

Pachymedusa dacnicolor tryptophyllin-1: structural characterization, pharmacological activity and cloning of precursor cDNA

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Abstract

Tryptophyllins are a heterogeneous group of amphibian skin peptides originally identified in skin extracts of Neotropical leaf frogs, *Phyllomedusa* sp., by chemical means. Until now, biosynthetic precursor structure and biological activity remain unreported. Here we describe the isolation of a novel, post-translationally modified tryptophyllin, Lys-Pro-Hyp-Ala-Trp-Val-Pro.amide (PdT-1), from the skin secretion of the Mexican leaf frog, *Pachymedusa dacnicolor*. Using a 3'- and 5'-RACE strategy and an in vitro skin cDNA library, the PdT-1-encoding precursor was cloned and found to consist of an open-reading frame of 62 amino acids with a single copy of PdT-1 located towards the C-terminus. A synthetic replicate of PdT-1 was found to be a potent myoactive agent, relaxing mammalian arterial smooth muscle and contracting small intestinal smooth muscle at nanomolar concentrations. PdT-1 is thus the first amphibian skin tryptophyllin to be pharmacologically characterized and the first whose precursor cDNA has been cloned.

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1. Introduction

Amphibian skin is, in terms of morphology, biochemistry and physiology, a complicated organ that performs a plethora of functions dedicated to the survival of the organism in what are often extreme environmental conditions [1–3]. The complex cocktails of bioactive molecules contained in amphibian cutaneous secretions appear to have evolved for passive chemical defense against predators [1–3]. The individual components belong to many classes of biochemical including biogenic amines, peptides, proteins, alkaloids and heterocyclics and as a consequence, each secretion possesses a wide spectrum of bioactivities [1–3].

Amphibian skin peptides have been the object of intense research interest for many years from both academic and pharmaceutical groups due to their potential applications in biophysical research, biochemical taxonomy and in lead compound development for new pharmaceuticals [4–7].

More than 200 species of American amphibians have been subjected to biological screening to determine if their skin extracts contain peptides with activities on a variety of smooth muscle types, systemic blood pressure, exocrine secretion, anterior pituitary secretion and the central nervous system [8].

This study highlighted the New World phyllomedusine leaf frogs, represented by species distributed from Mexico to Argentina, as “huge storehouses of biologically active peptides” due to the fact that they contained the greatest variety and abundance of such molecules ever found in any amphibian stock from around the world [9]. The subfamily Phyllomedusinae contains three well-known genera, Phyllomedusa, Agalychnis, Pachymedusa, and three less well-known genera, Hylomantis, Phasmahyla and Phrynomedusa, found only thus far in Brazil and until now unstudied [10]. Numerous peptides have been isolated from the former genera of leaf frogs (predominantly *Phyllomedusa* sp.) and clearly these can be classified, on a primary structural basis, into seven main families [8]. These are the caeruleins (phyllocaerulein) [11], the tachykinins (phyllomedusin) [12,13], the bombesins (phyllolitorin, [Leu8]-phyllolitorin,

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rohdeilitorin) [14,15], the bradykinins (phyllokinin) [16], the sauvagines [17], the dermorphins (dermorphin, [Hyp6]-dermorphin) [18] and the tryptophyllins [19,20].

The tryptophyllins are a large but heterogenous peptide family first isolated from *Phyllomedusa rohdei* skin [19] and subsequently from the skin of *Phyllomedusa sauvagei* and *Phyllomedusa bicolor* [3]. Most tryptophyllins have a common tryptophanyl (Trp) residue at position 2 from the C-terminus and one or two prolyl (Pro) residues, at position 2 (and 3) from the N-terminus [9]. Tryptophyllins are of particular interest because they were the first example of frog skin peptides identified on a solely chemical basis by a colour reaction for indoles on paper chromatograms. Curiously, they were inactive when tested in smooth muscle and systemic blood pressure bioassays [3].

Here we describe the structure of a novel tryptophyllin (PdT-1) from the skin secretion of the Mexican leaf frog, *Pachymedusa dacnicolor*, and the structure of its biosynthetic precursor deduced from cloned skin cDNA. In addition, we describe for the first time, the potent myoactive effects of a synthetic replicate of PdT-1, using isolated mammalian arterial and small intestinal smooth muscle preparations.

