Cholecystokinin Stimulates Extracellular Signal-regulated Kinase through Activation of the Epidermal Growth Factor Receptor, Yes, and Protein Kinase C

SIGNAL AMPLIFICATION AT THE LEVEL OF Raf BY ACTIVATION OF PROTEIN KINASE Cϵ*

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Cholecystokinin (CCK) and related peptides are potent growth factors in the gastrointestinal tract and may be important for human cancer. CCK exerts its growth modulatory effects through G_q-coupled receptors (CCK$_A$ and CCK$_B$) and activation of extracellular signal-regulated protein kinase 1/2 (ERK1/2). In the present study, we investigated the different mechanisms participating in CCK-induced activation of ERK1/2 in pancreatic AR42J cells expressing both CCK$_A$ and CCK$_B$. CCK activated ERK1/2 and Raf-1 to a similar extent as epidermal growth factor (EGF). Inhibition of EGF receptor (EGFR) tyrosine kinase or expression of dominant-negative Ras reduced CCK-induced ERK1/2 activation, indicating participation of the EGFR and Ras in CCK-induced ERK1/2 activation. However, compared with EGF, CCK caused only small increases in tyrosine phosphorylation of the EGFR and Shc, Shc-Grb2 complex formation, and Ras activation. Signal amplification between Ras and Raf in a CCK-induced ERK cascade appears to be mediated by activation of protein kinase Cϵ (PKCϵ), because 1) down-modulation of phorbol ester-sensitive PKCs inhibited CCK-induced activation of Ras, Raf, and ERK1/2 without influencing Shc-Grb2 complex formation; 2) PKCϵ, but not PKCα or PKCδ, was detectable in Raf-1 immunoprecipitates, although CCK activated all three PKC isoenzymes. In addition, the present study provides evidence that the Src family tyrosine kinase Yes is activated by CCK and mediates CCK-induced tyrosine phosphorylation of Shc. Furthermore, we show that CCK-induced activation of the EGFR and Yes is achieved through the CCK$_B$ receptor. Together, our data show that different signals emanating from the CCK receptors mediate ERK1/2 activation; activation of Yes and the EGFR mediate Shc-Grb2 recruitment, and activation of PKC, most likely PKCϵ, augments CCK-stimulated ERK1/2 activation at the Ras/Raf level.

Cholecystokinin (CCK)[1] and related peptides are potent growth factors in the gastrointestinal tract (1, 2). CCK stimulates

normal growth of the pancreas (3–6) and might be involved in growth of human pancreatic cancer (7, 8). Exogenous administration of CCK can lead to pancreatic hyperplasia, dysplasia, and malignancies (9) and accelerates the growth of malignant pancreatic tissue (10–12). In several cancer cell lines, CCK promotes growth (13). Furthermore, endogenous hypercholecystokininemia promotes carcinogenesis in the hamster (14). CCK binds to and activates receptors (CCK$_A$ and CCK$_B$) belonging to the seven-transmembrane-spanning family of G protein-coupled receptors (GPCRs) (15, 16). A major signaling cascade stimulated by both CCK$_A$ and CCK$_B$ receptors is pertussis toxin-insensitive activation of phospholipase C-β and subsequent activation of protein kinase C (PKC) (2, 16).

Activation of extracellular signal-regulated kinase 1/2 (ERK1/2) plays a key role in mediating proproliferative effects of both receptor tyrosine kinases (RTKs) such as the epidermal growth factor (EGF) receptor (EGFR) and GPCR (17). Depending on receptor and cell type, GPCR-induced ERK1/2 activation may involve stimulation of nonreceptor tyrosine kinases of the Src family and Pyk-2, receptor tyrosine kinases (most notably the EGFR), and phosphatidylinositol 3-kinase, leading to activation of Ras (17–24). Moreover, G_q-coupled receptors can activate ERK1/2 by a PKC-dependent Ras-independent pathway involving direct activation of Raf-1 (17, 25, 26).

CCK stimulates ERK1/2 by a mechanism depending on activation of phospholipase C and phorbol ester-sensitive PKCs (27, 28). In agreement with a PKC-dependent, Ras-independent mechanism of CCK-induced ERK1/2 activation, expression of dominant-negative Ras did not inhibit CCK-induced ERK1/2 activation in primary pancreatic acini (29). However, down-modulation of PKC by long term treatment with phorbol ester only partially inhibits CCK-induced ERK1/2 activation (28, 30), indicating that additional mechanisms are involved in CCK-induced ERK1/2 activation. CCK has been shown to induce tyrosine phosphorylation of Shc and Pyk-2 as well as complex formation of Grb2 with Shc and Pyk-2 in native rat pancreatic acini (27, 31), events occurring in Ras-dependent ERK1/2 activation (17, 19). In CCK$_B$-transfected Chinese hamster ovary

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[1] The abbreviations used are: CCK, cholecystokinin; CCK$_A$, CCK-A receptor; CCK$_B$, CCK-B receptor; DMEM, Dulbecco's modified Eagle's

medium; EGF, epidermal growth factor; EGFR, EGF receptor; GPCR, G protein-coupled receptor; HB-EGF, heparin-binding EGF-like growth factor; HGF, hepatocyte growth factor; PKC, protein kinase C; ERK, extracellular-regulated kinase; RTK, receptor tyrosine kinase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PP1, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo-D-3,4-pyrimidine; MOPS, 4-morpholinepropanesulfonic acid; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.

cells, the CCK$_B$ receptor agonist gastrin activates ERK1/2 by a Shc- and Src-dependent mechanism (32). In CCK$_B$ receptor-transfected gastric epithelial cells, gastrin induces EGFR tyrosine phosphorylation (33). Phosphatidylinositol 3-kinase may represent an additional signaling intermediate in gastrin-induced ERK1/2 activation (34, 35). Thus, a number of possible signaling intermediates of the Ras-dependent pathway have been assigned to CCK receptor-induced ERK1/2 activation, but it is unclear whether these signaling pathways operate within one cell type and whether cross-talk exists to mediate CCK-induced ERK1/2 activation.

In the present study, we sought to delineate the contribution of the EGFR, Src family tyrosine kinases, and PKC as well as their cross-talk in CCK-induced activation of the ERK1/2 pathway in the pancreatic acinar carcinoma cell line AR42J. Our data show that different signals emanating from the CCK receptor cooperate to mediate stimulation of ERK1/2; activation of Yes and the EGFR mediate Shc-Grb2 recruitment and Ras activation, and activation of PKC, most likely PKCε, potentiates ERK1/2 activation at the Ras/Raf level.

