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ORIGINAL ARTICLE

PD-sauvagine: a novel sauvagine/corticotropin releasing factor analogue from the skin secretion of the Mexican giant leaf frog, *Pachymedusa dacnicolor*

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Abstract Sauvagine is a potent and broad-spectrum biologically active peptide of 40 amino acid residues originally isolated from the skin of the South American frog, *Phyllomedusa sauvagei*. Since its discovery, no additional sauvagine structures have been reported. Following the discovery of sauvagine, peptides with similar primary structures/activities were identified in mammalian brain [corticotropin-releasing factor (CRF) and urocortin]. Here, we report the identification of a second sauvagine from the Mexican giant leaf frog, *Pachymedusa dacnicolor*, which displays primary structural features of both sauvagine and CRF. A cDNA encoding the peptide precursor was "shotgun" cloned from a cDNA library constructed from lyophilised skin secretion by 3′- and 5′-RACE reactions. From this, the primary structure of a 38-mer peptide was deduced and this was located in reverse phase HPLC fractions of skin secretion and both its mass and structure were confirmed by mass spectrometry. The biological activities of synthetic replicates of PD-sauvagine and sauvagine were compared using two different mammalian smooth muscle preparations and the novel peptide was found to be more potent in both. Bioinformatic analyses of PD-sauvagine revealed that it shared different regional sequence identities with both sauvagine and CRF.

Keywords Amphibian · Skin · Peptide · Molecular cloning · Smooth muscle

Introduction

The amphibian skin peptide, sauvagine, represents the first described member of a fundamentally important family of vertebrate neuropeptides, comprising corticotropin-releasing factor (CRF), urotensin-I (U1) and urocortin (UCN), which probably arose from an ancestral peptide early in the history of metazoans (Erspamer 1994; Dautzenber and Hauger 2002; Denver 2006; Liapakis et al. 2011). CRF, first found in extracts of ovine hypothalamus, has been subsequently located in brain tissues from a large number of mammals, birds and amphibians (Erspamer 1994; Dautzenber and Hauger 2002; Denver 2006; Liapakis et al. 2011). A major functional role of this peptide, after which it was named, is in releasing adrenocorticotropic hormone (ACTH), β-endorphin and α-melanocyte-stimulating hormone from the pituitary gland (Rivier et al. 1983; Denver 2006). Subsequently, two non-mammalian CRF-related peptides, the amphibian skin peptide sauvagine (SVG) and the fish peptide, urotensin-I (UI), were isolated and functionally characterised (Montecucchi and Henschen 1981; Denver 2006). Both peptides exhibited approximately 50% amino acid sequence identity with mammalian CRF and

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both were found to possess effects in releasing ACTH from mammalian pituitary glands (Boorse and Denver 2006). Later, a second mammalian peptide named urocortin (UCN) (now urocortin-I), with a high sequence similarity to sauvagine (SVG) and urotensin-I and two novel urocortin-like peptides, named urocortin-II (UCN-II) and urocortin-III (UCN-III), were identified in mammalian brain (Erspamer 1994; Boorse and Denver 2006; Fekete and Zorrilla 2007). All of these UCN-like peptides exhibit potent effects on appetite regulation and on the cardiovascular systems of mammals (Suda et al. 2004; Takahashi et al. 2004; Fekete and Zorrilla 2007). Meanwhile, other researchers identified two similar peptides, which were named stresscopin (a homologue of urocortin-III) and stresscopin-related peptide (a homologue of urocortin-II) (Hsu and Hsueh 2001; Boorse and Denver 2006). The broad spectrum of biological effects of sauvagine, that include a hypotensive action within the cardiovascular system, inhibition of release of prolactin, thyrotropin and growth hormone and stimulation of release of corticotropin and β-endorphin, were responsible for its original identification in the skin of the frog, *Phyllomedusa sauvagei* (Montecucchi and Henschen 1981; Erspamer 1994). Despite the broad spectrum of effects of sauvagine, its relatedness to CRF and its established central role in induction and maintenance of endogenous responses to stress, no further sequences of sauvagines have been reported from amphibian skin sources.

Here, we describe a second sauvagine/CRF-related peptide, named PD-sauvagine, isolated from the skin secretion of a phyllomedusine frog—the Mexican giant leaf frog, *Pachymedusa dacnicolor*, and the structure of its biosynthetic precursor deduced from cloned skin secretion cDNA. Bioinformatic analyses showed that both the mature peptide and biosynthetic precursor differed significantly in primary structure from the prototype amphibian peptide (hereafter referred to as PS-sauvagine) and that the mature peptide displayed a closer N-terminal structural identity with CRF. An intriguing feature of the mature peptide is that it possesses the critical CRF-binding protein (CRFBP) residue Ala (present in all known CRFs), rather than the Glu residue, that occupies this position (#21) in PS-sauvagine (Eckart et al. 2001).

Materials and methods

Specimen biodata and acquisition of skin secretion

Specimens of the Mexican giant leaf frog, *P. dacnicolor* ($n = 3$), were obtained from a commercial source and had been captive bred in the USA. 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 skin by transdermal electrical stimulation (6v DC, 4 ms pulse-width, 50 Hz) through platinum electrodes for two periods of 15 s duration (Tyler et al. 1992). The obvious viscous white secretion was washed from the skin using deionised water, snap-frozen in liquid nitrogen and lyophilised. Lyophilisate was stored at −20°C prior to analysis. All procedures involving the procurement of skin secretion from amphibians using transdermal electrical stimulation were subjected to ethical approval and carried out under appropriate UK personal and project licences for animal research.

