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Second messenger systems underlying amine and peptide actions on cardiac muscle in the horseshoe crab, Limulus polyphemus

Winsor H. Watson III
University of New Hampshire, Durham, win.watson@unh.edu

J. R. Groome
University of New Hampshire, Durham

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SECOND-MESSENGER SYSTEMS UNDERLYING AMINE AND PEPTIDE ACTIONS ON CARDIAC MUSCLE IN THE HORSESHOE CRAB *LIMULUS POLYPHEMUS*

BY JAMES R. GROOME* AND WINSOR H. WATSON III
Zoology Department, University of New Hampshire, Durham, NH 03824, USA

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Summary

The biochemical mechanisms by which octopamine, catecholamines and the peptide proctolin exert their actions on *Limulus* cardiac muscle were investigated. Amines produced long-lasting increases in the amplitude of contractions evoked by electrical stimulation. At 10^{-5} mol l^{-1}, the apparent order of potency for amine-induced increases in evoked contraction amplitude was dopamine ≈ octopamine > norepinephrine ≈ epinephrine. At this dose, amines produced long-lasting increases in the levels of cyclic AMP (octopamine > dopamine ≈ norepinephrine ≈ epinephrine), but not of cyclic GMP, in *Limulus* cardiac muscle.

Like the amines, the adenylate cyclase activator forskolin enhanced cardiac muscle contractility and increased levels of cyclic AMP, but not of cyclic GMP. The phosphodiesterase inhibitor IBMX produced a transient increase in cardiac muscle contractility, but typically produced long-lasting negative inotropy. This agent increased levels of both cyclic AMP and cyclic GMP in *Limulus* cardiac muscle.

Proctolin and the protein kinase C activator phorbol dB increased the contraction amplitude of the intact heart and the electrically stimulated myocardium. These compounds, as well as dopamine, elicited sustained contractures and rhythmic contractions when applied to deganglionated *Limulus* cardiac muscle rings. Unlike the amines, proctolin and phorbol dB did not increase cardiac muscle cyclic AMP levels.

These results suggest that several second-messenger systems may be utilized by amines and peptides to produce excitatory actions on cardiac muscle fibers of the *Limulus* heart. Cyclic AMP appears to be an important second messenger underlying the effects of amines to enhance cardiac muscle contractility. Pharmacological data suggest that proctolin may alter cardiac muscle contractility and excitability by a mechanism which involves the phosphatidylinositol pathway. Dopamine, unlike the other amines, produces a number of proctolin-like effects and may activate both the cyclic AMP and the phosphatidylinositol systems in *Limulus* cardiac muscle.

* Present address: Biology Department, Utah State University, Logan, UT 84322, USA.

Key words: *Limulus*, amines, cyclic AMP, proctolin, protein kinase C.

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 a review, see Watson & Augustine, 1982). Amines, which include octopamine (OCT), dopamine (DA), norepinephrine (NE) and epinephrine (EPI), act on multiple cellular targets within the cardiac ganglion/cardiac muscle network to produce increases in both the rate and the strength of heart contractions. Heart rate is increased by aminergic actions on pacemaker neurons in the cardiac ganglion (Augustine *et al.* 1982; Augustine & Fetterer, 1985); and the strength of heart contractions is enhanced through presynaptic actions on the neuromuscular junction to increase transmitter release (Watson & Hoshi, 1981) and postsynaptic actions to increase cardiac muscle contractility (Watson *et al.* 1985).

The excitatory actions of the amines on *Limulus* heart involve cyclic AMP as a second messenger (Groome & Watson, 1987). Amines increase the concentration of cyclic AMP, but not of cyclic GMP, in the *Limulus* cardiac ganglion and in cardiac muscle. Drugs which cause increases in intracellular levels of cyclic AMP, such as forskolin and 3-isobutyl 1-methyl xanthine (IBMX), have effects on the intact heart which are similar to those produced by the amines. In this study we investigated the role of cyclic AMP as a second messenger mediating the inotropic actions of amines on cardiac muscle.

Like the amines, the peptide proctolin and a proctolin-like peptide partially purified from *Limulus* nervous tissue produce increases in the strength of heart contractions (Benson *et al.* 1981). Proctolin produces its inotropic actions by enhancing cardiac muscle contractility (Watson *et al.* 1983). It also induces rhythmic contractions in the normally quiescent deganglionated *Limulus* heart (Watson & Hoshi, 1985). The biochemical mechanisms underlying these inotropic and myogenic effects have not been investigated in *Limulus*.

In this paper we present evidence indicating that most, but not all, of the actions of amines on *Limulus* cardiac muscle involve cyclic AMP as a second messenger. Additionally, proctolin and, to some extent, dopamine may act on cardiac muscle through a second-messenger system involving protein kinase C. Therefore, peptide and amine modulation of cardiac muscle contractility in *Limulus* involves at least two second-messenger systems.

Materials and methods

Measurement of cardiac muscle contractility

The contraction amplitude of intact *Limulus* hearts was monitored as described previously (Groome & Watson, 1987). To examine the direct effect of various compounds on cardiac muscle contractility, cardiac muscle rings (1–2 cm) were excised from deganglionated hearts and contractions were evoked by electrical stimulation. One 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 by a thread to a force transducer (Grass FT.03C). Contractions were

evoked by passing current (1–2 V, 80 ms duration at 0·1–0·2 Hz) between vertical stainless-steel plate electrodes located 2 mm from either side of the myocardial ring. Test solutions were added *via* the perfusion reservoir. To examine the effect of compounds on cardiac muscle fiber excitability in the absence of neuronal or electrical stimulation, tension was recorded from deganglionated myocardial rings pinned out in 5 ml perfusion chambers. Preparations were perfused continuously (5 ml min^{-1}) with sea water at room temperature.

Radioimmunoassay for cyclic nucleotides

The tissue extraction and radioimmunoassay (RIA) 'protocols for *Limulus* tissues have been described previously (Groome & Watson, 1987). Briefly, cardiac muscle rings were incubated in test solutions, homogenized in 6 % trichloroacetic acid and centrifuged (10 000 *g* for 30 min at 0°C). The supernatants were extracted in diethyl ether and dried. Cyclic AMP and cyclic GMP levels in cardiac muscle extracts were then determined by RIA. The tissue pellet from each homogenate was boiled in 1 mol l^{-1} NaOH for determination of protein content (Lowry *et al.* 1951).

