Cholecystokinin Stimulates Formation of Shc-Grb2 Complex in Rat Pancreatic Acinar Cells through a Protein Kinase C-dependent Mechanism

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Cholecystokinin (CCK) has recently been shown to activate the mitogen-activated protein kinase (MAPK) cascade (Ras-Raf-MAPK kinase-MAPK) in pancreatic acini. The mechanism by which the G<sub>G</sub> protein-coupled CCK receptor activates Ras, however, is currently unknown. Growth factor receptors are known to activate Ras by means of adaptor proteins that bind to phosphotyrosine domains. We therefore compared the effects of CCK and epidermal growth factor (EGF) on Tyr phosphorylation of Shc and its association with Grb2 and the guanine nucleotide exchange factor SOS. Three major isoforms of Shc (p46, p52, p66) were detected in isolated rat pancreatic acini with p52 being the predominant form. CCK and EGF increased tyrosyl phosphorylation of Shc (251 and 337% of control, respectively). CCK-stimulated tyrosyl phosphorylation of Shc as well as Shc-Grb2 complex formation was significant at 2.5 min, maximal at 5 min, and persisted for at least 30 min. Finally, SOS was found to be associated with Grb2 as assessed by probing of anti-Grb2 immunoprecipitates with anti-SOS. Since MAPK in pancreatic acini is activated via protein kinase C (PKC), we studied the effect of phorbol esters on Shc phosphorylation and found 12-O-tetradecanoylphorbol-13-acetate to be as potent as CCK. Furthermore, GF-109203X, a PKC inhibitor, abolished the effect of 12-O-tetradecanoylphorbol-13-acetate and also the effect of CCK but not the effect of EGF on Shc tyrosyl phosphorylation. CCK-induced tyrosyl phosphorylation of Shc was found to be phosphatidylinositol 3-kinase-independent, and CCK but not PKC inhibitor, abolished the effect of 12-O-tetradecanoylphorbol-13-acetate and also the effect of CCK but not the effect of EGF on Shc tyrosyl phosphorylation. CCK-induced tyrosyl phosphorylation of Shc was found to be phosphatidylinositol 3-kinase-independent, and CCK did not cause EGF receptor activation. These results suggest that formation of an Shc-Grb2-SOS complex via a PKC-dependent mechanism may provide the link between G<sub>G</sub> protein-coupled CCK receptor stimulation and Ras activation in these cells.

CCK<sup>¶</sup> regulates a variety of pancreatic functions, including secretion of pancreatic enzymes (1), stimulation of pancreatic growth (2, 3), and digestive enzyme synthesis (4). It is thought that some of these nonsecretory effects are a result of the ability of CCK to regulate expression of transcriptional factors, such as c-myc, c-jun, and c-fos (5). The CCK<sub>G</sub> receptor on rat pancreatic acinar cells is a member of the seven-transmembrane domain superfamily of receptors (6). Its actions on digestive enzyme secretion are mediated by heterotrimeric G proteins of the G<sub>i/G</sub> class which couple to phospholipase C and thereby lead to an increase in intracellular Ca<sup>2+</sup> concentration and activation of PKC (7).

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 (8, 9). The mechanism of tyrosine kinase receptor-stimulated mitogenic signaling involves formation of complexes of the guanine nucleotide exchange factor SOS, and the SH2 and SH3 domain-containing adaptor protein Grb2 with either autophosphorylated growth factor receptors or another Tyr-phosphorylated adaptor protein Shc (10–14). These protein-protein interactions result in the translocation of SOS from the cytosol to the plasma membrane, where its substrate Ras is localized (15, 16). Recent studies have shown that some G protein-coupled receptors utilize the same effectors as the tyrosine kinase receptor pathway (e.g. Shc-Grb2-SOS), resulting in Ras and MAPK activation (17–20). However, it was suggested that G<sub>i</sub>-coupled receptors generally initiate a Ras-independent pathway involving PKC, whereas the pertussis toxin-sensitive G<sub>i</sub>-coupled receptors utilize a pathway that induces Ras activation in a PKC-independent manner (21, 22).

In previous studies, we have found that CCK activates p42<sup>mapk</sup> and p44<sup>mapk</sup>, as well as other upstream components of the MAPK signaling cascade, including MEK and Ras, in isolated rat pancreatic acini (23–25). The aim of our present study was to evaluate the mechanism by which the G<sub>G</sub> protein-coupled CCK receptor activates Ras.

