Cholecystokinin and EGF activate a MAPK cascade by different mechanisms in rat pancreatic acinar cells

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Dabrowski, Andrzej, Guy E. Groblewski, Claus Schäfer, Kun-Liang Guan, and John A. Williams. Cholecystokinin and EGF activate a MAPK cascade by different mechanisms in rat pancreatic acinar cells. *Am. J. Physiol.* 273 (*Cell Physiol.* 42): C1472–C1479, 1997.—The effects of activating the G_q protein-coupled cholecystokinin (CCK) receptor on different proteins/signaling molecules in the mitogen-activated protein kinase (MAPK) cascade in pancreatic acinar cells were analyzed and compared with the effects of activating the tyrosine kinase-coupled epidermal growth factor (EGF) receptor. Both EGF and CCK octapeptide rapidly increased the activity of the MAPKs [extracellular signal-regulated kinase (ERK) 1 and ERK2], reaching a maximum within 2.5 min when 3.9- and 8.5-fold increases, respectively, were observed. The EGF-induced increase of MAPK activity was transient, with only a slight elevation after 30 min, whereas CCK-stimulated MAPK remained at a high level of activation to 60 min. The protein kinase C inhibitor GF-109203X abolished the activation by phorbol ester and inhibited the effect of CCK by 78% but had no effect on EGF-activated MAPK activity. EGF and CCK activated both forms of MAPK kinase (MEK), with CCK having a much larger effect, activating MEK1 by 6-fold and MEK2 by 10-fold, whereas EGF activated both MEKs by only 2-fold. Immunoblotting revealed three different forms of Raf in pancreatic acinar cells. Of the total basal Raf kinase activity, 3.7% was Raf-A, 89.0% was Raf-B, and 7.3% was c-Raf-1. All three forms of Raf were stimulated to a greater extent by CCK than by EGF, which was especially evident for Raf-A and c-Raf-1. The effect of CCK in activating Rafs was at least partially mimicked by stimulation with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate. EGF significantly increased GTP-bound Ras by 183 and 164% at 2.5 and 10 min, respectively; CCK and TPA had no measurable effect. Our study suggests that CCK and EGF activate the MAPK cascade by distinct mechanisms in pancreatic acinar cells.

Ras; Raf; mitogen-activated protein kinase kinase; protein kinase C; epidermal growth factor

MITOGEN-ACTIVATED PROTEIN kinases (MAPKs), also known as extracellular signal-regulated kinases (ERKs), are protein serine/threonine kinases that are rapidly activated by a variety of cell surface receptors (7, 10, 30, 39). They function in signal cascade pathways that control the expression of genes involved in many cellular processes, including cell growth and differentiation (24, 30, 34, 39). Blocking the function of the MAPK cascade-activating ERKs prevents cell proliferation in response to a number of growth-stimulating agents (33). Many extracellular signals leading to cell growth and differentiation are transmitted by two major classes of cell surface receptors: tyrosine kinase growth factor receptors and G protein-coupled receptors (30). Recent studies have shown that some G protein-coupled receptors utilize the same effectors as the tyrosine kinase receptor pathway [e.g., *src* homology/collagen-growth factor receptor bound 2-son of sevenless (Shc-Grb2-SOS], resulting in Ras and ERK activation (6, 21, 41, 43). However, it has also been suggested that the pertussis toxin-sensitive G_i-coupled receptors utilize a pathway that induces Ras activation in a protein kinase C (PKC)-independent manner, whereas G_q-coupled receptors generally initiate a Ras-independent pathway involving PKC (17).

The cholecystokinin (CCK)-A receptor on rat pancreatic acinar cells is a member of the seven transmembrane domain superfamily of receptors (45). Its actions on digestive enzyme secretion are mediated by heterotrimeric G proteins of the G_q/G_{11} class that couple to phospholipase C, leading to increases in intracellular Ca^{2+} concentration and activation of PKC (49). On the other hand, the epidermal growth factor (EGF) receptor is a classical tyrosine kinase growth factor receptor. Pancreatic acinar cells are known to bear EGF receptors, and EGF as well as CCK stimulate the growth of acinar cells in culture (29). Previous studies utilizing rat pancreatic acini have demonstrated that CCK strongly activates ERKs ($p42^{MAPK}$ and $p44^{MAPK}$) as well as other upstream components of this MAPK signaling cascade, including MAPK kinase (MEK) and Ras (8, 13, 14). Moreover, the activation of MAPK appears mediated by activation of PKC (13, 14). More recently, we have found that CCK is able to activate a Shc-Grb2-SOS complex in rat pancreatic acini, thereby providing a possible mechanism for Ras activation (9). EGF also induced this complex of adaptor proteins in acini, and this action was more potent than CCK (9). However, in preliminary studies, EGF was much weaker than CCK in activating ERKs. Therefore, in the present work, we compared the effects of CCK and EGF on the activation of components of the ERK pathways, including MEK, Raf, and Ras. The results indicate that the major mechanism for the activation of the ERKs by CCK in pancreatic acini involves a PKC-mediated activation of multiple forms of Raf and is distinct from the action of EGF that activates Ras and is PKC independent.

