Characterization of the antiproliferative signal mediated by the somatostatin receptor subtype sst5

cyclic GMP/cholecystokinin receptor/mitogen-activated protein kinase/somatostatin analogue RC-160/decreased cell growth

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ABSTRACT We investigated cell proliferation modulated by cholecystokinin (CCK) and somatostatin analogue RC-160 in CHO cells bearing endogenous CCK receptors and stably transfected by human subtype sst5 somatostatin receptor. CCK stimulated cell proliferation of CHO cells. This effect was suppressed by inhibitor of the soluble guanylate cyclase, LY 83583, the inhibitor of the cGMP dependent kinases, KT 5823, and the inhibitor of mitogen-activated protein (MAP) kinase kinase, PD 98059. CCK treatment induced an increase of intracellular cGMP concentrations, but concomitant addition of LY 83583 virtually suppressed this increase. CCK also activated both phosphorylation and activity of p42-MAP kinase; these effects were inhibited by KT 5823. All the effects of CCK depended on a pertussis toxin-dependent G protein. Somatostatin analogue RC-160 inhibited CCK-induced stimulation of cell proliferation but it did not potentiate the suppressive effect of the inhibitors LY 83583 and KT 5823. RC-160 inhibited both CCK-induced intracellular cGMP formation as well as activation of p42-MAP kinase phosphorylation and activity. This inhibitory effect was observed at doses of RC-160 similar to those necessary to occupy the sst5 recombinant receptor and to inhibit CCK-induced cell proliferation. We conclude that, in CHO cells, the proliferation and the MAP kinase signaling cascade depend on a cGMP-dependent pathway. These effects are positively regulated by CCK and negatively influenced by RC-160, interacting through CCKA and sst5 receptors, respectively. These studies provide a characterization of the antiproliferative signal mediated by sst5 receptor.

Somatostatin and cholecystokinin are both regulatory hormones widely distributed in the body. Both these hormones participate in a variety of biological processes including neurotransmission and control of pancreatic secretion. They induce their biological effects by interacting with specific G protein-linked receptors (1, 2). The role of the two peptides in the regulation of cell growth also has been demonstrated in studies carried out both in vitro and in vivo (2–6). The effects of CCK and its related peptide, gastrin, are transduced through at least two distinct subtypes of specific cell-surface receptors, CCKA (alimentary) and CCKB (brain, also called CCK8/gastrin receptors), that have been cloned in human and rodents (1). CCKA/gastrin receptors bind CCK and gastrin with the same high affinity. In contrast, the CCKB receptors exhibit a 100- to 500-fold higher affinity for CCK than gastrin. Gastrin has trophic effects on normal digestive mucosa and stimulates the growth of colon, gastric, and pancreatic cancer cell lines in culture (7, 8). This proliferative effect has been shown to be mediated by the gastrin/CCKB receptors in different cell models (8, 9).

In the rat exocrine pancreas that expresses CCKA type receptor, CCK is known to stimulate pancreatic growth both in vitro and in vivo (3–5). The CCKA receptor interacts with G proteins and thereby activates phospholipase C that hydrolyses phosphatidylinositol biphosphate, generating inositol 1,4,5-triphosphate and diacylglycerol, which mobilize intracellular calcium and activate protein kinase C, respectively (1). These two pathways act through the pertussis-insensitive G protein Gq and are involved in CCK-stimulated pancreatic enzyme secretion. Studies performed on pancreatic acini from rodents demonstrated that CCK can activate different intracellular pathways, including stimulation of intracellular calcium mobilization, and increase in cAMP, cGMP, arachidonic acid, and phosphatidic acid levels (1, 10). However the mechanism involved in the proliferative effect induced by CCK through CCKA receptor is not completely elucidated.

Somatostatin exerts an antiproliferative effect both in vitro and in vivo in normal as well as in tumoral cells. Somatostatin and its stable analogues inhibit tumor growth in vivo by indirectly inhibiting the action of hormones and growth factors (2, 11). Much evidence exists for directly mediated responses, as we and others have shown in pancreatic cancer cells of human or rat origin (2, 6, 11, 12).

