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

Andrea Pace a,b, Jose A. Tapia a,c, Luis J. Garcia-Marín c, Robert T. Jensen a,*

a Digestive Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892-1804, USA
b Universitätsklinikum, Hamburg Eppendorf, 20246 Hamburg, Germany
c Departamento de Fisiología, Universidad de Extremadura, Cáceres 10074, Spain

Received 27 September 2005; received in revised form 14 March 2006; accepted 15 March 2006

Available online 7 April 2006

Abstract

Src family kinases (SFK) play a central signaling role for growth factors, cytokines, G-protein-coupled receptors and other stimuli. SFKs play important roles in pancreatic acinar cell secretion, endocytosis, growth, cytoskeletal integrity and apoptosis, although little is known of the specific SFKs involved. In this study we demonstrate the SFK, Lyn, is present in rat pancreatic acini and investigate its activation/signaling. Ca2+-mobilizing agents, cAMP-mobilizing agents and pancreatic growth factors activated Lyn. CCK, a physiological regulator of pancreatic function, rapidly activated Lyn. The specific SFK inhibitor, PP2, decreased Lyn activation; however, the inactive analogue, PP3, had no effect. Inhibition of CCK-stimulated changes in [Ca2+]i decreased Lyn activation by 55%; GFX, a PKC inhibitor by 36%; and the combination by 95%. CCK activation of Lyn required stimulation of high and low affinity CCKA receptor states. CCK stimulated an association of Lyn with PKC-δ, Shc, p125FAK and PYK2 as well as with their autophosphorylated forms, but not with Cbl, p85, p130CAS or ERK 1/2. These results show Lyn is activated by diverse pancreatic stimulants. CCK’s activation of Lyn is likely an important mediator of its ability to cause tyrosine phosphorylation of numerous important cellular effectors such as p125FAK, PYK2, PKC-δ and Shc, which play central roles in CCK’s effects on acinar cell function.

Published by Elsevier B.V.

Keywords: Pancreas; src; src kinase; lyn; Cholecystokinin; Pancreatic secretion; Pancreatic growth; Pancreatic growth factor

1. Introduction

The Src family of kinases (SFK) consists of nine structurally-related peptide members [1,2]. They are activated by a wide range of stimuli including antigens, growth factors, oxidative stress, integrins, and some G protein-coupled receptors [1,2]. SFK’s function as key signaling molecules for the changes in cellular function produced by these stimuli including growth, gene transcription, secretion, adhesion, and apoptosis [1]. The SFK members (c-Src, Fyn and Yes) are widely expressed in different tissues, whereas the others (including Lyn, Hck, Fgr, Blk, Yrk) have a more restrictive distribution [2]. In pancreatic acinar cells, SFKs are reported to be important in cell growth and the cellular effects of various growth factors [3]. In addition, in pancreatic acini, SFKs play an important role in enzyme secretion, membrane recycling, endocytosis, apoptosis, protein synthesis in regulating cellular calcium levels, in regulation of PKCs, in response to injury such as pancreatitis, and in maintaining cytoskeletal organization [4–11]. At present little is known about the specific SFKs involved in these different pancreatic cellular responses. In pancreatic acinar cells the SFKs reported to be present are Yes [7,10]; pp60c-Src in some studies [7] but not others [10]; and in two studies, Lyn [9,10]. Whereas a number of studies have examined activation of pp60 Src or Yes in gastrointestinal tissues including pancreas [4,5,7,8,10,11], there is almost no information on Lyn activation by gastrointestinal hormones/neurotransmitters or growth factors that alter acinar pancreatic cell function.

The SFK, Lyn, was originally discovered in hematopoietic cells [12] and almost all the early studies of Lyn were in these...
cells in which Lyn was shown to have positive and negative effects on proliferation, differentiation, immune responses and receptor-mediated signaling [12]. Recent studies report Lyn’s presence in a number of nonhematopoietic cells [9,13]. In general, little is known of Lyn’s function in nonhematopoietic/immune tissues such as the pancreas. Some recent studies suggest Lyn may have important effects on survival/growth in some of these cells because it has been shown to function as a negative regulator of apoptosis in a number of cell systems [13,14].

The purpose of the present study was to address these issues by studying the activation of Lyn by various gastrointestinal hormones/neurotransmitters and growth factors and its cell signaling in response to one of the main pancreatic hormones, CCK, in pancreatic acinar cells. This cell system is an excellent model to examine these relationships because it has been shown to be highly responsive to many gastrointestinal hormones/neurotransmitters and growth factors which alter pancreatic acinar function and has been extensively used to study the effects of these stimulants on cell signaling cascades including activation of phospholipases (A, C, D), adenylyl cyclase and tyrosine kinase cascades [6,9,15–17].