EXPERIMENTAL PROCEDURES

Materials—CCK octapeptide, human recombinant EGF, [Glu^{52}]diphtheria toxin (CRM197), and pertussis toxin were obtained from Sigma. pUSEamp expression vector containing dominant negative Ras (N17Ras), agarose-conjugated Ras-binding domain, and glutathione *S*-transferase-MEK1 were from Upstate Biotechnology, Inc. (Lake Placid, NY). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, LipofectAMINE 2000, and penicillin/streptomycin were from Invitrogen. The EGFR-specific tyrosine kinase inhibitor 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG1478), the PKC inhibitors bisindoylmaleimide (GF109203X), Gö6976 and rottlerin, the Src family kinase inhibitor 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo-D-3,4-pyrimidine (PP1), 1,2-bis(2-aminophenoxy)ethene *N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), all dissolved in dimethyl sulfoxide, hepatocyte growth factor (HGF), and gastrin were obtained from Calbiochem. The ERK1/2 activity assay was from Cell Signaling (Beverly, MA). pEGFP-C1 was from Clontech (Palo Alto, CA). The CCK$_A$ receptor antagonist L364,717 and the CCK$_B$ receptor antagonist L365,260 were kind gifts of ML Laboratories PLC (Liverpool, UK).

Antibodies—Monoclonal anti-β-actin, affinity-purified horseradish peroxidase-conjugated anti-mouse, anti-rabbit, and anti-sheep IgG were obtained from Sigma. Sheep anti-EGFR used for immunoblotting and polyclonal anti-Shc IgG used for immunoprecipitation, anti-pan-Ras IgG, and agarose-conjugated Ras-binding domain were from Upstate Biotechnology. The antibody against Tyr(P)^{418}-Src was from BIO-SOURCE (Camarillo, CA). The monoclonal anti-Grb2, anti-Fyn, anti-Yes, anti-PKCα, anti-PKCδ, and anti-PKCε as well as monoclonal anti-Shc IgGs were from Transduction Laboratories (Lexington, KY). The anti-c-Src IgG (clone 327) was obtained from Calbiochem. The antibody raised against dually phosphorylated activated ERK1/2 was from Cell Signaling. Monoclonal anti-phosphotyrosine, polyclonal goat anti-EGFR and anti-Raf-1 used for immunoprecipitation, rabbit anti-Raf-1 IgG used for reprobing of the immunoblots, and horseradish peroxidase-conjugated anti-goat IgG were from Santa Cruz. Neutralizing and anti-HB-EGF was from R & D Systems (Minneapolis, MN).

Cell Culture and Transfection—AR42J cells were cultured in DMEM containing 10% fetal calf serum and antibiotics (36). For transient transfection, cells were cultured in 8-cm^2 dishes and transfected with 3 μg of Csk (C̲-terminal S̲rc k̲inase) in pSG5 or pUSEamp containing dominant negative Ras (N17Ras) using LipofectAMINE 2000 according to the instructions of the manufacturer. The efficiency of transfection was monitored by transfecting the cells with a plasmid coding for the green fluorescent protein (pEGFR-C1). Pertussis toxin treatment was carried out by incubating the cells with 200 ng/ml pertussis toxin for 12 h prior to the experiments (28).

Immunoprecipitation and Immunoblotting—AR42J cells were incubated with appropriate agents at 37 °C. At specified times, the incubation was stopped by the addition of lysis buffer (50 mM Hepes, pH 7.0, 100 mM NaCl, 0.2 mM MgSO$_4$, 0.5 mM Na$_3$VO$_4$, 0.4 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml aprotinin). The extracts were clarified by centrifugation and incubated sequentially (2 h for each incubation at 4 °C) with antibodies as indi-

cated and protein A/G-Sepharose (Amersham Biosciences) with gentle agitation. Immunoprecipitates were washed three times with lysis buffer, boiled for 3 min in 4× Laemmli sample buffer, separated on SDS-polyacrylamide gels under reducing conditions, and electrotransferred to nitrocellulose membranes (16, 28). Antigen-antibody complexes were visualized using horseradish peroxidase-conjugated IgGs and the enhanced chemiluminescence system. Where appropriate, a 50-μl aliquot of clarified whole-cell lysate was mixed with an equal volume of 4× Laemmli sample buffer and resolved by SDS-PAGE for confirmation of identity of proteins after immunoprecipitation. For reprobing, blots were incubated in stripping buffer (62.5 mM Tris-HCl, pH 6.7, 2% SDS, and 100 mM 2-mercaptoethanol) at 50 °C for 30 min, washed extensively with phosphate-buffered saline, reblocked as described (28), and reprobed with appropriate antibodies. To determine Src family kinase activity, cell lysates were either subjected to immunoprecipitation with anti-Src, anti-Yes, or anti-Fyn or directly analyzed by immunoblotting with an antibody recognizing Tyr(P)^{418}-Src. Tyr^{418} is an autophosphorylation site and thus reflects activation of Src family kinases (37). Blots were stripped and reprobed with appropriate antibodies to verify the amount of immunoprecipitated protein.

Ras Activation Assay—Ras activation was determined by affinity precipitation of activated Ras from cell lysates using agarose-conjugated Ras-binding domain. Cells were stimulated, washed once with phosphate-buffered saline, and lysed in a buffer (Ras lysis buffer) containing 25 mM Hepes, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM MgCl$_2$, 1 mM EDTA, 2% glycerol, and 8 μl of agarose-conjugated Ras-binding domain. After a 30-min incubation at 4 °C, immune complexes were washed three times with Ras lysis buffer, followed by analysis of the immune complexes by anti-Ras immunoblotting.

Raf-1 Assay—The Raf-1 assay was carried out similarly as described previously (28) with some modifications. Cells were stimulated and lysed, and Raf-1 was immunoprecipitated with anti-Raf-1 antibody as described above in the immunoprecipitation and immunoblotting protocol. The immunoprecipitates were washed twice in lysis buffer and once in assay buffer (20 mM MOPS, pH 7.2, 25 mM 2-glycerol phosphate, 5 mM EGTA, 1 mM Na$_3$VO$_4$, 1 mM dithiothreitol). The immune complexes were incubated with inactive fusion proteins glutathione *S*-transferase-MEK1 (0.4 μg) in a buffer containing 15 mM MOPS, pH 7.2, 20 mM 2-glycerol phosphate, 5 mM EGTA, 1 mM Na$_3$VO$_4$, 1 mM dithiothreitol, 150 μM ATP, and 25 μM MgCl$_2$ in a final volume of 45 μl for 30 min at 30 °C with gentle agitation. Reactions were stopped by the addition of 15 μl of 4× sample buffer, followed by boiling of the samples and SDS-PAGE. Phosphorylation of MEK1 by immunoprecipitated Raf-1 was determined by immunoblotting with an antibody specific for the phosphorylated form of MEK.

PKC Translocation Assay—The PKC translocation assay was carried out as described recently (38). Cells were incubated for 2 days in serum-free DMEM and stimulated as indicated. Thereafter, cells were washed and scraped into 1 ml of homogenization buffer (20 mM Tris/HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 2 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml aprotinin), lysed by sonication, and centrifuged for 1 h at 100,000 × *g* at 4 °C. Supernatants were used as a source of cytosolic protein. Pellets were resonicated in 1 ml of homogenization buffer supplemented with 1% Triton X-100 and centrifuged for 1 h at 100,000 × *g*, yielding the solubilized particulate fractions. Protein concentration was determined, and the fractions were analyzed by immunoblotting.

