Cholecystokinin activates G_{i1} -, G_{i2} -, G_{i3} - and several G_s -proteins in rat pancreatic acinar cells

Susanne SCHNEFEL,* André PRÖFROCK,* Klaus-Dieter HINSCH† and Irene SCHULZ*‡

*Max Planck Institut für Biophysik, Kennedyallee 70, 6000 Frankfurt am Main 70, and †Institut für Pharmakologie der FU Berlin, Thielallee 69/73, 1000 Berlin 33. Federal Republic of Germany

On separation of rat pancreatic plasma membrane proteins by two-dimensional gel electrophoresis, 15 GTP-binding protein (G-protein) α -subunits could be detected immunochemically using an α_{common} antibody. These consisted of five 48 kDa proteins (pI 5.70, 5.80, 5.90, 6.10 and 6.25) and five 45 kDa proteins (pI 5.90, 6.05, 6.25, 6.30 and 6.70), presumably corresponding to low- and high-molecular mass forms of the G_s -protein, as well as three 40/41 kDa proteins (pI 5.50, 5.70 and 6.00) and two 39 kDa proteins (pI 5.50 and 6.00). All of these proteins except for the more acidic 39 kDa protein were ADP-ribosylated by cholera toxin (CT). In addition, the three 40/41 kDa proteins and the more alkaline 39 kDa protein were also ADP-ribosylated by pertussis toxin (PT). CT- and PT-induced ADP-ribosylation changed the pI values of G-protein α -subunits by 0.2 pI units to more acidic values. Preincubation of isolated pancreatic membranes with cholecystokinin octapeptide (CCK-OP), which stimulates phospholipase C in acinar cells, decreased CT-induced as well as PT-induced ADP-ribosylation of the three 40/41 kDa proteins, whereas CT-induced ADP-ribosylation of one 45 kDa (pI 5.80) and all 48 kDa proteins was enhanced in the presence of CCK. Carbachol, another stimulant of phospholipase C, had no effect. The three 40/41 kDa proteins and one 48 kDa protein could be labelled with the GTP analogue [α - 32 P]GTP- γ -azidoanilide. CCK, but not carbachol, stimulated incorporation of the GTP analogue into all of these four proteins. Using different anti-peptide antisera specific for α -subunits of G-proteins we identified the three 40/41 kDa G_i -proteins as G_{i1} (pI 6.00), G_{i2} (pI 5.50) and G_{i3} (pI 5.70). The G_{i3} -protein was found to be the major G_i -protein of pancreatic plasma membranes. One of the 39 kDa proteins (pI 6.0) was identified as G_o . These results indicate that CCK receptors functionally interact with six G_s -proteins and with G_{i1} , G_{i2} and G_{i3} -proteins. Since evidence suggests that a 40/41 kDa CT substrate is involved in the stimulation of phospholipase C in pancreatic acinar cells, it is likely that one, two or all three 40/41 kDa G_i -proteins are involved in the coupling of CCK receptors with phospholipase C.

INTRODUCTION

Heterotrimeric GTP-binding proteins (G-proteins) play an important role in receptor-mediated signal transduction in the plasma membrane. Hormonal stimulation of phosphatidylinositol 4,5-bisphosphate-hydrolysing phospholipase C (PLC) is mediated by G-proteins [1]. Whereas α -subunits are unique for each G-protein, $\beta\gamma$ -complexes are very similar or identical [1]. At present, G-proteins involved in hormonal activation of PLC are divided into three classes, according to their different apparent sensitivities to bacterial toxins. They are either inactivated by pertussis toxin (PT)-mediated and/or by cholera toxin (CT)-mediated ADP-ribosylation of their α -subunits, or they are insensitive to both bacterial toxins [1].

Previously we have obtained evidence that, in rat pancreatic plasma membranes, a CT-sensitive 40 kDa G-protein is involved in functional coupling of cholecystokinin (CCK) receptors, but not of acetylcholine (ACh) receptors, to PLC [2,3]. So far, four α -subunits of G-proteins (α_{i1} , α_{i2} , α_{i3} and α_z) with molecular masses of 40–41 kDa have been identified by cDNA cloning and sequence analysis [4]. Whereas the α -subunits of the G_i -like proteins, α_{i1} , α_{i2} and α_{i3} are substrates for both CT and PT, α_z [4] lacks the site for PT-catalysed ADP-ribosylation.

