PROCTOLIN'S ROLE AS A COTRANSMITTER AT THE OVIDUCTS OF THE LOCUST *LOCUSTA MIGRATORIA*

by

KONRAD F. NORONHA

A thesis submitted in conformity with the requirements for the degree of Master of Science Graduate Department of Zoology in the University of Toronto

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ABSTRACT

PROCTOLIN'S ROLE AS A COTRANSMITTER AT THE OVIDUCTS OF THE LOCUST LOCUSTA MIGRATORIA by Konrad F. Noronha, Master of Science, 1997, Department of Zoology, University of Toronto. The oviducts of the locust Locusta migratoria provide an ideal system for studying neuromuscular transmission as it is innervated by a relatively small number of neurons and appears to be modulated by a few peptides and amines. In the present study, the locust oviducts were used to study the role of the pentapeptide proctolin (RYLPT) as a cotransmitter. Several proctolin analogues were assayed as potential agonists and antagonists of proctolin. Cycloproctolin was identified as a proctolin antagonist and used to further define proctolin's effects on neurally-evoked contractions. Proctolin was found to enhance basal tension, amplitude and relaxation rate of neurally-evoked contraction and the size of a post-contraction relaxation during the low stimulus regimes (1 and 2 stimuli). These responses of the oviduct muscle to the peptide proctolin were either significantly decreased or absent during the higher stimulus regimes (5 and 10 stimuli). The proctolin antagonist cycloproctolin was capable of inhibiting the proctolin-induced increase in neurally-evoked amplitude, basal tonus and post-contraction relaxation. The results of the present study support previous hypotheses in which proctolin was reported to act as a cotransmitter at the oviducts of Locusta.
ACKNOWLEDGEMENTS

I wish to relay sincerest gratitude to Dr. Angela B. Lange for her continued support and guidance of this research project. As well, I thank Dr. Richard H. Osborne for providing the proctolin analogues used in the present study. Special thanks to David A. Nykamp and Patricia Quigley for their help and encouragement.

Lastly, I thank my parents, Dr. Xavier and Angela Noronha as well as my brother, Trevor, without whom I would not be able to undertake such endeavours.
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<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic Guanosine Monophosphate</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DUM</td>
<td>Dorsal Unpaired Median</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EJP</td>
<td>Excitatory Junction Potential</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>IP₁</td>
<td>Inositol Monophosphate</td>
</tr>
<tr>
<td>IP₂</td>
<td>Inositol Diphosphate</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol Triphosphate</td>
</tr>
<tr>
<td>OAG</td>
<td>1-oleoyl-2-acetylglycerol</td>
</tr>
<tr>
<td>LPT</td>
<td>Leucine-Proline-Threonine</td>
</tr>
<tr>
<td>MEPP</td>
<td>Miniature End Plate Potential</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>RYLPT</td>
<td>Arginine-Tyrosine-Leucine-Proline-Threonine</td>
</tr>
<tr>
<td>SETi</td>
<td>Slow Excitatory Motorneuron</td>
</tr>
<tr>
<td>TPA</td>
<td>12-myristate 13-acetate 4-O methyl ether</td>
</tr>
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ORGANIZATION OF THESIS

Since the research presented in this thesis has been either published or submitted for publication, the thesis is organized as a collection of two scientific papers (Sections II and III). A general introduction (Section I) and discussion (Section IV) have been included to review and discuss some of the general concepts involved in this work.

A version of Section II of this thesis has been accepted for publication by the journal Peptides and is currently in press. It was submitted as a three-author paper (Noronha, Lange and Osborne) but the research and writing were done by the present author with the proctolin analogues kindly provided by Dr. Richard H. Osborne.

Section III of this thesis has been submitted to the Journal of Neurobiology as a two author paper (Noronha and Lange). All phases of the research and writing of this section were done by the present author.

In all cases, Dr. Angela B. Lange contributed to experimental design, editing of manuscripts and financial support of the research.
I. GENERAL INTRODUCTION.

COTRANSMISSION

In cotransmission, a "classical" neurotransmitter, such as acetylcholine, glutamate, dopamine or 5-hydroxytryptamine, is released along with another regulatory compound, such as a peptide, to influence the postsynaptic membrane (Boarder, 1989). Although it would seem natural that the function of multiple transmitters is to elicit a response that is more adaptive than that elicited by a single transmitter, it is possible that a coexisting substance need not always serve a functional role. It has been suggested that substances that are released into the synaptic cleft as a result of neural activity but that do not have receptors in the synaptic region be termed a "nontransmitter" (Chubb, 1977). One possibility is that multiple messengers were used by lower animals but have been replaced by more efficient single messengers in more advanced animals (Hokfelt et al., 1987).

Interactions between neurotransmitter systems have been the topic of research for the last two decades but in mammals the progress of understanding these interactions has been hampered by the complexity of the nervous system (Dyakonova et al., 1995). With this in mind, the easily accessible and relatively simple nervous systems of many invertebrates provide valuable preparations to study how the interactions of neurotransmitters underly behaviour. Additionally, evidence for cotransmission appears to be very common in invertebrate neurons (Adams and O'Shea, 1983; Bishop et al., 1987; O'Shea et al., 1985; Siwicki, et al., 1985)

The peptide proctolin (RYLPT) has been implicated as a cotransmitter at several neuromuscular junctions (Adams and O'Shea, 1983; Worden et al., 1985; Lange et al., 1986).
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Bishop and O’Shea (1982) have shown proctolin to be associated with some excitatory motorneurons in the cockroach *Periplaneta americana*. In studying the proctolin-containing slow coxal depressor motorneuron they found that stimulating this neuron in a short train produces a biphasic contractile response. There was a delayed and persistent rise in tension that was not associated with EJPs or with a membrane depolarization and the relaxation phase of transient contractions became progressively prolonged. The temporal separation of these effects from the usual transient responses and the absence of an associated muscle depolarization suggest that a non-glutamate-mediated mechanism is responsible. The fact that proctolin is released at this neuromuscular junction and that when applied in low concentrations the pentapeptide produces similar effects suggests that proctolin acts as a cotransmitter in this system.

Worden *et al.* (1985) have provided several lines of evidence to suggest that proctolin acts as a cotransmitter at the slow excitatory motorneuron (SETi) of the locust extensor tibialis muscle. Staining with an antibody to proctolin reveals proctolin immunoreactive axon colaterals and terminals on the fibers of the muscle. Furthermore, proctolin is released from potassium-depolarized nerve terminals in a calcium-dependent mechanism. Lastly, proctolin can be isolated by HPLC from regions of the muscle innervated by SETi where it is fifty times more concentrated than in non-SETi innervated regions. Physiologically, stimulation of SETi at a high frequency produces two effects that outlast the transient contractures normally attributed to glutamate. These include an increase in the frequency of a myogenic rhythm of contraction and an increase in tension that decays slowly.
General Introduction

The neuromuscular junctions at the oviduct of the locust, *Locusta migratoria*, are also believed to be modulated by glutamate and proctolin. The association, mode of action and effects of proctolin at the oviduct neuromuscular junction of *Locusta* are discussed below.

Oviducts of *Locusta migratoria*

Morphology

The internal organs of reproduction in the female locust, *Locusta migratoria*, consist of a pair of ovaries made up of a number of ovarioles and two lateral oviducts which converge posteriorly (from the ovaries) and unite to form a common oviduct. The common oviduct opens posteriorly to the exterior at the gonopore. Extensions of the common oviduct may include a genital chamber or bursa copulatrix and, in addition to these primary parts, two types of glands open into the female reproductive tract (Lange, 1992). These glands are the spermatheca, which is a storage receptacle for spermatozoa until they are needed for fertilization, and a pair of accessory glands, the function of which is poorly understood (Lange, 1992).

The walls of the lateral oviduct, common oviduct and spermatheca are usually covered by a muscular sheath consisting of circular and longitudinal fibres arranged in various layers (Lange, 1992). Although insect visceral muscles are striated like vertebrate skeletal muscle (Davey, 1964) they contract in a spontaneous, rhythmical manner when isolated from the central nervous system. The muscles of insect oviducts are therefore considered to be myogenically active (Lange et al., 1986; Lange, 1990) with complex movements believed to
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be responsible not only for transporting sperm within the oviduct but also for carrying mature eggs from the ovary into the lateral oviduct, and eventually through the common oviduct (Okelo, 1971).

Physiology

As mentioned above, insect visceral muscle and vertebrate smooth muscle both exhibit contractions that are slow and rhythmic and often coordinated into peristaltic waves (Davey, 1964; Miller, 1975). However, the similarities between insect visceral muscle and vertebrate smooth muscle extends further as both require calcium to initiate contraction.

In smooth muscle, the mechanism by which calcium regulates contraction is through myosin-linked regulation (Hill and Wyse, 1989). Smooth muscle myosin can interact with actin filaments only when the myosin light chains are phosphorylated. Calcium ions regulate the phosphorylation of myosin light chains indirectly, by combining with the calcium-binding protein calmodulin. The calcium/calmodulin complex then activates the enzyme myosin light-chain kinase, which phosphorylates the myosin light chains, initiating contraction and allowing continued crossbridge cycling as long as calcium is present. Some of the calcium ions that activate contraction may come from the sarcoplasmic reticulum, but much of the calcium enters the smooth muscle cell directly across the other plasma membrane (Eckert et al., 1988). Smooth muscles can be excited spontaneously, by nerves, hormones and in some cases by stretch of the muscle. All these sources of excitation act ultimately by increasing the concentration of intracellular calcium ions (Hill and Wyse, 1989).
**General Introduction**

**Proctolin**

Proctolin (H-Arg-Tyr-Leu-Pro-Thr-OH) was the first insect neuropeptide to be sequenced and was proposed to be a visceral muscle neurotransmitter (Brown and Starratt, 1975). Since that time, proctolin has been shown to be much more widely distributed with diverse physiological functions.

**History**

Proctolin has proven to be a significant regulatory substance in insects. While there is more evidence for the existence of and physiological roles of proctolin in Orthoptera than in any other insect group there is substantial evidence which suggests its presence in Hemiptera, Diptera, Hymenoptera and Coleoptera (Orchard et al., 1989). Proctolin appears to be involved in the control of visceral (Brown and Starratt, 1975; Lange et al., 1986), cardiac (Benson et al., 1981) and skeletal (O’Shea, 1985) muscles in Orthopterans, as well as possibly being involved in central processes (Fitch and Djamgoz, 1988).

Evidence for proctolin, or proctolin-like peptides, among non-insect invertebrates is also strong (O’Shea and Adams, 1986). For example, among the Crustacea proctolin has been detected in pericardial organs of crab *Cardisoma carnifex* (Sullivan, 1979) and lobster *Homonurus americanus* (Schwarz et al., 1984) as well as in crayfish and lobster central nervous system (Bishop et al., 1984). Proctolin’s occurrence in non-arthropod invertebrates has also been suggested by immunohistochemical studies in the nervous system of the leech ganglia, *Hirudo medicinalis* (Li and Calabrese, 1985).
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Lastly, proctolin immunoreactivity has been detected in mouse brain (Bernstein, et al., 1984), rat CNS (Hokfelt et al., 1987) and human brain stem (Bernstein et al., 1986). In some cases, especially where immunochemical detection methods are used in the absence of biochemical criteria, authentic proctolin may not be the identified compound (O’Shea and Adams, 1986), however, it is clear that proctolin or proctolin-like peptides are widely distributed throughout the animal kingdom.

Association of proctolin with the oviducts of Locusta

In order to classify proctolin’s role in contraction of the oviducts of Locusta, it is important to define what are neurotransmitters, neurohormones and neuromodulators. A neurotransmitter may be defined as a neuroactive substance which is released at a discrete synapse and transiently alters the ionic permeability of the postsynaptic membrane. A neurohormone is released into the general circulation and acts over a prolonged period on both proximal and distal targets. Lastly, a neuromodulator is released at or near a synapse or group of synapses and alters synaptic transmission by acting, with a variable time course, either pre- or postsynaptically, or both (Orchard et al., 1989).

