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Isolation and structural characterisation of a novel 13-amino acid insulin-releasing peptide from the skin secretion of *Agalychnis calcarifer*

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Abstract

We describe the isolation and characterisation of an insulinotropic peptide from the skin secretions of *Agalychnis calcarifer* frogs. Peptides in crude secretions obtained by mild electrical stimulation from the dorsal skin surface were purified by reversed-phase HPLC, yielding fractions in two zones with insulin-releasing activity ($p<0.001$). The peaks showing greatest *in vitro* insulin-releasing activity were subsequently purified to homogeneity, revealing a novel insulinotropic 13-amino-acid (1653.2 Da) peptide with the primary structure RRKPLFPLIPRPK (RK-13). A database search for RK-13 showed 53.8% similarity with the N-terminal region of proline-arginine-rich antimicrobial peptide (PR-39). Synthetic RK-13 stimulated insulin release in a dose-dependent, glucose-sensitive manner, exerting its effects through a cyclic AMP-protein kinase A pathway independent of pertussis toxin-sensitive G proteins. Unlike PR-39, RK-13 lacks antimicrobial effects on the growth of yeast, and Gram-positive and Gram-negative bacteria. Our data indicate that skin secretions of *Agalychnis calcarifer* frogs contain insulin-releasing peptides, including RK-13, which merit further investigation as insulin secretagogues.

Keywords: *Agalychnis calcarifer*; amphibian peptides; insulin secretion; insulinotropic peptide, RK-13.

Introduction

The skin of amphibians contains several types of secretory glands, either mucus or granular, distributed throughout the dorsal and body region. Mucous glands provide the moist coating necessary for cutaneous respiration, whereas granular glands synthesise and expel a variety of biologically active peptides (Erspamer and Melchiorri, 1983; Bevins and Zasloff, 1990). These compounds are thought to play various roles, either in the regulation of physiological functions of the skin or in defence against predators or microorganisms (Barthalmus, 1994; Barra and Simmaco, 1995).

Interest in frog skin peptides has increased following the observation that some have primary structures that are identical to endogenous mammalian counterparts (Erspamer, 1983; Bevins and Zasloff, 1990) or share discrete bioactive domains (Anastasi et al., 1971). The discovery of trachykinins, bombesin and caerulein, for example, aided the elucidation of substance P, gastrin-releasing peptide and cholecystokinin, respectively, and led to the concept of a brain-gut-skin peptide triangle (Walsh et al., 1979; Erspamer and Melchiorri, 1980). The granular secretions of the subfamily of Phyllomedusinae are known to contain large numbers of pharmacologically active peptides, such as sauvagine and dermorphin from *Phyllomedusa sauvagei* (Erspamer et al., 1980; Montecucchi et al., 1981), and tryptophillin from *Phyllomedusa rohdei* (Montecucchi, 1985).

The subfamily of Phyllomedusinae is divided into *Phyllomedusa*, *Agalychnis* and *Pachymedus* species (Duellman and Trueb, 1986). *Agalychnis calcarifer* is a leaf frog that occurs specifically in the humid lowlands of Central America (Caribbean, Costa Rica and Panama) and the north-western part of South America (Pacific Colombia and Ecuador) (Wallis, 1996). The sides, belly, and hidden surfaces of the limbs of this species are bright yellow or orange, while the back is dark green with whitish flecks (Wallis, 1996). Another noteworthy feature of this species is the presence of a short, pointed skin flap (the calcar) on the heel. Recent studies have shown that the skin secretions of the anurans *Agalychnis litodryas* (Marenah et al., 2004c), *Rana palustris* (Marenah et al., 2004a) and *Bombina variegata* (Marenah et al., 2004b) contain insulin-releasing peptides.

In the present paper we describe the isolation and characterisation of peptides with insulin-releasing activity from the skin secretion of *A. calcarifer*. This species of frog belongs to the subfamily of Phyllomedusinae (Duellman and Trueb, 1986), the skin of which is known to contain numerous peptides with diverse biological activity (Erspamer et al., 1985; Lazarus and Attila, 1993). The complete amino acid sequence of one novel insulinotropic peptide was determined and mechanistic studies were performed on its insulin secretory action.

