Cholecystokinin-stimulated Protein Kinase C-δ Kinase Activation, Tyrosine Phosphorylation, and Translocation Are Mediated by Src Tyrosine Kinases in Pancreatic Acinar Cells*

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Protein kinase C-δ (PKC-δ) is involved in growth, differentiation, tumor suppression, and regulation of other cellular processes. PKC-δ activation causes translocation, tyrosine phosphorylation, and serine-threonine kinase activity. However, little is known about the ability of G protein-coupled receptors to activate these processes or the mediators involved. In the present study, we explored the ability of the neurotransmitter/hormone, CCK, to stimulate these changes in PKC-δ and explored the mechanisms. In rat pancreatic acini under basal conditions, PKC-δ is almost exclusively located in cytosol. CCK and TPA stimulated a rapid PKC-δ translocation to membrane and nuclear fractions, which was transient with CCK. CCK stimulated rapid tyrosine phosphorylation of PKC-δ and increased kinase activity. Using tyrosine kinase (B44) and a tyrosine phosphatase inhibitor (orthovanadate), changes in both CCK- and TPA-stimulated PKC-δ tyrosine phosphorylation were shown to correlate with changes in its kinase activity but not translocation. Both PKC-δ tyrosine phosphorylation and activation occur exclusively in particulate fractions. The Src kinase inhibitors, SU6656 and PP2, but not the inactive related compound, PP3, inhibited CCK- and TPA-stimulated PKC-δ tyrosine phosphorylation and activation. In contrast, PP2 also had a lesser effect on CCK- but not TPA-stimulated PKC-δ translocation. CCK stimulated the association of Src kinases with PKC-δ, demonstrated by co-immunoprecipitation. These results demonstrate that CCK_A receptor activation results in rapid translocation, tyrosine phosphorylation, and activation of PKC-δ. Stimulation of PKC-δ translocation precedes tyrosine phosphorylation, which is essential for activation to occur. Activation of Src kinases is essential for the tyrosine phosphorylation and kinase activation to occur and plays a partial role in translocation.

The protein kinase C (PKC) family of proteins consists of 12 members that are phospholipid-dependent serine/threonine kinases (1–5). This family is divided based on their structure and allosteric requirements, into three general subgroups including the calcium-dependent conventional PKCs (α, β1, β2, and γ), two calcium-independent subgroups including the novel PKCs (δ, ε, η, θ, and τ) and atypical PKCs (ζ, λ1, and μ) (2–4). The phorbol ester activates all subgroups except the atypical subgroup. Different cells frequently possess different PKC isoforms and recent studies suggest the PKC isoforms may have different functions in various cells (6, 7).

Recent studies demonstrate the novel PKC, PKC-δ, is widely expressed and plays an important function in numerous diverse cellular processes including the modulation of transduction cascades (prostaglandin formation and phosphoinositide hydrolysis), regulation of various channels (Na+–H+ exchanges, L-channels, Glut-4), and numerous growth-related roles (cell growth, differentiation, apoptosis, and tumor suppression) (1, 8–17). PKC-δ can be activated by a wide range of stimuli including oxidative stress, growth factors, tumor promoters, immunoglobulins (IgE), chemotherapeutic agents (etoposide), Ras, and a few G protein-coupled receptors (1, 8, 11–13, 16, 18–22). Similar to other PKCs, activation of PKC-δ both stimulates its translocation to cellular membranes and increases its serine threonine kinase activity. However, in contrast to the other PKCs, PKC-δ is also tyrosine-phosphorylated upon stimulation (1, 19). With the G protein-coupled receptors (GPCRs) and to a varying degree with the other stimuli, the relationship between these three processes (translocation, tyrosine phosphorylation, and serine-threonine kinase activation) upon PKC-δ stimulation as well as the cellular mechanisms mediating these changes, remains unclear. Studies have concluded that with various stimuli, stimulation of PKC-δ tyrosine phosphorylation can increase kinase activity (1, 8, 23, 24), decrease kinase activity (1, 10, 18), is a necessary precursor for activation of kinase activity (23, 25), or the two processes are independent of each other (26, 27). Furthermore, some studies suggest Srsks kinases may play a key role, whereas others suggest they are not involved (10, 24–26, 28–32).

Recent studies demonstrate that activation of the CCK_A receptor, a heptahedral GPCR that mediates the action of neuropeptide/hormone, CCK, causes rapid tyrosine phosphorylation of PKC-δ in pancreatic acinar cells (19) as well as tyrosine phosphorylation of a number of important intracellular proteins that function as adaptors and effectors in mediating cellular responses (p125FAK, PYK2, p130Cas, mitogen-activated protein kinase, paxillin) (33–35). CCK functions both as

acetate; MOPS, 3-(N-morpholino)propanesulfonic acid; Ab, antibody; mAb, monoclonal antibody; PDGF, platelet-derived growth factor; PP2, 4-amino-5-(4-chlorophenyl)-7-(tr-buty1)pyrazolo[3,4-d]pyrimidine; PP3, 4-amino-7-phenylpyrazolo[3,4-d]pyrimidine.
a peptide neurotransmitter and neuromodulator, as well as a hormone in the gastrointestinal tract (36). In the central nervous system, CCK is one of the most abundant neuropeptides and has such diverse effects as functioning as a modulator of dopamine release, stimulating panic attacks, stimulating vagal transmission, and functioning as a regulator of satiety and morphine-induced analgesia (36, 37). In the gastrointestinal tract, CCK functions as a physiological mediator of pancreatic secretory and gallbladder contraction, and gastric and colonic motility and plays an important role in pancreatic acinar cell growth (36, 38–40). The cellular basis of action of CCK_{48} receptor stimulation has been extensively studied in pancreatic acinar cells, with studies demonstrating that CCK_{48} receptor activation causes stimulation of phospholipase C_{34} as well as stimulation of phospholipase C and D activation, resulting in mobilization of phosphatidyl inositol 4,5-bisphosphate, which results in the generation of diacylglycerol and IP_{3}, which activate protein kinase C and D, respectively (41, 42) as well as the role of Src kinases in mediating each process. 

