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Protein kinase C-mediated inhibition of transmembrane signalling through CCK_A and CCK_B receptors

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1 The rat CCK_A and CCK_B receptors were stably expressed in Chinese hamster ovary (CHO-09) cells in order to compare modes of signal transduction and effects of protein kinase C (PKC) thereupon.

2 Spectrofluorophotometry of Fura-2-loaded cells revealed that both receptors retained their pharmacological characteristics following expression in CHO cells. Sulphated cholecystokinin-(26-33)-peptide amide (CCK-8-S) increased the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in CCK_A cells, measured as an increase in Fura-2 fluorescence emission ratio, 1000 fold more potently than its non-sulphated form (CCK-8-NS) (EC_{50} values of 0.19 nM and 0.18 μM, respectively). By contrast, CCK-8-S and CCK-8-NS were equally potent in CCK_B cells (EC_{50} values of 0.86 nM and 1.18 nM, respectively). The CCK_A receptor agonist JMV-180 increased $[Ca^{2+}]_i$ only in CCK_A cells. Likewise, pentagastrin increased $[Ca^{2+}]_i$ only in CCK_B cells. Finally, CCK-8-S-induced Ca^{2+} signalling through the CCK_A receptor was most potently inhibited by the CCK_A receptor antagonist L364,718, whereas the CCK_B receptor antagonist L365,260 was more potent in CCK_B cells.

3 Receptor-mediated activation of adenylyl cyclase was measured in the presence of the inhibitor of cyclic nucleotide phosphodiesterase activity, 3-isobutyl-1-methylxanthine. CCK-8-S and, to a lesser extent, CCK-8-NS, but not JMV-180 or pentagastrin, stimulated the accumulation of cyclicAMP in CCK_A cells. By contrast, none of these agonists increased cyclicAMP in CCK_B cells.

4 Short-term (3 min) pretreatment with the PKC activator 12-*O*-tetradecanoylphorbol 13-acetate (TPA) evoked a rightward shift of the dose-response curve for the Ca^{2+} mobilizing effect of CCK-8-S in both cell lines. In addition, short-term TPA pretreatment markedly reduced CCK-8-S-induced cyclicAMP accumulation in CCK_A cells. In both cases, the inhibitory effect of TPA was abolished by the PKC inhibitors, GF-109203X and staurosporine, whereas no inhibition was observed with the inactive phorbol ester, 4-α-phorbol 12-myristate 13-acetate.

5 During prolonged TPA treatment, the cells gradually recovered from phorbol ester inhibition and in the case of CCK-8-S-induced Ca^{2+} mobilization complete recovery was achieved after 24 h of TPA treatment. Western blot analysis revealed that this recovery was paralleled by down-regulation of PKC-α, suggesting the involvement of this PKC isotype in the inhibitory action of TPA.

6 This study demonstrates that following expression in CHO cells (i) both CCK_A and CCK_B receptors are coupled to Ca^{2+} mobilization, (ii) only CCK_A receptors are coupled to cyclicAMP formation and (iii) with both receptors signalling is inhibited by PKC.

Keywords: $Cholecystokinin_A$ receptor; $cholecystokinin_B$ receptor; Chinese hamster ovary cells; CCK-8; JMV-180; calcium mobilization; cyclicAMP formation; TPA; protein kinase C down-regulation

Introduction

Receptors interacting with the cholecystokinin (CCK) and gastrin families of peptides have been demonstrated to be widely distributed throughout the gastrointestinal and nervous system (Wank, 1995). Initially, three distinct CCK receptors were identified on the basis of differences in relative affinities for cholecystokinin-(26-33)-peptide amide (CCK-8) and gastrin-like peptides. However, recent cloning and subsequent primary amino acid sequence deduction revealed the existence of only two CCK receptor subtypes, CCK_A and CCK_B, the latter being identical to the originally postulated gastrin receptor (Kopin *et al*., 1992; Pisegna *et al*., 1992; Wank *et al*., 1992b; Wank, 1995).

Hydropathy plots of predicted amino acid sequences of cloned CCK_A and CCK_B receptors revealed the presence of 7 transmembrane domains (Wank *et al*., 1992a). This is in agreement with the idea that both receptors belong to the superfamily of G protein-coupled receptors. It is well established that both receptors couple to phospholipase C to promote the hydrolysis of phosphatidylinositol 4,5-bisphosphate, leading to the increased production of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$), releasing Ca^{2+} from intracellular stores, and 1,2-diacylglycerol, activating protein kinase C (PKC) (Wank, 1995).

In addition, CCK-induced increases in adenosine 3′:5′-cyclic monophosphate (cyclicAMP) have been demonstrated in intact pancreatic acinar cells as well as in isolated pancreas membranes, suggesting G protein-mediated coupling of CCK_A receptors to adenylyl cyclase (for references, see Willems *et al*., 1984; 1987). This idea is supported by the observation that CCK readily increased cyclicAMP in Chinese hamster ovary (CHO) cells stably transfected with the CCK_A receptor (Yule *et al*., 1993). Although CCK-stimulated digestive enzyme secretion is markedly potentiated by cyclicAMP, the physiological relevance of CCK-induced stimulation of adenylyl cyclase activity has been questioned, since CCK-induced increases in cyclicAMP require the presence of an inhibitor of cyclic nucleotide phosphodiesterase activity and stimulation with relatively high CCK concentrations (Gardner & Jensen, 1993; Williams & Yule, 1993). However, in a recent study, CCK-induced increases in protein kinase A activity were

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measured in the absence of such an inhibitor and at physiological CCK concentrations (Marino *et al*., 1993). At present, information on cyclicAMP signalling through the CCK_B receptor is scarce. Galas *et al*. (1992) found that CCK did not increase adenylyl cyclase activity in a preparation of synaptoneurosomes. However, in the same study CCK was found to be unable to increase the activity of phospholipase C.

We have previously shown that activation of PKC by the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) inhibits high-affinity Ca^{2+} signalling through the CCK_A receptor in freshly isolated pancreatic acinar cells (Willems *et al*., 1993b; 1995) and stably transfected CHO-K1 cells (Smeets *et al*., 1996). The aim of the present work was to compare modes of signal transduction and regulation of receptor functioning by PCK of pure CCK_A and CCK_B receptors after functional expression in the same eukaryotic expression system, namely the CHO-09 cell.

Methods

Development of stable CCK_A and CCK_B cell lines

Full-length cDNAs encoding the rat CCK_A (Wank *et al*., 1992a) and CCK_B (Wank *et al*., 1992b) receptor were provided by Dr S.A. Wank (National Institutes of Health, Bethesda, MD, U.S.A.). Because of poor expression of the full-length cDNA of the CCK_A receptor, it was decided to use a cDNA truncated to within three nucleotides of the first in frame ATG (Yule *et al*., 1993). This truncated cDNA, subcloned into the mammalian expression vector pTEJ8, was kindly provided by Dr C.D. Logsdon (University of Michigan, Ann Arbor, MI, U.S.A.). Transcription of the CCK_A receptor was driven by a ubiquitin promoter. Resistance against G418 was provided by a neomycin phosphotransferase encoding gene under the control of the SV40 early promoter. Full-length cDNA of the CCK_B receptor was subcloned into the *EcoRI*-site of the mammalian expression vector pcDNA3 (Invitrogen Corporation, Carlsbad, CA, U.S.A.). Transcription of the CCK_B receptor was driven by the enhancer-promoter sequences of the early gene of human cytomegalovirus. Resistance against G418 was provided by a neomycine resistance gene under the control of an SV40 promoter. CHO-09 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and 50 μg ml^{-1} gentamicin in a humidified atmosphere of 5% CO_2 at 37°C. For transfection, cells were grown to 70% confluency, trypsinized and transferred to a cuvette (3×10^6 cells 300 μl^{-1}). The cells were electroporated (250 V, 960 μF) in the presence of 20 μg linearized pTEJ8-CCK_A or pcDNA3-CCK_B and replated in 100 mm plates (5 μl cell suspension/plate). At 40 h after electroporation, the plates were washed to remove dead cells. G418 was added at a concentration of 0.6 mg ml^{-1} and resistant colonies were selected at 14 days after electroporation. In order to obtain clonal cell lines, cells were seeded at a density of 0.5 cells per well in a 12-well plate. The cell lines were tested on the presence of functional CCK receptors by digital imaging microscopy.