Results

Isolation of insulin-releasing peptides

Crude venom was purified by HPLC, yielding 80 fractions (Figure 1). Significant insulin-releasing activity was observed with fractions 35–45 (zone A) and 53 (zone B) compared with 5.6 mM glucose ($p<0.001$, $n=3$) (Figure 2). The individual fractions in zone A were pooled and further purified, yielding peaks 1.1–1.20 (Figure 3A). Similarly, zone B was purified, yielding peaks 2.1–2.5 (Figure 3B). Subsequent *in vitro* testing demonstrated that peaks 1.3, 1.10, 1.17, 1.18, 2.4 and 2.5 increased insulin release

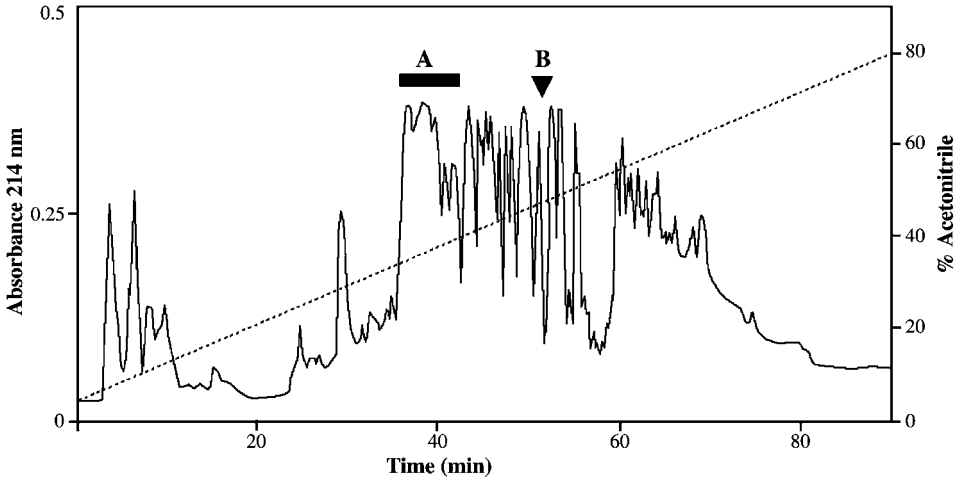

Figure 1 Reversed-phase HPLC of the crude venom of *Agalychnis calcarifer*.
Crude venom (20 mg) was dissolved in 0.12% trifluoroacetic acid/water (2 ml) and 1 ml was chromatographed on a semi-preparative C18 column as described in the materials and methods section. Absorbance was monitored at 214 nm, with collection of 2-ml fractions. Zones A and B correspond to fractions eluting at 35–45 and 53 min, respectively. The dashed lines show the concentration of acetonitrile in the eluting solvent.

by 1.3–2.7-fold compared with 5.6 mM glucose ($p<0.001$; $n=3$) (Figure 4). As assessed by a modified neutral red assay, peaks 2.4 and 2.5 caused an approximate 30% decrease in cell viability (data not shown; $p<0.01$; $n=3$). The other insulin-releasing peptides were free from toxic action and did not affect BRIN-BD11 cell viability.

Mass spectrometry and sequence analysis

The most potent insulin-releasing peptide without toxic effects on cell viability (peak 1.10) was subjected to mass spectral analysis, revealing a mass of 1653.2 Da (Figure 5). N-Terminal amino acid sequence analysis gave the primary sequence as RRKPLFPFIPRPK (RK-13 peptide). The calculated (theoretical) mass of the RK-13 peptide was 1652.1 Da. As shown in Table 1, a search in the Swiss-Prot FASTA database using the GCG sequence analysis programme (www.hgmp.mrc.ac.uk/Registered/Option/gcg.html) revealed 53.8% similarity of the novel 1653.2-Da peptide with the N-terminus of the proline-arginine-rich antimicrobial peptide, PR-39, originally isolated from pig intestine (Agerbeth et al., 1991). This peptide kills Gram-negative bacteria.