EXPERIMENTAL PROCEDURES

Materials. CCK octapaptide (CCK-8) was a gift from Squibb Research Institute (Princeton, NJ) or purchased from Research Plus (Bayonne, NJ). Mouse natural EGF was purchased from Collaborative Biomedical Products (Bedford, MA), 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was from LC Laboratories (Woburn, MA), and chromatographically purified collagenase was from Worthington Biochemicals

(Freehold, NJ). Aprotinin and leupeptin were from Boehringer Mannheim (Mannheim, Germany), prestained molecular weight standards were from Bio-Rad (Hercules, CA), and nitrocellulose membranes were from Schleicher & Schuell (Keene, NH). The enhanced chemiluminescence (ECL) detection system, protein A conjugated to horseradish peroxidase, and X-ray film were from Amersham (Arlington Heights, IL). ImmunoPure immobilized protein A agarose was from Pierce (Rockford, IL). PEI-cellulose F thin-layer chromatography plates were from Merck (Darmstadt, Germany). Ultrafree-MC 5,000 NMWL filter units for centrifugal filtration were from Millipore (Bedford, MA). Affinity-purified polyclonal antibodies to MEK [αMEK-1 (C-18) and αMEK-2 (N-20)] and antibodies to Raf [αRaf-A (C-20), αRaf-B (C-19), and αc-Raf-1 (C-20)] for immunoprecipitation and Western blotting procedures were from Santa Cruz Biotechnology (Santa Cruz, CA). An agarose conjugate of αRas antibody (Y13-259), v-H-*ras* (antibody 1), was purchased from Oncogene Science (Cambridge, MA). The antibody specific for the dual phosphorylated form of ERKs was from Promega (Madison, WI). All other reagents were obtained from Sigma (St. Louis, MO).

Preparation of pancreatic acini and cell-free extract. The preparation of pancreatic acini has been described previously (9, 13, 14). Briefly, pancreases from Sprague-Dawley rats were digested with purified collagenase, mechanically dispersed, and passed through a 150-µm mesh nylon cloth. Acini were then purified by centrifugation at 50 *g* for 3 min in a solution containing 4% bovine serum albumin (BSA) and were resuspended in incubation buffer that consisted of an *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered Ringer solution supplemented with 11.1 mM glucose, Eagle's minimal essential amino acids, 0.1 mg/ml soybean trypsin inhibitor, and 1% BSA. Acini were preincubated at 37°C with minimal shaking for 180 min and then stimulated with different agonists in 1-ml aliquots in 25 × 55 mm polystyrene vials for indicated times. When GF-109203X was studied, it was included for the last 30 min of preincubation and in the incubation solution. Acini were then pelleted in a microcentrifuge, washed once with 1 ml of ice-cold phosphate-buffered saline containing 1 mM Na_3VO_4 (pH 7.4) and sonicated for 5 s in 0.5 ml of ice-cold lysis buffer [50 mM β-glycerophosphate, 1.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 1 mM dithiothreitol, 10 µg/ml leupeptin, and 10 µg/ml aprotinin (pH 7.4)]. The lysates were then centrifuged in a microcentrifuge at 4°C for 15 min, and the supernatant was assayed for ERK activity. The amount of protein in cell extracts was assayed by the Bio-Rad protein assay reagent. For immunoprecipitation of MEK, acini were sonicated in phosphate-buffered saline containing 0.5% Triton X-100, 1 mM Na_3VO_4, 50 mM β-glycerophosphate, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. For immunoprecipitation of Raf, acini were sonicated in ice-cold lysis buffer containing 50 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 5 mM EDTA, 1 mM dithiothreitol, 0.2 mM Na_3VO_4, 25 mM NaF, 10 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. The lysates were then centrifuged at 15,000 *g* for 10 min at 4°C, and the supernatant was diluted to 2 mg/ml protein. Aliquots (0.5 ml) of the supernatants were subjected to immunoprecipitation.

In-gel MAPK assay. Kinase assays in sodium dodecyl sulfate (SDS)-polyacrylamide gels were carried out by a modified method of Kameshita and Fujisawa (23), using myelin basic protein (0.5 mg/ml polymerized in the gel) as substrate as described previously (8). The concentration of ATP in the kinase buffer was 20 µM, and added radioactivity was 1.5 µCi/ml.

Immunoprecipitation and Western blotting. Cell lysates were incubated with 1.5 µg of αMEK or αRaf antibody for 2 h, and then immobilized protein A agarose was added for an additional 1 h with shaking at 4°C. The immunoprecipitates were washed three times with the appropriate lysis buffer and once with 20 mM HEPES (pH 7.5), 0.05% 2-mercaptoethanol, and 0.2 mM EDTA and boiled for 5 min in a mixture (80:20) of lysis buffer and 250 mM Tris (pH 6.8), 5% SDS, 10% 2-mercaptoethanol, and 40% glycerol. The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis, followed by Western blot analysis with the indicated antibody using an ECL detection system. Primary antibodies for Western blotting were used at a concentration of 0.2 µg/ml.

Kinase assay. Recombinant human ERK1 was expressed as a glutathione-*S*-transferase (GST) fusion protein (16). MEK1 and MEK2 were also expressed as GST fusion proteins and purified as described previously (53). To measure Raf activity, immunoprecipitated Raf was used to activate 0.08 µg of GST-MEK1 in 20 µl of kinase buffer (18 mM HEPES, pH 7.4, 10 mM magnesium acetate, and 50 µM ATP). The reaction mixture was incubated at 30°C for 30 min with gentle shaking. The samples were briefly spun in a microcentrifuge, and 10 µl of the activated GST-MEK1 (0.04 µg) were used to activate 0.3 µg (10 µl) of ERK1 (53). After a 10-min incubation at 30°C, 20 µg of myelin basic protein dissolved in 20 µl of kinase buffer and 5 µCi [γ-^{32}P]ATP were added to initiate the kinase reaction for an additional 20 min at 30°C. One-half of the reaction mixture (20 µl) was transferred onto a 2.5-cm-diameter p81 phosphocellulose paper (Whatman). The filters were washed five times with 180 mM phosphoric acid and then rinsed with 95% ethanol. Phosphorylation was quantitated by scintillation counting.

