even though the limadrin sample was washed off the preparation within a few minutes after introduction into the bath. The initial increase in contraction amplitude (+25.0%) was followed by long-lasting inhibition of contraction amplitude (-68.5%, at 10 min after initial exposure to limadrin).

All of the effects of limadrin on the intact Limulus heart were mimicked by 10^-3 M IBMX (Fig. 1 (Chapter One), Fig. 6 (Chapter Two), Fig. 6). Additionally, the magnitude of the positive inotropic and chronotropic effects, as well as that of the long-lasting negative inotropic effect, were comparable for limadrin and IBMX. The similarity of these effects suggested that cyclic nucleotides might be involved in some of the actions of limadrin on the Limulus heartbeat.

Limadrin (5 brain equiv., fraction 23 from Sephadex G-25, experiment one) and IBMX (10^-3 M) significantly increased cardiac ganglion cAMP and cGMP levels (Table 6, Fig. 7). These effects were apparent within 3-10 min of incubation. Limadrin and IBMX also elicited substantial increases in levels of cardiac muscle cAMP and cGMP (Fig. 8). These findings suggest that their similar physiological effects may be explained by similar biochemical actions.

In experiment two, the physiological and biochemical actions of partially purified limadrin from Sephadex G-15 were examined in tests in
TABLE 6 - Effects of FMRFamide-like peptides on cAMP levels and physiology in Limulus cardiac ganglia and cardiac muscle

Cardiac Ganglion

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose</th>
<th>pmol cAMP (n)</th>
<th>Percent change in Heart Rate (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td></td>
<td>28.0 ± 10.6 (8)</td>
<td></td>
</tr>
<tr>
<td>FMRFamide</td>
<td>10^{-5} M</td>
<td>27.6 ± 4.5 (8)</td>
<td>-0.3 ± 0.7 (8)</td>
</tr>
<tr>
<td>pQPDLRFamide</td>
<td>10^{-5} M</td>
<td>28.1 ± 6.2 (5)</td>
<td>+13.2 ± 5.3 (8)</td>
</tr>
<tr>
<td>LIMADRIN&lt;sub&gt;1&lt;/sub&gt;</td>
<td>5, 3 b. e.</td>
<td>79.0 ± 24.9 (5)*</td>
<td>+90.0 (1)</td>
</tr>
<tr>
<td>LIMADRIN&lt;sub&gt;2&lt;/sub&gt;</td>
<td>10, 8 b. e.</td>
<td>49.8 ±10.0 (6)*</td>
<td>+25.6 (1)</td>
</tr>
</tbody>
</table>

Cardiac Muscle

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose</th>
<th>pmol cAMP (n)</th>
<th>Percent change in Contraction Ampl. (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td></td>
<td>6.1 ± 1.1 (10)</td>
<td></td>
</tr>
<tr>
<td>FMRFamide</td>
<td>10^{-5} M</td>
<td>6.4 ± 0.4 (8)</td>
<td>-2.3 ± 3.3 (8)</td>
</tr>
<tr>
<td>pQPDLRFamide</td>
<td>10^{-5} M</td>
<td>5.4 ± 0.5 (6)</td>
<td>-0.3 ± 3.7 (8)</td>
</tr>
<tr>
<td>LIMADRIN&lt;sub&gt;1&lt;/sub&gt;</td>
<td>5, 3 b. e.</td>
<td>31.6 ± 2.6 (6)†</td>
<td>+25.0 (1) -68.5 (1)</td>
</tr>
<tr>
<td>LIMADRIN&lt;sub&gt;2&lt;/sub&gt;</td>
<td>10, 8 b. e.</td>
<td>8.6 ± 1.2 (6)</td>
<td>-3.3 (1)</td>
</tr>
</tbody>
</table>

Table 6. Chronotropic and inotropic effects of FMRFamide-like peptides on the isolated Limulus heart and effects on levels of cardiac ganglion or cardiac muscle cAMP. Doses of limadrin are expressed as brain equivalents (b. e.) from a Sephadex fraction. Measurements were taken after 10 min exposure to a particular agent and are expressed as the mean ± S. E. Level of significant difference from control values was determined by a Student's t-test.

Level of significance: † (p ≤ .02); * (p ≤ .05).
Fig. 6. Similar inotropic and chronotropic actions of limadrin (top) and the phosphodiesterase inhibitor IBMX (bottom) on the intact Limulus heart. An extract of limarin (5 brain equiv.) was reconstituted in 2 ml seawater, adjusted to pH 7.6 and added to the preparation at the arrow. Limadrin caused a long-lasting increase in heart rate and a biphasic alteration of heart contraction amplitude. All of these effects were mimicked by perfusion (at arrow) of the heart preparation with $10^{-3}$ M IBMX. Calibration: vertical, 1.5 g; horizontal, 1 min.
Fig. 7. Limadrin (5 brain equiv.) and IBMX (10^-3 M) increased levels of cAMP (A) and cGMP (B) in the *Limulus* cardiac ganglion. Each area represents the mean content in cyclic nucleotide ± S. E. in 5 to 8 experiments. Level of significance was determined using an unpaired Student’s t-test.

Level of significance: ** (p ≤ .03); * (p ≤ .05).
Fig. 8. Limadrin (5 brain equiv.) and IBMX (10\(^{-3}\) M) increased levels of cAMP (A) and cGMP (B) in Limulus cardiac muscle rings. Each area represents the mean content in cyclic nucleotide \(\pm\) S. E. in 6 to 8 experiments. Level of significance was determined using an unpaired Student's t-test.

Level of significance: ** (p \(<\) .03); * (p \(<\) .05).
which TCA and Sep-Pak C₁₈ cartidges were employed in the extraction protocol. Under these conditions, limadrin had only modest effects on the intact Limulus heart (Table 6). Limadrin (3 brain equiv.) produced only a 20.0% increase in heart rate, and 8 brain equivalents caused only a 25.6% increase. Heart contraction amplitude was not affected at either dose.

