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Review

Proctolin, an Insect Neuropeptide

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Abstract: Synthetic, biological and conformational studies on the insect neuropeptide proctolin (Arg-Tyr-Leu-Pro-Thr) and some of its analogues are reviewed. Copyright © 1999 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: proctolin; proctolin analogues; neuropeptides; insect peptides

INTRODUCTION

Proctolin, the first structurally characterized myotropic insect neuromodulator, is a pentapeptide, L-arginyl-L-tyrosyl-L-leucyl-L-prolyl-L-threonine (Arg-Tyr-Leu-Pro-Thr), which was isolated in 1975 by Brown and Starratt [1] from whole-body extracts of the American cockroach *Periplaneta americana*. The peptide has been shown to occur in species of six insect orders, and in other invertebrates [2–10], including the crab *Cardisoma carnifex* [2], the lobster *Homarus americanus* [5], and the leech *Hirudo medicinalis* [7]. Proctolin-like immunoreactivity has also been found in the rat central nervous system [9]. Proctolin was first detected in the cockroach (*P. americana*) hindgut and then in different areas of neural and non-neural tissues [11–16]. It has been detected and quantified in the brain, essentially in the tritocerebrum, and in the suboesophageal ganglion of *P. americana* [11], as well as in *Leucophaea maderae* [13,15]. Proctolin-like immunoreactivity is confined to cell bodies and processes from ganglia belonging to the ventral nerve cord. The highest number of positive cell bodies is found in the terminal ganglion [11,16–19]. Proctolin is a neuroactive substance which is found in various neuronal cell types, including motoneurons inervating visceral and skeletal muscles [11,18–24], neurosecretory neurons [2,18–25], and sensory neurons [25].

On the basis of myotropic properties manifested in stimulation of contractions of visceral and skeletal [16,18,20–22] muscles [8] of insects and other invertebrates [24], proctolin was classified as an neurotransmitter. Several years ago, however, with the accumulation of more data concerning its biological properties, Orchard and co-workers [10] expressed the opinion that proctolin fulfills a rather neuromodulatory function in insects.

Proctolin-like peptides have been isolated from various insect species. The first example of a natural analogue was Ala-Tyr-Leu-Pro-Thr. This peptide

Abbreviations: Abbreviations for amino acid residues (all of which are of the L configuration unless otherwise stated) and their mode of use follow IUPAC-IUB conventions; Ach, α-aminocyclohexane carboxylic acid; Acp, α-aminocyclopentane carboxylic acid; Afb, β-amino-γ-phenylbutyric acid, i.e. β*homo*-Phe, or Phe elongated by insertion of a CH_2 group between the α-carbon and the carboxyl group; Can, canavanine; Gac, α-guanidino-ω-aminocaproic acid; Gap, α-guanidino-β-(*para*-aminophenyl)-propionic acid; Gav, α-guanidino-ω-aminovaleric acid; Har, homoarginine, i.e. arginine with a CH2 side-chain extension; MePhe, *N*-methylphenylalanine and so on; α-MeTyr, α-methyltyrosine and so on; Nal, β-(naphth-2-yl)-alanine; Phe(*p*-Cl), *para*-chlorophenylalanine and so on; β*homo*-Pro, pyrrolidin-2-ylacetic acid or Pro elongated by insertion of a CH_2 group between the α-carbon and the carboxyl group; Sar, sarcosine; Thz, thiazolidine-4-carboxylic acid; Gn, guanidino; Nic, nicotinyl.

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was isolated from brain extracts of Colorado potato beetle *Leptinotarsa decemlineata* [26], which has a weak stimulatory effect (at 10^{-7} M concentration) on the oviduct contracting muscles of locust *Locusta migratoria*. The synthetic [Ala^1]-proctolin described earlier by Starratt and Brown [27] did not exhibit any stimulatory effect at physiological concentrations in the hindgut of *P. americana*. Another two, the pentapeptides with the following sequences, Ala-Glu-Pro-Tyr-Thr-NH_2 and Asp-Ile-Pro-Pro-Arg-NH_2, have been isolated from the midgut of larvae of *Manduca sexta* [28,29]. They also stimulated contraction of the oviduct muscles of *L. migratoria*.

Proctolin has been a subject of numerous studies to:

- define its distribution in insect tissues;
- estimate its stability both *in vivo* and *in vitro*;
- evaluate its possible insecticidal activity;
- determination its myotropic effects;
- characterize receptors and determine the mechanism of its action;
- search for biological effects in vertebrates;
- investigate its conformation; and
- examine its structure–function relationship.

The last mentioned area has stimulated a demand for the synthesis of proctolin and its analogues.

BIOLOGICAL ACTIVITY IN INSECTS

Proctolin is the best known and most investigated insect myotropic neuropeptide. In addition to the cockroach proctodeum [3,8,10,12], proctolin stimulates contractions of foregut of the locust *S. gregaria* [27,29–31] and the midgut of the cockroach *Diploptera punctata* [32], and it is now regarded as the main neuromuscular transmitter/modulator in the gut of most insect species [8,10,12,15,32]. Further, it induces myogenic contractions of the extensor tibiae in *Locusta* [33], and the of coxal depressor in *Periplaneta* [34]; it stimulates oviduct contractility in *Periplaneta* [35], *Leucophaea* [8,36] and *Locusta* [37]; and it increases the rate of contraction of the hyperneural muscle in *Periplaneta* [38] and the dorsal longitudinal muscles of *Teleogryllus oceanicus* [39]. It also increases the rate and amplitude of contractions of semi-isolated heart preparations in several insects [22,40,41] and the antenal heart of the cockroaches, *Periplaneta* [42], *D. punctata* [43] and *Gromphadorhina coquereliana* [41]. However, the myocardia of *M. sexta* [44] and *Stomoxys calcitrans* [45] are not sensitive to proctolin, where even pharmacological concentrations of peptide (10^{-6}–10^{-3} M) are without effect. More recently, Coast [46] has found that proctolin increases the spontaneous writhing movements of malpighian tubules in *L. migratoria*. Because the tubules are not innervated, any regulation of their spontaneous contractile activity must be by factors in the circulation. These results suggest that there is a possible neurohormonal role for proctolin.