Proctolin does not appear to function as a classical neurotransmitter as it does not alter the ionic permeability of the post-synaptic membrane in most systems. In the few systems where proctolin does alter postsynaptic conductance, it does so with a prolonged, rather than a transient, time course (Sullivan and Miller, 1984). Proctolin’s presence in the haemolymph of Leucophaea maderae (Kingan and Titmus, 1983) suggests that it may act as a
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neurohormone in some insects, but proctolin is perhaps best described as a neuromodulator. Many proctolinergic neurons appear to be motorneurons and utilize proctolin as a cotransmitter (O'Shea and Bishop, 1982; Adams and O'Shea, 1983; Worden et al., 1985; Lange et al., 1986; Bishop et al., 1987).

Proctolin-like immunoreactivity has been observed in a number of neurons in the VIIth abdominal ganglion of the locust, Locusta migratoria. In particular, three positively staining bilaterally paired neurons are located in a position similar to the oviduct motorneurons (Lange et al., 1986). Proctolin is present in the area of oviduct muscle which receives extensive innervation (approximately 1.9±1 pmol/mg protein), whereas the area which receives little or no innervation (anterior region of the oviduct) contains about tenfold less proctolin. Measurable levels of proctolin are also found in the oviducal nerves and in the VIIth abdominal ganglion (Lange et al., 1986). Electrical stimulation of the oviducal nerves results in the calcium-dependent release of proctolin. The release is frequency-dependent, with maximum release occurring at about 30 Hz (Orchard and Lange, 1987). About 6.3% of the total store of proctolin can be released by five minutes of stimulation, whereas only 0.4% can be released during a five minute incubation in high potassium saline (Orchard and Lange, 1987).

Effects of proctolin on oviducts

Physiologically, neural stimulation results in a contraction of the oviducts which is sustained throughout the stimulation period (Orchard and Lange, 1986). The force of the
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contraction is dependent upon the stimulus frequency. Proctolin itself produces a number of effects. Proctolin results in dose-dependent increases in the amplitude of neurally-evoked contractions, the amplitude and frequency of myogenic contractions and the amplitude of a tonic contraction. These effects occur between $5 \times 10^{-11}$ M and $10^{-10}$ M proctolin (Lange and Orchard, 1984) and are produced in the absence of any major change in muscle membrane potential or EJP amplitude (Orchard and Lange, 1986). It seems likely that the release of a conventional neurotransmitter (perhaps glutamate) is responsible for the EJP and that proctolin produces its effects upon the muscle contraction somewhere in the excitation/contraction coupling pathway.

**Mode of action**

With the use of various calcium channel blockers, it has been shown that proctolin acts via calcium which enters through both voltage-dependent and receptor-operated channels (Lange et al., 1987). Both the myogenic contractions and the proctolin-induced sustained contractions are reversibly abolished in calcium-free saline (containing either high magnesium or EGTA) and by normal saline containing cobalt ions. It has been postulated that there could be two pools of calcium involved in mediating the changes of contraction, with a freely accessible pool involved in the myogenic contractions and a loosely bound pool involved in the proctolin-induced sustained contractions (Lange et al., 1987).

Activation of the proctolin receptor has also been shown to lead to the activation of phospholipase C to form inositol 1,4,5-triphosphate ($IP_3$) and diacylglycerol (DAG) (Lange,
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1988). The liberation of calcium from internal calcium stores, via IP$_3$, has therefore been implicated in the mediation of proctolin-induced contractions of the locust oviducts (Lange, 1988). Further evidence implicating inositol phospholipids in contraction of locust oviduct muscle (Lange, 1988) and foregut (Hinton and Osborne, 1995) was found using lithium chloride which, when added with proctolin, leads to an increase in IP$_1$ due to the inhibition of myo-inositol-1-phosphatase.

Nykamp et al. (1994) have shown that increases in cytosolic calcium levels (via intracellular and extracellular sources) leads to the phosphorylation of myosin-light chain kinase, via calmodulin. These findings are consistent with previous work since insect visceral muscle is believed to contract in a manner similar to vertebrate smooth muscle (Davey, 1964; Miller, 1975), which is mediated through myosin-linked regulation.

To obtain further physiological evidence for the involvement of inositol phospholipid hydrolysis in the action of proctolin, Lange (1988) examined the effects of the monoacyl derivative of DAG, 1-oleoyl-2-acetylglycerol (OAG), and two phorbol esters, one of which activates protein kinase C and one which is inactive. The active phorbol ester, 12-myristate 13-acetate 4-O methyl ether (TPA), and OAG were found to induce a slow developing basal contraction with intense phasic contractions superimposed. It is unlikely that these agents are leading to a release of proctolin from presynaptic stores since reduced calcium saline, which abolishes neurally-evoked contractions, did not prevent the actions of TPA or OAG. Therefore, the likely action of TPA and OAG would be via an action on protein kinase C in the muscle.
General Introduction

To further examine the actions of proctolin, Lange et al. (1987) looked at the second messengers cAMP and cGMP. The results of these studies suggest that proctolin has no effect on the content of cAMP or cGMP in the locust oviducts.

Metabolism

Isaac (1987) investigated the hydrolysis of proctolin by enzyme preparations from the nervous tissue of the desert locust, *Schistocerca gregaria*. In this study the ability of nervous tissue to degrade proctolin was examined by incubating proctolin (100 μM) with a crude enzyme preparation. Primary hydrolysis of the Arg-Tyr bond yielded Tyr-Leu-Pro-Thr as the predominant degradation fragment and an intermediate degradation product, free tyrosine, was also produced. Interestingly, 71% of the proctolin-degrading aminopeptidase activity was localized in synaptosomal fractions while the cleavage of the Tyr-Leu bond was associated (66%) with mitochondrial fractions. These results are similar to those obtained by Quistad et al. (1984) who showed that membrane fractions from brain and terminal ganglia of *Periplaneta americana* also generated Arg-Tyr and Tyr-Leu-Pro-Thr fragments, albeit in approximately equal amounts. Again, the significance of this partial degradation of proctolin is reflected by the work of Starratt and Brown (1979) who found proctolin to be almost devoid of biological activity unless the full pentapeptide structure is maintained. The results of the study by Isaac (1987) demonstrate the localization of a membrane aminopeptidase activity in a locust synaptic-membrane preparation which is consistent with a role for this enzyme in proctolin activation.
**General Introduction**

**PROCTOLIN ANALOGUES**

To date, various proctolin analogues have been synthesized in attempts to elucidate the structure-activity requirements of proctolin and its receptors. Starratt and Brown (1979) investigated a series of two, three, four and five amino acid chains with sequences which were substituted or represented parts of the proctolin sequence. Their work showed that the activity of proctolin depended upon the full pentapeptide being present with only the natural L-forms of the amino acid constituents. Slight modification to the peptide sequence resulted in an almost complete loss of activity. Subsequently, Sullivan and Newcomb (1982) showed that the presence and the size of the aromatic group of [tyrosine²] was necessary for full activity and that increasing the size of the aromatic ring by substitution with [tryptophan²] caused a marked decrease in the activity on the analogue. Konopinska et al. (1986) also substituted the hydroxyl group of the [tyrosine²] with various nitrogen containing groups and tested the [p-methoxy-phenylalanine²] supra analogue. All of these analogues were found to be more active than proctolin, demonstrating that a polar group is needed in this position for high agonist potency.

Through the assaying of several proctolin analogues, it is now believed that there is more than one proctolin receptor subtype. Support for this hypothesis comes from work done by Konopinska et al. (1988) in which the effects of a variety of proctolin analogues have been examined for cardioexcitatory action on two insect species, the cockroach, *Periplaneta americana* and the mealworm, *Tenebrio molitor*. For example, the N-terminally modified analogue Lys-Tyr-Leu-Pro-Thr was slightly more active than proctolin when assayed on the
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cockroach heartbeat but was much less effective on the mealworm heartbeat (Konopinska et al., 1988). More recently, Lange et al. (1993) have assayed several of the proctolin analogues used by Konopinska and her colleagues on the oviducts of Locusta migratoria and developed a rank order based on potency. The results of this study suggest that receptors between these three species of insect (Locusta migratoria, Tenebrio molitor and Periplaneta americana) and between each preparation are different. Overall the results of such studies support the suggestion that there are sub-types of proctolin receptors in insects and that synthesis of effective antagonists to proctolin may be useful for classification of these receptors.

With this in mind, several new proctolin analogues (see Fig. 1) have been synthesized and tested for potential antagonism to proctolin's response on the foregut (Gray et al., 1994). Of these analogues, three were tripeptides, namely Arg-Tyr-Thr, Tyr-Arg-Thr and Leu-Pro-Thr, and showed no agonistic effects on the foregut of the desert locust Schistocerca gregaria, with only Arg-Tyr-Thr being able to antagonize proctolin-induced contractions. However, it has been suggested that this ability of Arg-Tyr-Thr to partially inhibit proctolin's response may be caused by enzyme inhibiting effects similar to those of endogenous Arg-Tyr (Gray et al., 1994). Additionally, proctolin analogues modified at position two were assayed and while [N-methyl-L-tyrosine2]-proctolin showed no significant agonistic or antagonistic effects, [α-methyl-L-tyrosine2]-proctolin proved to be a potent antagonist of proctolin responses. Lastly, a cyclic proctolin molecule, cycloproctolin, was found to be devoid of agonistic activity and was only a weak proctolin antagonist.

The synthesis of specific and effective proctolin antagonists is particularly important to
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Figure 1. The molecular structure of proctolin (A) and three of the proctolin analogues (B, C and D) synthesized by Gray et al. (1994) and used in the present study. Additionally, three tripeptides were also used: Leu-Pro-Thr (the degradation product of endogenous proctolin hydrolysis), Tyr-Arg-Thr and Arg-Tyr-Thr.
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![Chemical Structures]

- [N-methyl-L-tyrosine²-proctolin (B)]
- Proctolin (RYLPT) (A)
- [alpha-methyl-L-tyrosine²-proctolin (C)]
- Cycloproctolin (D)
elucidate proctolin's role as a cotransmitter in a system like the oviduct neuromuscular junction of *Locusta*.

**OBJECTIVES**

The aim of this thesis is to elucidate proctolin’s role in cotransmission at the locust oviduct neuromuscular junction. To this end, the following proctolin analogues were tested for potential antagonism to the proctolin response on the oviducts of *Locusta*: \([N\text{-methyl-L-tyrosine}^2]\text{-proctolin, [\alpha\text{-methyl-L-tyrosine}^2]\text{-proctolin, cycloproctolin, and the triptides Arg-Tyr-Thr, Tyr-Arg-Thr and Leu-Pro-Thr. Secondly, because there has been no detailed description of the effects of proctolin on neurally-evoked contractions of the oviducts, the effects of proctolin during different stimulus regimes were examined and the proctolin antagonist cycloproctolin was used to further define proctolin's actions in cotransmission at this neuromuscular junction. Therefore, with the use of proctolin antagonists the peptide proctolin was studied in order to further define its physiological role in contraction of the oviducts of *Locusta*.}**

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II. PROCTOLIN ANALOGUES AND A PROCTOLIN METABOLITE AS ANTAGONISTS OF THE PEPTIDE PROCTOLIN.

ABSTRACT

The locust oviduct bioassay was used to assess a variety of proctolin analogues as possible agonists and antagonists of the peptide proctolin. Both [α-methyl-L-tyrosine²]-proctolin and [N-methyl-L-tyrosine²]-proctolin were agonists of proctolin with thresholds of 5x10⁻⁹ M. Interestingly, at these threshold doses the analogues were antagonists when applied along with proctolin, being capable of shifting the dose-response curve for proctolin an order of magnitude to the right. Of the three tripeptides tested Tyr-Arg-Thr and Arg-Tyr-Thr showed no agonistic effects and were incapable of antagonizing proctolin-induced contractions. The third tripeptide, Leu-Pro-Thr, showed minimal agonistic effects and when applied with proctolin, significantly decreased the maximum response and increased the ED₅₀ values of the parent compound. Interestingly, this tripeptide is a degradation product of proctolin. Cycloproctolin possessed no agonistic activity up to 10⁻⁶ M but did antagonize proctolin's response in a dose-dependent manner with 2x10⁻³ M cycloproctolin shifting the proctolin curve nearly two orders of magnitude to the right. Simultaneous application of 10⁻⁹ M [α-methyl-L-tyrosine²]-proctolin and 10⁻⁵ M cycloproctolin showed some synergistic effect as the maximum response to the peptide was decreased by 21.6% and the dose-response curve shifted further to the right. These proctolin antagonists will be useful tools for examining the physiological importance of proctolin in insects as well as helping to identify receptor subtypes.
**Proctolin Analogenes as Antagonists**

**INTRODUCTION**

Since its discovery in the cockroach *Periplaneta americana* (Brown and Starratt, 1975), the myotropic peptide proctolin (H-Arg-Tyr-Leu-Pro-Thr-OH) has been found to be widely distributed among arthropods (Puiroux and Loughton, 1992) and arachnids (Groome *et al.*, 1991). In insects, proctolin has been shown to stimulate muscle contraction (Groome *et al.*, 1991; Hertel *et al.*, 1985; Holman and Cook, 1985; Lange *et al.*, 1986; Washio and Koga, 1990) by acting as both a neuromodulator and possibly as a neurohormone (Orchard *et al.*, 1989).

