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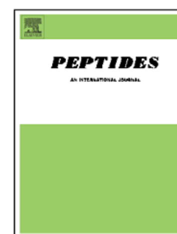

A peptide of the phylloseptin family from the skin of the frog *Hylomantis lemur* (Phyllomedusinae) with potent *in vitro* and *in vivo* insulin-releasing activity

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ABSTRACT

A peptide with the ability to release insulin from the rat BRIN-BD11 clonal β cell line was isolated from norepinephrine-stimulated skin secretions of the Lemur leaf frog *Hylomantis lemur* Boulenger,1882. Determination of the primary structure (FLSLIPHVISALSSL.NH_2) demonstrated that the peptide belongs to the phylloseptin family whose members have previously been identified in other Phyllomedusinae species. A synthetic replicate of the peptide, termed phylloseptin-L2, produced a significant stimulation of insulin release (134% of basal rate, $P < 0.01$) from BRIN-BD11 cells at a concentration of 30 nM, with a maximum response (301% of basal rate, $P < 0.001$) at a concentration of 3 μM. Phylloseptin-L2 did not stimulate release of the cytosolic enzyme, lactate dehydrogenase at concentrations up to 3 μM, indicating that the integrity of the plasma membrane had been preserved. The stimulatory action was maintained in the absence of extracellular Ca^{2+} and in the presence of verapamil (50 μM) and diazoxide (300 μM) suggesting that mechanism of action of the peptide did not primarily involve influx of Ca^{2+} or closure of ATP-sensitive K^+ channels. Administration of phylloseptin-L2 (50 nmol/kg body weight) into mice significantly ($P < 0.05$) increased total release of insulin and improved glucose tolerance during the 60 min period following an intraperitoneal injection of glucose (18 mmol/kg body weight). It is concluded that the peptide shows potential for development into a therapeutically valuable agent for the treatment of Type 2 diabetes.

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1. Introduction

The skins of anurans (frogs and toads) constitute an important source of biologically active peptides with potential for development into therapeutically valuable pharmaceutical agents. Cationic α-helical peptides with broad-spectrum antibacterial and antifungal activities are synthesized in the skins of many, but by no means all, species of frog and represent a component of the animal's system of innate immunity [7,18,28]. The Phyllomedusinae sub-family of the Hylidae family comprises 57 species organized in seven genera that are distributed from tropical Mexico to Argentina [14]. The skin secretions of phyllomedusid frogs have proved to be a rich source of peptides with a range of antimicrobial activities. These peptides may be grouped together on the basis of limited structural similarity and the following families have

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been identified: (a) dermaseptins, (b) dermatoxins, (c) phylloxins, (d) phylloseptins, (e) plasticins, and (f) hyposins (reviewed in refs. [4,26]). The members of the different families differ appreciably in primary structures but strong conservation of the amino-acid sequences of the signal peptide and N-terminal pro-regions of the biosynthetic precursors of the peptides demonstrates that they are related evolutionarily [30].

More recently, it has been shown that several frog skin peptides that were first identified on the basis of their ability to inhibit the growth of bacteria also have the ability to release insulin from rat clonal BRIN-BD11 β cells at concentrations that are not toxic to the cells (reviewed in ref. [3]). Examples of such peptides from phyllomedusid frogs include a 13 amino-acid-residue peptide from *Agalychnis calcarifer* with structural similarity to the N-terminal domain of proline–arginine-rich peptide PR-39 [2], and peptides from *Agalychnis litodryas* [21] and *Phyllomedusa trinatis* [20] that are structurally related to the dermaseptins. These peptides have excited interest because of the possibility that they may be developed into agents for treatment of Type 2 diabetes.

