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PROCTOLIN AND AN ENDOGENOUS PROCTOLIN-LIKE PEPTIDE ENHANCE THE CONTRACTILITY OF THE *LIMULUS* HEART

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SUMMARY

1. Synthetic proctolin increases the force but not the rate of heart contractions of *Limulus* in a time- and dose-dependent manner. The threshold of this effect is 3×10^{-10} M and the ED_{50} is approximately 10^{-8} M.

2. At concentrations up to 10^{-7} M, proctolin has no effect on the rhythmic electrical activity of the isolated cardiac ganglion, and it does not change the simple and compound postsynaptic potentials recorded at the cardiac neuromuscular junction.

3. Proctolin acts directly on the cardiac muscle fibres. Electrically stimulated myocardia show a proctolin-induced increase in contraction amplitude with the same concentration dependence as the intact heart.

4. A compound with an apparent molecular weight of 700–800 occurs in the *Limulus* nervous system, particularly in the cardiac ganglion. This compound resembles proctolin in being heat-stable, resistant to trypsin and chymotrypsin cleavage, and losing activity in a time-dependent manner in response to treatment with leucine aminopeptidase or pronase. This peptide induces spontaneous contractions and a contracture of the cockroach hindgut in a manner similar to proctolin. Moreover, the *Limulus* inotropic peptide, like proctolin, increases the force of contraction of the *Limulus* heart without affecting beat frequency.

5. It is concluded that an endogenous, proctolin-like peptide is an inotropic modulator of the *Limulus* heart, acting directly on the muscle fibres and not affecting cardiac ganglion activity.

INTRODUCTION

Invertebrate nervous systems, with their relatively simple organization and identifiable neurones, have provided valuable opportunities for the isolation and characterization of neuropeptides and neuroactive amines (Haynes, 1980). Considerable attention has been given to the invertebrate neurohormones, particularly those of the

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arthropods (crustaceans, Kleinholz & Keller, 1979; insects, Mordue & Stone, 1979) and the molluscs (Greenberg & Price, 1980). Several peptide factors, as well as catecholamine neurohormones, have been isolated from different species, and have a powerful influence on the frequency and force of heart contraction (reviewed by Haynes, 1980).

In the arthropod *Limulus*, which occupies a subphylum separate from crustaceans and insects, octopamine and several catecholamines increase the rate and amplitude of heart contractions (Augustine, Fetterer & Watson, 1982), and dopamine, octopamine, norepinephrine and epinephrine are endogenous to the *Limulus* nervous system (O'Connor, Watson & Wyse, 1982). The amines act by modulating activity of the *Limulus* cardiac ganglion (Augustine *et al.* 1982) and cardiac neuromuscular transmission (Watson & Hoshi, 1981; W. H. Watson, T. Hoshi & G. J. Augustine, in preparation). Similar effects are observed in crustacean cardiac ganglia (Cooke, 1966; Miller, Sullivan, Benson & Berlind, 1981) and skeletal and foregut neuromuscular junctions (Florey & Rathmayer, 1978; Lingle, 1979, 1981).

In this report we consider the possible role of a peptide, proctolin, in the control of the *Limulus* heartbeat. Proctolin is a pentapeptide (Arg-Tyr-Leu-Pro-Thr) first isolated from the insect hindgut (Brown & Starratt, 1975). It induces myogenic contractions in locust muscle (Piek & Mantel, 1977), contracture of lobster skeletal and cardiac muscle (Lingle, 1979; Schwarz, Harris-Warwick, Glusman & Kravitz, 1980; Miller & Sullivan, 1981), and both rhythmic contractions and sustained contracture of the cockroach hindgut (Brown, 1975; Cook & Holman, 1979). This peptide has also been implicated in the neural or hormonal control of the hearts of insects (Miller, 1979; O'Shea & Adams, 1981) and crustaceans (Sullivan, 1979; Miller & Sullivan, 1981), suggesting that it could be a widespread regulator of cardiac function among the arthropods. Recent evidence indicates that proctolin also has effects on mammalian smooth muscle (Schulz, Schwartzberg & Penzlin, 1981; Penzlin, Agricola, Eckert & Kusch, 1981).

The experiments presented here describe the action of proctolin on the neurogenic *Limulus* heart and the properties of a proctolin-like peptide present in the *Limulus* nervous system. Preliminary reports of this work have appeared elsewhere (Benson, Sullivan, Watson & Augustine, 1981; Benson, Watson, Augustine & Sullivan, 1981).

METHODS

Limulus *heart preparation*

Dissection of the heart, and methods of recording, were generally as given by Augustine *et al.* (1982) with the following modifications.

When examining the effect of cardiac ganglion extracts on the *Limulus* heart (Fig. 12), it was necessary to dilute the extracts as little as possible. For this reason, hearts from smaller animals (approximately 15 cm carapace width) were bathed in approximately 0·5 ml of saline. In all other experiments hearts from large animals were used and continuously perfused with saline.

To obtain intracellular recordings from neurones and muscle fibres, while simultaneously monitoring muscle tension, it was generally necessary to stabilize a small area of the heart and allow the remainder to move freely. This was achieved by pinning

5 mm × 5 mm piece of nylon mesh over the recording site (Sawada, Blankenship & McAdoo, 1981).

Natural sea water was used as standard physiological saline. Most drugs were prepared as stock solutions in distilled water and then diluted at least 100-fold with sea water on the day of the experiment. All drugs were obtained from Sigma Chemical Co., St. Louis, MO, with the exception of proctolin (United States Biochemical Corp., Cleveland, OH and Vega Biochemical, Tuscon, AZ) and tetrodotoxin (Calbiochem, La Jolla, CA).

Cumulative dose response curves for normal and deganglionated hearts were obtained by treating preparations with 10^{-11} M-proctolin for 10–20 min, and then progressively increasing the bath concentration by $\frac{1}{2}$ log unit increments until a maximal increase in contraction amplitude was observed. No desensitization was evident during this procedure, and data obtained in this manner agreed well with results from non-cumulative application of single doses.

Data from intracellular experiments were used only if it was possible to record continuously from the same nerve or muscle cell for at least 30 min. Control data were obtained for 15 min prior to the addition of proctolin, and were compared to data obtained once a maximal change in contraction amplitude was reached (usually 15 min).

Extraction of Limulus *nervous tissue*

Portions of the *Limulus* nervous system (protocerebrum, circumoesophageal ring, abdominal ganglia, cardiac ganglion, lateral nerves) were dissected, frozen on solid CO_2, lyophylized and stored at ambient temperature. They were screened for proctolin-like activity by acid extraction, gel filtration (Bio-Gel P2, 500 × 19 mm column; see below) and bioassayed using the cockroach hindgut. Initial tests revealed high concentrations of a hindgut-stimulating material in the cardiac ganglion whose molecular weight was similar to proctolin (Benson, Watson *et al.* 1981). Further biochemical analyses were, therefore, confined to this tissue, and detailed methods are described for the cardiac ganglion extractions only. Procedures were largely the same for all other tissues.

Twenty lyophylized cardiac ganglia (wet weight, 0·55 g) were homogenized with 1 ml of redistilled concentrated acetic acid (AcOH). The homogenate was heated to 100 °C for 1 min after which an additional 1 ml of AcOH was added. The homogenate was centrifuged and the yellowish supernatant decanted and saved. The low speed pellet was re-extracted twice with 0·5 ml AcOH and the supernatants combined. Two ml of 50 % ethanol were added to the combined supernatants. The mixture was extracted with water-saturated, chloroform-methanol (2:1) and centrifuged. The aqueous phase was saved and the organic phase was re-extracted (twice) with 1 ml of 50 % ethanol. The aqueous phases were combined, diluted with 3 ml water, frozen, and lyophylized. The residue was taken up in 300 ml of AcOH and filtered (0·2 μm, TFE; Bioanalytical Systems, Inc.) to yield the 'lipid-free' extracts. The latter were injected directly onto the gel-filtration columns.