Fluorescence measurements in individual CHO cells

Fluorescence measurements in individual CHO cells were carried out as described previously (Smeets *et al*., 1996; 1997). Briefly, cells were plated on a glass cover slip (2×10^4 cells 30 μl^{-1}) and allowed to attach for 30 min. Medium was added and cells were grown to subconfluency for another 24 h. Cells were loaded with Fura-2 in the presence of 2 μM Fura-2/AM and 0.025% (w/v) pluronic F-127 for 25 min at 37°C. After loading, excess Fura-2/AM was removed by washing the cells 3 times with a physiological salt solution (PSS) containing in mM NaCl 137, KCl 4.7, $MgCl_2$ 0.56, $CaCl_2$ 1.28, Na_2HPO_4 1, L-glutamine 2, D-glucose 5.5, 0.1% (w/v) BSA and HEPES 10 (pH 7.4). Cover slips were mounted in a thermostatic (34°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^{-1}. An epifluorescent 40 × magnification oil immersion objective was used to allow simultaneous monitoring of an average of close to 80 individual cells. Dynamic video imaging was carried out as described previously (Willems *et al*., 1993a) by use of the MagiCal hardware and TARDIS software provided by Joyce Loebl (Dukesway, Team Valley, Gateshead, U.K.). The fluorescence emission ratio at 492 nm was monitored as a measure of the cytosolic free calcium concentration ($[Ca^{2+}]_i$) after excitation at 340 and 380 nm.

Binding of [^{3}H]-CCK-8-S to membranes of CHO-09-CCK_A and CHO-09-CCK_B cells

Binding studies were performed according to the method described by Van Dijk *et al*. (1984). Briefly, cells were trypsinized and resuspended in a homogenization medium containing 10 mM HEPES (pH 7.4), 130 mM NaCl, 5 mM $MgCl_2$ and 0.01% (w/v) soybean trypsin inhibitor. After homogenization, the equivalent of 375,000 cells was transferred to glass tubes and preincubated for 30 min at 25°C. The binding reaction was started by addition of [^{3}H]-CCK-8-S at concentrations ranging from 0.199 nM to 3.181 nM. At 90 min, the reaction was stopped by rapid filtration through presoaked Whatman GF/B filters. The filters were washed twice with 3 ml ice-cold homogenization buffer and counted for radioactivity. Nonspecific binding, determined in the presence of 0.1 μM CCK-8-S, was less than 10% of total binding. After correction for nonspecific binding, the equilibrium dissociation constant (K_d) and binding capacity (B_{max}) were evaluated by Scatchard analysis.

Fluorescence measurements in suspensions of CHO cells

Fluorescence measurements in suspensions of CHO cells were performed as described previously (Smeets *et al*., 1997). Briefly, CHO cells were seeded in 25 cm^2 culture flasks (1×10^6 cells/flask) and grown for 48 h. Cells were trypsinized and washed twice in a HEPES/Tris medium containing in mM NaCl 133, KCl 4.2, $CaCl_2$ 1, $MgCl_2$ 1, D-glucose 5.8, soybean trypsin inhibitor 0.2 mg ml^{-1}, an amino acid mixture according to Eagle, 0.1% (w/v) BSA and HEPES 10, adjusted with Tris to pH 7.4. Cells were resuspended in HEPES/Tris medium containing 1% (w/v) BSA and loaded with Fura-2 in the presence of 2 μM Fura-2/AM and 0.025% (w/v) pluronic F-127 for 20 min at 37°C. Excess Fura-2/AM was removed by washing twice with HEPES/Tris medium containing 0.1% (w/v) BSA. Cells were transferred to a cuvette placed in a Shimadzu RF-5000 spectrofluorophotometer equipped with a magnetic stirrer and a thermostated (37°C) cuvette holder. The fluorescence emission ratio at 490 nm was monitored as a measure of the average $[Ca^{2+}]_i$ after excitation at 340 and 380 nm.

CyclicAMP measurements in CHO cells

For cyclicAMP measurements, CHO cells were seeded at a density of 200,000 cells per well in a 12-well plate and grown for 24 h. Cells were washed twice and preincubated with 250 μl

of the above HEPES/Tris medium (pH 7.4) containing 0.1% (w/v) BSA, 0.1 mM 3-isobutyl-l-methylxanthine and, where mentioned, the indicated concentrations of TPA, 4-α-phorbol 12-myristate 13-acetate, staurosporine, GF-109203X, CGP-41251 or JMV-180 for 10 min. At 10 min, the preincubation medium was replaced by 250 μl of medium identical to the preincubation medium and containing in addition the indicated concentrations of either CCK-8-S, CCK-8-NS, pentagastrin, JMV-180 or forskolin. At 10 min, the reaction was quenched by the addition of 250 μl of 10% (w/v) trichloroacetic acid. The cells were scraped off, transferred to a micro test tube (Eppendorf) and immediately frozen in liquid nitrogen. After being thawed, the samples were vigorously mixed and centrifuged for 4 min at $10{,}000 \times g$ (Eppendorf minifuge). A 350 μl aliquot of the supernatant was removed and extracted 3 times with water-saturated diethyl ether. The cyclicAMP content of the supernatant was determined by saturation assay with cyclicAMP binding protein as described previously (Willems *et al*., 1984; 1987).

PKC-isotype measurements in CHO cells

PKC-isotype measurements in CHO-09 cells were performed as described previously (Smeets *et al*., 1996). Briefly, total cell lysates were subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred overnight to polyvinylidine 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-isotype-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 being stained with 0.1 M diethanolamine, 0.34 mg ml^{-1} Nitroblue tetrazolium, 0.18 mg ml^{-1} 5-bromo-4-chloro-3-indolylphosphate and 1.0 M $MgCl_2$. Membranes were scanned by an Imaging Densitometer (BioRad, Münich, Germany) and the optical density values were calculated with the computer programme Molecular Analyst (BioRad, Münich, Germany).

Materials

Staurosporine and cyclicAMP were purchased from Boehringer (Mannheim, Germany) and sulphated and non-sulphated cholecystokinin-(26-33)-peptide amide (CCK-8), pentagastrin, 3-isobutyl-l-methylxanthine, bovine serum albumin (BSA), soybean trypsin inhibitor and TPA from Sigma Diagnostics (St. Louis, MO, U.S.A.). Fura-2/AM and pluronic F-127 were obtained from Molecular Probes Inc. (Eugene, OR, U.S.A.) and *t*-butyloxycarbonyl-Tyr(SO_3H)-Nle-Gly-Trp-Nle-Asp-2-phenylethyl ester (JMV-180) from Research Plus Inc. (Bayonne, NJ, U.S.A.). Activated charcoal (Norit, SX-1) was purchased from Norit (Amersfoort, The Netherlands) and 4-α-phorbol 12-myristate 13-acetate from LC Services Corporation (Woburn, MA, U.S.A.). [^{3}H]-CCK-8-S (67 Ci $mmol^{-1}$) and [^{3}H]-cyclicAMP (41 Ci $mmol^{-1}$) were obtained from Amersham International plc (Little Chalfont, Buckinghamshire, U.K.) and CGP-41251 (N-benzoylstaurosporine) from Ciba-Geigy (Basel, Switzerland). Forskolin and GF-109203X (2[1-(3-dimethylaminopropyl)-I*H*-indol-3-yl]-3-(I*H*-indol-3-yl)-maleimide) were purchased from Calbiochem (La Jolla, CA, U.S.A.) and isotype-specific PKC antibodies and tissue culture medium with additives from Gibco (Paisley, Scotland). I-block reagent was obtained from Tropix (Bedford, MA, U.S.A.). The cyclicAMP binding protein was isolated from bovine adrenal cortex as described by Brown *et al*. (1971). All other chemicals were of reagent grade.

Analysis of data

Half-maximal agonist concentrations for the peak increase in average cytosolic free Ca^{2+} concentration were calculated by means of the nonlinear regression computer programme InPlot (Graphpad Software for Science, San Diego, CA, U.S.A.). Student's *t* test was used to determine statistical differences ($P < 0.05$).