The lesser chronotropic or inotropic effects of limadrin in experiment two was associated with the finding that 10 brain equivalents of this factor had a smaller effect on levels of cAMP in cardiac ganglia or cardiac muscle than was elicited by 5 brain equivalents in experiment one (Table 6). Therefore, the reduction in the cardioexcitatory effect for this factor, using a different extraction protocol, was paralleled by a drop in its capacity to increase cAMP in the cardiac ganglion or muscle.

In experiment three, the relationship between levels of iR-FMRFamide and cardioexcitation of Sephadex fractions was investigated. First, an extract of 15 brains were passed through Sephadex G-25 without employing TCA. Fraction 23 was the peak of cardioexcitation and FMRFamide activity, and contained 1.7 pmol iR-FMRFamide / brain equiv. Ten brain equivalents in 1 ml were applied to the intact heart preparation, and this dose caused a 55.2% increase in heart rate. Next, the other 15 brains from the original 30 brain extract was subjected to TCA extraction prior to gel filtration. Fraction 23 was again the peak of cardioexcitation and FMRFamide-like immunoreactivity, and this fraction contained 0.9 pmol iR-FMRFamide/brain equiv. Ten brain equivalents elicited a 68.4% increase in heart rate. In either experiment, rate excitation was quite long-lasting, as heart rate was elevated above control for 1 hr or more.

These results indicate that FMRFamide levels in Sephadex fractions may have been decreased by employing TCA, but there was not a
corresponding drop in cardioexcitation. Additionally, recovery of iR-FMRFamide from Sep-Pak was tested. Virtually 100% of the original FMRFamide loaded onto the Sep-Pak cartridge was recovered in the 80% acetonitrile wash.

Effect of synthetic FMRFamide-like peptides on the Limulus heartbeat and on cAMP levels

Synthetic FMRFamide (10^{-5} M) or pQDPFLRFamide (10^{-5} M) had much lesser effects on heart contraction rate or strength than the limadrin extract tested in experiment one (Table 6). At this dose, pQPDFLRFamide had slightly greater effects than FMRFamide on the intact Limulus heart (Table 6). Occasionally, these peptides produced transient increases in heart rate of 40-50%. However, neither peptide elicited a prolonged chronotropic or inotropic response like that elicited by limadrin.

These results were consistent with the finding that neither of these peptides (10^{-5} M) significantly increased cAMP in the cardiac ganglion or in cardiac muscle when these tissues were incubated in peptide solution for 10 min (Table 6).
DISCUSSION

Several second messenger systems may be involved in the expression of peptide and amine actions on the neurogenic Limulus heart. Proctolin, which has no effect on cyclic nucleotide metabolism, appears to produce its positive inotropic (and myogenic) actions on Limulus cardiac muscle by a process involving the activation of protein kinase C. Limadrin, on the other hand, may elicit its varied actions by activation of both the cAMP and cGMP second messenger systems. Finally, dopamine may activate both cAMP and phosphatidylinositol (PI) metabolism in Limulus cardiac muscle. These findings suggest that the biochemical basis for peptide and amine modulation of the neurogenic Limulus heart consists of multiple second messenger systems acting either in concert, or in opposition, to produce characteristic cellular responses.

Proctolin and cyclic nucleotides

Proctolin failed to stimulate cAMP or cGMP synthesis in Limulus cardiac muscle at doses which have profound effects on cardiac muscle contractility. For example, at a concentration of $10^{-6}$ M, proctolin had no effect on cyclic nucleotide levels in Limulus cardiac muscle. At $10^{-5}$ M, OCT has pronounced effects on cardiac muscle cAMP. However, the positive inotropic effect of $10^{-6}$ M proctolin is significantly greater than that of $10^{-5}$ M octopamine (unpublished results).

The lack of increased cAMP or cGMP levels in Limulus cardiac muscle in response to proctolin was not an artifact of dose range or of time of incubation. Proctolin failed to influence cyclic nucleotide metabolism at
doses from $10^{-7}$ M to $10^{-5}$ M. Additionally, proctolin did not elicit a transient increase in either cAMP or cGMP, as has been described in other peptidergic systems (e.g. the action of SCPB in the *Aplysia* ventricle; Lloyd et al., 1985). These findings clearly demonstrate that proctolin does not increase cardiac muscle contractility via a cAMP-dependent mechanism.

Several previous studies have investigated the relationship between the actions of proctolin (or a proctolin-like peptide) on arthropod smooth, skeletal or cardiac muscle and the effects of this peptide on cyclic nucleotide metabolism. Proctolin was shown by S.-Rosza and Miller (1980) to have a mild stimulatory effect on adenylate cyclase activity in locust heart muscle. However, while the physiological effects of proctolin in this system were significantly greater than either DA or OCT, its effect on cyclase activity was markedly lower than these or other amines. The action of proctolin, to accelerate the myogenic rhythm in locust extensor tibia muscle, is mimicked by both forskolin and IBMX (Evans, 1984a). However, cyclic nucleotide analogues have the opposite (OCT-like) effect. Other studies indicated that cAMP mediates the inhibitory effect of octopamine, and not the excitatory effect of proctolin (Evans 1984 b,c). Cyclic AMP has also been proposed as a possible messenger in proctolinergic actions on the cockroach hindgut. While cAMP was shown to have proctolin-like excitatory effects on the *Lecophaeae* hindgut in one study (Cook et al., 1975), another study indicated that other cAMP agents produced inhibition of hindgut motility in the same species (Jennings et al., 1983). Therefore, in these and the present studies, a cAMP-dependent mechanism does not appear to be involved in the actions of proctolin on invertebrate muscle.
Proctolinergic activation of protein kinase C in Limulus cardiac muscle

The mechanism by which proctolin increases contractility in Limulus cardiac muscle appears to involve activation of protein kinase C and not of protein kinase A. The effects of phorbol 12,13 dibutyrate (which activates protein kinase C) and proctolin on Limulus cardiac muscle contractility were similar in two separate preparations (intact heart and electrically stimulated myocardium). First, both Ph dB and proctolin increased the amplitude of contractions in these preparations. Second, these effects were long-lasting. Finally, neither of these compounds significantly altered heart rate, as do the amines.

