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Modulation of the *Limulus* Heart[1]

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SYNOPSIS. In this paper we briefly review recent studies concerning amine and peptide modulation of the neurogenic *Limulus* heart. We have organized the presentation of our most recent data in terms of patterns that have emerged from our work which also appear to be true in several other systems. The comparative and perhaps evolutionary patterns which we have focused on are as follows:

1. A given modulator often acts at a number of sites in the system, or the organism, to produce an organized change in behavior or function.
2. Some modulators use the same second messenger to mediate their effects at each site in a system.
3. Modulators that have the same effect on a target tissue can use different cellular mechanisms.
4. The physiological state of a particular tissue *in vivo* may be markedly different from an isolated preparation due to local and circulating neuromodulators.
5. Each member of a family of modulators may play a unique role *in vivo*.

INTRODUCTION

There are a number of invertebrate and vertebrate systems that have proven invaluable as models for investigating neuromodulation. Although each system is unique there are a number of general patterns that have emerged which appear to cross traditional phylogenetic boundaries. This conference and the resulting volume of publications indicates that we have accumulated enough comparative data to begin to address these issues. In time we may be able to gain insight into the mechanisms that shaped the evolution of animal nervous systems and behavior.

The model system that we have concentrated on during the last 10 years, and the subject of this paper, is the neurogenic heart of the horseshoe crab, *Limulus polyphemus*. It has been the focus of many experimental analyses since Patten and Redenbaugh published a beautiful description of its anatomy in 1899 and Carlson demonstrated that it was neurogenic in 1904 (Carlson, 1904*b*). Much of the recent research on the *Limulus* heart has concentrated on determining the cellular mechanisms underlying modulation of the cardiac rhythm by a variety of neuroactive substances. Within the past few years research in our lab has elucidated the role of second messengers in the process of modulation and thus extended our understanding to the intracellular domain.

The first part of the paper is an overview of this particular model system and the modulators which affect it. In the second part of the paper we will summarize some of our most recent findings within the context of the cellular, comparative and evolutionary theme of this symposium. We will focus on general patterns which have emerged from our work which also appear to be true in other systems.

ORGANIZATION OF THE NEUROGENIC *LIMULUS* HEART

Basic anatomy and circulation

The *Limulus* heart is a long cylindrical structure which runs just underneath the dorsal carapace for most of the length of the animal (Fig. 1). The cardiac rhythm is controlled by the cardiac ganglion which lies on the dorsal surface of the heart muscle. Heart contractions begin near the middle of the heart and blood is pumped out through several large arteries to the remainder of the tissues. These vessels branch extensively to form a very complex arterial system, which surrounds most of the nervous system. Blood collects in the ventral sinus, just anterior to the gills. When

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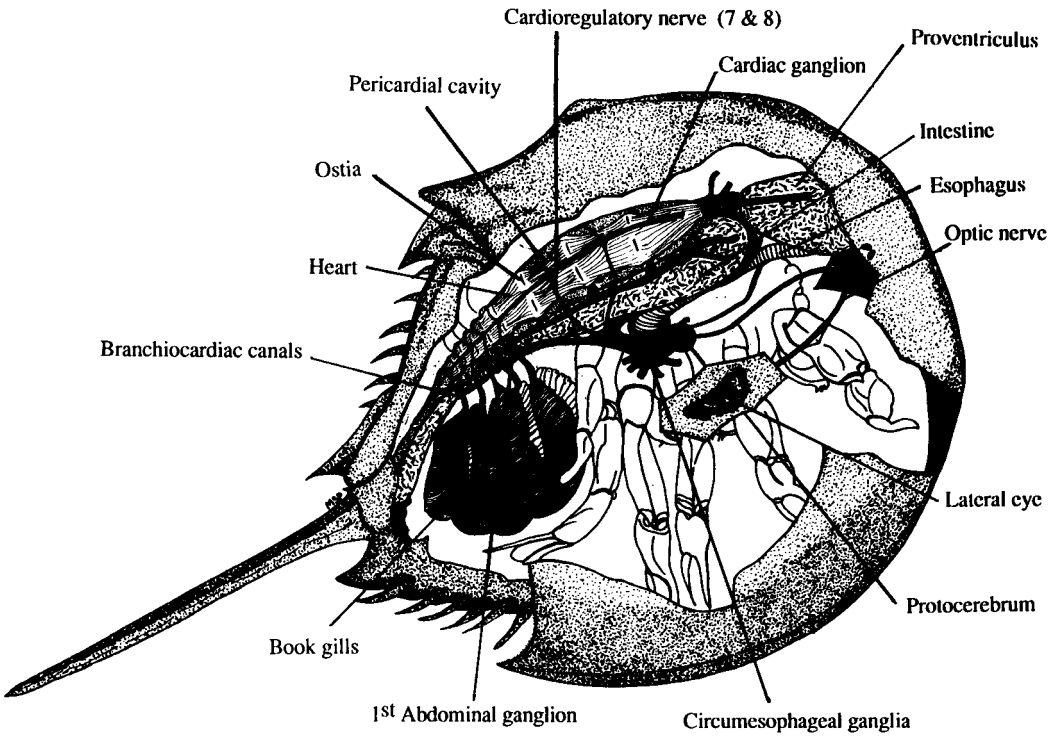


FIG. 1. Overview of *Limulus* anatomy.

the 5 gill plates contract rhythmically to ventilate the book gill lamellae they also act as a secondary heart, pumping oxygenated blood up to the pericardial cavity, through the branchiocardiac canals (Freadman and Watson, 1989; Fig. 1). Blood in the pericardial cavity is sucked into the heart through the 8 ostia when the heart relaxes. Valves in the ostia ensure that blood does not leak back into the pericardial cavity when the heart contracts.

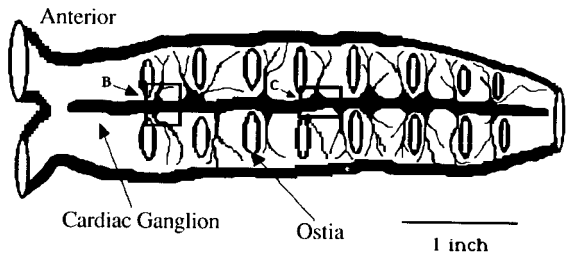
The location of the heart, just underneath the dorsal carapace, makes it convenient to monitor heart activity in freely moving animals by inserting fine wire electrodes through small holes drilled in the dorsal carapace. By correlating EMG and heart recordings one can demonstrate that the activity of the heart is coordinated with a number of stereotyped behavioral processes in the animal. For example, during gill cleaning the heart rate slows and during swimming the heart rate increases (Watson and Wyse, 1978). In other words, although the cardiac rhythm is not really considered a behavior by itself, modulation of heart activity is an important part of more complex behaviors, as is the case in a number of other animals represented in this symposium.