The Lemur leaf frog *Hylomantis lemur* Boulenger, 1882 (formerly known as *Phyllomedusa lemur* [12]) is a small (length 30–45 mm) anuran that inhabits humid, mid-elevation regions in Panama, Costa Rica, and Colombia. The animals are nocturnal and can be found on vegetation a few meters above ground in rainforest swamps. Although once considered to be relatively common, populations have declined drastically during the past 20 years and the species is now listed as endangered. A previous study has described the isolation from norepinephrine-stimulated skin secretions of *H. lemur* of two peptides with varying degrees of cytolytic activity against bacteria, fungi, and mammalian cells that were identified as belonging to the dermaseptin and phylloseptin families and so were termed dermaseptin-L1 and phylloseptin-L1 [8]. The current study has re-examined these skin secretions for the presence of peptides with the ability to stimulate insulin release from BRIN-BD11 cells and has identified a second member of the phylloseptin family (phylloseptin-L2) with potent insulinotropic properties both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Collection of skin secretions

The collection of norepinephrine-stimulated skin secretions from adult specimens of *H. lemur* ($n = 9$) in Omar Torrijos National Park, Coclé, Panama and partial purification on Sep-Pak C-18 cartridges has been described in detail previously [8,31]. After collection of secretions, all animals were released at the exact site of collection. Permits for collection of samples were provided through the Smithsonian Tropical Research Institute from the Autoridad Nacional del Ambiente, Republic of Panama.

2.2. Determination of insulin-releasing activity

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% (v/v) fetal calf serum, antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin) and 11.1 mM glucose as described [1–3]. The origin and characteristics of these cells have been provided in detail previously [24]. The cells were pre-incubated for 40 min at 37 °C in 1.0 ml Krebs Ringer bicarbonate buffer, pH 7.4 (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 $NaHCO_3$) supplemented with 5.6 mM glucose and 0.1% (w/v) bovine serum albumin. Incubations ($n = 8$) with synthetic phylloseptin-L2 (3×10^{-9} to 3×10^{-6} M) or with endogenous peptides were performed for 20 min at 37 °C using the same buffer. After incubation, aliquots of cell supernatant were removed for insulin radioimmunoassay [13]. For the purposes of comparison, incubations under the same conditions were carried out in the presence of the established insulinotropic agents listed in Table 1. All peptides were supplied by American Peptide Company (Sunnyvale, CA).

In order 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 Krebs Ringer bicarbonate buffer supplemented with 5.6 mM glucose (1.0 ml). Test incubations with synthetic phylloseptin-L2 (3×10^{-8} to 3×10^{-6} M) and with endogenous peptides were performed for 20 min at 37 °C. Lactate dehydrogenase (LDH) concentrations in the cell supernatants were measured using a CytoTox96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI) according to the manufacturer's protocol.

2.3. Peptide purification

The lyophilized skin secretions, after partial purification on Sep-Pak cartridges, were redissolved in 0.1% (v/v) trifluoroacetic acid (TFA)/water (2 ml) and injected onto a (1.0 cm × 25 cm) Vydac 218TP510 (C-18) reversed-phase HPLC column (Separations Group, Hesperia, CA) equilibrated with 0.1% (v/v) TFA/water at a flow rate of 2.0 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 21% (v/v) over 10 min and to 63% (v/v) 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 (50 μl) of the fractions to stimulate release of insulin from BRIN-BD11 cells were determined as described in Section 2.2.

Table 1 – A comparison of the effects of phylloseptin-L2, tolbutamide, glucagon-like peptide-1 (GLP-1), gastric inhibitory polypeptide (GIP) and cholecytokinin-8 (CCK-8) on insulin release from BRIN-BD11 cells.

Test agent	Insulin release (ng/10^6 cells/20 min)
None (control)	1.13 ± 0.10
Phylloseptin-L2 (10^{-7} M)	2.20 ± 0.20***
Tolbutamide (2×10^{-4} M)	2.50 ± 0.30***
GLP-1 (10^{-7} M)	2.6 ± 0.30***
GIP (10^{-7} M)	2.2 ± 0.20***
CCK-8 (10^{-7} M)	1.90 ± 0.10***

Values are the mean ± S.E.M. for eight independent observations.
*** $P < 0.001$ compared with 5.6 mM glucose alone (control).