To measure MEK activity, immunoprecipitated MEK was used to activate 0.3 µg of ERK1 in 20 µl of kinase buffer by incubation at 30°C for 30 min with gentle shaking. Myelin basic protein was then added to the reaction mixture along with [γ-^{32}P]ATP, and the assay was completed as for the Raf activity measurement.

Determination of GTP-bound Ras. Freshly prepared acini were preincubated at 37°C for 60 min in phosphate-free HEPES-Ringer buffer (1% BSA) and then resuspended in similar buffer containing 0.1% BSA and 0.25 mCi/ml of carrier-free [^{32}P]orthophosphate and incubated for 120 min with gentle swirling every 30 min. Cells were then either left untreated (control) or stimulated with CCK, EGF, or TPA for the indicated times. Acini were next quickly pelleted, washed with ice-cold phosphate-buffered saline containing 1 mM Na_3VO_4 and sonicated in 50 mM HEPES, 1 mM sodium phosphate (pH 7.4), 1% Triton X-100, 100 mM NaCl, 20 mM $MgCl_2$, 1 mg/ml BSA, 0.1 mM GTP, 0.1 mM GDP, 1 mM ATP, 0.4 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml soybean trypsin inhibitor, and 10 mM benzamide, as described by others (48). Extracts, containing the same amount of protein (1–1.5 mg in 0.5 ml), were immunoprecipitated with agarose-conjugated αRas antibody overnight, and the immune complexes were washed five times with lysis buffer and five times with wash buffer (50 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 150 mM NaCl, and 0.005% SDS) (20). Ras-associated guanylnucleotides were eluted in 30 µl of 20 mM HEPES (pH 7.5), 20 mM EDTA, 2% SDS, 0.5 mM GDP, and 0.5 mM GTP at 90°C for 3 min (20). Eluates were then transferred into Ultrafree-MC 5,000 NMWL filter units for centrifugal filtration at 2,000 *g* for 16 min at 21°C. Next, 10 µl of each filtrate were spotted on PEI-cellulose F plates, and the nucleotides were separated by thin-layer chromatography using 1 M KH_2PO_4 (pH 3.4) as the solvent. Labeled nucleotides were visualized and quantified by a GS-250 molecular imager (Bio-Rad). The use of these described elution conditions and centrifugal filtration increased resolution of the assay by eliminating streaks of nonspecific radioactive material in the background of separated nucleotides.

Data Analysis. Values are reported are means ± SE. Where appropriate, significance of difference between means was analyzed by Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS

Effect of EGF and CCK on the activity of MAPK in rat pancreatic acini. In a previous study (8) with an in-gel kinase assay, we found that CCK induced strong and prolonged activation of ERK1 and ERK2 ($p44^{MAPK}$ and $p42^{MAPK}$) in rat pancreatic acini. In the present study, with the same assay, we evaluated the effect of a classical growth factor and the known ERK stimulant EGF on ERK activity in rat pancreatic acini and compared it with the effect of CCK. Figure 1 presents the time course of EGF- and CCK-induced activation of ERKs; the integrated densities of the ERK1 and ERK2 bands were calculated and illustrated in Fig. 1, *B* and *C*. EGF and CCK-8 rapidly increased the activity of both ERKs, which reached a maximum activity within 2.5 min at 3.9-fold and 8.5-fold increases, respectively, over the activity at *time 0*. EGF-induced ERK activity diminished significantly within 10 min but remained slightly elevated above the control level to 60 min. In CCK-stimulated acini, ERK activity decreased slightly at 15 min but remained at a high level throughout the 60 min of stimulation. The integrated response to CCK over 60 min was almost six times that of EGF.

Previous work has shown that phorbol ester mimicked the effect of CCK in activating ERKs and that the effect of CCK could be inhibited by the protein kinase inhibitor staurosporine (13), but effects on EGF stimulation were not evaluated. We therefore used the newer and more specific PKC inhibitor, GF-109203X, to evaluate the role of PKC in the effect of CCK and EGF (Fig. 2). TPA increased the tyrosine phosphorylation of ERKs and ERK activity similar to CCK, and the effect on both parameters was totally blocked by GF-109203X in a dose-dependent manner. The effect of CCK on tyrosine phosphorylation of ERKs and enzyme activity at its initial peak was largely inhibited (78%), whereas the effect of EGF was not altered by GF-109203X. GF-109203X almost completely abolished the effect of CCK at 30 min (data not shown). Thus, at the level of ERKs, the effect of CCK is largely mediated by PKC, whereas the effect of EGF is not.