2. Experimental procedures

2.1. Materials

Male Sprague-Dawley rats (150–200 g) were obtained from the Small Animals Section, Veterinary Resources Branch, National Institutes of Health (Bethesda, MD). Purified collagenase (type CLSPA) was from Worthington; COOH-terminal octapeptide of cholecystokinin (CCK-8) was obtained from Peninsula Laboratories (Belmont, CA); CCK-JMV was obtained from Research Plus Inc., (Bayonne, NJ); Secretin, VIP, Endothelin-1, Hepatocyte Growth Factor (HGF), bFGF, Insulin, IGF-1, were from Bachem (Torrence, CA); phosphate-buffered saline, pH 7.4, was from Biofluids (Rockville, MD); Tyrosine kinase assay kit (non-radioactive) was from Upstate (Lake Placid, NY); phosphosylation-site specific pAb (anti-pY402 PYK2, anti-pY397 FAK, anti-pY418 Src) were from Biosource International (Camarillo, CA); goat anti-rabbit IgG horseradish peroxidase conjugate, mAb anti-Lyn, pAb anti-Lyn and Lyn Blocking Peptide, pAb anti-pp60Src, pAb anti-Yes, mAb anti-pp60Src, mAb anti-Yes were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phosphotyrosine mAb, anti-PKC-δ pAb, anti-FAK mAb and anti-PYK2 mAb and anti-Shc Ab were from BD Transduction Laboratories (Lexington, KY); platelet derived growth factor (PDGF), epidermal growth factor (EGF), PP2 (4-amino-5-[4-chlorophenyl]-7-pyrazol [3,4-d]pyrimidine) and PP3 (4-amino-7-phenylpyrazol [3,4-d]pyrimidine) were from Calbiochem (La Jolla, CA). Other reagents were from sources described previously [9,17,18].

2.2. Methods

2.2.1. Tissue preparation

Dispersed rat pancreatic acini were prepared according to the modifications of the procedure published previously [19]. Unless otherwise stated, the standard incubation solution contained 25.5 mM HEPES (pH 7.4), 98 mM NaCl, 6 mM KCl, 2.5 mM NaH2PO4, 5 mM sodium pyruvate, 5 mM sodium fumarate, 5 mM sodium glutamate, 11.5 mM glucose, 0.5 mM CaCl2, 1 mM MgCl2, 2 mM glutamine, 1% (w/v) albumin, 1% (w/v) trypsin inhibitor, 1% (v/v) vitamin mixture and 1% (w/v) amino acid mixture. The incubation solution was equilibrated with 100% O2 and all incubations were performed with 100% O2 as the gas phase. Dispersed acini from one or two rats were preincubated with standard incubation solution without or with different inhibitors for 3 h at 37 °C. Aliquots (1 ml) were then incubated at 37 °C with different agonists at the concentrations and times indicated. Acinar lysates were obtained as described previously [16,20]. Protein concentration was measured by the Bio-Rad protein assay reagent as described previously [19].

2.3. Immunoprecipitation tyrosine kinase assay

Immunoprecipitation of tyrosine phosphorylated proteins was performed as described previously [16]. For detection of p60 Src, Yes and Lyn, acinar lysates (900 μg/ml for p60 Src, 600 μg/ml for Yes, 300 μg/ml for Lyn) were incubated with 5 μg p60Src pAb, 4 μg Yes pAb, and 4 μg Lyn pAb. For the Lyn-blocking peptide experiments, a 120-min preincubation period with 8 μg/ml Lyn blocking peptide was performed. For detection of tyrosine phosphorylation of Lyn, acinar lysates (200–400 μg/ml) were incubated with 3.75 μg of anti-Lyn mAb or with 4 μg of anti-phosphotyrosine mAb (PY20) and 30 μl of Protein G-agarose overnight at 4 °C. For Lyn-co-immunoprecipitation studies acinar lysate (200–400 μg/ml) were incubated with 3.75 μg of anti-Lyn mAb and 30 μl of Protein-G-agarose overnight. The immunoprecipitates were washed three times with phosphate buffered saline and further analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting.

In vitro tyrosine kinase assay- After incubation with CCK-8 (1 or 10 nM) for 30s or with TPA (1 μM) for 5 min, pancreatic acini were lysed and Lyn was immunoprecipitated as described above. The immunoprecipitates were washed two times with lysis buffer and two times with tyrosine kinase reaction buffer (20 mM Tris–HCl pH 7.4, 10 mM MgCl2, 1 mM MnCl2, 1 mM DTT, 0.2 mM ATP) and resuspended in 50 μl of tyrosine kinase reaction buffer supplemented with 0.25 μg poly (Glu4-Tyr) peptide, biotin conjugated and 1 mM sodium orthovanadate. After 30 min incubation at 30 °C with vigorous shaking the reaction was stopped by heat inactivation (5 min at 95 °C). Phosphotyrosine was detected in a direct ELISA assay using a 1:100,000 dilution of anti-phosphotyrosine, recombinant 4G10, HRCP conjugate as described by the manufacturer (Tyrosine Kinase Assay Kit, Upstate #17-315).

