The VGF-derived peptide TLQP-62 modulates insulin secretion and glucose homeostasis

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Abstract

Insulin secretion control is critical for glucose homeostasis. Paracrine and autocrine molecules secreted by cells of the islet of Langerhans, as well as by intramural and autonomic neurons, control the release of different hormones that modulate insulin secretion. In pancreatic islets, the abundant presence of the granin protein VGF (nonacronymic; unrelated to VEGF) suggests that some of its proteolytically derived peptides could modulate hormone release. Thus, in the present study, we screened several VGF-derived peptides for their ability to induce insulin secretion, and we identified the VGF C-terminal peptide TLQP-62 as the most effective fragment. TLQP-62 induced a potent increase in basal insulin secretion as well as in glucose-stimulated insulin secretion in several insulinoma cell lines. We found that this peptide stimulated insulin release via increased intracellular calcium mobilization and fast expression of the insulin 1 gene. Moreover, the peripheral injection of TLQP-62 in mice improved glucose tolerance. Together, the present findings suggest that TLQP-62, acting as an endocrine, paracrine, or autocrine factor, can be considered a new, strong insulinotropic peptide that can be targeted for innovative antidiabetic drug discovery programs.

Key Words

- ▶ neuropeptide
- ▶ intracellular calcium
- ▶ signaling
- ▶ GSIS
- ▶ diabetes

*Journal of Molecular
Endocrinology*
(2015) **54**, 227–239

Introduction

Impaired insulin secretion and insulin resistance play major roles in the development of type 2 diabetes (T2D). The most important regulators of insulin secretion are plasma nutrients, among which glucose is the most effective; however, peripherally and locally secreted hormones, neurotransmitters, and many neuropeptides of the autonomic nervous system and intramural pancreatic ganglia also strongly affect insulin release (Braun *et al.* 2012). Understanding the complex mechanisms of

insulin secretion is a main goal of identifying new drugs that are able to prevent and/or ameliorate the negative metabolic aspects of T2D.

The *Vgf* (nonacronymic) gene has been identified as a nerve growth factor target gene (Levi *et al.* 1985, Salton *et al.* 1991). The VGF protein has biochemical features of the granin family of pro-peptide precursors (Helle 2004, Bartolomucci *et al.* 2011). VGF-derived peptides play an important role in metabolic homeostasis, insofar as they

balance feeding behavior and energy expenditure: germ-line deletion of the *Vgf* gene resulted in a hypermetabolic, lean, and obesity-resistant phenotype in mice (Hahm *et al.* 1999, 2002, Watson *et al.* 2005, 2009). Notably, *Vgf* knockout mice became sensitive to insulin and resistant to developing hyperinsulinemia when they were placed on a high-calorie diet or when they were crossed with different diabetes/obese mouse models (Watson *et al.* 2005).

VGF is a precursor molecule that is processed into an array of various peptides stored in large, dense core granules and secreted via the regulated secretory pathway in response to different stimuli, such as membrane depolarization and intracellular signaling activation (Possenti *et al.* 1989, 1999, Trani *et al.* 1995, 2002, Severini *et al.* 2009, Ferri *et al.* 2011). At least seven peptides have been shown to possess non-redundant biological functions in rodents, and VGF fragments are increasingly being recognized as useful biomarkers for human diseases (Bartolomucci *et al.* 2011). VGF is expressed and the peptides are stored in β cells (Possenti *et al.* 1989) and other cell types of the islet of Langerhans as well as in intramural fibers and ganglia of the pancreas (Cocco *et al.* 2007).

The C-terminal internal fragment TLQP-21 – which was originally investigated for its role in energy balance and lipolysis (Bartolomucci *et al.* 2006, 2009, Fargali *et al.* 2012, Possenti *et al.* 2012) – has recently been implicated in glucose homeostasis (Stephens *et al.* 2012). TLQP-21 has been shown to modulate glucose-stimulated insulin secretion (GSIS) and glucose homeostasis and to protect β cells from degeneration in rats (Stephens *et al.* 2012). Indeed, TLQP-21 has been shown to be released from the islets of Langerhans, which suggests that it works through a paracrine or autocrine mechanism (Stephens *et al.* 2012). Another VGF-derived peptide, NERP-2, has been reported to enhance GSIS in the mouse pancreatic β cell line MIN6 and isolated pancreatic islets (Moin *et al.* 2012). Because we have previously shown that primary neuronal cell cultures (Trani *et al.* 2002) and tumor-derived β cell lines (Possenti *et al.* 1999) contain and secrete the C-terminal VGF peptides upon depolarization stimuli, in the present study, we aimed to analyze their ability to modulate insulin secretion. We found that the C-terminal fragment TLQP-62 – which was previously identified in the CNS (Trani *et al.* 1995, Bartolomucci *et al.* 2006) and showing activity on synaptic plasticity (Alder *et al.* 2003, Bozdagi *et al.* 2008, Thakker-Varia *et al.* 2014) – was the most potent in inducing insulin secretion and in improving glucose tolerance *in vivo*.

Materials and methods

Materials

Media and serum were purchased from EuroClone (Paignton, Devon, UK); antibodies were raised against VGF by our group (Ferri *et al.* 2011); antibodies against phosphorylated protein kinases were purchased from Cell Signaling Technology (Danvers, MA, USA); all of the other chemicals, where not specified, were purchased from Sigma–Aldrich.

