Differential regulation of cholecystokinin- and muscarinic-receptor-mediated phosphoinositide turnover in Flow 9000 cells

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We have explored the hypothesis that the apparent greater efficiency of cholecystokinin (CCK-8) receptor–second messenger coupling compared with that of muscarinic receptor in Flow 9000 cells is due to differential feedback inhibitory control mechanisms. Pretreatment of Flow 9000 cells with the tumour-promoting protein kinase C (PKC)-activating agent 12-*O*-tetradecanoylphorbol 13-acetate (TPA) produced a time- and dose-dependent inhibition of CCK-8 and acetylcholine (ACh) stimulation of inositol phosphate production. The inhibition by TPA of ACh-induced PI (phosphoinositide) response involved reduction of the maximal response, but no change in the concentration of ACh required to evoke a half-maximal response. In contrast, TPA inhibition of CCK-8 responses could be overcome by increasing the CCK-8 concentrations. Flow 9000 cells pretreated with TPA exhibited a 52–68% reduction in [³H]quinuclidinyl benzilate ([³H]QNB) binding capacity, whereas [¹²⁵I]CCK-8 binding was unchanged. In saponin-permeabilized Flow 9000 cells, TPA pretreatment had no effect on guanosine 5′-[γ-thio]triphosphate (GTP[S])-induced inositol phosphate formation, indicating that G-protein linkage to phosphoinositidase C (PIC) was not affected. However, TPA significantly inhibited the potentiating effect of GTP[S] on CCK-8 and ACh activation of PI response, suggesting that the coupling between the receptors and the G-protein was impaired. The PKC-activator 1-oleoyl-2-acetyl-glycerol (OAG), a diacylglycerol analogue, also significantly reduced CCK-8 and ACh stimulation of inositol phosphate accumulation in these cells. Our results are consistent with the hypothesis that muscarinic activation of PI hydrolysis is subjected to rapid feedback inhibition via the 1,2-diacylglycerol–PKC pathway. CCK-receptor activation of PI turnover is modulated to a lesser extent, and this may partially explain apparent differences in the efficiency of receptor–second messenger coupling. It is proposed that TPA acting through PKC exerts its inhibitory action on muscarinic-agonist-mediated PI response mainly at the receptor level, whereas the inhibitory effect on CCK-8 response is at a site close to the receptor–G-protein coupling step.

INTRODUCTION

We have recently shown that activation of CCK-8 and muscarinic cholinergic receptors are linked to a rapid and transient increase in inositol polyphosphate accumulation in the human embryonic pituitary cell line Flow 9000. This is resulted from an increased breakdown of phosphatidylinositol 4,5-bisphosphate (PIP₂) by a GTP-regulatory protein (G-protein)-linked enzyme, phosphoinositidase C (PIC) (Clark *et al.*, 1987). A feature of the Flow 9000 cells is that, although the maximal increase in inositol phosphate formation elicited by both CCK-8 and muscarinic ligands was similar, the muscarinic-receptor density was more than ten times that of the CCK receptor. Comparison between the ability of CCK-8 and ACh in stimulating PI hydrolysis and in displacing specific binding of [¹²⁵I]CCK-8 and [³H]QNB respectively in Flow 9000 cells revealed that little or no spare receptor is involved in either case. In addition, maximal responses elicited by CCK-8 and muscarinic agonists are additive, indicating that neither the trans-

ducer G-protein or the effector enzyme PIC is limiting. A possible explanation for the apparent difference in receptor–response coupling efficiency could thus reside in the existence of differential inhibitory regulatory mechanisms at the two receptor sites. We decided to explore the possibility that the muscarinic receptor might be subjected to rapid feedback inhibition.