Gel filtration

The above extract (178 μl; 20 ganglion equivalents) was injected onto a [illegible]0 × 19 mm, Bio-Gel P2 gel filtration column, pre-equilibrated and developed with

1·2 M-acetate-formate (8 %/12 %) buffer. The flow rate was 0·13 ml/min and fractions were collected every 10 min. Five, 100 mm columns were developed in an identical manner. Fractions were frozen, lyophylized and redissolved in 1 ml of water containing 1 % AcOH and 10 % propanol. Ten μl of the solution were removed from each fraction (54 total) and subjected to the fluorometric assay (see below). An additional 300 μl aliquot was removed, lyophylized and redissolved in 210 μl cockroach saline for the proctolin bioassay procedure (see below). In the latter case, a 50 μl dose represented 0·7 ganglion equivalents. Additional aliquots of active fractions were removed for enzymatic studies and bioassay on *Limulus* hearts.

When fractions from the gel filtration of cardiac ganglia were bioassayed (as in Fig. 10) low molecular weight fractions were tested first because preliminary experiments indicated the elution of high molecular weight compound(s) near the excluded volume which gave rise to prolonged spontaneous contractions and which interfered with the hindgut assay. Immediately after the elution of extract components, the gel columns were calibrated with a mixture of blue dextran ($M_r = 2 \times 10^6$), vitamin B12 ($M_r = 1355$), proctolin ($M_r = 648$), reduced glutathione ($M_r = 307$), glycine ($M_r = 75$) and 2-mercaptoethanol ($M_r = 78$). Elution volumes of standards were determined by u.v. and visible spectroscopy, thin-layer chromatography, and by bioassay. The elution volume of glycine was regarded as the total permeation volume.

Protein estimation

The protein content of gel filtration fractions was estimated by fluorometric assay of primary amines with fluorescamine (Roche Diagnostics, Nutley, NJ). Aliquots (0·1 ganglion equivalents) of the gel filtration fractions were lyophylized and redissolved in 30 μl of 0·5 M-sodium borate buffer (pH = 9·4). 10 μl of 2 mg/ml fluorescamine in acetone were added and the mixture was immediately vortexed. After dilution to the appropriate volume with borate buffer, the relative fluorescence was measured with a Turner (Model 430) spectrofluorometer. Gamma-aminobutyric acid was used as a primary amine standard. Under the conditions of this assay the relative fluorescence of standards was linear in the range of 50–1500 pmol/sample.

Cockroach hindgut assay

The posterior portions of cockroach (*Periplaneta americana*, collected in the Kaimuki district, Honolulu) hindguts were isolated, removing the terminal abdominal ganglion while leaving the hindguts attached to the posterior abdominal segments. Hindguts were pinned at the abdomen to a Sylgard perfusion chamber (0·75 ml in volume) and the anterior ends were attached (basic tension 100–200 mg) via a thread to a Grass force transducer (FT. 03). The preparations were superfused (1 ml/min) at 18–22 °C with buffered (10–20 mM-Tris-HCl; pH 7·5) cockroach saline (Yamaski & Narahashi, 1959). Lyophilized aliquots of test solutions were redissolved in cockroach saline (50–200 μl) and 50 or 100 μl aliquots were pipetted directly onto preparations for the bioassay of proctolin-like activity. Synthetic proctolin was used as a standard (Vega Biochem, Tuscon, Arizona). Proctolin-like activity was usually characterized as the ability to effect a contracture of the hindgut. However, threshold concentrations were determined by increased spontaneous contractions (e.g., Fig. 9

Enzymatic tests

100 μl of the crude 'lipid-free' extract were removed, lyophylized and redissolved in 200 μl of 25 mM-Tris-HCl buffer at pH 7·5. Aliquots (0·3 ganglion equivalents) were placed in 100 μl of cockroach saline containing 0·5 mg/ml trypsin or 0·05 mg/ml chymotrypsin and incubated for up to 1 h at 29 °C. The test solutions were boiled (2 min) and cooled. 50 μl aliquots were tested for hindgut stimulating activity. Controls were treated in the same manner except for the omission of the enzyme.

Aliquots of active fractions ($0{\cdot}89\,V_t$–$0{\cdot}94\,V_t$; Bio-Gel P2 750 mm column) were tested for susceptibility to leucine aminopeptidase (LAP) and pronase treatment. The LAP experiments were carried out on fraction 22 only, while pronase tests were performed with combined fractions 22–25. Lyophilized aliquots (0·75 ganglion equivalents) of fraction 22 were incubated with 0·6 units of leucine aminopeptidase in 0·35 M-$(NH_4)_2SO_4$ buffer (pH 7·7) containing 1 mM-$MgCl_2$ at 36 °C for 0, 10, 30 and 60 min. Boiled enzyme served as a control at zero time. For pronase treatments 50 μl aliquots were incubated in 100 μl cockroach saline containing 10 μl C. B. Pronase (10 mg/ml, Calbiochem) for 0–120 min at 29 °C. Controls were treated in the same manner except for omission of the enzyme. After the incubation period samples were boiled, lyophylized and redissolved in 100 μl of cockroach saline for subsequent bioassay. Other aliquots (0·5 ganglion equivalents) of the 'lipid-free' extract were subjected to 1–15 h of leucine aminopeptidase treatment. In the latter case boiled enzyme served as control at all time points and samples were redissolved in 200 μl of cockroach saline before bioassay.

RESULTS

Bath application of proctolin enhanced the strength of *Limulus* heart contractions, without altering heartbeat rate (Fig. 1). Detectable changes in amplitude often occurred at concentrations as low as 10^{-10} M-proctolin, and at the ED_{50} ($\sim 2 \times 10^{-8}$ M) proctolin consistently increased contraction amplitude by more than 200 % (Fig. 2). Although tyramine has been reported to antagonize the increase in spontaneous contractions of the cockroach hindgut induced by proctolin (Brown, 1975;

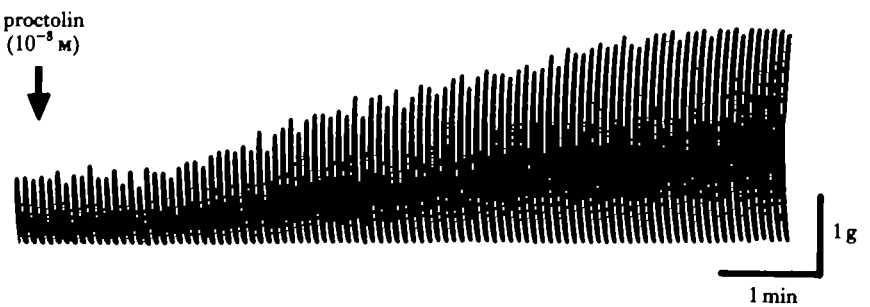

Fig. 1. The inotropic effect of proctolin on the isolated *Limulus* heart. The record shows heart contractions, as measured by a force transducer attached to the lateral margin of the heart muscle. Proctolin was perfused through the bath continuously, beginning at the arrow.

