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PHARMACOLOGICAL CHARACTERIZATION OF THE INTRACELLULAR SIGNALLING PATHWAY ACTIVATED BY TLQP-21

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To Mom & Dad,
and Luca,
for their constant support
and infinite patience.
Thanks for loving me
unconditionally.

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1. Abstract

Obesity is a global epidemic for which the current weight loss therapies are relatively ineffective. Many central and peripheral factors are involved in the mechanisms controlling eating behaviour, and the integration of these signals within the hypothalamus results in the generation of specific responses aimed at regulating energy balance.

TLQP-21 is a novel neuropeptide that has been implicated in the regulation of energy homeostasis, nociception, gastric function and several other physiologic functions. Although recent studies identified different receptors as the targets for TLQP-21, its molecular mechanisms of action at the cellular level remain largely unknown. Thus, since TLQP-21 is emerging as a novel target for obesity-associated disorders, diabetes, neuropathic pain, and other human pathologies, the purpose of this study was to better investigate the intracellular signalling pathway activated by the peptide-receptor interaction.

Here, using intracellular calcium mobilization assay and western blot analysis, we have pharmacologically characterized the intracellular signalling pathway activated by TLQP-21 in ovary, macrophage and microglial cells.

TLQP-21 dose-dependently stimulated a rapid and transient intracellular Ca^{2+} increase in CHO, RAW264.7 and N9 cells, and repeated exposure to the peptide resulted in a reduced response, indicating a possible desensitization mechanism of TLQP-21 receptor.

In particular, TLQP-21 stimulation induced an increase of cytoplasmic Ca^{2+} levels that was sustained by Ca^{2+} release from the ER, since treatment of the cells with thaspigargin reduced the TLQP-21-mediated increase of intracellular Ca^{2+}.

The release of Ca^{2+} from the ER store is regulated by the activation of PLCs and the subsequent production of IP_3 that binds to its receptors on the surface of the ER. In our cellular systems, TLQP-21 activity was reduced by the treatment with the PLC inhibitor U73122 and the IP_3R antagonist 2-APB, confirming a PLC-dependent mechanism of action for the peptide. Furthermore, TLQP-21 induced a rapid dephosphorylation of PLCγ1 in CHO cells, suggesting that Ca^{2+} response to TLQP-21

is mediated by the binding of the peptide to a G_q-coupled receptor that in turn activates PLCβ.
Ca^{2+} release from the ER activated Ca^{2+} entry from the extracellular environment, as demonstrated by the treatment of the cells with SKF-96365 and YM-58483, two specific inhibitors of the SOCE pathway.
In CHO cells, TLQP-21 induced also an increase of PKC phosphorylation and, afterwards, of ERK1/2 phosphorylation. Moreover, the increase of cytosolic Ca^{2+} concentration following TLQP-21 administration, stimulated the activation of Akt/PKB.

Our results suggest that the receptor stimulated by TLQP-21 belongs to the family of the G_q-coupled receptors, that activates membrane-lipid derived second messengers which thereby induce Ca^{2+} mobilization from the ER followed by a slower store-operated Ca^{2+} entry from outside the cell.
In conclusion, our research provides additional evidences about the molecular mechanisms of action of TLQP-21, and could be useful to open new approaches to improve the treatment of several human disorders, including obesity and diabetes.

2. Introduction

2.1. Obesity and diabetes, an overview

Overweight and obesity, defined by the World Health Organization (WHO) as abnormal or excessive fat accumulation, represent a risk factor for a number of chronic disorders, including diabetes, cardiovascular diseases and cancer.

The most common method used for classifying overweight and obesity in adults is the Body Mass Index (BMI), a simple index of weight-for-height. BMI is calculated by dividing a person's weight in kilograms for the square of his height in meters (kg/m^2). Individuals with BMI $\geq$ 25 kg/m^2 are considered overweight and those with BMI $\geq$ 30 kg/m^2 are considered obese.

Once believed a problem only in high income countries, overweight and obesity are now dramatically on the rise in low- and middle-income countries, particularly in urban settings. Worldwide obesity has more than doubled since 1980 reaching epidemic conditions. In 2014, 39% of adults aged 18 and over were overweight, and 13% were obese (*Global Health Observatory (GHO) data*).

As reported above, excess weight is an established risk factor for diabetes, a chronic disease that occurs when the pancreas does not produce insulin (Type 1 diabetes) or when the body cannot effectively use the insulin it produces (Type 2 diabetes) (*Fact sheet, WHO*). Most patients with type 2 diabetes are obese, and the global epidemic of obesity largely explains the increase in the incidence and prevalence of type 2 diabetes over the past 20 years (*Eckel et al., 2011*).

The etiology of obesity is multifactorial, involving a complex interaction among genetics, hormones and the environment (*Kaila and Raman, 2008*). However, the primary cause of obesity and overweight remain an energy imbalance between calories assumed and calories expended, due to an increased intake of energy-dense foods and a decreased physical activity.

In order to maintain normal energy balance, the body integrates both central and peripheral information to coordinate food intake and energy expenditure.

2.2. Central and peripheral regulation of energy homeostasis

The human brain plays a central role in integrating internal and external inputs to modulate appetite and energy expenditure (*Farr et al., 2016*). At the centre of energy homeostasis there is the hypothalamus, that was initially implicated in the control of food intake with the first clinical description of hypothalamic-pituitary injury resulting in obesity (*Williams and Elmquist, 2011*). Central signals from the hypothalamus are integrated with peripheral signals from the gastrointestinal tract, adipose tissue and pancreas, creating a complex network of neural circuits that regulates the individual response to nutrient absorption.

2.2.1. The hypothalamus: nuclei and peptides

The hypothalamus, which forms the anterior part of the diencephalon, is located below the thalamus and right above the brain stem. It is composed by distinct nuclei, including the arcuate nucleus (ARC), the paraventricular nucleus (PVN), the lateral hypothalamic area (LHA), the dorsomedial nucleus (DMN) and the ventromedial nucleus (VMN), which mediate numerous processes, like food intake, body temperature, emotional responses, circadian rhythms and reproduction.
Several studies highlighted the importance of the hypothalamus in the regulation of appetite and energy balance (*Brobeck, 1946; Anand and Brobeck, 1951a; Anand and Brobeck, 1951b*). Nuclei within the hypothalamus produce both orexigenic and anorexigenic neuropeptides that are integrated with peripheral signals to regulate food intake and energy expenditure (*Loh et al., 2015; Sahu, 2004*).

2.2.1.1. The arcuate nucleus (ARC)

The ARC is located into the mediobasal hypothalamus, adjacent to the third ventricle and the median eminence (ME). This area has a semi-permeable blood brain barrier (BBB) and thus is strategically positioned to sense circulating signals such as leptin, ghrelin and insulin (*Broadwell and Brightman, 1976; Cornejo et al., 2016*). Within the ARC there are two major neuronal populations controlling appetite and energy

expenditure: i) a subset of neurons that coexpress neuropeptide Y (NPY) and agouti-related peptide (AgRP) (orexigenic) and ii) a population of neurons that coexpress the cocaine- and amphetamine-regulated transcript (CART) and proopiomelanocortin (POMC) (anorexigenic).

Neuropeptide Y (NPY)

NPY is a 36-amino acid peptide originally isolated in the porcine brain (*Tatemoto et al., 1982*) that, together with the structurally similar peptide YY (PYY) and pancreatic polypeptide (PP), forms the NPY family (*Loh et al., 2015*). NPY is abundantly distributed in the central and peripheral nervous system (CNS; PNS). The expression and release of NPY in the ARC respond to changes in the energy status, being reduced under feeding conditions and increased under food deprivation conditions (*Schneeberger et al., 2014*). Consistent with this concept, intracerebroventricular (i.c.v.) injection of NPY causes rises in food intake and body weight and, with chronic administration, can result in morbid obesity (*Sainsbury et al., 1997; Stanley et al., 1986*). Although five G-protein-coupled receptors (GPCRs) (Y_1, Y_2, Y_4, Y_5 and Y_6) have been identified for NPY and its related peptides, it has been reported that Y_1 and Y_5 receptors mediate the feeding effects of NPY (*Sobrino Crespo et al., 2014*).

Agouti-related peptide (AgRP)

AgRP is a 132-amino acid peptide that is expressed exclusively in the ARC, where it co-localizes with NPY and the neurotransmitter γ-aminobutyric acid (GABA). As for NPY, AgRP is an orexigenic neuropeptide. Its central administration stimulates food intake, reduces energy expenditure and causes obesity, probably inhibiting melanocortin receptors (MCRs) 3 and 4 (*Schneeberger et al., 2014; Sternson and Atasoy, 2014*). AgRP neurons express receptors for peripheral hormonal signals, like insulin, ghrelin and leptin, and send projections into the other hypothalamic nuclei, including PVN, DMN and LHA (*Schneeberger et al., 2014*).

Cocaine- and amphetamine-regulated transcript (CART)

CART is an anorexigenic neuropeptide abundantly expressed in the hypothalamus, and co-localizes with POMC in the ARC. CART mRNA was originally identified on the basis of its increase following treatment with cocaine or amphetamine in rats (*Douglass et al., 1995*). Its expression is enhanced under feeding conditions and reduced under fasting conditions. Accordingly, i.c.v. injection of CART inhibits food intake, while antibodies against CART reverse this effect (*Kristensen et al., 1998*). To date any receptor has been identified for CART, but there is evidence that CART signalling can be blocked by pertussis toxin, suggesting the involvement of an inhibitory GPCR that couples to $G_{i/o}$ proteins (*Lau and Herzog, 2014*).

Proopiomelanocortin (POMC)

POMC is a prohormone precursor that is cleaved into several bioactive molecules, including α-melanocyte stimulating hormone (α-MSH), which represents the main regulator of energy balance in this family (*Valassi et al., 2008*). The POMC gene is expressed in the pituitary and to a lesser amount in numerous peripheral tissues, such as gut, skin, kidney and ovary (*Mountjoy, 2015*). As for CART, POMC is increased under feeding conditions and decreased under fasting conditions. The i.c.v. administration of α-MSH suppresses food intake and reduces body weight (*Schneeberger et al., 2014*). These anorexigenic effects of melanocortin are mediated by two receptors: MC3R and MC4R, highly expressed in the ARC.

2.2.1.2. The paraventricular nucleus (PVN)

The PVN, located in the anterior hypothalamus just above the third ventricle, is a characteristic anorexigenic center in addition to POMC neurons and the melanocortin pathways (*Sohn, 2015*). Its involvement in the regulation of energy balance is mediated by i) a subset of neurons that expressed thyrotropin-releasing hormone (TRH) and ii) a populations of neurons that expressed corticotropin-releasing hormone (CRH). The PVN receives innervation not only from the ARC NPY/AgRP and POMC/CART

neurons but also from extra-hypothalamic regions, such as the nucleus of the tractus solitarius (NTS).

Thyrotropin-releasing hormone (TRH)

TRH is synthetized in various brain areas and in several hypothalamic nuclei. TRH neurons receive afferents from various brain areas that transmit signals from the internal milieu or from the environment, and send projections into the external layer of the ME, from where they release TRH into the portal venous system (*Joseph-Bravo et al., 2016*). TRH expression is enhanced by α-MSH and inhibited by NPY/AgRP (*Valassi et al., 2008*). Central injection of TRH decreases food intake in rats, promoting a negative energy balance (*Vettor et al., 2002*).

Corticotropin-releasing hormone (CRH)

CRH is a 41-amino acid peptide highly expressed in PVN neurons. Central administration of CRH inhibits food intake and reduces body weight in rats (*Vettor et al., 2002*), whereas peripheral administration of CRH increases energy expenditure and fat oxidations in humans (*Smith et al., 2001*).

2.2.1.3. The lateral hypothalamic area (LHA)

The LHA is a heterogeneous structure located posteriorly to the preoptic area and anteriorly to the ventral tegmental area. LHA contains the primary orexigenic nucleus within the hypothalamus that projects not only within the LHA, ARC and PVN, but also into other regions involved in additional physiological functions (*Schneeberger et al., 2014*). The orexigenic effects of LHA are mediated by orexin and melanin-concentrating hormone (MCH) neurons.

Orexin

Orexin A and orexin B are two neuropeptides derived from the precursor prepro-orexin, whose expression is increased under fasting conditions (*Sakurai et al., 1998*). Orexins bind to specific GPCRs, known as orexin receptor 1 (OXR1) and OXR2,

highly expressed in the PVN, LHA and DMN. Central injection of orexins increases food intake, while central administration of an antagonist decreases feeding in rats (*Sakurai et al., 1998*).

Melanin-concentrating hormone (MCH)

MCH is a cyclic 19-amino acid neuropeptide, originally isolated from the pituitary gland of teleost fish, that can bind to two related GPCRs, MCH receptor 1 (MCHR1) and MCHR2 (*O'Leary, 2014*). MCH expression is enhanced under fasting conditions and its i.c.v. administration causes orexigenic effects (*Sobrino Crespo et al., 2014*).

2.2.1.4. The dorsomedial nucleus (DMN)

DMN, located dorsally to the VMN, receives projections especially from the ARC, and send projections into the PVN and LHA. The DMN is involved in different physiological processes, including feeding, body weight regulation and circadian activity, and its destruction results in hyperphagia and obesity (*Simpson et al., 2009*). The DMN contains high levels on NPY and CRH, as well as receptors for this peptides.

2.2.1.5. The ventromedial nucleus (VMN)

VMN receives projections from the AgRP and POMC neurons of the ARC, and in turn its neurons project into hypothalamic and extra-hypothalamic areas. Brain-derived neurotrophic factor (BDNF) is highly expressed in the VMN. Its administration reduces food intake and body weight (*Simpson et al., 2009*), while the lack of BDNF or its receptors lead to hyperphagia and obesity in humans and mice (*Lyons et al., 1999*).

2.2.2. The gastrointestinal tract: satiety signals

The intestine, being the first organ to process and absorb nutrients, is essential for informing the CNS regarding the types and quantity of nutrient entering the body (*Sisley and Sandoval, 2011*). The intestinal tract also releases an array of peptides and

hormones that provide feedback to the hypothalamus to regulate energy intake and expenditure (*Farr et al., 2016*). Most of the gastrointestinal peptides, including cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1) and PYY, operate as anorexigenic agents, resulting in a smaller meal size when administered exogenously (*Sisley and Sandoval, 2011*), while ghrelin stimulates food intake and is the most potent known circulating orexigen.

Cholecystokinin (CCK)

CCK is released post-prandially from the small intestine in response to saturated fats, long chain fatty acids and small peptides that would normally result from protein digestion (*Sobrino Crespo et al., 2014*). CCK binds to a specific receptor (CCK1R) located on vagal sensory nerve terminals providing to NTS a sense of fullness (*Valassi et al., 2008*). CCK was the first gut hormone demonstrated to have an effect on food intake. Its exogenous administration elicits satiety and reduces meal size (*Geary, 2004*), while the administration of CCK1R antagonists results in obesity (*Simpson et al., 2009*).

Glucagon-like peptide-1 (GLP-1)

GLP-1 is a gut-brain peptide produced by enteroendocrine L-cells located in the ileum and colon. It is released into the circulation following a meal and acts stimulating insulin secretion via GLP-1 receptors (GLP-1R) located in pancreatic β-cells (*Sisley and Sandoval, 2011*). Central administration of GLP-1 decreases food intake and body weight (*Lenard and Berthoud, 2008*) and activates *c-fos* expression in the ARC and PVN.

Peptide YY (PYY)

PYY is a 36-amino acid peptide secreted by L-cells in the gut and released into the circulation after a meal. Peripheral administration of PYY reduces food intake in rodents and humans and PYY knock-out mice develop obesity (*Batterham et al., 2002*). Its effect might be mediated via the Y_2 receptors, since this is abolished in Y_2R knock-out mice (*Batterham et al., 2002*).

Ghrelin

Ghrelin is a 28-amino acid acylated hormone produced by the stomach that exerts its action through the growth hormone secretagogues receptor (GHSR). GHSR is expressed in AgRP neurons of the ARC that are essential for mediating the orexigenic effects of ghrelin (*Schneeberger et al., 2014*). To stimulate the release of orexigenic peptides, ghrelin increases the number of projections on NPY and AgRP neurons, and inhibits the number of projections on POMC neurons (*Sobrino Crespo et al., 2014*). Central and peripheral administration of ghrelin promotes food intake in rodents, and enhances appetite in humans (*Nakazato et al., 2001; Wren et al., 2001*).

