The G-protein-coupled CCK2 receptor associates with phospholipase Cγ1

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Abstract In ElasCCK2 transgenic mice expressing cholecystokinin (CCK2) receptor in acinar cells, pancreatic phenotypic alterations and preneoplastic lesions are observed. We determined whether activation of phospholipase C gamma1 (PLCγ1), known to contribute to the tumorigenesis pathophysiology, could take place as a new signaling pathway induced by the CCK2 receptor. Overexpression and activation of the PLCγ1 in response to gastrin was observed in acinar cells. The possibility that the C-terminal tyrosine 438 of the CCK2 receptor associates with the SH2 domains of PLCγ1 was examined. A specific interaction was demonstrated using surface plasmon resonance, confirmed in a cellular system and by molecular modeling.

Keywords: G-protein-coupled receptor; Phospholipase C; CCK2 receptor; Precancerous condition; Surface plasmon resonance; Gastrin

1. Introduction

We recently demonstrated that transgenic expression of the cholecystokinin (CCK2) receptor (CCK2R), a G-protein-coupled receptor (GPCR), in pancreatic acini of ElasCCK2 mice stimulates pancreatic growth and induces phenotypic changes and tumorigenesis [1]. Molecular mechanisms leading to these effects are unresolved but logically result from the introduction of the CCK2 receptor on a CCK1 receptor background and its activation by post-prandial release of gastrin, its specific ligand. Signaling pathways specific of the CCK2R may contribute to the observed changes. Indeed, it is well established that gastrin modulation of cellular growth, differentiation and adhesion is linked to multiple signaling systems, that depend or not on G-protein coupling, including phospholipase C (PLC)β and PKC activation, Ca^{2+} mobilization and induction of several kinases activity [2].

An additional possibility is that CCK2R intrinsic specificities may also be responsible. Analysis of the CCK1 and CCK2 receptor protein sequences shows the presence of consensus immunoreceptor tyrosine-based inhibition motif (ITIM) [3] in the C-terminal intracellular cytoplasmic tail as well as in the third intracellular loop. Tyrosine residues of the ITIM sequences are phosphorylable candidate residues that may bind SH2 domains signaling molecules and initiate alternate signal transduction cascades. Interestingly, the CCK2R contains one ITIM sequence in the C-terminal intracellular tail that is absent in the CCK1R sequence.

We examined whether this phosphorylable tyrosine residue (Tyr438) would associate with the PLCγ1. Indeed, PLCγ1 has been implicated in pathways leading to cellular morphological changes, migration and proliferation [4]. Several reports also indicate that PLCγ1 is overexpressed in many cancers and may be implicated in the pathophysiology of tumorigenesis [5,6]. Like PLCβs, PLCγ1 acts as an early effector in signal transduction of many growth factors and mitogens, hydrolyzing phosphatidylinositol 4,5-biphosphate (PIP2) in inositol triphosphate (IP3) and diacylglycerol (DAG). Unlike PLCβs, PLCγ1 may have an additionnal pathway to propagate mitogenic signals via its SH2, SH3 and PH domains [4].

In this study, we show that CCK2R activates and associates with PLCγ1. Moreover, data from surface plasmon resonance analysis, mutational analysis and molecular modeling demonstrate that the association implicates the C-terminal Tyr438.

2. Materials and methods

2.1. Immunohistochemistry

ElasCCK2 mice, their controls (non transgenic littermates) and preparation of pancreas for immunohistochemistry studies have previously been described [1,7]. Immunohistochemistry was done on 5 μm tissue sections using an indirect immunoperoxidase protocol with a rabbit polyclonal antibody against PLCγ1 (1:1500, Santa Cruz Biotechnology, Heidelberg, Germany).

2.2. Preparation of isolated acini

Dispersed acini from mice pancreas were prepared by collagenase dissociation and incubated in Krebs–Ringer HEPES medium, pH 7.4, with or without the CCK2R agonist, sulfated [Nle^{11}]human gastrin 13, in the presence of the CCK1R antagonist SR 27897 (1.6 μM) [7] and

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Abbreviations: PLCγ1, phospholipase C gamma1; HA, hemagglutinin-A; CCK, cholecystokinin; GPCR, G-protein-coupled receptor; ITIM, immunoreceptor tyrosine-based inhibition motif

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solubilized in 50 mM Tris buffer, pH 7.4, containing 140 mM NaCl, 1 mM EDTA, 1.5% (w/v) CHAPS (3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate), 0.1% soybean trypsin inhibitor (w/v) and 0.5 mM orthovanadate.