TLQP-62 was synthesized at the BioMedical Genomics Center, University of Minnesota (Minneapolis, MN, USA). All of the other peptides used were purchased from PRIMM Srl (Milan, Italy). See Supplementary Figure 1 for amino acid sequences (see section on supplementary data given at the end of this article) (NM_030997.1). We also used two different scrambled peptides that had the amino acid content of TLQP-21 (Petrocchi Passeri *et al.* 2013) and LQEQ-19.

Cell cultures

The rat INS1 and INS1E cell lines were kindly provided by Prof. C Wollheim and Dr P Maechler (Rubì *et al.* 2002). MIN6B1 cells (Lilla *et al.* 2003) were provided by Dr P Halban (University of Geneva, Switzerland), with permission from Dr J-I Miyazaki (University of Osaka, Japan), who produced the maternal MIN6 cell line (Miyazaki *et al.* 1990). β TC6 cells were originally obtained from Dr S Efrat (Efrat *et al.* 1988).

INS1 and INS1E cells were cultured in RPMI 1640 supplemented with 5% fetal bovine serum (FBS), 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 50 μ M 2-mercaptoethanol. INS1E cells were cultured between passages 87 and 100 and were grown at 37 °C under 95% humidified air:5% CO₂. β TC6 cells were cultured in DMEM containing 15% FBS and 25 mM HEPES. MIN6B1 cells were maintained in DMEM containing 25 mM glucose, 15% FBS, and 70 μ M β -mercaptoethanol. For experiments, cells were cultured on poly-L-lysine-coated 96-well plates or in 35 mm dishes and maintained at 37 °C under 5% CO₂. Cells grown on glass cover slides were used for immunofluorescence studies.

Western blotting

Cells were harvested in lysis buffer (20 mM Tris–HCl, 0.5% NP40, and 10 mM EDTA in the presence of protease

inhibitors). Equivalent amounts of cell extracts (1/10 of the plate, $\sim 1 \times 10^5$ cells) were mixed with sample buffer according to Invitrogen NuPAGE. Following heating at 70 °C for 10 min, proteins were subjected to Nu-PAGE 4–12% Bis-Tris gels and then transferred electrophoretically to a PVDF membrane (Amersham, GE Healthcare, Little Chalfont, Buckinghamshire, UK). After staining with Ponceau S to verify the uniformity of protein load/transfer, the membranes were blocked with 5% low-fat milk in buffer for 2 h at room temperature and then analyzed for immunoreactivity. Incubation with primary rabbit C-terminal VGF antibodies (1:5000) was performed overnight at 4 °C. Incubation with peroxidase-coupled secondary goat-anti-rabbit antibodies (1:20 000, Sigma–Aldrich) was performed at room temperature for 1 h and developed with ECL system (GE Healthcare).

Immunofluorescence

Insulinoma cells were fixed with 4% paraformaldehyde (w/v in PBS) for 15 min at room temperature. Fixed cells were permeabilized with 0.2% Triton X-100 in Tris–HCl (pH 7.4) for 5 min, then incubated with the rabbit polyclonal antibody against the C-terminal sequence of VGF (1:2000) at room temperature for 2 h in a humidified chamber. TRITC-conjugated secondary antibodies (1:2000, Sigma–Aldrich) were incubated for 30 min at room temperature. Confocal microscopy was performed with a Leica TCS 4D system (Leica Biosystems, Nussloch, Germany) equipped with a 100 $\times$ 1.3–0.6 oil immersion objective.

TLQP-21 EIA

Material secreted into the extracellular culture milieu was precipitated with trichloro acetic acid (TCA) and resuspended in EIA buffer according to the manufacturer's protocol (TLQP-21 ELISA Kit; EK-003-89, Phoenix Pharmaceuticals, Burlingame, CA, USA). Briefly, 50 μ l of media, rehydrated primary antibody, and biotinylated TLQP-21 were loaded onto a pre-coated secondary antibody plate. Each sample was tested in duplicate. Following incubation and several washes, streptavidin–HRP was added and incubated for 1 h with orbital shaking to detect bound secondary antibody. After washing and blocking the reaction with 2 M HCl, the ELISA reaction was measured at 450 OD on a microplate reader (1420 Multilabel Counter, VICTOR 3, Perkin-Elmer, Waltham, MA, USA).

Insulin secretion

Insulin concentrations were measured using an ELISA kit specific for rat/mouse insulin (Millipore Corporation, Billerica, MA, USA). The cells were washed four times with a glucose-free buffer in modified Krebs–Ringer–bicarbonate–HEPES buffer (KRBH buffer: 134 mM NaCl, 4.7 mM KH_2PO_4 , 1.4 mM MgSO_4 , 1 mM CaCl_2 , and 10 mM HEPES (pH 7.4)) without BSA. Fresh KRBH containing the different VGF-derived peptides (TLQP-62, AQEE-30, or TLQP-21 at concentrations of 1, 3, and 10 μ M), 10 μ M forskolin (Frsk), 1 mM dibutyryl-cAMP (dbcAMP), glucose at either a low (2.5 mM) or high (20 mM) concentration, 100 nM tetra phorbol myristate (TPA), or 56 mM KCl were applied, and the supernatants were collected after 1 h of stimulation. Thereafter, the supernatant was centrifuged at 1200 g for 5 min in order to pellet the suspended material, and the supernatant was utilized for insulin ELISA and for 15% TCA precipitation according to the manufacturer's instructions.

To verify the integrity of the cells, we performed western blotting on TCA-precipitated supernatants to confirm the presence of actin or tubulin in the medium. We eliminated the samples that contained these proteins, which indicated the presence of cell lysis or secretory bodies such as exosomes. Preliminary experiments led us to be confident that no lysis was present with up to 3 h of secretion stimulation under standard conditions.

