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Insulin-releasing properties of the frog skin peptide pseudin-2 and its [Lys^{18}]-substituted analogue

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Abstract

Pseudin-2 is a cationic α-helical peptide that was first isolated from the skin of the paradoxical frog *Pseudis paradoxa* on the basis of its antimicrobial activity. We have investigated the insulin-releasing properties and cytotoxicity of the peptide, together with selected analogues with increased cationicity and hydrophobicity. At concentrations in the range 10^{-9}–10^{-6} M, pseudin-2, and its [Lys^{18}], [Phe^{8}], and [D-Lys^{3},D-Lys^{10},D-Lys^{14}] derivatives, stimulated insulin release from the BRIN-BD11 clonal β-cell line without increasing release of lactate dehydrogenase. The [Lys^{18}] analogue was the most potent (46% increase in insulin release at 10^{-9} M) and the most effective (215% increase in insulin release at 10^{-6} M). The more cationic [Lys^{3},Lys^{10},Lys^{14}] and [Lys^{3},Lys^{10},Lys^{14},Lys^{21}] analogues lacked insulinotropic action and the more hydrophobic [Phe^{16}] analogue was cytotoxic at concentrations $\geq 10^{-7}$ M. Pseudin-2 and [Lys^{18}]-pseudin-2 had no effect on intracellular calcium concentrations and stimulated insulin release in the absence of external calcium. [Lys^{18}]-pseudin-2 (10^{-8} M) stimulated insulin release in the presence of diazoxide and verapamil. Our results demonstrate that pseudin-2 stimulates insulin secretion from BRIN-BD11 cells by a mechanism involving Ca^{2+}-independent pathways and identify [Lys^{18}]-pseudin-2 as a peptide that may have potential for development as a therapeutically valuable insulinotropic agent for the treatment of type 2 diabetes.

Keywords: frog skin; insulin secretion; pseudin-2; type 2 diabetes.

Introduction

Granular glands present in the skins of many species of Anura (frogs and toads) are a rich source of bioactive molecules that serve to protect the animal against infection by pathogenic microorganisms and being eaten by predators (Rinaldi, 2002). As a result, amphibian skin secretions and extracts are considered a potential source of compounds with pharmaceutical and medical utility (Clarke, 1997; Conlon, 2004). In addition, amphibian skin secretions may also contain peptides that are structurally related, but not orthologous, to those synthesised in neuroendocrine tissues of mammals that have been shown to exhibit insulin-releasing activity (Marenah et al., 2004a). Examples include bombesin from *Bombina variegata*, which is structurally similar to gastrin-releasing peptide, and caerulein from *Hyla caerulea*, which is structurally similar to cholecystokinin (Marenah et al., 2004b). Similarly, peptides from *Rana palustris* (Marenah et al., 2004a), *Agalychnis litodryas* (Marenah et al., 2004c), *Phyllomedusa trinitatis* (Marenah et al., 2004d), *Agalychnis calcarifer* (Abdel-Wahab et al., 2005), *Rana pipiens* (Marenah et al., 2005), and *Rana saharica* (Marenah et al., 2006) have been shown to possess insulinotropic properties and thus can be considered as possible candidates for development as agents for the treatment of type 2 diabetes.

The 24-aa peptide pseudin-2 (GLNALKKVFQGIHEAIKLINNHVQ), first isolated from an extract of the skin of the paradoxical frog *Pseudis paradoxa* (Olson et al., 2001), has been investigated as a potentially valuable non-toxic antimicrobial agent (Pal et al., 2005). Pseudin-2 shows potent growth inhibitory activity against clinically relevant Gram-negative bacteria, but has weak cytolytic activity against human erythrocytes. In common with most frog-skin antimicrobial peptides, pseudin-2 exists in aqueous solution predominantly as a random coil, but in solvents that mimic the hydrophobic environment of the cell membrane, such as 50% trifluoroethanol/water, the peptide adopts an α-helical conformation. A preliminary study demonstrated that pseudin-2 stimulated release of insulin from clonal BRIN-BD11 insulinoma-derived cells at concentrations that did not stimulate release of the cytosolic enzyme lactate dehydrogenase (LDH) (Conlon et al., 2006). This lack of stimulation of LDH release was taken as evidence that the integrity of the plasma membrane was maintained. The BRIN-BD11 cell line is a well-established and convenient model to study insulin secretion in response to a range of nutrients, hormones, neurotransmitters, and drugs (McClenaghan et al., 1996; McClenaghan and Flatt, 1999). The aim of the present study was to investigate structure-activity relationships by evaluating the effects on release of insulin and LDH of selected analogues of pseudin-2 with either increased cationicity or increased hydrophobicity. The primary structures, molecular charges at pH 7, and calculated isoelectric points of the peptides used are shown in Table 1. In addition, the mechanism of action of pseudin-2 was studied by determining its effects on intracellular calcium concentration and whether known modulators of insulin secretion affect its insulinotropic action.