2. Materials and methods

2.1. Specimen biodata and secretion acquisition

Specimens of *P. dacnicolor* ($n=3$) were obtained from a commercial source and had been captive bred in the United States. The frogs were metamorphs (2-cm snout to vent length) on receipt and were grown to adult size (8-cm snout to vent length) over a 2-year period prior to secretion harvesting. They were maintained in our purpose-designed amphibian facility at 20–25 °C under a 12-h/12-h light/dark cycle and fed multivitamin-loaded crickets three times per week. Skin secretion was obtained from the dorsal paratoid folds by transdermal electrical stimulation (6 v DC, 4-ms pulse-width, 50 Hz) through platinum electrodes for two periods of 15-s duration [21]. The obvious viscous white secretion was washed from the skin using deionized water, snap-frozen in liquid nitrogen and lyophilized. Lyophilizate was stored at –20 °C prior to analysis.

2.2. Identification and structural analysis of PdT-1

Five milligrams of freeze-dried secretion was reconstituted in 0.5 ml of trifluoroacetic acid (TFA)/water (0.1:99.9, v/v), clarified by centrifugation and subjected to LC/MS using an LCQTM electrospray ion-trap mass spectrometer interfaced with a gradient HPLC system (both supplied by ThermoFinnegan, San Jose, CA, USA). The gradient employed was formed from (TFA)/water (0.1:99.9, v/v) to TFA/water/acetonitrile (0.1:19.9:80.0, v/v/v) in 240 min at a flow rate of 1 ml/min. The effluent from the chromatographic column was flow split with 10% of flow entering

the mass spectrometer and 90% routed to a fraction collector. Fractions (900 µl) were collected automatically at minute intervals. Following MS/MS fragmentation of peptides in individual chromatographic fractions by direct infusion onto a Q-TOF Ultima mass spectrometer (Micromass, UK), fraction #67 was selected for further study due to its tryptophyllin-like characteristics. These were a low mass (808 Da) and the presence of tryptophanyl and prolyl residues in its structure as implied by identification of corresponding immonium ions in MS/MS spectra. The primary structure of this peptide was deduced by automated Edman degradation using an Applied Biosystems 491 Procise sequencer. *P. dacnicolor* tryptophyllin-1 (PdT-1) was subsequently synthesised by solid-phase fmoc chemistry using an Applied Biosystems 433 peptide synthesiser. Products were purified and structures confirmed by LC/MS/MS prior to lyophilization. For pharmacological experiments, standardization of each synthetic peptide was achieved by acid hydrolysis of a known gravimetric quantity of lyophilizate followed by amino acid analysis using an Applied Biosystems PTH-amino acid analyzer.

2.3. Cloning of PdT-1 cDNA

Dorsal skin was excised from two euthanized adult *P. dacnicolor*, frozen in liquid nitrogen and subsequently ground to a fine powder in this medium. Polyadenylated mRNA was isolated using magnetic oligo-dT beads as described by the manufacturer (Dynal Biotec, UK). The isolated mRNA was subjected to 5' and 3'-rapid amplification of cDNA ends (RACE) procedures to obtain full-length PdT-1 nucleic acid sequence data using a SMART-RACE kit (Clontech, UK) essentially as described by the manufacturer. Briefly, the 3'-RACE reactions employed a UPM primer (supplied with the kit) and a degenerate sense primer (S: 5'-AARCCICCGITGG-3') that was complementary to the amino acid sequence, Lys-Pro-Pro-Ala-Trp-, of PdT-1. The discrete band of approximately 180 bp that was obtained following the 3'-RACE reaction was gel-purified (Fig. 1) and cloned using a pGEM-T vector system (Promega) and sequenced using an ABI 3100 automated sequencer. The sequence data obtained from this 3'-RACE product was used to design a specific PdT-1 cDNA antisense primer (AS: 5'-GCTCCTCAGGGTTATGACATGTA-3') to a region of the 3'-non-translated region. 5'-RACE was carried out using this specific primer in conjunction with the UPM RACE primer and this produced a discrete band of approximately 300 bp that was gel-purified (Fig. 1), cloned and sequenced.

