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Mechanisms of action of the antidiabetic peptide [S4K]CPF-AM1 in db/db mice

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Abstract

The antidiabetic effects and mechanisms of action of an analogue of a frog skin host-defence peptide belonging to the caerulein-precursor fragment family, [S4K]CPF-AM1 were investigated in db/db mice with a genetically inherited form of degenerative diabetes-obesity. Twice-daily treatment with the peptide (75 nmol/kg body weight) for 28 days significantly decreased blood glucose ($P < 0.01$) and HbA1c ($P < 0.05$) and increased plasma insulin ($P < 0.05$) concentrations with no effect on body weight, energy intake, body composition or plasma lipid profile. Peptide administration improved insulin sensitivity and intraperitoneal glucose tolerance. Elevated biomarkers of liver and kidney function associated with the db/db phenotype were significantly lowered by [S4K]CPF-AM1 administration. Peptide treatment significantly ($P < 0.05$) increased pancreatic insulin content and improved the responses of isolated islets to established secretagogues. Elevated expression of genes associated with insulin signalling ($Slc2a4$, $Insr$, $Irs1$, $Akt1$, $Pik3ca$, $Ppm1b$) in the skeletal muscle of db/db mice were significantly downregulated by peptide treatment. Genes associated with insulin secretion ($Abcc8$, $Kcnj11$, $Slc2a2$, $Cacn1c$, $Glp1r$, $Gipr$) were significantly upregulated by treatment with [S4K]CPF-AM1. Studies with BRIN-BD1I clonal β-cells demonstrated that the peptide evoked membrane depolarisation, increased intracellular Ca$^{2+}$ and cAMP and activated the protein kinase C pathway. The data indicate that the antidiabetic properties of [S4K]CPF-AM1 mice are mediated by direct insulinotropic action and by regulation of transcription of genes involved in both the secretion and action of insulin.

Introduction

A sedentary lifestyle, consumption of energy-dense foods and physical inactivity has led to an epidemic of obesity and dramatic increases in the prevalence, mortality and morbidity associated with Type 2 diabetes mellitus (T2DM). This situation means that T2DM is considered to be one of the most global health care challenges of the 21st century (Basu et al. 2013). Existing glucose-lowering drugs are often associated with poor glycaemic control and do not prevent severe and often life-threatening complications. This has necessitated the development of novel antidiabetic agents that improve impaired glycaemic responses and delay the related complications in the diabetic patient. The success of the antidiabetic peptide exendin-4 (generic name exenatide) that was first isolated from the venom of the Gila monster lizard Heloderma suspectum and functions as a long-acting agonist at the glucagon-like peptide-1 (GLP-1) receptor (Parkes et al. 2013) has intensified the search for other peptides with...
anti-diabetic properties from animal sources. Antidiabetic agents whose mechanism of action is different from those of existing drug classes are of particular interest.

Skin secretions of frogs have proved to be a rich source of peptides with therapeutic potential. Amphibian host-defence peptides have demonstrated anti-bacterial, anti-fungal, anti-viral, anti-cancer, wound healing and immunomodulatory activities (reviewed in Conlon et al. 2014, Xu & Lai 2015, Pantic et al. 2017). In addition, several such peptides that were first identified on the basis of their antimicrobial properties have subsequently been shown to stimulate insulin release in vitro using BRIN-BD11 rat clonal β-cells and mouse pancreatic islets and improve glycaemic responses in both lean and high fat-fed mice (reviewed in Conlon et al. 2018, 2019). In particular, peptides belonging to the caerulein precursor fragment (CPF) family of peptides, found in skin secretions of frogs belonging to the genus Xenopus (Conlon & Mechkarska 2014, Conlon et al. 2019), display both insulinotropic properties in vitro and a range of antidiabetic properties in animal models of T2DM (Srinivasan et al. 2013, 2015). For example, CPF-SE1 from Xenopus epitropicalis stimulates the in vitro release of insulin from BRIN-BD11 cells at low concentrations that do not compromise the integrity of the plasma membrane of the cell. Long-term administration of the peptide (twice-daily administration of 75 nmol/kg body weight for 28 days) to high fat-fed mice significantly increased circulating insulin and decreased plasma glucagon and triglyceride concentrations, pancreatic glucagon content and total body fat (Srinivasan et al. 2015). Thus, it was concluded that CPF-SE1 showed therapeutic promise for development into an agent for the treatment of patients with obesity-related T2DM.