It should be noted that Goudey-Perriere *et al.* [47] have discovered novel biological properties of proctolin on a non-excitable cell, the oocyte. They demonstrated for the first time that proctolin promoted the onset of vitellogenesis in an imaginal moult decapitated female cockroach of *Blaberus craniifer*. The effect of proctolin is reflected by the appearance of vitelline in the periphery of the basal oocyte. The authors suggest that proctolin induces vitellogenin uptake in the oocyte cockroach via a membrane depolarizing action.

As opposed to the above proctolin activities in insect preparations *in vitro*, knowledge of its neuropeptide activity *in vivo* is rather scanty. Slama *et al.* [48] showed that proctolin injections increase the amplitude of extracardiac haemocoelic pulsation (e.g. increase physiological contractions of the somatic intersegmental abdominal muscles) in *T. molitor* pupae. Very recently, Zornik *et al.* [49] reported that proctolin decreases the heart rate in the larva, pupa and adult of the fruitfly *Drosophila melanogaster*. These results are in conflict with the known strong cardioexcitatory action of proctolin *in vitro* [22,41,50–53] and with its stimulatory effect on extracardiac haemocoelic pulsation *in vivo* [54].

Proctolin stability in insects has been studied both *in vitro* and *in vivo*. Rapid *in vivo* degradation of proctolin ^{3}H or ^{14}C labelled in Tyr^2 [20,55] afforded the constituent amino acids (at least Tyr), the tetrapeptide Tyr-Leu-Pro-Thr and the dipeptide Arg-Tyr. In further biological investigation of proctolin stability, Issac [56] demonstrated that membrane peptidases exist in the nervous tissue of *S. gregaria* which can degrade proctolin by cleavage of the Tyr-Leu and the Arg-Tyr bonds yielding Arg-Tyr as well as Arg and Tyr. This breakdown pathway by membrane proteases has also been found in the ovaries and hindgut of *L. migratoria* [57].

An evaluation of proctolin insecticidal activity has been performed by topical application on larvae of *M. sexta*. However, proctolin does not penetrate the

larval cuticle [55], thus eliminating the possibility of its practical use for insect control.

BIOLOGICAL ACTIVITY IN VERTEBRATES

Effects on Human Blood Cells

The influence of proctolin the on the biological properties of impaired blood cells derived from leukaemic patients [58,59] has been studied *in vitro*. These studies indicated that it has a positive effect in restoring the phagocytic activity of human granulocytes [58] and has a stimulatory effect on the transformation rate of cultured lymphocytes [59].

Effects in Rats

Several years ago we became interested in studies on the influence of proctolin and selected analogues on various physiological processes in rats. We evaluated their cardiovascular and antinociceptive effects after intracerebroventricular (icv) injection [60]. Cardiovascular effects were evaluated (by icv application) of proctolin and some of its analogues modified in positions 1 or 2 of peptide chain, such as [Lys^1]-, [His^1]-, [Cit^1]-, [Cha(4-OMe)^2]- and [Phe(p-NH$_2$)^2]-proctolin. Doses of 10, 100 and 500 nmol of each peptide were used. The arterial blood pressure, heart-beat frequency and respiratory rate of mammals after icv administration of proctolin and its analogues were determined according to the method of Jędrusiak *et al.* [60]. Among the peptides tested only proctolin, [Phe(p-NH$_2$)^2]- and [Cha(4-OMe)^2]-proctolin (at a dose of 10 or 100 nmol) induced a significant increase of arterial blood pressure, which was blocked by naloxone injection, whereas other peptides were inactive. These results suggest that the hypertensive effect of proctolin and its derivatives is due to their stimulation of the opioid receptor in the rat central nervous system. Moreover, the peptides investigated here did not have any influence on heart-beat frequency, except [Phe(p-NH$_2$)^2]- and [Cha(4-OMe)^2]-proctolin. In contrast to proctolin itself, both of these peptides induced a transient increase of heart-beat frequency in the rat [61] (Unpublished data, 1991). The antinociceptive action in rats was bioassayed for proctolin and [Lys^1]-, [Phe(p-NH$_2$)^2]-, [Cha(4-OMe)^2]- and [Dopa^2]-proctolin, which were evaluated at doses of between 10 and 100 nmol by icv administration according to the method O'Callaghan and Holtzman [62]. Proctolin, [Phe(p-NH$_2$)^2]- and [Cha(4-OMe)^2]-proctolin showed antinociceptive activity, which was prevented by naloxone injection. [Cha(4-OMe)^2]-Proctolin was not only but also long term an antinociceptive effect lasting for 1 h.

The results of these studies showed that the cardiovascular as well as antinociceptive effects of proctolin and some of its analogues are connected with their interaction with opioid receptors. Recently, Plech and Konopińska [63] have found that the antinociceptive effect of proctolin in rats is a result of its interaction with μ- and κ-receptors in the brain. Moreover, the lack of cardiovascular and antinociceptive effects of proctolin analogues modified in position 1 of the peptide chain, support our earlier hypothesis [64,65] that the presence of an *N*-terminal Arg-residue in proctolin peptide skeleton is essential for its biological activity not only in insects but also mammals.