2.3.1. Western blotting

Western blotting was performed as described previously [16,17,20]. Immunoprecipitates were subjected to 10% SDS-PAGE; proteins were transferred to nitrocellulose membranes (0.45 and 0.2 μm) for proteins higher and lower than 60 kDa, respectively. Membranes were blocked for one h at room temperature and incubated for 2 h at room temperature or overnight at 4 °C with 1 μg/ml anti-PYK2 pAb, 0.07 μg/ml anti-pAb, 0.5 μg/ml anti-PYK2 mAb, 0.25 μg/ml anti-Src mAb, 4 μg/ml anti-c-Src mAb, 0.2 μg/ml anti-Lyn mAb, 1 μg/ml anti-Yes pAb or for 3 h at room temperature with 0.5 μg/ml anti-pAb or 1 μg/ml anti-Shc-Ab overnight. Membranes were washed twice and incubated 1 h at 25 °C with goat anti-rabbit IgG-HRP conjugate. When a polyclonal antibody was used, or for 45 min with anti-Shc Ab were from BD Transduction Laboratories (Lexington, KY); platelet derived growth factor (PDGF), epidermal growth factor (EGF), PP2 (4-amino-5-[4-chlorophenyl]-7-pyrazol [3,4-d]pyrimidine) and PP3 (4-amino-7-phenylpyrazol [3,4-d]pyrimidine) were from Calbiochem (La Jolla, CA). Other reagents were from sources described previously [9,17,18].
processed as described previously [17,19,21]. Equal protein loading was verified using Lyn mAb.

3. Results

To verify that the antibodies used detected SFKs in pancreatic acini, we immunoprecipitated whole cell lysates with specific polyclonal antibodies (anti-pp60Src-, anti-Yes- and anti-Lyn-Ab) and then analyzed by Western blotting with specific monoclonal antibodies (Fig. 1). We identified pp60Src, Yes and Lyn using these specific antibodies (Fig. 1). We distinguished Lyn in pancreatic acini by its different molecular weight from the other two Src-kinases, pp60Src and Yes (Fig. 1). Lyn showed the characteristic double band corresponding to its two isoforms at 53 kDa and 56 kDa, as seen in other tissues. A specific blocking peptide for Lyn abolished its signal (Fig. 1, compare lanes 3 and 4).

Numerous studies show that Lyn and the other SFK members share two tyrosine phosphorylation sites, one (Y418), causing the activation of these kinases, the second (Y527), causing its inhibition, both through a conformational change of the kinases [1]. Y418 phosphorylation of SFKs has been shown to reflect the degree of activation of SFKs [22] and therefore was used to assess specific Lyn activation by first specifically immunoprecipitating Lyn. Receptor-mediated secretagogues known to activate phospholipase C (PLC) (CCK, carbachol, bombesin) [15,23] as well as post-receptor stimulants directly activating PKC (TPA) or mobilizing cellular calcium [thapsigargin (TG) or calcium ionophore (A23187)] (bottom panel) [15], each activated Lyn (Fig. 2). The combination of a calcium-mobilizing agent (TG, A23187) with a PKC activator (TPA) caused no greater activation of Lyn than with either agent alone (Fig. 2, bottom panel, lanes 1-6). Specifically, TPA (1 μM) and TPA (0.3 μM) caused a 4.3±0.5 (Fig. 2) and 2.6±0.2 (data not shown) fold activation of Lyn, respectively; A23187 (1 μM) caused a 3.9±1.4 fold increase (Fig. 2) and the combination of A23187 and TPA (1 μM or 0.3 μM) caused a 3.6±0.3 fold increase (Fig. 2) and a 2.9±0.2 fold increase (data not shown) in Lyn activation. Endothelin-1 (Et-1), which interacts with Et-1 and Et-3 receptors on acini but does not activate PLC cascades or cause a known change in cellular signaling [15,24], caused minimal activation of Lyn (Fig. 2, top panel, lane 7). Secretagogues interacting with G protein-coupled receptors and activating adenylate cyclase in pancreatic acini (VIP,
secretin) [15] as well as 8Br-cAMP, each also stimulated weak activation of Lyn (Fig. 2, top panel, lanes 5 and 6; bottom panel, lane 7). Various growth factors known to alter pancreatic acinar

Fig. 3. Time course of site-specific tyrosine phosphorylation of Lyn stimulated by CCK-8 in rat pancreatic acini. Rat pancreatic acini were treated with CCK-8 (10 nM) for the indicated times and then lysed. After immunoprecipitation with anti-Lyn mAb, Western blotting was performed with anti-pSrc (pY418) pAb and visualized as described in the legend to Fig. 1. The upper part shows results from a representative experiment and the protein loading control assessed with Lyn mAb after stripping membranes. The values shown in the bottom part are the means±S.E. of nine experiments and are expressed as fold increase over the pretreatment level (experimental/control).