Results

Binding of [^{3}H]-CCK-8-S to membranes of CHO-09-CCK_A and CHO-09-CCK_B cells

Digital imaging microscopy of fura-2-loaded cells was used to screen G418-resistant colonies of CHO-09 cells, transfected with the cDNA of either the CCK_A or CCK_B receptor, for the presence of functional CCK receptors. With this procedure, two cell lines were selected, one for each receptor subtype, in which 100% of the cells displayed an increase in $[Ca^{2+}]_i$ in response to stimulation with 10 nM CCK-8-S (Figure 1). Mock-transfected CHO-09 cells did not respond to CCK-8-S concentrations as high as 1 μM (data not shown).

Binding of [^{3}H]-CCK-8-S to membranes prepared from the two cell lines was performed to examine possible differences in equilibrium dissociation constant (K_d) and/or binding capacity (B_{max}). Scatchard analysis of the binding data showed a similar K_d value for the CCK_A and CCK_B receptor (K_d values of 0.73 nM and 0.93 nM, respectively). Evaluation of the B_{max} values revealed that CHO-09-CCK_A cells expressed more receptors than CHO-09-CCK_B cells (B_{max} values of 270 and 13 fmol mg^{-1} protein, respectively).

Pharmacological characterization of the rat CCK_A and CCK_B receptor expressed in CHO-09 cells

The selected CCK_A and CCK_B cell lines were further characterized by spectrofluorophotometry. We have previously demonstrated that addition of the sulphated form of CCK-8 (CCK-8-S) to a suspension of fura-2-loaded cells expressing the CCK_A receptor results in a transient increase in fluorescence emission ratio, the peak value of which is a function of the concentration of the agonist (Willems *et al*., 1993b). The concentration at which CCK-8-S half-maximally increased the peak value (EC_{50}) was calculated to be 0.19 nM and 0.86 nM for CCK_A and CCK_B cells, respectively (Table 1). Similarly to CCK-8-S, the non-sulphated form of CCK-8 (CCK-8-NS) evoked a transient increase in both cell lines. In CCK_B cells, CCK-8-NS and CCK-8-S were equally potent (EC_{50} values of 1.18 nM and 0.86 nM, respectively). Conversely, in CCK_A cells the EC_{50} for the stimulating effect of CCK-8-NS, calculated to be 0.18 μM, was 1000 fold lower than that of CCK-8-S. The CCK_A receptor agonist

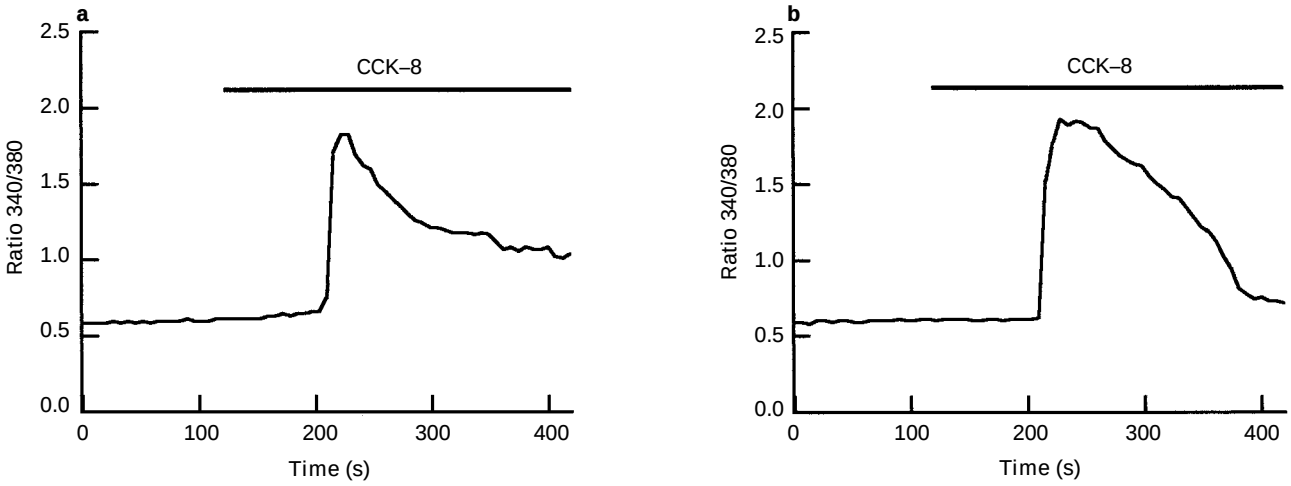

Figure 1 CCK-8-S-induced increase in cytosolic free Ca^{2+} concentration in individual CHO-09-CCK_A (a) and CHO-09-CCK_B (b) cells. CHO-09 cells were loaded with Fura-2 and stimulated with 10 nM CCK-8-S for the indicated period of time. Changes in $[Ca^{2+}]_i$ were monitored by digital imaging microscopy.

Table 1 EC_{50} values for the agonist-induced increase in $[Ca^{2+}]_i$ in CHO-09-CCK_A and CHO-09-CCK_B cells

Ligand	*EC_{50} CHO-CCK_A cells*	*EC_{50} CHO-CCK_B cells*
CCK-8-S	0.19 nM	0.86 nM
CCK-8-NS	0.18 μM	1.18 nM
JMV-180	25 nM	ND
pentagastrin	ND	1.20 nM

ND = no detectable increase in $[Ca^{2+}]_i$ after stimulation with high concentrations of ligand. CHO-09 cells, resuspended in incubation medium, were loaded with Fura-2 and transferred to a cuvette placed in a spectrofluorophotometer. Dose-response curves for the effects of the various agonists on the peak increase in $[Ca^{2+}]_i$ were prepared as described in the caption of Figure 2. EC_{50} values were calculated by means of a nonlinear regression computer programme.

Table 2 IC_{50} values for the inhibiting effect of L364,718 and L365,260 on the CCK-8-S-evoked increase in $[Ca^{2+}]_i$ in CHO-09-CCK_A and CHO-09-CCK_B cells

Antagonist	*IC_{50} CHO-CCK_A cells*	*IC_{50} CHO-CCK_B cells*
L364,718	1.5 nM	410 nM
L365,260	901 nM	76 nM

CHO-09 cells, resuspended in incubation medium, were loaded with Fura-2 and transferred to a cuvette placed in a spectrofluorophotometer. Cells were incubated in the presence of the indicated concentration of antagonist for 3 min before being stimulated with 1 nM CCK-8-S. In each experiment the CCK-8-S-induced peak increase in $[Ca^{2+}]_i$ measured in the absence of antagonist is set at 100%, to which all other values were related. IC_{50} values were calculated by means of a nonlinear regression computer programme.

JMV-180 readily increased $[Ca^{2+}]_i$ in CCK_A cells. The EC_{50} was calculated to be 25 nM. JMV-180 did not affect the cytoplasmic free Ca^{2+} concentration in CCK_B cells. Similarly, pentagastrin increased $[Ca^{2+}]_i$ in CCK_B cells without having an effect on the cytoplasmic free Ca^{2+} concentration in CCK_A cells. The EC_{50} for the stimulating effect of pentagastrin in CCK_B cells was calculated to be 1.20 nM.

Preincubation of CCK_A cells with the CCK_A receptor antagonist L364,718 (3S(−)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3-yl)-1H-indole-Z-carboxamide) resulted in a dose-dependent reduction of the peak increase in fluorescence emission ratio in response to subsequent stimulation with 1 nM CCK_8. Half-maximal inhibition was obtained with 1.5 nM L364,718 (Table 2). L364,718 was considerably less potent in inhibiting the CCK-8-S-evoked increase in fluorescence emission ratio in CCK_B cells, as was indicated by a 270 fold higher IC_{50} value of 410 nM. The CCK_B receptor antagonist L365,260 (3**R**(+)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3-yl)-N′-(3-methylphenyl-urea) reduced the CCK-8-S-evoked peak increase in fluorescence emission ratio most effectively in CCK_B cells. The IC_{50} value was calculated to be 76 nM. In CCK_A cells half-maximal inhibition was reached at a 12 fold higher concentration of 901 nM.