The large size of horseshoe crabs also makes it possible to attach EMG and EKG acoustic transmitters to their dorsal carapace without impeding their behavior. This work, which is just beginning, will enable us to firmly establish the relationship between cardioregulation and the normal behavior of horseshoe crabs in their natural habitat.

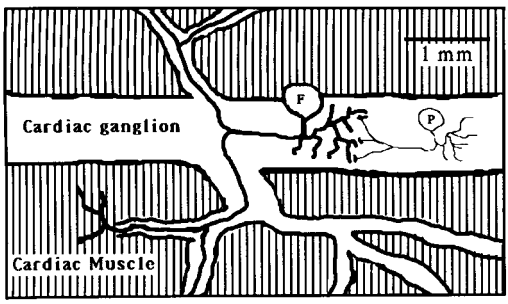
The neurogenic heart

The *Limulus* heart is comprised of circular and longitudinal heart muscle which is innervated and controlled by the cardiac ganglion, which consists of approximately 230 neurons (Bursey and Pax, 1970). While anatomical studies indicate that there are 5 different types of neurons in the ganglion (Bursey and Pax, 1970), only 2 functionally distinct classes of neurons have been dem-

A. Overview of the *Limulus* Heart



B. Schematic of the cardiac ganglion circuit



C. Lucifer fill of a follower neuron

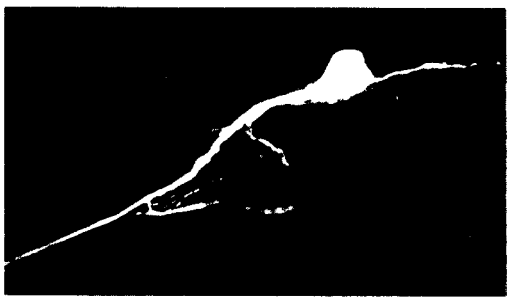


FIG. 2. Anatomy of the neurogenic *Limulus* heart. A. Overview of an entire heart. B. A schematic diagram of one section of the cardiac ganglion, showing the relationship between the pacemaker neurons (P), follower or motor neurons (F) and the cardiac muscle. C. A lucifer yellow fill of a follower neuron.

onstrated using electrophysiological methods (Abbott *et al.*, 1969; Lang, 1971; Augustine and Fetterer, 1985). The pacemaker neurons (20–40 μm) fire a single action potential at the beginning of each heartbeat (Figs. 2, 3). The action potentials in the pacemaker cells drive the 60–150 μm follower cells, which respond with a slow plateau potential upon which rides a burst of action potentials (Figs. 2, 3). The follower cells send axons out of the cardiac ganglion and appear to be motor neurons. Each follower cell burst leads to a compound EJP in the electrically inexcitable muscle fibers (Fig. 3). The prolonged (1–3 sec) depolarization resulting from the compound EJP causes a slow, steady heart contraction.

The basic mechanisms underlying the activity of the neurogenic *Limulus* heart are markedly different from those used by myogenic hearts. However, there exist a number of functional similarities between these two systems. First, both types of heart have a pacemaker system that communicates with the major contractile system via single, short duration action potentials. In the myogenic heart this pacemaker consists of specialized muscle cells, while in the neurogenic heart the pacemaker system is neuronal. Second, in each type of heart the pacemaker signal triggers a much longer duration electrical event which ultimately controls the duration, and to some degree the amplitude, of the heart contraction. In the myogenic heart, the ventricular muscle fibers produce a very long duration action potential which directly causes release of intracellular calcium and thus muscle contraction. In the neurogenic heart the follower cells produce a long burst of action potentials which indirectly, through the production of a compound EJP, causes a prolonged cardiac muscle contraction.

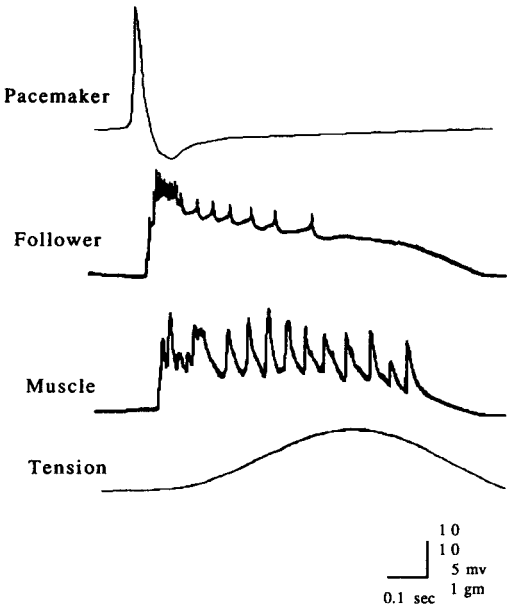


FIG. 3. Summary of the electrical and mechanical activity of the elements of the *Limulus* heart, during a single heartbeat. The cardiac cycle is triggered by a single, overshooting action potential in the pacemaker cells. Synaptic input from the pacemaker cells causes the follower cells to produce a plateau potential underlying a burst of action potentials. There is polyneuronal and multiterminal innervation of the cardiac muscle cells by the follower neurons, leading to a compound EJP during each cardiac ganglion burst. These compound EJPs lead to a slow contraction of the cardiac muscle. The bottom three traces were recorded simultaneously from one heart. The pacemaker trace was reproduced from a separate experiment.

Variations in the duration of the ventricular action potentials or follower cell bursts have the same effect on cardiac output in both systems. Thus, although the anatomical differences are dramatic there are a number of functional similarities between neurogenic and myogenic hearts.

As an experimental preparation for examining neuromodulation, the neurogenic heart offers a number of advantages. First, it is possible to monitor the responses of all of the elements of the system in an intact, isolated heart. Second, one can easily remove the entire cardiac ganglion from the heart and record from it, or the deganglionated heart muscle, for many hours. This makes it possible to independently examine the actions of a particular modulator on the cardiac ganglion neurons, the neuromuscular junction, or the heart muscle. The ability to isolate various parts of the system for physiological analysis is also an advantage for biochemical studies. Thus, one can determine how a given substance alters the system as a whole, and then demonstrate how the same substance affects each component of the system.

