

# Brevinin-1 and multiple insulin-releasing peptides in the skin of the frog *Rana palustris*

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## Abstract

Few studies have comprehensively examined amphibian granular gland secretions for novel insulinotropic peptides. This study involved isolation and characterisation of biologically active peptides from the skin secretions of *Rana palustris* frogs. Crude secretions obtained by mild electrical stimulation from the dorsal skin surface were purified by reversed-phase HPLC on a semipreparative Vydac C18 column, yielding 80 fractions. These fractions were assayed for insulin-releasing activity using glucose-responsive BRIN-BD11 cells. Acute 20 min incubations were performed in Krebs Ringer bicarbonate buffer supplemented with 5.6 mmol/l glucose in the absence (control) and presence of various fractions. Fractions 29–54 and fractions 68–75 showed significant 2.0–6.5-fold increases in insulin-releasing activity ( $P < 0.001$ ). The fractions showing most prominent insulinotropic activity were further purified to single homogeneous peaks, which, on

testing, evoked 1.5–2.8-fold increases in insulin release ( $P < 0.001$ ). The structures of the purified peptides were determined by mass spectrometry and N-terminal amino acid sequencing. Electrospray ionisation ion-trap mass spectrometry analysis revealed molecular masses of 2873.5–8560.4 Da. Sufficient material was isolated to determine the primary amino acid sequence of the 2873.5 Da peptide, revealing a 27 amino acid sequence, ALSILRGLEKLAKMGIALTNCKATKKC, repressing palustrin-1c. The database search for this peptide showed a 48% homology with brevinin-1, an antimicrobial peptide isolated from various *Rana* species, which itself stimulated insulin release from BRIN-BD11 cells in a concentration-dependent manner. In conclusion, the skin secretions of *R. palustris* frogs contain a novel class of peptides with insulin-releasing activity that merit further investigation.

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## Introduction

Amphibian skin secretions are a rich source of biologically active peptides which participate in mechanisms of frogs and toads to defend against microbial infection and being eaten by predators (Bevins & Zasloff 1990). These peptides generally belong to families of bioactive peptides, which through evolution have given rise to counterparts in mammals, such as bradykinins (Chen *et al.* 2002), caerulein/cholecystokinin (Anastasi *et al.* 1968), bombesin/gastrin-releasing peptide (Anastasi *et al.* 1972), exendin-4/glucagon like peptide-1 (Chen & Drucker 1997), tachykinins (Falconieri Erspamer *et al.* 1984), calcitonin gene-related peptide (Seon *et al.* 2000), and opioids and neuropeptides (Broccarda *et al.* 1981).

Frogs of the genus *Rana* represent an extremely diverse and widely distributed group with 250 estimated species worldwide, including 36 species that have been identified in North America (Duellman & Trueb 1994). Previous work on the skin secretions of different *Rana* species has led to the characterisation of various antimicrobial peptides

(Clark *et al.* 1994, Goraya *et al.* 1998, 2000). Most of these peptides are cationic in nature and have the potential to form amphipathic  $\alpha$ -helices either in aqueous solution or upon interaction with the lipid bilayers of bacterial cell membranes (Hancock *et al.* 1995, Hancock & Lehrer 1998). As well as antimicrobial peptides, the skin secretions of *Rana* species have yielded peptides that are either identical or structurally related to peptides synthesised in neuroendocrine tissues of mammals (Erspamer *et al.* 1986, Roseghini *et al.* 1988, 1989, Basir *et al.* 2000).

The pickerel frog *Rana palustris* is widely distributed from eastern Texas to South Carolina and to south-eastern Canada (Hardy & Raymond 1991, Moler 1992). These frogs commonly inhabit cool, wooded streams and springs, although they are also found in many other habitats (Conant & Collins 1998). The body of *R. palustris* varies in colour from yellow green to light bronze. These frogs produce toxic skin secretions that are irritating to humans and can be fatal to some predators such as snakes and birds (Duellman & Trueb 1994). This study describes the bioassay-led isolation, purification and structural

characterisation of insulin-releasing peptides from electrically derived skin secretions of *R. palustris*. Such peptides may be of therapeutic interest, as illustrated by enthusiasm for the treatment of diabetic patients with exendin-4 and related peptides isolated from the venom of the lizard *Heloderma suspectum* (Chen & Drucker 1997, Kolterman *et al.* 2003).

## Materials and Methods

### Reagents

Reagents of analytical grade and deionized water (Purite, Thame, UK) were used throughout the study. RPMI-1640 tissue-culture medium, fetal bovine serum, penicillin and streptomycin were all purchased from Gibco (Paisley, UK). Brevinin-1 was obtained from the Sigma Chemical Company (Poole, UK). HPLC grade acetonitrile was obtained from Rathburn (Waikersburn, UK). Sequencing grade trifluoroacetic acid (TFA) was obtained from Aldrich (Poole, UK). All chemicals employed in the operation of the 491 Procise gas phase sequencer were supplied by Perkin Elmer Applied Biosystems, (Warrington, UK). All other chemicals used were of the highest purity available.

### Collection of venom

Four young, captive-bred *R. palustris* frogs were maintained *in terraria* at 24 °C with 12 h light:12 h darkness cycle and were fed on crickets. The skin secretions were obtained from the frogs by gentle electrical stimulation (4 ms pulse width, 50 Hz, 5 V), using platinum electrodes rubbed over the moistened dorsal skin surface for 10 s. Secretions were then washed off into a glass beaker, using deionised water. The resultant secretions were freeze-dried in a Hetosicc 2.5 freeze-dryer (Heto, UK). Approximately 50 mg, dry weight, skin secretion were obtained.