Solutions

Natural sea water or artificial *Limulus* saline was used in these experiments. Normal *Limulus* saline was of the following composition: 445 mmol l^{-1} NaCl, 12 mmol l^{-1} KCl, 10 mmol l^{-1} $CaCl_2$, 46 mmol l^{-1} $MgCl_2$, with 10 mmol l^{-1} Hepes buffer at pH 7·5. Zero-calcium saline was made by omitting $CaCl_2$ and adding 2 mmol l^{-1} EGTA. Pharmacological agents utilized in this study were obtained from the following sources: 3-hydroxytyramine hydrochloride (dopamine, DA), (−)adrenalin (epinephrine, EPI) 3-isobutyl 1-methyl xanthine (IBMX), (−)-arterenol (norepinephrine, NE), DL-octopamine (OCT), 1-oleyl-2-acetyl sn-glycerol, papaverine, phorbol 12,13-dibutyrate (phorbol dB), phorbol 12-myristate 13-acetate (Sigma Chemical Co., St Louis, Missouri); forskolin (Calbiochem, San Diego, California); proctolin (Peninsula Laboratories, Belmont, California); RO-20-1724 (a gift from Hoffman LaRoche, Nutley, New Jersey); SQ 20009 (Squibb, Princeton, New Jersey).

Drug solutions were made either by dissolving agents directly in sea water, or by adding a sample of a stock drug solution to sea water. Carriers for stock solutions were: 0·1 mol l^{-1} acetic acid for amines, 95 % ethyl alcohol for forskolin, 100 % dimethyl sulfoxide for RO-20-1724 and distilled water for others. All stocks were diluted ≥1000 times. Control experiments indicated that these carrier concentrations did not affect the contractile or electrical properties of cardiac muscle fibers.

Results

Amine effects on electrically evoked contractions of cardiac muscle

Direct effects of amines on cardiac muscle contractility are difficult to quantify

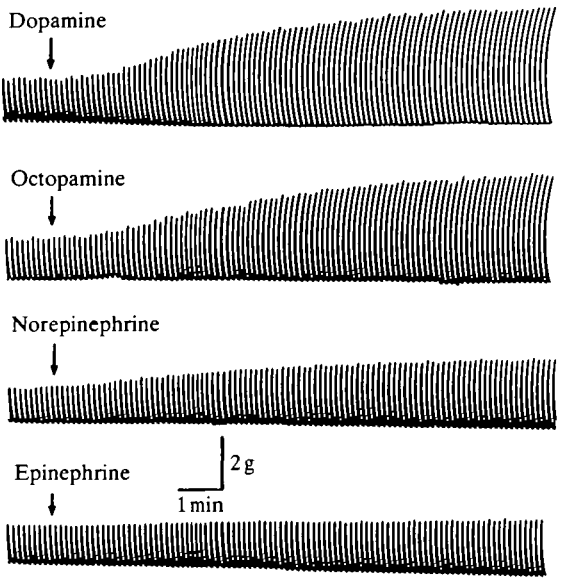

Fig. 1. Amines, at 10^{-5} mol l^{-1}, increased the amplitude of contractions of deganglionated cardiac muscle rings stimulated by electrical current pulses of constant frequency. Amines were added at the arrow and continuously perfused over the preparation. Responses to dopamine or octopamine were more rapid in onset and larger in magnitude than to either norepinephrine or epinephrine.

using the intact *Limulus* heart preparation, since amine-induced increases in heart rate decrease their overall inotropic action (Watson *et al.* 1985). Therefore, to assess the excitatory effect of amines on cardiac muscle contractility, deganglionated myocardial rings were prepared and contractions were evoked by electrical stimulation. All the amines tested, at 10^{-5} mol l^{-1}, produced a gradual increase in the amplitude of evoked heart contractions (Fig. 1, Table 1). Both DA and OCT had greater 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. 2A). 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 amplitude after 1–2 h.

Amine effects on levels of cyclic nucleotides in cardiac muscle

Levels of cyclic AMP in *Limulus* cardiac muscle were elevated by all the amines tested, at 10^{-5} mol l^{-1} (Table 1). At this dose, OCT had a significantly greater effect on cardiac muscle cyclic AMP levels than any of the other amines tested, producing a 795·1% (±139·8 S.E.M.) increase in cyclic AMP content. Amine-induced increases in cardiac muscle cyclic AMP content were long-lasting, persisting for at least 20 min after removal of amine from the incubation solution (Fig. 2B). None of the amines increased cyclic GMP levels in *Limulus* cardiac muscle (Table 1). Removal of calcium from the incubation medium did not reduce

Table 1. *Physiological and biochemical effects of amines and pharmacological agents on* Limulus *cardiac muscle*

Agent	Dose ($mol\,l^{-1}$)	Percent of control contraction amplitude	Cyclic AMP ($pmol\,mg^{-1}$ protein)	Cyclic GMP ($pmol\,mg^{-1}$ protein)
Control		100	6·1 ± 1·1 (6)	1·3 ± 0·2 (6)
Amines				
OCT	10^{-5}	659·6 ± 135·4 (14)†	54·6 ± 9·6 (6)†	0·8 ± 0·1 (6)
DA	10^{-5}	680·7 ± 163·0 (18)†	16·4 ± 2·6 (7)*	0·9 ± 0·1 (7)
NE	10^{-5}	227·7 ± 53·7 (12)†	14·9 ± 1·3 (8)*	1·1 ± 0·2 (6)
EPI	10^{-5}	217·6 ± 99·9 (12)*	14·4 ± 0·5 (8)*	1·1 ± 0·3 (6)
Peptides				
Proctolin	10^{-7}	211 ± 37·2 (9)†	–	–
	10^{-6}	–	6·3 ± 1·6 (6)	0·8 ± 0·1 (6)
	10^{-5}	–	7·7 ± 0·5 (6)	0·6 ± 0·2 (6)
Pharmacological agents				
Forskolin	5×10^{-6}	341·1 ± 100·4 (15)*	11·8 ± 0·8 (8)*	1·6 ± 0·2 (6)
IBMX	10^{-3}	109·1 ± 5·8 (14) 65·4 ± 5·0 (14)*	10·6 ± 0·5 (7)*	5·2 ± 1·2 (6)†
SQ 20009	10^{-4}	191·3 ± 24·7 (6)*	7·4 ± 0·6 (6)	–
RO-20-1724	10^{-3}	135·5 ± 9·8 (5)*	6·9 ± 0·1 (6)	–
Papaverine	10^{-3}	110·8 ± 6·4 (4)	7·7 ± 1·0 (6)	–
Phorbol dB	3×10^{-7}	351·9 ± 103·6 (10)†	5·5 ± 0·4 (10 min) (8) 5·3 ± 0·2 (20 min) (8)	– –

The mean (±S.E.M.) of N experiments (shown in parentheses) is represented for measurements of peak physiological effect (for IBMX both positive and negative values are given), and for radioimmunoassay measurements obtained after exposure of cardiac muscle rings to these agents for 10 min.