Insulin secretory actions of the synthetic RK-13 peptide

Synthetic RK-13 peptide exerted dose-dependent 1.5–1.9-fold stimulatory effects on insulin secretion from BRIN-BD11 cells ($n=8$) evident from a concentration of 10^{-8} M (Figure 6). The stimulatory effects induced by RK-13 (10^{-8} M) were enhanced at increasing concentrations over the range 1.1–16.7 mM glucose (Figure 7). Further-

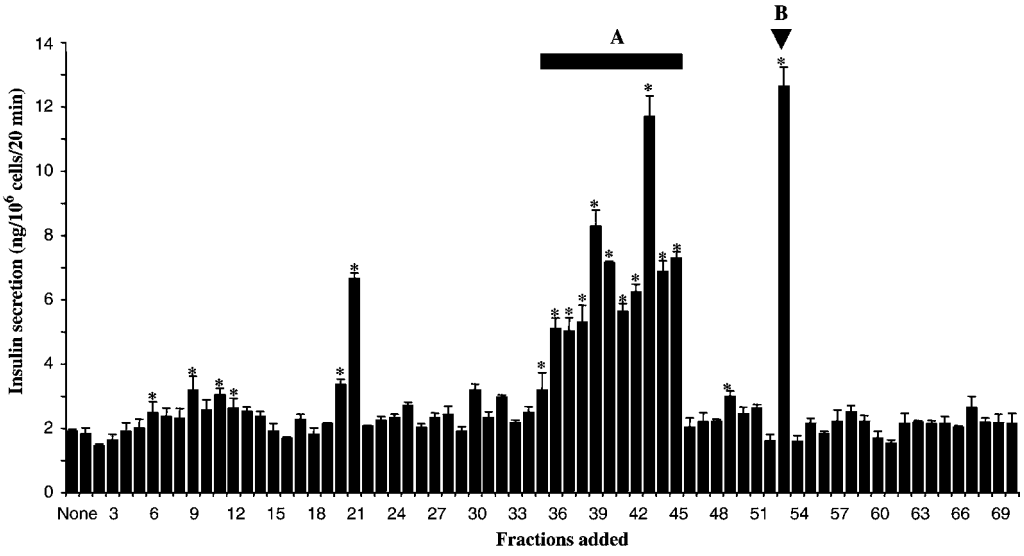

Figure 2 Effects of various semi-preparative C18 HPLC fractions of *Agalychnis calcarifer* crude venom on insulin secretion from BRIN-BD11 cells.
Incubations were performed at 5.6 mM glucose. Values are the mean±SEM for three separate observations. *$p<0.001$ compared with 5.6 mM glucose alone. Zones A and B correspond to fractions eluting at 35–45 and 53 min, respectively.

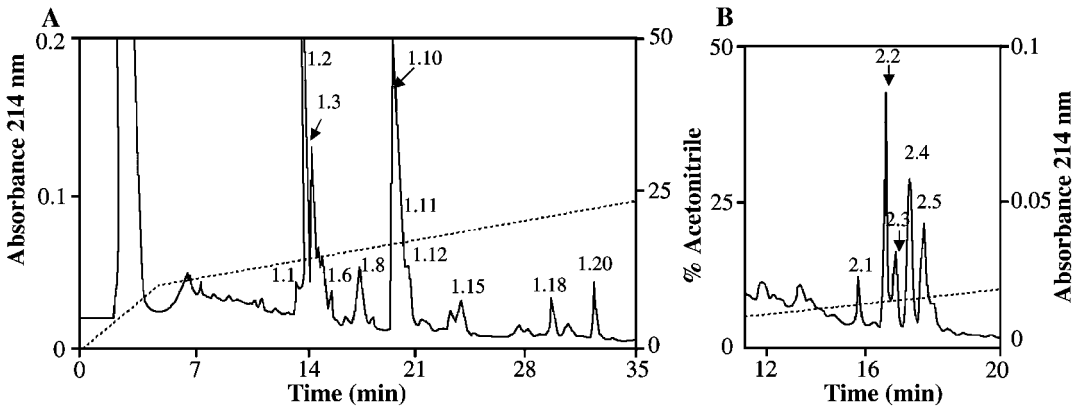

Figure 3 Reversed-phase HPLC purification of the pooled fractions of *Agalychnis calcarifer* from zones A and B in Figure 2. Fractions were applied to an analytical Vydac C18 column as described in materials and methods. Absorbance was monitored at 214 nm. The dashed lines show the concentration of acetonitrile in the eluting solvent.

