Biochimica et Biophysica Acta 1823 (2012) 1285–1294

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

The Src kinase Yes is activated in pancreatic acinar cells by gastrointestinal hormones/neurotransmitters, but not pancreatic growth factors, which stimulate its association with numerous other signaling molecules ☆

Veronica Sancho [1], Bernardo Nuche-Berenguer [1], R.T. Jensen *

Digestive Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892-1804, USA

ARTICLE INFO

Article history:
Received 15 February 2012
Received in revised form 25 April 2012
Accepted 14 May 2012
Available online 19 May 2012

Keywords:
Src
Yes activation
Pancreatic acinus
CCK
Signaling
Pancreatic growth factor

ABSTRACT

For growth factors, cytokines, G-protein-coupled receptors and numerous other stimuli, the Src Family of kinases (SFK) play a central signaling role. SFKs also play an important role in pancreatic acinar cell function including metabolism, secretion, endocytosis, growth and cytoskeletal integrity, although the specific SFKs involved are not fully known. In the present study we used specific antibodies for the SFK, Yes, to determine its presence, activation by pancreatic secretagogues or growth factors, and interaction with cellular signaling cascades mediated by CCK in which Yes participates in to cause acinar cell responses. Yes was identified in acini and secretagogues known to activate phospholipase C (PLC) [CCK, carbachol, bombesin] as well as post-receptor stimulants activating PKC [TPA] or mobilizing cellular calcium [thapsigargin/calcium ionophore (A23187)] each activated Yes. Secretin, which activates adenylate cyclase did not stimulate Yes, nor did pancreatic growth factors. CCK activation of Yes required both high- and low-affinity CCK_1-receptor states. TPA-/CCK-stimulated Yes activation was completely inhibited by thapsigargin and the PKC inhibitor, GF109203X. CCK/TPA stimulated the association of Yes with focal adhesion kinases (Pyk2, FAK) and its autophosphorylated forms (pY397FAK, pY402Pyk2). Moreover, CCK/TPA stimulated Yes interacted with a number of other signaling proteins, including Shc, PKD, $p130^{Cas}$, PI3K and PTEN. This study demonstrates that in rat pancreatic acini, the SFK member Yes is expressed and activated by CCK and other gastrointestinal hormones/neurotransmitters. Because its activation results in the direct activation of many cellular signaling cascades that have been shown to mediate CCK's effect in acinar cell function our results suggest that it is one of the important pancreatic SFKs mediating these effects.

Published by Elsevier B.V.

1. Introduction

The Src Family of kinases (SFK) play a central signaling role for growth factors, cytokines and G protein-coupled receptors [1]. The SFKs are also important in the signaling in many cellular processes such as cell secretion, endocytosis, growth, cytoskeletal integrity and apoptosis [1] which are mediated by these stimuli.

In pancreatic acinar cells the SFKs play an important role in enzyme secretion, membrane recycling, endocytosis, apoptosis, protein synthesis in regulating calcium cellular levels, in regulation of PKCs, in pancreatic acinar cell responses to injuries such as pancreatitis and in maintaining cytoskeletal organization [2–8]. Various studies report that of the nine SFKs [1], pancreatic acinar cells possess Yes [4,7,9], Lyn [6,7,9] and in some studies [4,9], but not others [7], pp60Src. Whereas the specific participation of Lyn in various cellular processes stimulated by gastrointestinal hormones/neurotransmitters and pancreatic growth factors in pancreatic acinar cells has been studied in detail [9], there is only limited information about the ability of these stimulants to activate pp60Src and Yes and even less information on their ability to stimulate different cellular pathways that have been

Abbreviations: CCK, COOH-terminal octapeptide of cholecystokinin; TPA, 12-O-tetradecanoylphorbol-13-acetate; SFK, Src family of kinases; PKC, protein kinase C; PYK2, proline-rich tyrosine kinase 2; FAK, focal adhesion kinase; SHC, Src homology 1 domain containing transforming protein; PKD, protein kinase D; CAS, Crk-associated substrate; PI3K, phosphatidylinositol-3-CAKβ, cell adhesion kinase β; PTEN, phosphatase and tensin homolog; Cbl, Casitas B-lineage lymphoma proto-oncogene; RAF, proto-oncogene serine/threonine-protein kinase proto-oncogene; HRP, horseradish peroxidase; HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor 1; bFGF, basic fibroblast growth factor; VIP, vasoactive intestinal peptide; CCK-JMV, CCK-JMV-180; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; 8-Br-cAMP, 8-Bromo-cyclic adenosine monophosphate; IP, immunoprecipitation; Co-IP, co-immunoprecipitation; PLC, phospholipase C; ET, endothelin; AKT, protein kinase B; GFX, GFX109203X, PKC inhibitor; MAPK/ERK, mitogen-activated protein kinase; CRK, CT10 Regulator of Kinase; NFκβ, nuclear factor-kappa B; PKA, protein kinase A; CCK_1 receptors, CCK type 1 receptors (high affinity for CCK); CCK_2 receptors, CCK type 2 receptors (high affinity for gastrin and CCK)

☆ This work is partially supported by the Intramural Research Program of the NIDDK, NIH.

* Corresponding author at: NIH/NIDDK/DDB, Bldg. 10, Rm. 9C-103, 10 Center Dr. MSC 1804, Bethesda, MD 20892-1804, USA. Tel.: +1 301 496 4201; fax: +1 301 402 0600.
E-mail address: robertj@bdg10.niddk.nih.gov (R.T. Jensen).
[1] Both authors contributed equally to this work.

0167-4889/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.bbamcr.2012.05.015

shown important in mediating the effects of these stimulants in pancreatic acinar cell function.

To address this question in the case of the SFK, Yes, in the present study we sought to determine whether activation of the SFK, Yes, is involved in mediating the cellular signaling of gastrointestinal hormones/neurotransmitters and of various gastrointestinal growth factors, which are known to alter pancreatic acinar cell function. We also sought to determine in pancreatic acinar cells whether these stimulatory effects have possible interactions with other signaling proteins that are reported to participate in different cellular processes. The pancreatic acini are a very useful model to examine these issues as they have been reported to be highly responsive to many gastrointestinal hormones/neurotransmitters and growth factors which alter pancreatic acinar function and have been used to study effects of these stimulants on cell signaling cascades [3,6,9–12].

2. Materials and methods

2.1. Materials

Male Sprague–Dawley rats (150–250 g) were obtained from the Small Animals Section, Veterinary Resources Branch, National Institutes of Health (NIH), Bethesda, MD. Rabbit anti-phospho-Src family (Tyr416), rabbit anti Src family, rabbit phospho-Pyk2 (Tyr402), rabbit anti-Pyk2, rabbit phospho-FAK (Tyr397), rabbit anti FAK, rabbit anti-protein kinase δ (PKCδ), rabbit phospho-PKCδ (Tyr311), rabbit phospho Shc (Tyr239/240), rabbit anti Shc, rabbit phospho-PKD (Ser744/748), rabbit anti-protein kinase D (PKD), rabbit anti phospho $p130^{Cas}$ (Tyr 410), phospho p85 PI3K (tyr458), rabbit anti-RafA, rabbit anti PTEN, rabbit anti-c-cbl, rabbit monoclonal anti-phospho c-Raf (56A6) antibodies and nonfat dry milk were purchased from Cell Signaling Technology, Inc. Rabbit $p130^{CAS}$ antibody, and anti-rabbit-HRP-conjugate antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal anti-Yes was purchased from BD Biosciences (San Jose, CA). Tris/HCl pH 8.0 and 7.5 were from Mediatech, Inc. (Herndon, VA). 2-Mercaptoethanol, protein assay solution, sodium lauryl sulfate (SDS) and Tris/Glycine/SDS (10×) were from Bio-Rad Laboratories (Hercules, CA). $MgCl_2$, $CaCl_2$, Tris/HCl 1 M pH 7.5 and Tris/Glycine buffer (10×) were from Quality Biological, Inc. (Gaithersburg, MD). Minimal essential media (MEM) vitamin solution, amino acids 100×, Dulbecco's phosphate buffered saline (DPBS), glutamine (200 mM), Tris–Glycine gels, L-glutamine, and fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA). COOH-terminal octapeptide of cholecystokinin (CCK), hepatocyte growth factor (HGF), bombesin, insulin-like growth factor 1 (IGF-1), basic fibroblast growth factor (bFGF), vasoactive intestinal peptide (VIP), endothelin and secretin were from Bachem Bioscience Inc. (King of Prussia, PA). CCK-JMV-180 (CCK-JMV) was obtained from Research Plus Inc., Bayonne, NJ. Epidermal growth factor (EGF), thapsigargin, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and deoxycholic acid were from Calbiochem (La Jolla, CA). Carbachol, insulin, dimethyl sulfoxide (DMSO), 12-O-tetradecanoylphobol-13-acetate (TPA), 8-bromoadenosine 3′5′ cyclic monophosphate sodium (8-Bromo-cAMP), L-glutamic acid, glucose, fumaric acid, pyruvic acid, trypsin inhibitor, HEPES, TWEEN® 20, Triton X-100, GFX (GFX109203X), phenylmethanesulfonylfluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), sucrose, sodium-orthovanadate, sodium azide and CelLytic™M Cell Lysis Reagent were from Sigma-Aldrich, Inc. (St. Louis, MO). Albumin standard, Protein G agarose beads and Super Signal West (Pico, Dura) chemiluminescent substrate were from Pierce (Rockford, IL). Protease inhibitor tablets were from Roche (Basel, Switzerland). Purified collagenase (type CLSPA) was from Worthington Biochemicals (Freehold, NJ). Nitrocellulose membranes were from Schleicher and Schuell Bioscience, Inc. (Keene, NH). L-364,718 (3S(−)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3-yl-1H-indole-2-carboxamide)) and L-365,260 (3R(+)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin3-yl)-N′-(3-methylphenyl)urea) were from Merck, Sharp and Dohme (West point, PA). YM022 ((R)-1-[2,3-dihydro-1-(2′-methyl-phenacyl)-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl]-3-(3-methylphenyl)urea) and SR27897 (1-[[2-(4-(2-chlorophenyl)-thiazol-2-yl)aminocarbonyl] indolyl]acetic acid) were from Tocris Bioscience (Ellisville, MO). Biocoat collagen I Cellware 60 mm dishes were from Becton Dickinson Labware (Bedford, MA). Albumin bovine fraction V was from MP Biomedical (Solon, OH). NaCl, KCl and NaH_2PO_4 were from Mallinckrodt (Paris, KY).