Reverse-phase HPLC fractionation of skin secretion

A 5 mg sample of lyophilised skin secretion was dissolved in 0.5 ml of 0.05 aqueous trifluoroacetic acid (TFA) and clarified of microparticulates by centrifugation (3,500×g for 5 min). The supernatant was carefully decanted and pumped directly onto an analytical reverse phase HPLC column (Jupiter C_5; 250 mm × 4.6 mm, Phenomenex, UK) and bound peptides were eluted using a gradient formed from 0.05/99.5 (v/v) TFA/water to 0.05/19.95/80.0 (v/v/v) TFA/water/acetonitrile in 80 min at a flow rate of 1 ml/min. Fractions (1 ml) were collected at minute intervals and the effluent absorbance was continuously monitored at λ214 nm. Samples (100 µl) were removed from each fraction in triplicate, lyophilised and stored at −20°C prior to smooth muscle pharmacological analysis.

Bioactivity screening using rat urinary bladder smooth muscle

Male Wistar rats (250–300 g) were killed by carbon dioxide asphyxiation followed by cervical dislocation. These procedures were performed under appropriate UK animal licences. The rats were placed dorsal surface down and the abdomen was opened by means of an incision along the mid ventral line and subcutaneous fat was carefully dissected. The exposed urinary bladder was removed from each rat, emptied of urine, placed in ice-cold Kreb's solution (118 mM NaCl, 4.7 mM KCl, 25 mM $NaHCO_3$, 1.15 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 1.1 mM $MgCl_2$ and 5.6 mM glucose) and equilibrated with 95% O_2, 5% CO_2. Muscle strips, 2 mm × 10 mm, were dissected from the bladder under a dissection microscope. These were tied at each end with a fine silk thread (0.2 mm diameter) with one end subsequently attached to a fixed pin and the other to a transducer in a 2 ml organ bath containing Kreb's solution at 37°C flowing at 2 ml/min with constant

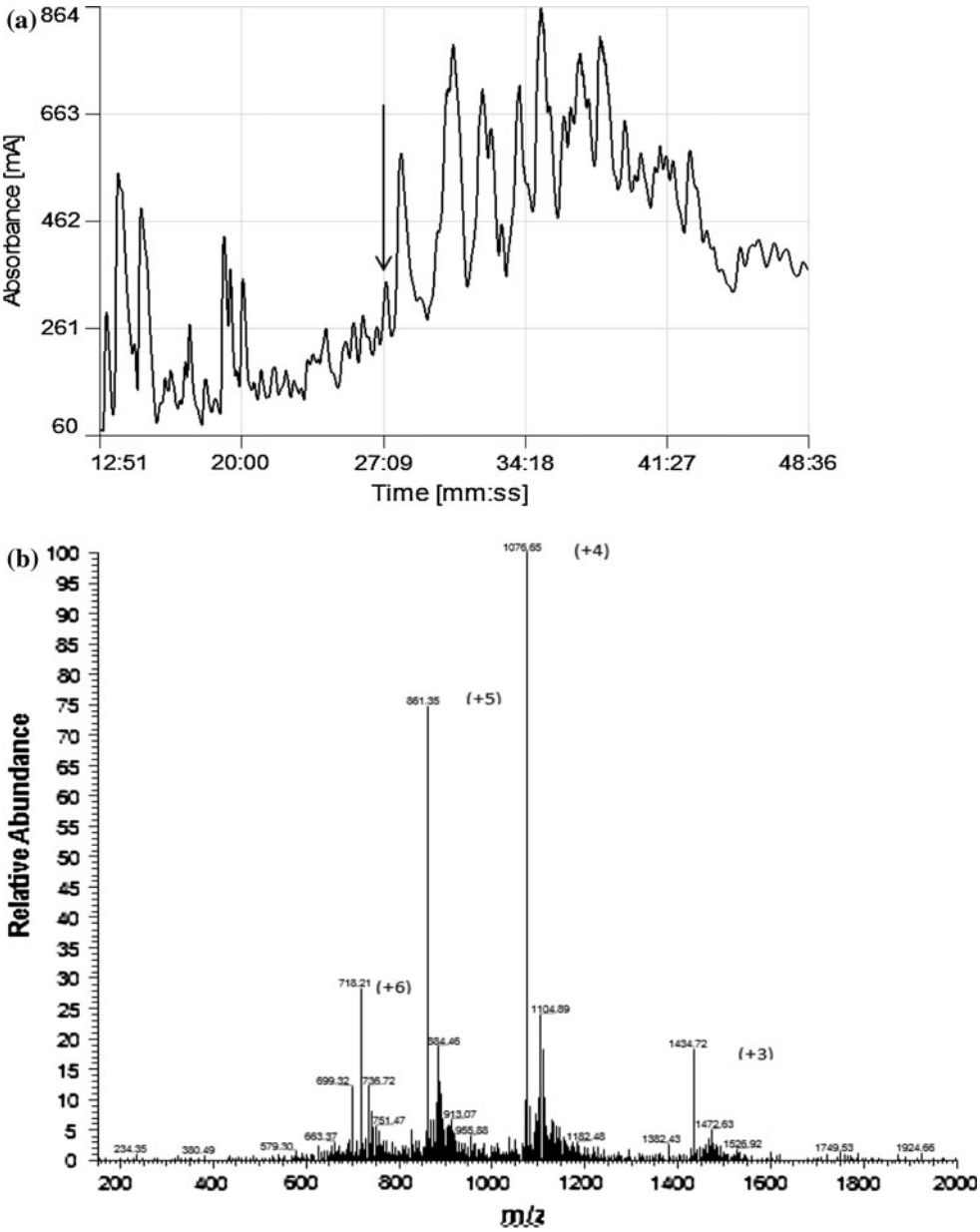

Fig. 1 **a** Reverse phase HPLC chromatogram of fractionated *P. dacnicolor* skin secretion. The elution position of the novel peptide PD-sauvagine is indicated by an *arrow*. **b** Electrospray ionisation mass spectrum of purified PD-sauvagine. Triply charged $(M + 3H)^{3+} = m/z$ 1,434.72, quadruply charged $(M + 4H)^{4+} = m/z$ 1,076.55, fivefold charged $(M + 5H)^{5+} = m/z$ 861.35 and sixfold charged $(M + 6H)^{6+} = m/z$ 718.2, ions are labelled