Inhibition of Ca^{2+} signalling through the CCK_A and CCK_B receptor expressed in CHO cells by phorbol ester treatment

Short-term (3 min) treatment of CCK_A cells with 0.1 μM 12-*O*-tetradecanoylphorbol 13-acetate (TPA) resulted in a significant reduction of the peak increase in fluorescence emission ratio evoked by submaximal (< 10 nM) concentrations of CCK-8-S (Figure 2a). As a result, the EC_{50} value for the stimulating effect of CCK-8-S increased approximately 12 fold from a value of 0.19 nM in untreated control cells to a value of 2.26 nM in TPA-treated cells. However, during prolonged treatment with TPA the cells gradually recovered from the inhibitory action of the phorbol ester. Thus, at 3 h following the onset of TPA treatment the cells had already partly regained their sensitivity to CCK-8-S, as was indicated by a 3.6 fold decrease of the EC_{50} value to 0.62 nM. Full recovery was observed at 24 h of TPA treatment. Cells treated with phorbol ester for 24 h displayed a slightly increased sensitivity to CCK-8-S in that the EC_{50} value, calculated to be 0.07 nM, was 2.8 fold lower than the value obtained with untreated control cells.

In CCK_B cells, short-term (3 min) pretreatment with 0.1 μM TPA not only reduced the increase in fluorescence emission ratio in response to submaximal (< 10 nM), but also to maximal ($\geqslant$ 10 nM), concentrations of CCK-8-S (Figure 2b). As a result, the EC_{50} increased from 0.86 nM in untreated control cells to

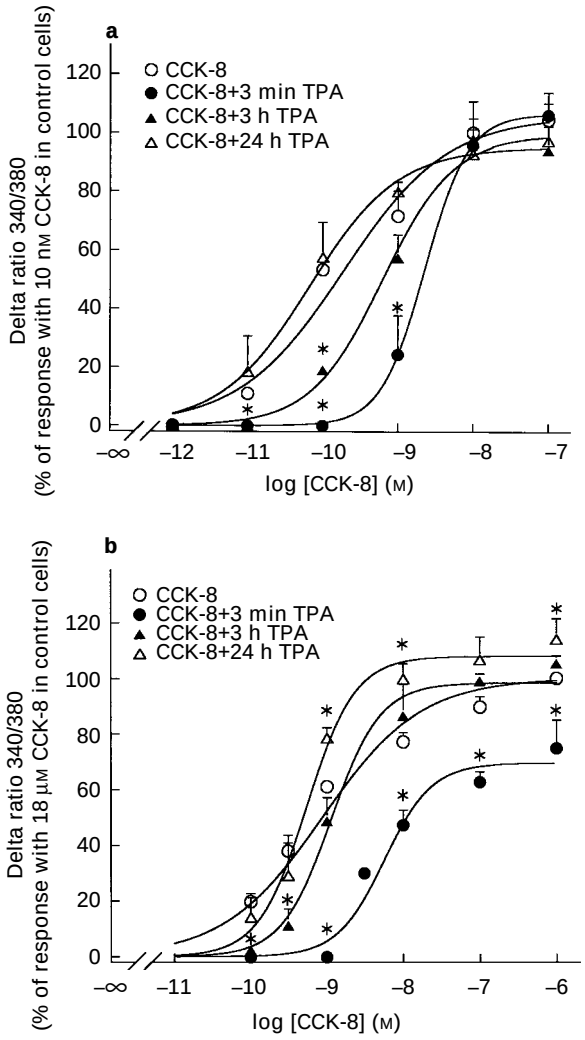

Figure 2 Time-dependence of the inhibitory effect of TPA pretreatment on the CCK-8-S-induced peak increase in $[Ca^{2+}]_i$ in suspensions of CHO-09-CCK_A cells (a) and CHO-09-CCK_B cells (b). CHO-09 cells, preincubated in the absence or presence of 0.1 μM TPA for 3 min, 3 h or 24 h, were loaded with Fura-2 and transferred to a cuvette placed in a spectrofluorophotometer. In each experiment, the peak increase in $[Ca^{2+}]_i$ obtained with 10 nM CCK-8-S (a) or 1 μM CCK-8-S (b) was set at 100% to which all other values were related. The data presented are the mean of 3–4 experiments; vertical lines show s.e.mean. *Significantly different from untreated cells ($P<0.05$).

5.63 nM in phorbol ester-treated cells, whereas the efficacy of the hormone was calculated to be decreased by 25%. At 3 h of TPA treatment, the stimulating effect of maximal CCK-8-S concentrations was completely restored. By contrast, the response to CCK-8-S concentrations below 1 nM, albeit partly restored, was still significantly reduced. The EC_{50} was calculated to be decreased 5 fold to 1.12 nM. With the lower CCK-8-S concentrations, control values for the increase in fluorescence emission ratio were reached at 24 h following the onset of TPA treatment. In these cells, the values for the peak increase in fluorescence emission ratio obtained with 1 nM and 10 nM CCK-8-S were significantly higher than the corresponding values in untreated control cells. Compared to untreated control cells, the EC_{50} value was slightly decreased to 0.54 nM.

Table 3 shows that the inhibiting effect of short-term (3 min) treatment with 0.1 μM TPA was not mimicked by the inactive phorbol ester, 4-α-phorbol 12-myristate 13-acetate (0.1 μM). Moreover, Table 3 shows that the two

Table 3 Reversal by staurosporine and GF-109203X of the inhibitory action of TPA on the CCK-8-S-induced peak increase in $[Ca^{2+}]_i$ in suspensions of CHO-09-CCK_A and CHO-09-CCK_B cells

	CHO-CCK_A cells	*CHO-CCK_B cells*
CCK-8-S-induced peak increase in $[Ca^{2+}]_i$ in untreated cells	100%	100%
CCK-8-S-induced peak increase in $[Ca^{2+}]_i$ (% of untreated)		
TPA + CCK-8-S	30.6 ± 4.6% [a]	25.4 ± 9.3% [a]
4-α-PMA + CCK-8-S	82.9 ± 10.4%	89.1 ± 19.5%
GF-109203X + CCK-8-S	138.4 ± 7.8% [a]	93.5 ± 14.5%
GF-109203X + TPA + CCK-8-S	100.6 ± 4.7%	96.7 ± 7.2%
Staurosporine + CCK-8-S	108.0 ± 11.0%	101.9 ± 13.6%
Staurosporine + TPA + CCK-8-S	94.1 ± 7.4%	84.7 ± 9.8%

[a]Compared with untreated CCK-8-S-stimulated cells ($P<0.01$). Cells, loaded with Fura-2, were transferred to a cuvette placed in a spectrofluorophotometer and preincubated in the absence or presence of TPA (0.1 μM) or 4-α-phorbol 12-myristate 13-acetate (4-α-PMA 0.1 μM) and/or staurosporine (1 μM) or GF-109203X (10 μM) for 3 min. CHO-09-CCK_A and CHO-09-CCK_B cells were then stimulated with 0.1 nM and 1 nM CCK-8-S, respectively. In each experiment, the peak increase in $[Ca^{2+}]_i$ obtained with CCK-8-S in untreated cells was set at 100%, to which all other values were related. The data presented are the mean ± s.e.mean of 3–4 experiments.

potent inhibitors of PKC activity staurosporine (1 μM) and GF-109203X (10 μM) completely reversed the inhibitory action of TPA. Neither staurosporine nor GF-109203X affected basal $[Ca^{2+}]_i$. The effect of the PKC inhibitor CGP-41251 could not be tested due to its autofluorescent properties.