2.3. Surface plasmon resonance analysis

Surface plasmon resonance experiments were performed on a BIAcore 3000 instrument (BIAcore AB). The biotinylated peptides containing the CCK2R ITIM sequences (in boldface) (peptide Y238: FIPGVVMA**VAYGLIS**RELYL, peptide Y390: SYASASVNP**LVYCFM**HRRFRQ, peptide Y438: SIASLSR**LSYTTIS**TLGPG) were synthesized on chlorotrityl resin by automated SPPS according to the Fmoc/tBu chemistry. For the synthesis of the phosphotyrosine peptides, Fmoc-Tyr(PO_3H_2)-OH was used and couplings were performed with HOBt/HBTU/DIPEA (1:1:2). The biotinylated peptides were immobilized on streptavidin-coated carboxymethylated dextran chips [8]. Different concentrations of the PLCγ1 protein corresponding to the two SH2 and the SH3 domains (amino acids 530–850 of rat PLCγ1, Santa Cruz Biotechnology, Heidelberg, Germany) were passed over the immobilized ITIM peptides and kinetic and affinity constants were determined using BIAevaluation software (BIAcore).

2.4. Site-directed mutagenesis and transfection of COS-7 cells

N-terminal hemagglutinin-A (HA)-tagged wild-type (WT) and tyrosine mutated human CCK2R cDNAs were constructed by oligonucleotide-directed mutagenesis [9]. COS-7 cells were transfected with vectors containing the cDNA for the WT or mutated CCK2R, using a modified DEAE–dextran method [10] and incubated with 10 nM gastrin during indicated times, then solubilized for immunoprecipitation and Western blot studies as previously described [11].

2.5. Receptor binding assays

(Thr,Nle)-CCK-9 was conjugated with Bolton–Hunter reagent and radioiodinated as described previously [12]. Transfected COS-7 cells were transferred to 24-well plates, washed with phosphate-buffered saline, pH 6.95, 0.1% BSA and then incubated for 60 min at 37 °C with 60 pM ^{125}I-BH-(Thr,Nle)-CCK-9 in the presence or the absence of competing agonists for binding experiments [10].

2.6. Immunoprecipitation and Western blot analysis

Solubilized acinar proteins (500 μg) were immunoprecipitated with anti-PLCγ1 polyclonal antibody or control IgG (2 μg/ml, Santa Cruz Biotechnology, Heidelberg, Germany), separated by SDS–PAGE, blotted on nitrocellulose membranes and probed with anti-phosphotyrosine (PY20, Transduction Laboratories) or anti-PLCγ1 monoclonal antibody (clone D-7-3, Upstate Biotechnology). Solubilized COS-7 cells proteins (1 mg) were immunoprecipitated with anti-PLCγ1 polyclonal antibody or anti-HA antibody (clone 12CA5, Boehringer Mannheim, IN, USA). Immunoblots were performed with anti-PLCγ1 polyclonal antibody or polyclonal anti-HA antibody (Clontech, Palo Alto, USA). Membranes were incubated with peroxidase-coupled secondary antibodies (1:10,000, Pierce, Bezons, France) and proteins were detected using the enhanced chemiluminescence system (Amersham Biosciences) or ^{125}I-protein A [11].

2.7. Molecular modeling

Available NMR structure of the complex comprising the C-terminal SH2 of PLCγ1 and a phosphopeptide from PDGF receptor (residues 1018–1029) was used as template. The C-terminal part of the CCK2R containing phosphorylated Tyr438 (residues 433–447) was constructed by homology modeling on the basis of the PDGF receptor phosphopeptide [13]. Then, CCK2R fragment containing phosphorylated Tyr438 was docked to C-terminal SH2 domain of PLCγ1. This docking was achieved by manual substitution of the phosphopeptide from PDGF receptor by modelled CCK2R fragment containing phosphorylated Tyr438 in the protein complex. Procedures of energy minimization were then applied. Insight II modules were used (Homology, Discover and Biopolymer; Accelrys, San Diego, CA, USA).

3. Results

As shown in Fig. 1 pancreatic acini expressed PLCγ1. While expression was moderate in control pancreas, we found an increased staining at the inner plasma membrane of ElasCCK2 acini consistent with an overexpression of PLCγ1 in these cells.