2.4. Smooth muscle pharmacology

Male albino Wistar rats (200–350 g) were euthanized by asphyxiation followed by cervical dislocation. The tail artery was prepared as previously described [22]. Incubation buffer was 95% O₂/5% CO₂ oxygenated Krebs' solution (NaCl 118 mM, KCl 4.7 mM, NaHCO₃ 25 mM, NaH₂PO₄ 1.15 mM,

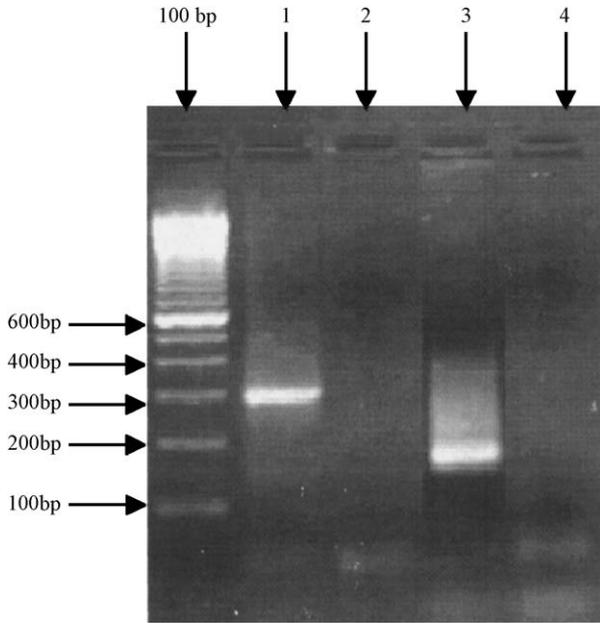


Fig. 1. Gel electropherogram of PdT-1 RT-PCR products generated from an in vitro skin library of *P. dancicolor*. Lane 1–5': RACE product from gene-specific primer. Lane 3–3': RACE product from degenerate primer. Lanes 2 and 4: respective non-template controls.

CaCl₂ 2.5 mM, MgCl₂ 1.1 mM, glucose 5.6 mM). Constriction or dilation of the arterial smooth muscle preparation was detected by an increase or decrease in pressure generated by water column displacement using pressure transducers connected to a MacLab System (AD Instruments, Australia). Data were displayed graphically on a Macintosh computer. Viability was determined using a range of bolus phenylephrine (5×10^{-6} – 1×10^{-3} M) exposures and the endothelial

layer of the artery was removed by bubbling with oxygen for 10 s. Absence of the endothelial layer was confirmed by the lack of relaxation in response to a 30-min perfusion of acetylcholine (5×10^{-5} M) after precontraction with phenylephrine (1×10^{-5} M). After perfusion of arterial preparations with 1×10^{-5} M phenylephrine to obtain constriction plateaux, relative relaxation was recorded following applications of PdT-1 in the range of 1×10^{-10} – 1×10^{-5} M. Peptide was added consecutively in increasing concentrations, each addition being followed by a wash out and equilibration phase.

For intestinal smooth muscle preparations, 1-cm segments of ileum were carefully placed onto the pins of a MacLab force transducer, one pin acting as a stationary fixed point while the second pin was free, permitting application of tension to the smooth muscle. The muscle segments were gradually exposed to 0.1 g increments in resting tension until the spontaneous contractions originated from a resting tension of 0.5 g. The contracting muscle preparations were allowed to stabilize for 25 min before the application of PdT-1. The intestinal smooth muscle preparations were exposed to peptide in the concentration range, 1×10^{-11} – 1×10^{-6} M, and relative changes in tension were recorded. Six replicates for each experimental data point were performed.

3. Results

3.1. Identification and structural analysis of PdT-1

A novel component of the skin secretion, identified by LC/MS, was a peptide of molecular mass 808 Da (Fig. 2)