Previously it has been demonstrated that the oviducts of the locust, *Locusta migratoria*, receive innervation from proctolin-containing neurons (Lange *et al.*, 1986) and are also very sensitive to proctolin. A 50 pM concentration of the peptide is capable of increasing the amplitude of neurally-evoked contractions, increasing the frequency and amplitude of myogenic contractions, and inducing a sustained increase in basal tonus (Lange and Orchard, 1984; Lange *et al.*, 1986). In addition, at the same neuromuscular junctions it acts as a cotransmitter with a more conventional neurotransmitter such as glutamate (Adams and O'Shea, 1983; Orchard and Lange, 1986).

The synthesis of a selective and potent proctolin antagonist is integral to elucidate proctolin's role in modulating contraction of oviduct muscle. Previously, Starratt and Brown (1979) have shown that the full pentapeptide structure is necessary for activity of proctolin, and slight modifications to the sequence can render a decrease in potency. Since then, Sullivan and Newcomb (1982) have suggested that the presence and size of the aromatic
**Proctolin Analogues as Antagonists**

group of the [tyrosine$^2$] residue is necessary for full activity and that increasing the size of the aromatic ring by substitution with [tryptophan$^2$] decreases the activity.

A number of proctolin analogues have been synthesized and their effects tested on the oviducts of locusts. Lange et al. (1993) examined the ability of selected analogues, modified in the [Arg$^1$]-position and [Tyr$^2$]-position, to mimic the basal contraction induced by proctolin. All of the analogues tested had thresholds, 50% maxima and maxima at higher concentrations than proctolin indicating that modification at positions 1 or 2 produced an analogue with decreasing potency. Additionally, King et al. (1995) tested analogues modified at position three and found them to be less effective than proctolin at inducing oviduct contractions, even though they all bound the receptor with high affinities. Based on the binding characteristics of proctolin and its known analogues, it is believed that there are two different proctolin receptors on the oviducts (King et al., 1995).

Gray et al. (1994) have also tested the effects of several proctolin analogues on the foregut of the locust *Schistocerca gregaria*. Of these analogues, three were tripeptides, namely Arg-Tyr-Thr, Tyr-Arg-Thr and Leu-Pro-Thr, and showed no agonistic effects, with only Arg-Tyr-Thr being able to antagonize proctolin-induced contractions. However, it has been suggested that this ability of Arg-Tyr-Thr to partially inhibit proctolin's response may be caused by enzyme inhibiting effects similar to those of endogenous Arg-Tyr (Gray et al., 1994). Additionally, proctolin analogues modified at position two were assayed and while [N-methyl-L-tyrosine$^2$]-proctolin showed no significant agonistic or antagonistic effects, [$\alpha$-methyl-L-tyrosine$^2$]-proctolin proved to be a potent antagonist of proctolin responses. Lastly,
a cyclic proctolin molecule was found to be devoid of agonistic activity and was only a weak proctolin antagonist.

The following paper describes effects of the proctolin analogues \( [N\text{-}N\text{-}methyl\text{-}L\text{-}tyrosine^2]\)-proctolin, \( [\alpha\text{-}N\text{-}methyl\text{-}L\text{-}tyrosine^2]\)-proctolin, cycloproctolin, and the tripeptides Arg-Tyr-Thr, Tyr-Arg-Thr and Leu-Pro-Thr on contractions of the oviducts of \textit{Locusta}. While a potent antagonist to the proctolin response would help to clearly identify proctolin's role in muscle contraction in this preparation, such antagonists may also help to distinguish different proctolin receptors throughout insects.

\textbf{MATERIALS AND METHODS}

\textit{Animals}

All experiments were performed on mature adult females of \textit{Locusta migratoria} reared at 30°C under crowded conditions on a 12 h light-12 h dark regime and fed fresh wheat seedlings supplemented with bran.

\textit{Bioassay}

Lateral and common oviducts (with the ovaries attached) were dissected out under physiological saline (composition in mM: NaCl, 150; KCl, 10; CaCl\(_2\), 4; MgCl\(_2\), 2; NaHCO\(_3\), 5; HEPES, 5; pH 7.2; sucrose, 90; trehalose, 5) through a mid-ventral incision. For isotonic recording of isolated oviducts, the anterior end of the preparation was pinned with minuten pins to a sylgard-lined dish while the posterior end of the common oviduct was attached via
Proctolin Analogues as Antagonists

fine thread to an AE875 miniature force transducer (Aksjeselskapet Mikro-Elektronikk, Oslo, Norway). Recordings were made on a flatbed recorder (Baxter, Mississauga, ON). Dose-response curves were obtained by applying 20 µl of saline containing known concentrations of proctolin or proctolin analogues directly to the oviducts, which were only flooded with saline for washing between applications of peptides. The response was quantified by measuring the amplitude of the tonic contraction induced by the peptides (when the contraction reached a plateau) and was expressed as a percentage relative to the maximum contraction induced by proctolin.

To test for antagonistic effects of the proctolin analogues, the oviduct preparation was first pre-incubated with the analogue, at the desired concentration, for 1 minute. A 20 ml aliquot of saline containing the desired concentrations of proctolin and proctolin analogue was next added followed by a wash with saline once the maximum response was observed. Dose-response curves were then obtained as described above. Data are reported as mean±S.E. and statistical significance determined using the Student's t test for unpaired samples, where p<0.05 was considered significant.

Materials

Proctolin (Peninsula Laboratories Incorporated, Belmont, CA) and proctolin analogues (Shell Research Ltd., Sittingbourne, Kent) were prepared as a 10⁻³ M stock solution in distilled water and diluted with saline prior to use.
Proctolin Analogues as Antagonists

RESULTS

Agonistic effects of proctolin analogues

Proctolin caused a dose-dependent contraction of the isolated oviduct of the locust Locusta migratoria at concentrations ranging from $10^{-11}$ M to $5 \times 10^{-7}$ M (Fig. 1). The maximal response to the pentapeptide was induced at a concentration of $3 \times 10^{-8}$ M with an $ED_{50}$ value of $2.58 \pm 0.78 \times 10^{-10}$ M. At doses greater than $10^{-7}$ M the contractions produced were weaker than the maximum, perhaps indicating desensitization.

Both $[N$-methyl-L-tyrosine$^2]$-proctolin and $[\alpha$-methyl-L-tyrosine$^2]$-proctolin induced dose-dependent contractions of the oviducts between $10^{-10}$ M to $10^{-5}$ M (Fig. 1). Addition of $[N$-methyl-L-tyrosine$^2]$-proctolin or $[\alpha$-methyl-L-tyrosine$^2]$-proctolin resulted in an increase in the amplitude and frequency of spontaneous contractions and an increase in basal tonus as indicated by a sustained contraction (Fig. 1A). These effects were similar to those seen when proctolin was added to the locust oviducts. Of these two analogues $[N$-methyl-L-tyrosine$^2]$-proctolin was a more potent analogue having an $ED_{50}$ of $1.07 \pm 0.55 \times 10^{-8}$ M and reaching a maximum contraction which was 85.5% that of proctolin (Fig. 1B). Cycloproctolin did not exhibit any agonistic effects up to a concentration of $10^{-6}$ M (Fig. 1). A contraction equivalent to $12.0 \pm 2.6\%$ of the maximum proctolin contraction was elicited by application of $2 \times 10^{-5}$ M cycloproctolin. Of the three tripeptides assayed, only Leu-Pro-Thr induced contractions of the oviduct muscle, albeit minimally, eliciting only a 14.4% contraction at $2 \times 10^{-5}$ M (Fig. 1B). The other two tripeptides, Arg-Tyr-Thr and Tyr-Arg-Thr showed no agonistic effects at concentrations ranging from $10^{-11}$ M to $10^{-4}$ M (data not shown).
Proctolin Analogues as Antagonists

Antagonistic effects of peptide analogues

Two of the three tripeptides, Arg-Tyr-Thr and Tyr-Arg-Thr, had no antagonistic effects when tested against proctolin-induced contractions of the oviducts. The third tripeptide, Leu-Pro-Thr, antagonized proctolin's response (p<0.05), but did not do so in a dose-dependent manner (Fig. 2A). When proctolin was applied in the presence of 10⁻⁵ M Leu-Pro-Thr, the maximum response to the parent compound was decreased by 26.6%, while the proctolin curve was shifted an order of magnitude to the right (Fig. 2B).

[α-methyl-L-tyrosine²]-proctolin, [N-methyl-L-tyrosine²]-proctolin and cycloproctolin were antagonists of the proctolin-induced contractions of locust oviducts. Application of 10⁻¹⁰ M, 10⁻⁹ M or 10⁻⁸ M [α-methyl-L-tyrosine²]-proctolin to the oviducts in the presence of proctolin resulted in a reduction in the response of the oviducts to proctolin as is seen by the shift in the curve to the right (Fig. 3; Table 1). This antagonism was maximal at 10⁻¹⁰ M [α-methyl-L-tyrosine²]-proctolin with higher doses of the analogue not increasing the ED₅₀ value. While 10⁻¹⁰ M [α-methyl-L-tyrosine²]-proctolin did not have any effect on the proctolin-induced maximum, 10⁻⁹ M and 10⁻⁸ M were capable of reducing the proctolin-induced maximum by 14.7% and 13.2% respectively (p<0.05) (Fig. 3; Table 1).

The antagonistic effects of [N-methyl-L-tyrosine²]-proctolin on proctolin-induced contractions were similar to that seen for [α-methyl-L-tyrosine²]-proctolin. Addition of 10⁻¹⁰ M, 10⁻⁹ M or 5x10⁻⁹ M [N-methyl-L-tyrosine²]-proctolin to the oviducts reduced the proctolin-induced contractions indicating that [N-methyl-L-tyrosine²]-proctolin was also an antagonist of proctolin (p<0.05) (Fig. 4; Table 1). The antagonistic effects of [N-methyl-L-
Proctolin Analogues as Antagonists

tyrosine\(^2\)-proctolin were maximal at 10\(^{-10}\) M and the ED\(_{50}\) was not increased when the concentration of antagonist was increased to 5\(\times\)10\(^{-9}\) M. [\(N\)-methyl-L-tyrosine\(^2\)]-proctolin (5\(\times\)10\(^{-9}\) M) was capable of reducing the proctolin-induced maximum by 19.0\% (p<0.05).

Cycloproctolin proved to be the best antagonist of proctolin (Fig. 5). Concentrations ranging from 5\(\times\)10\(^{-6}\) M to 2\(\times\)10\(^{-5}\) M, shifted the proctolin-induced curve to the right in a dose-dependent manner (Fig. 5; Table 1). This shift led to an increase in the ED\(_{50}\) value from 2.58\(\pm\)0.78 \(\times\) 10\(^{-10}\) M for proctolin to 1.08\(\pm\)0.19 \(\times\) 10\(^{-8}\) M for proctolin-induced contractions in the presence of 2\(\times\)10\(^{-5}\) M cycloproctolin. To examine for additive effects of the antagonists the combined application of 10\(^{-5}\) M cycloproctolin + 10\(^{-9}\) M [\(\alpha\)-methyl-L-tyrosine\(^2\)]-proctolin was tested on proctolin-induced contractions. The ED\(_{50}\) increased from 6.75\(\pm\)1.45 \(\times\) 10\(^{-9}\) M for proctolin in the presence of cycloproctolin (10\(^{-5}\) M) to 1.26\(\pm\)0.26 \(\times\) 10\(^{-8}\) M when both cycloproctolin (10\(^{-5}\) M) and [\(\alpha\)-methyl-L-tyrosine\(^2\)]-proctolin (10\(^{-9}\) M) were present (Fig. 6).