Stimulation of the phosphatidylinositol system involves the production of inositol trisphosphate (IP₃) and diacylglycerol as second messengers (Berridge, 1987). Phorbol esters mimic the C-kinase stimulating action of diacylglycerol in many cells (Kishimoto et al., 1980; Castagna et al., 1982). However, another class of C-kinase activators such as arachidonic acid have been proposed as second messengers of an alternative pathway resulting in the activation of this enzyme (McPhail et al., 1985; Snider et al., 1984). Therefore, while proctolin most likely activates protein kinase C in Limulus cardiac muscle, any one of several second messengers could conceivably be utilized in the inotropic action of proctolin.

Proctolin appears to exert its actions in at least one other system by stimulation of phosphatidylinositol metabolism. In locust skeletal muscle, proctolin increases levels of inositol trisphosphate and enhances glutamate-induced contractions (Worden and O'Shea, 1986). Inositol trisphosphate causes the release of internal calcium from the sarcoplasmic
reticulum in invertebrate (Rojas et al., 1987; Tublitz and Trombley, 1987) and vertebrate (Streb et al., 1983; Somlyo et al., 1985; Vergara et al., 1985; Nosek et al., 1986) muscle. Phorbol esters also increase muscle contractility in some systems (Sybertz, 1986; Dale and Obianime, 1987; Itoh and Lederis, 1987), presumably as a result of activation of protein kinase C. While proctolin does appear to utilize the PI system in its inotropic actions in the Limulus heart, further studies are needed to determine the relationship between stimulation of the PI system by proctolin, protein kinase C activation and inotropic modulation.

Activation of protein kinase C in many systems is closely tied to calcium movements within the cell or across the cell membrane (for review see Berridge and Irvine, 1984). In the present study, experiments with proctolin and Ph dB on deganglionated heart muscle indicate that C-kinase is important in several actions of proctolin which are dependent on calcium. First, proctolin and the phorbol ester elicited prolonged, calcium-dependent contractures in Limulus cardiac muscle. A similar dependence on calcium has been noted for the postsynaptic action of proctolin in skeletal muscle of Limulus (Rane et al., 1984) and Homarus (Schwarz et al., 1980). In contrast, the contractile effect of this peptide on smooth muscle in insects is not dependent on external calcium (Holman and Cook, 1985). Similarly, contractures of the Limulus hindgut induced by proctolin or phorbol esters are independent of external calcium (Groome, in preparation). These findings suggest that smooth and striated muscle forms of C-kinase are activated by proctolin in Limulus, and perhaps in other arthropods as well, by regulatory mechanisms with distinct dependencies for external calcium.

A second calcium-dependent effect of proctolin or Ph dB on Limulus
cardiac muscle was the production of rhythmic contractions in the absence of neuronal input. Contractions produced by proctolin are associated with 10-20 mV spikes (Watson and Hoshi, 1985). The pharmacological profile of this effect of proctolin-induced contractions (sensitivity to Ca\(^{++}\) channel blockers but not to TTX) suggested to these authors that proctolin might elicit its myogenic action by unmasking voltage-sensitive calcium channels in normally unexcitable cardiac muscle fibers.

A proctolin-like effect upon cardiac muscle excitability can be elicited following the application of phorbol ester to deganglionated preparations. Intracellular recordings from phorbol-treated cardiac muscle indicate that contractions of heart musculature are, like those produced by proctolin, associated with small (10-15 mV) spike-like potentials in cardiac muscle fibers (unpublished results). In Aplysia, protein kinase C activation by phorbol esters results in the enhancement of a voltage-sensitive calcium current in the bag cell neurons at the onset of afterdischarge (DeRiemer et al., 1985b; Kaczmarek, 1986). Protein kinase C activation by proctolin or phorbol ester in Limulus cardiac muscle may result in a similar effect on a voltage-sensitive calcium current which is not expressed under normal conditions.

Protein kinases involved in the actions of dopamine

Protein kinase C activation may be an important process in the expression of both peptide and amine actions on the Limulus heart. Increased cardiac muscle contractility in response to proctolin appears to involve activation of the PI system, while cyclic nucleotides are not involved. On the other hand, it seems probable that increased muscle
contractility in response to DA involves activation of cardiac muscle DA receptors linked to the PI/protein kinase C messenger system as well as cardiac muscle DA receptors linked to the cAMP/protein kinase A messenger system.

Dopamine has an excitatory inotropic effect which is disproportionate with its capacity to elevate cardiac muscle cAMP. Dopamine, at $10^{-5}$ M, produced a positive inotropic effect on electrically stimulated cardiac muscle comparable to that produced by $10^{-5}$ M octopamine (Fig. 2, Chapter Two). However, DA produced significantly lesser increases in cardiac muscle cAMP than did OCT (Figs. 3-5, Chapter Two). This difference does not appear to be simply a consequence of excess cAMP, and thus an oversaturation of protein kinase A, produced by OCT. This hypothesis is supported by the observation that there was no significant difference in elevation of cardiac muscle cAMP by DA, NE or EPI, while the effect of DA on evoked contractions was much greater than that of either NE or EPI. Therefore, the overall inotropic effect of DA must involve some other mechanism, in addition to activation of protein kinase A, in Limulus cardiac muscle.

