STUDIES ON THE MODE OF ACTION OF PROCTOLIN

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ABSTRACT

The hindgut response of Leucophaea maderae to glutamate resembles contractions evoked by brief neural stimulation. Proctolin induces substantial contractions of the hindgut after depolarization in 158 mM potassium, but glutamate does not cause contractions after depolarization. Proctolin's response on the hindgut of L. maderae is calcium dependent, and is inhibited by trifluoroacetazine.

Brown (1967) isolated a myotropic gut factor from the proctodeum and the stomodeum of the cockroach Periplaneta americana. He showed that this factor was dialyzable and insensitive to heat and chymotrypsin digestion. Brown (1975) identified his myotropic gut factor as a basic pentapeptide which he named proctolin; its amino acid sequence is (Arg-Tyr-Leu-Pro-Thr). Proctolin exerts a strong myotropic action on insect muscle (Miller 1979; Piek and Mantel 1977; May et al. 1979; Brown 1975). Holman and Cook (1970) found three myotropic agents from hindgut extracts of the cockroach Leucophaea maderae. Two of these agents were identified as L-glutamate and L-aspartate; the third substance was not identified. Holman and Cook (1972) further characterized this unknown substance as a basic peptide.

Although Brown (1975) conceptualized proctolin as an excitatory neurotransmitter in the viscera of insects, certain response characteristics could not be explained by a conventional neurotransmitters hypothesis (Cook and Holman 1979). For example, the hindgut response to proctolin in L. maderae and P. americana lasted many minutes instead of seconds. Proctolin's failure to fit the classical criteria for an excitatory neurotransmitter in the hindgut of L. maderae is best illustrated by comparing the actions of proctolin and monosodium glutamate (MSG) (Cook and Holman 1979).

Resemblance of Neurally and Chemically Evoked Responses. Electrical stimulation of the proctodeal nerve can evoke three types of responses from the hindgut: (a) a single phasic contraction; (b) a series of phasic responses with an elevated tonus; and (c) a sustained tonic contraction (Fig. 1, A1-3). By simply altering frequency and duration of stimuli a whole spectrum of responses can be achieved. MSG (1 X 10^{-5}M) elicited a maximum phasic contraction of the hindgut similar to the brief neurally evoked response (compare Fig. 1, A1 and B1). Proctolin response resembled contractile events evoked by recurrent stimulation of the proctodeal nerve (compare Fig. 1, A2 and B2). Figure 1, B3 demonstrates that after treatment with MSG the hindgut rapidly returned to normal responsiveness. A subsequent challenge with proctolin produced normal responsiveness despite the absence of a rinse between applications.

Threshold of Sensitivity to Proctolin. Starratt and Brown (1975) first reported the response threshold in P. americana to be in the 1 X 10^{-9}M range, but in this study the threshold was found to be 3 X 10^{-11}M in L. maderae (Fig. 2). Initially this two-fold difference in sensitivity was first considered to be a species difference, but tests showed a sensitivity in the 1-6 X 10^{-11}M range for both P. americana and L. maderae. Not only does proctolin have a very strong myotropic action on visceral muscle, but its effects have an extended duration. As seen in Fig. 2, at 3 X 10^{-11}M the proctolin stimulated response lasted 65 min. The persistent rhythmicity was the most consistent feature of these prolonged responses, not the increase in tonus. Even at higher concentrations of proctolin the initial increase in tonus stopped long
FIG. 1. Comparison of neurally and chemically evoked events in the hindgut of L. maderae. (A₁) A single phasic contraction of the longitudinal muscles of the hindgut caused by recurrent stimulation (bar) at 20 Hz for 2 sec. (A₂) Response of another hindgut to recurrent stimulation (bar) at 10 Hz for 1 sec every 2 sec. (A₃) Response of the same preparation to continuous stimulation (bar) at 5 Hz for 2 min. (B₁) A single phasic contraction evoked by the addition of glutamate (1.4 x 10⁻⁴M) (upright arrow) to the muscle chamber. (B₂) Sustained response of the same preparation to proctolin (3 X 10⁻¹⁰M). (B₃) Response of the same hindgut (after another rinse in saline solution) to a sequential treatment of glutamate (1 X 10⁻⁴M, 1st arrow) and then proctolin (3 X 10⁻¹⁰M, 2nd arrow). Arrows pointing downward signify a rinse in saline solution. Vertical calibration 2 cm chart = 2 mm tissue displacement; horizontal calibration 1 cm chart = 1 min.

FIG. 2. The character and duration of the response of a hindgut preparation from L. maderae to proctolin. (A) Effect of 3 X 10⁻¹¹M proctolin (upright arrow) on hindgut activity. (B) Effect of 7 X 10⁻¹¹M proctolin (arrow) on normal activity. (C) Extended response of the hindgut to 3 X 10⁻¹⁰M proctolin (arrow). Vertical calibration 2 cm chart = 2 mm tissue displacement; horizontal calibration 1 cm chart = 1 min. The arrows directed downward indicate a saline rinse.
before the augmented rhythmicity did. These responses strongly suggest that proctolin does not function strictly as a neurotransmitter.

