CCK_A Receptor Activation Stimulates $p130^{Cas}$ Tyrosine Phosphorylation, Translocation, and Association with Crk in Rat Pancreatic Acinar Cells†

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ABSTRACT: $p130^{Cas}$ (Crk-associated substrate), because of its structure as an adapter protein, can interact when tyrosine-phosphorylated with a large number of cellular proteins and therefore be an important modulator of downstream signals. A number of growth factors, lipids, and a few G protein-coupled receptors can stimulate $p130^{Cas}$ tyrosine phosphorylation. Recent studies show that tyrosine phosohorylation of intracellular proteins by the hormone/neurotransmitter cholecystokinin (CCK) in rat pancreatic acinar cells may be an important signaling cascade. In this study, we show in rat dispersed pancreatic acini CCK-8 rapidly stimulates tyrosine phosphorylation of $p130^{Cas}$, reaching a maximum (6.6 ± 1.4)-fold increase with a half-maximal effect at 0.3 nM. Activation of protein kinase C by TPA or increases in $[Ca^{2+}]_i$ by the calcium ionophore A23187 stimulated $p130^{Cas}$ phosphorylation. Blockade of CCK increases in $[Ca^{2+}]_i$ or PKC activity did not alter CCK-8-stimulated $p130^{Cas}$ phosphorylation; however, simultaneous blockage of both cascades caused a 50% inhibition. Partial inactivation by *C. botulinum* toxin of the small GTP-binding protein Rho caused a 41 ± 12% decrease in the CCK-stimulated $p130^{Cas}$ phosphorylation. Disruption of the actin cytoskeleton with cytochalasin D, but not the microtubule network with colchicine, completely inhibited CCK-8-stimulated $p130^{Cas}$ phosphorylation. Total $p130^{Cas}$ under basal conditions was largely localized (70 ± 2%) in the membrane fraction, and stimulation with CCK-8 induced total $p130^{Cas}$ translocation from the cytosolic fraction. CCK stimulation also caused a (5 ± 1)-fold increase in $p130^{Cas}$ tyrosine phosphorylated in the plasma membrane. Treatment with tyrphostin B44 inhibited CCK-8-stimulated $p130^{Cas}$ phosphorylation, but it had no effect on $p130^{Cas}$ translocation. CCK-8 caused rapid formation of a $p130^{Cas}$−Crk complex. In conclusion, our results demonstrate CCK_A receptor activation causes rapid tyrosine phosphorylation of $p130^{Cas}$ through PLC-dependent and -independent mechanisms that require the participation of the small GTP-binding protein Rho and the integrity of the actin cytoskeleton, but not the microtubule network. Moreover, CCK_A receptor activation causes translocation of $p130^{Cas}$ to the membrane and an increase in membrane tyrosine-phosphorylated $p130^{Cas}$. The translocation to the membrane does not require antecedent tyrosine phosphorylation. CCK_A activation promotes the rapid formation of a $p130^{Cas}$−Crk complex. These results suggest that $p130^{Cas}$ is likely an important modulator of downstream signals activated by CCK-8, possibly involved in regulating numerous cellular effects, such as effects on cell growth or cell shape.

Cholecystokinin (CCK) is a hormone/neurotransmitter, having far-ranging effects including acting as a physiological regulator of gallbladder contraction, gastric emptying (*1*, *2*) and pancreatic secretion (*2*, *3*), as well as having effects in the central nervous system (appetite regulation, anxiogenic effects, potentiation of morphine analgesia) (*2*, *4*−*7*), and potent growth effects both in normal tissues (pancreas) (*2*, *6*, *8*) and in neoplastic tissues (i.e., adenocarcinomas of the pancreas, stomach) (*2*, *6*, *9*). Recent studies demonstrate its physiologic effects on the gallbladder and pancreas are mediated by the CCK_A receptor (*2*), a member of the G protein-coupled heptahelical superfamily (*10*, *11*).

The cellular transduction pathways of CCK_A receptor activation have been extensively studied in pancreatic acinar cells where it has been shown to be coupled to activation of the phospholipase C cascade, phospholipase D, and phospholipase A_2 (*2*, *12*−*14*). Evidence suggests coupling to these pathways is particularly important in mediating enzyme secretion in these cells (*2*, *12*−*14*). A few recent studies demonstrate that with the CCK_A receptor, similar to integrins (*15*, *16*), growth factors (*17*−*21*), bioactive lipids such as lysophosphatidic acid and sphingosine (*22*−*27*), oncogenes (*16*, *28*), and a few other G protein-coupled receptors, stimulation of tyrosine phosphorylation of various intracellular proteins may also be an important transduction cascade (*26*, *29*−*32*). Activation of this cascade has been shown to be important in mediating cellular motility and growth (*28*,

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33). Recent studies have described one such protein, $p130^{Cas}$, a cellular protein originally identified as a tyrosine-phosphorylated substrate in $p60^{v\text{-}Src}$ or $p47^{v\text{-}Crk}$ transformed cells (*34*, *35*), as likely having a particularly important role, functioning as a molecular switch modulating downstream signals (*35*−*37*). This role is proposed because p130 has a unique structure, containing SH3 domains and SH2 domains as well as recognition sequences for SH3 domains (*35*, *38*), and therefore it can interact with the large number of cellular proteins known to interact with these motifs including Crk, Src, Abl, Nck, $p125^{FAK}$, and protein tyrosine phosphatase-1B (*36*−*40*). Recent studies provide evidence that integrins, oncogenes, some growth factors, and a few G protein-coupled receptors can stimulate tyrosine phosphorylation of $p130^{Cas}$ (*22*, *41*−*44*). It is unknown whether tyrosine phosphorylation of $p130^{Cas}$ occurs with CCK_A receptor activation or, if it does occur, whether it is coupled to activation of either limb of the phospholipase C cascade, as seen with some growth factors, bioactive lipids, or G protein-coupled receptors (*42*), but not with others (*22*, *23*, *44*). Furthermore, $p130^{Cas}$, similar to $p125^{FAK}$ and paxillin, are all components of focal adhesions (*36*, *45*, *46*), and recent studies show that tyrosine phosphorylation of these proteins can involve participation of the small GTP-binding protein, Rho (*27*, *31*, *47*), and the actin cytoskeleton (*15*, *16*, *25*−*27*, *47*, *48*). Tyrosine phosphorylation of $p130^{Cas}$ by growth factors and integrins can result in the formation of a complex of the $p130^{Cas}$ with the important adaptor protein c-Crk (*22*, *39*, *41*, *49*, *50*). Crk activation has been shown to result in coupling to a number of guanine nucleotide exchange factors which in turn can activate a number of transduction cascades (*51*−*57*). It is unknown whether similar intracellular processes might be involved if CCK_A receptor activation stimulates tyrosine phosphorylation of $p130^{Cas}$. In the present study, the effect of CCK on each of these transduction processes was investigated by studying the intracellular changes in these cascades caused by CCK_A receptor activation in dispersed pancreatic acinar cells, one of its main physiological sites of action (*2*).

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats (150−200 g) were obtained from the Small Animals Section, Veterinary Resources Branch, NIH, Bethesda, MD; purified collagenase (type CLSPA) was from Worthington Biochemicals, Freehold, NJ; COOH-terminal octapeptide of cholecystokinin (CCK-8) was obtained from Peninsula Laboratories, Belmont, CA; phosphate-buffered saline (PBS), pH 7.4, was from Biofluids, Rockville, MD; anti-p130-Crk-associated substrate ($p130^{Cas}$) monoclonal antibody (mAb), anti-p125 focal adhesion kinase ($p125^{FAK}$) mAb, anti-paxillin mAb, anti-Crk mAb, and anti-phosphotyrosine mAb (PY20) were from Transduction Laboratories, Lexington, KY; recombinant protein A−agarose was from Upstate Biotechnology Inc., Lake Placid, NY; GF109203X, Thapsigargin, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), *Clostridium botulinum* C3 transferase (C3 transferase), and deoxycholic acid were from Calbiochem, La Jolla, CA; soybean trypsin inhibitor (SBTI), dimethyl sulfoxide (DMSO), and Triton X-100 were from Sigma, St. Louis, MO; phenylmethanesulfonyl fluoride (PMSF) was from Fluka, Ronkonkoma, NY; basal medium eagle (BME) amino acids and BME vitamin solution were from Gibco Laboratories, Grand Island, NY; bovine serum albumin (BSA) fraction V was from Miles Inc., Kankakee, IL; aprotinin, leupeptin, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were from Boehringer Mannheim Biochemicals, Indianapolis, IN; rabbit anti-mouse IgG and anti-mouse IgG−horseradish peroxidase conjugate were from Pierce, Rockford, IL; sodium dodecyl sulfate (SDS), 2-mercaptoethanol, protein assay dye reagent, Tris/glycine/SDS buffer (10 times concentrated), and Tris/glycine buffer (10 times concentrated) were from BIO-RAD, Richmond, CA; Hyperfilm ECL and enhanced chemiluminescence detection reagents were from Amersham, Arlington Heights, IL; and nitrocellulose membrane was from Schleicher & Schuell, Keene, NH.

