Some Effects of Proctolin on the Cardiac Ganglion of the Maine Lobster, Homarus americanus (Milne Edwards)

Received March 9, 1981; revised July 10, 1981

SUMMARY

The neuropeptide proctolin has excitatory effects on the isolated lobster cardiac ganglion. Selective application to the anterior cell body region produces a dose-dependent (10^{-8}–$10^{-5}M$) prolonged depolarization of large anterior cells as well as marked increases in burst frequency and/or duration. In ganglia which have been silenced with tetrodotoxin, proctolin application to anterior cells elicits long-lasting depolarizing responses which are accompanied by a 10–30% increase of the apparent membrane input resistance. Higher proctolin concentrations produce high-frequency trains of driver potentials. It is proposed that a proctolinlike peptide may serve a neurohumoral role in the lobster cardiac ganglion and that the anterior motor neurons exhibit endogenous rhythmicity in its presence.

INTRODUCTION

Proctolin (Arg-Tyr-Leu-Pro-Thr), a neuropeptide, was originally isolated from cockroach hindguts (Brown, 1975; Starratt and Brown, 1975). In the class Insecta proctolin-containing nerve cell bodies represent a segmentally distributed system of central somata whose axons innervate visceral musculature including that of the hindgut and heart (Brown, 1975; O'Shea and Adams, 1980). Physiological and biochemical evidence from additional classes of arthropods including crustaceans (Sullivan, 1979) and xiphosurans (Benson et al., 1981), and studies with proctolin analogs (Piek, Visser, and Mantel, 1979; Starratt and Brown, 1979; Sullivan and Newcomb, 1980) strongly suggest that proctolin and "proctolinlike" molecules extracted from various arthropod nervous tissues represent a distinct and heretofore unrecognized family of neuropeptides.

In the arthropods, one physiological role for these peptides is the modulation of muscular contractions. It appears that proctolin has a direct effect on the musculature of all arthropods investigated in detail (Brown, 1975; Piek and Mantel, 1977; Cook and Holman, 1979; Lingle, 1979; May, Brown, and Clements, 1979; Miller, 1979; Schwarz et al., 1980).

While it has not been established firmly that proctolinlike peptides play a hormonal role in crustaceans, their presence in the neurosecretory pericardial organs and their reported cardioexcitatory activity have raised this possibility (Sullivan, 1979). We report here the first observations of effects of proctolin on an isolated nervous network, the lobster cardiac ganglion. The ganglion is a well-characterized nine-cell motor pattern generator whose rhythmic output controls heart contractions [see reviews by Hagiwara (1961); Hartline (1979)]. The nine cells usually are classified into two groups: (1) four small cells which are thought to serve as pacemakers, and (2) five large motor neurons which receive excitatory synaptic input from the small cells. In the following is described the effects of bath-applied proctolin as recorded from the anterior large cells.

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CCC 0022-3034/81/060629-11$01.10

MATERIALS AND METHODS

Maine lobsters *Homarus americanus* (0.5 kg, males) were obtained from local markets and maintained in a circulating seawater tank at 11°C. Lobster saline solutions consisted of NaCl, 462m*M*; KCl, 16m*M*; $MgCl_2$, 8m*M*; $CaCl_2$, 26m*M*; and 2m*M* Hepes buffer at pH 7.4. Tetrodotoxin (TTX) was obtained from Sigma Chemical Co. and used at a concentration of $3 \times 10^{-7}M$. Proctolin was obtained from Vega Biochemicals.

Our dissection procedure was essentially identical to that of Cooke and Hartline (1975). After careful dissection the ganglion was transferred to the two-chambered Sylgard dish (see below) on top of a microscope cover slip, and subsequently pinned out with cactus spines.

These experiments utilized a two-chambered Sylgard (Dow-Corning) dish (modified after Watanabe, 1958). The preparation was pinned out in such a manner that the anterior portion of the ganglion (somata 1–4) lay in one chamber and the remaining large-cell and small-cell somata lay in the other [cell nomenclature follows Hartline (1967)]. The central trunk of the ganglion passed through a small opening connecting the chambers. When the opening was filled with Vaseline, each chamber (volume, 400 μL) was perfused separately, permitting selective drug administration. Moreover, the Vaseline gap provided the electrical resistance necessary to record extracellular action potentials passing through the central trunk by simply placing platinum wires in each chamber. Electrical signals were amplified by a Grass P15 ac preamplifier. Figure 1(A) is a schematic representation of the two-pooled chamber and recording arrangement. All experiments were carried out at 18–22°C.

