CCK2 (CCK_B/gastrin) receptor mediates rapid protein kinase D (PKD) activation through a protein kinase C-dependent pathway

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Abstract Addition of gastrin or cholecystokinin octapeptide (CCK-8) to cultures of Rat-1 cells stably transfected with the CCK2 (CCK_B/gastrin) receptor induced protein kinase D (PKD) activation that was detectable within 1 min and reached a maximum (~10-fold) after 2.5 min of hormonal stimulation. Half-maximal PKD activation for both CCK-8 and gastrin was achieved at 10 nM. Treatment with various concentrations of the selective PKC inhibitors Ro 31-8220 or GF-I potently blocked PKD activation induced by subsequent addition of CCK-8 in a concentration-dependent fashion. Our results indicate that PKC-dependent PKD activation is a novel early event in the action of gastrin and CCK-8 via CCK2 receptors.

Key words: Gastrin; Cholecystokinin; Rat-1 cell; Protein kinase C; Protein kinase D

1. Introduction

Gastrin, produced by G cells in the gastric antrum, has been identified as the circulating hormone responsible for stimulation of acid secretion from the parietal cell [1]. Gastrin also acts as a potent cellular growth factor that has been implicated in a variety of normal and abnormal biological processes including maintenance of the gastric mucosa, proliferation of enterochromaffin-like (ECL) cells and neoplastic transformation [2]. Gastrin, cholecystokinin (CCK) and CCK-related peptides exert their characteristic effects on cellular processes by binding to specific guanine nucleotide-binding regulatory protein (G protein)-coupled receptor (GPCR) subtypes. The CCK2 (CCK_B/gastrin) receptor binds gastrin and CCK with similar affinity whereas the CCK1 (CCK_A) receptor exhibits a 500-fold higher affinity for CCK than for gastrin (see [3] for review). One of the earliest signal transduction events induced by activation of CCK2 receptors is the Gαq-mediated stimulation of β isoforms of phospholipase C (PLC) leading to Ins(1,4,5)P_3-mediated Ca^{2+} mobilization from internal stores and diacylglycerol (DAG) stimulation of classic and novel isoforms of the protein kinase C (PKC) family. Accordingly, gastrin stimulates rapid mobilization of Ca^{2+} from intracellular stores in a variety of cell types including fibroblasts stably transfected with the CCK2 receptor e.g. Rat-1 [4], NIH 3T3 [5] and CHO [6] and other cell types that express endogenous CCK2 receptors, including ECL [1] and small cell lung carcinomas [7,8]. In contrast, direct activation of PKC isoforms in response to gastrin has been less frequently measured and the molecular events occurring downstream of specific isoforms of PKC remain elusive.

Protein kinase D (PKD)/PKCμ is a serine/threonine protein kinase [9,10] with structural, enzymological and regulatory properties distinct from other members of the PKC family [11]. For example, the catalytic domain of PKD is distantly related to Ca^{2+}-regulated kinases and the regulatory region of this kinase contains a putative transmembrane domain, a pleckstrin homology (PH) domain that regulates enzyme activity [12,13], and lacks a sequence with homology to a typical PKC autoinhibitory pseudosubstrate motif [9,10]. In particular, PKD is rapidly activated in intact cells through a mechanism that involves phosphorylation [11]. Exposure of intact cells to phorbol esters, bryostatin or mitogenic agonists including bombesin and platelet-derived growth factor induces rapid PKD phosphorylation and activation, which is maintained during cell lysis and immunoprecipitation [12,14–17]. Several lines of evidence generated by using PKC-specific inhibitors and co-transfection of PKD with constitutively active PKC mutants, suggest that PKD is activated through a novel PKC-dependent signal transduction pathway in vivo [13–17]. The heterotrimeric G proteins Gq and Gi have been implicated in mediating PKC-dependent PKD activation in response to heptahelical receptor activation [17,18].