Fig. 4. Concentration-dependence of CCK-8 and CCK-JMV stimulation of the site-specific tyrosine phosphorylation of Lyn in rat pancreatic acinar cells. Rat pancreatic acini were treated for 0.5 min with the indicated CCK-8 and CCK-JMV concentrations and then lysed. After immunoprecipitation with anti-Lyn mAb, Western blotting was performed as described in the legend to Fig. 1. The upper part shows results from a representative experiment and the protein loading control assessed with Lyn mAb after stripping membranes. The values shown in the bottom part are the means±S.E. of nine experiments and are expressed as the percentages of maximal increase caused by 10 nM CCK-8 above control unstimulated values. The maximal increase was 4.4±1.3 fold.

Fig. 5. Ability of CCK-8 and TPA to increase relative Lyn kinase activity in rat pancreatic acini. Rat pancreatic acini were incubated for 30 s with 1 or 10 nM CCK-8 or for 5 min with 1 μM TPA and then lysed. Lyn immunoprecipitated were resuspended in tyrosine kinase reaction buffer with 0.25 μg poly(Glu4-Tyr) peptide biotinylated and incubated for 30 min at 30 °C. In vitro kinase assay and phosphotyrosine detection were performed as described in Methods. The values of relative Lyn kinase activity are the means±S.E. of four experiments in duplicate and are expressed as the fold-increase as a ratio of the experimental value over the control value without addition. The maximal increase was 4.5±0.65 fold. *P<0.05 compared to control.

Fig. 6. Calcium-and PKC-dependence of CCK-8 stimulation of site-specific tyrosine phosphorylation of Lyn. Rat pancreatic acini were pretreated for 30 min at 37 °C in a calcium-free medium in presence of thapsigargin alone (1 μM) or with GF109203X (5 μM) and in a calcium-containing medium with or without GF109203X. Acini were then stimulated with 1 nM CCK-8 and then lysed. After immunoprecipitation with anti-Lyn mAb, Western blotting was performed as described in the legend to Fig. 1. The upper part shows results from a representative experiment and the protein loading control assessed with Lyn mAb after stripping membranes. The values shown in the bottom part are the means±S.E. of seven experiments and are expressed as the percentages of maximal increase caused by 1 nM CCK-8 above control unstimulated values.
cell function (HGF, EGF, bFGF, insulin, IGF, PDGF) [3,15] all caused activation of Lyn (Fig. 2, middle panel).

Because CCK is one of the principal physiological stimuli of pancreatic acinar function [25], in the remainder of the study, we concentrated on its ability to activate Lyn. The increase in pY418 tyrosine phosphorylation caused by CCK was rapid and its time course of tyrosine phosphorylation was biphasic, with an initial rapid increase and then a decrease (Fig. 3). The maximal increase occurred after 30 s and was 4.1 ± 0.4-fold; however, even after a 15-min incubation with CCK-8 when tyrosine phosphorylation of Lyn had decreased 63%, it still had not returned to control values, remaining 80 ± 10% over the control value (Fig. 3). By 30 min, the CCK-induced Lyn tyrosine phosphorylation was not different from the basal level (Fig. 3) and a similar result was seen 60 min after stimulation (data not shown). The effect of CCK-8 on pY418 tyrosine phosphorylation of Lyn was concentration-dependent (Fig. 4). CCK-8 caused a detectible effect at 1 pM, a maximal effect at 1 nM and a half-maximal effect at 0.08 ± 0.01 nM (Fig. 4) on Lyn activation. CCK-JMV, an agonist at only the CCKA high affinity receptor state [26] stimulated activation of Lyn in a concentration-dependent manner (Fig. 4), causing a detectible effect at 0.1 nM, a maximal effect at 0.1 µM and a half-maximal effect at 1.2 ± 0.4 nM (Fig. 4). CCK-JMV was not as efficacious as CCK-8, with a CCK-JMV concentration of 0.1 µM, causing a maximal response that was 58% ± 6% of the maximal CCK-8-stimulated pY418 tyrosine phosphorylation of Lyn (Fig. 4).

