NEUROPEPTIDE TLQP-21, A VGF INTERNAL FRAGMENT, MODULATES HORMONAL GENE EXPRESSION AND SECRETION IN GH3 CELL LINE

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VGF • Chromogranin/secretogranin • Neuropeptides Hormone secretion • Prolactin • Signaling

Abstract
In the present study we demonstrated that TLQP-21, a biologically active peptide derived from the processing of the larger pro-VGF granin, plays a role in mammotrophic cell differentiation. We used an established in vitro model, the GH3 cell line, which upon treatment with epidermal growth factor develops a mammotrophic phenotype consisting of induction of prolactin expression and secretion, and inhibition of growth hormone. Here we determined for the first time that during mammotrophic differentiation, epidermal growth factor also induces Vgf gene expression and increases VGF protein precursor processing and peptide secretion. After this initia l observation we set out to determine the specific role of the VGF encoded TLQP-21 peptide on this model. TLQP-21 induced a trophic effect on GH3 cells and increased prolactin expression and its own gene transcription without affecting growth hormone expression. TLQP-21 was also able to induce a significant rise of cytoplasmic calcium, as measured by Fura2AM, due to the release from a thapsigar-gin-sensitive store. TLQP-21-dependent rise in cytoplasmic calcium was, at least in part, dependent on the activation of phospholipasefollowed by phosphorylation of PKC and ERK. Taken together, the present results demonstrate that TLQP-21 contributes to

differentiation of the GH3 cell line toward a mammotrophic phenotype and suggest that it may exert a neuroendocrine role in vivo on lactotroph cells in the pituitary gland.

Introduction

The Vgf gene encodes for a 617- or 615-VGF amino acid precursor protein (in rat/mouse and in man, respectively, with over 85% identity) [1], with a tissue-specific pattern of expression limited to neurons in the central and peripheral nervous systems and to specific populations of endocrine cells [2]. The precursor protein in the rat is processed by the neuroendocrine-specific prohor-mone convertases PC1/3 and PC2, producing a number of peptides, which are stored in dense core granules and secreted through the regulated pathway [3-5]. Due to structural and biochemical properties similar to other granular proteins, VGF recently was included in the extended granin family, which also includes chromogra-nins and secretogranins [6]. In rodents, the inducible VGF protein and/or its peptide-processed products have been implicated in the neuroendocrine regulation of reproduction and in metabolic homeostasis.

An increase of Vgf expression was demonstrated in the hypothalamus during lactation [7]. In the female rat anterior pituitary, VGF immunoreactivity was apparently restricted to a gonadotroph and lactotroph cell population, and it was shown that, through the estrous cycle phases, VGF peptide/s degranulation occurs at estrous pick in association with an increase in Vgf mRNA [8]. In female sheep, different VGF peptide immunoreactivity varies in pituitary cells also through the different reproductive seasons [9].

Biochemical detection and biological activity of intermediate and small-molecular-weight products from VGF processing have been demonstrated in vitro and in vivo [5, 10, 11].

One of the best characterized VGF-encoded peptides is TLQP-21, which spans from residue 556 to residue 576 of the precursor sequence [12]. TLQP-21 exerts a number of biological functions including: chronic i.c.v. infusion increases energy expenditure and prevents the early phase of diet-induced obesity [12], peripheral infusion induces prolipolytic effect [13], it regulates food intake and body weight in Syrian hamsters but not in mice [14, 15], it regulates gastric functions in vitro and in vivo [16-18], and data report the modulation of this peptide by feeding in gastric endocrine cells [19]. TLQP-21 did not show any activity on GH expression and secretion in porcine pituitary slices in vitro [20] and in vivo after chronic TLQP-21 i.v. injection in mice [21].

Importantly, a recent study has provided the first evidence of a direct effect of TLQP-21 peptide on the reproductive function demonstrating, in pubertal male rats, that central administration of TLQP-21

induced acute gonadotropin responses and that TLQP-21 stimulated LH secretion directly at the pituitary level [22].

In view of localization and striking modulation of Vgf mRNA and VGF peptides in the hypothalamus and pituitary cells [2], and the biological activity in the pituitary gland [22], the aim of the present study was to investigate the role exerted by TLQP-21 in GH3 cells, a rat tumor cell line that presents somatotroph and lactotroph pheno-types and can synthesize and release growth hormone (GH) and prolactin hormone [23], but also expresses VGF
[5]. These cells in the presence of epidermal growth factor (EGF) and estrogens differentiate toward the mammo-trophic phenotype inhibiting GH expression and stimulating prolactin expression and secretion [24, 25].

In particular, the purpose of this study was to test the hypothesis that (1) Vgf expression is modulated during EGF-induced cell differentiation and (2) TLQP-21 treatment may affect prolactin or GH expression and secretion.

Materials and Methods

Cells and Materials

GH3 rat pituitary tumor cell lines were obtained from ATCC (Rockville, Md., USA). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Invitrogen, N.Y., USA) supplemented with 100 U/ml penicillin, 100 jxg/ml streptomycin, L-glutamine, and 10% fetal bovine serum (FBS, Gibco). GH3 were cultured at 37° C in a humidified incubator containing 95% air and 5% CO_2 and seeded into 35-mm plates at a density of 10 ! 10^5 cells/well in DMEM medium supplemented with 10% FBS. After overnight incubation in complete medium, the cells were shifted to DMEM containing 2% FBS and then treated with EGF (10 ng/ml), TLQP-21 (10 JJLM), or vehicle, and maintained at 37°C for different times to induce differentiation.

Rabbit polyclonal antibodies (anti-phospho-kinases) were from Cell Signaling Technology (Beverly, Mass., USA).

TLQP-21 (TLQPPASSRRRHFHHALPPAR) and the scrambled control peptide Scr-21 (LRPSHTRPAHQSFARPLHRPA) were synthesized by PRIMM (Milan, Italy).

Antibodies for prolactin were kindly provided by the American Endocrinology Association. Polyclonal antibodies against GH were purchased from Chemicon, which is now Millipore (Bil-lerica, Mass., USA). Rabbit polyclonal antibodies raised against the VGF C-terminal fragment were obtained from our laboratory and largely characterized [3, 5]. The other reagents, when not specified, were purchased from Sigma (St. Louis, Mo., USA).

Cell Viability Assay

Cells were plated into poly-L-lysine-treated 96 -well plates (5 ! 10^4 per well) and cultured overnight in complete medium. This was then changed to DMEM containing test substances applied in 8 wells each. Cells were then cultured for different periods (up to 6 days) with a medium change (including test substances) every 2 days. Metabolic activity of GH3 cells was determined by a col-orimetric methyl-thiazolyl-tetrazolium (MTT) cell proliferation assay. This assay, although defined as a proliferative assay, measures cell trophism or viability of cells since it measures mito-chondrial activity (we then used the terms viability or trophic effect interchangeably).

Briefly, 20 JJLI of MTT (3-/4,5-dimethylthiazol-2-yl/-2,5,di-phenyltetrazolium bromide, Sigma) solution (5 mg/ml MTT in PBS) were added to each well containing GH3 cells treated with EGF (10 ng/ml), TLQP-21 (10 JJLM), or vehicle for different times. After 1 h incubation at 37° C, the MTT solution was removed, the precipitates were dissolved in 150 jxl of dimethyl sulphoxide (DMSO), and their absorption at 570 nm (A_{570nm}) was measured using a multiwell plate spectrophotometer. The experiments wereperformed three times. The $A_{570\ nm}$ value of control was used as 100% standard and all individual measurements were compared to this standard as percentage value.

For the assessment of cellular morphology, cells were cultured in differentiating medium, DMEM supplemented with 2% FCS. TLQP-21 (10 (XM) or EGF (10 ng/ml) were given for 6 days with medium change every other day and then photographed in a phase-contrast microscope for visual analysis of cell morphology.

Western Blot Analysis

Different experimental procedures were used for the preparation of the samples as described below.