more, the stimulatory effects of RK-13 (10^{-8} and 10^{-12} M) were reduced, but not abolished in the absence of Ca^{2+} in the incubation buffer (Figure 8). In addition, the stimulatory effects of RK-13 (10^{-8} M) were abolished in cells cultured overnight with 25 μM forskolin (Figure 9). Overnight culture with 10 nM PMA or pertussis toxin (0.1 μg/ml) did not affect the insulin-releasing ability of the peptide (Figure 9). Interestingly, the insulin-releasing action of RK-13 was not affected by 50 μM verapamil and was clearly evident in cells depolarised with 30 mM KCl (Table 2).

Antimicrobial assays

The minimum inhibitory concentration (MIC) values for the synthetic RK-13 were determined in the range 5×10^{-3}–1×10^{-1} M (data not shown). The results indicate that the peptide does not significantly inhibit the growth of the Gram-negative bacteria *Escherichia coli,* and *Pseudomonas aeruginosa*, the growth of the Gram-positive bacterium *Staphylococcus aureus* or the yeast *Candida albicans* over this concentration range. Slight inhibition of *E. coli* was detected over the range 2.5×10^{-2}–1×10^{-1} M.

Discussion

In this study, the crude skin secretion of *Agalychnis calcarifer* was fractionated by reverse-phase HPLC, yielding four peptides capable of eliciting 1.3–2.7-fold increases in insulin release without affecting cell viability as assessed by neutral red assay (Hunt et al., 1987). This insulin output is approximately equivalent to that induced by the gut peptides GLP-1 and GIP (O'Harte et al., 1998a,b; McClenaghan and Flatt, 1999). The most potent peptide (peak 1.10) retained insulinotropic activity at a further 1:500 dilution, possibly making it one of the most potent frog peptides isolated. However, the effectiveness of other peptides isolated was lost at more than 1:10 dilution of the original sample.

Structural analysis of the most potent insulinotropic peptide isolated (peak 1.10) was carried out by electrospray ionisation mass spectrometry and automated Edman degradation. This revealed a 13-amino-acid 1653.2-Da peptide with the novel sequence RRKPLFPLIPRPK. The theoretical (calculated) molecular mass (1652.1 Da) of this peptide, named RK-13, closely corresponds to the experimental mass. This peptide shows identity in seven of the 13 amino acids to the N-terminal sequence of the proline-arginine-rich peptide PR 39 originally isolated by Agerbeth et al. (1991) from the small intestine of the pig.

Many antibacterial peptides are cationic, binding to the exposed negative charges of bacterial membranes to induce complete lysis of the organism by rupturing the plasma membrane or disturbing the membrane lipid bilayer. This causes leakage of cellular components, as well as dissipating the electrical membrane potential

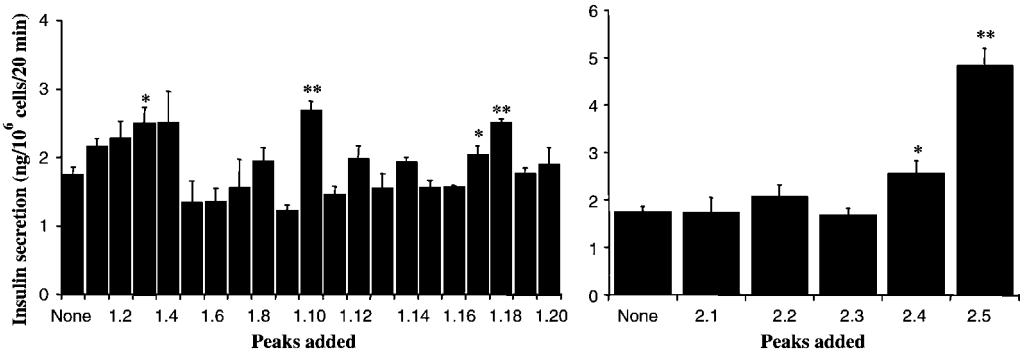

Figure 4 Effects of various purified peaks of *Agalychnis calcarifer* venom on insulin secretion from BRIN-BD11 cells. Incubations were performed at 5.6 mM glucose using fractions shown in Figure 3. Values are the mean±SEM for three separate observations. *$p<0.05$ and **$p<0.001$ compared with 5.6 mM glucose alone.