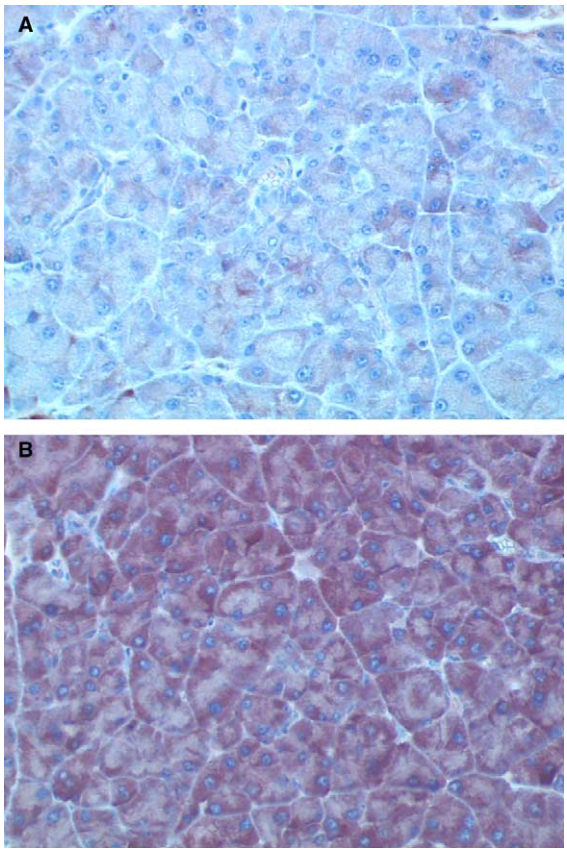

Fig. 1. PLCγ1 expression in exocrine pancreas. A: Immunostaining in control pancreas. B: Immunostaining in ElasCCK2 pancreas (original magnification, 40×).

We next examined whether gastrin treatment would stimulate PLCγ1 phosphorylation in isolated ElasCCK2 pancreatic acini. Western blot analysis of proteins immunoprecipitated with polyclonal anti-PLCγ1 antibody shows that a protein migrating at 143 kDa was phosphorylated following gastrin (Fig. 2A). This protein was absent in negative control experiments using rabbit preimmune serum for immunoprecipitation. Immunoblotting with monoclonal anti-PLCγ1 antibody yielded staining of a single protein migrating at 143 kDa and

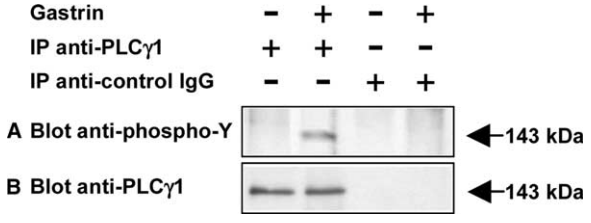

Fig. 2. Gastrin activates PLCγ1 in ElasCCK2 acinar cells. A: Acinar cells were incubated with (+) or without (−) sulfated gastrin (0.1 μM) during 1 min at 37 °C, followed by immunoprecipitation of the cell lysate with polyclonal anti-PLCγ1 antibodies and Western blotting with anti-phosphotyrosine antibodies. Negative control used rabbit preimmune serum for immunoprecipitation. B: The same filters were stripped and reprobed with monoclonal anti-PLCγ1 antibodies. The data shown are representatives of three independent experiments.