2.2.3. Adipocytes and pancreas: adiposity signals

Adiposity signals, including leptin and insulin, are a second type of feedback signals sensitive to nutrient intake that are modulated by adipose tissue mass (*Baskin et al., 1999*). In according to this concept, adiposity signals are hormones whose circulating concentration is proportional to fat mass.

Leptin

Leptin is an anorexigenic 167-amino acid protein secreted by adipocytes that is transported through the BBB, gaining access to neurons in the hypothalamus to influence energy homeostasis (*Woods and D'Alessio, 2008)*. In the brain, leptin receptors are concentrated in the ARC (*Sisley and Sandoval, 2011*), where leptin exerts its anorectic effect activating POMC/CART neurons and inhibiting NPY/AgRP neurons, resulting in reduced food intake. Accordingly, leptin deficiency results in severe obesity and hyperphagia, confirming the important role that this hormone plays in the CNS in the control of energy balance.

Insulin

Insulin is produced in pancreatic β-cells in response to nutrient and in direct proportion to body fat. Insulin, crossing the BBB, acts on ARC neurons in the hypothalamus reducing energy intake (*Sobrino Crespo et al., 2014*). Central infusion of insulin

results in a dose-dependent suppression of food intake in animals (*Vettor et al., 2002*), and the specific deletion of insulin receptors increases weight gain in mice (*Bruning et al., 2000*).

As has been outlined above, the control of energy balance depends critically on the CNS, with the hypothalamus playing an important role in integrating numerous central and peripheral nutrient signals.

Beside the peptides already discovered to be implicated in the control of energy balance, novel genes are continuously identified with a role in food intake. Among these, the *vgf* gene and its derived peptides are gaining increasing interest thanks to their implication in the development of obesity.

2.3. *vgf*, a neurotrophin-inducible gene

The identification of the *vgf* gene occurred thanks to the rising interest on neurotrophins following the discovery of the nerve growth factor (NGF), a molecule required for the development and maintenance of sensory and sympathetic nerve cells (*Levi-Montalcini, 1966*). The *vgf* gene was identified in 1985 as a NGF responsive gene in PC12 rat pheochromocytoma cells and its non-acronymic name is based on the selection of this clone from plate *V* of a nerve *G*rowth *F*actor-induced PC12 cell cDNA library (*Levi et al., 1985*). In response to NGF, PC12 cells differentiate into sympathetic neuron-like cells, as indicated by the expression of a number of morphological and biochemical markers of differentiation, including the formation of processes and the increase in neurotransmitter synthesis (*Greene and Tischler, 1976*). During differentiation, *vgf* has been characterized as one of the many genes that is deeply upregulated in expression in response to NGF treatment (*Levi et al., 1985*). Therefore, the PC12 cell line has been employed as a model for the investigation of the mechanisms that regulate *vgf* expression (*Possenti et al., 1989; Salton et al., 1991*). Later studies demonstrated that *vgf* exhibits a selective, but only marginal, inducibility to a diverse range of stimuli, including basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), cyclic adenosine monophosphate (cAMP) and insulin (*Hawley et al., 1992; Possenti et al., 1992; Salton et al., 1991*), despite the capacity of some of these factors to promote PC12 cell differentiation (*Hawley et al., 1992; Salton et al., 1991*).

2.3.1. *vgf* gene structure: from mouse to man

Comparison of *vgf* genomics clones from mouse (*Hahm et al., 1999*), rat (*Salton et al., 1991*) and human (*Canu et al., 1997a; Canu et al., 1997b*) reveals a highly conserved gene structure. *vgf* is a single copy gene (*Salton et al., 1991*) located on human chromosome 7q22 (*Canu et al., 1997a*) and on mouse chromosome 5 (*Hahm et al., 1999*). It is composed of three exons, the last of which encodes for the entire protein sequence, and of two small introns that interrupt the *vgf* gene in a region that encodes the 5’ untranslated sequence of the mRNA (*Salton et al., 1991*). In the rat, two different

mature mRNAs, that exhibit similar stability, are produced by alternative splicing that skips the second exon (*Hawley et al., 1992*).

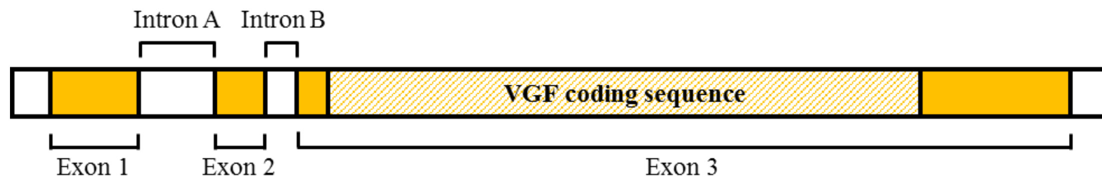

Figure A: Schematic representation of *vgf* gene structure.

The evolutionary conserved sequence can be found not only in the coding region, but also within the untranslated region and the promoter region. Indeed, the degree of homology between human and rodent within the 0.8-kbp promoter sequence examined exceeds 80% (*Canu et al., 1997b*), suggesting that critical regulatory elements are present in this part of the *vgf* promoter.

The 200-bp sequence immediately upstream of the transcription initiation site contains numerous consensus binding sites for known transcriptional regulators and include a TATA box (-33-bp), a CCAAT box (-141-bp) and a cAMP-response element (CRE; -76-bp) embedded in a 14-bp long palindromic sequence (*Hawley et al., 1992*). Some of these elements appear to contribute to the transcriptional regulation of *vgf* by NGF. For example, mutations of CRE abolish NGF and cAMP responses (*Hawley et al., 1992*) while disruption of the palindromic sequence, in the presence of an intact CRE, prevents induction by NGF but non by cAMP (*Possenti et al., 1992*). In addition to the CRE, the CCAAT element is necessary but not sufficient for *vgf* induction, whereas a G(S)G element (-6-bp) is required for maximal induction by NGF but not by cAMP (*D'Arcangelo et al., 1996*). The G(S)G motif may bind NGFI-A, a gene product that is robustly induced by NGF and Ras (*Milbrandt, 1987*). Therefore, it is likely that these elements are responsible for the tissue-specific expression of the *vgf* gene.

2.3.2. The VGF polypeptide

The *vgf* gene encodes a neuropeptide precursor comprising of 615 amino acids in human and 617 amino acids in rodents. VGF has a typical secretory leader sequence of 22 amino acids at the N-terminal that promotes translocation into the endoplasmic reticulum (ER). In fact, in PC12 cells VGF is stored inside and released from secretory vesicles through the regulated pathway (*Possenti et al., 1989*). Cleavage of the secretory leader sequence between residues 23 and 24 results in a polypeptide precursor rich in proline (11%) and glutamic acid (14%) residues (*Ferri and Possenti, 1996*) with a predicted mass of 68 kDa. Nevertheless, in Western blots analysis of PC12 cell extracts VGF is detected as a doublet of 80-90 kDa, probably due to the absence of post-translational modifications and to the high proline content that results in the reduced electrophoretic mobility of the VGF polypeptide (*Levi et al., 1985*).

A major feature of the VGF precursor sequence is the presence of paired basic amino acids residues, highly conserved from mouse to man, that represent potential cleavage sites for prohormone convertases (PCs) 1/3 and 2, that are members of the family of kexin/subtilisin-like serine proteinases (*Steiner, 1998).* Using a panel of antibodies against the C-terminal nonapeptide of rat VGF protein, several smaller peptides, called VGF-derived peptides, were identified in rat brain homogenates and in extracts of neuronal, endocrine and pancreatic β-cell lines (*Possenti et al., 1999; Trani et al., 1995*). The main products have apparent molecular masses of 20 kDa (VGF20 or NAPP-129) and 10 kDa (VGF10 or TLQP-62), while others of 18 kDa (VGF18) and 6 kDa (VGF6) are also often detected. The first VGF-derived peptide identified, named AQEE-30 or Peptide V, was purified from bovine posterior pituitary (*Liu et al., 1994*) and corresponds to the last 30 amino acids of the C-terminal of human VGF. By convention the VGF-derived peptides are identified by the first four amino acids followed by the overall length of the peptide (*Levi et al., 2004*).

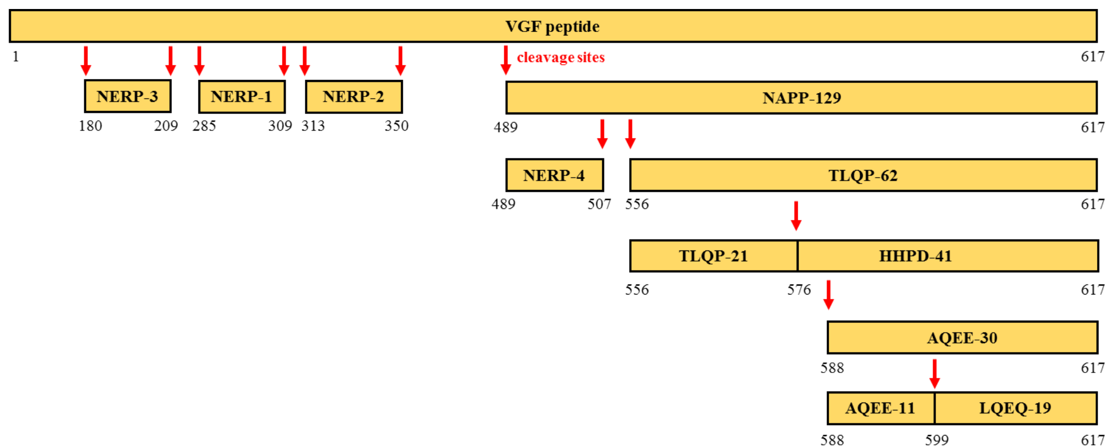

Figure B: Schematic representation of VGF-derived peptides.

2.3.3. Tissue distribution and regulation of VGF mRNA

VGF mRNA expression is restricted to subpopulations of neurons and neuroendocrine cells (*van den Pol et al., 1989*) and is regulated in a developmentally specific manner (*Snyder et al., 1998*). *In situ* hybridization studies in rat revealed that VGF expression is noted at embryonic day 11.5 (E11.5) in regions containing neural crest-derived cells destined to become cranial, sympathetic and dorsal root ganglia (DRGs) (*Snyder et al., 1998*). As development proceeds, VGF mRNA is additionally found in the ventral spinal cord, basal forebrain, thalamus, hypothalamus and pituitary (E13.5-E15.5). By the end of gestation (E17.5-E19.5), VGF expression can also be detected in the developing olfactory bulbs and hippocampal formation as well as in the retina, stomach and small intestine. The trend of expansion of VGF expression continues into the postnatal period: VGF mRNA expression expands into additional cortical regions and also increases dramatically in the cerebellum (*Snyder et al., 1998; 2003*). In the adult rat brain, VGF mRNA is widely distributed in the olfactory system, cerebral cortex, thalamus and hypothalamus and also in septal, amygdaloid and brainstem nuclei (*Snyder et al., 1998; van den Pol et al., 1989*). In addition, VGF mRNA has been detected in α- and γ-motoneurons in the adult rat spinal cord (*Snyder and Salton, 1998*).

Since VGF was initially identified as a neurotrophin-inducible gene, VGF regulation in different tissues has been examined. Further to its response to NGF, VGF is also upregulated by BDNF both *in vitro* and *in vivo* (*Alder et al., 2003*; *Eagleson et al., 2001*). Several studies indicate that VGF mRNA in hypothalamus responds to feeding (*Hamh et al., 1999*), adrenalectomy (*Mahata et al., 1993a*) and salt loading (*Mahata et al., 1993b*). Synthesis of VGF mRNA is also regulated in NTS and dorsal motor nucleus of the vagus (DMV) in response to experimental gastric damage (*Kanemasa et al., 1995*). VGF levels were found to be reduced in animal models of depression (*Thakker-Varia et al., 2007*), whereas its expression is induced by conditions that reflect increase in activity such as seizure, learning and synaptogenesis (*Bozdagi et al., 2008; Snyder et al., 1998*). In golden hamster and in mouse, VGF mRNA levels are also increased in the suprachiasmatic nucleus (SCN) in response to light and by the circadian rhythm (*Hamh et al., 1999*; *Wisor and Takahashi, 1997*), further associating VGF regulation with the diurnal control of feeding, activity and metabolic rate (*Levi et al., 2004*).

2.3.4. Functions of VGF

The studies of structure, distribution and regulation of VGF suggest that this molecule plays different physiological roles in the developing and/or adult nervous and neuroendocrine systems (*Salton et al., 2000*).

VGF-deficient mice

VGF function was assessed through the development of genetically engineered knock-out mice ($VGF^{-/-}$), in which the entire VGF coding sequence was removed by homologous recombination (*Hahm et al., 1999*).

At birth, $VGF^{-/-}$ pups were indistinguishable from heterozygous or wild-type littermates. However, several days following birth, VGF deficient mice showed a significant lack of body weight gain, and adults were found to weigh 50-70% less than wild-type.

In addition to being smaller than normal mice, $VGF^{-/-}$ mice were also hypermetabolic. The basal metabolic rate was significantly elevated, as measured by oxygen consumption and carbon dioxide output (*Hahm et al., 1999*). Although hypermetabolic, $VGF^{-/-}$ mice had reduced circulating thyroid hormone and leptin levels, normal core body temperature and apparently normal sympathetic activity. Peripheral fat stores, however, were dramatically reduced, as well as total body fat. Faecal fat levels were similar to control mice, suggesting the absence of alteration of the gastrointestinal tract.

VGF mutant mice were also found to be hyperactive compared to wild-type mice. Moreover, analysis of hypothalamic gene expression showed elevated AgRP and NPY mRNA levels and a reduced content of POMC mRNA. Serum insulin and glucose levels were reduced, while serum corticosterone level was increased when compared to that in the wild-type (*Hahm et al., 1999*).

As a consequence of the VGF mutation or secondary to low circulating leptin levels, 85-90% of $VGF^{-/-}$ mice had reduced fertility (*Hahm et al., 1999*). Onset of puberty and sexual maturation were delayed, sexual behaviour was abnormal and reproductive organs were shown to be much smaller compared to controls. Moreover, mutant females were unable to nurse their offspring, perhaps as a result of incomplete mammary gland development due to low circulating sex steroid hormone levels.

Energy balance and food intake

The lean, hypermetabolic and hyperactive phenotype of $VGF^{-/-}$ mice suggest that VGF and its derived peptides play an important role in the regulation of energy homeostasis (*Hahm et al., 1999*).

It has been demonstrated that i.c.v. injection of TLQP-62 and its cleaved product HHPD-41 possess a positive role on feeding, by increasing food intake in overnight fasted mice (*Bartolomucci et al., 2007*). Moreover, i.c.v. injection of NERP-2, one of two novel N-terminal peptides, has been reported to increase food intake, body temperature, locomotor activity and oxygen consumption through the orexin pathway (*Toshinai et al., 2010*).

Surprisingly, however, i.c.v. injection of TLQP-21 increased energy expenditure and prevented high-fat diet induced obesity in mice (*Bartolomucci et al., 2006*). In Siberian hamsters, too, TLQP-21 has been reported to decrease food intake (*Jethwa et al., 2007*), supporting a role for TLQP-21 as a catabolic neuropeptide.

Water balance

Water deprivation and salt loading increase VGF mRNA levels in rats (*Mahata et al., 1993b*). Upon i.c.v. injection, NERP-1 and NERP-2 appeared to suppress vasopressin release induced by hypertonic NaCl. Additionally, i.c.v. infusion of both NERPs attenuated the increase in vasopressin as a result of water deprivation in rats, an effect that was prevented by NERP antibodies (*Toshinai et al., 2009*).

Reproduction

Since VGF deficient mice were sexually immature and almost completely infertile (*Hahm et al., 1999*), the role of VGF in reproduction was examined in different *in vivo* works.

