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Mutational analysis of the potential phosphorylation sites for protein kinase C on the CCK_A receptor

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1 Many G protein-coupled receptors contain potential phosphorylation sites for protein kinase C (PKC), the exact role of which is poorly understood. In the present study, a mutant cholecystokinin$_A$ (CCK_A) receptor was generated in which the four consensus sites for PKC action were changed in an alanine. Both the wild-type (CCK_AWT) and mutant (CCK_AMT) receptor were stably expressed in Chinese hamster ovary (CHO) cells.

2 Binding of [^{3}H]-cholecystokinin-(26-33)-peptide amide (CCK-8) to membranes prepared from CHO-CCK_AWT cells and CHO-CCK_AMT cells revealed no difference in binding affinity (K_d values of 0.72 nM and 0.86 nM CCK-8, respectively).

3 The dose-response curves for CCK-8-induced cyclic AMP accumulation and inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) formation were shifted to the left in CHO-CCK_AMT cells. This leftward shift was mimicked by the potent inhibitor of protein kinase activity, staurosporine. However, the effect of staurosporine was restricted to CHO-CCK_AWT cells. This demonstrates that attenuation of CCK-8-induced activation of adenylyl cyclase and phospholipase C-β involves a staurosporine-sensitive kinase, which acts directly at the potential sites of PKC action on the CCK_A receptor in CCK-8-stimulated CHO-CCK_AWT cells.

4 The potent PKC activator, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), evoked a rightward shift of the dose-response curve for CCK-8-induced cyclic AMP accumulation in CHO-CCK_AWT cells but not CHO-CCK_AMT cells. This is in agreement with the idea that PKC acts directly at the CCK_A receptor to attenuate adenylyl cyclase activation.

5 In contrast, TPA evoked a rightward shift of the dose-response curve for CCK-8-induced Ins(1,4,5)P_3 formation in both cell lines. This demonstrates that high-level PKC activation inhibits CCK-8-induced Ins(1,4,5)P_3 formation also at a post-receptor site.

6 TPA inhibition of agonist-induced Ca^{2+} mobilization was only partly reversed in CHO-CCK_AMT cells. TPA also inhibited Ca^{2+} mobilization in response to the G protein activator, Mas-7. These findings are in agreement with the idea that partial reversal of agonist-induced Ca^{2+} mobilization is due to the presence of an additional site of PKC inhibition downstream of the receptor and that the mutant receptor itself is not inhibited by the action of PKC.

7 The data presented demonstrate that the predicted sites for PKC action on the CCK_A receptor are the only sites involved in TPA-induced uncoupling of the receptor from its G proteins. In addition, the present study unveils a post-receptor site of PKC action, the physiological relevance of which may be that it provides a means for the cell to inhibit phospholipase C-β activation by receptors that are not phosphorylated by PKC.

Keywords: Cholecystokinin$_A$ receptor; protein kinase C; receptor phosphorylation; mutagenesis; Chinese hamster ovary cells; calcium mobilization; cyclic AMP formation; phorbol ester; cholecystokinin; mastoparan

Introduction

Repeated or continuous stimulation of G protein-coupled receptors generally results in progressively smaller responses. Phosphorylation of receptors by G protein-coupled receptor kinases and/or second messenger-dependent kinases constitutes one of the earliest events in this process of desensitization (Freedman & Lefkowitz, 1996). Whereas the former kinases phosphorylate only the agonist-occupied form of the receptor (homologous phosphorylation), second messenger-dependent kinases act also on the non-occupied form of the receptor (homologous phosphorylation) and on other receptors (heterologous phosphorylation).

Receptor phosphorylation, by itself, may significantly attenuate transmembrane signalling in response to physiological agonist concentrations. However, when assessed at receptor-saturating levels of agonist no more than modest attenuation is observed (for references, see Freedman & Lefkowitz, 1996). Only when the receptor is phosphorylated by a G protein-coupled receptor kinase (GRK) is substantial desensitization achieved under conditions where agonist concentrations are high. This then is the result of binding of a member of the arrestin family of inhibitory proteins. Arrestins uncouple receptors from their respective G proteins and, hence, effectors. Although second messenger-dependent kinases also evoke uncoupling of receptors from their G proteins, they do so by phosphorylation alone (Pitcher *et al*., 1992).

The cholecystokinin$_A$ (CCK_A) receptor is a G protein-coupled receptor, which, among other things, mediates pancreatic exocrine secretion (Wank, 1995). Elucidation of

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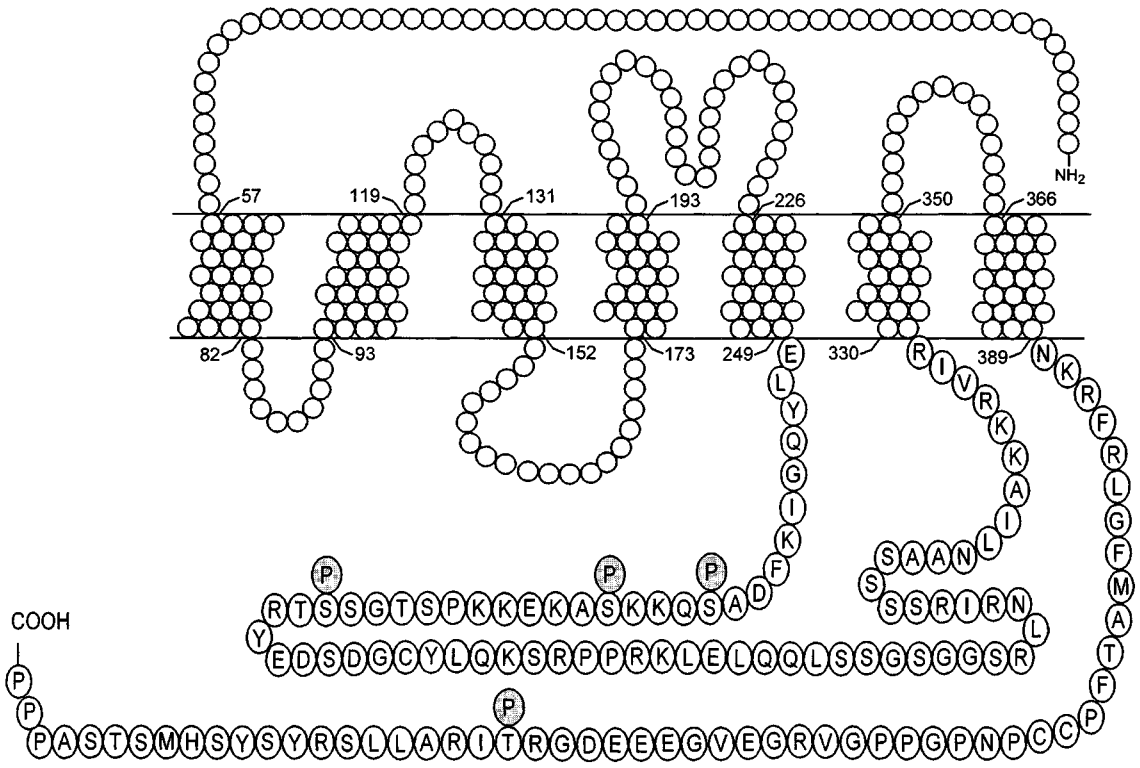

Figure 1 Schematic presentation of the CCK_A receptor. The indicated potential phosphorylation sites (Ser260, Ser264, Ser275 and Thr424) were mutated in an alanine.