The similarity of proctolin, phorbol ester and dopamine effects on several preparations suggests that DA, like proctolin, activates protein kinase C in Limulus cardiac muscle. All of these agents elicited contracture and rhythmic contractions of deganglionated cardiac muscle. Additionally, the action of dopamine to induce the deganglionated myocardium to beat rhythmically is consistent with earlier findings in which dopamine produced myogenic activity during glutamate-induced contractures of deganglionated cardiac muscle (Watson et al., 1985).

Dopamine may exert its proctolin-like actions independent of its
effect on cardiac muscle cAMP. However, the observation that OCT or forskolin produce slight proctolin-like or DA-like effects in some preparations raises the possibility that direct second messenger interaction may occur between the protein kinase A and protein kinase C systems in Limulus cardiac muscle. In other systems, protein kinase C activation directly influences adenylate cyclase activity through guanine nucleotide-binding proteins (Proll et al., 1985; Aiyar et al., 1986).

Several facts suggest, however, that DA-induced cAMP synthesis in Limulus cardiac muscle is independent of its actions on protein kinase C. First, proctolin, which activates C-kinase, had no effect on levels of cardiac muscle cAMP. Second, removal of external calcium (which blocks the effects of DA, proctolin or phorbol ester on deganglionated preparations) does not influence increases in cardiac muscle cAMP produced by DA (unpublished results). Finally, OCT and forskolin, agents which increase cardiac muscle cAMP, rarely produced contracture of Limulus cardiac muscle or the appearance of rhythmic contractions, while DA consistently elicited these effects. These findings suggest that the similarity of inotropic actions of amines and peptides on the Limulus heart is a consequence of a common substrate for protein kinases A and C (e.g. the sarcoplasmic reticulum), rather than a result of direct second messenger interaction.

The inotropic actions of octopamine, dopamine and proctolin on the Limulus heart are overtly similar, yet appear to occur via several independent mechanisms. Synergy of cAMP and PI action has been demonstrated in the vertebrate heart (Suzuki and Wang, 1987). The calcium pump protein phospholamban of the sarcoplasmic reticulum is phosphorylated by cAMP-dependent protein kinase, but this phosphorylation

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is also stimulated by activation of the PI system. Additional studies may reveal that multiple kinase activation is a common regulatory scheme in the control of heart contraction strength.

Cyclic nucleotides and limadrin actions on the Limulus heart

Cyclic AMP may underly the excitatory chronotropic actions of limadrin on the Limulus heart, as suggested by the physiological and biochemical data presented. The actions of limadrin and the phosphodiesterase inhibitor IBMX were alike in several respects. First, both agents produced a long-lasting increase in heart rate. Second, the positive chronotropic effect of either limadrin (White and Watson, 1984) or IBMX (Table 1, Fig. 7, Chapter One) is the result of the action of these agents to increase burst frequency in the cardiac ganglion. Third, both IBMX and limadrin elevate cardiac ganglion cAMP within 3-10 min of application to the isolated cardiac ganglion, a finding consistent with the time course of their effects on heart rate. Therefore, limadrin-induced increases of cAMP in the cardiac ganglion are probably responsible for its positive effect on heart rate, while the significance of limadrin- and IBMX- induced increases in cardiac ganglion cGMP remains uncertain.

The biphasic inotropic actions of limadrin and IBMX may be a consequence of activation of both cAMP- and cGMP- dependent protein kinases in Limulus cardiac muscle. Both limadrin and IBMX increase cardiac muscle cAMP and have short-term positive inotropic effects. The subsequent decrease in heart contraction amplitude in preparations exposed to these agents may be a consequence of elevated heart rate, increased cardiac muscle cGMP, or both.
The similar inotropic actions of limadrin and IBMX provide further
evidence that cAMP, and perhaps cGMP, are important in the overall control
of heart contraction strength. These findings also suggest that an
endogenous cardioactive agent may utilize both of these second messengers
to produce its overall effect on the cardiac rhythm. Limadrin, like IBMX,
may produce its characteristic actions on the Limulus heart as a
consequence of its effects on cyclic nucleotide metabolism at several loci
within this system (cardiac muscle, cardiac ganglion and neuromuscular
junction) which contribute to the strength of heart contractions. However,
in comparison to IBMX, little has been done to characterize the site of
inotropic actions of limadrin.

**Limadrin as a FMRFamide-like peptide in Limulus**

FMRFamide is a cardioactive peptide in many species of molluscs
(reviewed by Greenberg and Price, 1980; Price et al, 1987). Additionally,
cAMP mediates the effects of FMRFamide, as well as serotonin, on a number
of molluscan hearts (Higgins et al., 1978; Painter, 1982a; Greenberg et al.,
1983).

In *Limulus*, partially purified limadrin increased levels of cyclic
nucleotides in *Limulus* cardiac ganglia and cardiac muscle. Earlier work
(White and Watson, 1984) indicated that this cardioactive FMRFamide-like
factor (MW 4400 daltons) in the *Limulus* brain is subject to enzymatic
degradation by pronase and trypsin, and that it elevates heart rate by
increasing the burst rate of the cardiac ganglion. Therefore, FMRFamide or
FMRFamide-like peptides, as well as several amines, may act on the
neurogenic (*Limulus*) and myogenic (molluscan) heart via the second
messenger cAMP.

The FMRFamide-like peptides isolated from the Limulus brain, cardiac ganglion and ventral nerve cord were similar in hydophobicity to either FMRFamide or pQDPFLRFamide, as evidenced by their retention time on HPLC (Fig. 12, Chapter Three). However, the synthetic peptides act on the Limulus heart only at very high concentrations (10⁻⁵ M peptide produced increases in heart rate of only 10 to 25%), while greater effects (50 to 70% increases in heart rate) were observed after application of brain extracts which contained only 10⁻⁸ M iR-FMRFamide in one ml (experiment three).

