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Frog skin peptides (tigerinin-1R, magainin-AM1, -AM2, CPF-AM1, and PGla-AM1) stimulate secretion of glucagon-like peptide 1 (GLP-1) by GLUTag cells

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ABSTRACT

Skin secretions of several frog species contain host-defense peptides with multiple biological activities including *in vitro* and *in vivo* insulin-releasing actions. This study investigates the effects of tigerinin-1R from *Hoplobatrachus rugulosus* (Dicroglossidae) and magainin-AM1, magainin-AM2, caerulein precursor fragment (CPF-AM1) and peptide glycine leucine amide (PGLa-AM1) from *Xenopus amieti* (Pipidae) on GLP-1 secretion from GLUTag cells. Tigerinin-1R showed the highest potency producing a significant ($P < 0.05$) increase in GLP-1 release at a concentration of 0.1 nM for the cyclic peptide and 0.3 nM for the reduced form. All peptides from *X. amieti* significantly ($P < 0.05$) stimulated GLP-1 release at concentrations $\geqslant$300 nM with magainin-AM2 exhibiting the greatest potency (minimum concentration producing a significant stimulation = 1 nM). The maximum stimulatory response (3.2-fold of basal rate, $P < 0.001$) was produced by CPF-AM1 at a concentration of 3 μM. No peptide stimulated release of the cytosolic enzyme, lactate dehydrogenase from GLUTag cells at concentrations up to 3 μM indicating that the integrity of the plasma membrane had been preserved. The data indicate that frog skin peptides, by stimulating GLP-1 release as well as direct effects on insulin secretion, show therapeutic potential as agents for the treatment of type 2 diabetes.

1. Introduction

Incretins are factors released by the gastrointestinal tract, usually in response to feeding, with the ability to modulate insulin secretion and play a regulatory role in glucose homeostasis [1]. This gut-associated potentiation of insulin release, commonly referred to as the incretin effect, accounts for between 25% and 60% of postprandial insulin release in healthy subjects [2]. The physiologic actions of the two major incretin hormones, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) have been studied extensively (reviewed in [1–3]). The 42-amino acid peptide GIP is produced by K cells in the duodenum and is released in response to ingestion of a high-fat and high-carbohydrate diet [4]. GLP-1 is a 30-amino acid peptide produced in L cells found predominantly in the jejunum and ileum by post-translational processing of proglucagon [5–7].

GLP-1 has been identified as an important target in the treatment of type 2 diabetes. The peptide stimulates insulin release in a glucose-dependent fashion to normalize both fasting and postprandial glycaemia in individuals with type 2 diabetes [8,9]. In addition to its insulinotropic effects, GLP-1 is involved in the maintenance of β-cell mass by promoting cell proliferation and inhibiting apoptosis [10,11]. Other actions of GLP-1 include suppression of hepatic glycogenolysis and gluconeogenesis [9,12,13], inhibition of glucagon secretion [14–16] and gastric motility [13,14] and enhancement of insulin sensitivity [17–19]. These potentially beneficial effects of GLP-1 type 2 in diabetes therapy have motivated the development of analogs of the hormone that are resistant to degradation by the proteolytic enzyme, dipeptidyl peptidase 4 (DPP4) [20].

The discovery of exendin 4, an agonist at the GLP-1 receptor isolated from the venom of a lizard, the Gila monster *Heloderma suspectum* [21], and its development into a clinically valuable anti-diabetes drug [22] has led to the search for other natural agents with potential for treatment of type 2 diabetes. Skin secretions from a range of frog species have proved to be a valuable source of biologically active peptides with therapeutic potential [23,24]. Several frog skin host-defense peptides that were first identified on the basis of their ability to inhibit the growth of microorganisms display incretin activities [25]. Peptides with the ability to stimulate the release of insulin from BRIN-BD11 rat clonal β-cells at low concentrations that are not cytotoxic have been isolated from several frogs belonging to the families Bombinatoridae, Dicroglossidae, Hylidae, Leptodactylidae, Pipidae, and Ranidae (reviewed in [26]). However, reports of the effects of these