An assessment of in vitro Lyn kinase activity was performed in immunoprecipitates of pancreatic acini to show the correlation between pY418 tyrosine phosphorylation of Lyn, measured by Western blotting, with the relative Lyn kinase activity, measured by a direct ELISA assay. CCK-8 (1 nM) caused maximal increase in Lyn kinase activity (Fig. 5) similar to the maximal increase observed in pY418 tyrosine phosphorylation of Lyn cause by the same CCK-8 concentration (Fig. 4), 4.5 ± 0.65 vs. 4.4 ± 1.3, respectively. Moreover, TPA (1 µM) caused a similar fold increase in pY418 tyrosine phosphorylation of Lyn (4.3 ± 0.5 Fig. 2) and in Lyn kinase activity (3.5 ± 0.45 Fig. 5). Our results shown that pY418 tyrosine phosphorylation of Lyn reflect the degree of activation of Lyn, as previously described for this family of tyrosine kinases [22].
Ca²⁺-ATPase and depletes calcium from intracellular compartments. An agent that specifically inhibits the endoplasmic reticulum 

The maximal stimulation caused by CCK-8 (Fig. 6, lane 5).

Recent studies demonstrate CCK can stimulate activation and tyrosine phosphorylation of focal adhesion kinases (p125FAK and PYK2) in pancreatic acini [16,17,19]. Src family kinases can bind to the autophosphorylation sites of these kinases [p125FAK (pY397) and PYK2 (pY402)] and stimulate their activation in a number of other tissues [27]. To determine whether CCKA receptor activation could stimulate an association of Lyn with the focal adhesion kinase, p125FAK and PYK2, we assessed the formation of complexes between Lyn and these proteins by performing co-immunoprecipitation studies (Fig. 7). Stimulation with CCK-8 or the phorbol ester, TPA stimulated Lyn association with both focal adhesion kinases p125FAK and PYK2 (Fig. 7). CCK-8 and TPA also stimulated the association of Lyn with the autophosphorylated form of p125FAK (pY397) as well as the autophosphorylated form of PYK2 (pY402) (Fig. 7). To investigate whether in pancreatic acinar cells association of Lyn kinase with focal adhesion kinases is dependent on activation of Lyn, we used PP2, a specific inhibitor for Src tyrosine kinases, and its inactive analog, PP3, as a negative control [28]. Acini were pretreated for 60 min with PP2 (20 μM) or with PP3 (20 μM) and then incubated with 1 nM CCK-8 for 0.5 min or with 1 μM TPA for 5 min. Pretreatment of pancreatic acini with PP2 caused a complete inhibition of the association of Lyn with p125FAK (pY397) (Fig. 8, left panel) or PYK2 (pY402) (Fig. 8, right panel) induced by both CCK-8 and TPA. Pretreatment with PP3 under identical experimental conditions did not modify both basal or CCK-8- and TPA-stimulated association of Lyn with p125FAK (pY397) (Fig. 8, left panel) and PYK2 (pY402) (Fig. 8, right panel), demonstrating the specificity of the PP2. To confirm that PP2, and not PP3, were actually inhibiting activation of Lyn by CCK or TPA, pY418 tyrosine phosphorylation of Lyn was assessed after stimulation with CCK or TPA in the presence or absence of PP2 or PP3 (Fig. 9). PP2 (20 μM) caused a 68±17% inhibition of CCK and 60±10% inhibition of TPA-stimulated pY418 tyrosine phosphorylation of Lyn.

CCKA receptor stimulation activates phospholipase C, which promotes the hydrolysis of phosphatidylinositol 4,5-bisphosphate, leading to the generation of inositol phosphates that stimulate cellular calcium mobilization and to the production of diacylglycerol which activates PKCs [15,26]. We next examined whether CCK-8 induced activation of PKC or increase in intracellular calcium, or both, were needed for its ability to cause pY418 tyrosine phosphorylation of Lyn in pancreatic acini (Fig. 6). To determine the role of intracellular calcium changes [Ca²⁺], pancreatic acinar cells were pretreated for 30 min in a calcium-free medium with thapsigargin (10 μM), an agent that specifically inhibits the endoplasmic reticulum Ca²⁺-ATPase and depletes calcium from intracellular compartments [17]. These conditions have been shown to completely inhibit the [Ca²⁺]i increase stimulated by CCK-8 in rat pancreatic acini [17]. To determine whether PKC activation might be involved in mediating CCK-8-stimulated increases in pY418 tyrosine phosphorylation of Lyn, we examined the effect of a PKC inhibitor, GF109203X [17]. Thapsigargin in a calcium-free media inhibited CCK stimulation of pY418 tyrosine phosphorylation of Lyn by 55±8% (Fig. 6, compare lanes 2 and 3). The inhibition of PKC activation by CCK reduced pY418 tyrosine phosphorylation of Lyn by 35±26% of the maximal stimulation caused by CCK-8 (Fig. 6, compare lanes 2 and 4). The simultaneous inhibition of both a CCK-stimulated increase in intracellular calcium and PKC activation by the combination of GFX109203X and thapsigargin in a calcium-free media caused an additive effect, inhibiting the pY418 tyrosine phosphorylation of Lyn by 95±4% of the maximal stimulation caused by CCK-8 (Fig. 6, lane 5).