Major disadvantages of naturally occurring frog skin peptides as therapeutic agents, particularly for systemic administration, are their relatively low potency, short half-life in the circulation and their toxicity to mammalian cells. The strategy of increasing cationicity of these peptides by substituting one or more acidic or neutral amino acids by d- or L-lysine has led to the development of more stable analogues with increased antimicrobial potency and reduced haemolytic activity (Conlon et al. 2007, 2019). Application of this strategy to the development of potential peptide-based antidiabetic drugs has led to the design of analogues of alyteserin-2a (Ojo et al. 2013a) brevinin-2-related peptide (Abdel-Wahab et al. 2010), esculentin-2CHA (1-30) (Vasu et al. 2017), hymenochirin 1B (Owolabi et al. 2016a,b), PGLa-AM1 (Owolabi et al. 2017), pseudin-2 (Abdel-Wahab et al. 2008) and tigerinin-1R (Srinivasan et al. 2014, Ojo et al. 2016) with enhanced cationicity that exhibit both increased insulinotropic activity in vitro and improved glucose-lowering properties in vivo compared with the naturally occurring peptides.

Previous studies have reported broad-spectrum antimicrobial properties of CPF-AM1 (GLGVLGKALKIGANLL.NH2), first isolated from skin secretions of the Volcano clawed frog Xenopus amnieti (Conlon et al. 2010), including activity against oral pathogens (McLean et al. 2014) Further investigations showed that the peptide produced concentration-dependent increases in insulin release from BRIN-BD11 cells and GLP-1 release from GLUTag enteroendocrine cells without disrupting the integrity of the cell plasma membrane (Ojo et al. 2013b). Preliminary data indicated that the [L-Lys(d)]-substituted analogue of CPF-AM1 was more potent than the naturally occurring peptide in stimulating insulin-releasing activity in BRIN-BD11 cells and improved glycaemic responses in lean mice against a glucose challenge (B Owolabi & V Musale, unpublished observations). Based on these promising results, the present study examined the effects of twice-daily administration of [S4K]CPF-AM1 (75 nmol/kg body weight) for 28 days on glycaemic control, insulin concentration, lipid profile, islet morphology and function and expression of key genes in muscle and islets involved in glucose homeostasis in genetically obese db/db mice with degenerative diabetes. The effects of the peptide were compared with those of the established antidiabetic agent exenatide.

**Materials and methods**

**Peptide synthesis**

[S4K]CPF-AM1 (GLGVLGKALKIGANLL.NH2) was supplied in crude form by Sympeptide Co. Ltd. (Shanghai, China) and purified to near homogeneity (>98% purity) by reversed-phase HPLC as previously described (Srinivasan et al. 2015). The identity of purified peptide was confirmed by matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry using a Voyager DE PRO instrument (Applied Biosystems) (observed molecular mass 1664.2 Da, calculated molecular mass 1664.1 Da).

**Animals**

All animal experiments were carried out in compliance with the UK Animals (Scientific Procedures) Act 1986.

and EU Directive 2010/63EU for animal experiments and approved by Ulster University Animal Ethics Review Committee. Male C57.BLKSj db/db mice (BKS. Cg+Leprdb/+Leprdb/OlaHsd strain) together with male, non-diabetic C57BLKSj/+ control mice (BKS.Cg-Lean)/ OlaHsd strain) were supplied by Envigo (Huntingdon, UK) and housed individually with access to water and standard laboratory chow (10% fat, 30% protein, 60% carbohydrate; total energy 12.99 kJ/g; Trouw Nutrition, Cheshire, UK) in an air-conditioned room (22 ± 2°C) with a 12 h light:12 h darkness cycle. All necessary steps were taken to prevent any animal discomfort. Mice were kept for a 1 week acclimation period and were 8-week-old at the start of the experiment. Mice were allocated into four groups of eight animals with matching average body weights and blood glucose levels: (A) db/db mice treated with [S4K]CPF-AM1 (75 nmol/kg body weight), (B) db/db mice treated with exenatide (25 nmol/kg body weight), (C) control db/db mice treated with saline (0.9%, w/v NaCl) and (D) control lean +/+ mice treated with saline.

Effects of twice-daily peptide administration on body weight, metabolic parameters and islet morphology

Before initiation of treatment, mice were injected intraperitoneally twice-daily with saline for 3 days to facilitate adaptation to handling and injection stress. Mice were injected twice-daily (09:30 and 17:00 h) with either [S4K]CPF-AM1, exenatide or saline over a 28-day period. Body weight, food intake, water intake, blood glucose and plasma insulin concentrations were monitored at 3-day intervals. After the treatment period, intraperitoneal glucose tolerance (18 mmol/L arginine, 1 mmol/L human GLP-1 and 1 μmol/L human glucose-dependent insulinotropic polypeptide, GIP) were determined as previously described (Vasu et al. 2017). Following 60-min pre-incubation with 1.4 mmol/L glucose, islets were incubated at 37°C with Krebs-Ringer bicarbonate buffer (500 μL/tube) supplemented with 0.5% (w/v) bovine serum albumin, 16.7 mmol/L glucose and the test agents. After 60 min, the supernatants were collected and stored at −20°C for insulin RIA (Flatt & Bailey 1981).