Several possibilities exist to explain this discrepancy. First, the FMRFamide-like peptides in Limulus may not be identical to FMRFamide or pQDPFLRFamide. For example, numerous FMRFamide-like peptides are present in molluscs which differ from the Helix peptide by a single amino acid substitution (reviewed by Price et al, 1987). Second, the peptides isolated by HPLC may exist in Limulus as part of a larger molecule. This hypothesis is suggested by the fact that these peptides co-elute on gel filtration columns very soon after the void volume, and that an approximate MW of 4400 daltons has been reported for limadrin (White and Watson, 1984). The endogenous limadrin peptide may contain the amino acid sequences of both of these peptides. During extraction and purification procedures this polypeptide could be fragmented, producing smaller peptides which have little or no effect on the Limulus heart. Finally, extracts of limadrin after gel filtration might also contain other cardioexcitatory molecules, such as acetylcholine. Therefore, purification and sequencing of limadrin are necessary to fully characterize the physiological and biochemical actions of this cardioexcitatory FMRFamide-like peptide in Limulus.
In summary, proctolin and the Limulus FMRFamide-like peptide limadrin appear to utilize discrete second messenger systems to produce amine-like excitation of heart contraction strength (proctolin, which activates protein kinase C) or rate (limadrin, which activates protein kinase A). Dopamine appears to activate both C- and A-kinases in Limulus cardiac muscle. These findings indicate that peptides and amines activate a number of second messenger systems to produce characteristic alterations in the Limulus heartbeat.
CHAPTER FIVE
CONCLUSIONS AND FUTURE DIRECTIONS

The present study demonstrates the importance of cyclic nucleotide and phosphatidylinositol (PI) metabolism in multiple actions of amines and peptides on the neurogenic Limulus heart. Amines and the Limulus FMRFamide-like peptide limadrin increase heart contraction rate by increasing cAMP in the cardiac ganglion. Amines also utilize cAMP to enhance cardiac muscle contractility and neuromuscular transmission. Proctolin, and apparently dopamine as well, activate the PI second messenger system to produce several characteristic effects on Limulus cardiac muscle. Apparently, amines and peptides exert similar excitatory chronotropic and inotropic effects on the neurogenic Limulus heart by co-utilizing several second messenger systems in the cardiac ganglion and in cardiac muscle.

Future experiments on amines, peptides and second messengers

1. Determination of the role of cAMP in amine actions on pacemaker neurons.

As discussed in Chapter One, amine excitation of burst rate in the Limulus cardiac ganglion involves a cAMP-dependent mechanism. Since amines excite pacemaker neurons to produce their positive chronotropic actions (Augustine and Fetterer, 1985), it seems more than likely that
amines increase cAMP within these pacemaker neurons to increase their frequency of firing. It is not possible, at present, to assay individual pacemaker neurons for cAMP content. Additionally, attempts to record from pacemaker neurons have been unsuccessful up to this point. The demonstration that forskolin and IBMX produce amine-like increases in the activity of these neurons, as is true of their actions on heart contraction rate and on cardiac ganglion burst rate, is necessary to confirm that cAMP elevation in these neurons is indeed responsible for the chronotropic action of amines on the Limulus heart.

Another cAMP-dependent action of amines in the Limulus cardiac ganglion was suggested by experiments in this study in which manganese was applied to the cardiac ganglion to block pacemaker input onto follower neurons. Apparently, manganese does not completely block transmitter released by pacemaker neurons onto follower cells. Application of amines, forskolin or IBMX elicited burst-like potentials in follower neurons, suggesting that amines may utilize cAMP to enhance synaptic efficacy between pacemaker and follower neurons. Increased cAMP in pacemaker terminals may result in increased transmitter release. A rigorous electrophysiological analysis of pacemaker neurons and of the pacemaker/follower synapse is necessary to characterize cAMP-dependent actions of amines on the Limulus cardiac ganglion. These studies are clearly needed before an investigation of the substrate-level effects of amines on cardiac ganglion neurons is warranted (see number 4 below).

2. Further characterization of neuropeptides in Limulus

In Chapter Three, several proctolin-like and FMRFamide-like peptides
in various Limulus tissues were purified by high pressure liquid chromatography (HPLC). The determination of amino acid sequences of these neuropeptides will indicate whether they are structurally identical to known peptides, or exist as novel peptides in Limulus, an essential piece of information.

The actions of proctolin on the Limulus heart have been described in detail only for the synthetic peptide (H-Arg-Tyr-Leu-Pro-Thr-OH), which was originally purified from the cockroach Periplaneta (Brown and Starrat, 1975). If a Limulus proctolin-like peptide (e. g. PP1) has the same amino acid sequence as cockroach proctolin, then the physiological and biochemical experiments using synthetic proctolin in Limulus can be considered physiologically relevant. Additionally, proctolin-like peptides of a different structure (e. g. PP2, and possibly PP1) may be synthesized in order to characterize their actions in Limulus.

Sequence analysis is of critical importance in the determination of the relationship between Limulus iR-FMRFamide and limadrin. Specifically, are either of the two FMRFamide-like peptides purified by HPLC responsible for the cardioexcitatory actions of the FMRFamide-like limadrin molecule? If these two peptides are identical to clam FMRFamide and Helix pQDPFLRFamide, then they probably are not, since these synthetic peptides have little effect on the Limulus heart (Table 6, Chapter Four). Alternatively, the Limulus FMRFamide-like peptides may be more active analogs of the synthetic peptides, or limadrin may be active only as a high molecular weight polypeptide. All of these possibilities underline the necessity to sequence the Limulus FMRFamide-like peptides purified by HPLC and characterize the structure and action of limadrin.
3. **Determination of particular second messengers involved in specific actions of proctolin and dopamine on Limulus cardiac muscle**

   Activation of either protein kinase A (by amines, Chapter Two) or protein kinase C (by proctolin and dopamine, Chapter Four) is important in the enhancement of cardiac muscle contractility. The myogenic actions of proctolin and dopamine appear to be a result of activation of protein kinase C and not of protein kinase A (Chapter Four). Cyclic AMP is involved in the inotropic actions of dopamine, but other second messengers of the PI signalling system appear to be important in the effects of dopamine, as well as proctolin, on *Limulus* cardiac muscle.