It has been demonstrated that certain VGF C-terminal derived peptides promote penile erection in male rats when injected into the hypothalamic paraventricular nucleus. Four out of five peptides (HHPD-41, AQEE-30, LQEQ-19 and AQEE-11, but not TLQP-21) showed a positive effect on penile erection in a dose-dependent manner (*Succu et al., 2004*). Moreover, NERP-1 has a pro-erectile effect when injected into the lateral ventricles of rats (*Melis et al., 2012*). The effect on penile erection is thought to be *via* nitric oxide-mediated activation of oxytocinergic neurons (*Succu et al., 2004*).

Although TLQP-21 was shown to be ineffective on penile erection in rats, it has been shown to modulate both rat male and female reproductive axis at different levels (*Pinilla et al., 2011; Aguilar et al., 2013*). Central administration of TLQP-21 stimulated luteinizing hormone (LH) secretion both in male and female rats, while increased gonadotropin-releasing hormone (GnRH) secretion only in male rat hypothalamic explants.

Depression

VGF has antidepressant-like activity in rodents. It has been demonstrated that VGF is downregulated in the hippocampus in animal models of depression (*Thakker-Varia et al., 2007*), and exogenous infusion of TLQP-62 reduced the depressed state, possibly via a BDNF-dependent mechanism (*Bozdagi et al., 2008; Lin et al., 2014*). The antidepressant effect of VGF has been also confirmed by the exercise model (*Hunsberger et al., 2007*). Exercise regulates VGF mRNA and protein expression in the rodent hippocampus and induces an antidepressant response.

Interestingly, VGF mRNA level has been found to be significantly reduced in leukocytes of drug-free depressed patients, and its expression was restored in response to effective antidepressant treatment (*Cattaneo et al., 2010*).

Gastrointestinal tract

In addition to its role in energy balance regulation, TLQP-21 has been shown to induce contraction of gastric fundic strips in a dose-dependent manner, by releasing prostaglandin (PG) E_2 and PGF_{2a} from the mucosal layer (*Severini et al., 2009*). In addition, central injection of TLQP-21 decreased gastric emptying by about 40% (*Severini et al., 2009*) and reduced ethanol induced gastric lesion in rat, through nitric oxide and PGE_2 production (*Sibilia et al., 2010*).

Pain

Data from a number of pain models revealed a significant up-regulation of VGF in sensory neurons (*Chen et al., 2013*). Furthermore, VGF has been shown to be increased in DRG and spinal cord in rat models of neuropathic pain (*Maratou et al., 2009*).

Other evidences suggested a functional role for VGF-derived peptides in pain signalling. Indeed, intrathecal injection of TLQP-62 induced long lasting mechanical and cold allodynia (*Moss et al., 2008*). Peripheral injection of TLQP-21 resulted in hyperalgesia in the formalin model of inflammatory pain, while its central administration produced an analgesic effect in the second phase of the test (*Rizzi et al.,*

2008). Additionally, both AQEE-30 and the shorter peptide LQEQ-19 have been shown to induced thermal hyperalgesia through the activation of the mitogen-activated protein (MAP) kinase p38 in spinal microglia (*Riedl et al., 2009*).

Neuronal apoptosis

A further effect for VGF was shown in rat cerebellar granule cell (CGC) cultures, in which TLQP-21 increased, in a dose- and time-dependent manner, survival of CGCs by preventing apoptotic cell death induced by serum and potassium deprivation (*Severini et al., 2008*). Moreover, in primary mixed cultures of spinal cord neurons from SOD-1 mice, the exogenous expression of VGF attenuated excitotoxic injury (*Zhao et al., 2008*). Recently, it has been shown that SUN N8075, a small molecule inducer of VGF mRNA, exerts protective effect against endoplasmic reticulum-induced cell death (*Shimazawa et al., 2010*).

Human disease conditions

A small number of reports has reported alteration of VGF peptides in different human disease conditions. In particular, a reduction of VGF-derived peptides was found in the parietal cortex of Alzheimer's disease (AD) patients and a reduction of NERP-1 in the parietal cortex of Parkinson's disease (PD) patients (*Cocco et al., 2010*). Similarly, VGF peptides has been found to be decreased in the cerebrospinal fluid of patients affected by AD (*Carrette et al., 2003; Selle et al., 2005*).

2.4. The VGF-derived peptide TLQP-21

Among several bioactive peptides derived from VGF, TLQP-21 is the best-studied and characterized fragment.

TLQP-21, which spans from residue 556 to residue 576 of the precursor sequence, has been originally identified in rat brain tissue extracts by immunoprecipitation, microcapillary liquid chromatography-tandem mass spectrometry and database searching algorithms (*Bartolomucci et al., 2006*). TLQP-21 emerges as a multifunctional peptide involved in different biological processes. It has been shown to regulate energy balance (*Bartolomucci et al., 2006*; *Jethwa et al., 2007*), pain (*Chen et al., 2013; Fairbanks et al., 2014; Rizzi et al., 2008*), gastric function (*Severini et al., 2009; Sibilia et al., 2010*) and reproduction (*Aguilar et al., 2013; Pinilla et al., 2011*).

NH3-Tyr-Leu-Gln-Pro-Pro-Ala-Ser-Ser-Arg-Arg-Arg-His-Phe-His-His-Ala-Leu-Pro-Pro-Ala-Arg-COOH

Figure C: Schematic representation of TLQP-21 sequence.

TLQP-21 and metabolism

In the paper by Bartolomucci *et al.* (2006), chronic i.c.v. treatment of TLQP-21 in standard chow fed mice increased resting energy expenditure and rectal temperature, without affecting body weight and food intake. Furthermore, in the brown adipose tissue (BAT), TLQP-21 upregulated β2 adrenergic receptors (β2-AR) mRNA, in the presence of increased serum epinephrine level, while, in the white adipose tissue (WAT), TLQP-21 increased peroxisome proliferator-activated receptor δ (PPAR- δ) and uncoupling protein 1 (UCP1) mRNAs. In mice fed with a high-fat (HF) diet for 14 days, TLQP-21 conferred resistance to obesity: HF-TLQP-21 mice showed unaffected body and WAT weight and caloric efficiency, despite HF- and standard-fed mice consumed similar amount of kilocalories and showed similar locomotor activity. Moreover, TLQP-21 treatment normalized ghrelin and lowered leptin and triglycerides levels, changes that appeared to be independent from hypothalamic anorexigenic and orexigenic neuropeptides.

Similar catabolic effects were reported in the Siberian hamster, an animal model used to investigate the long-term central control of caloric intake and expenditure (*Ebling, 2014*). It has been shown that VGF mRNA is induced in specific hypothalamic areas of the Siberian hamster upon exposure to short photoperiods, which is associated with a seasonal decrease in appetite and weight loss (*Noli et al., 2015*). In Jethwa *et al.* (2007), single i.c.v. infusion of TLQP-21 to Siberian hamsters decreased food intake and body weight throughout the 24-hour period investigated, without affecting locomotor activity or feeding. Chronic i.c.v. administration of TLQP-21 caused a sustained and additive reduction in food intake, accompanied by a sustained and progressive reduction in body weight, due to a decrease in epididymal WAT.

Further evidence supporting a catabolic role for TLQP-21 were reported by Possenti *et al*. (2012). Chronic subcutaneous (s.c.) infusion of TLQP-21 in obese mice decreased perigonadal WAT adipocyte diameter and increased triacylglycerol lipolysis. These effects were paralleled by increased sympathetic tone in the adipose fat pads as demonstrated by increased tyrosine hydroxylase-enzymatic activity and noradrenaline content in both visceral and subcutaneous fat pads.

TLQP-21 and diabetes

Beside its role in the regulation of metabolism, TLQP-21 has been shown to attenuate the development of type 2 diabetes in rats. Stephens *et al*. (2012) reported that TLQP-21 potentiates glucose-stimulated insulin secretion in rat and human islets and improves glucose tolerance *in vivo*. Moreover, chronic administration (4 weeks) of TLQP-21 in pre-diabetic Zucker Diabetic fatty (ZDF) rats preserves islet mass and slows diabetes onset.

T*LQP-21 and pain*

TLQP-21 has been proposed as a modulatory peptide for pain (*Rizzi et al., 2008*). s.c. injection of TLQP-21 into the dorsal surface of the right hind paws of mice determined a hyperalgesic response, increasing the licking activity with a maximal effect 5-10 minutes after injection. Conversely, i.c.v. infusion of TLQP-21, 5 minutes before the

local treatment of formalin, reduced the licking activity in the second phase of the test (15-40 minutes), producing an analgesic effect.

Additional data regarding pain modulation were reported by Chen *et al.* (2013), using the partial sciatic nerve ligation (PSNL) as a model to study neuropathic pain. Intraplantar injection of TLQP-21-stimulated macrophages in rats subjected to PSNL caused mechanical hypersensitivity by decreasing the paw withdrawal threshold throughout the 48-hour period investigated.

Recently, Fairbanks *et al*. (2014) demonstrated spinal pronociceptive actions for TLQP-21, providing evidence that the peptide participates in mechanisms associated with the development and maintenance of persistent pain. Intrathecal injection of TLQP-21 evoked dose-dependent thermal hyperalgesia in mice subjected to the warm-water tail-immersion assay, with a peak 60-90 minutes after injection. This effect was inhibited by p38 MAP kinase, cyclooxygenase (COX) and lipoxygenase inhibitors. Moreover, intrathecal administration of anti-TLQP-21 attenuated the development and maintenance of tactile hypersensitivity in the spared nerve injury model of neuropathic pain.

T*LQP-21 and gastrointestinal functions*

As previously reported, TLQP-21 plays a role in the regulation of gastric functions. Severini *et al*. (2009) reported in *in vitro* studies that TLQP-21 in a concentration-dependent manner induced muscle contraction of rat longitudinal forestomach strips and only weak, not concentration-dependent contractions of the oesophagus or stomach antrum. This effect was mediated by the release of PGs, and was completely abolished by indomethacin pretreatment. Moreover, i.c.v. injection of TLQP-21 delayed gastric empting of a phenol red meal by about 40% both in male and female rats. Again, this effect was blocked by i.c.v. injection of indomethacin, suggesting that, also *in vivo*, TLQP-21 acts in the brain by stimulating PGs release.

Further indications of a TLQP-21 involvement in gastrointestinal functions have been reported by Sibilia *et al*. (2010). Acute i.c.v. but not intraperitoneal (i.p.) injection of TLQP-21 exhibited a dose-dependent protective effect against ethanol-induced gastric

lesions in rats. The TLQP-21 gastroprotective effect was accompanied by an increase in gastric PGE_2 production linked to an increase of COX-1 expression. Additionally, capsaicin treatment completely prevented TLQP-21 gastroprotective activity, suggesting that TLQP-21 may require the integrity of capsaicin-sensitive vagal and/or spinal afferents to exert its effect.

Afterwards, it has been shown that i.c.v. injection of TLQP-21 significantly inhibited gastric acid secretion in pylorus-ligated rats (*Sibilia et al., 2012*). This decrease resulted from a dose-dependent reduction in acid concentration and to a lesser extent to a fall in the volume of gastric secretion. In agreement with the previous data on gastric emptying, the TLQP-21 antisecretory activity was prevented by the somatostatin depletor cysteamine, by indomethacin and by functional ablation of sensory nerve by capsaicin, confirming that the central inhibitory effect of TLQP-21 is mediated by somatostatin and PGs and requires the integrity of sensory nerve fibres.

T*LQP-21 and reproduction*

In Pinilla *et al.* (2011), central administration of TLQP-21 induced acute gonadotropin responses in pubertal and adult male rats, likely via stimulation of GnRH secretion. Additionally, in pubertal (but not adult) males, TLQP-21 stimulated LH secretion directly at the pituitary level. Moreover, repeated central administration of TLQP-21 to pubertal males subjected to chronic undernutrition was able to ameliorate the hypogonadotropic state induced by food deprivation, whereas in fed pubertal males delayed the onset of puberty.

Additional data regarding TLQP-21 reproductive effects were described by Aguilar *et al*. (2013). Systemic administration of TLQP-21 directly stimulated LH and follicle stimulating hormone (FSH) secretion at the pituitary level in prepubertal female rats, whereas acute central injection of TLQP-21 was unable to modify LH secretion at this age. In contrast, acute i.c.v. administration of TLQP-21 in adult female rats increased LH secretion on diestrus and decreased the amplitude of the preovulatory LH surge on proestrus. Furthermore, repeated central administration of TLQP-21 to female rats fed

ad libitum during the pubertal transition increased food intake without increasing body weight and slightly advanced puberty onset.

Overall, these findings demonstrate that TLQP-21 is a pleiotropic peptide with multiple effects. This peptide by activating adipose tissue catabolism and regulating the brain-gut axis may open promising drug discovery perspective to prevent weight gain and obesity.

2.4.1. TLQP-21 receptor/s

In the last years, the effects of TLQP-21 have been well characterized, but little is known about the pathways and the molecular targets of the peptide.

TLQP-21 was found to bind to adipocyte membranes, stimulating a pro-lipolytic effect (*Possenti et al., 2012*), and to a unique binding site on the surface of Chinese Hamster Ovary (CHO) cells, using Atomic Force Microscopy (AFM) (*Cassina et al., 2013*). Recently, complement C3a receptor-1 (C3aR1) (*Hannedouche et al., 2013; Cero et al., 2014*) and globular head of the complement C1q receptor (gC1qR) (*Chen et al., 2013*) have been identified as receptors for TLQP-21.

TLQP-21 binding-site in adipocyte membranes

Possenti *et al*. (2012) were the first to identify a saturable receptor-binding activity for TLQP-21. A radiolabelled analogue of TLQP-21 (^{125}I-YA-TLQP-21) was used in the receptor-binding assay on adipocyte membranes, showing that the maximum binding capacity for TLQP-21 was higher in white adipocytes compared with other tissues, and it was selectively up-regulated in the adipose tissue of obese mice.

TLQP-21 binding-site on CHO cells

Cassina *et al*. (2013), by performing force measurement, showed the presence and the plasma membrane distribution of a TLQP-21 binding site on CHO cell line.

TLQP-21 biological activity was firstly assessed on the basis of its ability to induce a calcium response in CHO cells. TLQP-21-mediated intracellular calcium mobilization increased in a dose-dependent manner, reaching maximal levels within 10 seconds of stimulation and returning to basal levels in about 60 seconds. To establish the specificity of TLQP-21 effects on calcium release in CHO cells, was used the scramble peptide LRPS-21 that did not show any activity on intracellular calcium mobilization. For AFM force measurements, the individual forces between the biotinylated-TLQP-21 functionalized cantilever and the cell surface were analysed, demonstrating the presence of specific TLQP-21 binding sites on the surface of CHO cells. This binding site interacted with the peptide with an average force of 39 ± 7 pN indicating the existence of a single class of binding sites.

C3aR1

C3aR1 is a member of the GPCRs family, characterized by an extracellular N-terminal, followed by seven transmembrane domains connected by alternating intracellular and extracellular loops, and an intracellular C-terminal (*Shenker, 1995*). G proteins are heterotrimeric complexes consisting of αβγ complex with guanosine diphosphate (GDP) bound to the Gα-subunit. Receptor activation leads to a conformational change in Gα, resulting in an exchange of GDP for guanosine triphosphate (GTP). This interaction causes the dissociation of βγ complex which, in turn, mediates signal transduction. Following the binding of C3a to C3aR1, the primary signaling mechanism activated is through the G protein $G\alpha_i$ in human and mouse immune cells. C3a has also been reported to decrease cAMP levels in murine dendritic cells (*Klos et al., 2013*).

The human and mouse C3aR1 appear to be broadly expressed, with mRNA detected in lung, spleen, ovary, placenta, small intestine, spinal cord and brain (*Klos et al., 2013*). C3aR1 has also been observed on myeloid cells, such as, dendritic cells, macrophages and microglia, and on neurons, where it may have a role in CNS inflammation and during development (*Klos et al., 2013*). Although its role in the innate immune response, C3aR1 appears to be involved in metabolism. It has been

demonstrated that C3aR1 is highly expressed in the WAT and is strongly increased upon HF diet feeding in mice. Furthermore, a C3aR1$^{-/-}$ mouse model has been shown to prevent diet-induced obesity and insulin-resistance, by modulating adipose tissue macrophages infiltration and activation (*Mamane et al., 2009*).