Here, we examined whether PKD activation is also an early event in the action of the hormonal peptides gastrin and sulfated CCK octapeptide (CCK-8) using Rat-1 cells expressing the human CCK2 receptor. These cells have provided a useful model system to elucidate signal transduction pathways activated through CCK2 [4], bombesin [19,20] and neuromedin B [21] receptors. Our results demonstrate that treatment of quiescent cultures of these cells with either gastrin or CCK-8 induces a rapid and striking dose-dependent increase in PKD activity through a PKC-dependent pathway.

2. Materials and methods

2.1. Cell culture

The generation and properties of Rat-1 cells stably transfected with

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Abbreviations: AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride; CCK-8, sulfated cholecystokinin octapeptide; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; G proteins, guanine nucleotide-binding regulatory proteins; GF-I, bisindolylmaleimide I (GF 109203X); GPCR, G protein-coupled receptor; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PKC, protein kinase C; PKD, protein kinase D

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human CCK2 receptor (Rat-1CCK2R) have been previously described [4]. The cells were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 0.5 mg/ml of geneticin (G418) in a humidified atmosphere. For experimental purposes, cells were plated in 100 mm dishes at 3×10^5 cells/dish and grown in DMEM containing 10% FBS and were allowed to grow to confluency (5–7 days) and then changed to serum-free DMEM for 18–24 h prior to the experiment.

2.2. Immunoprecipitation

Cultures of Rat-1CCK2R cells, treated as described in the individual experiments, were washed and lysed in 50 mM Tris–HCl pH 7.6, 2 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF) and 1% Triton X-100 (lysis buffer A). Cell lysates were clarified by centrifugation at $15\,000\times g$ for 10 min at 4°C. PKD was immunoprecipitated at 4°C for 2–4 h with the PA-1 antiserum (1:100), as previously described [14]. The immune complexes were recovered using protein A coupled to agarose.

2.3. Kinase assay of PKD

PKD autophosphorylation was determined in an in vitro kinase assay by mixing 20 μl of PKD immunocomplexes with 10 μl of a phosphorylation mixture containing (final concentration) 100 μM [γ-^{32}P]ATP (specific activity 400–600 cpm/pmol), 30 mM Tris–HCl pH 7.4, 10 mM $MgCl_2$ and 1 mM DTT. After 10 min of incubation at 30°C, the reaction was stopped by washing with 200 μl of kinase buffer and then adding an equal volume of 2×SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer (200 mM Tris–HCl pH 6.8, 2 mM EDTA, 0.1 M Na_3VO_4, 6% SDS, 10% glycerol and 4% 2-mercaptoethanol), followed by SDS–PAGE analysis [14,22]. The gels were dried and the 110 kDa radioactive band corresponding to autophosphorylated PKD was visualized by autoradiography. Autoradiographs were scanned in a GS-710 calibrated imaging densitometer (Bio-Rad), and the labeled band was quantified using the Quantity One™ software program.

Exogenous substrate phosphorylation by immunoprecipitated PKD was carried out by mixing 20 μl of the washed immunocomplexes with 20 μl of a phosphorylation mixture containing 2.5 mg/ml syntide 2 (PLARTLSVAGLPGKK), a peptide based on phosphorylation site two of glycogen synthase. After 10 min of incubation at 30°C, the reaction was stopped by adding 100 μl of 75 mM H_3PO_4 and spotting 75 μl of the supernatant on P-81 phosphocellulose paper. Free [γ-^{32}P]ATP was separated from the labeled substrate by washing the P-81 paper four times for 5 min, in 75 mM H_3PO_4. The papers