In the present study we have combined methods using binding of specific antibodies against G-protein α -subunits, ADP-ribosylation with bacterial toxins, photoaffinity labelling with the GTP analogue [α - 32 P]GTP- γ -azidoanilide and high-resolution two-dimensional gel electrophoresis. This has enabled us to characterize the 40/41 kDa G-proteins that are sensitive to

CCK. We have found that CT as well as PT ADP-ribosylates three 40/41 kDa G-proteins in acinar plasma membranes, which we identified as G_{i1} , G_{i2} and G_{i3} . For all three of these proteins CCK, but not ACh, decreased CT- as well as PT-induced ADP-ribosylation. CCK, but not ACh, stimulated incorporation of the GTP photoaffinity analogue GTP- γ -azidoanilide into these three proteins. We therefore conclude that CCK receptors, but not ACh receptors, functionally interact with G_{i1} , G_{i2} and G_{i3} -proteins. Which of these G-proteins is involved in the activation of PLC cannot be decided from these data.

MATERIALS AND METHODS

Materials

CT, PT, carbachol, CCK-octapeptide (CCK-OP) and 5-bromo-4-chloro 3-indolyl phosphate were purchased from Sigma (München, Germany). Nitroblue Tetrazolium and goat anti-rabbit IgG (H + L)-alkaline phosphatase conjugate were purchased from Bio-Rad (München, Germany). *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodi-imide hydrochloride was obtained from Merck (Darmstadt, Germany). 4-Azidoanilide was from Serva (Freiburg, Germany). [32 P]NAD⁺ (800 Ci/mmol) and [α - 32 P]GTP (800 Ci/mmol) were purchased from NEN (Dreieich, Germany).

Antisera used in this study were raised against synthetic peptides corresponding to defined regions of G-protein α - or β -subunits (Table 1). AS/8 antiserum was generated against a region common to all G-protein α -subunits except that of α_z .

Abbreviations used: CCK-OP, cholecystokinin octapeptide; ACh, acetylcholine; G-protein, guanine-nucleotide-binding protein; CT, cholera toxin; PT, pertussis toxin; PLC, phospholipase C; DTT, dithiothreitol.

‡ To whom correspondence should be addressed.

Table 1. Antisera used in this study

Synthetic peptides were used as antigens for antibody production; the numbers in parentheses refer to the amino acid residues of cDNA-predicted sequence of peptide antigens.

Antiserum	Antigen	Specificity	Ref.
AS/8	α_{common} cDNA (47–61)	All α -subunits except for α_z	[5]
AS/7	Transducin- α (341–350)	α_i , α_i1 , α_i2	[9]
AS/6	α_0 cDNA (29–42)	α_0	[5]
AS/75	α_s cDNA (115–127)	α_s	[6]
AS/98	α_z cDNA (38–47)	α_z	[8]
LD	α_i1 cDNA (159–168)	α_i1	[9]
LE	α_i2 cDNA (160–169)	α_i2	[9]
SQ	α_i3 cDNA (159–168)	α_i3	[9]
AS/12	β_{36} cDNA (177–185)	β_{36}	[6]
AS/36	β_{35} cDNA (176–185)	β_{35}	[6]

(α_{common} peptide, amino acid sequence 47–61) [5]. The α_0 antiserum (AS/6) was raised against amino acids 29–42 of the α_0 -subunit [5]. AS/12 and AS/36 antisera were made against synthetic peptides corresponding to amino-acids 177–185 of the β_{36} -subunit and 176–185 of the β_{35} -subunit respectively [6]. Peptides used for preparation of α_{common} , α_0 and β_{36} antisera were identical with those used by Mumby *et al.* [7]. AS/75 antiserum was generated against amino acids 115–127 of the α_s -subunit and raised as described [6]. The antiserum recognized exclusively purified α_s (45 kDa) and α_s (50 kDa) and did not react with any other known G-protein α -subunit. AS/98 was raised against amino acids 38–47 of α_z [8]. AS/7 antiserum was made against the C-terminus (amino acids 388–397) of the α -subunit of transducin; it recognizes α_i , α_i1 and α_i2 , but not α_i3 [9]. Affinity-purified antisera LD specific for α_i1 was raised against amino acids 159–168 of α_i1 [9], LE specific for α_i2 was generated against amino acids 160–169 of α_i2 [9] and SQ specific for α_i3 was raised against amino acids 159–168 of α_i3 [9]. LD, LE, SQ and AS/7 antisera (see Table 1) were kindly provided by Dr. Allan Spiegel (National Institutes of Health, Bethesda, MD, U.S.A.).

Preparation of rat pancreatic plasma membranes

Acinar cells were isolated from the pancreas from male Wistar rats (200–250 g) by collagenase digestion as previously described [10]. Plasma membranes were prepared from isolated acinar cells using a MgCl_2 precipitation method [11].