**EXPERIMENTAL PROCEDURES**

**Materials—**CCK octapeptide (CCK-8) was from Squibb Research Institute (Princeton, NJ). Epidermal growth factor (EGF) was from Collaborative Biomedical Products (Bedford, MA). 12-O-Tetradecanoylphorbol-13-acetate (TPA), 4α-12-O-tetradecanoylphorbol-13-acetate (4α-TPA), and GF-109203X were from LC Laboratories (Woburn, MA). Bombesin was from Bachem (Torrance, CA), and chromatographically purified collagenase was from Worthington Biochemicals. Recombinant DNA-derived 22-kD rGH was a gift of Eli Lilly Co., and recombinant protein A-agarose was from Repligen (Cambridge, MA). Triton X-100, aprotinin, and leupeptin were purchased from Boehringer Mannheim, and PAGE, polyacrylamide gel electrophoresis.
CCK and EGF Induce Tyrosyl Phosphorylation of Shc and Its Association with Grb2 in Rat Pancreatic Acinar Cells

Shc proteins in pancreatic acinar cells. Acini were stimulated for different time periods with 1 nM CCK-8, and the cell extracts were then immunoprecipitated with α-Shc along with cell extracts of 3T3-F442A fibroblasts stimulated with GH, which were used as a positive control. As assessed by Western blotting with α-PY, stimulation with CCK induced rapid tyrosyl phosphorylation of a protein corresponding to p52 Shc (Fig. 1, upper panel). Phosphorylation reached a maximum (251 ± 21% of the control, n = 4) within 5 min and remained elevated at 15 and 30 min of CCK stimulation. Two other Shc proteins (p46 and p66) are tyrosyl phosphorylated upon GH stimulation in 3T3-F442A fibroblasts. In acinar cells, p46 Shc tyrosyl phosphorylation was increased slightly (137 ± 21% of control, n = 3) after stimulation with CCK, whereas no tyrosyl phosphorylation of p66 Shc was detected. Since in many different cell types EGF is a strong activator of Shc tyrosyl phosphorylation, through the EGF-R tyrosine kinase, we examined its effect in pancreatic acinar cells. Stimulation of the acini with 10 nM EGF for 5 min resulted in tyrosyl phosphorylation of p52 Shc which appeared to be significantly stronger (337 ± 21% of the control, n = 4) than that resulting from CCK stimulation. As with CCK, a slight increase in tyrosyl phosphorylation of p46 Shc was also detected. Reprobing of the blot with α-Shc revealed the presence of three bands in pancreatic acinar cells corresponding to p46, p52, and p66 Shc with the p52 being the predominant and the p66 barely expressed. Compared with pancreatic acini, 3T3-F442A fibroblasts express significant amounts of all three forms of Shc (Fig. 1, middle panel). The low level of p66 and p46 Shc in pancreatic acini may explain why we are not able to detect any Tyr phosphorylation of p66 Shc in these cells. Association of tyrosyl-phosphorylated Shc with Grb2 was subsequently examined by probing of α-Shc immunoprecipitates with α-Grb2 (Fig. 1, bottom panel). Stimulation with both CCK and EGF resulted in increased, by 2- and 3.5-fold, respectively, association of Shc and Grb2 with the time course of association similar to that of tyrosyl phosphorylation of Shc.

Effect of CCK on the Amount of mSOS-1 and Shc Associated with Grb2 in Rat Pancreatic Acinar Cells—Upon activation of tyrosine kinase receptors such as the EGF-R or the insulin receptor, tyrosine-phosphorylated Shc associates with Grb2...
and the guanine nucleotide exchange factor SOS thereby leading to Ras activation (10–14). Therefore, we next examined whether CCK receptor activation promotes Grb2 forming a complex with SOS in pancreatic acini. Grb2 immunoprecipitates were subjected to Western blotting with α-SOS. A band corresponding to SOS was observed in immunoprecipitates from control (not stimulated) acinar cells, suggesting constitutive association of Grb2 with SOS, as reported in other cell types (Fig. 2, upper panel). CCK did not increase the amount of SOS associated with Grb2. The same blot was then reprobed twice with α-Shc and α-PY, respectively, showing that upon CCK stimulation Grb2 associates in a complex with increased amount of tyrosyl-phosphorylated Shc (Fig. 2, middle and bottom panels). These data confirm the results shown in Fig. 1, indicating the ability of CCK to induce Shc-Grb2 complex formation.