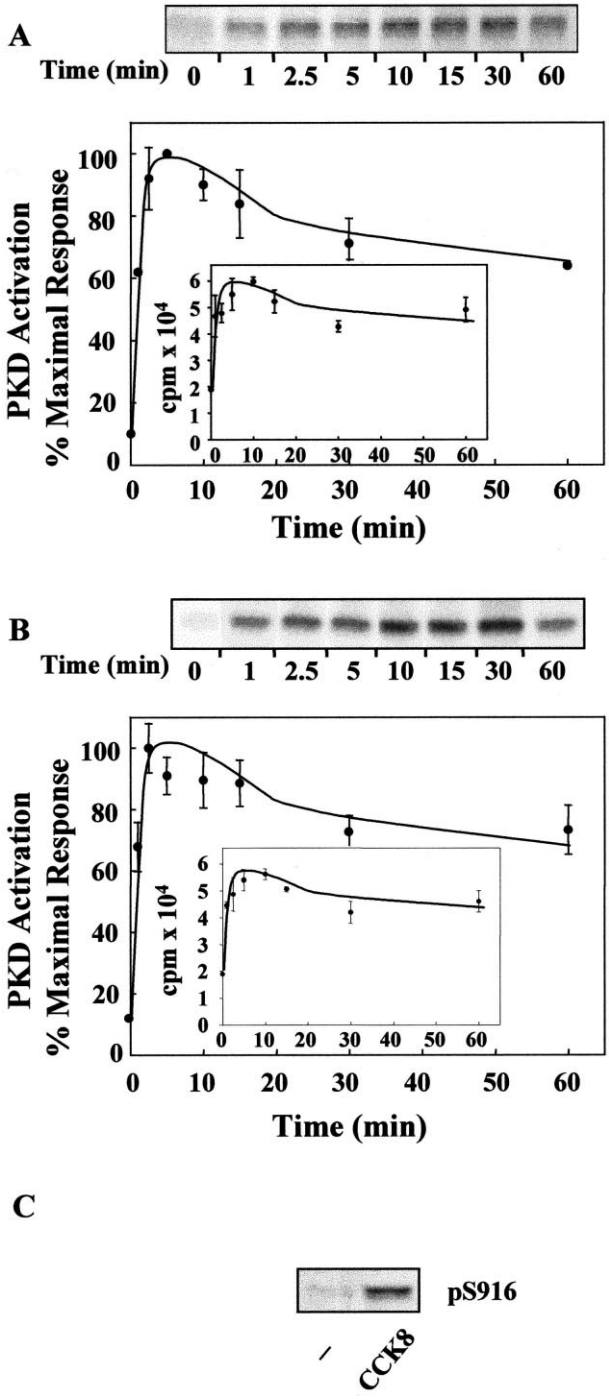

Fig. 1. Gastrin and CCK-8 stimulate PKD activation in a time-dependent manner. A, upper panel: Time course of gastrin-induced PKD activation. Confluent and quiescent cultures of Rat-1CCK2R cells were washed twice in DMEM and then incubated with 100 nM gastrin for various times at 37°C as indicated. Cells were lysed with lysis buffer A and immunoprecipitated with PA-1 antiserum. PKD activity was determined by an in vitro kinase assay as described in Section 2 followed by SDS–PAGE and autoradiography. The autoradiogram shown is representative of three independent experiments. Lower panel: Scanning densitometry of gastrin time course. The results shown are the values (means ± S.E.M., $n=3$) of the level of PKD activation by IVK obtained from scanning densitometry expressed as a percentage of the maximum increase in phosphorylation obtained with 100 nM gastrin. Insert: Confluent and quiescent cultures of Rat-1CCK2R cells in serum-free DMEM were treated with 100 nM gastrin for various times at 37°C. PKD activity in the immunocomplexes was then measured by syntide 2 phosphorylation, as described in Section 2. B, upper panel: Time course of CCK-8-induced PKD activation. Confluent and quiescent cultures of Rat-1CCK2R cells were washed twice in DMEM and then incubated with 100 nM CCK-8 for various times at 37°C as indicated. Cells were lysed with lysis buffer A and immunoprecipitated with PA-1 antiserum. PKD activity was determined by an in vitro kinase assay as described in Section 2 followed by SDS–PAGE and autoradiography. The autoradiogram shown is representative of three independent experiments. Lower panel: Scanning densitometry of CCK-8 time course. The results shown are the values (means ± S.E.M., $n=3$) of the level of PKD activation by IVK obtained from scanning densitometry expressed as a percentage of the maximum increase in phosphorylation obtained with 100 nM CCK-8. Insert: Confluent and quiescent cultures of Rat-1CCK2R cells in serum-free DMEM were treated with 100 nM CCK-8 for various times at 37°C. PKD activity in the immunocomplexes was then measured by syntide 2 phosphorylation, as described in Section 2. C: Detection of PKD activation by Western blot analysis using a phospho-specific antibody directed against the phosphorylated state of the C-terminal serine 916 (pS916). Confluent and quiescent cultures of Rat-1CCK2R cells in serum-free DMEM were treated with 100 nM CCK-8 for 10 min at 37°C. Western blot analysis using pS916 antiserum was performed following lysis of the cells with 2×sample buffer as described in Section 2. The Western blot shown is representative of three independent experiments.