RNA purification, RT, and gene expression evaluation by real-time PCR

Total RNA was extracted using TRIzol (Invitrogen), according to the manufacturer's instructions. RNA yield and purity were determined by spectrophotometry absorption at 260 and 280 nm. To obtain cDNA, 2 μ g total RNA was reverse transcribed in MLV RT buffer (Promega), 1 mM dNTP, 40 U/ml of Recombinant RNasin Ribonuclease Inhibitor (Promega), and MLV reverse transcriptase (Promega) in a final volume of 25 μ l. The reaction was incubated at 37 °C for 60 min, and the resulting cDNA was stored at –20 °C until it was used for analysis. Relative mRNA expression was measured with quantitative real-time PCR (qPCR) using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Austin, TX, USA); SYBR Select Master Mix fluorescence (Applied Biosystems) was used to quantify the relative amplicon amount. Cycle time (Ct) values for all of the samples were normalized with Tata Binding Protein (TBP; Invitrogen) using the $\Delta\Delta\text{Ct}$ formula. Each cDNA sample from treated

cells was assayed in triplicate for each point. For each set of primers, a no-template control and a no-reverse transcriptase control were included. The thermal cycling conditions were: denaturation at 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. Post-amplification dissociation curves were performed to verify the presence of a single amplification product and the absence of genomic DNA contamination.

The primer sequences used in the present study were as follows for rat genes: *Vgf* (GenBank NM_030997.1) forward: 5'-AGACGGGTCCGATTTTC-3', reverse: 3'-CAGAAG-AGGACGGATGCTG-5'; *Ins1* (GenBank NM_019129.3) forward: 5'-AGACCATCAGCAAGCAGGTC-3', reverse: 3'-CTTGGCTCCCAGAGGAC-5'; *Ins2* (GenBank NM_019130.2) forward: 5'-CGAAGTGGAGGACCCACA-3', reverse: 3'-TGCTGGTGCAGCACTGAT-5'; *Tbp* (GenBank NM_001185084.1) forward: 5'-CAGGTGGCAGCATGAA-GTGAC-3', reverse: 5'-AAGTAGCAGCACAGAGCAAGC-3'.

For mice genes, the sequences were: *Vgf* (GenBank NM_001039385.1) forward: 5'-CGACCCTCCTCTC-CACCT-3', reverse: 5'-CCCAACCCCTGGATCAGTA-3'; *Ins1* (GenBank NM_008386.3) forward: 5'-CAGAGAG-GAGGTACTTTGGACTATAAA-3', reverse: 5'-GCCATGTT-GAAACAATGACCT-3'; *Ins2* (GenBank NM_001185084.1) forward: 5'-GCCCTAAGTGATCCGCTACA-3', reverse: 5'-GCCATGTTGAAACAATAACCTG-3'; *Tbp* (GenBank NM_001185084.1) forward: 5'-CCAATGACTCCTATG-ACCCCTA-3', reverse: 5'-CAGCCAAGATTACGGTA-GAT-3'.

Signaling

INS1 cells were plated (10×10^5) on 35 mm poly-L-lysine-treated culture dishes and cultured overnight in complete medium. We assessed kinase activation as previously described (Petrocchi *et al.* 2010). Briefly, cells were starved for 1 h in serum-free RPMI with HEPES, sodium pyruvate, β -mercaptoethanol, and the substances to be tested, and incubated at 37 °C under 5% CO₂. At the end of the incubation period, the reaction was quickly stopped with ice by removing the medium and adding cold lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM EDTA) in the presence of a phosphatase and protease inhibitor mixture (Sigma-Aldrich). Harvested cells were kept at -80 °C until they were gel-run.

For each experiment, equivalent amounts of cell extracts (one-quarter of the plate, which was equivalent to $\sim 5 \times 10^5$ cells) were run as described in the western blot section. Membranes were incubated overnight at 4 °C with the specific primary antibodies – anti-pPKC, anti-pAMPK,

or anti-p-Erk1/2 (1:1000; Cell Signaling Technology) – in 5% BSA Tris buffer saline plus 1% Tween 20 (the following were also tested: anti-p-AKT, anti-p-P38, anti-p-JNK, anti-p-PKA substrate, anti-p-PKD substrate, anti-p-PLC, and anti-p-PLA). Then, cells were incubated for 1 h at room temperature with secondary goat-anti-rabbit peroxidase-conjugated antibodies (1:5000, GE Healthcare) in 5% BSA Tris buffer saline plus 1% Tween 20. After extensively washing the membranes, band detection was carried out by the ECL system (GE Healthcare). To normalize for sample loading, the blots were stripped (Restore Western Blot Stripping, Thermo Scientific, Rockford, IL, USA) and reprobed with an antibody against α -tubulin or β -actin (1/10 000; Sigma-Aldrich) overnight at 4 °C and used as internal controls. Blots were analyzed by scanning densitometry and quantified using ImageJ Software (NIH, Bethesda, MA, USA).

To investigate receptor identity, the C3a peptide agonist C3a70-77 (Anaspec, Fremont, CA, USA) and the C3a receptor antagonist SB290157 (Calbiochem, San Diego, CA, USA) were used.