**EXPERIMENTAL PROCEDURES**

**Materials**

Male Wistar 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). Anti-PKC-δ mAb and anti-phosphotyrosine mAb (PY20) were from BD-Transduction Laboratories (Lexington, KY). Anti-PKC-λ polyclonal Ab and goat anti-rabbit IgG was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phosphotyrosine mAb (4G10) and recombinant protein A-agarose were from Upstate Biotechnology, Inc. (Lake Placid, NY). Histone H1, 4-aminophosphonatophosphatidylethanolamine, 13-acytate (TPA), phenylmethanesulfonate fluoride, deoxycholic acid, EDTA, EGTA, sucrose, sodium pyrophosphate, sodium fluoride (NaF), sulfonyl fluoride, deoxycholic acid, EDTA, EGTA, sucrose, sodium pyrophosphate, sodium fluoride (NaF), β-glycerophosphate, and dithiothreitol were from Sigma. Phosphate-buffered saline, pH 7.4, was from Biofluids (Rockville, MD). Basal small and basal medium Eagle vitamin solution were from Invitrogen. Aprotinin, pepstatin, leupeptin, and PMSF were from Roche Applied Science. 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride and bovine serum albumin fraction V were from ICN Biomedicals Inc. (Aurora, OH). Goat anti-mouse IgG-agarose peroxidase conjugate, recombinant protein G-agarose, and enhanced chemiluminescence detection reagents were from Pierce. SDS, 2-mercaptoethanol, protein assay dye reagent, Tris/glucose/SDS buffer (10 times concentrated), and Triton X-100 were from Bio-Rad. Nonidet P-40 and Red ice were from Amersham Biosciences, and nitrocellulose membrane was from Schleicher & Schuell.

**Methods**

**Tissue Preparation**—Dissected rat pancreatic acini were obtained by collagenase digestion (45). Unless otherwise stated, the standard incubation solution contained 25.5 mM HEPES (pH 7.4), 98 mM NaCl, 6 mM KCl, 2.5 mM NaHPO_{4}, 5 mM sodium succinate, 5 mM sodium fumarate, 5 mM sodium glutamate, 11.5 mM glucose, 0.5 mM CaCl_{2}, 1 mM MgCl_{2}, 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% O_{2} and all incubations were performed with 100% O_{2} as the gas phase. 

**Immunoprecipitation and Co-immunoprecipitation**—Immunoprecipitation of PKC-δ or tyrosine-phosphorylated proteins was performed as described previously (35, 46). Briefly, dispersed acini from one rat were preincubated for up to 3 h at 37 °C in standard incubation solution. After preincubation, cellular aliquots of 1 ml were incubated at 37 °C with different agonists at the concentrations and times indicated and washed with ice-cold phosphate-buffered saline. Lysates were obtained from these aliquots using lysis buffer. When immunoprecipitation was made prior to a kinase assay, lysates were obtained without sonication using lysis buffer specific for the kinase assay (see composition below). For assessment of tyrosine phosphorylation and in the co-immunoprecipitation studies, lysates were obtained by sonication in Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% (w/v) NaN_{3}, 1 mM EDTA, 2.5 μg/ml aprotinin, 2.5 μg/ml pepstatin, 2.5 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 0.2 mM Na_{3}VO_{4} as described previously (46). For tyrosine phosphorylation studies, lysates (1 ml; 500 μg) were incubated with 4 μg of anti-phosphotyrosine mAb (PY20) or 4 μg of anti-PKC-δ polyclonal Ab and 25 μl of protein A-agarose overnight at 4 °C. For co-immunoprecipitation studies, lysates (1 ml; 800 μg) were preincubated with 6 μg of anti-PKC-δ polyclonal Ab or 6 μg of anti-PKC-δ mAb for a further 60 min at 4 °C. The immunoprecipitates were washed with phosphate-buffered saline and analyzed by SDS-PAGE and Western blotting.

**Subcellular Fractionation**—Acinar cell were fractionated into cytosolic, membrane, and nuclear fractions, according to the procedures published previously (35, 46, 47). Briefly, acinar cells were resuspended in 1 ml of lysis buffer without detergents and homogenized using a Polytron homogenizer (Brinkmann Instruments). Lysates were first centrifuged at low speed (500 × g) for 10 min at 4 °C to precipitate nuclei, debris, and fat. The supernatant was centrifuged for 30 min at 60,000 × g at 4 °C to separate the membrane fraction (pellet) and cytosol fraction (supernatant). The nuclear fraction was purified using ultracentrifugation in sucrose gradient (47) from pellets obtained after the initial centrifugation (500 × g). Pellets were resuspended in 1 ml of ice-cold KCl-containing lysis buffer (50 mM Tris/NaCl, pH 7.5, 150 mM NaCl, 0.1% (w/v) Na_{3}PO_{4}, 1 mM EDTA, 2.5 μg/ml aprotinin, 2.5 μg/ml pepstatin, 2.5 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 0.2 mM Na_{3}VO_{4}), deposited on top of tubes with 10 ml of KCl-containing lysis buffer and high sucrose concentration (500 × g). The supernatant was centrifuged for 4 h at 150,000 × g at 4 °C. After their isolation, both nuclear and plasma membranes were washed with phosphate-buffered saline, resuspended in regular lysis buffer, sonicated for 5 s at 4 °C, and centrifuged at 15,000 × g for 15 min. Protein concentration was estimated using the Bio-Rad protein assay reagent, and an equal amount of proteins per sample of each subcellular fraction was further analyzed by SDS-PAGE and Western blotting with or without previous immunoprecipitation.