2.2. Methods

2.2.1. Tissue preparation

Pancreatic acini were obtained by collagenase digestion as previously described [12]. Standard incubation solution contained 25.5 mM HEPES (pH 7.45), 98 mM NaCl, 6 mM KCl, 2.5 mM NaH_2PO_4, 5 mM sodium pyruvate, 5 mM sodium glutamate, 5 mM sodium fumarate, 11.5 mM glucose, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM glutamine, 1% (w/v) albumin, 0.01% (w/v) trypsin inhibitor, 1% (v/v) vitamin mixture and 1% (v/v) amino acid mixture.

2.2.2. Acini stimulation

After collagenase digestion, dispersed acini were pre-incubated in standard incubation solution for 2 h at 37 °C with or without inhibitors as described previously [12,13]. After pre-incubation 1 ml aliquots of dispersed acini were incubated at 37 °C with or without stimulants. Cells were lysed in lysis buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% sodium azide, 1 mM EGTA, 0.4 mM EDTA, 0.2 mM sodium orthovanadate, 1 mM PMSF, and one protease inhibitor tablet per 10 ml). After sonication, lysates were centrifuged at 10,000×*g* for 15 min at 4 °C and protein concentration was measured using the Bio-Rad protein assay reagent.

2.2.3. Immunoprecipitation

Immunoprecipitation of tyrosine phosphorylated proteins was performed as described previously [11]. For detection of Yes, 1 mg/ml of whole acinar lysate was incubated with 4 μg BD Yes antibody and 25 μl of protein G agarose beads overnight at 4 °C. Immunoprecipitates were washed once with lysis buffer and twice in phosphate buffered buffer 0.2 mM sodium orthovanadate and analyzed by SDS-PAGE and Western blotting.

2.2.4. Co-immunoprecipitation (Co-IP)

After stimulation, acini were washed with ice-cold phosphate-buffered saline, and then resuspended in 1 ml of CelLytic Buffer (CelLytic™M Cell Lysis Reagent 0.2 mM sodium orthovanadate, 1 mM PMSF, and one protease inhibitor tablet per 10 ml), mixed for 20 min at 4 °C and centrifuged for 15 min, 12,000×*g*. Supernatant containing the solubilized protein complexes was kept at −80 °C until protein determination by Bio-Rad protein assay reagents. For Co-IP studies, lysates (750 μg/μl) were incubated with 4 μg of the BD antibody and with 25 μl of protein G-agarose at 4 °C, overnight. The immunoprecipitates were washed once with CelLytic buffer and twice with phosphate-buffered saline and analyzed by SDS-PAGE and Western blotting.

2.2.5. Western blotting

Western blotting was performed as described previously [11,14]. The intensity of the protein bands was measured using Kodak ID Image Analysis, which were assessed in the linear detection range. When re-probing was necessary membranes were incubated in Stripping buffer (Pierce, Rockford, IL) for 30 min at room temperature,

washed twice for 10 min in washing buffer, blocked for 1 h in blocking buffer at room temperature and re-probed as described above.

2.2.6. Statistical analysis

All experiments were performed at least 3 times. Data are presented as mean ± SEM and were analyzed using the one-way ANOVA analysis with Dunnett's or Bonferroni multiple test as posttests using the GraphPad 5.0 software. p values <0.05 were considered significant. Curve fitting, EC_{50} and t_{max} were also calculated using the GraphPad 5.0 software.

3. Results

3.1. Specificity of the antibodies used in the study

In order to check the specificity of the antibodies to be used in this study, whole pancreatic acinar lysates, in parallel with human recombinant samples of the 3 SFK family members found in pancreatic acinar cells (Lyn, Yes, and Src) and the related SFK, Fyn, were immunoprecipitated with 4 μg anti-Yes BD monoclonal antibody and detected with pY416 Src family antibody (Fig. 1, *Panel B*, Cell Signaling: CS) or total Yes Ab (Fig. 1, *Panel A*, CS). We observed a signal only in the lane corresponding to the Yes kinase human recombinant (Fig. 1).

3.2. Ability of various pancreatic secretagogues but not pancreatic growth factors to stimulate Yes phosphorylation (pY416) in rat pancreatic acini

In order to establish whether Yes kinase is activated by known pancreatic secretagogues or growth factors [10], rat pancreatic acini were incubated in the absence and presence of several gastrointestinal hormones (CCK, carbachol, bombesin, secretin, VIP) known to activate pancreatic acinar cells and cause enzyme secretion [10]. As a measurement of Yes activity the phosphorylation of Y416 of Yes was assessed. Numerous studies show that all members of the Src family share 2 tyrosine phosphorylation sites, one at Y416 that causes the activation of these kinases and the other one, Y527 that produces their inhibition [1]. The pancreatic secretagogues that activate phospholipase C (CCK, carbachol, bombesin) stimulated an increase in Yes phosphorylation in tyrosine 416 (pY416) (334 ± 53, 207 ± 28, 213 ± 17% of control, respectively, all $p<0.05$ vs control) (Fig. 2, *Panel A*, Rows 4–6; Table 1). Secretin and VIP, which activate

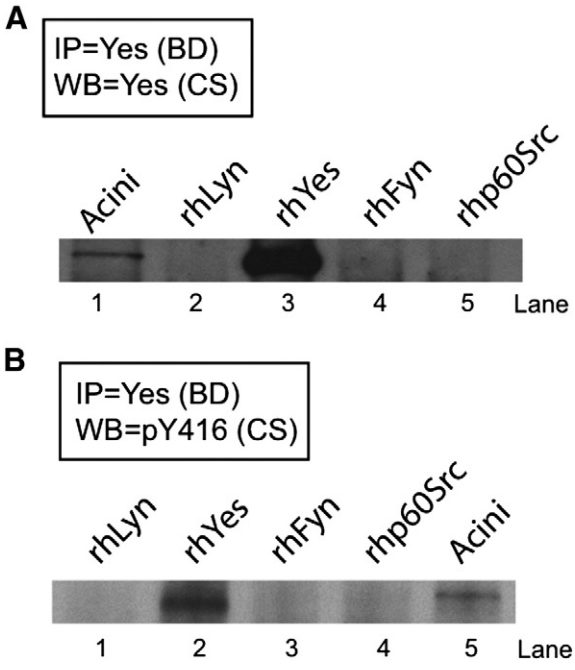

Fig. 1. Specificity of the antibodies used in the study. Lysates from pancreatic acini and equal amounts of human recombinant Src family members present in the pancreatic acinar cells (Lyn, Yes, and Src) or the related SFK, Fyn were immunoprecipitated (IP) in parallel with 4 μg of anti-Yes BD antibody and the resultant immunocomplexes were detected by Western blotting (WB) with a specific anti-Yes antibody (Cell signaling = CS), *Panel A*; or with a specific anti-pY416 Src family antibody (Cell signaling), *Panel B*.

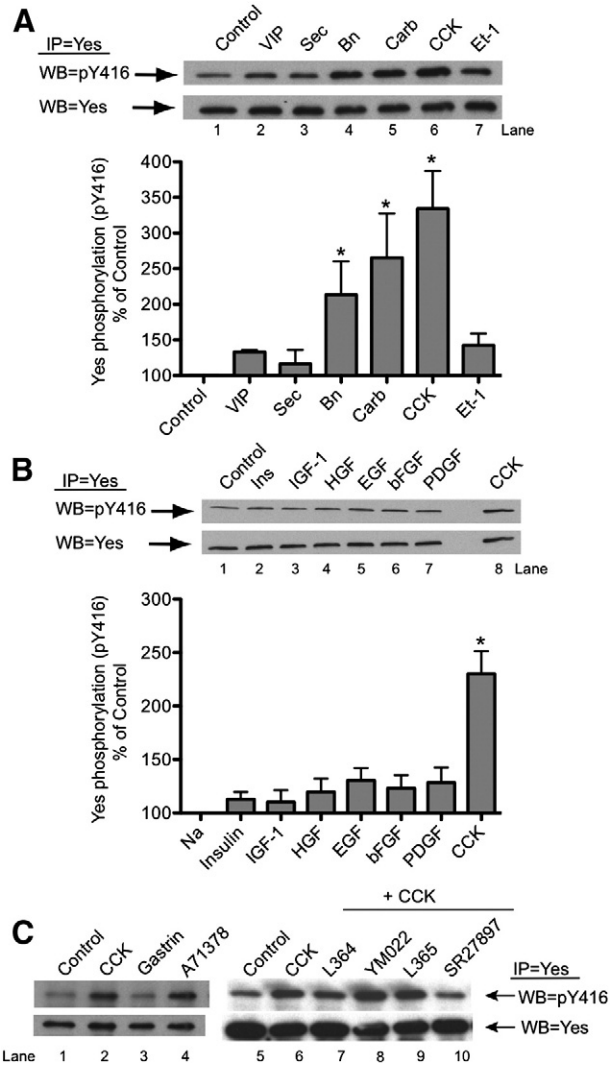

Fig. 2. Ability of various pancreatic secretagogues and pancreatic growth factors to stimulate Yes phosphorylation (pY416) in rat pancreatic acini. *Panel A*: Activation of pY416 Yes induced by CCK (100 nM), carbachol (10 μM), bombesin (1 nM), secretin (10 nM), VIP (10 nM) in isolated pancreatic acini. Isolated pancreatic acini were incubated with the indicated agents for 1 min, and then lysed. Yes kinase was immunoprecipitated as established in Materials and methods and Western blots were analyzed using anti-pY416 Src family and, as loading control, anti-Total Yes. Bands were visualized using chemiluminescence and quantified by densitometry. *Top*: Results of a representative blot of 6 independent experiments are shown. *Bottom*: Means ± S.E. of 6 independent experiments. Results are expressed as % of basal stimulation of the control group taking total Yes as a loading control. $^*p<0.05$ compared to the control group. *Panel B*: Action of CCK (100 nM, 1 min), insulin (1 μM, 10 min), EGF (10 nM, 5 min), PDGF (100 ng/ml, 10 min), bFGF (100 ng/ml, 5 min), IGF (100 nM, 10 min) and HGF (1 nM) upon the activation of Yes in the pancreatic acini. The experiment was performed as described in Panel A above. Results of a representative blot of 6 independent experiments are shown. $^*p<0.05$ compared to the control group. *Panel C*: Isolated pancreatic acini were incubated in the absence or presence of CCK (100 nM), gastrin (10 nM) or A71378 (30 nM) for 1 min, or preincubated for 5 min in the presence of L364,718 (1 μM), YM022 (1 μM), L365,260 (1 μM) or SR27897 (1 μM) and then in the additional presence of CCK (100 nM) for 1 min. Yes kinase activity was determined as established in Materials and methods.