bubbling of 95% O_2, 5% CO_2. After a 20 min equilibration period, muscle strips were tested for viability using 60 mM KCl. Following this, viable preparations were used to screen samples of reverse phase HPLC fractions for myoactivity.

Structural analyses and chemical synthesis of peptides

The PD-sauvagine peptide displaying the novel myoactivity, as determined by bioassay above, was subjected to structural analysis by MS/MS fragmentation sequencing using a Thermo Fisher LCQ Fleet electrospray ion-trap mass spectrometer (Thermo Fisher, San Jose, CA, USA).

PD-sauvagine and PS-sauvagine were synthesised by solid-phase fmoc chemistry using an Applied Biosystems 433 peptide synthesiser. Products were purified by reverse phase HPLC and the primary structures of the major components in each synthetic mixture were confirmed by MS/MS fragmentation. For pharmacological experiments, standardisation of synthetic peptides was achieved by acid hydrolysis of a known gravimetric quantity of lyophilisate followed by amino acid analysis using an Applied Biosystems PTH-amino acid analyser.

cDNA library construction from lyophilised skin secretion and molecular cloning of cDNAs

A 5 mg sample of lyophilised skin secretion was dissolved in 1 ml of cell lysis/mRNA stabilisation solution (Dynal, UK). Polyadenylated mRNA was isolated using magnetic

oligo-dT beads as described by the manufacturer (Dynal Biotech, UK). The isolated mRNA was subjected to 3′-RACE procedures to obtain full-length preprosauvagine nucleic acid sequence data using a SMART-RACE kit (Clontech, UK) essentially as described by the manufacturer. Briefly, the 3′-RACE reactions employed an NUP primer (supplied with the kit) and a sense primer (S1, 5′-CARGGIACIWSIYTIGAYYT-3′) that was complementary to the N-terminal PD-sauvagine amino acid sequence, QGTSLDL–. The products of 3′-RACE reactions were gel-purified and cloned using a pGEM-T vector system (Promega Corp.) and sequenced using an ABI 3100 automated sequencer. Following acquisition of these data, another specific antisense primer (AS1, 5′-CTGTGCAGA CTAGTGATGTTCCAGT-3′) was designed to a site in the 3′-untranslated region and was employed in 5′-RACE reactions. Products were likewise gel-purified, cloned and sequenced as described above.

Fig. 2 Nucleotide and translated open-reading frame amino acid sequences of cloned cDNA encoding the biosynthetic precursor of PD-sauvagine The putative signal peptide domain is double-underlined, the mature peptide is single-underlined and the stop codon is indicated by an *asterisk*

Pharmacological characterisation of PD-sauvagine using rat urinary bladder and guinea-pig colon smooth muscle

Smooth muscle preparations were prepared and treated as described previously (Chen et al. 2011; Li et al. 2011). Following this, a series of experiments was performed to address the pharmacological characterisation of the novel peptide. From 10^{-3} M stock solutions of the novel PD-sauvagine and PS-sauvagine, dose–response curves were constructed consisting of peptide concentrations ranging from 10^{-5} to 10^{-11} M. Peptides were added to the urinary bladder muscle strips, which were put under 0.1 g of tension, in increasing concentrations with 5 min flow-throughs between each dose. Each concentration of peptides was applied to a minimum of six muscle strips. Changes in tension of the bladder muscle strips were recorded and amplified through pressure transducers connected to a PowerLab System (AD Instruments Pty Ltd.).

Male adult guinea-pigs (250–300 g) were killed by carbon dioxide asphyxiation followed by cervical dislocation under appropriate UK animal licences. For rat colon smooth muscle preparations, 1 cm segments of colon 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 stabilise for 20 min before the application of peptides. The colon smooth muscle preparations were exposed to peptides in the concentration range of 10^{-5} to 10^{-11} M, and relative changes in tension were recorded. Six replicates for each experimental data point were performed. Urinary bladder muscle strips were used as described in a previous section.

Results

Identification and structural analyses of PD-sauvagine

The novel peptide was effectively resolved in crude skin secretion by the reverse phase HPLC parameters employed (Fig. 1a). Following fractionation, the purity and molecular mass (4301 Da) of the mature novel peptide was assessed by electrospray mass spectrometry (Fig. 1b). The sequence of the first 20 amino acid residues of the polypeptide was established by automated Edman degradation following incubation with pyroglutamate aminopeptidase (Chen et al. 2005) as: pGlu-GTSLDLTFDLLRHNLEIAK–. These data established that the novel peptide was a member of the sauvagine/CRF family.

Molecular cloning of the PD-sauvagine precursor-encoding cDNA from the skin secretion-derived cDNA library and bioinformatic analyses

The employment of the 3′- and 5′-rapid amplification of cDNA ends (RACE) strategies described here, resulted in the successful cloning of PD-sauvagine precursor-encoding cDNA and subsequent establishment of its unequivocal nucleotide and translated amino acid sequence (Fig. 2).