Methods

Tissue Preparation. Dispersed rat pancreatic acini were prepared according to the modifications (*58*) of the procedure published previously (*59*). Unless otherwise stated, the standard incubation solution contained (mM): HEPES (25.5) [pH 7.4]; NaCl (98); KCl (6); NaH_2PO_4 (2.5); sodium pyruvate (5); sodium fumarate (5); sodium glutamate (5); glucose (11.5); $CaCl_2$ (0.5); $MgCl_2$ (1); glutamine (2); albumin 1% (w/v); trypsin inhibitor 1% (w/v); vitamin mixture 1% (v/v); and amino acid mixture 1% (w/v). The incubation solution was equilibrated with 100% O_2, and all incubations were performed with 100% O_2 as the gas phase.

Immunoprecipitation. Immunoprecipitation was performed as described previously (*29*, *31*). Briefly, dispersed acini from one rat 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 agonists at the concentrations and times indicated. Acini were then separated by centrifugation, washed with phosphate-buffered saline with 0.2 mM Na_3VO_4 at 4 °C, centrifuged and sonicated for 5 s at 4 °C in 1 mL of Lysis Buffer, which contained 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% (w/v) NaN_3, 1 mM EGTA, 0.4 mM EDTA, 2.5 μg/mL aprotinin, 2.5 μg/mL leupeptin, 1 mM PMSF and 0.2 mM Na_3VO_4. Lysates were centrifuged at 15,000 g for 15 min. For tyrosine phosphorylation determination lysates (500 μg/mL) were incubated with 4 μg of anti-phosphotyrosine monoclonal antibody (PY20), 4 μg of anti-$p130^{Cas}$ mAb, 4 μg of anti-$p125^{FAK}$ mAb or 4 μg of anti-paxillin mAb and 4 μg of rabbit anti-mouse IgG and 30 μL of protein A-agarose overnight at 4 °C. For co-immunoprecipitation studies, lysates (400 μg) were incubated with 4 μg of anti-Crk mAb for 1 h at 4 °C. The immune complexes were incubated with 30 μL of protein A-agarose for 1 h at 4 °C. The immunoprecipitates were washed three times with PBS and further analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting.

Subcellular Fractionation. Acinar cell fractionation was carried out according to the procedure published previously (*60*) with minor modifications. Briefly, acinar cells were separated by centrifugation, washed with phosphate-buffered saline with 0.2 mM Na_3VO_4 at 4 °C, centrifuged, and resuspended in 1 mL of lysis buffer without Triton X-100 or deoxycholate and homogenized with the use of a Polytron

homogenizer (Brinkmann Instruments, Westburg, NY) for 20 s at power level 6 at 4 °C. Homogenates were centrifuged first at 500*g* for 10 min at 4 °C to remove nuclei, debris, and fat and then for 30 min at 100000*g* at 4 °C to obtain membrane and cytosol fractions. Membranes were washed with phosphate-buffered saline with 0.2 mM Na_3VO_4 at 4 °C, resuspended in 1 mL of lysis buffer, and sonicated for 5 s at 4 °C. Lysates were centrifuged at 15000*g* for 15 min to remove insoluble substances. Protein concentration was measured by the Bio-Rad protein assay reagent.

Western Blotting. Western blotting was performed as described previously (*29*, *31*). Briefly, anti-phosphotyrosine immunoprecipitates or subcellular fractions (15 μg of protein/well) were fractionated by SDS−PAGE. Proteins with molecular masses higher than 60 kDa 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, 0.05% (v/v) Tween 20, 0.02% NaN_3] and incubated for 2 h at 25 °C with 0.25 μg/mL anti-p130^{Cas} mAb, with 0.25 μg/mL anti-Crk mAb, or with anti-p125^{FAK} mAb, anti-paxillin mAb, or anti-phosphotyrosine mAb as described previously (*29*, *31*). After incubation with the primary antibody, membranes were incubated with anti-mouse IgG−horseradish peroxidase conjugate and developed with enhanced chemiluminescence detection reagents (ECL) for 60 s and exposed to Hyperfilm ECL. The densities of bands on the film were measured using a scanning densitometer (Molecular Dynamics, Sunnyvale, CA).

Assessment of Changes in Cytosolic Calcium [(Ca^{2+})$_i$] Levels. To determine changes in $[Ca^{2+}]_i$ after preincubation in a calcium-free medium with 10 μM thapsigargin, pancreatic acini were loaded with 2 μM fura-2/AM during the preincubation as described previously (*31*). Pancreatic acini were then washed 3 times in Na-Hepes medium. For measurement of $[Ca^{2+}]_i$, 2 mL of acinar cells was placed in quartz cuvettes in a Delta PTI scan and spectrometer (PTI Instruments, Gaithersburg, MD), and values for $[Ca^{2+}]_i$ were calculated as described previously (*61*, *62*) using a dissociation constant for the $[Ca^{2+}]_i$−fura-2 complex of 224 nM.

RESULTS

Kinetics and Dose-Dependence of the Ability of CCK-8 To Stimulate Tyrosine Phosphorylation of p130^{Cas} in Rat Pancreatic Acini. Pancreatic acinar cells were treated with 10 nM CCK-8 for 2.5 min, a concentration and time that cause a maximal stimulation of tyrosine phosphorylation (*29*, *31*), and lysed. Parallel lysates were immunoprecipitated with anti-tyrosine phosphorylated mAb, anti-p130^{Cas} mAb, anti-p125^{FAK} mAb, or anti-paxillin mAb and analyzed by Western blotting with the same antibodies (Figure 1). CCK-8 caused an increase in the tyrosine phosphorylation of at least three phosphotyrosine-containing bands above molecular mass of 60 kDa, which was the range examined in the present study (Figure 1, panel A, lanes 1 and 2). When the lysates were immunoprecipitated with anti-phosphotyrosine mAb and analyzed by Western blotting with p130^{Cas} mAb (Figure 1, panel B, lanes 1 and 2), it was evident that CCK-8 causes a marked increase in the tyrosine phosphorylation of p130^{Cas} which was also demonstrated when the acinar lysates were first immunoprecipitated with anti-p130^{Cas} and then analyzed

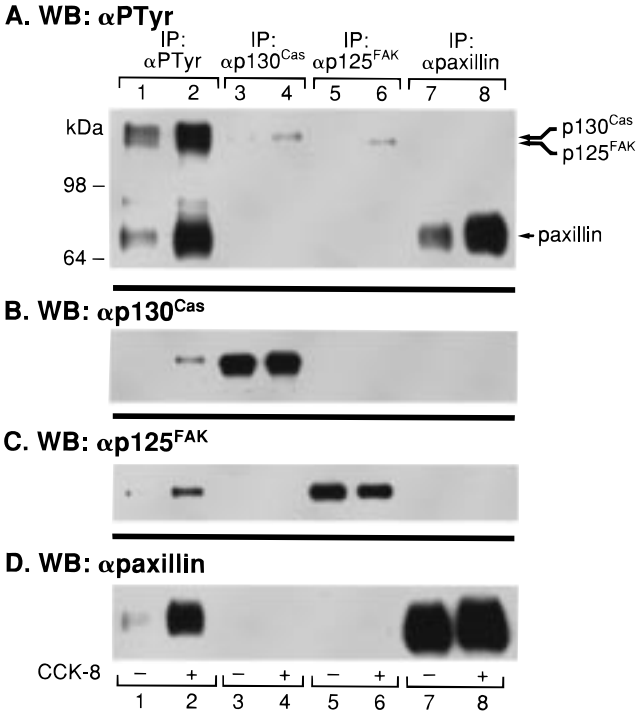

FIGURE 1: CCK-8 stimulation of p130^{Cas}, p125^{FAK}, and paxillin tyrosine phosphorylation in rat pancreatic acinar cells. Rat pancreatic acinar cells were treated for 2.5 min with no additions (lanes 1, 3, 5, and 7) or with 10 nM CCK-8 (lanes 2, 4, 6, and 8) and then lysed. Whole cell lysates were immunoprecipitated (IP) with anti-phosphotyrosine mAb (αPTyr, lanes 1 and 2), anti-p130^{Cas} mAb (αp130^{Cas}, lanes 3 and 4), anti-p125^{FAK} mAb (αp125^{FAK}, lanes 5 and 6), or anti-paxillin mAb (αpaxillin, lanes 7 and 8). Immunoprecipitates were analyzed by Western blotting (WB) using either anti-phosphotyrosine mAb (panel A, αPTyr), anti-p130^{Cas} mAb (panel B, αp130^{Cas}), anti-p125^{FAK} mAb (panel C, αp125^{FAK}), or anti-paxillin mAb (panel D, αpaxillin). The positions of p130^{Cas}, p125^{FAK}, and paxillin are indicated in the right upper panel. Positions of molecular mass markers are shown in the *y* axis on the left. The autoradiograms shown are representative of three independent experiments in duplicate.

with anti-phosphotyrosine mAb (Figure 1, panel A, lanes 3 and 4). Immunoprecipitation with anti-p130^{Cas} mAb followed by Western blotting with the same antibody showed that the recovery of p130^{Cas} from cell lysates was not altered by treatment with CCK-8 (Figure 1, panel B, lanes 3 and 4). Moreover, the p130^{Cas} mAb specifically identified p130^{Cas} because when the acinar lysates were immunoprecipitated with anti-p130^{Cas} mAb and developed with anti-p125^{FAK} mAb (Figure 1, panel C, lanes 3 and 4) or anti-paxillin mAb (Figure 1, panel D, lanes 3 and 4) only the p130^{Cas} protein was seen. Conversely, the p125^{FAK} and paxillin mAbs did not interact with the p130^{Cas} because when lysates were immunoprecipitated with anti-p125^{FAK} mAb or anti-paxillin mAb and developed with anti-p130^{Cas} mAb (Figure 1, panel B, lanes 5−8) or anti-phosphotyrosine mAb (Figure 1, panel A, lanes 5−8) no p130^{Cas} protein band was seen.