GH, Prolactin, and VGF Cellular Expression. Cells were plated (2 ! 10^5) into 35-mm poly-L-lysine-treated culture dishes and cultured overnight in complete medium. The medium was then changed to DMEM containing 2% FCS and tested substances. After the treatment period and replacing medium with a tested substance every other day, the medium was aspirated and the cells washed once with ice-cold PBS and then solubilized in lysis buffer [50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 1% NP40] containing protease inhibitor mixture (Sigma). After removing nuclei with short spin, the supernatants were collected for Western blots according to NuPAGE (Invitrogen, now Life Technologies, USA) protocols. The gels were loaded with the same amount of proteins analyzed by BCA Protein Assay Reagent (Pierce). Four different sets of experiments were analyzed in duplicate.

GH, Prolactin, and VGF Secretion. Cells were plated (10 ! 10^5) into 35-mm poly-L-lysine-treated culture dishes and cultured overnight in complete medium. After 3 washes in Ringer's solution, cells were stimulated with 1 ml of Ringer's solution for 1 h. Media collected at the end of the incubation periods were centri-fuged (1,200 rpm, 5 min), the supernatants precipitated with tri-chloroacetic acid (15%), and proteins assayed for GH, prolactin and VGF peptide secretion by Western blot analysis. The experiments were performed after EGF differentiated GH3 cells for 6 days (day 6) in order to increase VGF peptides expression. Three different sets of experiments in duplicate were used for the semi-quantitative analysis, not evaluating other blots in which

tubulin detection indicated cell lysis. Additionally, 3-hour and the 24-hour incubation times were not included in the analysis, which indeed showed cell damage by tubulin detection. ECL exposures were analyzed for semiquantitative data by Image J Scan Analysis software.

Phosphorylation of Cellular Kinases. Cells were plated (10 ! 10^5) into 35-mm poly-L-lysine-treated culture dishes and cultured overnight in complete medium. To assess kinase activation, cells were serum-starved incubated in DMEM for 1 h and the substances to be tested added at 37° directly into the plates without removing the plates from the incubator [26]. At the end of the incubation period, the reaction was quickly stopped, the cell culture dishes were placed on ice, the medium was removed, and cold lysis buffer was added (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, containing phosphatase and protease inhibitor mixture). For every experimental procedure, equivalent amounts of cell extracts (about one quarter of the cell extract obtained from each culture plate, equivalent to 15-20 (xg, approximately 2.5 ! 10^5 cells) were mixed with SDS-reducing sample buffer according to NuPAGE protocols. Following heating at 70°C for 10 min, proteins were subjected to SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to a poly vinylidene difluoride membrane (Amersham). After staining with Ponceau S to verify uniformity of protein load/transfer, the membranes were analyzed for immunoreactivity. Incubation with primary antibodies was performed overnight at 4°C (rabbit anti-p-Erk1/2, p-Akt, p-PKC, p-PLCp, p-PLA, p-AMPK, p-P38, p-JNK 1:1,000, and anti-hormones GH 1:5,000, PrL 1:5,000 and C-terminal VGF, 1:5,000).

Incubation with peroxidase-coupled secondary antibodies (Amersham, Arlington Heights, Ill., USA; now GE; 1:5,000) was performed for 1 h at room temperature. Immunoreactivity was developed by enhanced chemiluminescence (ECL system; Amersham; now GE). To normalize the loaded sample, blots were stripped (Restore Western Blot Stripping; Pierce, USA) and re-probed with an antibody against a-tubulin (Sigma, 1/10,000, overnight at 4°C). For analysis of the Western blotting data, den-sitometry analysis was performed using Scan Analysis software.

Three to five different performed experiments gave similar results. Figure 3 shows a representative Western blot analysis of a single time course experiment, probed for the different phospho-proteins.

Immunofluorescence

GH3 cells were fixed with 4% (w/v in PBS) paraformaldehyde for 15 min at room temperature. Fixed cells were permeabilized with 0.2% Triton X-100-Tris-HCl, pH 7.4, for 5 min, then incubated with the rabbit

polyclonal antibody against the C-terminal sequence of VGF (1:2,000) at room temperature for 2 h in a humid chamber. TRITC-conjugated secondary antibodies (Sigma, 1:2,000) were incubated for 30 min at room temperature.

Confocal microscopy was performed with a Leica TCS 4D system (Nussloch, Germany), equipped with 100! 1.3-0.6 oil immersion objective.

Cellular Ca^{2+} Assay

Optical fluorometric recordings with Fura2AM were used to evaluate the intracellular calcium concentration ([Ca^{2+}]i). Fura2AM stock solutions were obtained by adding 50 jxg of Fura2AM to 50 jxl of 75% DMSO plus 25% pluronic acid. Cells were bathed for 60 min at room temperature in 1 ml of extracellular solution (125 mM NaCl, 1 mM KCl, 5 mM $CaCl_2$, 1 mM $MgCl_2$, 8 glucose, and 20 HEPES, pH 7.35) with a final Fura2AM concentration of 5 (xM. This solution was then removed and replaced with extracellular solution, and the dishes were quickly placed on the microscope stage. The change of intracellular fluorescence intensity, meaning cytoplasm-free calcium level ([Ca^{2+}]i) of GH3 cells after treatment with EGF or TLQP-21 was observed by fluorescence microscopy. To measure fluorescence changes, a Hamamatsu Argus 50 computerized analysis system (Shizouka, Japan) was used, recording the ratio between the values of light intensity at 340 and 380 nm stimulation every 6 s [27]. Data are presented as the peak Ca^{2+} rise 8 SE. Quantitative data were obtained analyzing 10 values/min at the peak of induction, averaging 10-20 cells for each experiment (3 independent experiments).

RNA Preparation, Reverse Transcription, and Real-Time

Quantitative PCR Procedure

Total RNA from untreated or treated cells was extracted using TRIzol (Invitrogen, Carlsbad, Calif., USA), according to the manufacturer's instructions. To obtain cDNA, 2 (xg of total RNA was reverse transcribed in reverse transcription buffer using an oligo-dT primer (Promega, Madison, Wisc., USA) and the First Strand

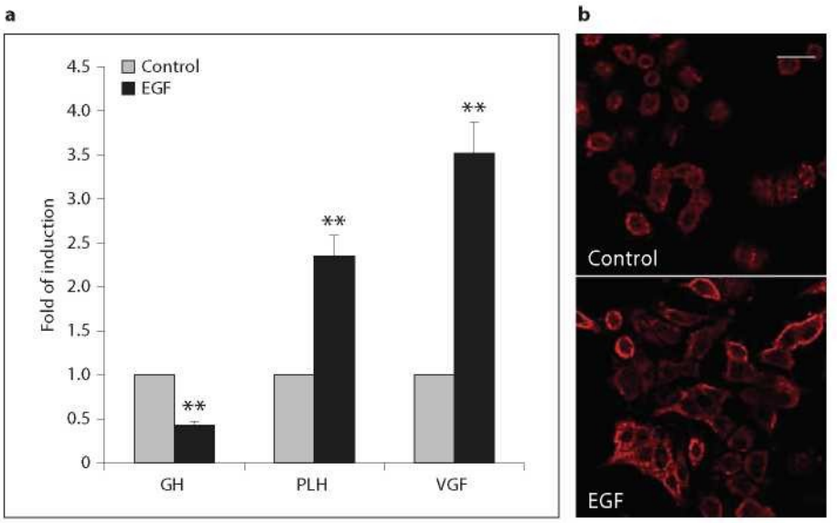


Fig. 1. Effect of EGF on VGF expression in GH3 cells. a Real-time PCR analysis. After 6 days of EGF exposure, GH, prolactin hormone (PLH), and VGF mRNA levels were measured with quantitative PCR. Data were calculated as fold of induction over control (arbitrary value of 1). Data represent mean (8 SEM) from five independent experiments run in duplicate (** p ! 0.01). b Immunofluorescence analysis, using an antibody raised against the VGF C-termi-nal region, shows the increase after EGF treatment of immunostaining for VGF, which is stored in secretory granules as indicated by punctuated staining. Change of GH3 morphology after EGF treatment is evident as large flat cells. Three separate experiments gave qualitatively similar results. Scale bar 30 jxM.

cDNA obtained with Synthesis Kit (Invitrogen). The preparation was supplemented with 0.01 M dithiothreitol and 1 mM each of dNTP and 200 units of human placenta ribonuclease inhibitor (Code No. 2310, Takara, Tokyo, Japan) in a final volume of 25 (xl. The reaction was incubated at 37°C for 60 min. Messenger RNA expression was quantitatively measured with real-time quantitative PCR (ABI Prism 7700 Sequence Detector; Perkin Elmer Applied Biosystems, Foster City, Calif., USA) utilizing Brilliant SYBR Green QPCR Master Mix (Qiagen).