   Proctolin and dopamine may interact with cardiac muscle receptors coupled with phospholipase C to produce phosphoinositides, such as IP$_3$, IP$_4$ and diacylglycerol. It remains to be seen which of these metabolites is increased in *Limulus* cardiac muscle by either proctolin or dopamine. Biochemical studies focusing on the effects of peptides and amines on inositol lipids in *Limulus* cardiac muscle should be complemented with an investigation of the respective roles played by particular second messengers in specific inotropic and myogenic actions. For example, it may be that increased muscle contractility produced by proctolin is a consequence of any or all of the following events: internal calcium release promoted by IP$_3$, calcium flux across the sarcolemma induced by IP$_4$, or activation of a voltage-sensitive Ca$^{++}$ channel by DAG / C-kinase.

4. **Determination of the subcellular substrates for activation of protein kinase A and protein C in Limulus cardiac muscle.**
As mentioned above, dopamine shares the cAMP system with other amines in actions on cardiac muscle contractility, but shares the PI system with proctolin in actions on contractility as well as on myogenicity. The biochemical basis for the overtly similar responses of proctolin and DA on cardiac muscle may be a convergence of these second messenger systems on particular cellular substrates. A first step in the analysis of the subcellular sites of inotropic action of amines and peptides might be the determination of the effects of the amines and proctolin on protein phosphorylation in cardiac muscle. Extracts of $^{32}$P-treated cardiac muscle exposed to amines, peptides or specific pharmacological agents may be subjected to gel electrophoresis in order to elucidate the effect of these agents on particular protein bands. For example, forskolin may phosphorylate the same cardiac muscle proteins as octopamine, suggesting that these proteins are normally regulated by the activity of protein kinase A. The comparison of proctolin-, OCT- and DA-induced protein phosphorylation may give some insight into the subcellular basis for similarities and differences in the action of amines and peptides on Limulus cardiac muscle.

5. Investigation of the physiological significance of cGMP in Limulus cardiac muscle

Cyclic GMP is not involved in excitatory amine actions in the Limulus heart (Chapters One and Two). Amines and forskolin had no effect on cGMP metabolism in the Limulus cardiac ganglion. The phosphodiesterase inhibitor IBMX significantly increased cardiac ganglion cGMP, but its effect
on burst activity appears to be limited to its capacity to increase cAMP.

In cardiac muscle, on the other hand, IBMX and the FMRFamide-like peptide limadrin may exert a cGMP-dependent effect to decrease muscle contractility (Chapter Four). This peptide, like IBMX, increased cardiac muscle cGMP. However, while IBMX clearly inhibits electrically evoked contractions, the direct action of limadrin on cardiac muscle contractility has not been determined. In addition, the physiological significance of decreased cardiac muscle contractility, especially in response to an agent which increases heart contraction rate, is not obvious. Purification of limadrin is necessary in order to characterize the physiological and biochemical actions of limadrin in *Limulus* cardiac muscle.

The neurogenic *Limulus* heart is a useful model system in which to study the mechanisms underlying coordinated and multiple actions of endogenous amines and peptides on particular cellular targets. Amines and peptides produce their characteristic effects on the *Limulus* heartbeat by activation of particular intracellular signalling systems. The variety of second messenger systems which may be involved in neuromodulation of the *Limulus* heart presents an interesting opportunity to study second messenger interaction and synergy in the expression of similar cellular responses.
LIST OF REFERENCES


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Animal Maintenance

Horseshoe crabs (*Limulus polyphemus*) were obtained from three sources: they were purchased from the supply department of the Marine Biological Laboratories at Woods Hole, Massachusetts, or collected from either Great Bay, New Hampshire or from Raritan Bay, New Jersey. Horseshoe crabs were maintained on a diet of mussels in flow-through sea tables (ambient temperature) located at the Jackson Estuarine Laboratories, UNH. Several weeks prior to use, crabs were transferred to recirculating sea tables (10-15 °C) located at the Zoology Department, UNH. Adult horseshoe crabs of either sex (carapace width 15-25 cm) were used in this study.

Whelks (*Busycon contrarium*, 6-10 cm length) were purchased from Gulf Marine Specimens Co. (Panacea, Florida) and kept in recirculating sea tables (10-15 °C) at UNH. Whelks remained healthy for several months in this system. Cockroaches (*Periplaneta americana*) were purchased from Carolina Biological Supply Co. (Burlington, North Carolina) and maintained on a diet of cereal at room temperature (20-23 °C).

Dissections (*Limulus*)

*Limulus* hearts were removed according to the method of Pax and
Sanborn (1967). For each preparation, a section of the dorsal carapace was removed, and the heart with associated cardiac ganglion was excised from underlying tissues. The heart was placed in natural sea water (obtained from Portsmouth Harbor, New Hampshire) at room temperature and cleaned of adhering tissues.

Hearts were then either placed in perfusion chambers for pharmacological experiments or dissected further to yield the isolated cardiac ganglion and the deganglionated myocardium. The cardiac ganglion was removed by grasping its anterior end, severing the motor roots and teasing the ganglion away from the underlying muscle fibers. Cardiac ganglia were then placed in perfusion chambers for extracellular or intracellular recording, or used for biochemical experiments (see tissue incubation and extraction protocol below).

The deganglionated myocardium was stripped of residual motor nerves and cut into 1-2 cm cardiac muscle rings, which were used either in pharmacological experiments examining muscle contractility or for biochemistry. The residual motor nerves were left attached to the myocardium in those experiments involving recordings from the neuromuscular junction.

