original article

ORIGINAL ARTICLE

Tigerinin-1R: a potent, non-toxic insulin-releasing peptide isolated from the skin of the Asian frog, *Hoplobatrachus rugulosus*

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Aim: Characterization of peptides in the skin of the Vietnamese common lowland frog *Hoplobatrachus rugulosus* with the ability to stimulate insulin release *in vitro* and improve glucose tolerance *in vivo*.

Methods: Peptides in an extract of skin were purified by reversed-phase HPLC, and their abilities to stimulate the release of insulin and the cytosolic enzyme lactate dehydrogenase were determined using BRIN-BD11 clonal β cells. Insulin-releasing potencies of synthetic peptides and their effects on membrane potential and intracellular Ca^{2+} concentration were also measured using BRIN-BD11 cells. Effects on glucose tolerance and insulin release *in vivo* were determined in mice fed a high-fat diet to induce obesity and insulin resistance.

Results: A cyclic dodecapeptide ($RVCSAIPLPICH.NH_2$), termed tigerinin-1R, was isolated from the skin extract that lacked short-term cytotoxic and haemolytic activity but significantly ($p < 0.01$) stimulated the rate of release of insulin from BRIN-BD11 cells at concentrations ≥ 0.1 nM. The maximum response was 405% of the basal rate at 5.6 mM ambient glucose concentration and 290% of basal rate at 16.7 mM glucose. C-terminal α-amidation was necessary for high potency and a possible mechanism of action of the peptide-involved membrane depolarization and an increase in intracellular Ca^{2+} concentration. Administration of tigerinin-1R (75 nmol/kg body weight) to high fat–fed mice significantly ($p < 0.05$) enhanced insulin release and improved glucose tolerance during the 60-min period following an intraperitoneal glucose load.

Conclusion: Tigerinin-1R is a potent, non-toxic insulin-releasing peptide that shows potential for development into an agent for the treatment of type 2 diabetes.

Keywords: antidiabetic drug, beta cell, dose–response relationship, incretins, insulin secretagogue, type 2 diabetes

Introduction

The current pandemic of type 2 diabetes mellitus has necessitated new approaches to treatment and management. Natural products, including biologically active peptides from both vertebrate and invertebrate species, are being examined for their glucose-lowering properties. One such approach to treatment is the use of agents based on physiological incretins [1]. An incretin is a factor released by the gut in response to nutrients that facilitates uptake of glucose by peripheral tissues by stimulating secretion of insulin. The most potent incretin in the human is glucagon-like peptide-1 (GLP-1), and several analogues of this peptide are already in clinical use [2]. Similarly, peptides based on the structure of a second endogenous insulin-releasing peptide, glucose-dependent insulinotropic peptide (GIP), show promise [3]. The comparative approach to the discovery of new incretin-based drugs has been vindicated by the identification in the venom of a lizard, the Gila monster *Heloderma suspectum* [4], of exendin-4, a long-acting agonist

at the GLP-1 receptor that is in current clinical use under its trade name Byetta [5].

The skins of many, although by no means all, species of Anura (frogs and toads) synthesize peptides with the ability to inhibit growth of pathogenic bacteria and fungi [6]. The peptides are released into skin secretions, often in very high concentrations, in response to stress or infection and constitute a component of the animal's system of innate immunity [7]. Recent studies have shown that several frog skin peptides that were first identified on the basis of their ability to inhibit the growth of bacteria also stimulate insulin release *in vitro* from BRIN-BD11 rat clonal β-cells at low concentrations that are not cytotoxic to the cells. Such insulin-releasing peptides have been identified in skin secretions and/or skin extracts from the species *Hylarana güntheri* [8], *Lithobates catesbeianus* [9], *Lithobates palustris* [10], *Lithobates pipiens* [11], *Lithobates septentrionalis* [12] and *Pelophylax saharicus* [13] belonging to the family Ranidae and from *Hylomantis lemur* [14] and *Pseudis paradoxa* [15] belonging to the family Hylidae. In addition, brevinin-2GUb from *H. güntheri* [8], phylloseptin-L2 from *H. lemur* [14] and brevinin-2–related peptide (B2-RP) from *L. septentrionalis* [12] have been shown to be active *in vivo*, increasing

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plasma insulin concentrations and improving glucose tolerance in mice. Structure–activity studies of synthetic replicates of peptides belonging to the temporin [16], pseudin-2 [15] and B2-RP [12] families have led to the design of analogues with increased insulin-releasing potency. In all these cases, the frog skin peptides were active at concentrations that did not produce release of the cytosolic enzyme, lactate dehydrogenase (LDH), from the cells showing that the integrity of the plasma membrane had been maintained. Thus, these compounds may have therapeutic potential for the treatment of diabetes mellitus.

As part of a program of investigation to examine frog species systematically for the presence of dermal antimicrobial and insulin-releasing peptides, this study describes the purification and structural characterization of an insulin-releasing peptide from an extract of the skin of the Vietnamese common lowland frog *Hoplobatrachus rugulosus* Wiegmann, 1834. This species, also known as the Chinese edible frog, was formerly classified as *Rana rugulosa* in the family Ranidae but is now included with three other species (*Hoplobatrachus crassus*, *H. occipitalis* and *H. tigerinus*) in the genus *Hoplobatrachus* in the subfamily Dicroglossinae of the family Dicroglossidae [17]. The frog is widely distributed in Southeast Asia, and its preferred habitats include paddy fields, irrigation ditches, floodplain wetlands, forest pools and other wet areas. It is currently listed as a species of 'least concern' by the IUCN Red List of Threatened Species but has suffered population declines because of loss of habitat, predation by introduced bullfrogs and by being overexploited as a food source [18].