Analysis of intracellular Ca²⁺ cycling

Optical fluorometric recordings with membrane-permeable forms of the calcium indicator Fura2AM (F1221) were used to evaluate intracellular calcium variations. Stock solutions were obtained by adding 50 μ g of the dye to 50 μ l of 75% DMSO plus 25% pluronic acid (F-127, Molecular Probes, Life Technologies, Carlsbad, CA, USA) and were stored frozen at -20 °C. Cells plated 48 h before experiments were bathed for 60 min at room temperature in 1 ml of extracellular solution (HBSS: 125 mM NaCl, 1 mM KCl, 5 mM CaCl₂, 1 mM MgCl₂, and 20 mM HEPES (pH 7.35), with 4.5 mM glucose; EuroClone), with a final Fura2AM concentration of 5 μ M. This solution was then removed and replaced with fresh Ringer solution, and the plates were quickly placed on the microscope stage. When not otherwise specified, Ringer solution with 4.5 mM glucose was used for experiments. The samples were analyzed for a 15–60 min period during which the ratio between the values of light intensity at 340 and 380 nm stimulation (F340/380) was recorded every 6 s. The change in intracellular fluorescence intensity, which indicates the cytoplasm-free calcium level ([Ca²⁺]_i) of cells after treatment with VGF-derived peptides and control substances, was measured by fluorescence microscopy with single-cell records: to measure fluorescence changes, a computerized analysis system was used (ImagEM; Hamamatsu Photonix, Hamamatsu, Japan). After each

experiment, cells were stimulated with 56 mM KCl to confirm cell reactivity. Cells that did not respond to the application of KCl were eliminated from the analysis.

Experiments were performed at least three times, and 35–45 single cells were recorded and averaged per experiment. Data presented in the figures are means $\pm$ S.E.M. of one representative experiment per group.

Cell proliferation assays

Cells were plated on poly-L-lysine-treated 96-well plates (3×10^4 cells/well) and cultured overnight in complete medium. Medium was then removed, and RPMI containing the test substances was applied to eight wells each. Cells were then cultured for 24 or 48 h. The metabolic activity of the cells was determined by a colorimetric methyl thiazolyl tetrazolium (MTT) assay as previously described (Petrocchi Passeri *et al.* 2013).

Proliferation was analyzed by BrdU incorporation (Cell Proliferation ELISA BrdU, Roche Diagnostics) according to the manufacturer's instructions. We performed 4 h of BrdU incorporation after the substance application.

Statistical analysis, which was performed on data from six wells (after eliminating the highest and the lowest points), was based on a paired two-tailed *t*-test between the mean values of the treated cells and the control cells. Experiments were performed in triplicate.

In vivo glucose tolerance test

Male CD1 mice (Charles River, USA) weighing 25–30 g were acclimatized for 10 days and housed under conventional housing conditions. A glucose tolerance test (GTT) was performed after an overnight fast. Mice were injected i.p. with TLQP-62 (1 or 5 mg/kg) or saline vehicle 30 min before an i.p. dose of 2 g/kg D-glucose (Stephens *et al.* 2012). Blood glucose levels from tail bleedings were monitored with an Accucheck Aviva glucometer (Roche Diagnostics) at –30, 0, 30, 60, and 120 min from the glucose injection. Protocols were approved by the IACUC, University of Minnesota.

Statistical analysis

Data are expressed as means $\pm$ S.E.M. and analyzed with unpaired two-tailed *t*-tests between the mean values of the treated cells and the control cells; GTT data were analyzed with ANOVA followed by Tukey's honestly significant difference (HSD) *post hoc* test. Differences were considered significant at $P < 0.05$.

Results

VGF protein expression in insulinoma cell lines

We first aimed to confirm the expression of VGF in different β cell lines (Possenti *et al.* 1999, Cocco *et al.* 2007) that are known to fully process the VGF precursor (~ 70 kDa), as well as its smaller fragments, using selective C-terminal antibodies (Ferri *et al.* 2011). VGF was constitutively expressed in all of the tested cell lines, and its expression became markedly increased after overnight treatment with Frsk (Fig. 1A) or dbcAMP (Possenti *et al.* 1999). In contrast, TPA, which first activates and then down-regulates PKC (Supplementary Figure 2), only slightly induced VGF protein expression (Fig. 1A). Positive immunostaining for the C-terminal portion (Ferri *et al.* 2011) in perinuclear (Golgi apparatus) and punctuated peripheral (secretory vesicles) areas confirmed the intra-granular localization of VGF for all of the cell lines tested (Fig. 1B).

The observation that cAMP up-regulated VGF processing suggested that VGF could be strongly modulated by incretine peptide signaling via PKA (e.g., glucagon and glucagon peptide-like 1) as well as by neurotransmitters (e.g., norepinephrine).

Figure 1

Expression of VGF and its fragments in the insulinoma cell lines. (A) Representative western blots of the VGF precursor and C-terminal peptides in INS1E, β TC-6, and MIN6B1 cells, cultured overnight with 10 μ M forskolin (Frsk) or 100 nM tetra phorbol myristate (TPA), and control cells (Cntr). Loading controlled with tubulin (TUB). Frsk up-regulated VGF expression more than TPA did. (B) Immunofluorescence analysis revealed that VGF was present in a punctuated pattern characteristic of secretory vesicles in all three cell lines. Scale bars = 5 μ m. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0313>.

Western blotting and EIA demonstrated that VGF-derived peptides were secreted (Supplementary Figure 3). Moreover, we noticed that 3 h of secretory stimuli could affect cell viability, as demonstrated by the detection of actin in the medium (Supplementary Figure 3). We thus performed all secretory experiments with a 1 h exposure time.

