Proctolin degradation by membrane peptidases from nervous tissues of the desert locust (*Schistocerca gregaria*)

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The hydrolysis of the insect neuropeptide proctolin (Arg-Tyr-Leu-Pro-Thr) by enzyme preparations from the nervous tissue of the desert locust (*Schistocerca gregaria*) was investigated. Neural homogenate degraded proctolin (100 μ M) at neutral pH by cleavage of the Arg-Tyr and Tyr-Leu bonds to yield Tyr-Leu-Pro-Thr, Arg-Tyr and free tyrosine. Arg-Tyr was detected as a major metabolite when the aminopeptidase inhibitors amastatin and bestatin were present to prevent Arg-Tyr breakdown. Around 50% of the proctolin-degrading activity was isolated in a 30000 g membrane fraction and was shown to be almost entirely due to aminopeptidase activity. The aminopeptidase had an apparent K_m of 23 μ M, a pH optimum of 7.0 and was inhibited by 1 mM-EDTA and amastatin [IC_{50} = 0.3 μ M], but was relatively insensitive to bestatin, actinonin and puromycin. Phenylmethanesulphonyl fluoride (1 mM) and *p*-chloromercuriphenylsulphonic acid (1 mM) had no effect on this enzyme activity. Although the bulk of the Tyr-Leu hydrolytic activity was located in the 30000 g supernatant, some weak activity was detected in a washed membrane preparation. This peptidase displayed a high affinity for proctolin (K_m = 0.35 μ M) and optimal activity at around pH 7.0. Synaptosome- and mitochondria-rich fractions were prepared from crude neural membranes. The aminopeptidase activity was concentrated in the synaptic-membrane preparation, whereas activity giving rise to Arg-Tyr was predominantly localized in the mitochondrial fraction. The subcellular localization of the membrane aminopeptidase is consistent with a possible physiological role for this enzyme in the inactivation of synaptically released proctolin.

INTRODUCTION

Proctolin (Arg-Tyr-Leu-Pro-Thr) is an insect neuropeptide that is considered to have a neurotransmitter/neuromodulator role in visceral (hindgut, oviduct, heart) and skeletal muscle of insects (Miller, 1983; Cook & Holman, 1985; Brown, 1975). This peptide was first isolated from the hindgut of the American cockroach (*Periplaneta americana*), on which it has a potent excitatory action (Brown, 1975; Starratt & Brown, 1975) and has since been found in insects from six orders (Brown, 1977). Proctolin has been detected throughout the insect nervous system (Bishop *et al.*, 1981; Kingan & Titmus, 1983), and neurons containing proctolin-like material have been identified and shown to have a widespread distribution within the central nervous system (Bishop *et al.*, 1981; Eckert *et al.*, 1981). The structural and functional diversity of the proctolin-containing neurons and the demonstration that the peptide can excite neurons of the terminal ganglion (Walker *et al.*, 1980) suggest that proctolin may have a neuroeffector role in both the central and peripheral nervous systems (Miller, 1983; O'Shea, 1982). A neurohaemal role for proctolin appears unlikely, as the peptide is rapidly degraded by soluble haemolymph enzymes (Quistad *et al.*, 1984; Starratt & Steele, 1984; Steele & Starratt, 1985). Studies *in vitro* on *P. americana* showed that the route of degradation can depend upon the pH of the haemolymph (Steele & Starratt, 1985). A carboxypeptidase was the principal activity at pH 6,

whereas at pH 8 the hydrolysis of the Tyr-Leu bond was of importance. Aminopeptidase activity was also significant, irrespective of the pH conditions.

In vertebrate tissues, extracellular membrane enzymes are considered to be involved in the termination of neuropeptide signals (Kreiger, 1983). Aminopeptidase, endopeptidase and dipeptidyl peptidase activities of cell membranes have been implicated in the inactivation of neuropeptides [for a review, see Turner *et al.* (1985)], and recent studies suggest that a few peptidase enzymes may be responsible for the primary hydrolysis of a broad spectrum of peptide messengers (Fulcher *et al.*, 1982; Matsas *et al.*, 1983). Many of these enzymes display maximum activity at around neutral pH and have K_m values in the micromolar range (Hudgin *et al.*, 1981; Schwartz *et al.*, 1981; Fulcher *et al.*, 1982; Hersh, 1982, 1985; Matsas *et al.*, 1984). Whether similar membrane enzymes are important in insects for neuropeptide inactivation is unclear. A recent study on the metabolism of proctolin by tissue homogenates of *P. americana* suggested that two soluble enzyme activities, which cleave the Arg-Tyr and Tyr-Leu bonds, might have a role in the degradation of proctolin (Quistad *et al.*, 1984). However, a function for these soluble enzymes in the inactivation of proctolin cannot be assumed. Similar, but much lower, enzyme activities were also associated with unwashed membrane preparations. There is no information available concerning the metabolism of proctolin in other insects.