Materials and Methods

All experiments with live frogs were approved by the Animal Research Ethics committee of United Arab Emirates University (protocol no. A21-09) and were carried out by authorized investigators. Three adult specimens of *H. rugulosus* of unknown sex were purchased in Vietnam in a border region adjacent to Hekou County, China. The animals were anesthetized by immersion in ice-water and sacrificed by decapitation. Skin was immediately removed and freeze-dried for shipment to United Arab Emirates University. The tissue (4.17 g) was extracted by homogenization in ethanol/0.7 M HCl (3 : 1 vol/vol; 100 ml) at 0 °C using a Waring blender. The homogenate was stirred for 1 h at 0 °C and centrifuged (4000 g for 30 min at 4 °C). Ethanol was removed from the supernatant under reduced pressure, and after further centrifugation (4000 g for 30 min at 4 °C), the extract was pumped onto 6 Sep-Pak C-18 cartridges (Waters Associates, Milford, MA, USA) connected in series at a flow rate of 2 ml/min. Bound material was eluted with acetonitrile/water/trifluoroacetic acid (TFA) (70.0 : 29.9 : 0.1, vol/vol/vol) and freeze-dried.

Peptide Purification and Characterization

The skin extract, after partial purification on Sep-Pak cartridges, was re-dissolved in 0.1% (vol/vol) TFA–water (2 ml) and injected onto a (2.2 × 25 cm) Vydac 218TP1022 (C-18) reversed-phase high performance liquid chromatography (HPLC) column (Grace, Deerfield, IL, USA) equilibrated with

0.1% (vol/vol) TFA–water at a flow rate of 6.0 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 21% (vol/vol) over 10 min and to 63% (vol/vol) over 60 min using linear gradients. Absorbance was monitored at 214 and 280 nm, and fractions (1 min) were collected. The abilities of freeze-dried aliquots (100 μl) of the fractions to stimulate the release of insulin from BRIN-BD11 cells were determined. Fractions containing peptides with insulin-releasing activity were pooled and chromatographed on a (1 × 25 cm) Vydac 208TP510 (C-8) column. The concentration of acetonitrile in the eluting solvent was raised from 0 to 50% over 60 min, and the flow rate was 2.0 ml/min, and individual peaks were collected by hand. The peak associated with insulin-releasing activity was chromatographed on a (0.46 × 25 cm) Vydac 218TP54 (C-18) column. The concentration of acetonitrile in the eluting solvent was raised from 21 to 56% over 50 min, and the flow rate was 1.5 ml/min.

The primary structure of the peptide was determined by automated Edman degradation using an Applied Biosystems model 494 Procise sequenator (Foster City, CA, USA) at the University of Nebraska Medical Center Protein Structure Core Facility (Omaha, NE, USA). Matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry was carried out using a Voyager DE-PRO instrument (Applied Biosystems) operated in reflector mode with delayed extraction as described [8,9]. The instrument was calibrated with peptides of known molecular masses in the range of 1–4 kDa, and the accuracy of mass determination was ±0.02%.

Peptide Synthesis

Tigerinin-1R (Arg-Val-Cys-Ser-Ala-Ile-Pro-Leu-Pro-Ile-Cys-His.NH$_2$) and its non-amidated derivative (Arg-Val-Cys-Ser-Ala-Ile-Pro-Leu-Pro-Ile-Cys-His.COOH) were supplied in crude, uncyclized form by GL Biochem (Shanghai) Ltd (China). Formation of the disulfide bridge was accomplished by the method of Tam et al. [19], and the peptides were purified to near homogeneity by reversed-phase HPLC on a (2.2 × 25 cm) Vydac 218TP1022 (C-18) reversed-phase HPLC column equilibrated with 0.1% (vol/vol) TFA/water at a flow rate of 6.0 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 21% (vol/vol) over 10 min and to 56% (vol/vol) over 60 min using a linear gradient. The identities of the synthetic peptides were confirmed by electrospray mass spectrometry, and the purity of all peptides tested was 98%.

Determination of Insulin-releasing Activity *In Vitro*

The BRIN-BD11 rat clonal β-cell line is a well-established and convenient model to study insulin secretion in response to a range of nutrients, hormones, neurotransmitters and drugs [20]. BRIN-BD11 cells were grown at 37 °C in an atmosphere of 5% CO$_2$ and 95% air in RPMI-1640 tissue culture medium containing 10% (vol/vol) foetal calf serum, antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin) and 11.1 mM glucose as described [8–10]. The origin and characteristics of these cells have been provided in detail previously [21]. The cells were pre-incubated for 40 min at 37 °C in 1.0 ml Krebs–Ringer bicarbonate (KRB) buffer, pH 7.4 supplemented with either

5.6 or 16.7 mM glucose and 0.1% (w/v) bovine serum albumin [8]. Incubations with purified endogenous peptides ($10^{-12} - 3 \times 10^{-6}$ M; $n = 8$) were performed for 20 min at 37 °C using the same buffer. After incubation, aliquots of cell supernatant were removed for insulin radioimmunoassay [22].