C-terminal VGF-derived peptides stimulate insulin secretion in insulinoma cells

After finding that VGF is expressed and smaller fragments are secreted upon increased cAMP, we assessed the insulin secretion activity of three main C-terminal VGF-derived peptides (namely, TLQP-62, AQEE-30, and TLQP-21), several smaller fragments, and scrambled peptides (Supplementary Table 1). To this end, INS1 cells cultured in a low-glucose condition (Jones & Persaud 1998) were stimulated with the different VGF peptides. We found that TLQP-62 was the most potent insulin secretagogue (Fig. 2A and B), whereas TLQP-21 and AQEE-30 had no, or a weak, effect under this experimental condition. We thus used peptides at a high dose (10 μ M) for all subsequent experiments to maximize stimulations.

According to previous work (Hohmeier *et al.* 2000), variations in insulin secretion in the different cell lines are the result of glucose sensitivity either to the voltage-dependent calcium channels or to cell confluence. Therefore, we used the INS1E subclone cell line for follow-up experiments because of their higher sensitivity to glucose and peptide stimulation and because of the very low effect that confluence has on them. We found that TLQP-62 was a potent insulin secretagogue independently of glucose concentration (Fig. 2C). Conversely, TLQP-21 had only a very weak effect at the low-glucose concentration, but it nevertheless potentiated GSIS, which is consistent with what has been reported previously (Stephens *et al.* 2012). Moreover, none of the smaller fragments of TLQP-62 (TLQP-8, AQEE-10, or YIEH-10) or the scrambled peptides affected the insulin secretion of insulinoma cell lines under either low- or high-glucose conditions (data not shown).

Gene expression regulation by TLQP-62 in INS1E cells

Modulation of *Ins1*, *Ins2*, and *Vgf* mRNA expression was assessed with time-course experiments for 2, 6, and 18 h (overnight) (Fig. 3). High glucose slightly but significantly stimulated transcription of *Ins1* and *Vgf* at 2 h but not at the later time points (Fig. 3A); moreover, it up-regulated

Figure 2

Stimulation of insulin secretion by VGF fragments in insulinoma cells.

(A) Insulin secretion was assessed in INS1 cells after 1 h of static incubation in medium containing a low glucose concentration (2.5 mM) plus 10 μ M of one of three C-terminal VGF-derived peptides (TLQP-62, TLQP-21, or AQEE-30). The positive controls were: Glc, 20 mM glucose; Frsk, 10 mM forskolin; TPA, 100 nM tetra phorbol myristate; and KCl, 56 mM potassium chloride. Graph of means $\pm$ S.E.M. of six independent experiments run in duplicate. (B) Insulin secretion by INS1 cells after 1 h of static incubation in medium containing low glucose (2.5 mM) and different concentrations (1, 3, or 10 μ M) of a C-terminal VGF-derived peptide (TLQP-62, TLQP-21, or AQEE-30). Graph of means $\pm$ S.E.M. of three independent experiments run in duplicate. (C) Insulin secretion by INS1E cells after 1 h of static incubation in media containing 10 μ M of a C-terminal VGF-derived peptide (TLQP-62, TLQP-21, or AQEE-30), 10 μ M Frsk, 100 nM TPA, or 56 mM KCl in either low (2.5 mM) or high (20 mM) glucose (Gluc). Insert, the ratio of insulin secretion in INS1E cell in high glucose (20 mM) to that in low glucose (2.5 mM). Graph of means $\pm$ S.E.M. of six independent experiments run in duplicate. Statistical significance between mean values of treated cells and control cells (Cntr: 100%) was analyzed using the paired two-tailed *t*-test: **P* < 0.01 and ***P* < 0.001.

Figure 3

Modulation of insulin and *Vgf* mRNAs by TLQP-62. (A) qPCR for insulin 1 (*Ins1*), *Ins2*, and *Vgf* genes after exposure to high glucose (20 mM) for 2 h, 6 h, or 18 h (o/n). Insert, western blot demonstrating that the increase in VGF precursor after 2 h of exposure to high glucose was countered by pretreatment for 15 min with 20 μg/ml cyclohexamide (CHX). TUB, tubulin. (B) qPCR for *Ins1*, *Ins2*, and *Vgf* after exposure to 10 μM TLQP-62. (C) qPCR for *Ins1*, *Ins2*, and *Vgf* genes in the presence of 10 μM forskolin (Frsk). Statistically significant differences between mean values of treated cells and control cells (Cntr: 100%) were analyzed using the paired two-tailed *t*-test: **P* < 0.05 and ***P* < 0.001.

expression of the VGF precursor protein (Fig. 3A, inset), an effect blocked by pretreatment with cyclohexamide. This suggested that the effect was transcriptionally dependent, but we also cannot exclude the glucose-mediated translational regulation of mRNAs, which has been previously reported (Melloul *et al.* 2002). In a similar fashion, TLQP-62 increased *Ins1* and *Vgf* gene expression but not that of *Ins2* at 2 h (Fig. 3B). As expected, Frsk induced transcription of *Ins1* (Philippe & Missotten 1990) and strongly

increased *Vgf* mRNA as well (Fig. 3C) (Possenti *et al.* 1999); moreover, the effect of Frsk was not affected by the glucose concentration in the medium.