ADP-ribosylation

PT- and CT-catalysed ADP-ribosylation of membrane proteins was performed as previously described [2] with the following modifications. PT was preactivated in the presence of dithiothreitol (DTT; 20 mmol/l) and ATP (1 mmol/l), and the incubation mixture contained 0.05% Lubrol PX. ADP-ribosylation was carried out in the presence of NADP⁺ (0.3 mmol/l). ADP-ribosylation of isolated permeabilized acinar cells was performed for 90 min at 25 °C as described [2], except that the incubation medium contained 1 mmol of NAD⁺/l and 0.3 mmol of NADP⁺/l, and the free MgCl_2 concentration was reduced to 0.1 mmol/l.

Photoaffinity labelling

Synthesis of [α -³²P]GTP- γ -azidoanilide and photoaffinity labelling of membrane proteins was carried out as described previously [3].

SDS/PAGE and immunoblotting

For immunochemical analysis, membrane proteins were alkylated with *N*-ethylmaleimide before electrophoresis according to Evans *et al.* [12]. Proteins were precipitated with 500 μ l of methanol/chloroform/water (4:3:1, by vol.). Electrophoresis was carried out in the presence of urea (4 mol/l) as described previously [2]. Proteins were electrophoretically transferred to nitrocellulose according to Towbin *et al.* [13]. Transferred proteins were revealed with Ponceau S. Nitrocellulose blots were incubated in a solution containing potassium phosphate, pH 7.4 (20 mmol/l), NaCl (200 mmol/l) and 5% (w/v) BSA. Incubation with the specific antisera was performed in a buffer containing potassium phosphate, pH 7.4 (20 mmol/l), NaCl (200 mmol/l) EDTA (1 mmol/l) 0.3% (v/v) Tween 20, 1% (w/v) BSA and 10% (v/v) newborn calf serum. Antibody binding was detected by an alkaline phosphatase immunoassay (Bio-Rad).

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed according to O'Farrell [14], with modifications according to Woolkalis *et al.* [15]. Briefly, membranes were solubilized in a solution containing: K_2CO_3 (5 mmol/l), DTT (20 mmol/l) and 1% (v/v) Lubrol PX for 1 h at 4 °C. After centrifugation at 10000 *g* for 15 min at 4 °C, the supernatant was diluted in 2 vol. of 'dilution buffer' containing urea (9.5 mol/l), 2% (v/v) ampholine (1.6% pH 5–7; 0.4% pH 3–10), 5% (v/v) β -mercaptoethanol and 8% (v/v) Triton X-100. The concentration of urea in the solution was adjusted to 8 mol/l. Proteins were applied to the basic end of the gel. Isoelectric focusing was performed at 200 V for 1 h and at 650 V for 17 h. The established pH gradient was linear between pH 5.0 and 7.0. Gels were subjected to electrophoresis in the second dimension as described above.

Other methods

Proteins were assayed as described by Bradford *et al.* [16], using BSA as a standard. Autoradiography of dried gels or nitrocellulose blots was performed by exposure to 3M R2 films at –70 °C. Values given for the isoelectric points of G-proteins are mean values $\pm$ S.D. from three separate experiments.

RESULTS

Two-dimensional analysis of CT and PT substrates

We have previously shown that, in isolated permeabilized rat pancreatic acinar cells, preactivated CT ADP-ribosylates proteins with molecular masses of 40, 45 and 48–50 kDa [2]. With CCK-

Fig. 1. Effect of CCK on CT- and PT-induced [32 P]ADP-ribosylation of rat pancreatic plasma membrane proteins

Pancreatic plasma membrane proteins (150 μ g of protein) were incubated with CT (100 μ g/ml; *a,b*) or PT (1.6 μ g/ml; *c,d*) and [32 P]NAD $^{+}$ in the absence (*a,c*) or presence (*b,d*) of CCK-OP (1 μ mol/l) as indicated. Proteins were subjected to two-dimensional PAGE and transferred to nitrocellulose, and autoradiography of the dried blot was performed. The experiment shown is representative of four similar experiments.

