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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 GTPγS 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; GTPγS

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 (PIP_2) and the subsequent generation of two intracellular messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol [1–3]. In permeabilized cells, GTP, the GTP analog GTPγS and F^- stimulate phospholipase C activity [2,4–7]. In some cells [8,9], pertussis toxin inhibits agonist-induced IP_3 production, whereas in other cells [10,11] including the exocrine pancreas [12,13] pertussis toxin has no effect on IP_3 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, GTPγS-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 GTPγS-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 [^{32}P]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; IP_3, inositol trisphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); VIP, vasoactive intestinal polypeptide; CT, cholera toxin; PT, pertussis toxin; f-MLP, formyl Met-Leu-Phe

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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*-[^{3}H]inositol and [^{32}P]NAD$^+$ from New England Nuclear Chemicals (Dreieich, FRG). A [^{3}H]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 μCi/ml *myo*-[2-^{3}H]inositol (approx. 6 μmol/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 K_2H 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 μmol/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 μg/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 GTPγS-induced IP_3 production*

Pretreatment of permeabilized acinar cells with preactivated CT (40 μg/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 GTPγS-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 μg/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

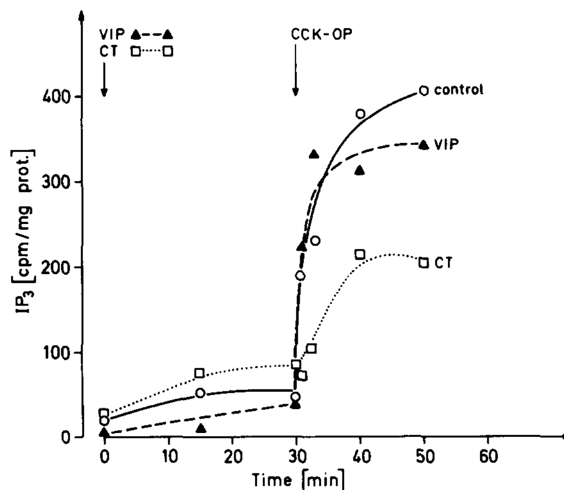

Fig.1. Effects of CT and VIP on CCK-OP-induced IP_3 production in permeabilized pancreatic acinar cells. *myo*-[^{3}H]Inositol-prelabeled, permeabilized cells (6.9 mg/ml of protein) were incubated under standard conditions without addition (○—○) or with VIP (0.1 μmol/l) (▲---▲), or with CT (40 μg/ml)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

Table 1

Effect of CT on basal, CCK-OP-, CCh- and GTPγS-induced IP_3 production in isolated permeabilized pancreatic acinar cells

	A (no addition)	B (CCK)	C (CCh)	D (GTPγS)
Control	100 ± 18 (10)	342 ± 74 (7)	187 ± 11 (3)	516 ± 161 (3)
With CT	107 ± 6 (10)	218 ± 26 (7)	196 ± 37 (3)	204 ± 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 μg/ml). CCK-OP (0.3 μmol/l), CCh (50 μmol/l) and GTPγS (10 μmol/l) were added after 30 min of preincubation. (A) IP_3 production during 30 min of preincubation (control, 100% = 105 ± 18 cpm/mg protein). (B,C) IP_3 production 10 min after addition of the stimulant in % of the control. (D) GTPγS-induced IP_3 production which was maximal at 25 min after addition of GTPγS (in % of the control). Values show means in % of the control ± 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 GTPγS 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, GTPγS

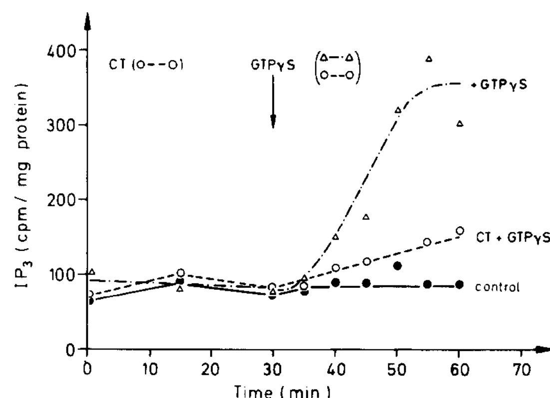

Fig.2. Effects of CT on GTPγS-induced IP_3 production in permeabilized pancreatic acinar cells. *myo*-[^{3}H]Inositol-prelabeled, permeabilized cells (6.5 mg of protein/ml) were incubated without additions (●—●) or in the presence (○---○) of CT (40 μg/ml). GTPγS (10 μmol/l) was added to the incubation medium (Δ----- Δ ; ○---○) where indicated. The experiment shown is representative for 3 experiments.