### Purification of peptides

The crude venom (20 mg) was dissolved in 0.12% TFA/water (2 ml), and 1 ml was chromatographed on a Vydac 218TP510 semi-preparative C-18 column (25 × 1 cm, Hesperia, CA, USA). The column was equilibrated with 0.12% (v/v) TFA/water at a flow rate of 2 ml/min. With 0.1% (v/v) TFA in 70% acetonitrile/water, the concentration of acetonitrile in the eluting solvent was raised to 80% (v/v) over 80 min, using a linear gradient. Absorbance was monitored at 214 nm. Fractions (2 ml) were collected and assayed for insulin-releasing activity. Fractions, which showed major insulinotropic effects were pooled and rechromatographed on a Vydac 208TP54 analytical C-18 column (25 × 0.46 cm). The column was equilibrated with 0.12% (v/v) TFA/water at a flow rate of

1 ml/min. With 0.1% (v/v) TFA in 70% acetonitrile/water, the concentration of acetonitrile in the eluting solvent was raised to 15% (v/v) over 5 min and to 80% (v/v) over 70 min, using linear gradients. Absorbance was monitored at 214 nm.

### Culture of insulin-secreting cells

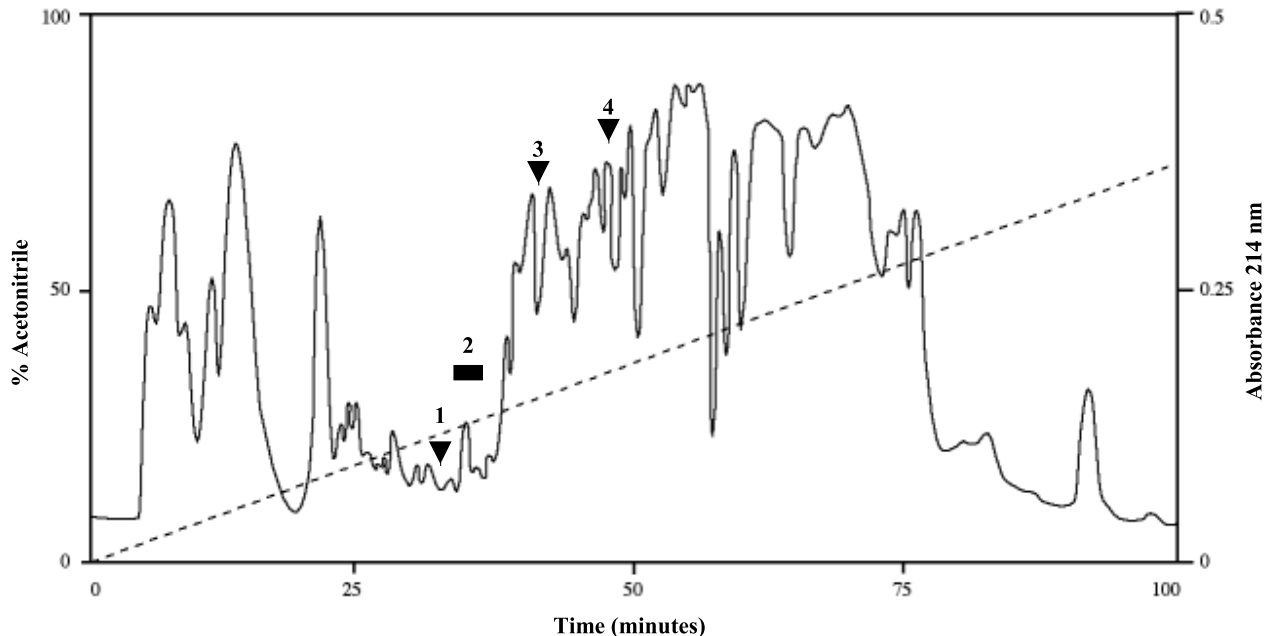
BRIN-BD11 cells were cultured in RPMI-1640 tissue-culture medium containing 10% (v/v) fetal calf serum, 1% (v/v) antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin) and 11.1 mM glucose. The production and characterisation of BRIN-BD11 cells are described elsewhere (McClenaghan *et al.* 1996). Cells were maintained in sterile tissue-culture flasks (Corning Glass Works, Sunderland, UK) at 37 °C in an atmosphere of 5% CO<sub>2</sub> and 95% air, using the LEEC incubator (Laboratory Technical Engineering, Nottingham, UK).

### Acute insulin-release studies

BRIN-BD11 cells were harvested with the aid of trypsin/EDTA (Gibco), seeded into 24-multiwell plates (Nunc, Roskilde, Denmark) at a density of  $1.5 \times 10^6$  cells per well, and allowed to attach during overnight culture at 37 °C. Acute studies of insulin release were preceded by 40-min preincubation at 37 °C in a 1.0 ml Krebs Ringer bicarbonate (KRB) buffer (115 mM NaCl, 4.7 mM KCl, 1.28 mM CaCl<sub>2</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 10 mM NaHCO<sub>3</sub> and 5 g/l BSA, pH 7.4) supplemented with 5.6 mM glucose. This glucose concentration potentially enables detection of both stimulators and inhibitors of insulin secretion. Established secretagogues, including GIP, GLP-1, CCK and acetylcholine, evoke good insulin responses to 5.6 mM glucose (McClenaghan & Flatt 1999). Test incubations were performed for 20 min at 37 °C, using the same buffer supplemented with 5.6 mM glucose in the absence (control) and presence of various venom peaks or synthetic brevinin-1, as indicated in the figures. To assess physiological relevance, incubations with brevinin-1 were repeated with Ca<sup>2+</sup>-free KRB buffer supplemented with 0.5 mM EGTA.