Table 1
Y416 Yes Kinase Phosphorylation characteristics and interactions of Yes in rat pancreatic acini[a].

Variable	pY416 phosphorylation
Stimulation by pancreatic secretagogues[b]	
Yes	CCK, carbachol, bombesin,
No	Secretin, Endothelin-1, VIP
Stimulation by pancreatic growth factors[b]	
Yes	–
No	Insulin, EGF, PDGF, bFGF, IGF-1, HGF
Stimulation by post-receptor activators	
Yes	A2387, Thapsigargin, TPA
No	8-Bromo-cAMP
Potency/time-course	
EC_{50} of CCK (nM)[c]	2.11 ± 0.15
EC_{50} of CCK-JMV (nM)[c]	19.45 ± 1.19
$t_{1/2}$ of CCK (min)[d]	0.31 ± 0.04
t_{max} of CCK (min)[d]	0.98 ± 0.14
$t_{1/2}$ of TPA (min)[e]	1.25 ± 0.24
t_{max} of TPA (min)[e]	2.49 ± 0.29
Interactions with other kinases	
Co-immunoprecipitation with[f,g,h]	PYK2, $p125^{FAK}$, $p130^{Cas}$, Shc, PKD, PKCδ, PI3K, PTEN

[a] Results are calculated from the data shown in Figs. 2–8.
[b] Concentration and incubation times are reported in Fig. 2.
[c] Concentrations and incubation times are reported in Fig. 3.
[d] Concentration and incubation times are reported in Fig. 4.
[e] Concentration and incubation times are reported in Fig. 5.
[f] Concentration and incubation times are reported in Fig. 6.
[g] Concentration and incubation times are reported in Fig. 7.
[h] Concentration and incubation times are reported in Fig. 8.

adenylate cyclase in rat pancreatic acini [10], did not increase phosphorylation of Yes Y416 (Fig. 2, *Panel A*, Rows 2 and 3; Table 1). Endothelin-1 (ET-1), which interacts with ET-1 and ET-3 receptors in the pancreatic acinar cell, but does not activate PLC cascades nor activate adenylate cyclase [15] did not produce a significant increment in Y416 Yes phosphorylation (Fig. 2, *Panel A*, Row 7; Table 1).

None of the known pancreatic growth factors tested (insulin, EGF, PDGF, bFGF, IGF, and HGF) [10] was able to activate Yes kinase and stimulate tyrosine 416 phosphorylation of Yes (Fig. 2, *Panel B*, Rows 1–7; Table 1). This lack of stimulation was not due to the inability of the growth factors to activate acinar cells because each stimulated pS473 Akt phosphorylation (data not shown).

In order to determine if the CCK stimulating effect on pY416 Yes phosphorylation was due to the presence/activation of CCK_1 or CCK_2 receptors in the acinar cell suspension, isolated acinar cells were first incubated with either CCK, gastrin, or a known CCK_1 receptor agonist (A71378) [16] (Fig. 2, *Panel C*, Lanes 1–4). Gastrin did not produce any increase in pY416 Yes phosphorylation, and the CCK activation of Yes was mimicked by the incubation of the cells with the selective CCK_1 receptor agonist, A71378. Moreover, when the acinar cells were incubated with CCK and two different CCK_1 receptor antagonists [L364,718 or SR27897] [17,18] the increment in pY416 Yes phosphorylation observed in the sole presence of CCK was largely inhibited, but not in the presence of CCK_2 receptor antagonists [L365,260 or YM022] [18] (Fig. 2, *Panel C*, Lanes 5–10). These results demonstrate that the observed effect of CCK in pY416 Yes phosphorylation is only due to the activation of the CCK_1 receptors.

3.3. Dose–response effect of CCK and CCK-JMV on Yes kinase Y416 phosphorylation in rat pancreatic acini

As CCK has an important role in both the physiology and pathophysiology of the pancreas [10,19], we focused our study in the activation of Yes kinase exerted by this hormone in rat pancreatic acini. Increasing concentrations of CCK produced a monophasic increase in Y416 phosphorylation of Yes, detectable at 0.01 nM concentration (Fig. 3), maximal stimulation occurred with 100 nM CCK (280 ± 16% of control) and CCK's half-maximal effect (EC_{50}) occurred with 2.11 nM ± 0.15 nM (Fig. 3, Table 1). The CCK_1 receptor in pancreatic acini can exist in two different activation states, a low and a high-affinity state, and the activation of the different states activates different cell signaling cascades [20–22]. In order to determine the contribution of each activation receptor state to the activation of Yes kinase by CCK, pancreatic acini were incubated in the presence of increasing concentrations of CCK-JMV, known to be an agonist of the CCK_1 high affinity state and an antagonist of the low affinity CCK_1 receptor state in the rat pancreatic acini [20,22,23]. CCK-JMV stimulated tyrosine 416 phosphorylation of Yes kinase in a monophasic manner with concentrations from 100 nM to 1000 nM (Fig. 3) with an EC_{50} of 19.45 ± 1.19 nM (Fig. 3, Table 1), and therefore was 9 times less potent than CCK. CCK-JMV caused 63% of the maximal stimulation of pY416 Yes phosphorylation caused by CCK (Fig. 3). These results support the conclusion that CCK stimulation of Yes kinase activation is mediated 60% by the high affinity state CCK_1 receptor and 40% by activation of the low affinity CCK_1 receptor state. As a CCK concentration of 100 nM produces the maximal stimulation of Yes phosphorylation, it was selected for use in the next part of the study.

3.4. Time course of CCK and TPA stimulation of Yes kinase Y416 phosphorylation in rat pancreatic acini

Stimulation of Yes pY416 by CCK was time-dependent, with a significant increment after 30-second incubation time (278 ± 27% of control, $p<0.05$), and maximum after 1 min (310 ± 43% of control, $p<0.05$) (Fig. 4), with $t_{1/2}$ of 0.309 ± 0.042 min (Table 1). The increase in phosphorylation of pY416 Yes by CCK was maintained in time and lasted over 15 min (all $p<0.05$) (Fig. 4).

Because phospholipase C (PLC) stimulating pancreatic hormones, which subsequently activate PKC, stimulated Yes Y416 phosphorylation, the ability of TPA, a known PKC activator, to stimulate Yes Y416 phosphorylation at different times, was studied (Fig. 4). TPA produced, as in the case of CCK, a rapid increase (1 min: 153 ± 25% of control) in Yes kinase pY416 phosphorylation (Fig. 4), maximum

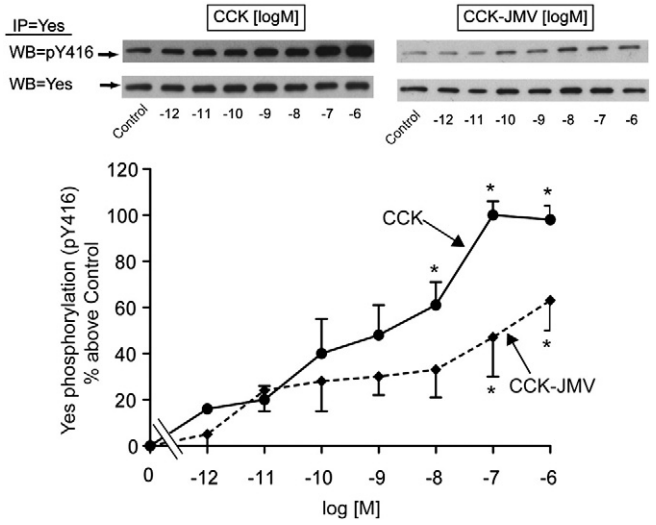

Fig. 3. Dose–response effect of CCK and CCK-JMV on Yes kinase phosphorylation in rat pancreas acini. Experiments were performed as described in Fig. 1 legend and Materials and methods. *Top*: Results of a representative blot of 5 independent experiments are shown. *Bottom*: Means ± S.E. of 5 independent experiments. Results are expressed as % of maximal CCK stimulation (CCK 100 nM: 280 ± 16% of control). *$p<0.05$ compared to the control group.