Fig. 3 Predicted *b*- and *y*-ion MS/MS fragment ion series (singly-, doubly charged and triply charged) of PD-sauvagine. Observed ions are indicated in *bold typeface*

#1	b(1+)	b(2+)	b(3+)	Seq.	y(1+)	y(2+)	y(3+)	#2
1	113.02333	57.01530	38.34596	E-Gln->pyro-Glu				38
2	170.04480	85.52604	57.35312	G	4189.38214	2095.19471	**1397.13223**	37
3	271.09248	136.04988	91.03568	T	4132.36067	2066.68397	1378.12507	36
4	358.12451	179.56589	120.04635	S	4031.31299	2016.16013	**1344.44251**	35
5	471.20858	236.10793	157.74104	L	3944.28096	1972.64412	1315.43184	34
6	586.23553	293.62140	196.08336	D	3831.19689	1916.10208	**1277.73715**	33
7	**699.31960**	350.16344	233.77805	L	3716.16994	1858.58861	**1239.39483**	32
8	**800.36728**	**400.68728**	267.46061	T	3603.08587	1802.04657	1201.70014	31
9	947.43570	474.22149	316.48342	F	3502.03819	1751.52273	1168.01758	30
10	**1062.46265**	**531.73496**	354.82573	D	3354.96977	**1677.98852**	**1118.99477**	29
11	**1175.54672**	588.27700	392.52042	L	3239.94282	**1620.47505**	**1080.65246**	28
12	**1288.63079**	644.81903	430.21511	L	3126.85875	**1563.93301**	**1042.95777**	27
13	1444.73191	**722.86959**	482.24882	R	3013.77468	**1507.39098**	1005.26308	26
14	1581.79082	791.39905	**527.93512**	H	2857.67356	**1429.34042**	953.22937	25
15	**1695.83375**	**848.42051**	565.94943	N	2720.61465	**1360.81096**	907.54307	24
16	**1808.91782**	904.96255	603.64412	L	2606.57172	1303.78950	**869.52876**	23
17	**1937.96042**	**969.48385**	646.65832	E	2493.48765	**1247.24746**	**831.83407**	22
18	2051.04449	**1026.02588**	684.35301	I	2364.44505	**1182.72616**	788.81987	21
19	2122.08161	**1061.54444**	**708.03205**	A	2251.36098	1126.18413	**751.12518**	20
20	2250.17658	1125.59193	**750.73038**	K	2180.32386	1090.66557	727.44614	19
21	2378.23516	**1189.62122**	**793.41657**	Q	2052.22889	**1026.61808**	684.74781	18
22	2507.27776	**1254.14252**	836.43077	E	**1924.17031**	**962.58879**	**642.06162**	17
23	2578.31488	**1289.66108**	860.10981	A	**1795.12771**	**898.06749**	599.04742	16
24	2691.39895	**1346.20311**	**897.80450**	L	1724.09059	**862.54893**	575.36838	15
25	2819.49392	**1410.25060**	**940.50282**	K	**1611.00652**	806.00690	537.67369	14
26	2947.58889	**1474.29808**	983.20115	K	**1482.91155**	**741.95941**	**494.97537**	13
27	3075.64747	**1538.32737**	**1025.88734**	Q	**1354.81658**	677.91193	**452.27704**	12
28	3146.68459	**1573.84593**	**1049.56638**	A	**1226.75800**	**613.88264**	409.59085	11
29	3217.72171	**1609.36449**	1073.24542	A	**1155.72088**	578.36408	385.91181	10
30	3345.81668	**1673.41198**	1115.94374	K	**1084.68376**	542.84552	362.23277	9
31	3459.85961	**1730.43344**	1153.95805	N	**956.58879**	478.79803	319.53445	8
32	3615.96073	**1808.48400**	**1205.99176**	R	**842.54586**	421.77657	281.52014	7
33	3729.04480	1865.02604	**1243.68645**	L	686.44474	343.72601	229.48643	6
34	3842.12887	1921.56807	**1281.38114**	L	573.36067	287.18397	191.79174	5
35	3955.21294	1978.11011	**1319.07583**	L	460.27660	230.64194	154.09705	4
36	4070.23989	2035.62358	**1357.41815**	D	347.19253	174.09990	116.40236	3
37	4171.28757	2086.14742	**1391.10071**	T	232.16558	116.58643	78.06004	2
38				I-Amidated	131.11790	66.06259	44.37748	1

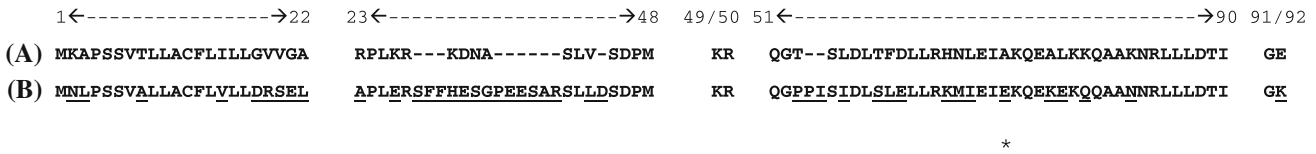

Fig. 4 Comparison of amino acid sequences and domain organisations of **a** PD-sauvagine from the skin of *Pachymedusa dacnicolor* and **b** sauvagine from *Phyllomedusa sauvagei* (Accession No. AY943910). Domain 1 (1–22)—putative signal peptide. Domain 2 (23–48)—spacer peptide. Domain 3 (49, 50)—propeptide convertase processing site. Domain 4 (51–90)—mature peptide, Domain 5 (91, 92)—C-terminal extension containing glycyl (G) residue amide donor. Differences in amino acid sequences are *underlined* and the site of Ala (A) for Glu (E) residue substitution is indicated by an *asterisk* (*)