8, but not with the Ach analogue carbachol, both of which stimulate PLC in acinar cells, ADP-ribosylation of the 40 kDa protein was decreased. In order to find out if the 40 kDa CT substrate observed in one-dimensional SDS/PAGE consists of more than one protein, [32 P]ADP-ribosylated membrane proteins were subjected to two-dimensional PAGE. Autoradiography of the CT substrates revealed that the 40 kDa protein can be separated into three differently charged proteins with isoelectric points (pI) of 5.30 ± 0.04 , 5.60 ± 0.08 and 5.75 ± 0.08 (Figs. 1*a* and 1*b*). The radiolabelled 45 kDa CT substrate, which presumably corresponds to the low-molecular-mass α -subunit of G_s , was found to exist in at least five differently charged species with pI values of 5.65 ± 0.04 , 5.80 ± 0.05 , 5.95 ± 0.05 , 6.10 ± 0.03 and 6.30 ± 0.06 . The 48 kDa CT substrates, presumably corresponding to the high-molecular-mass α -subunits of G_s , were resolved into at least five differently charged proteins with pI values of 5.30 ± 0.04 , 5.55 ± 0.07 , 5.65 ± 0.04 , 5.70 ± 0.03 and 5.80 ± 0.05 on two-dimensional PAGE and autoradiography.

We have previously shown that in rat pancreatic plasma membranes only a weak PT-induced ADP-ribosylation of a 40/41 kDa protein was obtained [2]. However, in more recent experiments PT effectively ADP-ribosylated G-proteins of pancreatic plasma membranes if it was activated in the presence of ATP (1 mmol/l) and if ADP-ribosylation was performed in the presence of 0.05% Lubrol PX. As shown in Figs. 1(*c*) and 1(*d*), three differently charged proteins with molecular masses of 40/41 kDa were ADP-ribosylated by PT. The 40/41 kDa PT substrates had the same isoelectric points as the 40/41 kDa CT substrates (see Fig. 1). With both CT- and PT-induced ADP-ribosylation, major amounts of radioactivity were incorporated into the protein of pI 5.60 and the smallest amount into the protein of pI 5.75 (see Figs. 1*a* and 1*c*). These data indicate that CT and PT ADP-ribosylate the same 40/41 kDa proteins in pancreatic plasma membranes. On longer exposure of gels to radiosensitive films (up to 6 days), an additional 39 kDa protein

with a pI value of 5.7 could be detected in the autoradiograms after CT- and PT- induced ADP-ribosylation (results not shown). No effect of CCK on CT- or PT- induced ADP-ribosylation of this protein could be detected (results not shown). In order to decide whether or not PT-induced ADP-ribosylation had occurred in isolated cells, we prepared plasma membranes from control and PT-pretreated cells and incubated them under ADP-ribosylation conditions with PT and [32 P]NAD $^{+}$. Since no difference in labelling of the 40/41 kDa PT substrates could be observed in the two membrane preparations, we assume that PT-induced ADP-ribosylation had not occurred in intact cells (results not shown).

Effect of CCK-OP and carbachol on CT- and PT-induced ADP-ribosylation of G-proteins

When pancreatic plasma membranes were preincubated with CCK-OP (1 μ mol/l) and subsequently ADP-ribosylated with CT in the presence of [32 P]NAD $^{+}$, incorporation of [32 P]ADP-ribose into all three 40/41 kDa proteins was markedly reduced in comparison with membranes preincubated in the absence of CCK-OP (see Fig. 1). In contrast, CCK-OP stimulated ADP-ribosylation of one of the five low-molecular-mass α -subunits (pI 5.8) and all five high-molecular-mass α -subunits (see Fig. 1). Pretreatment of plasma membranes with carbachol (10 μ mol/l) had no effect (results not shown). Preincubation of plasma membranes with CCK-OP (1 μ mol/l) reduced not only CT- but also PT-induced ADP-ribosylation of all three 40/41 kDa PT substrates (see Fig. 1). Again, this effect of CCK-OP could not be mimicked by carbachol (results not shown).

Effect of CCK-OP and carbachol on photolabelling of 40/41 kDa G-proteins with GTP- γ -azidoanilide

We have previously shown that in rat pancreatic plasma membranes the photoreactive GTP analogue [α - 32 P]GTP- γ -azidoanilide is mainly incorporated into a 40 kDa protein [3]. On

Fig. 2. Effect of CCK-OP on photolabelling of G-proteins from rat pancreatic acinar cell membranes with [α - 32 P]GTP- γ -azidoanilide

Plasma membranes (100 μ g of protein) were incubated with 0.24 μ mol of [α - 32 P]GTP- γ -azidoanilide/l in the absence (a) or presence (b) of CCK-OP (1 μ mol/l). Proteins were subjected to two-dimensional PAGE, blotted on to nitrocellulose and autoradiography was performed. The experiment shown is representative of four similar experiments.

two dimensional PAGE of plasma membrane proteins labelled with GTP- γ -azidoanilide, the 40/41 kDa protein band was resolved into three different proteins with pI values of 5.40 ± 0.07 , 5.70 ± 0.06 and 5.90 ± 0.07 (Fig. 2). In addition, the GTP analogue was incorporated into a 48 kDa protein (pI 5.40 ± 0.09), presumably corresponding to a high-molecular-mass α_s -subunit. CCK-OP (1 μ mol/l) stimulated incorporation of the photolabel into all three 40/41 kDa proteins and into the 48 kDa protein (see Fig. 2). In addition, in the presence of CCK-OP, incorporation of the photolabel into three less abundant 40/41 kDa proteins could be detected.