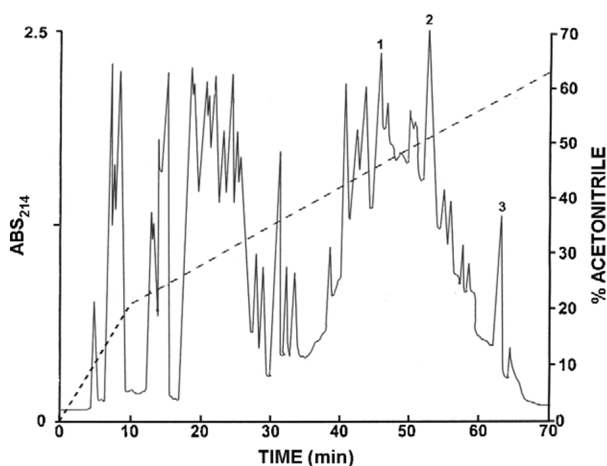

Fig. 1 – Reversed-phase HPLC on a semipreparative Vydac C-18 column of skin secretions from *H. lemur* after partial purification on Sep-Pak cartridges. Peak 2 contained material that stimulated the release of insulin from BRIN-BD11 cells. Peak 1 was previously shown to contain dermaseptin-L1 and peak 3 contained phylloseptin-L1 [8]. The dashed line shows the concentration of acetonitrile in the eluting solvent.

The peptide with insulin-releasing activity (designated peak 2 in Fig. 1) was purified to near homogeneity by sequential chromatography on (1.0 cm × 25 cm) Vydac 214TP510 (C-4) and (1.0 cm × 25 cm) Vydac 219TP510 (phenyl) columns. The concentration of acetonitrile in the eluting solvent was raised from 21% to 56% over 50 min and the flow rate was 2.0 ml/min.

2.4. *Structural characterization*

The primary structure of the peptide was determined by automated Edman degradation using a model 494 Procise sequenator (Applied Biosystems, Foster City, CA). Amino-acid composition analysis was performed by the University of Nebraska Medical Center Protein Structure Core Facility (Omaha, NE). MALDI-TOF MS was carried out using a Voyager DE-PRO instrument (Applied Biosystems) that was operated in reflector mode with delayed extraction and the accelerating voltage in the ion source was 20 kV. The instrument was calibrated with peptides of known molecular mass in the 2000–4000 Da range. The accuracy of mass determinations was ±0.02%.

2.5. *Peptide synthesis*

Phylloseptin-L2 was supplied in crude form by GL Biochem Ltd. (Shanghai, China) and was purified to near homogeneity by reversed-phase HPLC on a (2.2 cm × 25 cm) Vydac 218TP1022 (C-18) column equilibrated with acetonitrile/water/TFA (21.0/78.9/0.1, v/v/v) at a flow rate of 6 ml/min. The concentration of acetonitrile was raised to 56% (v/v) over 60 min using a linear gradient. Absorbance was measured at 214 and 280 nm and the major peak in the chromatogram was collected manually. The monoisotopic molecular mass of the peptide determined by electrospray mass spectrometry was consistent with the mass calculated from the proposed structure. The final purity of the peptide was >98%.

2.6. *Measurement of* $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined by a fluorometric method using monolayers of BRIN-BD11 cells and a FLIPR Ca^{2+} assay kit (Molecular Devices, Sunnyvale, CA) as described previously [1,3,22]. Phylloseptin-L2 was tested at a concentration of 3×10^{-7} M and alanine at a concentration of 1×10^{-2} M.

2.7. *In vivo studies*

NIH male Swiss mice (age 45–55 weeks) were housed individually in an air-conditioned room (22 ± 2 °C) with a 12 h light:12 h dark cycle. Animals had free access to water and standard laboratory chow. All experiments were carried out by authorized investigators in accordance with the UK Animals (Scientific Procedures) Act 1986. No adverse effects were observed following administration of the peptide.

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 phylloseptin-L2 (50 nmol/kg body weight). All solutions were administered in 0.9% NaCl (5 ml/kg body weight). Blood samples were collected from the cut tip of the tail vein of unanesthetized animals into chilled fluoride/heparin-coated microcentrifuge tubes (Sarstedt, Nümbrecht, Germany) at the times indicated in Fig. 6. After centrifugation (13,000 × g for 30 s), plasma glucose concentrations were measured by an automated glucose oxidase procedure using a Beckman glucose analyser II. Plasma insulin concentrations were measured by radioimmunoassay as previously described [13].

2.8. *Statistical analysis*

Results are expressed as mean ± S.E.M. and values were compared using ANOVA followed by Newman–Keuls post-hoc test. Groups of data were considered to be significantly different if $P < 0.05$.