The simplicity of the *Limulus* neurogenic heart can also be a major advantage when trying to unravel the complexities of neuromodulation. Most researchers take advantage of the simple ganglia and large identifiable cells in invertebrates to study the actions of modulators on isolated ganglia. This approach has revealed a great deal about how certain substances can alter the strength of particular synapses (Klein and Kandel, 1978; Pellmar, 1981), change the probability that a given behavior will occur in response to a given stimulus (Prior and Watson, 1988), and modify the output of an entire neural network through selective excitation and inhibition of identifiable neurons and their synapses (Nagy and Dickinson, 1983; Harris-Warrick and Flamm, 1986). However, in many of these model systems the relationship between the output of an isolated ganglion and the actual behavior controlled by that ganglion is difficult to assess. For example, for years people have debated whether certain outputs of the *Pleurobranchaea* buccal ganglion represent ingestion or egestion (McClellan, 1982*a*, *b*; Gillette and Gillette, 1983; Croll and Davis, 1987), and many of the motor patterns produced by isolated crustacean stomatogastric ganglia in response to various neuropeptides and transmitters cannot be linked to a given feeding behavior or behavioral state. The circuitry of the *Limulus* cardiac ganglion is very simple and the output of the system, at any level, is fairly easy to interpret. This enables one to demonstrate where and how a particular compound affects the system and also interpret the functional consequences of a given pharmacological treatment.

The modulators

Modulatory substances probably reach the *Limulus* heart through release from cardioregulatory nerves. There are 7 pairs of cardioregulatory nerves in the horse-

shoe crab (Fig. 1). They emanate from the last 3 pairs of circumesophageal ganglia and from each abdominal ganglion. They appear to project to the area surrounding the heart and in some preparations apparent cardioregulatory nerve axons have been observed entering the cardiac ganglion. However, their terminals have never been visualized.

Stimulation of these nerves leads to inhibition and excitation of the heart (Pax and Sanborn, 1964; Pax, 1969; Corning and Von Burg, 1969), with the anterior nerves producing more inhibition. Recordings from cardioregulatory nerves 9 and 10 in semi-intact animals have revealed that there are distinct units which fire during cardioinhibition and others which are active during heart acceleration (Watson, 1978). Thus, neuronal control of heart activity is certainly present although it is not known what substances are released by the cardioregulatory nerves and where they are released.

A variety of substances have been proposed as possible cardioregulatory substances in *Limulus.* Since Carlson's initial pharmacological studies in 1904, scientists have been perfusing the hardy isolated *Limulus* heart with a number of drugs, hormones and neurotransmitters (Carlson, 1904*a*). We will focus on those compounds for which the evidence most strongly suggests a role in cardioregulation.

There is a variety of evidence suggesting that acetylcholine is the excitatory cardioregulatory compound in *Limulus.* Acetylcholine receptors are present in the cardiac ganglion (Thomas and Townsel, 1981). In addition, the cardiac ganglion, its lateral nerves and the cardioregulatory nerves stain for acetylcholinesterase (Stephens and Greenberg, 1973). However, the pharmacological actions of applied Ach are equivocal, perhaps due to presence of the abundant acetylcholinesterases (Townsel *et al.,* 1977).

The most likely candidates for inhibitory transmitters in *Limulus* are serotonin and GABA. Studies by Pax and Sanborn (1967*a, b*) demonstrated that both substances inhibit the heart, but in each case it was difficult to block the effects of cardioinhibitory nerve stimulation with the same compound that blocked the applied substance. In order to resolve this controversy it will be necessary to 1) repeat their experiments using some of the more recently developed pharmacological agents that are more potent and specific and 2) demonstrate the presence of the putative cardioinhibitory transmitter in the cardioregulatory nerves.

For the past decade, in collaboration with Drs. Augustine, Fetterer, Wyse and O'Connor, we have been studying the possible role of octopamine and the catecholamines as cardioregulatory agents in *Limulus.* In 1979 we demonstrated that all the major catecholamines, L-dopa, dopamine (DA), norepinephrine (NE) and epinephrine (E) are present in the *Limulus* nervous system, cardiac ganglion and cardioregulatory nerves (O'Connor *et al.,* 1982). Furthermore, octopamine is synthesized by isolated cardiac ganglia (Sullivan, personal communication) and released in response to hypoosmotic stress (Edwards *et al.,* 1979). In no case have we been able to establish that particular cells in the ganglion contain any of these amines. However, in the lobster, *Homarus americanus,* there is evidence that the large motor neurons in the cardiac ganglion contain either dopamine or norepinephrine (Ocorr and Berlind, 1983). All of the aforementioned amines increase the rate and strength of heart contractions (Augustine *et al.,* 1982). We have now determined where and how these amines act and we will discuss it in detail later in the paper.

There are also several neuropeptides located throughout the *Limulus* nervous system which appear to play a role in cardioregulation. The first of these to be discovered was a proctolin-like peptide (Benson *et al.,* 1981). Proctolin, or a proctolin-like peptide, is present throughout the *Limulus* nervous system, including the cardiac ganglion (Watson *et al.,* 1983; Groome, 1988; Groome, deTschaschell, Watson, Townley, Tillinghast and Wyse, in preparation). When applied to the heart, synthetic proctolin or the *Limulus* proctolin-like peptide increases the strength of heart contractions without altering the heart rate.

There are also one or more FMRFamide-like peptides in the *Limulus* nervous system (Watson *et al.,* 1984). Immunoreac-

tive FMRFamide has been visualized in the cardiac ganglion and fibers have also been observed on cardiac muscle. At least one of the *Limulus* FMRFamide-like peptides (tentatively named limadrin) appears to be different from synthetic clam FMRFamide (Watson *et al.*, 1984; White and Watson, 1984; Groome, 1988). While synthetic clam FMRFamide has weak excitatory effects, limadrin produces marked cardioacceleration. However, unlike the amines, FMRFamide-like peptides have little, if any, inotropic activity.

In crustaceans there is good evidence for the existence of neurohormones which are released from a pericardial organ (Cooke and Sullivan, 1982). Many of the substances that have been identified in extracts of the pericardial organs, such as octopamine (Evans *et al.*, 1976), serotonin (Cooke and Goldstone, 1970), dopamine (Cooke and Goldstone, 1970) and proctolin (Sullivan, 1979), have potent effects on the heart (Cooke, 1966; Cooke and Sullivan, 1982) and other tissues (Kravitz *et al.*, 1980; Cooke and Sullivan, 1982). However, while these neurohormones circulate at levels that are near the threshold to produce physiological effects there are no data to our knowledge demonstrating a causal relationship between fluctuations in circulating hormone levels and a given behavior or behavioral state. Thus, it is still not clear whether these substances are just acting locally on the neurogenic heart or both locally and systemically to produce a coordinated change in behavior or physiology.

In *Limulus* we have observed tissue over each pair of ostia. These tissues contain substantial quantities of proctolin-like and FMRFamide-like substances (Groome, 1988). The cardioregulatory nerves pass through this area and give off numerous processes as in the crustacean POs. It would be interesting to find out if two distantly related arthropods possess a similar neurosecretory organ.