Deduction of the full predicted primary structure of PD-sauvagine from these data enabled confirmation of this structure present in HPLC fractions of skin secretion, through MS/MS fragmentation sequencing (Fig. 3). The open-reading frame of the precursor-encoding cDNA consisted of 80 amino acid residues and an NCBI BLAST search found that this structure exhibited only a 54% amino acid sequence identity with the sauvagine precursor from the skin of the waxy monkey frog, *P. sauvagei* (Fig. 4). An interesting observation was that the two mature peptide structures displayed a higher degree of structural similarity (62%) (Fig. 5). When examined more closely, however, the

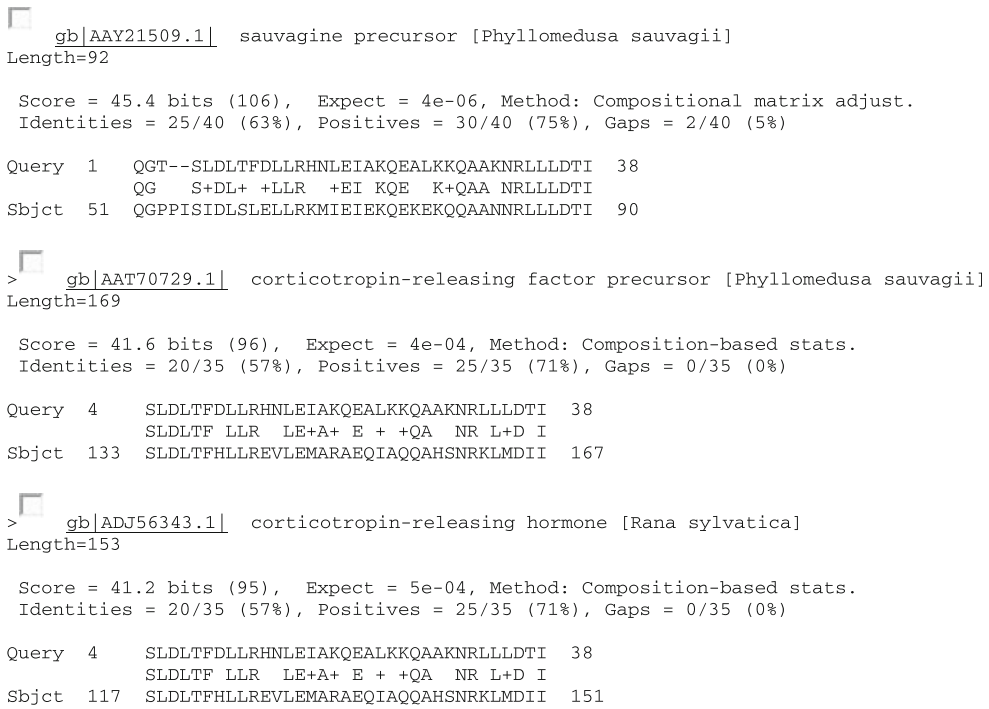

Fig. 5 Results of NCBI BLAST analysis using the full primary structure of PD-sauvagine as the query sequence. The first hit was with sauvagine from *Phyllomedusa sauvagei* indicating a 63% overall identity. The second and third hits were with the CRF/CRH peptides from *Phyllomedusa sauvagei* and *Rana sylvatica*, respectively, both showing 57% identity in a 35-mer segment. In fact, the same degree of identity was shown for most CRF/CRH peptides in the database indicating the high degree of sequence identity of this peptide across the vertebrates

N-terminal half of the PD-sauvagine structure was found to be closer to CRFs in structure (Fig. 6), while the C-terminal half was closest to the corresponding region of PS-sauvagine (Fig. 7). The N-terminal residue of both PD- and PS-sauvagines is a Gln (Q) and it is this that spontaneously cyclises in the acidic environment of the secretory granule to form the post-translationally modified pyroglutamic acid residue. This must be removed enzymatically prior to Edman degradation as this technique requires a free N-terminal amino group. The mature peptide sequence in both precursors is flanked N-terminally by a classical -Lys-Arg- (-KR-) propeptide convertase processing site and C-terminally by a glycyl (G) residue that serves as an amide donor to generate the common C-terminal isoleucinamide. A major difference in the structures of both sauvagines is the lack of the -Pro-Pro-(-PP-) doublet sequence at the N-terminus of PD-sauvagine. This feature would have major implications in the secondary structure of this region and this was found to be the case following secondary structure predictions of PD-sauvagine, PS-sauvagine and PS-CRF (Fig. 8) using the SWISS-MODEL workspace (Arnold et al. 2006). The nucleotide sequence of the PD-sauvagine precursor has been deposited in the EMBL Nucleotide Sequence Database under the accession code FR846380.

Following the acquisition of the full and unequivocal primary structure of PD-sauvagine obtained through a combination of Edman degradation, molecular cloning of biosynthetic precursor-encoding cDNA and MS/MS fragmentation sequencing, the peptide was successfully chemically-synthesised by solid-phase fmoc methodology and purified using semi-preparative LC/MS to >95% purity. A synthetic replicate of PS-sauvagine was also successfully chemically synthesised to a similar degree of purity.