Reproducibility of Results—Results are representative of at least three experiments on different occasions giving similar results.

RESULTS

Inhibition of EGFR Signaling Reduces CCK- and Gastrin-induced ERK1/2 Activation in AR42J Cells—The pancreatic cell line AR42J is a well established model for CCK-induced signaling and expresses both CCK$_A$ and CCK$_B$ receptors, both of which are activated by CCK and stimulate ERK1/2 by a PKC-dependent mechanism (27, 28, 30, 39). CCK activated ERK1/2 with a maximum after 3 min as determined by immunoblotting of cell lysates with an antibody recognizing specifically the dually phosphorylated activated form of ERK1/2 (Fig. 1*A*). To determine whether the EGFR is involved in CCK-induced activation of ERK1/2, we tested the effect of AG1478, a well established inhibitor of the EGFR tyrosine kinase, on CCK-induced ERK1/2 activation. As shown in Fig. 1, *B* and *C*, AG1478 strongly inhibited CCK-induced ERK1/2 phosphoryla-
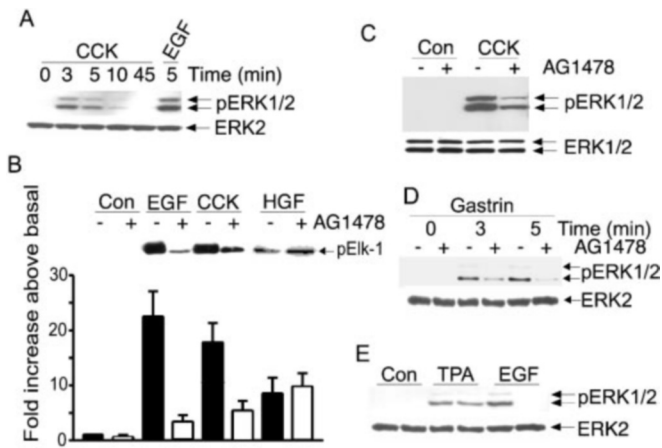
FIG. 1. **The EGFR-specific tyrosine kinase inhibitor AG1478 inhibits CCK-induced ERK1/2 activation.** AR42J cells were incubated with CCK (10 nM), EGF (10 nM), HGF (10 nM), or TPA (100 nM) for 3 min or the indicated time in the absence or presence of AG1478 (250 nM) or vehicle (Me$_2$SO). *A* and *C–E*, ERK1/2 activation was determined by immunoblotting of cellular lysates with an antibody that specifically recognizes the dually phosphorylated activated form of ERK1 and ERK2. Before reprobing with anti-ERK1/2, filters were stripped. *B*, following cell lysis, ERK1/2 was immunoprecipitated, and its activity was assayed with recombinant Elk-1 as a substrate by anti-phospho-Elk-1 immunoblotting. *Con*, control.

tion and activation. AG1478 had no effect on ERK1/2 activation in response to HGF, which activates the c-Met/HGF receptor tyrosine kinase, showing the specificity of this compound for the EGFR tyrosine kinase (Fig. 1*B*). AG1478 had only a small if any effect on ERK1/2 phosphorylation in response to TPA (Fig. 1*D*), an activator of PKC. These data indicate that maximal ERK1/2 activation in response to CCK requires EGFR tyrosine kinase, whereas TPA-induced ERK1/2 activation is mainly EGFR-independent in AR42J cells.

To differentiate which CCK receptor subtype mediates EGFR tyrosine kinase-dependent ERK1/2 activation, we investigated the effect of AG1478 on ERK1/2 phosphorylation in response to gastrin, which activates only the CCK$_B$ receptor. As shown in Fig. 1*E*, AG1478 strongly inhibited gastrin-induced ERK1/2 phosphorylation, indicating that ERK1/2 activation in response to CCK$_B$ receptor stimulation is EGFR-dependent.

CCK Induces Tyrosine Phosphorylation of the EGFR and Shc as Well as Complex Formation of the EGFR with Grb2 and Shc—Certain GPCRs have been reported to induce tyrosine phosphorylation and activation of the EGFR (24, 40). To investigate if CCK induces tyrosine phosphorylation of the EGFR, cells were stimulated with CCK or EGF followed by immunoprecipitation of the EGFR and analysis of the immunoprecipitates by anti-phosphotyrosine immunoblotting. As shown in Fig. 2, incubation of the cells with CCK caused rapid tyrosine phosphorylation of the EGFR. CCK-induced tyrosine phosphorylation of the EGFR was, however, considerably smaller than the effect of EGF. The EGFR-specific tyrosine kinase inhibitor AG1478 abolished CCK-induced EGFR tyrosine phosphorylation, indicating that the EGFR tyrosine kinase domain mediates CCK-induced EGFR tyrosine phosphorylation.

EGFR activation involves complex formation of the EGFR with the adaptor proteins Grb2 and Shc and tyrosine phosphorylation of Src homology 2 domain-containing substrates such as Shc (17). Determination of Grb2 immunoreactivity in EGFR immunoprecipitates showed that CCK increased complex formation between the EGFR and Grb2 (Fig. 2*B*). Analysis of Shc immunoprecipitates by anti-phosphotyrosine, anti-Grb2, and anti-EGFR immunoblotting revealed that CCK induced tyrosine phosphorylation of p47^{Shc} and p52^{Shc} as well as complex
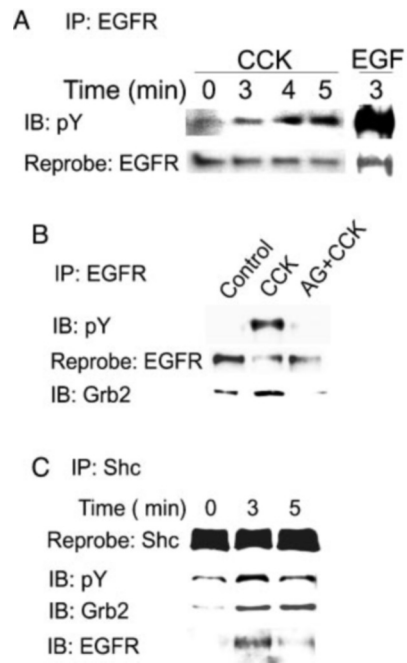

FIG. 2. **CCK causes tyrosine phosphorylation of the EGFR and Shc and complex formation of Shc with both EGFR and Grb2.** Cells were exposed to CCK (10 nM) or EGF (20 nM) in the absence or presence of AG1478 or vehicle (Me$_2$SO) for 3 min or the indicated time periods. Following cell lysis, immunoprecipitation (*IP*) was carried out with anti-EGFR or anti-Shc, and the immunoprecipitates were analyzed by anti-phosphotyrosine, anti-EGFR, and anti-Grb2 immunoblotting (*IB*). Following stripping, blots were reprobed with anti-EGFR (*A–C*) or anti-Shc (*B*).

formation of Shc with Grb2 and the EGFR (Fig. 2*C*). Taken together, these data demonstrate that CCK induces tyrosine phosphorylation of the EGFR and Shc as well as formation of a complex between Shc, Grb2, and the EGFR, events closely related to EGFR activation.