Table 1 Primary structure of pseudin-2 and its analogues.

Peptide	Amino acid sequence	Net charge	pI
Pseudin-2	GLNALKKVFQGIHEAIKLINNHVQ	+2	10.32
Lys[18]	GLNALKKVFQGIHEAIK**K**INNHVQ	+3	10.56
Lys[3,10,14]	GL**K**ALKKVF**K**GIH**K**AIKLINNHVQ	+6	11.22
D-[Lys[3,10,14]]	GL**k**KLKKVF**k**GIH**k**AIKLINNHVQ	+6	11.22
Lys[3,10,14,21]	GL**K**ALKKVF**K**GIH**K**AIKLIN**K**HVQ	+7	11.29
Phe[8]	GLNALKK**F**FQGIHEAIKLINNHVQ	+2	10.32
Phe[16]	GLNALKKVFQGIHEA**F**KLINNHVQ	+2	10.32

pI: calculated isoelectric point.

Results

Effects of pseudin-2 peptides on insulin secretion and β-cell cytotoxicity

At concentrations in the range 10^{-9}–10^{-6} M, pseudin-2, and its [Lys[18]], [Phe[8]], and [D-Lys[3],D-Lys[10],D-Lys[14]] analogues, stimulated the rate of insulin secretion from BRIN-BD11 cells compared to the basal rate in the presence of 5.6 mM glucose alone (Figures 1–4). This effect was not associated with a loss of integrity of the plasma membrane, as demonstrated by lack of stimulation of LDH release from the cells (Figures 1–4). The [Lys[18]] analogue was the most potent (46% increase in insulin release at 10^{-9} M) and the most effective (215% increase in insulin release at 10^{-6} M) (Figure 2). [Phe[16]]-pseudin-2 also stimulated insulin release at 10^{-6} M, but showed cell cytotoxicity at concentrations $\geq 10^{-7}$ M (Figure 5). Increasing the molecular charge on the peptide to +6 in [Lys[3],Lys[10],Lys[14]]-pseudin-2 and to +7 in