A major product of PIP₂ hydrolysis is 1,2-diacylglycerol (DAG), which has been shown previously to activate a calcium- and phospholipid-dependent kinase, protein kinase C (PKC) (for review, see Nishizuka, 1986). PKC has been implicated in the regulation of a variety of cellular responses via phosphorylation of specific substrate proteins (Niedel & Blackshear, 1986). These include, for example, many membrane receptors, such as muscarinic receptor (Liles *et al.*, 1986), α₁-adrenoceptor (Leeb-Lundberg *et al.*, 1985), β-adrenoceptor (Sibley *et al.*, 1984), epidermal-growth-factor receptor (Hunter *et al.*, 1984), transferrin receptor (May *et al.*, 1986) and interleukin-2 receptor (Shackelford & Trowbridge, 1984), G-proteins (Katada *et al.*, 1985), and

Abbreviations used: CCK-8, cholecystokinin octapeptide (sulphated); ACh, acetylcholine; [³H]QNB, [³H]quinuclidinyl benzilate; PI, phosphoinositide(s); [³H]IP, total [³H]inositol phosphates; DAG, 1,2-diacylglycerol; PIC, phosphoinositidase C; PKC, protein kinase C; GTP[S], guanosine 5′-*O*-(3′-thiotriphosphate); TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PDA, phorbol 13,20-diacetate; OAG, 1-oleoyl-2-acetyl-glycerol; DMSO, dimethyl sulphoxide; KRB, Krebs–Ringer bicarbonate; IC₅₀, concentration causing half-maximal inhibition; PIP₂, phosphatidylinositol 4,5-bisphosphate.

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enzymes such as guanylate cyclase (Zwiler *et al.*, 1985). By phosphorylating various receptor proteins and transducers such as G-proteins, PKC is proposed to exert an important feedback regulatory action on receptor-mediated events such as PIP_2 hydrolysis (Nishizuka, 1986). In the present study we have employed the potent tumour-promoting phorbol ester TPA, a PKC activator, to explore the possible role of PKC in regulating CCK-8- and muscarinic-receptor-mediated inositol phospholipid turnover in Flow 9000 cells.

MATERIALS AND METHODS

Materials

CCK-8 was purchased from Bachem (Saffron Walden, Essex, U.K.). ACh was obtained from Sigma. [^3H]QNB (sp. radioactivity 42.6 Ci/mmol), [^{125}I]CCK-8 (sp. radioactivity 2200 Ci/mmol) and *myo*-[2- ^3H]inositol (sp. radioactivity 17.1 Ci/mmol) were purchased from New England Nuclear (Stevenage, Herts., U.K.). TPA and PDA were obtained from Cambridge Research Biochemicals (Harston, Cambridge, U.K.). GTP[S] was bought from Boehringer Mannheim (Lewes, East Sussex, U.K.). Culture media and antibiotics were obtained from either Gibco (Paisley, Renfrewshire, Scotland, U.K.) or Flow Laboratories (Rickmansworth, Herts., U.K.). All other chemicals were ordered from Fisons (Loughborough, Leics., U.K.).

Cell culture

The human embryonic pituitary cell line Flow 9000 (passages 17–26) was purchased from Flow Laboratories. The cells were cultured in a O_2/CO_2 (19:1) atmosphere at 37 °C in Ham's F10 medium supplemented with antibiotics and sera [10% (v/v) donor horse serum and 2.5% (v/v) foetal-bovine serum]. Cells were plated at approx. 1.5×10^5 cells/22 mm well in a 12-well tissue-culture cluster (Costar) (Lo *et al.*, 1986a).

