

Acetylcholine and cholecystokinin receptors functionally couple by different G-proteins to phospholipase C in pancreatic acinar cells

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We have studied the involvement of GTP-binding proteins in the stimulation of phospholipase C from rat pancreatic acinar cells. Pretreatment of permeabilized cells with activated cholera toxin inhibited both cholecystokinin-octapeptide (CCK-OP) and GTP γ S but not carbachol (CCh)-induced production of inositol trisphosphate. Pertussis toxin had no effect. Neither vasoactive intestinal polypeptide, a stimulator of adenylyl cyclase, nor the cAMP-analogue, 8-bromo cAMP, mimicked the inhibitory effect of cholera toxin on agonist-induced phospholipase C activation. This indicates that inhibition by cholera toxin could not be attributed to a direct interaction of cholera toxin activated G_s with phospholipase C or to an elevation of cAMP. In isolated rat pancreatic plasma membranes cholera toxin ADP-ribosylated a 40 kDa protein, which was inhibited by CCK-OP but not by CCh. We conclude from these data that both CCK- and muscarinic acetylcholine receptors functionally couple to phospholipase C by two different GTP-binding proteins.

Cholera toxin; Pertussis toxin; Inositol trisphosphate; ADP-ribosylation; GTP γ S

1. INTRODUCTION

G-proteins appear to play a role in the functional coupling of receptors to the phospholipase C which leads to the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP₂) and the subsequent generation of two intracellular messengers, inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol [1–3]. In permeabilized cells, GTP, the GTP analog GTP γ S and F[−] stimulate phospholipase C activity [2,4–7]. In some cells [8,9], pertussis toxin inhibits agonist-induced IP₃ production, whereas in other cells [10,11] including

the exocrine pancreas [12,13] pertussis toxin has no effect on IP₃ formation. Conflicting results have been obtained concerning the effect of cholera toxin on agonist-induced phospholipase C activity in the exocrine pancreas [12,14]. In a human T-cell line [15] and in clonal human pituitary cells, cholera toxin was shown to inhibit receptor-mediated activation of phospholipase C. In the latter study, however, GTP γ S-induced stimulation of phospholipase C was not inhibited by cholera toxin [16].

Here we report that in isolated permeabilized acinar cells from rat pancreas, cholecystokinin-, as well as GTP γ S-stimulated but not carbachol-stimulated activation of phospholipase C is inhibited by preincubating cells in the presence of activated cholera toxin. Furthermore, cholera toxin induced [³²P]ADP-ribosylation of a 40 kDa protein in isolated plasma membranes from pancreatic acinar cells. This ADP-ribosylation was reduced in the presence of CCK-OP but remained unaffected in the presence of carbachol. We, therefore, con-

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Abbreviations: CCK-OP, cholecystokinin octapeptide; ACh, acetylcholine; CCh, carbamylcholine chloride (carbachol); G-protein, guanine nucleotide binding protein; IP₃, inositol trisphosphate; GTP γ S, guanosine 5'-O-(3-thiotriphosphate); VIP, vasoactive intestinal polypeptide; CT, cholera toxin; PT, pertussis toxin; f-MLP, formyl Met-Leu-Phe

clude that two different, cholera toxin-sensitive and cholera toxin-insensitive GTP-binding proteins couple the CCK and the acetylcholine receptor, respectively, to phospholipase C.

2. MATERIALS AND METHODS

2.1. Materials

VIP, CT and synthetic octapeptide of CCK-OP were obtained from Sigma (München, FRG), *myo*-[^3H]inositol and [^{32}P]NAD $^+$ from New England Nuclear Chemicals (Dreieich, FRG). A [^3H]cAMP radioassay kit was purchased from Amersham Buchler (Braunschweig, FRG). Pertussis toxin was a gift of Dr M. Yajima (Kyoto, Japan).

2.2. Methods

Isolated acinar cells from rat pancreas (male Wistar rats of approx. 200 g) were prepared by collagenase digestion and permeabilized by washing them in a nominally Ca^{2+} -free solution as described by Streb and Schulz [17]. Inositol-phospholipids were prelabeled by addition of 100 $\mu\text{Ci}/\text{ml}$ *myo*-[2- ^3H]inositol (approx. 6 $\mu\text{mol}/\text{l}$) to the cells during the last hour of collagenase digestion.