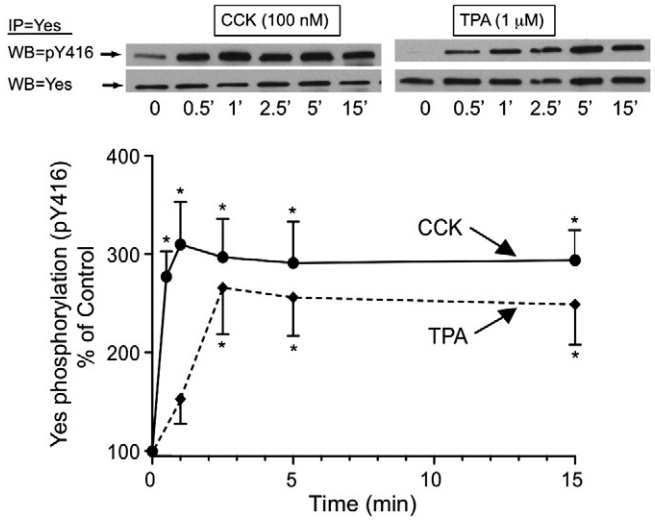

Fig. 4. Time course of CCK and TPA stimulation of Yes pY416 phosphorylation in rat pancreatic acini. Experiments were performed as described in Fig. 1 legend and Materials and methods. *Top*: Results of a representative blot of 7 independent experiments are shown. *Bottom*: Means ± S.E. of 7 independent experiments. Results are expressed as % of basal stimulation of the control group. *$p<0.001$ compared to the control group (i.e. 0 time).

at 2.5 min (266 ± 47% of control, $p<0.05$) with a $t_{1/2}$ of 1.25 ± 0.24 min (Table 1), and the increase was maintained for 15 min. TPA stimulation of Yes kinase pY416 phosphorylation was delayed in time compared to the CCK time course, likely due to the fact that TPA has to cross the cell's plasma membrane prior to PKC activation.

3.5. Effect of post-receptor stimulants, PKC inhibition or Ca^{2+} depletion on Y416 Yes kinase phosphorylation by CCK and TPA

When the ability of post-receptor stimulants to stimulate tyrosine 416 phosphorylation of Yes was tested, the PKC activating agent (TPA) produced a significant increment in tyrosine 416 phosphorylation of Yes (TPA = 202 ± 34% of control $p<0.05$). However, the apparent increase of Yes phosphorylation induced by the cellular calcium-mobilizing agent, thapsigargin (TG) did not reach statistical significance (171 ± 14% of control). The calcium ionophore A23187 and the AMP cyclic analog 8-Br-cAMP did not stimulate Yes (137 ± 18% or 116 ± 11% of control, respectively). The combination of the calcium mobilization agent (thapsigargin) with a PKC activator did not cause a greater activation than the addition of these two agents alone (Fig. 5, *Panel A*, Rows 3, 4, 6; Table 1). On the other hand, the combination of the calcium ionophore (A23187) with the PKC activator (TPA) increased significantly the activation of Yes as compared with the addition of these two agents alone ($p<0.05$) (Fig. 5, *Panel A*, Rows 2, 3, 5; Table 1).

We next examined whether CCK-induced activation of PKC or increase in intracellular calcium was needed for its ability to cause pY416 tyrosine phosphorylation of Yes in pancreatic acini (Fig. 5, *Panel B*, Rows 1–6). To determine the role of intracellular calcium changes [Ca_i], pancreatic acinar cells were pretreated for 1 h in a calcium-free medium with thapsigargin (10 μM), an agent that specifically inhibits the endoplasmic reticulum Ca^{2+}-ATPase and depletes calcium from intracellular compartments in a calcium free medium [12]. These conditions have been previously shown to completely inhibit the [$Ca^{2+}{}_i$] increase stimulated by CCK in rat pancreatic acinar cells [12]. In order to examine the implication of PKC in the CCK stimulated tyrosine 416 Yes phosphorylation, isolated acinar cells were treated for 1 h with a general PKC inhibitor GFX109203X [12] prior to incubation with CCK or TPA.

When PKCs activation was inhibited, no increment in the tyrosine phosphorylation of Yes was produced by CCK or TPA (CCK alone: 293 ± 24% of control, $p<0.05$ vs control; CCK + GFX: 139 ± 21% of control, $p<0.05$ vs CCK alone; TPA alone: 369 ± 41% of control, $p<0.05$ vs control; TPA + GFX: 93 ± 4% of control, $p<0.05$ vs TPA alone) (Fig. 5, *Panel B*, Rows 1–6). Inhibition of increments in intracellular Ca^{2+} produced a significant decreased in the pY416 tyrosine phosphorylation of Yes by CCK (CCK alone: 261 ± 32% of control, $p<0.05$ vs control; TG + CCK: 146 ± 11% of control, $p<0.05$ vs CCK alone) or TPA (TPA alone: 393 ± 76% of control, $p<0.05$ vs control; TG + TPA: 174 ± 20% of control, $p<0.05$ vs TPA alone) (Fig. 5, *Panel B*, Rows 7–12).

3.6. Ability of CCK and TPA to stimulate the association of Yes kinase with various cellular proteins in rat pancreatic acinar cells

Src kinases are reported to interact with a number of other important cellular signaling proteins in different cell types including the focal adhesion kinases $p125^{FAK}$ and PYK2 [1,9,24,25], PI3K [1], Cbl [1], PKD [26], Raf [27] Shc [1,28], PKCδ [1], and $p130^{Cas}$ [1], PTEN [29], kinases/phosphatases known to be particularly important in regulating processes such as growth and apoptosis, adhesion or secretion. In order to determine the possible interaction of Yes with these proteins in the pancreatic acini, we studied the ability of CCK or TPA to stimulate a direct interaction of Yes with these proteins by performing co-immunoprecipitation (Co-IP) studies (Figs. 6–8). We first investigated the Co-IP of Yes kinase with the different proteins after incubation with CCK (2.5 min, 100 nM) or TPA (5 min, 1 μM), concentrations that caused a maximal Yes activation [CCK (100 nM) (Fig. 3) and TPA (1 μM)]. We observed that CCK and TPA (Fig. 6–7, *Panel A–D*, Rows 1–3, Lanes 1–3; Table 1) produced an increase in the association of Yes with $p125^{FAK}$ and PYK2, PI3K, PKD, $p130^{Cas}$, Shc and the novel PKC kinases δ, but not with Raf, Cbl or ERK1/2 (data not shown). In order to determine if the Co-IP of Yes kinase with these different proteins also occurred at more physiological concentrations of CCK, we performed the same type of CO-IP experiments using CCK at concentrations of 100 pM and 1 nM (Fig. 8). We observed that at both concentrations CCK (Fig. 8, Rows 1–3, Lanes 1–13; Table 1) produced an increase in the association of Yes with PYK2, PI3K, PKD, $p130^{Cas}$, Shc and the novel PKC kinases δ, but not with $p125^{FAK}$ (Fig. 8, Row #3) (which did interact with Yes at higher concentrations) (Fig. 6, Row #3, Panel A) or with Raf, Cbl or ERK1/2 (data not shown). To ensure that the antibody used for immunoprecipitation was not the cause of lack of association, we repeated the Co-IP experiments in the reverse direction: immunoprecipitating with Raf, Cbl or ERK1/2 antibodies and immunodetection with Yes antibodies, and we did not obtained any stimulated direct association with Yes after incubation with CCK or TPA.

4. Discussion

In pancreatic acinar cells, Src family of kinases (SFKs) are reported to be important for cell growth, to play an important role in enzyme secretion, endocytosis, membrane recycling, apoptosis, protein synthesis, regulation of calcium levels, regulation of PKCs or in response to acinar cell injury, as occurs in pancreatitis [3,4,6–9]. The SFK family consists of nine structurally related members [1], of which 3 are reported in pancreatic acinar cells (Lyn, pp60Src, Yes) [4,6,7,9,28], however little is known in pancreatic acinar cells, except for the case of Lyn [9], of the individual SFK members participation in the cellular signaling cascades mediating these various pancreatic cellular processes.

The present study was designed to address the question of whether the activation of the SFK, Yes, is involved in mediating the cellular signaling of gastrointestinal hormones/neurotransmitters and of various gastrointestinal growth factors that are known to alter pancreatic acinar cell function. The presence of Yes and its specific activation was assessed by using a specific Yes antibody for immunoprecipitating and the panSrc phospho-specific antibody