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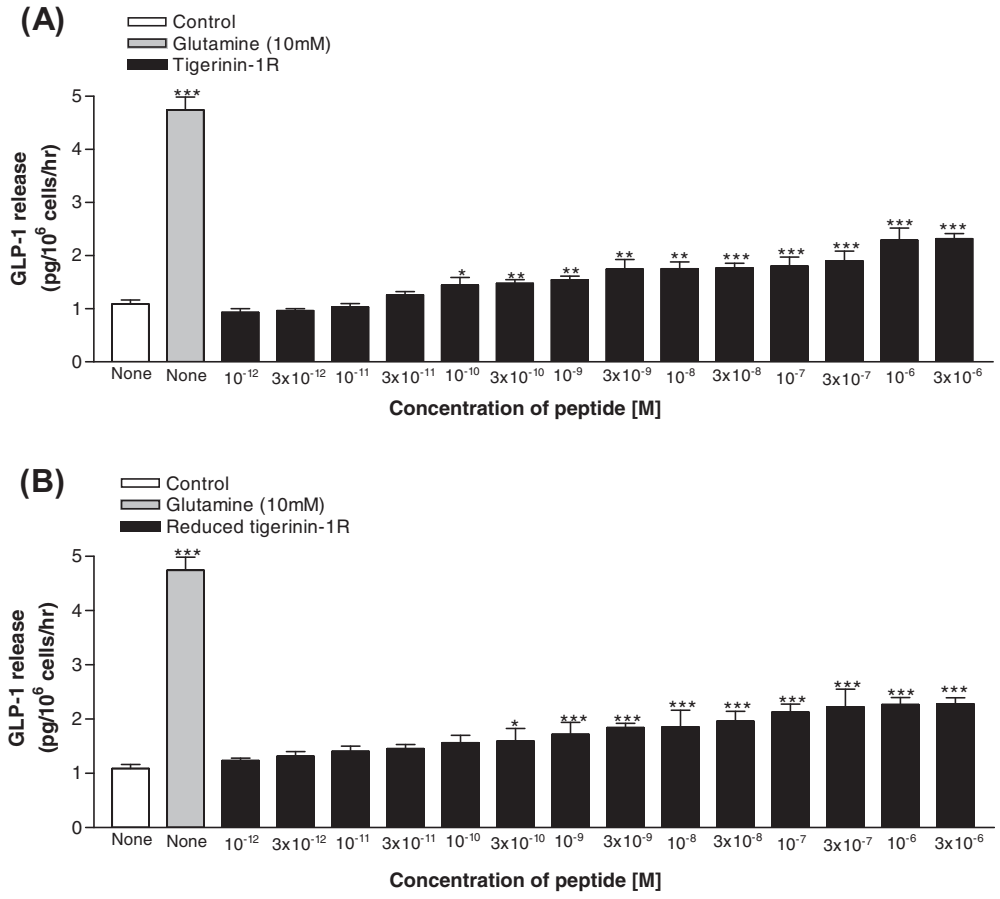

Fig. 1. Effects of (A) tigerinin-1R and (B) reduced tigerinin-1 on the rate of release of GLP-1 from GLUTag cells. Values are mean ± SEM ($n = 8$) $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$ compared to control.

insulinotropic or any other amphibian skin peptides on GLP-1 release are lacking. The present study provides evidence for stimulatory effects of characterized amphibian skin peptides on GLP-1 secretion.

We investigated the effects of synthetic replicates of the oxidized and reduced forms of tigerinin-1R, first isolated from the skin secretions of *H. rugulosus* (Dicroglossidae) [27], and magainin-AM1, magainin-AM2, CPF-AM1 and PGLa-AM1 first isolated from the skin secretions of *X. amieti* (Pipidae) [28] on the rate of release of GLP-1 from the enteroendocrine GLUTag cell-line. This cell line is derived from a colonic tumor excised from a transgenic mouse expressing SV40 large T antigen and expresses the proglucagon gene at high levels and secretes multiple fully processed glucagon-like peptides including GLP-1 [29–31].

2. Materials and methods

2.1. Peptide synthesis

The oxidized and reduced forms of tigerinin-1R (RVCSAIPLPICH.NH_2), magainin-AM1 (GIKEFAHSLGKFG KAFVGGILNQ), magainin-AM2 (GVSKILHSAGKFG KAFLGEIMKS), CPF-AM1 (GLGSVL GKALKIGANLL.NH_2) and PGLa-AM1 (GMASKAGSVLGK VAKV ALKAAL.NH_2) were supplied in crude form by GL Biochem Ltd. (Shanghai, China). Purification to near homogeneity (>98% purity) was performed by reversed-phase HLPC on a (2.2 cm × 25 cm) Vydac 218TP1022 (C18) column equilibrated with acetonitrile/water/triflouroacetic acid (21/78.9/0.1 v/v) as previously described [27]. Identities of the peptides were confirmed by MALDI-TOF mass spectrometry.