were dried, and the radioactivity incorporated into syntide 2 was determined by Cerenkov counting.

2.4. Western blot analysis

Quiescent cultures of Rat-1CCK2R cells grown on 100 mm dishes were washed twice with DMEM and then treated as described. The cells were lysed in 2×SDS–PAGE sample buffer. Following SDS–PAGE on 8% gels, proteins were transferred to Immobilon-P membranes (Millipore) and blocked by 3–6 h incubation with 5% non-fat milk in phosphate-buffered saline (PBS), pH 7.2. Membranes were then incubated overnight with an antiserum that specifically recognizes the phosphorylated state of serine 916 of PKD at a dilution of 1:500 in PBS containing 5% non-fat dried milk. Horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:5000, Amersham) was then applied for 1 h at room temperature after washing three times with PBS containing 0.05% Tween. Immunoreactive bands were detected by enhanced chemiluminescence Western blotting ECL™ reagents (Amersham).

2.5. Materials

[γ-^{32}P]ATP (370 MBq/ml) was from Amersham Pharmacia Biotech (Piscataway, NJ, USA). GF-I (also known as bisindolylmaleimide I or GF 109203X) and Ro 31-8220 were purchased from Calbiochem (San Diego, CA, USA). Sulfated gastrin II, CCK-8, wortmannin, rapamycin, PD 098059, genistein, and DMEM were purchased from Sigma. Protein A-agarose was from Boehringer Mannheim (Indianapolis, IN, USA). Geneticin G418 was purchased from Gibco BRL. PA-1 antiserum was raised against the synthetic peptide EEREMKALSERVSIL that corresponds to the C-terminal region of the predicted amino acid sequence of PKD, as previously described [14,22]. An antiserum that specifically recognizes the phosphorylated state of serine 916 of PKD (pS916) was generously provided by Dr. Doreen Cantrell, Imperial Cancer Research Institute, London, UK. Other items were from standard suppliers or as indicated in the text.

3. Results and discussion

3.1. CCK2 receptor mediates PKD activation

To determine whether gastrin can induce PKD activation in Rat-1CCK2R cells, quiescent cultures of these cells were treated with this hormone for increasing times, lysed and PKD was immunoprecipitated with PA-1 antiserum. The resulting immunocomplexes were incubated with [γ-^{32}P]ATP and the incorporation of ^{32}P into PKD was analyzed by SDS–PAGE and autoradiography. As shown in Fig. 1A, PKD isolated from unstimulated Rat-1CCK2R cells had low catalytic activity. Treatment of Rat-1CCK2R cells with gastrin induced a rapid and striking increase in PKD kinase activity which was maintained during cell lysis and immunoprecipitation. PKD activation was detectable within 1 min and reached a maximum (~10-fold) after 2.5 min of gastrin stimulation.