Hannedouche *et al*. (2013) reported the identification of C3aR1 as the target of TLQP-21 in two different rodent ovary cell lines, CHO-K1 and O-342 cells. CHO-K1 cells responded to rat and, to a lesser extent, to human TLQP-21 showing an increase in intracellular calcium, though ATP priming was necessary to observed a robust signal, suggesting that TLQP-21 receptor was not a G_q-coupled GPCR. The lack of cAMP modulation and the sensitivity to pertussis toxin (PTX) indicated that the G protein mediating these effects was likely to be G_0.

To gain deeper insight into the molecular mechanisms activated by TLQP-21, RNA sequencing was carried out on CHO-K1 cells (TLQP-21-responsive) and CCL39 cells (TLQP-21-nonresponsive). As they hypothesised that TLQP-21 receptor was a G_0-coupled receptor, they restricted their transcriptional analysis to GPCRs and identified a panel of 21 candidates, supposing that the candidate receptor expression would be higher in CHO-K1 cells compared to CCL39 cells.

To confirm the identity of the receptor, a set of well characterized GPCRs antagonists was used to inhibit TLQP-21 calcium response both in CHO-K1 and O-342 cells. The most effective antagonist was SB290157, a compound described as a C3aR1 antagonist (*Ames et al., 2001*). This result was further validated by siRNA screening in CHO-K1 cells. Of the 63 siRNAs tested against the 21 candidate genes, the only gene knockdown that consistently reduced TLQP-21 response was achieved with siRNAs targeting C3aR1.

Recombinant receptor expression in human embryonic kidney (HEK) 293 cells further demonstrated that both the hamster and rat C3aR1 receptors conferred responsiveness to TLQP-21. Finally, direct biochemical evidence showing TLQP-21 binding to membrane preparations containing recombinantly expressed C3aR1 further supported the hypothesis that TLQP-21 is a selective natural agonist of chemoattractant C3aR1 in rodents.

Afterwards, Cero *et al.* (2014) confirmed C3aR1 as the target of TLQP-21. By photoaffinity labelling experiments, TLQP-21 was shown to crosslink with a protein of approximately 55 kDa, corresponding to the nominal molecular weight of C3aR1, in CHO and 3T3L1 cells. The presence of the C3aR1 antagonist SB290157 reduced the crosslinking efficiency, indicating that SB290157 competes with TLQP-21 for the binding to the receptor. To provide additional evidence that TLQP-21 targets C3aR1, a β-arrestin recruitment assay was performed in HTLA cells transfected with human C3aR1. The half-maximal effective concentration (EC_{50}) for mouse TLQP-21 was approximately 3 times lower than that of C3a, while the EC_{50} for the human TLQP-21 was 22 times lower than that of C3a, indicating that mouse and human TLQP-21 are active toward human C3aR1, although with different potency.

Nuclear magnetic resonance (NMR) experiments showed that TLQP-21 is an unstructured peptide. However, when incubated with cells expressing C3aR1, TLQP-21 underwent a folding-upon-binding transition, adopting a well-defined α-helical conformation. This structural transition did not occur in the presence of $C3aR1^{-/-}$ cells and was inhibited upon treatment of 3T3L1 cells with the C3aR1 antagonist SB290157.

In conclusion, this study reinforced the finding by Hannedouche *et al.* (2013), confirming C3aR1 as the target of the VGF-derived peptide TLQP-21.

gC1qR

gC1qR/p33, originally isolated from Raji cells (*Ghebrehiwet et al., 1994*), is a multi-compartment protein expressed both intracellularly and on the cell surface, with a unique capacity to recognize in a specific manner diverse ligands, including microbial, cellular and plasma proteins (*Ghebrehiwet and Peerschke, 2004*). gC1qR is expressed in endothelial cells, lymphocytes, phagocytic cells and smooth muscle cells (*Peerschke et al., 2004*). It binds the globular heads of the complement factor C1q, inducing a varied range of biological responses, which include inositol-1,4,5-trisphosphate (IP_3) production and generation of pro-coagulant activity on platelets; chemotaxis on mast cells, eosinophils and fibroblast; generation of anti-proliferative response on B and T

cells; and enhancement of phagocytosis and superoxide production by neutrophils (*Ghebrehiwet et al., 2001*).

Chen *et al*. (2013) identified gC1qR as the receptor for TLQP-21 using chemical crosslinking combined with mass spectrometry (MS) analysis. Western blotting showed a band when the crosslinker-peptide complex was applied on membrane proteins from adult rat brains and spinal cord. Additional experiments using avidin monomeric column and MS analysis identified the ~30 kDa band as gC1qR.
A panel of siRNAs against gC1qR was used to corroborate the identity of the receptor. siRNAs significantly reduced both the protein levels of gC1qR and the number of macrophage cells responding to TLQP-21 analysed by live cell calcium imaging. Moreover, incubation of macrophages with neutralizing gC1qR monoclonal antibodies resulted in the reduction of the response to TLQP-21.
Finally, to examine the role of gC1qR in pain modulation, gC1qR antibody was applied to the site of nerve ligation in a rat model of PSNL. Application of the antibody against gC1qR delayed the onset of hypersensitivity caused by TLQP-21, suggesting that macrophages stimulated with TLQP-21 via gC1qR initiate hyperexcitation of sensory neurons.

All these studies demonstrate the existence of different cell membrane receptors for TLQP-21 in rodents, although up to now little is known about the molecular mechanism by which this peptide exerts its action. Improving knowledge about the intracellular pathway activated by TLQP-21 and the identification of the human TLQP-21 receptor could help to improve the treatment of different human disorders, including obesity and diabetes.

2.5. Intracellular calcium homeostasis

The calcium ion (Ca^{2+}) is a very versatile second messenger involved in a variety of cellular processes, including gene regulation, muscle contraction, proliferation and cell death. Ca^{2+} was recognized for the first time as a carrier of signals in 1883, when Ringer demonstrated its importance in the contraction of isolated frog hearts (*Ringer, 1883*). Unlike other second-messenger molecules, Ca^{2+} cannot be metabolized, so cells regulate Ca^{2+} intracellular levels through the simultaneous interplay of four main processes: i) Ca^{2+} influx from the extracellular space across the plasma membrane (PM) into the cytoplasm; ii) Ca^{2+} extrusion from the cytoplasm across the PM; iii) Ca^{2+} release from intracellular stores into the cytosol; and iv) Ca^{2+} sequestration into the intracellular stores (*Bootman and Lipp, 2001; Clapham, 1995; Möller, 2002*). In resting cells, the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is maintained at 100 nM, compared with the 2 mM found extracellularly. However, when cells are stimulated $[Ca^{2+}]_i$ increases to levels of 1 μM and more.

2.5.1. Ca^{2+} influx mechanisms

Cells use different types of Ca^{2+} influx channels, that can be classified on the basis of their activation mechanisms into three main groups: i) voltage-operated Ca^{2+} channels (VOCs); ii) receptor-operated Ca^{2+} channels (ROCs); and iii) store-operated Ca^{2+} channels (SOCs) (*Bootman and Lipp, 2001; Möller, 2002*).

Voltage-operated Ca^{2+} channels (VOCs)

VOCs, largely utilised by excitable cell types such as muscle and neuronal cells (*Bootman and Lipp, 2001*), are the primary mediators of depolarization-induced Ca^{2+} entry (*Simms and Zamponi, 2014*). VOCs are heteromultimers composed by different subunits (α_1, $\alpha_2\delta$, β, γ), with the α_1 subunit forming the ion conduction pore and the others serving to modulate channel gating. The α_1 subunit pore is formed by four homologous domains (I-IV), with six transmembrane α-helices (S1-S6) in each. Several isoforms for each subunit have been identified, and their combination gives rise to different types of VOCs with distinct pharmacological characteristics.

Receptor-operated Ca^{2+} channels (ROCs)

ROCs are rapidly opened by the binding of an external ligand to the extracellular domain of the channel. This type of channels is prevalent on secretory cells and at nerve terminals (*Bootman and Lipp, 2001*). Well-known ROCs include *N*-methyl-D-aspartate (NMDA) receptor, nicotinic acetylcholine (nAch) receptor and ATP receptors.

Store-operated Ca^{2+} channels (SOCs)

SOCs are Ca^{2+} channels located on the PM that are activated by any procedure that empties the Ca^{2+} stores within the ER (*Parekh and Putney, 2005*). It is now accepted that store-operated Ca^{2+} entry (SOCE) requires members of two families of proteins: the *st*romal-*i*nteracting *m*olecule (STIM) proteins, that functions as the sensor of Ca^{2+} levels within the ER, and the Orai proteins, which are the pore-forming channel subunits (*Hewavitharana et al., 2007*). STIM are single-pass transmembrane proteins located in the membrane of the ER, that bind Ca^{2+} through a pair of low-affinity EF hand binding domains. Upon depletion of Ca^{2+} stores, Ca^{2+} dissociates from the EF hand domains, causing a conformational change and oligomerization of STIM. STIM multimers translocate to region of the ER in close proximity with the PM where they activate Orai Ca^{2+} channels (*Smyth et al., 2010; Verkhratsky and Parpura, 2014*). Replenishment of the stores results in the dissociation of STIM from Orai and in the inactivation of Ca^{2+} release-activated Ca^{2+} (CRAC) current.

2.5.2. Ca^{2+} extrusion mechanisms

Cells utilize two main mechanisms to extrude calcium ions and return the $[Ca^{2+}]_i$ to baseline levels (*Morgans et al., 1998*). One is the Na^+/Ca^{2+} exchanger, a high capacitance calcium antiporter, located on the PM, which passively use the Na^+ gradient to extrude Ca^{2+} from the cytoplasm (*Herchuelz et al., 1998; Möller, 2002*). The other is the plasma membrane Ca^{2+} ATPase (PMCA), a transport protein that hydrolyse one ATP molecule for each Ca^{2+} ion pumped outside.

2.5.3. Ca^{2+} release mechanisms

The release of Ca^{2+} from the ER into the cytosol is primarily mediated by the IP_3 receptors (IP_3Rs) (*Möller, 2002*), which are composed of four subunits that assemble into both homo- and hetero-tetrameric structures (*Foskett et al., 2007; Saleem et al., 2014*). IP_3Rs are the link between extracellular signals and the initiation of cytosolic Ca^{2+} signals (*Saleem et al., 2014*). The binding of a ligand to the specific receptor on the PM leads to the activation of phospholipase C (PLC), that catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into IP_3. IP_3 is water soluble and diffuses into the cytosol where it binds to IP_3R causing the release of Ca^{2+} from the ER into the cytoplasm.

A second type of intracellular Ca^{2+} releasing channels located on the ER are the ryanodine receptors (RyRs), which are expressed in excitable cell types, such as muscle and neurons. RyRs, that are present in three different isoforms, are so called because of their high affinity for the plant alkaloid ryanodine (*Bootman and Lipp, 2001*).

2.5.4. Ca^{2+} sequestration mechanisms

The last mechanism that cells utilize to regulate $[Ca^{2+}]_i$ is the sequestration of Ca^{2+} ions into the intracellular stores of the ER (*Movsesian and Schwinger, 1998*). This process is mediated by the sarcoplasmic/endoplasmic Ca^{2+} ATPase (SERCA) located on the surface of the ER, that uses ATP to pump Ca^{2+} ions into the ER, where they are sequestered by high concentrations of specialized buffer molecules, like calsequestrin (*Clapham, 1995*).

3. Aim

Obesity is a major health problem that is becoming a global epidemic (*Loh et al., 2015*). Being overweight or obese is the main risk factor for type 2 diabetes, and both obesity and diabetes are important independent risk factors for the development of other chronic disorders, including cardiovascular disease and cancer.
Current weight loss therapies targeting the reduction of food intake have proven to be relatively ineffective, and the ability to reduce the risk of obesity-related complications is limited (*Loh et al., 2015*). Understanding how the CNS mechanisms underlying eating behaviour are dysregulated is necessary in order to develop effective therapeutics for this epidemic conditions (*Farr et al., 2016*).
Many central and peripheral factors have been implicated in the regulation of energy homeostasis (*Schneeberger et al., 2014*). The integration of these signals within the hypothalamus results in the generation of specific and coordinated physiological responses aimed at regulating energy balance through the modulation of appetite and energy expenditure.
A novel gene, highly expressed in the hypothalamus, that has been proposed to play an important role in the regulation of food intake and body weight is *vgf*. Previous *in vivo* studies revealed that $VGF^{-/-}$ mice are thin, small, hypermetabolic and hyperactive compared to their wild-type littermates (*Hahm et al., 1999*), suggesting an anabolic function for *vgf* and its derived peptides. However, data from subsequent studies utilizing peptides derived from VGF did not support this concept. In particular, TLQP-21 ($VGF^{556-576}$) was demonstrated to prevent the development of obesity induced by hypercaloric diet in mice (*Bartolomucci et al., 2006*), and to reduce food intake in Siberian hamsters (*Jethwa et al., 2007*). All together these data indicate that TLQP-21 exerts a catabolic action, and thus it could be a lead compound to develop agonists and/or antagonists as new therapeutic tools for the treatment of obesity and other human disorders, including diabetes and pain.
The interest for TLQP-21 as a potential drug target is increasing also thanks to the recent discovery of a number of binding sites for the peptide. TLQP-21 was found to bind to adipocytes membranes (*Possenti et al., 2012*) and to a binding site on the surface of CHO cells (*Cassina et al., 2013*). Recently, also the receptors for the

complement components C3a (C3aR1) and C1q (gC1qR) have been identified as a possible binding sites for TLQP-21 (*Hanneddouche et al., 2013; Chen et al., 2013*).

Despite the effects of TLQP-21 have been well characterized, its molecular mechanisms at the cellular level are still an object of debate. Thus, since TLQP-21 is emerging as a novel target for obesity-associated disorders, diabetes, neuropathic pain, and other human pathologies, the purpose of this study was to better investigate, from a pharmacological point of view, the intracellular signalling pathway activated by the peptide-receptor interaction.

To this aim, the biological activity of TLQP-21 will be tested in different immortalized cell lines, including: i) CHO, a cell line derived from the ovary of the Chinese hamster; ii) RAW264.7, a murine macrophage cell line; iii) N9, a microglia murine cell line; and iv) the embryonic mouse hypothalamic cell lines N-38, N-41 and N-42. Cells will be treated with TLQP-21, at different times and concentrations, and different cellular functions will be evaluated.
Using the intracellular calcium mobilization assay, a fluorimetric test widely used for intracellular free calcium measurement of agonist-stimulated and antagonist-inhibited calcium signalling, in a first series of experiments it will be detected the ability of different TLQP-21 concentrations to induce an increase of intracellular calcium levels. Then, in TLQP-21-responsive cells, experiments aimed to identify the mechanisms of action of the peptide will be carried out using known antagonists and inhibitors of several signalling molecules.
A second series of experiments, which require the use of Western blotting, will be performed to investigate the effects of TLQP-21 treatment on phosphorylation levels of different signalling molecules.

In conclusion, this study will allow us to characterize the intracellular signalling pathways activated by TLQP-21 in distinct cellular systems. Furthermore, the results obtained from our research will be of great relevance to develop new drugs for the treatment of human pathologies.

4. Materials and methods

4.1. Peptides

TLQP-21 and LRPS-21 (**Table 1**) were synthesized by conventional solid phase synthesis and then purified on a C18 reversed phase column.

Peptides	
TLQP-21	TLQPPASSRRRHFHHALPPAR
LRPS-21	LRPSHTRPAHQSFARPLHRPA

Table 1: TLQP-21 and LRPS-21 amino acid sequences

4.2. Cell cultures

CHO cells were cultured in HAM'S F12 Medium (Sigma Aldrich) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Euroclone).
The murine microglial cell line N9 (*Corradin et al., 1993*) were grown in Iscove's Modified Dulbecco's Medium (IMDM) (Sigma Aldrich) supplemented with 5% heat-inactivated FBS.
N-38, N-41 and N-42 murine hypothalamic cell lines and RAW264.7 murine macrophage cell line were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich) supplemented with 10% heat-inactivated FBS.
Cells were cultured onto 100 mm tissue culture dish (Euroclone) and all the media were supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (all Euroclone). Cells were grown under standard culture conditions at 37°C in a 5% CO_2 humidified incubator. The cells were always used at less than 15 passages.