To determine cytotoxicity, BRIN-BD11 cells were seeded into 24-multiwell plates and allowed to attach during overnight culture at 37 °C. Prior to the test, cells were pre-incubated for 40 min at 37 °C in KRB buffer supplemented with 5.6 mM glucose (1.0 ml). Test incubations with chromatographic fractions or synthetic peptides (0.1–3 μM; $n = 4$) were performed for 20 min at 37 °C. LDH concentrations in the cell supernatants were measured using a CytoTox 96 non-radioactive cytotoxicity assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Haemolytic activity was measured as previously described [8]. Peptides in the concentration range 16–500 μM were incubated with washed human erythrocytes (2×10^7 cells) from a healthy donor in Dulbecco's phosphate-buffered saline, pH 7.4 (100 μl) for 1 h at 37 °C. After centrifugation (12 000 g for 15 s), the absorbance at 450 nm of the supernatant was measured. A parallel incubation in the presence of 1% (vol/vol) Tween-20 was carried out to determine the absorbance associated with 100% haemolysis. The LD_{50} value was taken as the mean concentration of peptide producing 50% haemolysis in three independent experiments.

Intracellular Calcium ($[Ca^{2+}]_i$) and Membrane Potential Studies

Changes in membrane potential and $[Ca^{2+}]_i$ were determined fluorometrically using monolayers of BRIN-BD11 cells as previously described [16,23] using a membrane potential assay kit or a Ca^{2+} assay kit (Molecular Devices, Sunnyvale, CA, USA) according to the manufacturer's recommended protocols. Data were acquired using a FlexStation scanning fluorometer with integrated fluid transfer workstation (Molecular Devices). The cells were incubated at 37 °C for 10 min with either tigerinin-1R or its non-amidated derivative at a concentration of 1 μM. Control incubations in the presence of 5.6 mM glucose alone, 30 mM KCl and 10 mM alanine were also carried out.

In Vivo Studies

All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and EC Directive 86/609/EEC for animal experiments. Male National Institutes of Health (NIH) Swiss mice were used at age of 17 weeks, having been maintained for 3 months on a high-fat diet (45% kcal from fat, 20% kcal from protein, 35% kcal from carbohydrate; total energy 19.5 kJ/g) (Harlan Laboratories, Indianapolis, IN, USA) to induce obesity, insulin resistance and glucose intolerance [24]. Age-matched groups ($n = 6$) of overnight fasted mice received an intraperitoneal injection of either glucose alone (18 mmol/kg body weight) or in combination with synthetic tigerinin-1R (75 nmol/kg body weight). All solutions were administered in 0.9% NaCl (5 ml/kg body weight). Blood samples were collected as previously described [12] at the times indicated in figures 7 and 8. Plasma glucose concentrations were

measured by an automated glucose oxidase procedure using a Beckman glucose analyser II, and plasma insulin concentrations were measured by radioimmunoassay as described [22]. No adverse effects were observed following administration of the peptide.

Statistical Analysis

Results are expressed as mean ± standard error of mean, and values were compared using two-way analysis of variance followed by Newman–Keuls post-hoc test. Groups of data were considered to be significantly different if p < 0.05.

Results

Purification and Characterization of the Peptide

The elution profile on a preparative Vydac C-18 column of an extract of skin from *H. rugulosus*, after partial purification on Sep-Pak cartridges, is shown in figure 1. The fractions denoted by bar A in figure 1 contained material that stimulated the release of insulin from BRIN-BD11 cells but did not increase the rate of release of LDH. The fractions denoted by bar B in figure 1 also contained insulin-releasing activity, but these fractions were cytotoxic to the cells, as indicated by an increased rate of release of LDH, and so were not investigated further. The fractions from zone A were pooled and re-chromatographed on a semi-preparative Vydac C-8 column (figure 2A). The late eluting peak denoted by the cross contained the insulin-releasing peptide that was purified to near homogeneity by further chromatography on an analytical Vydac C-18 column (figure 2B). The major peak designated by the cross was associated with activity. The approximate yield of the purified peptide was 12 nmol.
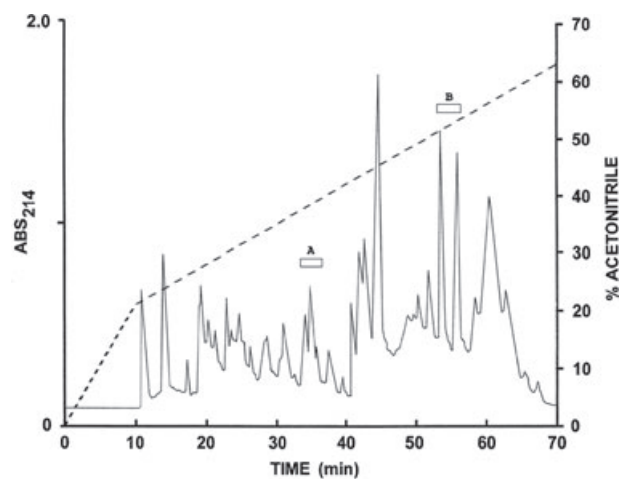
Figure 1. Reversed-phase HPLC on a preparative Vydac C-18 column of an extract of *H. rugulosus* skin after partial purification on Sep-Pak cartridges. The fractions in zone A contained components that stimulated the release of insulin, but not LDH, from BRIN-BD11 cells. The fractions in zone B contained components that stimulated release of both insulin and LDH from BRIN-BD11 cells. The dashed line shows the concentration of acetonitrile in the eluting solvent.
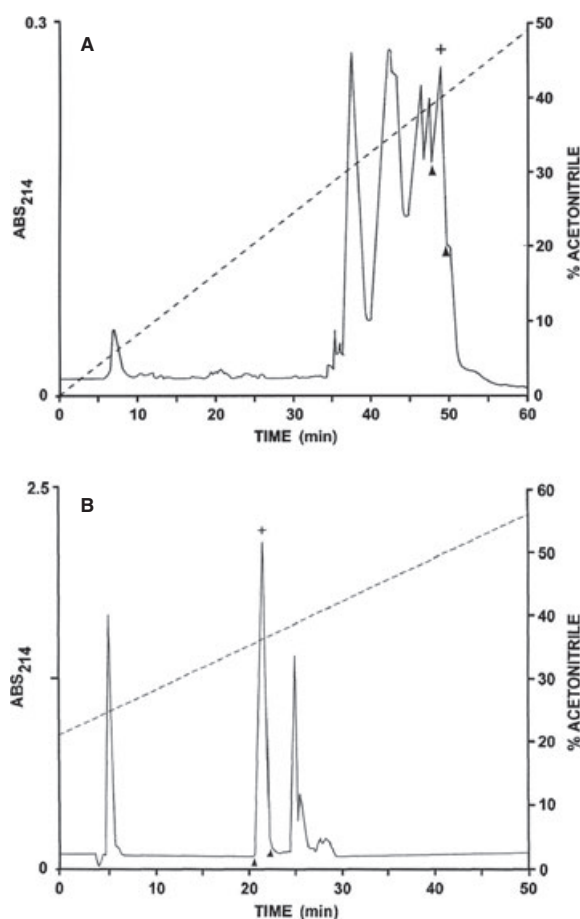
Figure 2. Reversed-phase HPLC on (A) a semi-preparative Vydac C-8 column and (B) an analytical Vydac C-18 column of the pooled fractions from zone A in figure 1. The peaks denoted by (+) contained the peptide with insulin-releasing activity. The arrowheads show where peak collection began and ended.