Intracellular recordings from large cells were made with 10–20-MΩ electrodes filled with 3*M* KCl connected to WPI M707 or VF-1 preamplifiers. Two electrodes were placed in a large cell for determining *I–V* relationships. Constant currents were injected via the bridge circuit of the M-707 preamplifier controlled by a Grass S4 stimulator and monitored by means of a WPI model 180 virtual ground system.

Proctolin was either bath-applied to one or both chambers or concentrations were introduced as 50- or 100-μL aliquots (by means of an Eppendorf pipette) upstream from the ganglion while maintaining a continuous flow of saline. While the latter method provided a rapid exposure of the ganglion and afforded better estimates of the time course of proctolin effects, it inevitably resulted in a slight dilution (approximately a factor of 2) of the effective concentration. In most experiments, saline flow rate was approximately 200–300 μL/min. However, in experiments specifically designed to examine the time course of proctolin effects the flow rate was two to three times faster.

RESULTS

Properties of anterior large cells

The following observations are based upon intracellular recordings from 15 anterior large cells (cell No. 1, 2, or 3) in 13 intact preparations. The observed resting potentials fell within a range of 42–60 mV. The mean resting potential (RP) was 52 ± 6 mV (S.D.; $n = 14$). Figure 1(B) shows a selected burst. Its duration of 250 ms and amplitude of 22 mV is representative of our observations. These values are in agreement with those reported previously for *Homarus* (Cooke, 1966; Connor, 1969). Below, we report the effects of bath application of proctolin to the anterior large-cell region.

Effects of proctolin on bursting ganglia

Proctolin (10^{-7}–$10^{-4}M$) was observed to have multiple effects upon ganglia when applied selectively to the anterior cell body region (cell Nos. 1–4). Figures 2(A) and 2(B) illustrate two typical observations. Proctolin was noted to produce a dose-dependent slow depolarization in all preparations tested [see Figs. 1(C), 2(A)–2(C)]. Effects upon burst parameters appeared to depend upon the rate of ganglionic bursting prior to application. In slowly bursting ganglia (<30