PI hydrolysis assay

After reaching confluence, Flow 9000 cells were labelled with *myo*-[2- ^3H]inositol at 2 μCi /well for 48 h, by which isotopic equilibrium would have been achieved (Lo *et al.*, 1986a). Phorbol esters, dissolved in dimethyl sulphoxide (DMSO), were then added for 2–10 min. The final concentration of DMSO in the incubation medium was 1%, and an equal amount of DMSO was added to control wells. After phorbol pretreatment, cells were washed three times with Krebs–Ringer bicarbonate (KRB, supplemented with glucose) buffer and then exposed to agonists at various concentrations in the presence of 10 mM-LiCl for 30 min in a final incubation volume of 400 μl . In permeabilized-cell studies, cells were pretreated with saponin (50 $\mu\text{g}/\text{ml}$) at room temperature for 15 min [after this treatment, $95 \pm 5\%$ ($n = 4$) of the cells were unable to exclude Trypan Blue, in contrast with control cells incubated in KRB but with saponin omitted, where only $17 \pm 3\%$ ($n = 4$) were labelled with Trypan Blue] and then washed thoroughly before the incubation with drugs. After exposure, the incubation was stopped by adding 1 ml of ice-cold chloroform/methanol (1:2, v/v) solution. Cell extracts were then transferred to BioVials (Beckman, High Wycombe, Bucks., U.K.), and equal volumes (0.3 ml) of water and chloroform were added to each vial for phase separation. A 0.9 ml portion of upper aqueous phase was transferred

into an Econo-column (Bio-Rad) containing 0.8 ml of anion-exchange resin (Bio-Rad AG 1-X8, formate form) and total [^3H]inositol phosphates ([^3H]IP, including inositol mono-, bis-, tris- and tetrakis-phosphate) were eluted with 4×2 ml of 1.0 M-ammonium formate/0.1 M-formic acid.

Radioligand binding studies

Initial experiments have established that binding parameters [both B_{max} (maximal binding capacity) and K_d (apparent equilibrium dissociation constant)] are similar in both [^3H]QNB and [^{125}I]CCK-8 binding studies performed with broken cell membranes and intact-cell preparations. This is especially important regarding the permeable nature of [^3H]QNB used in the present study. However, the similar binding characteristics of [^3H]QNB binding to both intact cells and membrane preparations suggest that specific [^3H]QNB binding to intact cells is likely to represent cell-surface binding sites.

Intact Flow 9000 cells pretreated with TPA or DMSO (vehicle) for 10 min were used for binding studies. Control or TPA-pretreated Flow 9000 cells (2×10^5 cells for 0.06 mg of protein per well) were rinsed with 1 ml of fresh KRB buffer after removal of the culture medium and were then incubated with increasing concentrations of either [^3H]QNB or [^{125}I]CCK-8 in the presence or absence of either 10^6 M-atropine or unlabelled CCK-8 respectively, at 37 °C for 1 h in saturation experiments. In [^3H]QNB inhibition assays, 0.04 nM-[^3H]QNB was incubated with various concentrations of ACh, in a final incubation volume of 0.5 ml, for 1 h. The incubations were terminated by rapidly aspirating the incubation medium. The radioligand-bound monolayer of cells was rinsed three times with 3 ml of ice-cold Tris/HCl (50 mM-Tris/HCl, pH 7.4). The cells were then trypsin-treated with 0.5 ml of trypsin/EDTA (Gibco) solution and transferred to scintillation vials. Each well was then rinsed with another 0.5 ml of trypsin/EDTA solution. The solutions from the same well were pooled, 10 ml of scintillant was added, and the radioactivity was measured by liquid-scintillation spectrometry with an efficiency of about 40%. Protein content was estimated by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Presentation of results

All experiments presented in the present study were repeated at least three times. Unless otherwise stated, experimental data points are usually expressed as means $\pm$ S.E.M. for three or more separate experiments. Statistical significance of results was assessed by Student's *t* test.

RESULTS

Effects of phorbol esters on agonist-induced [^3H]IP formation

When [^3H]inositol-prelabelled Flow 9000 cells were pretreated with TPA (1 μM , 10 min), the cells showed a significantly reduced response to both CCK-8 (1 nM) and ACh (25 μM) in the stimulation of [^3H]IP production (Table 1). The weak tumour-promoter PDA (1 μM , 10 min) had no effect on [^3H]IP accumulation induced by both agonists. These results are consistent with PKC being the target of TPA action, since the potencies of

Fig. 1. Effects of TPA pretreatment on (a) ACh and (b) CCK-8 stimulation of [³H]IP formation

[³H]Inositol-prelabelled Flow 9000 cells were pretreated with either 10⁻⁶ M (■) or 10⁻⁸ M (●)-TPA or -DMSO (vehicle, ○) for 10 min. Cells were then washed thoroughly with buffer and challenged with various concentrations of ACh (a) or CCK-8 (b) for 30 min. Total [³H]IP were extracted and quantified as described in the Materials and methods section. Each data point represents mean for four separate experiments performed in duplicate. The S.E.M. was less than 10% for each data point.