Recently, in further studies *in vivo* in rats we have found that the proctolin analogues H-D-Arg-D-Tyr-D-Leu-D-Pro-D-Thr-OH and [D-Tyr^2, D-Thr^5]-proctolin block the antinociceptive effect of proctolin [66] and two enkephalins in rats in a manner like naloxone (a blocker of opiate receptors in rat brain). These results indicate that proctolin analogues modified by more than one D-amino acid residue in the peptide chain have a potent antagonistic activity in relation to the opiate receptors of rat brain.

STRUCTURE/FUNCTION RELATIONSHIP STUDIES

Structure–myotropic function studies in insects have prompted the elaboration of many syntheses of proctolin and its analogues [1,4,27,31,40,51,52, 54,58,66–70]. Structural modifications have included consecutive replacement residues in positions 1–5, as well as syntheses of truncated or elongated analogues [27,54,67,71]. (Tables 1–3). Cyclo-proctolin [67] and an analogue with the peptide bond isostere –CH$_2$–O– between Tyr^2 and Leu^3 (Tyr^2-ψ-Leu^3) [72] have also been synthesized. Biological studies of proctolin analogues have focused on the contraction of the hindgut muscles of the cockroach *P. americana* [27,54], heart-beat frequency in *P. americana* or *T. molitor* [22,50–53], the oviduct of *L. migratoria* [10,24,68], and foregut contraction in *S. gregaria* [31,67,73]. Out of 100 proctolin analogues, 45 had an agonistic effect in selected insect species, whereas five compounds showed antagonistic properties (Tables 2 and 3).

The significance of particular amino acid residues in the proctolin molecule for its myotropic properties has been assessed [74]. At physiological concentrations (10^{-10}–10^{-7} M) most of the proctolin analogues in Table 1 were inactive [74]. On the other hand the pentapeptides listed in Tables 2 and 3 showed activity comparable to or higher than proctolin as agonists; while only a few of them showed antagonistic properties. All proctolin analogues modified at positions 3, 4 or 5 (except [Val^3]-, [Hyp^4]-proctolin) were without myotropic properties (Table 1), whereas some modified in positions 1 or 2 retained agonistic activity or had antagonistic activity (Tables 2 and 3).

Table 1 Proctolin Analogues without Myotropic Activity in Insects at Physiological Concentrations

Peptide analogues modified in	Reference
Position 1	
X^1-Tyr-Leu-Pro-Thr; X^1 =	
D-Arg; AcArg, Orn	[27,75]
Glu; Glp; Gly	[21]
Gac; Gav; Gap, Cit; His; γ-Abu	[91]
Arg(NO_2)	[8]
Orn(NMe_2), Lys(NMe_2), Lys(iPr), Lys(Nic), D-Lys(Nic)	[75]
Position 2	
Arg-X^2-Leu-Pro-Thr; X^2 =	
D-Tyr, Ala, Phe	[27]
His; Trp	[21]
Cha(4-OMe)	[91]
Afb(p-OH); Afb(p-NH_2)	[91]
TyrψLeu	[72]
Ser; Tyr(PO_3H_2)	[68]
MeTyr(Me), Lys(Nic), D-Lys(Nic)	[83]
Position 3	
Arg-Tyr-X^3-Pro-Thr; X^3 =	
D-Leu, Ala	[27]
Ser	[79]
Acp, Ach, Pro	[74,93]
Positions 4 or 5	
Arg-Tyr-Leu-X^4-Thr; X^4 =	
D-Pro	[27]
Pro(4-OMe); Thz; β*homo*-Pro; Ach; Sar	[74,82]
Arg-Tyr-Leu-Pro-X^5; X^5 =	
D-Thr; Ser; Thr-NH_2	[27,79,83]
MeThr	[67]
D-Val	[83]
Other proctolin analogues	
Arg-Tyr-Leu-Pro-Thr-NH_2, Tyr-Leu-Pro-Thr, Leu-Pro-Thr	[27]
Arg-Tyr-Leu-Pro-Thr-Ala	[79]
Tyr-Arg-Tyr, Leu-Pro-Thr, Tyr-Leu	[67]
H-Cys-Arg-Tyr-Leu-Pro-Thr-Cys-OH, Arg-Tyr-Leu-Pro	[79]
Phe(p-NH_2)-Leu-Pro-Thr, Phe(p-NO_2)-Leu-Pro-Thr, Phe(3-CO_2Et,4-OMe)-Leu-Pro-Thr	[69,74]
Arg-D-Tyr-Leu-D-Pro-Thr, Arg-Tyr-D-Leu-D-Pro-Thr	[83,91]

Functional Significance of the Amino Acid Residues in the Peptide Chain

Arg-1. In order to investigate the role of the *N*-terminal Arg residue of the proctolin for myotropic effect in insects, several groups have performed syntheses of analogues modified at position 1 (Tables 1 and 3). The myotropic effects were investigated *in vitro* on selected insects such as the cockroach hindgut or heart, yellow mealworm heart, locust (*L. migratoria*) oviduct or *S. gregaria* foregut.