**Western Blotting**—Western blotting was performed as described previously (34, 35, 46). Anti-phosphotyrosine or anti-PKC-δ immunoprecipitates, whole cell lysates (10 μg of proteins/well), or subcellular fraction lysates (10 μg of proteins/well) were fractionated by SDS-PAGE (10% polyacrylamide gels). Proteins with molecular masses higher than 60 kDa (20 kDa in co-immunoprecipitation studies) were transferred to nitrocellulose membranes. Membranes were blocked overnight at 4 °C using blotto (5% nonfat dried milk in a solution containing 50 mM Tris/HCl, pH 8.0, 2 mM CaCl_{2}, 80 mM NaCl, and 0.05% (v/v) Tween 20) and incubated for 90 min at 25 °C with 0.9 μg/ml of anti-phosphotyrosine mAb (4G10) or 0.2 μg/ml of anti-PKC-δ polyclonal Ab. After incubation with the primary antibody, membranes were washed twice for 4 min with blotto and incubated for 45 min at 25 °C with anti-mouse or anti-rabbit IgG-agarose peroxidase conjugate. Membranes were washed with washing solution (50 mM Tris/HCl, pH 8.0, 2 mM CaCl_{2}, 80 mM NaCl, and 0.05% (v/v) Tween 20), incubated for 5 min with enhanced chemiluminescence detection reagents (SuperSignal West Dura; Pierce), and, finally, exposed to Biomax AR films (Eastman Kodak Co.) or directly measured in a Kodak Image Station 440CF (PerkinElmer Life Sciences). The intensity and molecular weight of bands on films or directly measured in a Kodak Image Station 440CF (PerkinElmer Life Sciences). The intensity and molecular weight of bands on films or directly measured in a Kodak Image Station 440CF (PerkinElmer Life Sciences). The intensity and molecular weight of bands on films or directly measured in a Kodak Image Station 440CF (PerkinElmer Life Sciences).
the immunoprecipitates using the PKC assay kit from Upstate Biotechnology following the directions provided by the company with minor modifications. Immune complexes bound to the protein G-agarose were washed two times with 1 ml of lysis buffer for the kinase assay and two times with assay dilution buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM dithiothreitol, and 5 mM EGTA). Following the last wash, the pelleted beads were resuspended in 30 μl of assay dilution buffer containing 5 μg of phosphatidylinerine and 0.5 μg of diacylglycerol. The kinase reaction was initiated with the addition of a magnesium/ATP mixture (75 mM MgCl2 and 0.5 mM ATP) containing 10 μCi of [γ-32P]ATP (3000 Ci/mmol) and the substrate, either histone H1 (10 μg) or the PKC substrate peptide (QKRPSQRSKL) (80 μg). The reaction mixtures (final volume 90 μl) were briefly vortexed and then incubated at 30 °C for 30 min with occasional mixing. After incubation, when histone H1 was used as the substrate, the kinase reaction was terminated by adding 15 μl of 4× SDS sample buffer and boiling the samples for 5 min at 95 °C. Samples were resolved using 4–20% SDS-PAGE gels. Finally, gels were stained, destained, and analyzed in a Phosphor imager (InstantImager; Packard Instrument Co.). When PKC-δ–20% SDS-PAGE gels. Finally, gels were dried and analyzed in a boiling the samples for 5 min at 95 °C. Samples were resolved using precipitation by SDS-PAGE followed by Western blotting with anti-PKC-δ polyclonal Ab, as described under “Methods.” Bands were visualized using chemiluminescence, and quantification of band intensities was performed by densitometry. The upper panel shows results from a representative experiment with unstimulated control cells (time 0) and cells stimulated by CCK-8 (10 nM) or TPA (1 μM) for the indicated times. These results are representative of three others in duplicate. The values shown in the bottom panel are mean ± S.E. of four independent experiments expressed as the percentage of maximal existence of PKC-δ (100%) in membrane fraction reached by CCK-8-stimulated acinar cells within a 1-min incubation or by TPA-stimulated acini within a 5-min incubation. Maximal stimulation by 10 nM CCK-8 was a 19.8 ± 4.2-fold increase.

**RESULTS**

**PKC-δ Subcellular Localization in Rat Pancreatic Acinar Cells and CCK-8 and TPA Stimulation of PKC-δ Translocation**—Previous studies (9, 48, 49) demonstrate that pancreatic acinar cells possess PKC-δ and that the phorbol ester TPA, as well as activation of some G-protein-coupled receptors, can cause its tyrosine phosphorylation (8, 19, 24, 50, 51). In the present study, we found that CCK-8 and TPA caused a translocation of PKC-δ (Figs. 1–3) as well as activation of PKC-δ (Figs. 4 and 5) and stimulated its tyrosine phosphorylation (Figs. 6–8). In rat pancreatic acini under basal conditions, PKC-δ was almost exclusively localized (95 ± 2%) in the cytosolic fraction (Fig. 1, lane 1). Upon the addition of 1 μM TPA for 5 min, there was almost a complete translocation of PKC-δ from the cytosolic fraction to the membrane or nuclear fractions (Fig. 1, lane 2), with the amount of PKC-δ immunodetected in cytosol being reduced by >95% (Fig. 1, lane 2). After TPA treatment, 55 ± 6% of PKC-δ was localized in the plasma membrane fraction and 45 ± 6% in the nuclear fraction (Fig. 1, lane 2). Treatment with the neuropeptide CCK-8 also induced translocation of PKC-δ to membrane and nuclear fractions in pancreatic acini but to a less extent than TPA (Fig. 1). Specifically, with 10 nM CCK-8 treatment for 2.5 min, there was a 37 ± 6% decrease in cytosolic PKC-δ levels (Fig. 1, lane 3), and there was a simultaneous increase by 27 ± 9% in the membrane fraction and by 15 ± 4% in the nuclear fraction (Fig. 1, lane 3).

**Time Course of CCK-8 and TPA Stimulation of PKC-δ Translocation from Cytosol to the Membrane and Nuclear Fractions in Rat Pancreatic Acini**—To assess the kinetics of PKC-δ translocation following stimulation by TPA or CCK, we investigated the time course of PKC-δ subcellular localization in target membranes after CCK-8 or TPA stimulation (Figs. 2 and 3). With CCK-8 stimulation, translocation from cytosol to the membrane fraction was maximal by 1 min and then rapidly decreased (Fig. 2, circles). After 15 min of incubation with CCK-8, only 25 ± 9% of the amount of PKC-δ seen at 2.5 min remained in the membrane fraction (Fig. 2, circles). Identical
CCK Stimulation of PKC-δ

Fig. 3. Time course of CCK-8 and TPA stimulation of PKC-δ translocation to the nuclear fraction in rat pancreatic acini. Rat pancreatic acini were stimulated with 10 nM CCK-8 or 1 μM TPA for the indicated times. Nuclear fraction lysates were obtained by ultracentrifugation as described in Methods. Proteins were fractionated by SDS-PAGE, and total PKC-δ was detected by anti-PKC-δ immunoblotting as described in Methods and in the legend to Fig. 2. The upper panel are results from a representative experiment, showing nuclear PKC-δ in control acini (time 0) and acini stimulated with CCK-8 (10 nM) or with TPA (1 μM) for the indicated times. Results are representative of three others in duplicate. Values shown in the lower panel are mean ± S.E. of four independent experiments expressed as the percentage of maximal PKC-δ translocation to nuclear fraction, which in pancreatic acinar cells was induced by CCK-8 (10 nM) within 1 min of stimulation and by TPA (1 μM) within 5 min of stimulation. Maximal stimulation by CCK-8 (10 nM) was a 13.4 ± 7.2-fold increase.

Kinetics were found in the CCK-8-induced translocation of PKC-δ to the nuclear fraction (Fig. 3, circles). However, TPA-induced PKC-δ translocation to either the membrane (Fig. 2) or to the nuclear fraction (Fig. 3) showed a different pattern. Specifically, after treatment with 1 μM TPA, PKC-δ in the membrane fraction was 72 ± 5% of maximal by 1 min and maximal by 5 min and remained relatively unchanged for up to 15 min (Fig. 2, triangles). This pattern for TPA-induced PKC-δ translocation was similar in the nuclear fraction, where PKC-δ was found with comparable levels for at least 10 min after maximal (Fig. 3, triangles).