CCK-8 (10 nM) caused rapid tyrosine phosphorylation of p130^{Cas} (Figure 2, left panel). Tyrosine phosphorylation after addition of the peptide reached a maximum within 1 min with a (6.6 ± 1.4)-fold increase (Figure 2, left panel). At later times, the tyrosine phosphorylation of p130^{Cas} decreased such that it was only 17.5 ± 4.5% of maximum stimulation (i.e., at 1 min) 40 min after CCK-8 addition (Figure 2, left panel). The effect of CCK-8 on p130^{Cas} tyrosine phospho-

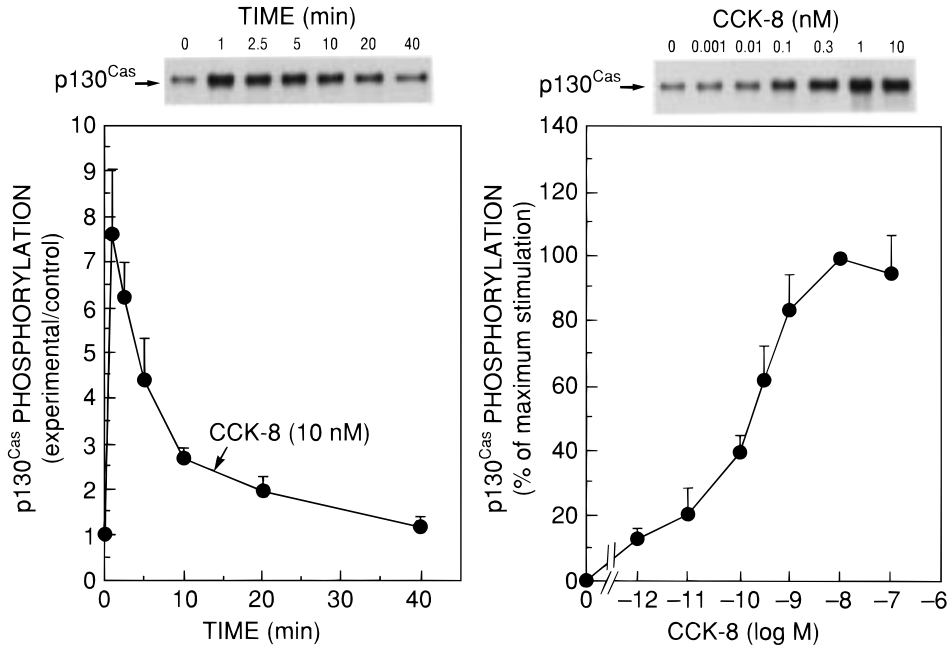

FIGURE 2: Time course (left panel) and concentration dependence (right panel) of CCK-8 stimulation of $p130^{Cas}$ tyrosine phosphorylation in rat pancreatic acinar cells. Rat pancreatic acinar cells were treated with the indicated concentrations of CCK-8 at the indicated times and then lysed. Whole cell lysates were immunoprecipitated with anti-phosphotyrosine mAb (PY20). Immunoprecipitates were analyzed by SDS−PAGE followed by transfer of proteins of molecular mass >60 kDa to nitrocellulose membrane and Western blotting using anti-$p130^{Cas}$ mAb as described under Methods. Bands were visualized using ECL, and quantification of phosphorylation was performed by scanning densitometry. Left panel: The upper panel shows results from a representative experiment with CCK-8 (10 nM) at the indicated times. These results are representative of three others performed in duplicate. The values shown in the bottom panel are mean ± SEM of four independent experiments and are expressed as fold increase over the pretreatment level (experimental/control). Right panel: Rat pancreatic acinar cells were incubated for 2.5 min with the indicated concentrations of CCK-8. The upper panel shows $p130^{Cas}$ tyrosine phosphorylation results from a representative experiment with no additions or with various concentrations of CCK-8. These results are representative of three others performed in duplicate. The bottom panel shows the quantification of $p130^{Cas}$ tyrosine phosphorylation. Values are the means ± SEM ($n = 6$) expressed as the percentage of maximal increase caused by 10 nM CCK-8 above control unstimulated values. In these experiments, 10 nM CCK-8 caused an (8 ± 3)-fold increase over the unstimulated control value.

rylation was concentration-dependent (Figure 2, right panel). CCK-8 caused a detectable increase at 1 pM, half-maximal effect at 0.3 nM, and maximal effect at 10 nM for $p130^{Cas}$ tyrosine phosphorylation (Figure 2, right panel).

Role of CCK-Induced Changes in $[Ca^{2+}]_i$ or PKC Activation in Mediating CCK-Stimulated Increases in $p130^{Cas}$ Tyrosine Phosphorylation. In addition to increasing the production of inositol phosphate, activation of phospholipase C by CCK-8 promotes the hydrolysis of phosphatidylinositol 4,5-bisphosphate, leading to production of diacylglycerol, which in turn activates protein kinase C (PKC) (*12*). We next studied whether CCK-8's activation of one or both of these second messengers was needed for its ability to cause tyrosine phosphorylation of $p130^{Cas}$. To determine whether direct activation of PKC increased the tyrosine phosphorylation of $p130^{Cas}$, pancreatic acinar cells were treated with the phorbol ester TPA. TPA (1 μM) stimulated a rapid increase in $p130^{Cas}$ tyrosine phosphorylation which reached a maximum at 5 min with a (2.6 ± 0.6)-fold increase, and was maintained for at least 20 min (Figure 3). To determine whether increased cytosolic calcium either alone or in combination with activation of PKC could alter the tyrosine kinase pathway, we compared the ability of the calcium ionophore A23187 to cause $p130^{Cas}$ tyrosine phosphorylation in pancreatic acinar cells when present alone or with TPA (Figure 4). The calcium ionophore A23187 (1 μM) caused only a (0.7 ± 0.3)-fold increase in $p130^{Cas}$ tyrosine phosphorylation, which was 56 ± 7% of that caused by TPA (1 μM) (Figure 4, top panel, lane 4). The increase in $p130^{Cas}$ tyrosine phosphorylation after simultaneous stimulation with both TPA (1 μM) and A23187 (1 μM) was not significantly different from the increase obtain with TPA (1 μM) alone (103 ± 20% vs 100%, respectively) (Figure 4, top panel, lanes 2 and 5, and bottom panel). Moreover, simultaneous stimulation of $p130^{Cas}$ tyrosine phosphorylation with a submaximal concentration of TPA (3 nM) and A23187 (1 μM) did not result in an increase greater than that seen by either TPA (3 nM) alone or A23187 (1 μM) alone (Figure 4, top panel, lanes 3, 4, and 6).

To determine whether PKC activation might be involved in mediating the CCK-8-stimulated changes in $p130^{Cas}$ tyrosine phosphorylation, we examined the effect of a protein kinase C inhibitor, GF109203X (*63*). Previously, we have shown that pretreatment of pancreatic acinar cells with 5 μM GF109203X for 2 h caused complete inhibition of tyrosine phosphorylation induced by activation of PKC with TPA (*31*). Pretreatment of pancreatic acinar cells with GF109203X (5 μM) for 2 h did not inhibit tyrosine phosphorylation of $p130^{Cas}$ by 10 nM CCK-8 (Figure 5, top right panel, lane 6). To examined the effect of changes in $[Ca^{2+}]_i$ alone or in combination with PKC activation in mediating the CCK-8 stimulation of $p130^{Cas}$ tyrosine phosphorylation, the effect of thapsigargin, an agent that specifically inhibits the endoplasmic reticulum Ca^{2+}-ATPase and thereby depletes Ca^{2+} from intracellular compartments (*61*), was examined alone or with the PKC inhibitor GF109203X present (Figure 5). Treatment with 10 μM thapsigargin for 1 h in a calcium-free medium (with EGTA, 5 mM), alone or in combination

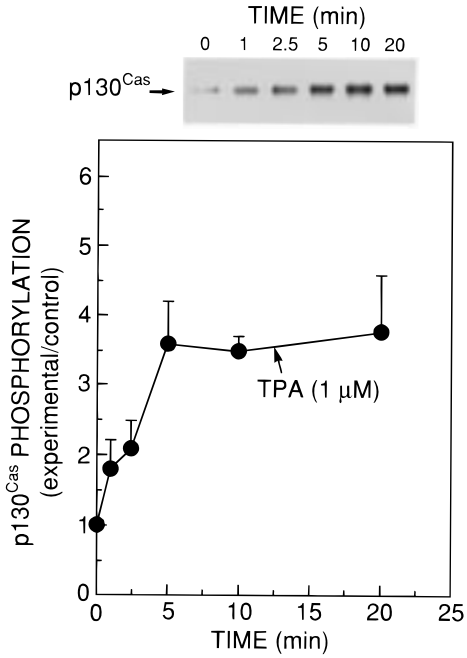

FIGURE 3: Time course of the ability of the phorbol ester TPA to stimulate p130Cas tyrosine phosphorylation in rat pancreatic acinar cells. Rat pancreatic acinar cells were treated with 1 μM TPA at the indicated times and then lysed. p130Cas tyrosine phosphorylation was determined by immunoprecipitation and Western blotting as described in the Figure 2 legend. The top panel shows p130Cas tyrosine phosphorylation results from a representative experiment with TPA (1 μM) at the indicated times. These results are representative of three others performed in duplicate. The bottom panel shows the quantification of p130Cas tyrosine phosphorylation. Values are the mean ± SEM (n = 4) expressed as fold increase over the pretreatment level (experimental/control). In these experiments, maximal stimulation seen at 20 min was a (2.8 ± 0.8)-fold increase over the unstimulated control value.