3. Results

3.1. *Purification of the peptides*

The skin secretions, after concentration and partial purification on Sep-Pak cartridges, were chromatographed on a Vydac C-18 semipreparative reversed-phase HPLC column (Fig. 1). An aliquot of the prominent peak designated 2 (subsequently shown to contain phylloseptin-L2) increased the rate of release of insulin from BRIN-BD11 cells without affecting the rate of release of LDH. Peak 1 has been previously shown to contain dermaseptin-L1 and peak 3 to contain phylloseptin-L1 [8].

Partial purification of phylloseptin-L2 on a semipreparative Vydac C-4 column is shown in Fig. 2a and the peptide was purified to near homogeneity, as assessed by a symmetrical peak shape and mass spectrometry, by a final chromatography on a semipreparative Vydac phenyl column (Fig. 2b). The final yield of purified peptide was 95 nmol.

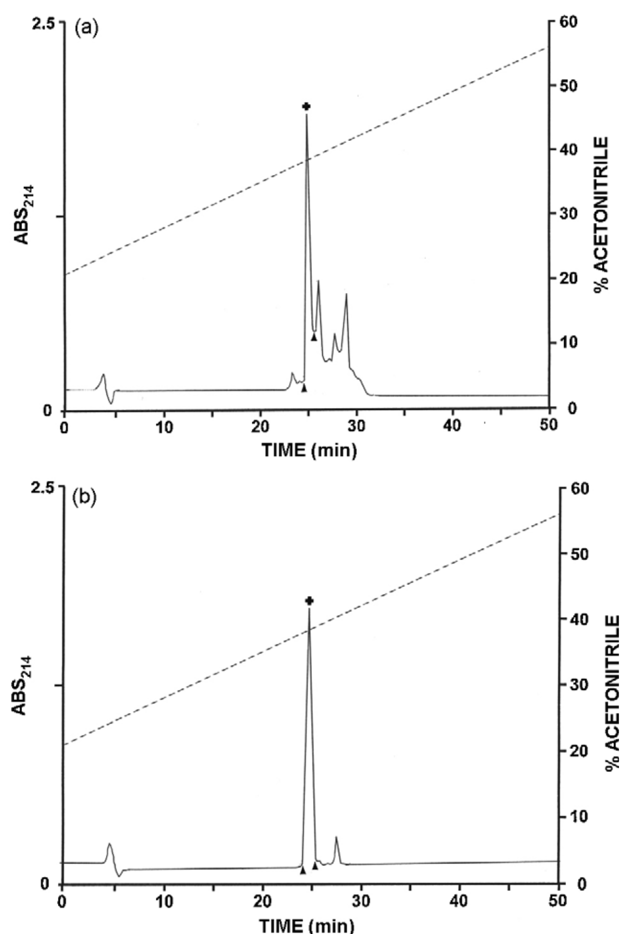

Fig. 2 – Purification of phylloseptin-L2 on a semipreparative (a) Vydac C-4 column and (b) Vydac phenyl column. The arrowheads show where peak collection began and ended and the peak with insulin-releasing activity is denoted by (+).

3.2. Structural characterization

The primary structure of phylloseptin-L2 was established by automated Edman degradation as FLSLIPHVISALSSL. This sequence was confirmed by amino-acid composition analysis [Found: Ser 4.1 (4), Pro 1.0 (1), Ala 1.2 (1), Val 0.5 (1), Ile 1.4 (2), Leu 4.1(4), Phe 1.0 (1), His 1.0 (1) residues/mol peptide]. The figures in parentheses show the number of residues predicted from the proposed sequence. The low values for the amounts of Val and Ile are a consequence of the resistance of the sterically hindered Val–Ile bond to hydrolysis. The observed molecular mass of the peptide, determined by MALDI-TOF mass spectrometry was consistent with the proposed sequence and demonstrates that phylloseptin-L2 is C-terminally α-amidated (observed monoisotopic relative molecular mass 1594.8; calculated molecular mass 1594.9 for the amidated form of the peptide).