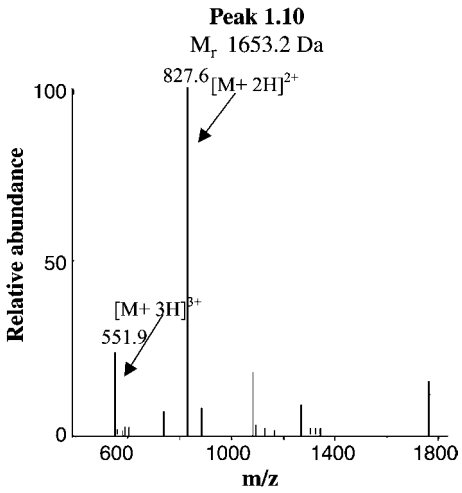

Figure 5 Electrospray ion-trap mass spectrometry analysis of purified peak (1.10) of *Agalychnis calcarifer* eluted from HPLC as shown in Figure 3.
The molecular mass was determined from the ESI-MS profile as described under materials and methods.

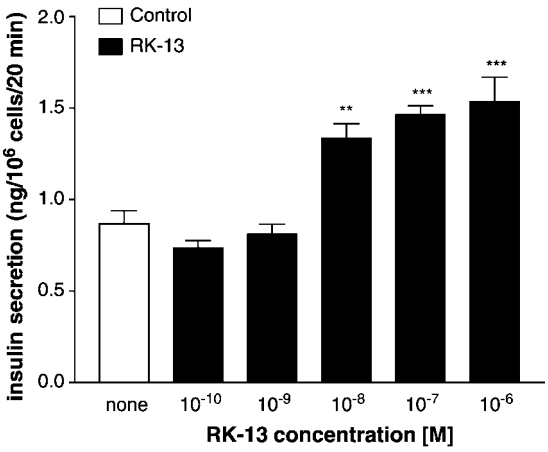

Figure 6 Dose-dependent effects of synthetic RK-13 peptide on insulin secretion from BRIN-BD11 cells.
Incubations were performed at 5.6 mM glucose. Values are the mean±SEM of eight separate observations. $^{**}p<0.01$ and $^{***}p<0.001$ compared to 5.6 mM glucose alone.

(Epand and Vogel, 1999). PR-39, on the other hand, has been shown to kill bacteria by inhibiting DNA and protein synthesis (Boman et al., 1993). The sequence homology of the novel insulin-releasing peptide at seven of the 39 amino acids might not be sufficient to mimic the actions of PR-39. Indeed, RK-13 not only lacked effects on BRIN-BD11 cell viability, but also failed to inhibit the growth of Gram-positive or -negative bacteria or yeast over the concentration ranges tested.

The lack of effect of the novel RK-13 peptide on cell viability indicates that the insulin-releasing activity cannot be simply attributed to cell lysis or toxicity. It therefore follows that this peptide stimulates insulin release through a regulated pathway. Notably, the effects on insulin release were both concentration-dependent and glucose-sensitive. Removal of extracellular calcium or blockade of voltage-dependent Ca^{2+} channels with verapamil decreased but did not abolish secretory effectiveness. Similarly, a powerful insulin response was observed using cells depolarised with 30 mM KCl, indicating a degree of independence from changes in ion permeability. However, down-regulation of cyclic AMP-protein kinase A pathways by overnight culture of BRIN-BD11 cells with forskolin (Altman et al., 1987; Gromada et al., 1998) blocked the acute stimulatory effects of both the novel peptide and forskolin. Although similar overnight culture with PMA blocked protein kinase C-dependent pathways and the acute stimulatory effects of PMA as expected (Hii et al., 1987; Yamatani et al., 1988; Persaud et al., 1989; Wolf et al., 1989), the insulinotropic actions of the novel peptide and forskolin persisted in full. This pattern of responsiveness shares certain similarities with the actions of GLP-1 and GIP in the β-cell. In addition, the stimulatory actions were not inhibited by overnight culture with pertussis toxin (Seaquist et al., 1992), indicating the involvement of a G-protein-independent pathway in the stimulatory actions of the novel peptide.