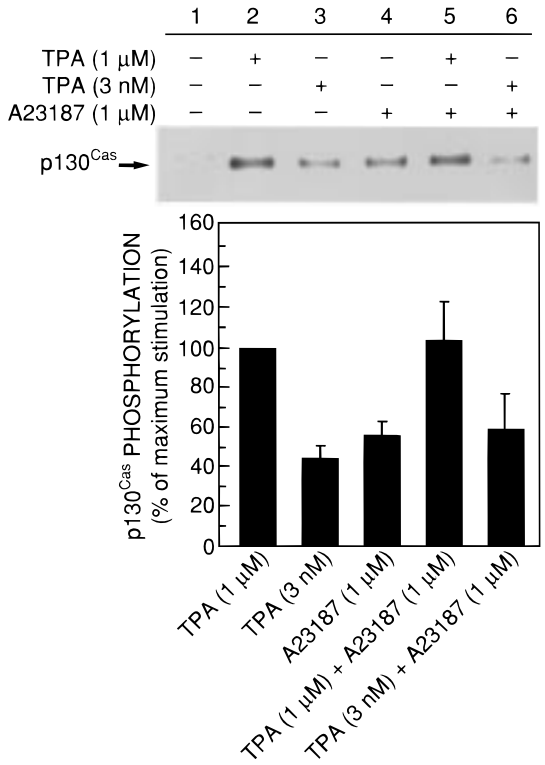

FIGURE 4: Effect of the calcium ionophore A23187, alone or in combination with the phorbol ester TPA, on stimulation of p130Cas tyrosine phosphorylation in rat pancreatic acinar cells. Pancreatic acinar cells were treated with the indicated agents for 2.5 min, and p130Cas tyrosine phosphorylation was determined as described in the Figure 2 legend. Results shown in the top panel are from a typical experiment representative of three others performed in duplicate. In the bottom panel are the means ± SEM of four experiments expressed as the percentage of the maximal increase of p130Cas tyrosine phosphorylation caused by 1 μM TPA above control. In these experiments, 1 μM TPA caused a (2.5 ± 1)-fold increase over the unstimulated control value. Numbers at the top of the upper panel are the lane numbers.

with GF109203X, inhibited completely the increase in $[Ca^{2+}]_i$ induced by subsequently added CCK-8 (*31*) (Figure 5, left panel). Pretreatment with thapsigargin in a calcium-free medium had no effect on the increase in p130Cas tyrosine phosphorylation caused by CCK-8 (10 nM) (Figure 5, top right panel, lane 6). However, the combination of GF109203X and thapsigargin decreased CCK-8-stimulated tyrosine phosphorylation of p130Cas by 53.5 ± 2.5% (Figure 5, top right panel, lane 8, and last bottom right panel). Finally, neither pretreatment with GF109203X or thapsigargin nor pretreatment with the combination of both significantly modified p130Cas basal tyrosine phosphorylation in unstimulated acinar cells (Figure 5, top right panel, lanes 1−4).

Role of Activation of p21rho in CCK-Stimulated Tyrosine Phosphorylation of p130Cas. Previous studies (*31*, *64*) show that activation of p21rho (Rho), a small GTP-binding protein, is involved in the tyrosine phosphorylation of a number of intracellular proteins. Moreover, recent studies (*31*, *32*) demonstrated that the ability of CCK to cause tyrosine phosphorylation of p125FAK and paxillin in pancreatic acinar cells is critically dependent on the activation of Rho. To determine whether activation of Rho could be involved in the ability of CCK to stimulate tyrosine phosphorylation of p130Cas in pancreatic acinar cells, we examined the effect of pretreatment for 3 h with the exoenzyme *Clostridium botulinum* C3 transferase on CCK-8-stimulated changes in p130Cas tyrosine phosphorylation, because the C3 transferase has been shown both in other cell systems (*31*) and in pancreatic acini (*32*, *65*) to enter the cell and to ADP-ribosylate Rho which results in its inactivation (*31*, *65*). Pretreatment with 25 μg/mL C3 transferase for 3 h decreased CCK-8-stimulated p130Cas tyrosine phosphorylation by 41 ± 12% (Figure 6, top panel, lanes 3 and 4, and bottom panel). C3 transferase pretreatment of control cells in the absence of CCK-8 did not modify significantly basal tyrosine phosphorylation (Figure 6, top panel, lanes 1 and 2).

Requirement for Integrity of the Actin Microfilament or the Microtubular Network for CCK-Stimulated Tyrosine Phosphorylation of p130Cas. Recent studies (*15*, *23*−*27*, *66*) show that the integrity of the actin cytoskeleton, but not the microtubule network, is important for tyrosine phosphorylation of some cellular proteins such as p125FAK and paxillin. To test the possibility that these proteins might be important in CCK-8-increased p130Cas tyrosine phosphorylation, we pretreated pancreatic acinar cells for 2 h with cytochalasin D (3 μM), a selective disrupter of the actin filament network (*67*), or colchicine, a selective inhibitor of microtubules synthesis (*68*), and then incubated with CCK-8 (10 nM) for another 2.5 min (Figure 7). Treatment with cytochalasin D inhibited CCK-8-stimulated p130Cas tyrosine phosphorylation by 74 ± 5% (Figure 7, lane 5). In contrast, pretreatment with colchicine (0.3 μM) had no effect on p130Cas tyrosine phosphorylation stimulated by CCK-8 (10 nM) (Figure 7,

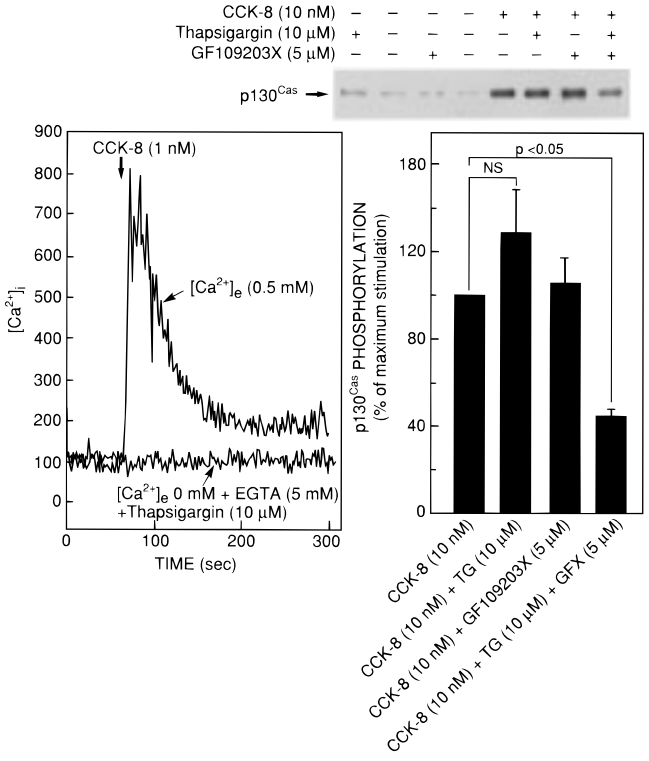

FIGURE 5: Effect of preincubation with thapsigargin on CCK-8-stimulated changes in cytosolic calcium $[Ca^{2+}]_i$ (left panel) or with the protein kinase C inhibitor GF109203X, either alone or in combination, on CCK-8 stimulation of $p130^{Cas}$ tyrosine phosphorylation in rat pancreatic acinar cells (right panel). (Left panel) Pancreatic acini were pretreated with or without 10 μM thapsigargin for 1 h in a calcium-free medium (with 5 mM EGTA) with 2 μM Fura-2/AM, washed, and then stimulated with CCK-8 (arrow). $[Ca^{2+}]_i$ was determined as described under Methods. This result is representative of three others. (Right panel) Pancreatic acinar cells were pretreated with 10 μM thapsigargin for 1 h in a calcium-free medium (with EGTA, 5 mM) or with GF109203X (5 μM) for 2 h either alone or in combination. Acini were then incubated for a further 2.5 min with no additions (control) or with CCK-8 (10 nM). $p130^{Cas}$ tyrosine phosphorylation was determined as described in the Figure 2 legend. The top right panel shows a single experiment representative of three others performed in duplicate. In the lower right panel are shown the means ± SEM from four experiments, and the data are expressed as the percentage of the maximal increase in phosphorylation caused by 10 nM CCK-8 above the control level. In these experiments, 10 nM CCK-8 caused a (6 ± 0.5)-fold increase over the unstimulated control value.