Potentiation of Neural Evoked Events. MSG at 1.2 X 10^{-5} M can evoke a contraction of the hindgut in L. maderae that is readily visible over background myogenesis (Holman and Cook 1970). However, when this amino acid was introduced into the preparation chamber about 5 sec before neural stimulation (Fig. 3, A_1 and B_1), as little as 1.5 X 10^{-6} M MSG produced a brief potentiation that never lasted more than 30 sec. This same preparation, containing 1.5 X 10^{-6} M MSG, gave only a barely perceptible response in the absence of neural stimulation (Fig. 3, A_2 and B_2). This observation suggests that the nerve-muscle junction is more sensitive than the muscle fiber membrane to MSG stimulation.

The threshold for proctolin’s myotrophic action on the hindgut was 1-3 X 10^{-10} M in both P. americana (Brown 1975) and L. maderae (Cook and Holman 1979). Moreover, the small amplitude changes in neurally evoked responses that occurred after the addition of proctolin were in no way comparable to the responses evoked by the same concentration of the peptide after suspension of neural stimulation (Fig. 3, C_1-C_3). These results suggest that the visceral muscle fibers themselves are more sensitive than is the nerve muscle junction to stimulation by proctolin.

![Graphs of neural evoked events](image)

**Fig. 3.** Potentiations of neurally evoked events with sub-threshold amounts of L-glutamate on the hindgut of L. maderae. Innervated hindguts were stimulated every 20 sec by a 1-sec train of pulses at 15 Hz. (A_1) Potentiation of neurally evoked responses by 1.5 X 10^{-6} M glutamate (upright arrow). (A_2) Response of the same hindgut to 1.5 X 10^{-6} M glutamate. (B_1) Potentiation in another hindgut during neural stimulation by 1.5 X 10^{-6} M glutamate. (B_2) Response of the same preparation to glutamate without neural stimulation. (C_1 and C_2) show the duration of potentiation by 1.5 X 10^{-10} M proctolin in C_1 and by 3 X 10^{-10} M in C_2. (C_3) shows the response of the same preparation to 3 X 10^{-10} M proctolin after suspension of neural stimulation. Vertical calibration 2 cm chart = 2 mm tissue displacement; horizontal calibration 1 cm chart = 1 min.

Potassium Depolarization Effects on the Action of MSG and Proctolin. Cook and Holman (1980) discovered that proctolin can cause substantial contractions of the hindgut after depolarization in high potassium salines. Isotonic potassium saline solutions have been used for a number of years to differentiate membrane electrical phenomena from the mechanical events of muscle contraction. This principle seemed appropriate for use with insect visceral muscle, since the muscles of the hindgut in P. americana were progressively depolarized by elevating the potassium in the bathing medium (Nagaï 1972). The completeness of depolarization in the case of L. maderae was indicated by the total failure of
neurally evoked events after treatment with 158 mM KCl containing 2 mM calcium (Fig. 4, B4). The exposure of the isolated hindgut to high potassium solutions generally caused an immediate phasic contraction that lasted from 1 to 3 min (Fig. 4, A3). This was followed by a slow drop in tension that continued until a baseline tension was reached in 10 to 12 min. Although 158 mM KCl initially caused a substantial contraction of the hindgut, successive rinses in 158 mM potassium solutions often failed to evoke additional contractions (Fig. 4, A3).

In figure 4, A1-3 we show the response of a hindgut to proctolin and L-glutamic acid before and after membrane depolarization with 158 mM KCl. Although glutamate (1 X 10^-4M) evoked a substantial contraction in normal saline (Fig. 4, A2), the hindgut did not respond to the amino acid (1 X 10^-4M) after depolarization in 158 mM potassium (Fig. 4, A3). Such results provide clear evidence that the action of glutamate is restricted to the

FIG. 4. Effect of 158 mM potassium depolarization on the action of glutamate and proctolin on the hindgut of L. maderae. (A1) Response of the hindgut to 5 X 10^-3M proctolin (arrow) in normal saline solution. (A2) Response of the same preparation to 10^-3M monosodium glutamate after a rinse in fresh saline solution. (A3) Once the hindgut had been depolarized by the addition of 158 mM potassium containing 2 mM calcium (first bar), it failed to respond to 10^-3M (first arrow) and 10^-2M (second arrow) glutamate. After a rinse with 158 mM potassium (second bar), 5 X 10^-3M proctolin (last arrow) caused a contracture. (B1) Response of an innervated hindgut to proctolin (10^-3M). (B2) Phasic contractions evoked by neural stimulation at 40 Hz for 2 sec every 45 sec (dots) and a tonic contraction by 40 Hz for 30 sec (dash). (B3) A contracture induced by 158 mM potassium containing 2 mM calcium (bar). (B4) The same depolarization preparation 9 min later showing the failure of neurally evoked responses (dots and dash) and the effect of 5 X 10^-3M and 5 X 10^-2M proctolin, 1st and 2nd arrows, respectively. (C1) Effect of proctolin 10^-3M (arrow) on another hindgut in normal saline solution. (C2) Contractions induced by proctolin (1st arrow, 10^-3M; 2nd arrow and last arrow, 10^-2M) after depolarization in 158 mM potassium (bars). Vertical calibration 2 cm chart = 2 mm tissue displacement; horizontal calibration 2 cm chart = 2 min.
surface membrane. However, proctolin (1 X $10^{-8}M$) produced noticeable contraction of visceral muscle after depolarization with KCl (Fig. 4, A3 and C3). These responses suggest that proctolin directly affects mechanical events in the excitation-contraction coupling sequence. Proctolin caused a sustained tonic response which could be increased by additional amounts of the peptide (Fig. 4, C3). A comparison of these responses suggests that proctolin has a site of action other than the myoneural junction (MNJ).

