Recovery from TPA inhibition of receptor-mediated Ca\(^{2+}\) mobilization is paralleled by down-regulation of protein kinase C-\(\alpha\) in CHO cells expressing the CCK-A receptor

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Summary  Digital-imaging microscopy of Fura-2-loaded Chinese hamster ovary cells, stably expressing the cholecystokinin-A receptor, revealed that both the C-terminal octapeptide of cholecystokinin (CCK\(_\alpha\)) and its analogue JMV-180, which acts as an agonist at the high-affinity CCK-A receptor, recruited CHO-CCK-A cells dose-dependently in terms of receptor-mediated Ca\(^{2+}\) mobilization. Agonist-evoked cell recruitment was inhibited by short-term (10 min) pretreatment with 0.1 \(\mu\)M 12-O-tetradecanoylphorbol 13-acetate (TPA). In the case of CCK\(_\alpha\) inhibition was overcome with increasing of the hormone concentration. In contrast, increasing of the JMV-180 concentration did not reverse the inhibitory action of TPA. CHO-CCK-A cells gradually regained their responsiveness to JMV-180 during prolonged TPA pretreatment. Complete recovery was observed within 1 h following addition of TPA. Western blot analysis using antibodies directed against the various PKC isotypes revealed that recovery was paralleled by the disappearance of PKC-\(\alpha\). Surprisingly, short-term (10 min) TPA pretreatment virtually completely inhibited the formation of inositol 1,4,5-trisphosphate \([\text{Ins}(1,4,5)P_3]\) in response to CCK\(_\alpha\) concentrations at which the effect on cell recruitment was not affected by short term phorbol ester pretreatment. Together with the finding that JMV-180 does not detectably increase the cellular \([\text{Ins}(1,4,5)P_3]\) content, this suggests a large overproduction of this second messenger by CCK\(_\alpha\) concentrations supramaximal in terms of cell recruitment. Again, full responsiveness was observed after long term TPA pretreatment. The present observations are in agreement with the idea that in CHO-CCK-A cells activation of PKC-\(\alpha\) leads to inhibition of agonist-evoked Ca\(^{2+}\) mobilization through inhibition of receptor-stimulated \([\text{Ins}(1,4,5)P_3]\) formation.

INTRODUCTION

Receptors interacting with the cholecystokinin (CCK) family of peptides are widely distributed throughout the gastrointestinal and nervous system [1]. CCK receptors are classified into two subtypes on the basis of their difference in affinity for sulphated versus nonsulphated CCK [1]. Thus, the CCK-B receptor displays similar affinities for both forms of the hormone, whereas the CCK-A receptor has a considerably higher affinity for sulphated CCK-A receptor.

Abbreviations: CCK\(_\alpha\), C-terminal octapeptide of cholecystokinin; JMV-180, Boc-Tyr(SO\(_3\))-Nle-Gly-Trp-Nle-Asp-2-phenylethylester; TPA, 12-O-tetradecanoylphorbol-13-acetate; \([\text{Ins}(1,4,5)P_3]\), inositol 1,4,5-trisphosphate; [Ca\(^{2+}\)], free cytosolic calcium concentration; PKC-\(\alpha\), protein kinase C-\(\alpha\); CHO-CCK-A cells, Chinese hamster ovary cells expressing the cholecystokinin-A receptor.
CCK. In addition, the CCK-B receptor does not discriminate between CCK and gastrin, whereas the CCK-A receptor is highly selective for CCK [1]. Finally, antagonists have been developed, which can effectively discriminate between both subtypes of the CCK receptor [1]. The present study concerns the CCK-A receptor, which, among others, is present on the pancreatic acinar cell [2,3]. Hormonal stimulation of these epithelial cells, which synthesize and store the digestive enzymes, results in the rapid release of these acinar products by means of exocytosis. In the case of CCK, triggering of the secretory event is paralleled by the rapid breakdown of phosphatidylinositol 4,5-bisphosphate to yield inositol 1,4,5-trisphosphate \([\text{Ins}(1,4,5)P_3]\) and 1,2-diacylglycerol (DAG) [4]. \([\text{Ins}(1,4,5)P_3]\) releases Ca\(^{2+}\) from the endoplasmic reticulum, leading to a rapid increase in cytosolic free Ca\(^{2+}\) concentration \([\text{Ca}^{2+}]\), and DAG activates protein kinase C (PKC). Recently, the CCK-A receptor has been cloned and hydropathy plots of its predicted amino acid sequence revealed the presence of 7 transmembrane domains characteristic for members of the G protein-coupled superfamily of receptors [5].