3.3. In vitro insulin-releasing activity of phylloseptin-L2

A synthetic replicate of phylloseptin-L2 produced a concentration-dependent stimulation of the rate of insulin secretion

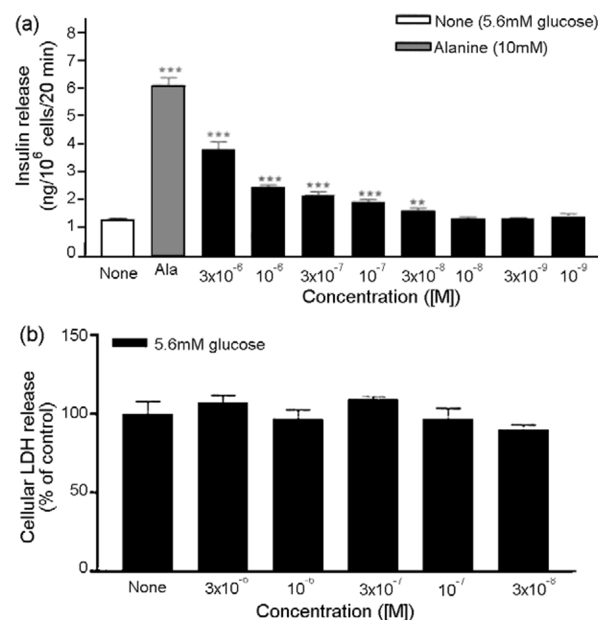

Fig. 3 – Effects of synthetic phylloseptin-L2 and alanine on (a) insulin release and (b) lactate dehydrogenase (LDH) release from BRIN-BD11 cells. Values are mean ± S.E.M., $n = 8$ for insulin release and $n = 4$ for LDH release. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to 5.6 mM glucose alone.

from BRIN-BD11 cells compared with the basal rate in the presence of 5.6 mM glucose only (Fig. 3a). A significant effect was produced (134% of basal rate, $P < 0.01$) at a concentration of 3×10^{-8} M, with a maximum response (301% of basal rate, $P < 0.001$) at a concentration of 3×10^{-6} M.

Phylloseptin-L2 (3×10^{-7} M) also stimulated insulin release at a glucose concentration of 16.7 mM (2.08 ± 0.24 ng/10^6 cells/20 min vs 1.87 ± 0.24 ng/10^6 cells/20 min basal release; $P < 0.05$). The increased rate of insulin release was not associated with a loss of integrity of the plasma membrane as demonstrated by lack of stimulation of LDH release from the cells at concentrations up to 3×10^{-6} M (Fig. 3b). Phylloseptin-L2 (3×10^{-7} M) maintained its ability to release insulin from BRIN-BD11 cells in the absence of extracellular calcium (Fig. 4). In the presence of 5.6 mM glucose, the peptide (3×10^{-7} M) significantly ($P < 0.05$) stimulated insulin release in the presence of 50 μM verapamil (insulin release in the presence of verapamil = 1.33 ± 0.10 ng/10^6 cells/20 min and in the presence of verapamil + peptide = 1.68 ± 0.11 ng/10^6 cells/20 min; $n = 8$). Similarly, the peptide (3×10^{-7} M) significantly ($P < 0.05$) stimulated insulin release in the presence of 300 μM diazoxide (insulin release in the presence of diazoxide = 1.15 ± 0.11 ng/10^6 cells/20 min and in the presence of diazoxide + peptide = 1.62 ± 0.14 ng/10^6 cells/20 min; $n = 8$). The insulinotropic action of phylloseptin-L2 is compared with that of tolbutamide, and the incretin peptides GLP-1 and GIP [16] in Table 1.

3.4. Effect of phylloseptin-L2 on $[Ca^{2+}]_i$

Alanine (10 mM) produced a rapid increase in $[Ca^{2+}]_i$ in BRIN-BD11 cells that was sustained throughout the experiment

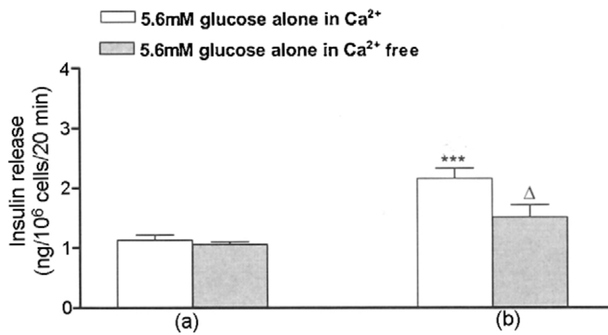

Fig. 4 – Effects of synthetic phylloseptin-L2 on insulin release from BRIN-BD11 cells in the presence (open bars) and absence (shaded bars) of extracellular calcium. (a) 5.6 mM glucose only, (b) 5.6 mM glucose + phyllosptin-L2 (3×10^{-7} M). Values are the mean ± S.E.M. for eight independent experiments. ***P < 0.001 compared to 5.6 mM glucose only in the presence of Ca^{2+} and $^{\Delta}$P < 0.05 compared to 5.6 mM glucose in the absence of Ca^{2+}.