AG1478 abolished CCK-induced complex formation with Grb2 and the tyrosine-phosphorylated EGFR (Fig. 2*B*), indicating the involvement of the EGFR tyrosine kinase activation in this process. In contrast, AG1478 had no effect on tyrosine phosphorylation of Shc and its complex formation with Grb2 in response to HGF (data not shown), which activates the c-Met/HGF receptor tyrosine kinase, thus confirming that AG1478 specifically antagonized the effect of the EGFR tyrosine kinase in our system.

The addition of GF109203X, a chemical inhibitor of PKC, or loading of the cells with the intracellular calcium chelator 1,2-bis(2-aminophenoxy)ethene *N,N,N′,N′*-tetraacetic acid acetoxymethyl ester did not prevent CCK-induced tyrosine phosphorylation of the EGFR (data not shown), indicating that an increase in intracellular calcium or activation of PKC is not essential for CCK-induced EGFR transactivation. Moreover, since cleavage of pro-HB-EGF by metalloproteinases has been shown to mediate EGFR transactivation by G$_i$- and G$_q$-coupled receptors in several different cell lines (41), we investigated the effect of neutralizing anti-HB-EGF antibody and of the HB-EGF inhibitor [Glu^{52}]diphtheria toxin (CRM197) on CCK-induced tyrosine phosphorylation of the EGFR and ERK1/2 activation. Anti-HB-EGF or CRM197 had no effect on CCK responses (data not shown), indicating that HB-EGF is not involved in CCK-induced EGFR and ERK1/2 activation in AR42J cells.

Ras Is Involved in CCK-induced ERK1/2 Activation— Whereas there is agreement concerning the requirement of Ras in EGF- and G$_i$-coupled receptor-induced ERK1/2 activation,
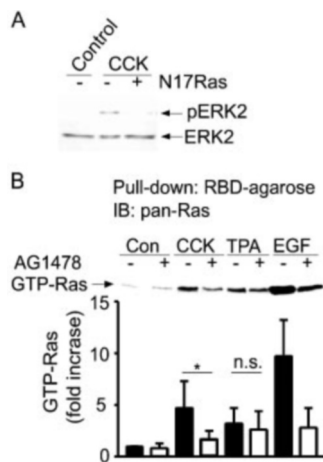
FIG. 3. **CCK induces activation of Ras, which is essential for CCK-induced ERK1/2 activation.** *A*, AR42J cells were transiently transfected with N17Ras or empty vector. After 24 h, cells were serum-starved for 5 h and were then exposed to CCK (10 nM) for 3 min at 37 °C. Following lysis, cellular proteins were analyzed by anti-phospho-ERK1/2 immunoblotting (*IB*). The blots were stripped and reprobed with anti-ERK2. *B*, cells were stimulated with CCK (10 nM), TPA (10 nM), or EGF (10 nM) in the presence or absence of AG1478 (250 nM) for 3 min. Following lysis, equal amounts of cellular extracts were incubated with Ras binding domain (*RBD*) peptide coupled to agarose. Affinity precipitates were analyzed for GTP-bound activated Ras by anti-pan-Ras immunoblotting. The *asterisk* represents a significant difference ($p < 0.05$) according to analysis of variance; *n.s.*, not significant; *Con*, control.

G_q-coupled receptor-induced ERK1/2 activation has been reported to be mediated by both Ras-dependent or -independent pathways (17, 23, 42–44). Adenoviral expression of dominant negative Ras was found to have no effect on CCK-induced activation of ERK1/2 in cultured primary pancreatic acinar cells (29), and CCK appears to have no significant effect on the amount of activated GTP-bound Ras in freshly prepared pancreatic acinar cells (39, 45), suggesting that Ras may not be involved in CCK-induced ERK1/2 activation in native pancreatic acinar cells. If CCK-induced activation of ERK1/2 is mediated by EGFR transactivation in pancreatic AR42J cells, CCK-induced ERK1/2 activation should depend on Ras activation. Involvement of Ras in CCK-induced ERK1/2 activation was determined in AR42J cells transiently transfected with dominant-negative Ras (N17Ras) or empty vector. As shown in Fig. 3*A*, N17Ras inhibited CCK-induced ERK1/2 phosphorylation.

The findings that dominant-negative Ras and AG1478 inhibited CCK-induced ERK1/2 activation suggest involvement of both the EGFR and Ras in CCK-induced ERK1/2 activation. Therefore, we studied the effect of CCK, EGF, and AG1478 on Ras activity. Both CCK and EGF increased the amount of GTP-bound Ras in an AG1478-sensitive fashion (Fig. 3*B*), indicating that CCK induces Ras activation through an EGFR-dependent mechanism. TPA also caused activation of Ras (Fig. 3*B*). However, TPA-induced Ras activation was less affected by AG1478 than the CCK response, indicating that phorbol ester-induced Ras activation occurs mainly through an EGFR-independent mechanism.

Src Family Tyrosine Kinases Are Involved in CCK-induced Ras and ERK1/2 Activation—In the majority of studies, GPCR-induced ERK1/2 activation depends on activation of Src family tyrosine kinases (17). To study the role of Src family tyrosine kinases in CCK-induced ERK1/2 activation, we first investigated the effect of PP1, a specific inhibitor of Src family kinases, on CCK-induced activation of ERK1/2 and Src family tyrosine kinases. Because there is a correlation between autophosphorylation of Tyr[418] and activation of Src kinase (37), Src
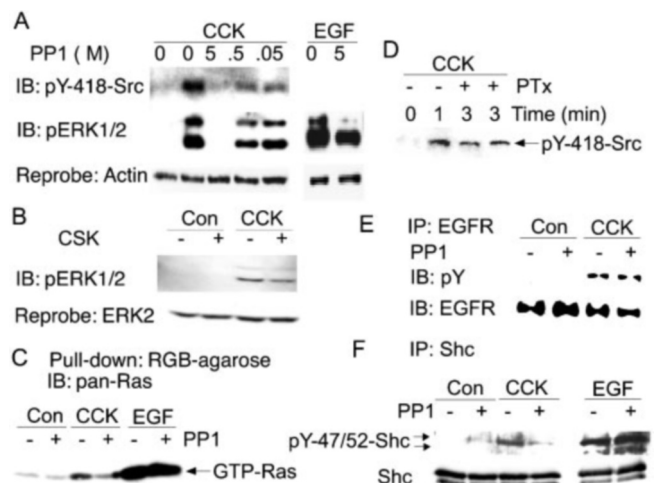