EGF and CCK activate MEK1 and MEK2 in rat pancreatic acinar cells. In a previous study (14), MEK1 and MEK2 were identified in rat pancreatic acinar cells by immunoblotting, and total MEK activity was found to be rapidly activated by CCK and TPA. In the present study, we compared the effect of EGF and CCK on the

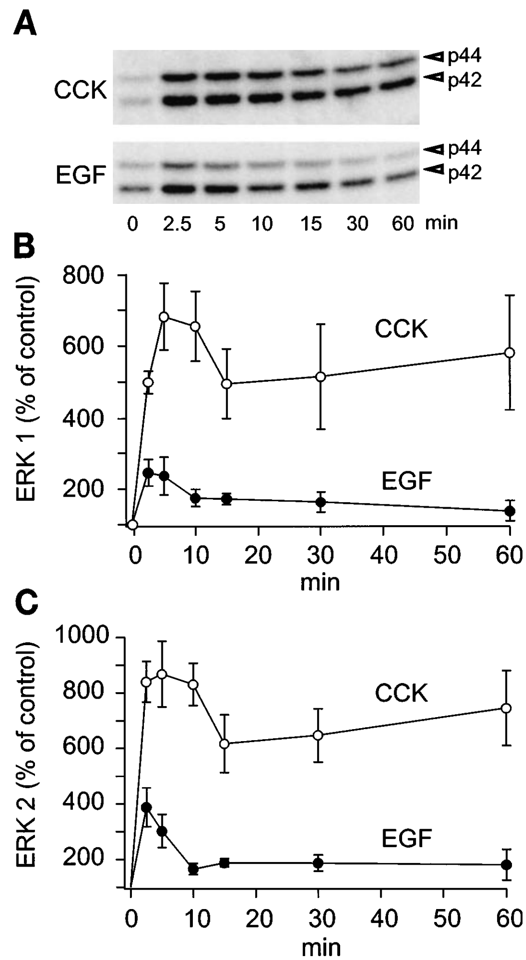

Fig. 1. Effect of cholecystokinin (CCK) and epidermal growth factor (EGF) on the activity of extracellular signal-regulated kinases (ERKs) in rat pancreatic acini. Acini were stimulated for different time periods with 1 nM CCK octapeptide (CCK-8) or 100 nM EGF and then sonicated in lysis buffer and boiled in stop solution. Samples were run on gels containing myelin basic protein, and in-gel kinase assays were subsequently performed (a representative experiment is shown in *A*). Intensity of phosphorylation was measured with the GS-250 molecular imager and results are expressed as a percentage of the average value at *time 0* for ERK1 [p44 mitogen-activated protein kinase (MAPK)] in *B* and for ERK2 (p42 MAPK) in *C*. For *B* and *C*, each point represents mean ± SE of 3–5 independent experiments, each performed in duplicate.

individual kinase activities of MEK1 and MEK2. The specificity of αMEK antibodies was determined by Western blotting and immunoprecipitation. No cross-reactivity of αMEK1 and αMEK2 antibodies with GST-MEK1 and GST-MEK2 fusion proteins was detected (Fig. 3). In addition, there was no cross-reactivity of tested antibodies with MEK1 or MEK2 immunoprecipitated from acini (Fig. 3). Acini were stimulated for 2.5 min with 100 nM EGF or 1 nM CCK, and the cell extracts were immunoprecipitated with αMEK1 or αMEK2 to measure kinase activity. Under the conditions of our assay, we found a basal kinase activity of MEK1 that was seven times higher than that of MEK2 (Table 1). EGF and CCK activated both forms of MEK, with CCK being much more potent, activating MEK1 6-fold and MEK2 10-fold, whereas EGF activated both