Cellular, Comparative and Evolutionary Patterns

Now that we have presented some background information about the neurogenic *Limulus* heart and the compounds that modulate its output we are going to discuss some of the cellular, comparative and evolutionary patterns that have emerged from our work. Where possible we will also point out other systems where these same trends appear to be true.

A given modulator often acts at a number of sites in the system, or the organism, to produce an organized change in behavior or function

The major effect of dopamine and octopamine on the *Limulus* heart is cardioacceleration. Increases in heart rate are the result of direct effects of the amines on cardiac ganglion pacemaker cells (Augustine and Fetterer, 1985). When the heart rate increases in response to application of an amine there is a concomitant decrease in the duration of the follower cell plateau potential. This decrease in the plateau potential appears to be a result of being driven faster by the pacemaker cells. Entrainment of the cardiac ganglion to high burst rates by stimulating groups of pacemaker cells immediately changes burst duration in the follower cells (Fig. 4; Watson and Groome, in preparation). In both entrained preparations and hearts exposed to excitatory amines this change in burst duration results in fewer spikes per burst in the follower cells. Because the amplitude of the compound EJPs in the muscle are related to the number of follower spikes per burst, then at high heart rates one would expect the amplitude of the heartbeats to go down. However, the amines also act at other sites in the system to compensate for their negative inotropic action. The two other sites of action are the neuromuscular junction and the cardiac muscle (Fig. 5).

Dopamine and octopamine both increase the amplitude of compound and single EJPs recorded from cardiac muscle cells (Watson *et al.*, 1985). While amines do not alter muscle fiber input resistance, they produce an increase in the frequency of mEJPs, suggesting that amines act presynaptically to cause an increase in transmitter release (Watson *et al.*, 1985). Both compounds also increase the contractility of isolated cardiac muscle (Watson *et al.*, 1985; Groome,

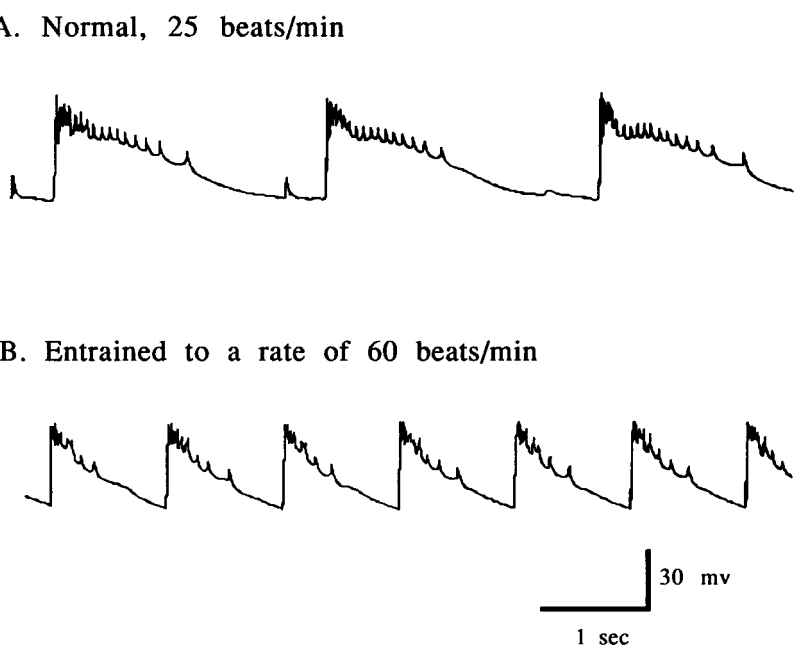


FIG. 4. Entrainment of a cardiac ganglion to a higher rate reduces the size of the plateau potential and the number of spikes/burst. Intracellular recordings were obtained from a follower cell in an isolated cardiac ganglion while it was bursting at a normal rate of approximately 25/min. Then the bursting rate was increased to 60/min by stimulating the pacemaker region of the ganglion with a suction electrode. The burst rate changed as soon as stimulation was initiated and the record shown at the bottom of this figure was obtained 5 sec after the onset of entrainment. There is an immediate reduction in burst duration and the number of spikes/burst.

1988). These actions lead to increases in contraction amplitude which partially compensate for the decrease in contraction amplitude that would result if amines acted only on the pacemaker cells. The net result is an increase in both the frequency and amplitude of heart contractions, which together produce an increase in cardiac output and blood pressure. It should be noted that the increase in the strength of contractions is particularly important because it has been demonstrated in several invertebrates that changes in stroke volume have the greatest impact on cardiac output (Smith, 1987; Wilkens, 1987).

There are many other examples of modulators or hormones producing a coordinated change in function by acting on multiple sites, at the cell, tissue and organ level of organization. From a comparative perspective it is interesting to note that catecholamines modulate cardiac output in the myogenic vertebrate heart in a manner that is analogous to amine cardioexcitation in *Limulus*, by increasing heart rate and strength of contractions. As the heart rate goes up the duration of the plateau potentials in the Purkinje cells is reduced. Due to the fact that contraction strength is strongly influenced by the duration of the Purkinje cell action potentials, decreases in their duration can reduce stroke volume. However, catecholamines also act on numerous sites within the Purkinje cells, to enhance the contractility of the heart muscle and assure a coordinated increase in cardiac output. Thus, as in the neurogenic *Limulus* heart, the combined actions of a single compound at multiple sites in the system result in a net increase of the rate *and* strength of heart contractions.

In this symposium there are numerous other examples of this general concept. The leech heart is driven by a neural network located in the segmental ganglia (Calabrese and Arbas, 1985; Calabrese and Norris,