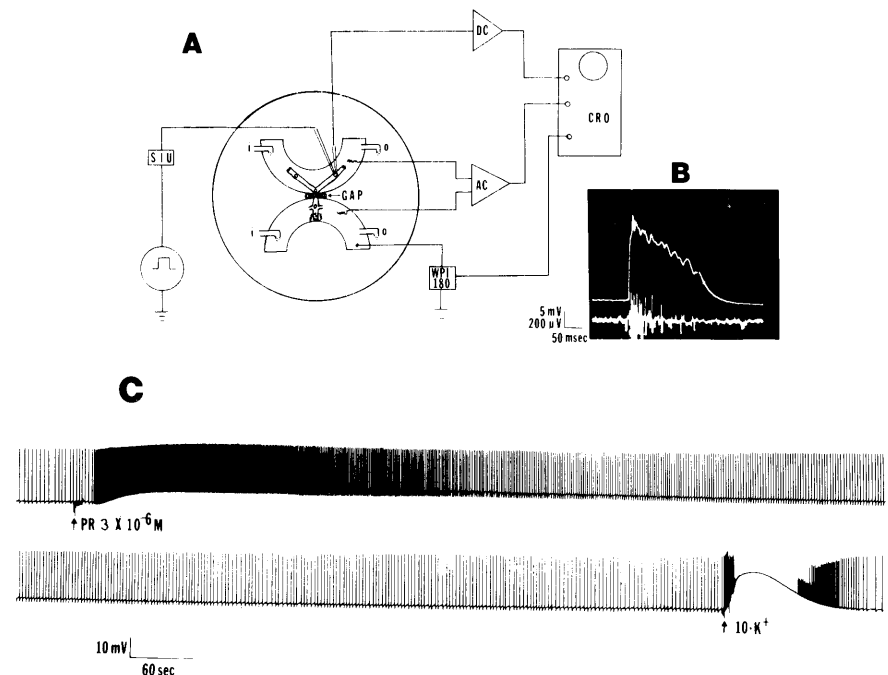

Fig. 1. Schematic representation of the perfusion and recording systems utilized and typical recordings from large cells. (A) Two-chamber perfusion dish. Each chamber contained approximately 0.4 mL of saline and was perfused continuously (i = in; o = out) at a rate of approximately 0.3 mL/min. A small opening between the two chambers permitted the ganglia to be pinned out in such a manner that the anterior large-cell bodies (Nos. 1–4) lay in one chamber and small-cell somata and large-cell No. 5 lay in the other. A Vaseline gap (GAP) separated the two pools, allowing selective drug administration and creating a high-resistance pathway across which extracellular signals were recorded. (B) A typical recording from anterior cell No. 3 showing intra- and extracellular potentials. See text for further information. (C) A penwriter recording of the response (cell No. 2; RP = 50 mV) of a bursting ganglion to a test aliquot (100 μL of 1.5×10^{-6} *M* proctolin) applied to the anterior chamber approximately 0.5 cm upstream from the preparation. Each vertical deflection represents a burst. The record is continuous. Note that the proctolin response (membrane depolarization and concurrent increase in burst frequency) is maximal after ca. 60 s and thereafter decays over a period of 20 min. Following washout, a 100-μL test aliquot of $10 \times K^+$ saline was administered in an identical manner. Its time course serves as a calibration for the perfusion system. Calibration: (B) 5 mV, 200 μV, 50 ms; (C) 10 mV, 60 s.

bursts/min) proctolin produced a marked increase in burst frequency [Fig. 2(A)] with little effect upon burst duration as measured at the base of the burst. Although the maximal frequency generally coincided with the peak depolarization, the two effects did not decay in parallel (see below). Figure 2(C) shows the dose–response relationships for burst frequency and depolarization obtained from a slowly bursting ganglion. Apparent dissociation constants of 2×10^{-7} and 5×10^{-6} were estimated from the depolarizing and frequency responses, respectively.

In contrast, ganglia which had high burst rates (>50 bursts/min) rarely responded with increases in burst frequency. Such preparations were noted to exhibit an increase in burst duration [Fig. 2(B)]. This increase, as measured at the base of the burst, was dose-dependent and could exceed 50%. It was most prominent at the peak of the proctolin-induced depolarization and appeared to decay concurrently with the repolarization of the membrane potential.