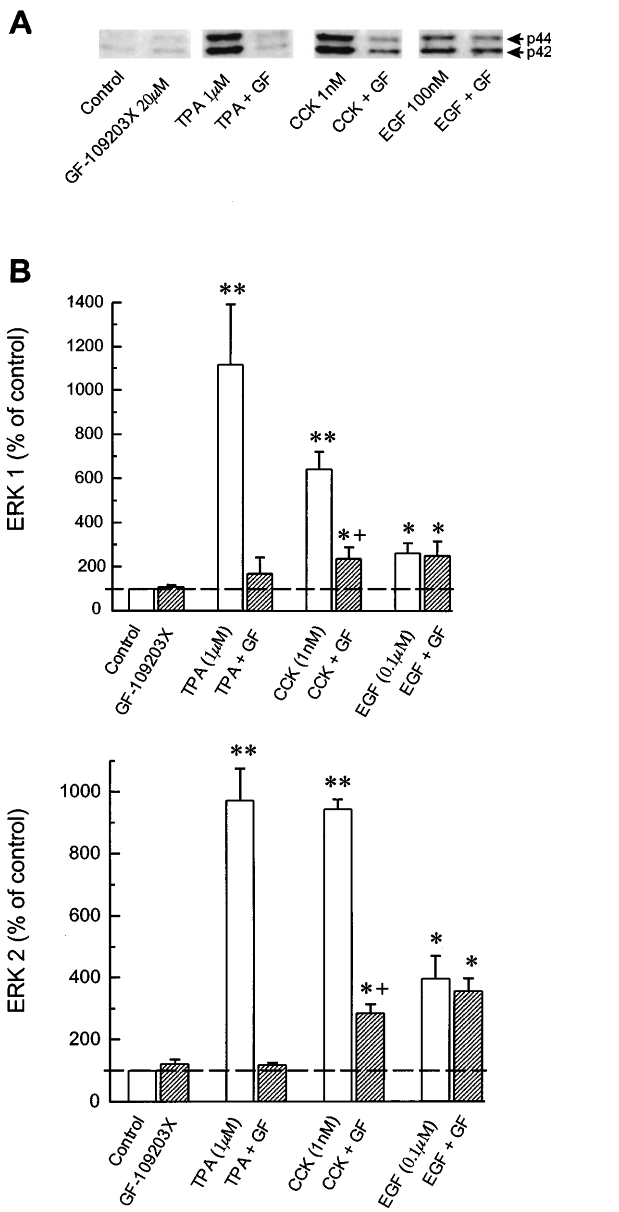

Fig. 2. Effect of the protein kinase C (PKC) inhibitor, GF-109203X, on tyrosine phosphorylation and activity of ERKs in rat acini. Acini were pretreated for 30 min with 20 μM GF-109203X (GF) as specified and then stimulated for 2.5 min with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), CCK, or EGF; acini were then sonicated in lysis buffer and subjected to gel electrophoresis and Western blotting (*A*) or subjected to in-gel kinase assay using myelin basic protein (*B*). *A:* typical result using anti-active MAPK antibody. *B:* means and SE for ERK activity from 3 independent experiments performed similarly to Fig. 1. * $P < 0.05$ vs. control; ** $P < 0.01$ vs. control; $^+$ $P < 0.01$ vs. CCK alone.

MEKs 2-fold. In other experiments, acini were stimulated for 5 min with the same concentrations of EGF and CCK. Similar MEK activation was observed except for a significantly weaker MEK1 response to EGF (139 ± 13% of control, complete data not shown).

Effect of EGF, CCK, and TPA on the activity of different forms of Raf in rat pancreatic acinar cells. Using immunoprecipitation and Western blotting, we identified the presence of three different forms of Raf in the rat pancreatic acinar cells (Fig. 4). In other cell types, Raf-A, Raf-B, and c-Raf-1 are known to exist, respectively, as 68-, 93- to 95-, and 72- to 76-kDa proteins. Therefore, the three different forms of Raf existing in pancreatic acinar cells have molecular masses similar to their counterparts from other cell types. The observation of different molecular masses also ensures that the antibodies are not cross-reacting with the other forms of Raf. Acini were then stimulated for 2.5 min with 100 nM EGF, 1 nM CCK, or 1 μM TPA, and the cell extracts were immunoprecipitated with αRaf antibodies followed by assay of Raf kinase. Total basal Raf kinase activity was accounted for as being 3.7% Raf-A, 89.0% Raf-B, and 7.3% c-Raf-1 (Fig. 5). All three forms of Raf were stimulated to a greater extent by CCK than by EGF, which was especially evident for Raf-A and c-Raf-1 (Fig. 5). The effect of CCK on Raf-A stimulation was mimicked by the phorbol ester TPA

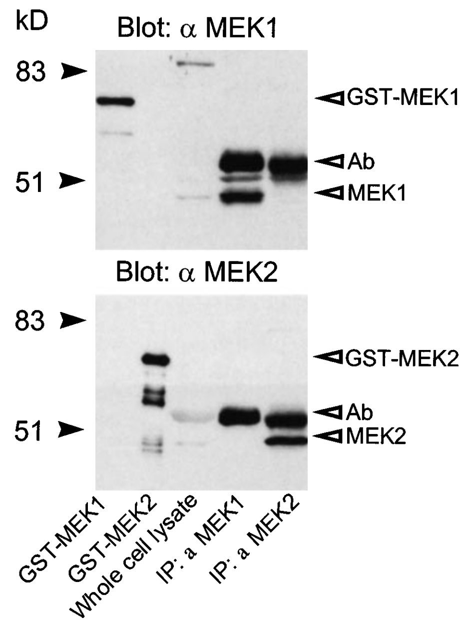

Fig. 3. Specific immunoprecipitation and Western blotting of recombinant MEK1 and MEK2 (100 ng of each), whole cell acinar lysates (20 μg), and immunoprecipitated MEK1 and MEK2 (from 1 mg acinar lysate). Samples were subjected to SDS polyacrylamide gel electrophoresis (PAGE), and then Western blot with αMEK1 or αMEK2 antibody was performed. Filled arrowheads on *left* indicate positions of prestained, low-range molecular markers. Open arrowheads on *right* indicate positions of glutathione-*S*-transferase (GST)-MAPK kinase (MEK), MEK1, MEK2, and the heavy chain of the antibody (Ab) used for immunoprecipitation.

Table 1. *Effect of EGF and CCK stimulation on kinase activity of MEK1 and MEK2 in pancreatic acinar cells*

	MEK1	MEK2
Basal kinase activity, cpm	82,574 ± 15,533	12,307 ± 1,995
EGF stimulation, % of control	217 ± 30	210 ± 17
CCK stimulation, % of control	587 ± 82	989 ± 361

Values are means ± SE of 3 independent experiments, each performed in duplicate. EGF, epidermal growth factor; CCK, cholecystokinin; MEK, mitogen-activated protein kinase kinase; cpm, counts/min.