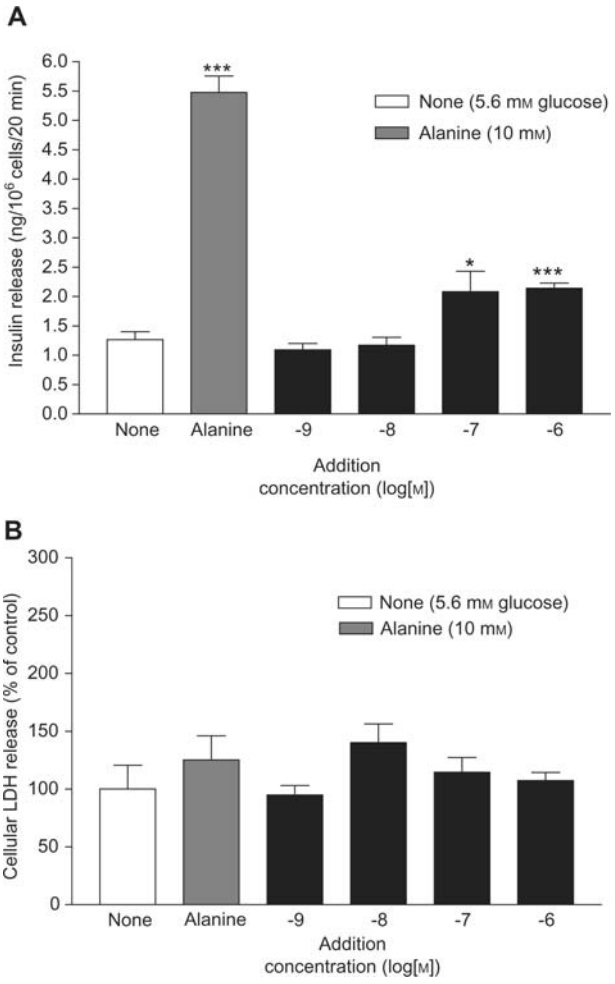

Figure 1 Effects of pseudin-2 on (A) insulin release and (B) LDH release from BRIN-BD11 cells.
Values are mean±SEM with n=8 for insulin release and n=4 for LDH release. *$p<0.05$, ***$p<0.001$ compared to 5.6 mM glucose alone.

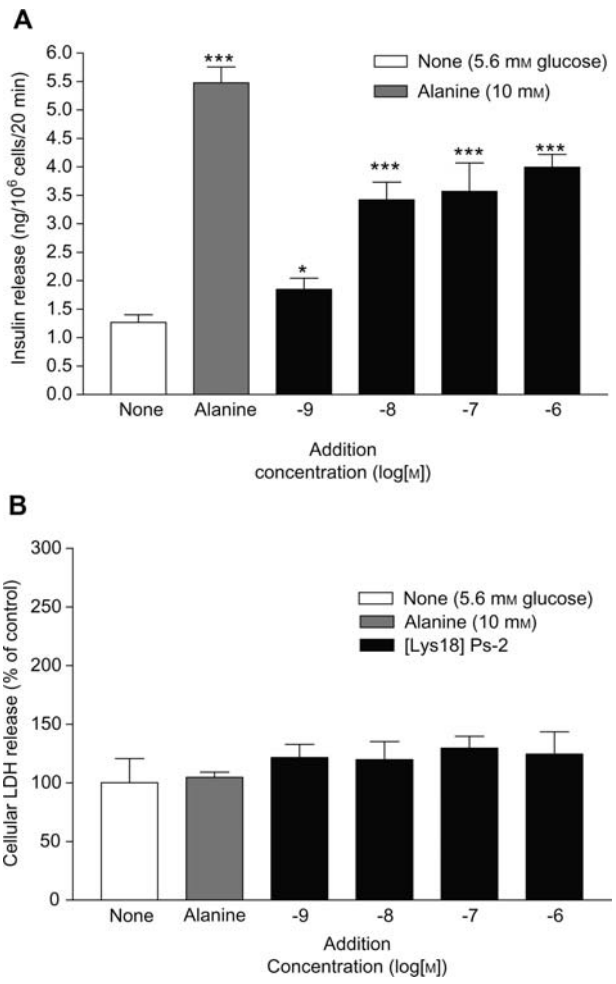

Figure 2 Effects of [Lys[18]]-pseudin-2 on (A) insulin release and (B) LDH release from BRIN-BD11 cells.
Values are mean±SEM with n=8 for insulin release and n=4 for LDH release. *$p<0.05$, ***$p<0.001$ compared to 5.6 mM glucose alone.