### Molecular mass determination

The molecular masses of the purified individual non-toxic peaks exhibiting insulin-releasing activity were determined by Matrix Assisted Laser Desorption Ionisation-Time of Flight (MALDI-TOF; Applied Biosystems, Foster City, CA, USA) mass spectrometry. An aliquot of 1 µl of the sample was mixed with 0.5 µl of a matrix solution (10 mg/ml solution of  $\alpha$ -cyano-4-hydroxycinnamic acid in acetonitrile/ethanol (1/1)), placed on one well of a 100-well stainless steel sample plate and allowed to dry at room temperature. Masses were recorded as a mass/charge ratio against abundance,



**Figure 1** Reversed-phase HPLC of the crude venom of *R. palustris*. The crude venom (20 mg) was dissolved in 0.12% (v/v) trifluoroacetic acid/water (2 ml), and 1 ml was applied to a semi-preparative Vydac C18 column, as described in Materials and Methods. The dashed line shows the concentration of acetonitrile in the eluting solvent. 1, 2, 3 and 4 correspond to fractions eluted at 32, 36–38, 45 and 49 min respectively.

and compared with theoretical values calculated by the peptide calculator, a computer package included in the MALDI-TOF software (Voyager 5 data explorer; Applied Biosystems).

#### Structural analysis of the purified peptides by automated Edman degradation

The primary structures of the purified peptide were determined by automated Edman degradation, using an Applied Biosystems Procise 491 microsequencer (Warrington, UK). Standard operating procedures were used (Applied Biosystems Model 491 Protein Sequencers Users' Manual). The limit for detection of phenylthiohydantoin amino acids was 0.2 pmol. The primary structure was compared with those deposited in the SWISSPROT database.

#### Statistical analysis

Results are expressed as mean  $\pm$  S.E.M. Values were compared using Student's unpaired *t*-test. Groups of data were considered to be significantly different if  $P < 0.05$ .

## Results

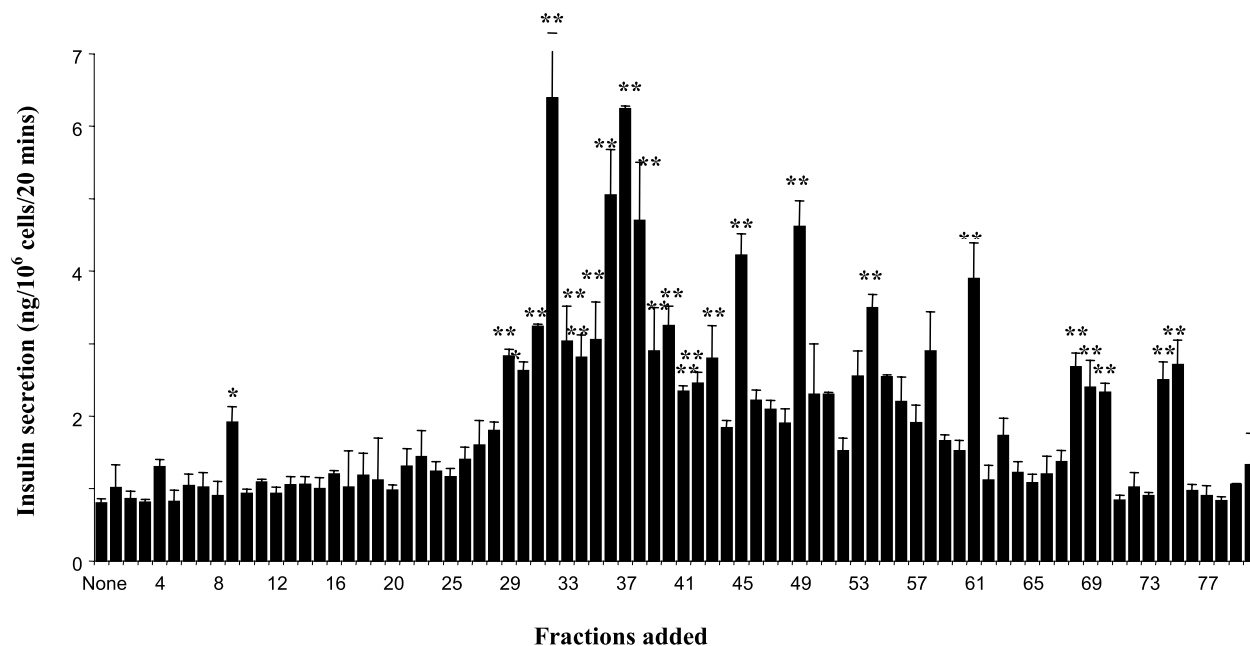
#### Isolation of insulin-releasing peptides

The crude venom was purified as described in Materials and Methods, yielding 80 fractions (Fig. 1). Significant

insulin-releasing activity was observed within fractions 29–54 and 68–75 compared with 5.6 mM glucose ( $P < 0.001$ ,  $n = 3$ ) (Fig. 2). The fraction in band 1 (fraction 32) (Fig. 1) was further purified, yielding peaks 1.1–1.5 (Fig. 3A). The fractions (36–38) in band 2 were pooled and rechromatographed, yielding peaks 2.1–2.7 (Fig. 3B). Fractions in bands 3 and 4 corresponding, to fractions 45 and 49 respectively, were also repurified, yielding peaks 3.1–3.8 and 4.1–4.4 respectively (Fig. 3C and D). The purified peaks obtained were tested for insulin-releasing activity. Peaks 2.6, 2.7, 3.1, 3.8, 4.3 and 4.4 showed 1.5–2.8-fold increases in insulin release compared with 5.6 mM glucose alone ( $P < 0.01$ ;  $n = 3$ ) (Fig. 4). However, none of the peaks from the repurification of fraction 32 (1.1–1.5) showed any significant insulin-releasing activity (Fig. 4).