FIG. 4. **Role of Src family tyrosine kinases in CCK-induced ERK1/2 activation, tyrosine phosphorylation of the EGFR and Shc, and recruitment of Shc and Grb2 to the EGFR.** *A*, cells were exposed to CCK (10 nM) in the presence or absence of the indicated concentration of PP1 for 3 min. Cell lysates were analyzed by anti-phospho-418-Src, anti-phospho-ERK1/2, and anti-β-actin immunoblotting. *B*, Csk- or mock-transfected cells were stimulated with CCK for 3 min followed by lysis of the cells and analysis of the ERK phosphorylation by anti-ERK1/2. The blot was stripped and reprobed with anti-ERK2. *C*, cells were stimulated with CCK or EGF (10 nM) in the presence or absence of PP1 (10 μM) for 3 min. Following cell lysis, equal amounts of cellular protein extracts were incubated with Ras binding domain (*RBD*) peptide coupled to agarose. Affinity precipitates were analyzed for GTP-bound activated Ras by anti-pan-Ras immunoblotting. *D*, pertussis toxin (*PTx*)-pretreated and -untreated cells were exposed to CCK (10 nM) for the indicated time. Following cell lysis, autophosphorylation of Src family tyrosine kinases was detected by immunoblotting with anti-Tyr(P)[418]-Src. *E* and *F*, immunoprecipitation was carried out with anti-EGFR (*E*) or anti-Shc (*F*), and immunoprecipitates were analyzed by anti-phosphotyrosine, anti-EGFR, anti-Grb2, and anti-Shc immunoblotting. Blots were stripped and reprobed with anti-EGFR (*E*) or anti-Shc (*F*). *Con*, control.

kinase activation can be detected by immunoblotting with an antibody recognizing the autophosphorylated form of Src (Tyr(P)[418]-Src). As shown in Fig. 4*A*, CCK caused an increase in the proportion of autophosphorylated Src family kinase; this was inhibited by PP1. The inhibitory effect of PP1 on CCK-induced Src family kinase autophosphorylation closely correlated with its effect on CCK-induced ERK1/2 phosphorylation, providing evidence that PP1 indeed inhibited CCK-induced ERK1/2 activation by interfering with activation of Src family kinases. The involvement of Src family tyrosine kinase in CCK-induced ERK activation was confirmed by experiments in which Csk, which phosphorylates and thereby blocks activation of Src family tyrosine kinases (46, 47), was transfected into the cells, followed by detection of CCK-induced ERK1/2 activation. As shown in Fig. 4*B*, expression of Csk reduced CCK-induced ERK1/2 phosphorylation significantly.

PP1 also reduced EGF-induced ERK1/2 phosphorylation (Fig. 4*A*), but this effect was clearly smaller than its effect on CCK-induced ERK1/2 phosphorylation. PP1 inhibited CCK-induced Ras activation, whereas the response to EGF remained unchanged (Fig. 4*C*). Together, these results demonstrate that CCK-induced, but not EGF-induced, ERK1/2 activation depends on stimulation of Src family tyrosine kinases, which are involved in CCK-induced ERK1/2 activation upstream of Ras activation.

Since CCK receptor activation can lead to activation of pertussis toxin-sensitive G proteins (48–51) and G_i-coupled receptors can activate Src by Gβγ, which then induces tyrosine phosphorylation of Shc, we studied the effect of pertussis toxin, which inactivates $G_{i/o}$-type G proteins, on CCK-induced auto-
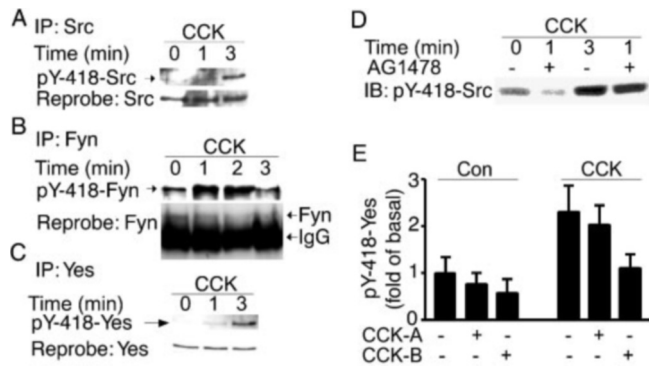
FIG. 5. **CCK induces activation of Src, Fyn, and Yes.** Cells were exposed to CCK (10 nM) in the presence or absence of AG1478 (250 nM), CCK-A, or CCK-B receptor blocker for the indicated time or 3 min (*E*). Following cell lysis, immunoprecipitation was carried out with anti-Src (*A*), anti-Fyn (*B*), or anti-Yes (*C* and *E*), and immunoprecipitates were analyzed by anti-Tyr(P)^{418}-Src. Blots were stripped and reprobed with anti-Src (*A*), anti-Fyn (*B*), and anti-Yes (*C*). Cell lysates were analyzed by anti-Tyr(P)^{418}-Src (*D*).
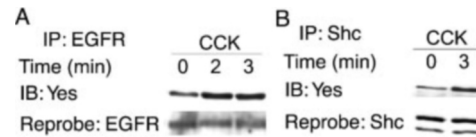

FIG. 6. **CCK increases Yes immunoreactivity in EGFR and Shc immunoprecipitates.** Cells were exposed to CCK (10 nM) for the indicated time. Following cell lysis, immunoprecipitation was carried out with anti-EGFR (*A*) or anti-Shc (*B*), and immunoprecipitates were analyzed by anti-Yes. Blots were stripped and reprobed with anti-EGFR (*A*) or anti-Shc (*B*).

phosphorylation of Src family kinase. As shown in Fig. 4*D*, treatment of the cells with pertussis toxin had no effect on CCK-induced autophosphorylation of Src family kinase. Furthermore, pertussis toxin treatment did not alter CCK-induced tyrosine phosphorylation of Shc (data not shown). These data suggest that $G_{i/o}$ proteins do not participate in CCK-induced tyrosine phosphorylation of Shc.

Recent studies provided heterogeneous data concerning the involvement of Src family kinases in GPCR-induced EGFR transactivation (42, 43, 52–57). Src family kinases might be involved in GPCR-induced assembly of the Ras activation complex either by mediating tyrosine phosphorylation of the EGFR (53, 55, 57) and/or Shc (20, 58). To investigate whether activation of Src family tyrosine kinases is involved in CCK-induced EGFR tyrosine phosphorylation and/or recruitment of the docking proteins Shc and Grb2 to the EGFR, cells were incubated with CCK or EGF in the presence or absence of PP1 followed by immunoprecipitation of the EGFR or Shc and analysis of the immunoprecipitates by anti-phosphotyrosine, anti-Shc, anti-Grb2, and anti-EGFR immunoblotting. As shown in Fig. 4*E*, PP1 had no effect on CCK-induced tyrosine phosphorylation of the EGFR but completely inhibited CCK-induced tyrosine phosphorylation of Shc (Fig. 4*F*). These data indicate that CCK-induced EGFR tyrosine phosphorylation is Src family kinase-independent, whereas CCK-induced recruitment of Shc and Grb2 to the EGFR and tyrosine phosphorylation of Shc require Src family kinase activity. PP1 had no effect on EGF-induced tyrosine phosphorylation of Shc (Fig. 4*F*).