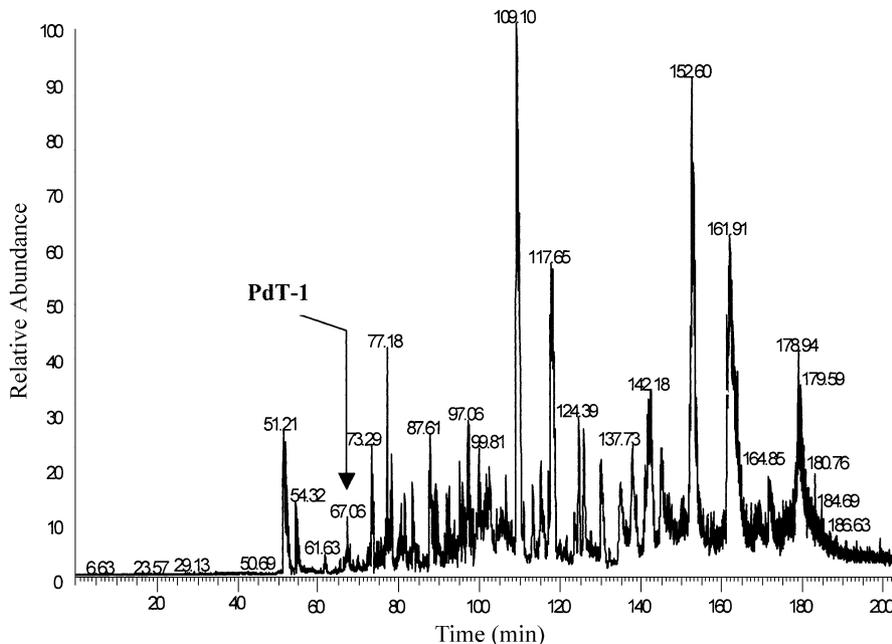


Fig. 2. LC/MS total ion count (TIC) spectrum of fractionated *P. dancicolor* skin secretion. The elution position of the novel peptide PdT-1 is indicated.

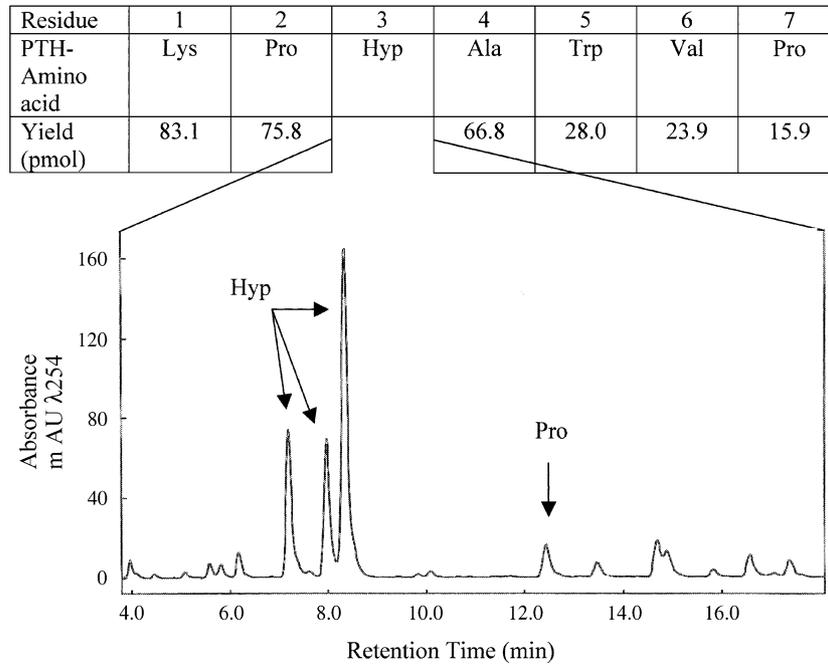


Fig. 3. Automated Edman degradation of the novel peptide PdT-1. The chromatogram of cycle #3 is shown to illustrate the unequivocal triplet-peak signature of PTH-hydroxyproline. The carry over of PTH-proline from cycle #2 is also indicated for reference.

whose primary structure was established by both MS/MS fragmentation and automated Edman degradation as: Lys-Pro-Hyp-Ala-Trp-Val-Pro.amide (SwissProt accession no. P83455) (Fig. 3). This novel tryptophyllin-related heptapep-

tide was named *P. dacnicolor* tryptophyllin-1 (PdT-1), reflecting the specific name of the species of origin. Comparison of the primary structure of PdT-1 with other previously reported tryptophyllins is shown in Fig. 4. From this,

T-1 peptides

Pachymedusa dacnicolor (PdT-1)
Phyllomedusa rohdei
Phyllomedusa sauvagei

Lys-Pro-Hyp-Ala-Trp-Val-Pro.NH₂
Lys-Pro-Hyp-Ser-Trp-Ile-Pro.NH₂
Lys-Pro-Pro-Pro-Trp-Val-Pro-Val
Lys-Pro-Pro-Pro-Trp-Ile-Pro-Val