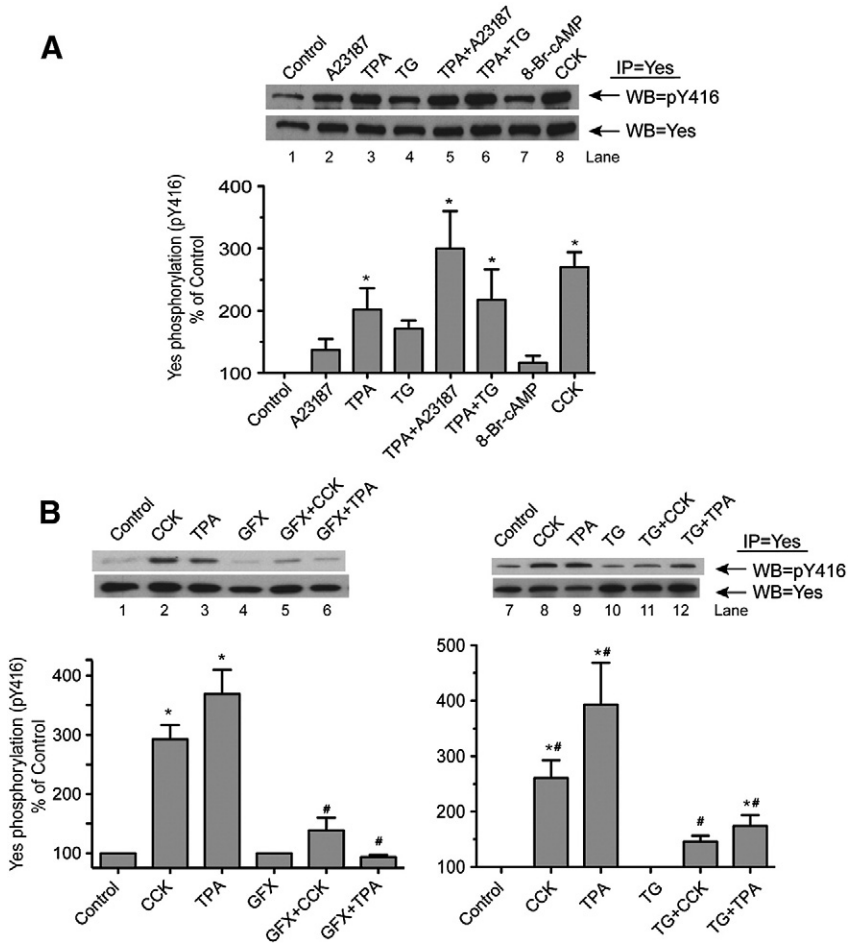

Fig. 5. Effect of post-receptor stimulants, PKC inhibition or Ca^{2+} depletion on pY416 Yes phosphorylation by CCK and TPA. *Panel A*: Effect of A23187 (1 μM), TPA (1 μM), thapsigargin (TG) (1 μM), 8-Br-cAMP (1 mM) on Yes phosphorylation (at 5 min), and of CCK (100 nM), (at 1 min). *Top*: Results of a representative blot of 4 independent experiments are shown. *Bottom*: Means ± S.E. of 4 independent experiments. Results are expressed as % of basal stimulation of the control group. $^{*}p<0.05$ compared to the control group. *Panel B*: Isolated acini were preincubated for 2 h, alone or in the additional presence of GF109203X (5 μM, 30 min, *Panel B Left*) or thapsigargin (TG) (1 μM, 2 h, *Panel B Right*) in calcium free media and then incubated with or without 100 nM CCK or 1 μM TPA for 1 or 2.5 min, respectively. *Top*: Results of a representative blot of 4 independent experiments are shown. *Bottom*: Means ± S.E. of 4 independent experiments. Results are expressed as % of basal stimulation of the control group. $^{*}p<0.05$ compared to the control group, $\#p<0.05$ compared to the stimulant alone.

pY416 for specific detection of the Y416 phosphorylated form of Yes. This later finding is important because Y416 phosphorylation reflects the degree of SFKs activation [1] and it has been widely used to specifically assess SFK activation when combined with specifically immunoprecipitating individual SFKs, such as Lyn or pp60Src [9]. Our results demonstrate that the SFK, Yes is present in pancreatic acini and that its activated pY416 form can be specifically detected.

In this the present study we first investigated the ability of various pancreatic stimulants to activate Yes by assessing their ability to stimulate pY416 tyrosine phosphorylation of Yes. Previous results have demonstrated that numerous growth factors (HGF, EGF, bFGF, insulin, IGF and PDGF) are able to interact with specific receptors on pancreatic acinar cells and stimulate growth and/or protein synthesis as well as alter other cellular functions [10]. In our study, we used concentrations that were able to activate Akt in acini, under identical experimental conditions, that previous studies reported the particular pancreatic growth factors we studied activated a number of pancreatic acinar cell responses [9,10,14,20,23]. We did not observe activation of Yes induced by any of these growth factors. This result contrasts with a previous study in pancreatic acini in which, the SFK, Lyn was activated by the same pancreatic growth factors used in the present study [9]. Our results are similar with bFGF to a previous study in pancreatic acinar cells in which bFGF was reported to not activate SFK in pancreatic acinar cells [3]. Our results have both similarities and differences from studies in other tissues, reporting SFK activation by these growth factors [30]. EGF, PDGF, and bFGF activate the SFK, cSrc in murine fibroblasts [31], and in skeletal muscle cells insulin stimulates SFK activation [32]. In ovarian cells insulin stimulates Fyn activation [33]; in differentiating preadipocytes IGF stimulates Src activation [34]; and EGF and PDGF stimulated activation of Yes in hepatocytes and fibroblasts in some studies [35,36]. The ability of the growth factors to stimulate Lyn in the pancreatic acinar cells, but not Yes, illustrates the potential for diversity in the function of the different isoforms of the SFKs in these cells. From studies in other tissues it has been concluded that cSrc and other SFKs are activated in response to many of the same cellular signals, but it is not always clear where Yes performs unique functions and when it functions redundantly with other members of the Src family of kinases [37]. In pancreatic acini, our results indicate that, unlike the SFK Lyn [9], Yes, is not involved in the cellular response to growth factors.

Our results show that pancreatic secretagogues which activate G protein-coupled receptors on pancreatic acinar cells and stimulate phospholipase C (PLC)-mediated cascades, [10], activate Yes in pancreatic acini. This was demonstrated by the fact that CCK, the muscarinic cholinergic agonist carbachol, and bombesin, each of which interacts with specific G-protein coupled receptors on pancreatic

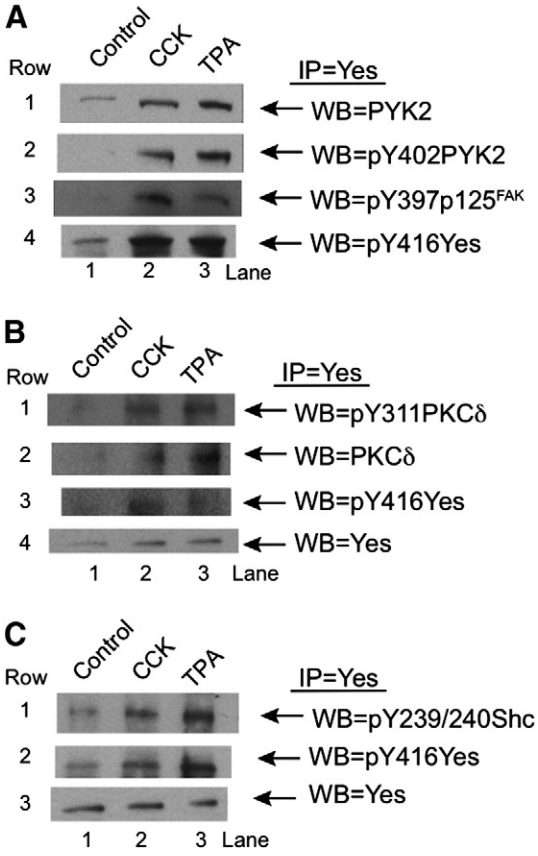

Fig. 6. Ability of CCK (100 nM) or TPA (1 μM) to stimulate the association of Yes kinase with PYK2, p125^{FAK}, PKCδ, and Shc. Isolated pancreatic acini were incubated in the absence and presence of CCK (2.5 min) or TPA (5 min). Equal amounts of proteins were immunoprecipitated with an anti-Yes (BD) and then subjected to Western blotting, using as first antibody anti-p125^{FAK}, anti-PYK2, anti-PKCδ and anti-Shc or anti-Src, total or phosphorylated form of the proteins. The bands were visualized using chemiluminescence. Results are representative of 8 independent experiments.

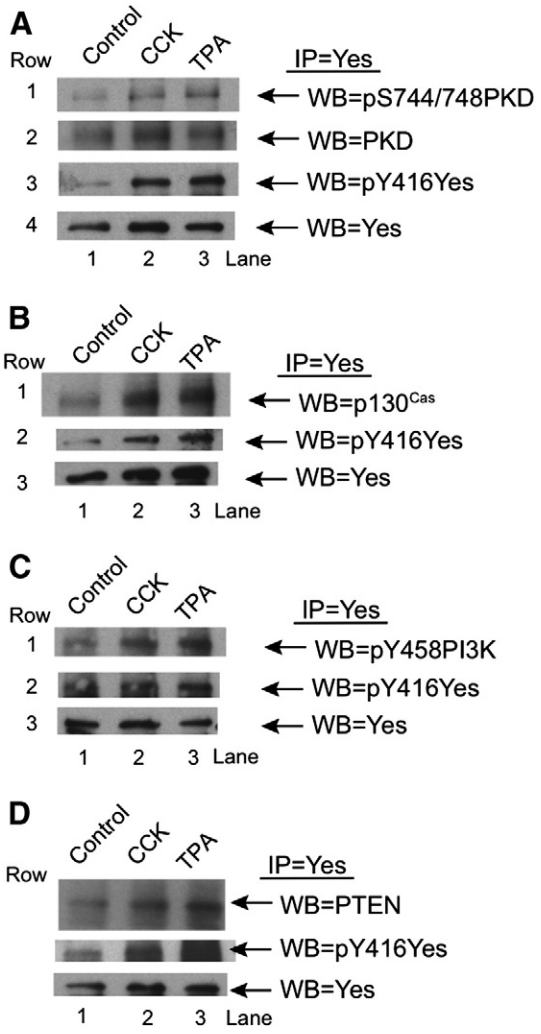

Fig. 7. Ability of CCK (100 nM) or TPA (1 μM) to stimulate the association of Yes kinase with PKD, p130^{Cas} PI3K and PTEN. Isolated pancreatic acini were incubated in the absence and presence of CCK (2.5 min) or TPA (5 min). Equal amounts of proteins were immunoprecipitated with an anti-Yes (BD) and then subjected to Western blotting, using as first antibody anti-PKD, anti-p130^{Cas}, anti-PI3K, anti-PTEN or anti-Src, total or phosphorylated form of the proteins. The bands were visualized using chemiluminescence. Results are representative of 8 independent experiments.

acini to activate phospholipase C, resulting in changes in cytosolic calcium and PKC activation [10], each activated the SFK, Yes in pancreatic acinar cells. However, other secretagogues stimulating PKA-mediated pathways, or other signaling cascades like VIP, secretin or endothelin, which interacts with GPCRs receptors on pancreatic acini, but does not stimulate phospholipase C or adenylate cyclase [15], did not activate the SFK, Yes in pancreatic acini. These results have some similarities and differences from previous studies of the ability of these GI hormones/neurotransmitters to activate SFKs in pancreatic acini and other tissues. They are similar in that the SFK Lyn was also activated in pancreatic acinar cells by bombesin, CCK, and carbachol, however they differ from Lyn activation, which was weakly stimulated by endothelin or secretin [9], whereas Yes in the same cells is not. They are similar to a previous study which demonstrated that supramaximal concentrations of CCK both stimulate kinase activity of Yes, and also its phosphorylation [7]. In other tissues, and in agreement with our study, bombesin-related peptides and muscarinic cholinergic agents activated SFKs [1], however, in other studies and in contrast to our results, secretin also activated the SFKs [1], which is different from our results with Yes in pancreatic acinar cells. Also, in contrast to our results, endothelin activates SFKs in different cells in some studies [38,39] however, in endothelial cells endothelin activated the SFK Fyn, but Src and Hck were inhibited and Yes was unaltered [40]. These results demonstrate that the different SFKs are activated differentially by pancreatic secretagogues/neurotransmitters in acinar cells and also that the ability of a given gastrointestinal secretagogue/neurotransmitter to activate SFKs can differ in different tissues.