It was shown in earlier studies, that only [Lys^1]-proctolin and L-arginyl-proctolin (Arg-Arg-Tyr-Leu-Pro-Thr) [21,27] had full proctolin activity at 10^{-8} M in the cockroach hindgut. Other proctolin analogues were practically inactive (Table 3). These observations were explained by Sullivan and Newcomb [21] in terms of the presence of two basic areas of the *N*-terminal amino acid (Arg or Lys) which are not required for full intrinsic activity although they may promote high affinity binding with a receptor on the target tissue. Earlier work of Starratt and Brown [27] seemed to support this hypothesis. It was also supported by further investigations [52,54] performed on proctolin modified at position 1 (Tables 1 and 3). *N*-terminal substitution and addition of analogues L-arginyl-proctolin, [Lys^1]-, [Har^1]-, [Can^1]- and [Orn(iPr)^1]-proctolin [74,75] were active in insect hindgut or heart. Among them, [Lys^1]-and [Har^1]-proctolin stimulated the hindgut and foregut of the cockroach *B. craniifer* [76,77]. The lack of cardiotropic activity in *P. americana* and *T. molitor* was observed in the case of analogues containing two basic areas (provided by α- and ω-amino functions or a ω-guanidine and α-amino group) in position 1, such as His, Phe(p-NH_2), Gac, Gav or Gap. On the other hand, *N*-terminally modified proctolin analogues as L-arginyl-proctolin, Har^1 and Can^1-derivatives (contaning a guanidine system in the side chain) preserved full proctolin myostimulatory properties in insects (hindgut, heart or oviduct) [40,52–54,78]. In a further series of proctolin analogues [75] containing an Arg isostere, only

Table 2 Myotropic Activities of Proctolin Analogues Modified at Position 2, in Insects (*in Vitro*)

Peptide	Myotropic effect in insects						
Arg-X-Leu-Pro-Thr	*P. americana*		*T. molitor*	*L. migratoria*	*S. gregaria*	*B. craniifer*	
	Hindgut[a]	Heart[a]	Heart[b]	Oviduct[a]	Foregut[c]	Hindgut[b]	Foregut[a]
X =							
Phe(OMe)	sag [27]	sag [51]	sag [51]	ag [78]	np	np	np
Phe(OEt)	–	ag [69]	in [69]	np	sag [69]		
Phe(*p*-NH_2)	–	sag [51]	sag [51]	ag [78]	sag [69]	ag [77]	ag [77]
Phe(*p*-NO_2)	–	sag [51]	sag [51]	ag [78]	ag [31]	–	–
Phe(*p*-NMe_2)	–	sag [51]	ag [51]	ag [78]	–	–	–
Dopa	–	sag [85]	ag [85]	ag [78]	wag [55]	ag [76]	ag [76]
Tyr(*m*-NH_2)	–	ag [31]	ag [31]	–	–	–	
Tyr(*m*-NO_2)	–	ag [31]	ag [31]	–	–	–	–
Tyr(*m*-I), Tyr(3,5-di-I)	–	–	–	–	ag [67]	–	–
Phe(*p*-F)	–	–	sag [67]				
Afb(*p*-NO_2)	–	–	–	–	ant [85]	–	–
α-MeTyr	–	–	–	–	ant [67,70]	–	–
Phe(*p*-Cl)	–	–	wag [94]	–	wag [94]	–	–
D-Phe(*p*-Cl)	–	–	in	–	wag [94]	–	–
D-Phe(*p*-NH_2)	–	–	wag [94]	–	ant [94]	–	–
MeTyr	–	–	in	–	ag [67]	–	–
D-3-Pal	–	–	in	–	ag [94]	–	–
Nal	–	–	in	–	wag [94]	–	–
D-Nal	–	–	wag [94]	–	wag [94]	–	–
D-Phe(*p*-NO_2)	–	–	wag [94]	–	wag [94]	–	–

sag, super-agonist – more active than proctolin; ag, 50–100% of proctolin activity; wag, retained 20–40% proctolin activity; in, not active; ant, antagonist.
Biological effects (at max. concentration): [a] 10^{-9} M; [b] 10^{-8} M; [c] 10^{-7} M.

[Orn(iPr)[1]]-proctolin showed agonistic activity in the heart of *T. molitor* and foregut of *S. gregaria*. Arg-1 is clearly important for the myotropic activity of proctolin in insects.

Tyr-2. The role of the aromatic acid residue at position 2 has been more fully discussed in the literature [21,27,50,67,79]. Thus, replacement of the Tyr[2] by Phe or D-Tyr resulted in loss of stimulatory activity on the proctodeal muscle on *P. americana* [21,27]. A surprising result was that substitution of Phe(*p*-OMe) for Tyr increased activity almost threefold, suggesting that the free hydroxyl group of Tyr is not essential for myotropic action [27] (Table 2). All these observations enabled a hypothesis that the myotropic activity of proctolin depends on the residue in position 2, and on the presence of an oxygen atom in a *para* position on a phenyl ring [21]. To verify this assumption, syntheses of further proctolin analogues were carried out [50–52,54,64,69] by successive replacement of the Tyr[2] residue by Phe(*p*-NH_2), Phe(*p*-NMe_2), Phe(*p*-NO_2), Phe(*p*-OMe), Phe(*p*-OEt), Phe(*p*-Gn), Afb(*p*-OH), Afb(*p*-NO_2), Dopa, Tyr(*m*-NH_2), Tyr(*m*-NO_2) and Cha(4-OMe) (Table 2). When evaluated for cardioexcitatory activity in the heart of *P. americana* and *T. molitor* according to the method of Rosiński and Gäde [40], the majority of the analogues increased the heart-beat frequency of both insect species in a manner comparable with, or stronger than proctolin itself. [Dopa[2]]-proctolin [54] exhibited particularly high activity at the physiological concentrations (Table 2), causing a stronger chronotropic effect against the *P. americana* heart than proctolin, whereas in the case of the *T. molitor* heart or oviduct and *L. migratoria* oviduct [78] this type of action was considerably decreased. Similar species specificity was noticed for [Tyr(*m*-NO_2)[2]]-proctolin, which accelerated *P. americana* heart contractions but did not have any effect on the heart-beat frequency of *T. molitor*. On the other hand, [Tyr(*m*-NH_2)[2]]-proctolin has proctolin activity against the heart of both insect species [31]

Table 3 Other Proctolin Analogues with Myotropic Effects in Insects (*in Vitro*)