(Fig. 5a). In contrast to the effect of this well-established beta-cell stimulator, phylloseptin-L2 (10^{-6} M) did not produce a significant increase in $[Ca^{2+}]_i$ compared with 5.6 mM glucose alone (Fig. 5b).

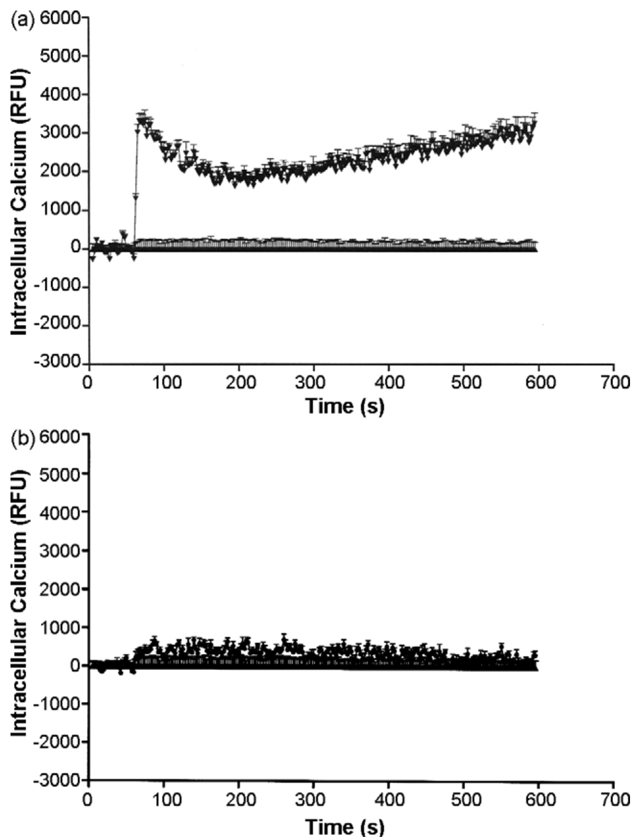

Fig. 5 – Effects of (a) alanine and (b) synthetic phylloseptin-L2 (3×10^{-7} M) on intracellular calcium concentrations ($[Ca^{2+}]_i$) in BRIN-BD11 cells compared with the effect of glucose only. Values are the mean ± S.E.M. for eight independent experiments. RFU: relative fluorescence units.

3.5. *Effect of phylloseptin-L2 on glucose tolerance and insulin release in vivo*

As shown in Fig. 6A, plasma glucose concentrations in mice receiving phylloseptin-L2 (50 nmol/kg body weight) were significantly less ($P < 0.01$) at 15 min ($P < 0.01$) and 30 min ($P < 0.05$) after intraperitoneal injection of glucose compared to animals receiving vehicle only. The integrated response of plasma glucose (area under the curve) was significantly ($P < 0.01$) less after administration of phylloseptin-L2 compared with vehicle only (Fig. 6C). Plasma insulin concentrations were significantly ($P < 0.05$) higher at 60 min after intraperitoneal injection of glucose in animals receiving phylloseptin-L2 (Fig. 6B) and the integrated response (total amount of insulin released over 60 min) was significantly ($P < 0.05$) greater (Fig. 6D).

4. Discussion

This study has led to the purification of an insulin-releasing peptide present in relatively high abundance in skin secretion of the Panamanian frog, *H. lemur* that has been identified as a member of the phylloseptin family of peptides. The phylloseptins are a group of structurally related peptides previously identified in the skin secretions of the Brazilian tree frogs, *P. hypochondrialis* [6,9,19] and *P. oreades* [19] on the basis of their ability to inhibit the growth of bacteria. Their primary structures are compared with that of phylloseptin-L1 and phylloseptin-L2 in Fig. 7. Although many insulinotropic peptides that were first identified in frog skin have structurally related counterparts in mammalian nervous and gastrointestinal tissues (e.g. bombesin/gastrin releasing peptide and caerulein/cholecystokinin), there is no evidence at this time for the existence of a phylloseptin-related peptide in mammals.