Stimulation of PKC-δ Kinase Activity by CCK-8 and TPA and Subcellular Distribution of the Active Form of PKC-δ in Rat Pancreatic Acinar Cells—To investigate the ability of CCK-8 to stimulate PKC-δ activation, we assessed PKC-δ kinase activity using two different substrates. First, we pretreated acini with 10 nM CCK-8 or with the phorbol ester TPA (1 μM) as a positive control and measured the kinase activity of PKC-δ in whole cellular lysates using histone H1 as substrate. Incubation of pancreatic acinar cells with 1 μM TPA for 5 min or 10 nM CCK-8 for 2.5 min increased PKC-δ kinase activity 2.10 ± 0.08- and 2.09 ± 0.13-fold, respectively (Fig. 4, top panel, lanes 2 and 3).

We also performed kinase assays using a peptide with the sequence QKRPSQRSKYL optimized for PKC kinase assays (Upstate Biotechnology). A similar degree of activation of PKC-δ was found with this substrate, compared with using histone H1 as the substrate, with an average -fold increase of 2.34 ± 0.19 with CCK-8 stimulation (n = 16) and 2.48 ± 0.16 with TPA treatment (n = 16) (Fig. 5, top panel).

In previous studies in pancreatic acini, a redistribution of PKC activity was only detectable with TPA treatment but not after CCK stimulation (9). In order to investigate in pancreatic acinar cells the basal activity of PKC-δ in different cellular compartments and the effect of CCK-8 or TPA stimulation on PKC-δ activity, we assessed PKC-δ kinase activity under basal conditions and after either CCK-8 or TPA stimulation in the cytosol or membrane fractions (Fig. 4). No PKC-δ kinase activity was detected in cytosolic fractions from either basal or stimulated (CCK-8 or TPA) pancreatic acini (Fig. 4, middle and bottom panels, lanes 1–3). However, incubation of pancreatic
FIG. 5. Inhibition of PKC-δ kinase activity by the tyrosine kinase inhibitor, tyrphostin B44, and stimulation of PKC-δ tyrosine phosphorylation and PKC-δ kinase activity by the tyrosine phosphatase inhibitor, sodium orthovanadate. Top panel, rat pancreatic acinar cells were pretreated with or without 300 μM tyrphostin B44 for 60 min and then incubated with 10 nM CCK-8 or with 1 μM TPA as described in the legend to Fig. 6. After incubation, acini were lysed, PKC-δ was immunoprecipitated with anti-PKC-δ mAb from total cell lysates, and kinase activity was assayed on immunoprecipitates using a PKC peptide as substrate. Reaction products were spotted on p81 papers and kinase activity was quantified by scintillation counting and expressed as cpm. Values shown are mean ± S.E. of four independent experiments in duplicate expressed as -fold increase over background activities (experimental/control). In the middle and lower panels, rat pancreatic acini were stimulated with the indicated concentrations of sodium orthovanadate (Na₃VO₄) for 30 min or with CCK-8 10 nM for 2.5 min and then lysed. Tyrosine phosphorylation was determined as described in the legend to Fig. 6, and kinase activity was determined as described above. In the middle panel, results shown are from a typical experiment representative of three others in duplicate. In the lower panel are shown the mean ± S.E. of four independent experiments in duplicate for kinase activity expressed as -fold increase over background activities (**, p < 0.01 compared with the control values; Student’s t test for unpaired samples).

acini with 10 nM CCK-8 for 2.5 min (Fig. 4, lower panels, lane 6) or 1 μM TPA for 5 min (Fig. 4, lower panels, lane 5) increased the PKC-δ activity in membrane fractions, reaching 4.2 ± 1.1- and 4.8 ± 0.5-fold increase, respectively. This finding agrees with the subcellular distribution of PKC-δ described above, which was consistently only detected in cytosol before stimulation (Fig. 1, control lane on top and middle panels) and translocated to particulate components with CCK-8 and TPA stimulation (Fig. 1). The relatively higher PKC-δ activity stimulated by CCK-8 or TPA in subcellular fractions compared with the activity obtained in assays performed in whole cell lysates (4.2 ± 1.1 versus 2.09 ± 0.13 and 4.8 ± 0.5 versus 2.10 ± 0.08, respectively; Fig. 4, compare top and bottom panels) can be attributed to the significantly lower basal values of PKC-δ activity in membrane fraction, values that resulted in a greater -fold increase of PKC-δ kinase activity.

FIG. 6. CCK-8 and TPA stimulation of PKC-δ tyrosine phosphorylation in whole cell lysates and cytosolic and membrane fractions from rat pancreatic acini and the effect of the tyrosine kinase inhibitor, tyrphostin B44. Rat pancreatic acinar cells were pretreated without (lanes 1, 3, and 5) or with 300 μM tyrphostin B44 for 60 min (lanes 2, 4, and 6) and then incubated with no additions, with 10 nM CCK-8 for an additional 2.5 min, or with 1 μM TPA for 5 min and finally lysed. Whole (Total) cell lysates and subcellular fractions were immunoprecipitated (IP) with anti-PKC-δ polyclonal Ab. Immunoprecipitates were analyzed by SDS-PAGE followed by transfer of proteins of molecular mass >60 kDa to nitrocellulose membrane and Western blotting (WB) using anti-phosphotyrosine mAb (4G10) as described under “Methods.” Bands were visualized using chemiluminescence and quantified by densitometry. The top panel shows representative results for total cell lysates, the middle panel shows results for the cytosolic fraction, and the lower panel shows results for the membrane fraction. These results are representative of three others performed in duplicate.