As an independent measure of PKD activation induced by gastrin in Rat-1CCK2R cells, we also examined phosphorylation of an exogenous substrate using syntide 2 [23,24], a peptide identified as an excellent model substrate for PKD [9,13,22,25]Consistent with the results of autophosphorylation assays, syntide 2 phosphorylation assays also showed that Rat-1CCK2R cells stimulated with gastrin had dramatically increased PKD activity (Fig. 1A, inset).

The CCK2 receptor binds gastrin and CCK-8 with similar high affinity [3]. Accordingly, addition of CCK-8 instead of gastrin to cultures of Rat-1CCK2R cells also induced a rapid increase in PKD activity, as judged by autophosphorylation assays (Fig. 1B) and by syntide 2 phosphorylation assays (Fig. 1B, inset). These results demonstrate that PKD activation is one of the early events induced by gastrin or CCK-8 in this model system.

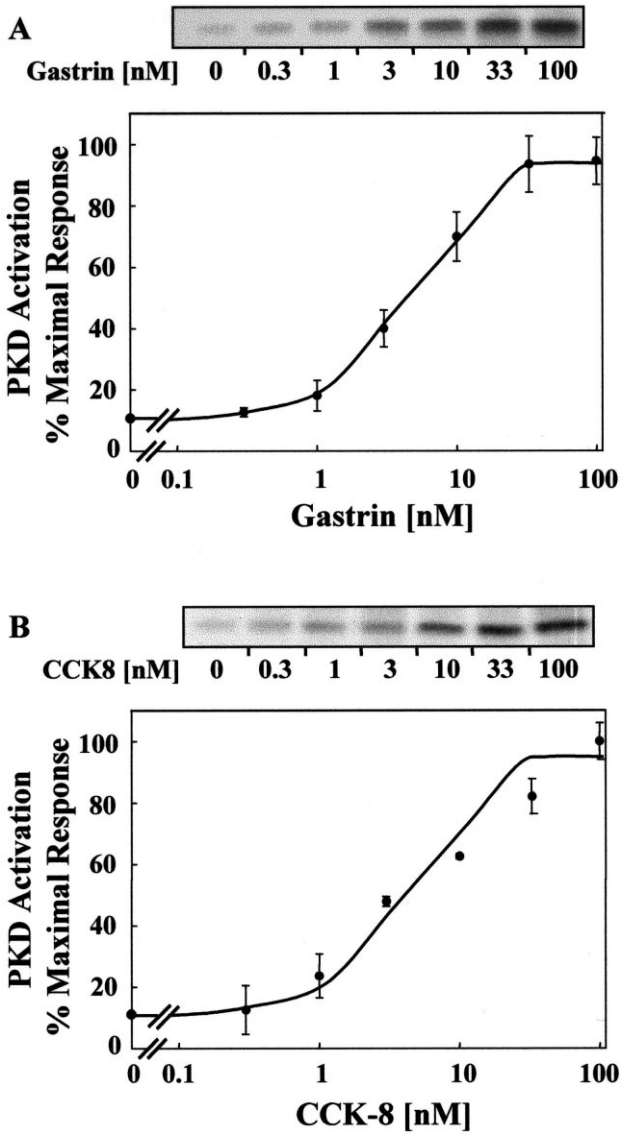

Fig. 2. Gastrin and CCK-8 induce PKD activation in a dose-dependent manner. A, upper panel: Dose response of gastrin-induced PKD activation. Confluent and quiescent cultures of Rat-1CCK2R cells were treated with various concentrations of gastrin for 10 min at 37°C, as indicated. The cultures were lysed in lysis buffer A and immunoprecipitated with PA-1 antiserum, and PKD activity determined by an in vitro kinase assay as described in Section 2. The autoradiogram shown is representative of three independent experiments. Lower panel: Scanning densitometry of gastrin dose response. The results shown are the values (means ± S.E.M., $n = 3$) of the level of PKD activation from IVK obtained from scanning densitometry expressed as a percentage of the maximum increase in phosphorylation obtained with 100 nM gastrin. B, upper panel: Dose response of CCK-8-induced PKD activation. Confluent and quiescent cultures of Rat-1CCK2R cells were treated with various concentrations of CCK-8 for 10 min at 37°C, as indicated. The cultures were lysed in lysis buffer A and immunoprecipitated with PA-1 antiserum, and PKD activity determined by an in vitro kinase assay as described in Section 2. The autoradiogram shown is representative of three independent experiments. Lower panel: Scanning densitometry of CCK-8 dose response. The results shown are the values (means ± S.E.M., $n = 3$) of the level of PKD activation from IVK obtained from scanning densitometry expressed as a percentage of the maximum increase in phosphorylation obtained with 100 nM CCK-8.