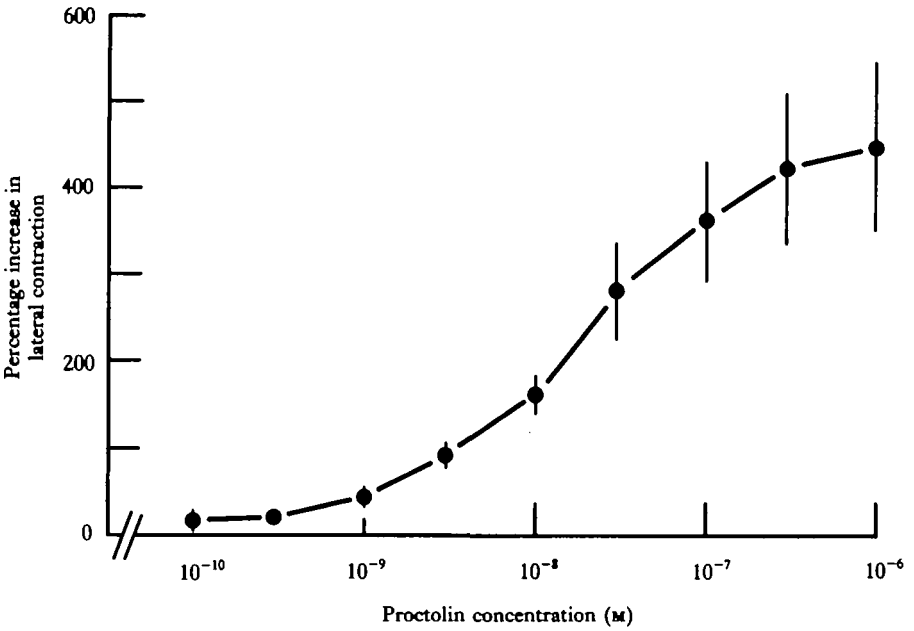

Fig. 2. Cumulative dose-response curve for the inotropic responses of six intact hearts. Points represent mean steady state changes in the amplitude of heart contractions following a 20 min exposure to increasing concentrations of proctolin. Increases are expressed as percentage of control amplitude, and bars indicate S.E.M. The abscissa is a log scale.

Sullivan & Newcomb, 1980), at a concentration of 10^{-4} M, it did not alter the inotropic effect of 10^{-8} M-proctolin, or the myogenic effect of 10^{-6} M-proctolin on the *Limulus* heart.

The response to proctolin had a gradual onset, reaching a maximum in approximately 15 min (Fig. 3A). This maximum level was sustained for up to 30 min during continuous exposure to proctolin with no evidence of desensitization. The effects of proctolin were long-lasting, with most hearts requiring prolonged exposure to proctolin-free sea water before contraction strength returned to control levels (Fig. 3B). The long duration of the effect of proctolin following application is a characteristic and striking aspect of the action of this and many other neuropeptides.

Site of action

The site of action of proctolin on the isolated *Limulus* heart may be one or more of the following: the neurones which comprise the cardiac ganglion; the muscle fibres of the myocardium; the neuromuscular junctions between the two.

Proctolin at concentrations as high as 10^{-6} M had no effect on the bursting activity recorded extracellularly from isolated cardiac ganglia. To test further the effect of proctolin on cardiac ganglion neurones, intracellular recordings were made from follower cell somata. These are uni- and multipolar neurones which send axons out to the myocardium (Fedele, 1942; P. Riordan & G. J. Augustine, in preparation) and which have a pattern of electrical activity suggesting that they are motoneurones (Palese, Becker & Pax, 1970; Lang, 1971). Simultaneous recordings of follower cell electrical activity and muscle tension revealed that there were no significant chang

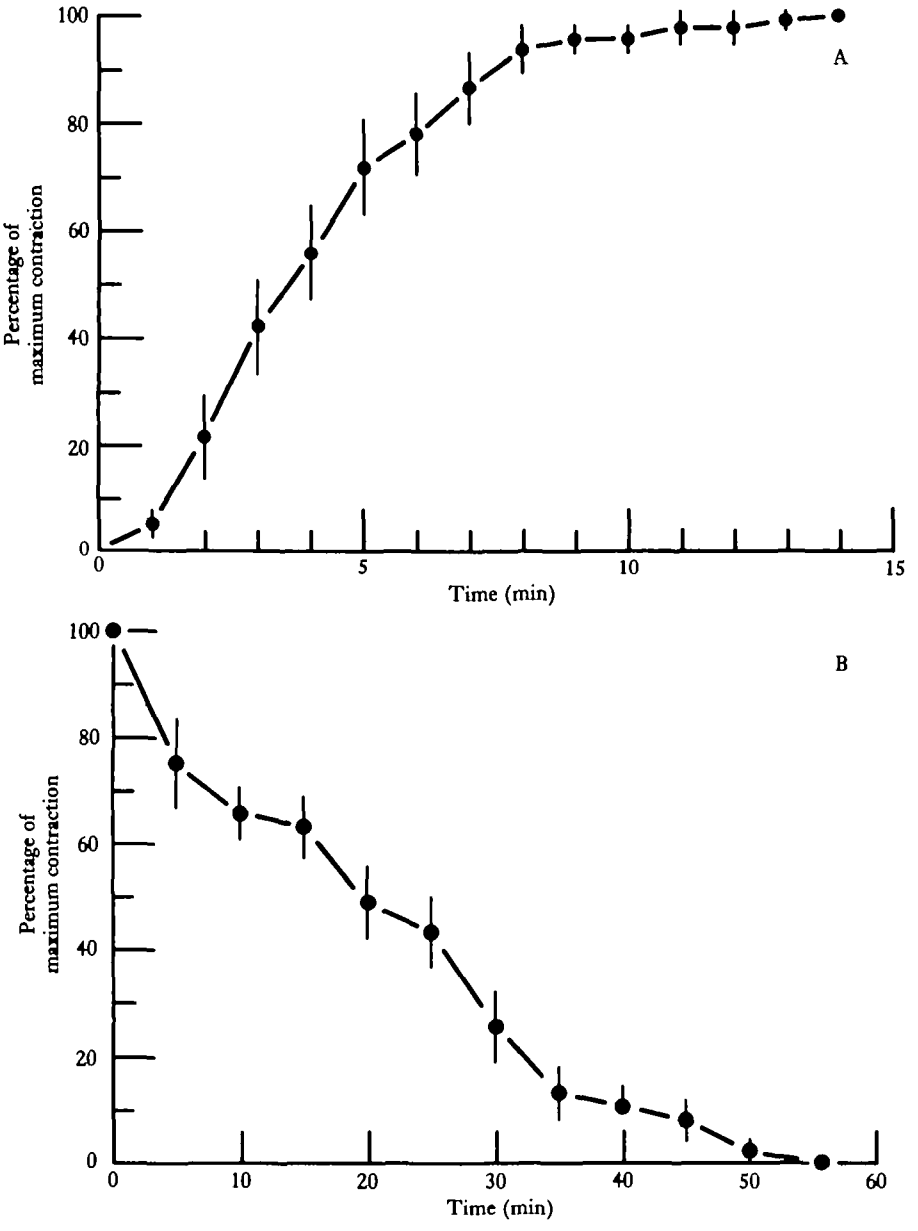

Fig. 3. Time course of proctolin effects. A. Onset kinetics. Proctolin (10^{-8} M) was added to the bath at time zero. The amplitude of five heartbeats was measured every minute and averaged. Strength of contraction was expressed as a percentage of the maximum amplitude obtained after 20 min exposure to 10^{-8} M-proctolin. Bars represent S.E.M. B. Wash-off. Following 20 min exposure to 10^{-8} M-proctolin, hearts were perfused continuously [~ 20 ml (2 bath vol)/min] with sea water at room temperature. Contraction amplitude was determined every 5 min as in A.

in the electrical properties of the presumptive cardiac ganglion motoneurones during increases in contraction amplitude resulting from the addition of proctolin, or decreases in tension during the 1 h wash-off period (Fig. 4). We conclude that the [redacted]ion of proctolin on the *Limulus* heart is not mediated via the cardiac motoneurones.