Stimulation of PKC-δ Tyrosine Phosphorylation by CCK-8 and TPA and Subcellular Distribution of Tyrosine-phosphorylated PKC-δ in Pancreatic Acinar Cells: Effects of B44—in a recent study (19), we reported that CCK-8 can stimulate tyrosine phosphorylation of PKC-δ through occupation of the low affinity CCK₄ receptor in rat pancreatic acini. To explore the relationship between CCK-8 or TPA stimulation of PKC-δ tyrosine phosphorylation in pancreatic acini and their ability to cause PKC-δ translocation, we performed studies with the tyrosine kinase inhibitor, tyrphostin B44. CCK-8 (Fig. 6, top panel, lane 5) or TPA (Fig. 6, top panel, lane 3) caused a marked increase in PKC-δ tyrosine phosphorylation in whole cell lysates. An identical result was obtained with the acini lysates when they were first immunoprecipitated with anti-phosphotyrosine mAb (PY20) and then analyzed with anti-PKC-δ polyclonal Ab (data not shown) instead of the reversed order as was done in Fig. 6 (top panel). No PKC-δ tyrosine phosphorylation could be detected in the cytosolic fractions from either basal or stimulated (CCK-8 or TPA) pancreatic acinar cells (Fig. 6, middle panel, lanes 1–6). However, both CCK-8 (Fig. 6, bottom panel, lane 5) and TPA (Fig. 6, bottom panel, lane 3) stimulated an increase in PKC-δ tyrosine phosphorylation in the membrane fraction. Previous studies we have reported that pretreatment of pancreatic acini with the tyrosine kinase inhibitor tyrphostin B44 caused a nearly complete inhibition of CCK-8-stimulated tyrosine phosphorylation of several proteins (34, 35). Pretreatment of pancreatic acini for 90 min with 300 μM
B44 had no effect on basal tyrosine phosphorylation of PKC-δ (Fig. 6, lanes 1 and 2); however, it inhibited by >80% the PKC-δ tyrosine phosphorylation induced by CCK-8 or TPA in both whole cell and membrane lysates (Fig. 6, lanes 6 and 4, respectively, in top and bottom panels). Inhibition of PKC-δ tyrosine phosphorylation by tyrphostin B44 was not due to differences in protein loading, because when membranes used in phosphorylation studies were stripped from anti-phosphotyrosine antibodies and incubated later with anti-PKC-δ mAb, equivalent loading of PKC-δ in acinar lysates was seen (data not shown).

**Effect of the Tyrosine Kinase Inhibitor, Tyrphostin B44, on CCK-8 or TPA Stimulation of PKC-δ Activity and the Effect of the Tyrosine Phosphatase Inhibitor, Sodium Orthovanadate, on PKC-δ Tyrosine Phosphorylation and Its Kinase Activity—**To determine whether the PKC-δ tyrosine phosphorylation state altered or regulated its enzymatic activity, we pretreated pancreatic acinar cells with 300 μM B44, a concentration that markedly inhibited CCK-8- or TPA-stimulated PKC-δ tyrosine phosphorylation (Fig. 6, top panel) and then measured kinase activity in whole cell lysates (Fig. 5). Pretreatment of pancreatic acini with B44 did not affect basal PKC-δ kinase activity (Fig. 5, top panel). However, this pretreatment inhibited almost completely PKC-δ activation induced by CCK-8 or TPA (Fig. 5). These results demonstrate that inhibition of PKC-δ tyrosine phosphorylation also resulted in inhibition of its activation, suggesting a relationship exists between tyrosine phosphorylation and kinase activity in pancreatic acinar cells for PKC-δ. To study further the nature of this relationship, we investigated whether a similar coupled effect could be detected by the reverse study by inhibiting tyrosine phosphatase activity. To accomplish this, we incubated acini with sodium orthovanadate (Na3VO4), an agent that inhibits protein-tyrosine phosphatases (52), and assessed its effect on PKC-δ tyrosine phosphorylation and kinase activity (Fig. 5, middle and lower panels). Treatment of pancreatic acini for 30 min with different concentrations of Na3VO4 (10, 100, and 1000 μM) stimulated PKC-δ tyrosine phosphorylation at concentrations higher than 10 μM, reaching at 1000 μM a 4-fold increase (22.85 ± 2.72) equivalent to that obtained with 10 nM CCK-8 (Fig. 5, middle panel). Sodium orthovanadate, at concentrations higher than 10 μM, increased significantly PKC-δ activity, reaching a 1.49 ± 0.15-fold increase at 1000 μM (Fig. 5, bottom panel). Similar to stimulation of tyrosine phosphorylation, the increase of PKC-δ kinase activity obtained with 1000 μM sodium orthovanadate was equivalent to that obtained by treatment with 10 nM CCK-8 (Fig. 5, bottom panel). These results further support the conclusion that the PKC-δ tyrosine phosphorylation state depends not only on tyrosine kinase activity but also on the combined action of tyrosine kinases and tyrosine phosphatases and reinforce the conclusion that tyrosine phosphorylation can regulate PKC-δ kinase activity in pancreatic acinar cells.

**Effect of PKC-δ Tyrosine Phosphorylation State on Its Translocation Stimulated by CCK-8 and TPA—**To study the relationship between PKC-δ translocation, tyrosine phosphorylation, and activation, we analyzed the effect of the tyrosine kinase inhibitor, B44, on CCK-8- or TPA-induced PKC-δ translocation (Fig. 7). Pretreatment with 300 μM tyrphostin B44 for 90 min did not alter the basal distribution of PKC-δ (Fig. 7, compare lanes 1 and 2) and did not modify either the pattern of translocation to membrane or nuclear fractions, induced by CCK-8 (Fig. 7, top panel, lanes 5 and 6, and bottom panel) or TPA (Fig. 7, top panel, lanes 3 and 4, and bottom panel). These results show that PKC-δ translocation is independent of and not regulated by tyrosine phosphorylation or activation states of PKC-δ. These results, coupled with the finding that PKC-δ tyrosine phosphorylation (Fig. 6) and activation (Fig. 4) were only found in the particulate fraction after CCK-8 or TPA stimulation and support the conclusion that PKC-δ translocation induced by these agents precedes its tyrosine phosphorylation and activation.

**CCK Stimulation of PKC-δ**

![Figure 7. Effect of tyrphostin B44 on CCK-8 or TPA stimulation of PKC-δ translocation.](image-url)
Involvement of Src Kinases in CCK-8 or TPA Stimulation of PKC-δ Tyrosine Phosphorylation and Activity—In some cells, members of Src family of kinases (Src kinases) are involved in PKC-δ tyrosine phosphorylation (10, 18, 23, 26, 28, 29, 32, 53–55). At least two Src kinases (i.e. pp60src and c-Yes) are present in pancreatic acinar cells (43, 44, 56–58) and are activated by CCK-8 stimulation (43, 44, 56). To investigate whether in pancreatic acinar cells Src kinases are involved in tyrosine phosphorylation or activation of PKC-δ, we used PP2, a specific inhibitor for Src tyrosine kinases, and its inactive analog PP3 as negative control (59). Acini were pretreated for 60 min with PP2 (20 μM) or with PP3 (20 μM) and then incubated with 10 nM CCK-8 for 2.5 min or with 1 μM TPA for 5 min. Pretreatment of pancreatic acini with PP2 caused a complete inhibition of PKC-δ tyrosine phosphorylation induced by both CCK-8 and TPA (Fig. 8, middle panel), indicating that in pancreatic acini Src activation is required for PKC-δ tyrosine phosphorylation by these stimulants. Pretreatment with PP3 under identical experimental conditions did not modify both basal or CCK-8- and TPA-stimulated tyrosine phosphorylation of PKC-δ (Fig. 8, middle panel), demonstrating the specificity of the PP2. Because it has been reported that PP2 could also inhibit other tyrosine kinases (such as PDGF receptor) (29, 60), we used another Src kinase inhibitor, SU6656, which has a higher specificity than PP2 for Src kinases (60). Similar to the effect seen with PP2, preincubation of pancreatic acini with 5 μM SU6656 for 120 min inhibited by more than 65 ± 15% PKC-δ tyrosine phosphorylation stimulated by CCK-8 (data not shown). These results provide additional evidence supporting the importance of the role of Src-related kinases in mediating PKC-δ tyrosine phosphorylation.

