Biochem. J. (1986) 236, 337–343 (Printed in Great Britain)

Evidence suggesting that a novel guanine nucleotide regulatory protein couples receptors to phospholipase C in exocrine pancreas

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The initial response of many cells to 'Ca^{2+}-mobilizing' agonists is phospholipase C-mediated hydrolysis of phosphatidylinositol bisphosphate to inositol trisphosphate (IP_3) and diacylglycerol. It has been suggested, by analogy with receptor regulation of adenylate cyclase, that 'Ca^{2+}-mobilizing' receptors may interact with a guanine nucleotide-binding protein (G protein) to regulate phospholipase C activity. Here we report increased accumulation of IP_3 in response to caerulein or carbachol in electrically permeabilized rat pancreatic acinar cells. The stable analogues of GTP (guanosine 5'-[γ-thio]trisphosphate and guanosine 5'-[β,γ-imido]triphosphate) stimulate IP_3 accumulation and potentiate the effects of caerulein and carbachol. This synergism demonstrates an interaction between receptors, a G protein and phospholipase C. These responses are unaffected by pretreatment of the cells with pertussis or cholera toxins under conditions that produce substantial covalent modification of G_i and G_s, the proteins that couple receptors to adenylate cyclase. We therefore conclude that the G protein that couples receptors to phospholipase C in exocrine pancreas is probably neither G_i nor G_s; instead, we propose that a different G protein mediates this effect.

INTRODUCTION

Phospholipase C-mediated hydrolysis of PIP_2 to IP_3 and diacylglycerol is the initial response of many cells to 'Ca^{2+}-mobilizing' agonists. (1,4,5)IP_3 enters the cytosol and, in many cell types, including exocrine pancreas (Streb *et al.*, 1983), it has been shown to mobilize Ca^{2+} from intracellular pools (Berridge & Irvine, 1984). Diacylglycerol remains in the plasma membrane, and activates protein kinase C (Nishizuka, 1984). In many tissues, activation of both branches of this signalling pathway (an increase in cytosolic Ca^{2+} concentration and activation of protein kinase C) synergistically stimulates a cellular response (Nishizuka, 1984; Berridge, 1984), for example enzyme secretion from exocrine pancreas (Merritt & Rubin, 1985). These signals provide links between PIP_2 hydrolysis at the plasma membrane and intracellular responses. However, the mechanisms whereby receptor occupation leads to activation of phospholipase C are not established. By analogy with receptor regulation of adenylate cyclase (Codina *et al.*, 1984; Smigel *et al.*, 1984), it has been suggested that 'Ca^{2+}-mobilizing' receptors may interact with a guanine nucleotide-binding protein to regulate phospholipase C activity (Haslam & Davidson, 1984; Cockcroft & Gomperts, 1985; Litosch *et al.*, 1985).

Here, we report increased accumulation of IP_3 in response to caerulein (acting at the cholecystokinin receptor) or carbachol (acting at the muscarinic cholinergic receptor) in electrically permeabilized rat pancreatic acinar cells. We provide evidence that a G protein couples these receptors to phospholipase C activation, and that this activation is unaffected by pretreatment of the cells with cholera or pertussis toxins. We therefore propose that this G protein is probably distinct from those involved in receptor regulation of adenylate cyclase.

EXPERIMENTAL

Materials

Collagenase (*Clostridium histolyticum*) and guanine nucleotides were obtained from Boehringer Mannheim. ATP (from horse muscle), phosphocreatine and creatine kinase, from Sigma, were used without further purification. Peninsula Laboratories (Belmont, CA, U.S.A.) supplied the caerulein. [^{3}H]Inositol (10–30 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). Purified pertussis toxin was supplied by List Biological Laboratories (Campbell, CA, U.S.A.) and cholera toxin was from Calbiochem. [α-^{32}P]NAD^+ was obtained from ICN Radiochemicals. All other reagents were from Sigma.