The PCR primers were designed based on the published sequences using a NCBI-oligo research program: VGF (GenBank NM030997.1) forward 5'-AGACGGGTCCGGATTTTC-3' and reverse 5'-CAGAAGAGGACGGATGCTG-3'; GH (GenBank NC005109.2) forward 5'-TGTTTGCCAATGCTGTGC-3' and reverse 5'-TGTCCTCGGGAATGTAGG-3'; and prolactin (GenBank NM012629.1) forward 5'-CCATGAACAGCCAAGTGT-CA-3' and reverse 5'-TGGCAGAACAGAAGGTTTGA-3'

The internal reference TATA-binding protein primer was purchased from Invitrogen. Real-time PCR amplification and product detection was performed using an ABI PRISM 7900 Fast Real-Time PCR (Applied Biosystems, Foster City, Calif., USA). Each assay included a standard curve sample in duplicate, a no template control, and the cDNA sample from the treated GH3 cells 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: 92°C for 2 min for de-naturation, followed by 40 cycles of 92°C for 30 s, 54°C for 30 s, 75°C for 30 s, and

extension at 75°C for 5 min. Postamplification dissociation curves were performed to verify the presence of a single amplification product and the absence of genomic DNA contamination.

Statistical Analysis

Statistically significant differences were calculated by one-way analysis of variance (ANOVA) for repeated measures followed by Bonferroni's test, analyzing data from treated cultures and the corresponding control; * p ! 0.05; ** p ! 0.01.

Results

VGF Is Induced during GH3 Differentiation GH3 cells constitutively synthesize both GH and prolactin [23]. GH3 cells were treated with EGF (10 ng/ml) for 6 days. In accordance with previous data [24, 25], we demonstrated that EGF treatment decreased GH mRNA expression approximately threefold and increased prolactin mRNA expression more than twofold, as compared to unstimulated cells (fig. 1a). In addition, since in the female rat anterior pituitary lactotroph cells express high VGF immunoreactivity [8] and the Vgf gene increases in pituitary of lactating dams (unpubl. data), we tested the effect of EGF on the expression of VGF mRNA. As shown in figure 1a, real-time PCR determination showed that after 6 days of exposure, VGF mRNA expression was increased by EGF (about 3.5-fold) compared to nonstim-ulated cells, suggesting that both prolactin and VGF are stimulated during mammotrophic cell differentiation. Moreover, we studied the presence and distribution of VGF immunoreactivity by immunocytochemistry, using antibodies raised against the VGF C-terminal region [5]. As shown in figure 1b, EGF treatment in GH3 cells that constitutively express VGF caused an increase in immunoreactivity accompanied by significant morphological changes from small roundish cells to flat polyhedric form. The immunofluorescence appears like punctuated staining, excluding nuclear area, characteristic of secretory granules.

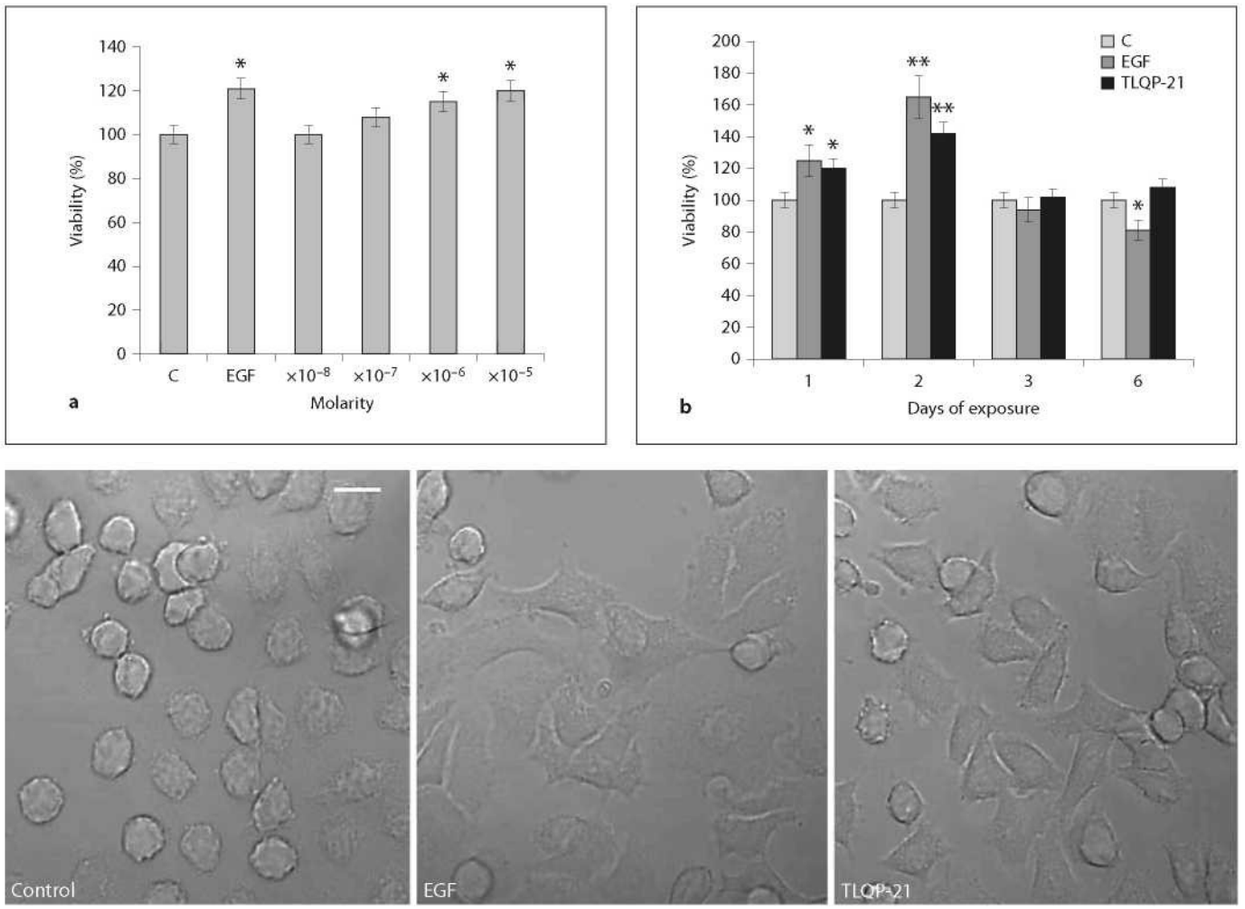


Fig. 2. Trophic and differentiative effect of TLQP-21 on GH3 cells. GH3 cell viability was determined by 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay after different times of incubation. a Cell viability obtained after 24 h of treatment with TLQP-21 at different concentrations and with EGF (10 ng/ml). Data represent the mean (8 SEM) from two independent experiments run 6 times. A significant effect was obtained at 1 and 10 (xM (* p ! 0.05). b Trophic activity of TLQP-21 (10 JXM) and EGF (10 ng/ml) upon varying treatment length. Data represent

the mean (8 SEM) from three independent experiments run 6 times (* p ! 0.05; ** p ! 0.01). It is worth noting that initially, on the first and second day, both EGF and TLQP-21 induced a trophic effect that was blunted during differentiation and EGF showed an inhibitory effect on day 6. c Representative phase-contrast photomicrographs of GH3 cells under control conditions treated for 6 days with EGF (10 ng/ml) or TLQP-21 (10 JJLM). Scale bar: 12 jxm. Cells treated with EGF and TLQP-21 appeared more present in the dish, with a polyhedric shape and more clustered.