Immunochemical characterization of G-proteins present in pancreatic plasma membranes

In order to characterize G-protein α - and β -subunits present in plasma membranes from rat pancreatic acinar cells we probed for reactions of membrane proteins with various antisera raised against synthetic peptides corresponding to defined regions of G-protein α - and β -subunits. AS/8 antiserum raised against the α_{common} peptide (Table 1) reacted strongly with membrane peptides of 39 kDa, 40–41 kDa, 45 kDa and 48 kDa (Fig. 3). Interaction of the AS/8 antiserum with these proteins was blocked by incubation of AS/8 with the α_{common} peptide (5 μ g/ml) overnight at room temperature. The 45 kDa doublet proteins and the 48 kDa protein band detected by the α_{common} antibody could be identified as low- and high-molecular-mass α -subunits of the G_s -protein using antiserum AS/75 specific for α_s -subunits (Fig. 3). Using three different antisera against different α -subunits of the G_i family we could demonstrate the presence of α_{i1} , α_{i2} and α_{i3} immunoreactivity in pancreatic plasma membranes (Fig. 3). The affinity-purified antibody LD, specific for the α_{i1} -subunit, recognized a single protein of 41 kDa. Affinity-purified antibody LE, specific for an amino acid sequence in α_{i2} , detected a 40 kDa protein. An additional 36 kDa protein was detected by the LE antibody; the nature of this 36 kDa protein is not known. The

Fig. 3. Immunochemical detection of α - and β -subunits of different G-proteins in plasma membranes of rat pancreatic acinar cells

Plasma membrane proteins (50 μ g of protein) were separated by SDS/PAGE and transferred to nitrocellulose. Nitrocellulose filters were incubated with different diluted antisera: lane 1, pre-immune serum (1:300); 2, α_{common} (AS/8; 1:500); 3, α_s (AS/75; 1:300); 4, $\alpha_{i1} + 2$ (AS/7; 1:250); 5, α_{i1} (LD; 5 μ g/ml); 6, α_{i2} (LD; 5 μ g/ml); 7, α_{i3} (SQ; 5 μ g/ml); 8, α_o (AS/6; 1:300); 9, α_x (AS/98; 1:300); 10, β_{36} (AS/12; 1:200); 11, β_{35} (AS/36; 1:200). Filter-bound antibodies were detected by an alkaline-phosphatase-conjugated goat anti-(rabbit IgG) (1:1670 dilution). The experiment is representative of six similar experiments.

affinity-purified SQ antibody, which is specific for the α_{i3} -subunit, strongly reacted with the 40/41 kDa proteins. The AS/6 antibody, specific for the α -subunit of G_o , detected only a 39 kDa protein which also reacted with the α_{common} antibody. Antibody AS/98, specific for α_x , failed to detect any proteins (Fig. 3). These results indicate that rat pancreatic plasma membranes contain all three G_i -like proteins, i.e. G_{i1} , G_{i2} and G_{i3} , as well as at least one G_o -protein and low- and high-molecular-mass subunits of the G_s -protein, but no detectable amounts of G_{12} -protein.

We also tested the occurrence of β -subunits in rat pancreatic plasma membranes. AS/12 antibody, directed against an amino acid sequence of the 36 kDa β -subunit, and the AS/36 antibody, specific for the 35 kDa β -subunit of G-proteins, detected 36 kDa and 35 kDa proteins respectively (Fig. 3). The reactions with the 30 kDa and the 66 kDa bands recognized by the AS/36 and AS/12 antibodies respectively could not be blocked by preincubation of the antibodies with the corresponding peptides and are, therefore, assumed to be unspecific. The data indicate that both 36 and 35 kDa β -subunits of G-proteins are present in pancreatic plasma membranes.