To determine whether Src kinases activation also was required for PKC-δ activation by CCK-8 or TPA, we performed kinase assays in immunoprecipitates obtained from cells preincubated with 20 μM PP2 for 60 min or its inactive control PP3 (20 μM) (Fig. 8, bottom panel). PP2 almost completely inhibited PKC-δ activation by CCK-8 or TPA, inhibiting by more than 88 ± 8% the activation by either stimulant (Fig. 8, bottom panel). This inhibitory effect of PP2 was specific because cells preincubated with PP3 did not displayed differences in PKC-δ kinase activity compared with control cells (Fig. 8, bottom panel). Preincubation of pancreatic acini for 120 min with 5 μM SU6656 also inhibited by more than 61 ± 7% the PKC-δ kinase activity stimulated by CCK-8 (data not shown).

Relationship between Src Tyrosine Kinase Activity and CCK-8 or TPA Stimulation of PKC-δ Translocation—To determine whether Src kinases were important in mediating PKC-δ translocation stimulated by CCK-8 or TPA, we performed studies of subcellular distribution of PKC-δ with and without a preincubation with PP2 or PP3. TPA-stimulated PKC-δ translocation did not require activation of an Src kinase, because PP2 pretreatment did not modify the translocation pattern for PKC-δ stimulated by the phorbol ester (Fig. 9, top and bottom panels). PP2 pretreatment, however, significantly inhibited (p < 0.01) PKC-δ translocation stimulated by CCK-8. Specifically, PP2 pretreatment caused a 35.3 ± 5.7% increase of PKC-δ remaining in the cytosol after CCK-8 stimulation and a 39.9 ± 7.9% decrease in the amount of PKC-δ accumulating in the membrane with CCK-8 stimulation (Fig. 9, top and bottom panels). The inhibition by PP2 of CCK-8-stimulated PKC-δ translocation is not due to a general or nonspecific inhibition within the cell, because translocation with TPA was not affected and also because PP3, the inactive analogue of PP2, did not have an effect on either CCK-8- or TPA-stimulated PKC-δ translocation (Fig. 9, top and bottom panels). These results show that Src kinases are involved also in CCK-8-stimulated PKC-δ translocation in pancreatic acini, whereas TPA-induced PKC-δ translocation is independent of Src kinase activation.

Ability of CCK-8 to Stimulate Association of PKC-δ with Src Family Members—In some cells (18, 26, 30, 55), but not in others (10, 24), various stimulants induce an association between PKC-δ and Src kinases. To determine whether CCKα receptor activation could induce such an association in pancreatic acini, we assessed the formation of this complex by performing co-immunoprecipitation studies (Fig. 8, top panel). After immunoprecipitation of PKC-δ with a specific polyclonal Ab, Western blotting was performed with two different antibodies against Src kinases: a specific anti-Lyn monoclonal antibody (Lyn (H-6); Santa Cruz Biotechnology) (Fig. 8, top left panel), and the general monoclonal anti-Src family antibody (c-Src (B-12); Santa Cruz Biotechnology) that recognizes all Src kinase members (Fig. 8, top right panel). With the addition of 10 nM CCK-8 for 2.5 min, the formation of a PKC-δ-Src complex was stimulated, reaching a 2.47 ± 0.77-fold increase with both antibodies (Fig. 8, top panel). A similar effect was detected after
In the present study, we have investigated the ability of this GPCR to cause activation, tyrosine phosphorylation, and translocation of PKC-δ as well as the relationship between these processes. We have also studied the role of Src family kinases in each of these processes.

In the present study, we demonstrate that under basal conditions in rat pancreatic acini, PKC-δ is almost entirely cytosolic in location (i.e. 95%) and not tyrosine-phosphorylated, whereas with TPA or CCKA receptor activation, PKC-δ rapidly translocates to nuclear and other membranes, where it undergoes tyrosine phosphorylation and becomes activated. These results have similarities with and differences from findings with PKC-δ in other cells with different stimulants as well as findings reported previously with pancreatic acini. The finding that under basal conditions, PKC-δ is almost entirely cytoplasmic in location in pancreatic acini is similar to that reported in PC-12 cells (61), 32D hematopoietic cells (6), and NIH-3T3 fibroblasts (6). Our results are also consistent with one previous study on pancreatic acini (9) but differ from another, which reported that 35% of PKC-δ under basal conditions was localized to the membrane fraction (49). These results differ from parotid acinar cells, epidermal keratinocytes, and platelets in which only 30–50% of the PKC-δ is in the cytosol in unstimulated cells (8, 10, 62, 63). Similar to our findings, in almost all cells examined, under basal conditions PKC-δ kinase activity is low and, if present, localized to the cytosol (6). Similarly, under basal conditions, PKC-δ tyrosine phosphorylation is generally not detected in either membranes or cytosol (6, 8, 10, 21, 26, 63). Little information exists on the kinetics or magnitude of PKC-δ translocation with GPCR or growth factor activation by native ligands. In pancreatic acini with CCKA receptor activation, there is rapid (<2-min) tyrosine phosphorylation of PKC-δ (19) as well as the rapid (within 2 min) translocation of PKC-δ to membranes, which is similar to the rapid PKC-δ translocation reported with bombesin stimulation in Swiss 3T3 cells (62) and PDGF in 32D hematopoietic cells (6). The time course of PKC translocation to plasma and nuclear membrane by CCKA receptor activation was biphasic in contrast to translocation induced by TPA, which was monophasic and did not decrease with time. The results with CCKA are similar to those reported with PDGF stimulation in 32D hematopoietic cells (6), but differ from the monophasic time course seen with IgE cross-linking to RBL-2H3 cells (21) or bombesin stimulation of Swiss 3T3 cells (62). The prolonged membrane translocation of PKC-δ induced by TPA in the present study is similar to that reported in a number of other cells (6, 27, 64, 65) as well as pancreatic acini (49). With CCKA receptor activation, there was a 27% increase in PKC-δ in the membrane fraction and a 15% increase in the nuclear fraction, whereas with TPA, all of the PKC-δ translocated to these membrane fractions. This magnitude of PKC-δ translocation with CCKA receptor activation is similar to that reported with PDGF stimulation of 32D hematopoietic cells (6) or NIH 3T3 cells (6); however, it differs from the 0–2% increase reported by others with carbachol, secretin, or bombesin stimulation in pancreatic acini (9) and the 60–80% increase caused by neural growth factor stimulation in PC-12 cells (61) or by bombesin in Swiss 3T3 cells (62). No previous studies have reported the ability of activation of a GPCR to cause PKC-δ translocation to the nucleus; however, numerous studies have reported that TPA (66) as well as etoposide (22) or radiation (67) can induce such translocation in various cells, similar to our finding with CCKA receptor activation in the present study. The finding that, with CCKA receptor activation or TPA stimulation in pancreatic acini, tyrosine-phosphorylated PKC-δ and PKC-δ kinase activity were only detected in membrane fractions is generally similar to