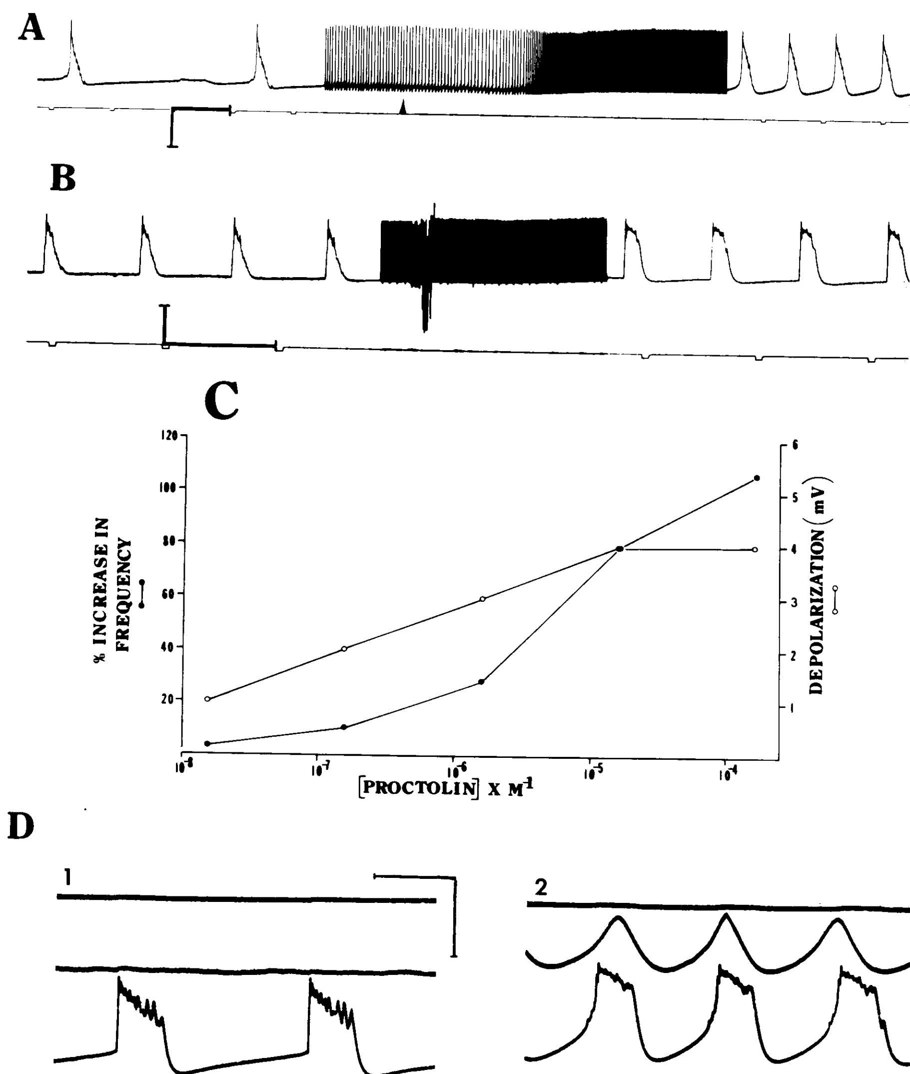

Fig. 2. Multiple effects of proctolin on bursting ganglia. (A) Intracellular recording from cell No. 3; (RP = 52 mV). Pipette application of 100 μL of 1.5 × $10^{-6}M$ proctolin (arrow) to a slowly bursting ganglion. The chart recorder was run at two different speeds: the faster speed (calibration, 1 s) shows burst parameters while the slower speed (calibration, 1 min) shows the slow depolarization and frequency change if present. Note here that proctolin causes a 3-mV depolarization, a 3.6-fold increase in frequency, and the enhancement of the interburst pacemaker potential. A slight increase in the plateau of the burst is also evident. Vertical calibration, 20 mV. (B) Effects of proctolin on a ganglion with a high burst rate. Intracellular recording from cell No. 2 (RP = 58 mV). Pipette application of 100 μL of 1.5 × 10^{-6} M proctolin to anterior chamber (artifact). Chart recording in the same manner as (A), but note different time scale; calibration same as in (A). In addition to a 3-mV depolarization, this cell exhibited a 28% increase in burst duration. No change in frequency occurred. Calibration bar, 20 mV. (C) Dose–response relationship of proctolin effects. Measurements were obtained from intracellular recording from cell No. 3 in a slowly bursting ganglion. Ordinates are peak percent increase in burst frequency and maximum depolarization (mV). Abscissa is proctolin concentration applied as 100-μL aliquots to the anterior chamber; 1.5 × 10^{-9} M proctolin was never observed to affect burst parameters. (D) The effect of proctolin on a ligatured anterior cell. Oscilloscope traces from ligatured cell No. 1 (middle trace) and cell No. 3 (lower trace). Top trace is zero potential for cell No. 3 and −20 mV for cell No. 1. (D1) Control: note the absence of ganglionic input to cell No. 1. (D2) The effects of anterior bath application of 1.5 × 10^{-6} M proctolin (approximately 3 min after switching the perfusion channel). Note that both cells were depolarized 3–4 mV, the appearance of sustained rhythmic driver potentials in the ligatured cell, and the marked enhancement of the pacemaker potentials in cell No. 3. Calibration: 20 mV; 400 ms.

The time course of a typical response to 100 μL of 3 × $10^{-6}M$ proctolin is shown in the slow, continuous record of Figure 1(C) (each vertical stroke represents a burst). The maximum membrane depolarization (6 mV as estimated from the maximum displacement of the postburst hyperpolarization) occurred approximately 70 s after proctolin application, plateaued for 2 min, and then waned in a more or less linear fashion during the next 20–30 min of washout with normal saline. The proctolin effect on burst frequency was maximal (105 bursts/min) within 45–60 s and plateaued for 1 min. The decay of the burst frequency response was notably different from that of the membrane potential. During the first 3 min following its plateau phase, the frequency response decayed in an exponential fashion (R^2 = 0.97), exhibiting a $t_{1/2}$ of 2.2 min. Thereafter the frequency response declined in an irregular manner, returning to its original value within 30 min.