Intracellular signaling pathways activated by TLQP-62

It has been well established that insulin is secreted in response to high glucose, but many other secretagogues can modulate its secretion through the activation of different intracellular signal pathways (Jones & Persaud 1998). To investigate the signaling activity of TLQP-62 in INS1E cells, we measured its effect on the phosphorylation levels of endogenous protein kinases (Fig. 4). TLQP-62 induced a significant early decrease (2–5 min) in AMPK phosphorylation, followed by an increase (10–15 min) and then another down-regulation (30–60 min). There are conflicting findings on the role of AMPK activation on β cell function (Fu *et al.* 2013). The fragment also significantly increased ERK phosphorylation within 5–15 min, and this was followed by a decrease at later time points (30–60 min). It also induced a slightly delayed (15–30 min) and variable modulation of PKC phosphorylation. We did not detect modulation in the phosphorylation of AKT, PLCγ, PLA2, JNK, p38 (Supplementary Figure 4), or in the substrates of PKA or PKD (data not shown). Very similar effects were observed for the other insulinoma cell lines (Supplementary Figure 5).

TLQP-62-induced signaling pathways were further probed after exposure to specific inhibitors for 30 min before stimulation for 1 h. The ERK inhibitor UO126 (1 μM), the PKC inhibitor Go6983 (1 μM), and the phospholipase inhibitor U73122 (2 μM) did not affect TLQP-62-induced (10 μM) insulin secretion (Table 1). This finding indicated that activated kinases were downstream from the calcium influx necessary for insulin secretion (see also the calcium mobilization section). In fact, the non-competitive inhibitor of sarco/endoplasmic reticulum Ca^{2+} ATPase thapsigargin (500 μM), which was used to deplete intracellular ER calcium storage, significantly decreased constitutive and TLQP-62-induced insulin secretion (by about 40%), which suggests the involvement of Ca^{2+} being released from intracellular storage (see also the next section). Indeed, thapsigargin did not decrease the release of TLQP-62 (Supplementary Figure 6), which suggests that there is a different secretory mechanism for cargo vesicles (Giordano *et al.* 2008).

Recently, C3aR1 was identified as the TLQP-21 receptor (Hannedouche *et al.* 2013). We therefore used the C3a peptide agonist C3a70-77 and the C3a receptor antagonist SB290157 to analyze whether this receptor

was also involved in TLQP-62-mediated signaling events. We did not obtain inhibition of TLQP-62-induced insulin secretion activity in the presence of SB290157 (Supplementary Figure 7), which indicates that TLQP-62 and

Figure 4

Early signaling of TLQP-62 in INS1E cells. (A) After 1 h of serum starvation, cells were exposed to 10 μ M TLQP-62 for different amounts of time and analyzed for phosphorylation of different kinases by western blotting. (B, C and D) Semiquantitative densitometric analysis of individual phosphorylated activated kinases. Graphs of means $\pm$ S.E.M. of five experiments, with data from the indicated time intervals grouped together. Statistically significant differences between values of treated cells and control cells (Cntr: 1, arbitrary units) were analyzed using the paired two-tailed *t*-test: **P* < 0.05.

Table 1 Insulin secretion in the INS1E cell line. Cells were pretreated with specific inhibitors for 30 min before stimulation with TLQP-62 (10 μ M) for 1 h

Inhibitor	TLQP-62	Insulin level (arbitrary units)
–	–	1.0 $\pm$ 0.1
–	+	3.8 $\pm$ 0.5
Go6983	–	1.0 $\pm$ 0.3
Go6983	+	4.0 $\pm$ 0.6
U73122	–	1.8 $\pm$ 0.3
U73122	+	3.8 $\pm$ 0.4
UO126	–	1.0 $\pm$ 0.1
UO126	+	3.5 $\pm$ 0.3
Thapsigargin	–	0.6 $\pm$ 0.1
Thapsigargin	+	2.3 $\pm$ 0.4*

**P* < 0.05 vs respective no TLQP-62.

TLQP-21 ligate different receptors. This finding is in line with the recently identified mechanism of C3aR1 activation by TLQP-21 (Cero *et al.* 2014).

TLQP-62 increases intracellular calcium mobilization in INS1E cells

To further dissect the mechanism of action of TLQP-62-mediated insulin release, we analyzed calcium influx ($[Ca^{2+}]_i$) with a FURA-2 fluorescence assay. We found that TLQP-62 increased intracellular calcium to a degree that was comparable with that of the TPA stimulation and greater than that produced by Frsk or KCl (Fig. 5A).

As for insulin secretion, the dose-dependent activity of TLQP-62 on calcium mobilization produced a low, but detectable, calcium increase at 1 μ M, which reached a plateau at 5–10 μ M (Supplementary Figure 8). The recovery of physiological calcium concentrations was achieved a few minutes after stimulus (Supplementary Video 1). Calcium influx upon exposure to TLQP-62 was detected also in β TC6 and MIN6B1 cell lines (Supplementary Figure 9).

Cells that had been depleted of glucose for 60 min before exposure to TLQP-62 had reduced calcium mobilization (Fig. 5B). When kept in high glucose (Fig. 5C), the basal intracellular calcium concentration was increased (compare the basal levels of the no-glucose condition in Fig. 5B with that in the high-glucose condition in Fig. 5C) and stimulation with TLQP-62 produced a normal response. However, calcium remained elevated after the stimulation (with a ratio higher than 1.5), which indicated a difficulty in extruding calcium from the cytosol.