4.3. Intracellular calcium mobilization assay

Cells were plated at different density (15.000 cells/well for N-38, N-41 and N-42; 20.000 cells/well for CHO and N9; 40.000 cells/well for RAW264.7) into black walled clear bottom 96-well plates (Greiner) and cultured up to 90-100% of confluence. Before assay, the medium was removed and cells were incubated in dark conditions with 100 µl of Hank's Balanced Salt Solution (HBSS) containing 20 mM HEPES, 2.5 mM Probenecid and FLUO-4 No Wash (NW) dye mix (Molecular Probes) at 37°C

and 5% CO_2 for 40-45 minutes. Fluorescence was monitored every 0.5 s for the 20 s preceding and the 60 s following the stimulation using the multilabel spectrophotometer Victor[3] (Perkin Elmer) (excitation, 485 nm; emission, 535 nm). The instrument calculates the fluorescence of resting cells and subsequently stimulates the same cells dispensing 25 µl of the solution into the well by an automated injector system without interrupting fluorescence measurements. The change in fluorescence recorded corresponds to the change in intracellular calcium levels after treatment.
TLQP-21 and LRPS-21 were diluted in HBSS solution and injected into the wells. When indicated, antagonists and inhibitors were added at different times before the end of the incubation with FLUO4-NW: 2 µM thaspigargin (TG), 20 min; 2 µM Cyclosporine A (CsA), 15 min; 10 µM U73122, 10 min; 75 µM 2-Aminoethoxydiphenyl borate (2-APB), 15 min; 10 µM SKF-96365, 20 min; 10 µM YM-58483, 20 min; and 1 mM EGTA, 30 min (all Sigma-Aldrich).
All the experiments were performed at 37°C and the values (F) were normalized against the baseline acquired at the time of the stimulation (F_0).

4.4. Western blot

Cells were plated 24 h before time course experiments in 35 mm dishes at 70% of confluence. After three washes with medium without serum, cells were serum-starved for 1 h. Time course experiments (10 µM TLQP-21 for 0, 1, 2, 5, 10, 15 and 30 min) were performed and after quick removal of the medium the reaction was stopped by placing the dish on ice and adding 100 µl of ice-cold lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM EDTA) containing a protease inhibitor cocktail and PhosphoStop inhibitor cocktail (Roche Diagnostic). Cells were harvested and equivalent amounts of cell extracts (corresponding to approximately 200.000 cells) were run on NuPAGE precast 4-12% gradient gels (Invitrogen) and transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham). After staining with Ponceau S to verify uniformity of protein load/transfer, membranes were blocked with 5% milk powder in phosphate-buffered saline/0.1% Tween-20 (PBS-T) for 1 h at room temperature (RT), and then incubated overnight at 4°C with the following primary

antibodies diluted 1:1000 in 5% BSA: anti-phospho-Akt Ser473, anti-phospho-ERK1/2 Thr202/Tyr204, anti-phospho-PKC Ser660, anti-phospho-PLCγ1 Tyr783 (Cell Signalling) and anti-β-actin (Sigma-Aldrich). Incubation with peroxidase-coupled secondary antibodies (1:5000; Amersham) was performed for 1 h at RT. Immunoreactivity was developed by enhanced chemiluminescence (ECL) method (Amersham). Two parallel gels were run for each experiment and with probes for different anti-phosphorylated protein antibodies as indicated, avoiding stripping protocols. Densitometry analysis was performed using Image J software and the data were normalized against β-actin.

4.5. Statistical analysis

Values are expressed as mean ± SEM. The statistical significance of differences between groups was evaluated with Tukey-Kramer's t-test for multiple comparisons, preceded by the analysis of variance (ANOVA). A P value of less than 0.05 was considered significant.

5. Results

5.1. Effects of TLQP-21 on intracellular calcium levels in different cell types

Ca^{2+} is a highly versatile second messenger involved in the activation of several intracellular signalling pathways. Starting from this observation, we first assessed whether TLQP-21 was capable to stimulate an increase in the intracellular Ca^{2+} levels in different immortalized cell lines in order to operate a first screening of the mechanisms of action activated by the peptide.
To this aim, cells were incubated *in vitro* with increasing concentration of TLQP-21, ranging from 1 nM to 10 μM. **Figure 1** shows the dose response curves for TLQP-21 and LRPS-21, a scramble control peptide, in the different cell lines tested.

As shown in **Figure 1A**, TLQP-21 evoked a significant increase in intracellular Ca^{2+} levels in CHO cells (EC_{50}: 0.85 μM), as previously described by Cassina *et al.* (2013). In our experimental setting, 10 nM was the lowest concentration of TLQP-21 that induced a significant increase of $[Ca^{2+}]_i$ compared to the control group (*$p<0.05$). Intracellular Ca^{2+} levels rose when cells were stimulated with TLQP-21 in the concentration interval of 500 nM – 10 μM (**$p<0.01$; ***$p<0.001$), apparently reaching a plateau phase for concentrations higher than 5 μM. By comparison, LRPS-21 tested at the same concentrations did not stimulate Ca^{2+} levels in CHO cells, thereby confirming the specificity of TLQP-21 action.

TLQP-21 caused a significant rise of intracellular Ca^{2+} levels also in RAW264.7 cells (EC_{50}: 0.36 μM) (**Figure 1B**). In particular, TLQP-21 in the concentration interval 1 nM – 100 nM did not induce any significant increase of intracellular Ca^{2+} levels compared to the control group. However, 500 nM TLQP-21 effectively stimulated an increase of intracellular Ca^{2+} levels (***$p<0.001$), and higher concentrations of TLQP-21 were even more effective, with stimulation of intracellular Ca^{2+} reaching a plateau at the 10 μM concentration (***$p<0.001$). In RAW264.7 cells, LRPS-21 did not induce any increase of intracellular Ca^{2+} levels at every concentration tested.

Figure 1C shows the effect of TLQP-21 treatment on intracellular Ca^{2+} levels in N9 microglial cells. At the concentration interval of 1 nM – 100 nM, TLQP-21 did not

stimulate any increase of intracellular Ca^{2+} levels. However, in the concentration interval 500 nM – 10 µM, TLQP-21 induced an elevation of $[Ca^{2+}]_i$ that was dose-dependent (EC_{50}: 0.96 µM), and was statistically significant compared to the control group (***$p<0.001$). By contrast, intracellular Ca^{2+} levels were not stimulated by LRPS-21, confirming again the specificity of TLQP-21.

Finally, both TLQP-21 and LRPS-21 did not induce a significant increase of intracellular Ca^{2+} levels in N-38, N-41 and N-42 cells (**Figure 1D-F**), indicating the absence of a binding site for TLQP-21 in these hypothalamic cell lines.

5.2. Effects of repeated stimulation with TLQP-21 on intracellular calcium levels

In a second series of experiments, we investigated whether TLQP-21-responsive cells could respond to repeated TLQP-21 stimulations given at 5 minutes' intervals from each other (**Figure 2**). Keeping into considerations the EC_{50} values of TLQP-21 in CHO, RAW264.7 and N9 cells, we decided to stimulate cells with 1 µM TLQP-21.

In CHO cells (**Figure 2A**), levels of intracellular Ca^{2+} increased sharply upon the first challenge with 1 µM TLQP-21, whereas 5 minutes later a second application of 1 µM TLQP-21 was ineffective in increasing cell fluorescence, and also the third challenge, 5 minutes later, was ineffective. Cells were fully responsive to a final challenge with 10 µM ATP to check for cell viability.

Figure 2B shows the effect of repeated TLQP-21 treatments on intracellular Ca^{2+} levels in RAW264.7 cells. The first administration of 1 µM TLQP-21, as expected, induced a robust increase of $[Ca^{2+}]_i$. However, 5 minutes later a second application of 1 µM TLQP-21 stimulated only a blunted increase in intracellular Ca^{2+} levels, whereas no effects were observed when TLQP-21 was applied for the third time. 10 µM ATP, applied after 5 minutes from the last stimulation with TLQP-21, induced a robust increment in cell fluorescence, indicating that cells were still viable.

Also in N9 cells, intracellular Ca^{2+} levels increased significantly after the first administration of 1 µM TLQP-21 (**Figure 2C**). The second stimulation with the peptide given 5 minutes later induced a moderated increase in cell fluorescence, that was still present, but to a lower extent, when 1 µM TLQP-21 was administered a third time 5 minutes later. A final administration of 10 µM ATP to examine for cell viability was not affected.

In order to investigate the possibility that 5 minutes' intervals were not sufficient for cells to recover from the first administration of TLQP-21, in a different experiment the second stimulation with the peptide was applied 30 minutes after the first one (**Figure 3**).

In CHO (**Figure 3A**) and RAW264.7 (**Figure 3B**) cells, 1 μM TLQP-21 given 30 minutes after the first administration failed to stimulate an increase of intracellular Ca^{2+} levels, indicating a possible desensitization mechanism of the receptor after repeated stimulations with the peptide.

In N9 cells (**Figure 3C**), the second stimulation with 1 μM TLQP-21 applied 30 minutes after the first one, induced a blunted but significant increase of intracellular Ca^{2+} levels, suggesting that in this cells TLQP-21 receptor undergoes a less complete desensitization process upon TLQP-21 stimulation.

5.3. Effects of TLQP-21 on the release of calcium from intracellular organelles

It is known that Ca^{2+} stores, such as ER and mitochondria, dynamically participate in the generation of Ca^{2+} signals into the cytoplasm (*Verkhratsky et al., 1998*). We have therefore studied the possible implication of these intracellular organelles in the increase of $[Ca^{2+}]_i$ induced by TLQP-21 in responsive cells.

The involvement of mitochondria was investigated using CsA, an inhibitor of the mitochondrial permeability transition pore (mPTP). The incubation of CHO cells for 15 minutes with 2 µM CsA did not modify the increase of intracellular Ca^{2+} levels induced by 1 µM TLQP-21 (**Figure 4A**). The same effect was observed also in RAW264.7 (**Figure 4B**) and in N9 cells (**Figure 4C**), in which the increase of intracellular Ca^{2+} induced by 1 µM TLQP-21 was not significantly modified by the 15-minute incubation with 2 µM CsA.

To evaluate the possibility that Ca^{2+} released from the ER could also be involved, we measured the effects of TLQP-21 in the presence of TG, which inhibits the Ca^{2+}-ATPase pump responsible for sequestering Ca^{2+} in the ER and depletes the store by irreversibly preventing its refilling.
As shown in **Figure 4A**, 20 minutes of incubation with 2 µM TG were effective in blunting the TLQP-21-mediated increase of intracellular Ca^{2+} with a reduction of about 52.41 ± 4.14% (***$p<0.001$) compared to TLQP-21 maximal effects.
Figure 4B and **4C** show the effects of the pretreatment with TG for 20 minutes on TLQP-21 activity in RAW264.7 and N9 cells, respectively. In both these cell lines, 2 µM TG induced a reduction of intracellular Ca^{2+} mobilization induced by 1 µM TLQP-21, that was of 62.86 ± 1.48% (***$p<0.001$) in RAW264.7 (**Figure 4B**) cells and of 81.84 ± 1.84% (***$p<0.001$) in N9 microglial cells (**Figure 4C**).

5.4. Effects of TLQP-21 on phospholipase C-dependent calcium release

In many cellular systems, PLC activation and subsequent IP_3 production is the intracellular transduction pathway regulating the release of Ca^{2+} from the ER.
To ascertain whether TLQP-21 induces Ca^{2+} mobilization through a PLC-dependent mechanism, cells were incubated for 10 minutes with 10 µM U73122, an amino steroid that is reported to behave as a specific inhibitor of PLC (*Bleasdale et al., 1990*) and is widely used to test the involvement of PLC in signalling pathways.

Results show that U73122 induced a strong reduction, about 82.81 ± 1.82% (***p<0.001), of 1 µM TLQP-21 stimulation of intracellular Ca^{2+} mobilization in CHO cells (**Figure 5A**). In RAW264.7 cells the incubation with 10 µM U73122 caused a partial, approximately 37.61 ± 6.73% (***p<0.001), but significant decrease in TLQP-21-induced Ca^{2+} release from the ER (**Figure 5B**). Also in N9 cells the ability of 1 µM TLQP-21 to increase $[Ca^{2+}]_i$ was lowered of 57.44 ± 6.23% (***p<0.001) after the 10-minute treatment with U73122 (**Figure 5C**).

As a further confirmation, cells were preincubated for 15 minutes with 75 µM 2-APB, which rapidly inhibits IP_3R-mediated Ca^{2+} release from the ER.
As shown in **Figure 5A**, treatment with 2-APB strongly reduced TLQP-21 activity, causing a reduction of intracellular Ca^{2+} mobilization of about 74.91 ± 1.65% (***p<0.001) in CHO cells. The TLQP-21-mediated increase of $[Ca^{2+}]_i$ was diminished also in RAW264.7 and N9 cells, where the preincubation with 75 µM 2-APB caused a reduction of 65.35 ± 3.29% (***p<0.001) and 62.07 ± 8.22% (***p<0.001), of TLQP-21 stimulation, respectively (**Figure 5B and 5C**).

5.5. Effects of TLQP-21 on the Store-Operated Calcium Entry

Reportedly, depletion of Ca^{2+} from the ER causes the activation of STIM proteins that, translocating into junctions formed by the ER and the PM, activate the highly calcium-selective Orai channels to balance $[Ca^{2+}]_i$ (*Soboloff et al., 2012*).
To assess the role of this pathway in TLQP-21 mechanism of action, cells were treated with a STIM-mediated Ca^{2+} inhibitor (SKF-96365), an inhibitor of Orai channels (YM-58483) or an extracellular Ca^{2+} chelator (EGTA) before the stimulation with 1 µM TLQP-21.

In CHO cells, we observed a significant reduction in TLQP-21-mediated intracellular Ca^{2+} mobilization after the treatment with the inhibitors (**Figure 6A**). In particular, we obtained a reduction of 34.41 ± 7.47% (**$p<0.01$) after the preincubation with 10 µM SKF-96365, of 26.72 ± 4.86% (*$p<0.05$) after the treatment with 10 µM YM-58483, and of 42.61 ± 12.72% (*$p<0.05$) after the treatment with 1 mM EGTA.

Figure 6B shows the effect of the preincubation with 10 µM SKF-96365, 10 µM YM-58483 and 1 mM EGTA in RAW264.7 macrophages. The treatment with these inhibitors caused a reduction of intracellular Ca^{2+} mobilization induced by TLQP-21 stimulation of 41.00 ± 5.69% (***$p<0.001$), 26.22 ± 4.49% (*$p<0.05$) and 70.58 ± 4.79% (***$p<0.001$), respectively.

The increase of $[Ca^{2+}]_i$ was reduced by the treatment with SKF-96365, YM-58483 and EGTA also in N9 cells (**Figure 6C**). In particular, incubation with 10 µM SKF-96365 caused a drop in TLQP-21-mediated Ca^{2+} response of about 42.88 ± 3.50% (***$p<0.001$), effect that was induced also by 10 µM YM-58483, causing a reduction of 59.03 ± 6.00% (***$p<0.001$). The treatment of N9 cells with the extracellular Ca^{2+} chelator EGTA lowered the rise in $[Ca^{2+}]_i$ only 36.72 ± 8.75% (**$p<0.01$).

5.6. Effects of TLQP-21 on the phosphorylation levels of intracellular signalling molecules in CHO cells

In order to investigate the molecular targets activated by TLQP-21, we have measured the effects of TLQP-21 treatment on phosphorylation of intracellular signalling effectors in CHO cells (**Figure 7**).

After 1 hour of serum starvation, cells were exposed to 10 μM TLQP-21 for 0-30 minutes. TLQP-21 induced a transient and significant dephosphorylation of phospho-PLCγ1 (**Figure 7A**), that occurred between 1 and 10 minutes from the stimulation with the peptide. After 1 minute from the exposure to TLQP-21, the level of phospho-PLCγ1 was reduced by 50.92 ± 11.18% (**$p<0.01$), reaching a nadir of about 70.76 ± 4.63% (***$p<0.001$) after 2 minutes, and remaining significantly lower than basal (36.69 ± 5.33%; *$p<0.05$) until the end of the 30-minute treatment.