Five somatostatin (sst1–sst5) receptor subtypes have been cloned from human (h), mouse, and rat (13, 14), but the biological functions mediated by these five sst(s) subtypes have not yet been completely established. Recently, after stably expressing sst(s) in NIH 3T3 and CHO cells, we demonstrated that only sst1, sst2, and sst5 mediated the antiproliferative effect of the somatostatin analogue RC-160 (15, 16). However, these receptor subtypes are coupled to distinct signal transduction pathways. Sst1 and sst2 mediated the stimulation of a tyrosine phosphatase activity, which is involved in the antiproliferative effect of the analogues (15, 17).

In CHO cells expressing sst5, RC-160 inhibited the proliferation induced by serum or by CCK-8 (16). The inhibitory effect of RC-160 was not abolished by specific inhibitors of tyrosine and serine/threonine phosphatases, such as orthovanadate or okadaic acid, respectively, indicating that a protein phosphatase was not involved in the negative growth signal coupled to sst5. We also observed that in sst5-expressing CHO cells, RC-160 inhibited cyclic AMP production and CCK-induced intracellular calcium mobilization (16). Production of cyclic AMP is not involved in CHO cell proliferation.

Abbreviations: CCK, cholecystokinin; cGMP, cyclic GMP; MAP, mitogen-activated protein; FCS, fetal calf serum.

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and the role of calcium mobilization in the growth regulation of CHO cells evoked by CCK or RC-160 has not been demonstrated. The present work was thus undertaken to elucidate the mechanisms by which CCK and the somatostatin analogue RC-160 regulate cell proliferation in CHO cells stably expressing sst5 receptor.

MATERIALS AND METHODS

Reagents. Sulfated cholecystokinin-8 (CCK-8), KT 5823, 6-anilino-5,8 quinolonequinone (LY 83583), and PD 98059 were purchased from Bachem (Voisin le Bretonneux, France). α-Minimal essential medium (αMEM), fungizone, streptomycin, penicillin, trypsin, and fetal calf serum (FCS) were purchased from Life Technologies and Gibco/BRL (Eragny, France). Pertussis toxin, dibutyryl cGMP, soybean trypsin inhibitor, and genetin (G418) were from Sigma. RC-160 was synthesized and purified as described (18). The CCKA receptor antagonist SR 27897 was kindly provided by Sanofi (Paris). Pertussis toxin, dibutyryl cGMP, soybean trypsin inhibitor, and genetin (G418) were from Sigma. RC-160 was synthesized and purified as described (18). The CCKA receptor antagonist SR 27897 was kindly provided by Sanofi (Paris). Pertussis toxin, dibutyryl cGMP, soybean trypsin inhibitor, and genetin (G418) were from Sigma. RC-160 was synthesized and purified as described (18).

CHO-K1 cells. Binding studies. To determine the number of CCK receptors in CHO-K1 cells, CHO cells were plated in 100-mm-diameter dishes until subconfluence and, after a 18 h period of serum deprivation, were incubated with CCK-8 and with or without RC-160 or other agents tested. The cells were then washed once with phosphate buffer saline (pH 7.0) and once with lysis buffer (50 mM Hepes, 150 mM NaCl/10 mM EDTA/10 mM NaF/F2/2 mM sodium orthovanadate, pH 7.4), and lysed in 500 µl of lysis buffer containing 1% Triton X-100 and protease inhibitors (1 mM phenylmethylsulfonyl fluoride/2 mg/ml aprotonin/20 mM leupeptin). After a 15-min incubation at 4°C, the lysate was collected and centrifuged at 13,000 × g for 10 min at 4°C to remove insoluble material. Phosphorylated proteins were immunoprecipitated by incubating the solubilized cells for 2 h at 4°C with a monoclonal antiphosphotyrosine antibody (Santa Cruz Biotechnology) prebound to Sepharose-protein G beads (Sigma) (dilution 1:200). The immune complexes were collected by centrifugation, washed twice with a buffer containing 30 mM Hepes, 30 mM NaCl, and 0.1% Triton X-100, pH 7.4, and boiled for 5 min in 50 µl of a SDS/sample buffer. The samples were separated on 10% SDS/polyacrylamide gels and electroblotted onto nitrocellulose membranes. The membrane was incubated for 45 min at 37°C in 10 mM Tris, 140 mM NaCl, pH 7.4 containing 3% BSA and 1% gelatin and then blotted overnight at 4°C with anti-p42-MAP kinase (ERK-2) polyclonal antibodies (Santa Cruz Biotechnology) (dilution 1:200). Bound antibodies were detected with horseradish peroxidase-conjugated anti-mouse IgG followed by ECL detection (Amersham).