**Sources of Drugs**

Pharmacological agents utilized in this study were obtained from the following sources:

1. **Amines**: dopamine, epinephrine, norepinephrine, octopamine (Sigma Chemical Co., St. Louis, Missouri).
2. **Peptides**: proctolin, FMRFamide (Peninsula Laboratories, Belmont, California), *Helix* FMRFamide-like peptide pQDPFLRFamide (Bachem Laboratories, Torrence, California).

3. **Cyclic nucleotide analogues**: dibutyryl cAMP, dibutyryl cGMP, 8-bromo cAMP, 8-bromo cGMP (Sigma); 8-benzylthio cAMP, 8-parachloro phenylthio cAMP (ICN Pharmaceuticals, Irvine, California).

4. **Phosphodiesterase inhibitors**: 3-isobutyl 1-methyl xanthine, papaverine, theophylline (Sigma); RO-20-1724 (a gift from Hoffman LaRoche, Nutley, New Jersey); SQ 20,009 (Squibb, Princeton, New Jersey).

5. **Phorbol esters**: phorbol 12,13 dibutyrate, phorbol 12-myristate 13-acetate, 1-oleyl 2-acetyl sn-glycerol (Sigma).

6. **Others**: forskolin (Calbiochem, San Diego, California); phentolamine (CIBA-GEIGY, Summit, New Jersey).

**Preparation and Storage of Drugs**

Drug solutions were either made by mixing powder in seawater or by adding an aliquot of a stock drug solution to seawater and mixing. Carriers for stock solutions were: dH$_2$O for peptides, 0.1N acetic acid for amines, 95% alcohol for forskolin and dimethyl sulfoxide for RO-20-1724. All stocks were diluted $\geq$ 1000 times. Control experiments indicated that the
equivalent carrier concentration did not affect the cardiac ganglion or cardiac muscle by itself.

_Tissue incubation and extraction - cyclic nucleotides_

Isolated cardiac ganglia or pieces of heart muscle (1 cm rings excised from the region between the 2nd to 6th ostia) were placed in vials containing 5 ml (cardiac ganglia) or 10 ml (cardiac muscle) of seawater at room temperature. The vials were continuously shaken for 1 hour, at which time tests were initiated by the addition of drug solutions. The tissues were removed after incubation and homogenized in ice-cold 6% trichloroacetic acid (TCA). The homogenates were centrifuged (cardiac ganglia at 10,000 x g; cardiac muscle at 5,000 x g) for 30 min at 4 °C. The supernatants were extracted with four, 5 ml washes of diethyl ether, and the aqueous phases dried on a vacuum centrifuge (Savant Instr., Hicksville, New York) and stored at -20 °C until assay. The tissue pellet from each homogenate was boiled in 1N NaOH for determination of protein content (Lowry et al., 1951).

_Radioimmunoassay for cyclic nucleotides_

Levels of cAMP and cGMP in _Limulus_ cardiac ganglia or cardiac muscle were determined by radioimmunoassay (RIA). Duplicate cAMP or cGMP standards (Sigma) and samples were diluted in 50 μL RIA buffer B (.05 M sodium acetate at pH 6.0, containing 0.1% gelatin and 0.1% sodium azide) and added to 10 x 75 mm incubation tubes. Tracer (10,000 cpm, 125I-labeled cAMP or cGMP, Chemicon Inc., La Jolla, Cal.) in 100 μL buffer B
containing 1% normal rabbit serum (NRS) was added, and the volume brought to 600 μL with buffer B. Cyclic AMP or cyclic GMP antibodies (Chemicon) were diluted (cAMP, 1/400; cGMP, 1/200) in 100 μL buffer A (.02 M sodium phosphate at pH 7.4, containing 0.1% gelatin and 0.1% sodium azide) with 1% NRS and added to each tube, except blanks. All tubes were then vortexed and incubated for 1 hour at room temperature.

Separation of bound and free 125I was accomplished by adding 100 μL secondary antibody (25% goat anti-rabbit gamma globulin in buffer A with 1% NRS) to each tube. After 30 min, the tubes were centrifuged (1800 x g for 30 min at 4 °C) and the supernatants were decanted. The pellets were solubolized (100 μL Protosol and 30 μL glacial acetic acid), mixed with 1 ml scintillation fluor, and counted (Beckman LS 7000).

Counts were converted to pmol cAMP or cGMP by linear regression of the working portion of the standard RIA curve. The zero standard cpm (35-50% of total cpm added) was used as the reference, or 100% bound. A series of dilutions from extracts of Limulus cardiac ganglia and cardiac muscle tissue displayed parallel inhibition of tracer binding as compared to standards, for either the cAMP or the cGMP RIA (Fig. 1). Both assays were sensitive to 0.1 pmol cyclic nucleotide. Neither cAMP or cGMP antibodies react with other cyclic nucleotides or cyclic nucleotide metabolic products (Chemicon).

**Tissue extraction - peptides**

*Limulus* tissues were excised, weighed and boiled for 10 min in 0.1N acetic acid containing .01% thiodiglycol. Tissues were stored at -20 °C until they were homogenized. The supernatants were centrifuged at 12,000
Fig. 1. Radioimmunoassay (RIA) for cyclic nucleotides in Limulus cardiac tissues. A. Cardiac ganglion and cardiac muscle TCA extracts displayed parallel binding affinity towards the cAMP antibody (1/400 dilution) in comparison to cAMP standards. B. Parallel inhibition was also observed for these extracts in the RIA for cGMP (1/200 dilution).
x g for 30 min at 0 °C. Peptide distribution measurements were performed on these supernatants. Further purification of selected tissues was initiated by re-extracting the dried supernatants with ice-cold TCA followed by centrifugation (12,000 x g for 30 min at 0 °C). The supernatants were further extracted with diethyl ether, and the aqueous phases were dried and stored at -20 °C until chromatographic purification techniques were applied.