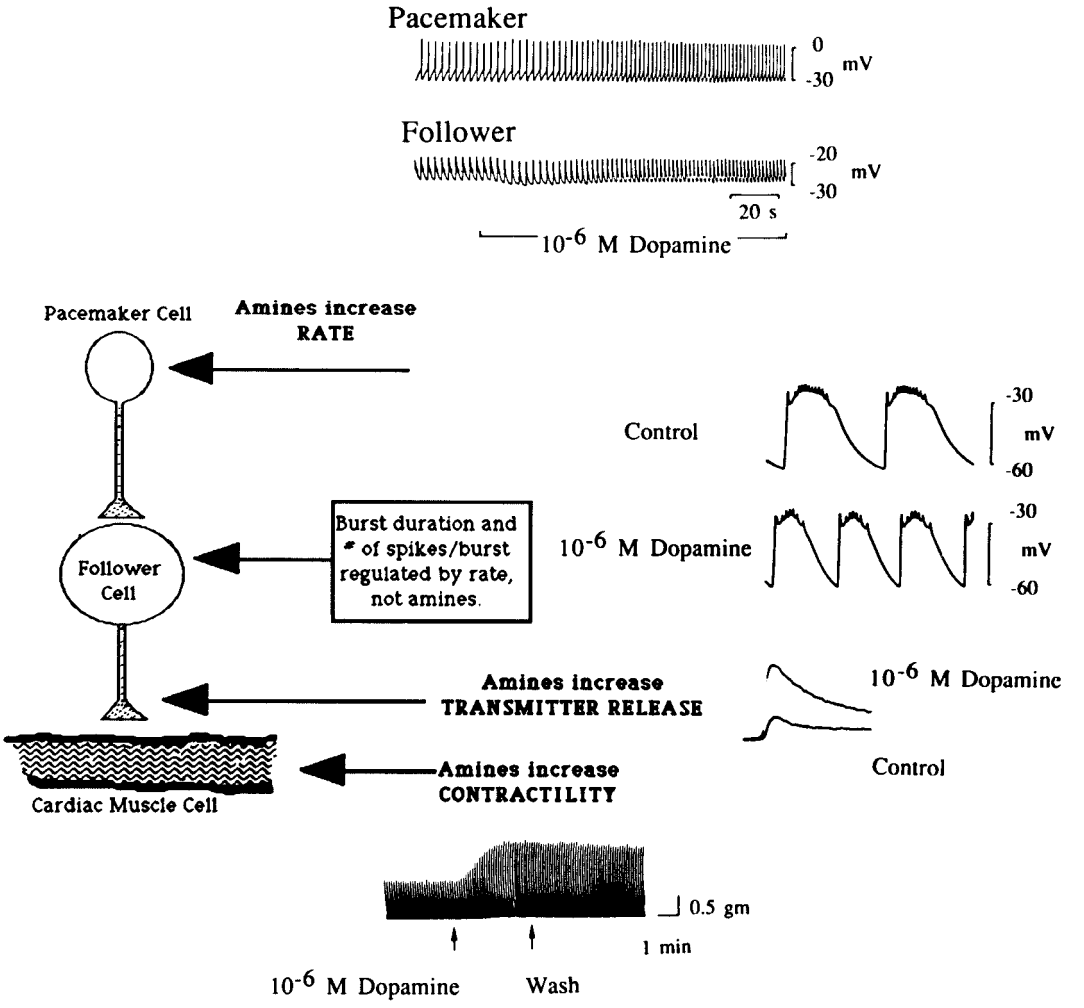


FIG. 5. Amines act at numerous sites in the neurogenic heart. The diagram on the left is a schematic of the cardiac ganglion circuitry. The figures on the right were taken from a number of different preparations. In the top figure intracellular recordings were obtained from a pacemaker and a follower cell during application of dopamine. The next figure demonstrates the differences in follower cell bursts before and after addition of dopamine. Beneath that figure are averaged evoked EJPs recorded from cardiac muscle before and after addition of dopamine. The bottom trace illustrates the response of deganglionated, electrically stimulated cardiac muscle to application of dopamine.

1989) and thus it could be considered a neurogenic heart with the cardiac ganglion located at some distance from the cardiac muscle. In the leech FMRFamide acts both on the central pattern generator and the cardiac muscle to increase cardiac output (Kuhlman *et al.*, 1985; Calabrese and Arbas, 1985).

In the feeding system of *Limax*, and most other mollusks as well, the central pattern generator for feeding is located in the paired buccal ganglia. SCP-like peptides are located in identified cells in the ganglion, in fibers innervating the salivary glands, and in numerous varicosities on the gut (Prior and Watson, 1987). Application of SCP_B to the intact preparation leads to enhancement of feeding behavior by making the feeding motor program in the buccal ganglia more responsive to feeding

stimuli, activating one of the salivary bursters, altering gut contractility, and stimulating the heart (Prior and Watson, 1987; Prior and Welsford, 1989). In *Aplysia,* where the involvement of SCPs in modulation of feeding has been investigated in more detail, SCP_A and SCP_B also modulate feeding behavior by acting at numerous sites (Lloyd, 1989; Lloyd *et al.,* 1988).

One of the most interesting examples concerns a single cell, the serotonergic metacerebral cell (MCC). The MCC in *Aplysia* and *Pleurobranchaea* influences the feeding central pattern generator in the buccal ganglia, the feeding motor neurons, and the feeding muscles (Gillette and Davis, 1977; Kupfermann *et al.,* 1979; reviewed in Ram, 1981). This provides an excellent example of an identifiable pair of cells using a single compound to modulate a certain behavior by acting simultaneously on pattern generating interneurons, motor neurons and appropriate muscle groups.

Hormones and transmitters could easily, and perhaps more efficiently, regulate behavior by simply acting on single key elements such as command fibers. However, this does not appear to be the pattern that has emerged from comparative studies (Ram, 1981). It is unlikely that we all discovered exceptions to the rule. Instead, it is more likely that compounds modulate motor systems at several different sites in order to produce complete, coordinated, and efficient changes in behavior.

Modulators often use the same second messenger to mediate their effects at different sites in a system

In the previous section we pointed out numerous examples of modulators acting at several different sites to produce a given coordinated change in behavior or physiology. We now want to extend that concept by suggesting that in some cases these modulators use the same second messenger at each of their target sites. As in the previous section, we will begin with a discussion of our own data and then compare it to findings in other organisms.

The primary excitatory amines in *Limulus,* octopamine and dopamine, appear to use the second messenger cAMP to mediate their intracellular actions at each site in the neurogenic heart system. Amines increase cAMP levels in both the cardiac ganglion and cardiac muscle (Groome and Watson, 1987). Agents, such as IBMX, that inhibit the phosphodiesterases that breakdown cAMP enhance the excitatory response to the amines. Moreover, agents such as forskolin, that increase levels of intracellular cAMP, mimic all of the actions of amines on the cardiac ganglion, the neuromuscular junction, and the cardiac muscle fibers (Fig. 6; Groome and Watson, 1989; submitted).

In the vertebrate myogenic heart activation of beta-adrenergic receptors by norepinephrine or epinephrine results in an increase in intracellular cAMP. Cyclic AMP then increases Ca^{++}-channel availability, enhances K^+-channels, activates phospholamban Ca^{++} pumps in the sarcoplasmic reticulum, and decreases the affinity of troponin for Ca^{++} (Reuter and Scholz, 1977; Tsien, 1977, 1987; Katz, 1979). While the anatomical differences are substantial, the functional consequences are very similar to those observed following activation of alpha-receptors by amines in the neurogenic *Limulus* heart. Ligand-induced elevation of cAMP leads to an increase in heart rate, a decrease in the duration of the electrical and mechanical events underlying each contraction, and an enhancement of the strength of heart contractions. In *Limulus,* the amines are acting through the same second messenger on different cells in the neurogenic heart while in the myogenic heart the amines are using the same second messenger at numerous sites *within* a given cell.