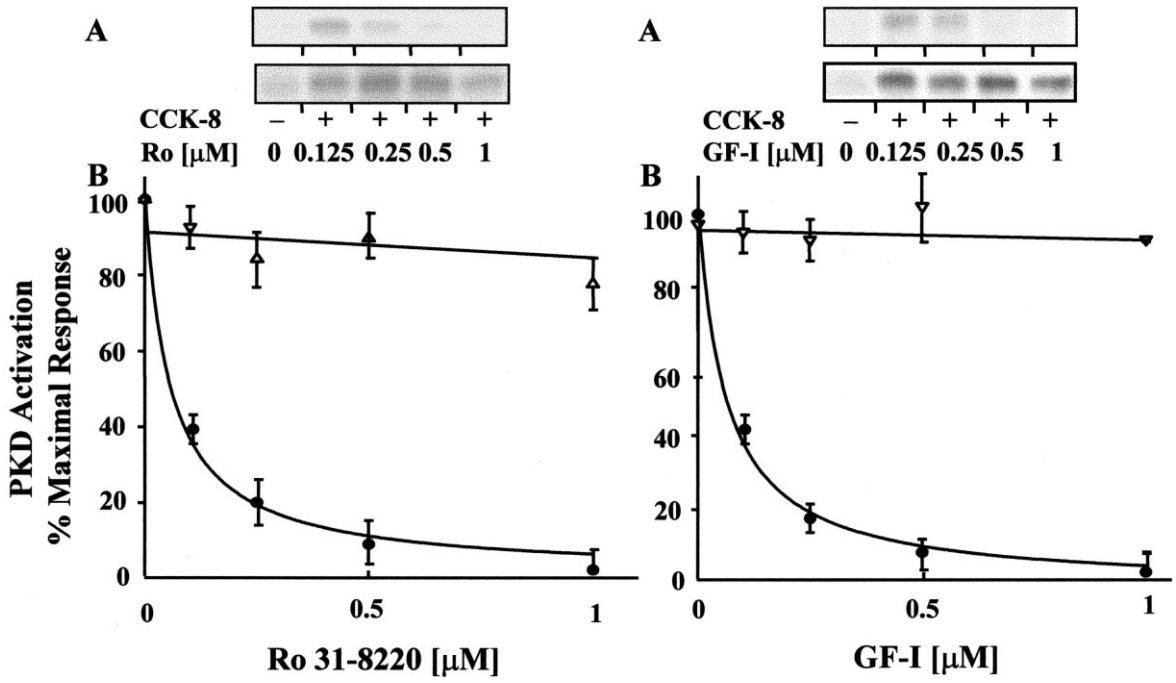

Fig. 3. CCK2R induces PKD activation through a PKC-dependent pathway. A: PKC inhibitors Ro 31-8220 and GF-I inhibit CCK-8-induced PKD activation. Confluent Rat-1CCK2R cells were incubated for 1 h with different concentrations of the PKC inhibitor Ro 31-8220 (A, left upper panel) or GF-I (A, right upper panel). Control cells (−) received equivalent amount of solvent. The cultures were subsequently stimulated for 10 min with 100 nM CCK-8 at 37°C. Cells were lysed with lysis buffer A and immunoprecipitated with PA-1 antiserum. PKD activity was then determined by an in vitro kinase assay, as described in Section 2. Parallel cultures were treated for 10 min with 100 nM CCK-8 at 37°C (A, lower panels). Cells were lysed with lysis buffer A and immunoprecipitated with PA-1 antiserum. PKD activity was then determined by an in vitro kinase assay in absence or presence of the indicated concentrations of Ro 31-8220 or GF-I added to the incubation mixture. B: Scanning densitometry. The results shown are the values (means ± S.E.M., $n = 3$) of the level of PKD activation obtained from scanning densitometry expressed as a percentage of the maximum increase in phosphorylation obtained from cells incubated with 100 nM CCK-8 for 10 min at 37°C. Values corresponding to PKD activity from cells incubated with Ro 31-8220 or GF-I are represented by circles. Values corresponding to PKD activity that was determined by an in vitro kinase assay in the presence of the indicated concentrations of GF-I or Ro 31-8220 added to the incubation mixture are represented by triangles.

Recently, an antiserum specifically recognizing the phosphorylated form of a PKD C-terminal residue, serine 916, was developed and used to detect in vivo autophosphorylation at this site by active PKD [26]. Thus, the pS916 antiserum provides a novel approach for detecting conversion of PKD to an active form within cells. Here, lysate from Rat-1CCK2R cells stimulated with 100 nM CCK-8 for 10 min was analyzed by SDS–PAGE followed by Western blot analysis using the pS916 antiserum. CCK-8 stimulation induced a dramatic increase in the immunoreactivity of the PKD band indicative of phosphorylation at Ser 916 (Fig. 1C).