the primary structure of this receptor (Figure 1) revealed the presence of four potential phosphorylation sites for the second messenger-dependent kinase, protein kinase C (PKC), three on serine (S260, S264 and S275) in the large intracellular third loop and one on threonine (T424) in the cytoplasmic carboxyl-terminal tail (Wank *et al*., 1992). Previous work suggested the concerted action of PKC and a putative receptor-specific kinase in homologous phosphorylation of the CCK_A receptor (Gates *et al*., 1993). In the same type of experiment, using the putative PKC inhibitor staurosporine, evidence was provided that heterologous phosphorylation of the CCK_A receptor occurs entirely through PKC (Zhu *et al*., 1994). This conclusion was furthermore substantiated by the observation that the potent PKC activator, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), readily increased the amount of phosphorylated CCK_A receptor protein (Gates *et al*., 1993; Zhu *et al*., 1994). The observation that TPA potently inhibits cell activation by physiological concentrations of CCK is in agreement with the idea that receptor phosphorylation leads to desensitization (for a review, see Willems *et al*., 1997). In a recent study, using freshly isolated pancreatic acinar cells in which PKC activity was down-regulated by the prolonged action of TPA, we provided evidence that a basal level of PKC-mediated receptor phosphorylation is required for the action of a putative receptor kinase (Smeets *et al*., 1998b). Together with the finding that CCK receptors engineered to have mutations in two (S260 and S264) of the four consensus sites for PKC action were not phosphorylated in response to CCK (Rao *et al*., 1997), this led us to speculate that CCK_A receptors are phosphorylated in a hierarchical way, with PKC sites phosphorylated initially, making potential phosphorylation sites for a putative receptor kinase more accessible.

Despite the fact that CCK_A receptors in which both S260 and S264 were changed to an alanine were no longer phosphorylated by a high concentration of CCK, CCK-induced inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) formation remained significantly inhibited following short-term pretreatment with a high concentration of CCK (Rao *et al*., 1997). However, proper evaluation of the data obtained was seriously hampered by the fact that the mutated receptors, similarly to the wild-type receptors, were rapidly internalized. The recent observation that TPA does not stimulate internalization of the CCK_A receptor (Toledo *et al*., 1997), shows that the latter problem can be circumvented by using TPA rather than CCK.

In the present study we generated a mutated CCK_A receptor in which the four potential sites for PKC phosphorylation were changed to an alanine. This mutant receptor was stably expressed in Chinese hamster ovary (CHO) cells in order to investigate the role of these sites in TPA-induced, and therefore PKC-mediated, inhibition of cell activation. The data presented demonstrate the existence of at least two sites of PKC inhibition, one at the receptor level and another one downstream of the receptor, in the pathway leading to phospholipase C activation. The absence of a post-receptor site of PKC action in the pathway leading to adenylyl cyclase activation allowed us to demonstrate that TPA inhibition at the receptor level was completely abolished following removal of the four potential PKC phosphorylation sites.

Methods

Mutagenesis of CCK_A receptor cDNA

Full-length cDNA encoding the rat CCK_A receptor (Wank *et al*., 1992), was originally provided by Dr S.A. Wank (National Institutes of Health, Bethesda, MD, U.S.A.). However, because of poor expression, a cDNA truncated to within three

nucleotides of the first in frame ATG (Yule *et al*., 1993) was used. 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.). Oligonucleotides for mutagenesis were: 5′-ATCAA-ATTTGATGCTGCGCAGAAGAAATCTG-3′ (S260A), 5′-GCGCAGAAGAAAGCGGCCAAAGAGAAG-3′ (S264A), 5′-CACTGGCAGCGCCACCCGATATGA-3′ (S275A) and 5′-AGGATGGGAGGGCCATAAGGGCAT-3′ (T424A). Mutations were introduced by the T7-GEN site-directed mutagenesis kit from US Biochemical (Cleveland, OH, U.S.A.) and confirmed by sequencing. For expression, cDNA was subcloned into the *Hind*III and *Bam*HI sites of the mammalian expression vector pTEJ8.

Stable expression of wild-type and mutant CCK_A receptors in CHO cells

Chinese hamster ovary (CHO)-K1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FCS 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 (wild-type or mutant). At 48 h after electroporation, G418 was added at a concentration of 1.2 mg ml^{-1}. G418-resistant colonies were selected at 14 days after electroporation. To obtain a clonal cell line, cells were seeded in 12-well plates at a density of 0.5 cells per well. Cells were tested for the presence of functional CCK_A receptors by digital imaging microscopy.

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

Binding studies were performed according to the method described by van Dijk *et al*. (1984). Briefly, CHO-CCK_A 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 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, 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 individual CHO-CCK_A cells

Fluorescence measurements in individual CHO cells were carried out as described previously (Smeets *et al*., 1996). Briefly, cells were trypsinized, seeded on a glass coverslip (2×10^4 cells 30 μl^{-1}), and allowed to attach for 30 min. Culture medium was added and the cells were grown for another 24 h. Cells were loaded with 2 μM fura-2/AM for 20 min at 37°C. Excess fura-2/AM was removed by washing the cells 3 times with physiological salt solution (PSS) containing (mM) NaCl 137, KCl 4.7, $MgCl_2$ 0.56, $CaCl_2$ 1.28, Na_2HPO_4 1.0, L-glutamine 2, D-glucose 5.5; 0.1% (w/v) bovine serum albumin and 10 mM HEPES (pH 7.4). Coverslips were mounted in a thermostatic (34°C) perfusion chamber, placed on the stage of an inverted microscope (Nikon Diaphot). An epifluorescent 40× magnification oil immersion objective was used to allow simultaneous monitoring of an average of close to 80 individual cells. Digital imaging microscopy was carried out as described previously (Willems *et al*., 1993a) using the MagiCal hardware and TARDIS software provided by Joyce Loebl (Dukesway, Team Valley, Gateshead, U.K.). 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.

Fluorescence measurements in suspensions of CHO-CCK_A cells

Suspension measurements were performed as described previously (Smeets *et al*., 1997). Briefly, CHO cells expressing the CCK_A receptor were seeded in 25 cm^2 culture flasks (5×10^5 cells/flask) and grown for 48 h. Cells were trypsinized and washed twice in a HEPES/Tris medium containing (mM): NaCl 133, KCl 4.2, $CaCl_2$ 1.0, $MgCl_2$ 1.0, glucose 5.8, soybean trypsin inhibitor 0.2 mg ml^{-1}, an amino acid mixture according to Eagle, 1% (w/v) bovine serum albumin and 10 mM HEPES, adjusted with Tris to pH 7.4. Cells were resuspended in HEPES/Tris medium and loaded with 2 μM fura-2/AM for 20 min at 37°C. Non-hydrolysed dye was removed by washing the cells twice in HEPES/Tris medium containing 0.1% (w/v) bovine serum albumin. Cells were resuspended in the latter medium and transferred to a cuvette, placed in a Shimadzu RF-5000 spectrofluorophotometer equipped with a magnetic stirrer and a thermostated cuvette holder. Fluorescence measurements were carried out at 37°C as described previously (Willems *et al*., 1993a). 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.

Determination of cyclic AMP accumulation

For adenosine 3′:5′-cyclic monophosphate (cyclic AMP) measurements, cells were seeded in a 12-well plate (200,000 cells/well) and grown for 24 h. Cells were washed twice with HEPES/Tris medium containing 0.1% (w/v) bovine serum albumin and 0.1 mM 3-isobutyl-1-methylxanthine and preincubated in the absence or presence of either 1 μM staurosporine or 0.1 μM TPA for 10 min. At 10 min, CCK-8 was added at the indicated concentration and the cells were incubated for another 10 min. At the end of the stimulation period, the reaction was quenched by the addition of trichloroacetic acid at a final concentration of 5% (w/v). Cells were scraped off, transferred to a micro test tube (Eppendorf) and immediately frozen in liquid nitrogen. After thawing, the samples were vigorously mixed and centrifuged for 4 min at 10,000 *g* (Eppendorf minifuge). An aliquot of the supernatant was removed and extracted 3 times with water-saturated ether. The cyclic AMP content of the extract was determined by saturation assay with cyclic AMP binding protein as described previously (Willems *et al*., 1984).