Immunochemical identification of G_i -protein α -subunits by two-dimensional PAGE

In order to determine isoelectric points of G_{i1} , G_{i2} and G_{i3} , membrane proteins were subjected to two-dimensional PAGE and immunoblotting. Using two-dimensional PAGE, α_{common} immunoreactivity could be resolved into three 40/41 kDa proteins with pI values of 5.50 ± 0.01 , 5.70 ± 0.06 and 6.00 ± 0.08 , two 39 kDa proteins with pI values of 5.50 ± 0.14 and 6.00 ± 0.13 , and five 45 kDa and five 48 kDa proteins with pI values of 5.90, 6.05, 6.25, 6.30 and 6.70, and 5.70 ± 0.09 , 5.80 ± 0.07 , 5.90 ± 0.03 , 6.10 ± 0.03 and 6.25 respectively (Fig. 4). The 45 kDa proteins and the 48 kDa protein (pI 6.25) could only be detected in two out of three experiments. The 40/41 kDa protein predominantly recognized by the α_{common} antiserum (pI 5.70) could be identified as α_{i3} by use of the SQ antibody. The next most abundant 40/41 kDa protein as estimated by the α_{common} immunoreactivity (pI 5.5) was detected by the LE antibody and corresponds to α_{i2} . The least abundant 40/41 kDa protein (pI 6.0) could be identified as α_{i1} by the LD antibody. These

Fig. 4. Two-dimensional analysis of α -subunits of different G-proteins of rat pancreatic acinar cell membranes by enzyme-linked immunotransfer blotting

Plasma membrane proteins (150 μ g of protein) were separated by two-dimensional PAGE and transferred to nitrocellulose. Nitrocellulose filters were incubated with antisera at appropriate dilutions as described in the legend to Fig. 3. Filter-bound antibodies were detected by alkaline-phosphatase-conjugated goat anti-(rabbit IgG) (1:1670 dilution). The experiment shown is representative for three similar experiments. Antisera: (a) α_{common} ; (b) α_1 ; (c) α_2 ; (d) α_3 ; (e) α_1+2 ; (f) α_6 .

results are confirmed by the AS/7 antibody which detected only α_1 and α_2 . The AS/6 antibody specific for α_6 -subunits detected only one of the 39 kDa proteins (pI 6.0) which reacted with the α_{common} antibody.

ADP-ribosylation with either PT or CT resulted in a shift of about 0.2 pI units in the migration of all ADP-ribosylated α -subunits of G-proteins (as detected with the α_{common} antibody) to more acidic positions, probably due to the negative charge of the ADP-ribose (results not shown).

DISCUSSION

In rat pancreatic acinar cells CCK stimulates both PLC and adenylate cyclase through G-proteins [1]. Whereas receptor-stimulated activation of adenylate cyclase is mediated by G_s -proteins having molecular masses of 45 and 48–50 kDa, the G-proteins involved in the hormonal activation of PLC have not yet been identified.

We have previously shown that a 40 kDa G-protein, which can be ADP-ribosylated by CT, is involved in functionally coupling CCK receptors, but not Ach receptors, to PLC in rat pancreatic acinar cells [2,3]. In the present study we demonstrate that this 40 kDa CT substrate can be separated into three differently charged proteins by two-dimensional PAGE. CT also ADP-ribosylates five differently charged 45 kDa and 48 kDa proteins, presumably corresponding to the low- and high-molecular-mass forms respectively of the G_s -proteins [15,17,18]. Preincubation of pancreatic plasma membranes with CCK-OP leads to decreased ADP-ribosylation of all of the 40/41 kDa proteins and enhanced ADP-ribosylation of one 45 kDa and all 48 kDa G_s -proteins. In addition, CCK stimulates incorporation of the GTP analogue GTP- γ -azidoanilide into the three 40/41 kDa proteins and into a 48 kDa G_s -protein. GTP- γ -azidoanilide was incorporated neither into the 45 kDa G_s -protein nor into some of the 48 kDa proteins. This may be due to structural differences between the GTP analogue and GTP, the natural ligand of G-proteins. The results suggest that activated CCK receptors functionally interact with three differently charged 40/41 kDa G-proteins, with one 45 kDa protein and with five 48 kDa forms of the G_s -protein.

We improved the conditions for PT-induced ADP-ribosylation and showed that PT effectively ADP-ribosylates the three 40/41 kDa CT substrates in isolated rat pancreatic plasma membranes. PT-induced ADP-ribosylation occurs preferentially with the holomeric $\alpha\beta\gamma$ -complex [19]. Preincubation of membranes with CCK-OP decreased PT-induced ADP-ribosylation of all three 40/41 kDa G-proteins. This indicates that the three 40/41 kDa G-proteins functionally interacting with the CCK receptor are substrates for both CT and PT.