Peptides	Myotropic effect in insects						
	P. americana		*T. molitor*	*L. migratoria*	*S. gregaria*	*B. craniifer*	
	Hindgut[a]	Heart[a]	Heart[b]	Oviduct[a]	Foregut[c]	Hindgut[b]	Foregut[a]
Arg-Arg-Tyr-Leu-Pro-Thr	ag [21]	–	–	–	–	–	–
Arg-Tyr-Thr	in [28]	–	–	–	ant [67]	–	–
Cyclo-Arg-Tyr-Leu-Pro-Thr	–	–	–	–	ant [67]	–	–
Tyr(*m*-NH_2)-Leu-Pro-Thr	–	ant [91]	ant [51]	–	–	–	–
[Ala^1]-proctolin	in [28]	–	–	ag [26]	–	–	–
[Har^1]-proctolin	–	ag [91]	ag [51]	ag [78]	–	ag [76]	ag [76]
[Lys^1]-proctolin	ag [21]	ag [91]	ag [51]	ag [78]	–	ag [76]	ag [76]
[Can^1]-proctolin	–	ag [82]	ag [82,94]	–	–	–	–
[Orn(Nδ-iPr)^1]-proctolin	–	–	ag [75]	–	ag [75]	–	–
[$MeArg^1$]-proctolin	–	–	ag [75]	–	–	–	–
[Val^3]-proctolin	–	ag [36]	ag [93]	–	–	–	–
[Gly^3]-proctolin	–	wag [36]	wag [93]	in [79]	–	–	–
[Ala^3]-proctolin	–	–	–	ag [79]	–	–	–
[Hyp^4]-proctolin	–	ag [93]	–	–	–	–	–
[Ala^5]-proctolin	–	–	–	ag [79]	–	–	–
[$MeThr^5$]-proctolin	–	–	–	–	ag [67]	–	–
[Val^5]-proctolin	–	–	ag [83]	–	ag [83]	–	–
[Ile^5]-proctolin	–	–	wag [83]	–	wag [83]	–	–
[D-Ile^5]proctolin	–	–	in [83]	–	wag [83]	–	–
[Asn^5]-proctolin	–	–	wag [83]	–	wag [83]	–	–
[Gln^5]-proctolin	–	–	in [83]	–	ag [83]	–	–
[Ser^5]-proctolin	–	–	ag [83]	–	in [83]	–	–
D-Arg-D-Tyr-D-Leu-D-Pro-D-Thr	–	–	sag [95]	–	–	–	–
D-Arg(NO_2)-Tyr-D-Leu-Pro-Thr	–	–	sag [95]	–	–	–	–
D-Arg-Tyr-D-Leu-Pro-Thr	–	–	sag [95]	–	–	–	–
Arg-D-Tyr-Leu-Pro-D-Thr	–	–	ag [95]	–	–	–	–

sag, super-agonist – more active than proctolin; ag, 50–100% of proctolin activity; wag, retained 20–40% proctolin activity; in, not active; ant, anagonist.
Biological effects (at max. concentration): [a] 10^{-9} M; [b] 10^{-8} M; [c] 10^{-7} M.

and the locust oviduct [78]. For [Cha(4-OMe)^2]-proctolin [54] and [Afb(*p*-X)^2]-proctolin, where X = –OH, $–NH_2$, or $–NO_2$, no cardiotropic action was observed in *P. americana* and *T. molitor* heart. Thus, substitution of the Afb(*p*-OH) residue for Tyr elongated the proctolin peptide chain by one $–CH_2–$ group which led to the loss of myotropic activity in insects. In further investigations Hinton *et al.* [31] found that [Afb(*p*-NO_2)^2]-proctolin had an antagonistic effect against the foregut of *S. gregaria*. Proctolin analogues modified in position 2 have also been the subject of recent investigations by other groups [31,67,68,72,73,78] (Table 2). In a myotropic test performed on the *L. migratoria* oviduct [78] only [Phe(*p*-NO_2)^2]-, Phe(*p*-NH_2)^2- and [$Dopa^2$]-proctolin showed proctolin agonistic activity, whereas the

[Phe(*p*-OMe)^2]-analogue revealed super-agonistic properties [27] similar to effects described for other bioassays [50] in insects.