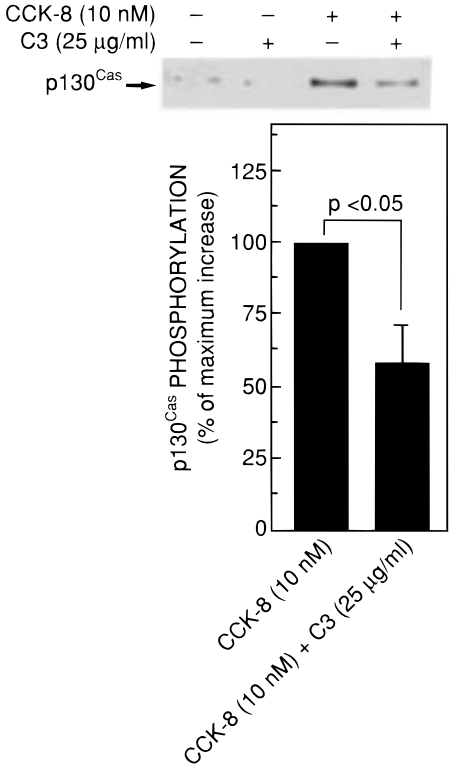

FIGURE 6: Effect of *Clostridium botulinum* C3 transferase on CCK-8 stimulation of $p130^{Cas}$ tyrosine phosphorylation in rat pancreatic acinar cells. Pancreatic acinar cells were pretreated for 3 h at 37 °C either in the absence or in the presence of 25 μg/mL C3 transferase. Acini were then incubated for a further 2.5 min with no additions or with 10 nM CCK-8 and then lysed. $p130^{Cas}$ tyrosine phosphorylation was determined as described in the Figure 2 legend. The top panel shows results from a typical experiment representative of three others performed in duplicate. The bottom panel shows the quantification of $p130^{Cas}$ tyrosine phosphorylation. Values are means ± SEM ($n = 4$) expressed as the percentage of the maximal increase caused by 10 nM CCK-8 above control unstimulated values. In these experiments, 10 nM CCK-8 caused a (6.5 ± 1.5)-fold increase over the unstimulated control value.

lane 6). Moreover, pretreatment with neither cytochalasin D nor colchicine significantly modifies $p130^{Cas}$ basal tyrosine phosphorylation in unstimulated acinar cells (Figure 7, lanes 1−3).

Subcellular Localization of $p130^{Cas}$ and Effect of CCK Stimulation on Subcellular Localization. In unstimulated pancreatic acinar cells, $p130^{Cas}$ was found in both the cytosolic and membrane fractions (Figure 8, panel A, lanes 1 and 3). Densitometry of immunoblots ($n = 8$) indicated that in unstimulated pancreatic acini total $p130^{Cas}$ was largely localized (70 ± 2%) in the membrane fraction. Upon the addition of 10 nM CCK-8 for 2.5 min, there was a 44 ± 8% decrease in the amount of total $p130^{Cas}$ in the cytosolic fraction (Figure 8, panel A, compare lanes 3 and 4). However, the amount of total $p130^{Cas}$ in the corresponding membrane fraction did not increase after CCK-8 stimulation (Figure 8, panel A, compare lanes 1 and 2). Similar studies were performed after immunoprecipitation with anti-phosphotyrosine mAb to allow changes in tyrosine-phosphorylated $p130^{Cas}$ in cytosol and membrane fractions to be assessed (Figure 8, panel B). In contrast to total $p130^{Cas}$ in the unstimulated state, phosphotyrosinated $p130^{Cas}$ was primarily in the cytosolic fraction (i.e., 69 ± 8%, mean ± SEM, $n = 6$) (Figure 8, panel B, compare lanes 1 and 3). After the addition of 10 nM CCK-8, there was a (5 ± 1)-fold increase in phosphotyrosinated $p130^{Cas}$ in the membrane fraction (Figure 8, panel B, lanes 1 and 2), and a (2.7 ± 0.3)-fold increase in the cytosolic fraction (Figure 8, panel B, lanes 3 and 4).

Pretreatment of pancreatic acinar cells for 30 min with the tyrosine kinase inhibitor tyrphostin B44 (*69*) at a concentration of 300 μM inhibited basal and CCK-8-stimulated $p130^{Cas}$ tyrosine phosphorylation [65 ± 16% and 81 ± 7%, respectively ($n = 3$)] (Figure 8, panel C, compare lanes 1 and 3 and compare lanes 2 and 4, respectively). However, tyrphostin B44 pretreatment had no effect on the CCK-8-stimulated decrease in the amount of total $p130^{Cas}$ in cytosolic fractions (Figure 8, panel D, compare lanes 5 and 6 to lanes 7 and 8). Specifically, with tryphostin B44 pretreatment with subsequent stimulation with 10 nM CCK-8, cytosolic $p130^{Cas}$ levels decrease 48 ± 11% (Figure 8, panel D, lanes 5 and 6), which was not significantly different

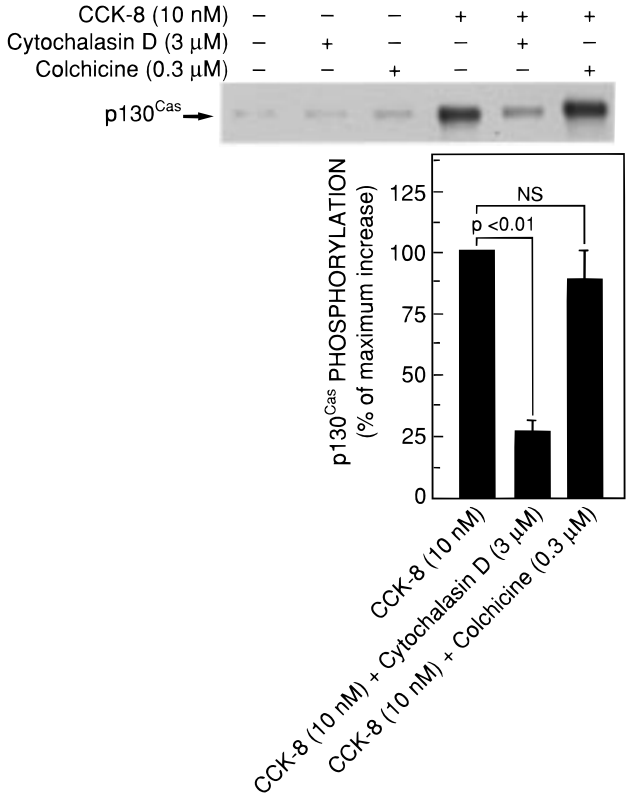

FIGURE 7: Effect of cytochalasin D or colchicine on CCK-8 stimulation of p130Cas tyrosine phosphorylation in rat pancreatic acinar cells. Pancreatic acinar cells were pretreated for 2 h at 37 °C either in the absence or in the presence of 3 μM cytochalasin D or 0.3 μM colchicine. Acini were then incubated for a further 2.5 min with no additions or with 10 nM CCK-8 and then lysed. p130Cas tyrosine phosphorylation was determined as described in the Figure 2 legend. The top panel shows results from a typical experiment representative of three others performed in duplicate. The bottom panel shows the quantification of p130Cas tyrosine phosphorylation. Values are means ± SEM ($n = 4$) expressed as the percentage of the maximal increase caused by 10 nM CCK-8 above control unstimulated values. In these experiments, 10 nM CCK-8 caused a (6.5 ± 2)-fold increase over the unstimulated control value.

from the 44 ± 8% ($n = 3$) decrease in cytosolic p130Cas levels in cells not pretreated with B44 (Figure 8, panel D, lanes 7 and 8). Pretreatment with tryphostin B44 decreased the relative total p130Cas concentration in cytosolic fractions by 49 ± 5% ($n = 3$) in unstimulated acinar cells (Figure 8, panel D, compare lanes 5 and 7).

Interaction of p130Cas with c-Crk. Recent studies with integrins and growth factors provide evidence that tyrosine phosphorylation of p130Cas can promote the formation of a p130Cas−Crk complex (*22*, *39*, *41*, *44*, *51*). To assess whether CCK_A receptor activation could promote the association of tyrosine-phosphorylated p130Cas with the adapter molecule, c-Crk, we assessed the formation of this complex by determining the results of coimmunoprecipitation of p130Cas with c-Crk after immunoprecipitation with c-Crk mAb (Figure 9, top panel). With the addition of 10 nM CCK-8, there was rapid stimulation of the formation of a p130Cas−c-Crk complex with a maximal effect seen at 1 min (Figure 9, top and bottom panels). This change was not due to differential recovery of c-Crk from CCK-8-treated cells because when c-Crk mAb was used for Western blotting after immunoprecipitation with c-Crk mAb similar amounts of c-Crk were seen in all samples (Figure 9, top panel). Similar results with p130Cas−c-Crk complex formation were obtained if lysates were first immunoprecipitated with p130Cas and Western blotting was performed using the c-Crk mAb (data not shown).