2.2. GLUTag cell culture

GLUTag cells were maintained at 37 °C in an atmosphere of 5% CO_2 and 95% air in glutamine-free Dulbecco's Modified Eagle's medium (DMEM) containing 5.5 mM glucose (pH 7.4) supplemented with 10% (v/v) fetal bovine serum, 1% antibiotic (100 U/ml penicillin and 0.1 mg/ml streptomycin) and 2 mM L-glutamine. The cells were seeded at a density of 10^5 per well into a matrigel (~1/100 dilution) coated 24-well tissue culture plates and incubated overnight at 37 °C prior to the experiment. After 24 h incubation, culture medium was removed and replaced with 1 ml of Krebs Ringer Bicarbonate (KRB) buffer containing 115 mM NaCl, 4.7 mM KCl, 1.28 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM HEPES, 8.4% (w/v) $NaHCO_3$, 1% bovine serum albumin (BSA) supplemented with 1.0 mM glucose (pH 7.4) and incubated for 45 min at 37 °C. After the pre-incubation process, the assay buffer were removed and replaced with test solutions appropriate to each experiment. All test solutions were prepared using KRB buffer supplemented with 2 mM glucose unless otherwise stated. Cells were incubated for 2 h at 37 °C with test solutions containing GLP-1 secretagogues at appropriate concentrations. An aliquot (950 μl) was collected from each well and stored at −20 °C until used for radioimmunoassay and cytotoxicity assay.

GLP-1 concentrations were measured using ^{125}I-labelled human GLP-1 (7–36) amide (specific activity 2000 Ci/mmol) as tracer, antiserum 2135 directed against a site in the central region of GLP-1 (7–36) amide, and human GLP-1 (7–36) amide as standard as previously described [20,32]. The concentration of lactate dehydrogenase (LDH) in aliquots (50 μl) of supernatant retrieved from acute GLP-1-release studies was measured as an indicator of