Pharmacological characterisation of PD-sauvagine using smooth muscle bioassays

Application of the synthetic sauvagine peptides to rat urinary bladder and guinea-pig colon smooth muscle preparations in the molar concentration range of 1×10^{-11} to 1×10^{-5} M, revealed their ability to contract both smooth muscle preparations (Fig. 9). Compared with PS-sauvagine (EC_{50} 6.7×10^{-6}M), PD-sauvagine was slightly more potent in contracting rat urinary bladder smooth muscle (EC_{50} 1.4×10^{-7}M) (Fig. 9a). In contrast, PD-sauvagine was considerably more potent (EC_{50} 7.2×10^{-9}M) in contracting guinea-pig colon smooth muscle than PS-sauvagine (EC_{50} 1.2×10^{-7}M) (Fig. 9b).

Discussion

The corticotropin-releasing factor (CRF) system plays a key role in the coordination and regulation of endocrine, autonomic, behavioural, immune and visceral responses to stress (Boorse and Denver 2006). Sauvagine, isolated from the skin of *P. sauvagei,* is a linear peptide of 40 amino acid residues (Montecucchi and Henschen 1981), and as the

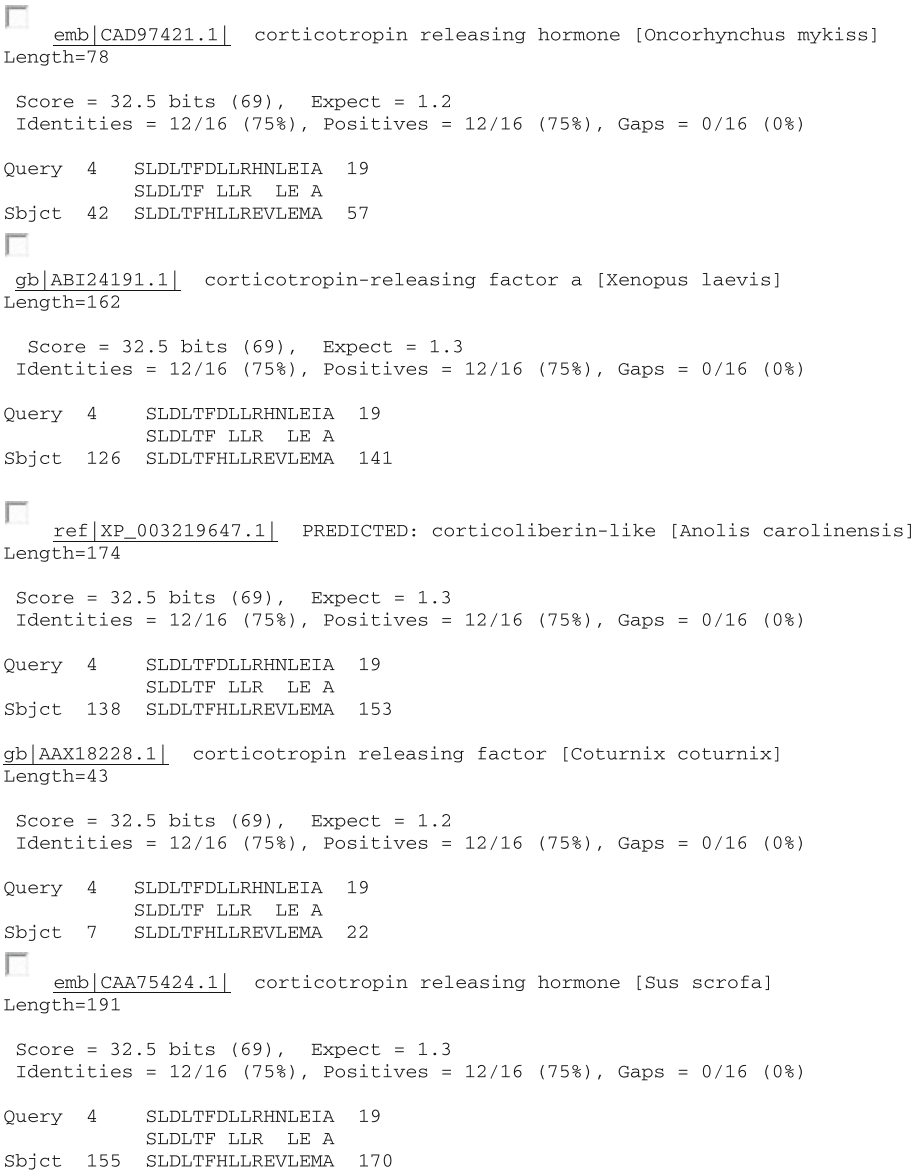

Fig. 6 Results of NCBI-BLAST using the N-terminal 19 residues of PD-sauvagine as the query sequence. The 4–19 residue segment of the query sequence was found to exhibit 75% sequence identity with the homologous region of most CRF/CRH peptides archived in the database representing fishes (*Oncorhynchus mykiss*), amphibians (*Xenopus laevis*), reptiles (*Anolis carolinensis*), birds (*Coturnix coturnix*) and mammals (*Sus scrofa*)

prototype of the amphibian CRF family of peptides, it displays a high degree of primary structural similarity to ovine hypothalamic corticotropin releasing factor (CRF) and urotensin-I (UI) from the urophyses of bony fishes (Boorse and Denver 2006). However, despite the historical significance of the discovery of sauvagine in the skin of a phyllomedusine frog and the demonstration of typical sauvagine bioactivity in skin extracts of many additional species from this taxon (Erspamer 1994), to date, there has been no report of the primary structure of any additional sauvagine from these sources.