Using freshly isolated pancreatic acinar cells, we [6–9] and others [10,11] demonstrated that short-term activation of PKC by 12-O-tetradecanoylphorbol 13-acetate (TPA) results in inhibition of CCK-stimulated enzyme secretion and Ca\(^{2+}\) mobilization. The inhibitory action of the phorbol ester appeared to be restricted to the lower concentrations of CCK, which led to the conclusion that PKC, directly or indirectly, converts the CCK-A receptor from a high-affinity state into a low-affinity state [8,9]. This idea is supported by the observation that TPA causes the complete loss of high-affinity CCK binding sites [12] and the finding that inhibition by TPA of the stimulatory action of the CCK analogue JMV-180, which acts as an agonist only at the high-affinity CCK-A receptor, is not overcome by increasing the agonist concentration [8,9]. Moreover, TPA has been demonstrated to induce phosphorylation of the receptor in intact acinar cells [13–16] and together with the finding that the intracellular domains of the receptor contain 4 potential phosphorylation sites for PKC [5], this suggests that PKC might act directly, i.e. without the intermediation of a receptor kinase, at the level of the CCK-A receptor.

The aim of the present study was to investigate whether signaling through the CCK-A receptor is still inhibited by TPA when the receptor is expressed in Chinese hamster ovary (CHO) cells. If so, CHO-CCK-A cells would provide a suitable mean to assess the role of the 4 potential phosphorylation sites for PKC by site-directed mutagenesis. Using a video imaging technique, we have previously demonstrated that CCK dose-dependently recruits freshly isolated pancreatic acinar cells in terms of receptor-mediated Ca\(^{2+}\) mobilization [17]. Moreover, we have demonstrated that TPA pretreatment causes a rightward shift of the dose-recruitment curve [9]. Since this technique, if applicable to CHO-CCK-A cells, would enable rapid screening of mutated forms of the CCK-A receptor with respect to inhibition of signaling by TPA, it was investigated whether CCK recruits CHO-CCK-A cells also in a dose-dependent manner and whether TPA causes a similar rightward shift of the dose-recruitment curve. The level at which PKC interferes with signaling through the stably expressed CCK-A receptor was determined by investigating the effect of TPA pretreatment on the kinetics of the CCK-induced formation of \([\text{Ins}(1,4,5)P_3]\). Finally, in order to identify the PKC isotype(s) involved in the inhibitory action of TPA, CHO-CCK-A cells were treated with the phorbol ester for prolonged periods of time and the rate of disappearance of the various PKC isotypes, estimated by means of immunoblotting, was correlated with the recovery from TPA-induced inhibition of CCK-stimulated Ca\(^{2+}\) mobilization and \([\text{Ins}(1,4,5)P_3]\) formation.

The data presented demonstrate that both CCK and JMV-180 dose-dependently recruit CHO cells stably expressing the CCK-A receptor and that short-term TPA pretreatment causes a rightward shift of the dose-recruitment curve for CCK and complete inhibition of JMV-180 evoked cell recruitment. In addition, short-term TPA pretreatment was found to effectively inhibit CCK-evoked \([\text{Ins}(1,4,5)P_3]\) formation. In the continuous presence of TPA, the cells gradually recovered from these inhibitory actions of TPA. The latter process was paralleled by the selective disappearance of PKC-\(\alpha\), suggesting that this PKC isotype intermediates in the inhibitory action of TPA on signaling through the CCK-A receptor stably expressed in CHO cells.