To investigate which Src family tyrosine kinases are activated in response to CCK and could therefore participate in CCK-induced ERK1/2 activation, cells were stimulated with CCK followed by immunoprecipitation of Src, Fyn, and Yes and analysis of the immunoprecipitates for Tyr(P)^{418}-Src immunoreactivity. As shown in Fig. 5, *A–C*, CCK caused rapid autophosphorylation of Src, Fyn, and Yes, indicating that CCK induces activation of these Src kinases. Since EGF can also induce activation of Src family tyrosine kinases (59), we investigated the effect of AG1478 on CCK-induced Src family Tyr^{418} phosphorylation. As shown in Fig. 5*D*, AG1478 had no significant effect on CCK-induced Src family tyrosine kinase autophosphorylation, showing that CCK-induced Src family kinase activation does not require activation of the EGFR.

It has recently been shown that gastrin induces complex formation between Src and Shc in CCK$_B$/gastrin receptor-expressing Chinese hamster ovary cells (32). Similarly, lysophosphatidic acid, which activates a G_i-coupled receptor, causes

complex formation of Shc with Src but not with Fyn or Yes (20), whereas angiotensin II induces association of Shc with Fyn in cardiac myocytes (21) or with Src in vascular smooth muscle cells (57), indicating that GPCR-induced signaling toward Shc can involve several different members of the Src family tyrosine kinases depending on receptor and cell type. To examine which Src family kinases might be involved in CCK-induced tyrosine phosphorylation of Shc and complex formation between the EGFR, Grb2, and Shc, EGFR and Shc immunoprecipitates from CCK-pretreated and untreated cells were examined for the presence of Src, Fyn, and Yes immunoreactivity. As illustrated in Fig. 6, CCK led to an increase in Yes immunoreactivity in both EGFR and Shc immunoprecipitates, indicating that Yes could be involved in tyrosine phosphorylation of Shc and its complex formation with the EGFR and Grb2. Src immunoreactivity was detected in both EGFR and Shc immunoprecipitates, but there was no clear increase in Src immunoreactivity in EGFR and Shc immunoprecipitates upon treatment of the cells with CCK (data not shown). Fyn immunoreactivity was undetectable in Shc or EGFR immunoprecipitates. These data point to a critical role of Yes in CCK-stimulated tyrosine phosphorylation of Shc and its complex formation with Grb2.

Because Yes appears to play a critical role in CCK-induced signal transduction, we examined which CCK receptor subtype mediates CCK-induced tyrosine phosphorylation of Yes using the CCK receptor subtype-specific inhibitors. As shown in Fig. 5*E*, CCK-induced tyrosine phosphorylation of Yes was inhibited by CCK$_B$, but not by CCK$_A$ receptor antagonist, indicating that the CCK$_B$ receptor mediates CCK-induced Yes activation.

PKC Augments CCK-induced ERK1/2 Signaling at the Ras/Raf Level—Fig. 7 compares the effects of CCK with that of EGF with respect to induction of tyrosine phosphorylation of Shc, Shc-Grb2 complex formation, and activation of Ras, Raf, and ERK1/2. CCK was a much weaker stimulator of the ERK cascade than EGF up to the level of Ras, whereas at the level of Raf-1, the effects of CCK and EGF were almost equal. This indicates signal amplification between Ras and Raf-1. Activation of PKC appears to mediate this signal amplification, because CCK-induced Ras, Raf-1, and ERK1/2 activation were inhibited by down-modulation of PKC by TPA, whereas more proximal responses (activation of Shc/Grb2) were not influenced by this treatment.

Since PKC has been proposed as a modulator of Ras-Raf activation and activation of PKC is clearly involved in CCK-induced ERK1/2 activation (25, 26, 60), we studied the effect of prolonged treatment of the cells with TPA, which leads to down-modulation of diacylglycerol-sensitive classical and novel PKC isoenzymes, on CCK-induced tyrosine phosphorylation of Shc, Shc-Grb2 complex formation, and activation of Ras, Raf, and ERK1/2. As shown in Fig. 7, TPA long term treatment had no effect on CCK- or EGF-induced tyrosine phosphorylation of Shc or its complex formation with Grb2 (Fig. 7), although CCK-induced ERK1/2 phosphorylation tested in the same cell lysates was clearly inhibited (Fig 7). A similar result was obtained when PKC activation was inhibited by GF109203X
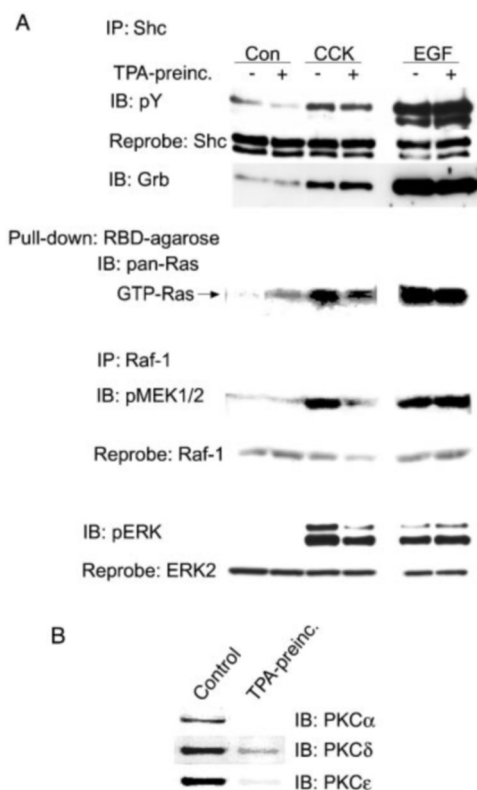
FIG. 7. **Effect of prolonged TPA treatment on CCK- or EGF-induced tyrosine phosphorylation of Shc and Shc-Grb2 complex formation.** *A*, TPA-pretreated and control cells were exposed to CCK (10 nM) or EGF (3 nM) for 3 min. Cell lysates were immunoprecipitated with anti-Shc followed by anti-phosphotyrosine and anti-Grb2 immunoblotting, followed by stripping and reprobing of the blot with anti-Shc. The same lysates were used for immunoprecipitation with anti-Raf-1. The Raf-1 immune complexes were incubated with inactive fusion proteins glutathione *S*-transferase-MEK1. Phosphorylation of MEK1 by immunoprecipitated Raf-1 was determined by immunoblotting with an antibody specific for the phosphorylated form of MEK. The blot was reprobed with anti-Raf-1. Aliquots of the lysates were analyzed by anti-phospho-ERK1/2 immunoblotting, followed by reprobing of the blot with anti-ERK2. Furthermore, equal amounts of cellular extracts were incubated with Ras binding domain (*RBD*) peptide coupled to agarose. Affinity precipitates were analyzed for GTP-bound activated Ras by anti-pan-Ras immunoblotting. *B*, cells preincubated with 10 nM TPA or vehicle (dimethyl sulfoxide) for 18 h were analyzed by anti-PKCα, -PKCδ, and -PKCε immunoblotting. *Con*, control.