TLQP-21 Trophic and Differentiative Effect on GH3

Cells

Having established that GH3 cells express and can modulate Vgf gene, we wanted to examine the role of TLQP-21 on this model.

We first examined the trophic activity of the VGF-de-rived peptide TLQP-21 (10 and 1 (xM, 100 and 10 nM) on cultured GH3 cells. As positive controls, we used 10 ng/ ml of EGF, which is known to induce, in absence or in low serum, proliferation and morphological changes in lacto-trope and GH3 cell line [24].

As shown in figure 2a, MTT analysis revealed that 24-hour incubation of GH3 with different concentrations of TLQP-21 significantly increased the cell viability at 10 and 1 (xM, inducing about a 20 and 15% increase, respectively, as compared to untreated cells. EGF (10 ng/ml), used as positive control, provoked an increase in cell viability of about 22%.

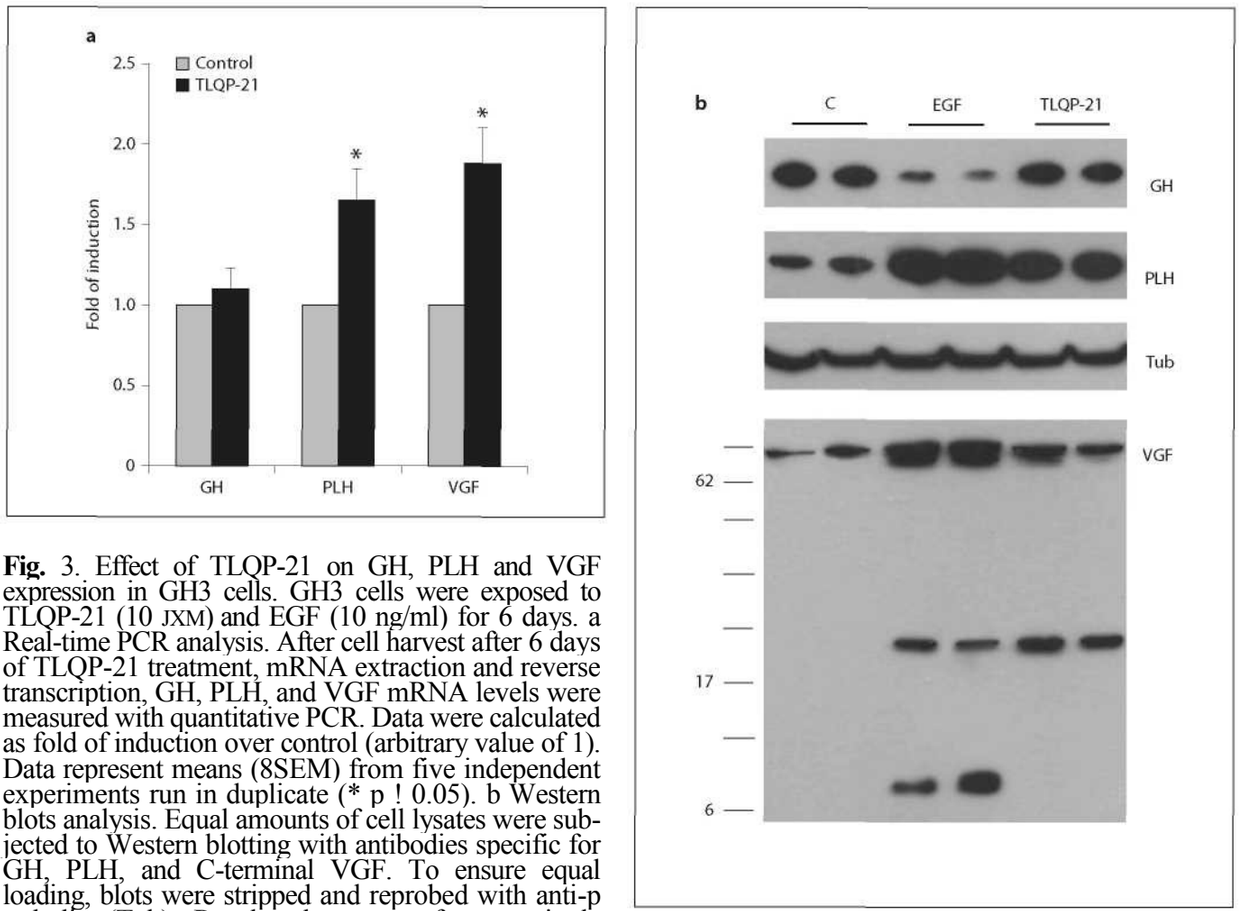


Fig. 3. Effect of TLQP-21 on GH, PLH and VGF expression in GH3 cells. GH3 cells were exposed to TLQP-21 (10 JXM) and EGF (10 ng/ml) for 6 days. a Real-time PCR analysis. After cell harvest after 6 days of TLQP-21 treatment, mRNA extraction and reverse transcription, GH, PLH, and VGF mRNA levels were measured with quantitative PCR. Data were calculated as fold of induction over control (arbitrary value of 1). Data represent means (8SEM) from five independent experiments run in duplicate (* p ! 0.05). b Western blots analysis. Equal amounts of cell lysates were subjected to Western blotting with antibodies specific for GH, PLH, and C-terminal VGF. To ensure equal loading, blots were stripped and reprobed with anti-p -tubulin (Tub). Results shown are from a single experiment and are representative of three separate experiments run in duplicate.

Based on these preliminary data, the TLQP-21 concentration of 10 (xM was used for the following experiment. For growth analyses, we then examined the prolif-erative activity of this peptide at different days of treatment (1, 2, 3, and 6 days). As shown in figure 2b, at day 1, 10 (xM TLQP-21 caused a significant increase of cell proliferation of about 20 8 2%, which is comparable to the effect of the EGF response (22 8 4%). Both TLQP-21 and EGF showed a maximal activity at day 2 (50% of increase for TLQP-21 and 70% for EGF), while no effect was observed by increasing days of exposure. No proliferative activity was observed using the scrambled TLQP-21 peptide (Scr-21; 10 (xM; data not shown).

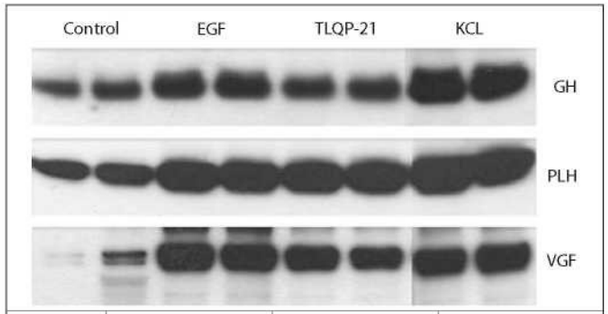
In GH3 cells seeded on polylysine-coated dishes in presence of a 2% serum concentration, EGF causes robust and time-dependent morphological modifications consisting of a change from a normally rounded morphology

to a much more flattened and elongated morphology with extensive processes, sometimes assembled in small clusters with polyhedric shape [24]. Six days of incubation with EGF caused the expected morphological change in GH3 cells, and comparable effects were observed with TLQP-21 incubation (fig. 2c).