T-2 peptides

P. rohdei

Phe-Pro-**Pro-Trp**-Val.NH₂
 Phe-Pro-**Pro-Trp**-Leu.NH₂
 Phe-Pro-**Pro-Trp**-Met.NH₂
 pGlu-**Pro-Trp**-Val.NH₂
 pGlu-**Pro-Trp**-Met.NH₂
 pGlu-Phe-**Pro-Trp**-Leu.NH₂
 Phe-**Pro-Trp**-Leu.NH₂
 Phe-Leu-**Pro-Trp**-Tyr.NH₂
 pGlu-Ile-**Pro-Trp**-Phe-His-Arg.NH₂

Litoria rubella

T-3 peptides

P. rohdei **pGlu-Glu-Lys-Pro-Tyr-Trp-Pro-Pro-Pro-Ile-Tyr-Pro-Met**
P. sauvagei **pGlu-Asp-Lys-Pro-Phe-Trp-Pro-Pro-Pro-Ile-Tyr-Pro-Val**
P. bicolor **pGlu-Glu-Lys-Pro-Phe-Tyr-Pro-Pro-Pro-Ile-Tyr-Pro-Val**

- conserved residues in each group in bold type.

Fig. 4. Comparison of the amino acid sequence of PdT-1 with those of other previously described tryptophyllins. These have been classified into three discrete groups, T-1, T-2 and T-3 peptides, based upon obvious structural similarities that are indicated in bold type. Previously sequenced tryptophyllins were either not appended a trivial name or named TPH-X, where X=the number of amino acid residues in the sequence.

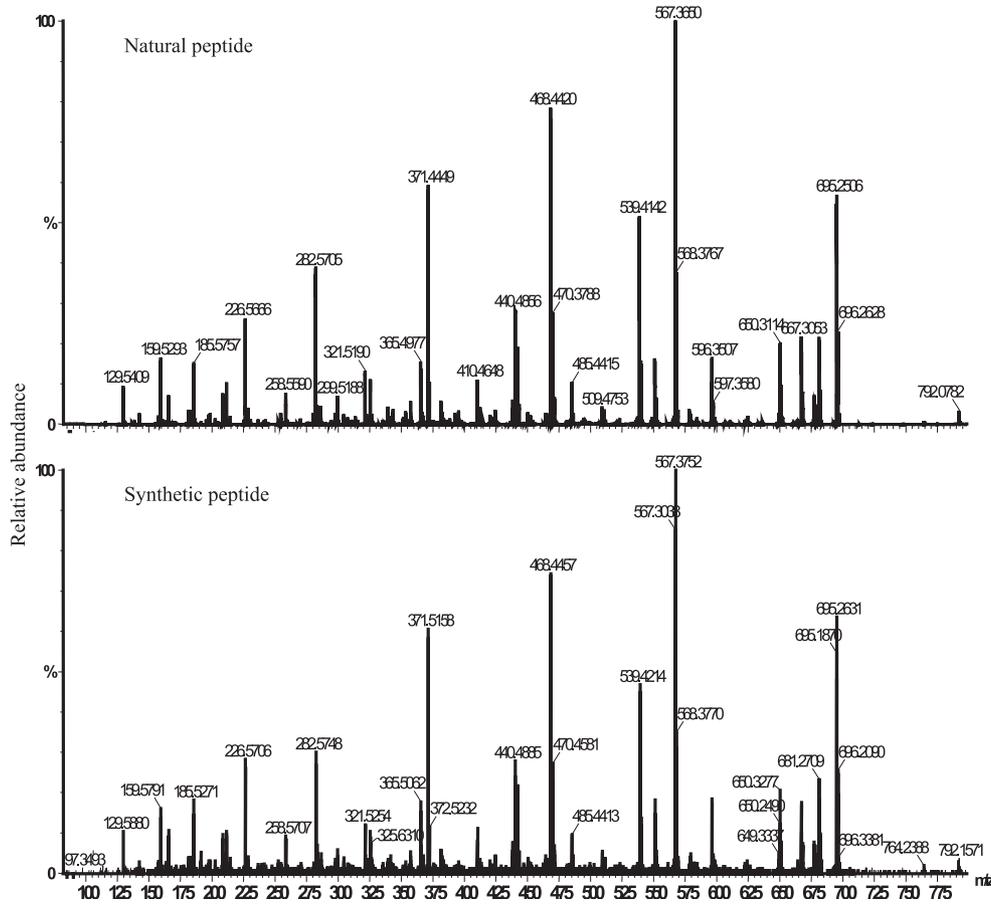


Fig. 5. MS/MS spectra of natural PdT-1 (upper panel) and synthetic PdT-1 (lower panel) obtained using a QTOF-Ultima mass spectrometer to confirm identity of both peptides.