Stimulation of Rat-1CCK2R cells with increasing concentrations of either gastrin or CCK-8 for 10 min induced a striking dose-dependent increase in PKD activation, as judged by assays of in vitro PKD kinase activity after immunoprecipitation (Fig. 2B). Half-maximal and maximal PKD activation for both CCK-8 and gastrin was achieved at 10 and 100 nM, respectively (Fig. 2B). In contrast, addition of CCK-8 or gastrin to cultures of Rat-1 cells untransfected or transfected with vector did not induce any significant PKD activation.

Taken together, the results presented in Figs. 1 and 2 provide multiple lines of evidence indicating that stimulation of Rat-1CCK2R cells with gastrin or CCK-8 induces rapid and striking PKD activation.

3.2. CCK-8/gastrin-induced PKD activation is PKC-dependent

In order to elucidate the role of PKCs in PKD activation induced by gastrin or CCK-8, cultures of Rat-1CCK2R cells were treated with various concentrations of the selective PKC inhibitors Ro 31-8220 [27] or GF-I [28] prior to gastrin/CCK-8 stimulation. (Fig. 3A, upper panels, left and right) As shown in Fig. 3A (upper panel), treatment with the PKC inhibitors potently blocked PKD activation induced by subsequent addition of CCK-8 in a concentration-dependent fashion. Maximal inhibition of PKD activation (~90%) was achieved at 1 μM of each inhibitor. In contrast, Ro 31-8220 or GF-I added directly to the in vitro kinase assay, even at the concentrations (0.25–1 μM) that abrogated CCK-8-induced PKD activation in intact Rat-1CCK2R cells, did not inhibit PKD activity (Fig. 3A, lower panel). These results imply that Ro 31-8220 and GF-I do not inhibit PKD activity directly but interfere with gastrin/CCK-8-mediated PKD activation in intact cells by blocking PKC.

In contrast to the results obtained with GF-I or Ro 81-3220, treatment of Rat-1CCK2R cells with the broad spectrum protein tyrosine kinase inhibitor genistein, the MEK inhibitor PD098059 [29] which prevents ERK activation, the phosphoinositide 3-kinase inhibitor wortmannin [30] or rapamycin [31] which interfere with activation of the phosphoinositide 3-kinase downstream target p70 ribosomal S6 kinase ($p70^{S6K}$) did not interfere with gastrin-induced PKD activation. (Fig. 4). These results demonstrate the specificity of the PKC inhibitors and indicate that these kinases are not upstream regulators of PKD.