Here, we report the primary structure of a novel sauvagine and have successfully cloned its biosynthetic precursor-encoding cDNA from the skin secretion of the Mexican giant leaf frog, *P dacnicolor*. Representing just the second sauvagine-related peptide from an amphibian skin source, PD-sauvagine exhibits significant differences in primary structure from the prototype peptide from *P. sauvagei* skin. This divergence in primary structure is also observed in respective biosynthetic precursors. Of the structural differences observed between PD-sauvagine and the prototype PS-sauvagine, two are worthy of special mention. PD-sauvagine lacks the prolyl residue doublet found in the N-terminal region of sauvagine and many CRFs. In addition to this, BLAST analysis using the N-terminal half of PD-sauvagine, revealed a greater degree of sequence similarity with CRFs whereas a similar analysis with the C-terminal half of PD-sauvagine, revealed the highest degree of sequence similarity with PS-sauvagine (Figs. 6, 7). Secondary structure prediction analyses using the SWISS-MODEL workspace (Fig. 8) suggested that this primary structural difference indeed induces a secondary

sp|P01144.1|SAUV_PHYSA RecName: Full=Sauvagine; AltName: Full=Sauvagine; Short=SVG
Length=40

Score = 41.4 bits (90), Expect = 6e-04
Identities = 15/19 (79%), Positives = 15/19 (79%), Gaps = 0/19 (0%)

```
Query  1   KQEALKKQAAKNRLLLDTI  19
           KQE  K QAA NRLLLDTI
Sbjct  22  KQEKEKQQAANNRLLLDTI  40
```

> gb|AAY21509.1| sauvagine precursor [Phyllomedusa sauvagii]
Length=92

Score = 41.4 bits (90), Expect = 0.001
Identities = 15/19 (79%), Positives = 15/19 (79%), Gaps = 0/19 (0%)

```
Query  1   KQEALKKQAAKNRLLLDTI  19
           KQE  K QAA NRLLLDTI
Sbjct  72  KQEKEKQQAANNRLLLDTI  90
```

Fig. 7 Results of NCBI-BLAST using the C-terminal 19 residues of PD-sauvagine as the query sequence. The only two relevant hits are illustrated. Both indicate the high degree of identity (79%) of this region of PD-sauvagine with the homologous region of the original sauvagine sequence. No significant similarity was found in an additional 120 hits with any CRF/CRH sequence

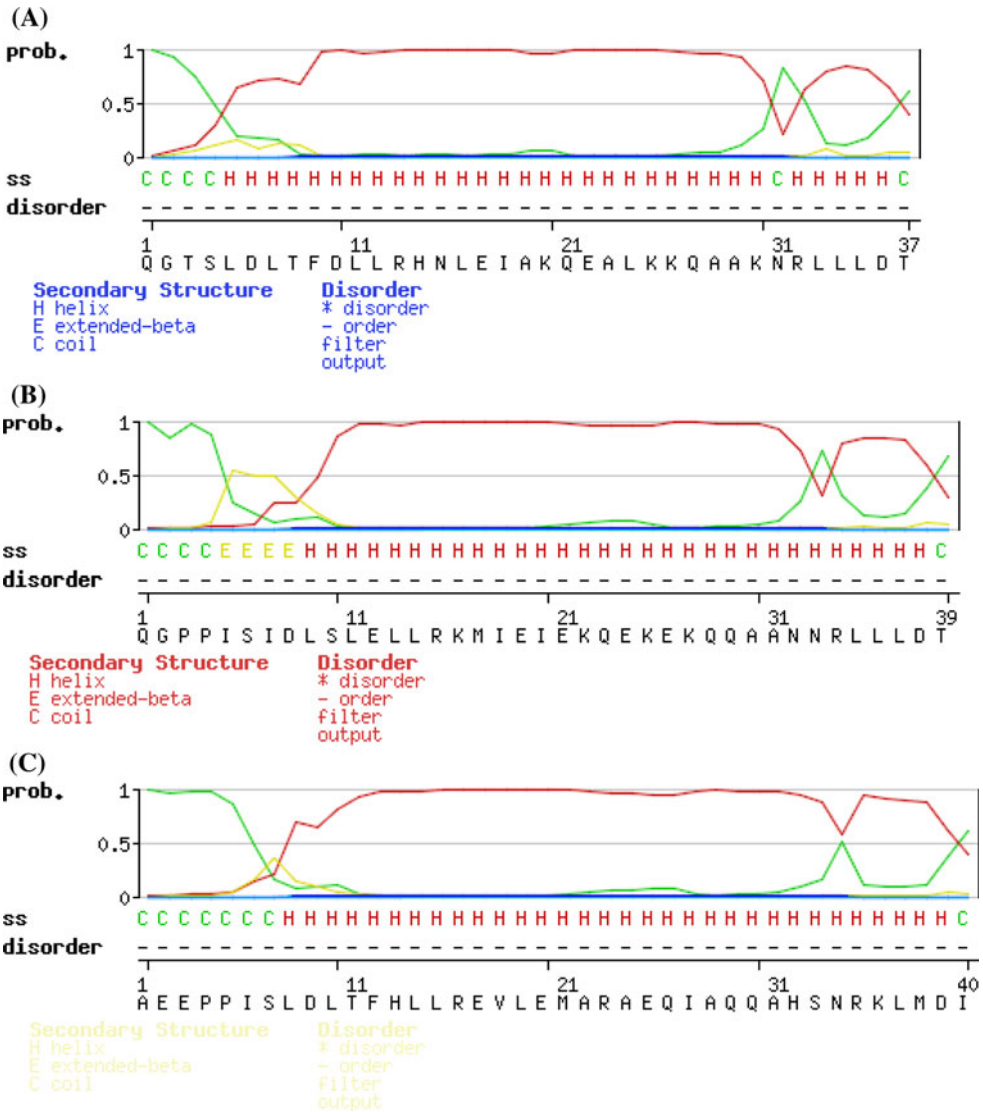

Fig. 8 Secondary structure prediction analyses of **a** PD-sauvagine, **b** sauvagine and **c** *Phyllomedusa sauvagei* CRF. Note that all three peptides are essentially composed of α-helix (H) and that PD-sauvagine (**a**) and *P. sauvagei* CRF (**c**) lack the short extended β-sheet region in the N-terminal domain of sauvagine (**b**)

structural alteration in this region with the extended beta sheet region of PS-sauvagine not being present in either PD-sauvagine or PS-CRF (Fig. 8). The second significant difference in primary structure between the two sauvagines was the substitution of the Glu (E) residue at position 21 in PS-sauvagine with the canonical CRF residue Ala (A) at