Gene expression studies

Islets and skeletal muscle for gene expression studies were stored at −72°C. mRNA, extracted using TRIzol Reagent (Sigma-Aldrich), was converted to cDNA using SuperScript™ II Reverse Transcriptase (Invitrogen Life Technologies) (Vasu et al. 2014). Gene amplification was carried out by real-time PCR using an MJ Mini Thermal Cycler (Bio-Rad Laboratories). PCR reaction mixture (containing 4.5 μL of Quantifast SYBR green PCR mix (Qiagen), 1 μL of forward and 1 μL reverse primers, 3 μL of cDNA and 1 μL of nuclease-free water) was added to the PCR tube strips. The forward and reverse primers nucleotide sequences have been provided previously (Ojo et al. 2016, Owolabi et al. 2016b). All the genes were normalised to β-actin (Actb) expression and analysed using the ΔΔCt method.

Biochemical analyses

Blood samples were collected in fluoride/heparin microcentrifuge tubes (Sarstedt, Numbrecht, Germany) by tail bleeding and centrifuged for 30 s at 13,000 g.
Glucose concentrations were measured using an Ascenacia Counter Blood Glucose Meter (Bayer). Blood HbA1c concentrations were measured using an A1cNow™ kit (PTS Diagnostics, Indianapolis, IN, USA) following the manufacturer’s instructions. Hepatic and renal toxicity was assessed by measuring plasma aspartic acid transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (AP) activities and creatinine concentrations, using commercially available kits (Randox Laboratories, Crumlin, UK) following manufacturer’s instructions. Plasma cholesterol concentrations were determined using an I-lab 650 automated clinical chemistry analyser (Diamond Diagnostics, Holliston, MA, USA). LDL-cholesterol concentrations were calculated using the de Cordova equation: 3/4 (total cholesterol – HDL-c) (Martins et al. 2015). Plasma amylase activity was determined using a commercially available assay kit following manufacturer’s instructions (Abcam).

Mechanistic studies with BRIN-BD11 clonal β-cells

The effects of [S4K]CPF-AM1 (1 µmol/L) on membrane potential and intracellular calcium in BRIN-BD11 cells were determined fluorometrically using a FLIPR Membrane Potential Assay Kit and a FLIPR Calcium 5 Assay Kit (Molecular Devices, Sunnyvale, CA, USA) as previously described (Ojo et al. 2013a). Control incubations were carried out using 5.6 mmol/L glucose only, 5.6 mmol/L glucose + 30 mmol/L KCl and 5.6 mol/L glucose +10 mmol/L alanine. Incubations to assess effects on insulin secretion were carried out in the presence of known modulators of insulin release (the K+ channel activator, diazoxide (300 µmol/L) and the blocker of l-type voltage-dependent Ca2+ channels, verapamil (50 µmol/L) and in Ca2+ media containing 1 mmol/L EGTA.

Effects of the peptide (1 µmol/L) on cAMP production in BRIN-BD11 cells was determined as previously described (Owolabi et al. 2017) using an R&D Systems Parameter assay kit (Abingdon, UK) following the manufacturer’s recommended protocol. Effects of downregulation of the protein kinase A (PKA) and protein kinase C (PKC) pathways on [S4K]CPF-AM1 (1 µmol/L)-mediated insulin release were determined in BRIN-BD11 cells that had been incubated overnight with either or both forskolin (25 µmol/L), an activator of PKA, or phorbol 12-myristate 13-acetate (PMA; 10 nmol/L), an activator of PKC, as previously described (Owolabi et al. 2017). Control incubations with GLP-1 (10 nmol/L), CCK-8 (10 nmol/L), forskolin (25 µmol/L), PMA (10 nmol/L) and forskolin (25 µmol/L) + PMA (10 nmol/L) were also carried out.

Effects of [S4K]CPF-AM1 on GLP-1 release from GLUTag cells

The effects of [S4K]CPF-AM1 (10−12–10−6 mol/L) on the release of GLP-1 from GLUTag enteroendocrine cells were determined as previously described (Ojo et al. 2013b). Glutamine (10 mmol/L) was used as a positive control and GLP-1 concentrations were measured using an assay kit (Millipore) according to the manufacturer’s instructions.