The present paper describes the metabolism of

Fig. 1. Proctolin degradation by locust nervous tissue

Degradation products, generated by incubating 100 μ M-proctolin with the 1000 g supernatant (S_1) from *S. gregaria* in the absence (a) or presence (b) of a mixture of 0.1 mM-amastatin and 1 mM-bestatin, were resolved by reversed-phase h.p.l.c. using an 8 min linear gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid (5–35%). Proctolin hydrolysis was 20 and 13% in (a) and (b) respectively. Retention times for proctolin (1), Tyr-Leu-Pro-Thr (2), Arg-Tyr (3) and tyrosine (4) are indicated.

proctolin *in vitro* by neural tissues of *Schistocerca gregaria*, and some properties of the proctolin-degrading activity of membrane-bound peptidases.

MATERIALS AND METHODS

Proctolin, [tyrosine-3,5- 3 H]proctolin and Arg-Tyr were purchased from Sigma (Poole, Dorset, U.K.), New England Nuclear (du Pont, Stevenage, Herts., U.K.) and Serva (Uniscience, London, U.K.) respectively. Tyr-Leu-Pro-Thr was obtained from the degradation of proctolin *in vitro* by nervous tissue from *S. gregaria*. The tetrapeptide was isolated by h.p.l.c. and characterized by amino acid analysis (Isaac, 1985). Actinonin was a gift from Dr. A. J. Kenny, Department of Biochemistry, University of Leeds, Leeds, U.K. Other inhibitors were obtained from Sigma.

Enzyme preparations

Nervous tissues (brain, thoracic ganglia, abdominal ganglia and nerve cord) were excised from male and female locusts. Contaminating trachea and fatty tissues were carefully removed before storage at -20°C . For the isolation of the membrane and supernatant fractions, tissues were homogenized in 50 mM-Tris/HCl buffer, pH 7.3 (0.1 g of tissue/ml of buffer), and the homogenate was then centrifuged at 1000 g for 10 min to give supernatant S_1 , which was used as a source of crude enzyme for initial studies. To obtain a membrane preparation, the supernatant (S_1) was centrifuged at 30000 g for 20 min (JA-21 rotor; Beckman J2-21 centrifuge). The resulting supernatant (S_2) was retained, and pellets were resuspended in 0.5 M-NaCl/50 mM-Tris/HCl, pH 7.3, and centrifuged again at 30000 g for 20 min to give a washed membrane pellet, which was resuspended in 1 ml of 10 mM-Tris/HCl, pH 7.3. Crude neural membranes were separated into synaptosome- and mitochondria-rich fractions by the methods developed by Breer and co-workers (Breer & Jeserich, 1980; Breer & Knipper, 1984). Briefly, locust ganglia were excised and homogenized in 0.25 M-sucrose, buffered with 0.1 M-Tris/HCl, pH 7.5. After centrifugation at

1000 g for 15 min, the supernatant was centrifuged at 17000 g for 25 min using a Beckman JA-21 rotor. The pellet was resuspended in Ficoll (10%, w/v, in 0.25 M-sucrose) and centrifuged at 10000 g for 45 min in a Beckman JS-13 swing-out rotor using adaptors to hold 0.5 ml centrifuge tubes. The pellet (resuspended in 5 mM-Tris/HCl, pH 7.3) was shown to be enriched in succinate dehydrogenase activity and was retained as the mitochondrial fraction. The supernatant was rich in acetylcholinesterase activity, as synaptosomes do not sediment under these conditions (Breer & Jeserich, 1980). Synaptosomal membranes were then isolated from the supernatant by centrifugation at 30000 g for 20 min in a Beckman JA 21 rotor after 10-fold dilution of the Ficoll with 0.25 M-sucrose. This pellet was resuspended in 5 mM-Tris/HCl, pH 7.5, for 30 min to lyse the synaptosomes, before centrifugation at 30000 g for 20 min in a Beckman JA-21 rotor. The pellet was resuspended in 5 mM-Tris/HCl, pH 7.3, and was retained as a synaptic-membrane fraction. All procedures were carried out at 4°C , and enzyme assays were performed within 12 h of the preparation of the membrane fractions.