GH, Prolactin, and VGF Expression in GH3 Cells

following TLQP-21 Stimulation

In order to test the effect of TLQP-21 on GH, prolactin, and VGF, GH3 cells were exposed to TLQP-21 (10 |xM) and EGF (10 ng/ml) for 6 days. TLQP-21 caused an approximate 1.5-fold induction of prolactin mRNA while leaving GH unaffected (fig. 3a). Vgf mRNA expression was also increased by TLQP-21 treatment (about twofold) compared to nonstimulated cells, suggesting that both prolactin and VGF are stimulated during mammotrophic cell



	GH	PLH	VGF
Control	1.26 ± 0.30	1.32 ± 0.41	1.07 ± 0.11
EGF	2.85 ± 0.15*	2.32 ± 0.22*	1.90 ± 0.63*
TLQP-21	2.46 ± 0.33*	2.45 ± 0.52*	1.64 ± 0.34*
KCl25	4.19 ± 1.12*	3.12 ± 0.64*	2.10 ± 0.25*

Fig. 4. Secretion of GH, PHL, and VGF after 1 h of stimulation. Relative hormone release from GH3 differentiated cells after 1 h of exposure to different secretagogues, average of 3 different experiments run in duplicate. The panel shows a representative Western blot and the table shows the semiquantitative analysis of five independent experiments.

differentiation. These data are consistent with data showing a high VGF immunoreactivity in female rat anterior pituitary lactotroph cells [8]. Gene expression data were confirmed at the protein level by performing a Western blot analysis using antibodies specific for GH, prolactin, and VGF (fig. 3b). As shown in figure 3b, EGF induced (as expected) a decrease of GH and an increase of prolactin hormones. Furthermore, EGF increased pro-VGF precursor form (70-kDa molecular mass) and the appearance of additional bands of approximately 20 and 6 kDa. On the other hand, TLQP-21 less extensively increased the constitutive VGF precursor form and induced the process only of the 20-kDa band. As previously reported, processing of VGF protein occurs in a cell-specific manner [3], depending on the presence of the different prohormone conver-tases [5, 28], reinforcing the assumption of specific functions of VGF-derived peptides in mammotrophic cells.

GH, Prolactin, and VGF Secretion in GH3 Cells following TLQP-21 Stimulation

GH, prolactin, and VGF-secreted peptides were measured by Western blot. EGF predifferentiated GH3-conditioned media were trichloroacetic acid precipitates. EGF (10 ng/ml), TLQP-21 (10 (xg/ml), and KCl (25 mM) were administrated for 1 h. As mentioned in Methods, after 3 and 24 h the conditioned media showed cell damage and these time points were not estimated. ECL expo-

sures were analyzed for semiquantitative data by ImageJ Scan Analysis software. Results were expressed as the ratio of stimulated secreted peptides over control. As shown in figure 4, after 1 h incubation time, GH, prolactin, and VGF secretion were stimulated by both EGF and TLQP-21: GH increased 2-3 times in EGF and TLQP-21 treatment, prolactin secretion was stimulated roughly twofold in EGF and TLQP-21 treatment, and VGF peptide secretion (70-kDa precursor form) was increased about twofold following EGF treatment or TLQP-21 addition. After KCl (25 mM) stimulation as control of regulated secretion, GH release increased about 4 times, prolactin secretion increased more than 3 times and VGF precursor 2 times. For VGF secretion, there was indeed a variation on constitutive and regulated secretion depending on the precursor form or the processed peptides (online suppl. fig. 1; for all online supplementary material, see www.karger.com/doi/10.1159/000339855). Moreover, these semiquantitative data obtained from Western blot scan analysis were confirmed by ELISA for GH secretion (GH kit ELISA, LINCO Research Inc., St. Charles, Mo., USA; not shown). Indeed GH medium content by ELISA after 1 h of treatment with EGF,TLQP-21, or KCl 25 mM confirmed the increase of secretion, although values were less severe (Control = 1; EGF = 1.24; TLQP-21 = 1.22; KCl = 1.68). The amount of GH secreted corresponds roughly to 2% of total intracellular contents.

Evaluation of Intracellular Signaling Molecule

Activations by TLQP-21

We measured the potential effect of TLQP-21 treatment on phosphorylation of signaling molecules, such as ERKs, PLA, PKC, AMPK, AKT, JNK, and p38 in GH3 cells. After 1 h of serum starvation in presence of DMEM, cells were exposed to TLQP-21 (10 (xM) for different times as indicated in figure 5.

TLQP-21 administration induced an early increase of PKC phosphorylation (2-10 min, 22%), followed by increases of ERK phosphorylation (5-30 min, 48%) and a decrease of AMPK phosphorylation (15-60 min, 28%; fig. 5 left part). Modulation of PLA signaling was occasionally observed; values were not statistically significant and had large variability

(5-60 min decreased phosphorylation, 10-15%). As a positive control, we used EGF (10 ng/ml). In accordance to published data [29], EGF administration transiently stimulated robust phosphorylation of ERK1/2 (5-15 min, 452%) and a slight increase in PKC phosphorylation (5-15 min, 24%; online suppl. fig. 2).

Phosphorylation of PLC7, AKT, JNK, or p38 was not affected by TLQP-21 administration (fig. 5 right part).

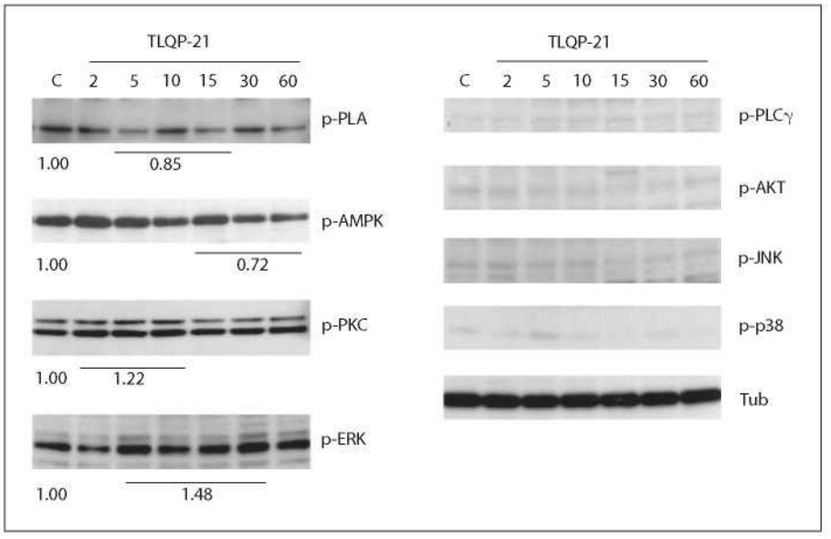


Fig. 5. Western blot analysis of signaling phospho-activated proteins in GH3 cells. Time course of TLQP-21 (10 JXM) treatment from 2 min up to 60 min. Equal amounts of cell lysates were processed for Western blot analysis, using specific antibodies for the phosphorylated (active) forms of signaling molecules. To ensure equal loading, blots were stripped and re-probed with anti-p-tubulin (Tub). Results shown are from a single experiment and are representative of three separate experiments. The blots on the left represent significant modulations of phospho-activated kinases, while the right part shows negative results.

TLQP-21 Increased Intracellular Calcium as Measured by Fura2AM

To elucidate the intracellular signaling activated by TLQP-21, we tested the ability of this peptide to increase intracellular calcium ([Ca^{2+}]i). Preliminary experiments were carried out to assess a concentration-response curve for TLQP-21. Figure 6a shows a representative trace of the [Ca^{2+}] i rise recorded from a single cell induced by increasing concentrations of TLQP-21 (3, 10 (xM) and by 10 (xM of the scrambled peptide (Scr-21). At the end of each experiment, cell reactivity was tested triggering the calcium entry by high potassium-dependent (KCl, 25 mM) membrane depolarization. As shown in the graph of figure 6a (average of 30 cells from 3 different experiments), 3 and 10 (xM TLQP-21 induced a marked rise in the Fura2AM ratio, from 0.75 8 0.05 to 0.89 8 0.03 and from 0.75 8 0.05 to 1.09 8 0.09 (value obtained calculating 1 min of exposure), respectively, while the Scr-21 was fully inactive. The effect of the peptide was compared to the increase in the Fura2AM ratio produced by application of KCl-25 (from 0.75 8 0.05 to 1.40 8 0.11). In contrast, as shown in figure 6b, EGF (up to 100 ng/ml) did not significantly modify [Ca^{2+}]i, although it was able to increase hormone expression.