Statistical analysis

Data were compared using either one-way ANOVA or two-way ANOVA as appropriate with Bonferroni post hoc test and unpaired Student’s t-test (non-parametric, with two-tailed P values and 95% CI) as appropriate (GraphPad PRISM Version 5.0). Area under the curve (AUC) analysis was performed using the trapezoidal rule with baseline correction. Data are presented as mean ± S.E.M. Where appropriate, outliers greater than two S.D. from the mean were removed from the data set.

Results

Effects of [S4K]CPF-AM1 administration on body weight, energy intake, blood glucose, and plasma insulin concentrations

Prior to the start of treatment, body weight (Fig. 1A and B), energy intake (Fig. 1C and D), non-fasting blood glucose (Fig. 1E and F) and plasma insulin (Fig. 1G and H) were significantly greater (P < 0.001) in all groups of db/db mice compared with lean mice. After 28 days of treatment with [S4K]CPF-AM1, no significant differences in body weight and energy intake were observed compared with control db/db mice treated with saline. In contrast, energy intake was significantly (P < 0.001) decreased in the exenatide-treated group although there was no change in body weight. This apparent contradiction suggests that energy expenditure may have been greater in this group. [S4K]CPF-AM1 administration produced a small but significant (P < 0.05–P < 0.01) decrease in blood glucose concentration throughout the treatment period but the magnitude of the decrease was appreciably less than that produced by exenatide. The hyperinsulinemia associated with the db/db phenotype progressively declined during the 28-day treatment period in control mice consistent with failing β-cell function. [S4K]CPF-AM1 treatment delayed the fall of circulating insulin concentrations but to a lesser extent than in mice treated with exenatide.
Effects of [S4K]CPF-AM1 administration on glucose tolerance and insulin sensitivity

Following an intraperitoneal glucose challenge, plasma glucose concentrations and integrated responses (AUC) were elevated until 60 min in db/db control mice compared with those in lean mice (Fig. 2A and C). [S4K]CPF-AM1 treatment significantly (P < 0.05–P < 0.001) decreased plasma glucose concentrations at all time points compared with db/db controls. The corresponding plasma insulin concentrations (Fig. 2B) and integrated insulin response (Fig. 2D) were also elevated (P < 0.05) in [S4K]CPF-AM1 treated mice but to a lesser extent than in animals treated with exenatide. Computing the insulin response in terms of the integrated change in insulin from 0 to 30 min divided by the change in glucose over the same period gives a ratio of 0.095 ± 0.037 for the [S4K]CPF-AM1-treated db/db mice and a comparable ratio of 0.128 ± 0.031 for exenatide-treated db/db mice. These values are significantly greater than the ratio for saline-treated db/db control mice (0.023 ± 0.004).

Administration of [S4K]CPF-AM1 over a 28-day period significantly decreased blood HbA1c concentrations from 80.0 ± 7.6 mmol/mol in db/db control mice to 57.2 ± 4.9 mmol/mol (P < 0.05) in [S4K]CPF-AM1 treated mice and to 41.8 ± 5.3 mmol/mol (P < 0.01) in exenatide treated mice (Fig. 3A). In order to evaluate effects on insulin

Figure 1
Effects of 28-day administration of [S4K]CPF-AM1 (75 nmol/kg) or exenatide (25 nmol/kg) on (A and B) body weight, (C and D) energy intake, (E and F) non-fasting blood glucose and (G and H) plasma insulin concentrations in db/db mice. The black horizontal bar represents the treatment period. Values are mean ± s.e.m. for eight mice. *P < 0.05, **P < 0.01, ***P < 0.001 compared with lean mice and ΔP < 0.05, ΔΔP < 0.01, ΔΔΔP < 0.001 compared with control db/db mice.

Figure 2
Effects of 28-day administration of [S4K] CPF-AM1 (75 nmol/kg) or exenatide (25 nmol/kg) on intraperitoneal glucose tolerance in db/db mice. Panels show (A) non-fasting plasma glucose concentration, (B) integrated glucose concentrations (area under the curve (AUC)), (C) plasma insulin concentrations and (D) integrated insulin concentrations. Values are mean ± s.e.m. for eight mice. *P < 0.05, **P < 0.01, ***P < 0.001 compared with lean mice and ΔP < 0.05, ΔΔP < 0.01, ΔΔΔP < 0.001 compared with db/db control mice.
sensitivity, exogenous insulin was administered to mice intraperitoneally. The rate of glucose clearance from the circulation was significantly ($P < 0.05$) greater in [S4K]CPF-AM1 treated mice compared with $db/db$ animals treated with saline (Fig. 3B and C). Similarly, QUICKI values, indicative of insulin sensitivity, were significantly ($P < 0.05$) improved in peptide treated mice compared to $db/db$ controls (Fig. 3D).