Stimulation of cyclicAMP formation through the CCK_A, but not CCK_B, receptor expressed in CHO cells

The ability of CCK_A and CCK_B receptors to couple functionally to adenylyl cyclase was investigated by measuring the accumulation of cyclicAMP 10 min following the onset of stimulation of CHO-09 cells expressing either one of the two CCK receptor subtypes with 1 μM CCK-8-S in the presence of the inhibitor of cyclic nucleotide phosphodiesterase activity, 3-isobutyl-1-methylxanthine (Figure 3). The values presented are expressed as a percentage of the amount of cyclicAMP that was formed in response to stimulation with 1 μM of the adenylyl cyclase activator, forskolin. CCK_A cells displayed an 11.3 fold increase in the amount of cyclicAMP following stimulation with 1 μM CCK-8-S. By contrast, the same concentration of CCK-8-S did not evoke any increase in the amount of cyclicAMP in CCK_B cells. The possibility of CCK-8-S being unable to increase adenylyl cyclase activity due to coupling of the CCK_B receptor not only to G_S but also to G_i was excluded by the observation that inhibition of the latter G protein, by pretreatment of the cells with 100 ng ml^{-1} pertussis toxin, did not lead to any increase in cyclicAMP accumulation in response to stimulation with 1 μM CCK-8-S (data not shown). Neither 1 μM pentagastrin nor 1 μM CCK-8-NS affected cyclicAMP accumulation in CCK_B cells (data not shown). By contrast, 1 μM CCK-8-NS evoked a 1.5 fold increase in the amount of cyclicAMP in CCK_A cells, whereas 1 μM pentagastrin was without effect on cyclicAMP formation (data not shown). The CCK_A receptor agonist JMV-180 (10 μM), albeit readily increasing $[Ca^{2+}]_i$, did not affect cyclicAMP formation in CCK_A cells (Figure 4).

Inhibition of cyclicAMP formation through the CCK_A receptor expressed in CHO cells by phorbol ester treatment

The stimulating effect of CCK-8-S on the accumulation of cyclicAMP in CCK_A cells was clearly dose-dependent with a lowest effective CCK-8-S concentration of 10 nM (Figure 4). Short-term (10 min) treatment of the cells with 0.1 μM TPA markedly reduced the increase in cellular cyclicAMP content in response to each stimulating concentration of CCK-8-S. The possibility that TPA inhibited cyclicAMP accumulation at or beyond the level of adenylyl cyclase-catalyzed cyclicAMP formation was excluded by the observation that TPA enhanced rather than reduced forskolin-stimulated cyclicAMP accumulation (Figure 5). It has been suggested that JMV-180 acts as an antagonist at the CCK_A receptor. The fact that JMV-180 (10 μM) does not stimulate cyclicAMP accumulation by itself offers the possibility to assess its antagonistic mode of action on the CCK_A receptor, by measuring its effect on CCK-8-S-induced cyclicAMP accumulation. Figure 4 shows that 10 μM JMV-180 completely inhibited cyclicAMP accumulation in response to subsequent stimulation with CCK-8-S concentrations as high as 1 μM.

Table 4 shows that short-term (10 min) treatment with 0.1 μM 4-α-phorbol 12-myristate 13-acetate did not inhibit CCK-8-S-induced cyclicAMP accumulation. Moreover, Table 4 shows that staurosporine (1 μM), GF-109203X (10 μM) and CGP-41251 (10 μM) completely abolished the inhibitory action of TPA. All three PKC inhibitors tended to increase the stimulating effect of CCK-8-S. However, only in the case of staurosporine was statistical significance reached.

Down-regulation of the various PKC-isotypes present in CHO-09 cells during prolonged phorbol ester treatment

Lysates of CHO-09 cells were used to identify PKC-isotypes by Western blotting. Membranes were probed with polyclonal antibodies directed against either the -α, -β, -γ, -δ, -ε, -η, -θ or -ζ isotype of PKC. In doing so, only the -α, -η, -θ and -ζ isotypes could be detected in this cell type. Figure 6 shows that the amount of PKC-α progressively decreased during phorbol ester treatment. At 6 h and 24 h following the onset of TPA treatment the amount of PKC-α was decreased by 54% and 73%, respectively. By contrast, the amounts of PKC-η, -θ and -ζ were still not decreased after 24 h of phorbol ester treatment.

Discussion

The present study compares pharmacology, modes of signal transduction and regulation of receptor functioning by PKC of pure CCK_A and CCK_B receptors after functional expression in the same eukaryotic expression system, namely the CHO-09 cell. The CHO-09 cell was chosen because this cell, in contrast to the CHO-K1 cell used in previous studies (Dal Forno *et al*., 1994; Smeets *et al*., 1996; 1997), did not display an increase in $[Ca^{2+}]_i$ when stimulated with high (>1 μM) concentrations of CCK-8-S.

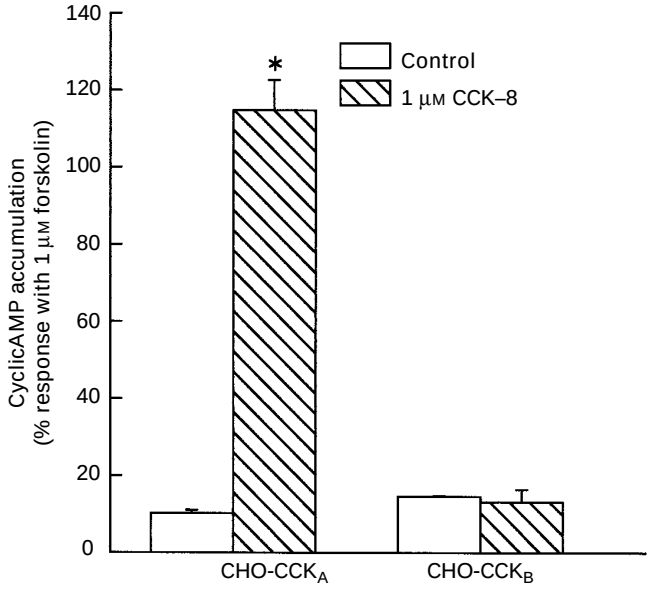

Figure 3 CCK-8-S-induced cyclicAMP accumulation in CHO-09-CCK_A cells and CHO-09-CCK_B cells. CHO-09 cells were seeded at a density of 200,000 cells per well in a 12-well plate and grown for 24 h. Cells were washed twice and stimulated with 1 μM CCK-8-S in the presence of 0.1 mM 3-isobutyl-1-methylxanthine for 10 min. In each experiment, the amount of cyclicAMP measured in cells stimulated with 1 μM forskolin was set at 100%, to which all other values were related. Absolute values for basal and forskolin-stimulated cyclicAMP accumulation were 8.5±3.8 (mean±s.e.mean) pmol/well and 86.4±3.0 pmol/well for CHO-09-CCK_A cells and 6.9±4.3 pmol/well and 37.2±8.1 pmol/well for CHO-09-CCK_B cells. The data presented are the mean±s.e.mean of 3–4 experiments. *Significantly different from unstimulated cells ($P<0.05$).

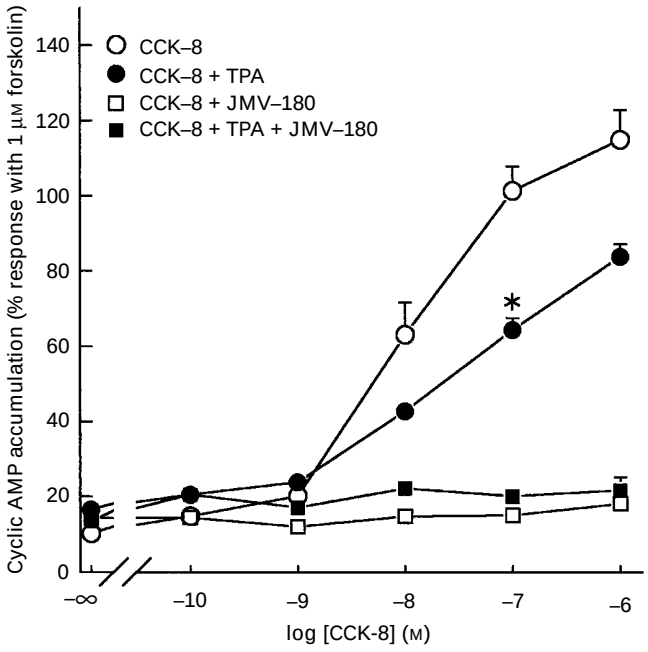

Figure 4 Effect of TPA and JMV-180 on CCK-8-S-induced cyclicAMP accumulation in CHO-09-CCK_A cells. Cells were preincubated in the absence and presence of 0.1 μM TPA and/or 10 μM JMV-180 for 10 min. Subsequently, cells were stimulated with the indicated concentrations of CCK-8-S in the presence of 0.1 mM 3-isobutyl-1-methylxanthine for another 10 min. The reaction was quenched and cyclicAMP was determined. In each experiment, the amount of cyclicAMP measured in cells stimulated with 1 μM forskolin was set at 100%, to which all other values were related. Absolute values for basal and forskolin-stimulated cyclicAMP accumulation are presented in the legend of Figure 3. The data presented are the mean±s.e.mean of 3–4 experiments. *Significantly different from corresponding CCK-8-S-stimulated untreated cells ($P<0.05$).