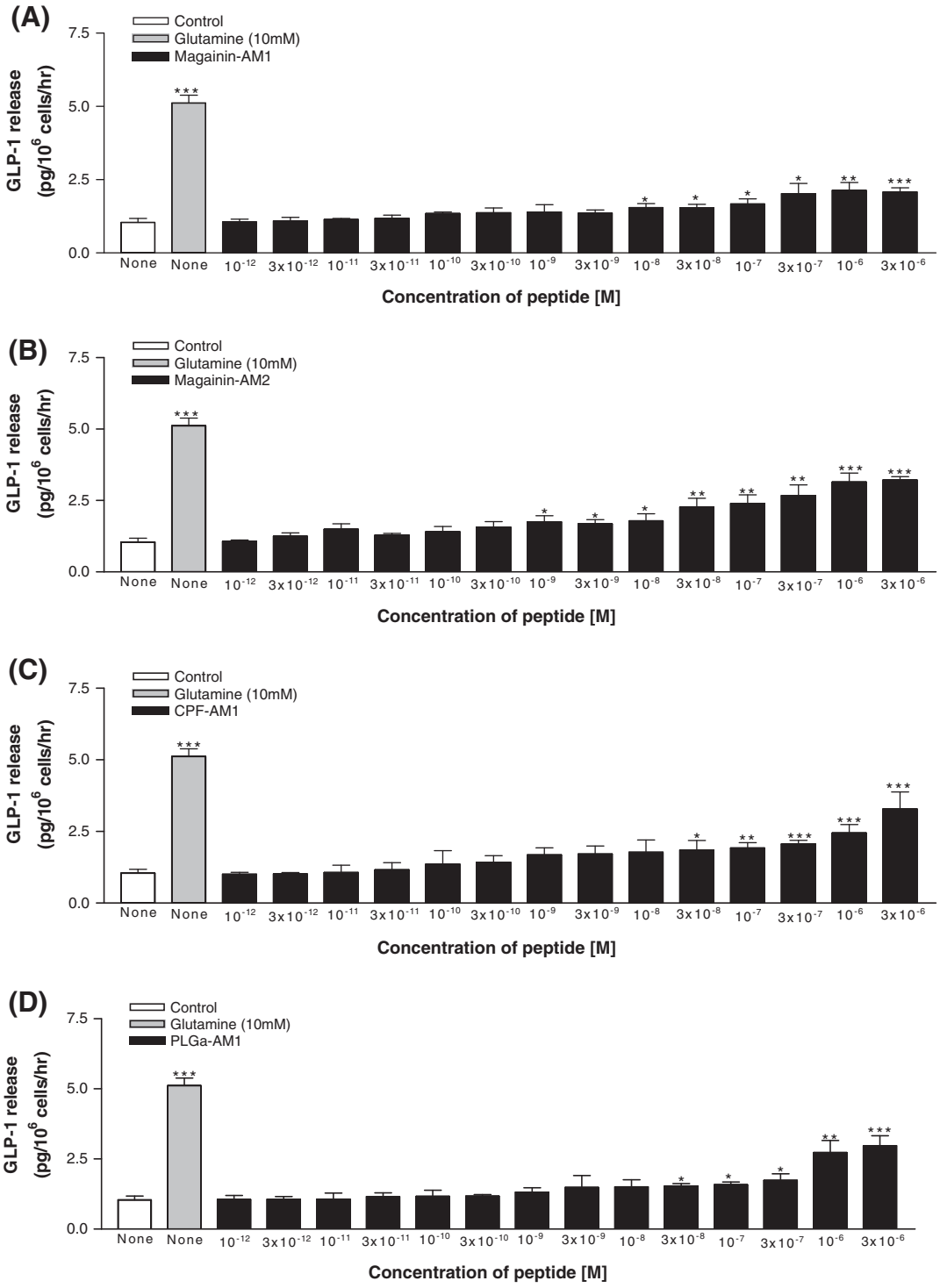

Fig. 2. Effects of (A) magainin-AM1, (B) magainin-AM2, (C) CPF-AM1, and (D) PGLa-AM1 on the rate of release of GLP-1 from GLUTag cells. Values are mean ± SEM ($n = 8$) $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$ compared to control.

cytotoxicity. LDH concentrations were measured using a CytoTox96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI) according to the manufacturer's protocol.

2.3. 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 $P < 0.05$.

3. Results

3.1. Effects of secretagogues on GLP-1 release

The basal rate of GLP-1 secretion from GLUTag cells was 1.1 ± 0.1 ρg/10^6 cells/h and this rate increased by 4.3-fold ($P < 0.001$) in the presence of 10 mM L-glutamine (Fig. 1). Addition of tigerinin-1R elicited a concentration-dependent stimulation of the rate of GLP-1 release with a concentration of 0.1 nM producing a significant ($P < 0.05$) increase over the basal rate (Fig. 1A). The

maximum response produced by a concentration of 3 μM was 2.1-fold greater ($P < 0.001$) than the rate of basal release. The reduced form of tigerinin-1R was less potent than the oxidized (disulphide-bridged) form producing a significant ($P < 0.05$) stimulation in the rate of GLP-1 release at a concentration of 0.3 nM (Fig. 1B). The maximum response of the reduced tigerinin-1R produced at a concentration of 3 μM was not significantly different from that of the oxidized form.

In a second series of experiments, the basal rate of GLP-1 secretion from GLUTag cells was 1.1 ± 0.2 ρg/10^6 cells/h and this rate increased by 4.9-fold ($P < 0.001$) in the presence of 10 mM L-glutamine (Fig. 2). Concentration-dependent effects of peptides isolated from the skin secretion of *X. amieti* (magainin-AM1, magainin-AM2, CPF-AM1, and PGLa-AM1) on the rate of GLP-1 release are shown in (Fig. 2). All peptides produced a significant ($P < 0.05$) increase over the basal rate of release at concentrations ⩾300 nM. Magainin-AM2 was the most potent peptide producing a significant ($P < 0.05$) increase at a concentration of 1 nM. CPF-AM1 was the most effective peptide producing a maximum response of 3.2-fold greater than basal rate ($P < 0.001$) at 3 μM concentration.

No peptide tested in this study produced a significant increase in the basal rate of release of LDH at concentrations up to 3 μM indicating the stimulation of GLP-1 release was not associated with loss of integrity of the plasma membrane of the GLUTag cells.