**Peptide assays**

FMRFamide-like and proctolin-like activities in extracts of *Limulus* nervous tissue were determined by both RIA and bioassay. Assays were performed at each stage of the purification sequence to quantify the level of immunoreactive (iR-) and bioactive FMRFamide-like or proctolin-like material in each fraction and to concentrate active fractions.

**FMRFamide RIA**

All RIA constituents were made up in RIA buffer of the following composition: .05 M sodium phosphate containing 0.1% bovine serum albumin and .02% sodium azide, at pH 7.4. Samples or standards were added to glass 10 x 75 mm incubation tubes in 50 μL aliquots. Tracer consisted of $^{125}$I-TMRF (iodinations performed by Hazleton and Meloy Laboratories, Springfield, Virginia); approximately 20,000 cpm in 100 μL buffer were added to each tube. Assay buffer was added to each tube to bring the volume to 1 ml and all tubes were vortexed. FMRFamide antibody was added to all tubes except blanks and totals (Ab 231 at 1/10,00 in RIA buffer). The
FMRFamide antibody was kindly provided by Tom O'Donohue (NIH, Bethesda, Maryland). Details of its characterization can be found in O'Donohue et al. (1984). All tubes were vortexed and incubated at 4 °C for 24 to 36 hr. The separation protocol and calculation technique employed were identical to those utilized for the RIA of cyclic nucleotides, except for the buffer used. This assay was sensitive to 5 to 10 pg of synthetic FMRFamide, and extracts of Limulus brains or abdominal ganglia displayed parallel binding characteristics with FMRFamide (Fig. 2b).

Proctolin RIA

The protocol for detection of proctolin in Limulus by RIA was similar to that for FMRFamide, utilizing proctolin antibody "E" (generously provided by Barbara Beltz, Harvard Medical School, Cambridge, Massachusetts). This antibody has been characterized previously (Schwarz, 1983). In this study, antibody "E" was prepared from frozen stock at a dilution of 1/5000 in RIA buffer. Iodinated proctolin was obtained from Hazelton and diluted to yield 20,000 cpm in 100 μL buffer. The proctolin RIA was sensitive to 10 pg of synthetic proctolin, and extracts of Limulus nervous tissue exhibited parallel inhibition of antibody / tracer binding in comparison to standard proctolin (Fig. 2a).

FMRFamide Bioassay

The radula protractor muscle (RPM) of the left-handed whelk (Busycon contrarium) was used to determine FMRFamide-like bioactivity in Limulus extracts. Details of the protocol for dissection of this muscle may
Fig. 2. Radioimmunoassay (RIA) for peptides in extracts of Limulus tissues. A. Acetic acid extracts of Limulus brain and ventral nerve cord inhibited binding of $^{125}\text{I}$-proctolin to antibody "E" (diluted 1/5000). Serial dilutions of tissue extracts displayed parallel inhibition with proctolin standards. B. In a similar fashion, these extracts displaced $^{125}\text{I}$-FMRFamide from antibody 231 (diluted 1/10,000).
be found in Watson et al., 1984. The RPM was placed in a vertical 1 ml seawater perfusion chamber and the radula tied to a Grass Ft. 03 force transducer. Sustained contractures of this muscle could be elicited by addition of synthetic FMRFamide to the chamber by syringe. Samples were adjusted to pH 7.6 and added in the same fashion as standards. The assay was viable for many hours. Sensitivity of this preparation was approximately 1 ng/ml. The quantity of FMRFamide-like activity in extracts was calculated by linear regression. Comparison of the tension produced by the sample, to a standard curve of contracture tension elicited by doses of synthetic FMRFamide, allowed expression of sample activity in terms of pg FMRFamide equivalents (Fig 3).

Proctolin Bioassay

The cockroach (Periplaneta americana) hindgut preparation was used to screen Limulus extracts for proctolin-like bioactivity. The proctodeum and rectum of the cockroach were isolated and tied off at either end. The preparation was placed in the same type of recording chamber used for the Busycon RPM and perfused with aerated cockroach saline of the following composition: 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 5 mM CaCl₂, 4 mM NaHCO₃, 5 mM Hepes buffer at pH 7.4 and containing 20 mM glucose and 2 mM trehalose sugars. Samples were adjusted to pH 7.4 and added in 1 ml aliquots by syringe in the same manner as for the RPM bioassay. Synthetic proctolin elicited both a sustained contracture and individual contractions of the hindgut at a threshold dose of approximately 3 ng/ml. Reproducible contractures of the cockroach hindgut could be elicited for many hours.
Fig. 3. *Busycon* radula protractor muscle bioassay. A. Standard and extract dilution curves for FMRFamide assay. FMRFamide-like activity in extracts was quantified by comparing the contracture tension produced by a given sample to the linear regression of the standard curve. B. Synthetic FMRFamide (5 ng) and an extract of *Limulus* brain (0.1 brain equiv. in 1 ml) elicited contracture of the *Busycon* RPM. Samples were added at the arrow and washed off after peak contracture was reached. Calibration: vertical, 150 mg; horizontal 2 min.
Proctolin-like activity was measured by the degree of contracture produced by a given sample using the standard curve shown in Fig. 4.
Fig. 4. Periplaneta hindgut bioassay for proctolin. A. Standard and extract dilution curves. The degree of hindgut contracture was used to quantify the amount of proctolin-like activity present in extracts. B. Application of either synthetic proctolin (10 ng in 1 ml) or an extract of Limulus brain (0.1 brain equiv. in 1 ml) caused a similar response in this preparation. Samples were added at the arrow and washed off after peak contracture was established. Calibration: vertical 250 mg; horizontal 2 min.