Determination of inositol 1,4,5-trisphosphate levels

For Ins(1,4,5)P_3 measurements, cells were seeded in a 12-well plate (200,000 cells/well) and grown for 24 h. Cells were washed twice with HEPES/Tris medium containing 0.1% (w/v) bovine serum albumin and preincubated in the absence and presence of either 1 μM staurosporine or 0.1 μM TPA for

10 min. At 10 min, the preincubation medium was replaced by identical medium containing in addition the indicated concentration of CCK-8. After a stimulation period of 20 s, the reaction was quenched by the addition of trichloroacetic acid at a final concentration of 10% (w/v). Cells were scraped off, transferred to a micro test tube (Eppendorf) and centrifuged for 4 min at 10,000 *g* (Eppendorf minifuge). An aliquot of the supernatant was removed and extracted 3 times with water-saturated ether. The Ins(1,4,5)P_3 content of the extract was determined by isotope-dilution assay as described previously (Willems *et al*., 1993b).

Materials

Cholecystokinin-(26-33)-peptide amide (CCK-8), inositol 1,4,5-trisphosphate, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), 3-isobutyl-1-methylxanthine, bovine serum albumin and soybean trypsin inhibitor were purchased from Sigma Diagnostics (St. Louis, MO, U.S.A.) and staurosporine and cyclic AMP from Boehringer (Mannheim, Germany). GF-109203X (2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide) and Mas-7 (H-Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Ala-Leu-Leu-NH_2) were obtained from Calbiochem (La Jolla, CA, 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.). Fura-2/AM and pluronic F-127 were purchased from Molecular Probes Inc. (Eugene, OR, U.S.A.) and GF/B filters from Whatman Int. Ltd. (Maidstone, U.K.). Tissue culture medium with additives was obtained from Gibco (Paisley, Scotland) and [^{3}H]-CCK-8 (67 Ci mmol^{-1}), [^{3}H]-cyclic AMP (41 Ci mmol^{-1}) and D-*myo*-[^{3}H]-inositol 1,4,5-trisphosphate (51.4 Ci mmol^{-1}) from Amersham International plc (Little Chalfont, Buckinghamshire, U.K.). Activated charcoal (Norit, SX-1) was purchased from Norit (Amersfoort, The Netherlands). The cyclic AMP binding protein was isolated from bovine adrenal cortex as described previously (Willems *et al*., 1984). All other chemicals were of reagent grade.

Analysis of data

Half-maximal agonist concentration (EC_{50}) and maximal effect of the agonist (intrinsic activity) 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 to membranes of CHO cells expressing wild-type or mutant CCK_A receptors

To investigate the role of PKC in CCK_A receptor desensitization, a mutant receptor was generated in which the four potential phosphorylation sites for PKC (Ser260, Ser264, Ser275 and Thr424), located on the third intracellular loop and the C-terminal tail of the receptor (Figure 1), were changed to an alanine. For functional analysis, the wild-type (WT) and mutant (MT) CCK_A receptor were stably expressed in CHO cells. Binding of [^{3}H]-CCK-8 to membranes prepared from CHO-CCK_AWT cells and CHO-CCK_AMT cells was performed to examine possible differences in equilibrium dissociation constant (K_d) and/or binding capacity (B_{max}). Scatchard analysis of the binding data revealed a similar K_d value for the WT and MT receptor (Table 1). This finding demonstrates that the mutations did not alter the binding affinity of the CCK_A receptor for CCK-8. Evaluation of the B_{max} values showed that the difference in receptor density was relatively small between the two cell lines (B_{max} values of 0.42 and 0.88 pmol mg^{-1} protein for CHO-CCK_AWT and CHO-CCK_AMT cells, respectively).

TPA inhibition of CCK-8-induced cyclic AMP formation in CHO cells expressing wild-type or mutant CCK_A receptors

It has been shown that CCK_A receptors are functionally coupled to adenylyl cyclase following expression in CHO cells (Yule *et al*., 1993; Wu *et al*., 1997; Smeets *et al*., 1998a). In the present study, CHO cells, preincubated in the presence of the inhibitor of cyclic nucleotide phosphodiesterase activity, 3-isobutyl-1-methylxanthine, for 10 min, were stimulated with CCK-8 for another 10 min following which the cellular cyclic AMP content was determined. CCK-8 (1 μM) did not increase cyclic AMP in mock-transfected CHO cells. This demonstrates the absence of endogenous receptors that can interact with CCK-8 to increase adenylyl cyclase activity. In both cell lines, CCK-8 increased cyclic AMP dose-dependently (Figure 2). However, the dose-response curve was significantly shifted to the left in CHO-CCK_AMT cells. Nonlinear regression analysis revealed EC_{50} values of 38.0 nM and 3.5 nM and intrinsic activities of 495 pmol mg^{-1} protein and 550 pmol mg^{-1} protein for CHO-CCK_AWT cells and CHO-CCK_AMT cells, respectively (Figure 2b).

To investigate the possibility that the leftward shift of the dose-response curve for the stimulating effect of CCK-8 on cyclic AMP accumulation in CHO-CCK_AMT cells was due to the absence of functional PKC phosphorylation sites, both cell lines were pretreated with the potent inhibitor of protein kinase activity, staurosporine (1 μM), for 10 min, before being stimulated with CCK-8 for another 10 min. Figure 2a shows that staurosporine markedly shifted the dose-response curve to the left in CHO-CCK_AWT cells (EC_{50} values of 41.0 nM and 5.4 nM for untreated and staurosporine-treated CHO-CCK_AWT cells, respectively). In contrast, staurosporine did not affect the EC_{50} in CHO-CCK_AMT cells (EC_{50} values of 2.2 nM and 1.5 nM for untreated and staurosporine-treated CHO-CCK_AMT cells, respectively). These data show that staurosporine markedly (7.5 fold) decreased the EC_{50} value in CHO-CCK_AWT cells to a value (5.4 nM) comparable to that obtained with either untreated (2.2 nM) or staurosporine-treated (1.5 nM) CHO-CCK_AMT cells. Consequently, the ratio of the EC_{50} values for CHO-CCK_AWT cells and CHO-CCK_AMT cells decreased from 18.6 in the untreated situation to 3.6 in the staurosporine-treated situation.

In CHO-CCK_AWT cells, cyclic AMP formation in response to 10 nM, 100 nM and 1 μM CCK-8 was

Table 1 Binding characteristics of the wild-type and mutant CHO-CCK_A receptor

CHO-CCK_A cells	B_{max} (pmol mg^{-1} protein)	K_d (nM)
Wild-type	0.42±0.01	0.72±0.06
Mutant	0.88±0.03	0.86±0.13

The K_d and B_{max} values were determined by scatchard-plot analysis. Values shown are the mean±s.d. from two independent experiments.

significantly reduced following pretreatment with 0.1 μM TPA for 10 min (Figure 2b). EC_{50} and intrinsic activity were calculated to be 70.0 nM and 405 pmol mg^{-1} protein, respectively. In contrast, no TPA inhibition of CCK-8-induced cyclic AMP production was observed in CHO-CCK_AMT cells. EC_{50} and intrinsic activity amounted to 3.2 nM and 510 pmol mg^{-1} protein, respectively.