Isolated cells were incubated in 2.5 ml of a buffer containing in mmol/l: 110 KCl, 7 MgCl_2 , 5 $\text{K}_2\text{H ATP}$, 10 creatine phosphate, 5 K_2 -succinate, 5 pyruvate, 8 units/ml creatine kinase, 25 HEPES, pH 7.4, at 25°C. The free Mg^{2+} concentration of this solution was calculated at 2.1 mmol/l, the contaminating Ca^{2+} at about 2 $\mu\text{mol}/\text{l}$. Before addition, CT or PT were preactivated by incubation with 20 mmol/l dithiothreitol. For control experiments, CT was inactivated by heating for 10 min at 95°C before addition of dithiothreitol.

For determination of IP_3 production, 200 μl aliquots were analyzed for inositol phosphates, using Dowex anion-exchange columns as described [18].

Cyclic AMP (cAMP) was assayed using a cAMP kit obtained from Amersham Buchler. Preparation of a fraction enriched in plasma membranes was performed as described recently, using a MgCl_2 precipitation method [19].

For the CT-induced ADP-ribosylation, about 50 μg of membrane protein was incubated for 30 min at 32°C in an incubation medium containing (in mmol/l) 340 K-phosphate, 10 thymidine, 1 EDTA, 0.005 NAD $^+$, 0.5 μCi [^{32}P]NAD $^+$, 0.1 mg/ml activated CT, pH 7.0, or for the ADP-ribosylation by pertussis toxin in (in mmol/l) 0.02 Tris-HCl, 1 EDTA, 10 thymidine, 0.005 NAD $^+$, 5 $\mu\text{g}/\text{ml}$ activated PT, pH 7.0. Proteins were precipitated with 500 μl methanol/chloroform/water (4:1:3). SDS-polyacrylamide gel electrophoresis using a 6%:12.5% gel was performed as described [20], and the dried gel was autoradiographed.

Protein content was determined according to Lowry et al. [21], using bovine serum albumin as standard.

3. RESULTS

3.1. Effect of CT on CCK-, carbachol- and $\text{GTP}\gamma\text{S}$ -induced IP_3 production

Pretreatment of permeabilized acinar cells with

preactivated CT (40 $\mu\text{g}/\text{ml}$) in the presence of NAD $^+$ did not affect basal phosphoinositide metabolism. However, CCK-OP-induced IP_3 production following 30 min of incubation of the cells was inhibited by 49% when preactivated CT was present in the incubation medium ($p < 0.001$) (fig.1, table 1). Similarly $\text{GTP}\gamma\text{S}$ -induced IP_3 production was inhibited by CT by 75% following 25 min of incubation ($p < 0.001$) (see table 1, fig.2). In contrast, CCh-induced IP_3 production was unaffected by pretreatment of the cells with CT (see table 1). In three experiments, preactivated PT (20–2000 $\mu\text{g}/\text{ml}$) in the presence of NAD $^+$ had no effect on basal nor on CCK-OP- or CCh-induced IP_3 production (not shown).

3.2. Effect of the cAMP-analog 8-bromo cAMP on CCK-OP-mediated activation of phospholipase C

CT stimulates cAMP production in pancreatic acinar cells [22] (fig.3) presumably by ADP-ribosylation of the G_s protein of the adenylyl cyclase system. In order to decide if inhibition of phospholipase C stimulation was due either to a direct effect of the activated G_s protein on

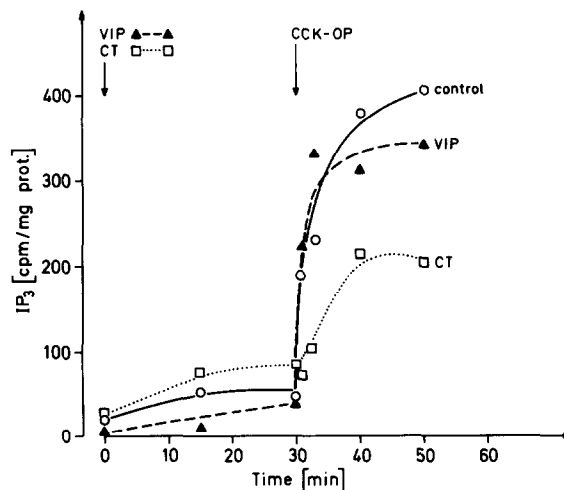


Fig.1. Effects of CT and VIP on CCK-OP-induced IP_3 production in permeabilized pancreatic acinar cells. *myo*-[^3H]inositol-prelabeled, permeabilized cells (6.9 mg/ml of protein) were incubated under standard conditions without addition (\circ — \circ) or with VIP (0.1 $\mu\text{mol}/\text{l}$) (\blacktriangle — \blacktriangle), or with CT (40 $\mu\text{g}/\text{ml}$) and NAD $^+$ (\square — \square) for 30 min, followed by addition of CCK-OP (0.3 $\mu\text{mol}/\text{l}$). The experiment shown is representative for 3 experiments.