Enzyme assays

Proctolin (10–150 μ M) or [3 H]proctolin (0.05–10 μ M) was incubated with enzyme in 0.1 M-Tris/HCl buffer, pH 7.3, at 30°C for up to 2 h (total incubation volume 50 μ l). Reactions were commenced by the addition of substrate and terminated by the addition of 8% (w/v) trichloroacetic acid (10 μ l). 100 μ M-Proctolin was used for the routine assay of aminopeptidase activity. Metabolites were resolved by h.p.l.c. using a 3 μ Ultrasphere ODS column (75 mm $\times$ 4.6 mm; Beckman), a flow rate of 1 ml/min and a linear gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid increasing either from 10 to 35% in 6 min or from 5 to 35% in 8 min. No Arg-Tyr-Leu-Pro standard was available; however, this peptide has been shown by other workers to be eluted after proctolin on similar reversed-phase h.p.l.c. systems and is well resolved from other tyrosine-containing metabolites (Starratt & Steele, 1984; Quistad *et al.*, 1984). Tyrosine and tyrosine-containing peptides were

Table 1. Ability of subcellular fractions from insect nervous system to degrade proctolin

Metabolites were quantified by reversed-phase h.p.l.c. as described in the text. Recoveries of enzyme activities in S₂ and P, determined in the absence or presence of the inhibitors amastatin (0.1 mM) and bestatin (1 mM), were 74 and 65% respectively of that in S₁. Values are an average for duplicate assays and values for a separate experiment are indicated in parentheses. Proctolin hydrolysis did not exceed 25%. Abbreviation: n.d., not detected.

Enzyme preparation	Inhibitor	Product released (nmol/h per 10 mg of tissue)		
		Tyrosine	Arg-Tyr	Tyr-Leu-Pro-Thr
1000 g supernatant (S ₁)	None	4.5 (7.4)	1.5 (0.8)	27.3 (24.2)
	Amastatin + bestatin	0.5 (n.d.)	8.0 (4.7)	4.5 (4.5)
30000 g supernatant (S ₂)	None	3.8 (6.0)	1.1 (0.2)	7.1 (8.6)
	Amastatin + bestatin	0.6 (0.8)	5.1 (4.9)	2.1 (4.6)
30000 g pellet (P)	None	n.d. (1.5)	n.d. (n.d.)	12.9 (11.7)
	Amastatin + bestatin	n.d. (n.d.)	0.4 (n.d.)	0.5 (n.d.)

detected and quantified by monitoring absorbance of the eluate at 280 nm. For the detection of radioactivity, samples were collected and, after the addition of 2 ml of Optiphase 'Safe' (LKB), were analysed for ³H in a liquid-scintillation counter. Counting efficiencies were determined by external-standards-ratio and internal-standard methods.

Acetylcholinesterase activity was measured by the method of Ellman *et al.* (1961) and succinate dehydrogenase activity was determined as described by Porteous & Clark (1965). The results of the enzyme assays are the average of at least duplicate assays.

RESULTS

Proctolin degradation by nervous tissue

The ability of nervous tissue to degrade proctolin was initially investigated by incubating proctolin (100 μ M) with the crude enzyme preparation, S₁, buffered at pH 7.3. Primary hydrolysis of the Arg-Tyr bond yielded Tyr-Leu-Pro-Thr as the predominant degradation fragment (Fig. 1a). Free tyrosine, a less important metabolite, resulted from the hydrolysis of Arg-Tyr, which was shown to be an intermediate degradation product at pH 7.3 by adding the specific aminopeptidase inhibitors amastatin and bestatin to the enzyme preparation before incubation with proctolin (Fig. 1b). Arg-Tyr may have resulted from primary cleavage of the Tyr-Leu bond of proctolin or from secondary metabolism of fragments produced by carboxypeptidase action. However, there was no evidence for the formation of Arg-Tyr-Leu-Pro as a metabolite at pH 7.3, in either the absence or presence of aminopeptidase inhibitors.