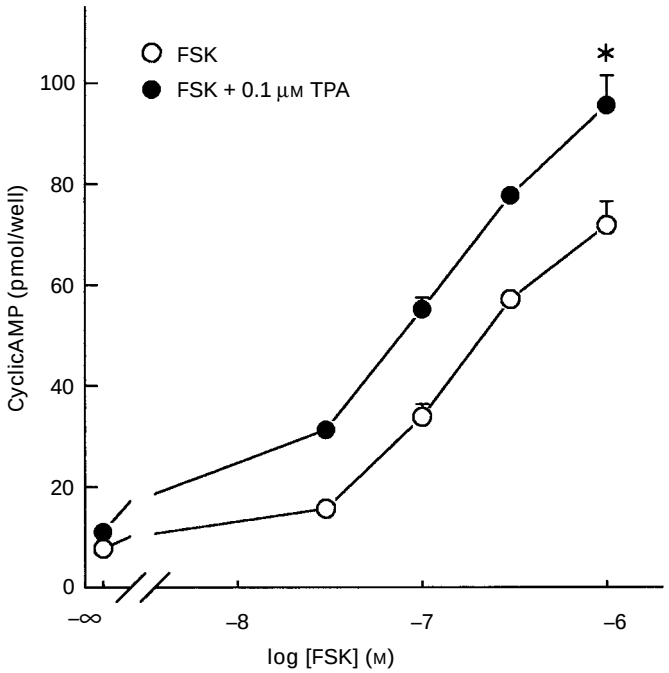

Figure 5 Effect of TPA on forskolin-induced cyclicAMP accumulation in CHO-09 cells. Cells were preincubated in the absence and presence of 0.1 μM TPA for 10 min. Subsequently, cells were stimulated with the indicated concentration of forskolin (FSK) in the presence of 0.1 mM 3-isobutyl-1-methylxanthine for another 10 min. The amount of cyclicAMP measured is expressed in pmol cyclicAMP per well. Where indicated by error bars, the data presented are the mean$\pm$s.e.mean of 3 experiments. *Significantly different from corresponding forskolin-stimulated untreated cells ($P<0.05$).

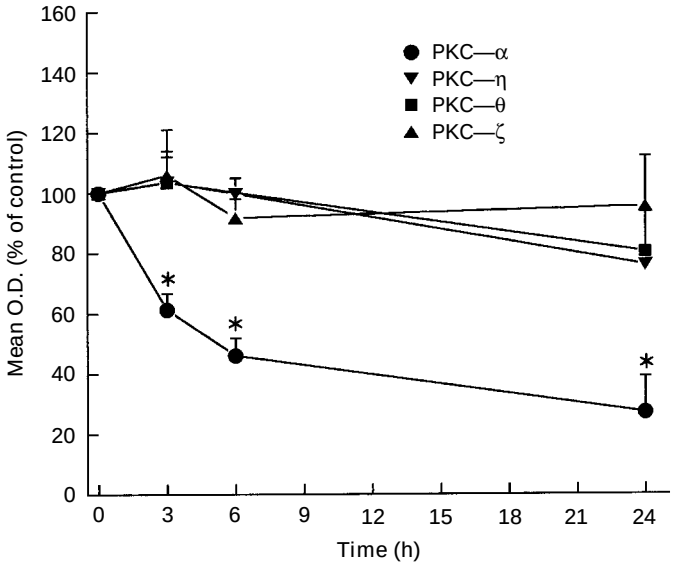

Figure 6 Time-dependence of TPA-induced down-regulation of protein kinase C isotypes in CHO-09 cells. CHO-09 cells were incubated in the absence and presence of 0.1 μM TPA for the indicated periods of time. Total cell lysates were subjected to SDS-polyacrylamide gel electrophoresis followed by Western blotting. Membranes were incubated with PKC-isotype-specific antibodies in the absence and presence of the corresponding PKC-isotype-specific peptides and PKC-specific bands were quantified densitometrically. In each experiment, the value obtained with the untreated cells was set at 100% to which all the other values were related. The data presented are the mean of 3–4 experiments; vertical lines show s.e.mean. *Significantly different from untreated cells ($P<0.05$).

Table 4 Reversal by staurosporine, GF-109203X and CGP-41251 of the inhibitory action of TPA on CCK-8-S-induced cyclicAMP accumulation in CHO-09-CCK_A cells

Untreated	11.9$\pm$0.4%
Untreated + CCK-8-S	114.0$\pm$5.9%
TPA	10.8$\pm$2.0%
TPA + CCK-8-S	64.6$\pm$3.2%[a]
4-α-PMA	9.3$\pm$1.3%
4-α-PMA + CCK-8-S	108.9$\pm$6.2%
Staurosporine	12.8$\pm$1.8%
Staurosporine + CCK-8-S	151.3$\pm$12.5%[a]
Staurosporine + TPA + CKK-8-S	147.2$\pm$11.4%
GF-109203X	7.9$\pm$0.2%[b]
GF-109203X + CCK-8-S	130.9$\pm$7.5%
GF-109203X + TPA + CCK-8-S	129.9$\pm$8.8%
CGP-41251	8.6$\pm$2.4%
CGP-41251 + CCK-8-S	133.6$\pm$12.5%
CGP-41251 + TPA + CCK-8-S	103.0$\pm$5.0%

[a]Compared with untreated CCK-8-S-stimulated cells ($P<0.05$). [b]Compared with untreated cells ($P<0.01$). Cells were preincubated in the absence or presence of TPA (0.1 μM) or 4-α-phorbol 12-myristate 13-acetate (4-α-PMA 0.1 μM) and/or staurosporine (1 μM), GF-109203X (10 μM) or CGP-41251 (10 μM) for 10 min. Subsequently, cells were stimulated with 0.1 μM CCK-8-S in the presence of 0.1 mM 3-isobutyl-1-methylxanthine for another 10 min. The reaction was quenched and cyclicAMP was determined. In each experiment, the amount of cyclicAMP measured in cells stimulated with 1 μM forskolin was set at 100%, to which all other values were related. Absolute values for basal and forskolin-stimulated cyclicAMP accumulation in CHO-09-CCK_A cells are presented in the caption of Figure 3. The data presented are the mean$\pm$s.e.mean of 3 experiments.

Pharmacology of CCK receptor subtypes

Consistent with the CCK receptor subtype pharmacology (Silvente-Poirot *et al*., 1993; Wank, 1995), CCK-8-S was 1000 fold more potent than CCK-8-NS in increasing the peak increase in $[Ca^{2+}]_i$ in cells expressing the CCK_A receptor, whereas CCK-8-S, CCK-8-NS and pentagastrin were equally potent in cells expressing the CCK_B receptor. Furthermore, the CCK_A receptor agonist JMV-180 did not increase $[Ca^{2+}]_i$ through the CCK_B receptor, whereas pentagastrin did not affect $[Ca^{2+}]_i$ through the CCK_A receptor. Finally, CCK-8-S-induced Ca^{2+} signalling through the CCK_A receptor was more potently (600 fold) inhibited by the CCK_A receptor antagonist L363,718, whereas signalling through the CCK_B receptor was more potently (5.4 fold) inhibited by the CCK_B receptor antagonist L365,260. The present findings are in agreement with previous studies, in which different expression systems were used to express functionally both CCK receptor subtypes (Yule *et al*., 1993; Denyer *et al*., 1994; Wank, 1995; Dunlop *et al*., 1996). CCK-8-S appeared to be slightly more potent (4.5 fold) in cells expressing the CCK_A receptor. Since the binding affinity for CCK-8-S was similar for both receptors, this difference is probably due to the higher number of CCK receptors expressed on CCK_A cells as compared to CCK_B cells.