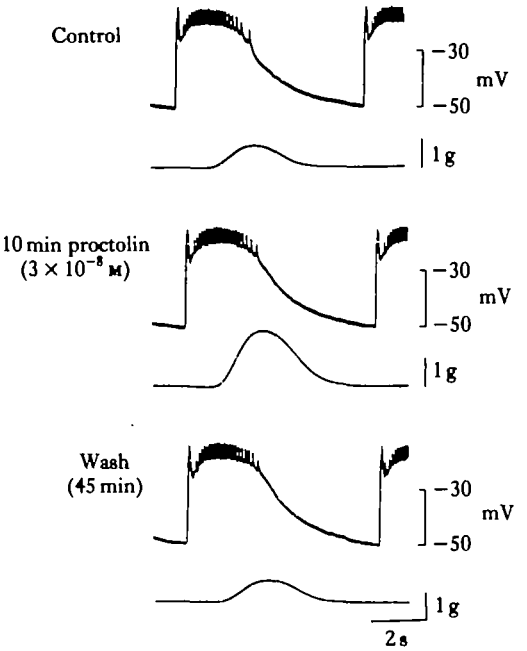

Fig. 4. Intracellular records from a follower cell and tension measurements from the heart musculature before, during, and after exposure to 3×10^{-8} M-proctolin. Follower cell potentials and apparent input resistance were unchanged by proctolin, although there was a significant increase in contraction amplitude.

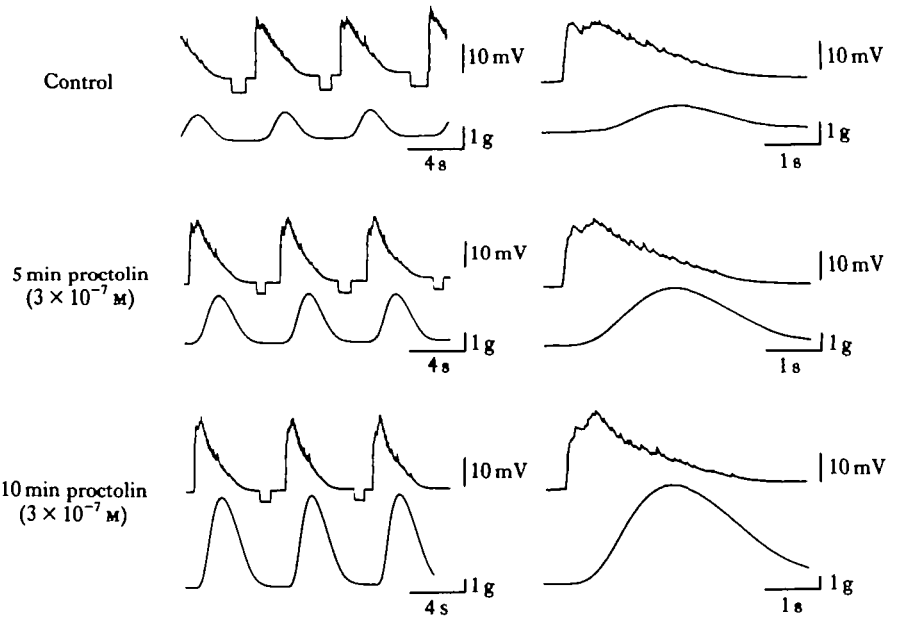

Fig. 5. Intracellular records from a cardiac muscle fibre, and tension measurements from the heart musculature before and after exposure to 3×10^{-7} M-proctolin. Traces on the right were obtained at the same time as those on the left, but were photographed at a faster sweep speed. Hyperpolarizing current pulses (0·5 nA) were passed between EJP bursts to measure input resistance. No wash-off record was obtained because the muscle contractions became so strong that they dislodged the electrode. Note the changes in calibration for tension recordings.

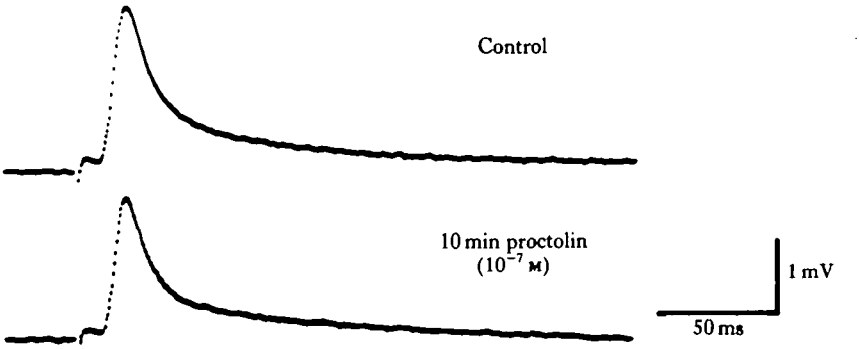

Fig. 6. Averaged (32 events) unitary EJPs before (top) and 10 min after (bottom) exposure to proctolin (10^{-7} M). EJPs were elicited by stimulating a cardiac ganglion motor nerve with a suction electrode. This was the smallest unit recorded from this fibre. There was a small decrease (0·27 mV) in the EJP amplitude in this experiment following proctolin application. However, in some experiments the EJP amplitude increased slightly and in seven experiments the mean decrease in amplitude was only 4·2 % (see text).

Although proctolin did not alter the electrical properties of follower neurones as recorded from the cell body, it is possible that it might enhance transmission at the cardiac neuromuscular junction. The effect of proctolin on neuromuscular transmission was tested in intact hearts. The typical electrical activity recorded from *Limulus* heart muscle consists of summating EJPs which give rise to a complex compound EJP (McCann, 1962; Abbott, Lang & Parnas, 1969*a*,*b*). In 10 experiments, neither the amplitude nor the duration of these compound EJPs was significantly affected by proctolin (Fig. 5), and no consistent changes in membrane potential or apparent input resistance were observed, even though contraction amplitude increased as much as 300 % (Fig. 5).

To examine the effect of proctolin on individual neuromuscular junctions, cardiac ganglia were removed from hearts and the remaining nerve bundles were electrically stimulated. This permitted unitary EJPs to be recorded intracellularly from cardiac muscle fibres (Abbott *et al.* 1969*a*,*b*). The amplitude and time course of these EJPs were unaffected (mean decrease in amplitude = 4·2 %, S.E.M. = 4·03 %, $N = 7$) by doses of proctolin capable of augmenting heart contractions by more than 500 % (Fig. 6). This indicates that neuromuscular transmission is not the site of action of proctolin. This lack of change in EJP amplitude is a further indication that muscle fibre input resistance does not change as a result of proctolin application (Gage, 1976).

The effect of proctolin on the muscle was investigated in deganglionated hearts that were induced to contract by direct electrical stimulation (Augustine *et al.* 1982). The contractions were enhanced by application of proctolin (Fig. 7A). To eliminate the possibility that the electrical stimulation was exciting residual axons, $2{\cdot}5 \times 10^{-7}$ M-tetrodotoxin (TTX) was added to the bath to block any possible axonal activity (Rulon, Hermsmeyer & Sperelakis, 1971; Augustine, 1979).