Table 1

Effect of CT on basal, CCK-OP-, CCh- and GTP γ S-induced IP $_3$ production in isolated permeabilized pancreatic acinar cells

	A (no addition)	B (CCK)	C (CCh)	D (GTP γ S)
Control	100 \pm 18 (10)	342 \pm 74 (7)	187 \pm 11 (3)	516 \pm 161 (3)
With CT	107 \pm 6 (10)	218 \pm 26 (7)	196 \pm 37 (3)	204 \pm 38 (3)

Permeabilized cells (4.8 mg of protein/ml) were prelabeled with *myo*-[3 H]inositol and incubated under standard conditions in the presence or absence of CT (40 μ g/ml). CCK-OP (0.3 μ mol/l), CCh (50 μ mol/l) and GTP γ S (10 μ mol/l) were added after 30 min of preincubation. (A) IP $_3$ production during 30 min of preincubation (control, 100% = 105 \pm 18 cpm/mg protein). (B,C) IP $_3$ production 10 min after addition of the stimulant in % of the control. (D) GTP γ S-induced IP $_3$ production which was maximal at 25 min after addition of GTP γ S (in % of the control). Values show means in % of the control \pm SE. The number of separate experiments is given in parentheses

phospholipase C or to elevation of cAMP levels following stimulation of adenylyl cyclase by CT, we investigated the effect of 8-bromo-cAMP, a poorly hydrolyzable cAMP analog, as well as of VIP, a stimulatory hormone for adenylyl cyclase, on CCK-OP-induced IP $_3$ production. The inclusion of 8-bromo-cAMP in the incubation buffer prior to the addition of permeabilized cells had no effect on either basal or CCK-OP-induced IP $_3$ production (table 2). VIP-induced cAMP production in pancreatic acinar cells was similar to that produced by CT (see fig.3). In contrast, VIP had no effect on CCK-OP-induced IP $_3$ production whether it was given 30 min (see table 2 and fig.1) or 1 min (not shown) before addition of CCK-OP to the incubation medium.

3.3. Effect of agonists on CT-dependent ADP-ribosylation of a 40 kDa protein

When pancreatic acinar cell membranes were treated with CT and [32 P]NAD $^+$, four proteins with molecular masses of 50, 48, 45 and 40 kDa were ADP-ribosylated (fig.4). Addition of GTP γ S to the incubation medium markedly stimulated the ADP-ribosylation of the 50, 48 and 45 kDa proteins, presumably corresponding to the α -subunits of the stimulatory G-protein of the adenylyl cyclase (see fig.4). In contrast, GTP γ S

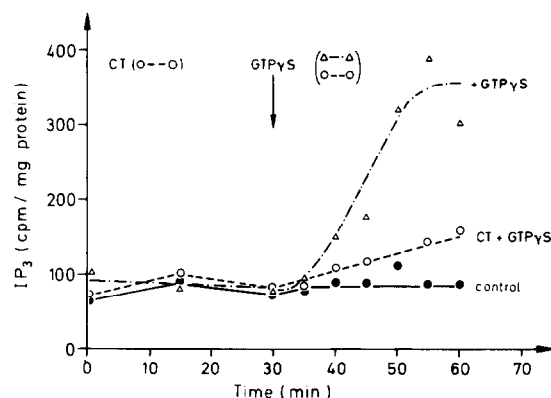


Fig.2. Effects of CT on GTP γ S-induced IP $_3$ production in permeabilized pancreatic acinar cells. *myo*-[3 H]inositol-prelabeled, permeabilized cells (6.5 mg of protein/ml) were incubated without additions (●—●) or in the presence (○---○) of CT (40 μ g/ml). GTP γ S (10 μ mol/l) was added to the incubation medium (Δ — Δ ; ○---○) where indicated. The experiment shown is representative for 3 experiments.