**MATERIALS AND METHODS**

**Materials**

Fura-2/AM was purchased from Molecular Probes Inc., Eugene, OR, USA and \(\beta\)-butyloxy carbonyl-Tyr(SO\(_4\))-Nle-Gly-Tyr-Nle-Asp-2-phenylethyl ester (JMV-180) from Research Plus Inc., Bayonne, NJ, USA. CCK\(_g\) inositol 1,4,5-trisphosphate, chelerythrine chloride and TPA were obtained from Sigma Diagnostics, St Louis, MO, USA and D-myo-[\(\text{H}\)]inositol 1,4,5-trisphosphate (51.4 Ci/mmol) from Amersham, UK. Isotype-specific PKC antibodies and tissue culture medium with additives were purchased from Gibco, Paisley, UK and staurosporine from Boehringer, Mannheim, Germany. 1-block Reagent was obtained from Tropix, Bedford, MA, USA and 4-\(\alpha\)-phorbol 12-myristate 13-acetate from LC Services Corporation, Woburn, MA, USA. All other chemicals were of reagent grade.
Development of a stable CHO-CCK-A cell line

Full-length cDNA encoding the rat CCK-A receptor [5] was originally provided by Dr S.A. Wank (National Institutes of Health, Bethesda, MD, USA). However, because of poor expression, it was decided to use a cDNA truncated to within three nucleotides of the first in frame ATG [18]. This truncated cDNA, subcloned into the mammalian expression vector pTEJ8, was kindly provided by Dr C.D. Logsdon (University of Michigan, Ann Arbor, MI, USA). Transcription of the CCK-A receptor is driven by a ubiquitin promoter. Resistance against G418 is provided by a neomycin phosphotransferase encoding gene under the control of the SV40 early promoter. CHO-K1 cells were originally provided by Dr SA. Wank (National Institutes of Health, Bethesda, MD, USA). However, CHO-K1 cells were grown to 70% confluency, trypsinized and transfected to a cuvette (3 x 10^6 cells/300 μl). The cells were electroporated (250 V, 960 μF) in the presence of 20 μg linearized pTEJ-CCK-A and replated in 100 mm plates (5 μl cell suspension/plate). At 40 h after electroporation, the plates were washed to remove dead cells and G418 was added at a concentration of 1.2 mg/ml. G418-resistant colonies were selected at 14 days after electroporation. The cells were tested on the presence of functional CCK-A receptors by digital imaging microscopy.

Fluorescence measurements in individual CHO-CCK-A cells

For fluorescence measurements, cells were plated on a glass cover slip (2 x 10^4 cells/30 μl) and allowed to attach for 30 min. Culture medium was added and the cells were grown to subconfluency for another 24 h. Cells were loaded with 2 μM Fura-2/AM for 25 min at 37°C. To remove non-hydrolysed dye, cells were washed 3 times with a physiological salt solution (PSS) containing 137 mM NaCl, 4.7 mM KCl, 0.56 mM MgCl_2_2, 1.28 mM CaCl_2, 1.0 mM Na_2HPO_4, 2 mM L-glutamine, 5.5 mM D-glucose, 0.1% (w/v) BSA and 10 mM HEPES (pH 7.4). Cover slips were mounted in a thermostatic (33°C) perfusion chamber, placed on the stage of an inverted microscope (Nikon Diaphot). Superfusion with PSS was at a flow rate of 1 ml/min. Routinely, an epifluorescent 40x magnification oil immersion objective was used to allow simultaneous monitoring of an average of close to 60 cells. Dynamic video imaging was carried out as described previously [17] using the MagiCal hardware and Tardis software provided by Joyce Loeb (Dukeway, Team Valley, Gateshead, UK). The fluorescence emission ratio at 492 nm was monitored as a measure of the cytosolic free Ca^{2+} concentration ([Ca^{2+}]c) after excitation at 340 and 380 nm.