RESULTS

Expression of CCKA Receptor by CHO-K1 Cells. We first characterized the CCK receptors endogenously expressed by CHO-K1 cells. Binding experiments showed that [125I]-labeled (Thr-Nle)-Tyr-CCK-9 bound specifically to both CHO and sst5 cells with pSV2neo plasmid (CLONTECH) using lipofectin reagent (Life Technologies/Gibco). Stable transfectants were selected in αMEM containing genetin at 600 ng/ml. Genetin-resistant clones expressing hss5 somatostatin receptor (sst5/CHO) were selected by somatostatin binding as described in ref. 15 using [125I]-Thr-Nle somatostatin-14 as tracer (16). Two clones were selected for subsequent experiments. CHO-K1 cells were concomitantly stably transfected with pSV2neo vector alone and were used as control clones (neo/CHO). After selection, cells were cultured in αMEM containing 10% FCS and genetin (200 µg/ml).

Cell Transfections. CHO cells (CHO K1 strain) were cultured in αMEM containing 10% FCS. The human sst5 cDNA, subcloned into the pCDNA1/Amp vector (Invitrogen) (19), was stably cotransfected in CHO cells with pSV2neo plasmid (CLONTECH) using lipofectin reagent (Life Technologies/Gibco). Stable transfectants were selected in αMEM containing genetin at 600 ng/ml. Genetin-resistant clones expressing hss5 somatostatin receptor (sst5/CHO) were selected by somatostatin binding as described in ref. 15 using [125I]-Thr-Nle somatostatin-14 as tracer (16). Two clones were selected for subsequent experiments. CHO-K1 cells were concomitantly stably transfected with pSV2neo vector alone and were used as control clones (neo/CHO). After selection, cells were cultured in αMEM containing 10% FCS and genetin (200 µg/ml).

Binding Studies. [125I]-labeled (Thr-Nle)-CCK-9 was radioiodinated and purified by HPLC as described (20). CHO cells were grown in 35-mm-diameter dishes for 72 h in αMEM containing 10% FCS until confluence (5 × 10⁵ cells per dish). After αMEM was removed, cells were washed twice with cold, serum-free αMEM. Binding was performed at 25°C for 90 min in a final volume of 1.5 ml of serum-free αMEM containing BSA (1 mg/ml), soybean trypsin inhibitor (0.5 mg/ml), bacitracin (0.5 mg/ml), 100 mM [125I]-labeled (Thr-Nle)-CCK-9, and unlabeled peptide analogues. Cells were then washed once with cold binding buffer and collected after a 10-min incubation in 0.1 M NaOH for determination of bound radioactivity. Nonspecific binding was determined in the presence of 1 µM CCK-8.

Cell Growth Assay. CHO cells were cultured in αMEM containing 10% FCS and plated in 35-mm dishes at 50 × 10⁴ cells per ml (2 ml per dish). After a 18 h period of serum deprivation, the cells were washed twice with Krebs-Ringer Hepes buffer containing 1.2 mM NaCl, 4.8 mM KCl, 0.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 5 mM glucose, 2 mM glutamine, essential amino acids, non-essential amino acids, 24.5 mM Hepes, and 0.2 mg/ml BSA, pH 7.4. After a 15-min equilibration phase at 37°C, cells were incubated with or without CCK-8 and other agents tested diluted in Krebs-Ringer Hepes buffer. Reaction was stopped by addition of 2 ml of 95% ethanol. After a 4-h precipitation at 4°C, cells were scraped and centrifuged at 2,750 × g for 15 min at 4°C. Supernatant was removed and lyophylized, and cGMP content was determined in the crude extracts using the radiolimmunobinding assay kit from DuPont/NEN using succinyl cGMP tyrosine methyl ester-[125I] as tracer. Results were expressed in fmol of cGMP per 10⁶ cells.