The primary structure of the insulin-releasing peptide isolated from *H. rugulosus* skin was established by automated Edman degradation as Arg-Val-Xaa-Ser-Ala-Ile-Pro-Leu-Pro-Ile-Xaa-His. The results of MALDI-TOF mass spectrometry

showed that Xaa represented cysteine and that the peptide contained a disulfide bridge and an α-amidated residue at its C-terminus (observed molecular mass: 1304.9 Da; calculated molecular mass: 1304.7 Da).

In Vitro Insulin-releasing Activities

The basal rate of release of insulin from BRIN-BD11 cells in the presence of 5.6 mM glucose alone was 1.00 ± 0.08 ng/10^6 cells/20 min, and this rate increased to 7.93 ± 1.15 ng/10^6 cells/20 min ($p < 0.001$; $n = 8$) in the presence of the well-established insulin secretagogue, alanine (10 mM). The effects of tigerinin-1R on the stimulation of insulin release from BRIN-BD11 cells in the presence of 5.6 mM glucose are shown in figure 3. The peptide produced significant ($p < 0.001$) and concentration-dependent stimulations of the rate of insulin secretion at concentrations ≥ 0.1 nM compared with basal rate in the presence of glucose alone. The maximum response (405% of basal rate) was produced by concentration of 3 μM. The peptide did not stimulate release of the cytosolic enzyme, LDH at concentrations up to 3 μM, indicating that the integrity of the plasma membrane had been preserved. The non-amidated form of tigerinin-1R also produced significant ($p < 0.05$) stimulation of the rate of insulin release in the presence of 5.6 mM glucose at concentrations ≥ 100 nM, but the magnitudes of the responses were less ($p < 0.001$) at all concentrations tested.

In a second series of incubations, the basal rate of release of insulin from BRIN-BD11 cells in the presence of 16.7 mM glucose alone was 2.82 ± 0.23 ng/10^6 cells/20 min, and this rate increased to 9.16 ± 0.62 ng/10^6 cells/20 min ($p < 0.001$; $n = 8$) in the presence of alanine (10 mM). The effect of increasing concentrations of tigerinin-1R (amidated and non-amidated) upon the rate of insulin release from BRIN-BD11 cells is shown in figure 4. Tigerinin-1R again produced significant ($p < 0.01$) and concentration-dependent stimulations of the rate of insulin secretion at concentrations ≥ 0.1 nM compared with basal rate in the presence of glucose alone. The maximum response (290% of basal rate at 3 μM) was significantly ($hboxp < 0.001$) less than the maximum response in the presence of 5.6 mM glucose.
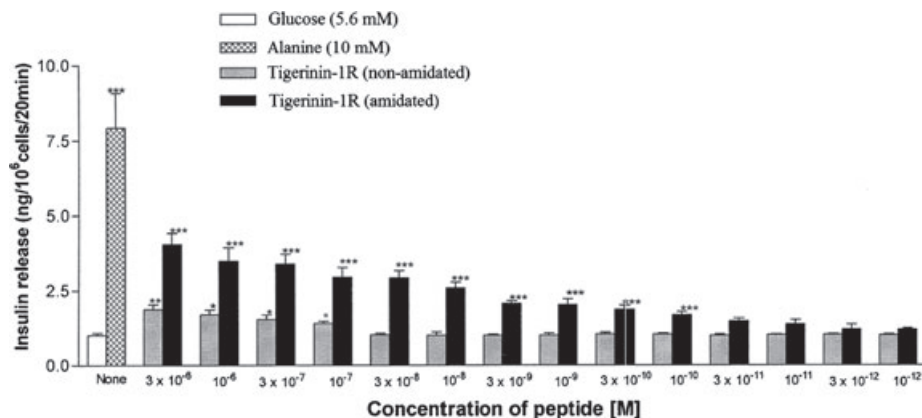
Figure 3. Effects of tigerinin-1R (amidated and non-amidated forms) on the release of insulin from BRIN-BD11 clonal β-cells in the presence of 5.6 mM glucose (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ compared with glucose; $n = 8$).
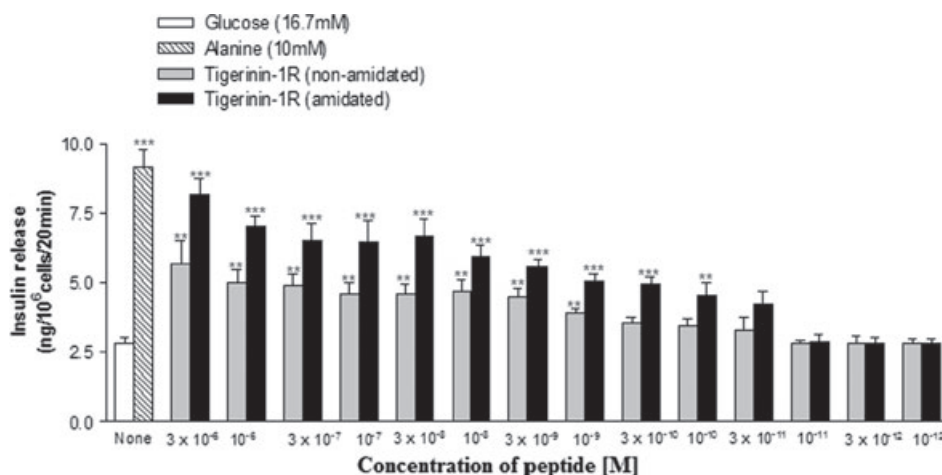
Figure 4. Effects of tigerinin-1R (amidated and non-amidated forms) on the release of insulin from BRIN-BD11 clonal β-cells in the presence of 16.7 mM glucose (***$p < 0.001$, **$p < 0.01$ compared with glucose alone; $n = 8$).