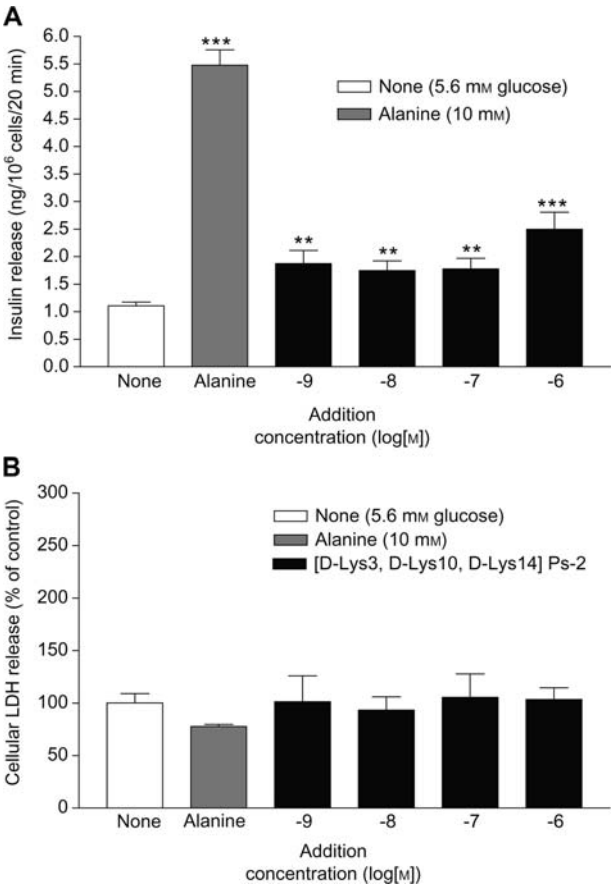

Figure 3 Effects of [D-Lys³,D-Lys¹⁰,D-Lys¹⁴]-pseudin-2 on (A) insulin release and (B) LDH release from BRIN-BD11 cells. Values are mean±SEM with n=8 for insulin release and n=4 for LDH release. $^{**}p<0.01$, $^{***}p<0.001$ compared to 5.6 mM glucose alone.

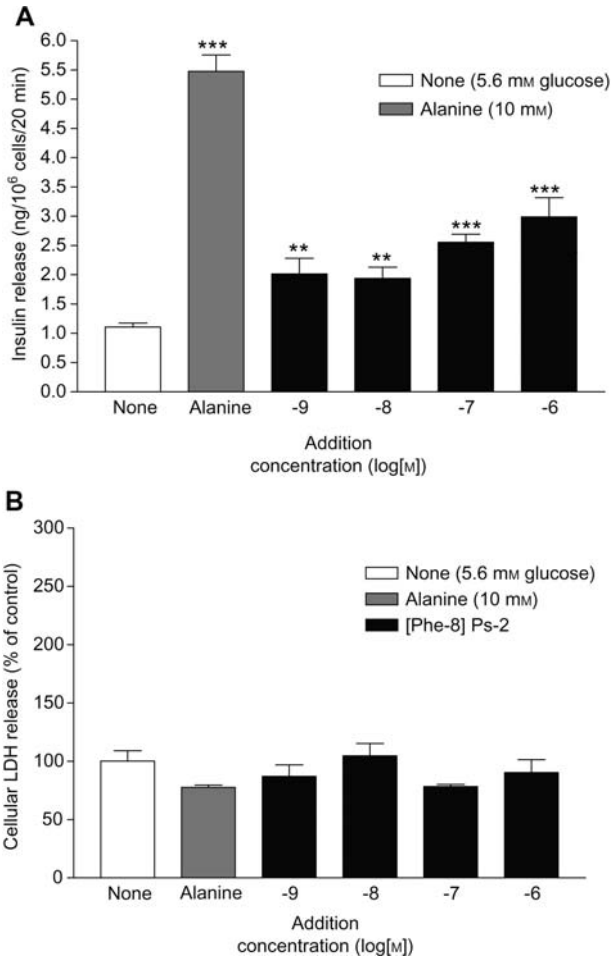

Figure 4 Effects of [Phe⁸]-pseudin-2 on (A) insulin release and (B) LDH release from BRIN-BD11 cells. Values are mean±SEM with n=8 for insulin release and n=4 for LDH release. $^{**}p<0.01$, $^{***}p<0.001$ compared to 5.6 mM glucose alone.

[Lys³,Lys¹⁰,Lys¹⁴,Lys²¹]-pseudin-2 produced peptides that had no significant stimulatory effect on insulin release (data not shown).

Effects of pseudin-2 peptides on $[Ca^{2+}]_i$

Glucose (16.7 mM), a depolarising concentration of KCl (30 mM) and alanine (10 mM) produced a rapid, but transient, reduction in intracellular calcium ($[Ca^{2+}]_i$) in BRIN-BD11 cells, followed by a sustained increase throughout the experiment (Table 2). In contrast to the effects of these well-established β-cell stimulators, pseudin-2, [Lys¹⁸]-pseudin-2, [D-Lys³,D-Lys¹⁰,D-Lys¹⁴]-pseudin-2 and [Phe⁸]-pseudin-2 did not produce a significant increase in $[Ca^{2+}]_i$ compared to 5.6 mM glucose alone (Table 2).