Immunoblotting Analysis of p42-Mitogen-activated protein (MAP) Kinase Phosphorylation. Sst5/CHO cells were plated in 100-mm-diameter dishes until subconfluence and, after a 18 h period of serum deprivation, were incubated with CCK-8 and with or without RC-160 or other agents tested. The cells were then washed once with phosphate buffer saline (pH 7.0) and once with lysis buffer (50 mM Hepes/150 mM NaCl/10 mM EDTA/10 mM NaF/2/2 mM sodium orthovanadate, pH 7.4), and lysed in 500 µl of lysis buffer containing 1% Triton X-100 and protease inhibitors (1 mM phenylmethylsulfonyl fluoride/2 mg/ml aprotonin/20 mM leupeptin). After a 15-min incubation at 4°C, the lysate was collected and centrifuged at 13,000 × g for 10 min at 4°C to remove insoluble material. Phosphorylated proteins were immunoprecipitated by incubating the solubilized cells for 2 h at 4°C with a monoclonal antiphosphotyrosine antibody (Santa Cruz Biotechnology) prebound to Sepharose-protein G beads (Sigma) (dilution 1:200). The immune complexes were collected by centrifugation, washed twice with a buffer containing 30 mM Hepes, 30 mM NaCl, and 0.1% Triton X-100, pH 7.4, and boiled for 5 min in 50 µl of a SDS/sample buffer. The samples were separated on 10% SDS/polyacrylamide gels and electroblotted onto nitrocellulose membranes. The membranes were incubated for 45 min at 37°C in 10 mM Tris, 140 mM NaCl, pH 7.4 containing 3% BSA and 1% gelatin and then blotted overnight at 4°C with anti-p42-MAP kinase (ERK-2) polyclonal antibodies (Santa Cruz Biotechnology) (dilution 1:200). Bound antibodies were detected with horseradish peroxidase-conjugated anti-mouse IgG followed by ECL detection (Amersham).

Statistical Analysis. Results are expressed as mean ± SE. Comparisons were performed using Student’s t test; P < 0.05 was considered statistically significant.

RESULTS

Expression of CCKA Receptor by CHO-K1 Cells. We first characterized the CCK receptors endogenously expressed by CHO-K1 cells. Binding experiments showed that [125I]-labeled (Thr-Nle)-Tyr-CCK-9 bound specifically to both CHO and sst5 and Ss5/neo cells. Competitive inhibition experiments in the presence of unlabeled analogs and specific receptor antagonists revealed that the concentrations giving half-maximal inhibition of binding (IC₅₀) were 3.2 ± 0.9 nM for CCK-8, 2.8 ± 1.6 µM for sulfated gastrin 1–17, 0.43 ± 0.2 nM for the CCK₄ antagonist SR 27 897, and 0.66 ± 0.17 µM for the CCK₈ antagonist PD 135 158, respectively (data not shown from three separate experiments in triplicate). This pharmacological profile, that is, high affinity for CCK-8 and SR 27 897 and low affinity for sulfated gastrin and PD 135 158, indicates the presence of CCK₄ receptors in CHO K1 cells.
Stimulation of CHO Cell Proliferation by CCK Through a Pertussis Toxin-Dependent Mechanism. We previously observed that CCK at a concentration of 0.1 μM stimulated the proliferation of sst5/CHO cells (16). To further characterize this effect, sst5/CHO cells were or were not treated for 24 h with increasing concentrations of CCK-8. As shown in Fig. 1A, the proliferation was stimulated in a dose-dependent manner with a maximal effect at a concentration of 0.1 μM CCK-8. The half-maximal inhibition was produced by 1 ± 0.2 nM, this concentration being related to the affinity of CCK-8 to its receptor. Similar results were obtained on neo/CHO cells, the half-maximal stimulation of cell growth being produced by 0.75 ± 0.2 nM CCK-8 (from three experiments in triplicate). As shown in Fig. 1B, a 24-h treatment of cells by pertussis toxin reduced the CCK-induced stimulation of CHO cell proliferation by 85 ± 14%. These results indicate that CCK stimulates the proliferation of CHO-K1 by a mechanism involving a pertussis-toxin sensitive Gi/Go protein.