While it is possible to find numerous examples in the literature where a single compound acts at several different target sites, data concerning the second messengers used at each of these target sites is scarce. In many preparations, it is difficult to determine the second messenger(s) used in the cells that comprise the central pattern generator because the CPG circuitry is only a small fraction of the total circuitry in a ganglion of hundreds or thousands of neurons. However, the MCCs provide another example in which cAMP is the sec-

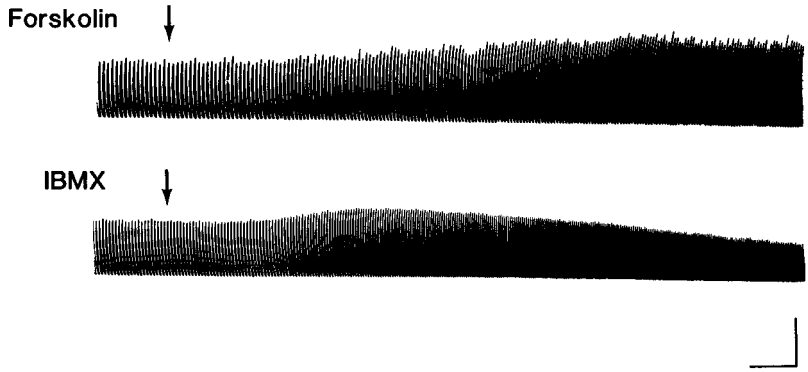


FIG. 6. Response of intact *Limulus* hearts to forskolin (5×10^{-6} M) and 10^{-3} M IBMX (3-isobutyl 1-methylxanthine). Both agents, like the amines, increase intracellular levels of cAMP and produce excitatory responses.

ond messenger used at both the neuromuscular junctions and in the ARM muscle (Ram, 1981). Additional comparative studies are clearly needed in order to determine whether this arrangement represents a unique situation or a common evolutionary pattern.

Modulators that have the same effect on a target tissue can use different cellular mechanisms

The role of a second messenger used to be quite straightforward when we were naive enough to believe that only a few existed. However, with the discovery of cGMP and a variety of phospholipid metabolites it has become clear that there is more than one way to transfer information across the plasma membrane. While some systems, such as the vertebrate heart, use the same second messenger to activate a number of different functionally related processes within a given cell, there are other preparations where it appears that different second messengers produce the same response.

Both amines and the pentapeptide proctolin cause an increase in contractility of *Limulus* cardiac muscle. Amines produce this increase in contractility by elevating intracellular cAMP (Groome and Watson, 1987; Groome and Watson, 1989). However, the overtly similar effect of proctolin *is not* mediated by cAMP or cGMP (Groome and Watson, 1989). Our most recent data suggest that proctolin acts through one or more of the phosphatidylinositol pathways. Application of a phorbol ester, which is known to activate protein kinase C, produces, like proctolin, an increase in contractility with no change in heart rate (Fig. 7). Thus, it appears that in *Limulus* cardiac muscle two different second messengers produce the same physiological effect (Fig. 8). What might be the adaptive significance of this arrangement? Perhaps it sets the stage for synergistic interactions between the two modulators. In *Aplysia,* two different second messengers, cAMP and the protein kinase C system, have a very similar effect on the firing of bag cells (Kaczmarek, 1986). While they modulate different ion channels it has been suggested that the two systems "act together to transform the firing pattern and shape of action potentials in the bag cell neurons" (Strong and Kaczmarek, 1987). As we learn more about the variety of second messengers and the way that they interact on different elements within the cell the functional significance of either using the same second messenger for multiple tasks, or different messengers for the same task, may become more evident.

The physiological state of a particular tissue in vivo *may be markedly different from that of an isolated preparation due to local and circulating neuromodulators*

In 1977 there was a Symposium at the American Society of Zoologists meeting entitled: "Comparative Physiology of

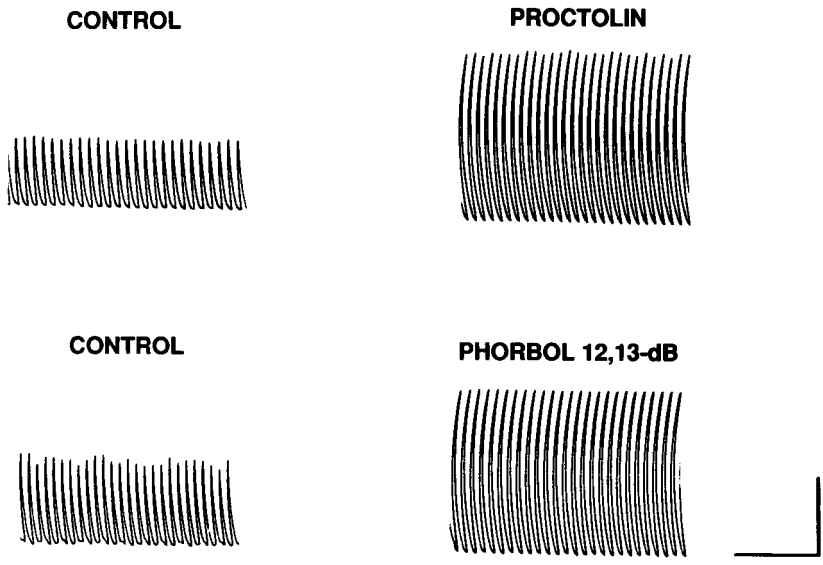


FIG. 7. Proctolin and the protein kinase C activator phorbol 12,13 dibutyrate elicit positive inotropic, but not chronotropic, responses when applied to the intact *Limulus* heart. Record of contractions before and during peak response to 10^{-7} M proctolin or 3×10^{-7} M phorbol ester. Calibration: vertical 1.5 g; horizontal, 1 min.

Invertebrate Hearts." One of the major items for discussion at this Symposium was the difference between neurogenic and myogenic hearts. The consensus was that there exists a continuum, since many species have hearts possessing both myogenic and neurogenic characteristics. Since that meeting the picture has become even more cloudy. In a number of different preparations it has become clear that the degree of stretch or the presence of certain modulators influences whether a given heart is myogenic or neurogenic. Smith (1987) has come up with the term "humourogenic" to describe situations where "the inherent myogenicity of the heart may depend on the level of a circulating peptide."