**Effects of [S4K]CPF-AM1 administration on in vitro insulin secretion**

Islets from $db/db$ mice were associated with impaired release of insulin in response to glucose (1.4 mmol/L, 5.6 mmol/L, 16.7 mmol/L) and other established insulin secretagogues (Fig. 3E). Twenty-eight-day treatment with either [S4K]CPF-AM1 or exenatide significantly ($P < 0.001$) increased the secretory responses relative to those of islets from the $db/db$ control group but not to the full extent of the responses of islets from the lean group.

**Effects of [S4K]CPF-AM1 administration on liver enzyme and amylase activities, creatinine concentration, lipid profile and body composition**

Activities of ALT, AST and ALP were significantly ($P < 0.001$) elevated in the plasma of saline-treated $db/db$ mice (76, 246 and 105%, respectively) compared to their non-diabetic littermates (Fig. 4A, B and C). Treatment with either [S4K]CPF-AM1 or exenatide significantly ($P < 0.05$– $P < 0.001$) lowered these parameters compared to $db/db$ controls. Elevated plasma creatinine concentrations in

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**Figure 3**

Effects of 28-day administration of [S4K]CPF-AM1 (75 nmol/kg) or exenatide (25 nmol/kg) on (A) blood HbA1c concentrations ($n = 4$), (B and C) plasma glucose concentrations and integrated responses after exogenous insulin administration ($n = 8$) and (D) Quantitative Insulin Sensitivity Check Index (QUICKI) ($n = 8$) in $db/db$ mice and (E) insulin release from pancreatic islets in the presence of established secretagogues ($n = 4$). All data are represented as mean ± S.E.M. $*P < 0.05$, $*P < 0.01$, $***P < 0.001$ compared with lean mice and $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$ compared with $db/db$ control mice.
Anti-diabetic actions of [S4K]CPF-AM1

**db/db** mice were also decreased by peptide treatment (Fig. 4D). The **db/db** phenotype was associated with elevated concentrations of plasma triglycerides (Fig. 5F) and low-density lipoprotein (LDL) (Fig. 5H) but these parameters were not significantly reduced by [S4K]CPF-AM1 treatment. Similarly, plasma cholesterol (Fig. 4E) and high-density lipoprotein (HDL) (Fig. 5G) concentrations were not significantly altered by treatment with the peptide. Plasma amylase was slightly but significantly elevated in mice treated with [S4K]CPF-AM1 compared with **db/db** controls (**P** < 0.01) and with lean mice (**P** < 0.05) (Fig. 5I). Treatment of **db/db** mice with either [S4K]CPF-AM1 or exenatide produced no significant change in bone mineral density, bone mineral content, lean body mass amount of body fat and body fat expressed a percentage of total body mass compared with **db/db** control mice (Fig. 5A, B, C, D, E and F).

**Effects of [S4K]CPF-AM1 administration on pancreatic insulin content and islet histology**

Pancreatic insulin content was significantly increased (**P** < 0.01) in mice treated with [S4K]CPF-AM1 relative to the **db/db** control group but not to the level found in the lean group (Fig. 6A). In saline treated **db/db** mice, number of islets/mm² (Fig. 6B) and β-cell area (Fig. 6D)
were significantly decreased and α-cell area significantly increased (Fig. 6E) compared with lean mice (Fig. 6E). There was no significant difference in islet area (Fig. 6C). Treatment with [S4K]CPF-AM1 produced no significant changes in these parameters compared with the db/db control group but an increase in the number of large- and medium-size islets was observed (Fig. 6F).

**Effects of [S4K]CPF-AM1 administration on gene expression in skeletal muscle and pancreatic islets**

Expression of genes associated with insulin signalling [Slc2a4 (glucose transporter 4, GLUT4), Insr (insulin receptor), Irs1 (insulin receptor substrate I) Pik3ca (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha), Akt1 (protein kinase B alpha) and Ppm1b (protein phosphatase 1B)] in the skeletal muscle of db/db mice was elevated relative to lean mice. This increase was completely reversed by [S4K]CPF-AM1 treatment in all cases (Fig. 7A, B, C, D, E and F). The magnitude of these effects was comparable to those observed in exenatide treated mice.