TLQP-21 caused also a prompt increase of phopsho-Akt/PKB, as shown in **Figure 7B**. Akt/PKB levels rose approximately of 191.18 ± 23.65% between 1 and 5 minutes from the stimulation (**$p<0.01$; ***$p<0.001$), remained significantly higher until 15 minutes (136 ± 16.34%; *$p<0.05$), and returned to basal levels at the end of the stimulation with TLQP-21.

Figure 7C shows the effect of TLQP-21 treatment on phosphorylation levels of PKC. Phopsho-PKC increased about 66.07 ± 8.87% (**$p<0.01$) over basal after 2 minutes of stimulation and the increase lasted until 15 minutes from TLQP-21 administration (54.86 ± 2.44%; **$p<0.01$).

Moreover, TLQP-21 administration transiently stimulated a robust phosphorylation of ERK1/2 (**Figure 7D**). After 2 minutes from the stimulation, phospho-ERK1/2 levels significantly increased by 150.33 ± 13.72% (***$p<0.001$), reached a peak at 5 minutes (211.77 ± 7.44%; ***$p<0.001$) and thereafter returned to basal at the end of the treatment with TLQP-21.

6. *Discussion*

Despite our knowledge of TLQP-21 activities is continuously expanding, its molecular mechanisms of action at the cellular level are still object of debate. Creating models to develop new analogs of TLQP-21 could be clinically important, since this peptide and its receptor could be an important target to counteract several human disorders, including obesity and diabetes.

TLQP-21 is one of the many bioactive products derived from post-translational cleavage of the C-terminal region of VGF, a pro-peptide involved in neurologic and metabolic disorders (*Bartolomucci et al., 2011*). TLQP-21 was initially identified in rat brain and further immunolocalized in gastric tissues (*Bartolomucci et al., 2006; Brancia et al., 2010*). TLQP-21 has been implicated in the regulation of energy balance, nociception, gastric function and several other physiologic functions (*Bartolomucci et al., 2006; Jethwa et al., 2007; Rizzi et al., 2008; Sibilia et al., 2010*). Although recent studies identified different receptors as the targets for TLQP-21 (*Chen et al., 2013; Hannedouche et al., 2013*), its cellular mechanisms of action remain largely unknown.

Our results demonstrate that TLQP-21 is a robust extracellular signal capable of promoting a Ca^{2+}-mediated transduction signal in different cell lines. Ca^{2+} is a highly versatile second messenger involved in several cellular processes. In immune cells, Ca^{2+} plays an important role for the activation of the immune response. In particular, in macrophages, Ca^{2+} was shown to regulate cytokines and nitric oxide (NO) synthesis, as well as phagocytosis (*Vaeth et al., 2015*). In microglial cells, intracellular Ca^{2+} influences multiple cellular functions, including enzyme activities and cytokines production (*Hoffmann et al., 2003*). Moreover, Ca^{2+} was demonstrated to be necessary to increase 2-arachidonoylglycerol (2-AG) production in microglia (*Färber and Kettenmann, 2006*). Ca^{2+} is an important intracellular signal also for cells involved in reproduction. It has been shown that ovarian cells regulate their rate of proliferation in response to changes in extracellular Ca^{2+} concentration *(McNeil et al., 1998*), and that mammalian fertilization is accompanied by oscillation in egg cytoplasmic Ca^{2+} concentrations that are responsible for driving the early developmental program (*Miao et al., 2012*).

In our experimental setting, TLQP-21 efficiently stimulated, in a dose-dependent fashion, a rapid and transient intracellular Ca^{2+} increase in CHO, RAW264.7 and N9 cells, and these data are consistent with those reported in recent studies on ovary and primary immune cells (*Cassina et al., 2013; Chen et al., 2013*). By contrast, no calcium response was found in hypothalamic cells N-38, N-41, and N-42 after TLQP-21 stimulation, despite TLQP-21 higher concentrations were found in the hypothalamic region (*Brancia et al., 2010*). The treatment with the scramble peptide LRPS-21 did not induce an increase in intracellular Ca^{2+} levels in the cell lines tested, confirming the specificity of TLQP-21 actions.

Repeated exposure to TLQP-21 resulted in a reduced response both in CHO, RAW264.7 and N9 cells, probably indicating a decrease in number of receptors available or a decreased activity of intracellular signalling pathways involved. Once activated, receptors are removed from the cell surface by internalization and often, after a prolonged stimulation, are completely lost from the cell surface (*Gainetdinov et al., 2004*). Alternatively, prolonged activation of the receptor can lead to an attenuation of signal transduction, a phenomenon common to many cells and called desensitization (*Countaway et al., 1992; Zastrow, 2002*). Two major types of desensitization have been characterized: 1) homologous, or agonist-specific desensitization and 2) heterologous, or agonist-nonspecific desensitization (*Chuang et al., 1996; Guo et al., 1997*). Homologous desensitization occurs when the agonist desensitizes the subsequent response of the same receptor only, with no effect on other receptor systems. Conversely, heterologous desensitization occurs when repeated stimulation of a receptor by an agonist results in desensitization of both the stimulated receptor and other receptors operating through similar mechanisms.
Interestingly, TLQP-21 induced an homologous desensitization to subsequent TLQP-21 administrations given at 5 or 30 minutes' intervals, but not to ATP. These data are consistent with those reported in rat primary macrophages and cerebellar granule cells (*Chen et al., 2013; Severini et al., 2008*), and suggest that the intracellular transduction pathway for TLQP-21 is distinct from that of ATP.

The rapid increase of intracellular Ca^{2+} concentration by an extracellular ligand is generated through a variety of cell-surface receptors, including G_q protein-coupled receptors and receptor tyrosine kinases (RTKs) (*Berridge et al., 2000; Seurin et al., 2003*).
GPCRs signalling is usually regulated through a desensitization mechanism that involves members of the family of G protein-coupled receptor kinases (GRKs). GRKs selectively phosphorylate agonist-activated receptors that, in turn, are bound by arrestins, which inhibit coupling of the receptor to the G protein preventing further stimulations (*Gainetdinov et al., 2004; Mnie-Filali and Piñeyro, 2012*).
On the other hand, internalization is the major regulator of RTKs signalling. Activated RTKs are endocytosed by clathrin-mediated and clathrin-independent mechanisms, controlling in this way the duration and intensity of downstream signalling events (*Goh and Sorkin, 2013; Li et al., 2008*). However, in certain cellar systems, RTKs were shown to activate signalling pathways even from within vesicles following their internalization (*Lemmon and Schlessinger, 2010*).

A universal and well established mechanism for Ca^{2+} signalling is the release from the intracellular compartments (*Clapham, 2007)*, where Ca^{2+} ions are held in reserve. Intracellular organelles, like endoplasmic reticulum and mitochondria, cross-talk each other to regulate basic cell biological processes (*Xu et al., 2015*), such as Ca^{2+} signalling, ion and lipid exchange.
In our cellular systems, TLQP-21 stimulation induced an increase of cytoplasmic Ca^{2+} levels that was caused by Ca^{2+} release from intracellular stores. In particular, our studies indicate that in CHO, RAW264.7 and N9 cells Ca^{2+} is released from the ER, as demonstrated by the treatment with TG, an inhibitor of SERCA, that depletes the ER store by preventing its refilling (*Thastrup et al., 1989*), whereas Ca^{2+} release from the mitochondria did not appear involved. These data are consistent with those reported in rat pituitary and insulinoma cells, in which TLQP-21 and TLQP-62, respectively, induced an increase of intracellular Ca^{2+} levels by opening the intracellular Ca^{2+} storage (*Petrocchi-Passeri et al., 2013; 2015*).

Our results also indicate that Ca^{2+} mobilization stimulated by TLQP-21 is triggered by the activation of PLCs, since pretreatment of the cells with the antagonist U73122 induced a significant reduction of intracellular Ca^{2+} release in CHO, RAW264.7 and N9 cells. U73122 was demonstrated to inhibit the hydrolysis of phosphatidylinositol (PPI) to IP_3 leading to a decrease in free cytosolic Ca^{2+} (*Yule and Williams, 1992*).
By inducing Ca^{2+} release from endoplasmic reticulum through PLC activation, GPCRs and RTKs provide a link between extracellular signals and the intracellular domain (*Seurin et al., 2003*). Reportedly, primary PLCs (β and γ) are activated directly by receptor stimulation: PLCβ subtypes are activated by GPCRs, whereas PLCγ subtypes are activated by RTKs (*Yang et al., 2013*).
It was previously reported that TLQP-21 administration did not affect PLCγ phosphorylation in GH3 pituitary cells (*Petrocchi-Passeri et al., 2013*). In the present study, we have detected a rapid and transient dephosphorylation of PLCγ1 in CHO cells after TLQP-21 stimulation. PLCγ1 is present in most cell types, and requires the phosphorylation of tyrosine residues (Tyr771, Tyr783 and/or Tyr1253) for being activated (*Sekiya et al., 2004*). Consequently, our data suggest that TLQP-21-Ca^{2+} response is mediated by the binding of the peptide with a G_q-coupled receptor rather than a RTK, since the treatment with TLQP-21 caused an inhibition of PLCγ1 phosphorylation.

PLCs are known to cleave PIP2 into diacylglycerol (DAG) and IP_3. DAG activates protein kinase C (PKC), while the binding of IP_3 to its receptor triggers the release of Ca^{2+} ions from the ER.
We have shown that pretreating the cells with 2-APB, an IP_3R antagonist, the increase of intracellular Ca^{2+} levels induced by TLQP-21 was greatly reduced, further confirming that the binding of the peptide with its receptor causes the release of Ca^{2+} from the endoplasmic reticulum.

It is known that Ca^{2+} depletion from the ER activates slower Ca^{2+} entry from outside the cell (*Hewavitharana et al., 2007*). STIM and Orai proteins are required for this store-operated Ca^{2+} entry process, that is responsible for replenishing Ca^{2+} lost from the ER (*Gregory et al., 2001; Liou et al., 2005; Roos et al., 2005*). The SOCE

mechanisms is common in microglia and represents a specific phenomenon of long-lasting activation following strong stimulation of the receptor, that is thought to be important for regulating various aspects of microglial activation (*Verkhratsky and Parpura, 2014*). It was demonstrated that the chronic increase in $[Ca^{2+}]_i$ mediated by SOCE is necessary for the release of NO and selected cytokines and chemokines (*Hoffmann et al., 2003*). Other studies have proposed a role for SOCE also in macrophages, where Ca^{2+} influx is supposed to be linked to the production of reactive oxygen species (ROS) and to be necessary for the engulfment of apoptotic cells (*Desai and Leitinger, 2014*).
Consistently, in CHO, RAW264.7 and N9 cells, we have found an inhibition of TLQP-21-mediated Ca^{2+} increase following the treatment with SKF-96365 and YM-58483, two specific STIM- and Orai-blockers. Moreover, also the treatment with the extracellular Ca^{2+} chelator EGTA only partially reduced the increase of intracellular Ca^{2+} concentration induced by the peptide. These data suggest that extracellular Ca^{2+} is not necessary for TLQP-21 activity since Ca^{2+} mobilization is still observed, and confirm that Ca^{2+} entry from the extracellular environment is a consequence of the release of Ca^{2+} from the endoplasmic reticulum, as previously demonstrated in primary macrophages (*Chen et al., 2013*).

Evidence suggests that SOCE activity could be modulated by the interaction between Orai and Ca^{2+}-activated potassium (KCa) channels (*Chen et al., 2016*). Indeed, it has been shown that persistent Ca^{2+} influx into the cell through Orai channel requires an electrical driving force that is sustained by efflux of K^+ ions *(Lin et al., 2014*).
These data are in agreement with our recent demonstration that a novel derivative of TLQP-21 was capable to trigger the activation of a Ca^{2+}-dependent K^+ outward current in N9 microglial cells (*Rivolta et al., 2017*). The opening of K^+ channels is important for the hyperpolarization of the PM that allows the persistence of the driving force for Ca^{2+} to entry from the extracellular environment to replenish the intracellular Ca^{2+} stores.

As previously reported, Ca^{2+} and DAG work together to activate PKC, which in turn regulate numerous cellular responses, including gene expression and protein secretion.

Our results show that, in CHO cells, TLQP-21 induced an increase of PKC phosphorylation and, afterwards, of ERK1/2 phosphorylation, effects that were also found in rat pituitary cells and in mouse 3T3-L1 adipocytes (*Petrocchi-Passeri et al., 2013; Possenti et al., 2012; Cero et al., 2017*).
The increase of cytosolic Ca^{2+} concentration stimulates also the activation of numerous signalling molecules. In particular, we have demonstrated that in CHO cells TLQP-21 induced a rapid phosphorylation of Akt/PKB, a protein kinase that functions as a critical regulator of cell survival and proliferation in response to extracellular signals (*Song et al., 2005).*

The data presented here clearly provide new evidences for the downstream effects of the binding of TLQP-21 to its receptor in ovary, macrophage and microglial cells.
Our results demonstrate that TLQP-21 probably activates PLCβ that in turn produces DAG and IP_3 as second messengers. DAG activates PKC, that in sequence stimulates ERK1/2 phosphorylation, while IP_3 induces intracellular Ca^{2+} release from the ER with the subsequent Ca^{2+} entry form outside the cell. Moreover, the increase of intracellular Ca^{2+} concentration induced by TLQP-21 causes Akt/PKB phosphorylation (**Figure 8**).
These data suggest that TLQP-21 activates a receptor that should belong to the family of G_q-coupled receptors, as previously proposed (*Petrocchi-Passeri et al., 2013*). In contrast to previously reported data (*Hannedouche et al., 2013*), TLQP-21 efficiently stimulated intracellular Ca^{2+} levels without the need of an ATP priming.
Our results also corroborate the previous assumption that fragments of VGF, once released from vesicles through the regulated secretory pathway, interact with a G protein-coupled receptor inducing release of Ca^{2+} from intracellular stores (*Salton et al., 2000*).

Recently, C3aR1 (*Hannedouche et al., 2013*) and gC1qR (*Chen et al., 2013*) have been indicated as possible receptors for TLQP-21 in rodents.
C3aR1 is a G_i-coupled receptor (*Klos et al., 2013; Zaidi and Ali, 2007*) that was identified as the target of TLQP-21 in CHO cells using RNA sequencing and siRNA screening (*Hannedouche et al., 2013*). Moreover, in these cells, TLQP-21 was found

to induce an increase in intracellular Ca^{2+} levels, though ATP priming was necessary to observed a robust signal.
Afterwards, C3aR1 was confirmed as receptor for TLQP-21 through a photoaffinity labelling method (*Cero et al., 2014*). Moreover, NMR experiments showed that TLQP-21 underwent to a folding-upon-binding transition, adopting a well-defined conformation, when incubated with cells expressing C3aR1.
Using chemical crosslinking combined with MS analysis (*Chen et al., 2013*), gC1qR was also identified in membrane fractions of rat brain as the receptor for TLQP-21. gC1qR is the receptor for the globular head of the complement factor C1q, and does not bind the C3a factor.

Given the identification of C3aR1 and gC1qR as possible binding sites for TLQP-21, we have attempted the pharmacological characterization in different cellular systems. Understanding the mechanism of action of TLQP-21 could be of great relevance since this neuropeptide is supposed to play important roles in the CNS and peripheral tissues.

In conclusion, our research provides additional evidences about the molecular mechanism of action of TLQP-21, and suggests the existence of different receptors for the peptide in rodent cells. Improving knowledge about the intracellular pathway activated by TLQP-21 and the identification of the human receptor could help to improve the treatment of several human disorders, including obesity and diabetes.