The potency of the non-amidated form was less than that of tigerinin-1R (threshold concentration for a significant increase in insulin release = 1 nM) and maximum response (202% of basal rate at 3 µM) was also significantly ($p < 0.001$) less. Neither peptide stimulated release of LDH at concentrations up to 3 µM.

Haemolytic Activity

Synthetic tigerinin-1R (amidated and non-amidated forms) at concentrations up to 500 µM did not produce significant haemolysis of human erythrocytes during a 60-min incubation.

Effect on Membrane Potential and Intracellular [Ca²⁺]

At a glucose concentration of 5.6 mM, both tigerinin-1R (amidated) and tigerinin-1R (non-amidated) produced a sustained membrane depolarization in BRIN-BD11 cells (figure 5). The magnitude of the effect was less than that produced by 30 mM KCl but was significantly ($p < 0.001$) greater than in the presence of glucose alone. Similarly, both amidated and non-amidated tigerinin-1R produced a rise in the concentration of intracellular Ca^{2+} that was significantly ($p < 0.001$) greater than in the presence of glucose alone (figure 6). Both the tigerinin-1R peptides caused a sharp initial increase in intracellular calcium, which was sustained throughout the 300-s period, whereas alanine caused a sharp increase that by 100 s had levelled out slightly above basal levels. The integrated responses (area under the curve) for the amidated peptide were significantly ($p < 0.05$) greater than that for the non-amidated derivative for effects on membrane potential (figure 5C) and intracellular Ca^{2+} concentrations (figure 6C).

## Effects on Insulin Release and Glucose Tolerance *In Vivo*

After intraperitoneal injection of glucose in obese, insulin-resistant mice, plasma insulin concentrations were significantly ($p < 0.05$) higher at 15 min in animals receiving tigerinin-1R (75 nmol/kg body weight) compared with animals receiving vehicle only (figure 7A). The integrated response (total amount of insulin released over 60 min) was significantly ($p < 0.05$) greater in animals receiving tigerinin-1R (figure 7B). The effect of the peptide on plasma glucose concentration was not significant at any time point (figure 8A), but the integrated response of plasma glucose (area under the curve) was significantly ($p < 0.05$) less after administration of tigerinin-1R compared with vehicle only (figure 8B). Estimation of the highest likely circulating concentration achieved after injection (assuming 100% bioavailability) gives a value between 1 and 2 µM, which is within the range of the *in vitro* concentrations tested.

Discussion

The amino acid sequence of the insulin-releasing dodecapeptide isolated in this study indicates structural similarity to peptides belonging to the tigerinin family that were first identified in the Indian frog *H. tigerinus* (formerly classified as *Rana tigerina*) [25], and so, the peptide has been termed tigerinin-1R (figure 9). More recently, cDNAs encoding biosynthetic precursors of tigerinins from the Chinese frog *Fejervarya cancrivora* (formerly *Rana cancrivora*), also in the family Dicroglossidae, have been characterized [26]. Like the tigerinin-1R isolated in this study, the four tigerinins isolated from *H. tigerinus* skin secretion contained a C-terminally α-amidated residue, whereas the peptides from *F. cancrivora* contained a C-terminal carboxyl group.