The *Limulus* heart is an interesting example of this phenomenon. This is somewhat ironic because it was the first neurogenic heart described (Carlson, 1904*b*) and has always been put forth as the classic example of neurogenicity. Shortly after we published our findings demonstrating that proctolin (10^{-8} M) caused a normal *Limulus* heart to beat more strongly (Benson *et al.*, 1981), we discovered that higher doses of proctolin (10^{-6} M) caused isolated *Limulus* heart muscle (cardiac ganglion removed) to beat rhythmically with approximately the same frequency as hearts with the cardiac ganglion intact (Watson and Hoshi, 1985). Normally, isolated *Limulus* heart muscle is electrically inexcitable (Abbott *et al.*, 1969). However, after the addition of proctolin small rhythmic spikes can be recorded from heart muscle and these appear to be responsible for triggering the rhythmic contractions. These actions of proctolin are calcium-dependent. Proctolin may be unmasking some voltage-sensitive Ca^{++}-channels and converting the normally quiescent myocardium into a myogenic state. It is not clear whether the mechanisms which underlay induction of myogenicity are an exaggerated form of the same mechanisms involved in inotropic modulation, whether they serve some other function, or if the concentration of proctolin surrounding cardiac muscle ever reaches a level capable of eliciting myogenicity.

As described earlier, the leech heart is similar to a neurogenic heart with a pattern generator that lies within the CNS. Under

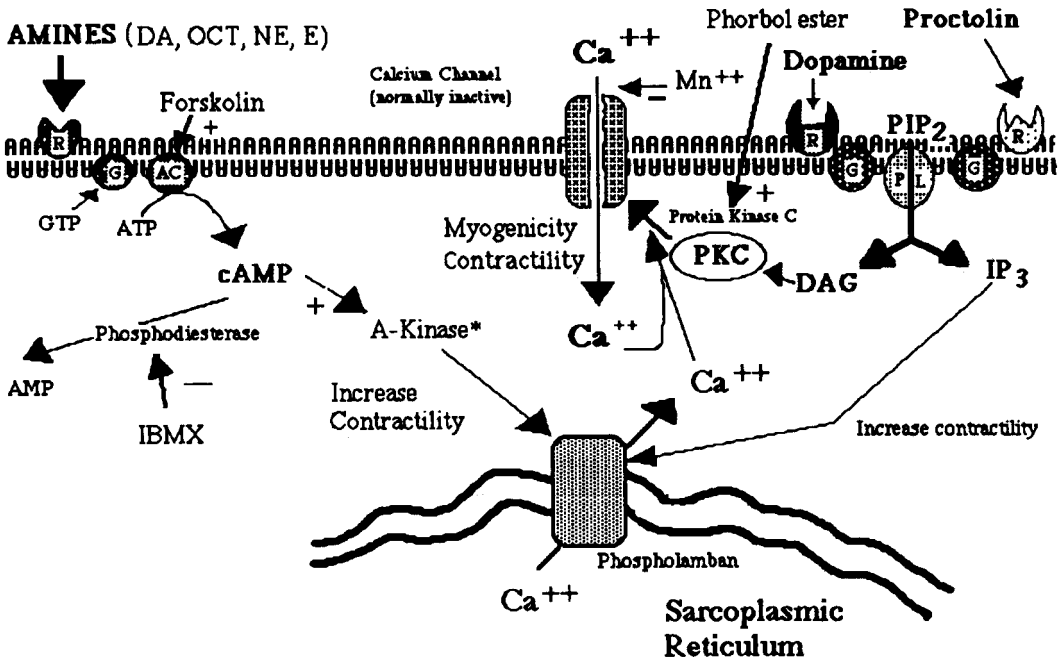


FIG. 8. Tentative model of how amines and peptides modulate *Limulus* cardiac muscle via second messenger pathways. There is strong evidence that several amines increase contractility using cAMP as a second messenger. The site of action of the protein kinase A, activated by cAMP, is not known. Proctolin appears to activate protein kinase C, which in turn may open calcium channels, leading to an increase in intracellular calcium and (at high doses) myogenicity. Proctolin may also produce an increase in IP_3 which could act directly on the sarcoplasmic reticulum to release intracellular calcium. There is also evidence that dopamine may act via either DAG or IP_3, in addition to producing an increase in cAMP. Abbreviations: R = receptor, G = G protein, AC = adenylate cyclase, DAG = diacylglycerol, PIP_2 = phosphatidylinositol 4,5-bisphosphate, IP_3 = inositol trisphosphate, PLC = phospholipase C.

normal circumstances the CPG drives the heart muscle via heart motor neurons such as the HE cells. Recently, Kuhlman *et al.* (1985) demonstrated that the peptide FMRFamide, or a close relative, is present throughout the leech CNS and in particular in the HE motor neurons and the HA modulatory cells. In addition, Calabrese and Maranto (1984) demonstrated that application of FMRFamide to the isolated leech heart caused it to beat rhythmically. Thus, as is true with the *Limulus* heart, application of a small peptide converts the heart from a neurogenic to a myogenic state. What is the normal state of the heart in *Limulus* and the leech *in vivo*, where it may be continuously perfused with a number of cardioactive substances? When we remove the heart from the hormones and modulators normally present in the blood and perfuse it with saline *in vitro* will it respond to other applied substances the same way that it would *in vivo*?

There are many other potential "humourogenic" systems. Smith (1987) describes a number of different molluscan hearts, most notably the *Aplysia* heart, which appear to require the presence of hemolymph in order to produce normal electrical and mechanical activity. Benson (1981) has described how the freshly isolated crab (*Portunus*) heart has electrically excitable muscle cells, but after perfusion with saline the fibers gradually become electrically inexcitable. We have witnessed a very similar phenomenon in horseshoe crabs (Fig. 4 in Watson *et al.*, 1985; unpublished data). The stomatogastric ganglion behaves much differently when its normal inputs from the CNS are blocked (Russell and Hartline,