Table 1. Effects of PKC activators on CCK-8 and ACh stimulation of [³H]IP formation in Flow 9000 cells

[³H]Inositol-prelabelled Flow 9000 cells were pretreated with either TPA, PDA, OAG or vehicle for 10 min. Cells were thoroughly washed and then exposed to either ACh (25 μM) or CCK-8 (1 nM) for 30 min. The reaction was stopped, and total [³H]IP were extracted as described in the Materials and methods section. Data are means ± S.E.M. for three independent experiments. **P* < 0.05; ***P* < 0.01 versus vehicle-treated cells.

Treatment	[³ H]IP formation (d.p.m./well)		
	Control	ACh	CCK-8
DMSO (vehicle)	1180 ± 55	5565 ± 240	6310 ± 250
TPA (1 μM)	1063 ± 80	1555 ± 120**	3650 ± 215**
PDA (1 μM)	1205 ± 57	5231 ± 292	6250 ± 110
OAG (10 μM)	1305 ± 112	2735 ± 136**	3088 ± 277*

these phorbol esters correlate with their action on purified PKC (Kikkawa *et al.*, 1983).

The effect of TPA (10⁻⁶ and 10⁻⁸ M) on ACh-stimulated [³H]IP formation are shown in Fig. 1(a). The TPA inhibition was dose-dependent and could not be reversed by increasing the concentrations of ACh. The IC₅₀ value of TPA in inhibiting a ACh (1 mM) stimulation of [³H]IP formation was 4.4 ± 0.9 nM (*n* = 4). In contrast, Fig. 1(b) shows that pretreatment of Flow 9000 cells with TPA (10⁻⁶ and 10⁻⁸ M) resulted in a different profile of inhibition of TPA on the dose-dependent activation of [³H]IP formation by CCK-8. Whereas the response to low concentrations of CCK-8 was significantly reduced by TPA, an increase in CCK-8 concentration overcame the TPA inhibition. The IC₅₀ value for the effect of TPA on CCK-8 (1 nM) stimulation of [³H]IP production was 6.9 ± 1.7 nM (*n* = 4). Both IC₅₀ values are in accord with the nanomolar *K_i* value of TPA in displacing [³H]-phorbol 12,13-dibutyrate binding to PKC (Leach & Blumberg, 1985), thus further support the assumption that PKC is the site of action of TPA.

1-Oleoyl-2-acetyl-glycerol (OAG), a synthetic cell-permeable analogue of DAG, is known to activate PKC in a similar manner to TPA (Nishizuka, 1986). Exposure of Flow 9000 cells to OAG (10 μM) for 10 min signi-

ficantly inhibited formation of [³H]IP production initiated by both CCK-8 (1 nM) and ACh (25 μM), while having no effect on the basal levels (Table 1). These results are consistent with the involvement of PKC in regulating agonist-mediated PIP₂ hydrolysis.

Time course of TPA inhibition

The inhibitory effect of TPA on agonist-activated [³H]IP formation was time-dependent, as shown in Fig. 2. Inhibition of ACh (25 μM) stimulation of [³H]IP production by TPA (100 nM) was rapid and nearly complete within 2 min pretreatment. In contrast, the inhibition by TPA of CCK-8 (1 nM)-mediated [³H]IP accumulation was progressive, with maximal response being achieved only after 10 min.