4. Discussion

Previous studies have shown the tigerinin-1R lacks short-term cytotoxic and hemolytic activity but stimulates the rate of release of insulin from BRIN-BD11 clonal β-cells at concentrations ⩾0.1 nM and administration of the peptide (75 nmol/kg body weight) to high fat-fed mice enhances insulin release and improved glucose tolerance during the 60 min period following an intraperitoneal glucose load [27]. Peptides belonging to the magainin, PGLa, and CPF families isolated from *X. laevis* and *Silurana epitropicalis* have also been shown to stimulate insulin release from BRIN-BD11 cells without loss of plasma membrane integrity [33]. We now extend these studies to demonstrate that tigerinin-1R and magainin, PGLa, and CPF peptides from *X. amieti* produce dose-dependent stimulations of GLP-1 release from GLUTag cells at concentrations that are not toxic to the cells. Tigerinin-1R was the most potent of the peptides tested eliciting a significant ($P < 0.05$) 1.3-fold increase in the rate of GLP-1 secretion at a concentration of 0.1 nM. The reduced form of tigerinin-1R was only slightly less potent and produced the same maximum response indicating that the disulfide bond between the two cysteine residues is not essential for stimulatory activity. This result is consistent with earlier studies showing the reduced form of tigerinin-1R also stimulated insulin release from BRIN-BD11 cells, albeit with reduced potency compared with the oxidized form [27]. For the *X. amieti* peptides, magainin-AM2 exhibited the greatest potency with a concentration of 1 nM producing a significant stimulation and CPF-AM1 was the most effective peptide tested producing the greatest maximum stimulation (3.2 times the basal rate of release) at a concentration of 3 μM.

The GLUTag cell-line represents one of the few *in vitro* cell culture models available to study the mechanisms of GLP-1 secretion and for screening of candidate therapeutic agents [34,35] and is an appropriate model for the investigations conducted in this study. It has been reported that glutamine is a more potent stimulator of GLP-1 release from GLUTag cells than glucose or other amino acids [36] and so 10 mM glutamine was used as a positive control to compare with effects produced by the peptides.

In explaining the mechanism by which compounds elicit their GLP-1 releasing effects, Reimann et al. [31] reported that amino acids, such as glutamine, trigger action potentials by co-transportation with Na^+ and produce a rise in intracellular calcium levels. They also reported that, while there is evidence for presence of a K_{ATP}-dependent pathway in GLUTag cells [30,36,37], K_{ATP} channel closure leading to increasing intracellular calcium might not represent the complete story regarding glucose sensing by L cells. An additional depolarising stimuli, provided by the coupling of Na^+ and glucose entry has been implicated to play a role in GLP-1 release from GLUTag cells [30]. Release of GLP-1 by L cells is also linked to the activation of PKA and PKC [8]. Tigerinin-1R [27] and CPF peptides [33] stimulate insulin release from BRIN-BD-11 cells by a mechanism that involves membrane depolarization and an increase in intracellular Ca^{2+} concentration consistent with their activity being mediated by the K_{ATP} channel-dependent pathway. Further studies are required to determine whether the GLP-1-releasing effects of amphibian skin peptides are dependent on membrane depolarization of GLUTag cells via the K_{ATP} channel-dependent pathway.