In contrast to phylloseptin-L1, that showed potent activity against the Gram-positive bacteria *S. aureus* (MIC = 8 μM) [8], the activity of phylloseptin-L2 against this microorganism was much weaker (MIC = 50 μM) and the peptide was inactive against *E. coli* (MIC > 100 μM) (unpublished data). Conversely, under the conditions of assay, phylloseptin-L1 did not stimulate insulin release from BRIN-BD11 cells. These observations are consistent with our, albeit limited, understanding of structure–activity relationships of the cationic, α-helical peptides. The cytolytic activities of such peptides against bacteria are determined by a complex interaction between cationicity, hydrophobicity, α-helicity and amphipathicity [15]. The bacterial cell membrane is rich in anionic phospholipids, such as phosphatidylglycerol, and negatively charged lipopolysaccharides so that an increase in peptide cationicity should promote interaction with the negatively charged bacterial cell membrane and increase antimicrobial potency. Unlike phylloseptin-L1 (charge at pH 7 = +2), phylloseptin-L2 lacks a basic residue (charge at pH 7 = +1) and so is incapable of adopting the stable amphipathic α-helical conformation required for high potency against microorganisms. On the other hand, structure–activity studies of peptides belonging to the temporin family [1] and pseudin-2 [3] have shown an inverse relationship between cationicity and insulin-releasing

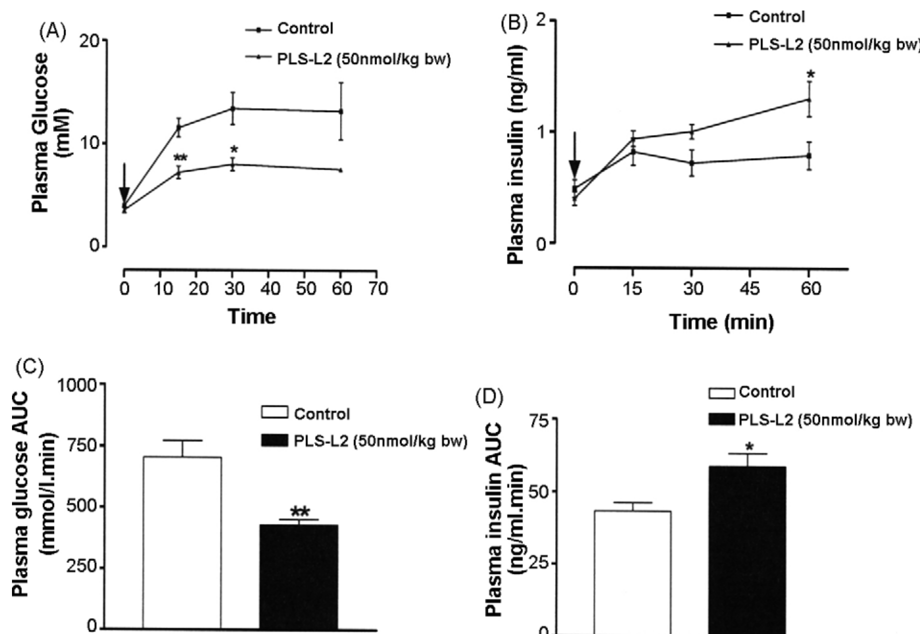

Fig. 6 – Effect of synthetic phylloseptin-L2 (50 nmol/kg body weight) on (A) plasma glucose concentrations, (B) plasma insulin concentrations, (C) integrated response of plasma glucose (area under curve, AUC), and (D) integrated response of plasma insulin, in lean mice. Glucose (18 mmol/kg body weight) was administered, with (closed triangles) and without (closed circles) the peptide, by intraperitoneal injection at the time indicated by the arrow. Values are mean ± S.E.M. (*n* = 6). *P < 0.05, **P < 0.01 compared to vehicle only.

activity. It was suggested that a less positively charged peptide can more easily traverse the plasma membrane of the BRIN-BD11 cells without permeabilization and interact with the secretory granules. ATP-dependent translocation of the antimicrobial peptide, buforin-2 across the intact plasma membrane of HeLa cells has been demonstrated [29]. Thus, the low cationicity of phylloseptin-L2 promotes insulin-releasing activity relative to antimicrobial activity. A recent study