Effects of TPA on [³H]QNB and [¹²⁵I]CCK-8 binding

In preliminary experiments we established that, in broken-cell-membrane preparations, TPA had no direct displacing activity towards either [¹²⁵I]CCK-8 or [³H]QNB binding.

In contrast, when we examined the displacement of [³H]QNB binding by ACh was well as the saturation binding of [³H]QNB in intact TPA-pretreated Flow 9000 cells, a 52–68% reduction in binding capacity was

Fig. 2. Time course of TPA inhibition of agonist-induced [3 H]IP accumulation

[3 H]Inositol-labelled Flow 9000 cells were preincubated with TPA (100 nM) for 2, 5 or 10 min. Cells were then washed free of TPA and exposed to either 25 μ M-ACh (●) or 1 nM-CCK-8 (○) for 30 min. Total [3 H]IP were extracted and separated as described in the Materials and methods section. Results are expressed as percentages of maximal response induced by the assigned concentration of ACh (450% of basal) and CCK-8 (480% of basal). Data represent means $\pm$ S.E.M. for three independent experiments performed in duplicate.

observed (Table 2 and Fig. 3a). The remaining muscarinic binding sites, however, had similar apparent affinity for both the radioligand and ACh.

In [125 I]CCK-8 saturation binding studies, TPA (1 μ M, 10 min), pretreatment had no effect on either the maximal

Table 2. Saturation analysis of [3 H]QNB binding in intact Flow 9000 cells pretreated with TPA or vehicle

Specific [3 H]QNB binding to intact Flow 9000 cells was determined as described in the Materials and methods section. Scatchard analyses of the saturation data yielded $B_{\max}$ and K_d . Binding-parameter estimates were determined by using a non-linear curve-fitting program. Hill coefficients are close to 1 in both control and TPA (1 μ M, 10 min)-treated cells. Results are means $\pm$ S.E.M. for three separate experiments, each in triplicate. * $P < 0.001$ versus vehicle-treated cells.

Binding parameter	DMSO (vehicle)-treated	TPA-treated
$B_{\max}$ (fmol/mg of protein)	187.8 $\pm$ 9.8	76.2 $\pm$ 5.9*
K_d (nM)	0.028 $\pm$ 0.007	0.030 $\pm$ 0.005

binding capacity ($B_{\max}$) or the apparent dissociation constant (K_d) in intact Flow 9000 cells (Fig. 3b). However, it is worth mentioning that our results here could not eliminate the possibility that CCK receptors were lost but recovered during the 1 h incubation.

Effects of TPA on GTP[S]-mediated [3 H]IP formation

The effects of TPA (1 μ M, 10 min) pretreatment on GTP[S] stimulation of PIP₂ hydrolysis are shown in Table 3. TPA pretreatment had no effect on GTP[S] (100 μ M) activation of [3 H]IP accumulation, but significantly reduced the potentiating action of GTP[S] on CCK-8- and ACh-activated [3 H]IP formation in saponin-permeabilized Flow 9000 cells. The inhibitory effect of TPA on the GTP[S]-potentiated ACh response was expected, since TPA reduces the number of muscarinic binding sites, as shown in Table 2. However, the effect of

Fig. 3. Effects of TPA pretreatment on (a) ACh-reversible [3 H]QNB binding and (b) saturable CCK-8 binding on intact Flow 9000 cells

Flow 9000 cells were pretreated with 1 μ M-TPA (●) or -DMSO (vehicle, ○) for 10 min. Cells were then incubated with (a) 0.04 nM-[3 H]QNB in the presence of various doses of ACh (10^{-6} – 10^{-2} M) or (b) increasing concentrations of [125 I]CCK-8 (0.05–1.40 nM) for 2 h, during which equilibrium binding would have been achieved. Procedures for binding experiments were as described in the Materials and methods section. Results were normalized to percentage of maximal specific binding in the case of [3 H]QNB experiments. Data are means $\pm$ S.E.M. for three separate experiments in (a) and means for four independent experiments in (b), for which the S.E.M. was less than 10% at each point.