Influence of Calcium. Since Ca$^{2+}$ generally serves as an important link between electrical and mechanical events in muscle contraction, it is necessary to look at the effects the absence of this cation might have on the action of proctolin after K$^+$ depolarization.

In figure 5 A, we illustrate the response of the hindgut to proctolin in 163 mM K$^+$ without calcium. Under these conditions the hindgut showed a rapid decline in response to the peptide. The addition of 2 mM calcium partially restored the contractile response caused by proctolin. Moreover, the calcium alone caused a contracture in the high potassium solution that was distinct from the contractions caused by proctolin (Fig. 5, B and C).

![Graph](image)

FIG. 5. Contraction depletion curve for proctolin ($10^{-8}M$) on hindgut of L. maderae after depolarization in 162 mM potassium without calcium and the partial restoration of these contractures on the addition of 2mM calcium. (A) Each point is the mean of at least 4 records and the vertical lines show the limits of the SE of the mean. Contractions are expressed as a percentage of maximum tension obtained for each stimulant in normal saline solution. (B) Representative potassium (bar) and proctolin (arrow) contractures. (C) Calcium (dot) and proctolin (arrow) responses after a 15 min depletion period in 162 mM potassium without calcium. Vertical calibration 2 cm chart = 2 mm tissue displacement; horizontal calibration 1 cm chart = 1 min.

The fact that calcium alone can cause the depolarized muscles of the hindgut to contract, and that this ion is also required for the response of proctolin, offers strong evidence that calcium functions as an intracellular mediator for contraction in insect visceral muscle. The qualitatively distinct and additive effect of proctolin, compared with the effects of calcium alone, suggests that this peptide releases bound calcium.

Calcium, in addition to its role in excitation-contraction coupling, has long been recognized as a second messenger in many cellular processes, even though the nature of the internal transducing mechanism is still unclear (Ebashi
et al. 1978). Over the last few years there has been a gradual realization that a single calcium binding protein may function as this calcium receptor, namely calmodulin (CAM) (formerly named calcium-dependent regulatory protein) (Cheung 1980).

Calmodulin is a ubiquitous protein found throughout the animal and plant kingdom. A few of the functions controlled by CAM are: (1) activation of phosphorylase kinase, causing glycogen breakdown; (2) disassembly of micro tubules, causing mitosis; (3) stimulation of membrane fusion, causing exocytosis; and (4) activation of myosin light chain kinase, causing muscle contraction in smooth muscle (Weiss et al. 1980).

We assayed for CAM in the hindgut of the cockroach Leurophaea maderae and found levels greater than 1.5% of the wet weight of the hindgut. We then attempted to determine if proctolin's response was CAM dependent. To do this we used trifluoperazine (TFP), an inhibitor of CAM dependent functions (Delorenzo 1981). After the addition of TFP, there was a marked reduction in frequency, moderate decrease in amplitude, and tendency toward extended duration of response, all of which are characteristic of a hindgut deprived of calcium ion (Fig. 6). When proctolin was readded there was no myotropism response, which

![Image of graphs A, B, C](image)

**FIG. 6.** Effect of trifluoperazine (TFP) on proctolin evoked contractions of the hindgut of L. maderae. (A) The up arrow indicates addition of 3 X 10^-10M proctolin; the down arrow indicates a rinse followed by normal hindgut myogenic activity. (B) The up arrow indicates the addition of 4.9 mm TFP. (C) At the up arrow 9 X 10^-10M proctolin was added. Vertical calibration 2 cm chart = 2 mm tissue displacement; horizontal calibration 1 cm chart = 1 min.
suggests that TPF alters the gut's ability to regulate calcium. As seen in Fig. 6, TPF blocked the proctolin response, indicating that the proctolin response is mediated through CAM.

In summary, proctolin does not match the sharply defined examples of rapid chemical transmission usually encountered in insects (Usherwood 1974). The peptide appears to have non-synaptic sites of action in the hindgut, and there is good reason to suppose that proctolin may regulate the mechanical threshold for excitation-contraction coupling in insect visceral muscle.

LITERATURE CITED