Inhibition of CCK-A receptor signaling by protein kinase C

Ins(1,4,5)P_3 measurements in CHO-CCK-A cells

Cells were plated out in 12-well plates (200,000 cells/well) 24 h previous to hormonal stimulation and Ins(1,4,5)P_3 measurement. TPA, at a final concentration of 0.1 μM, was added at the indicated times. Immediately before stimulation, cells were washed twice with a HEPES/Tris medium (pH 7.4) containing 133 mM NaCl, 4.2 mM KCl, 1.0 mM CaCl_2, 1.0 mM MgCl_2, 5.8 mM glucose, 0.2 mg/ml soybean trypsin inhibitor, an amino acid mixture according to Eagle, 1% (w/v) BSA and 10 mM HEPES, adjusted with Tris to pH 7.4. Each well was stimulated separately by the addition of 250 μl HEPES/Tris medium containing the indicated concentration of hormone. The reaction was stopped by addition of 62 μl 50% trichloroacetic acid at the times indicated. The cells were scraped off the bottom and transferred to a micro test tube (Eppendorf). The samples were centrifuged for 4 min at 10,000 g (Eppendorf minifuge) and a 240 μl aliquot of the supernatant was removed and extracted 3 times with 2 ml of water-saturated diethyl ether. A 150 μl aliquot was then removed and 4 μl 50% KOH was added to increase the pH to a value above 7.5. The Ins(1,4,5)P_3 content of the extract was determined by isotope dilution assay as previously described [8].

PKC-isotype measurements in CHO-CCK-A cells

Total cell lysates were subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred overnight to polyvinylidene difluoride membranes (Immobilon P, Millipore) by Western blotting. Membranes were blocked for 1 h with PBS (pH 7.4) containing 0.2% (w/v) I-Block reagent and 0.1% (v/v) Tween-20 and incubated overnight with PKC-isotype-specific antibodies diluted 1:500 in PBS (pH 7.4) containing 0.1% (w/v) I-Block reagent and 0.2% (v/v) Tween-20. To demonstrate the specificity of the reaction, control membranes were incubated with PKC-isotype-specific antibodies in the presence of corresponding PKC-isoform-specific peptides diluted 1:1000. Membranes were washed with PBS (pH 7.4) containing 0.3% (v/v) Tween-20 (washing buffer) and incubated for 1 h with goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase diluted 1:1000 in PBS (pH 7.4) containing 0.1% (w/v) I-Block reagent and 0.2% (v/v) Tween-20. Membranes were washed with the above washing buffer and PBS before staining with 0.1 M diethanolamine, 0.34 mg/ml Nitroblue tetrazonium, 0.18 mg/ml 5-bromo-4-chloro-3-indolylphosphate and 1.0 M MgCl_2. Membranes were scanned by an Imaging Densitometer (Biorad, München, Germany) and the optical density values were calculated with the computer program Molecular Analyst (Biorad, München, Germany).
Analysis of the data

Half-maximal CCK$_8$ and JMV-180 concentrations for the recruitment of CHO-CCK-A cells in terms of receptor-evoked Ca$^{2+}$ mobilization and maximal recruitment values were calculated by means of the nonlinear regression computer program InPlot (Graphpad Software for Science, San Diego, CA, USA). Scheffe’s F test was used to determine differences between the mean values.