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References

[1] J.D. Drucker, The biology of incretin hormones, Cell Metab. 3 (2006) 153–165.
[2] J.J. Meier, M. Nauck, Glucagon-like peptide-1 (GLP-1) in biology and pathology, Diabetes Metab. Res. Rev. 21 (2005) 91–117.
[3] L.L. Baggio, D.J. Drucker, GLP-1 and GIP, Gastroenterology 132 (2007) 2131–2157.
[4] J. Dupre, S.A. Ross, D. Watson, J.C. Brown, Stimulation of insulin secretion by gastric inhibitory polypeptide in man, J. Clin. Endocrinol. Metab. 37 (1973) 826–828.
[5] X. Zhu, A. Zhou, A. Dey, C. Norrbom, R. Carroll, C. Zhang, V. Laurent, I. Lindberg, R. Ugleholdt, J.J. Holst, D.F. Steiner, Disruption of PC1/3 expression in mice causes dwarfism and multiple neuroendocrine peptide processing defects, Proc. Natl. Acad. Sci. USA 99 (2002) 10293–10298.
[6] K. Mortensen, L.L. Christensen, J.J. Holst, C. Orskov, GLP-1 and GIP are colocalized in a subset of endocrine cells in the small intestine, Regul. Pept. 114 (2003) 189–196.
[7] R. Ugleholdt, X. Zhu, C.F. Deacon, C. Orskov, D.F. Steiner, J.J. Holst, Impaired intestinal proglucagon processing in mice lacking prohormone convertase 1, Endocrinology 145 (2004) 1349–1355.
[8] P.L. Brubaker, J. Schloos, D.J. Drucker, Regulation of glucagon-like peptide 1 synthesis and secretion in the GLUTag enteroendocrine cell-line, Endocrinology 139 (1998) 4108–4144.
[9] J.J. Meier, B. Gallwitz, S. Salmen, O. Goetze, J.J. Holst, W.E. Schmidt, M.A. Nauck, Normalization of glucose concentrations and deceleration of gastric emptying after solid meals during intravenous glucagon-like peptide 1 in patients with type 2 diabetes, J. Clin. Endocrinol. Metab. 88 (2003) 2719–2725.
[10] J. Buteau, S. Foisy, E. Joly, M. Prentki, Glucagon-like Peptide-1 induces pancreatic β-cell proliferation via transactivation of the epidermal growth factor receptor, Diabetes 52 (2003) 124–132.
[11] J. Buteau, W. El-Assaad, C.J. Rhodes, L. Rosenberg, E. Joly, Glucagon-like peptide-1 prevents beta cell glucolipotoxicity, Diabetologia 47 (2004) 806–815.
[12] H.C. Fehmann, R. Goke, B. Goke, Cell and molecular biology of the incretin hormones glucagon-like peptide-I and glucose-dependent insulin releasing polypeptide, Endocr. Rev. 16 (1995) 390–410.
[13] B. Willms, J. Werner, J.J. Holst, C. Orskov, W. Creutzfeldt, M.A. Nauck, Gastric emptying, glucose responses, and insulin secretion after a liquid test meal: effects of exogenous glucagon-like peptide-1 (GLP-1)-(7–36) amide in type 2 (noninsulin-dependent) diabetic patients, J. Clin. Endocrinol. Metab. 81 (1996) 327–332.
[14] R. Ritzel, C. Orskov, J.J. Holst, M.A. Nauck, Pharmacokinetic, insulinotropic, and glucagonostatic properties of GLP-1 [7–36 amide] after subcutaneous injection in healthy volunteers. Dose-response-relationships, Diabetologia 38 (1995) 720–725.
[15] J.F. Todd, J.P.H. Wilding, C.M.B. Edwards, F.A. Khan, M.A. Ghatei, S.R. Bloom, Glucagon-like peptide-1 (GLP-1): a trial of treatment in non-insulin-dependent diabetes mellitus, Eur. J. Clin. Invest. 27 (1997) 533–536.