#### Effects of synthetic brevinin-1 on insulin secretion

Synthetic brevinin-1 induced a concentration-dependent 1.5–3.5-fold increase of insulin release over the range  $10^{-8}$ – $10^{-6}$  M (Fig. 5A). To exclude the possibility that the insulin response to brevinin-1 was merely due to lysis of the cells, the experiment was repeated using  $\text{Ca}^{2+}$ -free KRB buffer. Under these conditions, the insulin-releasing activity at  $10^{-8}$  M and  $10^{-7}$  M brevinin-1 was totally abolished (Fig. 5B). At  $10^{-6}$  M, brevinin-1 induced a significant 2.5-fold increase in insulin release, but the response was 70% less ( $P < 0.001$ ;  $n = 8$ ) than observed with normal KRB buffer. Consistent



**Figure 2** Effects of various semi-preparative C18 HPLC fractions of *R. palustris* crude venom on insulin secretion from BRIN-BD11 cells. Incubations were performed at 5.6 mM glucose, using fractions shown in Fig. 1. Values are the mean  $\pm$  s.e.m. for three separate observations. \* $P < 0.05$  and \*\* $P < 0.001$  compared with 5.6 mM glucose alone.

with these observations, the viability of cells measured by modified neutral red assay (Hunt *et al.* 1987) was not affected by brevinin-1. In contrast, incubation with 16 mM alloxan, as positive control, decreased cell viability by 15% ( $P < 0.05$ ;  $n = 8$ ; data not shown).

#### Sequence analysis and mass spectrometry

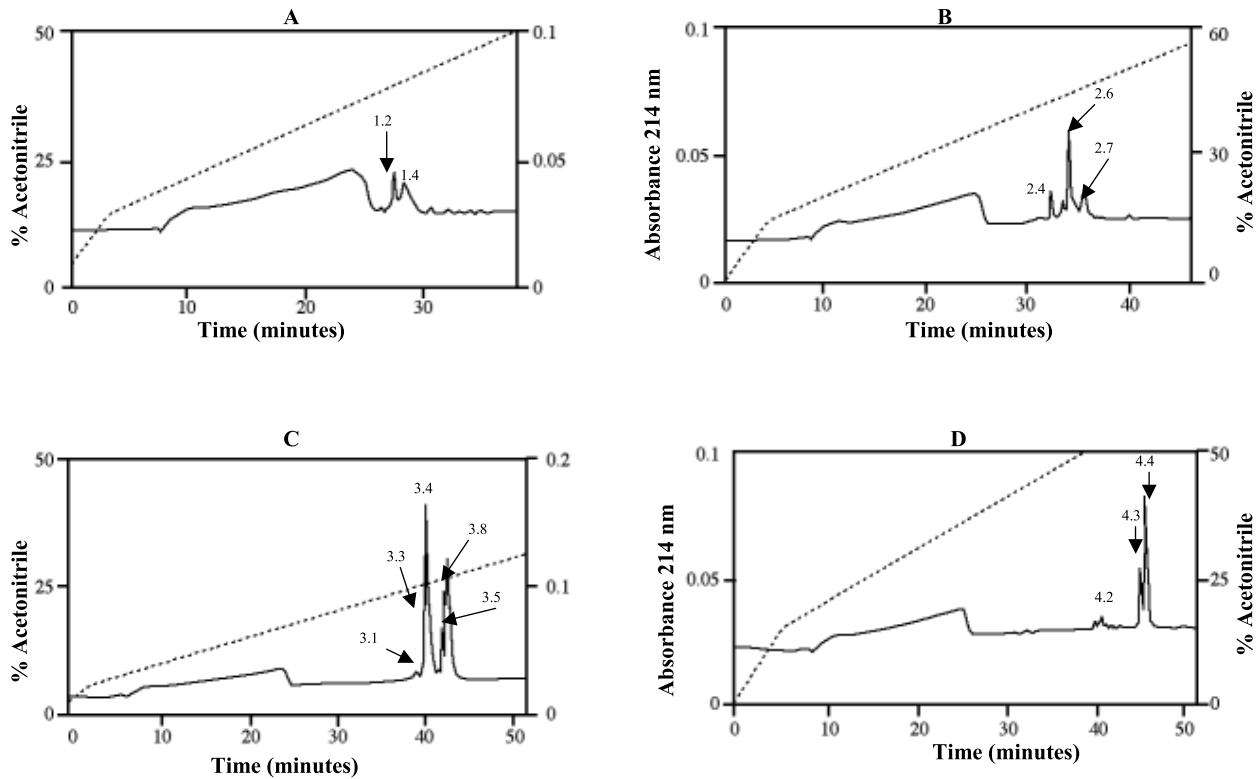
The purified peaks which showed major significant insulin-releasing activity (peaks 2.6, 2.7, 3.1, 3.8, 4.3 and 4.4) (Fig. 4) were subjected to mass spectral analysis. The mass spectrometry of peaks 2.7, 3.1, 3.8 and 4.3 revealed molecular masses of 8560.4 Da, 4919.9 Da, 2873.5 Da and 3848.7 Da respectively (Fig. 6). However, the masses of peak 2.6 and 4.4 could not be determined. Peptides with mass spectral data were subjected to N-terminal amino acid sequence analysis on Applied Biosystems 491 Procise Protein Sequencer. The primary amino acid sequence for peak 3.8 was successfully determined, and it comprised 27 amino acids: ALSILRGLEKLAKMGIALTNCKATKCC (Table 1). A search in the SWISSPROT FASTA data bank with the GCG sequence analysis programme ([www.hgmp.mrc.ac.uk/Registered/Option/gcg.html](http://www.hgmp.mrc.ac.uk/Registered/Option/gcg.html)) revealed that this peptide was identical to palustrin-1c isolated by the group of Conlon (Basir *et al.* 2000). As evident from the multiple sequence alignment in Table 1, this peptide with novel insulin-releasing activity is a member of the brevinin/esculentin/gaegurin/ranatuerin family of antimicrobial peptides (Park *et al.* 1994, Goraya

*et al.* 1998, 2000, Conlon *et al.* 1999, Basir *et al.* 2000). The sequences for peaks 2.7, 3.1 and 4.3 could not be determined due to the small amounts of peptides isolated.