Table 3. Effects of TPA pretreatment on GTP[S]-mediated [³H]IP accumulation in saponin-permeabilized Flow 9000 cells

Saponin-permeabilized Flow 9000 cells were pretreated with vehicle (DMSO) or TPA (1 μ M) for 10 min and were then exposed to GTP[S] with or without CCK-8 or ACh for another 30 min. Water-soluble [³H]IP were separated as described in the Materials and methods section. Data represent means $\pm$ S.E.M. for four independent experiments, each in duplicate. * P < 0.05 and ** P < 0.01 versus vehicle-treated cells.

Condition	[³ H]IP formation (d.p.m./well)	
	Vehicle	TPA
Control	1005 $\pm$ 65	1128 $\pm$ 101
GTP[S] (100 μ M)	1859 $\pm$ 93	1921 $\pm$ 77
CCK-8 (100 nM)	1016 $\pm$ 97	1005 $\pm$ 114
ACh (1 mM)	1417 $\pm$ 113	1360 $\pm$ 163
GTP[S] + CCK-8	3221 $\pm$ 217	2442 $\pm$ 122*
GTP[S] + ACh	3617 $\pm$ 281	2007 $\pm$ 131**

TPA on GTP[S] potentiation of CCK-8-induced inositol phospholipid breakdown was unexpected and may be important in considering the nature of the TPA interaction with the CCK receptor-response coupling system.

DISCUSSION

Activation of hydrolysis of PIP₂ by a variety of agonists leads to the production of two or more putative second messengers. One of these second messengers, namely 1,2-diacylglycerol, is an activator of PKC. PKC plays a vital role in initiating a variety of cellular functions such as neurotransmitter release (Zurgil & Zisapel, 1985), regulation of membrane ionic conductance (Baraban *et al.*, 1985) and cell differentiation (Vanderbark & Niedel, 1984). In addition, PKC has been implicated in the feedback regulation of receptor-mediated events such as PI hydrolysis, as well as feed-forward regulation of other second-messenger systems, for example, the adenylate cyclase-cyclic AMP pathway (Yoshimasa *et al.*, 1987). In the present study we have shown that TPA, a PKC activator, significantly inhibits both CCK-8 and muscarinic-receptor-mediated PI turnover in Flow 9000 cells.

Since the inhibitory effects of TPA on muscarinic-receptor-mediated [³H]IP accumulation could not be overcome by increasing the agonist concentrations, a loss of binding sites as indicated by a reduction in $B_{\max}$ in saturation analysis of [³H]QNB binding could conceivably account for the attenuated PI response. However, it is important to note that, whereas ACh-induced [³H]IP accumulation was inhibited 75–90% by 1 μ M-TPA (Fig. 1a), only a 52–68% reduction in muscarinic-receptor binding sites was observed with an identical treatment of the cells (Fig. 3a). It is therefore possible that the loss of muscarinic binding sites is only partially responsible (although it seems to be the major factor) in the attenuation of the PI response by TPA. A corollary of this is that TPA may have another site of action at one of the steps that occur between receptor occupation and

PI hydrolysis. One of these possible sites is the G-protein, modification of which by TPA (presumably through PKC) would impair the efficiency of coupling between the receptor and the G-protein. It is interesting to point out that, although phosphorylation of the muscarinic receptor by PKC is well documented in neuroblastoma cells (Liles *et al.*, 1986), in a number of cell systems PKC exerts its inhibitory action on muscarinic-receptor-mediated responses without affecting the binding characteristics of the receptor (Orellana *et al.*, 1985; Vicentini *et al.*, 1985; Ansah *et al.*, 1986). Recently, Ho *et al.* (1987) have demonstrated that purified muscarinic receptors from porcine synaptic membrane were phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase, resulting in a concomitant loss of [³H]QNB binding capacity of these receptors. Thus it appears that PKC can regulate muscarinic-receptor-initiated events through more than one mechanism.