Level of significant difference from control was determined by a Student's t-test.

Level of significance: † ($P \leq 0{\cdot}02$); * ($P \leq 0{\cdot}05$).

OCT, octopamine; DA, dopamine; NE, norepinephrine; EPI, epinephrine; IBMX, 3-isobutyl 1-methyl xanthine; phorbol dB; phorbol 12,13-dibutyrate.

the effect of amines on cyclic AMP levels in *Limulus* cardiac muscle. At $10^{-5}\,mol\,l^{-1}$, both DA (255·0% ± 23·1 S.E.M., $N = 6$) and OCT (681·4% ± 51·3 S.E.M., $N = 6$) produced increases in cardiac muscle cyclic AMP after 3 min that were significant and comparable to increases observed when calcium was present.

The increases in cardiac muscle cyclic AMP levels induced by OCT or DA were dose-dependent (Fig. 3A). Octopamine increased levels of cardiac muscle cyclic AMP to a larger extent than did DA at all doses tested (10^{-6} to $5\times10^{-5}\,mol\,l^{-1}$). Neither OCT nor DA altered cardiac muscle cyclic GMP content at any dose (Fig. 3B).

Effects of forskolin and IBMX

The adenylate cyclase activator forskolin, at $5\times10^{-6}\,mol\,l^{-1}$, induced a gradual and long-lasting increase in the size of evoked heart contractions (Fig. 4, Table 1).

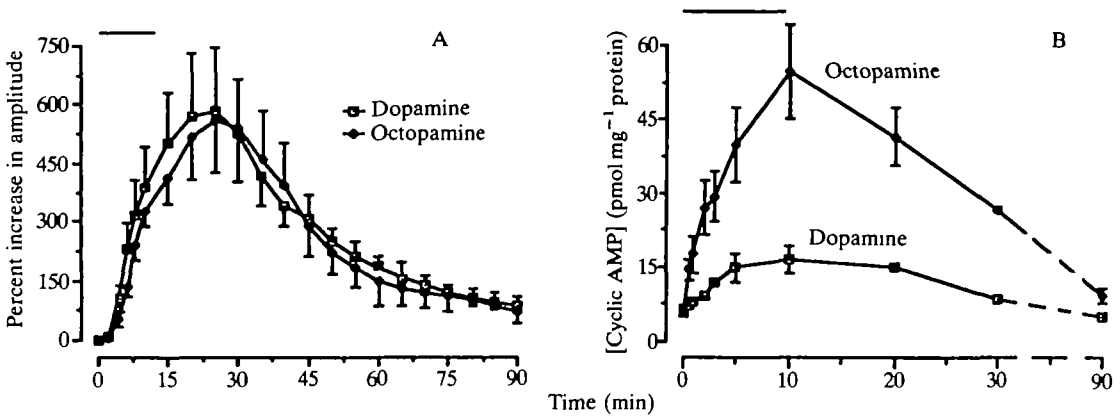

Fig. 2. (A) Dopamine (10^{-5} mol l^{-1}) and octopamine (10^{-5} mol l^{-1}) produced similar long-lasting increases in the size of electrically evoked contractions of deganglionated cardiac muscle. Amines were added for 10 min, indicated by the solid line, and contractions continued to increase for approximately 20 min after removal of amine from the perfusion solution. Each point represents the mean increase in contraction amplitude ± S.E.M. in 14–18 experiments. (B) Both amines (10^{-5} mol l^{-1}) elevated cardiac muscle cyclic AMP levels with a time course similar to their inotropic effect. However, the relative magnitude of the cyclic AMP increase for octopamine is much greater than that for dopamine. Each point represents the mean content of cardiac muscle cyclic AMP (±S.E.M.) in 6–7 experiments.

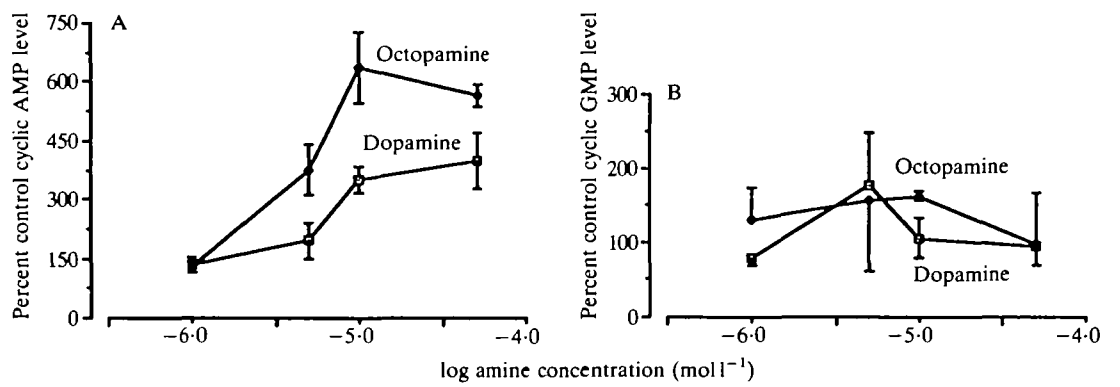

Fig. 3. Dose–response curve for amine effects on levels of cyclic nucleotides in *Limulus* cardiac muscle. Tests were performed by incubating cardiac muscle rings in various doses of octopamine or dopamine for 3 min. (A) Octopamine produced greater increases in cardiac muscle cyclic AMP than did dopamine at all doses above 10^{-6} mol l^{-1}. (B) Neither octopamine nor dopamine, at any dose, influenced cardiac muscle cyclic GMP levels in these experiments. In both A and B, each point represents the mean content of cardiac muscle cyclic AMP or cyclic GMP (±S.E.M.) in five experiments.