#### Discussion

The skin secretions of frogs of the genus *Rana* are known to contain various biologically active peptides (Clark *et al.* 1994, Duellman & Trueb 1994, Goraya *et al.* 2000), including a number with significant antimicrobial activity (Basir *et al.* 2000). The present study has demonstrated for the first time that the skin secretions of *R. palustris* contain a new class of peptides with insulin-releasing activity. Since amphibians produce a large array of peptides in their defensive skin secretions (Bevins & Zasloff 1990), this new class may deter attack by stimulating insulin secretion and causing hypoglycaemia in the predator species. More research is required to determine whether additional actions exist outside the pancreatic beta cells.

Purification of the crude venom of *R. palustris* by reverse-phase HPLC in association with *in vitro* testing of insulin secretion allowed isolation of four insulinotropic peptides with molecular masses of 2873.5–8560.4 Da. The primary structure of the 2873.5 Da peptide was successfully completed in a single analysis, revealing a 27 amino acid with the sequence: ALSILRGLEKLAKMGIALTNCKATKCC. The theoretical (calculated) mass (2873.7 Da) based upon the proposed amino acid sequence was almost



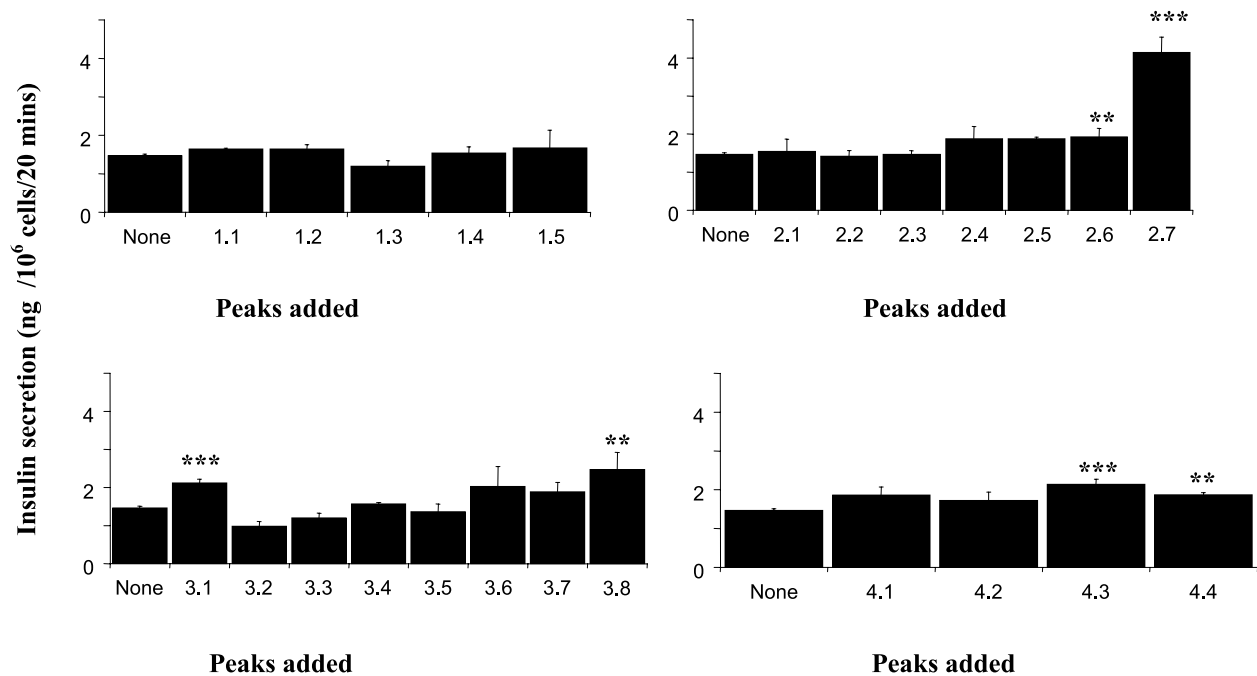
**Figure 3** Reversed-phase HPLC purification of the pooled fractions of *R. palustris* from zones 1, 2, 3 and 4 in Fig. 1, represented in panels A, B, C and D respectively. Fractions were applied to an analytical Vydac diphenyl column, as described in Materials and Methods. The dashed line shows the concentration of acetonitrile in the eluting solvent.

identical to the experimental mass, indicating the absence of any additional post-translational modifications (O- and/or N-glycosylation and phosphorylation) of the constitutive amino acid side chains. The amino acid sequence of the other three insulintropic peptides could not be determined due to shortage of isolated peptide and/or blockage of the N-terminus with pyroglutamate.

The SWISSPROT database search revealed that the 27 amino acid insulin-releasing peptide isolated corresponded to palustrin-1c (Basir *et al.* 2000). This exhibits 48% identity in 13 of the 27 amino acids with brevinin-1. Brevinins are part of a family of related peptides isolated from the skin secretions of various *Rana* species (Morikawa *et al.* 1992, Park *et al.* 1994, Conlon *et al.* 1999, Goraya *et al.* 2000) which contain a cysteine-bridge cyclic heptapeptide region at the C-terminus. In this region, the insulintropic peptide palustrin-1c exhibited homology at six amino acids, including both cysteine residues (Table 1). Although the precise mechanisms by which brevinins and associated peptides exert antimicrobial activity are poorly understood (Hancock & Lehrer 1998, Kwon *et al.* 1998), evidence suggests that they lyse the organism by rupturing the membrane or disturbing the membrane lipid bilayer by their ability to adopt amphipathic  $\alpha$ -helical conformations upon interaction

with the lipid bilayer of bacterial cell membranes (Hancock *et al.* 1995, Hancock & Lehrer 1998). The cysteine-bridge at the C-terminus of brevinin peptides has been proposed to play a significant role in this activity, since deletion of the C-terminal cysteine residue in ranalexin, an antimicrobial peptide from *Rana* species, resulted in a complete loss of antimicrobial activity (Clark *et al.* 1994). However, recent studies suggest that the intra-disulphide bridge and the N-terminal region in brevinin 1E are not essential for antimicrobial activity, but that they are important for hemolytic activity (Kwon *et al.* 1998). Thus, the decrease in antimicrobial activity due to deletion of the C-terminal cysteine seems to play a crucial role in the activity of brevinins rather than being a requirement for a disulphide bond.