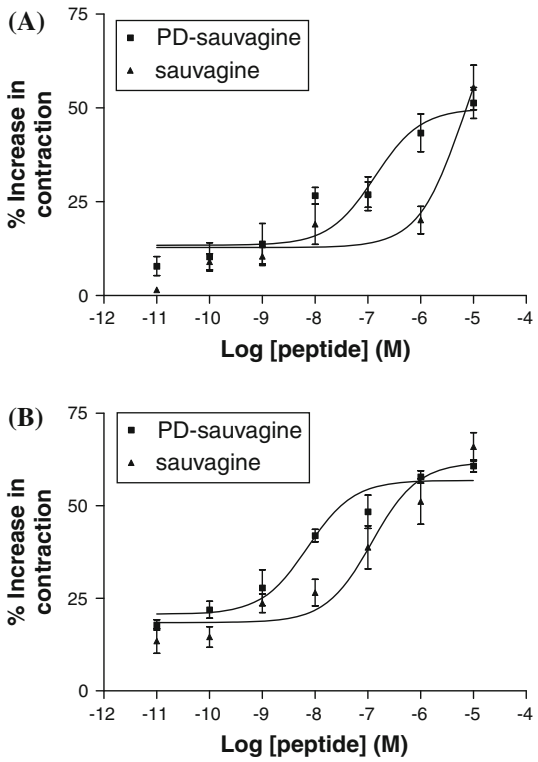

Fig. 9 Dose-response curves of PD-sauvagine (*filled square*) and sauvagine (*filled triangle*) using **a** rat urinary bladder smooth muscle and **b** guinea-pig colon smooth muscle. Each data point represents the mean and standard error of the mean of six replicates

the analogous position 19 in PD-sauvagine. Although this could be overlooked as a trivial substitution, it is of fundamental importance to the pharmacodynamics of CRF and related peptides as this residue has been shown to be an absolute requirement for binding to CRF binding protein (CRFBP)—a plasma protein that modulates the duration of action of such structurally related bioactive peptides (Dautzenber and Hauger 2002; Liapakis et al. 2011). The differences in primary and secondary structures between just two examples of amphibian skin-derived sauvagines, would suggest that more systematic studies on this peptide family would produce additional variants. Previous studies that simply examined phyllomedusine frog skin extracts for sauvagine bioactivity and assessed the quantities present in each species based upon relative activities, may not be an accurate reflection of abundance, as quantitation would be dependent on the discrete bioassay employed with in vitro assays producing different results from in vivo assays depending on the degree of interaction of structural variants with CRFBPs in whole animal models. In other words, activity would be dependent on the degree of partitioning and release of active peptides from plasma binding proteins.

CRF and related peptides have been shown to have a wide spectrum of biological effects including those related to gastrointestinal motility through actions on elements of both peripheral and central nervous systems. The direct peripheral effects of CRF and related peptides on gastrointestinal motility have been studied mainly using in vitro bioassays of longitudinal and/or circular smooth muscle strips from guinea-pig and rat intestine and have demonstrated increases in contraction through activation of myenteric neurons (Lázár et al. 2003; Sand et al. 2010). Moreover, both CRF itself and its cognate receptors have shown to be expressed in the rat urinary bladder (LaBerge et al. 2008). Despite this latter observation, the effects of CRF-related peptides on urinary bladder smooth muscle have not been widely reported. Here, following successful solid-phase chemical syntheses of both PD- and PS-sauvagines, we compared their biological effects by use of rat urinary bladder and guinea-pig colon smooth muscle bioassays—tissues which have been previously shown to be rich in CRF receptors (Lázár et al. 2003; LaBerge et al. 2008; Sand et al. 2010). Both sauvagines were found to be agonists of endogenous CRF receptors by nature of their observed dose-dependent contraction effects on both smooth muscle preparations and in both, PD-sauvagine was found to be more potent than PS-sauvagine (Fig. 9).

The present study reports the primary structure of a novel sauvagine (CRF-related peptide) and its biosynthetic precursor from the skin secretion of the phyllomedusine frog, *P dacnicolor*. The 38-mer peptide, named PD-sauvagine, is the second such CRF-related peptide structurally characterised from this source and both mature peptide and biosynthetic precursor differ significantly in primary structure from the prototype peptide, sauvagine (named PS-sauvagine in this study to indicate its species of origin, *P. sauvagei*). PD-sauvagine was found to a more potent agonist of CRF receptors in two types of mammalian smooth muscle and may prove to have further differential actions in other in vitro and in vivo bioassays due to several significant differences in primary structure when compared to the prototype peptide.

Amphibian skin secretions continue to provide both novel bioactive peptides per se and also a plethora of structural analogues of established peptide families that are proving a unique resource for pharmacologists and as potential leads for novel therapeutics.

Conflict of interest The authors declare that they have no conflict of interest.

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