DISCUSSION

A recent study demonstrates that in addition to integrins and oncogenes, activation of various growth factors, of bioactive lipids, and of a few G protein-coupled receptors can also stimulate tyrosine phosphorylation of p130Cas (*22*, *41*−*44*). A number of results in our study support the conclusion that activation of the G protein-coupled CCK_A receptor in rat pancreatic acini can cause tyrosine phosphorylation of this protein and that this event may be functionally important in the action of CCK. First, whether a mouse anti-phosphotyrosine monoclonal antibody is used for immunoprecipitation followed by Western blotting using a specific anti-p130Cas monoclonal antibody or whether the same antibodies are used in reverse order, CCK-8 caused a striking increase in the tyrosine phosphorylation of a single p130Cas immunoreactive band. Second, tyrosine phosphorylation of p130Cas was a rapid consequence of the addition of CCK-8 to pancreatic acini and similar in kinetics to the effect of bombesin in Swiss 3T3 cells (*22*) or angiotensin II in vascular smooth muscle cells (*44*) on p130Cas tyrosine phosphorylation. p130Cas tyrosine phosphorylation declined after 2.5 min but remained above base line levels for at least 40 min. Third, the dose−response curve for p130Cas tyrosine phosphorylation occurred over the same CCK-8 concentrations that cause CCK_A receptor activation (*11*, *70*) and generally over the concentration range in which CCK-8 stimulates changes in cellular calcium (*11*, *70*, *71*), generation of inositol phosphates (*11*, *70*, *72*), MAP kinase activation (*73*), and p125FAK and paxillin tyrosine phosphorylation (*29*, *31*).

Role of CCK-Induced Changes in $[Ca^{2+}]_i$ or PKC Activation in CCK-Stimulated Increases in p130Cas Tyrosine Phosphorylation. At present, very little is known about the intracellular pathways that link receptor activation to tyrosine phosphorylation of p130Cas, especially in the cases of G protein-coupled receptors such as the CCK_A receptor. Activation of the pancreatic CCK_A receptor is known to induce polyphosphoinositide breakdown to generate inositol 1,4,5-triphosphate and diacylglycerol, which in turn mobilize cellular calcium and activate PKC, respectively (*11*, *12*). Recent studies demonstrate that in SH-SY5Y human neuroblastoma cells, in dispersed vascular smooth muscle cells, and in some Swiss 3T3 cells (*22*, *25*, *42*, *44*), p130Cas rapidly becomes tyrosine-phosphorylated after activation of PKC by TPA. However, in Swiss 3T3 cells, stimulation of p130Cas tyrosine phosphorylation by bradykinin (*25*), but not by the neuropeptide bombesin (*22*), was reduced by PKC inhibitors. Furthermore, stimulation of p130Cas tyrosine phosphorylation by bioactive lipids or bombesin in Swiss 3T3 cells was not dependent on calcium mobilization from intracellular stores (*22*, *23*). However, in rat pancreatic acini, it was recently demonstrated that CCK stimulation of tyrosine phosphorylation of two closely related p130Cas proteins, p125FAK and paxillin, was mediated by both PKC- and calcium-dependent and PKC- and calcium-independent pathways (*31*). To investigate further the possible relationship between the activation of either the calcium or the PKC pathways of the phospholipase C (PLC) cascade by CCK and their ability to

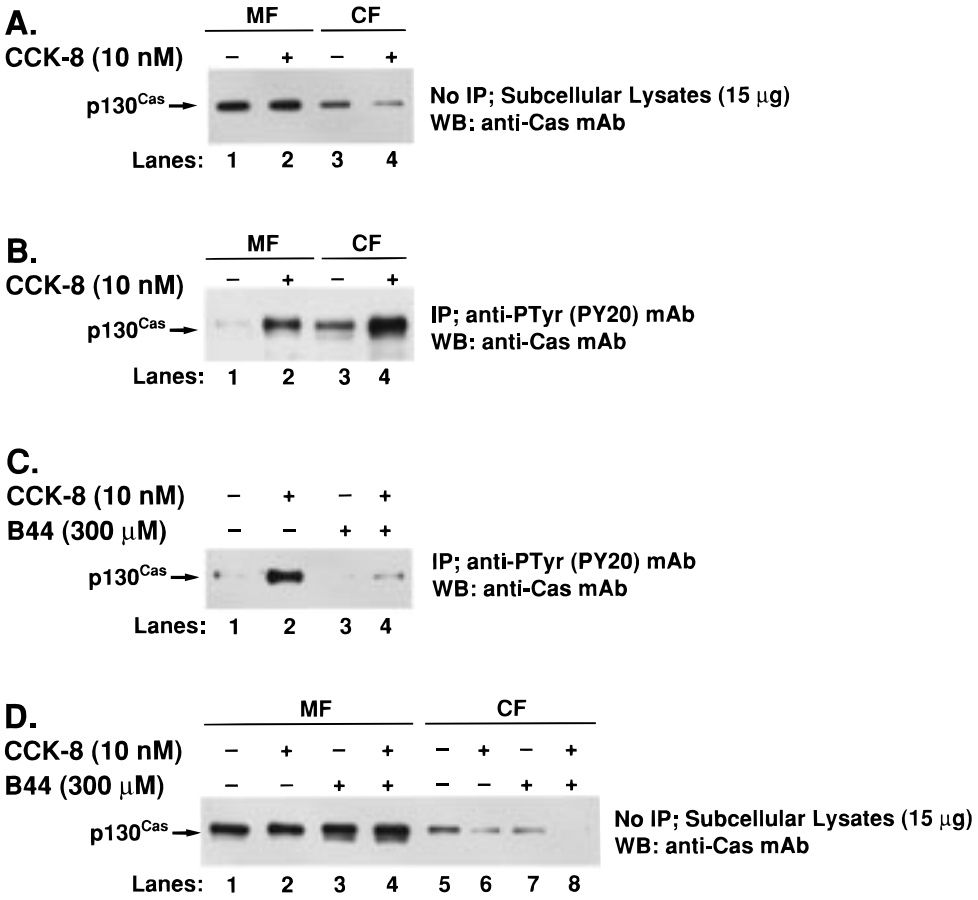

FIGURE 8: CCK-8 stimulation of p130^{Cas} translocation from cytosol and membrane fractions in rat pancreatic acinar cells and the effect of the tyrosine kinase inhibitor, tyrphostin B44, on stimulation of p130^{Cas} tyrosine phosphorylation and its translocation. Panel A: The ability of CCK-8 to stimulate translocation of total p130^{Cas}. Rat pancreatic acinar cells were incubated for 25 min either with or without 10 nM CCK-8. Cytosol fractions (CF) and membrane fractions (MF) were isolated as described under Methods. Lysates of the subcellular fractions (15 μg/well) were analyzed by Western blotting (WB) with anti-p130^{Cas} mAb as described under Methods without immunoprecipitation (IP) prior to Western blotting. Results shown are from a typical experiment representative of three others. Panel B: Ability of CCK-8 to stimulate changes in tyrosine-phosphorylated p130^{Cas} in the membrane fraction (MF) or cytosolic fraction (CF). The incubation conditions were the same as those described in the panel A legend. After isolation of the membrane and cytosolic fraction lysates, immunoprecipitation (IP) was performed with anti-phosphotyrosine mAb and Western blotting (WB) with anti-p130^{Cas} mAb. Results shown are from a typical experiment representative of five others. Panel C: Ability of the tyrosine kinase inhibitor, tyrphostin B44, to inhibit CCK-8 stimulation of tyrosine phosphorylation of p130^{Cas}. Rat pancreatic acini were pretreated with or without 300 μM tyrphostin B44, and then incubated with or without CCK-8 (10 nM) for 2.5 min. Cellular lysates were immunoprecipitated (IP) with anti-phosphotyrosine mAb, and Western blotting (WB) was performed with anti-p130^{Cas} mAb. Results shown are from a typical experiment representative of three others. Panel D: Effect of tyrphostin B44 pretreatment on CCK-8-stimulated translocation of total p130^{Cas}. Incubation conditions were identical to panel C. After incubation, membrane (MF) and cytosolic fractions (CF) were processed as described in panel A without immunoprecipitation (IP). Western blotting (WB) was performed with anti-p130^{Cas}. Results shown are from a typical experiment representative of five others.

stimulate p130^{Cas} tyrosine phosphorylation in rat pancreatic acini, we used three different approaches. First, we investigated whether activation of PKC by phorbol esters or increased cytosolic calcium by a calcium ionophore could increase the tyrosine phosphorylation of p130^{Cas}. Second, we investigated whether inhibition of PKC or depletion of Ca^{2+} from intracellular compartments could alter CCK-8 stimulation of p130^{Cas} tyrosine phosphorylation. Finally, because it has been shown that simultaneous activation of PKC and increases in cytosolic calcium in pancreatic acinar cells can have synergistic effects on a number of cellular responses including tyrosine phosphorylation of various intracellular proteins (*12*, *31*), we investigated whether simultaneous PKC activation and intracellular calcium increases or simultaneous inhibition of both pathways was required for CCK-8 stimulation of p130^{Cas} tyrosine phosphorylation.