one can see that all members of this heterogeneous family fall into three discrete groups (T1, T2 and T3). In other words, there are actually three different peptide families hitherto

considered as one. Following unequivocal establishment of primary structure and post-translational modifications, PdT-1 was subsequently successfully batch-synthesized (250

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1   GATCCTATTC TGTGGTGCC AGGACTCAGC ACTTCTGAA TCATCAGACC
       M N F L K K S L F L V L F L G
51  AAATATGAAT TTCTGAAGA AGTCGCTTTT CCTGTCTG TTCCTCGGAT
       F V S I S F C D E E K R Q D D D E
101 TCGTTTCAT TTCCTTCTGT GATGAAGAGA AAAGACAGGA TGACGATGAG
      G N E R E E K K E I Q E D G N Q E
151 GGAATGAGA GAGAGAAAA GAAAGAAAT CAAGAAGACG GAAATCAAGA
      E R R D K P P A W V P G K *
201 AGAAAGAAGA GATAAGCCTC CTGCTTGGGT TCCTGAAAA TAGTTACATG
251 TCATAACCCT GAGGAGCAA ATTATCGGTA ATAGTGCCGA AACTAATAA
301 AGCATGTGTA ACTGCAAAAA AAAAAAAAAA AAAAAAAAAA A
    
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Fig. 6. Nucleotide sequence of full-length PdT-1 cDNA. The putative signal peptide (single underline), mature PdT-1 (double underline) and stop codon (asterisk) are indicated.

mg). Both natural and synthetic PdT-1 were subjected to MS/MS fragmentation using a Q-TOF Ultima mass spectrometer and their identity was confirmed (Fig. 5).

3.2. cDNA cloning

The PdT-1-encoding precursor cDNA was successfully cloned from the in vitro skin library using the RACE protocol described. The 3'-RACE product (approx. 180 bp) contained the nucleotide sequence of only four, C-terminal open-reading frame amino acids, -Val-Pro-Gly-Lys, followed by a stop codon, a non-translated region and a poly-A tail. The 5'-RACE product encoded the entire open-reading frame of 62 amino acids (including a putative signal peptide sequence) and a 5'-non-translated region. The nucleotide sequence of the full-length PdT-1-encoding cDNA (EMBL accession no. AJ507318) (Fig. 6) was subsequently confirmed by repeated identity in at least 6 clones and by repeated PCR of the entire transcript.

3.3. Smooth muscle pharmacology

The synthetic replicate of PdT-1 induced a dose-dependent relaxation of rat arterial smooth muscle and contraction of small intestinal smooth muscle (Fig. 7). Bradykinin, known to have similar effects in both smooth muscle preparations, was included in parallel experiments as a comparison. PdT-1 ($EC_{50} = 5.30 \pm 0.22$ nM, mean/S.E.M., $n = 6$) was more potent than bradykinin ($EC_{50} = 218 \pm 0.27$ nM, mean/S.E.M., $n = 6$) in relaxing arterial smooth muscle (Fig. 7a), but contrastingly, PdT-1 ($EC_{50} = 8.17 \pm 0.31$ nM, mean/S.E.M., $n = 6$) was of a similar potency to bradykinin ($EC_{50} = 7.71 \pm 0.28$ nM, mean/S.E.M., $n = 6$), in contracting small intestinal smooth muscle (Fig. 7b).