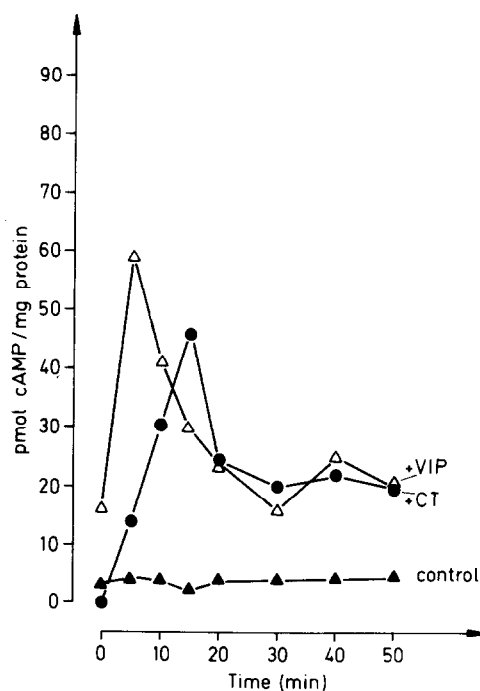


Fig.3. Effects of VIP and CT on cAMP production in permeabilized pancreatic acinar cells. Permeabilized cells (6.5 mg of protein/ml) were incubated without additions (\blacktriangle — \blacktriangle) or in the presence of CT (40 μ g/ml) (●—●), or VIP (0.1 μ mol/l) (Δ — Δ).

Table 2

Effects of 8-bromo-cAMP and VIP on IP₃ production in the absence (A) or presence (B) of CCK-OP in isolated permeabilized pancreatic acinar cells

	A (no addition)	B (CCK)	n
Control	100 ± 28	304 ± 54	8
8-Bromo-cAMP	100 ± 21	333 ± 80	5
VIP	100 ± 50	324 ± 150	3

Cells (6.5 mg of protein/ml) were preincubated under standard conditions in the absence or presence of VIP (0.1 μ mol/l) or 8-bromo-cAMP (0.1 mmol/l). CCK-OP (0.3 μ mol/l) was added after 30 min of preincubation time. (A) IP₃ production during 30 min of preincubation (control, 100% = 133 ± 37 cpm/mg protein). (B) CCK-OP-induced IP₃ production 10 min after addition of CCK-OP (in % of the control). Values give means in % of the control ± SE; n = number of separate experiments

(see fig.4) and GTP (not shown) inhibited the ADP-ribosylation of the 40 kDa protein. Addition of CCK-OP, which stimulates phospholipase C,

also inhibited the CT-dependent ADP-ribosylation of the 40 kDa protein, but was without effect on the labeling of the other protein bands. Simultaneous addition of GTP γ S and CCK-OP also inhibited CT-induced ADP-ribosylation (see fig.4). However, CCh, which stimulates phospholipase C (see table 1), had no effect on the CT-dependent ADP-ribosylation of the 40 kDa protein. CCK-OP had no effect on the PT-dependent ADP-ribosylation of the 41 kDa protein, which presumably corresponds to α -subunit of a G_i protein.

4. DISCUSSION

Our studies in isolated permeabilized pancreatic acinar cells show that CT, which activates G_s of adenylate cyclase by ADP-ribosylation of its α -subunit, inhibits CCK-OP-stimulated as well as GTP γ S-stimulated but not CCh-stimulated phospholipase C activity. This is in contrast to a previous report which showed that CT inhibits