RESULTS

Effect of short-term TPA pretreatment on agonist-evoked calcium mobilization in CHO-CCK-A cells

Chinese hamster ovary cells, stably expressing the CCK-A receptor and grown to subconfluency on glass cover slips, responded with a rapid increase in [Ca$^{2+}$]$_i$ when stimulated with CCK$_8$ (Fig. 1). At low CCK$_8$ concentrations, the response pattern consisted of repetitive increases in [Ca$^{2+}$]$_i$ (Fig. 1A). JMV-180, which acts as an agonist at the high-affinity CCK-A receptor, evoked such oscillatory changes in [Ca$^{2+}$]$_i$ at all concentrations tested. In contrast, high CCK$_8$ concentrations induced a sustained increase in [Ca$^{2+}$]$_i$ (Fig. 1B). CCK$_8$ recruited CHO-CCK-A cells in a dose-dependent manner in terms of receptor-mediated Ca$^{2+}$ mobilization (Fig. 2). Nonlinear regression analysis revealed that half-maximal recruitment was obtained with 0.8 pM CCK$_8$. Pretreatment of the cells with 0.1 μM TPA for 10 min resulted in complete inhibition of cell recruitment by 10 pM CCK$_8$. However, the inhibitory effect of TPA was overcome with increasing of the CCK$_8$ concentration. Thus, at a concentration of 10 nM, CCK$_8$ evoked an increase in [Ca$^{2+}$]$_i$ in 100% of the TPA-treated cells. Nonlinear regression analysis revealed a shift of the EC$_{50}$ value to 70 pM CCK$_8$ in TPA pretreated cells. Similarly, JMV-180 dose-dependently increased the number of responding CHO-CCK-A cells (Fig. 3B). The half-maximal stimulatory concentration of the agonist was calculated to be 0.3 nM. Short-term (10 min) pretreatment with TPA (0.1 μM) completely inhibited cell recruitment by the lower concentrations of JMV-.
Inhibition of CCK-A receptor signaling by protein kinase C

Table 1 Reversal by staurosporine and chelerythrine chloride of the inhibitory action of TPA on JMV-180-evoked recruitment of CHO-CCK-A cells

<table>
<thead>
<tr>
<th>Addition</th>
<th>Responding cells (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JMV-180 (10 nM)</td>
<td>99 ± 0.3</td>
</tr>
<tr>
<td>JMV-180 (10 nM) + TPA (0.1 μM)</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>JMV-180 (10 nM) + 4-α-phorbol 12-myristate 13-acetate (0.1 μM)</td>
<td>90 ± 10</td>
</tr>
<tr>
<td>JMV-180 (10 nM) + TPA (0.1 μM) + staurosporine (0.1 μM)</td>
<td>97 ± 1</td>
</tr>
<tr>
<td>JMV-180 (10 nM) + TPA (0.1 μM) + chelerythrine chloride (2 μM)</td>
<td>87 ± 8</td>
</tr>
</tbody>
</table>

CHO-CCK-A cells were loaded with Fura-2 and stimulated with 10 nM JMV-180 for 5 min. Phorbol ester-treated cells were incubated with 0.1 μM TPA in the absence or presence of either 0.1 μM staurosporine or 2 μM chelerythrine chloride for 10 min. In case of the inactive phorbol ester, cells were preincubated with 0.1 μM 4-α-phorbol 12-myristate 13-acetate for 10 min. The data presented are the mean ± SEM of 3 experiments. On average, 60 cells were analysed in each individual experiment.

The inhibitory action of 0.1 μM TPA was partially overcome with increasing of the JMV-180 concentration. JMV-180 maximally recruited 60% of TPA (0.1 μM) pretreated cells with a half-maximal stimulatory concentration of 50 nM. However, increasing of the TPA concentration to 1 μM completely blocked the recruitment of cells by JMV-180 in 3 independent experiments. Table 1 shows that the inhibitory effect of 0.1 μM TPA on cell recruitment by 10 nM JMV-180 was not observed with the inactive phorbol ester, 4-α-phorbol 12-myristate 13-acetate (0.1 μM). Moreover, Table 1 shows that the inhibitory action of TPA (0.1 μM) was completely reversed in the presence of either staurosporine (0.1 μM) or chelerythrine chloride (2 μM), two potent inhibitors of protein kinase C activity.