stimulation of pancreatic acini with 1 μM TPA for 5 min, showing a 3.20 ± 1.49-fold increase with the two Src family kinase antibodies (Fig. 8, top panel).

DISCUSSION

The novel protein kinase, PKC-δ, is expressed in many tissues and plays an important role in cell growth, cell differentiation, apoptosis, tumor suppression, regulation of ion channels (L-channels, GLUT-4, Na-H E exchangers), phosphoinositol hydrolysis, prostaglandin formation, and secretion (1, 8–17). Recent studies show that diverse stimuli can activate this serine threonine kinase including growth factors, oxidative stress, Ras, and a few GPCRs (1, 8, 10–13, 16, 19, 20). Similar to other PKCs, these stimuli increase the serine threonine kinase activity of PKC-δ and also stimulate its translocation to membranes, but specific to PKC-δ, they also stimulate its tyrosine phosphorylation (1, 19). In the case of GPCRs and to some extent with the other stimuli, their ability to activate each of these processes and the relationships of activation of these different processes upon PKC-δ stimulation remain unclear, as do the cellular mechanisms involved. Recently (19), activation of the CCKA receptor, a GPCR, in pancreatic acinar cells by the neurotransmitter hormone, cholecystokinin, has been shown to cause rapid tyrosine phosphorylation of PKC-δ.
that reported with other stimuli (26) in most cells (6, 8, 10, 21, 63). However, these results differ from other cells where the majority of the tyrosine phosphorylation or kinase activity with stimulation was located in the cytosol (6, 27, 54, 68). These results demonstrate that PKC-δ activity, cellular localization, and tyrosine phosphorylation can vary markedly in different cells under basal conditions and with stimulation by different agents.

Studies in various cells with different stimuli have shown that stimulation of PKC-δ translocation may or may not be required for stimulation of PKC-δ tyrosine phosphorylation or increased kinase activity (6, 8, 10, 19, 21, 26, 27). Numerous findings in our study support the conclusion that with stimulation of pancreatic acini by CCK_2 receptor activation or TPA, PKC-δ translocation to plasma or nuclear membranes occurs first and is required for PKC-δ tyrosine phosphorylation/kinase activation to subsequently occur (Fig. 10). First, the tyrosine kinase inhibitor, B44, had no effect on CCK- or TPA-stimulated PKC-δ translocation to membranes while inhibiting stimulated tyrosine phosphorylation and PKC-δ kinase activity by more than 80%. Second, the Src inhibitor, PP2, completely inhibited TPA- or CCK-stimulated PKC-δ tyrosine phosphorylation and PKC-δ kinase activity while having no effect on TPA stimulation of PKC-δ translocation and a minimal effect on translocation caused by CCK-8. Third, no PKC-δ tyrosine phosphorylation or kinase activity was detected in membranes under basal conditions prior to PKC-δ translocation from the cytosol with stimulation of the acini by CCK or TPA. However, with TPA or CCK stimulation, rapid translocation to membranes occurred with an increase in PKC-δ tyrosine phosphorylation and kinase activity in the membrane fraction. Last, the rapid time course of PKC-δ translocation to membranes with CCK or TPA stimulation is similar to that shown for increased tyrosine phosphorylation (19), which is occurring only in the membranes. Our result differs from that with transforming growth factor-α stimulation of keratinocytes (10), TPA in MCF-7 breast cancer cells (26), and H_2O_2 effect in PKC-δ transfected Chinese hamster ovary K1 cells (27) in which PKC-δ translocation was not required for either PKC-δ tyrosine phosphorylation or stimulation of kinase activity. However, our results are consistent with findings in a number of cells with various stimulants, where stimulation of PKC-δ translocation preceded increases in PKC-δ tyrosine phosphorylation and/or kinase activity (6, 8, 10, 21).

The relationship between the ability of stimulants to alter PKC-δ tyrosine phosphorylation and kinase activity remains controversial. Numerous in vitro studies have demonstrated that increased tyrosine phosphorylation of PKC-δ by Src family members (24, 28, 54), the insulin receptor (8), or the PDGF receptor (28) increased PKC-δ serine threonine kinase activity. Furthermore, in intact cells using tyrosine phosphatase inhibitors, stimulation of PKC-δ tyrosine phosphorylation is reported to increase PKC-δ kinase activity by carbachol or TPA in parotid acinar cells (24) or PC12 cells (24). Conversely, inhibiting the increased tyrosine phosphorylation with tyrosine phosphatases inhibited PKC-δ kinase activity (24). Consistent with this stimulant effect of PKC-δ tyrosine phosphorylation on PKC-δ activity, a number of studies have shown a close correlation between the ability of a stimulant to increase PKC-δ tyrosine phosphorylation and increase its kinase activity (6, 16, 63). Conversely, increased PKC-δ tyrosine phosphorylation is reported to decrease PKC-δ kinase activity with TPA stimulation of keratinocytes (10), SP-1 cells (53), or 3Y1-NY72 v-Src transformed fibroblasts (18). Furthermore, with ATP stimulation of PKC-δ transfected Chinese hamster ovary K1 cells (27) or TPA stimulation of MCF-7 breast cancer cells (26), tyrosine phosphorylation is reported to be independent of increased PKC-δ kinase activity. Last, two studies in β cells (23, 25) provide evidence that PKC-δ must be activated in order for PKC-δ tyrosine phosphorylation to occur. A number of findings support the conclusion that after TPA or CCK stimulates PKC-δ translocation to the membranes in pancreatic acini, stimulation of PKC-δ tyrosine phosphorylation occurs, which results in increased PKC-δ kinase activity (Fig. 10). First, the tyrosine kinase inhibitor, B44 (69, 70), resulted in a complete inhibition of PKC-δ serine threonine activation. This was not a nonspecific effect of B44, because under these conditions it had no effect on TPA- or CCK-stimulated PKC-δ translocation. Second, orthovanadate, which selectively inhibits tyrosine phosphatases (52), stimulated an increase in PKC-δ tyrosine phosphorylation as well as an increase in PKC-δ serine threonine kinase activity. Furthermore, the orthovanadate dose-response curve for stimulating each process was similar. Third, two Src kinase inhibitors caused the same degree of inhibition of CCK-
stimulated increases in PKC-δ tyrosine phosphorylation and PKC-δ kinase activity, supporting the conclusion that they were coupled processes. Last, CCK and TPA only stimulated increases in PKC-δ tyrosine phosphorylation and PKC-δ kinase activity in the membrane fraction, also consistent with the conclusion that these two processes were coupled and occurring in similar cell fractions.