Since proctolin is thought to be a cotransmitter at the neuromuscular junctions of the locust oviduct, it was important for future experiments to ensure that the analogue did not antagonize a glutamate-induced contraction. Therefore, 10\(^{4}\) M glutamate was applied to the oviducts in the presence of 10\(^{-6}\) M cycloproctolin (Fig. 7). No change in response to the transmitter was observed, suggesting that cycloproctolin is specific to the proctolin receptor.
Proctolin Analogues as Antagonists

Figure 1. A. Typical chart recordings of locust oviduct contractions. Application of $10^{-8}$ M proctolin (applied at upward triangle) resulted in a sustained contraction of the muscle. The addition of $10^{-8}$ M [N-methyl-L-tyrosine$^2$]-proctolin (N-Meth) or $10^{-8}$ M [$\alpha$-methyl-L-tyrosine$^2$]-proctolin (Alpha-Meth) produced a sustained contraction of the locust oviduct which was smaller than that seen with proctolin. Cycloproctolin (Cyclo) and Leu-Pro-Thr (LPT) showed no agonistic effects at $10^{-8}$ M. Downward triangles indicate times when the preparations were washed with saline.

B. Dose-response curves for proctolin- (□), [N-methyl-L-tyrosine$^2$]-proctolin- (▲), [$\alpha$-methyl-L-tyrosine$^2$]-proctolin-(▼), cycloproctolin- (○) and Leu-Pro-Thr- (♦) induced contractions of the locust oviducts. Cycloproctolin and Leu-Pro-Thr showed agonistic effects at concentrations above $10^{-6}$ M and $10^{-7}$ M respectively. Each symbol is a mean ± S.E. of 4-11 replicates.
Proctolin Analogues as Antagonists

A

10^{-8} M Proctolin
10^{-8} M N-Meth
10^{-8} M Alpha-Meth
10^{-8} M Cyclo

B

% of Max. Proctolin Contraction

Agonist [M]
Proctolin Analogues as Antagonists

Figure 2.A. Sample trace recordings of proctolin-induced contractions in the presence of Leu-Pro-Thr (LPT). Application of the analogue (applied at solid black bar) caused a slight contraction of the oviduct muscle but did not antagonize the proctolin-induced contractions dose-dependently. Proctolin (5x10⁻⁹ M) was applied at the upward triangles and the preparations were washed with saline at times indicated by the downward triangles.

B. Effects of 10⁻⁵ M Leu-Pro-Thr on proctolin-induced contractions. In the presence of the analogue (●) the proctolin curve (□) was shifted to the right one order of magnitude and the maximum response to the parent peptide was decreased by 26.6% (n=4-11).
Proctolin Analogues as Antagonists

A

\[10^{-7} \text{ M LPT} \quad 10^{-6} \text{ M LPT} \quad 10^{-5} \text{ M LPT}\]

B

% of Max. Proctolin Contraction

\[10^{-11} \quad 10^{-10} \quad 10^{-9} \quad 10^{-8} \quad 10^{-7} \quad 10^{-6}\]

Proctolin Concentration [M]
Figure 3. The effect of varying doses of [α-methyl-L-tyrosine\(^2\)]-proctolin on proctolin-induced contractions of locust oviduct. The analogue, at concentrations of \(10^{-10}\) M (■), \(10^{-9}\) M (▲) and \(10^{-8}\) M (○) partially antagonized proctolin's effects. A dose-response curve of proctolin alone (□) is shown for comparison. Data are expressed as the mean ± S.E. (n = 4-11).
Proctolin Analogues as Antagonists

% of Max. Proctolin Contraction vs Proctolin Concentration [M]

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Table 1. Antagonistic effects of proctolin analogues on the oviducts of Locusta.
### Proctolin Analogues as Antagonists

#### Bioassay

<table>
<thead>
<tr>
<th>Analogue</th>
<th>ED$_{50}$ [M] *</th>
<th>Maximum Increase * #</th>
</tr>
</thead>
<tbody>
<tr>
<td>[N-methyl-L-tyrosine$^2$]-proctolin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^{-10}$ M</td>
<td>$3.47\pm0.98 \times 10^{-9}$</td>
<td>$94.2\pm7.1%$</td>
</tr>
<tr>
<td>$10^{-9}$ M</td>
<td>$3.70\pm0.64 \times 10^{-9}$</td>
<td>$90.6\pm4.2%$</td>
</tr>
<tr>
<td>$5 \times 10^{-9}$ M</td>
<td>$1.28\pm0.27 \times 10^{-9}$</td>
<td>$81.0\pm3.0%$</td>
</tr>
<tr>
<td>$[\alpha$-methyl-L-tyrosine$^2$]-proctolin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^{-10}$ M</td>
<td>$3.75\pm0.86 \times 10^{-9}$</td>
<td>$96.3\pm7.1%$</td>
</tr>
<tr>
<td>$10^{-9}$ M</td>
<td>$2.19\pm1.97 \times 10^{-9}$</td>
<td>$85.2\pm1.6%$</td>
</tr>
<tr>
<td>$10^{-8}$ M</td>
<td>$3.39\pm1.70 \times 10^{-9}$</td>
<td>$86.7\pm3.8%$</td>
</tr>
<tr>
<td>Leu-Pro-Thr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^{-5}$ M</td>
<td>$2.50\pm0.67 \times 10^{-9}$</td>
<td>$73.4\pm3.6%$</td>
</tr>
<tr>
<td>Cycloproctolin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$5 \times 10^{-6}$ M</td>
<td>$1.68\pm0.45 \times 10^{-9}$</td>
<td>$79.6\pm4.3%$</td>
</tr>
<tr>
<td>$10^{-5}$ M</td>
<td>$6.75\pm1.45 \times 10^{-9}$</td>
<td>$84.3\pm1.8%$</td>
</tr>
<tr>
<td>$2 \times 10^{-5}$ M</td>
<td>$1.08\pm0.19 \times 10^{-8}$</td>
<td>$80.7\pm5.1%$</td>
</tr>
<tr>
<td>Cycloproctolin + [N-methyl-L-tyrosine$^2$]-proctolin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^{-5}$ M + $10^{-9}$ M</td>
<td>$1.26\pm0.26 \times 10^{-8}$</td>
<td>$78.3\pm5.2%$</td>
</tr>
</tbody>
</table>

* The concentration of peptide needed to produce a basal contraction which is 50% of the maximum contraction induced by the same peptide.

# Maximum increase in basal contraction induced by proctolin in the presence of the potential antagonist as compared to the maximum increase in contraction induced by proctolin alone.
Figure 4. The effects of varying doses of \([N\text{-methyl-L-tyrosine}^2]\)-proctolin at concentrations of \(10^{-10}\) M (△), \(10^{-9}\) M (■) and \(5 \times 10^{-9}\) M (▼) on proctolin-induced contractions of locust oviduct. All concentrations of \([N\text{-methyl-L-tyrosine}^2]\)-proctolin antagonized proctolin's effect (□), but not in a dose-dependent manner. Each point is a mean ± S.E. 4-11 replicates.
Proctolin Analogues as Antagonists

Proctolin Concentration [M]

% of Max. Proctolin Contraction

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Figure 5. A. Typical chart recordings of proctolin-induced contractions of locust oviduct in the presence of varying concentrations of cycloproctolin. Application of cycloproctolin (Cyclo), as indicated by the solid black bars, caused a slight contraction of the oviduct muscle but antagonized the proctolin-induced contraction in a dose-dependent manner. Upward triangles indicate the addition of 5x10^{-9} M proctolin and open downward triangles indicate washing with saline.

B. The effects of 5x10^{-6} M (▲), 10^{-3} M (⊙) and 2x10^{-5} M (▼) cycloproctolin on proctolin-induced contractions. A dose-response curve of proctolin alone (□) is shown for comparison. As can be seen, increasing concentrations of cycloproctolin shift the curve to the right. Each symbol is a mean ± S.E. of 4-11 replicates.
Proctolin Analogues as Antagonists

A

B

% of Max. Proctolin Contraction

110
100
90
80
70
60
50
40
30
20
10
0

10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Proctolin Concentration [M]

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Proctolin Analogues as Antagonists

Figure 6. Effects of simultaneous application of $10^{-9}$ M $[\alpha$-methyl-L-tyrosine$^2$]-proctolin and $10^{-5}$ M cycloproctolin (●). Simultaneous application of both analogues to the oviducts caused a shift in the curve to the right as compared to the effects of $10^{-9}$ M $[\alpha$-methyl-L-tyrosine$^2$]-proctolin (▼) or $10^{-5}$ M cycloproctolin (△). Dose-response curves showing the effects of proctolin in the presence of $10^{-9}$ M $[\alpha$-methyl-L-tyrosine$^2$]-proctolin and $10^{-5}$ M cycloproctolin are re-drawn from figures 3 and 5, respectively, for comparison. When applied together, these analogues decreased the maximum response to proctolin (□) by 21.62% and increased the ED$_{50}$ value from $2.58 \pm 0.78 \times 10^{-10}$ M (for proctolin) to $1.08 \pm 0.19 \times 10^{-8}$ M. Data are expressed as a mean ± S.E. of 4-11 replicates.
Proctolin Analogues as Antagonists

% of Max. Proctolin Contraction

Proctolin Concentration [M]
Proctolin Analogues as Antagonists

Figure 7. Typical chart recording of the glutamate-induced contraction of the locust oviduct in the presence and absence of cycloproctolin (Cyclo). Application of $10^6$ M cycloproctolin (solid black bar) did not antagonize the effect of glutamate (applied at upward triangle) ($n=4$). The preparations were washed with saline at times indicated by the downward triangles.
Proctolin Analogues as Antagonists

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Proctolin Analogues as Antagonists

DISCUSSION

Two of the three tripeptides used in this study, Arg-Tyr-Thr and Tyr-Arg-Thr, were incapable of eliciting contractions of the oviduct muscle, while the third, Leu-Pro-Thr, exhibited agonistic effects at high concentrations. This lends further support to the work of Starratt and Brown (1979) who have suggested that the full pentapeptide structure is necessary for activity of proctolin. Gray et al. (1994) have also tested these tripeptides on the foregut of the locust Schistocerca gregaria and found them to be devoid of agonistic action. While Leu-Pro-Thr was the only tripeptide tested which was able to antagonize proctolin-induced contractions in the oviducts of Locusta, Arg-Tyr-Thr was the only tripeptide antagonist of proctolin on the foregut of Schistocerca (Gray et al., 1994). The ability of Leu-Pro-Thr to inhibit a proctolin-induced contraction is of interest since membrane peptidases from nervous tissue (Isaac, 1987) and from the ovaries (Puiroux and Lough ton, 1992) have been shown to degrade proctolin by the cleavage of the Arg-Tyr bond and the Tyr-Leu bond leaving the tripeptide Leu-Pro-Thr. This degradation product of proctolin, the tripeptide Leu-Pro-Thr, would appear to act as an antagonist of proctolin, thereby limiting the effectiveness or duration of action of the released proctolin.

Although [α-methyl-L-tyrosine$^2$]-proctolin and [N-methyl-L-tyrosine$^2$]-proctolin were not agonists of proctolin in Schistocerca foregut (Gray et al., 1994), they were capable of inducing contractions of the oviducts of Locusta. [α-methyl-L-tyrosine$^2$]-proctolin and [N-methyl-L-tyrosine$^2$]-proctolin elicited maximum contractions 67.0% and 85.5% of that induced by proctolin. Neither of these tyrosine$^2$-substituted analogues were particularly
Proctolin Analogues as Antagonists

effective antagonists of proctolin at the doses tested. Higher doses could not be tested due to the agonistic actions of these analogues on the oviduct muscle.