Materials and methods

Reagents

RPMI-1640 tissue culture medium, foetal bovine serum, penicillin and streptomycin were all purchased from Gibco (Paisley, Strathclyde, UK). Phorbol-12-myrisate-13-acetate (PMA), forskolin, pertussis toxin and verapamil were obtained from the Sigma Chemical Company Ltd (Poole, Dorset, UK). HPLC-grade acetonitrile was obtained from Rathburn (Walkersburn, Scotland). Sequencing-grade trifluoroacetic acid was obtained from Aldrich (Poole, Dorset, UK). All chemicals employed in the operation of the 491 Procise gas-phase sequencer were supplied by Perkin Elmer Applied Biosystems (Warrington, UK). RK-13 (RRKPLFPLIPRPK) peptide was synthesised by Invitrogen Life Technologies (Paisely, UK) through utilisation of Fmoc chemistry (Maeji et al., 1995). The synthetic peptide had a molecular weight of 1653.2 Da and a purity of 70%. All other chemicals used were of the highest purity available.

Collection of venom

Four young captive-bred *Agalychnis calcarifer* were maintained *in terraria* at 24°C with a 12 h/12 h light/dark 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 washed off into a glass beaker using deionised water. The resultant secretions were freeze-

Table 1 Similarity to PR-39 of RK-13 isolated from *Agalychnis calcarifer*.

Peptide	Amino acid sequence	Similarity
RK-13	R R K P L F P F I P R P K	53.8%
	| | | | | | |	
PR-39	R R R P R P P Y L P R P R P P P F F P P R L P P R I I P G F P P R F P P R F P	

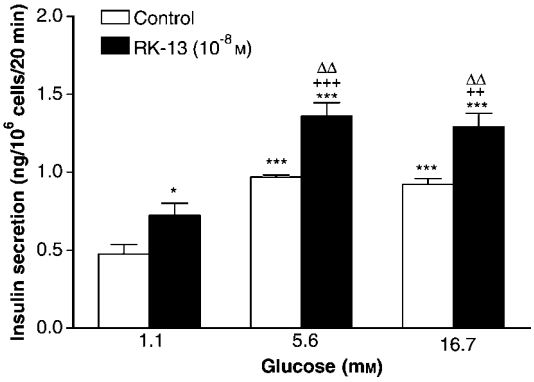

Figure 7 Effects of glucose on insulin secretion induced by RK-13 from BRIN-BD11 cells.
Values are the mean±SEM of eight separate observations. *$p<0.05$, ***$p<0.001$ compared to 1.1 mM glucose alone. $^{++}p<0.01$, $^{+++}p<0.001$ compared to RK-13 at 1.1 mM glucose. $^{\Delta\Delta}p<0.01$ compared to respective incubations in the absence of RK-13.

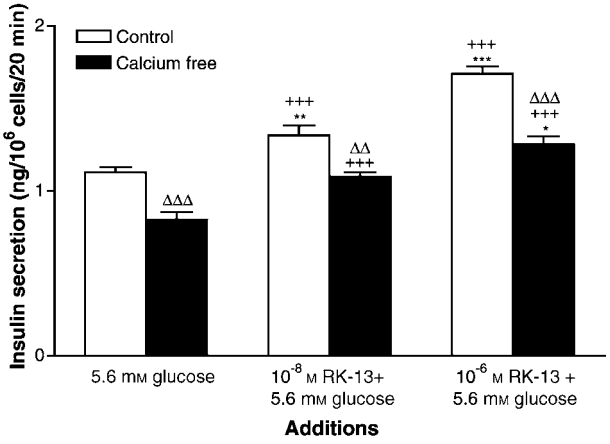

Figure 8 Effects of synthetic RK-13 peptide on insulin secretion from BRIN-BD11 cells in the presence or absence of Ca^{2+}. Values are the mean±SEM of eight separate observations. **$p<0.01$ and ***$p<0.001$ compared to 5.6 mM glucose alone in the presence of Ca^{2+}. $^{+++}p<0.001$ compared to 5.6 mM glucose alone in the absence of Ca^{2+}. $^{\Delta\Delta}p<0.01$ and $^{\Delta\Delta\Delta}p<0.001$ compared to respective incubations in the presence of Ca^{2+}.

dried in a Hetosicc 2.5 freeze dryer (Heto, Nottingham, UK). Samples of 50 mg (dry weight) of skin secretion were obtained.