In [³H]inositol-prelabelled Flow 9000 cells, TPA pretreatment also significantly attenuated PI responses elicited by CCK-8. However, this TPA inhibition could be reversed by increasing the concentration of CCK-8 (Fig. 1b). In addition, TPA-pretreated Flow 9000 cells had similar binding characteristics ($B_{\max}$ and K_d) in [¹²⁵I]CCK-8 saturation binding studies as compared with control vehicle-treated cells (Fig. 3b). This observation is reminiscent with those of A-10 vascular smooth-muscle cells (Aiyar *et al.*, 1987), rat renal mesangial cells (Pfeilschifter, 1986), rabbit neutrophils (Naccache *et al.*, 1985) and NG108-15 cells (Osugi *et al.*, 1987), in which TPA significantly inhibited vasopressin-, angiotensin II-, fMet-Leu-Phe- and bradykinin-stimulated PI turnover respectively, but with no effect on corresponding radio-ligand binding parameters. This again leads us to propose that TPA may exert an effect on receptor-effector coupling.

Two candidates for the post-receptor site of action of TPA (via PKC) are the G-protein and the effector enzyme PIC. Using saponin-permeabilized Flow 9000 cells, we have previously shown that one can by-pass the receptor and stimulate PIC by adding non-hydrolysable GTP analogues such as GTP[S] and guanosine 5'-[β -imidio]triphosphate ('Gp[NH]ppG') to activate the G-protein. Moreover, GTP[S] is able to potentiate the effect of CCK-8 on PI hydrolysis in these permeabilized cells (Lo *et al.*, 1986b). The fact that TPA pretreatment does not affect GTP[S]-induced [³H]IP formation in saponin-permeabilized cells suggests that neither PIC nor the G-protein-PIC coupling mechanism is affected by TPA. However, TPA pretreatment, before permeabilization, significantly reduced the potentiating effect of GTP[S] on CCK-8 stimulation of [³H]IP accumulation, suggesting that the coupling between the receptor and the G-protein might have been impaired. In this context, it is worth mentioning that Bauer & Jakobs (1986) have shown that phosphorylation of the G_i-protein by TPA in intact human platelets leads to a decrease in hormone-induced inhibition of adenylate cyclase, whereas that induced by GTP[S] was not affected. Thus it is possible that TPA pretreatment can phosphorylate the G-protein involved in receptor-PIC coupling in Flow 9000 cells. In doing so, TPA impairs the coupling between the receptor and the G-protein while having no effect on the interaction between G-protein and PIC. This may account for the additional site of action of TPA in the inhibition of ACh-stimulated PI response, as mentioned above. Our time-

course studies show another distinct difference between TPA inhibition of CCK-8- and ACh-mediated responses in that the former is much slower process. This may represent differences between the phosphorylation of the muscarinic receptor and the coupling G-protein. Additional experiments are needed to confirm this hypothesis.

The question now arises as to whether differential feedback inhibition via PKC can explain our original observation that there appears to be a greater efficiency of CCK receptor-response coupling in Flow 9000 cells. Our results are consistent with the concept that PKC-mediated down-regulation of the muscarinic receptor limits its response at an early time point. However, we have been unable to show that inhibition of PKC increases the muscarinic-receptor-mediated response, since, in our hands, the putative PKC inhibitors 1-(5-isoquinolinesulphonyl)-2-methylpiperazine ('H-7') and *N*-(2-aminoethyl)isoquinoline-5-sulphonamide ('H-9') (Hidaka *et al.*, 1984) were inactive in reversing the inhibitory effects of TPA on both ACh- and CCK-8-mediated responses (results not shown). We would also predict that a truly selective and potent PKC inhibitor would increase the muscarinic response in our system. Our results also have a more general significance in that co-storage and co-release of peptides and amines is a common event in neuroendocrine and neural systems. Thus, even if two primary co-messengers act through a common second-messenger system, a differential intracellular feedback mechanism will ensure that the primary co-messengers may have differential and interacting effects.

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