Numerous receptor and nonreceptor tyrosine kinases are reported to stimulate tyrosine phosphorylation of PKC-δ in different cells. These include growth factor receptors (28, 30) and several cytosolic tyrosine kinases, such as c-Abl (67, 71) and various Src kinase family members including Lyn (10, 23, 28, 53), Lck (54), Lyn (32, 55), or c-Src (10, 18, 23, 29, 53, 55). Although the Src family members have received the most attention, their role in altering PKC-δ tyrosine phosphorylation as well as altering PKC-δ kinase activity or even participating in PKC-δ translocation is highly variable with different stimuli in different cells. Furthermore, in general, only limited information exists from studies of GPCRs for gastrointestinal hormones/neuromodulators. In vitro studies using PKC-δ from keratinocytes (10), COS-7 cells (54), or RBL-2H3 cells (32) demonstrate that Src family members can stimulate PKC-δ tyrosine phosphorylation and kinase activity (28, 54). However, in various cells with different stimuli, it was reported that Src increased PKC-δ activity (24, 25, 31) or had no effect on PKC-δ kinase activity (26). In almost all studies in various cells, activation of Src family members by stimulants increased PKC-δ tyrosine phosphorylation (24, 25, 30, 55). Previous studies have demonstrated that pancreatic acini have a number of Src family kinases including Src (44, 57, 58) as well as Yes (58) and report that CCK receptor stimulation can cause Src activation (44, 56). In the present study, we demonstrate that the Src family member Lyn is also present in pancreatic acinar cells. A number of our results support the conclusion that these Src kinases play an important role in mediating CCKA receptor stimulation of PKC-δ tyrosine phosphorylation and increased PKC-δ kinase activity and play a major role in stimulation of PKC-δ translocation (Fig. 10). First, the Src kinase inhibitors PP2 and SU6656 inhibited both TPA and CCK stimulation of PKC-δ tyrosine phosphorylation as well as PKC-δ kinase activity. Second, both CCK and TPA stimulated the association of the Src family kinase Lyn with PKC-δ. Finally, PP2 caused a small (30%) but significant decrease in CCK-stimulated PKC-δ translocation but not translocation stimulated by TPA.

Our findings of association between PKC-δ and an Src family kinase member after TPA or CCKR activation are different from results with carbachol or TPA stimulation of PC12 cells (24) or parotid acinar cells (10, 24), where no co-localization was detected, but are similar to results with TPA stimulation of MCF-7 breast cancer cells (26) or v-Src-transformed 3Y1 cells (18), where co-localization was stimulated. Our finding that the Src kinase Lyn was stimulated to associate with PKC-δ by CCK or TPA is also reported with PDGF stimulation of C6 glioma cells (30) and stimulation of IgE receptor cross-linking in RBL-2H3 mast cells (55). Whereas the numerous studies reviewed above demonstrate that Src kinases can alter PKC-δ tyrosine phosphorylation and/or kinase activity, few studies have examined the effect of activation of Src kinases on PKC-δ translocation to membranes. Our finding that Src kinase activation plays an important role in mediating at least 30% of the maximal amount of PKC-δ translocation caused by CCK differs from a study in v-Ras-transformed keratinocytes (31) in which Src kinase activation inhibited PKC-δ translocation; however, our results are consistent with two studies (18, 72) of v-Src-transformed cells, where activation of Src kinases was reported to increase PKC-δ translocation.

In conclusion, our results, combined with those from other studies, lead us to propose the relationships shown in Fig. 10 for the ability of CCK or TPA to stimulate changes in PKC-δ in pancreatic acini. CCKR receptor activation results in the activation of both phospholipase C and D (38, 73, 74). Furthermore, CCKA receptor activation activates Src kinases in pancreatic acini (38, 44, 57). Both TPA and CCK cause the rapid translocation of PKC-δ from the cytosol to membranes (plasma and nuclear), whereas tyrosine phosphorylation and kinase activation of PKC-δ occurs. Although in other cells with different stimuli, PKC-δ activation or partial activation precedes PKC-δ tyrosine phosphorylation (23, 25), our results with complete inhibition of kinase activation with the tyrosine kinase inhibitor B44 are most consistent with the conclusion that tyrosine phosphorylation of PKC-δ in acini precedes PKC-δ activation and, in fact, stimulates it. Our results demonstrate that activation of a Src family kinase (at least Lyn and perhaps others) is essential for tyrosine phosphorylation of PKC-δ and its subsequent activation. Not shown in Fig. 10 is the fact that Src kinase activation also contributes, to a lesser extent, to the translocation of PKC-δ caused by CCK but not by TPA. Although the mechanism of this latter effect was not investigated in our study, it is consistent with the recently described ability of various Src kinases to stimulate diacylglycerol generation (75, 76). Although the Src kinase inhibitor PP2 completely inhibited CCK- and TPA-stimulated PKC tyrosine phosphorylation and activation, high concentrations of the more specific inhibitor, SU6656, only cause 60% inhibition. This result may be due to an inability to use sufficiently high concentrations of this compound to inhibit all Src kinase activity or to the possibility that 40% of the tyrosine phosphorylation is due to another non-Src kinase. The differences in the kinetics of TPA and CCK stimulation of PKC-δ translocation demonstrate that translocation is rapidly reversed with CCK but not with TPA stimulation. Since we did not detect PKC-δ tyrosine phosphorylation or activation in the cytosolic fraction of pancreatic acini, and the amount of cellular PKC-δ remains unchanged after CCK-stimulation at any time (data not shown), the return of PKC-δ to cytosol after CCK stimulation (Figs. 2 and 3) must involve inactivation and dephosphorylation of the PKC-δ by an unidentified protein-tyrosine phosphatase. This proposal is supported by the finding that inactivation of protein-tyrosine phosphatases by sodium orthovanadate increases PKC-δ tyrosine phosphorylation and activation (Fig. 10).

REFERENCES