The ability of tigerinin-1R to stimulate insulin release from BRIN-BD11 clonal β-cells at concentrations as low as 0.1 nM, coupled with its very-low short-term cytotoxicity to these cells and to human erythrocytes, indicate that it can be considered as a potential candidate for development into a therapeutically valuable agent for treatment of type 2 diabetes. The peptide is appreciably more potent than other naturally occurring frog skin peptides examined under the same experimental conditions. For example, the threshold concentration necessary to produce a significant increase in the rate of insulin release
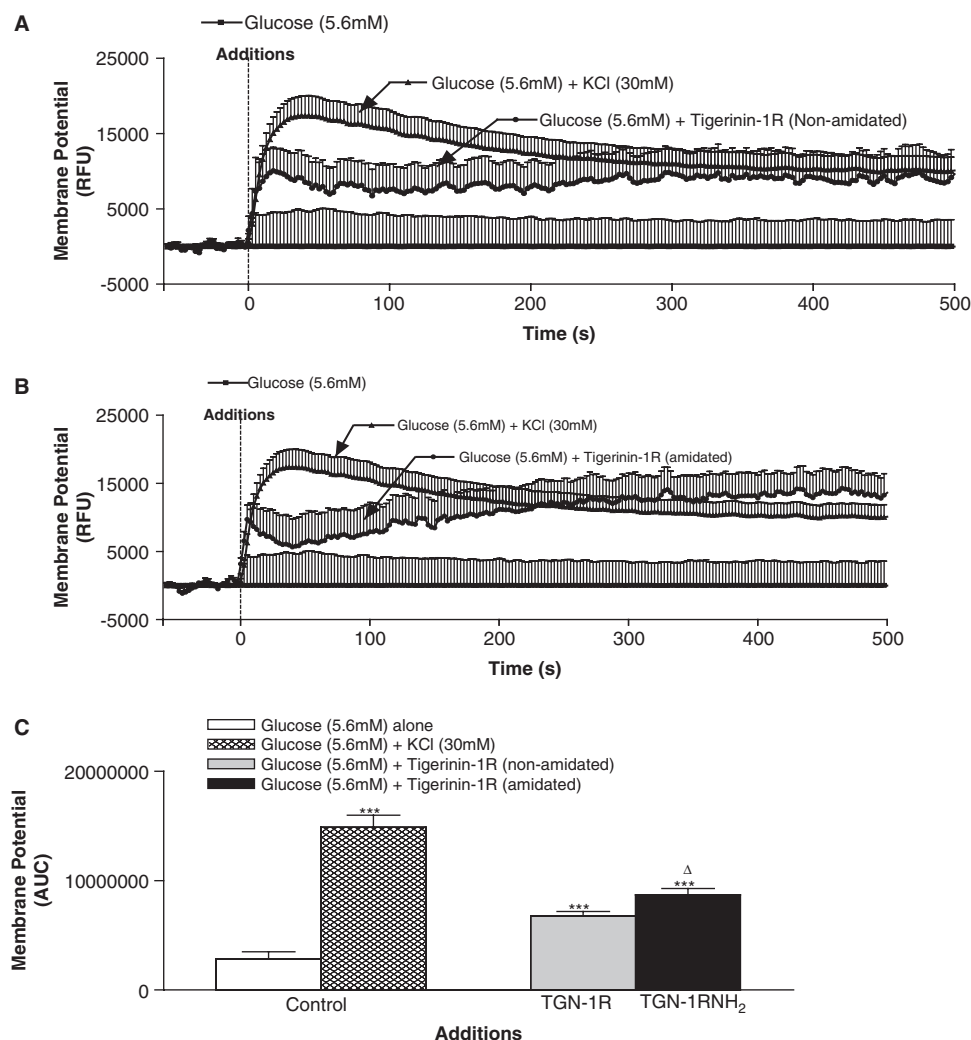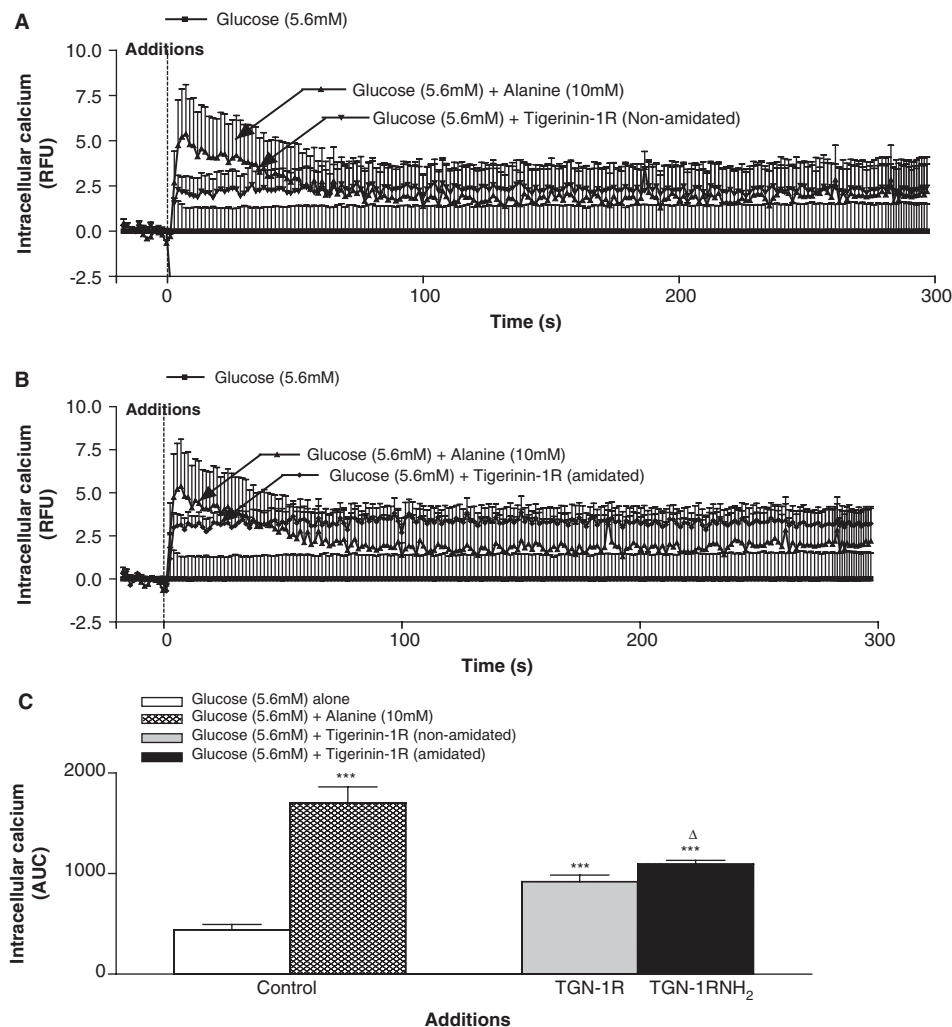
Figure 5. Effects of non-amidated (A) and amidated (B) tigerinin-1R (1 μM) on membrane potential of BRIN-BD11 in the presence of 5.6 mM glucose compared with the effects produced by 5.6 mM glucose alone and by 5.6 mM glucose containing 30 mM KCl. (C) The integrated responses [area under the curve (AUC)]. Values are mean ± standard error of mean (***$p < 0.001$ compared with glucose alone, $\Delta p < 0.05$ compared with non-amidated tigerinin-1R; $n = 6$).