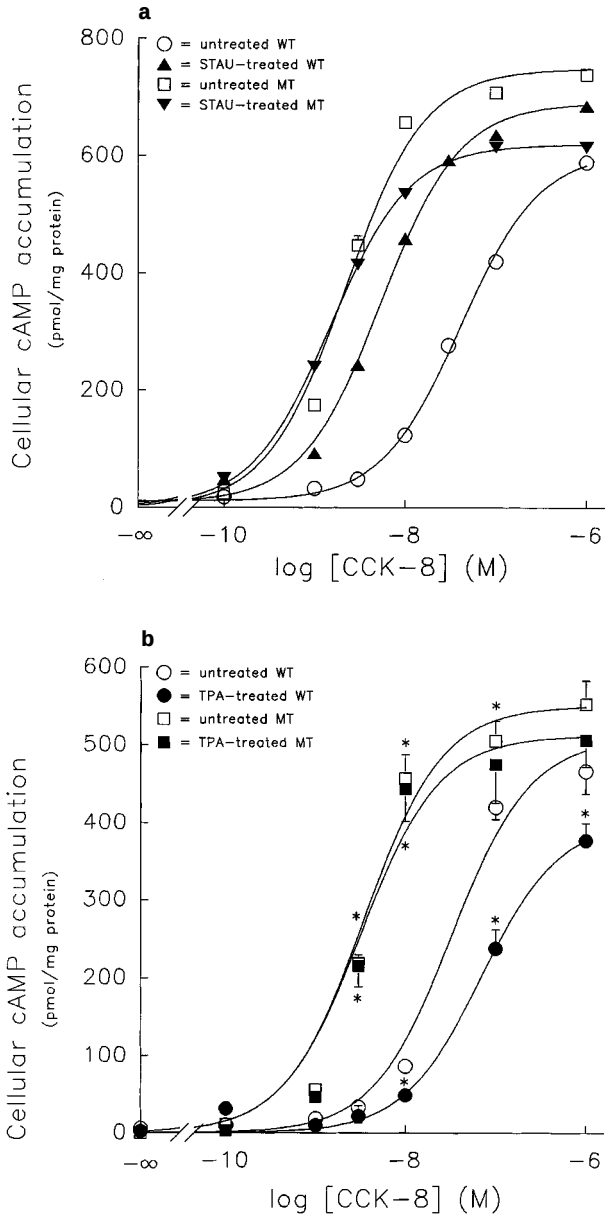

Figure 2 Effect of TPA and staurosporine on CCK-8-induced cyclic AMP accumulation in CHO-CCK_AWT and CHO-CCK_AMT cells. CHO cells expressing either the wild-type or mutant CCK_A receptor were incubated in the presence of 0.1 mM 3-isobutyl-1-methylxanthine and in the absence or presence of 1 μM staurosporine (a) or 0.1 μM TPA (b) for 10 min. Subsequently, the cells were stimulated with the indicated concentrations of CCK-8 for another 10 min. After rapid quenching of the reaction, the amount of cyclic AMP was determined and expressed in pmol mg^{-1} protein. The data presented are of a single experiment in which 3 nM and 30 nM CCK-8 were tested in triplicate in staurosporine-treated MT cells and WT cells, respectively (a), or the mean and s.e.mean (vertical lines) of 4–8 experiments (b). *Significantly different from corresponding untreated WT cells ($P<0.05$).

TPA inhibition of CCK-8-induced Ins(1,4,5)P_3 formation in CHO cells expressing wild-type or mutant CCK_A receptors

We have previously shown that CCK-8 effectively increases the cellular Ins(1,4,5)P_3 content in CHO-CCK_AWT cells (Smeets *et al*., 1996). The effect of the hormone was time-dependent and even with the lowest effective CCK-8 concentration a plateau was reached within 20 s following the onset of stimulation. The present study shows that CCK-8 increased the cellular Ins(1,4,5)P_3 content dose-dependently in both cell lines. Figure 3 shows that the dose-response curve was slightly shifted to the left in cells expressing the mutant receptor. Nonlinear regression analysis revealed EC_{50} values of 7.5 nM and 4.0 nM and intrinsic activities of 355 pmol mg^{-1} protein and 400 pmol mg^{-1} protein for CHO-CCK_AWT and CHO-CCK_AMT cells, respectively. The values obtained with 3 nM and 10 nM CCK-8 were significantly higher in CHO-CCK_AMT cells (see also, Figure 4).

To investigate the possibility that the shift to the left of the dose-response curve for the stimulant effect of CCK-8 on the plateau increase in Ins(1,4,5)P_3 in CHO-CCK_AMT cells was due to the absence of functional PKC phosphorylation sites, both cell lines were pretreated with staurosporine (1 μM) for 10 min, before being stimulated with CCK-8 for 20 s. Staurosporine markedly shifted the dose-response curve to the left in CHO-CCK_AWT cells (EC_{50} value of 2.7 nM) (Figure 3). The effect of staurosporine on the stimulant action of 10 nM CCK-8 (tested in triplicate) was statistically significant

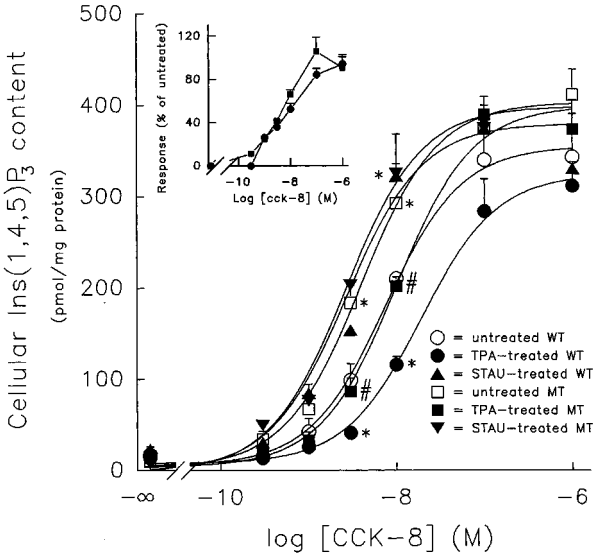

Figure 3 Effect of TPA and staurosporine on the CCK-8-induced plateau increase in Ins(1,4,5)P_3 in CHO-CCK_AWT and CHO-CCK_AMT cells. CHO cells expressing either the wild-type or mutant CCK_A receptor were incubated in the absence or presence of 1 μM staurosporine or 0.1 μM TPA for 10 min. Subsequently, the cells were stimulated with the indicated concentrations of CCK-8 for another 20 s. After rapid quenching of the reaction, the amount of Ins(1,4,5)P_3 was determined and expressed in pmol mg^{-1} protein. The insert shows for each agonist concentration the increase in TPA-treated cells as percentage of that in untreated cells. In the case of staurosporine-treated cells, the data presented are of a single experiment except for those obtained with 10 nM CCK-8, which are the mean and s.e.mean of three experiments. The data obtained with untreated and TPA-treated cells are the mean and s.e.mean of 3 experiments. *Significantly different from corresponding untreated WT cells ($P<0.05$). #Significantly different from corresponding untreated MT cells ($P<0.05$).

(see also, Figure 4). In contrast, staurosporine did not affect the EC_{50} in CHO-CCK_AMT cells (EC_{50} value of 2.7 nM). The drug did not alter the intrinsic activities (380 pmol mg^{-1} protein and 400 pmol mg^{-1} protein for CHO-CCK_AWT cells and CHO-CCK_AMT cells, respectively).

Short-term (10 min) pretreatment with TPA (0.1 μM) evoked a rightward shift of the dose-response curve for the effect of CCK-8 on the plateau increase in Ins(1,4,5)P_3 in both cell lines (Figure 3). EC_{50} values were calculated to be more than doubled to 19.1 nM and 10.0 nM for CHO-CCK_AWT and CHO-CCK_AMT cells, respectively. TPA treatment did not significantly alter the intrinsic activities (325 pmol mg^{-1} protein and 400 pmol mg^{-1} protein for CHO-CCK_AWT cells and CHO-CCK_AMT cells, respectively). In both cell lines, the values obtained with 3 nM and 10 nM CCK-8 were significantly decreased following TPA treatment (see also, Figure 4). The percentage inhibition was not different between CHO-CCK_AWT cells and CHO-CCK_AMT cells (Figure 3, insert).