Cycloproctolin was incapable of inducing contractions of the oviduct muscle at concentrations up to $10^{-6}$ M. Similarly, Gray et al. (1994) found this same analogue to be devoid of agonist activity in the foregut of Schistocerca and have suggested that this may be due to the increased rigidity of the analogue's molecular structure when compared to that of the parent molecule. This rigidity was introduced into the proctolin molecule by linking the N- and C-terminus groups, resulting in the formation of an amide bond and hence a different secondary structure (Odell et al., 1996). Cycloproctolin proved to be the most effective antagonist of proctolin on Locusta oviducts, with the antagonistic properties being dose-dependent. Simultaneous application of cycloproctolin and $[\alpha$-methyl-L-tyrosine$^2$]-proctolin resulted in a further antagonism of the response to applied proctolin. This suggests that the tissue contains two proctolin receptors, one of which is insensitive to $[\alpha$-methyl-L-tyrosine$^2$]-proctolin, since this analogue produced a maximal antagonism at $10^{-10}$ M and addition of cycloproctolin increased the ED$_{50}$ value significantly ($p<0.05$).

The differences between the results of the present study and work done by Gray et al. (1994) lend support to the hypothesis that there is more than one proctolin receptor subtype (Bartosz-Bechowski et al., 1990; Konopinska et al., 1986; Konopinska et al., 1988a; Konopinska et al., 1988b; Konopinska et al., 1990). For example, Konopinska et al. (1988b) found the proctolin analogue, Lys-Tyr-Leu-Pro-Thr, to be a more potent agonist on the cockroach heartbeat than the parent compound and much less potent on the mealworm.
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heartbeat. Furthermore, Lange et al. (1993) have examined the effects of a series of proctolin analogues on locust oviducts and developed rank orders, based upon doses required for half-maxima and maximal contraction. These rank orders were different from those reported using other preparations (Konopinska et al., 1986; Konopinska et al., 1988b).

Recently, Hinton and Osborne (1995, 1996) have used \([\alpha\text{-methyl-L-tyrosine}^2]\text{-proctolin and cycloproctolin to investigate the nature of second messenger systems activated following combination of proctolin with its receptors. Thus they demonstrated that the foregut of Schistocerca gregaria contains a proctolin receptor which is linked to a lithium-sensitive signal transduction pathway and, when activated, increases intracellular inositol triphosphate levels. This increase is reduced in the presence of \([\alpha\text{-methyl-L-tyrosine}^2]\text{-proctolin and cycloproctolin. Hinton and Osborne (1996) have also identified a second proctolin receptor in the foregut of Schistocerca gregaria which is insensitive to cycloproctolin. These observations suggest that cycloproctolin and lithium will be useful tools for the future classification of proctolin receptor subtypes. Such studies are currently being undertaken on oviducts of Locusta migratoria, which have previously been shown to use the phosphoinositol pathway for some of the actions of proctolin (Lange, 1988).}

ACKNOWLEDGEMENTS

This work was supported by grants to Angela B. Lange from the Natural Sciences and Engineering Research Council of Canada.
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REFERENCES


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III. PROCTOLIN'S ROLE IN NEURALLY-EVOKED CONTRACTIONS OF THE LOCUST OVIDUCTS.

ABSTRACT

The effects of proctolin (RYLPT) on neurally-evoked contractions of locust oviduct muscle were studied in order to examine its role as a cotransmitter. Increasing the number of stimuli in a burst (from 1-30 stimuli) resulted in an increase in amplitude of contraction of locust oviduct muscle. Proctolin increased the amplitude of neurally-evoked contractions at lower stimulus regimes (1 and 2 stimuli bursts) but did not do so at higher stimulus regimes (5 and 10 stimuli bursts). The effects of proctolin were dose-dependent within the 1 and 2 stimulus regimes, with thresholds at $10^{-9}$ M and maxima at $2.5 \times 10^{-4}$ M. Addition of proctolin increased the basal tonus and size of a post-contraction relaxation of the oviduct muscle in a dose-dependent manner during all stimulus regimes. However, the effect of proctolin on basal tonus and the post-contraction relaxation was less at the higher stimulus regimes. Previously, several proctolin analogues had been tested for their ability to antagonize proctolin-induced contractions of the oviduct muscle. Since proctolin is proposed to be a cotransmitter at this neuromuscular junction, one of these analogues, cycloproctolin, was used to antagonize proctolin's effects on neurally-evoked contractions. In the presence of the antagonist, the maximum amplitude induced by application of proctolin decreased by 22.7% while the proctolin-induced increase in basal tonus decreased by 45.8%. Finally, the maximum increase in size of the post-contraction relaxation caused by proctolin was lowered by 32.0%. The results of the present study show that exogenously applied proctolin is an excitant of the
ovoiduct muscle at lower, rather than higher, stimulus regimes, and this latter inaction may be due to the release of endogenous proctolin during increased neural stimulation.

**INTRODUCTION**

Although insect visceral muscles are striated, they display properties similar to smooth muscles of vertebrates in that they contract in slow and rhythmic waves. Insect oviduct muscles exhibit myogenic contractions which are often co-ordinated to form peristaltic waves and are presumed to be used to control the movement of mature eggs (Okelo, 1971; Thomas, 1979) and/or sperm (Davey, 1958) within the oviducts. Recently, it has also been postulated that the slow rhythmic contractions of the locust oviduct muscles aid in the circulation of haemolymph around the body (Orchard and Lange, 1995).

To date most of the studies investigating contraction of the oviduct muscle have centred around the neural control of the muscle rather than the hormonal control. The oviducts of *Locusta migratoria*, receive polyneural innervation from a relatively small number of neurons located in the VII abdominal ganglion (Lange and Orchard, 1984) and two of these neurons, the dorsal unpaired median neurons, have been found to contain octopamine (Orchard and Lange, 1985). Octopamine is a naturally occurring biogenic amine that reduces the amplitude of neurally-evoked contractions in addition to reducing the basal tonus and inhibiting myogenic activity of locust oviducts (Orchard and Lange, 1985; Lange and Orchard, 1986; Lange and Tsang, 1993). Additionally, three other pairs of motoneurons that project to the oviducts may be proctolin-containing neurons (Lange *et al.*, 1986). Proctolin is a
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pentapeptide (RYLPT) originally isolated from the proctodeum of the cockroach *Periplaneta americana* (Brown and Starratt, 1975) and, in insects, proctolin has been shown to stimulate muscle contraction (Groome *et al.*, 1991; Hertel *et al.*, 1985; Holman and Cook, 1985; Lange *et al.*, 1986; Washio and Koga, 1990) by acting as both a neuromodulator and possibly as a neurohormone (Orchard *et al.*, 1989).

When applied directly to the oviducts of *Locusta*, proctolin produces a dose-dependent tonic contraction, an increase in the amplitude of neurally-evoked contractions and an increase in the amplitude and frequency of myogenic contractions (Lange, 1993; Lange *et al.*, 1986). While the mode of action of proctolin still needs clarification, the peptide's actions have been shown to require extracellular calcium (Lange *et al.*, 1987; Wilcox and Lange, 1995) and there is evidence of proctolin-induced inositol phospholipid formation (Lange, 1988) suggesting the presence of IP$_3$-sensitive calcium stores. However, because proctolin appears to have little effect on the ionic permeability of the post-synaptic membrane, as judged by changes in membrane potential, the peptide does not appear to act as a conventional transmitter but rather as a cotransmitter in this system, along with a more conventional transmitter such as glutamate which produces the excitatory junction potential (Orchard and Lange, 1986).

Individual neurons can typically contain various combinations of cotransmitters, with some of these being neuropeptides (Bartfai *et al.*, 1986; Kupfermann, 1991). The neuropeptide proctolin has been implicated in cotransmission at a number of nerve terminals (Bishop *et al.*, 1991; Orchard and Lange, 1986; Adams and O'Shea, 1983). For example,
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proctolin is released along with L-glutamate in response to neural stimulation of the motorneurons at the tonic flexor neuromuscular junction in the crayfish (Bishop et al., 1987). It appears that proctolin is an important contributor to the magnitude of tension generated in this muscle. Additionally, proctolin has been shown to have several effects upon neuromuscular transmission at the ventral opener muscle synapse of the locust, Locusta migratoria (Orchard et al., 1989) and is thought to act as a cotransmitter at the neuromuscular junction (Belanger and Orchard, 1988). In this preparation, proctolin dose-dependently increases the amplitude of excitatory junction potentials and neurally-evoked tension, and at low concentrations increases the frequency of miniature postsynaptic potentials without causing any significant change in the muscle fibre resting potential or the basal tension.

Antagonists to proctolin would be useful for providing tools for deciphering the precise roles of proctolin during cotransmission. With this in mind, several proctolin analogues have been synthesized and tested for potential antagonism to proctolin's response (Lange et al., 1993; Gray et al., 1994; King et al., 1995; Noronha et al., 1996). Gray et al. (1994) found [α-Methyl-L-tyrosine²]-proctolin as well as a cyclic proctolin molecule, cycloproctolin, to be peptide antagonists to the putative neurotransmitter proctolin in the locust, Schistocerca gregaria. More recently Noronha et al. (1996) have shown cycloproctolin to be a specific and effective antagonist to proctolin in Locusta.

This paper describes the effects of proctolin on neurally-evoked contractions of the locust oviduct and outlines the effects of cycloproctolin as a peptide antagonist used to further
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discriminate proctolin's actions in cotransmission at this neuromuscular junction.

MATERIALS AND METHODS

Animals

Mature adult females of *Locusta migratoria* reared at 30°C under crowded conditions on a 12 h light-12 h dark regime were used for this study. The locusts were fed fresh wheat seedlings supplemented with bran.

Preparations

Lower lateral and common oviducts (without the ovaries attached), along with their oviducal nerves, were dissected out under physiological saline (composition in mM: NaCl, 150; KCl, 10; CaCl₂, 4; MgCl₂, 2; NaHCO₃, 5; HEPES, 5; pH 7.2; sucrose, 90; trehalose, 5) through a mid-ventral incision. Lateral branches of the oviducts were pinned down to a Sylgard lined dish with minuten pins and the posterior end of the common oviducts attached to a Grass FT.03 force transducer (Grass Medical Instruments, Quincy MA) via a fine thread.

Tension Recordings

Oviducal nerves were electrically stimulated via a suction electrode using pulses delivered through a Grass SIU5 stimulus isolation unit (Grass Medical Instruments, Quincy MA) driven by a Grass S88 stimulator (Grass Medical Instruments, Quincy MA). The stimulus regimes used for these experiments are schematically depicted in Fig. 1. A 0.5 msec. square pulse was
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delivered at a 30 Hz stimulus frequency and the duration of the burst was increased to allow for 1, 2, 5, 10, 20 and 30 stimuli to be delivered. Therefore, the size of burst increased as follows: 1 stimulus at 35 ms; 2 stimuli at 70 ms; 5 stimuli at 170 ms; 10 stimuli at 350 ms; 20 stimuli at 700 ms; 30 stimuli at 1100 ms. The interburst interval was also increased in order to maintain a constant number of stimuli over time. The data was analysed after 40 stimuli had been delivered, a time at which the effects of proctolin on the measured parameters would have reached maximum (See Appendix I, Figs. 2 and 3).

To test for the effects of proctolin the preparation was kept in a bath containing 400 µl of saline and the peptide was applied by replacing 200 µl of saline with 200 µl of proctolin (in saline) at twice the desired concentration. Once the maximum response to proctolin was observed the preparation was washed several times with saline and subsequently the next dose of proctolin was applied. In this manner dose-response curves were constructed to observe changes in response of the oviduct muscle to the peptide while different stimulus regimes were delivered.

To test for antagonistic effects of cycloproctolin, the preparations were pre-incubated for 1 minute in the antagonist prior to each addition of proctolin.

Data analysis

Data analysis was carried out using the Enhanced Graphics Acquisition and Analysis (EGAA) software (RC-Electronics Inc., Goleta, CA). To quantify the effects of proctolin and
**Figure 1.** Diagrammatic representation of the stimulus regimes used to electrically stimulate the oviducal nerves of the locust, *Locust migratoria*. The nerves were stimulated via a suction electrode using 0.5 msec pulses delivered through a Grass SIU5 stimulus isolation unit driven by a Grass S88 stimulator. A stimulus frequency of 30 Hz was delivered and the duration of the burst was increased to allow for 1, 2, 5, 10, 20 or 30 stimuli to be delivered. During each train of pulses, the stimuli were delivered 34 ms apart. Isotonic mechanical events were detected from the oviducts using fine thread attached to a Grass FT .03 force transducer.
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cycloproctolin, four parameters were measured: basal tonus, amplitude, post-contraction relaxation and relaxation rate of the contractions. The basal tonus response was measured by normalizing the changes in basal tonus produced by proctolin to the maximal increase induced by the peptide. In experiments where cycloproctolin was used, the neurally-evoked contractions were analyzed ignoring the effects of myogenic contractions which were induced by the proctolin antagonist. The post-contraction relaxation was measured as a percentage of the maximum increase induced by proctolin. The change in amplitude caused by proctolin was quantified as a percentage relative to the average amplitude in saline prior to proctolin addition and lastly the maximum rate of relaxation was calculated for all averaged contractions. All measurements were taken from an average of 5 to 15 contractions.