(data not shown). Prolonged TPA treatment inhibited CCK-induced Ras and Raf-1 activation without influencing the effect of EGF (Fig. 7), suggesting a role of PKC in CCK-induced activation of Ras and Raf-1.

Immunoblotting analysis revealed expression of three major phorbol ester- or diacylglycerol-responsive PKC isoforms (*i.e.* PKCα, -δ, and -ε) in AR42J cells and revealed that prolonged treatment of the cells with TPA led to down-modulation of these PKC isoenzymes (Fig. 7, *lower panel*). To provide evidence which PKC isoform might be involved in CCK-induced ERK1/2 activation, we studied the effect of CCK on PKC activation as detected by translocation of PKC from the cytosol to the membrane compartment. As illustrated in Fig. 8*A*, CCK caused rapid translocation of PKCα, -δ, and -ε from the cytosol to the membrane fraction, indicating that CCK causes activation of these PKC isoenzymes.

To examine which PKC isoenzyme mediates CCK-induced activation of Raf-1 and subsequently ERK1/2, we studied the effect of Gö6976, an inhibitor of calcium-dependent PKC isoenzymes, and rottlerin, an inhibitor of PKCδ, on CCK-induced ERK1/2 phosphorylation. As illustrated in Fig. 8*B*, Gö6976 and
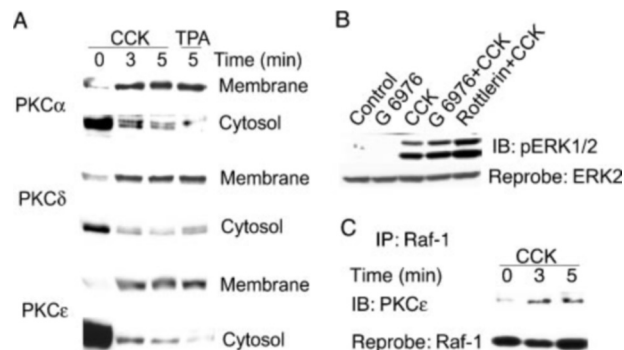

FIG. 8. **CCK induces activation of PKCε and its complex formation with Raf-1.** *A*, cells were incubated for 2 days in serum-free DMEM and stimulated as indicated with CCK (10 nM) or TPA (10 nM). Thereafter, cells were washed, scraped, homogenized, and separated into cytosol and membrane fractions as described under "Experimental Procedures." The fractions were analyzed by immunoblotting with anti-PKCα, -δ, and -ε. *B*, cells were exposed to CCK in the absence or presence of Gö6976 (10 μM) or rottlerin (10 μM) for 3 min. Cell lysates were analyzed by anti-phospho-ERK1/2, followed by stripping and reprobing of the blot with anti-ERK2. *C*, cells were stimulated with CCK for the indicated time. Cell lysates were immunoprecipitated with anti-Raf-1. The immunoprecipitates were analyzed by anti-PKCε immunoblotting.

rottlerin had no effect on CCK-induced ERK1/2 activation, suggesting that conventional PKCs and PKCδ are unlikely to be involved in CCK-induced ERK1/2 activation.

DISCUSSION

The mechanisms by which GPCRs activate ERK1/2 are characterized by considerable heterogeneity. Depending on receptor and cell type, GPCRs have been shown to mediate Ras-independent ERK1/2 activation via stimulation of PKC or Ras-dependent ERK1/2 activation via activation of receptor and non-receptor tyrosine protein kinases (17). In the present study, we provide evidence that activations of the EGFR, Yes, and PKCε are involved in CCK-induced ERK1/2 activation and cooperate to accomplish CCK-induced ERK1/2 activation.

We found that CCK caused activation of the EGFR, which is essential for full activation of ERK1/2 in response to CCK. The evidence for this is based on the findings that CCK caused tyrosine phosphorylation of the EGFR and Shc, Shc-Grb2 complex formation, recruitment of these docking proteins to the EGFR as well as Ras, and ERK1/2 activation through an EGFR tyrosine kinase-dependent pathway. CCK-induced AG1478-sensitive ERK1/2 phosphorylation was mimicked by the CCK$_B$ receptor agonist gastrin, indicating that CCK$_B$ receptor activation mediates EGFR-dependent ERK1/2 activation. This hypothesis is supported by a recent study showing that gastrin induces EGFR tyrosine phosphorylation in CCK$_B$/gastrin receptor-transfected gastric epithelial cells (33).

The mechanism of CCK receptor-induced EGFR tyrosine phosphorylation is unclear. The inhibitory effect of AG1478 suggests that CCK-induced EGFR tyrosine phosphorylation is due to EGFR autophosphorylation. It has recently been shown that cleavage of pro-HB-EGF by metalloproteinases mediates EGFR transactivation in response to G$_i$- and G$_q$-coupled receptors in several cell types (41). Moreover, in CCK$_B$ receptor-transfected gastric epithelial cells, gastrin induces release of HB-EGF into the medium, which causes EGFR tyrosine phosphorylation (33). Thus, it is possible that CCK also induces EGFR tyrosine phosphorylation by activation of metalloproteinase and proteolytic processing of EGFR ligand precursors in pancreatic AR42J cells. However, in the present study, neither the HB-EGF inhibitor [Glu^{52}]diphtheria toxin (CRM197) nor neutralizing anti-HB-EGF IgG had any effect on CCK-

induced ERK1/2 activation, suggesting that HB-EGF may not be involved in CCK-induced EGFR and ERK1/2 activation in AR42J cells. However, it is possible that proteolytic cleavage of an EGFR ligand precursor different from HB-EGF mediates the effects of CCK on EGFR tyrosine phosphorylation. Src family kinases have been implicated in GPCR-induced EGFR tyrosine phosphorylation, and GPCR can induce association of Src with the EGFR (53–55, 57). In other studies, GPCR-induced EGFR tyrosine phosphorylation was found to be Src-independent (52, 56). In the present study, inhibition of Src kinases had no significant effect on CCK-induced EGFR tyrosine phosphorylation, indicating that Src family kinases may not be involved in CCK-induced EGFR tyrosine phosphorylation. However, because CCK induced activation of Yes and its recruitment to the EGFR, Yes may modulate signaling from the CCK-stimulated EGFR.

Whereas there is consent for the requirement of Ras in EGF- and G_i-mediated ERK1/2 activation, G_q-mediated ERK1/2 activation can occur through Ras-dependent or -independent pathways depending on receptor and cell type (17, 23, 25). Adenoviral expression of dominant-negative Ras in primary rat pancreatic acini was found to have no effect on CCK-induced activation of ERK1/2 (29), and CCK has no significant effect on the amount of activated GTP-bound Ras, although it appears to enhance GTP turnover on Ras (39, 45), suggesting that Ras may not be involved in CCK-induced ERK1/2 activation in these cells. The present study shows that CCK caused activation of Ras and that CCK-induced ERK1/2 activation was abolished by dominant negative Ras in pancreatic AR42J cells, thus providing strong evidence for the notion that CCK stimulates ERK1/2 through a Ras-depending pathway in these cells.