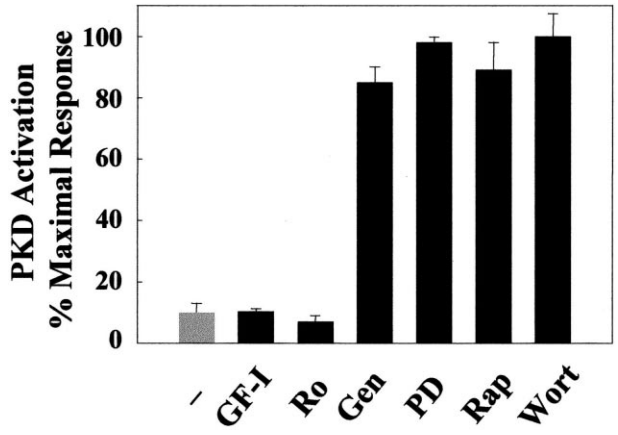

Fig. 4. Treatment with either Ro 31-8220 or GF-I but not with other kinase inhibitors prevents CCK2R-mediated PKD activation. Quiescent and confluent cultures of Rat-1CCK2R cells were incubated for 1 h with either 1 μM GF-I (GF-I), 1 μM Ro 31-8220 (Ro), 50 μM genistein (Gen), 20 μM PD098059 (PD), 20 nM rapamycin (Rap), 100 nM wortmannin (Wort) or an equivalent amount of solvent (−). Cells were subsequently challenged for 10 min with 100 nM gastrin. Cell lysates were immunoprecipitated with PA-1 antiserum, and PKD activity was determined by an in vitro kinase assay, followed by SDS–PAGE and autoradiography. The results shown are the values (means ± S.E.M., $n = 3$) of the level of PKD activation from IVK obtained from scanning densitometry expressed as a percentage of the maximum increase in phosphorylation obtained with 100 nM gastrin.

3.3. Conclusions

In addition to its role in gastric acid secretion, gastrin also acts as a potent cellular growth factor that has been implicated in a variety of normal and abnormal biological processes including maintenance of the gastric mucosa, proliferation of ECL cells and neoplastic transformation. An association between hypergastrinemia and proliferation of ECL cells, sometimes leading to formation of gastric carcinoid tumors, is now well established in rats [32] and in humans [33,34]. Epidemiological evidence also has been obtained that implicates hypergastrinemia as a factor in development of colorectal cancer [35]. Colorectal carcinomas have been found to produce progastrin-related peptides and CCK2 receptors [36–38]. Thus, there is considerable interest in the elucidation of the signal transduction pathways that mediate the biological effects of the multifunctional agonists gastrin and CCK.

PKC, a major target for the tumor promoting phorbol esters, has been implicated in the signal transduction pathways that mediate a wide range of biological responses in response to growth factors, hormones, neuropeptides and cellular oncogenes [39–41]. Although some studies, using pharmacological inhibitors, have implicated PKC in CCK2 receptor-mediated signal transduction [42,43], direct activation of PKC isoforms in response to gastrin has been less frequently measured. PKD is a novel serine/threonine protein kinase that can be distinguished from PKC isoforms by a variety of criteria including catalytic domain structure, substrate specificity, the presence of a PH domain and absence of a pseudosubstrate autoinhibitory motif which is present in all known PKCs upstream of the first cysteine-rich domain [11]. PKD has been implicated in the regulation of EGF receptor signaling [44], Na^+/H^+ antiport activity [45], Golgi organization and function [46,47], NFkB-mediated gene expression [48] and cell migration [49]. The results presented here demonstrate that stimulation of Rat-1CCK2R cells with gastrin or CCK-8 induces a rapid and striking dose-dependent increase in PKD activity through a PKC-dependent pathway. Our findings identify PKD activation as a novel early event in the action of gastrin and CCK-8 via the CCK2 receptor.

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