Fig. 9. The effect of the Src-inhibitor PP2 and its inactive analog PP3 on CCK-8 and TPA stimulation of site-specific Lyn tyrosine phosphorylation in rat pancreatic acinar cells. The experimental conditions were as described in Fig. 8 legend. After immunoprecipitation with anti-Lyn mAb, Western blotting was performed as described in the legend to Fig. 1. The upper part shows results from a representative experiment and the protein loading control assessed with Lyn mAb after stripping membranes. This experiment is representative of three others. The values shown in the bottom part are the means±S.E. of four experiments and are expressed as fold increase over the pretreatment level (experimental/control).

Fig. 10. CCK-and TPA-stimulated association of Lyn with Shc and PKC-δ. Rat pancreatic acini were treated with CCK-8 (10 nM) for 0.5 min or TPA (1 μM) for 5 min and then lysed. After immunoprecipitation with anti-Shc pAb (top panel) or anti-PKC-δ pAb (bottom panel), Western blotting (WB) was performed with anti-Lyn mAb. Results are representative of two other experiments.
whereas PP3 had no effect on stimulation by either CCK or TPA (Fig. 9).

CCK is reported to activate MAP kinases in pancreatic acini [29] as well as the novel protein kinase C, PKC-δ, in addition to stimulating its tyrosine phosphorylation and translocation [9]. In a number of tissues, Src kinases are reported to play a role in the activation of Shc and/or PKC-δ by various stimuli and increase the association of Src with these proteins [9,30]. Recently, both TPA and CCK in pancreatic acini are reported to stimulate the interaction of PKC-δ with a Src kinase and evidence was provided that Lyn was at least partially involved [9]. To determine whether CCKA receptor activation could stimulate an association in pancreatic acini between SFKs and Shc, and to confirm the previous study reporting an increased association between PKC-δ and Lyn [9], we performed co-immunoprecipitation studies (Fig. 10). Both TPA and CCK stimulated an association of Lyn with Shc and with PKC-δ (Fig. 10). Src kinases are reported to associate [31,32] with a number of other intracellular proteins in other cells including p130Cas, PI3K, and Cbl. We assessed whether CCKA receptor activation or TPA stimulation could stimulate such an association in pancreatic acini using immunoprecipitation studies. Immunoprecipitating with antibodies to p130Cas, Cbl, phosphoCbl, ERK 1/2, or the p85 subunit of PI3K, and Western blotting with anti-Lyn antibody, or performing studies with antibodies in the reverse order, did not demonstrate any CCKA receptor or TPA-stimulated association between Lyn kinase and these proteins (data not shown).

4. Discussion

The present study was designed to address the question of whether activation of the SFK, Lyn is involved in the cellular signaling by various gastrointestinal growth factors and gastrointestinal hormones/neurotransmitters that are known to alter pancreatic acinar cell function. The effect of the hormone/neurotransmitter, cholecystokinin (CCK), which is a physiological regulator of pancreatic acinar cell function [25], was investigated in detail since CCK is reported to activate other SFKs such as Yes and pp60Src [5,10,11,17,33]. A number of our results support the conclusion that the SFK, Lyn, is present in pancreatic acinar cells. Lyn’s presence was established by Western blotting after immunoprecipitating with specific Lyn antibodies. The expression of Lyn could be distinguished from the two other SFKs expressed in pancreatic acini, pp60Src and Yes, by their different molecular weights, and the detection of characteristic p53 and p56 isoforms of Lyn, which have been described in various tissues [12]. Furthermore, specific detection of Lyn in pancreatic acinar cells by the Lyn antibody used was supported by demonstrating the ability of a specific Lyn-blocking peptide to abolish the Western blotting signal. Our result is supported by findings in two other studies [9,10] which report that pancreatic tissues contains a Lyn-like protein.