Phylloseptin L1	LLGMIPLAIS AISALSKL
Phylloseptin-L2	FLSLIPHVIS ALSSL
Phylloseptin H1	FLSLIPHAIN AVSAIAKHN
Phylloseptin H2	FLS IPHAIN AVSTLVHHF
Phylloseptin H3	FLSLIPHAIN AVSALANHG
Phylloseptin H4	SLIPHAIN AVSAIAKHF
Phylloseptin H5	FLSLIPHAIN AVSAIAKHF
Phylloseptin H6	FLSLIPTAIN AVSALAKHF
Phylloseptin H7	FLGLLPSIVSGAVSLVKKL
Phylloseptin H8	FLSLLPSLVSGAVSLVKKL
Phylloseptin H9	FLSLLPSLVSGAVSLVIKL
Phylloseptin O1	FLSLIPHAIN AVSTLVHHS
Phylloseptin O2	FLSLIPHAIN AVSAIAKHS

Fig. 7 – A comparison of the primary structures of phylloseptins from *H. lemur* (L-1 and L-2), *P. hypochondrialis* (H1–H9) and *P. oreades* (O1 and O2). Conserved residues are shaded. Gaps have been introduced into some sequences to maximize sequence similarity.

involving circular dichroism and two-dimensional NMR spectroscopy has confirmed that the antimicrobial peptides, phylloseptin-1, -2, and -3 (now reclassified as phylloseptin-H1, -H2, and -H3 [4]) adopt α-helical conformations in membrane-mimetic environment that are stabilized by electrostatic interactions involving cationic residues [27].

The increase in insulin output from BRIN-BD11 cells produced by phylloseptin-L2 is comparable to that produced under the same experimental conditions by the antidiabetic drug, tolbutamide and the well-characterized insulinotropic peptides, glucagon-like peptide-1 and gastric inhibitory polypeptide [16] (Table 1). Insulin secretion from pancreatic beta cells is a complex process involving the integration and interaction of multiple external and internal stimuli. Numerous steps are involved in physiological regulation of insulin secretion by glucose, including GLUT-2 mediated transport into beta cells, stimulation of ATP-production, closure of K_{ATP} channels, and Ca^{2+} influx leading to exocytosis [23,25]. In the present study, the insulinotropic action of phylloseptin-L2 appears not to involve Ca^{2+} influx, at least as the primary mechanism, as activity was maintained in the presence of verapamil, a blocker of L-type voltage-dependent Ca^{2+} channels [17] and in the absence of extracellular calcium. Similarly, diazoxide, a inhibitor of ATP-sensitive K^+ channel closure [10] did not abolish the insulin-releasing activity indicating that the action of the peptide does not focus on ATP-production and participation of these channels. These observations are consistent with the results of related studies of the insulin-releasing properties of members of the temporin [1] and pseudin-2 [3] families of antimicrobial peptides.

The *in vivo* studies in mice demonstrated that an intraperitoneal injection of phylloseptin-L2, in a dose that was tolerated by the animals without apparent ill effects, significantly enhanced total insulin release and improved glucose tolerance after a glucose load. Consequently, phylloseptin-L2 may represent a candidate for the development of an antidiabetic agent. Although phylloseptin-L2 did not produce cell death or loss of integrity of the plasma membrane of BRIN-BD11 cells at concentrations up to 3 μM and is not hemolytic against human erythrocytes at concentrations up to 30 μM (unpublished data), further investigations of the toxicity of the peptide, both short-term and long-term, are required before it could be considered for systematic use. It is noteworthy that exendin-4, a peptide first isolated from the venom of a reptile [11], has been evaluated in clinical trials in patients with Type 2 diabetes and been shown to improve glycemic control, with few adverse effects [5]. However, exendin-4 acts as an agonist at the GLP-1 receptor and so its mechanism of action is more specific than that of phylloseptin-L2. At the time of writing 5602 species of frogs have been described [14] of which very few have been examined for the presence of antidiabetogenic peptides in their skin. The field is clearly wide open for the discovery for new components with therapeutic potential.

In view of the low antimicrobial potency of phylloseptin-L2, it is tempting to speculate that its presence in skin secretions, like that of the bradykinin- and tachykinin-related myotropic peptides, may be related to the animal's defense strategy against predators but evidence in support of this hypothesis is lacking.

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