4. Discussion

PdT-1 is a novel, tryptophan-containing heptapeptide amide (tryptophyllin), isolated and structurally characterized from the defensive skin secretion of the Mexican phyllomedusid leaf frog, *P. dacnicolor*. Tryptophyllins remain the only group of peptides isolated from amphibian defensive skin secretions solely on a chemical basis (indole-positive spots following paper electrophoresis) rather than by specific bioactivity [3]. For this reason, the bioactivity of this peptide group remains obscure and reference to their primary structures indicates a high degree of heterogeneity. However, in the course of comparing the primary structure of the novel peptide, PdT-1, with other tryptophyllins, it became apparent, when attempting multiple alignments, that all known structures partitioned into three discrete families, each displaying distinctive conserved motifs (see Fig. 4). Tryptophyllin-1 (T-1) peptides so far identified are either amidated heptapeptides (like PdT-1) or non-amidated octapeptides. The common motif appears to be a Lys-Pro-doublet at the N-terminus, a tryptophanyl residue at position 5 and a prolyl residue at position 7. Tryptophyllin-2 (T-2) peptides are more heterogenous (four to seven amino acid residues in length) but nevertheless exhibit a common internal -Pro-Trp-doublet. Although tryptophyllins per se were first identified in Neotropical phyllomedusine frog skin [19], peptides with obvious structural features of T-2 peptides have been isolated from the skin secretion of the Australian tree frog, *Litoria rubella* [23]. Tryptophyllin-3 peptides are tridecapeptides exhibiting the highest degree of structural similarity. The variable positions 2, 5, 6 and 13 are only marginally so as each demonstrates a conserved substitution (2-Glu/Asp; 5-Tyr/Phe; 6-Trp/Tyr; 13-Met/Val). In order to attempt some terminological clarification for this structurally heterogenous peptide family, we propose, on the basis of the more discrete structural similarities described here, that three families actually constitute the tryptophyllin superfamily and the designation of tryptophyllin-1 (T-1), tryptophyllin-2 (T-2) and tryptophyllin-3 (T-3) should be adopted. It should be noted that species such as *P. rohdei* contain members of all

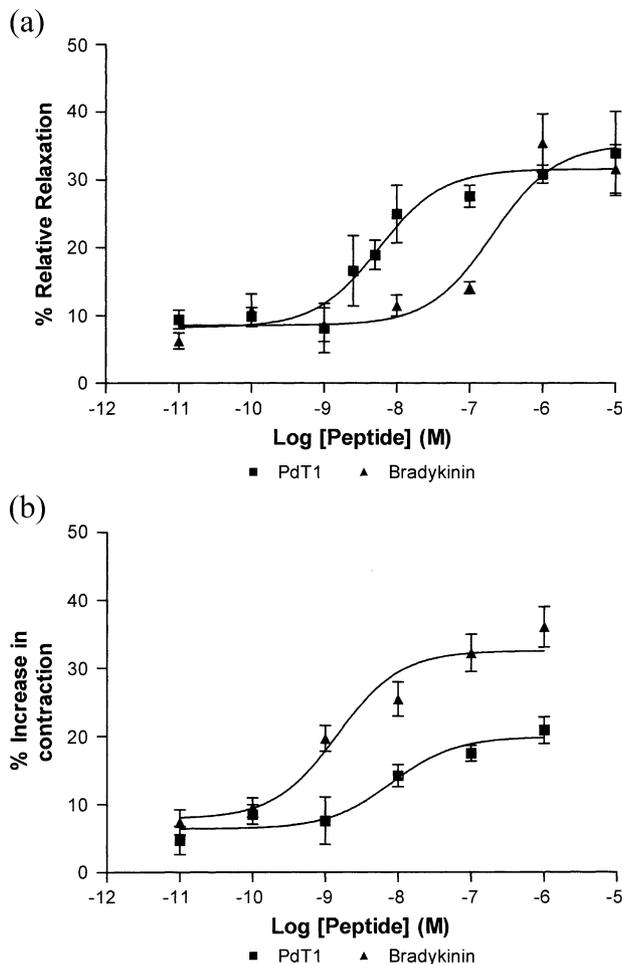


Fig. 7. Dose-response curves of PdT-1 (■) and bradykinin (▲) on rat arterial smooth muscle (a) and rat small intestinal smooth muscle (b). Each data point represents the mean \pm standard error of six consecutive replicates.

subfamilies and in the case of T-2 peptides, multiple structural isomers within this group [20]. It may well be that other species have representative peptides from additional groups that remain to be discovered.