Expression of the insulin I gene Ins1 and genes involved in insulin secretions Abcc8 (ATP binding cassette subfamily C member 8), Kcnj11 (potassium voltage-gated channel subfamily J member 11), Slc2a2 (glucose transporter 2), Cacna1c (calcium voltage-gated channel subunit alpha 1C) and Gck (glucokinase) were significantly (P < 0.001) decreased in db/db mice compared with lean mice (Fig. 8A, B, C, D, E and F). In all cases, treatment with [S4K]CPF-AM1 significantly (P < 0.01 – P < 0.001) upregulated gene expression compared with the db/db control animals but generally to a lesser extent than the effects produced by exenatide. [S4K]CPF-AM1 and exenatide treatments also reversed the downregulation of the Gipr (gastric inhibitory polypeptide receptor), Glp1r (glucagon-like peptide-1 receptor), Gcg (glucagon) and Pdx1 (pancreatic and duodenal homeobox 1) genes that was observed in the islets of db/db mice (Fig. 8G, H, I and J). The significantly
upregulated expression of the Stat1 (signal transducer and activator of transcription 1) gene that was observed in islets from db/db mice was also reversed by peptide treatment (Fig. 8K).

**Effects of [S4K]CPF-AM1 on membrane depolarisation, intracellular Ca\(^{2+}\) and cAMP concentration in BRIN-BD11 cells**

As shown in Fig. 9A, [S4K]CPF-AM1, at a concentration of 1 µmol/L, stimulated the rate of insulin release from BRIN-BD11 cells (McClenaghan et al. 1996) in the presence of 1.1 and 5.6 mmol/L glucose and the effect was maintained at high (16.7 mmol/L) glucose. The insulinotropic effect of the peptide was significantly (\(P < 0.05\)) attenuated in the presence of the K\(^+\) channel activator, diazoxide but not significantly so in the presence of the blocker of L-type voltage-dependent Ca\(^{2+}\) channels, verapamil (Fig. 9A). The rate of insulin release was stimulated by a 30 mmol/L KCl depolarising stimulus and this increase was significantly (\(P < 0.001\)) augmented by [S4K]CPF-AM1. When incubations were carried out in Ca\(^{2+}\)-free medium at 5.6 mmol/L glucose concentration, the ability of the peptide to stimulate release was attenuated but not completely abolished (Fig. 9B). Incubation of BRIN-BD11 cells with the peptide (1 µmol/L) produced a rapid and sustained increase in membrane potential (Fig. 9C) and an increase in intracellular Ca\(^{2+}\) concentrations (Fig. 9D).

In the presence of 200 µmol/L 3-isobutyl-1-methylxanthine (IBMX), [S4K]CPF-AM1 (1 µmol/L) produced a significant (\(P < 0.05\)) increase in cAMP production in BRIN-BD11 cells compared with the response at 5.6 mmol/L glucose alone (Fig. 10A). The magnitude of the increase was less than that produced by 10 nmol/L GLP-1. After downregulation of the PKA pathway by overnight incubation with forskolin, the insulin stimulatory activity of GLP-1, but not [S4K]CPF-AM1 and CCK-8, was strongly attenuated. In contrast, downregulation of the PKC pathway by overnight incubation with PMA was without effect on the stimulatory activity of GLP-1 but the effects of...
[S4K]CPF-AM1 and CCK-8 were abolished. Downregulation of both the PKA and PKC pathways by forskolin + PMA abolished the stimulatory responses of all peptides tested (Fig. 10B). The data suggest an involvement of the PKC pathway in the [S4K]CPF-AM-mediated stimulation of insulin release.

Effects of [S4K]CPF-AM1 on GLP-1 release from GLUTag cells

Incubation of GLUTag enteroendocrine cells with [S4K]CPF-AM1 at a concentration of 1 µmol/L produced a small but significant ($P < 0.05$) increase in the rate of GLP-1 release (Fig. 10C).

Discussion

Previous studies from the laboratory have investigated the antidiabetic and anti-obesity effects of acute and long-term administration of frog skin-derived peptides and their analogues in both lean mice and high fat-fed mice exhibiting obesity, insulin resistance and impaired glucose tolerance (reviewed in Conlon et al. 2018, 2019). These studies have now been extended to a study of the effect of an analogue of the frog skin peptide CPF-AM1 in genetically obese and diabetic $db/db$ mice. The leptin receptor deficient $db/db$ mouse is an animal model of degenerative T2DM characterised by early obesity, progressive hyperglycaemia and hyperinsulinaemia which on C57BL/KsJ genetic background is followed by a phase of $\beta$-cell destruction and severe hyperglycaemia (Yang et al. 2015).

In T2DM, a progressive decline in insulin biosynthesis and secretion results in increasing hyperglycaemia (Butler et al. 2003). In $db/db$ mice, the progressive decrease in non-fasting plasma insulin concentrations was delayed significantly by treatment with [S4K]CPF-AM1.
These findings correlate with enhanced pancreatic insulin content which illustrates the beneficial effects of the analogue on β-cell content and function. Consistent with previous studies in db/db mice (Wang & Brubaker 2002, Park et al. 2007), exenatide both delayed and attenuated the progressive decline in circulating insulin concentrations.