Figure 4 summarizes the effects of TPA and staurosporine on the stimulant action of 10 nM CCK-8 in both cell lines. The figure shows that the plateau increase in Ins(1,4,5)P_3 evoked by 10 nM CCK-8 was slightly (1.39 fold) but significantly higher in CHO-CCK_AMT cells. TPA (0.1 μM) markedly reduced the stimulant effect of 10 nM CCK-8 in both cell lines. However, the values calculated for the percentage inhibition did not differ significantly between the two cell lines ($47.5 \pm 5.9\%$, (mean $\pm$ s.e.mean), $n=3$ and $34 \pm 3.0\%$, $n=3$ for CHO-CCK_AWT cells and CHO-CCK_AMT cells, respectively). The inhibitory effect of TPA was completely reversed by either staurosporine (1 μM) or GF-109203X (10 μM). Similar to staurosporine, GF-109203X significantly increased the stimulant effect of 10 nM CCK-8 in CHO-CCK_AWT cells but not CHO-CCK_AMT cells. Importantly, the plateau increase in Ins(1,4,5)P_3 observed in staurosporine- or GF-109203X-treated CHO-CCK_AWT cells equalled that in (un)treated CHO-CCK_AMT cells.

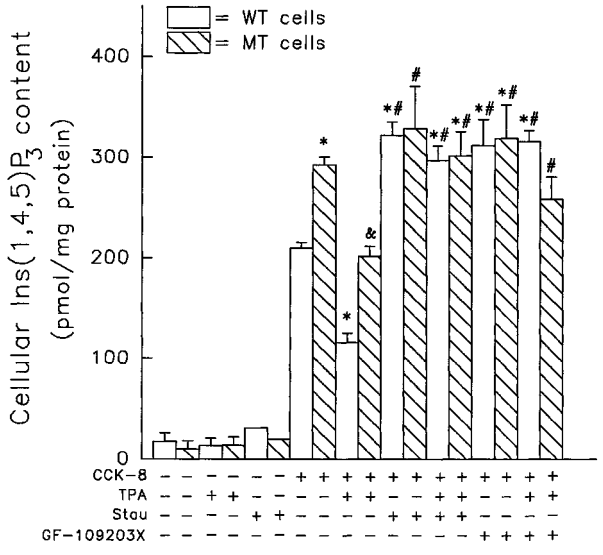

Figure 4 Effect of TPA, staurosporine and GF-109203X, alone and in combination, on the CCK-8-induced plateau increase in Ins(1,4,5)P_3 in CHO-CCK_AWT and CHO-CCK_AMT cells. CHO cells expressing either the wild-type or mutant CCK_A receptor were incubated in the absence or presence of 0.1 μM TPA and/or 1 μM staurosporine or 10 μM GF-109203X for 10 min. Subsequently, the cells were stimulated with 10 nM CCK-8 for another 20 s. After rapid quenching of the reaction, the amount of Ins(1,4,5)P_3 was determined and expressed in pmol mg^{-1} protein. The data presented are the mean and s.e.mean of 3 experiments. *Significantly different from untreated WT cells ($P<0.05$). &Significantly different from untreated CHO-CCK_AMT ($P<0.05$). #Significantly different from TPA-treated MT cells.

TPA inhibition of agonist-induced Ca^{2+} mobilization in CHO cells expressing wild-type or mutant CCK_A receptors

We previously showed that CCK-8 evokes a transient increase in average cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{i,av}$) in a suspension of CHO-CCK_AWT cells (Smeets *et al*., 1997). CCK-8 (10 nM) did not increase $[Ca^{2+}]_{i,av}$ in mock-transfected CHO cells. This demonstrates the absence of endogenous receptors that can interact with CCK-8 to activate phospholipase C. To measure $[Ca^{2+}]_i$, cells were loaded with the fluorescent Ca^{2+} indicator fura-2 and transferred to a cuvette placed in a spectrofluorophotometer to monitor the fluorescence emission ratio at 490 nm as a measure of $[Ca^{2+}]_i$ after excitation at 340 and 380 nm. The peak value of the $[Ca^{2+}]_{i,av}$ transient obtained with CHO-CCK_AWT cells increased when the CCK-8 concentration was increased and reached a maximum at 10 nM CCK-8. In each experiment, the latter value was set at 100%, to which all other values were correlated.

In both cell lines, the partial receptor agonist JMV-180, demonstrated previously to increase $[Ca^{2+}]_{i,av}$ without detectably stimulating the formation of Ins(1,4,5)P_3 in CHO-CCK_AWT cells (Smeets *et al*., 1996), maximally increased the peak increase in fluorescence emission ratio to approximately 60% of the maximal value obtained with CCK-8 (Figure 5). The EC_{50} values were calculated to be 4.3 nM and 5.0 nM in CHO-CCK_AWT and CHO-CCK_AMT cells, respectively. TPA (0.1 μM) virtually completely inhibited the stimulant effect of JMV-180 in CHO-CCK_AWT cells. The inhibitory action of the phorbol ester was partly reversed in CHO cells expressing the modified CCK_A receptor (Figure 5, insert). For instance, the percentage inhibition following stimulation of TPA-treated cells with a maximally effective JMV-180 concentration of 1 μM decreased from $91.9 \pm 2.4\%$ ($n=4$) in CHO-CCK_AWT cells to $57.5 \pm 10.3\%$ ($n=4$) in CHO-CCK_AMT cells.

Modification of the four potential PKC phosphorylation sites did not change the intrinsic activity of CCK-8, whereas it slightly decreased the EC_{50} from 33 pM in CHO-CCK_AWT cells to 11 pM in CHO-CCK_AMT cells (Figure 6). However, the values for the increase in fluorescence emission ratio did not differ statistically between CHO-CCK_AWT cells and CHO-CCK_AMT cells.

Pretreatment of CHO-CCK_AWT cells with 0.1 μM TPA for 3 min almost completely inhibited the stimulant effect of CCK-8 concentrations at or below 100 pM. However, beyond that concentration the inhibitory action of TPA was gradually overcome with the increasing CCK-8 concentration to become completely reversed at 10 nM CCK-8. As a result, the half-maximally stimulant CCK-8 concentration (EC_{50}) increased 12 fold from 33 pM in untreated CHO-CCK_AWT cells to 399 pM in TPA-treated CHO-CCK_AWT cells. TPA inhibition of the CCK-8-induced increase in $[Ca^{2+}]_{i,av}$ was significantly reduced in cells expressing the mutant CCK_A receptor (Figure 6, insert). Estimation of the EC_{50} revealed a 7.6 fold increase from 11 pM in untreated CHO-CCK_AMT cells to 84 pM in TPA-treated CHO-CCK_AMT cells.

TPA inhibition of Mas-7-induced Ca^{2+} mobilization in CHO cells

The finding that modification of the potential phosphorylation sites for PKC on the CCK_A receptor did not lead to complete

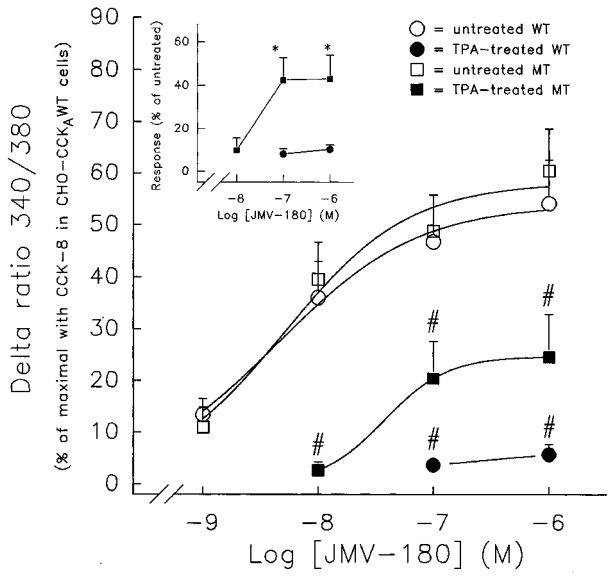

Figure 5 Effect of TPA on the dose-response curve for the stimulant effects of JMV-180 on the peak increase in average cytosolic free Ca^{2+} concentration in suspensions of CHO-CCK_AWT and CHO-CCK_AMT cells. CHO cells expressing either the wild-type or mutant receptor were loaded with Fura-2 and transferred to a cuvette placed in a spectrofluorophotometer. CHO-CCK_AWT cells and CHO-CCK_AMT cells were incubated in the absence or presence of 0.1 μM TPA for 3 min. The fluorescence emission ratio at 490 nm was monitored as a measure of the average cytosolic free Ca^{2+} concentration after excitation at 340 nm and 380 nm. Cells were stimulated with the indicated concentrations of JMV-180. In each experiment the maximal peak increase in fluorescence emission ratio obtained with 10 nM CCK-8 in CHO-CCK_AWT cells is set at 100%, to which all other values are related. The insert shows for each agonist concentration the responding TPA-treated cells as percentage of the responding untreated cells. The values presented are the mean and s.e.mean of 4–5 experiments. *Significantly different from corresponding CHO-CCK_AWT ($P<0.05$). #Significantly different from corresponding untreated ($P<0.05$).