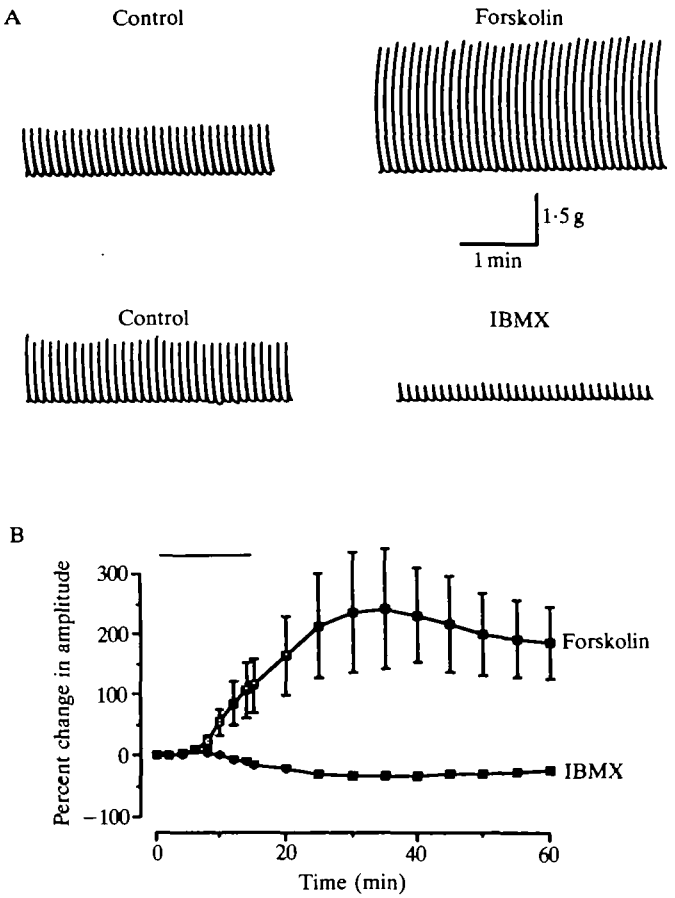

Fig. 4. Effect of forskolin (5×10^{-6} mol l^{-1}) and IBMX (10^{-3} mol l^{-1}) on evoked contractions in *Limulus* cardiac muscle rings. (A) Records of contractions before and 30 min after initial addition of agent to the preparation. (B) The effects of forskolin and IBMX (added for 15 min; solid line) on evoked contractions were long-lasting. Each point represents the mean change in amplitude ± S.E.M. in 14–15 experiments.

Enhancement of contraction amplitude was not blocked by pretreatment of the preparation with 3×10^{-7} mol l^{-1} tetrodotoxin (TTX). Since TTX appears to block residual or electrically evoked neuronal activity in this preparation (Watson *et al.* 1985), this result suggests a direct, amine-like effect of forskolin upon cardiac muscle fibers to increase contractility. At 5×10^{-6} mol l^{-1}, forskolin also increased levels of cardiac muscle cyclic AMP, but not of cyclic GMP (Table 1).

In contrast to the positive inotropic effect of forskolin, the phosphodiesterase inhibitor IBMX (10^{-3} mol l^{-1}) consistently decreased the amplitude of evoked contractions (Fig. 4, Table 1). The effect of IBMX was long-lasting and TTX-insensitive. However, in six of 14 experiments, IBMX elicited a transient increase in contraction amplitude prior to the onset of inhibition. IBMX, at 10^{-3} mol l^{-1}, significantly elevated levels of both cyclic AMP and cyclic GMP in cardiac muscle

(Table 1). The percentage increase in cardiac muscle cyclic GMP elicited by IBMX (292·3 % ± 74·4 S.E.M.) was much greater than the corresponding increase in cyclic AMP (73·8 % ± 3·6 S.E.M.) in this tissue. Other phosphodiesterase inhibitors, such as papaverine (10^{-3} mol l^{-1}), SQ 20009 (10^{-4} mol l^{-1}), and RO-20-1724 (10^{-3} mol l^{-1}), had lesser positive inotropic effects and negligible effects on levels of cardiac muscle cyclic AMP (Table 1).

Proctolin and phorbol dB actions on cardiac muscle contractions

Like the amines, the peptide proctolin increases the strength of heart contractions, but has a minimal effect on heart rate (Benson *et al.* 1981). The protein kinase C activator phorbol dB, at 3×10^{-7} mol l^{-1}, mimicked the positive inotropic action of 10^{-7} mol l^{-1} proctolin on the intact *Limulus* heart (Fig. 5). Both phorbol dB and proctolin produced a comparable increase in contraction amplitude, but the effect of the phorbol ester was slower in onset and in decay. In most experiments, the effects of proctolin and phorbol dB were exclusively inotropic, although occasionally these agents did produce slight increases in heart rate.

The inotropic effect of proctolin and phorbol dB was the result of an alteration in the properties of cardiac muscle fibers, since these agents also produced long-lasting increases in the amplitude of electrically evoked contractions of deganglionated myocardial rings (Fig. 6). The effect of neither agent was altered by pretreatment of the preparation with 3×10^{-7} mol l^{-1} TTX.

Phorbol 12-myristate 13-acetate and 1-oleyl 2-acetyl sn-glycerol were much less effective than phorbol dB in increasing the amplitude of cardiac muscle contractions. These agents produced only slight inotropic effects, and only at relatively high doses (10^{-6}–10^{-5} mol l^{-1}).

Effects of proctolin and phorbol dB on cyclic nucleotide levels in cardiac muscle

Levels of cyclic AMP or cyclic GMP in deganglionated cardiac muscle rings were not altered by a 10 min incubation in proctolin at 10^{-6}–10^{-5} mol l^{-1} (Table 1). Additionally, incubation of cardiac muscle rings in 10^{-6} mol l^{-1} proctolin solutions for periods ranging from 30 s to 10 min did not result in transient increases in levels of cyclic AMP or cyclic GMP. Like proctolin, phorbol dB (3×10^{-7} mol l^{-1}) had no effect on levels of cyclic AMP in cardiac muscle rings incubated in this drug for 10 or 20 min (Table 1).