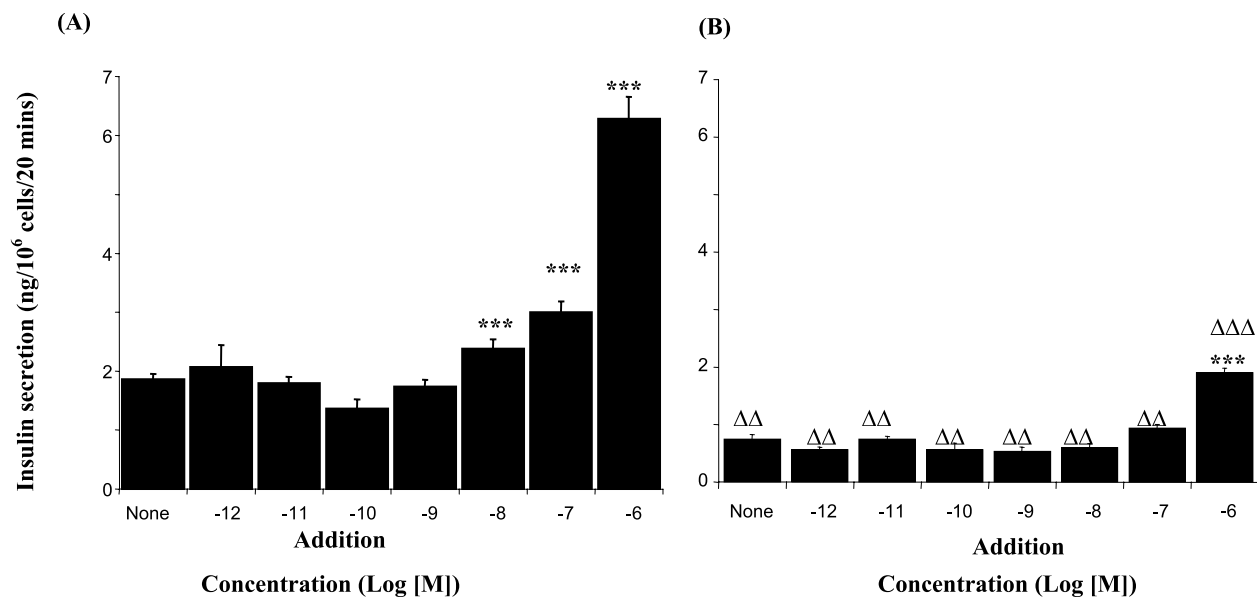
The mechanism through which the structurally characterised peptide, palustrin-1c, induces insulin secretion could not be determined due to the small amount of the peptide obtained and the use of a high proportion of the peak in determining its structure. Additionally, the presence of a cysteine disulphide bridge makes synthesis extremely difficult. However, there was no evidence that any of the insulintropic peptides isolated from the skin secretions of *R. palustris* exerted their effects by merely decreasing viability or causing lysis of BRIN-BD11 cells.



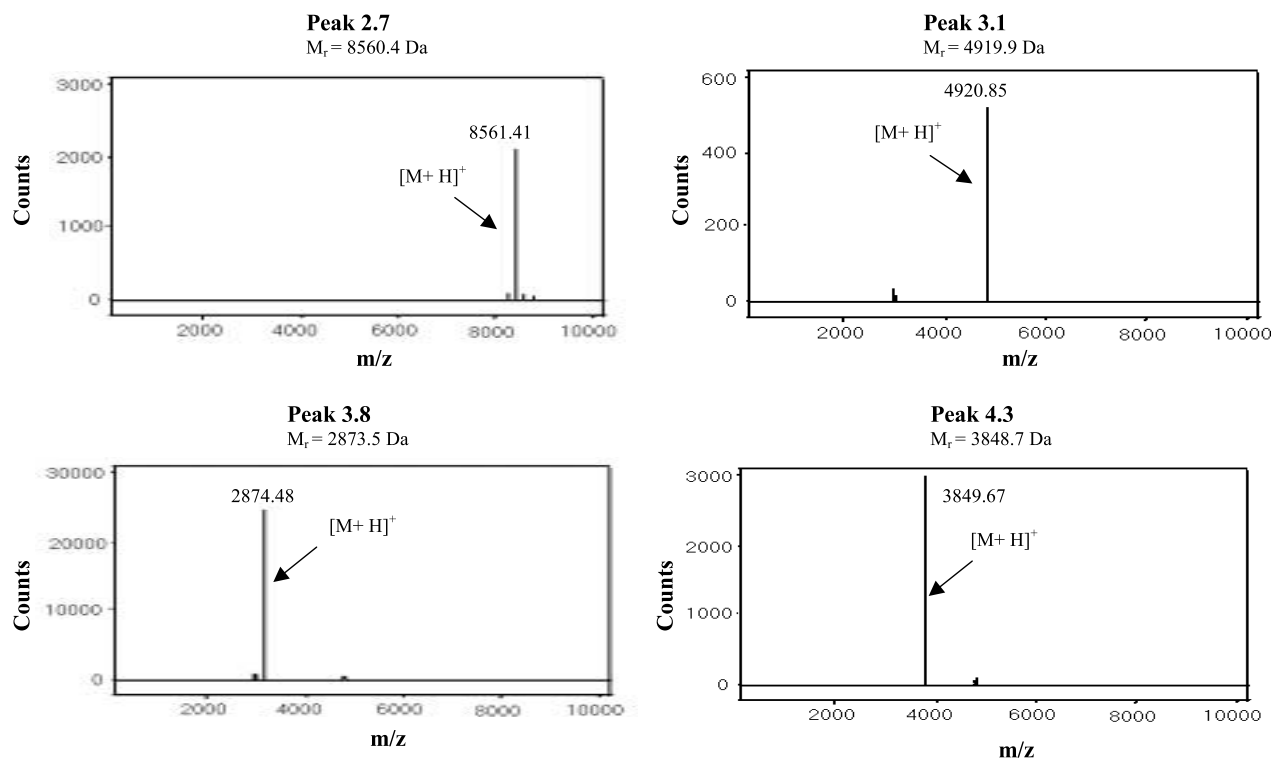
**Figure 4** Effects of various purified peaks of *R. palustris* venom on insulin secretion from BRIN-BD11 cells. Incubations were performed at 5.6 mM glucose, using hand-collected fractions shown in Fig. 3. Values are the mean  $\pm$  S.E.M. for three separate observations. \*\* $P < 0.01$  and \*\*\* $P < 0.001$  compared with 5.6 mM glucose alone.