Modes of transmembrane signalling of CCK receptor subtypes

Except that the present work confirms previous studies in the literature showing that the CCK_B receptor, similar to the CCK_A receptor, triggers the release of Ca^{2+} from intracellular stores (for references see Wank, 1995), it unequivocally demonstrates that only the CCK_A receptor triggers the formation of

cyclicAMP. CCK-8-S-induced increases in cyclicAMP have been observed before in cells over-expressing the CCK_A receptor (Marino *et al*., 1993; Yule *et al*., 1993), whereas information on cyclicAMP signalling through the CCK_B receptor is scarce. The CCK analogue JMV-180, which has been postulated to act as an antagonist at the low-affinity CCK_A receptor (Matozaki *et al*., 1989; 1990), did not affect cyclicAMP formation when added alone but effectively blocked CCK-8-S-evoked cyclicAMP formation (see also, Yule *et al*., 1993). This suggests that cyclicAMP signalling occurs through the low-affinity state of the receptor. We have previously shown that JMV-180 mimics the stimulating effect of CCK-8-S on Ca^{2+} mobilization without detectably affecting the production of Ins(1,4,5)P_3 in CHO-K1-CCK_A cells (Smeets *et al*., 1996). Since JMV-180 has been shown to release Ca^{2+} through the action of Ins(1,4,5)P_3 (Thorn & Petersen, 1993), this demonstrates that a non-detectable increase in Ins(1,4,5)P_3 is sufficient for maximal Ca^{2+} mobilization. By contrast, CCK-8-S was found to increase readily Ins(1,4,5)P_3 in CHO-K1-CCK_A cells, suggesting that, similar to the CCK-8-S-induced increase in cyclicAMP, the detectable CCK-8-S-evoked increase in Ins(1,4,5)P_3 occurs through the low-affinity state of the receptor. This latter idea is supported by the observation that JMV-180 causes a dose-dependent shift to the right of the dose-response curve for the effect of CCK-8-S on the peak increase in $[Ca^{2+}]_i$ in a suspension of pancreatic acinar cells, in which the Ca^{2+} mobilizing effect of JMV-180, but not the higher concentrations of CCK-8-S, was inhibited by short-term TPA treatment (Smeets *et al*., unpublished observations).

Regulation of receptor functioning by PKC

Effects on Ca^{2+} signalling We have previously shown that short-term activation of PKC by phorbol ester inhibits high-, but not low-, affinity Ca^{2+} signalling through the CCK_A receptor stably transfected in CHO-K1 cells (Smeets *et al*., 1996). The present study confirms this finding for CHO-09-CCK_A cells. However, in CHO-09-CCK_B cells, short-term TPA treatment not only inhibited the Ca^{2+} mobilizing effect of the lower concentrations of CCK-8-S, but also significantly reduced the peak increase in $[Ca^{2+}]_i$ evoked by the higher concentrations of CCK-8-S. A similar observation was recently reached with JMV-180 in CHO-K1-CCK_A cells and suggests that CCK-8-S acting through the CCK_B receptor does not produce sufficient amounts of Ins(1,4,5)P_3 to increase $[Ca^{2+}]_i$ maximally in a suspension of TPA-inhibited cells (Smeets *et al*., 1996). The inhibitory effect of TPA was completely abolished by the PKC inhibitors staurosporine and GF-109203X. The specificity of TPA was furthermore demonstrated by the lack of effect of the inactive phorbol ester, 4-α-phorbol 12-myristate 13-acetate.

We have previously shown that prolonged TPA treatment leads to a gradual recovery of CCK-8-S-evoked Ca^{2+} signalling in CHO-K1-CCK_A cells (Smeets *et al*., 1996). This recovery was paralleled by the disappearance of PKC-α and partial reduction of PKC-ε, whereas the amount of PKC-ζ remained unchanged. PKC-η and -θ were not detectable in CHO-K1 cells (R.R.L. Smeets, S.E. van Emst-de Vries, J.J.H.H.M. De Pont and P.H.G.M. Willems, unpublished observations). Based on this finding, PKC-α was concluded to be the most likely candidate for mediating the inhibitory action of TPA. This conclusion is strengthened by the current finding that in CHO-09 cells, which do not contain PKC-ε, recovery from phorbol ester inhibition was accompanied by a reduction of PKC-α and not the other (-η, -θ and -ζ) PKC isotypes present.

In CHO-09-CCK_B cells, recovery from TPA inhibition appeared to be faster with the higher CCK-8-S concentrations, whereas with the lower CCK-8-S concentrations recovery occurred equally fast in both cell lines. In line with the above idea that the CCK_B receptor produces less Ins(1,4,5)P_3 than the CCK_A receptor, this observation can be explained if the amount of Ins(1,4,5)P_3 that is produced upon stimulation with CCK-8-S gradually increases during the recovery phase, but does not reach the threshold value for Ca^{2+} mobilization with the lower CCK-8-S concentrations within the first 3 h following the onset of TPA treatment. However, with both cell lines this threshold value is reached after 24 h of TPA treatment. Finally, the fact that the response to CCK-8-S concentrations $\geqslant 1$ nM is significantly higher in CCK_B cells pretreated with TPA for 24 h suggests that, under normal conditions, the CCK_B receptor is already partly inhibited in a PKC-dependent manner.

Effects on cyclicAMP signalling The present study shows that CCK-8-S-stimulated cyclicAMP formation was significantly inhibited by TPA. The inhibitory effect of TPA was completely abolished by the PKC inhibitors staurosporine, GF-109203X and CGP-41251, whereas no inhibitory effect was observed with 4-α-phorbol 12-myristate 13-acetate. Together with the previous finding that short-term TPA treatment almost completely inhibited CCK-8-S-stimulated Ins(1,4,5)P_3 formation (Smeets *et al*., 1996), this finding demonstrates that as far as the CCK_A receptor is concerned not only high-affinity Ca^{2+} signalling, but also low-affinity cyclicAMP and Ins(1,4,5)P_3 signalling are inhibited by PKC. The present observation that TPA did not inhibit cyclicAMP formation in response to the adenylyl cyclase activator forskolin suggests that the inhibitory effect occurs at the receptor/G protein level.

Mechanism of action of PKC Based on the observation that activation of PKC leads to inhibition of high-affinity signalling through the high-affinity CCK_A receptor, leaving low-affinity signalling through this CCK receptor subtype unimpaired, we have previously postulated that PKC causes the transition of the CCK_A receptor from a high- to low-affinity state (Willems *et al*., 1993b; 1995; Smeets *et al*., 1996). Activation of PKC leads to rapid phosphorylation of the CCK_A receptor (Gates *et al*., 1993) and since elucidation of the primary structure of the receptor revealed the presence of four potential sites for PKC phosphorylation (Wank *et al*., 1992a), it was assumed that PKC acts directly at the level of the receptor protein. The present study shows that besides Ca^{2+} mobilization, cyclicAMP accumulation, measured in the presence of a cyclic nucleotide phosphodiesterase inhibitor, is also inhibited by PKC. This suggests that the same receptor phosphorylations interfere with coupling to two distinct G proteins. With respect to the CCK_B receptor, the data presented in this work show that, similar to the CCK_A receptor, high-affinity signalling is inhibited by PKC. Although PKC-mediated phosphorylation of the CCK_B receptor has not yet been demonstrated, the presence of one potential phosphorylation site for PKC (Wank *et al*., 1992b) suggests a similar mechanism of direct PKC phosphorylation of the receptor protein as that postulated for the CCK_A receptor.

Conclusions

This work shows that both CCK receptor subtypes, in addition to their pharmacological properties and modes of

transmembrane signalling, retain their ability to be regulated by PKC when expressed in CHO-09 cells. The latter offers the possibility of studying the role of the potential PKC phosphorylations site by mutational analysis.

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References

BROWN, B.L., ALBANO, J.D., EKINS, R.P. & SGHERZI, A.M. (1971). A simple and sensitive saturation assay method for the measurement of adenosine 3′:5′-cyclic monophosphate. *Biochem. J.,* **121,** 561 – 562.

DAL FORNO, G., PASTORINO, A.M., VAN AMSTERDAM, F.T.M., CORSI, M. & TRIST, D.G. (1994). Cholecystokinin increases intracellular calcium concentration in native CHO-K1 cells through the activation of CCK-A receptors. *Pharmacol. Commun.,* **4,** 307 – 316.