Stimulation of CHO Cell Proliferation by CCK Through cGMP-Dependent Pathway. To investigate the proliferative effect of CCK, we tested the possible involvement of the increase in intracellular concentrations of cGMP caused by the activation of guanylate cyclase pathway. As shown in Fig. 2A, the specific inhibitors of soluble guanylate cyclase and cGMP-dependent protein kinases, LY 83583 (0.1 μM) (22) and KT 5823 (0.1 μM) (23), respectively, significantly suppressed CCK-induced sst5/CHO cell proliferation. Similar results were obtained when inhibitors were administered at a concentration of 1 μM (data not shown). Moreover, the treatment of sst5/CHO cells cultured in serum-free medium with the cGMP analogue dibutyryl-cGMP resulted in a dose-dependent increase in cell proliferation when compared with control cells with a maximal effect at 1 μM (+42 ± 7% over control) and half-maximal stimulation at 0.4 μM (from three experiments in triplicate; P < 0.05). Treatment of sst5/CHO cells with 0.1 μM CCK-8 resulted in an increase of intracellular cGMP concentrations. The stimulation was maximal after 2 min of treatment and remained stable for 10 min (data not shown from two experiments in duplicate). Addition of an inhibitor of phosphodiesterase (2 mM 3-isobutyl-1-methylxanthine) to incubation medium did not modify the stimulatory effect of CCK (data not shown). The CCK-induced increase in intracellular cGMP concentrations was dose-dependent, the maximal effect being observed at 0.1 μM, and the half-maximal stimulation, at 1 ± 0.2 nM CCK (Fig. 2B). This latter concentration was related to that necessary to produce half-maximal stimulation of

Fig. 1. (A) Concentration-dependent stimulation by CCK-8 of proliferation of CHO cells expressing sst5. sst5/CHO were cultured in serum-free αMEM and then treated with increasing concentrations of CCK-8 for 24 h or not treated. Results are expressed as the percentage of control values obtained with untreated cells grown in FCS-free medium (0) (mean ± SE of three experiments in triplicate; *, P < 0.001). (B) Effect of pertussis toxin on CCK-8-induced stimulation of proliferation of CHO cells expressing sst5. Cell proliferation was induced by 0.1 μM CCK-8 for 24 h. Concomitantly, the cells were treated (hatched bars) with 100 ng/ml of pertussis toxin for 24 h or not treated (open bars). Results are expressed as the percentage of control values obtained with untreated cells. Under these conditions, 0.1 μM CCKs induced a cell growth stimulation of 37 ± 6% above basal values observed for cells grown in FCS-free medium (mean ± SE of three experiments in triplicate; *, P < 0.01).

Fig. 2. (A) Effect of soluble guanylate cyclase and protein kinase G inhibitors on CCK-8-induced stimulation and on RC-160-induced inhibition of proliferation of CHO cells expressing sst5. Cell proliferation was induced by 0.1 μM CCK-8 for 24 h. Concomitantly, cells were treated with LY 83583 (0.1 μM), KT 5823 (0.1 μM), and RC-160 (10 nM, hatched bars) for 24 h or were not treated. Results are expressed as the percentage of control values obtained with CCK-treated cells. Both inhibitors had no effect on control cells cultured in serum-free medium (data not shown). Under these conditions, 0.1 μM CCK-8 induced a cell growth stimulation of 46 ± 8% above basal values observed for cells grown in FCS-free medium (mean ± SE of three experiments in triplicate; **, P < 0.01). (B) Concentration-dependent stimulation of cGMP formation by CCK-8 in CHO cells expressing sst5. CHO cells stably expressing sst5 were cultured in αMEM containing 10% FCS during 18 h and treated with increasing concentrations of CCK-8 for 5 min in Krebs–Hepes buffer. cGMP content was determined in the crude extracts using radioimmunoassay. Basal value of cGMP level was 234 ± 12 fmol per 10⁶ cells. Results are expressed as the percentage of control values obtained with untreated cells (0). Mean ± SE of three experiments in duplicate. *, P < 0.05; **, P < 0.01.
proliferation of CHO cells and also to the affinity of CCK-8 for its receptor in CHO cells. In addition, when cells were concomitantly treated with CCK-8 (0.1 μM) and LY 83583 (0.1 μM), the increase of intracellular cGMP concentration induced by CCK was inhibited by 90 ± 8% (from three experiments in triplicate; P < 0.001). A 24-h pretreatment of sst5/CHO cells with 100 ng/ml of pertussis toxin inhibited the CCK-induced increase of intracellular cGMP concentration by 88 ± 8% (from three experiments in triplicate, P < 0.001). These results indicate that CCK stimulates the intracellular production of cGMP in CHO-K1 cells by activating a soluble guanylate cyclase through a Gi/Go pertussis toxin-sensitive G protein and that this pathway may be part of the proliferative signal induced by activation of CCKA receptors.