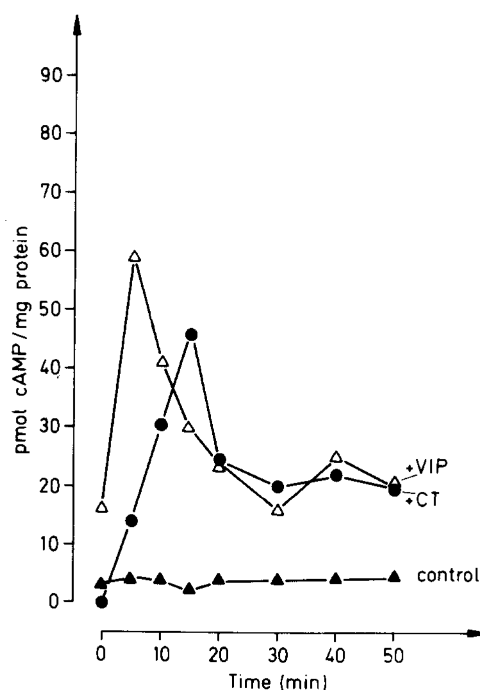

Fig.3. Effects of VIP and CT on cAMP production in permeabilized pancreatic acinar cells. Permeabilized cells (6.5 mg of protein/ml) were incubated without additions (▲—▲) or in the presence of CT (40 μg/ml) (●—●), or VIP (0.1 μmol/l) (Δ—Δ).

Table 2

Effects of 8-bromo-cAMP and VIP on IP_3 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 μmol/l) or 8-bromo-cAMP (0.1 mmol/l). CCK-OP (0.3 μmol/l) was added after 30 min of preincubation time. (A) IP_3 production during 30 min of preincubation (control, 100% = 133 ± 37 cpm/mg protein). (B) CCK-OP-induced IP_3 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 GTPγS 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 GTPγS-stimulated but not CCh-stimulated phospholipase C activity. This is in contrast to a previous report which showed that CT inhibits

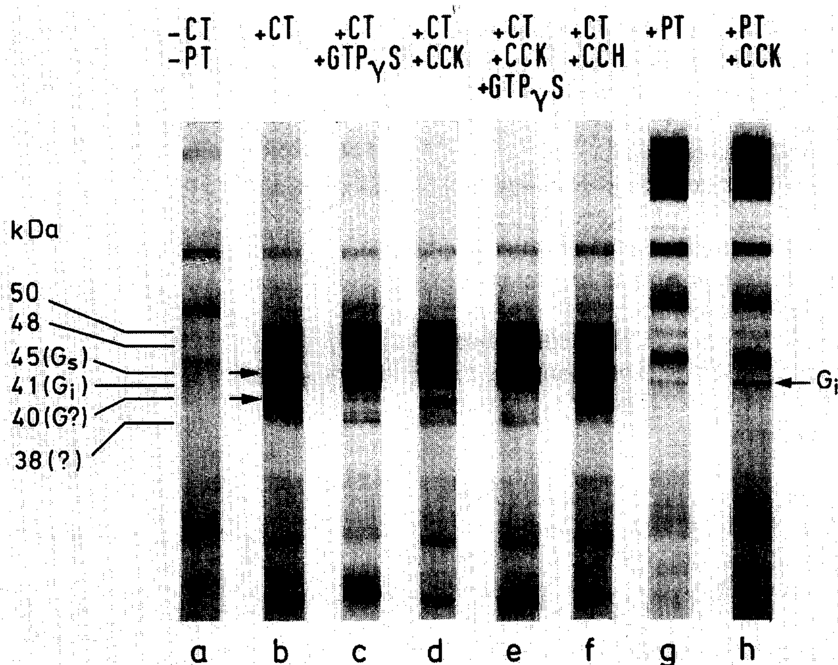

Fig.4. Autoradiography illustrating the effects of CCK-OP, CCh and GTPγS on the CT- and PT-dependent ADP-ribosylation of proteins from rat pancreatic membranes. Membranes (50 μg of protein) were incubated without toxin (lane a), with CT (lanes b–f) or with PT (lanes g and h) in the presence of $[^{32}P]NAD^+$ and with CCK-OP (0.3 μmol/l), CCh (1 μmol/l) or GTPγS (10 μmol/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 GTPγS-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 IP_3 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 GTPγS-induced IP_3 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, $[^{32}P]$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 GTPγS, ADP-ribosylation of the 40 kDa protein was inhibited by GTPγS. 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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