In the present study we assessed the ability of various pancreatic stimulants to activate Lyn by assessing their ability to stimulate pY418 tyrosine phosphorylation of Lyn. Our results show that CCK, as well as a number of other pancreatic secretagogues which activate G protein-coupled receptors on pancreatic acinar cells and stimulate either phospholipase C (PLC)-mediated, PKA-mediated pathways or other signaling cascades [15], can activate Lyn in pancreatic acini. These results have some similarities and differences from previous studies of the ability of these GI hormones/neurotransmitters and other stimulants to activate SFK in pancreatic acini and other tissues. They are similar in that CCK has been reported to stimulate SFKs in pancreatic acinar cells or AR42J pancreatic acinar cancer cells [4–8,11,34] as well as bombesin-related peptides, VIP, muscarinic cholinergic agents and secretin activate SFKs in other tissues [1,35]. Endothelin has been shown to activate SFKs in different cells [36,37]. Whereas in many cells, including pancreatic acini, agents that activate PLC cascades have been frequently shown to activate various SFKs, the ability of agents that stimulate adenylyl cyclase to activate various SFKs shows marked differences in different studies. In some cells agents that activate adenylyl cyclase, stimulate SFK activation [38–40]; whereas in other cells agents that increase cyclic AMP decrease SFK activation [41] or have no effect on SFK activation [42]. Our results demonstrate that Lyn is activated in pancreatic acini by G protein-coupled receptors acting through diverse signaling pathways including activation of adenylyl cyclase and, therefore, Lyn likely plays an important central role in signaling by many GI hormones/neurotransmitters that alter pancreatic acinar cell function.

Numerous growth factors (HGF, EGF, bFGF, insulin, IGF-1 and PDGF) have been shown to interact with specific receptors on pancreatic acinar cells and stimulate growth and/or protein synthesis as well as alter other cellular functions [15]. SFKs play an important role in mediating the action of growth factors in other tissues [1]. However little is known of the role of SFKs in growth factor signaling in normal pancreatic acinar cells. Our study demonstrates that each of the known pancreatic growth factors (HGF, EGF, bFGF, insulin, IGF-1 and PDGF) activate Lyn in rat pancreatic acini. Our results differ from a recent study [6] that reported bFGF failed to stimulate SFKs in pancreatic acini.

Numerous studies demonstrate that the CCK can activate both a high and low affinity receptor state which can mediate different cellular responses [17,26]. A number of results support the conclusion that CCK causes activation of Lyn by interacting with both CCKA receptor states. The CCK dose-response curve for Lyn activation extended over 5 log units which included the concentration ranges it activates, both high and low affinity receptor states [15], suggesting activation of both CCKA receptor states are involved in Lyn activation. This conclusion is supported by the results with CCK-JMV, a synthetic CCK analogue that stimulated 60% of the maximal Lyn activation caused by CCK-8. Because CCK-JMV in rat pancreatic acini functions as a full agonist at the high affinity state and an antagonist of the low affinity state [26], our results demonstrate 60% of Lyn activation is due to activation of the high affinity CCKA receptor state and 40% to activation of the low affinity CCK receptor state. These results have similarities and differences from that reported in other studies in pancreatic acini with CCKA receptor-mediated tyrosine phosphorylation of other proteins, or stimulation of SFK activation. Similar to CCK
stimulation of Lyn activation, CCK stimulation of tyrosine phosphorylation in pancreatic acini of the focal adhesion kinases (p125FAK, PYK2/CAβ) [17] or their phosphospecific sites (pY402, pY508, pY881-PYK2, pY397, pY577, pY925-FAK) [19]; and paxillin [16,17] is mediated by activation of both high and low affinity CCKA receptor state. In contrast, CCK-stimulated tyrosine phosphorylation of PKC-δ [9] or CrKII [34] is mediated by activation of only the low affinity CCKA receptor state. Also in contrast to a previous study [6] which reported CCK-stimulated SFK activity is mediated almost entirely by activation of the low affinity CCKA receptor state in pancreatic acini, we found with CCK stimulation of Lyn activation, at least 60% is through the high affinity CCKA receptor state. These results provide additional support to the proposal [43] that not only are the different CCKA receptor states on pancreatic acini differentially coupled to activation of phospholipase A2 and C [15], they are also differentially coupled to a number of tyrosine phosphorylation cascades including activation of Lyn.