Experiments were conducted at room temperature (approximately 22°C) and, where appropriate, data are reported as mean±S.E. Statistical significance was determined using the Student's t test for unpaired samples, where p<0.05 was considered significant.

Materials

Proctolin (Peninsula Laboratories Incorporated, Belmont, CA) and proctolin analogues (kindly given to us by Dr. Richard H. Osborne, University of the West of England, Frenchay Campus) were prepared as a 10⁻³ M stock solution in distilled water and diluted with saline prior to use.
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RESULTS

During increasing stimulus regimes the amplitude of neurally-evoked contractions of the oviduct muscle increased significantly (p<0.005; Fig. 2A, B). A 1 stimulus regime produced an average contraction that was 14.7±2.6% of the maximum contraction produced by a 30 stimuli regime. The subsequent increase in the number of stimuli delivered to the oviducal nerves caused a concomitant increase in the amplitude of contraction of the oviduct muscle (Fig 2B). When the area under the contraction curves were plotted against the log number of stimuli, the response was far from linear. A dramatic shift in slope of the curve was evident at stimuli above 5 and again at 10 (Fig. 2C), with the area being disproportionately higher than expected for a linear relationship. As neural stimulation was increased it was also observed that the myogenic activity of the oviduct muscle decreased and was absent between the 10, 20 and 30 stimuli bursts.

The effects of proctolin were also tested on the oviducts during various stimulus regimes. During the 1, 2, 5 and 10 stimuli regimes, proctolin dose-dependently increased the basal tonus of the muscle as measured as the percent of the maximum increase induced by the peptide (Fig. 3A). However, at the higher stimulus regimes (ie. both 5 and 10 stimuli bursts) the overall increase in the tonus of the muscle produced by proctolin was less pronounced when compared to that observed at the lower (ie. 1 and 2) stimulus regimes (Fig. 3B). When compared to the basal tonus change at a 2 stimuli regime, the effect of adding 5x10⁻¹⁰ M proctolin during 5 and 10 stimuli regimes produced changes in basal tonus that were 49.5±5.5% and 80.5±5.4% lower, respectively. This reduction in the effect of the peptide,
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when added at higher neural stimulation, was found to occur at all concentrations of proctolin (Fig. 3B).

Additionally, the changes in amplitude induced by the addition of proctolin to neurally-evoked contractions during different stimulus regimes were measured. Proctolin produced a dose-dependent increase in the amplitude of neurally-evoked contractions as well as a pronounced post-contraction relaxation (Fig. 4). The proctolin-induced increase in amplitude occurred during both the 1 and 2 stimuli bursts, with thresholds of $10^{-9}$ M (Fig. 4B). At these lower stimulus regimes, the maximum response to the peptide was observed at $2.5 \times 10^{-8}$ M with $5 \times 10^{-4}$ M proctolin being less effective, perhaps indicating that desensitization was occurring. While there is no statistical difference between the maxima elicited by $2.5 \times 10^{-8}$ M proctolin (during 1 and 2 stimuli bursts) the trend indicates a decreased potency of proctolin when the 2 stimuli regime was delivered. This decreased potency of proctolin is more evident during the 5 and 10 stimulus regimes where no change in amplitude was induced by the peptide. This suggests that the amplitude of the neurally-evoked contraction was already enhanced by the release of endogenous proctolin at the higher frequencies.

As mentioned above, addition of proctolin produced a dose-dependent increase in the size of the post-contraction relaxation (Figs. 4A and 5A). Of note is that the dose-response curves showing the effects of proctolin on the post-contraction relaxation were shifted almost one order of magnitude to the right when 5 or 10 stimuli were applied. The maximum increase induced by the peptide was observed at $5 \times 10^{-9}$ M (during 1 and 2 stimuli regimes) and $10^{-8}$ M (during 5 and 10 stimulus regimes). Although the size of the post-contraction relaxation was
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enhanced dose-dependently, the effects of the peptide were significantly greater at the lower stimulus regimes (p<0.05; Fig. 5B). Addition of 5x10^{-10} M proctolin during a 5 stimuli regime produced a post-contraction relaxation that was 44.7±4.2% lower than that produced after adding the same concentration of the peptide during the 2 stimuli regime. Moreover, the size of the post-contraction relaxation at 10 stimuli was 77.3±1.8% lower when compared to that of 2 stimuli. The increase in the size of the post-contraction relaxation elicited by 5x10^{-9} M and 5x10^{-8} M proctolin was also smaller during the 5 and 10 stimuli regimes.

Also observed was a progressive decrease in the rate of relaxation as the number of stimuli delivered was increased (Fig. 6). At a one stimulus regime there was a 202.7±22.5% increase in the rate of relaxation as compared to the control where no peptide was added. During the 2 and 5 stimuli regimes the maximum increase in the rate of relaxation was 187.8±24.9% and 165.1±10.2% over the control respectively. Interestingly, no change in the rate of relaxation was observed during delivery of the 10 stimuli bursts.

To help distinguish proctolin's role in contraction of the oviduct muscle, the proctolin antagonist cycloproctolin was used. During the 1 stimulus regime, cycloproctolin (10^{-5} M) lowered the maximal proctolin-induced increase in basal tonus by 45.8% (p<0.05; Fig. 7). The maximum increase in amplitude caused by proctolin was decreased by 22.7% when cycloproctolin was present (Fig. 8). Lastly, the ability of the peptide to elicit the post-contraction relaxation was also significantly reduced (p<0.05; Fig. 9). During the 1 stimulus burst, proctolin's maximum effect was observed at 5x10^{-9} M but in the presence of 10^{-5} M cycloproctolin this maximum was elicited at 10^{-8} M and was 32.0% lower.
Figure 2.A. Sample trace recordings of neurally-evoked contractions during varied stimulus regimes. The amplitude of contractions increased as the number of stimuli increased.

B. The effects of increasing the number of stimuli on the amplitude of neurally-evoked contractions of locust oviduct. Each data point is the mean±S.E. of 6-11 preparations.

C. The effects of increasing the number of stimuli on the area under the curve of neurally-evoked contractions of locust oviduct. Each data point is the mean±S.E. of 6-11 preparations.
A

B

C

Neurally-Evoked Contractions of Oviduct
Figure 3.A. The effects of varying concentrations of proctolin on the basal tonus of neurally-evoked contractions of the oviduct muscle. Regardless of the different stimulus regimes used there was a dose-dependent increase in basal tonus of the muscle in response to the application of proctolin. The data were analysed as a percent of the maximum increase in basal tonus induced by the peptide. Each symbol is the mean±S.E. of 4 preparations.

B. Effects of proctolin on basal tonus were analysed as a percent of the change induced when a 2 stimuli regime were delivered. While proctolin was capable of increasing the basal tonus of the muscle during all the stimulus regimes used, the effects of the peptide were less pronounced at the higher stimulus regimes. Inset: Sample trace recordings showing the effects of adding 5x10^{-9} M proctolin (bar) during the delivery of the 2, 5 or 10 stimuli regimes. Dotted line indicates basal tonus.
Neurally-Evoked Contractions of Oviduct

A

- Plot showing the relationship between Proctolin Concentration (M) and Basal Tone as a percentage of Max. Proctolin Increase. Different symbols represent various numbers of stimuli: 1, 2, 5, and 10 stimuli.

B

- Bar graph showing Basal Tonus Relative to Basal Tonus at 2 Stimuli. Different bars represent different Proctolin concentrations: 5x10^{-10} M, 6x10^{-8} M, and 5x10^{-8} M. Number of Stimuli tested are 2, 5, and 10.
**Neurally-Evoked Contractions of Oviduct**

**Figure 4.A.** An example of neural stimulation (5 stimuli regime) of the locust oviduct after the addition of $10^{-8}$ M proctolin. At this concentration no increase in the amplitude of neurally-evoked contractions was seen, however, a pronounced post-contraction relaxation was evident.

**B.** Dose-response curves showing the effect of proctolin on the amplitude of neurally-evoked contractions of the oviducts. Proctolin produced a dose-dependent increase in amplitude of neurally-evoked contractions during the 1 and 2 stimuli regimes respectively but this increase was not observed at the higher stimulus regimes (5 and 10 stimuli). Each point is the mean±S.E. of 4-5 preparations.
Neurally-Evoked Contractions of Oviduct

A

Amplitude

Post-Contraction Relaxation

0.35 sec

50 mg

B

Neurally-Evoked Amplitude: Percent of Control

Proctolin Concentration [M]

-67-
Neurally-Evoked Contractions of Oviduct

Figure 5.A. The effects of varying concentrations of proctolin and 4 different stimulus regimes on the size of the post-contraction relaxation. The post-contraction relaxation was measured as a percentage of the maximum increase induced by proctolin. The post-contraction relaxation increased with increasing concentrations of the peptide and the curve was shifted to the right with increasing stimulus frequencies (5 and 10 stimuli regimes). Each symbol is the mean±S.E. of 4 preparations.

B. Proctolin's effects on the post-contraction relaxation as analysed as a percent of the change induced when the 2 stimuli regime was delivered. Similar to proctolin's effect on basal tonus, during higher stimulus regimes (5 and 10 stimuli), the effect of the peptide on the size of the post-contraction relaxation was lower. See the inset of Fig. 4B for sample trace recordings.
Neurally-Evoked Contractions of Oviduct

A

Post-Contraction Relaxation:
Percent of Max. Proctolin Increase

Proctolin Concentration [M]

B

Post-Contraction Relaxation:
Percent Relative to 2 Stimuli

Number of Stimuli

-69-
Figure 6. Proctolin's effects on the relaxation rate of neurally-evoked contractions. At a threshold between $5 \times 10^{-10}$ M and $10^{-9}$ M, proctolin significantly increased the rate of relaxation during the 1 or 2 stimuli regimes ($p<0.05$). This effect was decreased during the delivery of the 5 stimuli regime and was completely absent at the 10 stimulus regime. Data are represented as the mean±S.E. of 4 preparations.
Figure 7. Inhibition of the proctolin-induced increase in basal tonus by cycloproctolin during delivery of the single stimulus regime. In the presence of $10^{-5}$ M cycloproctolin, the increase in basal tonus caused by the application of proctolin was lowered. Data are represented as the mean±S.E. of 4 preparations.
Neurally-Evoked Contractions of Oviduct

![Graph showing the effect of Proctolin and Proctolin + 10^{-5} M Cyclo on Basal Tonus as a percent of Max. Proctolin Increase vs. Proctolin Concentration [M].](image-url)
Figure 8. Dose-response curves showing proctolin's effect on amplitude of neurally-evoked contractions in the presence or absence of $10^{-5}$ M cycloproctolin during the 1 stimulus regime. In the presence of the antagonist, the maximal increase in the amplitude of contractions induced by proctolin was lowered by 22.7%. Each data point represents the mean ± S.E. of 4 preparations.
Neurally-Evoked Contractions of Oviduct

Proctolin Concentration [M]

Neurally-Evoked Amplitude Percent of Control

-●- Proctolin
-▲- Proctolin + 10^{-5} M Cyclo
Figure 9. Dose-response curves showing the inhibition of the proctolin-induced post-contraction relaxation by $10^{-5}$ M cycloproctolin. In the presence of the antagonist the proctolin-induced increase in the size of the post-contraction relaxation was inhibited by 32.0%. Data are represented as the mean±S.E. of 4 preparations.
Neurally-Evoked Contractions of Oviduct

- [Proctolin]
- [Proctolin + 10^{-5} M Cyclo]

- Percent of Max. Proctolin Increase

- Proctolin Concentration [M]

-77-
**Neurally-Evoked Contractions of Oviduct**

**DISCUSSION**

We have shown that proctolin increases the amplitude of neurally-evoked contractions of the oviduct muscle in the locust, *Locusta migratoria*. In addition, the presence of proctolin increased the basal tonus of the muscle, as well as the size of the post-contraction relaxation in a dose-dependent manner. However, at the higher stimulus regimes (5 and 10 stimuli bursts) the absolute increase in both the basal tonus and post-contraction relaxation was much lower when compared to those produced during the lower stimulus regimes (1 and 2 stimuli bursts) and at the higher stimulus regimes proctolin did not enhance the amplitude of neurally-evoked contractions. While the oviducts are innervated by proctolin containing neurons, they also receive innervation from octopamine containing DUM neurons (Orchard and Lange, 1985) and octopamine has been shown to be a muscle relaxant in this preparation (Orchard and Lange, 1986; Lange and Tsang, 1993). It is plausible that exposure to a high number of stimuli causes the release of octopamine from the DUM neurons, lowering the basal tonus of the muscle as well as decreasing the amplitude of neurally-evoked contractions and hence making proctolin’s effects appear to be lower. To test this, preparations were stimulated at 1, 2, 5 and 10 stimuli regimes and proctolin was applied in the presence of $10^{-5}$ M phentolamine, which has been shown to block the effects of octopamine (Lange and Orchard, 1986). Proctolin’s effects on basal tonus, post-contraction relaxation and amplitude of neurally-evoked contractions were the same regardless of the presence of phentolamine (data not shown) suggesting that the lowered effects of the pentapeptide during increased stimulation was not due to the release of the biogenic amine octopamine. Alternatively then,
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this decreased response to the peptide may be due to the release of proctolin from the oviducal nerves during the higher stimulus regimes (5 and 10 stimuli bursts) (Orchard and Lange, 1987) which subsequently masks the effects of adding proctolin.