To determine whether TLQP-21-induced [Ca^{2+}]i was influenced by Ca^{2+} store depletion, GH3 cells were prein-cubated with 500 ng/ml of the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin for 30 min [30]. Thapsigargin completely prevented TLQP-21 in induced rise of [Ca^{2+}]i, while it had only a slight effect on the rise

in [Ca^{2+}]i induced by KCl-25 mM (fig. 6c). These data allow us to conclude that TLQP-21 is able to induce calcium increase by opening the intracellular Ca^{2+} storage and not by activating membrane calcium channels.

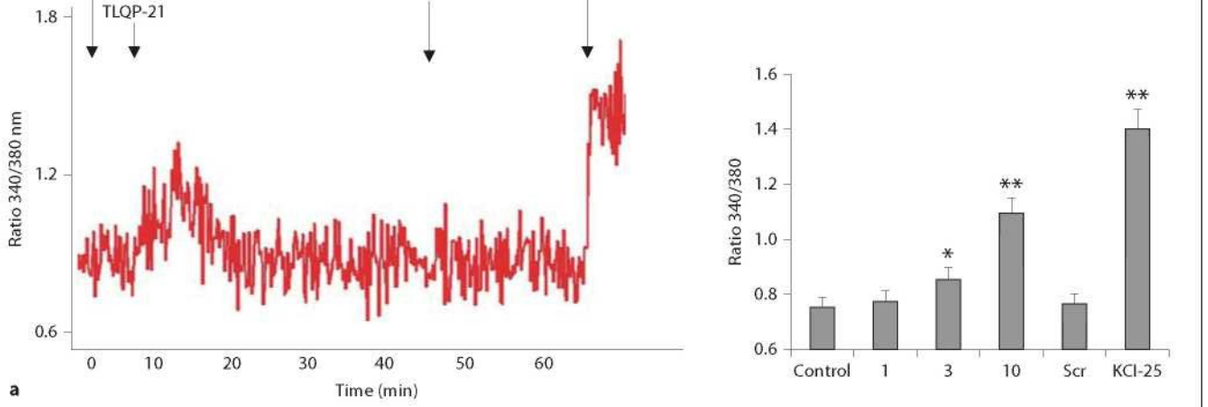
Intracellular Signaling Events Coupled to TLQP-21

Rise in [Ca^{2+}]i

We demonstrated that TLQP-21 raises [Ca^{2+}]i in GH3 cells and increases phosphorylation of PKC, ERK, and AMPK. Accordingly, we aimed to identify which of these signaling pathways was required for [Ca^{2+}]i effects of the peptide. GH3 cells were incubated with TLQP-21 after pretreatment with pharmacological inhibitors. The Fura2AM ratio was examined and compared to TLQP-21-stimulated control cells (fig. 7).

To test whether the ERK1/2 pathway was activated by TLQP-21, GH3 cells were pretreated (30 min) with the ERK inhibitors U0126 (10 (xM) or PD 98059 (50 (xM not shown) and the effects of TLQP-21 on the Fura2AM ratio were examined over the control (fig. 7a). Inhibition of ERK1/2 signaling did not block TLQP-21-induced rise of [Ca^{2+}]i, excluding the involvement of this pathway in the TLQP-21 mechanism of action on calcium influx. Although a light phosphorylation of ERK was indicated in Western blot, it can be explained as a secondary event.

Next, we examined whether TLQP-21 rise of [Ca^{2+}]i involved a PKC-dependent mechanism. For this study, GH3 cells were pretreated with PKC inhibitors, 12-O-tet-radecanoylphorbol-13-acetate (TPA) and Gö6976 (selective inhibitor of Ca^{2+}-dependent PKC isoforms). GH3


TLQP-21
Ratio 340/380 nm
1.8
1.2
0.6
0
10
20
30
40
50
60
Time (min)
a
Ratio 340/380
1.6
1.4
1.2
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0.8
0.6
*
**
**
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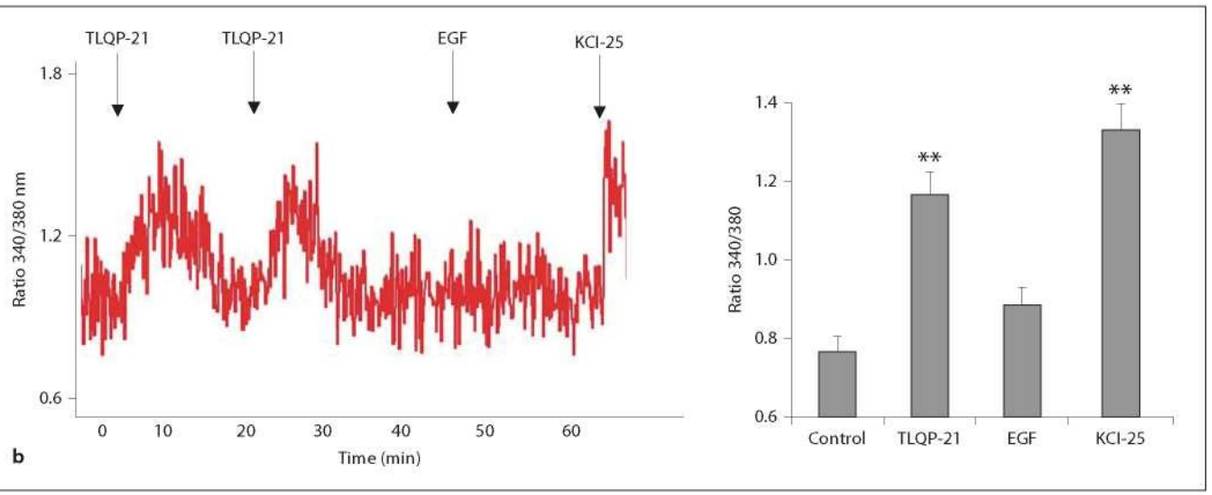
Control
1
3
10
Scr
KCl-25


TLQP-21
TLQP-21
EGF
KCl-25
Ratio 340/380 nm
1.8
1.2
0.6
0
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20
30
40
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60
Time (min)
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Ratio 340/380
1.4
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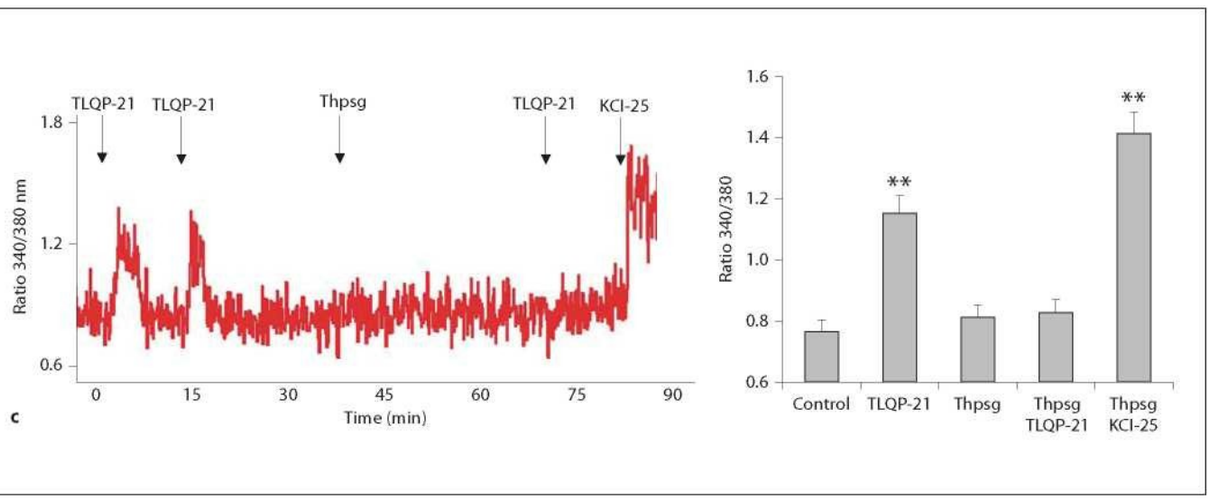
Control
TLQP-21
EGF
KCl-25