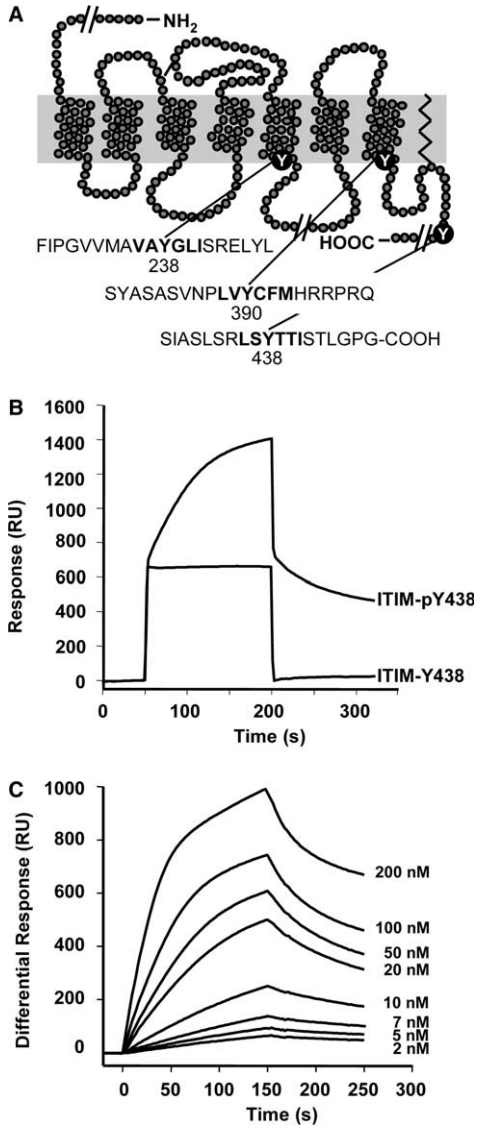

Fig. 3. Surface plasmon resonance analysis of PLCγ1 binding to CCK2 receptor ITIM peptides. A: Schematic representation of the CCK2 receptor. The three phosphorylable tyrosine residues (Y238, Y390, Y438) are localised. The sequences of the synthetic peptides that contain the ITIM sequences (shown in boldface) and that were biotinylated for surface plasmon resonance experiments are indicated. B: Sensorgrams obtained for the association of PLCγ1 (100 nM) using a chip either coupled to C-terminal phosphorylated ITIM peptide of the CCK2 receptor (pY438) or to the same unphosphorylated peptide (Y438). The extent of association is represented by the difference in RU (resonance units) that are measured at the beginning and the end of the PLCγ1 injection. C: Dose-dependent binding of PLCγ1 to the C-terminal phosphorylated ITIM peptide. Differential response represents phosphorylated tyrosine-dependent binding.

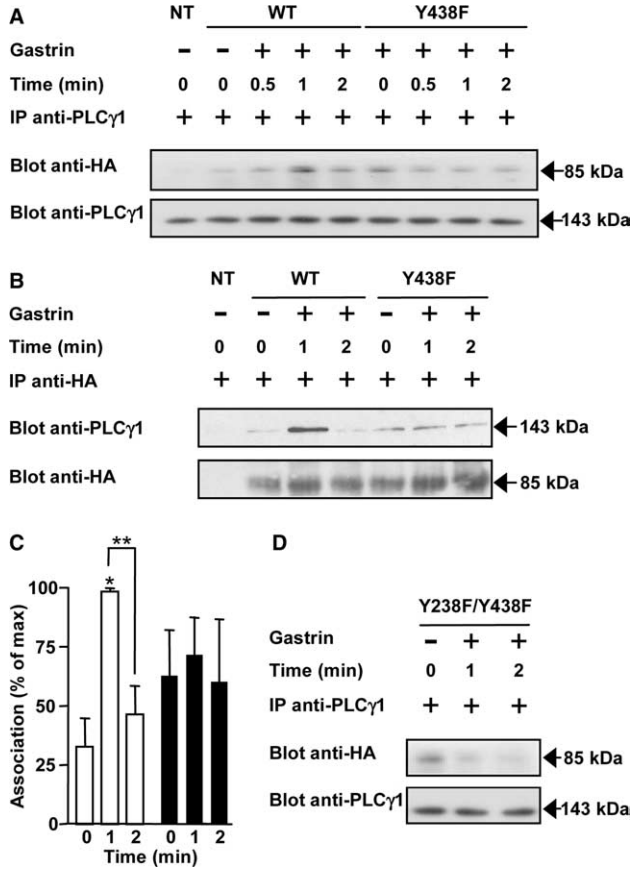

Fig. 4. Interaction between the PLCγ1 and the Tyr438 of the CCK2R. COS-7 cells were transfected or not (NT) with the WT or mutated HA-tagged CCK2R (Y438F), incubated with (+) or without (−) 10 nM sulfated gastrin during indicated times and solubilized. A: After immunoprecipitation with anti-PLCγ1 antibodies, proteins were blotted with anti-HA and anti-PLCγ1 antibodies. B: After immunoprecipitation with anti-HA antibodies, proteins were blotted with anti-PLCγ1 and anti-HA antibodies. C: Densitometric analysis of data shown in B. Results represent means ± S.E.M. of three independent experiments. * and ** significance at P from 0.01 to 0.05 and P from 0.001 to 0.01, respectively, determined using the Student's t test. D: COS-7 cells were transfected with the double mutated HA-tagged CCK2R (Y238F/Y438F), incubated with (+) or without (−) 10 nM sulfated gastrin during indicated times and solubilized. After immunoprecipitation with anti-PLCγ1 antibodies, proteins were blotted with anti-HA antibodies.

allowed its identification as the PLCγ1 (Fig. 2B). These results demonstrate that the CCK2R mediates PLCγ1 activation in ElasCCK2 acinar cells.