Effect of Different Stimuli on Tyrosyl Phosphorylation of Shc in Rat Pancreatic Acinar Cells—It is known that CCK, after binding its receptor, triggers hydrolysis of polyphosphoinositide by activation of a phospholipase C, thereby generating inositol 1,4,5-trisphosphate and diacylglycerol, which mobilize intracellular Ca²⁺ and activate PKC, respectively (7). We investigated whether some of these signal transduction pathways was responsible for activation of adaptor protein Shc-Grb2 complexes, by determining the effects of various agonists on tyrosyl phosphorylation of Shc. CCK, bombesin, and carbachol, all of which activate phospholipase C in acini, as well as the potent stimulator of PKC, TPA, stimulated tyrosyl phosphorylation of p52 Shc to a similar extent, whereas the biologically inactive analog of TPA, 4α-TPA, had no effect (Fig. 3). The Ca²⁺-ATPase inhibitor cyclopiazonic acid, which increases intracellular Ca²⁺, had little or no effect on tyrosyl phosphorylation of Shc. Ionomycin, which is another intracellular Ca²⁺-increasing agent, also had no effect on Shc-Grb2 complex formation in rat pancreatic acini (data not shown). These observations strongly indicate that CCK-induced activation of Shc-Grb2 complex may be PKC-dependent. These results are consistent with previous data reporting that CCK, bombesin, carbachol, and TPA significantly stimulated MAPK activity in pancreatic acini by a PKC-dependent mechanism, but increasing intracellular Ca²⁺ had a little or no effect (23, 25).

CCK Does Not Cause EGF-R Transactivation in Rat Pancreatic Acinar Cells—It was reported recently that the EGF-R is tyrosine phosphorylated rapidly upon stimulation of Rat-1 cells with the G protein-coupled receptor agonists endothelin-1, lysophosphatic acid, and thrombin, suggesting that there is an intracellular mechanism for transactivation (50). Therefore, we examined whether such a transactivation could account for the tyrosyl phosphorylation of Shc and Shc-Grb2 complex formation in pancreatic acinar cells stimulated with CCK. Cell extracts of acini stimulated with EGF or CCK were immunoprecipitated with α-EGF-R and probed, by Western blotting, with α-PY (Fig. 4). Although stimulation with EGF induced strong tyrosyl phosphorylation of the EGF-R, CCK had little or no effect.

CCK-induced Tyrosyl Phosphorylation of Shc Is Largely PKC-dependent in Rat Pancreatic Acinar Cells—To evaluate the role of PKC in tyrosyl phosphorylation of Shc, we determined the effect of a potent inhibitor of PKC, GF-109203X (31, 32), on CCK- or EGF-induced activation of the complex. This inhibition is specific for PKC at concentrations of 1–2 μM in cultured cells. Since freshly prepared pancreatic acini usually require much higher concentrations of different enzyme inhibitors than required for cultured cells, we first determined the concentration at which this inhibitor would block TPA-stimulated MAPK activity in isolated pancreatic acini. 20 μM GF-109203X was required to block totally the TPA-stimulated MAPK activity in pancreatic acini (data not shown). Since it is known that at concentrations higher than 2 μM this compound may inhibit other protein kinases, including EGF-R tyrosine kinase (31), we also evaluated the effect of different concentrations of GF-109203X on EGF-R tyrosine phosphorylation in pancreatic acini. As shown in Fig. 5A, the concentration of inhibitor found to block TPA-stimulated MAPK activity (20 μM) had no effect on EGF-induced receptor tyrosyl phosphorylation, suggesting specificity for PKC at this concentration.

For further assessment of the role of PKC in tyrosyl phosphorylation of Shc, pancreatic acini were pretreated or not with 20 μM GF-109203X and then stimulated for 15 min with CCK, TPA, or EGF. Both CCK and TPA induced similar and significant (2.5-fold) increases in tyrosyl phosphorylation of Shc, whereas the effect of EGF was notably stronger (Fig. 5B). Pretreatment with PKC inhibitor GF-109203X inhibited the CCK- and TPA-induced effect extensively, but it did not influence EGF-induced increase in tyrosyl phosphorylation of Shc. These data strongly suggest that CCK-induced tyrosyl phosphorylation of Shc but not the effect of EGF in rat pancreatic acinar cells is PKC-dependent.