The onset of secondary complications in T2DM can be prevented by maintaining strict glycaemic control (UK Prospective Diabetes Study (UKPDS) Group 1998). In the present study, [S4K]CPF-AM1 treatment of db/db mice significantly decreased circulating glucose concentrations without impacting body weight or energy intake. Glycated haemoglobin (HbA1c), reflecting average blood glucose concentration over several weeks, was also lowered by treatment with the analogue (29 %) but to a lesser extent than produced by exenatide (52%).

As early as 6 weeks of age, db/db mice show impaired glycaemic responses to a glucose load which deteriorate with age (Wang & Brubaker 2002). There have been previous reports of several frog skin peptides and their analogues showing improved glucose tolerance in high fat-fed mice (Srinivasan et al. 2015, Ojo et al. 2016, Owolabi et al. 2016b, Vasu et al. 2017). In the present study, db/db mice treated with saline only showed an impaired glycaemic response to an intraperitoneal glucose load. After 28-day treatment with [S4K]CPF-AM1, the response to a glucose challenge was improved significantly as was found in mice treated with exenatide. The improvement of glycaemic response in the [S4K]CPF-AM1 treated group correlated with improved insulin sensitivity as evidenced by a higher QUICKI index. Long-term administration of the structurally related peptide CPF-SE1 to high fat-fed mice using the same protocol as in the present study also did not affect body weight or energy intake but, along with decreased circulating glucose and increased insulin concentrations, decreased body fat and plasma triglyceride concentrations (Srinivasan et al. 2015). However, treatment of db/db mice with [S4K]CPF-AM1 had no significant effect on adiposity or lipid profile whereas exenatide treatment produced significant decreases in circulating triglyceride and LDL concentrations. The present study suffers from the limitation that the effects of an only single dose of peptide administered over 28 days were investigated but further studies using additional doses over different time periods will be performed to optimise therapy and that these may reveal superior effects.

Expression of the Slc2a4, Insr, Irs1, Akt1, Pik3ca and Ppm1b genes, associated with insulin action, was upregulated in skeletal muscle of db/db control mice compared with expression in +/+ lean controls. These observations are consistent with previous findings that an increase in Akt1 expression was observed in the liver and kidney of db/db mice (Feliers et al. 2001). Similarly, the activities of enzymes in the proximal insulin signalling

**Figure 10**
Effects of [S4K]CPF-AM1 on (A) cAMP concentrations in BRIN-BD11 cells (n = 6), (B) insulin release from BRIN-BD11 cells in the presence or absence of 25 µmol/L forskolin, 10 nmol/L PMA and 25 µmol/L forskolin + 10 nmol/L PMA (n = 8), (C) GLP-1 release from GLUTag cells (n = 4). All data are represented as mean ± S.E.M. *P < 0.05, **P < 0.01, ***P < 0.001 compared with 5.6 mmol/L glucose alone, ΔΔΔP < 0.001 compared with incubation under normal culture conditions. In panel C, •P < 0.05, •••P < 0.001 compared with 2.0 mmol/L glucose alone.
cascade are increased in the insulin-resistant skeletal muscle of mice with experimentally induced liver cirrhosis (Jessen et al. 2006). It is tempting to speculate that this increase in insulin signalling activity and the increase expression of SLC2A4 could be an attempt to compensate for an impaired ability in GLUT4 translocation which is a primary defect in the skeletal muscles of T2DM patients (DeFronzo & Tripathy 2009). Consistent with improved insulin sensitivity in [S4K]CPF-AM1 treated mice, the expression of these genes was normalised to the same extent as observed with exenatide.

Impaired insulin secretion is a significant hallmark of long-standing T2DM (Kahn et al. 2006). As found in the study of Do et al. (2014), islets isolated from db/db mice exhibited impaired insulin release in response to glucose and established insulin secretagogues. In [S4K]CPF-AM1 and exenatide treated mice, insulin secretion was improved significantly. The positive effects of the analogue on β-cell function were further demonstrated by its effects on islet gene expression. Consistent with other studies in db/db mice (Shimoda et al. 2011, Poitout 2013), genes associated with insulin secretory activity (Pdx1, Glp1r, Gipr, Abcc8, Kcnj11, Gck, Cacna1C and Ggc2) were significantly downregulated and the Stat1 gene, which regulates expression of multiple of genes involved with cell survival, was significantly upregulated compared to lean control mice. These diabetes-induced changes were attenuated by both [S4K]CPF-AM1 and exenatide treatments. Effects on islet morphology were minor but immunohistochemical analysis of pancreatic sections revealed that both peptides prevented the substantial decline of the large and medium-size islets seen in db/db mice.