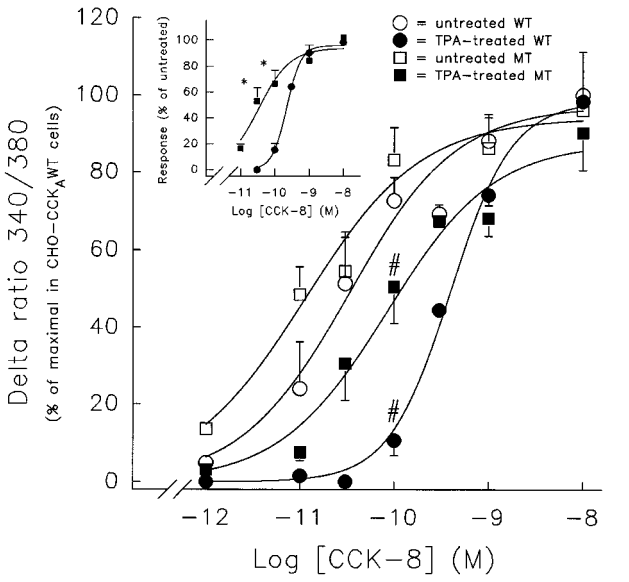

Figure 6 Effect of TPA on the dose-response curve for the stimulatory effects of CCK-8 on the peak increase in average cytosolic free Ca^{2+} concentration in suspensions of CHO-CCK_AWT and CHO-CCK_AMT cells. The fluorescence was measured as described in the caption of Figure 5. CHO cells expressing either the wild-type or mutant CCK_A receptor were incubated in the absence or presence of 0.1 μM TPA for 3 min. The cells were stimulated with the indicated concentrations of CCK-8. In each experiment the maximal peak increase in fluorescence emission ratio obtained with 10 nM CCK-8 in CHO-CCK_AWT cells is set at 100%, to which all other values are related. The insert shows for each agonist concentration the responding TPA-treated cells as percentage of the responding untreated cells. The values presented are the mean and s.e.mean of 2–4 experiments. *Significantly different from corresponding CHO-CCK_AWT ($P<0.05$). #Significantly different from corresponding untreated ($P<0.05$).

reversal of the inhibitory action of TPA on JMV-180-induced Ca^{2+} mobilization and CCK-8-induced Ins(1,4,5)P_3 formation suggested an additional effect of PKC downstream of the receptor. To test this idea, the effect of TPA on the increase in $[Ca^{2+}]_i$ evoked by the G protein activator Mas-7 was investigated. However, in contrast to CCK-8 and JMV-180, Mas-7 only poorly increased the fluorescence emission ratio in a suspension of CHO cells. Therefore, Mas-7-induced changes in $[Ca^{2+}]_i$ were measured at the single cell level using digital imaging microscopy. Figure 7 shows that addition of 10 μM Mas-7 evoked periodic $[Ca^{2+}]_i$ rises in CHO cells. Since Mas-7 has recently been shown to promote the influx of external Ca^{2+} (Suh *et al*., 1996), measurements were performed in nominally Ca^{2+} free medium. On average 83.7±7.4% ($n=3$) of the CHO cells responded to 10 μM Mas-7 (Figure 8). The percentage responding cells markedly dropped to 7.7±2.8% ($n=3$) following short-term (3 min) pretreatment with 0.1 μM TPA.

Discussion

The main conclusion of the present work is that PKC inhibits signalling through G protein-coupled receptors solely at the receptor level, where receptors linked to adenylyl cyclase are concerned, and at both the receptor level and a post-receptor site, where receptors linked to phospholipase C-β are concerned (Figure 9).

Phosphorylation sites for PKC have been identified on many G protein-coupled receptors, including the CCK_A receptor (Wank *et al*., 1992). The aim of this work was to assess the possible involvement of these sites in the regulation of receptor activity. To this end, CHO cells were stably transfected with a mutant CCK_A receptor in which the four potential sites for PKC action (Ser260, Ser264, Ser275 and Thr424), located on the third intracellular loop and carboxyl-terminal tail (Figure 1), were changed to an alanine.

It has been suggested that PKC acts in concert with a G protein-coupled receptor kinase (GRK) in homologous CCK_A receptor desensitization (Gates *et al*., 1993). On the other hand, evidence has been provided that PKC is the sole mediator in heterologous CCK_A receptor desensitization. Maximal activation of second messenger-dependent protein kinases occurs already at submaximal receptor activation. This suggests that second messenger-mediated desensitization may be most important under conditions of low receptor occupancy (Lohse *et al*., 1990). It is of importance to note that the present study, which uses TPA to activate PKC, mimics the condition of heterologous desensitization. In contrast, GRK-mediated desensitization, which is much stronger than second messenger-mediated desensitization, is quantitatively most important under conditions where agonist concentrations, and therefore receptor occupancy, are high (Hausdorff *et al*., 1990).

Using a CCK_A receptor antiserum, Miller and co-workers recently showed that the S260/264A mutant of the CCK_A receptor, which is not phosphorylated upon stimulation with TPA or CCK-8, is rapidly internalized in response to CCK-8 (Rao *et al*., 1997). Conceivably, correct interpretation of the

data obtained with CCK_A receptors with modified consensus sites for PKC action in a classical desensitization experiment using pretreatment with a high CCK-8 concentration, followed by thorough washing to remove the agonist and subsequent stimulation with a low CCK-8 concentration, is seriously hampered by this phosphorylation-independent form of desensitization. Moreover, possible effects of these mutations on the kinetics of internalization cannot be excluded. For instance, it has been demonstrated that PKC deficiency amplifies rather than attenuates agonist-induced desensitization (Shih & Malbon, 1996). TPA, in contrast to CCK-8, does not induce receptor internalization (Toledo *et al*., 1997). This

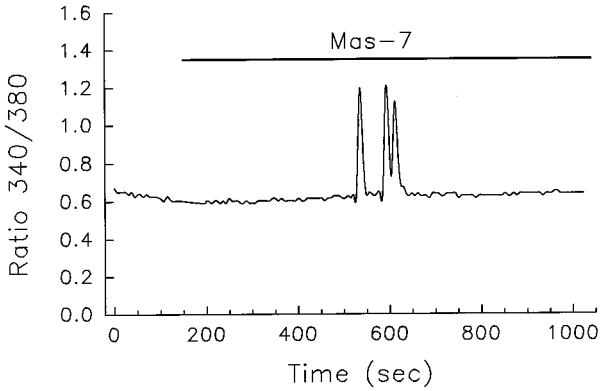

Figure 7 Effect of Mas-7 on the cytosolic free Ca^{2+} concentration in a single CHO-CCK_A cell. CHO-CCK_A cells were loaded with Fura-2 and changes in fluorescence emission ratio 340/380 nm were monitored by digital imaging microscopy. The cells were superfused with physiological salt solution and stimulated with 10 μM Mas-7 for the indicated period of time.