Proctolin responses from TTX-treated hearts were similar to those obtained in normal sea water (Fig. 7B). The dose-response curve for the inotropic effect of proctolin on the deganglionated heart (Fig. 7A) was similar to the curve obtained with intact hearts (Fig. 2), with a threshold around 10^{-10} M, ED_{50} between 10^{-8} M and

10^{-7} M, and saturating dose of approximately 10^{-6} M. We conclude that proctolin increases the strength of heart contractions by directly altering the contractility of the muscle fibres of the myocardium.

Proctolin at concentrations greater than 10^{-7} M often caused a contracture of the deganglionated myocardium and induced spontaneous rhythmic contractions. The amplitude and frequency of the rhythmic contractions were similar to the normal heartbeat (Fig. 8). This proctolin-induced myogenicity occurred in the presence of TTX (Fig. 8), or in sodium-free saline. It cannot be due to residual cardiac ganglion activity because both TTX and sodium depletion block evoked EJPs in *Limulus*

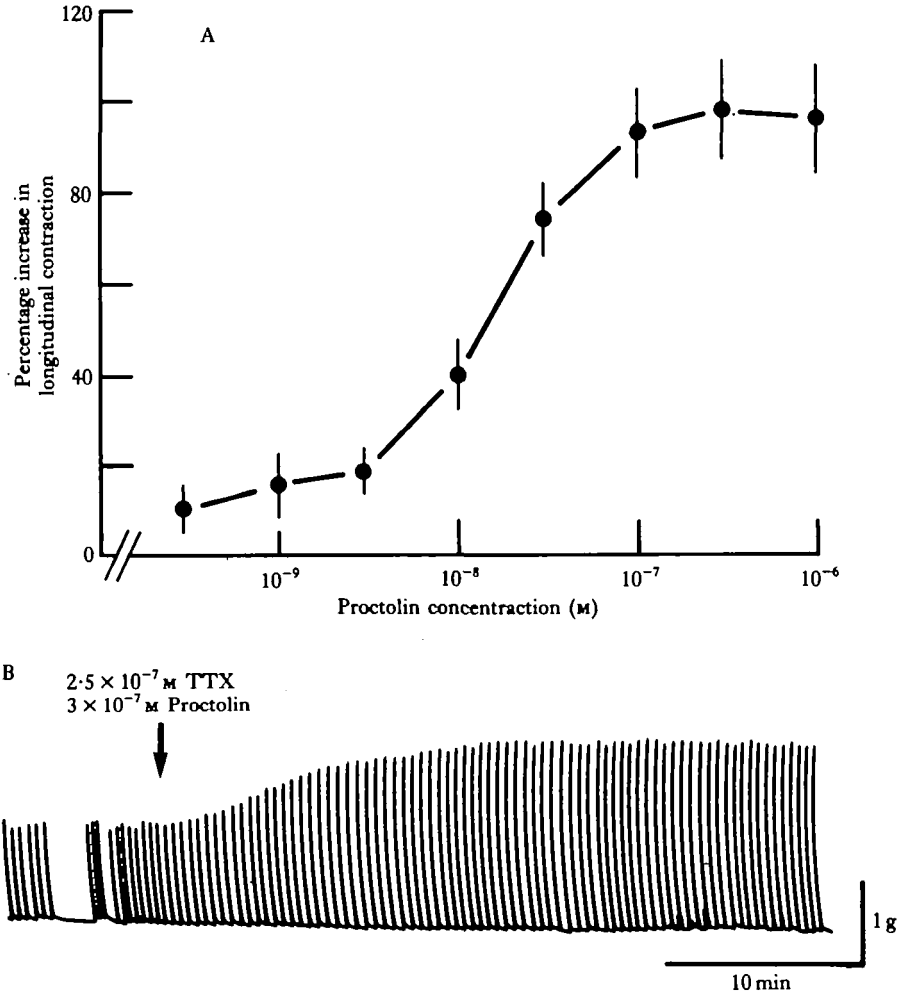

Fig. 7. Effect of proctolin on the deganglionated heart. A. Cumulative dose-response curve for the effects of proctolin on the amplitude of contractions recorded from 4–6 deganglionated hearts. The heart musculature was stimulated extracellularly via an array of electrodes (Augustine *et al.* 1982). Data were analysed and plotted as in Fig. 2. B. Proctolin effects on a deganglionated myocardium in the presence of TTX ($2{\cdot}5 \times 10^{-7}$ M). TTX was added to the bath 30 min before, and during, proctolin (3×10^{-7} M) application. The heart was stimulated as in A, except that the heart remained immersed in saline throughout the experiment. This record demonstrates the persistance of the proctolin effect in the presence of TTX.

cardiac muscle fibres (Parnas, Abbott & Lang, 1969; Hoshi & Watson, 1981; W. Watson & T. Hoshi, in preparation).

Isolation of a proctolin-like peptide

The low threshold and the magnitude of the proctolin effect described above suggest a physiological role for proctolin in the control of heart activity in *Limulus*. To test whether a proctolin-like peptide was present in *Limulus*, we screened extracts of *Limulus* nervous tissue, which were partially purified by gel filtration on Bio-Gel P2, 19 × 500 mm column, for proctolin-like activity using the cockroach hindgut bioassay. Hindgut-activating material was found in the brain, circumoesophageal ganglion, abdominal ganglia, and cardiac ganglion but not in the lateral nerves. The highest specific activity appeared in the cardiac ganglion extracts, especially in fractions which corresponded with the elution volume of synthetic proctolin (0·8–0·9 V_t, referred to as fraction 15; V_t = elution volume of glycine).

Endogenous cardiac ganglion proctolin-like activity was compared to synthetic proctolin with thin-layer chromatography (TLC). A 'lipid-free' extract of 10 cardiac ganglia was subjected to gel filtration and then fraction 15 was separated further with TLC (solvents: ButOH, AcOH, water). Bioassay of TLC fractions eluted from 1·0 cm scrapes of the TLC plates indicated that the major component effecting contracture of the hindgut ran with an R_f (reference value) of 0·33–0·50 (TLC fraction 3, Fig. 9A). The R_f of synthetic proctolin was 0·39 ± 0·02 ($N = 4$).

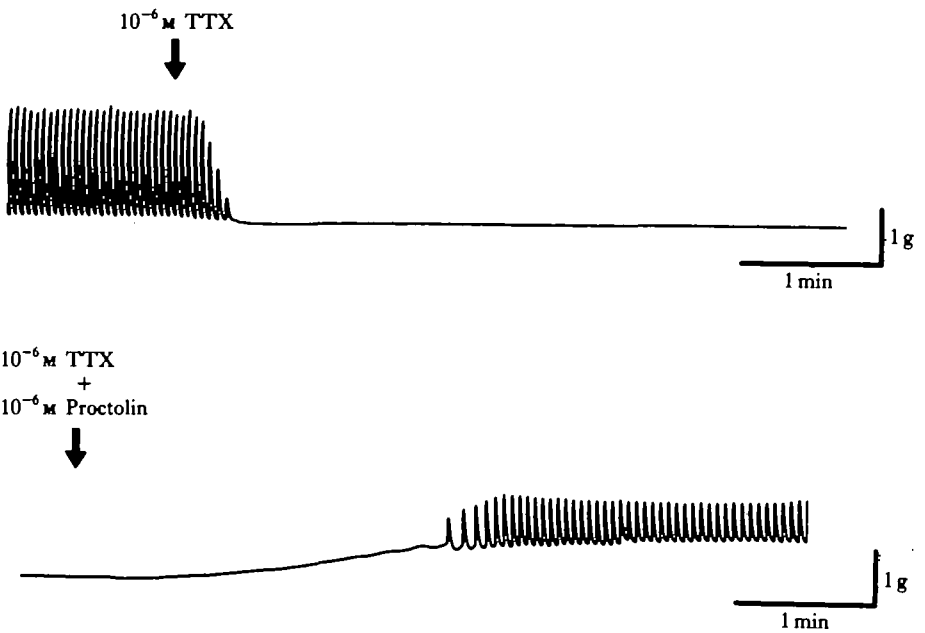

Fig. 8. Myogenic effects of proctolin. Top: TTX application eliminated beating in a normal heart by blocking the conduction of impulses in the cardiac ganglion. Bottom: 10 min later, application of 10^{-6} M proctolin in the presence of TTX induced a small sustained contracture followed by rhythmic contractions.