In order to distinguish temporal artifacts due to bulk flow parameters from time course of proctolin effects we applied a 100-μL aliquot of high potassium saline in a manner identical to the previous proctolin application [Fig. 1(C)]. The membrane response to high K^+ reached its maximum depolarization within 24 s, plateaued for 10–15 s, and decayed exponentially with a $t_{1/2}$ of 17 s (R^2 + 0.97). Assuming a linear (but not necessarily Nernstian) relationship between membrane potential and log external K^+ concentration, we interpret the high K^+ depolarization time course to be an accurate indicator of the time dependence of concentration profiles which obtain in the perfusion chamber. Thus, we consider the prolonged decay (30 min) of the proctolin response to be a specific property of the proctolin receptor–effector system. Although these data place an upper limit on the magnitude (45–60 s) of the delayed onset of proctolin's effect, complicating factors beyond our control (bulk flow parameters and limited diffusion regimes) do not permit conclusions concerning the rate-limiting factors responsible for this delay.

The effects of proctolin were also examined on anterior cells isolated by ligature [Fig. 2(D)]. This procedure has been shown to isolate branch cells from small-cell synaptic input while leaving the site for driver potential production functionally intact (Cooke and Tazaki, 1979). In Figure 2(D1) with a ligature placed between cell Nos. 1 and 3, all synaptic drive and spontaneous activity was eliminated in cell No. 1 (middle trace) while normal activity remained in cell No. 3 (lower trace). Anterior bath application of 1.5 × $10^{-6}M$ proctolin produced a typical large-cell response in cell No. 3. The ligatured cell depolarized and produced repetitive driver potentials. These were independent of activity in the rest of the ganglion (cf. cell No. 3 trace). Note that they also failed to produce spikes, presumably due to isolation by the ligature of the soma from the trigger zone. The time course of the proctolin action on both cells was similar to that noted above. Interestingly, the maximal frequency of repetitive driver potentials in the ligatured cell (115/min) exceeded that of ganglionic bursting (90/min).

Effects of TTX-treated ganglia

The above-described experiments suggested that one site of proctolin action may be upon the large cells. The authors therefore examined the effects of proctolin applications to ganglia which were exposed to tetrodotoxin (TTX). Tazaki and Cooke (1979a,b,c; see also Cooke and Tazaki, 1979) have demonstrated that TTX (3 × $10^{-7}M$) treatment eliminates ganglionic activity and

permits analysis of driver potentials (DP) produced in the large cells of crab and lobster cardiac ganglia. In cardiac ganglia, driver potentials are active (voltage- and time-dependent), Ca^{2+}-dependent (TTX-resistant), slow potentials which can be elicited by depolarizing current pulses (Tazaki and Cooke, 1979b; see also Tazaki, 1971). In spontaneously active ganglia pacemaker activity and/or excitatory synaptic inputs to the large cells are sufficient to elicit DP. Once elicited, the depolarizing phase of the DP would serve to drive spike trains at axon trigger zones. The subsequent repolarization and postburst hyperpolarization would terminate the excitatory drive at the trigger zone. In addition to their role in burst formation, driver potentials may affect burst frequency through network interactions (Tazaki and Cooke, 1979c; Benson, 1980).

TTX [(3–6) $\times 10^{-7}M$] was applied here to the entire ganglion by bath application to both chambers. Tazaki and Cooke (1979a) noted that TTX increases the input resistance of crab (*Portunus*) large cells and we observed a similar resistance increase in *Homarus* large cells [Fig. 3(D)]. Selective application of proctolin to anterior large-cell regions produced a depolarizing response which at higher concentrations (5×10^{-7}–$5 \times 10^{-5}M$) was accompanied by spontaneous, rhythmic driver potentials [Fig. 3(A), 3(B)]. The depolarization was dose-dependent [Fig. 3(B)] and appeared to be of greater magnitude than those observed in non-TTX-treated ganglia.

The proctolin-induced driver potentials occurred at frequencies as high as 1.5 Hz. The highest frequencies always occurred concurrently with the peak depolarization, and thereafter (30–60 s later) the DPs appeared to become refractory and damp out [Fig. 3(A)]. In contrast, the underlying depolarization generally outlasted the spontaneous driver potentials. It should be noted that step depolarizations by intracellularly injected current to membrane potentials equivalent to those produced by proctolin were never observed to elicit more than a single driver potential. Figure 3(C) shows a typical train of driver potentials induced by proctolin. A pronounced pacemaker potential preceding each driver potential is apparent (arrows).