Additional effects of proctolin, amines and pharmacological agents on Limulus *cardiac muscle*

Sustained contractures of cardiac muscle rings were often observed during application of 10^{-7} mol l^{-1} proctolin to preparations stimulated by electrical current pulses (Fig. 7). In several experiments DA, at 10^{-5} mol l^{-1}, produced a similar effect.

Proctolin, applied at a concentration of 10^{-6} mol l^{-1}, produces both contracture and rhythmic contractions in deganglionated *Limulus* hearts (Watson & Hoshi, 1985). In this study, similar effects were observed with the application of

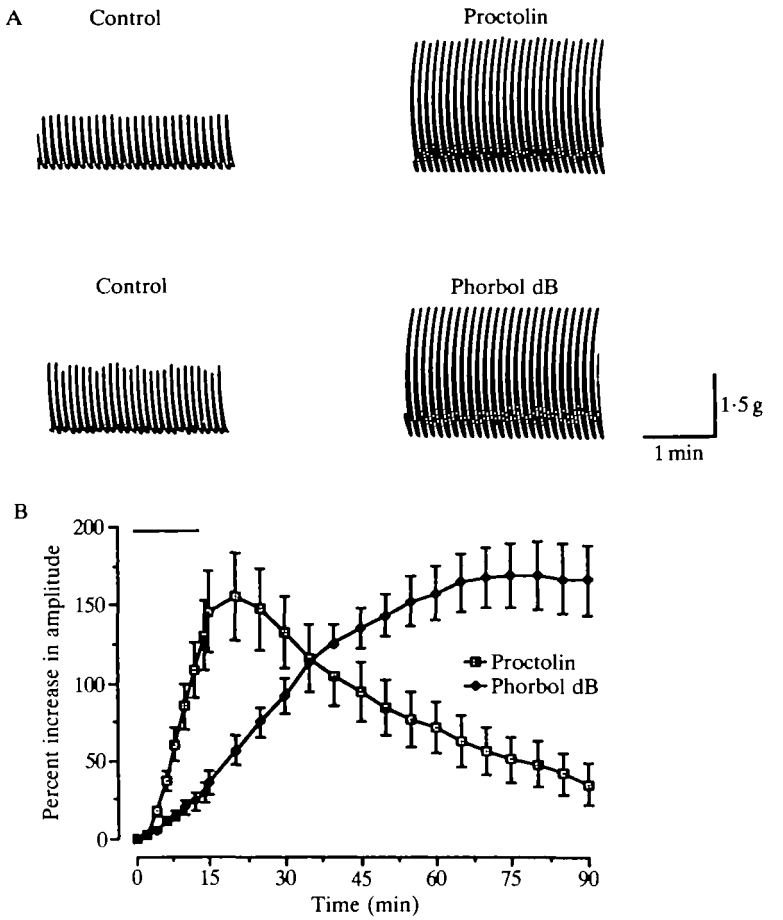

Fig. 5. Proctolin and the protein kinase C activator phorbol 12,13-dibutyrate (phorbol dB) elicit positive inotropic, but not chronotropic, responses when applied to the intact *Limulus* heart. (A) Record of heart contractions before and during peak response to 10^{-7} mol l^{-1} proctolin or 3×10^{-7} mol l^{-1} phorbol dB. (B) Time course of the inotropic effect of proctolin or phorbol dB (added to the preparation as indicated by the solid line).

10^{-6} mol l^{-1} proctolin to deganglionated 2 cm cardiac muscle rings (Fig. 8, Table 2). Dopamine (10^{-5} mol l^{-1}) and phorbol dB (5×10^{-7} mol l^{-1}) also elicited contracture and rhythmic contractions in deganglionated cardiac muscle rings (Fig. 8, Table 2). While the degree of contracture produced by phorbol dB was less than that elicited by either proctolin or DA, the phorbol ester more consistently produced rhythmic contractions than any agent tested. In contrast, OCT (10^{-5} mol l^{-1}) produced a modest contracture in only one of 15 preparations. Octopamine elicited rhythmic contractions in a few experiments, but these contractions were of much smaller amplitude and lower frequency than DA-

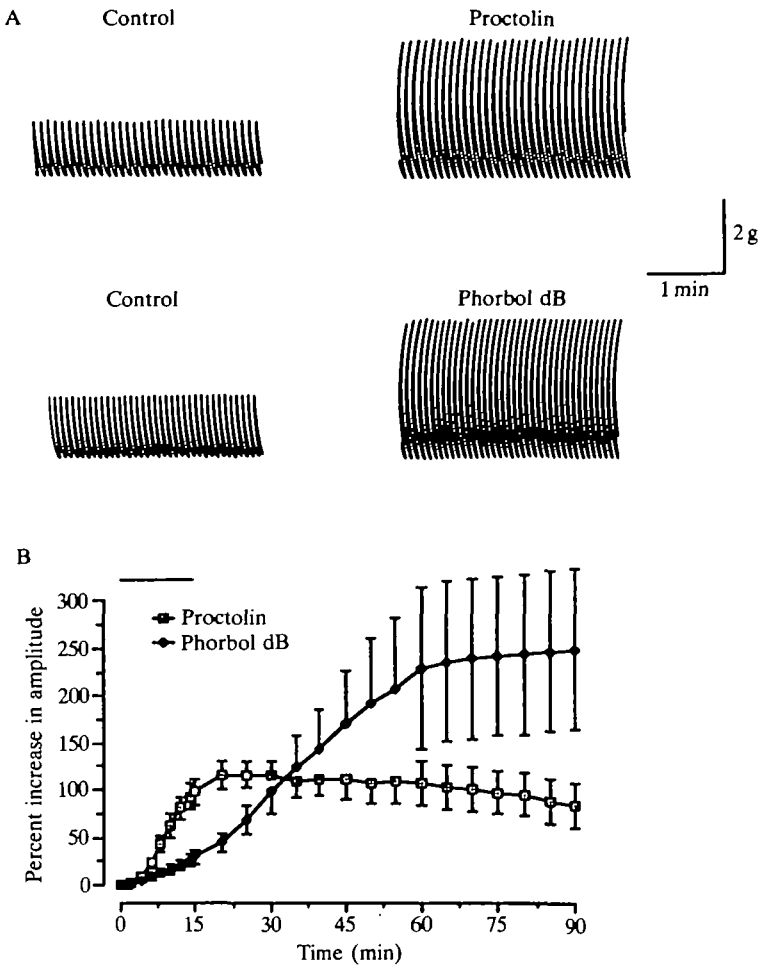

Fig. 6. The positive inotropic effects of proctolin and phorbol dB are the result of direct actions on cardiac muscle fibers. (A) Record of electrically evoked cardiac muscle contractions before and during peak response to 10^{-7} mol l^{-1} proctolin or 3×10^{-7} mol l^{-1} phorbol dB. (B) Time course of the increase in evoked contraction amplitude in response to proctolin or phorbol dB (solid line indicates application).

elicited contractions. Forskolin (10^{-5} mol l^{-1}) also rarely produced either of these effects.