Effects of modulators on insulin-releasing activity

As shown in Figure 6, pseudin-2 and [Lys¹⁸]-pseudin-2 maintained their ability to stimulate insulin release in the absence of extracellular Ca^{2+}. [Lys¹⁸]-pseudin-2 stimulated insulin release at glucose concentrations of 1.1 and 16.7 mM, as well as at 5.6 mM (Table 3). The insulinotropic effect of [Lys¹⁸]-pseudin-2 was maintained in the presence of diazoxide (300 μM) and verapamil (50 μM) (Table 3).

Discussion

A helical wheel projection (Schiffer and Edmundson, 1967) of the pseudin-2 structure indicates that the α-helical conformation adopted by the peptide has considerable amphipathic character, with the hydrophilic residues Lys⁶, Lys⁷, Glu¹⁴, and Lys¹⁷ segregating on one face of the helix and the hydrophobic residues Leu², Leu⁵, Val⁸, Phe⁹, Ile¹², Ile¹⁶, and Val²³ segregating on the opposite face (Olson et al., 2001). The aim of the present study was to evaluate effects on the release of insulin and LDH of selected analogues of pseudin-2 with either increased cationicity or increased hydrophobicity. The more cationic peptides used in this study contained either one ([Lys¹⁸]), three ([Lys³, Lys¹⁰, Lys¹⁴]) or four [Lys³, Lys¹⁰, Lys¹⁴, Lys²¹] L-lysine substitutions on the hydrophilic face of the α-helix. The more hydrophobic analogues contained either [Phe⁸] or [Phe¹⁶] on the hydrophobic face of the helix. The study also investigated the properties of

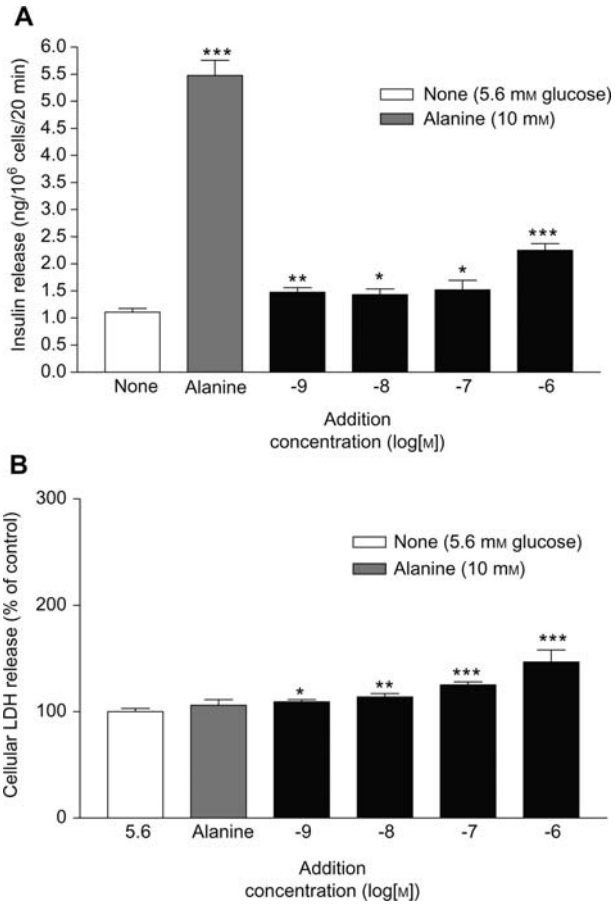

Figure 5 Effects of [Phe[16]]-pseudin-2 on (A) insulin release and (B) LDH release from BRIN-BD11 cells.
Values are mean±SEM with n=8 for insulin release and n=4 for LDH release. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to 5.6 mM glucose alone.

the analogue [D-Lys[3],D-Lys[10],D-Lys[14]]-pseudin-2 that is associated with increased cationicity, but in which the α-helix has been destabilised by the introduction of D-amino acids.