Figure 3(D) shows current–voltage relationships obtained from a large cell in normal saline, $3 \times 10^{-7}M$ TTX, and TTX plus $1.5 \times 10^{-7}M$ proctolin. In three experiments in which two electrodes were utilized for determining *I–V* relationships, we observed increases in apparent membrane resistance of 10, 16, and 32% in response to proctolin.

DISCUSSION

In this communication, we have demonstrated that the insect myogenic peptide proctolin has pronounced effects upon a crustacean motor pattern generator. These effects demonstrate that proctolin can produce marked changes in both the frequency and the duration of ganglionic bursting. Our observation that effects upon burst frequency are most prominent in slowly bursting ganglia is consistent with the results of Maynard (1961) who noted minimal effects of cardioaccelerator stimulation in ganglia bursting at high rates (1.7–2.0 Hz).

The authors propose that in slowly bursting ganglia, i.e., ganglia in which small cells are pacemaking at frequencies <0.5 Hz [see Fig. 2(A)], anterior application of proctolin effects high-frequency, rhythmic driver potentials in the large cells (see TTX experiments, Fig. 3) which entrain the network. That is to say, it is the augmented large-cell pacemaker potential which drives the small cell(s) to

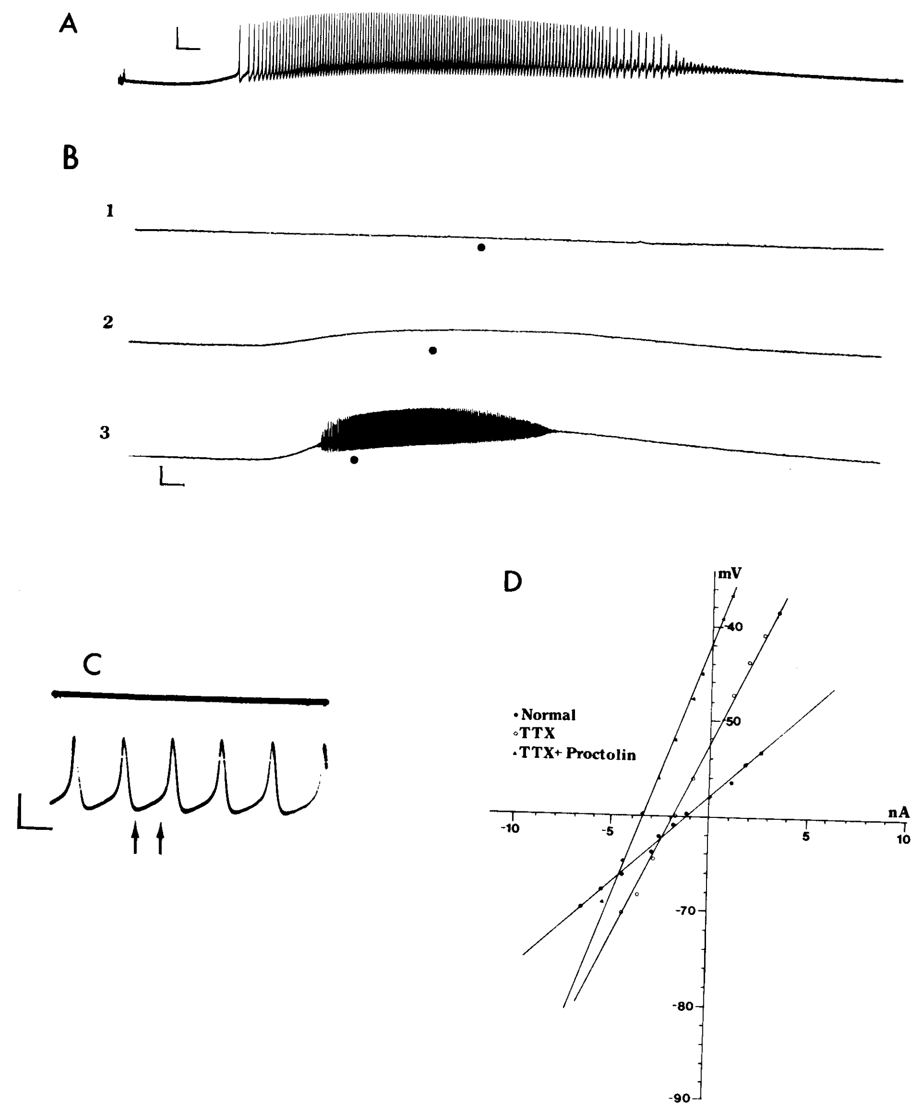

Fig. 3. Effects of proctolin on TTX-treated ganglia. (A) Pen recording of intracellular potential from cell No. 1; initial RP = 49 mV. Proctolin (100 μL; $1.54 \times 10^{-6}M$) was applied via a pipette to the anterior chamber. Artifact at the beginning of trace denotes time of application. The induced depolarization (10 mV) outlasts the appearance of spontaneous driver potentials. The latter appear to "damp out" in 2–3 min, diminishing in both frequency and amplitude. Calibration: 10 mV, 20 s. (B) Dose dependence of proctolin effects in the presence of TTX ($3 \times 10^{-7}M$). Cell No. 3: RP = 58 mV in normal saline and 49 mV in TTX. Proctolin [$1.5 \times 10^{-8}M$, $3.0 \times 10^{-7}M$, $1.5 \times 10^{-6}M$; (1)–(3), respectively] was bath-applied to the anterior chamber. At intermediate concentrations, proctolin produces a depolarization which is not accompanied by driver potentials. The dot in each record denotes onset of washout. Calibration: 10 mV, 24 s. (C) Oscilloscope trace from an intracellular recording of cell No. 1 [same preparation as in (A)] during the peak response to a proctolin pulse ($3 \times 10^{-7}M$; 100 μL) in the presence of TTX. Note the marked pacemaker activity between repetitive driver potentials (arrows). Calibration: 0.5 s, 10 mV. (D) (●—●) Current–voltage relationships in normal saline, (○—○) 3×10^{-7} M TTX, and (▲—▲) TTX plus $1.5 \times 10^{-7}M$ proctolin salines. Data represent steady-state potentials reached during 500 ms duration current pulses. Current was passed with one electrode and transmembrane potential was measured with a second electrode. In this preparation (cell No. 3) proctolin was observed to increase input resistance by 16% (5.1 $M\Omega$/4.4 $M\Omega$) as compared to the TTX saline.