Dependence on external calcium has previously been demonstrated for the action of proctolin on the deganglionated *Limulus* heart (Watson & Hoshi, 1985). In the present experiments, rhythmic contractions induced by the application of 10^{-6} mol l^{-1} proctolin, 10^{-5} mol l^{-1} DA or 5×10^{-7} mol l^{-1} phorbol dB were abolished with the introduction of the calcium channel blocker Mn^{2+} (20 mmol l^{-1}) to the recording chamber. Five to ten minutes after removal of Mn^{2+}, rhythmic contractions were again apparent. Addition of 3×10^{-7} mol l^{-1} TTX to these preparations did not abolish contractions. When cardiac muscle

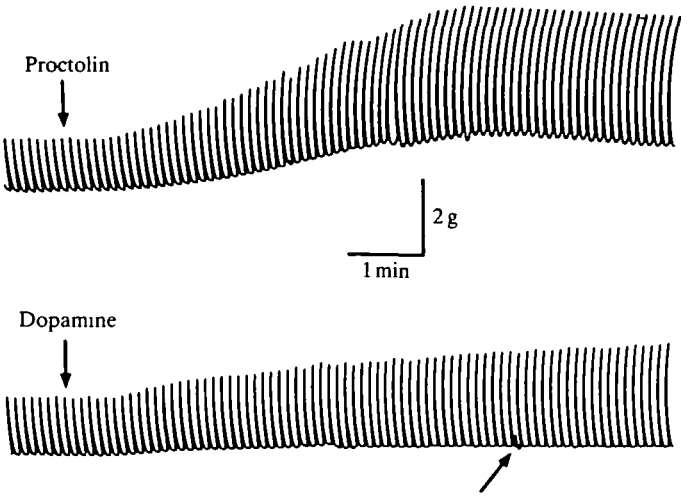

Fig. 7. Proctolin (10^{-7} mol l^{-1}) or dopamine (10^{-5} mol l^{-1}) often caused a sustained contracture when applied (continuously, starting at arrow) to cardiac muscle rings stimulated to contract by regular current pulses. The contractile effect of proctolin was observed more frequently and was more pronounced than that of dopamine. The bottom arrow points to a small after-contraction observed during application of dopamine. Both proctolin and dopamine produced after-contractions in some experiments.

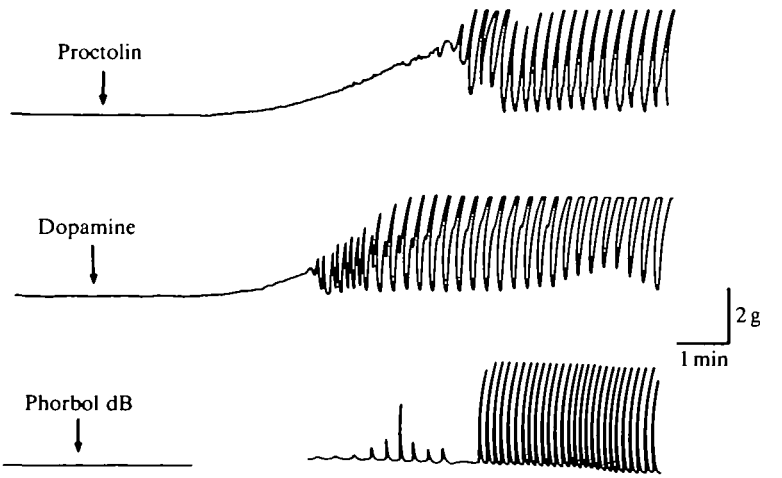

Fig. 8. Effect of proctolin, dopamine and phorbol dB on *Limulus* cardiac muscle excitability. Agents were applied to deganglionated cardiac muscle rings at the arrow and washed off after 10 min exposure. Proctolin (10^{-6} mol l^{-1}), dopamine (10^{-5} mol l^{-1}) and phorbol dB (5×10^{-7} mol l^{-1}) all produced rhythmic contractions of cardiac muscle. These agents often produced contracture of the myocardium prior to the initiation of contractions. The interruption in the bottom trace is equal to a 10 min period.

Table 2. *Physiological effects of proctolin, amines and pharmacological agents on unstimulated cardiac muscle*

Agent	Dose ($mol\,l^{-1}$)	(*N*)	Rhythmicity (% of preparations tested)	Increase in baseline tension (g)
Proctolin	10^{-6}	12	83·3	0·92 ± 0·26
DA	10^{-5}	30	63·3	0·34 ± 0·10
Phorbol dB	5×10^{-7}	22	95·2	0·13 ± 0·04
Forskolin	10^{-5}	15	23·3	0·02 ± 0·02
OCT	10^{-5}	15	23·3	0·01 ± 0·01

Agents were applied to the preparation for 10 min and then removed from the perfusion solution. Appearance or absence of rhythmic contractions of cardiac muscle rings 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 observed in response to particular agents were variable, but were most pronounced for proctolin, DA and phorbol dB.

The force of sustained contractures produced by a given agent prior to the onset of rhythmic contractions is expressed as the mean increase in baseline tension ± S.E.M.

See Table 1 for an explanation of the abbreviations.

rings were perfused with saline containing $0Ca^{2+}/2\,mmol\,l^{-1}$ EGTA, application of proctolin, phorbol ester or DA was without effect. Subsequent washing of the preparation with normal saline resulted in contracture and long-lasting rhythmic contractions.