**Activation by CCK of p42-MAP Kinase of CHO Cells Through a cGMP-Dependent Pathway.** MAP kinases, also known as extracellular signal-regulated kinases (ERKs), are a family of protein-serine/threonine kinases now considered as key molecules regulating cell proliferation and gene expression, because of their ability to phosphorylate many regulatory proteins including transcriptional factors. Activation of MAP kinases requires phosphorylation of both threonine and tyrosine regulatory sites by the specific upstream protein kinase MAP kinase kinase (MEK). In isolated rat pancreatic acini, CCK-8 is known to activate the MAP kinases as well as MEK (24, 25). In CHO cells, as shown in Fig. 3, CCK-8 induced a rapid phosphorylation of p42-MAP kinase. A kinetic study revealed that stimulation was maximal after 5 min of treatment, remained stable within 10 min, and decreased at 15 min (data not shown). Furthermore, a 18-h pretreatment of cells by pertussis toxin (100 ng/ml) inhibited this effect (Fig. 3).

Treatment of cells with 0.1 μM CCK-8 for 5 min also increased p42-MAP kinase activity by 146 ± 15% over basal value (from three experiments in triplicate, P < 0.001), and this effect was also inhibited by 84 ± 5% after an 18-h pretreatment of cells with pertussis toxin (100 ng/ml) (P < 0.001). These results indicate that CCK activates MAP kinase in CHO-K1 cells through a pertussis-toxin-dependent mechanism.

To further investigate the possible implication of cGMP pathway in the CCK-induced activation of MAP kinase, cells were treated with 0.1 μM CCK-8 in the presence or absence of the cGMP-dependent protein kinase inhibitor, KT 5823 (0.1 μM), and added 10 min prior to the CCK treatment. The inhibitor alone did not modify the phosphorylation of p42 MAP kinase but inhibited CCK-induced phosphorylation of p42-MAP kinase, suggesting that a cGMP-dependent protein kinase is implicated in the activation of MAP kinase induced by CCK in CHO cells (Fig. 3 A and B).

We also investigated the effect of the MEK inhibitor, PD 98059 (26), on CCK-induced cell proliferation. Cells were incubated for 24 h with 0.1 μM CCK-8 with or without 0.1 μM PD 98059. The inhibitor alone had no effect on control cells grown in serum-free medium but reduced the CCK-induced cell proliferation by 85 ± 6.4% (from two experiments in triplicate). These results suggest that activation of MAP kinase induced by CCK-8 is involved in the proliferative effect of CCK mediated through CCKA receptors in CHO cells.

Taken together, all these results demonstrate that CCKA receptors are coupled to a soluble guanylate cyclase through a Gi/Go pertussis toxin-sensitive G protein and that this pathway is implicated in the stimulation of MAP kinase and the resulting stimulation of cell proliferation induced by CCK.

**Inhibition by RC-160 of CCK-Induced Increase of Intracellular cGMP Concentrations in sst5/CHO Cells.** As we showed above, the somatostatin analogue RC-160 inhibited CCK-induced stimulation of sst5/CHO cell proliferation (Fig. 2A). We then tested the effect of RC-160 on intracellular cGMP concentration. Sst5/CHO cells were incubated with 0.1 μM CCK in the presence of increasing concentrations of RC-160. As shown in Fig. 4, RC-160 produced a dose-dependent inhibition of CCK-induced increase of intracellular cGMP concentrations; maximal effect was observed at 10 nM and half-maximal inhibition by 0.32 ± 0.1 nM. These analogue concentrations were similar to those necessary for the inhibition of CCK-induced cell proliferation and for the occupancy of the recombinant human sst5 receptor subtype (16). Addition of an inhibitor of phosphodiesterase (2 mM 3-isobutyl-1-methylxanthine) to the incubation medium did not modify the stimulatory effect of RC-160 (data not shown). Because pertussis toxin alone inhibited CCK-induced intracellular cGMP production of CHO-K1 cells, we could not evaluate the