Effect of long-term TPA pretreatment on JMV-180-evoked recruitment of CHO-CCK-A cells

Short-term (10 min) pretreatment of CHO-CCK-A cells with 0.1 μM TPA led to a complete loss of responsiveness to 10 nM JMV-180 in approximately 90% of the cells (Fig. 3A). However, an increasing number of cells regained responsiveness to this JMV-180 concentration during prolongation of phorbol ester pretreatment. Control recruitment values were obtained within 1 h of TPA pretreatment. Surprisingly, the number of cells that regained responsiveness to JMV-180, at concentrations submaximal in terms of cell recruitment, did not increase to preinhibitory levels (Fig. 3B). Thus, after 3 h of TPA pretreatment, markedly less cells responded to 0.1 nM and 1 nM JMV-180 than in the control situation and even after 24 h of phorbol ester pretreatment control values were still not reached with these lower concentrations of the agonist. In contrast, control values were already reached within 3 h following the onset of TPA pretreatment with a maximally effective JMV-180 concentration of 10 nM.

Effect of TPA pretreatment on agonist-evoked inositol trisphosphate formation in CHO-CCK-A cells

In the absence of any stimulus, the Ins(1,4,5)P₃ content of CHO-CCK-A cells amounted to 6.2 pmol/mg protein (SE = 1.6, n = 7). Addition of 10 nM CCK₉, a concentration supramaximal in terms of cell recruitment, rapidly increased the cellular Ins(1,4,5)P₃ content to a maximum of 42.7 pmol/mg protein (SE = 5.8, n = 6, Fig. 4). The maximal effect was reached at 10 s following the onset of stimulation and the cellular Ins(1,4,5)P₃ content remained elevated in the continuous presence of the hormone for at least 5 min. In contrast, 1 μM JMV-180, a concentration also supramaximal in terms of cell recruitment, did not significantly increase the cellular Ins(1,4,5)P₃ content. The stimulatory effect of CCK₉ was clearly dose-dependent in that the plateau level readied with 1 nM CCK₉, a concentration still supramaximal in terms of cell recruitment, amounted to 12.3 pmol Ins(1,4,5)P₃/mg protein (SE = 1.2, n = 3). Addition of 0.1 nM CCK₉, being the lowest maximally effective CCK₉ concentration in terms of cell recruitment, did not result in a significant increase in cellular Ins(1,4,5)P₃ content (data not shown).

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Agonist-evoked inositol trisphosphate formation in CHO-CCK-A cells

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**DISCUSSION**

The data presented are in agreement with the idea that short-term (10 min) activation of the α-subtype of protein kinase C leads to inhibition of signaling through the cholecystokinin-A receptor stably expressed in Chinese hamster ovary cells. In the present study, we made use of the ability of phorbol esters to down-regulate PKC. The data presented clearly indicate that the rate of disappearance differed greatly between the 3 PKC isotypes demonstrated in this and other studies [19,20] to be present in CHO cells. PKC-α was down-regulated by 95% at 3 h following the onset of phorbol ester treatment. In contrast, PKC-ε disappeared at a considerably slower rate, whereas
PKC-ζ was not down-regulated at all. The disappearance of PKC-α was paralleled by the recovery from the inhibitory action of TPA on JMV-180-evoked Ca²⁺ mobilization, suggesting that this PKC isotype is involved in phorbol ester-induced inhibition of Ca²⁺ signaling through the CCK-A receptor. Interestingly, complete recovery was observed only with JMV-180 concentrations ≥ 10 nM. As a result, the EC₅₀ value for JMV-180-evoked cell recruitment increased from a control value of 0.3 nM to values of 1.6 nM and 1.3 nM at 3 h and 24 h of TPA treatment, respectively. One explanation might be that low-level PKC-α activation, such as expected to occur during submaximal stimulation with JMV-180, sensitizes rather than inhibits cells with respect to receptor-evoked Ca²⁺ mobilization. A similar observation was reached in a recent study with freshly isolated pancreatic acinar cells [9]. Another explanation might be that prolonged TPA treatment leads to a loss of functional receptors as a result of receptor internalization [21].