Discussion

Several second-messenger systems appear to be involved in the expression of peptide and amine actions on *Limulus* cardiac muscle. The present study indicates that cyclic AMP is an important second messenger of amine actions on cardiac muscle contractility. While cyclic AMP may be the sole second messenger underlying the inotropic actions of OCT, NE and EPI, DA may utilize both the cyclic AMP and the phosphatidylinositol (PtdIns) systems to enhance the strength of heart contractions. Proctolin, which has no effect on cyclic nucleotide metabolism in *Limulus* cardiac muscle, may produce its positive inotropic (and myogenic) actions by a process involving the activation of protein kinase C. These findings suggest that the biochemical basis for peptide and amine modulation of cardiac muscle in *Limulus* consists of multiple second-messenger systems acting either in concert, or separately, to produce characteristic cellular responses.

Role of cyclic AMP in amine actions on cardiac muscle contractility

Octopamine, NE and EPI 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 cyclic AMP level. The adenylate cyclase activator forskolin also enhanced cardiac muscle contractility.

Although forskolin has been shown to produce effects in some systems by a non-cyclic-AMP mechanism (Hoshi *et al.* 1988; Wagoner & Pallotta, 1988), we believe that forskolin acts specifically on *Limulus* cardiac muscle fibers. Forskolin significantly elevated levels of cyclic AMP, but not cyclic GMP, in *Limulus* cardiac muscle. Additionally, forskolin has no effect on either the membrane potential or the input resistance of *Limulus* cardiac muscle fibers (J. R. Groome & W. Watson, unpublished results). Therefore, it appears that forskolin and the amines influence the process of excitation–contraction coupling in the *Limulus* heart by increasing cardiac muscle cyclic AMP level. The results from the present study are the first to suggest a cyclic-AMP-dependent mechanism underlying a specific action of amines to enhance cardiac muscle contractility in a neurogenic heart.

A cyclic-AMP-dependent mechanism appears to underlie the excitatory actions of amines on excitation–contraction coupling in the myogenic hearts of *Aplysia* (Sawada *et al.* 1984; Drummond *et al.* 1985) and *Mercenaria* (Higgins, 1974; Paciotti & Higgins, 1985). In addition, octopamine may utilize cyclic AMP to increase the contraction strength of the lobster heart (Battelle & Kravitz, 1978). The involvement of cyclic AMP in the positive inotropic actions of amines has also been documented for a wide variety of vertebrate species (Reuter, 1974; Tsien, 1977; Benfey, 1980; Kranias & Solaro, 1982; Earm *et al.* 1983; Macey *et al.* 1984). These studies and the present results suggest a general messenger role for cyclic AMP in amine modulation of cardiac muscle contractility.

Proctolin and cyclic nucleotides

Proctolin failed to stimulate cyclic AMP or cyclic GMP synthesis in *Limulus* cardiac muscle at doses (10^{-6} and 10^{-5} mol l^{-1}) which have profound effects on cardiac muscle contractility. Additionally, proctolin did not elicit a transient increase in either cyclic AMP or cyclic GMP level, as has been described in other peptidergic systems (e.g. the action of SCP_B in the *Aplysia* ventricle; Lloyd *et al.* 1985). These findings clearly indicate that proctolin does not increase cardiac muscle contractility in *Limulus via* a mechanism dependent on cyclic AMP or cyclic GMP.

Several previous studies have investigated the relationship between the actions of proctolin (or a proctolin-like peptide) on smooth, skeletal and cardiac muscle of arthropods, and the effects of this peptide on cyclic nucleotide metabolism (S.-Rosza & Miller, 1980; Jennings *et al.* 1983; Evans, 1984*a*,*b*). In these and the present studies, cyclic nucleotides were not shown to have a major role in the actions of proctolin on invertebrate muscle.

Possible second messengers underlying the effects of proctolin on Limulus *cardiac muscle*

The mechanism by which proctolin increases contractility in *Limulus* cardiac muscle may involve activation of protein kinase C. Several of the effects of proctolin and phorbol dB (which mimics the stimulatory action of diacylglycerol on protein kinase C) were similar in two separate preparations (intact heart and

electrically stimulated myocardium). First, both proctolin and phorbol dB 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 PI system involves the production of inositol trisphosphate ($Ins P_3$) and diacylglycerol (DAG) as second messengers (Berridge & Irvine, 1984). Inositol trisphosphate causes the release of internal calcium from the sarcoplasmic reticulum in invertebrate (Rojas *et al.* 1987; Tublitz & Trombley, 1987) and vertebrate muscle (Streb *et al.* 1983; Somlyo *et al.* 1985; Vergara *et al.* 1985; Nosek *et al.* 1986). Phorbol esters mimic the protein kinase C stimulating action of DAG in many cells (Kishimoto *et al.* 1980; Castagna *et al.* 1982). Phorbol esters also increase muscle contractility in some systems (Sybertz *et al.* 1986; Dale & Obianime, 1987; Itoh & Lederis, 1987). In locust skeletal muscle, proctolin may increase levels of inositol trisphosphate and enhances contractions induced by glutamate (Worden & O'Shea, 1986). While protolin may utilize the PtdIns system in its inotropic actions in the *Limulus* heart, further studies are needed to determine the relationship between stimulation of the PtdIns 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 cells membrane (for a review, see Berridge & Irvine, 1984). In the present study, experiments with proctolin and phorbol dB on deganglionated heart muscle suggest that protein kinase C may be 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. Calcium-dependence has been noted for the postsynaptic action of proctolin on skeletal muscle fibers in *Limulus* (Rane *et al.* 1984) and *Homarus* (Schwarz *et al.* 1980). A second calcium-dependent effect of proctolin or phorbol dB on *Limulus* cardiac muscle was the production of rhythmic contractions in the absence of neuronal input. Sensitivity to calcium channel blockers, but not to TTX, suggests a similar pharmacological profile of contractions induced by either proctolin or phorbol dB. Contractions produced by proctolin are associated with 10–20 mV spikes (Watson & Hoshi, 1985). Intracellular recordings from phorbol-treated cardiac muscle also reveal 10–15 mV spike-like potentials concomitant with contractions (J. R. Groome & W. H. Watson, unpublished results). These pharmacological results point out the necessity to determine the second messengers produced in *Limulus* cardiac muscle in response to proctolin. Biochemical studies may establish the specific role(s) for protein kinase C in the modulation of contractile and electrical properties of cardiac muscle fibers by proctolin.