from BRIN-BD11 cells was 30 nM for ranatuerin-2CBd [9], 30 nM for phylloseptin-L2 [14] and 100 nM for brevinin-2GUb [8]. The maximum increase in the rate of insulin release (405% of basal rate at a concentration of 3 μM) was greater than that produced under the same experimental conditions by the antidiabetic drug, tolbutamide and the well-characterized insulinotropic peptides, GLP-1 and GIP [14]. The study has shown that a C-terminally α-amidated amino acid residue is necessary for high potency. Structure–activity relationships among the frog skin insulin-releasing peptides are incompletely understood. An investigation of a series of peptides belonging to the temporin family suggested a possible correlation between hydrophobicity and insulin-releasing activity [16]. Consequently, the decrease in hydrophobicity produced by replacement of the C-terminal carboxamide group by a carboxyl group may be responsible for the decrease in potency and efficacy by reducing the magnitude of the interaction of

the peptide with the (phospho)lipids of the plasma membrane of the BRIN-BD11 cells.

Insulin secretion is regulated by two major signalling pathways: the K_{ATP} channel–dependent and K_{ATP} channel-independent augmentation pathway [27]. In the former pathway, insulin secretion is triggered by an increased [ATP]/[ADP] ratio, closure of ATP-sensitive potassium channels and opening of voltage-dependent calcium channels. The influx of extracellular calcium and elevation of cytoplasmic intracellular calcium induce exocytosis of insulin. The present observation that tigerinin-1R, like KCl and alanine [28], produces cellular depolarization and increases [Ca^{2+}]$_i$ in BRIN-BD11 cells is consistent with its insulin-releasing activity being mediated by the K_{ATP} channel-dependent pathway. It has been shown that an antimicrobial peptide of the brevinin-1 family from the frog *Glandirana emeljanovi* stimulates insulin release from rat RINm5F insulinoma-derived cells by a mechanism that involves an increase in intracellular calcium concentration [29].

Figure 6. Effects of non-amidated (A) and amidated (B) tigerinin-1R (1 μM) on the concentration of intracellular Ca^{2+} in BRIN-BD11 cells in the presence of 5.6 mM glucose compared with the effects produced by 5.6 mM glucose alone and by 5.6 mM glucose containing 10 mM alanine. (C) The integrated responses [area under the curve (AUC)]. Values are mean ± standard error of mean (***$p < 0.001$ compared with glucose alone, Δ$p < 0.05$ compared with non-amidated tigerinin-1R; $n = 6$).

The obese, insulin-resistant and glucose-intolerant mouse, developed by long-term feeding of a high-fat diet, is a valuable animal model with which to study type 2 diabetes mellitus [24]. A previous study has shown that an analogue of the frog skin peptide B2-RP ([D4K]B2RP) significantly improves glucose tolerance and enhances total insulin release in these animals in the 60-min period following a glucose load [12]. We have now extended these studies using the same protocol to show that tigerinin-1R also significantly enhances insulin release and improves glucose tolerance. In contrast to the sulfonylureas, the peptide failed to induce rebound hypoglycaemia even when administered at high concentrations *in vivo*. However, blood glucose measurements were made only up to 60 min after injection, and further studies are required to ascertain whether the peptide produces hypoglycaemia in the longer term. In view of the high potency of the peptide *in vitro*, the magnitude of the effects *in vivo* was less than expected. Although the integrated responses of plasma glucose and plasma insulin

concentrations in the animals receiving glucose plus tigerinin-1R were significantly ($p < 0.05$) less than that in animals receiving glucose alone, there was no significant difference in concentration of glucose at any time point (figure 8), and plasma insulin concentrations were significantly greater only after 15 min (figure 7). In contrast, there was a sustained decrease in plasma glucose and increase in plasma insulin after administration of [D4K]B2RP that persisted for the 60-min period of the experiment [12]. Further studies are clearly required to investigate the *in vivo* degradation and pharmacokinetic properties of tigerinin-1R.