This was determined by neutral red staining and simple observations of the cells under phase-contrast microscopy. Furthermore, experiments performed with synthetic brevinin-1 revealed a concentration-dependent stimula-

tion of insulin release, which could be abolished by removal of extracellular  $Ca^{2+}$ . This indicates clearly the involvement of regulated secretory pathway(s) in the insulinotropic effect. These observations suggest that



**Figure 5** Effects of various concentrations of synthetic brevinin-1 on insulin secretion from BRIN-BD11 cells. Incubations were performed at 5.6 mM glucose in (A) normal KRB buffer and (B)  $Ca^{2+}$ -free buffer. Values are the mean  $\pm$  S.E.M. for eight separate observations. \*\*\* $P < 0.001$  compared with 5.6 mM glucose alone.  $\Delta\Delta P < 0.01$  and  $\Delta\Delta\Delta P < 0.001$  compared with similar conditions in (A).



**Figure 6** Mass spectrometry analysis of purified peptides of *R. palustris* eluted from HPLC. An aliquot of 1  $\mu$ l of the sample was mixed with 0.5  $\mu$ l of a matrix solution, placed on a 100-well, stainless-steel sample plate and allowed to dry at room temperature. The mass spectra were recorded with the Voyager MALDI-TOF mass spectrometer.

**Table 1** A comparison of the primary structures of the isolated peptide, palustrin-1c (peak 3.8), from *R. palustris* with other members of the brevinin/esculentin/gaegurin/ranaturin family

*Palustrin-1c	-ALSILRG-----LEKLAKMGIALTNCATKKC	27
Brevinin-1	-FLPVLAAA---GIAAKVVP-----ALFCKITKKC	24
Esculentin-2P	GFSSIFRGVAKFASKGLGKDLARLGVNLVACKISKQC	37
Gaegurin-1	SLFSLIKAGAKFLGKNLLKQGACY----AACKASKQC	33
Ranaturin-1	SMLSVLK-----NLGKVGGLGFVACKINKQC	25

Sequences are brevinin-1 from *R. brevipoda porsa* (Morikawa *et al.* 1992), esculentin-2P from *R. pipiens* (Goraya *et al.* 2000), ranaturin-1 from *R. catesbeiana* (Goraya *et al.* 1998) and gaegurin-1 from *R. rugosa* (Park *et al.* 1994). The sequences are aligned so as to maximise structural similarity.

the mechanisms underlying the insulin-releasing activities of these molecules might be distinct from those mediating antimicrobial action. Indeed, other peptide regulators of insulin secretion, including gastric inhibitory polypeptide (GIP) and diazepam-binding inhibitor (DBI), have also been shown to exhibit antimicrobial effects (Agerberth *et al.* 1993).

In conclusion, the present study has demonstrated that the skin secretions of *R. palustris* contain various insulin-releasing peptides, including a 27 amino acid peptide, palustrin-1c, thought to act through physiological pathways. This peptide exhibited 46% homology with brevinin-1, which was also shown to trigger physiological

insulin release. Further studies are required to assess structural-functional relationships, mechanisms of action, extra pancreatic effects and whether mammalian counterparts of these interesting and potentially antidiabetic peptides exist.