The hypothesis of a direct interaction between the CCK2R and the PLCγ1 was then tested using surface plasmonic resonance to assess quantitatively the capacity of the phosphorylated

Table 1
Binding characteristics of the WT HA-tagged and mutated CCK2 receptors

Receptor	K_D1 (nM)	K_D2 (nM)	B_{max} 1 (pmol/10^6 cells)	B_{max} 2 (pmol/10^6 cells)
WT	0.3 ± 0.1	14.4 ± 1.2	1.9 ± 0.7	10.3 ± 0.8
Y438F	0.4 ± 0.1	11.2 ± 1.2	2.6 ± 0.1	15.9 ± 1.7
Y238F/Y438F	0.5 ± 0.1	10.6 ± 1.0	0.5 ± 0.7	7.2 ± 2.1

Binding parameters (K_D and B_{max}) were determined by Scatchard analysis (Kell Radlig software) of binding inhibition experiments of ^{125}I-Bolton-Hunter-(Thr,Nle)-CCK9 on COS-7 cells transfected with WT or mutated CCK2 receptors. Values are means ± S.E.M. from three separate experiments. Each experiment was realized in duplicate.

tyrosine of the three ITIM consensus sequences of the receptor (Fig. 3A) to interact with SH2 domains of the enzyme. A clear interaction between the immobilized C-terminal phosphorylated ITIM peptide containing Tyr438 and PLCγ1 was observed, whereas no binding was found for the unphosphorylated peptide (Fig. 3B) demonstrating that the association specifically depends on the phosphorylated tyrosine. Binding of the PLCγ1 protein was dose-dependent (2–200 nM) (Fig. 3C). Global fitting of these interactions results in an affinity constant of 38 nM and a maximal binding of 985 resonance units. A specific interaction between PLCγ1 and phosphorylated Tyr238 was also measured but affinity and maximal association were lower (not shown). No association was obtained with the phosphorylated Tyr390.

To determine whether the interaction between the CCK2R and PLCγ1 also exists in a cellular context, we performed immunoprecipitation experiments using COS-7 cells transfected with the N-terminal HA-tagged CCK2R. Cells were stimulated with gastrin for various times then lysed and the PLCγ1 was immunoprecipitated from the lysates. Immunoblot with the anti-HA antibody shows that gastrin induced a rapid and transient association of PLCγ1 with the receptor that was maximal within 1 min (Fig. 4A). This result was confirmed by performing immunoprecipitation of the CCK2R followed by anti-PLCγ1 blotting (Fig 4B and C). To test the role of C terminal ITIM site of the CCK2R, and more particularly that of the phosphorylable tyrosine residue present within this motif, we replaced Tyr438 with phenylalanine (Y438F). Binding properties of mutated receptor were similar compared with WT receptor (Table 1). In contrast, interaction with PLCγ1 in response to gastrin was modified. Indeed, while basal association was increased compared to WT, PLCγ1 was unable to interact with the stimulated mutated Y438F CCK2R (Fig. 4A–C). We then tested the hypothesis that increased basal association resulted from an interaction with the ITIM motif located in the third intracellular loop, investigating whether PLCγ1 would associate with a double mutated Y238F/Y438F CCK2R. This mutant exhibited same binding characteristics as WT receptor (Table 1). As shown in Fig. 4D, double mutation did not abolish basal interaction of PLCγ1 with the CCK2R. Taken together, these results demonstrate the essential role of Tyr438 for PLCγ1 association in response to CCK2R activation.

Interactions between PLCγ1 and the CCK2R were further studied using molecular modeling. In the refined model of complex obtained, phosphorylated Tyr438 of the CCK2R is stably inserted into a binding pocket through strong interactions with aminoacids Arg39 and His57 of SH2 domain of PLCγ1 (Fig. 5). These interactions were not found when Tyr438 was not phosphorylated (data not shown). According to the model, interaction of CCK2R with SH2 of PLCγ1 also involves residues at positions −1 (Ser437), +1 (Thr439) and +6 (Leu444) relative to the Tyr438 residue. Those residues interact, respectively, with Glu22, His57 and Arg91 of PLCγ1 SH2 domain (Fig. 5).