In this study, direct activation of PKC by TPA caused a significant increase in p130^{Cas} tyrosine phosphorylation. Thus, PKC activation could be a potential intracellular signaling pathway that could mediate CCK-stimulated p130^{Cas} tyrosine phosphorylation, as previously demonstrated for stimulation of p130^{Cas} tyrosine phosphorylation in Swiss 3T3 cells by bradykinin (*25*) or bFGF/IGF-1 in differentiating neuroblastoma cells (*42*). However, our results demonstrated that it is unlikely PKC activation alone by CCK-8 is involved in stimulating tyrosine phosphorylation of p130^{Cas}. This conclusion is strongly supported by the fact that pretreatment with the PKC inhibitor GF109203X, at a concentration that has previously been shown to completely inhibit TPA-stimulated p125^{FAK} and paxillin tyrosine phosphorylation in rat pancreatic acini (*31*), had no effect on stimulation of p130^{Cas} tyrosine phosphorylation caused by CCK-8. Furthermore, a number of results suggest that increases in cytosolic calcium by CCK-8 are not likely to be important in CCK-8-stimulated increases in p130^{Cas} tyrosine phosphorylation. First, increases in cytosolic calcium alone, by

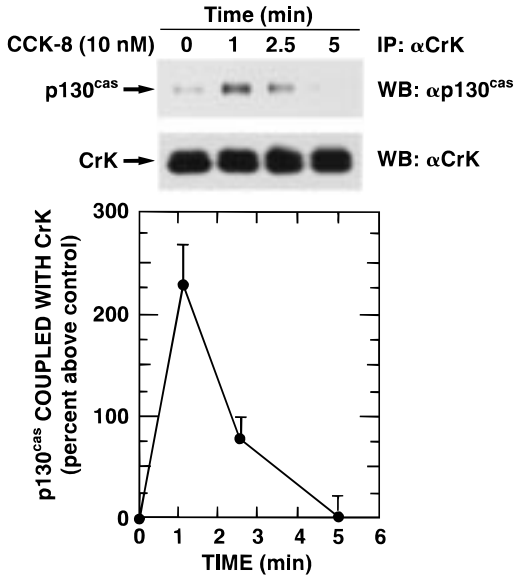

FIGURE 9: Ability of CCK-8 to stimulate association of p130^{Cas} with endogenous c-Crk. Time-dependent formation of the p130^{Cas}–Crk complex. Rat pancreatic acinar cells were incubated with 10 nM CCK-8 for the indicated times and then lysed. The lysates were immunoprecipitated (IP) with anti-Crk mAb (αCrk). The resulting immunocomplexes were analyzed by Western blotting (WB) with anti-p130^{Cas} mAb (αp130^{Cas}) or with anti-Crk mAb (αCrk). The top panels show total p130^{Cas} and c-Crk results from a given experiment representative of three others. In the bottom panel are the means ± SEM of four experiments with values expressed as a percentage of the maximal result at 1 min.

treatment of pancreatic acini with the calcium ionophore A23187 (1 μM), had only a minimal efficacy at causing tyrosine phosphorylation of p130^{Cas}. Second, that the increase in cytosolic calcium alone was not essential for the ability of CCK-8 to stimulate tyrosine phosphorylation of p130^{Cas} was shown directly by pretreatment with thapsigargin in a calcium-free medium to deplete the intracellular calcium stores. Under these conditions, in the present study and in a previous study (*31*) CCK-8 caused no changes in cytosolic calcium; however, tyrosine phosphorylation of p130^{Cas} in response to CCK-8 was not altered. These results are similar to those from studies of the action of bombesin or lysophosphatidic acid in Swiss 3T3 cells (*22*, *24*) or angiotensin II in cultured vascular smooth muscle cells (*44*), which also showed that inhibition of either limb of the phospholipase C cascade did not alter stimulation of p130^{Cas} tyrosine phosphorylation. However, they differ from the action of bradykinin (*25*) in Swiss 3T3 cells, which stimulates tyrosine phosphorylation of p130^{Cas} through both PKC-dependent and PKC-independent mechanisms, showing that various G protein-coupled receptor mechanisms of stimulation of p130^{Cas} tyrosine phosphorylation vary with different G protein-coupled receptors.

In a number of cellular responses including tyrosine phosphorylation, the full simultaneous activation of both limbs of the PLC cascade by G protein-coupled receptors is needed to obtain a maximal response with receptor activation (*12*, *31*). In the present study, the simultaneous activation of both arms of the PLC cascade, as occurs with CCK_A receptor activation in intact pancreatic acini (*12*) by the addition of TPA and the calcium ionophore, A23187, did not cause either synergistic or additive effects on p130^{Cas} tyrosine phosphorylation. However, simultaneous inhibition of both PKC activation by a PKC inhibitor and cytosolic calcium increases by preincubation with thapsigargin decreased CCK-8 stimulation of p130^{Cas} tyrosine phosphorylation by almost 50%. These results strongly suggest that CCK-8 stimulates p130^{Cas} tyrosine phosphorylation by both calcium- and PKC-dependent and calcium- and PKC-independent pathways and that each pathway contributes approximately equally. This result is similar to findings in a previous study in rat pancreatic acini (*31*) or in platelets (*15*) examining CCK- or thrombin-induced p125^{FAK} tyrosine phosphorylation, respectively; however, it differs from results of a study examining NMB stimulation of tyrosine phosphorylation of p125^{FAK} in rNMB receptor transfected cells (*27*). In a previous study (*31*) with CCK in pancreatic acini and with thrombin in platelets (*15*), tyrosine phosphorylation of p125^{FAK} was not impaired by inhibition of cytosolic calcium increases and only partially in the case of thrombin stimulation after PKC inhibition, whereas the simultaneous inhibition of both pathways completely abolished thrombin-stimulated p125^{FAK} tyrosine phosphorylation (*15*) and partially reduced CCK-induced p125^{FAK} tyrosine phosphorylation (*31*). In contrast with NMB receptor activation, tyrosine phosphorylation of p125^{FAK} was not inhibited by simultaneous inactivation of both the calcium and PKC pathways (*27*). These results demonstrate further that the role of activation of the two limbs of the phospholipase C cascade in causing tyrosine phosphorylation of p130^{Cas}, p125^{FAK}, or paxillin, three proteins which are found in focal adhesions (*36*, *46*), appears to differ with different G protein-coupled receptors.

Role of Activation of p21^{rho} in CCK-Stimulated Tyrosine Phosphorylation of p130^{Cas}. Recent studies demonstrate that tyrosine phosphorylation of a number of cellular proteins induced by various neuropeptides, lipids, and growth factors requires the involvement of the small GTP-binding protein, Rho (*27*, *31*, *47*). Furthermore, a previous study (*64*) demonstrates directly that activated Rho added by scrape loading to Swiss 3T3 cells stimulated p130^{Cas} tyrosine phosphorylation. Results in the present study, coupled with previous findings from another recent study (*31*), demonstrate that inactivation of Rho in rat pancreatic acini by ribosylation with *Clostridium botulinum* toxin markedly inhibits CCK-8-induced p130^{Cas} tyrosine phosphorylation. These results provide evidence that Rho is involved in the ability of CCK_A receptor activation to stimulate tyrosine phosphorylation of p130^{Cas} in rat pancreatic acinar cells. At present, it remains unclear in rat pancreatic acini whether Rho is necessary for all p130^{Cas} tyrosine phosphorylation after CCK_A receptor activation or if there are other pathways involved that do not involve Rho activation. This uncertainty exists because in the present study intact pancreatic acinar cells were used and even high concentrations and a prolonged incubation time with the *C. botulinum* C3 transferase, as used in the present study, do not result in complete inactivation of Rho (*31*).

Requirement for Integrity of the Actin Microfilament or Microtubular Network for CCK-Stimulated Tyrosine Phosphorylation of p130^{Cas}. Recent studies demonstrate that a number of neuropeptides such as bombesin and endothelin, as well as various bioactive lipids such as lysophosphatidic acid and sphingosylphosphocholine, can stimulate a rapid increase in stress fibers and focal adhesions (*16*, *64*, *74*).

Not only the small GTP-binding protein Rho but also the actin cytoskeleton has been shown to play an important role in this process (*15*, *16*, *25*−*27*, *47*, *48*). Therefore, in the present study we examined whether the integrity of the actin cytoskeleton was essential for p130^{Cas} tyrosine phosphorylation using cytochalasin D, which specifically disrupts actin microfilaments (*67*). We found that cytochalasin D almost completely inhibited CCK-8-stimulated p130^{Cas} tyrosine phosphorylation. This effect appeared specific for actin microfilaments because colchicine, which disrupts microtubules (*68*, *75*), had no effect on CCK-8-stimulated p130^{Cas} tyrosine phosphorylation. This result demonstrates that the integrity of the actin cytoskeleton is essential for CCK_A receptor activation to cause p130 tyrosine phosphorylation.

p125^{FAK}, paxillin, and p130^{Cas} are all components of focal adhesions (*36*, *46*), and with the results from the present study, combined with those from previous studies (*29*−*31*), each has now been shown to be tyrosine phosphorylated in rat pancreatic acini after CCK_A receptor activation. The exact relationship in the tyrosine phosphorylation of these three proteins in pancreatic acinar cells or other cells is not yet clear (*64*). Whereas studies suggest that paxillin is a substrate for p125^{FAK} (*64*, *76*), the relationship with tyrosine phosphorylation of p130^{Cas} is unclear. In other cell systems (*37*), p130^{Cas} associated both in vivo and in vitro with p125^{FAK}. Moreover, it has been demonstrated that p130^{Cas} is a p125^{FAK} substrate in Cos-1 and 293T cells (*77*). However, integrin-mediated phosphorylation of p130^{Cas} in fibroblasts has been shown not to depend on the presence of p125^{FAK} or tyrosine phosphorylation of p125^{FAK} (*51*). The present study combined with previous studies in pancreatic acini (*29*, *31*) demonstrates that in rat pancreatic acini, CCK_A receptor stimulation of the tyrosine phosphorylation of each of these three proteins (i.e., p125^{FAK}, p130^{Cas}, paxillin) is affected similarly by the intracellular processes studied. Tyrosine phosphorylation of each protein is mediated by both PKC- and calcium-dependent and PKC- and calcium-independent pathways; each requires the integrity of the actin cytoskeleton, but not the microtubular network; and activation of Rho is essential for the tyrosine phosphorylation for each. These results suggest that the tyrosine phosphorylation of each of these three proteins in rat pancreatic acini is closely coordinated and is affected similarly by these different cellular processes. It remains unclear whether this is because the tyrosine phosphorylation of each is sequentially dependent on the tyrosine phosphorylation of one of the others or whether each is acted on independently by the same processes.