Although the structures of many tryptophyllins have been known for some time, until now the nucleotide sequence of a precursor cDNA has not been reported. One of the possible major reasons for this is that the peptides are generally of short chain length—a factor that takes degenerate primer design and subsequent reaction-priming function to the limits of the technology. Here we describe the first precursor nucleotide sequence of a tryptophyllin-1 deduced by a 3'-and 5'-RACE technique. The degenerate primer employed in 3'-RACE was designed to the N-terminal five residues of PdT-1, Lys-Pro-Pro-Ala-Trp (note residue 3 is Pro as Hyp is a post-translational modification). This primer used the fact that Trp has a single triplet codon and would be assured to anneal for the RACE reaction. This initial strategy was successful and generated a 3'-RACE product (approx. 180 bp) containing only four residues of an open-reading frame, -Val-Pro-Gly-Lys-STOP, extending through a 3'-untranslated region to a poly-A tail. For 5'-RACE, a specific primer was designed to a site in the 3'-non-translated region, that produced a single discrete product of approximately 300 bp. Cloning and sequencing of this product revealed its authenticity indicated by a single copy of PdT-1 located near the C-terminus of an open-reading frame of 62 amino acid residues with a putative signal peptide located at the N-terminus. Typical of many amphibian venom gland peptide precursors, the intervening sequence between the putative signal peptide and the mature peptide coding sequence is rich in acidic amino acid residues and their secondary amines [2]. Several potential propeptide convertase processing sites in the form of paired basic amino acid residues (-Lys-Arg-; -Lys-Lys-; -Arg-Arg-) are present, the latter probably representing the near N-terminal cleavage site of PdT-1 with a subsequent aminopeptidase-mediated removal of an aspartyl residue generating the N-terminal lysyl residue of the mature peptide. The penultimate glycyl residue in the open-reading frame is appropriately positioned to act as an amide donor for generation of the C-terminal prolylamide residue present on the mature peptide. This prototype tryptophyllin precursor cDNA is thus highly condensed not only possessing a very small open-reading frame but also a remarkably short 3'-non-translated region prior to the poly-A tail. A distinct second possibility for the lack of report of tryptophyllin precursor cDNA structure may reside in the relatively small transcript that could easily have been regarded by previous researchers as an artefact or spurious product.

In an early report [9] and subsequently in a more contemporary review [3], the authors state that the pharmacological activity of the tryptophyllins remains to be established, although for T-2 peptides, some general metabolic effects, including stimulation of liver protein synthesis and body weight increase, have been documented [24]. In

addition, there is a single report of localization of immunoreactivity in pituitary gonadotrophs [25]. Here we present a novel and unequivocal biological activity for PdT-1. This peptide was found to be a potent relaxant of rat arterial smooth muscle displaying an EC₅₀ value (5.3×10^{-9} M) significantly lower than the control effector peptide, bradykinin (2.18×10^{-7} M) tested under the same conditions. Contrastingly, both peptides were of similar potency in contracting rat small intestinal smooth muscle as judged by their respective EC₅₀ values (PdT-1— 8.17×10^{-9} M vs. bradykinin— 7.71×10^{-9} M). PdT-1 is thus a potent arterial smooth muscle dilator—a biological effect that may be likely to extend to other T-1 peptides from other species, especially to *P. rohdei* tryptophyllin-1 (PrT-1), that displays highly conserved substitutions in positions 4 (Ser for Ala) and 6 (Ile for Val), when compared with PdT-1. Although PdT-1 represents a relatively minor component of the frog skin secretion (the majority of the more abundant and more hydrophobic peptides are antimicrobials), it is nevertheless active at low nanomolar concentrations on arterial smooth muscle. Thus, in keeping with many other active peptides found in frog skin secretions and indeed in snake venoms, low abundance is often compensated for by high biological potency and/or highly selective targeting. Whether PdT-1 acts via a new target receptor or on a known receptor expressed in mammalian arterial smooth muscle remains to be investigated. A novel target receptor for induction of arterial vasodilatation would be of considerable interest to the pharmaceutical industry as this tissue represents one of the most fundamental therapeutic targets for vascular disease in the developed world. Like many previously described amphibian skin peptides, the T-1 peptides may prove to have undiscovered structural and functional counterparts in mammalian neuroendocrine tissues and/or to function as superagonists at selected endogenous regulatory peptide receptors [3].

The enormous diversity of bioactive peptides in amphibian skin continues to be a source of novel structures that begs to be more exhaustively mined. Such molecular discovery programmes are unfortunately time-limited as global amphibian biodiversity continues in freefall [26].

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