This study has confirmed our previous observation that the antimicrobial peptide pseudin-2 exerts concentration-dependent stimulatory effects on insulin secretion from BRIN-BD11 cells without cytotoxicity, as assessed by cellular LDH release (Conlon et al., 2006). Increasing the cationicity of the peptide from +2 to +3 while maintaining amphipathicity by the substititution Leu[18]→Lys produced a non-toxic analogue with increased potency and effectiveness compared to the native peptide. The insulin-releasing properties of [Lys[18]]-pseudin-2 are broadly similar to those of the naturally occurring gut hormones GLP-1(7–36)amide and GIP (McClenaghan and Flatt, 1999). Increasing the molecular charge on the peptide to +6 in [Lys[3],Lys[10],Lys[14]]-pseudin-2 and to +7 in [Lys[3],Lys[10],Lys[14],Lys[21]]-pseudin-2 produced peptides that had no stimulatory effect on insulin release. However, the analogue [D-Lys[3],D-Lys[10],D-Lys[14]]-pseudin-2 with increased cationicity but with a destabilised α-helical conformation retained the ability to stimulate insulin release with potency and effectiveness comparable to pseudin-2. This analogue also showed high potency in inhibiting the growth of Gram-negative bacteria without haemolytic activity against human erythrocytes (Pal et al., 2005). Increasing the hydrophobicity of pseudin-2 while maintaining the amphipathic nature of the peptide by the substitutions Val[8]→Phe and Ile[16]→Phe had only minor effects on the insulin-releasing activity of pseudin-2. Both analogues stimulated insulin release at relatively high concentrations with an effectiveness similar to that of the native peptide, but [Phe[16]]-pseudin-2 was toxic to BRIN-BD11 cells at concentrations $\geq 10^{-7}$ M, as indicated by increased release of LDH.

Insulin secretion from pancreatic β-cells is a complex process involving the integration and interaction of multiple external and internal stimuli. Nutrients, hormones and neurotransmitters all modulate insulin release (McClenaghan and Flatt, 1999). Numerous steps are involved in physiological regulation of insulin secretion by glucose, including GLUT-2-mediated transport into β-cells, metabolism to ATP, closure of K_{ATP} channels and Ca^{2+} influx leading to exocytosis (Newgard and McGarry, 1995; Matschinsky, 1996). In the present study, glucose, alanine and KCl increased $[Ca^{2+}]_i$ and stimulated insulin release from BRIN-BD11 cells. The insulinotropic actions of pseudin-2, and its more potent and effective analogue [Lys[18]]-pseudin-2, did not involve an increase in $[Ca^{2+}]_i$ and were maintained in the absence of extracellular calcium. Similarly, diazoxide and verapamil, well-characterised modulators of insulin release, did not influence the insulin-releasing actions of [Lys[18]]-pseudin-2. Diazoxide and verapamil can inhibit insulin release in β-cells by activation of K_{ATP} channels and blockade of voltage-dependent Ca^{2+} channels, respectively (Ashcroft et al., 1984; Miki et al., 1999). Such observations suggest that the insulinotropic effects of the peptide are mediated via Ca^{2+}-independent pathways.

In conclusion, this study has identified an analogue of the frog skin antimicrobial peptide pseudin-2 ([Lys[18]]-pseudin-2) that displays insulin-releasing activity at concentrations as low as 10^{-9} M that do not produce toxic effects in BRIN-BD11 cells. Further studies *in vivo* are warranted to determine whether the peptide shows potential for development as a novel therapeutic agent for treatment of type 2 diabetes.

Table 2 Effects of pseudin-2 and its analogues on intracellular calcium in BRIN-BD11 cells.

Test agent	Peak (RFU)	
	Minimum	Maximum
Control (5.6 mM glucose)	0±475	0±475
Glucose (16.7 mM)	-381±88	3507±570*
KCl (30 mM)	-543±120	9589±478*
Alanine (10 mM)	-379±86	3266±563*
Pseudin-2 (10^{-7} M)	-419±80	1223±561
[Lys[18]]-pseudin-2 (10^{-8} M)	-592±341	957±431
[D-Lys[3],D-Lys[10],D-Lys[14]]-pseudin-2 (10^{-8} M)	-393±83	951±454
[Phe[8]]-pseudin-2 (10^{-8} M)	-325±163	1386±584

Results are expressed as relative fluorescence units (RFU). Values are mean±SEM for n=11. *$p<0.05$ compared to 5.6 mM glucose alone.