Material obtained from partially purified (Bio-Gel P2, 500 mm) fractions was used to estimate the amount of proctolin equivalents in a *Limulus* cardiac ganglion. Activity equivalent to 0·6 pmoles proctolin was present in each ganglion (Fig. 9B).

A clearer separation of cardiac ganglion extracts was obtained with a 750 mm gel column (Fig. 10A, B). The longer column separated the proctolin-like material into three apparent peaks: Peak 1 (long column 'LC' fraction 22) corresponded to material which eluted at 0·85 V_t; Peak 2 (LC fraction 25) = 0·96 V_t; and Peak 3 (LC fraction 34) = 1·31 V_t. Peak 1 eluted just prior to synthetic proctolin, suggesting that its molecular weight is slightly greater than 648. Peak 2 co-eluted with reduced glutathione ($M_r = 307$), and was very near V_t. The activity of peak 3 was not stable in saline, and its retarded elution pattern is similar to the indoleamine 5-HT which is also known to affect contractures of the cockroach gut (R. E. Sullivan, unpublished observations). Two additional experiments with lots of 10–20 ganglia confirmed the presence of a proctolin-like peptide in the region 0·85–0·96 V_t. However, as is apparent in Fig. 10A, the peaks were not well resolved. This may be due to the presence of two proctolin-like peptides.

Cardiac ganglion proctolin-like activity was sensitive to the same enzymes as synthetic proctolin. Leucine aminopeptidase (LAP) reduced the hindgut stimulating activity of lipid-free crude extracts (Fig. 11A), whereas incubation with trypsin (Fig. 11B) or chymotrypsin (Fig. 11C) had little or no effect. Treatment of LC fraction 22 with LAP for 1 h at 36 °C appeared to reduce the hindgut activity in a time-dependent manner. A similar incubation of fraction 25 with LAP gave inconclusive results. Pronase treatment of combined fractions 22 and 25 resulted in a rapid (< 1 min) loss

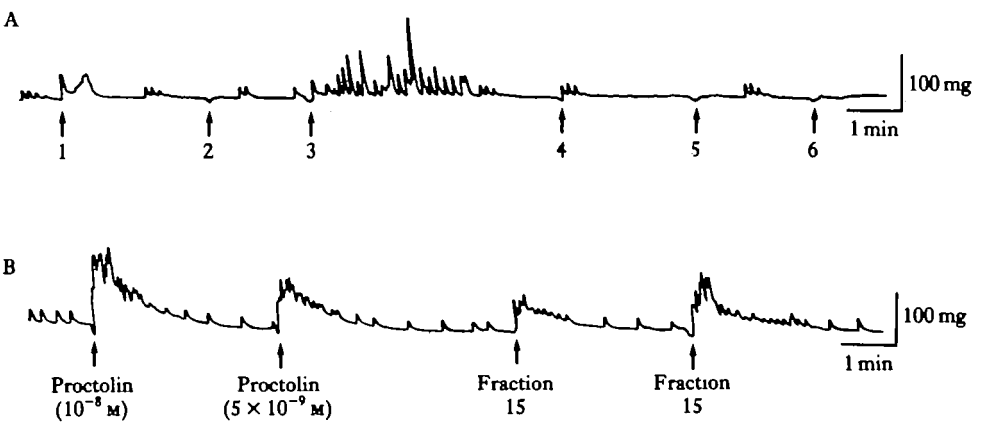

Fig. 9. Responses of cockroach hindguts to thin-layer chromatograph (TLC) fractions, gel filtration fractions and synthetic proctolin. A. Bioassays of TLC fractions. Following gel filtration on a 500 mm Bio-Gel P2 column an aliquot representing one cardiac ganglion equivalent of volume element 0·8–0·9 V_t (fraction 15) was spotted on a TLC plate and developed with butanol-AcOH-water (60:15:25). Plates were dried and separated into six 1 cm fractions which were then assayed for hindgut activity. Arrows indicate the time of application of TLC fractions (1 – origin, 6 – front). TLC fraction 3 (R_f 0·33–0·50) contained the major hindgut activating substance. B. Bioassays of synthetic proctolin and crude fraction 15. An aliquot (0·8 ganglia equivalents) of fraction 15 obtained from gel filtration of 10 cardiac ganglia was redissolved in 100 µl of cockroach saline and two 50 µl aliquots were bioassayed and compared with 50 µl test doses of synthetic proctolin (1×10^{-8} M and 5×10^{-9} M). Arrows denote time of application of each test fraction. Responses to fraction 15 were approximately equal to those produced by 50 µl of 5×10^{-9} M proctolin, indicating that each ganglion contains approximately 0·6 pmol equivalents of proctolin.