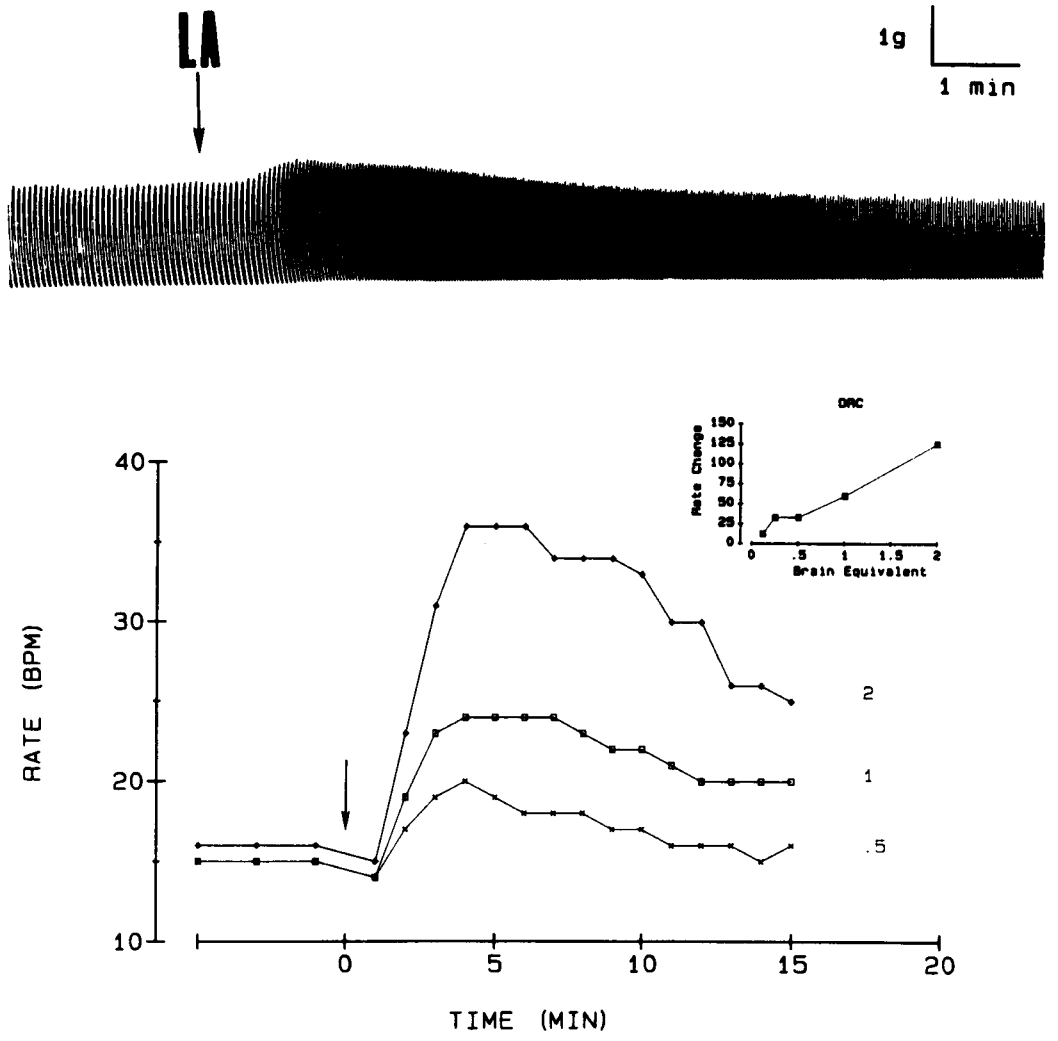


FIG. 9. Response of a *Limulus* heart to application of partially purified limadrin, a FMRFamide-like peptide. A. Application of two brain equivalents of limadrin leads to an increase in heart rate and a decrease in the amplitude of heart contractions. B. Time course and dose dependence of limadrin effects. Aliquots of active limadrin fractions from a G-50 Sephadex column were applied to a single *Limulus* heart (not the same heart illustrated in A), with at least 1 hour of washing between applications. Detectable excitation was achieved with 0.2 brain equivalents (B.E.s) and 2 B.E.s caused a 125% increase in heart rate.

1978). Much like the humourogenic hearts, normal stomatogastric bursting depends on substances released by inputs from the CNS. More recent work with the stomatogastric ganglion indicates that there are *at least* 7 different substances which are present in the neuropile and which modify the output of the system (Marder and Hooper, 1985; Harris-Warrick *et al.*, 1989). Which of these substances are normally present and released probably determines the normal state of the stomatogastric ganglion *in vivo*.

In almost all of the systems presented in this symposium there are examples of networks and target tissues that are modulated by several different substances. Most of us do our experiments on isolated preparations, perfused with saline. In order to interpret our data in terms of the situation

that exists *in vivo* we must make efforts to learn more about the circulating or local concentrations of the compounds of interest. In addition, we must also determine if the presence of one compound alters the system so that it is more or less responsive to another compound. These types of studies may help us to more fully comprehend the complexity of neuromodulation as it has evolved *in vivo*.

Each member of a family of modulators may play a unique role in vivo

Several years ago we demonstrated that there are 3 different catecholamines in the horseshoe crab nervous system (O'Connor *et al.*, 1982). Battelle and Evans (1984) have also reported that octopamine is present in neural structures. Our pharmacological studies on the heart indicate that all 4 of these amines have similar actions and all of their chronotropic effects are blocked by the alpha-adrenergic receptor blocker phentolamine (Augustine *et al.*, 1982). We tentatively concluded that they are all interacting with the same receptor. More work indicates that there are at least two dopamine receptors (Watson *et al.*, 1985; Groome and Watson, 1987). Further studies are necessary to determine which of the amines is the natural cardioexcitatory substance, and what roles the other amines play.

The situation is even more complex when dealing with the various peptide families. Here the families are larger and unfortunately peptide receptor pharmacology is in its infancy. Furthermore, studies in other organisms have demonstrated that even slight changes in the amino acid sequence of FMRFamide-like peptides dramatically alter their biological activity (Price *et al.*, 1985; Boyd and Walker, 1987; Anctil, 1987). In *Limulus* there are two or three different FMRFamide-like peptides (Watson *et al.*, 1984; Groome, 1988). Application of synthetic clam FMRFamide has only a slight cardioexcitatory effect, while one of the natural ligands, limadrin, produces marked cardioacceleration, accompanied by a decrease in contraction amplitude (Fig. 9). Even following purification of limadrin considerable work will be necessary to map the distribution of the different FMRFamide-like peptides and differentiate the relative physiological roles.

Now that peptide, transmitter, and even receptor families have become widely accepted, we must begin to ask why these families exist. Why is it adaptive to have several different FMRFamide-like peptides in one animal, or 5 different types of opiate receptors in the vertebrate brain, or two SCPs in one cell? Determining which member of a family is released at each location in the animal, and under what circumstances, may help us address these questions. Only when we know which ligand is naturally acting on our given experimental model system will we know that the physiological experiments we perform are valid. When we have a clearer picture of substance identity and the timing and site of release we can begin to truly integrate the effects of neuromodulators on isolated preparations with our understanding of normal behavior.

Closing Remarks

During the past 10 years we have opened up the pandora's box of neuromodulation. Despite the fact that we have been asking simple questions and using "simple" model systems each finding reveals additional complexity. Unlike traditional hormones and transmitters, modulators change the "state" of the system, often without producing a direct effect themselves. If neural networks like cardiac, buccal and stomatogastric ganglia are continuously bombarded with a wide array of neuromodulators, which each alter the probability that the network will function in a particular manner, then it makes it virtually impossible to predict how the system will respond to a given input *in vivo*. However, it is precisely this characteristic of neural networks that makes them capable of performing such a wide variety of tasks with relatively few elements. If our goal is to understand how the nervous system works then we must address this fundamental and complex problem.

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NOTE ADDED IN PROOF

We recently sequenced the proctolin-like peptide in the *Limulus* brain and it is identical to authentic cockroach proctolin.

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