Previously we have shown that PT has no effect on CCK-OP-induced activation of PLC as measured by CCK-OP-stimulated InsP_3 production in isolated rat pancreatic acinar cells [2]. Preincubation of cells with PT at ADP-ribosylation conditions and subsequent isolation of plasma membranes followed by PT ADP-ribosylation with [^{32}P]NAD $^+$ showed that ADP-ribosylation of the 40/41 kDa protein had not taken place in intact cells. There may be different reasons for the failure of PT to ADP-ribosylate G_i -proteins in whole acinar cells. Since ADP-ribosylation of acinar cells can only be performed in the absence of detergents, which are necessary for ADP-ribosylation in membranes of acinar cells, PT substrates may not be accessible to the toxin. Furthermore, an endogenous inhibitor activity of the PT-induced ADP-ribosylation might be present in whole acinar cells which could have been lost during membrane preparation [20,21].

Using different antisera specific for α - or β -subunits of G-proteins we were able to identify the three 40/41 kDa G-proteins interacting with the CCK receptor as G_{i1} , G_{i2} and G_{i3} . The G_{i3} -protein was found to be the major G_i protein of acinar cells as detected by PT- and CT-induced ADP-ribosylation and α_{common} immunoreactivity, whereas G_{i1} is only present in minor amounts. The demonstration of a functional interaction with CCK receptors provides strong evidence that one or more of the G_{i1} , G_{i2} - and G_{i3} -proteins are involved in hormonal regulation of PLC in acinar cells.

In HL 60 cells, chemoattractant receptors, which are coupled to PLC and do not interact with the adenylate cyclase system, functionally interact with G_{i2} - and G_{i3} -proteins [22]. Moreover, synthetic peptides corresponding to the C-terminal amino acid sequence of the α_{i3} -subunit inhibit guanosine 5'-[γ -thio]triphosphate (GTP[S])-induced as well as chemotactic factor (fMet-Leu-Phe)-induced activation of PLC [23]. Evidence points to the existence of at least two G-proteins (stimulatory and inhibitory) involved in hormonal regulation of PLC [24]. In GH_3

pituitary tumour cells and in brown adipocytes adenosine inhibits PLC via a PT-sensitive G-protein [25,26]. This raises the possibility that, in addition to an activating G_i -protein, one of the three G_i -proteins sensitive to CCK-OP may couple to inhibitory CCK receptors to PLC. G_i -proteins might also couple CCK receptors to other effector systems. Willems *et al.* [27] gave evidence that CCK receptors are coupled to an inhibitory G-protein of adenylate cyclase in rat pancreatic acinar cells [27]. Furthermore, interaction of G_{i1} , G_{i2} and/or G_{i3} with different CCK receptor subtypes is also possible.

From the present data we cannot ascribe any functional change induced by CCK to G_{i1} , G_{i2} and G_{i3} pancreatic plasma membranes. It is quite possible that CCK receptors may stimulate PLC through all three G_i -proteins.

Recently it was shown, using antibodies raised against the brain G_o -protein, that G_o is present in rat pancreas [28]. In the present study two 39 kDa proteins could be detected by the α_{common} antibody in two-dimensional PAGE of proteins from rat pancreatic acinar cell membranes. However, only one of these proteins was identified as α_o by the AS/6 antiserum raised against an amino acid sequence of the brain G_o -protein. In adipocytes and in myometrial membranes a novel 39 kDa protein, immunologically related to $G_o\alpha$, has been detected [29,30]. This protein is not recognized by the antibody used in this study [5]. We therefore assume that, in addition to the G_o -protein, another immunologically distinct 39 kDa protein is present in rat pancreatic acinar cells. Recently, Moriarty *et al.* [31] have shown that the G_o protein, but not the G_{i1} -, G_{i2} - or G_{i3} -proteins, could serve as the signal transducer of the receptor-regulated PLC in *Xenopus* oocytes. Since we did not observe any effect of CCK on either ADP-ribosylation or photolabelling of the 39 kDa protein, an involvement of G_o in receptor-PLC coupling in pancreatic acinar cells is quite unlikely.

In summary, we have identified fifteen G-protein α -subunits in rat pancreatic plasma membranes. CCK receptors functionally interact with nine G-proteins including all three G_i -proteins and with several, but not all, forms of the G_o -protein. The presence of different G-proteins sensitive to CCK suggests that the diversity of CCK-induced signal transduction pathways is apparent at the level of G-proteins in the plasma membrane. Three 40/41 kDa proteins have been identified as G_{i1} , G_{i2} and G_{i3} , G_{i3} being the major G_i -protein, and these are assumed to be involved in hormonal regulation of PLC activity.

We thank Dr. Allan Spiegel for kindly providing the $G_i\alpha$ antibodies and Ingrid Tychowiecka for technical assistance. This work was supported by grants from the Deutsche Forschungsgemeinschaft.