Purification of peptides

The lyophilised crude venom (20 mg) was dissolved in 0.12% trifluoroacetic acid (TFA)/water (2 ml) and a 1-ml aliquot was chromatographed on a Vydac 218TP510 semi-preparative C-18 column (25×1 cm; Hesperia, USA). The column was equilibrated with 0.12% (v/v) trifluoroacetic acid/water at a flow rate of 2 ml/min. Using 0.1% (v/v) TFA in 70% acetonitrile/water, the concentration of acetonitrile in the eluting solvent was increased to 80% (v/v) over 80 min in a linear gradient. Absorbance was monitored at 214 nm, with collection of 2-ml fractions. Fractions which showed major insulin-releasing activity were pooled and rechromatographed using 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. Using 0.1% (v/v) TFA in 70% acetonitrile/water, the concentration of acetonitrile in the eluting solvent was increased 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) foetal 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_2 and 95% air using a LEEC incubator (Laboratory Technical Engineering, Nottingham, UK). In one experimental series, cells were cultured overnight with 25 μM forskolin, 10 nM PMA or 0.1 μg/ml pertussis toxin prior to acute tests.

Acute insulin release studies

Insulin release from BRIN-BD11 cells was determined using cell monolayers (McClenaghan et al., 1996). The cells were harvested with the aid of trypsin/EDTA (Gibco), seeded into 24-well plates (Nunc, Rosklide, Denmark) at a density of 1.5×10^6 cells

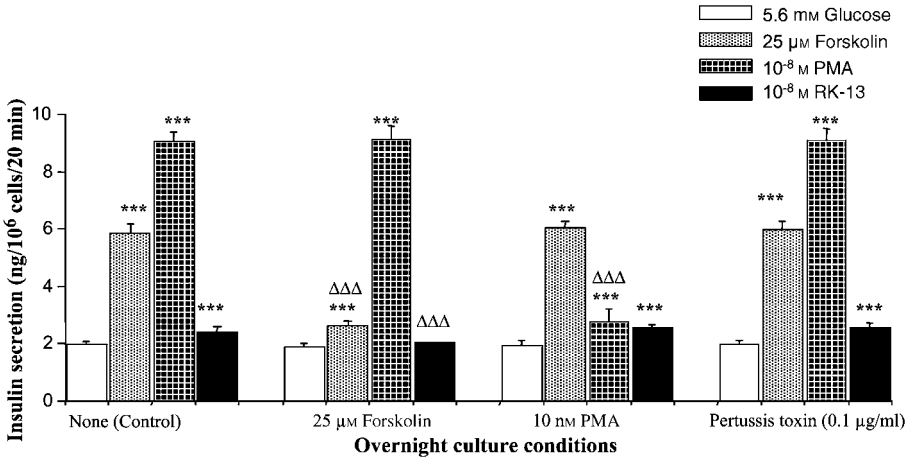

Figure 9 Effects of RK-13, forskolin and PMA on acute insulin release from BRIN-BD11 cells.
Cells were cultured overnight in the absence (control) or presence of 25 μM forskolin, 10 nM PMA and 0.1 μg/ml pertussis toxin. Acute incubations were performed at 5.6 mM glucose. Values are the mean±SEM for eight separate observations. ***$p<0.001$ compared with 5.6 mM glucose alone under same culture conditions; $^{\Delta\Delta\Delta}p<0.001$ compared with the respective test reagent following control culture.

Table 2 Effects of 10^{-8} M RK-13 on insulin secretion from BRIN-BD11 in the presence of verapamil or depolarising K^+ concentration.