### Acknowledgements

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## References

- Agerberth B, Boman A, Andersson M, Jornvall H, Mutt V & Boman HG 1993 Isolation of three antimicrobial peptides from pig intestine: gastric inhibitory polypeptides (7–42), diazepam-binding inhibitor (32–86) and a novel factor. *European Journal of Biochemistry* **216** 623–629.
- Anastasi A, Erspamer V & Endean R 1968 Isolation and amino acid sequence of caerulein, the active decapeptide of the skin of *Hyla caerulea*. *Archives of Biochemistry and Biophysics* **125** 57–68.
- Anastasi A, Erspamer V & Bucci M 1972 Isolation and amino acid sequences of alytesin and bombesin, two analogous active tetradecapeptides from the skin of European discoglossid frogs. *Archives of Biochemistry and Biophysics* **148** 443–446.
- Basir YJ, Floyd C, Knoop FC, Dulka J & Conlon JM 2000 Multiple antimicrobial peptides and peptides related to bradykinin and neuromedin N isolated from skin secretion of the pickerel frog, *Rana palustris*. *Biochimica et Biophysica Acta* **1543** 95–105.
- Bevins CL & Zasloff M 1990 Peptides from frog skin. *Annual Review of Biochemistry* **59** 395–414.
- Broccardo M, Erspamer V, Falconieri Erspamer G, Improta G, Linari G, Melchiorri P & Montecucchi PC 1981 Pharmacological data on dermorphins, a new class of potent opioid peptides from amphibian skin. *British Journal of Pharmacology* **73** 625–631.
- Chen T, Orr DF, Bjourson AJ, McClean S, O'Rourke M, Hirst DG, Rao P & Shaw C 2002 Novel bradykinins and their precursor cDNAs from European yellow-bellied toad (*Bombina variegata*) skin. *European Journal of Biochemistry* **269** 4693–4700.
- Chen YE & Drucker DJ 1997 Tissue-specific expression of unique mRNAs that encode proglucagon-derived peptides or exendin 4 in the lizard. *Journal of Biological Chemistry* **272** 4108–4115.
- Clark DP, Durell S, Maloy WL & Zasloff M 1994 Ranalexin. A novel antimicrobial peptide from bullfrog (*Rana catesbeiana*) skin, structurally related to the bacterial antibiotic, polymyxin. *Journal of Biological Chemistry* **269** 10849–10855.
- Conant R & Collins JT 1998 *A Field Guide to Reptiles and Amphibians of Eastern/Central North America*. Boston, MA: Houghton Mifflin.
- Conlon JM, Halverson T, Dulka J, Platz JE & Knoop FC 1999 Peptides with antimicrobial activity of the brevinin-1 family isolated from skin secretion of the southern leopard frog, *Rana sphenoccephala*. *Peptide Research* **54** 522–527.
- Duellman WE & Trueb L 1994 *Biology of Amphibians*. New York: McGraw-Hill.
- Eng J, Kleinman WA, Singh L, Singh G & Raufman JP 1992 Isolation and characterisation of exendin-4, an exendin-3 analogue, from *Heloderma suspectum* venom. Further evidence for an exendin receptor on dispersed acini from guinea pig pancreas. *Journal of Biological Chemistry* **267** 7402–7405.
- Erspamer V, Falconieri Erspamer G & Cei JM 1986 Active peptides in the skins of two hundred and thirty American amphibian species. *Comparative Biochemistry and Physiology. C: Comparative Pharmacology* **85** 125–137.
- Falconieri Erspamer G, Mazzanti G, Yasuhara T & Nakajima T 1984 Parallel bioassay of physalaemin and hylambatin on smooth muscle preparations and blood pressure. *Journal of Pharmacy and Pharmacology* **36** 284–286.
- Goraya J, Knoop FC & Conlon JM 1998 Ranatuerins: antimicrobial peptides isolated from the skin of the American bullfrog, *Rana catesbeiana*. *Biochemical and Biophysical Research Communications* **250** 589–592.
- Goraya J, Wang Y, Li Z, O'Flaherty M, Knoop FC, Platz JE & Conlon JM 2000 Peptides with antimicrobial activity from four different families isolated from the skins of the North American frogs *Rana luteiventris*, *Rana berlandieri* and *Rana pipiens*. *European Journal of Biochemistry* **267** 894–900.
- Hancock REW & Lehrer R 1998 Cationic peptides: a new source of antibiotics. *Trends in Biotechnology* **16** 82–88.
- Hancock REW, Falla T & Brown MH 1995 Cationic bactericidal peptides. *Advances in Microbial Physiology* **37** 135–175.
- Hardy L & Raymond LR 1991 Observation on the activity of the pickerel frog, *Rana palustris* (Anura: Ranidae), in northern Louisiana. *Journal of Herpetology* **25** 220–228.
- Hunt SM, Chrzanowska C, Barnett CR, Brand HN & Fawell JK 1987 A comparison of *in vitro* cytotoxicity assays and their application to water samples. *Alternatives to Laboratory Animals* **15** 20–29.
- Kolterman OG, Buse JB, Fineman MS, Gaines E, Heintz S, Bicsak TA, Taylor K, Kim D, Aisporna M, Wang Y & Baron AD 2003 Synthetic exendin-4 (exenatide) significantly reduces postprandial and fasting plasma glucose in subjects with type 2 diabetes. *Journal of Clinical Endocrinology and Metabolism* **88** 3082–3089.
- Kwon MY, Hong SY & Lee KH 1998 Structure-activity analysis of brevinin 1E amide, an antimicrobial peptide from *Rana esculenta*. *Biochimica et Biophysica Acta* **1387** 239–248.
- McClenaghan NH & Flatt PR 1999 Physiological and pharmacological regulation of insulin release: insights offered through exploitation of insulin secreting cell lines. *Diabetes Obesity and Metabolism* **3** 137–150.
- McClenaghan NH, Barnett CR, Ah-Sing E, Abdel-Wahab YH, O'Harte FP, Yoon TW, Swanson-Flatt SK & Flatt PR 1996 Characterization of a novel glucose-responsive insulin-secreting cell line, BRIN-BD11, produced by electrofusion. *Diabetes* **45** 1132–1140.
- Moler PE 1992 Pickerel frog, *Rana palustris*. In *Rare and Endangered Biota of Florida*, pp 66–69. Ed. PE Moler. Gainesville, FL: University Press of Florida.
- Morikawa N, Hagiwara K & Nakajima T 1992 Brevinin-1 and -2, unique antimicrobial peptides from the skin of the frog, *Rana brevipoda porsa*. *Biochemical and Biophysical Research Communications* **189** 184–190.
- Park JM, Jung JE & Lee BJ 1994 Antimicrobial peptides from the skin of a Korean frog, *Rana rugosa*. *Biochemical and Biophysical Research Communications* **205** 948–954.
- Roseghini M, Falconieri Erspamer G & Severini C 1988 Biogenic amines and active peptides in the skin of fifty-two African amphibian species other than bufonids. *Comparative Biochemistry and Physiology. C: Comparative Pharmacology* **91** 281–286.
- Roseghini M, Falconieri Erspamer G, Severini C & Simmaco M 1989 Biogenic amines and active peptides in extracts of the skin of thirty-two European amphibian species. *Comparative Biochemistry and Physiology. C: Comparative Pharmacology* **94** 455–460.
- Seon AA, Pierre TN, Redeker V, Lacombe C, Delfour A, Nicolas P & Amiche M 2000 Isolation, structure, synthesis, and activity of a new member of the calcitonin gene-related peptide family from frog skin and molecular cloning of its precursor. *Journal of Biological Chemistry* **275** 5934–5940.

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