Of note, Frsk produced a fast calcium influx that remained sustained (Fig. 5D) but had fluctuations in the

Figure 5

Calcium influx in INS1E cells. Comparison of cytoplasmic calcium levels measured with Fura2AM. (A) Signal after stimulation with 10 μ M TLQP-62, 100 nM tetra phorbol myristate (TPA), 10 μ M forskolin (Frsk), or 56 mM KCl, in 4.5 mM glucose HBSS. (B) Glucose responsiveness. Cells were glucose-starved for 60 min before stimulation with 20 mM glucose or 10 μ M TLQP-62. (C) Effect of 10 μ M TLQP-62 on cells kept in 20 mM glucose. (D) Pretreatment with 10 μ M Frsk desensitized the cells to TLQP-62-induced calcium influx but reinforced the effect of 56 mM KCl. (E) In contrast, pretreatment with 100 nM TPA had no effect on TLQP-62-induced calcium influx. The panels are of representative experiments and give the means $\pm$ s.e.m. of 35–45 individual single-cell recordings.

single-cell traces (Supplementary Figure 10). TLQP-62's effect was strongly negatively affected by Frsk-induced PKA activation, which suggests down-regulation of its receptor (Fig. 5D). Conversely, stimulation of the PKC pathway with TPA did not affect TLQP-62-induced stimulation of calcium influx (Fig. 5E). However, both pathways seemed to stimulate the effect of high potassium on calcium influx: under the normal condition, the peak was around a 1.5:2 ratio (Fig. 5A), whereas the peak increased to 3.0 in the presence of Frsk or TPA (Fig. 5D and E).

Multiple applications of TLQP-62 did not decrease calcium stimulation, which suggests that the receptor did not become down-regulated or that there was a very fast recovery (Fig. 6A). Depletion of intracellular calcium by thapsigargin strongly decreased the peak calcium signal upon stimulation with TLQP-62 (Fig. 6B); this also occurred with KCl, which opens voltage-dependent membrane channels and then activates ER calcium flux. Indeed, in the absence of a physiological extracellular calcium concentration (Fig. 6D), the activity of TLQP-62 was strongly decreased. Surprisingly, the absence of extracellular calcium weakly affected KCl-induced calcium influx (Fig. 6C and D). We hypothesize that sustained, self-reinforcing calcium-induced calcium release (Lemmens *et al.* 2001) is involved in TLQP-62- and KCl-induced insulin secretion.

Together, these findings clearly demonstrate that in INS1E cells, TLQP-62 induces calcium influx via the opening of membrane calcium channels and by releasing it from internal storage.

The trophic and proliferative effects of TLQP-62 on insulinoma cell lines

INS1 cells were exposed for 24 h to different agents and assessed for mitochondrial activity with the MTT assay and DNA replication by assessment of BrdU incorporation.

Figure 6

Intracellular storage and extracellular calcium influx in INS1E cells. (A) Multiple stimulations with 10 μ M TLQP-62 did not decrease calcium influx. (B) Pretreatment with 500 μ M thapsigargin (Tpsg) depleted intracellular calcium storage uptake, which reduced the response to 10 μ M TLQP-62 and then 56 mM KCl 20 min later. (C) The effects of TLQP-62 and KCl in the presence of a physiological concentration of extracellular calcium in the HBSS medium. (D) The effects of TLQP-62 and KCl in the absence of extracellular calcium. The panels are of representative experiments and give the means $\pm$ s.e.m. of 35–45 single-cell recordings.

TLQP-62 stimulated a small, but not statistically significant, increase of the signal in both assays (Supplementary Figure 11). Experiments performed on INS1E, β TC6, and MIN61B cells produced similar results, as did a 48 h exposure (data not shown), which indicates that TLQP-62 peptides do not significantly affect insulinoma cell viability.

TLQP-62 improves glucose tolerance *in vivo*

Finally, after having established the cell-autonomous effect of TLQP-62 as a novel insulin secretagogue *in vitro*, we aimed to translate the present findings *in vivo* by testing the hypothesis that TLQP-62 modulates glucose homeostasis in mice. The experiment was conducted in lean non-obese and non-diabetic WT mice.

To this end, mice were fasted overnight and glucose tolerance was investigated in the presence and absence of different doses of TLQP-62. We found that acute injection of TLQP-62 (5 mg/kg) significantly improved glucose tolerance in the glucose tolerance test (Fig. 7).

Discussion

Impaired insulin secretion and insulin resistance play major roles in the development of T2D. The presence of VGF peptides has been detected in β cells as well as in other cell types of the islets of Langerhans (Possenti *et al.* 1999, Cocco *et al.* 2007, Stephens *et al.* 2012), which most likely suggests a paracrine or autocrine mechanism of action. These peptides are also produced from the neurons of the autonomic nervous system and pancreatic intra-mural ganglia (Cocco *et al.* 2007).

Figure 7

Glucose tolerance test *in vivo*. (A) Male CD1 mice weighing ~30 g each were injected with a single dose of saline ($n = 12$), 1 mg/kg TLQP-62 ($n = 8$), or 5 mg/kg TLQP-62 ($n = 11$). Acute injection of TLQP-62 significantly improved glucose tolerance in mice only at the higher dose. (B) Area under the curve of glucose concentration during the GTT. * $P < 0.05$ and ** $P < 0.01$.

Figure 8

Proposed model of TLQP-62's mechanism of action. Released TLQP-62 ligates a yet-to-be-identified receptor (1), activating signal transduction cascades by first opening calcium channels on the membrane (2) and stimulating endoplasmic reticulum release (3), and then secondarily modulating the AMPK, PKC, and ERK pathways (4). The resulting increase in intracellular Ca^{2+} concentration is permissive for the secretion of insulin (5). TLQP-62 is released along with insulin and exerts a paracrine effect (6). TLQP-62 also has an effect on gene transcription (7).