Depending on receptor and cell type, GPCR-induced ERK1/2 activation depends completely or partially on EGFR or platelet-derived growth factor receptor activation or is independent from RTK transactivation (17). Even in cases where GPCR-induced ERK1/2 activation is completely dependent on RTK transactivation, the strong stimulatory effect of G_q-coupled receptors on ERK1/2 often contrasts with rather small effects on RTK tyrosine phosphorylation (56, 57, 61–63). In the present study, CCK activated ERK1/2 and Raf-1 to a similar extent as EGF, and activation of the EGFR and Ras was required for full activation of ERK1/2 by CCK. However, compared with EGF, CCK caused only small increases in tyrosine phosphorylation of the EGFR and Shc, Shc-Grb2 complex formation, and Ras activation. Thus, in CCK-induced activation of the ERK cascade, signal amplification occurs between Ras and Raf. This is concluded from our finding that down-modulation of phorbol ester-sensitive PKCs inhibited CCK-induced activation of Ras, Raf, and ERK1/2, but not tyrosine phosphorylation of Shc and its complex formation with Grb2. Recent studies have shown that activation of PKC may modulate Ras-dependent ERK activation by fine tuning Ras-Raf activation (25, 26, 60). The finding that phorbol ester-induced Ras and ERK1/2 activation was much less sensitive to EGFR tyrosine kinase inhibition compared with the effects of CCK is well in agreement with the assumption that PKC-activating phorbol esters act at the level of Ras-Raf in AR42J cells.

CCK induces activation of PKCα as well as of PKCδ and -ϵ (64) (present study). The present study reveals that inhibition of conventional PKCs did not prevent CCK-induced ERK1/2 activation, indicating that PKCα is not involved. The findings that PKCϵ complexed with Raf-1, that this complex formation was increased by CCK stimulation, and the lack of effect of the PKCδ inhibitor rottlerin on CCK-induced ERK1/2 activation suggest involvement of PKCϵ in CCK-induced ERK1/2 activation. However, the specificity of rottlerin has recently been challenged (65). Taking into account that PKCϵ forms a complex with Raf-1 in fibroblasts (66–68), the data of the present study suggest that CCK-induced activation of Raf-1 and subsequent stimulation of ERK1/2 by CCK is mediated by activation of PKCϵ, which then potentiates CCK stimulation of the ERK cascade at the level of Ras/Raf.

The PKC inhibitor GF109203X has recently been shown to inhibit CCK-induced tyrosine phosphorylation of Shc in native pancreatic acini (27), whereas TPA mimicked it (30), suggesting involvement of PKC in CCK-induced tyrosine phosphorylation of Shc. We also observed that TPA induces tyrosine phosphorylation of Shc and Shc-Grb2 complex formation (data not shown). However, GF109203X or PKC down-modulation by prolonged TPA treatment had no effect on CCK-induced tyrosine phosphorylation of Shc or its complex formation with Grb2, suggesting that activation of PKC is not involved in CCK-induced tyrosine phosphorylation of Shc in AR42J cells.

Src family tyrosine kinases can be activated by various extracellular signals, including GPCRs and RTKs (59, 69). The present study shows that CCK caused increase in tyrosine phosphorylation of three different Src family tyrosine kinases (*i.e.* Src, Fyn, and Yes on tyrosine 418), reflecting their activation. The mechanisms by which GPCRs activate Src family kinases are still incompletely understood. The β_2-adrenergic receptor activates Src by promoting its interaction with the adaptor protein arrestin (70). A number of studies indicate pertussis toxin-sensitive activation of Src, Fyn, Yes, and Lyn in various cell types (22, 71–73). It has previously been shown that α-subunits of G_s and G_i, but not $G\alpha_q$, can activate Src directly (74). Our finding that pertussis toxin had no effect on CCK-induced autophosphorylation of Src family tyrosine kinases argues against an involvement of G_i proteins in CCK-induced Src activation despite the ability of CCK receptors to activate pertussis toxin-sensitive G proteins (48–51). A recent study has shown that the G_q-coupled angiotensin II receptor activates Src directly without the contribution of G proteins (75). However, whether CCK receptors utilize similar mechanisms to activate Src family kinases remains to be established.

There is strong evidence that Src family tyrosine kinases can be involved in G_q-coupled receptor-induced ERK1/2 activation, but this is not universal (42, 52, 53, 55–57, 76, 77). These tyrosine kinases might be involved in GPCR-induced assembly of the Ras activation complex by mediating tyrosine phosphorylation of the EGFR (53, 55, 57) and/or Shc (20, 59). GPCR stimulation has been reported to induce complex formation of Src or Fyn with Shc and of Src with the EGFR (20, 21, 32, 57). The present study shows that GPCR activation can induce complex formation of Yes with both the EGFR and Shc. In particular, our data indicate that CCK-induced ERK1/2 activation requires a concerted action of both the EGFR and Yes that converge at the level of Shc-Grb2 complex formation. This assumption is based on the findings that complex formation between Shc and Grb2 as well as their association with the EGFR and the downstream events including Ras and ERK1/2 activation were sensitive to Src family kinase inhibition in addition to their dependence on the EGFR tyrosine kinase and that Yes was activated and recruited to both Shc and the EGFR in response to CCK. CCK also induced activation of Src and Fyn, but it did not cause robust increase in complex formation between Src and the EGFR or Shc, and Fyn immunoreactivity was undetectable in EGFR or Shc immunoprecipitates. Thus, Src and Fyn probably have roles in CCK signaling other than inducing tyrosine phosphorylation of Shc. Our finding that CCK_B, but not CCK_A receptor antagonist inhibited CCK-induced tyrosine 418 phosphorylation of Yes suggests that the CCK_B receptor mediates CCK-induced activation of Yes.

In contrast to CCK, EGF-induced activation of the ERK1/2 pathway appears to be less dependent on Src family kinase activation than the CCK-induced response, because inhibition of Src family kinase had no effect on EGF-induced tyrosine phosphorylation of Shc, its complex formation with Grb2, and Ras activation. PP1 significantly reduced EGF-induced activation of ERK1/2, but the inhibitory effect of PP1 on EGF-induced ERK1/2 activation was clearly smaller than its effect on CCK-induced ERK1/2 activation. In agreement with our data, a recent study shows that expression of a kinase-inactive mutant of Src does not alter EGF-induced tyrosine phosphorylation of Shc, Shc-Grb2 complex formation, and ERK1/2 activation in epithelial cells (78).

In conclusion, the present study demonstrates that three different signals emanating from the G_q-coupled CCK receptor mediate ERK1/2 activation, including transactivation of the EGFR and activation of Yes, which are recruited to and essential for formation of the EGFR-Grb2-Shc complex, which in turn mediates CCK-induced Ras activation. Activation of PKC, most likely PKCϵ, provides a third signal essential for full CCK-induced ERK1/2 activation at the Ras/Raf level.

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