Washed membrane and supernatant (S₂) fractions were prepared from nervous tissue by centrifugation of the crude enzyme preparation (S₁) at 30000 g. Of the total proctolin-degrading activity present in S₁, approx. 50% was located in the membrane pellet. Membrane peptidases gave rise to mainly Tyr-Leu-Pro-Thr, whereas the supernatant enzymes under the same conditions released Tyr-Leu-Pro-Thr, free tyrosine and minor quantities of Arg-Tyr (Table 1). In addition, each fraction was assayed for proctolin-degrading activity in the presence of a mixture of amastatin (0.1 mM) and bestatin (1 mM). The quantitative distribution pattern obtained in the presence of these aminopeptidase inhibitors indicated that the enzymes involved in the

Fig. 2. Effect of inhibitors on aminopeptidase activity of neural membranes

Membrane preparations (30000 g pellet) were pre-incubated with inhibitors for 10 min at 20 °C before the addition of 100 μ M-proctolin and incubation at 30 °C. Inhibition of aminopeptidase activity by amastatin (○), bestatin (●), actinonin (□) and puromycin (■) is expressed as a percentage of the uninhibited activity. Aminopeptidase was assayed by the routine method described in the text.

formation of Arg-Tyr were predominantly localized in the 30000 g supernatant (Table 1). In a separate experiment, a mixture of amastatin (0.1 mM) and bestatin (1 mM) was shown to protect proctolin from hydrolysis by membrane peptidases even under conditions which in the absence of inhibitors resulted in 50% degradation, thus confirming the importance of a neutral aminopeptidase activity in the metabolism of proctolin *in vitro* by membrane-bound enzymes.

Properties of the membrane-bound aminopeptidase

The membrane aminopeptidase activity (2.9 ± 0.43 nmol/min per mg of protein; $\pm$ S.E.M., $n = 8$) showed optimal activity at around pH 7.0. By using substrate concentrations ranging from 10 to 150 μ M, this activity was shown to have an apparent K_m of 23 μ M. Cleavage of the Arg-Tyr bond was strongly inhibited by

Fig. 3. Hydrolysis of (a) 0.1 μM - and (b) 0.01 μM - $[\text{³H}]$ proctolin by neural membranes

Hydrolysis products were resolved by reversed-phase h.p.l.c. using the same conditions as described for Fig. 1. Fractions were collected every 0.2 min for the measurement of radioactivity as described in the text. Retention times for proctolin (1), Tyr-Leu-Pro-Thr (2), Arg-Tyr (3) and tyrosine (4) are indicated.

amastatin (IC_{50} 0.3 μM). but was less sensitive to bestatin, actinonin and puromycin (Fig. 2). Phenylmethanesulphonyl fluoride (1 mM) and *p*-chloromercuriphenylsulphonic acid (1 mM) had no effect but the presence of EDTA (1 mM) resulted in 84% inhibition.

Proctolin degradation by neural membranes at low substrate concentration

A different metabolite pattern for the degradation of proctolin by neural membranes was obtained when the substrate concentration was lowered to below 1 μM . Arg-Tyr was detected as a significant degradation product in addition to Tyr-Leu-Pro-Thr and tyrosine (Fig. 3). It was clear that lowering the substrate concentration favoured the formation of Arg-Tyr rather than Tyr-Leu-Pro-Thr (Fig. 3b). Hydrolysis of the Tyr-Leu bond was measured in the presence of amastatin (2 μM) to minimize Arg-Tyr cleavage, and was shown to be maximal at pH 7.0 and have an apparent K_m of 0.35 μM . The rate of Arg-Tyr release from 1.0 μM - $[\text{³H}]$ proctolin by crude neural membranes was 0.047 ± 0.008 nmol/min per mg of protein (S.E.M., $n = 3$).

Subcellular localization of the membrane peptidases

A crude neural-membrane preparation was further fractionated into synaptosomal and mitochondrial fractions, which were assayed for acetylcholinesterase, succinate dehydrogenase and proctolin-degrading activity. Proctolin-degrading aminopeptidase activity followed a distribution similar to that of acetylcholinesterase (Table 2), with 71% of the membrane enzyme being localized in the synaptosomal fraction. The high-affinity cleavage of the Tyr-Leu bond was predominantly associated (66%) with the mitochondrial fraction. The remaining activity present in the synaptosomal membranes was not removed by washing with 0.4 M-NaCl.