7. *Figures*

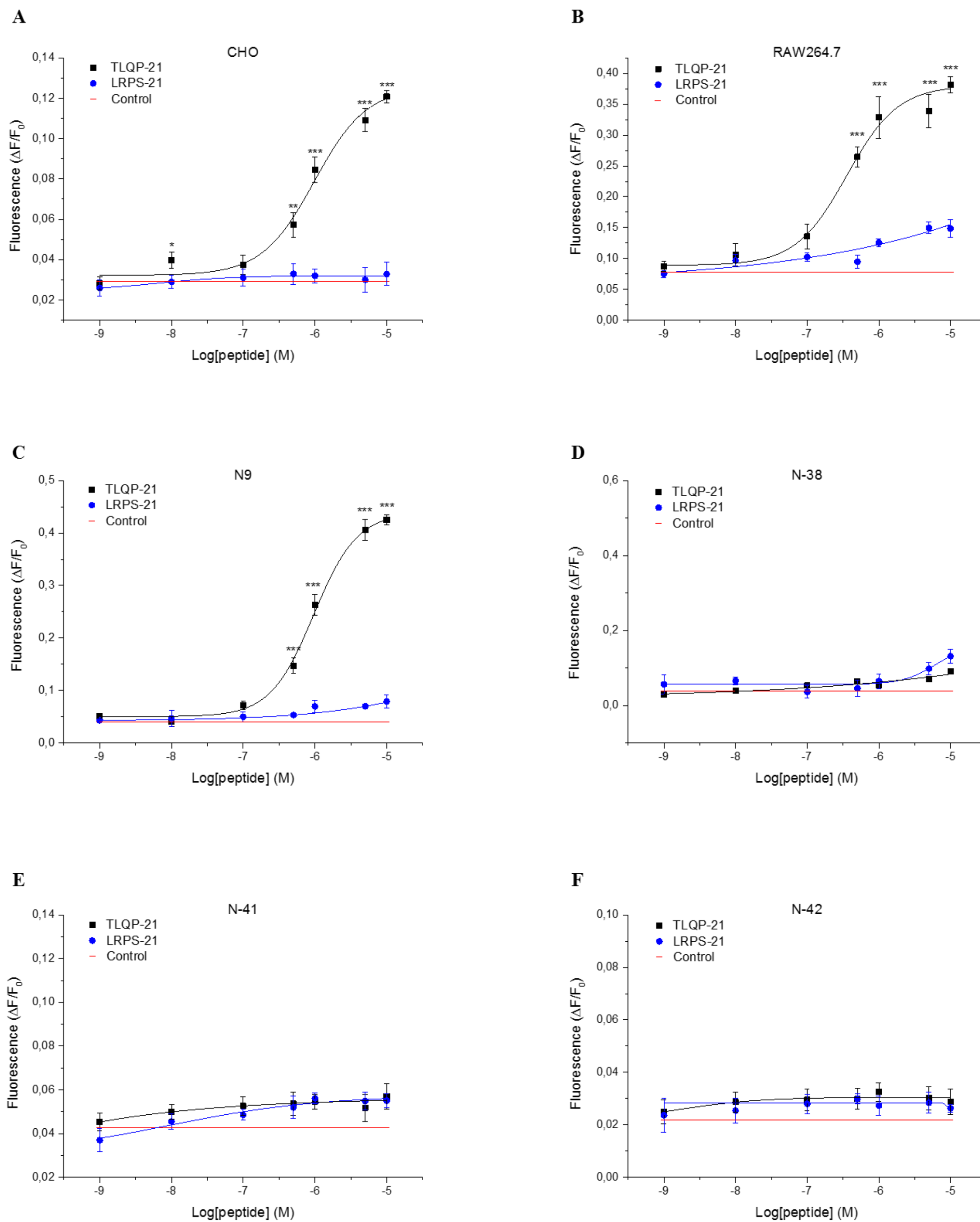

Figure 1. TLQP-21 stimulation of intracellular Ca^{2+} levels in different cell lines. Cells were loaded with FLUO-4 NW and fluorescence emissions were measured every 0.5 s for the 60 s following the injection of the stimuli. TLQP-21 was applied at concentrations ranging from 1 nM to 10 µM. Ca^{2+} mobilizing activity of TLQP-21 is plotted in terms of maximal response obtained at each given concentration in CHO (**A**), RAW264.7 (**B**), N9 (**C**), N-38 (**D**), N-41 (**E**) and N-42 (**F**) cells. To ascertain the specificity of the effects, cells were also challenged with 1 nM - 10 µM LRPS-21, a scrambled peptide of TLQP-21. The control group

is plotted as a red line and represents the basal fluorescence measured before the injection of the stimuli. Results are the means ± SEM of measurements obtained in at least 6 different wells for each experiment. All experiments have been repeated at least three times. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs Control.

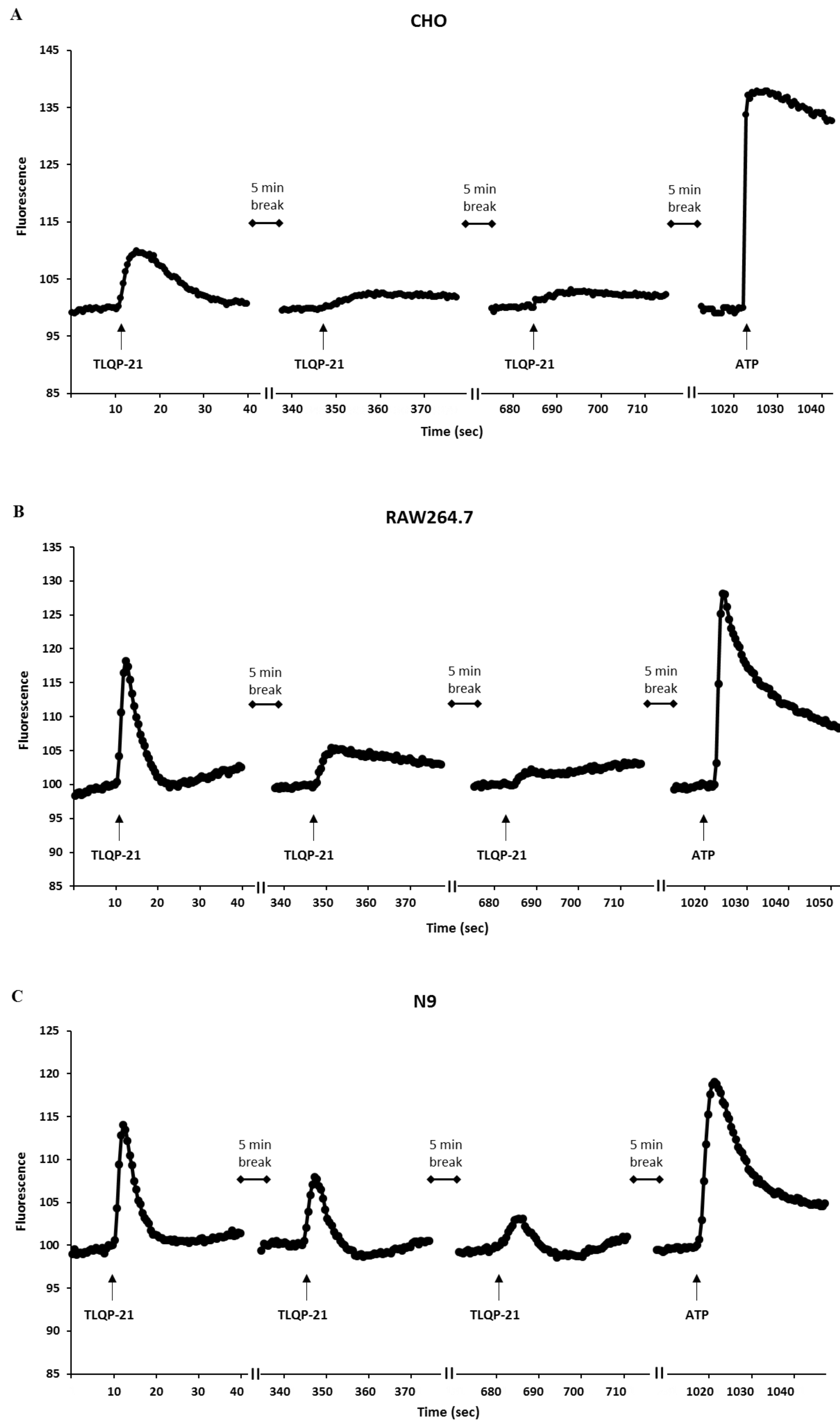

Figure 2. Effects of multiple stimulations with TLQP-21 on intracellular Ca^{2+} levels in CHO, RAW264.7 and N9 cells. Cells were loaded with FLUO-4 NW and fluorescence emissions were measured every 0.5 s for the 60 s following the injection of the stimuli. The

injection of 1 μM TLQP-21 was applied three times at 5 minutes' intervals to CHO (**A**), RAW264.7 (**B**) and N9 (**C**) cells. At the end of the experiment, a stimulation with 10 μM ATP was used to check for cell viability. Results are the means of measurements obtained in at least 6 different wells for each experiment. All experiments have been repeated at least three times.

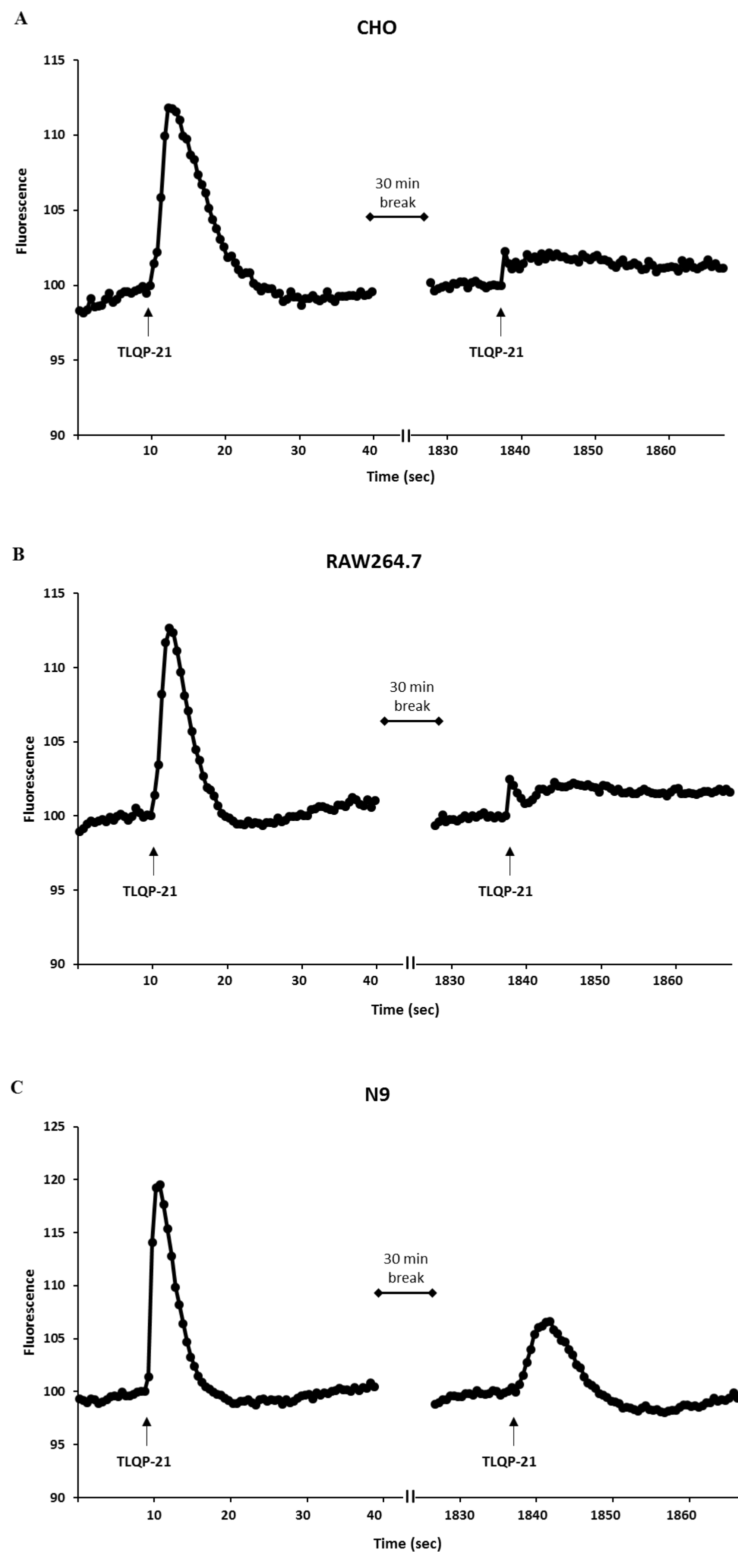

Figure 3. Repeated TLQP-21 stimulation on intracellular Ca^{2+} levels in CHO, RAW264.7 and N9 cells. Cells were loaded with FLUO-4 NW and fluorescence emissions were measured every 0.5 s for the 60 s following the injection of the stimuli. 1 µM TLQP-21 was applied

twice at 30 minutes' interval to CHO (**A**), RAW264.7 (**B**) and N9 (**C**) cells. Results are the means of measurements obtained in at least 6 different wells for each experiment. All experiments have been repeated at least three times.

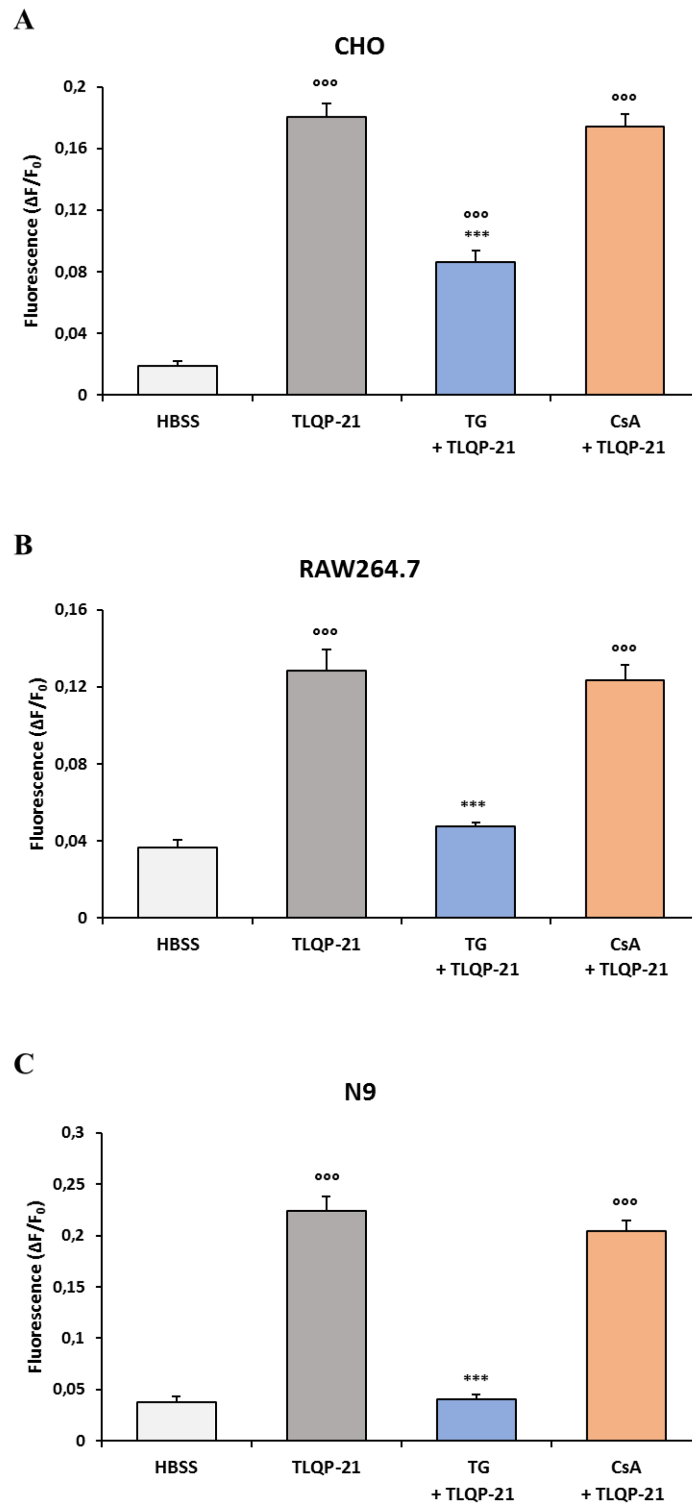

Figure 4. Modulation of intracellular Ca^{2+} levels by TLQP-21 in the presence of CsA and TG in CHO, RAW264.7 and N9 cells. Cells were loaded with FLUO-4 NW and treated with different inhibitors before the stimulation with 1 µM TLQP-21. CsA (2 µM – 15 min), a mPTP

inhibitor, did not affect TLQP-21 stimulation of intracellular Ca^{2+} levels as did TG (2 μM – 20 min), an inhibitor of the ER Ca^{2+}-ATPase, in CHO (**A**), RAW264.7 (**B**) and N9 (**C**) cells. Results are shown as the means ± SEM of measurements obtained in at least 6 different wells for each experiment. All experiments have been repeated at least three times. °°°$p<0.001$ vs HBSS; ***$p<0.001$ vs TLQP-21.