To provide a greater insight into the interaction of the SFK, Yes, with important cellular cascades mediating the action of pancreatic secretagogues/neurotransmitters, the hormone/neurotransmitter CCK, which is a physiological regulator of the pancreatic acinar cell function [41] and has been reported to stimulate SFKs in pancreatic acinar cells and other tissues [2,7,8,12,28], was investigated in detail. We found that CCK causes a rapid activation of Yes which is similar to that reported for its ability to activate Lyn or Yes in pancreatic acini in other studies [7,9]. However, the kinetics of Yes activation differed from other studies [7,9], because we found sustained Yes activation lasting up to 15 min, whereas in the other studies, activation of SFKs by CCK, rapidly decreased with time [7,9]. In various tissues, activation of CCK_1 and CCK_2 receptors stimulates activation of various SFKs [6,9,42–44]. Our results demonstrate that CCK activation of Yes in pancreatic acini was due only to CCK_1 receptor activation and not to CCK_2 receptors that could be present in accompanying cells or possibly on acini. CCK_2 receptors are reported in pancreatic acinar cells from other species and in the rat pancreatic acinar cell line AR42J, CCK is reported to activate Yes through CCK_2 receptors [10,45,46]. However, our results demonstrate

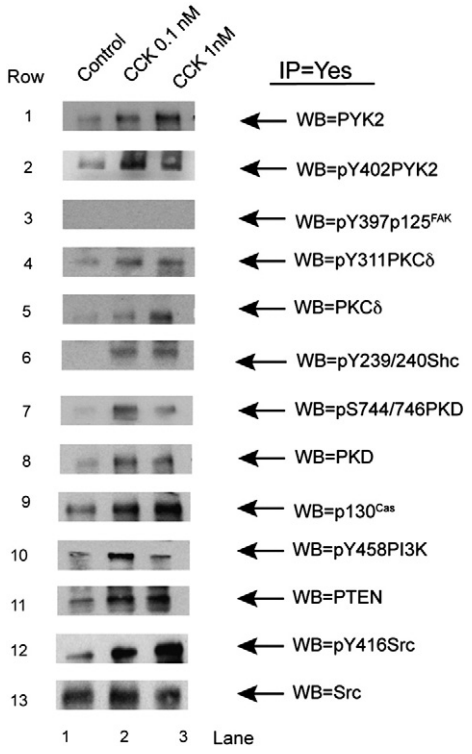

Fig. 8. Ability of low concentrations of CCK (100 pM, 1 nM) to stimulate the association of Yes kinase with PYK2, $p130^{Cas}$, Shc, PKD, PKCδ, PI3K and PTEN. Isolated pancreatic acini were incubated in the absence and presence of the indicated concentration of CCK for 2.5 min. Equal amounts of proteins were immunoprecipitated with an anti-Yes (BD) and then subjected to Western blotting, using as first antibody anti-$p125^{FAK}$, anti-PYK2, anti-PKCδ, anti-Shc, anti-PKD, anti-$p130^{Cas}$, anti-PI3K, anti-PTEN or anti-Src, total or phosphorylated form of the proteins. The bands were visualized using chemiluminescence. Results are representative of 4 independent experiments.

that pancreatic acinar cells differ from pancreatic tumoral AR42J cells in the CCK receptor subtype mediating Yes activation.

Numerous studies demonstrate that CCK can activate both a high and low affinity receptor state, which can mediate different cellular responses [12,47,48]. A number of our results support the conclusion that full activation of Yes by CCK requires activation of both CCK_1 receptor states. In our study, the CCK dose–response curve for Yes activation extended over concentration ranges that CCK activates both high and low affinity receptor states [10], suggesting activation of both CCK_1 receptor states, are involved in Yes activation. This conclusion is supported by the results with the CCK-JMV, a synthetic CCK analog, which stimulated only 64% of the maximal Yes activation caused by CCK-8. Because CCK-JMV in rat pancreatic acini functions as a full agonist at the high affinity receptor state and an antagonist of the low affinity state [22,48], our results demonstrate that 64% of maximal Yes activation by CCK is due to activation of the high affinity CCK_1 receptor state and 36% to activation of the low affinity CCK_1 receptor state. These results have similarities and differences from that reported in other studies in pancreatic acini with CCK_1 receptor-mediated activation of SFK and other proteins. Similar to its mechanism in the activation of Yes, CCK-induced activation of the SFK, Lyn in pancreatic acini [9], is mediated by activation of both high and low affinity receptor states, as is the stimulation of the tyrosine phosphorylation of focal adhesion kinases ($p125^{FAK}$, PYK2/CAβ) and their phospho-specific sites [12,13], the scaffolding protein, paxillin [11,12] and the novel PKC, pKCμ (PKD) [20]. However, the relative importance of activation of the high and low affinity CCK_1 receptor state differs among these proteins. In contrast to these results, CCK-mediated activation in pancreatic acini of phospholipase D or PI3K requires activation of only the high affinity receptor state [49], whereas in the case of PKC-δ [6] or CRK-II [50] only the low affinity CCK_1 receptor state is required for activation. Also, in contrast to our results with Yes activation, a previous study [3] reported that CCK-stimulated SFK activity is mediated almost entirely by activation of the low CCK_1 receptor state in pancreatic acini. Our results provide additional support to the proposal [51] that not only are the different CCK_1 receptor states on pancreatic acini differentially coupled to activation of phospholipase A2 and C [10], they are also differentially coupled to a number of tyrosine phosphorylation cascades including activation of the SFK, Yes.

Activation of the pancreatic CCK_1 receptor stimulates phospholipase C (PLC), resulting in the generation of inositol phosphates and diacylglycerol which in turn results in mobilization of cellular calcium and activation of PKCs, respectively [10,12]. A previous study reported that activation of the two limbs of the PLC cascade (PKC and calcium) in pancreatic acini and other cells by different stimuli, can have effects on the phosphorylation of the SFK Lyn [9]. A number of our results support the conclusion that in rat pancreatic acini maximal activation of Yes by CCK requires activation of both limbs of the PLC cascade. First, thapsigargin, an agent that increases cellular calcium acutely in a calcium-containing medium, activated Yes. Second, direct activation of PKCs by the phorbol ester TPA also activated Yes. Third, incubation with the Ca^{2+} ATPase inhibitor, thapsigargin, under conditions which deplete cellular calcium resulting in complete inhibition of CCK-mediated increases in $[Ca^{2+}]_i$ in pancreatic acini, did not completely inhibit CCK-stimulated Yes activation, resulting in a reduction of 71%. Furthermore, the PKC inhibitor, GF109203X, at a concentration that inhibited TPA-stimulated tyrosine phosphorylation of other non-receptor tyrosine kinases such as $p125^{FAK}$ and PYK2 [12,13] in rat pancreatic acini, caused an 80% decrease in CCK-stimulated activation of Yes. These results have similarities and differences from the role of PKC activation and/or changes in cytosolic calcium by different stimulants in other cells on activation of SFKs. Similar to the effect of CCK in pancreatic acini, PKC activation or changes in cytosolic calcium have been shown to be important for SFK activation by a number of stimulants in different cells [25,52]. However, activation of SFK in granulocytes is not dependent on PKC activation [52] and bombesin activation of Src pathway in 3T3 cells is calcium-independent [25]. These results support the conclusion that the importance of activation the two limbs of PLC in activating SFKs varies in different cells.

In pancreatic acini [2,7,9,28,43,53] and also in other cells [1], SFKs are important in modulating activation of a number of cellular signaling cascades involved in different cellular functions such as secretion, endocytosis, growth, cytoskeletal integrity and apoptosis. However, the specific Src isoform mediating the effect is usually unknown because there are no specific inhibitors/activators for the different SFKs. In some cases, SFKs are able to associate and interact directly with a wide range of substrates, such as kinases, cytoskeletal and structural proteins, adapters and receptors. An assessment of these associations can be used to determine the specific interaction with SFKs with different cellular signaling cascades in pancreatic acini [2,7,9,28,43,53] and also in other cellular systems [1]. Activated SFKs are reported to directly associate with focal adhesion kinases ($p125^{FAK}$, PYK2) [1,24,25], paxillin [1], $p130^{CAS}$ [1], PKC-δ [1], PI3-kinase [1], Shc [1,28] and Cbl [1]. Our results using specific Yes co-immunoprecipitation experiments, demonstrate that TPA and CCK at both physiological and supramaximal concentrations, stimulate the association of Yes with a number of cellular signaling cascades including with focal adhesion kinases, PKCδ, Shc, $p130^{Cas}$, PI3K, PTEN and PKD, but not with Cbl or Erk 1/2. A previous study [13] in pancreatic acini of Pyk-2 and $p125^{FAK}$, demonstrated that CCK and TPA stimulate tyrosine phosphorylation differentially at three of their most important regulatory sites. Our study demonstrates that CCK, as well as TPA, stimulates an association of Yes with each of their specific autophosphorylated forms (pY397