In clinical practice, hepatic parameters such as ALT, AST and ALP activities are measured to monitor the progression of disease and toxicity of drugs. In T2DM, the normal function of the liver is compromised by the accumulation of lipid, which induces hypertrophic changes in hepatocytes, as indicated by elevated levels of these enzymes (Son et al. 2015, Bora et al. 2016). In the present study, elevated activities of the liver enzymes associated with the db/db phenotype were reversed by both [S4K]CPF-AM1 and exenatide treatments. Furthermore, creatinine concentrations, a molecular marker of renal function, were markedly decreased in both treatment groups. These observations suggest an absence of toxicity of the analogue at the dose used and possible beneficial effects on liver and kidney function. However, the effects on the lipid profile produced by exenatide administration were superior to the corresponding effect produced by the frog peptide. Exenatide treatment significantly lowered the elevated concentrations of triglycerides and LDL associated with the db/db group whereas [S4K]CPF-AM1 had no effect on these parameters. In db/db mice treated with either [S4K]CPF-AM1 or exenatide, a small increase in amylase activity was observed compared with saline-treated db/db controls suggesting a potential issue with exocrine pancreatic function. Increased plasma amylase activities have been reported for T2DM patients treated with GLP-1 mimetics but a correlation with the development of acute pancreatitis has not been established (Filippatos et al. 2014, Steinberg et al. 2017).

In common with a number of other frog skin-derived peptides (reviewed in Conlon et al. 2018), [S4K]CPF-AM1 at a concentration of 1 μM produced sustained membrane depolarisation and elevation of intracellular Ca2+ in BRIN-BD11 clonal β-cells. The insulin-releasing activities of the peptide were attenuated in Ca2+-free media and in the presence of the potassium channel activator, diazoxide suggests that the effects of the peptides are mediated, at least in part, by the KATP channel-dependent pathway. However, the increase in insulin release produced by a depolarising stimulus (30 mM KCl) is augmented by the peptide suggesting that the peptides may also be acting by a KATP channel-independent pathway.

Overall, these results demonstrate that [S4K]CPF-AM1 exhibits a broad spectrum of actions in db/db mice with severe degenerative diabetes which are similar, although in most cases less pronounced, than the established antidiabetic drug exenatide. Such changes appear to be mediated by direct actions on the β-cell involving increases in intracellular Ca2+ and cAMP concentrations together with activation of the PKC pathway leading to increased release of insulin as well as by changes in expression of key signalling genes in both islets and skeletal muscle leading to increased insulin sensitivity. Despite the fact that [S4K]CPF-AM1 and exenatide display no similarity in primary structure, the peptides demonstrate rather similar biological activities in db/db mice although the frog peptide is generally less effective. The actions of exenatide are mediated through activation of the GLP-1 receptor (Thorens et al. 1993) and it is noteworthy that [S4K]CPF-AM1 treatment markedly increases expression of Glp1r and modestly stimulates GLP-1 release from GLUTag cells. The future therapy of T2DM will increasingly involve agents with the ability to activate multiple pathways. This is well illustrated by the successful development of unimolecular dual or triple agonists acting at GLP-1, GIP and glucagon.
receptors (Brandt et al. 2018). In this light, future studies will investigate the antidiabetic action of fusion peptides comprising [S4K]CPF-AM1 covalently linked to GLP-1, exenatide, or GIP which may overcome some of the disadvantages such as poor glycaemic control and undesirable side effects associated with the use of the mono agonist peptides alone. In addition, actions of long-acting analogues of such peptides containing albumin-binding moieties such as palmitate will be investigated.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References


DeFronzo RA & Tripathy D 2009 Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. *Diabetes Care* **32** (Supplement 2) S157–S163. (https://doi.org/10.2337/dc09-S302)

Do DH, Low JT, Gaisano HY & Thorn P 2014 The secretory deficit in islets from db/db mice is mainly due to a loss of responding beta cells. *Diabetologia* **57** 1400–1409. (https://doi.org/10.1007/s00125-014-3226-8)


Meenan P, McCrudden MT, Linden GJ, Irwin CR, Conlon JM & Lundy FI 2014 Antimicrobial and immunomodulatory properties of PGLa-AM1, CPF-AM1, and magainin-AM1: potent activity against...


Poitout V 2013 Lipotoxicity impairs incretin signalling. Diabetologia 56 231–233. (https://doi.org/10.1007/s00125-012-2788-6)