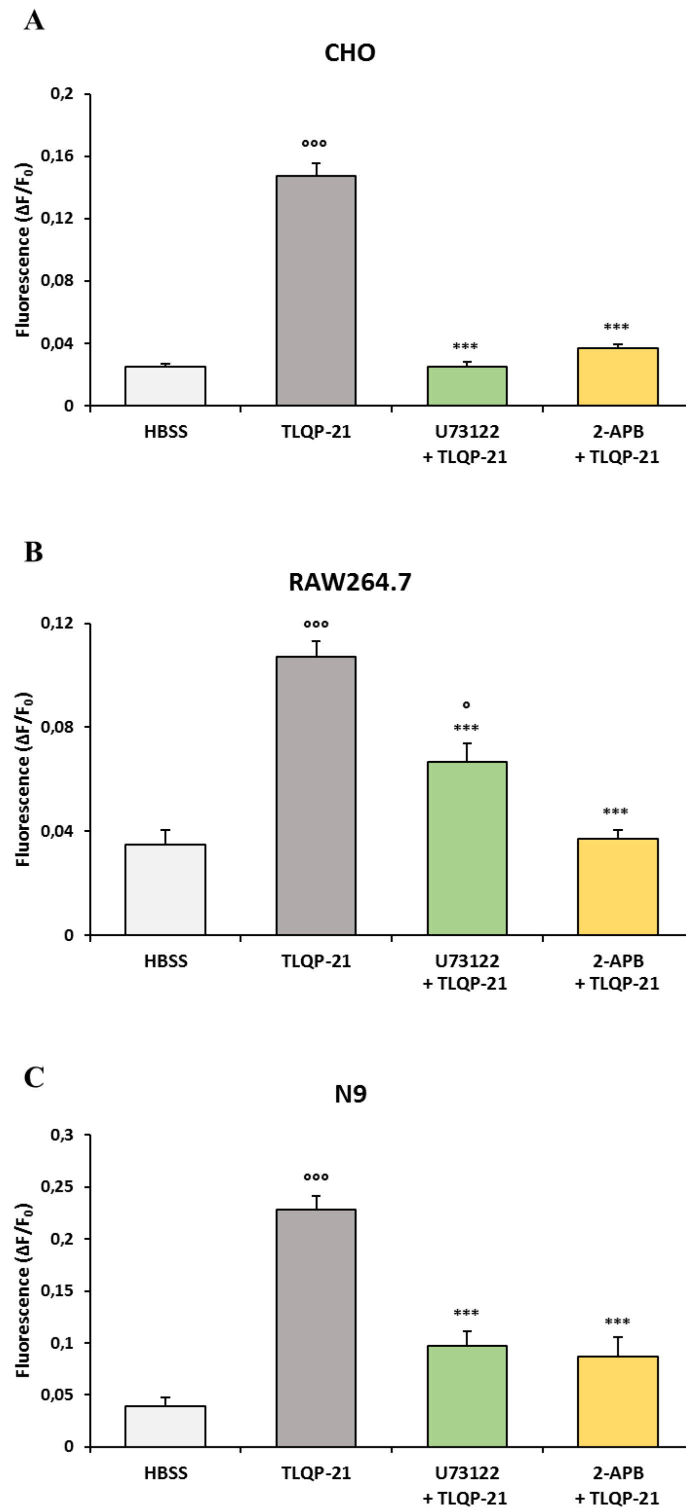

Figure 5. Modulation of intracellular Ca^{2+} levels by TLQP-21 in presence of U73122 and 2-APB in CHO, RAW264.7 and N9 cells. Cells were loaded with FLUO-4 NW and treated with different inhibitors before the stimulation with 1 µM TLQP-21. The PLC inhibitor

U73122 (10 μM – 10 min) and the IP_3R antagonist 2-APB (75 μM – 15 min) significantly reduced TLQP-21 stimulation of intracellular Ca^{2+} mobilization in CHO (**A**), RAW264.7 (**B**) and N9 (**C**) cells. Results are shown as the means ± SEM of measurements obtained in at least 6 different wells for each experiment. All experiments have been repeated at least three times. °$p<0.05$ and °°°$p<0.001$ vs HBSS; ***$p<0.001$ vs TLQP-21.

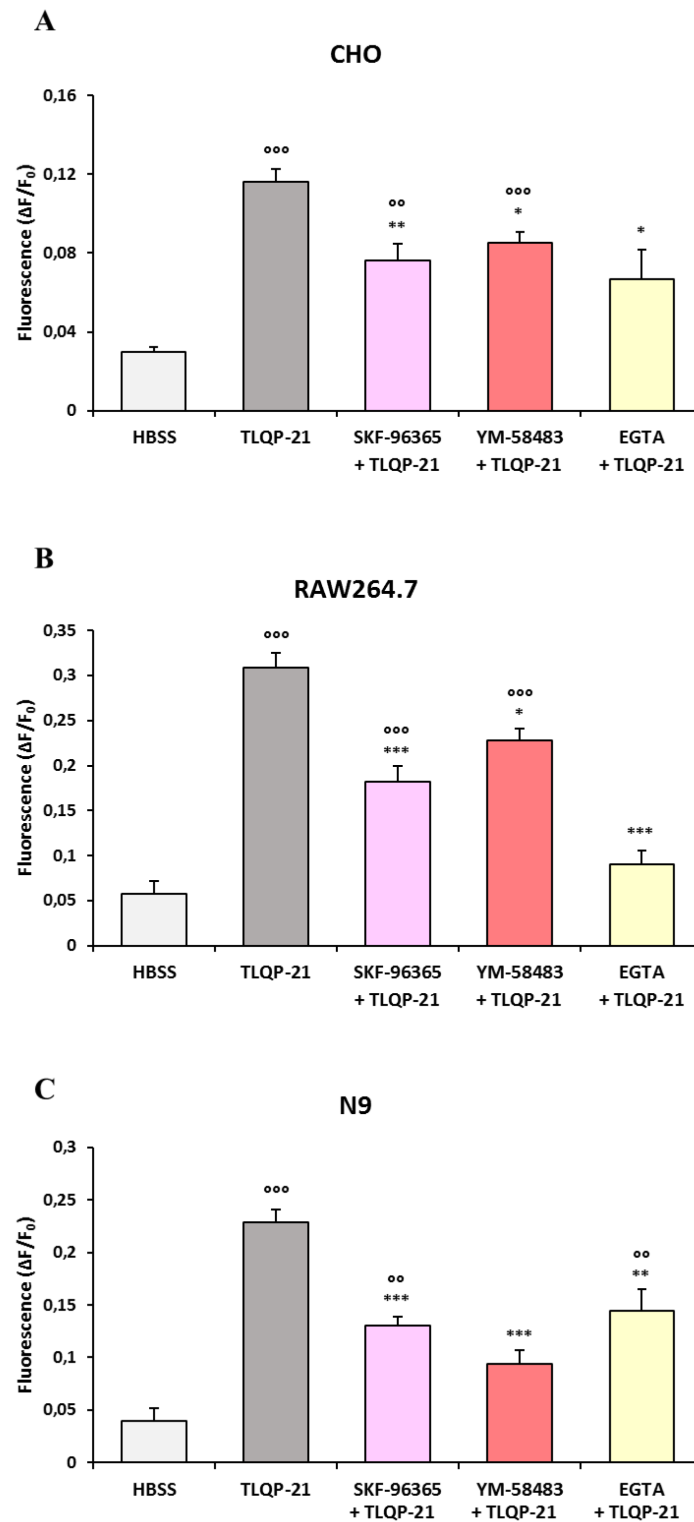

Figure 6. Modulation of intracellular Ca^{2+} levels by TLQP-21 in presence of SKF-96365, YM-58483 and EGTA in CHO, RAW264.7 and N9 cells. Cells were loaded with FLUO-4 NW and treated with different inhibitors before being stimulated with 1 μM TLQP-21. The

inhibitors of the SOCE process, SKF-96365 (10 μM – 20 min), YM-58483 (10 μM – 20 min) and EGTA (1 mM – 30 min), affect significantly TLQP-21 effects in CHO (**A**), RAW264.7 (**B**) and N9 (**C**) cells. Results are the means ± SEM of measurements obtained in at least 6 different wells for each experiment. All experiments have been repeated at least three times. °°$p<0.01$, °°°$p<0.001$ vs HBSS; *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs TLQP-21.

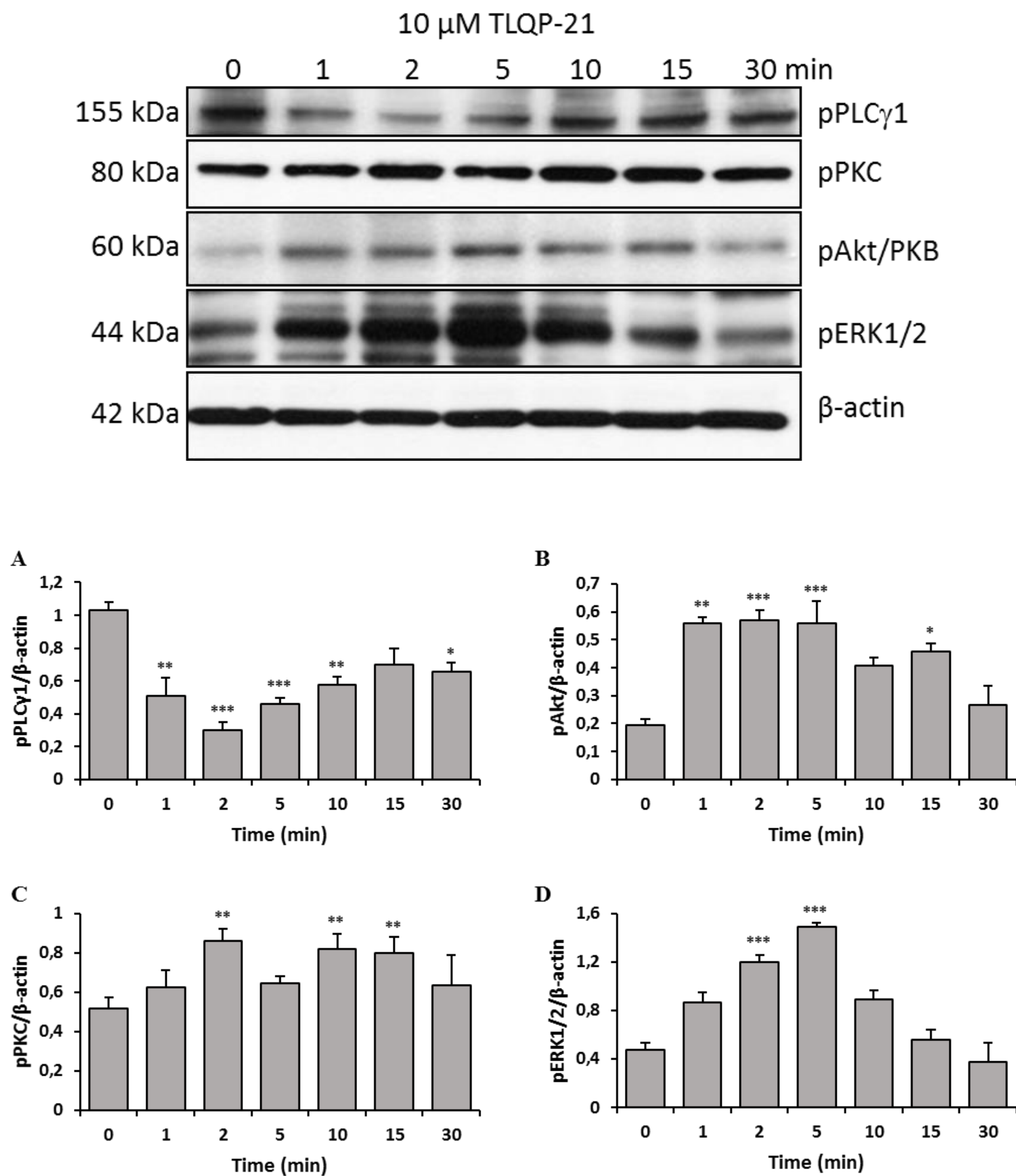

Figure 7. Western blot analysis of relevant phospho-activated proteins in CHO cells. Time course of 10 µM TLQP-21 treatment from 1 to 30 min. Equal amounts of cell lysates were processed for Western blot analysis, using specific antibodies for the phosphorylated (active) forms of signalling molecules. Abbreviations: pPLCγ1, phospho-PLCγ1 (Tyr783); pAkt/PKB, phospho-Akt/PKB (Ser473); pPKC, phospho-PKC (Ser660); and pERK1/2, phospho-ERK1/2 (Thr202/Tyr204). To normalize for loading, blots were also probed with anti-β-actin. Results shown are means ± SEM of at least 5 measurements obtained in five independent experiments. *$p<0.05$, **$p<0.01$ and **$p<0.001$ vs Time 0.

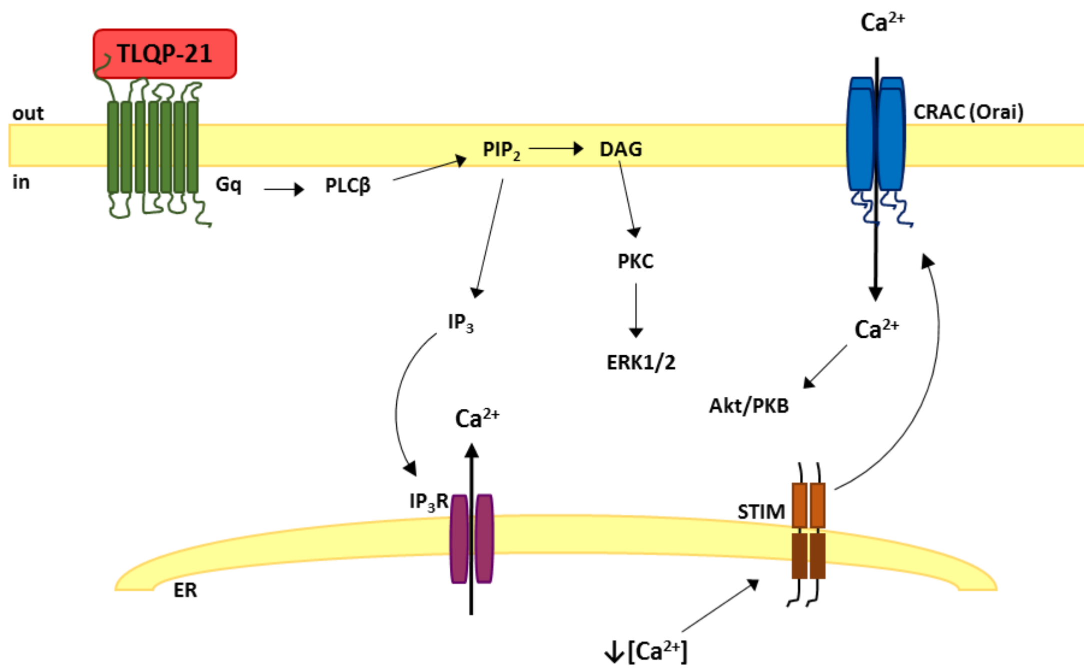

Figure 8. Schematic representation of TLQP-21 intracellular transduction mechanism in CHO, RAW264.7 and N9 cells. TLQP-21, by binding a G_q-coupled receptor, activates PLCβ that in turn produces DAG and IP_3 as second messengers. These molecules activate PKC, that stimulates ERK1/2 phosphorylation, and induce intracellular Ca^{2+} release from the ER and the subsequent Ca^{2+} entry form outside the cell. Phosphorylation of Akt is probably a result of the increase in intracellular Ca^{2+} concentrations.

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