The efficacy of an incretin peptide is dependent on the rate at which it is cleared from the circulation [3]. For example, exendin-4 [5] and the acylated analogue, liraglutide that contains a fatty acid group on Lys^{26} of GLP-1 [2,30] are cleared less rapidly than GLP-1 and so exhibit a more effective and longer-acting glucose-lowering response *in vivo* with few adverse effects. The modest effect of tigerinin-1R on glucose
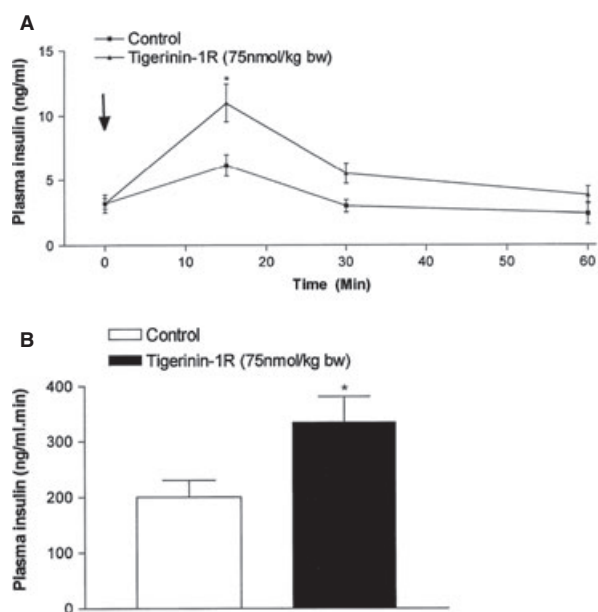
Figure 7. Effect of tigerinin-1R on plasma insulin concentrations in high fat−fed mice with dietary induced obesity and insulin resistance. Glucose (18 mmol/kg body weight) was administered by intraperitoneal injection at the time indicated by the arrow, either alone (control) or in combination with tigerinin-1R (75 nmol/kg body weight). (A) Plasma insulin concentrations and (B) the integrated response [area under the curve (AUC)] are shown. Values are mean $\pm$ standard error of the mean for six independent experiments. *p $<$ 0.05 compared with control.
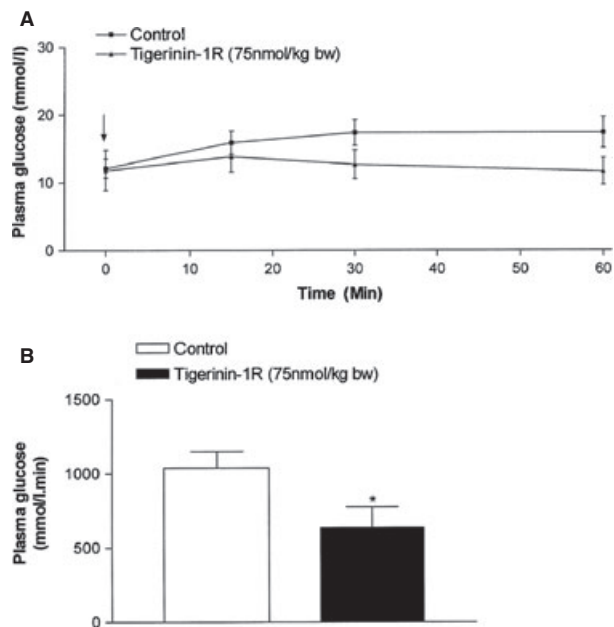
Figure 8. Effect of tigerinin-1R on glucose tolerance in high fat−fed mice with dietary induced obesity and insulin resistance. Glucose (18 mmol/kg body weight) was administered by intraperitoneal injection at time indicated by the arrow, either alone (control) or in combination with tigerinin-1R (75 nmol/kg body weight). (A) Plasma glucose concentrations and (B) the integrated response [area under the curve (AUC)] are shown. Values are mean $\pm$ standard error of the mean for six independent experiments. *p $<$ 0.05 compared with control.

H. rugulosus		RVCSAIPLPICH.NH₂
H. tigerinus	1	FCTMIPIPRCY.NH₂
H. tigerinus	2	RVCFAIPLPICH.NH₂
H. tigerinus	3	RVCYAIPLPICY.NH₂
H. tigerinus	4	RVCYAIPLPIC.NH₂
F. cancrivora	1	RVCSAIPLPICH
F. cancrivora	2	RVCMAIPLPLCH

Figure 9. A comparison of the primary structures of the tigerinin-related peptides from *H. rugulosus, H. tigerinus* and *F. cancrivora*. The shaded residues are conserved.

tolerance *in vivo* is probably a consequence of its short half-life in the circulation. In the case of peptides containing a disulfide bond such as tigerinin-1R, protein-disulfide oxidoreductases can disrupt the cysteine bridge, rendering the peptide inactive and more susceptible to proteolytic degradation. This problem was addressed in a previous study in which the disulfide bridge in the frog skin antimicrobial peptide brevinin-1BYa (FLPILASLAAKFGPKLFCLVTKKC) was replaced by a non-reducible and metabolically stable dicarba bond [31]. The resulting derivative showed increased growth inhibitory potency against a range of micro-organisms, but its therapeutic potential was limited by increased cytotoxicity against human erythrocytes. Future studies will investigate the glucose-lowering properties and toxicities of dicarba tigerinin-1R and acylated analogues containing a fatty acid moiety.

In conclusion, this study suggests that tigerinin-1R has the potential to act as structural template for development of an antidiabetic drug that may lack some of the disadvantages encountered with sulfonylureas such as hypoglycaemic rebound. However, future studies are needed to evaluate this issue further, to compare the glucose-lowering properties of the peptide in obese, glucose-intolerant mice with those in lean, healthy mice and to determine how its mechanism of action compares with that of other available drugs.

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Conflict of Interest

The authors declare that there is no competing interest for any author. P. R. F. and J. M. C. have designed this study. O. O. O., Y. H. A. A. and M. M. conducted the study. O. O. O., Y. H. A. A. and P. R. F. carried out the analysis. J. M. C. wrote the manuscript.

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