DISCUSSION

Neural enzymes from *S. gregaria* were able to degrade proctolin at neutral pH by cleavage of the Arg-Tyr and Tyr-Leu bonds. Neural membranes were enriched with a proctolin-degrading aminopeptidase activity that displayed properties (pH optimum and K_m) similar to those of membrane-bound aminopeptidases from vertebrate brain tissue which have been implicated in the inactivation of synaptically released neuropeptides, such as enkephalins and cholecystokinin-8 (Hersh, 1982; Deschodt-Lanckman, 1985; Giros *et al.*, 1986). Although most of the Tyr-Leu-cleaving activity was localized in the 30000 *g* supernatant, some weak activity with a high affinity for proctolin was associated with the crude membrane preparation. However, subcellular fractionation of the neural homogenate resulted in different distribution profiles for the latter activity and that of the aminopeptidase. The bulk of the Tyr-Leu-hydrolysing activity was localized in the mitochondrial fraction, and is thus unlikely to play a major role in the inactivation of proctolin. In contrast, the aminopeptidase, following a distribution pattern similar to that of acetylcholinesterase, was present at high specific activity in a synaptic membrane preparation.

The effect of EDTA and aminopeptidase inhibitors on the membrane aminopeptidase indicated that this enzyme is a metallopeptidase which is sensitive to inhibition by amastatin, but not bestatin, actinonin and puromycin. Puromycin is a general aminopeptidase inhibitor (Turner *et al.*, 1985); however, the degree of inhibition of different enzymes can vary greatly. One aminopeptidase that may be involved in the inactivation of [Met]enkephalin in rat brain has been shown, like the

Table 2. Specific activities of proctolin-degrading and marker enzymes in synaptosomal and mitochondrial membrane fractions

Specific activities are mean values for four separate experiments. Hydrolysis of the Tyr-Leu bond of proctolin was measured in the presence of 100 μ M-amastatin, using 1 μ M-[3 H]proctolin as described in the text. Amino-peptidase activity was determined by measuring the formation of Tyr-Leu-Pro-Thr as described in the text.

Membrane preparation	Enzyme activity (nmol/min per mg of protein) (mean $\pm$ S.E.M.)			
	Acetylcholinesterase	Succinate dehydrogenase	Amino-peptidase	Tyr-Leu hydrolysis
Synaptosomal	868 $\pm$ 74	3.18 $\pm$ 0.41	4.78 $\pm$ 0.28	0.018 $\pm$ 0.001
Mitochondrial	231 $\pm$ 27	15.2 $\pm$ 2.5	1.59 $\pm$ 0.21	0.033 $\pm$ 0.005

insect amino-peptidase described in the present study, to be relatively insensitive to puromycin (Hersh, 1981; Giros *et al.*, 1986). Bestatin and amastatin can non-competitively inhibit a range of amino-peptidase enzymes (Turner *et al.*, 1985), and actinonin is a strong inhibitor of amino-peptidase M and leucine amino-peptidase (Umezawa *et al.*, 1985).

The lack of inhibition of the amino-peptidase by phenylmethanesulphonyl fluoride and *p*-chloromercuriphenylsulphonic acid suggests that this enzyme is neither a serine nor a thiol proteinase. Inhibition of proctolin hydrolysis by substance P, molluscan cardio-excitatory neuropeptide and bradykinin (R. E. Isaac, unpublished work) indicated that other peptides may also bind to the active site of this amino-peptidase.

The present observations, that locust nervous tissue can efficiently degrade proctolin at neutral pH to release Arg-Tyr and Tyr-Leu-Pro-Thr, are in agreement with the results obtained by Quistad *et al.* (1984) with brain and terminal ganglia of *P. americana*. These workers, using a substrate concentration of 8 nM, showed that membrane fractions generated Arg-Tyr and Tyr-Leu-Pro-Thr fragments in approximately equal amounts. However, less than 10% of the proctolin-degrading activity of the cockroach homogenates was localized in the membrane pellet, and hydrolysis of the Tyr-Leu bond was shown to be the primary cleavage site in tissue homogenates. The use of a very low substrate concentration for the assay of the cockroach amino-peptidase is likely to have given an underestimation of the importance of this enzyme in the degradation of proctolin. Although soluble enzymes may be involved in neuropeptide degradation in insects, peptidases positioned on the surface of the plasma membrane are likely to have a more critical role in regulating peptide action at a synapse. In the case of proctolin, it appears that any degradation of the pentapeptide to smaller fragments will result in a severe decrease in physiological activity (Starratt & Brown, 1979). The results of the present study, demonstrating the localization of a membrane amino-peptidase activity in a locust synaptic-membrane preparation, is consistent with a role for this enzyme in proctolin inactivation.

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