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**Fig. 3.** Tyrosine phosphorylation of p42-MAPK induced by CCK-8 in CHO cells expressing sst5. sst5/CHO cells were plated in 100-mm-diameter dishes until subconfluence and, after a 18-h period of serum deprivation, were treated for 5 min with CCK-8 (0.1 μM) with or without a pretreatment with pertussis toxin (PTx, 100 ng/ml). Cells were also treated with 0.1 μM CCK-8 with or without KT 5823 (KT, 0.1 μM) added 10 min prior to CCK. The total lysate was immunoprecipitated with antiphosphotyrosine antibody coupled to protein A-Sepharose and subjected to immunoblotting analysis by using anti-p42-MAP kinase antibody. (A) Representative immunoblot analysis. (B) The intensities of tyrosine phosphorylation was quantified by an imager analyzer (Biocon, Paris). Values are expressed as a percentage of maximal effect obtained with CCK-8, KT 5823 or pertussis toxin given alone had no effect on control value (data not shown). Results are mean ± SE of three determinations, each obtained from different runs of cell treatment and immunoblotting experiments.

**Fig. 4.** Concentration-dependent inhibition by RC-160 of CCK-8-induced cGMP formation in CHO cells expressing sst5. Cells were treated with CCK-8 (0.1 μM) as described in legend to Fig. 2B and with increasing concentrations of RC-160. Under these conditions, 0.1 μM CCK-8 induced a stimulation of 101 ± 15% above basal values (220 ± 5 mol per 10⁶ cells) observed for cells incubated with CCK-free buffer. Results are expressed as the percentage of control values obtained with CCK-8-treated cells. Mean ± SE of three experiments in duplicate. *, P < 0.05; **, P < 0.001.
effect of this toxin on the inhibitory action of RC-160 on cGMP formation.

To further characterize the implication of CCK in the antiproliferative effect mediated by sst5 receptor, cells were incubated for 24 h with 0.1 μM CCK-8 in the presence or absence of LY 83583 (0.1 μM), KT 5823 (0.1 μM), and RC-160 (10 nM). The analogue had no additional influence on the effect of inhibitors on CCK-induced cell proliferation (Fig. 2A), suggesting that the same pathway could be involved in the inhibitory effect of LY 83583, KT 5823, and RC-160. All these results indicate that human sst5 stably expressed in CHO cells mediates the negative effect of RC-160 on CCK-induced cell proliferation through a cGMP-dependent pathway.

Inhibition by RC-160 of CCK-Induced Activation of MAP Kinase in sst5/CHO Cells. To further characterize the negative regulation of RC-160 on sst5/CHO cell proliferation, we examined the effect of the analogue on CCK-induced MAP kinase phosphorylation and activity. As shown in Figs. 5 and 6, RC-160 inhibited both the p42-MAP kinase phosphorylation and activity in a dose-dependent manner, the maximal effect being observed at a concentration of 1–10 nM RC-160. The concentrations of RC-160 required for half-maximal stimulation of MAP kinase phosphorylation and activity were 0.32 ± 0.07 nM and 23.3 ± 7 pM, respectively. These results demonstrate that activation of sst5 leads to an inhibition of CCK-induced MAP kinase activity and suggest that this effect may be part of the antiproliferative signal of somatostatin mediated by sst5 receptor.

DISCUSSION

In the present study, we have demonstrated that the proliferation of CHO cells stably expressing sst5 receptor was positively modulated by CCK. In this model, CCK interacted with endogenous CCK receptors that displayed a pharmacological profile indicative of the CCKA receptor subtype. By applying reverse transcriptase (RT)–PCR experiments using primers recognizing the first transmembrane domain and the third intracellular loop of the human CCKA receptor, we found that both wild and sst5-expressing CHO cells expressed CCKA mRNA. The cascade of events implicated in the proliferative effect of CCK in CHO cells may involve the successive activation of a soluble guanylate cyclase through a pertussis toxin-sensitive G protein, a cGMP-dependent protein kinase, and MAP kinases. We previously found that calcium mobilization induced by CCK in these cells was not dependent on a pertussis toxin-sensitive G protein (16). Based on these observations, we can eliminate intracellular calcium mobilization as a possible mechanism implicated in the CCK-mediated cell growth signaling. The G protein coupled to these receptors remains unknown, but CCKA receptors have been found to couple to G1, 1, 2, and 3 proteins in rat pancreatic acinar cells (27). One of these G proteins thus could be a candidate for coupling to CCKA receptors in CHO cells.