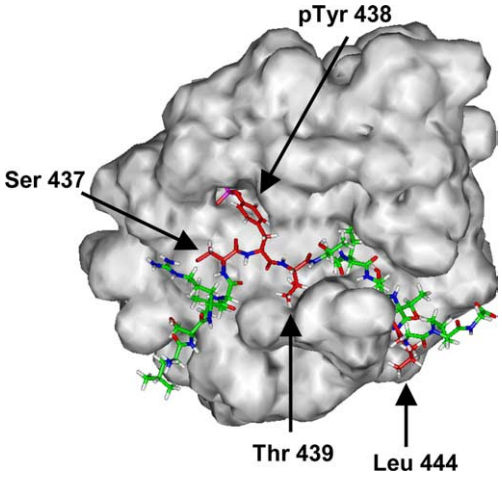

Fig. 5. Interactions between the PLCγ1 SH2 domain and the C terminal part of the CCK2R. Molecular surface of the PLCγ1 SH2 domain is shown in gray. Amino acids of the CCK2R fragment (residues 433–447) interacting with PLCγ1 are indicated in red (arrows).

4. Discussion

The CCK2 receptor is a GPCR that specifically mediates the actions of gastrin via association and activation of heterotrimeric G proteins and activation of many signaling enzymes such as the PLC, the protein kinases C and A as well as other kinases involved in the regulation of mitogenesis and cellular adhesion by growth factors [11,14–16].

Results of the current study show that targeting expression of the CCK2R in pancreatic acinar cells is associated with overexpression of PLCγ1 in these cells. Increased expression of PLCγ1 is known to occur in pathological situations including cancer [6] and kidney diseases [17] as well as in benign hyperproliferative epidermal diseases [18] but paradoxically, regulation of PLCγ1 expression has not been well characterized. In the case of ElasCCK2 mice, overexpression is consistent with efficient activation of the transgenic CCK2R and coupling to the phosphoinositide signaling [7]. This additional signaling pathway may initiate many activities contributing to increased pancreatic growth, phenotypic changes and cancer [1].

Second, our data are the first example of a possible direct interaction between the CCK2R and an intracellular effector, PLCγ1. These are in line with accumulating literature data describing association of GPCRs with a wide variety of proteins other than G proteins [19]. Among these proteins, PLCγ1 has been described to be recruited via its SH2 domains to the C-terminus tail of bradykinin B2 [20] and angiotensin II AT1 receptors [21]. Unlike these receptors, the CCK2R exhibits two consensus binding motifs, identified as ITIM sequences, that bind PLCγ1 in a tyrosine phosphorylation-dependent manner as verified by in vitro surface plasmon resonance studies. Interestingly, a direct interaction with high nanomolar binding affinity, similar to that reported for other effectors binding to ITIM-bearing molecules [22], is observed with the C-terminal motif. This association was also confirmed in cells expressing the CCK2R in response to gastrin activation. The role of the phosphorylable tyrosine residues of the ITIM motifs was assessed using site-directed mutagenesis as an alternative to the demonstration of phosphorylated CCK2R that remains an actual challenge. Mutation of the C-terminal ITIM tyrosine residue abolished the interaction in response to gastrin thus giving biological significance to in vitro data. Moreover,

molecular modeling data are in complete agreement with these experimental results. Our data show that increased basal interaction between PLCγ1 and the CCK2R-Y438F mutant does not result from association to phosphorylated Y238F. Contribution of other parts of the CCK2R, such as rich prolin sequences interacting with the SH3 domain of PLCγ1, or of scaffolding molecules are likely mechanisms to be considered.

Tyrosine phosphorylation of PLCγ1 in response to gastrin was demonstrated resulting in a PLC stimulation mechanism similar to that of classic growth factor receptors such as the epidermal growth factor receptor. While the CCK2R lacks intrinsic tyrosine kinase activity, it activates several kinases, among them Src family kinases are described as essential for B2- and AT1-receptors-stimulated PLCγ1 activation [20,21]. Moreover, arachidonic acid and phosphatidylinositol 3,4,5-triphosphate produced upon stimulation by gastrin of phospholipase A2 [23] and PI3-kinase [14], respectively, may also likely contribute to the activated state of PLCγ1 [24].

In conclusion, PLCγ1 is at the junction of numerous signal transduction pathways and activates different biological responses such as DNA synthesis, transformation, cell movement and migration. Therefore, the demonstration of an upregulation of PLCγ1 in ElasCCK2 acini and of its association with the CCK2 receptor is an important step towards the understanding of the changes of proliferation and differentiation that were observed in the pancreas of ElasCCK2 mice.

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