In principle, inhibition of receptor-evoked Ca²⁺ mobilization can occur at several levels including the CCK-A receptor, the G-protein involved in signal transduction, the phospholipase C involved in phosphatidylinositol 4,5-bisphosphate breakdown, the enzymes involved in Ins(1,4,5)P₃ metabolism, the Ca²⁺ release channels involved in Ins(1,4,5)P₃-stimulated Ca²⁺ release from internal Ca²⁺ stores and, finally, the mechanisms involved in Ca²⁺ extrusion from the cytosolic compartment. However, the present observation that CCK₈-stimulated Ins(1,4,5)P₃ formation was significantly inhibited in TPA-treated CHO-CCK-A cells suggests that PKC exerts its effect upstream to the process of Ins(1,4,5)P₃-stimulated Ca²⁺ release. It has been reported that phorbol esters can stimulate the phosphorylation of the CCK-A receptor in intact rat pancreatic acinar cells [14]. Moreover, recent elucidation of the primary structure of the CCK-A receptor has revealed the presence of several potential phosphorylation sites for PKC [5]. Taken together, most of the information presently available is in agreement with the idea that PKC directly phosphorylates the CCK-A receptor, thus inhibiting agonist-induced Ins(1,4,5)P₃ formation.

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and subsequent Ca\(^{2+}\) mobilization. It should be noted that this conclusion is not supported by the finding that, in rat pancreatic acinar cells, TPA effectively blocked the CHO-CCK-A cells used in the present study did not display a rise in [Ca\(^{2+}\)]\(_i\) in response to mastoparan (data not shown). Furthermore, the present observation that PKC inhibits signaling through the CCK-A receptor stably expressed in CHO cells does not favour the idea that PKC acts indirectly through a receptor-specific kinase.

Evidence in support of such a mechanism has recently been provided for the β-adrenergic receptor [23].

Comparison of the dose-response curves for the stimulatory effects of CCK\(_B\) on cell recruitment and Ins\((1,4,5)\)P\(_3\) formation revealed that the hormone, at a concentration of 0.1 nM, already maximally recruited CHO-CCK-A cells in terms of receptor-mediated Ca\(^{2+}\) mobilization without detectably increasing the cellular Ins\((1,4,5)\)P\(_3\) content. Significant stimulation of Ins\((1,4,5)\)P\(_3\) formation was only observed with CCK\(_B\) concentrations > 1 nM that are supramaximal in terms of cell recruitment. Similar results have previously been observed with freshly isolated rabbit pancreatic acinar cells [9]. In contrast, JMV-180, at concentrations supramaximal in terms of cell recruitment, did not detectably stimulate the production of Ins\((1,4,5)\)P\(_3\) in CHO-CCK-A cells. Similarly, JMV-180 has been demonstrated to poorly stimulate Ins\((1,4,5)\)P\(_3\) formation in pancreatic acinar cells [24,25]. Taken together, these observations suggest that Ins\((1,4,5)\)P\(_3\) is either overproduced during supramaximal stimulation with CCK\(_B\) or not involved in Ca\(^{2+}\) mobilization during supramaximal stimulation with CCK\(_B\) or stimulation with JMV-180. The latter explanation implies the presence of an alternative messenger system in both pancreatic acinar cells and CHO cells. However, recent experiments have provided substantial evidence that JMV-180 does act through the intermediation of Ins\((1,4,5)\)P\(_3\) to stimulate the release of Ca\(^{2+}\) from intracellular stores [26–28]. Unlike in pancreatic acinar cells [9], short-term (10 min) TPA pretreatment dramatically reduced Ins\((1,4,5)\)P\(_3\) formation in response to CCK\(_B\) concentrations supramaximal in terms of cell recruitment. Surprisingly, this reduction did not lead to any decrease in the number of cells recruited by these supramaximal CCK\(_B\) concentrations, indicating that Ins\((1,4,5)\)P\(_3\) is produced in sufficient amounts to promote the release of Ca\(^{2+}\) from internal stores.