Two additional aspects of the present results lend further support to this hypothesis. Firstly, plotting the residual values of the linear regression curves for the dose-response curves of proctolin’s effects on basal tonus shows that, as the number of stimuli delivered increases, the dose-response curves deviate from linearity and appear to approach a sigmoidal response, suggesting that the effects of adding low doses of proctolin were greater during lower (rather than higher) amounts of stimulation (See Appendix I, Fig. 4). Secondly, there was a dose-dependent increase in neurally-evoked amplitude and relaxation rate during the lower stimulus regimes but not at the higher stimulus regimes. Thus, one might anticipate that during higher stimulus regimes proctolin is released from the motoneurons resulting in an increase in amplitude of neurally-evoked contractions over that anticipated in the absence of released proctolin. This is evident in the non-linear response of area of contraction shown in Fig. 2C, where there is a dramatic and disproportionate increase in area above the 5 and again above the 10 stimuli regimes.

The ability of proctolin to produce a dose-dependent increase in the size of the post-contraction relaxation was unexpected. When the peptide was applied, the initial response was an almost instantaneous increase in the basal tonus of the muscle, which, after reaching a plateau, lowered slightly and then levelled off. At that point the maximum amplitude of contraction was observed and persisted until the wash off period. During the wash the basal
Neurally-Evoked Constrictions of Oviduct

tonus first lowered and subsequently the proctolin-induced increase in amplitude was lowered. The size of the post-contraction relaxation was directly proportional to the proctolin-induced increase in basal tonus. This pattern of effects suggests that the amplitude and basal tonus are enhanced independently, but the size of the post-contraction relaxation may be correlated to the increase in basal tonus produced by the pentapeptide.

In the presence of the proctolin antagonist cycloproctolin, proctolin’s effects on neurally-evoked contractions were reduced suggesting that proctolin enhances neurally-evoked contractions of the oviduct muscle. The ability of cycloproctolin to reduce the proctolin-induced increase in basal tonus and post-contraction relaxation is a further indication of the extent of proctolin’s natural role in modulating contraction of the oviduct muscle of Locusta.

Proctolin is released from the oviducal nerves (Orchard and Lange, 1987) and the question of what the pentapeptide’s precise role in oviducal contraction still remains. Locusta show adaptational oviposition behaviour (Engelmann, 1970) and can retain their ovulated eggs within the lateral oviducts until a suitable oviposition site is found. This ability to control the expulsion of eggs could be modulated by neural regulation and such neurogenic contractions may be involved in some aspect of oviposition behaviour. The muscles of the locust oviduct and the common oviduct, while still retaining myogenicity, are controlled by input from the oviducal nerves. Lange et al. (1984) found that large amplitude action potentials, passing from the CNS along the oviducal nerve, resulted in bioelectric potentials and sustained contractions of the posterior lateral and common oviducts. These contractions may be involved in a behaviour associated with oviposition as constriction of the lateral
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oviducts would prevent mature eggs from passing through to the common oviduct from which expulsion would occur. The release of proctolin which, as shown here, enhances nerve-evoked contractions of the oviducts, could play a significant role in the retention of eggs and subsequently the egg-laying behaviour of Locusta.

The oviducts are believed to be under both neural and hormonal control with the neuroendocrine system controlling the characteristics of the myogenic contractions (Girardie and Lafon-Cazal, 1972) and the seventh abdominal ganglion, via the oviducal nerves, providing direct neural control. This proposed dual-control system for oviposition of eggs has been suggested for other insects as well. Thomas (1979) found that the muscles controlling egg progression into the common oviduct of Carausius morosus may be under neural control, whereas egg expulsion by the genital valves depends upon a neuroendocrine factor. In the present study the myogenic activity of the oviduct muscle began to wane as the number of stimuli delivered to the oviducal nerves was increased. If the peristaltic waves of contractions are necessary for the passage of mature eggs through the lateral oviducts, then the loss of myogenicity in this muscle during increased stimulation could be explained by the locust’s need to retain the eggs during disruption of oviposition.

The effects of proctolin on myogenic contractions of the locust oviducts have been well documented, but this is the first paper to detail the pentapeptide’s effects on neurally-evoked contractions of the same muscle. Proctolin is believed to act as a cotransmitter at the oviduct neuromuscular junction, and the results of the present study not only lend further support to this hypothesis but provide some indication of the role this neuropeptide plays in the
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contraction of the oviducts of Locusta.

ACKNOWLEDGEMENTS

This work was supported by the Natural Sciences and Engineering Research Council of Canada. We are most grateful to Dr. Richard H. Osborne for providing the proctolin antagonist, cycloproctolin.
REFERENCES


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IV. GENERAL DISCUSSION

PROCTOLIN ANALOGUES

To date several proctolin analogues have been tested for potential antagonism to the peptide proctolin (Starratt and Brown, 1979; Lange et al., 1993; Puiroux et al., 1993; Gray et al., 1994; King et al., 1995). Gray et al. (1994) synthesized and assayed several proctolin analogues and found [α-Methyl-L-tyrosine²]-proctolin and cycloproctolin to be antagonists of the proctolin response in Schistocerca foregut. In the present study, both of these analogues, as well as [N-Methyl-L-tyrosine²]-proctolin and Leu-Pro-Thr, were found to be antagonists of proctolin on the oviducts of Locusta with only the tripeptide and cyclic molecule working dose-dependently. Interestingly, Starratt and Brown (1979) found the tripeptide Leu-Pro-Thr to be devoid of any biological activity on cockroach proctodeal muscle and have suggested that the full pentapeptide structure of proctolin is necessary for bioactivity. While the findings of Starratt and Brown (1979) concur with the results of the present study, as Leu-Pro-Thr was found to lack agonistic action on the oviducts of Locusta, the results of the present work also show that the tripeptide was capable of significantly reducing proctolin-induced contractions. Studies have shown that Leu-Pro-Thr is a degradation product of proctolin hydrolysis (Isaac, 1987; Quistad et al., 1984). The results of the present study suggest that the tripeptide is still capable of binding to a proctolin receptor on the oviduct muscle of Locusta, although it is incapable of initiating contraction. This interesting result suggests a possible function of this degradation product as it could act to modify the effects of proctolin or inhibit the effects of proctolin sometime after release.
General Discussion

The differences between results from the antagonist and agonist studies on different muscle systems could be attributed to the vast array of effects induced by proctolin as well as the existence of proctolin receptor sub-types in insects. Lange et al. (1993) examined the ability of some selected proctolin analogues to mimic the basal contraction induced by proctolin on locust oviducts and developed rank orders of these analogues based on potency and doses needed to induce maximal effects. These rank orders were found to differ between the cockroach and mealworm heart (Konopinska et al., 1986) as well as for locust oviduct. Results from the present study and binding studies (Puiroux et al., 1992) also suggest that there may be more than one proctolin receptor on the oviducts of *Locusta*. When [α-Methyl-L-tyrosine2]-proctolin and cycloproctolin were applied simultaneously the observed antagonism of the proctolin response was greater than that observed when a maximal dose of [α-Methyl-L-tyrosine2]-proctolin was applied alone.

COTRANSMISSION

The function of cotransmission is a much debated topic and several theories have been put forth outlining the purpose of both the coexistence and cotransmission of messengers. Specifically, it has been suggested (Kupferman, 1991) that cotransmission may serve to i) mediate biphasic responses ii) inhibit the release of the conventional transmitter from the nerve terminal iii) reduce receptor desensitization or iv) work synergistically with primary transmitter substances to enhance postsynaptic responses.
Mediation of biphasic responses

Cotransmitters can serve to carry increased information from an individual neuron, for example, a neuron may use a fast transmitter for conventional conveyance of information and may use a peptide to mediate slower trophic effects (Kupferman, 1991). Modulation of neuromuscular transmission at the locust oviducts has not been shown to be of a biphasic nature, although physiologically, the effects of glutamate are nearly spontaneous whereas the proctolin-induced increase in basal tonus takes slightly longer to reach plateau. With proctolin being presumed to be released during increased frequency of neural stimulation it is perhaps more accurate to say that proctolin produces a longer lasting effect and therefore is released during higher frequency stimulation when a sustained contraction is needed.

Presynaptic inhibition of transmitter release

In addition, it has been suggested that a cotransmitter may function to enhance the efficiency of transmission at some junctions by acting presynaptically to reduce the amount of the primary transmitter, while at the same time acting postsynaptically to enhance the effectiveness of the primary transmitter (Stjarne et al., 1986). Lundberg et al. (1986) have postulated that this improvement of the economy of function may work only when a large response is required and when the terminal fires at a frequency sufficiently high to release the peptide (second) transmitter.

It is unlikely that such a scenario exists at the oviducal nerve synapse of Locusta. Orchard
and Lange (1986) have reported that application of proctolin (at concentrations ranging from \(1.6 \times 10^{-10} \text{ M}\) to \(6 \times 10^{-9} \text{ M}\)) resulted in a dose-dependent, though relatively small depolarization, but only produced a small increase in amplitude of EJPs. If proctolin was acting to inhibit release of glutamate from this nerve terminal, a reduction in amplitude of EJP would presumably be expected. Alternatively, it is possible that proctolin acts presynaptically to enhance release of glutamate into the synapse and evidence of this comes from the slight increase in EJP amplitude produced by proctolin. However, both of these hypotheses are highly speculative as there has been no evidence of presynaptic proctolin receptors on the oviducal nerve.

Such a system, where a neuromodulator acts to inhibit release of transmitter, has been suggested for the locust oviducts. Cheung et al. (1994) have shown octopamine to reduce the amplitude of three EJP heights and in cases where summation occurred the resultant active response was either decreased or completely abolished by the biogenic amine. This decrease in EJP amplitude in response to octopamine may be due to postsynaptic effects that would reduce the responsiveness of the muscle to the neurotransmitter or alter the contractile machinery. Alternatively, this response may reflect presynaptic effects that would reduce neurotransmitter release (Cheung et al., 1994). Support for this latter hypothesis comes from the observation that octopamine reduced miniature endplate potential (MEPP) frequency in a dose-dependent manner and this effect was blocked by the \(\alpha\)-adrenergic blocking agent, phentolamine.

While there is no evidence to suggest that the release of proctolin from the oviducal
Genera
dlusion
nerves results in a reduction in the subsequent release of transmitter, the oviducts of *Locusta*
do appear to gain such flexibility as a result of the presence of octopamine.