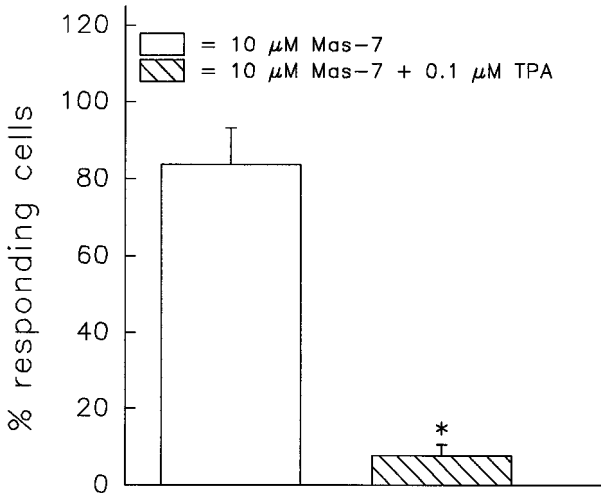

Figure 8 Effect of TPA on the Mas-7-induced increase in $[Ca^{2+}]_i$ in individual CHO-CCK_A cells. CHO-CCK_A cells were loaded with Fura-2 and the changes in fluorescence emission ratio 340/380 nm were monitored by digital imaging microscopy. The cells were superfused with physiological salt solution containing either 0.1% (v/v) dimethyl sulphoxide or 0.1 μM TPA and stimulated with 10 μM Mas-7 for 900 s. The figure shows the number of cells responding to Mas-7 as a percentage of total. The values presented are the mean $\pm$ s.e.mean of 3 experiments. *Significantly different from corresponding untreated ($P<0.05$).

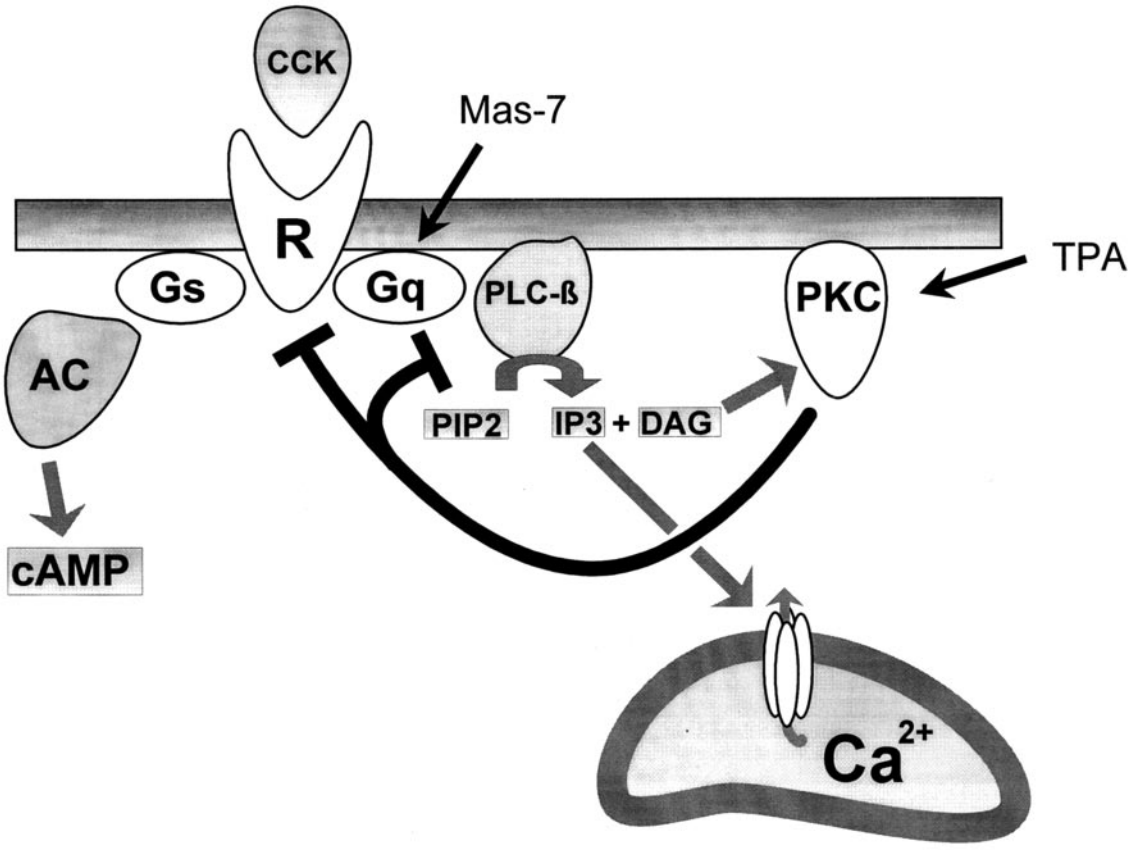

Figure 9 Proposed model of the inhibitory action of PKC on G-protein-coupled receptor signalling. The pathway leading to increased Ins(1,4,5)P_3 formation is inhibited at both the receptor and post-receptor level, whereas the pathway leading to cyclic AMP formation is inhibited only at the receptor level. Inhibition at the receptor occurs at physiological (low-level) PKC activation, while inhibition at the post-receptor site requires high-level PKC activation.

observation demonstrates that TPA can be used to study the role of the potential phosphorylation sites for PKC in the regulation of receptor activity without the interference of pretreatment-induced receptor internalization. In view of this, we have decided to use pretreatment with TPA rather than high CCK-8 in this study.

PKC acts solely at the receptor level to inhibit adenylyl cyclase activation

Although naturally expressed CCK_A receptors primarily interact with a G protein to activate phospholipase C-β, CCK-induced increases in $[Ca^{2+}]_i$ have repeatedly been demonstrated to be accompanied by relatively small increases in cyclic AMP (for references, see Wank, 1995). This suggests that the CCK_A receptor can also interact with a G protein to activate adenylyl cyclase. We (Smeets *et al*., 1998a) and others (Yule *et al*., 1993; Wu *et al*., 1997) have demonstrated that the latter interaction becomes manifest following overexpression of the CCK_A receptor in CHO cells. This is in agreement with the idea that permissiveness in the interaction of receptors with G proteins is most pregnant in the case of receptor overexpression (Raymond, 1995).

The present study shows that, despite a similar K_d, the dose-response curve for CCK-8-induced cyclic AMP accumulation was significantly shifted to the left in otherwise untreated CHO-CCK_AMT cells. Evidence that this increase in sensitivity is due to the absence of functional PKC phosphorylation sites comes from the observation that the potent inhibitor of protein kinase activity, staurosporine, evoked a comparable shift to the left in CHO-CCK_AWT cells but not CHO-CCK_AMT cells. This finding demonstrates that cyclic AMP signalling through the CCK_A receptor is inhibited by a staurosporine-sensitive kinase acting directly at one or more of the four predicted sites for PKC action on the receptor protein (Figure 9). At first sight, this observation seems to exclude any important role for a staurosporine-insensitive receptor kinase (GRK) in the attenuation of CCK-8-induced cyclic AMP formation in otherwise untreated CHO-CCK_AWT cells. However, in a previous study we provided evidence that PKC phosphorylation of the CCK_A receptor is required for the action of a GRK (Smeets *et al*., 1998b).

TPA significantly inhibited the CCK-8-induced accumulation of cyclic AMP in cells expressing the wild-type receptor. This inhibitory effect of high-level PKC activation was completely reversed in cells expressing the modified CCK_A receptor. This finding unequivocally demonstrates that PKC is the staurosporine-sensitive kinase involved in attenuation of CCK-8-induced cyclic AMP accumulation in otherwise untreated CHO-CCK_A cells. Abolishment of TPA-inhibition of agonist-induced cyclic AMP formation by modification of potential PKC phosphorylation sites on the receptor is generally observed with receptors that classically couple to adenylyl cyclase (Yuan *et al*., 1994; Widmann *et al*., 1996).