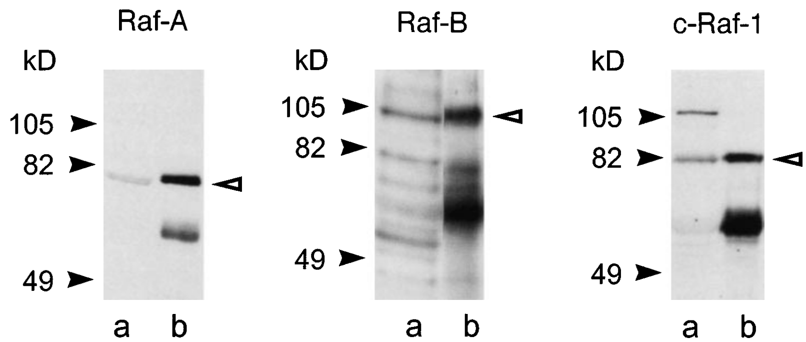

Fig. 4. Specific immunoprecipitation and Western blotting of 3 different forms of Raf from pancreatic acinar cells. Whole cell lysates (50 µg, *lane a*) and immunoprecipitates of Raf-α, Raf-B, and c-Raf-1 (from 1 mg protein lysate, *lane b*) were subjected to SDS-PAGE, and then Western blot was performed with the corresponding antibody. Filled arrowheads on *left* indicate positions of prestained, low-range molecular markers. Open arrowheads on *right* indicate the immunoidentified Raf.

and partly reproduced for Raf-B and c-Raf-1 stimulation.

Effect of EGF, CCK, and TPA on Ras activation in rat pancreatic acini. We previously reported that CCK and TPA increased the exchange rate of guanine nucleotides on Ras in rat pancreatic acini (14). To evaluate the steady-state activation of Ras, intact cells were incubated with ^{32}P to label cellular nucleotide pools and the relative amounts of GTP and GDP associated with Ras were determined. EGF significantly increased GTP-bound Ras by 183 and 164% at 2.5 and 10 min, respectively (Fig. 6). In contrast, CCK and TPA had no statistically significant effect at 2.5 or at 10 min on GTP binding. CCK and TPA also had no effect at 5 min, whereas EGF increased GTP-bound Ras to a range that was similar as that observed at 2.5 and 10 min (data not shown). Together, these data indicate that, in rat pancreatic acinar cells, EGF activates MAPK through a Ras-dependent mechanism, whereas CCK-induced activation of the MAPK cascade seems to be primarily Ras independent.

DISCUSSION

CCK is known to activate ERK1 and ERK2 ($p44^{MAPK}$ and $p42^{MAPK}$), as well as other upstream components of this MAPK signaling cascade, in isolated rat pancreatic acini (8, 13, 14). We have recently demonstrated in isolated rat pancreatic acini that CCK stimulates tyrosyl phosphorylation of Shc and the formation of a Shc-Grb2 complex through a PKC-dependent mechanism (9). Because Grb2 exists in a permanent complex with SOS, we concluded that formation of a Shc-Grb2-SOS complex via a PKC-dependent mechanism might provide the link between G_q protein-coupled CCK receptor stimulation and Ras activation in these cells (9). In the same study, we found that EGF was much more potent than CCK in inducing tyrosyl phosphorylation of Shc and induction of Shc-Grb2 complexes and that this action was PKC independent.

In the present work, we found that CCK was much more potent than EGF in activating ERKs. EGF-induced ERK activation was rapid and transient, with a peak at 2.5 min and a slightly elevated plateau from 10 to 60 min. On the contrary, after a rapid increase to a larger maximum at 2.5–5 min, CCK-induced ERK activity remained highly activated for up to 60 min. In different biological systems, MAPK activation is known to be correlated with more than one physiological response to a specific stimulus, and this raises the question of how the same MAPK cascade can affect different physiological responses (30, 34, 39). One of the best-studied differentiating systems is PC-12 cells in which both EGF and nerve growth factor (NGF) activate the MAPK cascade; however, EGF treatment induces proliferation, whereas NGF treatment induces differentiation (39). It has been hypothesized that the difference between the EGF and NGF responses may be caused by differences in the duration of the increased ERK activity (42). Similar to pancreatic acini, EGF-induced ERK activity in PC-12 cells is transient, whereas NGF-induced ERK activity is more sustained. A sustained pattern of MAPK activation, similar to that of CCK-induced MAPK in pancreatic acini, was also recently reported in NIH/3T3 mouse fibroblasts stimulated with serum (36). Interestingly, after stimulation with serum, as much as one-half of all detectable MAPK activity was associated with microtubules. Because MAPK has targets in different parts of the cell, it

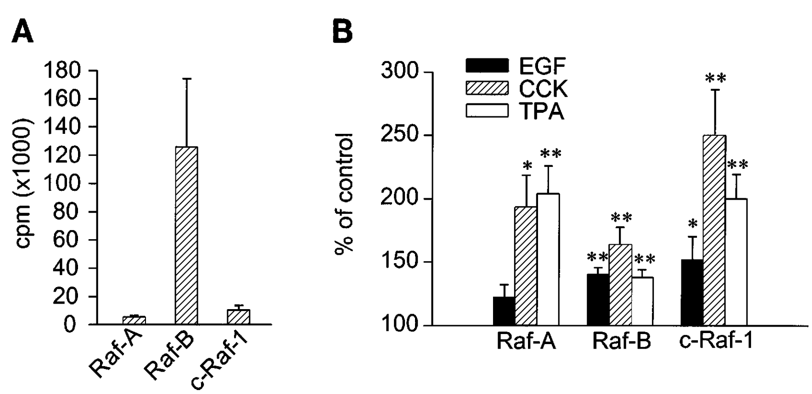

Fig. 5. Effect of EGF, CCK, and TPA on the kinase activity of 3 different forms of Raf in pancreatic acini. Immunoprecipitated Raf was subjected to kinase assay as described in EXPERIMENTAL PROCEDURES. *A:* basal Raf kinase activity. *B:* effect of 2.5-min stimulation with 100 nM EGF, 1 nM CCK-8, or 1 µM TPA on the kinase activity of 3 different forms of Raf, shown as percentage of control activity. Data represent means ± SE of 3 or 4 independent experiments, each performed in duplicate. * $P < 0.05$ vs. control; ** $P < 0.01$ vs. control.