We observed that the CCK-induced cell proliferation was inhibited by the MEK inhibitor, PD 98059. We also demonstrated that CCK-8 rapidly activated both MAP kinase phosphorylation and activity. This activation was also dependent on a pertussis toxin-sensitive G protein and the cGMP-dependent kinase pathway. Molecular events that linked G kinase and MAP kinases cascade remain to be elucidated.

CCK receptor activation is known to be associated with an intracellular production of cGMP as is the case for many G protein-coupled receptors such as neurotensin or bradykinin receptors. We observed that CCKA receptors mediate cell proliferation in vitro through the production of intracellular cGMP and activation of MAP kinase pathway. Cyclic GMP is a key regulatory molecule in visual transduction, integration of neuronal response to excitatory neurotransmission, relaxation of smooth muscle cells, salt transport, and reabsorption in intestine and distal tubule of kidney. Increase of intracellular levels of cGMP is also known to regulate the proliferation of many cell systems. A negative regulation of cell proliferation has been observed on rat mesangial cells and rabbit and rat vascular smooth muscle cells after treatment with cGMP analogues or stimulation with atrial natriuretic factor known to activate the intracellular production of cGMP (28, 29). Conversely, the positive effects of cGMP on cell proliferation have.

Fig. 5. Concentration-dependent inhibition by RC-160 of tyrosine phosphorylation p42-MAP kinase activated by CCK-8 in CHO cells expressing sst5. Sst5/CHO cells were plated in 100-mm-diameter dishes until subconfluence and, after a 18-h period of serum deprivation, cells were treated for 5 min with CCK-8 (0.1 μM) and increasing concentrations of RC-160 or not treated. The total lysate was immunoprecipitated with anti-p42-MAP antibody coupled to protein-A Sepharose and subjected to immunoblot analysis by using anti-p42-MAP kinase antibody as described in Materials and Methods. (A) Representative immunoblot analysis. (B) The intensities of tyrosine phosphorylation was quantified by an imager analyzer (Biorad). Values are expressed as percentage of maximal effect obtained with CCK-8 (0). RC-160 administered alone at a concentration of 10 nM had no effect (data not shown). Results are mean ± SE of three determinations, each obtained from different runs of cell treatment and immunoblotting experiments.
been rarely described (30). Cyclic GMP has several intracellular target proteins including cGMP-gated ion channel, cGMP-binding cyclic nucleotide phosphodiesterases, and cGMP-dependent protein kinases (or G kinases). The G kinases have been recently implicated in the transactivation of the c-fos promoter, the product of c-fos gene, that plays an important role in the regulation of cell growth and differentiation (31).

We previously demonstrated that the somatostatin analogue RC-160 inhibited CCK-induced cell proliferation of CHO cells stably expressing human sst5 subtype. This effect did not involve activation of protein phosphatases or inhibition of adenylyl cyclase activity. In the present work we observed that RC-160 inhibited CCK-induced increase of both intracellular cGMP levels and MAP kinase activity. These inhibitory effects occurred at concentrations of RC-160 related to those necessary to occupy sst5 receptor and to inhibit cell proliferation (16). We concluded that a negative coupling of sst5 to a soluble guanylate cyclase may be responsible for the antiproliferative effect of RC-160 in this cell system. To our knowledge, no prior observations exist concerning a negative coupling of somatostatin receptor to guanylate cyclase.

It has been found that sst5 mRNA is expressed not only in pituitary but also in brain and various peripheral tissues (19, 32). In man, sst5 mRNAs are expressed in pancreas, small intestine, colon mucosa, adrenal, skeletal muscle, placenta, heart, and vascular smooth muscle cells (refs. 19 and 33; unpublished observations). However, the implication of sst5 in the growth regulation of these tissues remains to be elucidated. We found that this subtype is also expressed in colon cancer and in the colon cancer cell line HT-29 (33). This receptor thus involves activation of protein phosphatases or inhibition of cGMP-binding cyclic nucleotide phosphodiesterases, and cGMP-dependent protein kinases (or G kinases). The G1181.