Addition	Insulin secretion (ng/10^6 cells/20 min)	
	Control	10^{-8} M RK-13
None	1.68±0.16	2.75±0.12*
Verapamil (50 μM)	1.74±0.17	$3.54\pm0.20^{\Delta\Delta}$***
KCl (30 mM)	$5.32\pm0.38^{\Delta\Delta\Delta}$	$16.45\pm0.10^{\Delta\Delta\Delta}$***

Acute incubations were performed at 5.6 mM glucose. Values are mean±SEM for eight separate observations. *$p<0.05$ and ***$p<0.001$ compared with control, $^{\Delta\Delta}p<0.01$ and $^{\Delta\Delta\Delta}p<0.001$ compared with no addition.

per well, and allowed to attach overnight. Prior to acute tests, cells were preincubated for 40 min at 37°C in 1.0 ml of a Krebs-Ringer bicarbonate buffer (KRB; 115 mM NaCl, 4.7 mM KCl, 1.28 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 10 mM $NaHCO_3$, 5 g/l bovine serum albumin, pH 7.4) supplemented with 1.1 mM glucose. 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 fractions, peaks (equivalent to 25 μl of dried HPLC fraction) or test agents as indicated in the figures. Further insulin release studies were performed using a synthetic version of the most potent peptide purified, RK-13. These included dose- and glucose-dependent effects of RK-13, together with studies on the mechanism of action using Ca^{2+}-free KRB buffer (supplemented with 0.5 mM EGTA) and other conditions affecting signal transduction pathways. In one experimental series, the insulin secretory effects of RK-13 were examined using cells cultured for 18 h with 25 μM forskolin, 10 nM PMA or pertussis toxin (0.1 μg/ml). Cell viability after 20-min test incubations was assessed by a modified neutral red assay (Hunt et al., 1987). After incubation, aliquots of buffer were removed and stored at −20°C for insulin radioimmunoassay (Flatt and Bailey, 1981).

Molecular mass determination

The molecular masses of peptides in the purified insulin releasing peaks were determined using electrospray ionisation quadrupole ion-trap mass spectrometry (ESI-MS). Samples were infused at a flow rate of 5 μl/min. Mass spectra were recorded on a Thermo Finnigan LCQ™ benchtop quadrupole ion-trap mass spectrometer (Thermo Finningan, Hemel Hempstead, UK). Spectra were collected using full ion scan mode over the mass/charge (m/z) range 150–2000. In the ESI source, nitrogen sheath and auxiliary gas flows were maintained at 50 and 5, respectively, and refer to arbitrary values set by the software. The heated capillary temperature was 220°C and the spray voltage was set to 5 kV. Nitrogen gas for the LCQ™ was delivered from a Whatman nitrogen generator (Whatman Inc, Haverhill, USA) while helium damping gas present in the ion-trap was obtained from BOC Medical Gases (Guildford, Surrey, UK). Ions were detected and analysed in the positive mode as a function of their m/z ratio. The molecular mass of the peaks/peptides were determined from ESI-MS profiles using prominent multiple-charged ions and the following equation: $M_r=iM_i-iM_h$, where M_r is the molecular mass, M_i is the m/z ratio, I is the number of charges and M_h is the mass of a proton.

Structural analysis by automated Edman degradation

The primary structure of purified peptide was determined by automated Edman degradation, using an Applied Biosystems (Foster City, USA) Procise 491 microsequencer. Standard operating procedures defined in the manufacturer's user manual were used. The limit for detection of phenylthiohydantoin amino acids was 0.2 pmol. The primary structure was compared with those deposited in the SWISSPROT™ database.

Antimicrobial assays

To evaluate the antimicrobial effects, the minimal inhibitory concentrations (MICs) of peptides were determined by a standard microdilution method using 96-well microtitre cell-culture plates (Barchiesi et al., 1994). Serial dilutions of peptide (5–100 mM) in Mueller Hinton broth were incubated for 22 h at 37°C in a humidified atmosphere of 5% CO_2 in air with an inoculum (105 CFU/ml) from overnight cultures of *Escherichia coli* (NCTC 11560), *Pseudomonas aeruginosa* (NCTC 10662) and *Staphylococcus aureus* (NCTC 6571). Incubations with *Candida albicans* (CBS 562) were carried out in Sabourand liquid medium for 22 h at 37°C. After incubation, the absorbance at 620 nm of each well was determined using a Safire Tecan spectrofluorimeter (Reading, UK). The MIC of each peptide was taken as the lowest concentration at which visible inhibition of growth was observed.

Statistical analysis

Results are expressed as mean±SEM and values were compared using Student's unpaired *t*-test. Groups were considered to be significantly different for $p<0.05$.

Acknowledgments

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