The secretory granules of β cells contain a diverse cargo of peptides and other biologically active molecules. Thus, stimuli that cause the secretion of insulin also cause the release of these molecules, which may interfere positively or negatively in an autocrine manner (Suckale & Solimena 2010). Because VGF-derived C-terminal peptides have been found to be preferentially produced by β cells (Possenti *et al.* 1999), we tested their capacity to stimulate insulin secretion. Among the C-terminal VGF-derived peptides analyzed, we found that the TLQP-62 fragment caused strong induction of insulin secretion *in vitro* in the presence of low glucose. This stimulatory effect was dose dependent, and it potentiated GSIS. TLQP-62 also quickly increased intracellular calcium mobilization, an event dependent on calcium influx from the extracellular compartment as well as from intracellular storage (Lemmens *et al.* 2001). The action of TLQP-62 required the presence of a physiological glucose level (4.5 mM), because calcium influx was decreased in glucose-free medium. Among the other analyzed VGF-peptides, TLQP-21 – as reported previously (Stephens *et al.* 2012) – did not strongly increase insulin release under the low-glucose condition but did produce a positive effect on GSIS in the INS1E cell line. Regarding the other C-terminal-derived peptides, AQEE-30 and LQEQ-19 but not YIEH-10 still maintained an ability to stimulate

insulin secretion, but they were less potent than the full-length C-terminal TLQP-62 peptide.

The receptor(s) for these peptides has(have) not yet been identified. Recently, TLQP-21 has been reported to bind to the C3a receptor (Hannedouche *et al.* 2013, Cero *et al.* 2014). C3aR1 can be activated by C3a and TLQP-21 with an intact C-terminal Ala-Arg moiety, which is absent in TLQP-62. Moreover, the C3aR1 antagonist SB290157 did not inhibit the pro-secretory effect of TLQP-62. Accordingly, we hypothesize that TLQP-62 activates a different receptor, but we do not exclude that AQEE-30 and LQEQ-19, which still retain some biological activity, could also be active on that receptor. It seems that the most C-terminal portion of the peptide is responsible for receptor binding, but more structural and pharmacological studies are required to determine this conclusively.

Intracellular signaling activated by TLQP-62 was fast (within 2–5 min) and produced reversible dephosphorylation of AMPK followed by phosphorylation of ERK and PKC. The involvement of AMPK in insulin secretion has been intensively discussed, but discrepancy among different papers exists. The present finding is similar to that of Fu *et al.* (2013): a fast down-regulation of p-AMPK mimics the effect of high intracellular ATP by inducing the cell to secrete insulin and by closing the potassium channels. We did not see activation of PKA-substrate phosphorylation by TLQP-62. Of note, Frsk – which activates the PKA pathway – blunted the fast effect of TLQP-62 on calcium. Conversely, TPA – which activates and then down-regulates PKC – did not inhibit TLQP-62-induced calcium mobilization.

We also assessed gene expression induced by high glucose, exposure to TLQP-62, and PKA activation (via Frsk). Slight increases in *Ins1* and *Vgf* – but not *Ins2* – genes were produced by high glucose and were statistically significant only early on (at 2 h). TLQP-62 was also able to stimulate *Ins1* and *Vgf* at this early time point, with weak *Ins1* up-regulation also occurring after 18 h of exposure. In contrast, Frsk induced a strong up-regulation of *Vgf* overnight. There was no synergistic effect with high glucose at any time point, which suggests a probable glucose transitory effect resulting from the replacement of intragranular protein storage after the secretory stimulus. This hypothesized exchange of secreted proteins is supported by the increase in VGF-protein precursor that occurs early after exposure to high glucose. Because cyclohexamide blocked accumulation of VGF-protein precursor, we assume that this effect is regulated at the transcriptional level.

It has been previously reported that TLQP-21 causes a protective and trophic effect on islet β cells (Stephens *et al.*

2012). In the present cell culture conditions, we detected a slight increase in cell trophism and proliferation after a 24 h stimulation with TLQP-62, but these data were not statistically significant.

After finding that TLQP-62 is a potent insulin secretagogue *in vitro*, we tested its effect on glucose tolerance *in vivo*. We found that administration of TLQP-62 improved glucose tolerance in mice, which suggests that the peptide exerts an insulin secretagogue effect *in vivo* as well. The significant improvement of glucose tolerance was particularly evident at the 30 min time point, which is suggestive of enhanced insulin secretion. Insulin levels were not directly determined in the present experiment; therefore, further studies are needed to support this conclusion.

Therefore, we propose a model in which TLQP-62 induces a fast increase in intracellular calcium mobilization via its release from intracellular storage and membrane calcium channel opening, and these two effects are self-reinforcing (Fig. 8). A secondary event involves the activation of kinases such as PKA that could regulate gene transcription and be responsible for down-regulation of the TLQP-62 signal. Moreover, the secretion of VGF-derived peptides by β cells could have a self-reinforcing paracrine effect on insulin secretion. We conclude that TLQP-62 is a new insulin secretagogue that improves glucose tolerance and could thus be considered a new target for drug discovery programs aimed at treating conditions characterized by impaired insulin secretion, such as T1D and the late phase of T2D.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-14-0313>.

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.

Funding

The work was supported in part by a Minnesota Partnership for Biotechnology and Medical Genomics' Decade of Discovery in Diabetes grant (to A B) and by MIUR (to R P).

Acknowledgements

We thank Dr P Halban for productive discussion and for providing the MIN6B1 cell line. We also thank Pierre Maechler for providing us with the INS1E cell line.

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Received in final form 20 March 2015

Accepted 9 April 2015