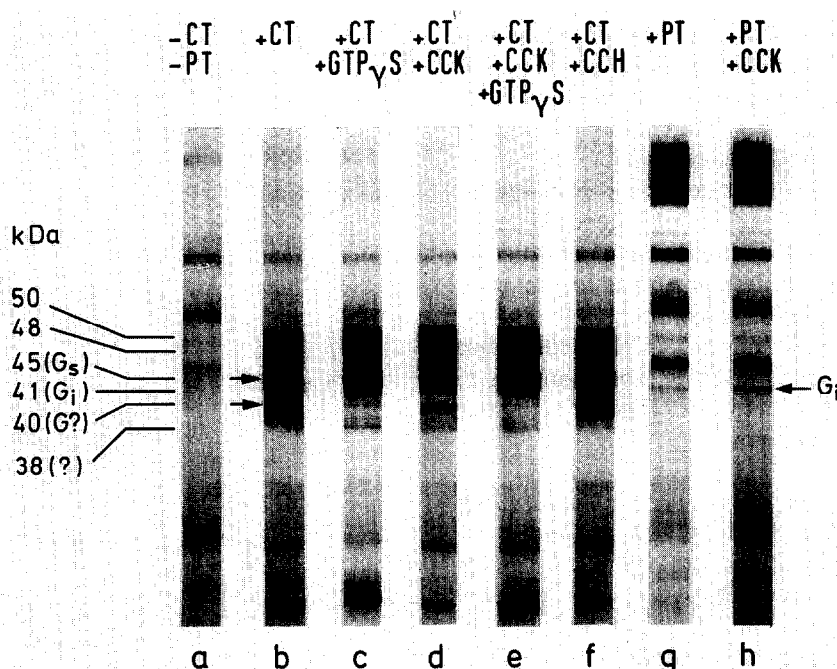


Fig.4. Autoradiography illustrating the effects of CCK-OP, CCh and GTP γ S on the CT- and PT-dependent ADP-ribosylation of proteins from rat pancreatic membranes. Membranes (50 μ g of protein) were incubated without toxin (lane a), with CT (lanes b-f) or with PT (lanes g and h) in the presence of [³²P]NAD⁺ and with CCK-OP (0.3 μ mol/l), CCh (1 μ mol/l) or GTP γ S (10 μ mol/l) as indicated in the figure. The samples were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography of the dried gel was performed. The experiment shown is representative for 6 experiments.

CCK-OP- and CCh-induced but not GTP γ S-induced phospholipase C activation in human pituitary clonal cells [16].

In several systems, cAMP has been shown to inhibit phospholipase C [23]. However, in the present system, neither the weakly hydrolyzable analog of cAMP, 8-bromo-cAMP nor VIP, which stimulates cAMP production in pancreatic acinar cells to the same extent as does CT, inhibit CCK-OP-induced phospholipase C activity. Direct interaction of the CT-activated G_s protein with phospholipase C could also be a possible explanation for the observed inhibition of IP₃ production. This is however unlikely, as VIP, which stimulates adenylate cyclase but not phospholipase C [14], had no effect on CCK-OP-induced stimulation of phospholipase C. Since GTP γ S-induced IP₃ production is also inhibited by CT, we assume that inhibition of phospholipase C is due to ADP-ribosylation of a G-protein which couples the CCK-receptor to phospholipase C, leading to functional inactivation of this G-protein and not to inhibition of receptor-G-protein interaction as has been assumed by Lo and Hughes [16] for pituitary Flow 9000 cells.

This assumption is further supported by studies on CT-induced ADP-ribosylation of membrane proteins. As shown in fig.4, [³²P]ADP-ribosylation of a 40 kDa protein was found in membranes of pancreatic acinar cells. In contrast to the enhanced ADP-ribosylation of G_s in the presence of GTP γ S, ADP-ribosylation of the 40 kDa protein was inhibited by GTP γ S. We further tested if the effect of CT on CCK-OP-induced stimulation of phospholipase C activity might coincide with any effects of this hormone on ADP-ribosylation of the 40 kDa protein. In fact, as demonstrated in fig.4, CCK-OP but not CCh decreased CT-induced ADP-ribosylation of the 40 kDa protein. It is unlikely that the 40 kDa protein represents either a member of the G_i family (since it is not ADP-ribosylated by PT) or a form of G_s, which have different molecular masses and which do not show a decreased ADP-ribosylation in the presence of CCK. The CCK-OP-mediated inhibition of CT-dependent ADP-ribosylation of the 40 kDa protein may indicate that this 40 kDa protein is involved in the functional coupling of CCK-OP receptors to phospholipase.

Recently, Gierschik and Jakobs [24] suggested

that a 40 kDa protein in membranes prepared from myeloid differentiated HL 60 cells may be involved in coupling of receptors to phospholipase C. This protein, however, is ADP-ribosylated by both CT and PT [24,25]. Furthermore, f-MLP, a stimulant of phospholipase C, increased CT-dependent ADP-ribosylation of the 40 kDa protein [24].

At present, we can distinguish three different classes of G-proteins, which are involved in receptor-mediated regulation of phospholipase C. These proteins can be differentiated by their different apparent sensitivities to bacterial toxins. They are either inactivated by PT (the G_i-like proteins), or by CT, or insensitive to both of them (review [3,26,27]).

Our data indicate that in pancreatic acinar cells CCK receptors and muscarinic ACh receptors are coupled to phospholipase C by two different G-proteins. Neither of them is a substrate for PT, and only the CCK receptor-coupling but not the ACh receptor-coupling G-protein is ADP-ribosylated by CT with consequent inhibition of phospholipase C. The molecular mass of the G-protein supposed to couple CCK receptors to phospholipase C is very similar to that of G_i-like proteins. Isolation of this protein and determination of its amino acid sequence will be needed to clarify whether or not this 40 kDa protein can be attributed to the G_i-like family of G-proteins.

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