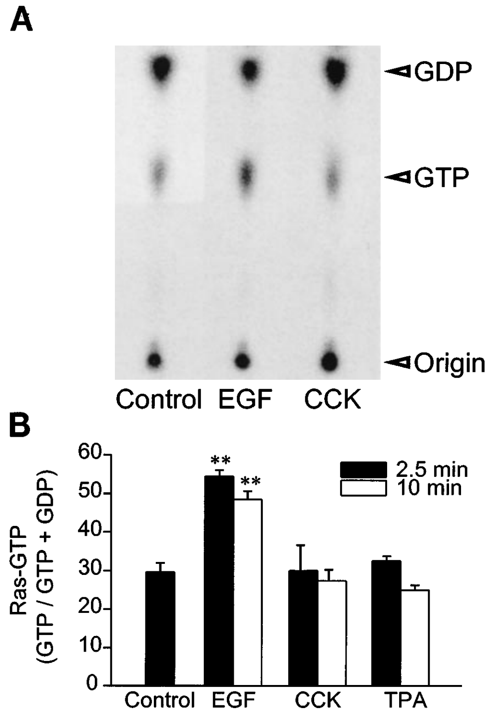

Fig. 6. Effect of EGF, CCK, and TPA on Ras activation in rat pancreatic acini as indicated by guanine nucleotide binding. Pancreatic acini labeled with ^{32}P were stimulated for indicated times with 100 nM EGF, 1 nM CCK-8, or 1 μM TPA. Cell lysates were then immunoprecipitated with αRas antibody, and guanine nucleotides were eluted. *A:* representative fragment of thin-layer chromatography separation of Ras-bound GTP and GDP. Origin, spot where sample was applied. *B:* quantitated data. Each bar represents mean ± SE of 3–5 independent experiments, each performed in duplicate. ** $P < 0.01$ vs. control.

is possible that EGF and CCK may activate distinct pools of MAPK in pancreatic acinar cells. Compartmentalization of the MAPK cascade is also suggested in a recent report (47) in which insulin and EGF regulate distinct pools of Grb2-SOS in the control of Ras activation.

The role of PKC in mediating the action of CCK to activate the acinar cell MAPK cascade is based on the mimicking effect of TPA and the blocking effect of PKC inhibitors. CCK, as well as carbachol and bombesin, is known to increase diacylglycerol and activate PKC in acinar cells (49). TPA, which also activates PKC in acini, has been shown to activate ERKs (13), MEK (14), Rafs (present study), and $p90^{RSK}$ (5). The activation of MAPK was previously shown to be blocked by staurosporin (13) and GF-109203X (5). In the present study, GF-109203X was shown to block MAPK activation by CCK but not EGF. Very recently, GF-109203X was shown to also inhibit $p90^{RSK}$ and $p70^{S6K}$ (2). These actions are unlikely to explain the inhibition of MAPK shown here, since $p90^{RSK2}$ is downstream of MAPK and rapamycin, a specific inhibitor of $p70^{S6K}$, had no effect of MAPK activation (5). Thus, although there are concerns about the specificity of the PKC antagonists, the bulk of the evidence is consistent with a role for PKC in activating the pancreatic MAPK cascade.

MEK1 and MEK2 are the only two identified ERK activators (51). In a previous study, we reported that CCK rapidly activated total MEK activity and that this effect was mimicked by TPA as well as by carbachol and bombesin (14); the latter two agents act on receptors that, similar to CCK, activate phospholipase C (49). MEK activity, immunoprecipitated with a monoclonal antibody raised against MEK1, was also increased by CCK and TPA, but the specificity of the antibody for MEK1 vs. MEK2 was not established (14). In the present study, we found that EGF and CCK were both capable of activating the two forms of MEK. However, as was observed at the MAPK level, CCK had a much larger effect than EGF in activating both forms of the enzyme.

Identification of c-Raf-1 as a MEK activator provided an essential link between the growth factor receptor tyrosine kinase and the MAPK cascade (11, 18, 26). Raf proteins are a family of protein kinases presently consisting of Raf-A, Raf-B, and c-Raf-1, with c-Raf-1 being the best characterized. Whereas c-Raf-1 is expressed in a wide range of tissues (3, 32, 37), both Raf-A and Raf-B expression are restricted to certain tissues. In contrast to c-Raf-1, the roles of Raf-A and Raf-B in the MAPK cascade remain unclear (3, 28, 32, 37). c-Raf-1 physically interacts with the activated Ras, which recruits the kinase to the cytoplasmic membrane (6, 44, 46, 52). At the membrane, c-Raf-1 becomes activated by a poorly understood mechanism reportedly involving phosphorylation at both tyrosine and serine/threonine residues (11, 22). All three forms of Raf are able to activate MEK1 (22, 35, 37, 51). Whereas c-Raf-1 can activate both MEK1 and MEK2, Raf-A has been reported to activate MEK1 but not MEK2 (51). We identified all three forms of Raf in pancreatic acinar cells. Interestingly, Raf-B was found to account for the