p125^{FAK} and pY402 PYK2) [13,24] suggesting that Yes action is important in their activation. These results are similar to previous observations in pancreatic acini [9,43] or other tissues [25,54,55], but differ from another study in rat pancreatic acinar cells in which the association of Yes with FAK was not observed [7]. Our findings of direct interactions of Yes with a number of important cell signaling molecules have similarities and differences with other studies of SFKs in pancreatic acini and other tissues. These results are similar to findings in pancreatic acini with the SFK, Lyn in that both CCK/TPA stimulated its association with PKC-δ, Shc, p125^{FAK} and Pyk-2, however, in contrast with Yes they differ from our results with Yes in that no direct interaction of Lyn with p130^{CAS} or PI3K was detected [9]. In our study the failure to stimulate an association of Yes with ERK 1/2 was not due to lack of stimulation by CCK of this signaling pathway in pancreatic acini, because previous studies demonstrate that CCK activates this pathway in pancreatic acinar cells under similar experimental conditions to those used in our study [23]. Our results suggest that activation of the SFK, Yes, could play an important role in CCK activation of MAP kinase in pancreatic acini, because CCK stimulated both an association of Yes with Shc as well as with Pyk2, each of which activates MAP kinases [12]. These results are similar to activation of a number of G-protein-coupled-receptors which have been shown to stimulate MAP kinases by a SFK dependent mechanism [44], and similar to results in pancreatic cell lines in which activation of CCK receptors stimulated a complex formation of Yes with Shc [28]. In pancreatic acini CCK also stimulates in a Src-dependent manner, the translocation, activation and tyrosine phosphorylation of PKCδ [6]. This activation of PKCδ stimulates NFk-B activity [56], which has been shown to be important in the development of pancreatitis. Our results raise the possibility that the SFK, Yes, could be involved together with Lyn [9] in this process, because in both cases, CCK stimulated the association of these SFKs with PKCδ. Similarly in other cell systems the association of PKCδ with other SFKs is seen with different stimulants [6,57,58]. However, these results differ from findings in PC-12 cells and parotid cells [59], where neither TPA nor carbachol stimulated the association of PKC-δ and Src, although each activated PKC-δ [6,57]. Our results with a lack of stimulated interaction of Cbl with Yes differ from those in several studies where an association between SFKs members and Cbl has been demonstrated [60,61] and this interaction can be a mechanism of activation of PI3K [60,61]. However, unlike the SFK Lyn, [9], CCK and TPA stimulated an association between Yes and PI3K, which suggests that Yes activation may be important for the PI3K activation through a mechanism different than the Cbl interaction.

In conclusion, this study demonstrates that in rat pancreatic acini the SFK, Yes is present and is activated by CCK and other gastrointestinal hormones/neurotransmitters that activate phospholipase C signaling cascade, but not by pancreatic growth factors. The activation of Yes by CCK, is very rapid, requires stimulation of both limbs of the phospholipase C pathway (calcium and PKC) and is mediated by activation of both high and low CCK1 receptor affinity states. This results in the direct association of Yes with a number of important CCK signaling cascades including focal adhesion kinases Pyk-2 and p125^{FAK}, PKCδ, Shc, p130^{Cas}, PI3K, PTEN and PKD. These results support the conclusion that activation of the SFK Yes plays a central role in activating pancreatic signaling cascades which have been shown to be important for mediating CCK changes in acinar cell function (growth, apoptosis, secretion). The results suggest that the Src kinase Yes in pancreatic acini may be one of the important pancreatic SFKs mediating many of the physiological and pathophysiological roles of SFKs described in numerous studies.

References

[1] S.M. Thomas, J.S. Brugge, Cellular functions regulated by Src family kinases, Annu. Rev. Cell Dev. Biol. 13 (1997) 513–609.

[2] F. Nozu, C. Owyang, Y. Tsunoda, Involvement of phosphoinositide 3-kinase and its association with pp 60src in cholecystokinin-stimulated pancreatic acinar cells, Eur. J. Cell Biol. 79 (2000) 803–809.

[3] Y. Tsunoda, H. Yoshida, F. Nozu, Receptor-operated Ca2 + influx and its association with the Src family in secretagogue-stimulated pancreatic acini, Biochem. Biophys. Res. Commun. 314 (2004) 916–924.

[4] E.M. Parker, M.M. Zaman, S.D. Freedman, GP2, A GPI-anchored protein in the apical plasma membrane of the pancreatic acinar cell, co-immunoprecipitates with src kinases and caveolin, Pancreas 21 (2000) 219–225.

[5] S.D. Freedman, M.H. Katz, E.M. Parker, A. Gelrud, Endocytosis at the apical plasma membrane of pancreatic acinar cells is regulated by tyrosine kinases, Am. J. Physiol. 276 (1999) C306–C311.

[6] J.A. Tapia, L.J. Garcia-Marin, R.T. Jensen, Cholecystokinin-stimulated protein kinase C-delta activation, tyrosine phosphorylation and translocation is mediated by Src tyrosine kinases in pancreatic acinar cells, J. Biol. Chem. 12 (2003) 35220–35230.

[7] G. Lynch, S. Kohler, J. Leser, M. Beil, L.J. Garcia-Marin, M.P. Lutz, The tyrosine kinase Yes regulates actin structure and secretion during pancreatic acinar cell damage in rats, Pflugers Arch. 447 (2004) 445–451.

[8] P.C. Redondo, A.I. Lajas, G.M. Salido, A. Gonzalez, J.A. Rosado, J.A. Pariente, Evidence for secretion-like coupling involving pp 60src in the activation and maintenance of store-mediated Ca2 + entry in mouse pancreatic acinar cells, Biochem. J. 370 (2003) 255–263.

[9] A. Pace, J.A. Tapia, L.J. Garcia-Marin, R.T. Jensen, The Src family kinase, Lyn, is activated in pancreatic acinar cells by gastrointestinal hormones/neurotransmitters and growth factors which stimulate its association with numerous other signaling molecules, Biochim. Biophys. Acta 1763 (2006) 356–365.

[10] R.T. Jensen, Receptors on pancreatic acinar cells, Third edition, in: L.R. Johnson, E.D. Jacobson, J. Christensen, D.H. Alpers, J.H. Walsh (Eds.), Physiology of the Gastrointestinal Tract, vol. 2, Raven Press, New York, 1994, pp. 1377–1446.

[11] L.J. Garcia, J.A. Rosado, A. Gonzalez, R.T. Jensen, Cholecystokinin-stimulated tyrosine phosphorylation of p125FAK and paxillin is mediated by phospholipase C-dependent and -independent mechanisms and requires the integrity of the actin cytoskeleton and participation of p21rho, Biochem. J. 327 (1997) 461–472.

[12] J.A. Tapia, H.A. Ferris, R.T. Jensen, L.J. Marin, Cholecystokinin activates PYK2/CAKβ, by a phospholipase C-dependent mechanism, and its association with the mitogen-activated protein kinase signaling pathway in pancreatic acinar cells, J. Biol. Chem. 274 (1999) 31261–31271.

[13] A. Pace, L.J. Garcia-Marin, J.A. Tapia, M.J. Bragado, R.T. Jensen, Phosphospecific site tyrosine phosphorylation of p125^{FAK} and proline-rich kinase 2 is differentially regulated by cholecystokinin receptor A activation in pancreatic acini, J. Biol. Chem. 278 (2003) 19008–19016.

[14] V. Sancho, M.J. Berna, M. Thill, R.T. Jensen, PKCtheta activation in pancreatic acinar cells by gastrointestinal hormones/neurotransmitters and growth factors is needed for stimulation of numerous important cellular signaling cascades, Biochim. Biophys. Acta 1813 (2011) 2145–2156.

[15] P. Hildebrand, J.E. Mrozinski Jr., S.A. Mantey, R.J. Patto, R.T. Jensen, Pancreatic acini possess endothelin receptors whose internalization is regulated by PLC-activating agents, Am. J. Physiol. 264 (1993) G984–G993.

[16] J.M. Qian, W.H. Rowley, R.T. Jensen, Gastrin and CCK activate phospholipase C and stimulate pepsinogen release by interacting with two distinct receptors, Am. J. Physiol. 264 (1993) G718–G727.

[17] C.M. Coussens, J.M. Williams, D.R. Ireland, W.C. Abraham, Tyrosine phosphorylation-dependent inhibition of hippocampal synaptic plasticity, Neuropharmacology 39 (2000) 2267–2277.

[18] S.C. Huang, L. Zhang, H.C. Chiang, S.A. Wank, P.N. Maton, J.D. Gardner, R.T. Jensen, Benzodiazepine analogues L-365,260 and L-364,718 as gastrin and pancreatic CCK receptor antagonists, Am. J. Physiol. 257 (1989) G169–G174.

[19] J.F. Rehfeld, L. Friis-Hansen, J.P. Goetze, T.V. Hansen, The biology of cholecystokinin and gastrin peptides, Curr. Top. Med. Chem. 7 (2007) 1154–1165.

[20] M.J. Berna, K.M. Hoffmann, J.A. Tapia, M. Thill, A. Pace, S.A. Mantey, R.T. Jensen, CCK causes PKD1 activation in pancreatic acini by signaling through PKC-delta and PKC-independent pathways, Biochim. Biophys. Acta 1773 (2007) 483–501.

[21] W.H. Rowley, S. Sato, S.C. Huang, D.M. Collado-Escobar, M.A. Beaven, L.H. Wang, J. Martinez, J.D. Gardner, R.T. Jensen, Cholecystokinin-induced formation of inositol phosphates in pancreatic acini, Am. J. Physiol. 259 (1990) G655–G665.

[22] H.A. Stark, C.M. Sharp, V.E. Sutliff, J. Martinez, R.T. Jensen, J.D. Gardner, CCK-JMV 180: a peptide that distinguishes high affinity cholecystokinin receptors from low affinity cholecystokinin receptors, Biochim. Biophys. Acta 1010 (1989) 145–150.

[23] M.J. Berna, J.A. Tapia, V. Sancho, M. Thill, A. Pace, K.M. Hoffmann, L. Gonzalez-Fernandez, R.T. Jensen, Gastrointestinal growth factors and hormones have divergent effects on Akt activation, Cell. Signal. 21 (2009) 622–638.

[24] D.D. Schlaepfer, C.R. Hauck, D.J. Sieg, Signaling through focal adhesion kinase, Prog. Biophys. Mol. Biol. 71 (1999) 435–478.

[25] E.P. Salazar, E. Rozengurt, Bombesin and platelet-derived growth factor induce association of endogenous focal adhesion kinase with Src in intact Swiss 3T3 cells, J. Biol. Chem. 274 (1999) 28371–28378.