Activation of the pancreatic CCKA 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 [15,17]. Recent studies show activation of the two limbs of the PLC cascade (i.e. PKC and cellular calcium) in pancreatic acini and other cells by different stimuli, can have different effects on the phosphorylation of non-receptor tyrosine kinases such as the focal adhesion kinases, p125FAK and PYK2 [16,17]. There is only minimal information available on the interaction with the PLC cascade and activation of SFKs in pancreatic acinar cells. In one study [6] it was concluded that CCK could activate SFKs in pancreatic acini only through its activation of the low affinity CCKA receptor state, resulting in mobilization of cellular calcium, whereas its activation of the high affinity CCKA receptor state or PKC activation had no effect on SFK activity. A number of our results support the conclusion that in pancreatic acini CCK activation of Lyn involves activation of both limbs of the PLC cascade. First, agents that increased cellular calcium activated Lyn. Second, activation of PKCs by TPA also activated Lyn. Third, incubation with the Ca2+ATPase inhibitor, thapsigargin, which has been shown to completely inhibit CCK-mediated increase in [Ca2+]i in pancreatic acini [17], inhibited CCK-stimulated Lyn activation by 55%. Furthermore, the PKC inhibitor, GF109203X, at a concentration that inhibited TPA-stimulated tyrosine phosphorylation of other non-receptor tyrosine kinases such as p125FAK and PYK2 [17,19] in rat pancreatic acini, caused a 36% decrease in CCK-stimulated activation of Lyn. Fourth, the simultaneous inhibition of both limbs of the PLC cascade by addition of GF109203X and thapsigargin, completely inhibited Lyn activation, supporting the conclusion that CCK’s activation of both limbs of the PLC cascade are essential for full Lyn activation. 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 [44,45]. In contrast, activation of SFKs in granulocytes is not dependent on PKC activation [45], and bombesin activation of Src pathway in 3T3 cells is calcium-independent [44].

In other cells, activated SFKs are known to associate with and alter the activity of many substrates including kinases, cytoskeletal proteins, structural proteins, enzymes, adapters and receptors [1]. Particularly important with some growth factors and G-protein-coupled receptors are interactions with focal adhesions kinases (p125FAK, PYK2) [1,27,44], paxillin [1], p130CAS [1], PKC-δ [1], P13-kinase [1], Shc [1,30,33,46,47], and Cbl [1]. A number of our results support the conclusion that CCK and TPA activation of Lyn in pancreatic acini stimulates association with focal adhesion kinases as well as with Shc and PKC-δ. First, CCK as well as TPA stimulated an association of Lyn with p125FAK and PYK2 as well as with pY397 p125FAK and pY402 PYK2, their specific autophosphorylated forms [19,27]. Second, PP2 [28], a specific SFK inhibitor, but not the related inactive analogue, PP3 [28], inhibited the pY418 tyrosine phosphorylation of Lyn by CCK and TPA and inhibited the association of Lyn with either pY397 p125FAK or pY402 PYK2. Third, immunoprecipitation studies showed both CCK and TPA stimulated an association of Lyn with Shc and PKC-δ but not with Cbl, ERK 1/2, the p85 subunit of PI3K, or with p130CAS. Previous studies demonstrate CCK activates ERK 1/2 [29], p130CAS [20], and PI3K [5] in pancreatic acinar cells; therefore, the failure to stimulate an association of Lyn with these signaling molecules as reported with Lyn and other SFKs in other cells [1] is not due to lack of stimulation by CCK of these signaling pathways in pancreatic acini. Previous studies have demonstrated in pancreatic acini CCK stimulates activation of MAP kinases [29] via a signaling cascade involving activation of PLC, resulting in mobilization of cellular calcium, PKC activation and stimulation of tyrosine kinases. CCK also stimulates formation of a Shc-Grb2-Sos complex in pancreatic acini [46], which has been shown to play a central role in MAP kinase activation. Lastly, a number of G-protein-coupled receptors activate MAP kinases via activation of PYK2 [17] and activation of CCKA receptor in pancreatic acini stimulates a Grb2-PYK2 complex formation [17]. Our results support the conclusion that the SFK, Lyn, may play a central role in CCK activation of MAP kinase in pancreatic acini because CCK stimulated both an association of Lyn with Shc as well as PYK2, each of which activate MAP kinases [17]. These results are similar to activation of a number of other G-protein-coupled receptors which have been shown to stimulate MAP kinases by similar mechanisms [48], and similar to results in neutrophils with activation of chemoattractant receptors which stimulated a complex formation between Lyn and Shc [47]. Recent studies demonstrate CCK stimulates the translocation, activation, and tyrosine phosphorylation of PKC-δ in pancreatic acini [9], which is mediated by a Src-dependent mechanism [9], and this activation stimulates NFκ-B activity [49] which is important in CCK-stimulated pancreatitis in these cells [49]. Our results support the conclusion that the SFK, Lyn, is likely the SFK involved because our results confirm the findings of a previous study [9] that CCK stimulated the association of Lyn with PKC-δ.
This result is consistent with studies in other cells where stimulation by insulin, PKC activators, or growth factors is reported to stimulate an association of Lyn or another SFK with PKC-δ [9,50] but differ from results in PC-12 cells [51] or parotid cells [51] where neither TPA nor carbochol stimulated an association of PKC-δ and Src, although each activated PKC-δ [9,50].

References