Osborne and co-workers have also performed studies of proctolin derivatives, modified at the Tyr^2 residue in position 2 [31,73]; they studied [MeTyr^2]-, [Phe(*p*-F)^2]-, [Tyr(*m*-I)^2]-, [Tyr(3,5-I_2)^2]- and [α-MeTyr^2]-proctolin. All these analogues, except [MeTyr^2]- and [α-MeTyr^2]-proctolin, showed agonistic activity. The agonistic properties of analogues iodinated at the aromatic ring of Tyr-2 suggest a possible use of these [^{125}I]-derivative to identify and characterize the proctolin receptor [67]. These remarks are supported by studies of Puiroux *et al.* [79] on the proctolin receptor in the oviduct of *L. migratoria*, where iodinated proctolin derivatives have high myostimulatory activity. [α-MeTyr^2]-Proctolin [67] (a peptide with no agonistic properties) has high antagonistic activity on the foregut of *S. gregaria*. Further, Osborne *et al.* [31] found that [Afb(*p*-NO_2)^2]-proctolin (an analogue synthesized earlier [50,69]) also showed the antagonistic activity. In further studies conducted by Loughton's group, [Ser^2]- and [Tyr-(PO_3H_2)^2]-proctolin were practically inactive in relation to the *L. migratoria* oviduct [68]. Lack of myotropic activity was also observed in the case of a proctolin derivative with the peptide bond isostere –CH_2–O– between Tyr-2 and Leu-3 [72]. Recently, we performed syntheses of further proctolin analogues, modified in position 2 by Phe(*p*-Cl), D-Phe(*p*-Cl), [MeTyr, D-Phe(*p*-NH_2), D-Phe(*p*-NMe_2), MeTyr(OMe), D-3-Pal, Nal, D-Nal, Lys(Nic), D-Lys(Nic) and D-Phe(*p*-NO_2) replacement [80] (Table 2). When applied to the heart of *T. molitor* at physiological concentrations ranging from 10^{-9} to 10^{-7} M, these peptides (Table 2) retained weak (15–20%) proctolin activity. The others were practically inactive. It is interesting that analogues containing D-Phe(*p*-X) residues, where X = –NH_2, –NMe_2 or –NO_2, weakly stimulated the heart-beat frequency in *T. molitor*. In our earlier work [74] we found that proctolin analogues containing L-Phe(*p*-X) derivatives showed a high cardioexcitatory effect in the yellow mealworm. Results presented here testify that the L-configuration of the amino acid residue in position 2 of the proctolin skeleton plays an important role for cardiostimulatory activity. On the other hand, a weak cardiotropic effect of [Nal^2]-proctolin in *T. molitor* showed that the presence of a *p*-substituted phenyl ring at position 2 of proctolin is also important for this activity. In a myotropic test on the foregut of *S. gregaria*, we found that the analogues [Phe(*p*-Cl)^2]-, [D-Nal^2]- and [D-Phe(*p*-NO_2)^2]-proctolin retained 15–25% of proctolin-like activity, whereas [D-Phe(*p*-Cl)^2]-, [Phe(*p*-NH_2)^2]- and [D-3-Pal^2]-proctolin had weak inhibitory properties on contraction of the locust foregut in the 10^{-7}–10^{-6} M range. Other peptides had neither agonistic nor antagonistic activity. In addition [Phe(*p*-NH_2)^2]- and [D-3-Pal^2]-proctolin reduced the maximum response to applied proctolin by 64 and 49%, respectively, at 10^{-6} M. In the [Phe(*p*-NH_2)^2]- and [D-3-Pal^2]-analogues the basic character of the two side chains is probably responsible for their myotropic effect in locust foregut.

In conclusion, one can infer that the myotropic effects of proctolin depend on the presence of a polar *para* substituent on the aromatic side chain at position 2, i.e. oxygen, nitrogen or halogen atoms. The presence of the oxygen and nitrogen lone pairs most likely facilitates interaction with the receptor site in both insects bioassayed. Some other substituents such as hydroxyl, amino, or nitro groups in the *m*-position of the phenyl ring cause a distinct increase in myotropic activity in the cockroach or locust, but lead to its impairment in the yellow mealworm. Species specificity is also shown by [Phe(*p*-OEt)^2]-proctolin, which had chronotropic activity against the heart of *P. americana* but in the case of *T. molitor* the heart was inactive [50]; it showed supra-agonistic activity in relation to the locust (*S. gregaria*) foregut [31]. On the other hand, elongation of the proctolin chain at position 2 by a methylene group at the α-position in the case of [Afb(*p*-NO_2)^2]-proctolin led to loss of cardiotropic activity in cockroach and mealworm, though this analogue had an antagonistic effect on the locust foregut. The antagonistic effects observed in [α-MeTyr^2]- and [Afb(*p*-NO_2)^2]-proctolin are probably a result of the presence of methyl or methylene groups at the α-C of Tyr-2 and that modification is sufficient to obtain structural analogues with antagonstic properties. This opinion finds support in Cameron and Khambay's work on a proctolin analogue with the peptide bond between Tyr-2 and Leu-3 was replaced by an isosteric system [72]. This modification led to an inactive compound in relation to the foregut of *S. gregaria*, perhaps due to a change in the accessibility of the biologically active proctolin conformation. Moreover, recent studies [80] show that the presence of a naphthalene system in the side chain in position 2 of the proctolin peptide chain is sufficient for preservation of agonistic activity in the locust. The antagonistic properties in the locust were mainly evoked by proctolin analogues modified in position 2 of the peptide skeleton with replacement

by *para* amino substituted D-Phe or a D-amino acid containing pyridine ring in its side chain.

Leu-3. The role of Leu-3, has so not far been discussed in detail. Two peptides [D-Leu^3]- and [Ala^3]-proctolin were inactive by the *P. americana* hindgut stimulatory test of [27]. Studies on the significance of Leu-3 for myotropic function in insects may shed some light on the hydrophobic interaction of proctolin with its receptor site. Moreover, it should be pointed out that the Leu residue in proctolin is situated between a Tyr-residue, which plays an important role in creating cardiotropic activity in insects [50,67,81] and a Pro-residue, which is responsible for stabilization of the biologically active proctolin conformation [81]. In further studies, a series of three modified proctolin analogues was synthesized by replacement of Leu-3 by the following: Val, Thr, Gly, Pro, Acp, Ach [50], and MeLeu [67] (Table 3). Their biological activities were evaluated in a myostimulatory test on the heart of *P. americana* or *T. molitor* [40] or the foregut of *S. gregaria* [67]. Species specificity was observed: [Val^3]- and [Gly^3]-proctolin preserved 55 and 30%, respectively, of proctolin cardiotropic activity in *T. molitor*, whereas the other analogues were inactive. In the case of *P. americana* the [Val^3]-proctolin had 25% of the native peptide's activity. On the other hand, [MeLeu^3]-proctolin preserved almost full proctolin myotropic activity in the foregut of *S. gregaria* [67]. These results show that the Leu-3 plays an essential role in the hydrophobic interaction of proctolin with its receptor site.