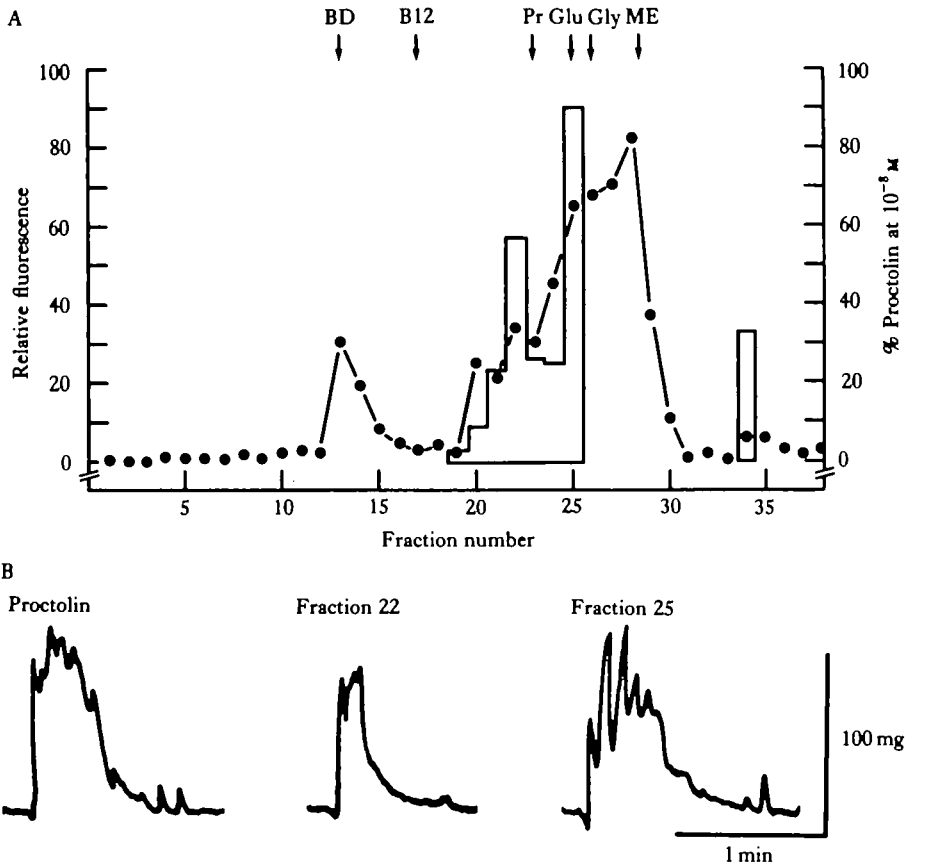

Fig. 10. Separation of hindgut activating factors from *Limulus* cardiac ganglia. A. Ten ganglion equivalents dissolved in pH 1·9 acetate-formate (8 %/12 %) buffer were injected onto a gel filtration column (750 × 9 mm Bio-Gel P2) which was pre-equilibrated and developed with the same buffer at a rate of 0·13 ml/min. 10-min fractions were collected. Abscissa: Fraction number. Ordinates: (1) Relative fluorescence (●—●—●—●), calculated by the fluorescamine technique, with GABA as a standard. Twenty relative fluorescence units = 1 nmol GABA. (2) Percent 10^{-8} M-proctolin (vertical bars) = response of a cockroach hindgut to a 50 µl dose (0·7 ganglia equivalents) of each fraction divided by the response to a 50 µl dose of 10^{-8} M-proctolin ×100. Each bar represents the average of three tests, using separate hindguts. Arrows indicate elution volumes of the following compounds: blue dextran (BD), Vitamin B12 (B12), proctolin (Pr), reduced glutathione (Glu), glycine (Gly, also indicates total permeation volume, V_t), 2-mercaptoethanol (ME). In two additional experiments under identical conditions the pronounced peak at fraction 22 was not as evident. B. Responses of cockroach hindgut to proctolin and fractions 22 and 25. Standard test doses of each (see above) were applied under bioassay conditions.

of activity. These observations suggest that LC fraction 22 contains a low molecular weight peptide with enzymatic and pharmacological properties characteristic of proctolin-like peptides (Brown & Starratt, 1975; Sullivan, 1979).

The proctolin-like activity present in the *Limulus* cardiac ganglion altered contractions of the *Limulus* heart in a manner similar to synthetic proctolin. Sample aliquots of LC fractions 22 and 25 were lyophylized and subsequently tested for their actions on the *Limulus* heart. LC fraction 22 produced a gradual increase in contraction amplitude, but not rate (Fig. 12), and LC fraction 25 caused a transient inhibition of

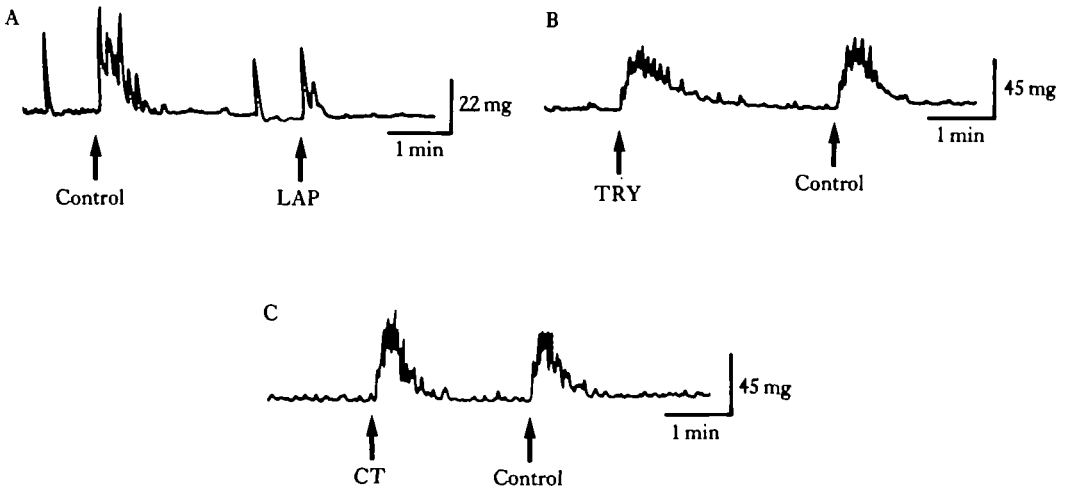

Fig. 11. Enzymatic susceptibility of hindgut stimulating substances isolated from the cardiac ganglion of *Limulus*. A. Treatment of crude, lipid free, extracts with leucine aminopeptidase (LAP) for 15 h at 29 °C resulted in a 61 % decrease in activity when compared to boiled enzyme control. B. Response of hindgut to crude extract after incubation with trypsin (TRY) for 1 h at 29 °C. C. Response to crude extract after incubation with chymotrypsin (CT) under same conditions as B. Arrows denote time of test dose application for A–C.

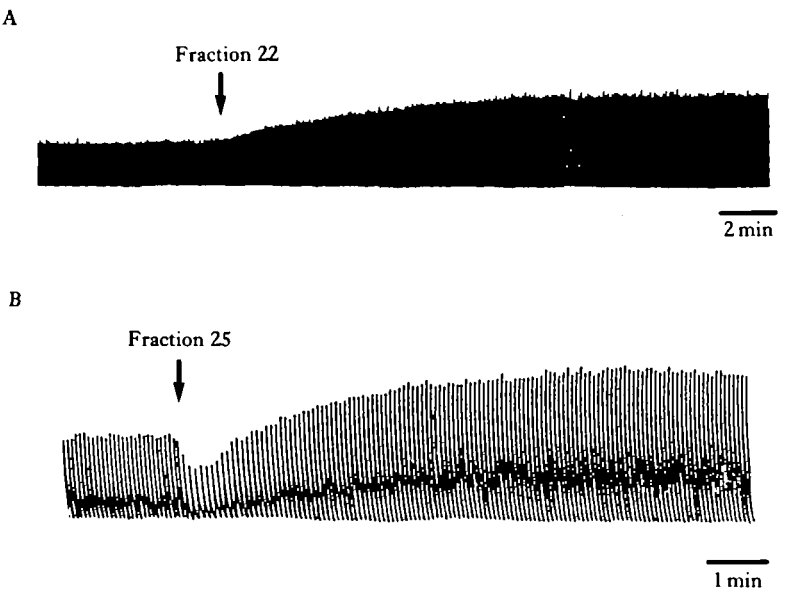

Fig. 12. Response of the *Limulus* heart to the proctolin-like peptide isolated from the *Limulus* cardiac ganglion. LC fractions 22 and 25 (see Fig. 10) were lyophylized, dissolved in sea water, and applied to spontaneously beating hearts (at arrow). Note the slow onset and large increase in contraction amplitude, but not rate, for fraction 22 (proctolin-like peptide), while fraction 25 evokes a complex change in both beat rate and amplitude.

the heart followed by excitation. Therefore, LC fraction 22 resembles synthetic proctolin in both its biochemical and pharmacological properties.

DISCUSSION

Proctolin enhances the strength of *Limulus* heart contractions in a time- and dose-dependent manner (Figs 2, 3A, B). In contrast to amine modulation of the *Limulus* heart (Augustine *et al.* 1982; Watson & Hoshi, 1981), this potentiation is due to a direct effect on cardiac muscle fibres, rather than an effect on the cardiac ganglion neurones or cardiac neuromuscular transmission. A peptide with chemical properties similar to synthetic proctolin is found in the *Limulus* central nervous system and cardiac ganglion, and this compound enhances muscle contraction in the insect hindgut and *Limulus* heart, as does synthetic proctolin. These results suggest that proctolin may be an endogenous inotropic modulator of the *Limulus* heart.