Reduction of receptor desensitization

A third hypothesis as to the function of cotransmission is that by releasing multiple transmitters to produce a similar action the concentration of any given transmitter can be relatively low, thereby reducing receptor desensitization and downregulation (Belcher and Ryall, 1977; Huidobro-Toro and Parada, 1988). Such a scenario could exist in the locust oviducts. To test this, successive applications of glutamate to the oviducts could be performed in order to observe any decreases in potency, thus, suggesting desensitization. If such a decrease exists, then the simultaneous application of proctolin and glutamate can be used to test if proctolin decreases this desensitization.

Enhancement of responses

Lastly, Kupferman (1991) has suggested that the peptide and other cotransmitters may have synergistic effects and so function to amplify the actions of relatively few synaptic terminals of a given presynaptic cell. The results of the present study suggest that proctolin may serve to enhance contractions of the oviduct muscle in *Locusta*. Using a 1 stimulus regime the application of proctolin to the locust oviducts was found to increase the amplitude and relaxation rate of neurally-evoked contractions, basal tension, as well as the size of the post-contraction relaxation. These findings lend further support to the notion that proctolin is
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acting as a cotransmitter at the oviducal neuromuscular junction since it is effective at inducing contraction but has no effect on amplitude of EJPs (Lange *et al.*, 1984). In the coxal depressor muscle of cockroaches proctolin had minimal effect upon membrane potential or EJPs (Adams and O'Shea, 1983) yet still induced contractions, whereas in slow tonic muscles of the extensor-tibiae in locust proctolin resulted in a large membrane depolarization and contraction (May *et al.*, 1979). Clearly, proctolin may possess more than one mode of action. Dunbar and Huddart (1982) have suggested a dual mode of action in locust proctodeal muscles; a quick acting pathway via an action on postsynaptic membranes and a long term action on extra junctional receptors.

In the present study, it was also observed that proctolin was incapable of enhancing neurally-evoked contractions during increased neural stimulation (ie. both 5 and 10 stimuli bursts). Presumably, proctolin was released endogenously from the oviducal nerves during such increased neural stimulation and therefore masked the effects of applied proctolin.

**PROCTOLIN'S ROLE UNDERLYING BEHAVIOUR**

The question that still remains then is what is the function of dual transmission in the locust oviducts? It has been proposed in locusts that during pre-ovipositional activities a hormone is released into the haemolymph, in anticipation of egg-laying, and this hormone increases the force of contractions of the muscles of the lateral oviducts (Okelo, 1971; Girardie and Lafon-Cazal, 1972). As a consequence it is pertinent during the time when the hormone is present and the eggs are being propelled down the lateral oviducts, to possess a
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mechanism for inhibiting egg progression until the appropriate time. In Locusta, electrical stimulation of the oviducal nerves produces a sustained contraction of in vitro oviducts which propels eggs back towards the ovaries (Lange et al., 1984) thereby providing a physiological basis for part of the adaptive ovipositional activities of locusts. Implanted electrodes have been used to monitor the electrical activity of the oviducal nerves during times of egg-laying (Lange et al., 1984). These nerves are only active at times when egg laying must be prevented such as during digging behaviour or following interruption of egg-laying (Lange, 1993).

As mentioned, proctolin is believed to be a cotransmitter for crayfish tonic muscles (Bishop et al., 1987) and in this system proctolin is believed to be needed for efficient production of tension by providing a means of generating tension with less neural activity. Postural muscles such as these are necessary to maintain a given position for long periods of time, but also need to adjust rapidly to changing conditions. Therefore, Bishop et al. (1987) have postulated that proctolin's role is probably to maintain tension, since the peptide's response reaches a maximum only after ten seconds.

Clearly the above description of proctolin's action on crustacean muscle differs from that of its actions in insects, where proctolin causes tension increases in resting muscle fibres and prolongs the tension produced by neural stimulation (Adams and O'Shea, 1983). Both of the differences can be simply explained by assuming that insect muscles are somewhat depolarized relative to crayfish muscles, or that the depolarization-tension curve is shifted (Bishop et al., 1987). In the crayfish muscle, tension requires that the muscle be depolarized from the resting
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potentid. Hence, the duration of tension is set by the stimulus duration and time constant of the membrane.

Future Work

Now that we have successfully identified a specific and relatively effective antagonist to proctolin on the oviducts of Locusta, this analogue can now be used to further discriminate proctolin's role from that of glutamate. This can be accomplished in many ways.

Lange (1988) reported that proctolin stimulated the production of inositol monophosphate (IP1), inositol 1,4-biphosphate (IP2) and inositol 1,4,5-triphosphate (IP3) in locust oviducts during a five minute incubation. These effects of the pentapeptide were enhanced when the tissue was incubated in a bathing solution containing lithium. Further evidence that inositol phospholipid hydrolysis may mediate the physiological actions of the peptide was provided by Baines et al. (1990), who demonstrated that proctolin enhancement of neurally-evoked contractions of the locust mandibular closer muscles was facilitated by an influx of extracellular calcium which could be blocked by the calcium channel blocker verapamil.

A time course of proctolin's actions can be determined by incubating oviducts in proctolin and stopping the reaction at various time (eg. 15, 30, 45, 60, 90 and 120 seconds) followed by measuring the production of radiolabel from [3H]myo-inositol incubated tissues. Furthermore, incubating tissue homogenates (or whole oviducts) with buffer containing cycloproctolin (5x10^-6 M - 10^-4 M) and/or [α-methyl-L-tyrosine^2]-proctolin (10^-9 M) prior to the addition of proctolin will determine the extent to which inositol phosphate levels are
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increased by proctolin. Additionally, if the presence of both cycloproctolin and [α-methyl-L-tyrosine\(^2\)]-proctolin resulted in a differential or additive ability to abolish \[^3\text{H}\]inositol phosphate production the data would then correspond to the physiological analyses of the present study to suggest that the locust oviduct contains at least two proctolin receptor subtypes.

A similar study has been reported by Hinton and Osborne (1996) in which proctolin was shown to be a powerful stimulant of the production of \[^3\text{H}\]inositol phosphates from locust foregut homogenates. In this study, the reduced ability of [α-methyl-L-tyrosine\(^2\)]-proctolin to stimulate phosphatidylinositol production (as compared to the parent peptide) was in agreement with an earlier study by Gray et al. (1994) where this proctolin analogue was shown to be a weak partial agonist. Moreover, [α-methyl-L-tyrosine\(^2\)]-proctolin was shown to significantly attenuate \[^3\text{H}\]IP\(_3\) and \[^3\text{H}\]IP\(_4\) production stimulated by proctolin.

A second way to further define proctolin's role in contraction of the oviducts of *Locusta* would be to examine the proctolin-induced post-contraction relaxation. It is possible that such an effect is caused by changes in potassium conductance in the effector cells and this hypothesis could be tested by measuring EJPs in the presence of a potassium channel blocker, such as Agitoxin-2 or Margatoxin. If such a change in conductance is what caused the post-contraction relaxation then one would expect that the relaxation would not be observed when the potassium channel blocker is applied. Since proctolin's actions have been shown to require calcium (Wilcox and Lange, 1995), it may also be possible that the post-contraction relaxation is mediated by calcium-activated potassium channels. Again, this can be tested with
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potassium channel toxin blockers such as Charybotoxin or Iberiotoxin which have been shown to block high conductance, calcium-activated potassium channels (Miller et al., 1985; Galvez et al., 1990).

CONCLUSION

It has been suggested that cotransmission exists as a form of evolutionary relic that was evolved for reasons not directly related to function. The cotransmitters in any given neuron may not have been selected against, since they may not interfere with neuronal function and may be linked to developmental programs that are expressed in other cells (Dumont and Robertson, 1986). In light of the results of the present study, along with several other studies (Adams and O’Shea, 1983; Bishop et al., 1987; Lange et al., 1986; Lloyd et al., 1984; O’Shea et al., 1985; Siwicki et al., 1985; Worden et al., 1985) it appears that there may be a functional significance to cotransmission as the flexibility of these systems is enhanced by the presence of multiple messengers.

Pharmacologically the proctolin receptor has been examined via structure-activity studies and the effects of a variety of substitutions and deletions of amino acids have indicated a number of important sites for receptor binding/activation. Ultimately, the identification and further classification of proctolin receptors is paramount to elucidating proctolin's role as a cotransmitter and the discovery of more specific and potent antagonists will help in this endeavour.
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REFERENCES


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APPENDIX I: THE EFFECTS OF PROCTOLIN ON NEURALLY-EVOKED CONTRACTIONS OF LOCUST OVIDUCTS

While the present results show that proctolin increases the basal tonus, amplitude, relaxation rate and size of the post-contraction relaxation on neurally-evoked contractions of the oviducts, there was no consistent effect on duration (Fig. 1). Duration was measured as the time to reach 50% of maximum contraction. During all stimulus regimes used, the duration of contraction varied greatly and there was no significant difference (p<0.05) in the values.

In the present study, parameters such as amplitude, basal tonus, relaxation rate and post-contraction relaxation were used to define the maximum effect proctolin had on neuromuscular transmission at the oviducts of Locusta. These parameters were measured at specific times after the addition of proctolin to the preparations. In order to determine the times at which to take such measurements, time courses were developed showing proctolin’s effects on neurally-evoked contractions during the various stimulus regimes (Figs. 2 and 3). During a 1 stimulus regime, the maximum effect of proctolin on basal tonus and post-contraction relaxation was observed 40 seconds after application of the peptide, while the proctolin-induced increase in amplitude of contraction reached maximum at 60 seconds. During a 2 stimuli regime the maximum effects of proctolin were observed 50 seconds after proctolin application. Lastly, during the higher stimulus regimes (ie. both 5 and 10 stimuli bursts) the oviducal nerves were stimulated every 5 and 10 seconds respectively and the
Appendix I

Maximum response to the peptide was observed between 30-50 seconds after application. Therefore, based on these time courses the time taken to reach maximum response to proctolin was determined for each stimulus regime and that time was subsequently used in all measurements of proctolin’s effects.

Proctolin’s effects on basal tonus were found to be much greater during the lower stimulus regimes rather than the higher regimes (See Section III, Fig. 3). To test the statistical significance of this, the data of the proctolin-induced percent increase in basal tonus were plotted as linear regressions. Next, the residual values (i.e. the difference between the regression line and the actually data points) were plotted (Fig. 4). As can be seen, the data obtained during the lower stimulus regimes was very close to the linear regression line suggesting that the response to the peptide was more linear. However, the data obtained during the higher stimulus regimes formed a more sinusoidal distribution suggesting that the response to the peptide was more sigmoidal during increased stimulation of the oviducal nerves. This decreased sensitivity to proctolin during the 5 and 10 stimuli regimes lends further support to the hypothesis that proctolin is being released endogenously from the oviducal nerves during increased neural stimulation.
Figure 1. The effects of proctolin on duration of contraction. During the 1 stimulus regime, proctolin dose-dependently increased the duration of contractions while no change was observed during the 2 stimuli regime. Only slight increases in duration of contraction were observed during the 5 and 10 stimuli regimes respectively. All data points are the mean±S.E. of 4 preparations.
Appendix I

A graph showing the duration (as a percent of control) for different proctolin concentrations at various stimulus levels.

- Line with circle symbols: 1 Stimulus
- Line with square symbols: 2 Stimuli
- Line with triangle symbols: 5 Stimuli
- Line with upward triangle symbols: 10 Stimuli

The x-axis represents Proctolin Concentration [M], ranging from $10^{-10}$ to $10^{-7}$.

The y-axis represents Duration: Percent of Control, ranging from 60 to 140.
Figure 2. A time course of proctolin’s effects on neurally-evoked contractions of locust oviducts during a 1 stimulus regime. Proctolin was added at time=0 and was washed off at times denoted by the downward arrows. Data are represented as the mean±S.E. of 4 preparations.
Figure 3. Time courses of proctolin's effect during a 2 stimuli regime. Proctolin was added at time=0 and was washed off at times denoted by the downward arrows. Data are shown as the mean±S.E. of 4 preparations.
Figure 4. A plot of the residual values for the proctolin-induced percent increase in basal tonus. As the stimulation was increased, proctolin's effects deviate from a linear distribution and appear to be more sigmoidal. Each data point is a mean±S.E. of 4 preparations.
IMAGE EVALUATION
TEST TARGET (QA-3)

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