In contrast to freshly isolated pancreatic acinar cells [8], CHO-CCK-A cells responded with a sustained increase in Ins\((1,4,5)\)P\(_3\), suggesting that either Ins\((1,4,5)\)P\(_3\) production continues at a high rate or Ins\((1,4,5)\)P\(_3\) metabolism occurs at a reduced rate in CHO-CCK-A cells. In case Ins\((1,4,5)\)P\(_3\) production continues at a high rate, this might suggest that CHO-CCK-A cells lack a receptor-specific kinase which rapidly desensitizes the receptor leading to a drop in Ins\((1,4,5)\)P\(_3\) production rate.

PKC-mediated inhibition of signaling through the CCK-A receptor appeared to be restricted to CCK\(_B\) concentrations at which the hormone submaximally stimulated the process of cell recruitment in terms of receptor-mediated Ca\(^{2+}\) mobilization and was completely overcome with increasing of the CCK\(_B\) concentration. In contrast, inhibition of the stimulatory effect of JMV-180, which acts as an agonist at the high-affinity CCK-A receptor, was not overcome with increasing of the agonist concentration. Similar observations, reached with freshly isolated rabbit pancreatic acinar cells, have led to the hypothesis that PKC activation results in inhibition of signaling through the high-affinity CCK-A receptor [8,9]. Moreover, from the observation that Ins\((1,4,5)\)P\(_3\) formation in response to the higher CCK\(_B\) concentrations was not inhibited by TPA, it was concluded that PKC did not affect signaling through the low-affinity CCK-A receptor. Therefore, the present study clearly demonstrates the ability of TPA to significantly inhibit Ins\((1,4,5)\)P\(_3\) formation in response to CCK\(_B\) acting through the low-affinity receptor in CHO-CCK-A cells. This observation suggests that in CHO cells, in contrast to pancreatic acinar cells, the low-affinity CCK-A receptor is affected in a PKC-dependent manner.

The EC\(_{50}\) value for the stimulatory effect of CCK\(_B\) on the recruitment of CHO-CCK-A cells was calculated to be 0.8 pM. This value is 20-fold lower as compared to the value of 16.8 pM obtained with rabbit pancreatic acinar cells [9]. Similarly, the EC\(_{50}\) value for the stimulatory effect of JMV-180 on the recruitment of CHO-CCK-A cells, calculated to be 0.3 nM, is 30-fold lower as compared to the value of 9 nM obtained with rabbit pancreatic acinar cells (R.L.L. Smeets, K.M. Garner, J.H.J.H.M. de Pont and P.H.G.M. Willems, unpublished observations). Moreover, JMV-180 maximally recruited 100% of the CHO-CCK-A cells, whereas in the case of rabbit pancreatic acinar cells this value amounted to 70%. These observations demonstrate that, for hitherto unknown reasons, CHO-CCK-A cells are more sensitive to stimulation by CCK\(_B\) than freshly isolated rabbit pancreatic acinar cells. Thus far, the mechanism underlying the dose-dependent recruitment of cells in terms of agonist-induced Ca\(^{2+}\) mobilization is unclear. Although it can be argued that the differences in sensitivity observed within a population of freshly isolated cells are the result of receptor damaging during the isolation procedure, this is not likely to be the case with the CHO-CCK-A cells used in this study.

In summary, the data presented are in agreement with the idea that high-level PKC-α activation by TPA inhibits signaling through the CCK-A receptor in CHO-CCK-A
cells at the level of receptor-stimulated Ins(1,4,5)P3 formation. It remains to be elucidated whether PKC-α acts directly at the receptor protein.

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