Pro-4 and Thr-5. In earlier work only two analogues modified in position 4, [Ala^4]- and [D-Pro^4]-proctolin, and three modified in position 5, [Ala^5]-, [D-Thr^5]- and [Thr^5]-proctolin amide, have been described. None of them had any myotropic activity in the cockroach hindgut [27] (Table 1). Theoretical conformational analysis of the proctolin molecule implied that the presence of the Pro-4 is necessary for stabilization of its biologically active conformation [81]. On this premise Konopińska *et al.* [82] have performed further studies on proctolin analogues modified in position 4 (Tables 1 and 3), with replacement such as β*homo*-Pro, Hyp and Pro(4′-OMe), Thz, Ach, or Sar. Only [Pro(4-OH)^4]-proctolin retained a weak agonistic proctolin activity, whereas the other analogues were inactive in the cardiotropic test. These results suggest that the presence of Pro-4 is essential for the cardiotropic properties of proctolin in insects. Among the proctolin analogues modified in position 5 of the peptide skeleton (Table 3) only *C*-terminally modified analogues with Ala [27], MeThr [67], Val, Ile, D-Ile, Asn, Gln or Ser [83] replacements showed agonistic or weak-agonistic effects on the oviduct of *L. migratoria* [79] or on the foregut of *S. gregaria* [67,83] or in the heart of *T. molitor* [83]. These data suggest that the *C*-terminal Thr residue plays an important role for myotropic activity in insects.

Other proctolin analogues. In Table 1 and Table 3 proctolin analogues with various modifications are presented. There are analogues with an elongated or truncated proctolin backbone; cyclo-analogues, such as 'cyclo-proctolin' cyclized head to tail and H-Cys-proctolinyl-Cys-OH; and analogues with native L-amino acids replaced by D-isomers in two or all positions. The aim was to search for structural agonists or antagonists in order to define the proctolin receptor site in insects. Proctolin agonistic properties in the cockroach hindgut were retained by the elongated analogue L-arginyl-proctolin [21] and by some analogues containing more than one D-amino acid residue, such as [D-Arg^1, D-Pro^4]-proctolin, whereas D-Arg-D-Tyr-D-Leu-D-Pro-D-Thr-OH, [D-Arg^1, D-Leu^3]-, [D-Arg(NO$_2$)^1, D-Leu^3]- and [D-Tyr^2, D-Thr^5]-proctolin acted as supra-agonistic peptides, in yellow mealworm heart [66]. But two truncated proctolin analogues, H-Tyr(*m*-NH$_2$)-Leu-Pro-Thr-OH [23] and H-Arg-Tyr-Thr-OH [67] showed weak antagonistic activity in relation to the cockroach and mealworm hearts or the locust foregut. Cyclo-proctolin [67,73] produced a weak antagonistic response on the foregut of *S. gregaria* and reduced the response to proctolin up to 37% at a 10^{-6} M concentration. The other analogues listed in Table 1 were inactive, including H-Cys-proctolinyl-Cys-OH [79]. It is clear that the search for proctolin antagonists remains an open field.

CONFORMATIONAL STUDIES ON PROCTOLIN

The first theoretical conformational analysis of proctolin was performed in 1979 by Bertins and Nikiforovich [81]. These studies suggested that proctolin prefers a folded conformation which is stabilized by a salt bridge between the –COOH group of Thr-5 and the ω-guanidine system of Arg-1. To confirm this, Nikiforowich synthesized an analogue cyclized between the L-lysine side chain and the *C*-terminal carboxyl group. This compound was completely inactive (Nikifiorovich GV, personal communication, 1992). Later, theoretical conformational analysis of proctolin and some of its active

analogues modified in positions 1 or 2 were carried out by Angielski *et al.* [84]. In these studies, five families of low-energy conformations common for most analogues were found, but it was difficult to propose a biologically active conformation for a small flexible peptide like proctolin. The similarity to another linear biologically important pentapeptide, enkephalin is profound [84]. Further conformational studies on proctolin and a series of selected analogues were performed by spectroscopic methods, NMR or CD [85,66,84] (Figure 1). The subject of our preliminary conformational studies on proctolin was a series of analogues containing D-residues, all D-, [D-Arg^1, D-Leu^3]-, [D-Arg(NO$_2$)^1]-, [D-Arg^1,Pro^4]-, and [D-Tyr^2, D-Thr^5]-proctolin [66]. These peptides showed unexpected super-agonistic activity in relation to the heart of insects and prompted us to perform the conformational studies by NMR and CD methods in D_2O. In these studies we observed that both ordered and unordered structures were present in the conformational equilibria of these peptides. The NMR results indicated that the all-D proctolin is a mirror image of the native proctolin. The conformational equilibria of the other proctolin analogues are studied apparently dominated by ordered structures of the β-turn type. Besides proctolin, Osborne's group [85] studied its *N*-methylated derivatives [MeTyr^2]-, [MeLeu^3]- and [MeThr^5]-proctolin in solution by NMR. They postulated from NOE measurements that proctolin exists in similar conformations in $(CD_3)_2SO$ and CD_3OH, with one predominant (90%) form. The proline residue was shown to have a *trans* geometry. The evidence for a *trans*-proline conformation is significant for the solution structure because of the strong conformation-directing properties of Pro. There was little secondary structure detectable for proctolin, although an ionic interaction between the α-NH_2 of Arg^1 and -COO^- of Thr^5 is possible. The conformation of [MeTyr^2]-proctolin is also stabilized by a salt bridge between the α-NH_2 of Arg-1 and the Thr-5 –COO^-. In conformational studies performed on cyclo-proctolin, Hinton *et al.* [70] postulated that cyclo-proctolin exists in a conformation with resembles a β-bulge loop and displays a *cis*-Pro geometry. At the moment it is very difficult to say which conformation of proctolin is important for the creation of myotropic properties in insects. Further work is obviously desirable.