It was suggested recently that Gβγ-mediated tyrosyl phosphorylation of Shc is inhibited by wortmannin, implying involvement of phosphatidylinositol 3-kinase (PI3K) (20). However, pretreatment of pancreatic acini with 1 μM wortmannin...
had no effect on CCK-induced tyrosyl phosphorylation of Shc (Fig. 6). At the same conditions, wortmannin totally inhibited PI3K-dependent activation of p70S6K in pancreatic acini.²

**DISCUSSION**

We recently reported that CCK activated p42/44 MAPK and p44/42 MAPK, as well as other upstream components of the MAPK signaling cascade, including Ras and MEK, in isolated rat pancreatic acini (23–25). In the present study we have demonstrated for the first time that CCK stimulates tyrosyl phosphorylation of Shc and formation of Shc-Grb2 complex in isolated rat pancreatic acini. We have also found Grb2 existing in a permanent complex with SOS, which may, therefore, provide a link between Gq protein-coupled CCK receptor stimulation and PI3K-dependent activation of p70S6K. In these experiments, each performed in duplicate, for EGF-R transactivation.

It is known that CCK, after binding to its receptor, triggers hydrolysis of polyphosphoinositide, generating inositol 1,4,5-trisphosphate and diacylglycerol, which mobilize intracellular Ca²⁺ and activate PKC, respectively (7). Although TPA was as potent as CCK in promoting tyrosyl phosphorylation of Shc in pancreatic acini, the Ca²⁺-ATPase inhibitor cyclopiazonic acid was much less effective. This suggests that formation of diacylglycerol and subsequent PKC activation may be the primary mechanism mediating phosphorylation of Shc and activation of MAPK pathway (23–25) in rat pancreatic acini. The formation of inositol 1,4,5-trisphosphate and subsequent increase in intracellular Ca²⁺ appears to have a minor or no role in Shc-Grb2 complex formation (this paper) and MAPK activation (23) in these cells. A role for PKC in Ras activation is also supported by the observation that TPA increased the rate of binding of GTP to Ras almost as effectively as CCK (24).

It was reported recently that the heterotrimeric Gq proteins have been shown to stimulate MAPK (34–37). However, it was suggested that Gq and Gq coupled receptors stimulate MAPK activation via distinct signaling pathways. In COS-7 or Chinese hamster ovary cells, Gβγ was reported to be responsible for mediating Gq-coupled receptor-stimulated MAPK activation through a mechanism utilizing Ras and p74MAPK independent of PKC. In contrast, Gα was reported to mediate Gq and Gq-coupled receptor-stimulated MAPK activation using a Ras-independent mechanism employing PKC and p74MAPK (21, 22). Additionally, in COS-7 cells Gβγ-induced phosphorylation of p52 Shc appears to be PI3K-dependent (20). Interestingly, in isolated rat pancreatic acini, inhibition of PI3K with wortmannin had no effect on CCK-induced tyrosyl phosphorylation of Shc, whereas a phorbol ester, TPA, was as potent as CCK in tyrosyl phosphorylation of this adaptor protein. Moreover, preincubation of acini with GF-109203X, a potent PKC inhibitor, almost totally prevented

² J. Bragado and J. A. Williams, unpublished data.
tyrosyl phosphorylation of Shc suggesting additionally that formation of the Shc-Grb2-SOS complex in isolated rat pancreatic acini is PKC-dependent. In addition, MAPK activation in pancreatic acini was also inhibited by the PKC inhibitor GF-109203X (38).

It is not known how CCK-induced activation of PKC may lead to tyrosyl phosphorylation of Shc in pancreatic acini. Recent reports suggest that tyrosine kinases of the Src family are responsible in many different cell types for activation of the Shc-Grb2-SOS pathway and/or MAPK cascade by G protein-coupled receptors (33, 39, 40). However, at least in certain cell types, PKC seems to have a negative regulatory effect on Src coupled receptors (33, 39, 40). It was reported recently the EGF-R becomes rapidly tyrosyl-phosphorylated upon stimulation of Rat-1 cells with the phosphatase inhibitor wortmannin for 40 min and stimulated with 1 nM CCK-8 for 15 min. The lower panel shows a representative immunoblot. The upper panel presents the quantitative data as the mean of two different experiments, each performed in duplicate, with the range of difference between the experiments (black dots).

FIG. 6. Tyrosyl phosphorylation of Shc is not PI3K-dependent in rat pancreatic acinar cells. Acinar cells were preincubated with 1 μM wortmannin for 40 min and stimulated with 1 nM CCK-8 for 15 min. The cell lysates were then immunoprecipitated with an α-Shc antibody and analyzed by Western blotting with a α-PY antibody. The upper panel shows a representative immunoblot. The lower panel presents the quantitative data as the mean of two different experiments, each performed in duplicate, with the range of difference between the experiments (black dots).
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