[26] N. Cabrera-Poch, L. Sanchez-Ruiloba, M. Rodriguez-Martinez, T. Iglesias, Lipid raft disruption triggers protein kinase C and Src-dependent protein kinase D activation and Kidins220 phosphorylation in neuronal cells, J. Biol. Chem. 279 (2004) 28592–28602.

[27] R.A. Klinghoffer, C. Sachsenmaier, J.A. Cooper, P. Soriano, Src family kinases are required for integrin but not PDGFR signal transduction, EMBO J. 18 (1999) 2459–2471.

[28] A. Piiper, R. Elez, S.J. You, B. Kronenberger, S. Loitsch, S. Roche, S. Zeuzem, Cholecystokinin stimulates extracellular signal-regulated kinase through activation of the epidermal growth factor receptor, Yes, and protein kinase C. Signal amplification at the level of Raf by activation of protein kinase Cepsilon, J. Biol. Chem. 278 (2003) 7065–7072.
[29] Y. Nagata, K.H. Lan, X. Zhou, M. Tan, F.J. Esteva, A.A. Sahin, K.S. Klos, P. Li, B.P. Monia, N.T. Nguyen, G.N. Hortobagyi, M.C. Hung, D. Yu, PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients, Cancer Cell 6 (2004) 117–127.
[30] D.A. Clump, I.H. Qazi, M. Sudol, D.C. Flynn, c-Yes response to growth factor activation, Growth Factors 23 (2005) 263–272.
[31] A.P. Belsches, M.D. Haskell, S.J. Parsons, Role of c-Src tyrosine kinase in EGF-induced mitogenesis, Front. Biosci. 2 (1997) d501–d518.
[32] T. Rosenzweig, S. Aga-Mizrachi, A. Bak, S.R. Sampson, Src tyrosine kinase regulates insulin-induced activation of protein kinase C (PKC) delta in skeletal muscle, Cell. Signal. 16 (2004) 1299–1308.
[33] X.J. Sun, S. Pons, T. Asano, M.G. Myers Jr., E. Glasheen, M.F. White, The Fyn tyrosine kinase binds Irs-1 and forms a distinct signaling complex during insulin stimulation, J. Biol. Chem. 271 (1996) 10583–10587.
[34] H. Sekimoto, J. Eipper-Mains, S. Pond-Tor, C.M. Boney, {Alpha}V{Beta}3 integrins and Pyk2 mediate IGF-I activation of Src and MAPK in 3T3-L1 cells, 2005.
[35] R.M. Kypta, Y. Goldberg, E.T. Ulug, S.A. Courtneidge, Association between the PDGF receptor and members of the src family of tyrosine kinases, Cell 62 (1990) 481–492.
[36] R. Reinehr, S. Becker, A. Hongen, D. Haussinger, The Src family kinase Yes triggers hyperosmotic activation of the epidermal growth factor receptor and CD95, J. Biol. Chem. 279 (2004) 23977–23987.
[37] J.M. Summy, M. Sudol, M.J. Eck, A.N. Monteiro, A. Gatesman, D.C. Flynn, Specificity in signaling by c-Yes, Front. Biosci. 8 (2003) s185–s205.
[38] M. Sumitomo, R. Shen, M. Walburg, J. Dai, Y. Geng, D. Navarro, G. Boileau, C.N. Papandreou, F.G. Giancotti, B. Knudsen, D.M. Nanus, Neutral endopeptidase inhibits prostate cancer cell migration by blocking focal adhesion kinase signaling, J. Clin. Invest. 106 (2000) 1399–1407.
[39] H. Kodama, K. Fukuda, E. Takahashi, S. Tahara, Y. Tomita, M. Ieda, K. Kimura, K.M. Owada, K. Vuori, S. Ogawa, Selective involvement of p130Cas/Crk/Pyk2/c-Src in endothelin-1-induced JNK activation, Hypertension 41 (2003) 1372–1379.
[40] A. Mandal, M. Shahidullah, C. Beimgraben, N.A. Delamere, The effect of endothelin-1 on Src-family tyrosine kinases and Na,K-ATPase activity in porcine lens epithelium, J. Cell. Physiol. 226 (2011) 2555–2561.
[41] R.T. Jensen, Involvement of cholecystokinin/gastrin-related peptides and their receptors in clinical gastrointestinal disorders, Pharmacol. Toxicol. 91 (2002) 333–350.
[42] A. Ferrand, S. Vatinel, A. Kowalski-Chauvel, C. Bertrand, C. Escrieut, D. Fourmy, M. Dufresne, C. Seva, Mechanism for Src activation by the CCK2 receptor: patho-physiological functions of this receptor in pancreas, World J. 12 (2006) 4498–4503.
[43] L. Daulhac, A. Kowalski-Chauvel, L. Pradayrol, N. Vaysse, C. Seva, Gastrin stimulates the formation of a p60Src/p125FAK complex upstream of the phosphatidylinositol 3-kinase signaling pathway, FEBS Lett. 445 (1999) 251–255.
[44] L. Daulhac, A. Kowalski-Chauvel, L. Pradayrol, N. Vaysse, C. Seva, Src-family tyrosine kinases in activation of ERK-1 and p85/p110-phosphatidylinositol 3-kinase by G/CCKB receptors, J. Biol. Chem. 274 (1999) 20657–20663.
[45] D.H. Yu, S.C. Huang, S.A. Wank, S. Mantey, J.D. Gardner, R.T. Jensen, Pancreatic receptors for cholecystokinin: evidence for three receptor classes, Am. J. Physiol. 258 (1990) G86–G95.
[46] D.H. Yu, M. Noguchi, Z.C. Zhou, M.L. Villanueva, J.D. Gardner, R.T. Jensen, Characterization of gastrin receptors on guinea pig pancreatic acini, Am. J. Physiol. 253 (1987) G793–G801.
[47] M.L. Villanueva, S.M. Collins, R.T. Jensen, J.D. Gardner, Structural requirements for the action of cholecystokinin on enzyme secretion from pancreatic acini, Am. J. Physiol. 242 (1982) G416–G422.
[48] S. Sato, H.A. Stark, J. Martinez, M.A. Beaven, R.T. Jensen, J.D. Gardner, Receptor occupation, calcium mobilization and amylase release in pancreatic acini: effect of CCK-JMV-180, Am. J. Physiol. 257 (1989) G202–G209.
[49] N. Rivard, G. Rydzewska, J.S. Lods, J. Martinez, J. Morisset, Pancreas growth, tyrosine kinase, PtdIns 3-kinase, and PLD involve high-affinity CCK-receptor occupation, Am. J. Physiol. 266 (1994) G62–G70.
[50] A.G. Andreoletti, M.J. Bragado, J.A. Tapia, R.T. Jensen, L.J. Garcia-Marin, Cholecystokinin rapidly stimulates CrK11 function in vivo in rat pancreatic acini: Formation of crk-11-protein complexes, Eur. J. Biochem. 270 (2003) 4706–4713.
[51] J.A. Tapia, M.J. Bragado, L.J. Garcia-Marin, R.T. Jensen, Cholecystokinin stimulated tyrosine phosphorylation of PKC-delta in pancreatic acinar cells is regulated bidirectionally by PKC activation, Biochim. Biophys. Acta (Mol. Cell Res.) 1593 (2002) 99–113.
[52] H. Welch, I. Maridonneau-Parini, Lyn and Fgr are activated in distinct membrane fractions of human granulocytic cells, Oncogene 15 (1997) 2021–2029.
[53] F. Nozu, Y. Tsunoda, A.I. Ibitayo, K.N. Bitar, C. Owyang, Involvement of RhoA and its interaction with protein kinase C and Src in CCK-stimulated pancreatic acini, Am. J. Physiol. 276 (1999) G915–G923.
[54] M. Jeschke, M.L. Brandi, M. Susa, Expression of Src family kinases and their putative substrates in the human preosteoclastic cell line FLG 29.1, J. Bone Miner. Res. 13 (1998) 1880–1889.
[55] M.J. Antonieta Cote-Velez, E. Ortega, A. Ortega, Involvement of pp 125FAK and p60SRC in the signaling through Fc gamma RII-Fc gamma RIII in murine macrophages, Immunol. Lett. 78 (2001) 189–194.
[56] A. Satoh, A.S. Gukovskaya, J.M. Nieto, J.H. Cheng, I. Gukovsky, J.R. Reeve Jr., T. Shimosegawa, S.J. Pandol, PKC delta and epsilon regulate NF-κB activation induced by cholecystokinin and TNF-α in pancreatic acinar cells, Am. J. Physiol. Gastrointest. Liver Physiol. 287 (2004) G582–G591.
[57] M. Shanmugam, N.L. Krett, C.A. Peters, E.T. Maizels, F.M. Murad, H. Kawakatsu, S.T. Rosen, M. Hunzicker-Dunn, Association of PKC delta and active Src in PMA-treated MCF-7 human breast cancer cells, Oncogene 16 (1998) 1649–1654.
[58] D. Crosby, A.W. Poole, Physical and functional interaction between protein kinase C delta and Fyn tryosine kinase in human platelets, J. Biol. Chem. 278 (2003) 24533–24541.
[59] C. Benes, S.P. Soltoff, Modulation of PKCδ tyrosine phosphorylation and activity in salivary and PC-12 cells by Src kinases, Am. J. Physiol. Cell Physiol. 280 (2001) C1498–C1510.
[60] P.M. Dombrosky-Ferlan, S.J. Corey, Yeast two-hybrid in vivo association of the Src kinase Lyn with the proto-oncogene product Cbl but not with the p85 subunit of PI 3-kinase, Oncogene 14 (1997) 2019–2024.
[61] S. Hunter, E.A. Burton, S.C. Wu, S.M. Anderson, Fyn associates with Cbl and phosphorylates tyrosine 731 in Cbl, a binding site for phosphatidylinositol 3-kinase, J. Biol. Chem. 274 (1999) 2097–2106.