threshold via electrotonic interactions, thereby initiating the burst. In contrast, we suggest that frequency control may be retained by the small-cell pacemaker in ganglia with high burst rates. Previous investigators have also proposed a role for large-cell slow potentials in the control of pacemaker activity in the cardiac ganglion (Watanabe and Bullock, 1960; Connor, 1969; Matsui, Ebara, and Ai, 1972).

We chose to limit our initial studies to applications of proctolin to the anterior cell body as previous investigators have noted regional distributions of pharmacological sensitivity in *Homarus* cardiac ganglion (Cooke and Hartline, 1975). According to their terminology our experimental procedures limited the site of action of proctolin to the following areas: (1) large-cell somata (1–4); (2) large cell (Nos. 1–5) trigger zones; (3) the pharmacologically sensitive areas of cell Nos. 1–5; (4) the distal synaptic areas of small cell axons; and (5) synaptic areas of regulatory nerves. The experimental procedure specifically excluded the known pharmacologically sensitive areas of the small cells from the anterior chamber. Although we cannot at this time conclusively rule out effects upon presynaptic elements, the simplest interpretation of our data is that proctolin acts directly on the anterior large cells. This is supported by (1) persistence of the proctolin-induced depolarization of large cell somata in 0 Ca^{2+}, Mg^{2+}-substituted saline in both the presence and absence of TTX (our unpublished observations); (2) the induction by proctolin of depolarization and sustained repetitive driver potentials in large cells isolated by ligature [Fig. 2(D)].

The above-described experiments suggest several mechanisms by which proctolin may be exerting its effects upon bursting. First, depolarization of large cells produces increases in burst frequency (Matsui, Ebara, and Ai, 1972) and may affect thresholds at small-cell trigger zones (Watanabe and Bullock, 1960). Since the proctolin-induced depolarization of large-cell somata is accompanied by an increase in apparent membrane resistance and persists in 0 Ca^{2+} and TTX salines, it would appear that a simple decrease in resting K conductance may underlie this aspect of the response. The depolarizing response may affect secondarily voltage-sensitive conductances, the net result of which may tend to enhance spontaneity. Although the increased resistance may account for an enhanced recording of pacemaker and driver potentials, it is also possible there is a direct and specific action of proctolin on large-cell voltage-dependent conductances (see Sullivan, Tazaki, and Miller, 1981).