TLQP-21
TLQP-21
Thpsg
TLQP-21
KCl-25
Ratio 340/380 nm
1.8
1.2
0.6
0
15
30
45
60
75
90
Time (min)
c
Ratio 340/380
1.6
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Control
TLQP-21
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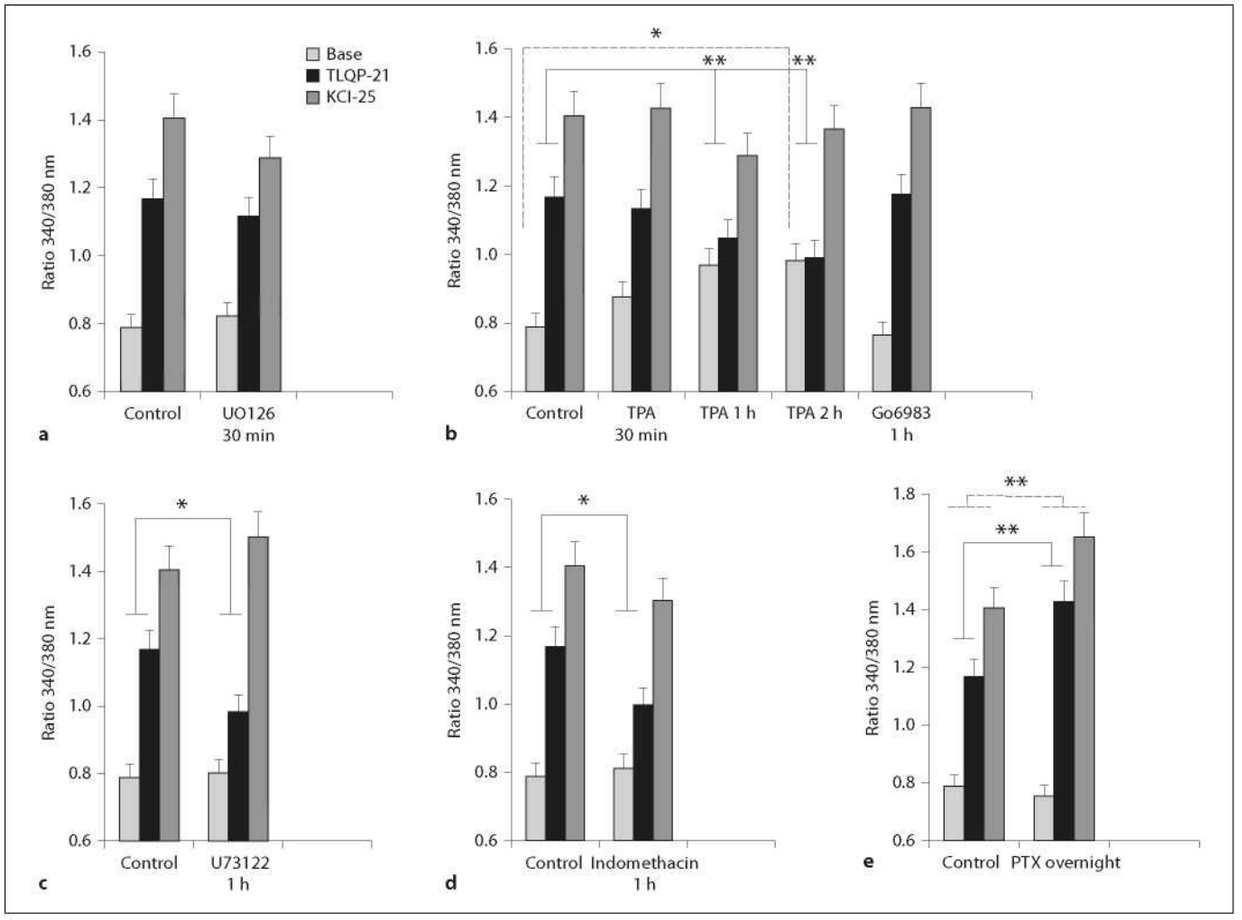
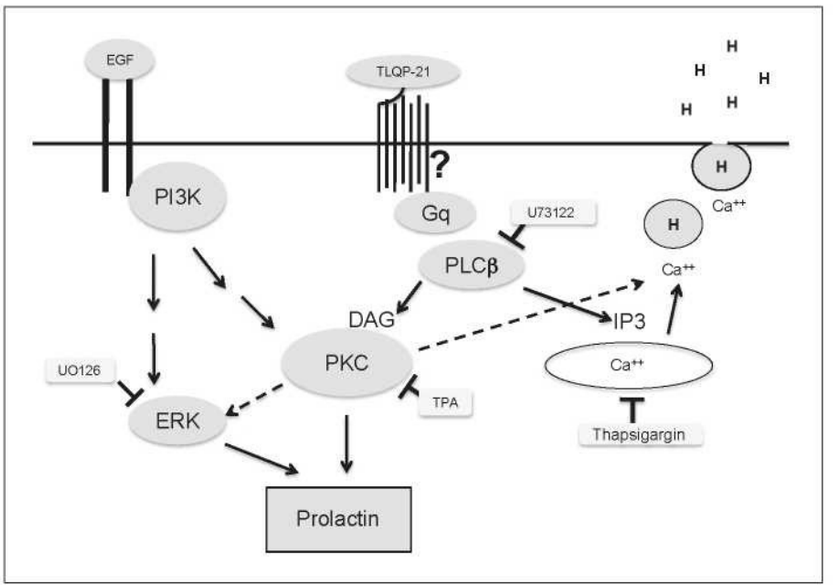


Fig. 6. Effect of TLQP-21 on $[Ca^{2+}]_i$ increase as measured by Fura2AM. GH3 cells were loaded with Fura2AM and stimulated for the indicated period of time. Fluorescence changes in $[Ca^{2+}]i$ were monitored by digital imaging microscopy. The scale indicates the values of ratio 340/380 nm over time expressed in minutes. a A representative trace showing the increase in cytosolic Ca^{2+} concentration in a single rat pituitary GH3 cell induced by TLQP-21, first 1 (xM and then 10 (xM, or the scrambled peptide (Scr) at 10 (xM. KCl 25 mM was used to confirm cell viability through activation of voltage-dependent Ca^{2+} channels. b A representative trace showing the increase in cytosolic Ca^{2+} concentration induced by subsequent TLQP-21 (10 JXM) or EGF (10 ng/ml) treatment and by KCl 25 mM. c A representative trace showing the increase in cytosolic Ca^{2+} concentration in a single cell induced by TLQP-21 (10 JXM) alone or after thapsigargin (500 ng/ml) pretreatment for 20 min. KCl 25 mM was used to prove that no damage has been induced to the cells, which still respond to opening membrane calcium channels. The corresponding panels indicate statistical analysis of the changes in $[Ca^{2+}]_i$ induced by different TLQP-21 concentrations, Scr (10 (xM), EGF (10 ng/ml), and KCl 25 mM. Row data were analyzed from the recovered files and 10 values for each cell (1 min) at the peak of secretion were considered (average of 40 cells from three different experiments; * p ! 0.05; ** p ! 0.01).

Fig. 7. Modulation of intracellular $[Ca^{2+}]_i$ by TLQP-21 andKCl25 mM in the presence of different drugs. GH3 cells were loaded with Fura2AMfor 1 hand exposed to different kinase inhibitors before testing TLQP-21 10 jxM and then KCl 25 mM. 10 values for each cell (1 min; average of 20 cells from two different experiments) at the peak of secretion were statistically evaluated (* p !0.05; ** p ! 0.01). a MEKK inhibitor UO126, which arrests ERK phos-phorylation, did not significantly affect TLQP-21 calcium stimulation, b TPA (1 JXM), a PKC transient activator and then a PKC downregulator after 30 min, 1 h, and 2 h exposure. Downregula-tion of PKC strongly affected intracellular calcium level increasing calcium level (dotted line) and annulling the TLQP-21 effect (solid line). Go6983 (10 (xM, 1 h exposure) is an inhibitor of calci-um-dependent-PKC species and it did not influence the TLQP-21 stimulation. c U73122 (10 (xM, 1 h exposure), a phospholipase inhibitor, partially influenced TLQP-21 stimulation. d Indomethacin (20 nM, 1 h exposure), a cyclooxygenase inhibitor, only partially and with cell variability inhibited TLQP-21 stimulation, e Pertussis toxin (PTX; 200 ng/ml, overnight treatment) inhibitor of Gi subunit of GPCR. The inhibition of Gi indeed increased response to TLQP-21 (solid line), but also increased the sensibility of the membrane K-dependent calcium channels (dotted line).