PKC acts at both the receptor level and a post-receptor site to inhibit phospholipase C activation

Effects on Ins(1,4,5)P_3 formation Native CCK_A receptors primarily couple to phospholipase C-β to promote the hydrolysis of phosphatidylinositol 4,5-bisphosphate leading to the increased formation of Ins(1,4,5)P_3. Similar to the dose-response curve for CCK-8-induced cyclic AMP accumulation that for the CCK-8-induced plateau increase in Ins(1,4,5)P_3 was shifted to the left in CHO-CCK_AMT cells. Also in this case, inhibition of protein kinase activity by staurosporine mimicked this leftward shift without having any effect on the dose-response curve for CCK-8-induced Ins(1,4,5)P_3 formation in CHO-CCK_AMT cells. These findings demonstrate that CCK-8-induced phospholipase C activation, similar to CCK-8-induced adenylyl cyclase activation, is attenuated by a staurosporine-sensitive kinase acting directly at the receptor protein in otherwise untreated CHO-CCK_AWT cells.

However, in contrast to CCK-8-induced cyclic AMP accumulation, CCK-8-induced Ins(1,4,5)P_3 formation was markedly inhibited as a result of high-level PKC activation by TPA in both cell lines. This may suggest the presence of other sites for PKC action on the receptor, which are then involved in its further uncoupling from the pathway leading to phospholipase C-β activation. However, this possibility is excluded by the recent finding that modification of Ser260 and Ser264 completely prevented TPA-induced phosphorylation of the CCK_A receptor following expression in CHO cells (Rao *et al*., 1997). Therefore, a more likely explanation is the existence of an additional site of PKC inhibition downstream of the receptor. However, in contrast to the sites for PKC action on the CCK_A receptor, this post-receptor site requires high-level PKC activation to become operative. The inhibitory action of TPA was reversed by staurosporine and GF-109203X. This is in agreement with the idea that TPA acts solely through PKC.

Effects on Ca^{2+} signalling Ins(1,4,5)P_3 interacts with specific receptors located on the endoplasmic reticulum to stimulate the release of Ca^{2+} into the cytosol resulting in a rapid increase in $[Ca^{2+}]_i$. The dose-response curves for the stimulant effects of JMV-180 and CCK-8 on the peak increase in $[Ca^{2+}]_i$ were not shifted to the left in otherwise untreated CHO-CCK_AMT cells. This is in agreement with the previous finding that neither staurosporine nor PKC down regulation evoked a leftward shift of the dose-response curve for CCK-8-induced Ca^{2+} mobilization in CHO-CCK_AWT cells (Smeets *et al*., 1998a). At first sight, this finding seems to contradict with the leftward shift of the dose-response curve for the stimulant effect of CCK-8 on the plateau increase in Ins(1,4,5)P_3 observed in these cells. However, it should be noted that $[Ca^{2+}]_i$ is already maximally increased at relatively low CCK-8 concentrations that do not detectably increase Ins(1,4,5)P_3. It is conceivable that the mechanism of CCK-8-induced receptor desensitization is only marginally active at these physiological (<0.1 nM) CCK-8 concentrations. The same holds true for the partial CCK_A receptor agonist JMV-180, which is unable to evoke a detectable increase in Ins(1,4,5)P_3 in these cells (Smeets *et al*., 1996).

Many studies have demonstrated that phorbol esters can effectively inhibit signalling through Ca^{2+} mobilizing G protein-coupled receptors (for references, see Willems *et al*., 1997). Using JMV-180, we now show that TPA inhibition of receptor-mediated Ca^{2+} mobilization is only partially reversed in CHO cells expressing the mutant receptor. Essentially the same results were obtained with the lower, more physiological, concentrations of CCK-8, which, similar to JMV-180, maximally increase $[Ca^{2+}]_i$ without detectably increasing Ins(1,4,5)P_3. These observations are in agreement with the present conclusion that one or more of the four potential phosphorylation sites for PKC are involved in TPA inhibition of Ins(1,4,5)P_3-mediated Ca^{2+} signalling through the CCK_A receptor. Moreover, the finding that TPA inhibition is reversed only in part in cells expressing the mutated receptor is in agreement with the idea of the presence of an additional inhibitory site of action of PKC downstream the receptor (Figure 9). The existence of such an additional site was demonstrated by the use of Mas-7, which directly acts at the

level of the G protein to activate phospholipase C-β and the stimulant effect, which on $[Ca^{2+}]_i$ was almost completely blocked by TPA.

Phorbol esters are widely used in mutational studies on the role of PKC in desensitization of G protein-coupled receptors. However, the present study shows that, as far as Ca^{2+} mobilizing receptors are concerned and activation of the phospholipase C-β pathway is measured, complete reversal of the inhibitory action of phorbol esters by modification of potential PKC phosphorylation sites can be masked by the presence of an additional inhibitory site of action of PKC downstream the receptor. Similar to the present study, partial reduction of TPA inhibition after modification of potential PKC phosphorylation sites was observed with the 5-hydroxytryptamine$_{1A}$ receptor expressed in Ltk^- fibroblasts (Lembo & Albert, 1995) and the platelet-activating factor receptor expressed in RBL-2H3 cells (Ali *et al*., 1997). In contrast, TPA inhibition of agonist-induced inositol phosphate formation was found to be abolished in Rat-1 cells expressing a C-terminal deletion mutant of the neurokinin$_2$ receptor (Alblas *et al*., 1995). This suggests that Rat-1 cells, in contrast with CHO-K1 cells, do not possess a post-receptor site of PKC inhibition. However, it should be noted that in the latter study a single agonist concentration was used. This leaves the possibility that abolishment rather than partial reversal of the inhibitory effect of TPA was due to a leftward shift of the dose-response curve for agonist-induced Ins(1,4,5)P_3 formation similar to that observed in the present study.

Model for the inhibitory action of PKC on transmembrane signalling through the CCK_A receptor

Based on the above observations we postulate that PKC inhibits Ins(1,4,5)P_3-mediated Ca^{2+} signalling through Ca^{2+} mobilizing receptors both at the receptor level, involving potential PKC phosphorylation sites, and at a level downstream of the receptor (Figure 9), whereas it inhibits cyclic AMP signalling only at the receptor level. Inhibition at the post-receptor site requires high-level PKC activation. In contrast, inhibition at the receptor site occurs already at modest levels of PKC activation. These modest levels of PKC activation are obtained with CCK-8 concentrations ranging from 0.1 nM to 30 nM, which readily increase Ins(1,4,5)P_3 and maximally increase $[Ca^{2+}]_i$. No inhibition is observed at physiological (<0.1 nM) CCK-8 concentrations, which readily increase $[Ca^{2+}]_i$ without detectably increasing Ins(1,4,5)P_3. At present, the molecular identity of the downstream site of action of PKC is unknown. However, the observation that phospholipase C-β is phosphorylated in a PKC-dependent manner demonstrates its candidacy (Ali *et al*., 1997; Ryu *et al*., 1990). A post-receptor site of PKC action may be of physiological relevance since it provides a means for the cell to inhibit phospholipase C-β activation by receptors that are not phosphorylated by PKC. TPA inhibition of CCK-8-induced Ca^{2+} signalling was gradually reversed with increasing CCK-8 concentration. This demonstrates that receptor phosphorylation attenuates rather than blocks receptor activity. This conclusion is supported by the finding that CCK-8-induced cyclic AMP formation was also inhibited only in part by TPA.

Conclusions

From the data presented in this work and in the literature the picture emerges that during excessive stimulation involving a receptor that is coupled to the activation of PKC (in this study mimicked by the action of TPA), signalling through other receptors (in this study the CCK_A receptor) is attenuated in a PKC-dependent manner at both the receptor level and the post-receptor level (this study) without these receptors being internalized (Toledo *et al*., 1997). Termination of the condition of excessive stimulation then leads to a rapid phosphatase-dependent resensitization of the latter receptors.

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