Application of proctolin to whole ganglia effects sequential changes in ganglionic motor output which include (1) increases in burst duration and burst frequency, (2) increased number of impulses per burst, and (3) alteration of intraburst impulse patterning. These effects are mediated by proctolin action on both small and large cells. The predominant small-cell effect (apparent within 5–10 s) is a dramatic prolongation of the small-cell burst which often produces a "doublet bursting" mode in large cells (Sullivan, Tazaki, and Miller, 1981). This effect is *not* observed upon anterior application of proctolin in the split chamber. While experiments with *in situ* and *in vivo* lobster hearts have demonstrated proctolin ($10^{-6}M$) responses (increases in force of contraction and beat frequency) which correspond to the observed ganglionic effects, such experiments are often complicated by a proctolin-induced contracture of the myocardium and systolic arrest. For this reason sequencing and synthesis of the crustacean proctolinlike peptide may be requisite to relevant behavioral analysis.

Previous investigators have noted that fresh extracts of crab pericardial organs cause changes in burst parameters of *Homarus* cardiac ganglion large cells similar to those reported here (Cooke, 1966; Cooke and Hartline, 1975; and Lemos and Berlind, 1981).* Recently, one of the pericardial organ cardioexcitatory peptides has been found to exhibit pharmacological and biochemical properties indistinguishable from proctolin, and it has been proposed that proctolinlike peptides play a neurohormonal role in decapod crustaceans (Sullivan, 1979). Other studies demonstrating the action of proctolin on crustacean musculature support this hypothesis (Lingle, 1979; Schwarz et al., 1980). The authors therefore suggest that the above proctolin responses represent normal physiological events rather than nonspecific receptor agonism.†

One additional point that needs clarification is the dose–response relationship of the proctolin effect. In contrast to the thresholds reported for neuromuscular effects (10^{-10}–$10^{-8}M$; see Schwarz et al., 1980; Lingle, 1979), thresholds obtained using bath applications of proctolin in TTX-treated ganglia were $10^{-8}M$ for depolarizing effect and $8 \times 10^{-8}M$ for induction of repetitive driver potentials. Dose–response curves obtained by the pipette application method [Fig. 2(C)] may be skewed to higher concentrations due to dilution factors. The dose–response relationship seems to support the neurohormone hypothesis; however, the possibility that a proctolinlike peptide serves a synaptic transmitter role in the cardiac ganglion cannot be ruled out completely.

This work was supported by an NIH postdoctoral fellowship (NS06191) to RES and NIH grants NS15453 and NS11808 to Ian M. Cooke. This work was also partially supported by funds from the University of Hawaii Foundation. The authors would like to thank Dr. Daniel Hartline, Dr. Allan Berlind, Dr. David Russell, and Dr. Kenro Tazaki at the Bekesy Laboratory for the helpful discussions. Special thanks to Elaine Sullivan and Tina Weatherby for photographic assistance.

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* Crab pericardial organs (PO) contain a number of cardioexcitatory substances: (1) the trypsin-sensitive "frequency peptide," (2) the proctolinlike peptide, (3) dopamine, and (4) serotonin (5-HT). The effects of fresh extracts on lobster hearts and ganglia are Paracelsian in nature, but as first noted by Maynard and Welsh (1959), the bulk (90%) of the cardioexcitatory activity is due to the trypsin-sensitive frequency peptide (see Sullivan, 1977, 1979).

† Experiments with proctolin analogs have provided evidence for the specificity fo the proctolin responses. The following analogs at each concentration notedogs were without effect: Gly-Tyr-Leu-Pro-Thr ($1 \times 10^{-6}M$); Lys-Phe-Ile-Gly-Leu-Met-NH_2 ($3 \times 10^{-5}M$); and Lys-Tyr-Leu-Pro-Thr ($2 \times 10^{-7}M$). In contrast, Arg-Phe-Leu-Pro-Thr ($2.8 \times 10^{-8}M$) elicited a 36% increase in burst frequency from a slowly bursting preparation (R. E. Sullivan, R. Newcomb, and M. W. Miller, unpublished observations).

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