Several lines of evidence indicate that proctolin acts by a direct effect upon *Limulus* cardiac muscle. Firstly, proctolin potentiated electrically evoked contractions in deganglionated myocardia (Fig. 7A). Secondly, high doses of proctolin also caused spontaneous rhythmic contractions in this preparation (Fig. 8). Finally, proctolin did not measurably alter cardiac ganglion activity or cardiac neuromuscular transmission (Figs 4, 5, 6). However, there are two possible complications in the experiments with deganglionated hearts. First, proctolin produced a smaller change in the amplitude of contraction of deganglionated hearts than in intact hearts (cf. Figs 2, 7A). This is probably due to the stimulation and recording techniques, which permitted measurement of longitudinal contractions in deganglionated preparations and only lateral contractions in intact hearts. The similar threshold and ED_{50} concentrations for the proctolin dose-response curves from these two preparations (Figs 2, 7A) is an indication that both are affected by proctolin in a similar manner. A second problem is that residual nerve trunks remain attached to deganglionated hearts, and may serve as a target for proctolin. This is unlikely because proctolin did not affect the EJPs produced by stimulation of these nerves (Fig. 6), and TTX, which blocks axonal conduction in *Limulus* cardiac neurones, did not block the proctolin-induced increase in contractions (Fig. 7B). Furthermore, the proctolin-induced myogenic contractions observed in deganglionated preparations were not affected by TTX (Fig. 8), or removal of sodium from the saline, which eliminates transmitter-induced postsynaptic potentials (Onodera & Takeuchi, 1976; W. Watson & T. Hoshi, in preparation). We conclude that proctolin is acting directly and exclusively upon cardiac muscle to enhance the amplitude of the *Limulus* heartbeat.

Proctolin has direct effects upon other arthropod muscles. It produces or enhances myogenic contractions of insect muscle (Brown, 1975; Piek & Mantel, 1977; Holman & Cook, 1979; Irving & Miller, 1980; O'Shea & Adams, 1981) and causes contractures in lobster skeletal and cardiac muscle (Lingle, 1979; Schwarz *et al.* 1980; Miller & Sullivan, 1981). Proctolin (10^{-9} M) enhances heartbeat contraction amplitude in the crab, *Portunus*, (Sullivan, 1979) without affecting its cardiac ganglion up to concentrations of 5×10^{-8} M (J. Benson, unpublished observations). However, proctolin does affect *Homarus* cardiac ganglion premotor and motoneurones directly (Miller & Sullivan, 1981), and there is preliminary evidence that it also excites neurones in the

cockroach terminal ganglion (Walker, James, Roberts & Kerkut, 1980). Therefore, although proctolin enhances muscular contraction in a variety of arthropods, its actions are not always limited to muscle cells.

Our experiments thus far do not reveal a mechanism for proctolin's ability to enhance the contractility of *Limulus* cardiac muscle. At low concentrations it has negligible effects on the electrical properties of the muscle fibres and thus it resembles the inotropic modulation of *Aplysia* buccal musculature produced by serotonin (Weiss, Cohen & Kupferman, 1975). However, at higher concentrations, proctolin produces a contracture followed by rhythmic spikes and contractions in the deganglionated myocardium (Fig. 8; Hoshi & Watson, 1981; W. Watson & T. Hoshi, in preparation). One mechanism that would account for the effects of both low and high concentrations of proctolin on *Limulus* heart muscle is an increase in intracellular calcium. This mechanism would be comparable to the action of strophanthidin on mammalian cardiac muscle (Kass, Lederer, Tsien & Weingart, 1978; Karagueuzian & Katzung, 1982). Low concentrations of strophanthidin increase intracellular calcium and produce a positive inotropic effect, while higher doses lead to spontaneous contractions accompanied by, but not always synchronized with, rhythmic action potentials. It has been proposed that both the rhythmic contractions and spikes are due to oscillating calcium-induced release of calcium from the sarcoplasmic reticulum (Reuben, Brandt & Grandfest, 1974; Fabiato & Fabiato, 1975; Kass *et al.* 1978; Karagueuzian & Katzung, 1982). Further analysis of proctolin's actions on *Limulus* cardiac muscle will be required in order to determine if this peptide is acting in a similar manner, or via one of several other feasible mechanisms.

The biochemical analyses reported here suggest the presence of a proctolin-like peptide in *Limulus* nervous tissue, especially within the cardiac ganglion. LC fraction 22 elutes close to synthetic proctolin on gel filtration columns (Fig. 10A) and has a similar R_f value, according to our TLC protocol. Both peptides are susceptible to enzymatic degradation by leucine aminopeptidase and pronase, but not trypsin or chymotrypsin (Fig. 11) and the responses of the *Limulus* heart and cockroach hindgut to LC fraction 22 and synthetic proctolin are almost identical. However, the cockroach bioassay is not absolutely specific for proctolin. For example, insect brain and corpus cardiacum contains an additional chromatographically distinct hindgut-stimulating peptide which is pharmacologically indistinguishable from proctolin (Holman & Cook, 1979). Thus, considering the lack of specificity of the hindgut assay, and that all our data are based on a total of 60–100 pmol of peptide (~ 100 ganglia equivalents), LC fraction 22 must be considered as a proctolin-like peptide only.

It is possible that LC fraction 22 is a structural analogue of proctolin. Structure-function analysis indicates that the proctolin molecule can accept specific amino acid substitutions and additions and still retain full intrinsic activity (Piek, Visser & Mantel, 1979; Starratt & Brown, 1979; Sullivan & Newcomb, 1980). Comparative pharmacological data suggest that proctolin may be a member of a family of structurally-related arthropod peptides (Sullivan, 1979; Benson *et al.* 1981; Sullivan, Tazaki & Miller, 1981). Further purification and sequence analysis will allow better appreciation of the relationship of LC fraction 22 to proctolin.

The observations reported here, together with findings from other arthropod

suggest that proctolin or a proctolin-like peptide acts as a modulator of heart contraction. Although proctolin is located in association with the cockroach hindgut (Brown & Starratt, 1975), and immunohistochemical studies suggest that it is released directly onto the hindgut muscles from terminal ganglion neurones, it also occurs in interneurones which do not directly connect with the hindgut (Eckert, Agricola & Penzlin, 1981). In the crustaceans (Sullivan, 1979; Miller & Sullivan, 1981) as well as the insects (O'Shea & Adams, 1981; Miller, 1979), proctolin is found in heart associated structures, and modulates cardiac function as we have observed in *Limulus*. In the decapod crustacean, *Portunus*, ~ 100 pmol of proctolin-like activity are localized in the pericardial organs (Sullivan, 1979) which are neurohaemal structures that release neurohormones into the bloodstream as it enters the heart (Sullivan, Friend & Barker, 1977). We recovered 0·6 pmol of proctolin-like activity from each *Limulus* cardiac ganglion, an amount which produced only a small increase in heart contraction. This suggests localized release rather than circulation as a neurohormone, and this could be the basis of the augmentation of heart contraction amplitude observed in *Limulus* when the lateral nerves are stimulated (Garrey & Knowlton, 1934). Precise cellular localization should reveal by which means proctolin reaches the cardiac muscle.

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