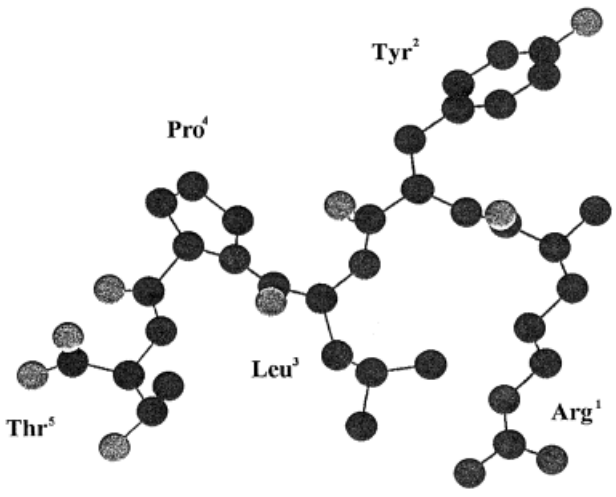

Figure 1 The conformation of proctolin in acetonitrile.

PROCTOLIN RECEPTOR(S) AND MODE OF ACTION

Proctolin analogue structure–activity relationship studies had demonstrated the importance of each amino acid residue for bioactivity [21,27,50–53,67,69,71,76–79,86,87]. However, these studies were unable easily to recognize binding in the absence of bioactivity. The development of a proctolin binding assay allowed a comparison of binding affinity with biological activity and provided further grounds to distinguish between proctolin receptors. Characterization of the proctolin receptor using ligand-binding studies began with Banner *et al.* [88], who showed that [^{125}I]-proctolin exhibited dose-dependent binding to membranes isolated from the hindgut of *S. gregaria*. Subsequently, studies on the binding of [^{3}H]-proctolin to hindgut and oviduct membranes from *L. migratoria* [31,79,89] and the binding of tritiated proctolin to foregut and hindgut membranes from the cockroach *Blaberus craniifer* [76] were made. These studies suggested the presence of two proctolin receptor subtypes on locust oviduct but not on hindgut membranes [31,79,89]. In *Blaberus* foregut and hindgut assays, Mazzocco-Manneval *et al.* [76,77] inferred the possible presence of two classes of proctolin receptors, pharmacologically distinguishable with analogues, but exhibiting the same binding characteristics (K_d and B_{max}) for tritiated proctolin.

The mode of action of proctolin has been investigated by studying the second messenger-pathways associated with proctolin receptors on several tissues and insects. In most tested skeletal and

visceral muscles, proctolin action is associated with a phospholipase C transduction pathway [61,79,90] and the peptide does not appear to act via c-AMP or c-GMP [37,76]. Lange [74] reported that proctolin stimulated the release of inositol 1-phosphate (IP_1), inositol 1,4-biphosphate (IP_2) and inositol 1,4,5-triphosphate (IP_3) from *Locusta* oviduct and the effects were enhanced significantly when the tissue was incubated in the presence of lithium ions. In another study Bains and Dower [91] showed that the effect of proctolin on maintained tension in locust mandibular closer muscles was facilitated by an influx of extracellular calcium, which could be blocked by the calcium channel blocker verapamil. This effect of proctolin was mimicked by incubation with inositol 1,4,5-trisphosphate and phorbol-12,13-dibutyrate [91]. Recently, Hinton and Osborne [92] have shown that proctolin-stimulated inositol 1,4,5-triphosphate and 1,3,4,5-tetrakisphosphate production in homogenates from the foregut of *S. gregaria* was potentiated significantly by Li^+ and blocked by [α-MeTyr^2]-proctolin. Moreover, Mazzocco-Manneval *et al.* [76] observed that proctolin induced hydrolysis of phosphoinositides in the foregut of the cockroach *B. craniifer* is increased in the presence of GTPs which confirms the notion that proctolin receptors belong to the group of receptors with seven transmembrane domains and associated to heterotrimeric G-proteins.

CONCLUSIONS

In summary:

1. of 100 synthetic proctolin analogues, 45 showed myotropic activity in insects;
2. 40 of these analogues are proctolin agonists and five are antagonists, [α-MeTyr^2]-proctolin being the most potent antagonist so far discovered;
3. both cardioexcitatory and myostimulatory effects depend on the presence and the type of the *para* substituent at position 2 of the proctolin sequence [50], and on the presence of the peptide bond hydrogen atom of the peptide bonds between Tyr-2 and Arg-1 [85] and between Tyr^2-Leu^3 [72];
4. additional substitution at the *meta* position of the Tyr phenyl ring probably facilitates interaction with the receptor site;
5. the Arg^1, Leu^3 and Pro^4 residues in proctolin molecule are essential for its myotropic activity;
6. the myotropic effect of selected analogues on the locust oviduct or foregut bioassay suggest that there are probably sub-types of proctolin receptors in insects;
7. highly potent antagonistic properties are shown by proctolin analogues with the CH_3– or -CH_2– groups at C-α of the Tyr^2 residue, or weakly antagonistic activity was observed in cyclo-proctolin (an analogue with a rigid conformation), H-Arg-Tyr-Leu-OH and H-Tyr(*m*-NH_2)-Leu-Pro-Thr-OH;
8. in conformational studies, an ordered structure is observed for proctolin and some of its analogues;
9. some proctolin analogues containing two or all D-residues have an unexpected super-agonistic activity on the heart of insects;
10. besides biological activity in insects, proctolin and some of its analogues have significant biological effects in mammals, manifested by analgesic or potent antagonistic activity in relation to the opiate receptor in rats.

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