Dopaminergic activation of multiple second-messenger systems in cardiac muscle

Dopamine significantly increased cyclic AMP levels in cardiac muscle with a time course similar to its effect on cardiac muscle contractility. However, in comparison with the other amines, DA had an excitatory inotropic effect which was disproportionate with its capacity to elevate cardiac muscle cyclic AMP level

(Table 1, Fig. 2). This discrepancy does not appear to be simply a consequence of excess cyclic AMP production (and thus an oversaturation of protein kinase A) by OCT. This hypothesis is supported by the observation that there was no significant difference in elevation of cardiac muscle cyclic AMP level 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.

The similarity in effect of proctolin, phorbol ester and DA on several preparations suggests that both DA and proctolin may activate protein kinase C in *Limulus* cardiac muscle. All these agents elicited contracture and rhythmic contractions of deganglionated cardiac muscle. Interestingly, DA has been shown to produce myogenic activity in deganglionated *Limulus* cardiac muscle during glutamate-induced contractions (Watson *et al.* 1985). Increased cardiac muscle contractility and excitability in response to DA may involve activation of cardiac muscle DA receptors linked to the PtdIns/protein kinase C messenger system as well as cardiac muscle DA receptors linked to the cyclic AMP/protein kinase A messenger system.

Cyclic AMP and cyclic GMP as opposing regulators of heart contraction strength

Cyclic GMP is not involved in excitatory actions of amines or proctolin on *Limulus* cardiac muscle. Rather, increases in cyclic GMP level may oppose the excitatory influences of cyclic AMP on cardiac muscle contractility. The phosphodiesterase inhibitor IBMX increased levels of both cyclic AMP and cyclic GMP in *Limulus* cardiac muscle (Table 1). It is possible that the biphasic inotropic response of the *Limulus* heart to IBMX (see Groome & Watson, 1987) is a consequence of the biochemical effect of IBMX on both cyclic AMP and cyclic GMP content of cardiac muscle. IBMX only transiently enhanced the strength of evoked contractions. This response was followed by prolonged inhibition. Positive inotropy in response to IBMX may be a consequence of increased levels of cardiac muscle cyclic AMP, while the negative inotropic effects of this compound may be a result of increased levels of cyclic GMP. It has been proposed that increased levels of muscle cyclic GMP might oppose the stimulatory effects of cyclic AMP on the myogenic rhythm in the extensor tibiae muscle of the locust *Schistocerca*, where millimolar concentrations of IBMX increase levels of both cyclic AMP and cyclic GMP (Evans, 1984*a*).

Recently, we have investigated the actions of a *Limulus* FMRFamide-like peptide (as yet unpurified) on the *Limulus* heart. This peptide, tentatively named limadrin (White & Watson, 1984), also produces a biphasic inotropic effect on the *Limulus* heartbeat and increases both cyclic AMP and cyclic GMP levels in cardiac muscle (Groome, 1988). The similarity of IBMX and limadrin actions on the physiology and biochemistry of *Limulus* cardiac muscle raises the possibility that cyclic GMP, like cyclic AMP, may be important in the neurohormonal regulation of cardiac muscle contractility.

Role of cardiac muscle cyclic AMP phosphodiesterase

A negative inotropic effect is sometimes observed during bath application of amines to the isolated, intact *Limulus* heart (Watson *et al.* 1985). This effect appears to be a consequence of increased heart rate, and not a direct, negative effect of amines on cardiac muscle contractility. Since cyclic AMP is important in excitatory chronotropic as well as inotropic actions of amines on the *Limulus* heart (Groome & Watson, 1987), the relative activities of cardiac ganglion and cardiac muscle cyclic AMP phosphodiesterases may be important in the fine tuning of simultaneous modulation of heart contraction rate and strength by amines.

Cyclic AMP elevation (and contractility increases) may be maximized in *Limulus* cardiac muscle by a weak cyclic-AMP-dependent phosphodiesterase, thereby partially offsetting the negative effects of increased rate. Cyclic AMP produced in cardiac muscle transiently exposed to amine was only slowly degraded to the inactive 5'-AMP metabolite. Contractility increases were likewise long-lasting. Additionally, IBMX is ineffective in potentiating the effects of sub-threshold doses of DA or OCT on heart contraction amplitude (Groome & Watson, 1987) or levels of cardiac muscle cyclic AMP (Groome, 1988).

Although SQ 20009 and, to some extent, RO-20-1724 increased evoked contraction amplitude, their biochemical actions are uncertain. For example, SQ 20009 produces marked irregularity in contractions of the intact *Limulus* heart preparation (Groome & Watson, 1987), unlike the amines or other pharmacological agents. In general, the phosphodiesterase inhibitors employed had minor effects on cardiac muscle cyclic AMP levels, suggesting that this enzyme does not effectively limit cyclic-AMP-dependent increases in contraction amplitude produced by the amines.

Amine-induced rate increases, however, may be limited by a highly active cyclic-AMP-dependent phosphodiesterase in the cardiac ganglion (Groome, 1988). Amines and pharmacological agents produce increases in the cyclic AMP content and burst rate of isolated *Limulus* cardiac ganglia which are of shorter duration than those observed in cardiac muscle. Additionally, IBMX potentiates the effects of subthreshold doses of DA or OCT to increase heart rate (Groome & Watson, 1987) or to elevate cardiac ganglion cyclic AMP levels (Groome, 1988). These findings suggest that a balance between the excitatory effects of amines on specific cellular targets in this system might be achieved by the relative activities of cardiac ganglion and cardiac muscle cyclic-AMP-dependent phosphodiesterases.

In summary, similar inotropic actions of amines and the peptide proctolin on the neurogenic *Limulus* heart may be achieved by activation of the protein kinase A system, the protein kinase C system, or both. Therefore, the *Limulus* myocardium represents a promising system for investigating the interactions between two classes of cardioregulatory agents and intracellular messenger systems. Finally, it will be interesting to discover the degree to which these second-messenger systems interact at other loci in the neurogenic *Limulus* heart network and to determine the molecular basis underlying the overall effects of these neurohormones on the cardiac rhythm.

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