DENYER, J., GRAY, J., WONG, M., STOLZ, M. & TATE, S. (1994). Molecular and pharmacological characterization of the human CCK_B receptor. *Eur. J. Pharmacol.,* **268,** 29 – 41.

DUNLOP, J., BRAMMER, N. & ENNIS, C. (1996). Pharmacological characterization of a Chinese hamster ovary cell line transfected with the human CCK-B receptor gene. *Neuropeptides,* **30,** 359 – 363.

GALAS, M.C., BERNARD, N. & MARTINEZ, J., (1992). Pharmacological studies on CCK-B receptors in guinea pig synaptoneurosomes. *Eur. J. Pharmacol.,* **226,** 35 – 41.

GARDNER, J.D. & JENSEN, R.T. (1993). Receptors for secretagogues on pancreatic acinar cells. In *The Pancreas: Biology, Pathobiology, and Disease*, 2nd edn. ed. Go, V.L.W., DiMagno E.P., Gardner J.D., Lebenthal, E., Reber, H.A. & Scheele, G.A. pp. 151 – 166. New York: Raven Press.

GATES, L.K., ULRICH, C.D. & MILLER, L.J. (1993). Multiple kinases phosphorylate the pancreatic cholecystokinin receptor in an agonist-dependent manner. *Am. J. Physiol.,* **264,** G840 – G847.

KOPIN, A.S., LEE, Y.-M., MCBRIDE, E.W., MILLER, L.J., LU, M., LIN, H.Y., KOLAKOWSKI, L.F. & BEINBORN, M. (1992). Expression, cloning and characterization of the canine parietal cell gastrin receptor. *Proc. Natl. Acad. Sci. U.S.A.,* **89,** 3605 – 3609.

MARINO, C.R., LEACH, S.D., SCHAEFER, J.F., MILLER, L.J. & GORELICK, F.S. (1993). Characterization of cAMP-dependent protein kinase activation by CCK in rat pancreas. *FEBS Lett.,* **316,** 48 – 52.

MATOZAKI, T., MARTINEZ, J. & WILLIAMS, J.A. (1989). A new CCK analogue differentiates two functionally distinct CCK receptors in rat and mouse pancreatic acini. *Am. J. Physiol.,* **257,** G594 – G600.

MATOZAKI, T., GÖKE, B., TSUNODA, Y., RODRIGUEZ, M., MARTINEZ, J. & WILLIAMS, J.A. (1990). Two functionally distinct cholecystokinin receptors show different modes of actions on Ca^{2+} mobilization and phospholipid hydrolysis in isolated rat pancreatic acini. *J. Biol. Chem.,* **265,** 6247 – 6254.

OZCELEBI, F., RAO, R.V., HOLICKY, E., MADDEN, B.J., MCCORMICK, D.J. & MILLER, L.J. (1996). Phosphorylation of cholecystokinin receptors expressed on Chinese hamster ovary cells. *J. Biol. Chem.,* **271,** 3750 – 3755.

PISEGNA, J.R., DE WEERTH, A. & WANK, S.A. (1992). Molecular cloning of the human brain and gastric cholecystokinin receptor: structure, functional expression and chromosomal localization. *Biochem. Biophys. Res. Commun.,* **189,** 296 – 303.

SILVENTE-POIROT, S., DUFRESNE, M., VAYSSE, N. & FOURMY, D. (1993). The peripheral cholecystokinin receptors. *Eur. J. Biochem.,* **215,** 513 – 529.

SMEETS, R.L.L., GARNER, K.M., HENDRIKS, M., VAN EMST-DE VRIES, S.E., PEACOCK, M.D., HENDRIKS, W., DE PONT, J.J.H.H.M. & WILLEMS, P.H.G.M. (1996). Recovery from TPA inhibition of receptor-mediated Ca^{2+} mobilization is paralleled by down-regulation of protein kinase C-α in CHO cells expressing the CCK-A receptor. *Cell Calcium,* **20,** 1 – 9.

SMEETS, R.L.L., IJZERMAN, A.P., HERMSEN, H.P.H., OPHORST, O.J.A.E., VAN EMST-DE VRIES, S.E., DE PONT, J.J.H.H.M. & WILLEMS, P.H.G.M. (1997). Mutational analysis of the putative devazepide binding site of the CCK_A receptor. *Eur. J. Pharmacol.,* **325,** 93 – 99.

THORN, P. & PETERSEN, O.H. (1993). Calcium oscillations in pancreatic acinar cells, evoked by the cholecystokinin analogue JMV-180, depend on functional inositol 1,4,5-trisphosphate receptors. *J. Biol. Chem.,* **268,** 23219 – 23221.

VAN DIJK, A., RICHARDS, J.G., TRECIAK, A., GILLESSEN, D. & MÖHLER, H. (1984). Cholecystokinin receptors: Biochemical demonstration and autoradiographic localization in rat brain and pancreas using [^{3}H]-cholecystokinin-8 as radioligand. *J. Neurosci.,* **4,** 1021 – 1033.

WANK, S.A. (1995). Cholecystokinin receptors. *Am. J. Physiol.,* **269,** G628 – G646.

WANK, S.A., HARKINS, R., JENSEN, R.T., SHAPIRA, H., DE WEERTH, A. & SLATTERY, T. (1992a). Purification, molecular cloning, and functional expression of the cholecystokinin receptor from rat pancreas. *Proc. Natl. Acad. Sci. U.S.A.,* **89,** 3125 – 3129.

WANK, S.A., PISEGNA, J.R. & DE WEERTH, A. (1992b). Brain and gastrointestinal cholecystokinin receptor family: structure and functional expression. *Proc. Natl. Acad. Sci. U.S.A.,* **89,** 8691 – 8695.

WILLEMS, P.H.G.M., FLEUREN-JAKOBS, A.M.M., DE PONT, J.J.H.H.M. & BONTING, S.L. (1984). Potentiating role of cyclicAMP in pancreatic enzyme secretion, demonstrated by means of forskolin. *Biochim. Biophys. Acta,* **802,** 209 – 214.

WILLEMS, P.H.G.M., TILLY, R.H.J. & DE PONT, J.J.H.H.M. (1987). Pertussis toxin stimulates cholecystokinin-induced cyclicAMP formation but is without effect on secretagogue-induced calcium mobilization in exocrine pancreas. *Biochim. Biophys. Acta,* **928,** 179 – 185.

WILLEMS, P.H.G.M., VAN EMST-DE VRIES, S.E., VAN OS, C.H. & DE PONT, J.J.H.H.M. (1993a). Dose-dependent recruitment of pancreatic acinar cells during receptor-mediated calcium mobilization. *Cell Calcium,* **14,** 145 – 159.

WILLEMS, P.H.G.M., VAN HOOF, H.J.M., VAN MACKELENBERGH, M.G.H., HOENDEROP, J.G.J., VAN EMST-DE VRIES, S.E. & DE PONT, J.J.H.H.M. (1993b). Receptor-evoked Ca^{2+} mobilization in pancreatic acinar cells: evidence for a regulatory role of protein kinase C by a mechanism involving the transition of high-affinity receptors to a low-affinity state. *Pflügers Arch.,* **424,** 171 – 182.

WILLEMS, P.H.G.M., SMEETS, R.L.L., BOSCH, R.R., GARNER, K.M., VAN MACKELENBERGH, M.G.H. & DE PONT, J.J.H.H.M. (1995). Protein kinase C activation inhibits receptor-evoked inositol trisphosphate formation and induction of cytosolic calcium oscillations by decreasing the affinity-state of the cholecystokinin receptor in pancreatic acinar cells. *Cell Calcium,* **18,** 471 – 483.

WILLIAMS, J.A. & YULE, D.J. (1993). Stimulus-secretion coupling in pancreatic acinar cells. In *The Pancreas: Biology, Pathobiology, and Disease*, 2nd edn. ed. Go, V.L.W., DiMagno, E.P., Gardner, J.D., Lebenthal, E., Reber, H.A. & Scheele, G.A. pp. 167 – 189. New York: Raven Press.

YULE, D.I., TSENG, M.-J., WILLIAMS, J.A. & LOGSDON, C.D. (1993). A cloned CCK-A receptor transduces multiple signals in response to full and partial agonists. *Am. J. Physiol.,* **265,** G999 – G1004.

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