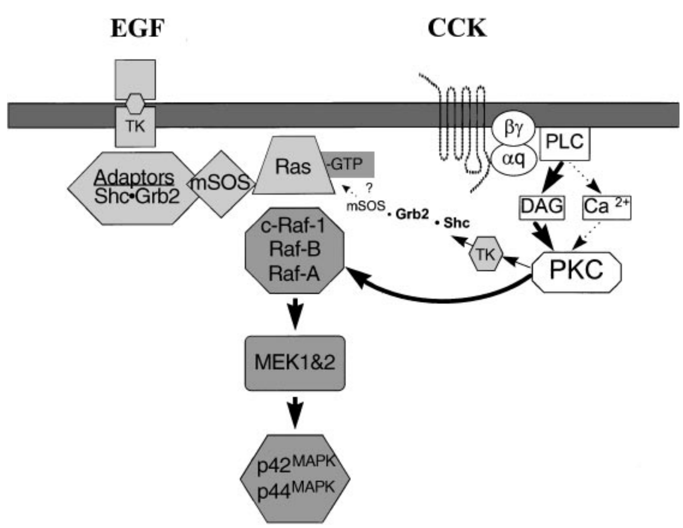

Fig. 7. Schematic diagram presenting different pathways of MAPK cascade stimulated by CCK and EGF in pancreatic acinar cells. EGF receptor is shown signaling through its tyrosine kinase (TK) activity to Ras, whereas CCK is shown with a minor potential pathway through Ras and a major pathway involving PKC acting through Raf bypassing Ras. DAG, diacylglycerol; PLC, phospholipase C; Shc, *src* homology/collagen; Grb2, growth factor receptor bound 2; SOS, son of sevenless; mSOS, mammalian SOS.

largest portion of total Raf kinase basal activity, with Raf-A and c-Raf-1 representing only 3.7% and 7.3% of total activity, respectively. A similar ratio of unstimulated c-Raf-1 to Raf-B kinase activity was recently reported in NIH/3T3 fibroblasts (37). It remains to be determined what the functional significance is for such a predominance of Raf-B in the cells. EGF and CCK activated all three forms of Raf in pancreatic acinar cells. However, CCK was more potent in activating each form of Raf, and its effect was largely reproducible by TPA. These results raise the possibility that in pancreatic acinar cells, PKC may directly activate Raf. It is already known that PKC may activate c-Raf-1 and Raf-A in some cell types (4, 25, 31). Our results also suggest that Raf-A and Raf-B in addition to c-Raf-1 may be activated by PKC.

The signaling pathways coupling G_q-linked receptors to MAPK activation are unclear (34). It was recently reported that the heterotrimeric G_q protein-coupled angiotensin II receptor has the ability to activate the Shc-Grb2-SOS pathway leading to Ras activation in cardiac myocytes (38). These authors (38) suggested that the Src family of tyrosine kinases but not PKC plays an essential role in angiotensin II-induced activation of Ras. Receptors other than CCK that couple to the heterotrimeric G_q and G_i proteins have also been shown to stimulate MAPK (1, 19, 50). However, it was suggested that G_q- and G_i-coupled receptors stimulate MAPK activation via distinct signaling pathways. In COS-7 or Chinese hamster ovary cells, $G\beta\gamma$ was reported to be responsible for mediating G_i-coupled receptor-stimulated ERK activation through a mechanism utilizing Ras and c-Raf-1, independent of PKC. In contrast, $G\alpha$ was reported to mediate G_q- and G_o-coupled receptor-stimulated MAPK activation using a Ras-independent mechanism that employed PKC and c-Raf-1 (17). Interestingly, in our study, EGF apparently activated Ras in pancreatic acinar cells, as assessed by an increase in the amount of Ras-bound GTP, whereas CCK and TPA had no effect. This is in contrast to our previous results in which both CCK and TPA increased the binding of GTP to Ras (14). CCK possibly increases Ras GAP activity as well as GTP binding to Ras. This would result in an increased turnover of Ras with no change in the steady-state level of GTP Ras. Because Raf activation is believed to be dependent on increased levels of GTP Ras, the present data suggest that the major component of CCK-induced activation of MAPK is Ras independent and that CCK-activated PKC may directly activate members of the Raf kinase family in pancreatic acinar cells (Fig. 7). Therefore, further work is necessary to evaluate the role of the CCK-induced and PKC-dependent activation of the Shc-Grb2 complex in pancreatic acinar cells. It is possible that this pathway is associated with other as yet unidentified effector proteins besides SOS in acini. On the basis of experiments investigating the activation of the Shc-Grb2-SOS complex by insulin and/or EGF (47), the existence of different Grb2 pools has been hypothesized. One smaller pool associates with Shc in a Grb2-SOS complex, and a second larger pool binds tyrosine-phosphorylated Shc independent of SOS. This observation is consistent with a recent study indicating the presence of distinct subcellular compartmentalized pools of Shc (40).

Together, our study suggests that the MAPK cascade leading to ERK can be activated by multiple mechanisms in pancreatic acinar cells. Whereas EGF appears to act in a classical Ras-dependent manner, the major component of CCK-induced activation of the MAPK cascade is activated at the level of Raf. Furthermore, stimulation of Raf by CCK appears mediated by PKC.

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