Subcellular Localization of p130^{Cas} and Effect of CCK Stimulation on Subcellular Localization. A number of studies (*45*, *46*) show that p130^{Cas} is a component of focal adhesions which are attached to the plasma membrane and in some cases was also detected along stress fibers which are anchored to the plasma membrane (*46*). In the present study, examination of the subcellular distribution of total p130^{Cas} in rat pancreatic acini shows that under basal conditions the majority (70 ± 2%) is present in plasma membranes. This result differs from a recent study (*45*) in NIH 3T3 cells where in the basal state total p130^{Cas} was primarily in the cytoplasm, which suggests the basal distribution varies in different cell types. In contrast to total p130^{Cas}, the majority (68 ± 8%) of the tyrosine-phosphorylated p130^{Cas} in the unstimulated state was present in the cytosolic fraction. Recent studies showed that p130^{Cas} translocates from the cytoplasm to membrane fractions with v-Crk-transformation of 3Y1 cells (*35*) and in 527F−c-Src-transformation of NIH 3T3 cells (*45*). Our results show that in rat pancreatic acini after CCK stimulation there is a decrease in the amount of total p130^{Cas} detectable in cytosolic fractions which likely reflects a translocation of p130^{Cas} from cytoplasm to membranes. However, a reciprocal increase in the amount of total p130^{Cas} in the corresponding membrane fractions was not detected, probably reflecting the failure to detect a smaller fraction undergoing translocation that was added to the much larger amount of total p130^{Cas} that was already present in the membrane. With CCK stimulation, there was also a marked increase, (5 ± 1)-fold, in tyrosine-phosphorylated p130^{Cas} in the plasma membrane fraction, with a lesser, (2.7 ± 0.3)-fold, but significant increase in the cytosolic tyrosine-phosphorylated fraction. This latter finding is consistent with results in IL-8 receptor-transfected cells (*78*) where the CXC chemokine melanoma growth stimulatory activity was shown to stimulate an increase in tyrosine-phosphorylated p130^{Cas} in both plasma membrane and cytosolic fractions after activation of this G protein-coupled receptor.

The question can be raised whether the tyrosine phosphorylation of p130^{Cas} is necessary for translocation of p130^{Cas} to the plasma membrane to occur because both increase with CCK_A receptor activation. Our findings suggest that p130^{Cas} tyrosine phosphorylation is not necessary for p130^{Cas} translocation after CCK stimulation because treatment with a tyrosine kinase inhibitor (tyrphostin B44) (*69*), at concentrations which markedly inhibited CCK-stimulated p130^{Cas} tyrosine phosphorylation, had no effect on the CCK-stimulated p130^{Cas} translocation. However, pretreatment with tyrphostin B44 reduced the amount of total p130^{Cas} in the cytosolic fraction and increased the amount of total p130^{Cas} in the membrane fraction under basal conditions. This finding demonstrates the total amount of p130^{Cas} in the cytosol in unstimulated cells is influenced by the basal rate of tyrosine phosphorylation. A possible model, based on data from the present study and others, could be proposed that could explain these observations as well as the changes in tyrosine-phosphorylated p130^{Cas} and total p130^{Cas} in cytosol and membrane fractions with CCK stimulation. In unstimulated pancreatic acinar cells, p130^{Cas} is mainly localized in the membrane, which would likely be dependent on interactions of membrane proteins with the SH3 domains of p130^{Cas} because studies in Src-transformed cells demonstrate this domain is essential for localization of p130 to focal adhesions attached to the membrane (*45*). After CCK stimulation, the p130^{Cas} localized in the cytosolic fractions in rat pancreatic acini is recruited to the membrane fractions, perhaps after binding to an adapter protein such as c-Src (*45*) or Crk (*35*) because each of these proteins has been shown in other cells to be a potent stimulant of p130^{Cas} translocation to the membrane (*41*, *45*). p125^{FAK} is localized to focal adhesions, and it is reported that the SH3 domain of p130^{Cas} can bind directly to p125^{FAK} (*36*, *49*). However, an association of p130^{Cas} with p125^{FAK} is unlikely to be required for the translocation of p130^{Cas} because p130^{Cas} has been shown to localize to focal adhesions in p125^{FAK} negative cells (*45*). It is likely in the membrane that the tyrosine phosphorylation of p130^{Cas} occurs because prior tyrosine phosphorylation of p130^{Cas} is not required for translocation of p130^{Cas} in

pancreatic acini; however, with CCK-8 stimulation, membrane-bound tyrosine-phosphorylated p130^{Cas} increases. Furthermore, in a recent study (*45*) it has been shown that the kinase activity of 527F−c-Src was not required for the translocation of p130^{Cas} in 527F−c-Src-transformed NIH 3T3 cells, and it was suggested that 527F−c-Src-mediated localization of p130^{Cas} to the focal adhesion is caused not by the tyrosine phosphorylation of p130^{Cas} with activated c-Src but by the binding of p130^{Cas} to 527F−c-Src itself. Following tyrosine phosphorylation, p130^{Cas} interacts with a number of signaling molecules, such as Crk, Nck, and several other proteins (*38*, *79*). The binding of these signaling molecules could account for the translocation of p130^{Cas} to cytoplasmic fractions where they become dephosphorylated by cytosolic protein tyrosine phosphatases, such as PTP-PEST for which p130^{Cas} tyrosine phosphorylation has been shown to be a specific substrate (*80*).

Interaction of p130^{Cas} with c-Crk. p130^{Cas} was originally identified as a major tyrosine-phosphorylated protein in cells transformed by p47^{v-Crk} (v-Crk) and also was shown to be tyrosine-phosphorylated in cells transformed by p60^{v-Src} (*34*, *35*). p130^{Cas} contains an SH3 domain at the amino terminus followed by a substrate domain, a protein-rich motif, and numerous tyrosines at the carboxyl terminus and has a cluster of 15 potential SH2 binding motifs (*34*, *35*). Because these varied structural features of p130^{Cas} allow it to bind proteins containing SH2 domains, SH3 domains and recognition sequences for SH3 domains, it has been proposed p130^{Cas} can behave as a molecular switch, turning downstream signals on and off (*35*−*37*). For example, the substrate domain of p130^{Cas} has potentially phosphorylated tyrosines and binds to v-Crk (*38*, *79*), whereas the protein-rich area near the carboxyl terminus and Y762 provides binding sites for the SH3 and SH2 domains of Src kinase (*79*). Recent studies show growth factors and integrin stimulation of tyrosine phosphorylation of p130^{Cas} stimulate formation of a p130^{Cas}−c-Crk complex (*22*, *39*, *41*, *49*, *50*). In the present study, we demonstrate by coimmunoprecipitation experiments that in rat pancreatic acini, CCK_A receptor activation results in the rapid formation of this complex. Crk belongs to a family of adapter proteins that consist almost entirely of SH2 and SH3 domains and include the oncogene v-Crk, two forms of C-Crk (Crk-I, Crk-II), and a Crk-like protein (CRKL). Crk binds to a number of signaling proteins through its SH3 domains such as the guanine nucleotide exchange factors, Sos and C3G (*51*−*54*). These guanine exchange factors can activate Ras (i.e., Sos) or RapI (C3G) which have been shown to be important in mitogenesis and in tumor growth in some tissues (*55*−*57*). This raises the possibility that this pathway could be important in mediating the known growth effects of CCK in pancreatic acinar cells (*8*) and deserves additional study in future experiments.

In conclusion, in the present study we demonstrate CCK_A receptor activation causes the rapid tyrosine phosphorylation of p130^{Cas}. This occurs through phospholipase C-dependent and -independent mechanisms that require the participation of the small GTP-binding protein, Rho, and the integrity of the actin cytoskeleton but not the microtubular network. CCK_A receptor activation causes translocation of total p130^{Cas} to the plasma membrane and increased plasma membrane tyrosine-phosphorylated p130^{Cas}. The p130^{Cas} also rapidly forms complexes with c-Crk, and therefore likely functions as a molecular switch in pancreatic acinar cells as suggested from studies in other systems (*35*−*37*). Because of this central role, p130^{Cas} likely functions as an important modulator of downstream signals involving other tyrosine phosphorylated proteins and adaptor proteins activated by CCK in pancreatic acinar cells, possibly involved in regulation of changes in cell shape or the growth effects of CCK, as has been demonstrated for other stimulants in other cell systems (*28*, *33*, *55*−*57*).

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