REFERENCES

- Graziano, M. P. & Gilman, A. G. (1987) Trends Pharmacol. Sci. **8**, 478–481
- Schnefel, S., Banfic, H., Eckhardt, L., Schultz, G. & Schulz, I. (1988) FEBS Lett. **230**, 125–130
- Schäfer, R., Christian, A.-L. & Schulz, I. (1988) Biochem Biophys. Res. Commun. **155**, 1051–1059
- Lochvie, M. A. & Simon, M. J. (1988) Biochemistry **27**, 4957–4965
- Hinsch, K.-D., Rosenthal, W., Spicher, K., Binder, T., Gausepohl, H., Frank, R., Schultz, G. & Joost, H. G. (1988) FEBS Lett. **238**, 191–196
- Hinsch, K.-D., Tychowiecka, I., Gausepohl, H., Frank, R., Rosenthal, W. & Schultz, G. (1989) Biochim Biophys Acta **1013**, 60–67
- Mumby, S. M., Kahn, R. A., Manning, D. R. & Gilman, A. G. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 265–269
- Spicher, K., Hinsch, K.-D., Gausepohl, H., Frank, R., Rosenthal, W. & Schulz, G. (1988) Biochem Biophys Res. Commun. **157**, 883–890
- Goldsmith, P., Rossiter, K., Carter, A., Simonds, W., Unson, C. G., Vinitsky, R. & Spiegel, A. (1988) J. Cell Biol. **263**, 6476–6479
- Streb, H. & Schulz, I. (1983) Am. J. Physiol. **245**, G347–G357
- Bayerdöffer, E., Eckhardt, L., Haase, W. & Schultz, I. (1985) J. Membr. Biol. **84**, 45–60
- Evans, T., Brown, M. L., Fraser, E. D. & Northeys, J. K. (1986) J. Biol. Chem. **261**, 7052–7059
- Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
- O'Farrell, P. H. (1975) J. Biol. Chem. **250**, 4007–4021
- Wookalis, M. J., Nakada, M. T. & Manning, D. R. (1986) J. Biol. Chem. **261**, 3408–3413
- Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
- Schleifer, L. S., Garrison, J. C., Sternweis, P. C., Northup, J. K. & Gilman, A. G. (1980) J. Biol. Chem. **255**, 2641–2644
- Deery, W. J., Rebeiro-Neto, F. & Field, J. B. (1987) Biochem Biophys. Res. Commun. **144**, 536–542
- Huff, R. M. & Neer, E. J. (1986) J. Biol. Chem. **261**, 1105–1110
- Hara-Yokoyama, M. & Furuyama, S. (1988) FEBS Lett. **234**, 27–30
- Hara-Yokoyama, M. & Furuyama, S. (1989) Biochem Biophys Res. Commun. **160**, 67–71
- Gierschik, P., Gierschik, S., Sidropoulos, D., Dieterich, K. & Jakobs, K. H. (1989) Biol. Cell. Transducing Signals '89: Int. Washington Spring Symp. Ninth, abstr. 184
- Verghese, M. W., Irsula, O. & Amarant, T. (1989) FASEB J. **3**, A228, abstr. 10
- Linden, J. & Delahunty, T. M. (1989) Trends Pharmacol. Sci. **10**, 114–120
- Delahunty, T. M., Cronin, M. J. & Linden, J. (1989) Biochem. J. **255**, 69–77
- Schimmel, R. J., Elliott, M. E. & McCarthy, L. (1987) in Topics and Perspectives in Adenosine Research (Gerlach, E. & Becker, B. F., eds.), pp. 261–274, Springer Verlag, Berlin
- Willems, P. H. G. M., Tilly, R. H. J. & de Pont, J. J. H. M. (1987) Biochim Biophys. Acta **928**, 179–185
- Asano, T., Morishita, R., Semba, R., Itoh, H., Kaziro, Y. & Kato, K. (1989) Biochemistry **28**, 4749–4754
- Milligan, G., Tariffin, Z., Goureau, O., Unson, C. & Harbon, S. (1989) FEBS Lett. **244**, 411–416
- Rouot, B., Carrette, J., Lafontan, M., Lan Tran, P., Fehrentz, J.-A., Bockaert, J. & Toutant, M. (1989) Biochem. J. **260**, 307–310
- Moriarty, T. M., Padrell, E., Carty, D. J., Omri, G., Landau, E. M. & Iyengar, R. (1990) Nature (London) **343**, 79–82

Received 21 December 1989/26 March 1990; accepted 2 April 1990