Fig. 8. Schematic representation of TLQP-21 intracellular signaling. Our data indicate that TLQP-21 stimulates GH3 cells through the binding of a most likely GPCR coupled to a Gq. It activates phospholi-pase, PI3 stimulates Ca^{+2} intracellular storage release, and DAG activates PKC. PKC stimulates, as a secondary event, ERK phosphorylation and it can eventually reinforce Ca^{+2} mobilization and then hormone secretion.



cells were pretreated with TPA (1 *xM)* for different times (30 min, 1 h, and 2 h). As shown in figure 7b, TPA time-dependently reduced the TLQP-21-mediated rise of $[Ca^{2+}]i$, eliciting about a 15% reduction after 30 min, 60% after 1 h, and 95% after 2 h preincubation time. Indeed, TPA alone induced a rise of Ca^{2+} from intracellular storage (about 22% after 2 h; fig. 7b dotted line), but did not modify the membrane voltage-dependent channels (KCl 25). Treatment with Gö6983 (10 (xM) for 1 h did not significantly affect the peptide activity (fig. 7b).

Treatment with U73122 phospholipase inhibitor (10 *xM)* for 1 h did affect the peptide activity, inducing a partial decrement of $[Ca^{2+}]i$ of about 42% compared to the control (fig. 7c).

In addition, since TLQP-21 regulated gastric functions via a prostaglandin-mediated mechanism [16, 17], we examined the effect of indomethacin pretreatment on TLQP-21 effect (fig. 7d). The TLQP-21-mediated rise of $[Ca^{2+}]i$ was partially abolished, about 38%, by indomethacin (20 nM) after 1 h pretreatment, resulting in some cells being more sensitive than others to indomethacin exposure.

Treatment with pertussis toxin 200 ng/ml for 16 h, which is able to inhibit the Gi subunit of GPCRs, did not affect calcium signaling by TLQP-21, eventually showing an increase of $[Ca^{2+}]i$ sensitivity for both TLQP-21 (fig. 7d, solid line) and potassium-dependent membrane calcium channel (fig. 7d, dotted line, KCl 25).

We confirmed the correct mechanism of action of these drugs on GH3 cells, analyzing, by Western blots, the phosphorylation of ERK, PKC, and AMPK induced by each drug after the specific time of exposure (online suppl. fig. 3).

Discussion

As previously reported, VGF appears to be widely expressed, but differentially processed in different brain areas and in pituitary cells [2, 3, 5]. The presence of VGF peptides in lactotroph cells has been clearly shown in different species [8, 9].

In the current study, we demonstrated that EGF in the GH3 cell line, used as model of lactotroph differentiation [25], besides a strong increase in prolactin content, is able to induce Vgf expression both as mRNA and VGF protein. The pro-VGF molecule appears to be cleaved, additionally suggesting an increase of prohormone conver-tases specific for VGF processing, most likely PC1/3 [5, 28].

We also established that the VGF-derived peptide, TLQP-21, exerts in GH3 cells a trophic and differentiative effect, as demonstrated by MTT and morphological cell analysis. TLQP-21 induces morphological changes of GH3 cells from predominantly small rounded cells to larger cells with a more flattened morphology and elongated processes when tested in reduced serum condition (2% FBS), showing similar effects to EGF used as a positive control [24].

EGF induces lactotrope differentiation in GH3 by causing decreased GH and increased prolactin expres-sion [24,25]. Results obtained indicate that TLQP-21, unlike EGF, does not modify GH expression but increases prolactin mRNA and protein expression. TLQP-21 treatment also significantly increases Vgf mRNA, thus suggesting that it might exert an autocrine feedforward mechanism. The resulting effect of pharmacological treatment with TLQP-21 is the development of a lacto-troph phenotype, which is similar to the effect exerted by EGF, but not completely superimposable.

Although the TLQP-21 receptor has not been identified yet, we tried to study the intracellular response of GH3 cells to TLQP-21 exposure. A slight increase of ki-nase phosphorylation was demonstrated early for PKC and successively for ERK, while a decrease in PLA, followed by a decrease in AMPK phosphorylation, was observed.

Indeed, EGF signaling in GH3 cells involves diverse cell-specific intracellular pathways that include PI3K, ERK, and PKC [31]. Both EGF and TLQP-21 induced rapid PKC and ERK phosphorylation, and this transient ERK activation maybe sufficient for the induction of prolactin expression. As demonstrated for the

neuropeptide TRH [32], EGF and TRH share a common intracellular mechanism of signal transduction that is mediated through stimulation of the PKC pathway and ultimately by release of calcium from intracellular storages. ERK phosphorylation is necessary for prolactin synthesis, but it is not directly involved in prolactin secretion and can be activated via PKC-dependent pathways activating directly MEK kinase [33]. In the same way, TLQP-21 induced an increase of PKC phosphorylation and, afterwards, of ERK phosphorylation, suggesting an analogous mechanism of action.

Our studies indicate that TLQP-21 treatment induced a rise in $[Ca^{2+}]i$, and this effect was determined by release from intracellular Ca^{2+} storage compartment, as demonstrated by treatment with the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin, which depletes sar-coplasmatic reticulum calcium storage [30]. The calcium increase, stimulated by TLQP-21 exposure, allows the fusion of secretory vesicles and the release of the stored hormones GH, prolactin, and VGF peptides.

Regarding the TLQP-21 mechanism of action, mobilization of intracellular Ca^{2+} stores was studied in the presence of inhibitors of different second messenger signaling

pathways. Considering these results, we focused on the potential role of the PKC pathway. Our data revealed that TLQP-21-mediated increase of cytosolic Ca^{2+} is triggered by activation of a phospholipase followed by phosphorylation of PKC (indicated by the time course experiments on kinase phosphorylation) since pretreatment with TPA, a downregulator of PKC, blocks TLQP-21-induced Ca^{2+} release in a time-dependent manner. A slight increase of kinase phosphorylation was demonstrated early for PKC and successively for ERK, while a decrease in PLA phosphorylation followed by a decrease in AMPK activation was observed. Unlike PACAP, which stimulates cAMP increase and PKA phosphorylation [34], TLQP-21 does not appear to stimulate this pathway (data not shown), as previously demonstrated in a 3T3-L1 cell line [13].

According to the data obtained from phosphorylation kinase activations and from the use of selective inhibitors, we conclude that the most likely TLQP-21 effect in GH3 cells is mediated by a G protein-coupled receptor activating a Gq that stimulates a phospholipase in order to obtain PI3 and DAG molecules. Those second messengers open the intracellular Ca^{2+} storage and stimulate PKC that eventually activate ERK phosphorylation. We attempted to indicate the intracellular signaling in figure 8.

Since pro-VGF and its byproducts are highly expressed in hypothalamic neurons, which project to the median eminence and in the posterior pituitary, and it is expressed in endocrine cells of anterior pituitary,

mainly in gonadotroph and lactotroph cells [2,9], we can speculate a neuromodulatory action of these molecules as demonstrated recently for TLQP-21 on reproductive function of male rats [22]. However, the dynamics of VGF secretion in the portal-pituitary circulation and a direct action on anterior pituitary are presently not completely elucidated and although more in vivo studies will account on the possible role of TLQP-21 as a real mammotrophic pep-tide, our studies indicate that TLQP-21 peptide could have a neuromodulatory effect as hormone, which is released from the hypothalamic area in median eminence, or acting as paracrine-autocrine hormone on the pituitary gland.

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