[16] B. Ahren, H. Larsson, J.J. Holst, Effects of glucagon-like peptide-1 on islet function and insulin sensitivity in non-insulin-dependent diabetes mellitus, J. Clin. Endocrinol. Metab. 82 (1997) 473–478.
[17] J. Rachman, F.M. Gribble, B.A. Barrow, J.C. Levy, K.D. Buchanan, R.C. Turner, Normalization of insulin responses to glucose by overnight infusion of glucagon-like peptide 1 (7–36) amide in patients with NIDDM, Diabetes 45 (1996) 1524–1530.
[18] D.A. D'Alessio, S.E. Kahn, C.R. Leusner, J.W. Ensinck, Glucagon-like peptide 1 enhances glucose tolerance both by stimulation of insulin release and by increasing insulin-independent glucose disposal, J. Clin. Invest. 93 (1994) 2263–2266.
[19] A. Mizuno, M. Kuwajima, K. Ishida, Y. Noma, T. Murakami, K. Tateishi, I. Sato, K. Shima, Extrapancreatic action of truncated glucagon-like peptide-1 in Otsuka Long-Evans Tokushima fatty rats, an animal model for noninsulin-dependent diabetes mellitus, Metabolism 46 (1997) 745–749.
[20] P.R. Flatt, C.J. Bailey, B.D. Green, Recent advances in antidiabetic drug therapies targeting the enteroinsular axis, Curr. Drug Metab. 10 (2009) 125–137.
[21] J. Eng, W.A. Kleinman, L. Singh, G. Singh, J.P. Raufman, Isolation and characterization of exendin-4, an exendin-3 analogue, from *Heloderma suspectum* venom, J. Biol. Chem. 267 (1992) 7402–7405.
[22] A. Barnett, Exenatide, Expert Opin. Pharmacother. 8 (2007) 2593–2608.
[23] T.L. Pukala, J.H. Bowie, V.M. Maselli, I.F. Musgrave, M.J. Tyler, Host-defence peptides from the glandular secretions of amphibians: structure and activity, Nat. Prod. Rep. 23 (2006) 368–393.
[24] J.M. Conlon, Structural diversity and species distribution of host-defense peptides in frog skin secretions, Cell. Mol. Life Sci. 68 (2011) 2303–2315.
[25] Y.H.A. Abdel-Wahab, S. Patterson, P.R. Flatt, J.M. Conlon, Brevinin-2-related peptide and its [D4K] analogue stimulate insulin release in vitro and improve glucose tolerance in mice fed a high fat diet, Horm. Metab. Res. 42 (2010) 652–656.
[26] O.O. Ojo, P.R. Flatt, Y.H.A Abdel-Wahab, J.M. Conlon, Insulin-releasing peptides, in: A.J. Kastin, (Ed.), Handbook of Biologically Active Peptides, second ed., Elsevier, San Diego, in press.
[27] O.O. Ojo, Y.H.A. Abdel-Wahab, P.R. Flatt, M. Mechkarska, J.M. Conlon, Tigerinin-1R: a potent, non-toxic insulin-releasing peptide isolated from the skin of the Asian frog, *Hoplobatrachus rugulosus*, Diabetes Obes. Metab. 13 (2011) 1114–1122.
[28] J.M. Conlon, N. Al-Ghaferi, E. Ahmed, M.A. Meetani, J. Leprince, P.F. Nielsen, Orthologs of magainin, PGLa, procaerulein-derived, and proxenopsin-derived peptides from skin secretions of the octoploid frog *Xenopus amieti* (Pipidae), Peptides 31 (2010) 989–994.
[29] D.J. Drucker, T. Jin, S.L. Asa, T.A. Young, P.L. Brubaker, Activation of proglucagon gene transcription by protein kinase A in a novel mouse enteroendocrine cell line, Mol. Endocrinol. 8 (1994) 1646–1655.
[30] F.M. Gribble, L. Williams, A.K. Simpson, F. Reimann, A novel glucose-sensing mechanism contributing to glucagon-like peptide-1 secretion from the GLUTag cell line, Diabetes 52 (2003) 1147–1154.
[31] F. Reimann, P.S. Ward, F.M. Gribble, Signaling mechanisms underlying the release of glucagon-like peptide 1, Diabetes 55 (2006) S78–S85.
[32] M.K. Gutniak, L. Juntti-Berggren, P.M. Hellström, A. Guenifi, J.J. Holst, S. Efendic, Glucagon-like peptide I enhances the insulinotropic effect of glibenclamide in NIDDM patients and in the perfused rat pancreas, Diabetes Care 19 (1996) 857–863.
[33] D. Srinivasan, M. Mechkarska, Y.H.A Abdel-Wahab, P.R. Flatt, J.M. Conlon JM Caerulein precursor fragment (CPF) peptides from the skin secretions of *Xenopus laevis* and *Silurana epitropicalis* are potent insulin-releasing agents, Biochimie (2012), http://dx.doi.org/10.1016/j.biochi.2012.10.026 [Epub ahead of print].
[34] D.J. Drucker, P.L. Brubaker, Proglucagon gene expression is regulated by a cyclic AMP-dependent pathway in rat intestine, Proc. Natl. Acad. Sci. USA 86 (1989) 3953–3957.
[35] P.L. Brubaker, Regulation of intestinal proglucagon-derived peptide secretion by intestinal regulatory peptides, Endocrinology 128 (1991) 3175–3182.
[36] F. Reimann, L. Williams, G.D. Xavier, G.A. Rutter, F.M. Gribble, Glutamine potently stimulates glucagon-like peptide 1 secretion from GLUTag cells, Diabetologia 47 (2004) 1592–1601.
[37] F. Reimann, F.M. Gribble, Glucose-sensing in glucagon-like peptide-1-secreting cells, Diabetes 51 (2002) 2757–2763.