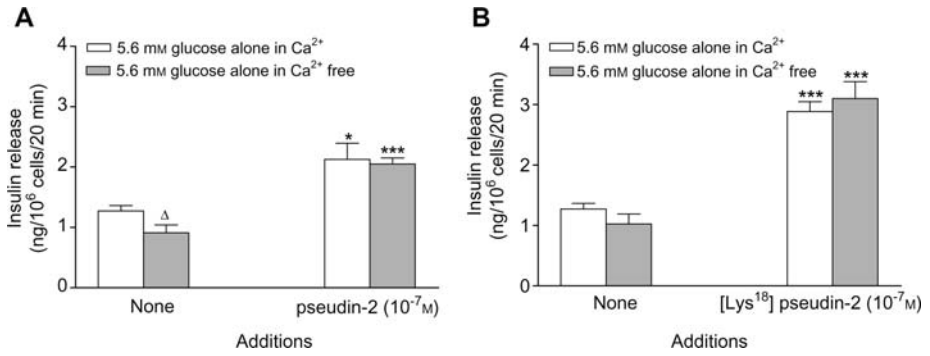

Figure 6 Effects of (A) pseudin-2, and (B) [Lys[18]]-pseudin-2 on insulin release in the presence (open bars) and absence (filled bars) of extracellular calcium.
Values are mean±SEM (n=8). *$p<0.05$, ***$p<0.001$ compared to effects in the absence of peptide. $^{\triangle}p<0.05$ compared to effects in the presence of calcium.

Table 3 Effects of [Lys[18]]pseudin-2 on insulin secretion from BRIN-BD11 cells in the presence of known modulators of β-cell functions.

Test solution	Insulin release (ng/10[6] cells/20 min)
1.1 mM glucose	0.60±0.08
5.6 mM glucose	1.27±0.09
16.7 mM glucose	1.80±0.20
5.6 mM glucose+300 μM diazoxide	0.90±0.07*
5.6 mM glucose+50 μM verapamil	1.14±0.12
1.1 mM glucose+10^{-8} M [Lys[18]]-pseudin-2	0.86±0.13
5.6 mM glucose+10^{-8} M [Lys[18]]-pseudin-2	2.90±0.20*
16.7 mM glucose+10^{-8} M [Lys[18]]-pseudin-2	2.19±0.19*
5.6 mM glucose+300 μM diazoxide+10^{-8} M [Lys[18]]-pseudin-2	2.53±0.16*$^{\triangle}$
5.6 mM glucose+50 μM verapamil+10^{-8} M [Lys[18]]-pseudin-2	2.30±0.15*$^{\triangle}$

Values are mean±SEM (n=8). *$p<0.05$ compared to the appropriate glucose-only control. $^{\triangle}p<0.05$ compared to controls in the presence of diazoxide and verapamil.

Materials and methods

Reagents

Pseudin-2 and its analogues were synthesised, purified, and characterised as previously described (Pal et al., 2005). RPMI-1640 tissue culture medium, foetal bovine serum, penicillin and streptomycin were all purchased from Gibco (Paisley, UK). The cytotoxicity assay kit was obtained from Promega (Madison, WI, USA). The FLIPR Ca^{2+} assay kit was purchased from Molecular Devices (Sunnyvale, CA, USA). All other chemicals used were of the highest purity available.

Insulin-release studies

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) foetal calf serum, antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin) and 11.1 mM glucose using a procedure that has previously been described in detail (McClenaghan et al., 1996). Prior to experimentation, monolayers of cells were preincubated for 40 min at 37°C in 1.0 ml of a Krebs-Ringer bicarbonate buffer (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 $NaHCO_3$) containing 0.1% (w/v) bovine serum albumin, pH 7.4, and supplemented with 5.6 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 peptides or test agents. Aliquots were removed and stored at -20°C for insulin radioimmunoassay (Flatt and Bailey, 1981). Lactate dehydrogenase was measured as previously described (Abdel-Wahab et al., 2007).

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

$[Ca^{2+}]_i$ was determined by a fluorimetric method using monolayers of BRIN-BD11 cells as previously described (Mathews et al., 2006; Abdel-Wahab et al., 2007). Pseudin-2 and its analogues were tested at a concentration of 10^{-8} or 10^{-7} M.

Statistical analysis

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

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