Preparation of pancreatic acinar cells

The basic medium was Krebs–Henseleit (1932) saline (pH 7.4; gassed with O_2/CO_2, 19:1), supplemented with essential amino acids, glucose (11.5 mM) and soya-bean trypsin inhibitor (0.1 mg/ml). Acinar cells were prepared from pancreases of male Sprague–Dawley rats (150–200 g) by modifications of methods previously described (Streb & Schulz, 1983; Merritt & Rubin, 1985). Minced tissue was incubated with collagenase (0.05 unit/ml) for 10 min at 37 °C in the above medium

Abbreviations used: BSA, bovine serum albumin; G protein, GTP-binding protein; G_i, G_s, inhibitory and stimulatory GTP-binding proteins of the adenylate cyclase system; α_i, α_s and $\beta\gamma$, subunits of G_i and G_s; GDP[S], guanosine 5'-[β-thio]diphosphate (trilithium salt); p[NH]ppG, guanosine 5'-[β,γ-imido]triphosphate (tetralithium salt); GTP[S], guanosine 5'-[γ-thio]triphosphate (tetralithium salt); IP, inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; PIP_2, phosphatidylinositol bisphosphate.

with BSA (0.25%) and lower Ca^{2+} (0.1 mM) and Mg^{2+} (0.1 mM). This was followed by two incubations, each of 5 min, in medium containing BSA (0.25%), EDTA (2 mM) and no Ca^{2+} or Mg^{2+}. The tissue was washed and then incubated for a further 10 min in the collagenase-containing medium. Cells were dispersed by vigorous shaking, filtered through gauze, and centrifuged through medium containing 4% BSA. At this and all subsequent stages, the Krebs–Henseleit medium contained Ca^{2+} (1.28 mM) and Mg^{2+} (1.18 mM). The cells were incubated for 10 min at 37 °C in medium supplemented with 1% BSA and then resuspended in the same medium with 0.1% BSA for use in experiments. Cell viability, assessed by Trypan Blue exclusion, exceeded 98%.

Permeabilization of acinar cells

Cells were resuspended at a cell density of about 4 mg of cell protein/ml in a Ca^{2+}-free medium whose composition otherwise resembled cytosol (Burgess *et al.*, 1983). This medium had the following composition: KCl, 100 mM; NaCl, 20 mM; $NaHCO_3$, 25 mM; $MgSO_4$, 5 mM; NaH_2PO_4, 0.96 mM; EGTA, 1.0 mM; ATP, 1.5 mM; phosphocreatine, 5 mM; creatine kinase, 5 units/ml; BSA, 2%; soya-bean trypsin inhibitor, 0.01%; pH 7.2 at 37 °C; the gas phase was O_2/CO_2 (19:1). After exposure to an intense electric field (ten exposures to a field strength of 2 kV/cm; time constant 100 μs) (Knight & Baker, 1982; Knight & Koh, 1984), more than 99% of cells were permeable to Trypan Blue and remained permeable throughout subsequent experiments. Exposure of cells to lower field strengths rendered them permeable to $^{86}Rb^+$, but not to higher-M_r substances (inositol phosphates or Trypan Blue). Immediately after permeabilization, dinitrophenol (0.5 mM), oligomycin (10 μM) and antimycin (10 μM) were added, and the cells were incubated for 5 min before use in experiments.

Measurement of [^{3}H]inositol phosphates

Intact cells (50 mg of cell protein/ml) were incubated with [^{3}H]inositol (100 μCi/ml) for 4–5 h at 37 °C, washed by centrifugation, resuspended at 4 mg of cell protein/ml in the Ca^{2+}-free cytosolic-type medium, and permeabilized. Incubations (in duplicate) were initiated by addition of 250 μl samples of permeabilized cells to the test substances in 250 μl of the cytosolic-type medium with the estimated final free [Ca^{2+}] buffered at 140 nM (Burgess *et al.*, 1983), the cytosolic free [Ca^{2+}] of unstimulated acinar cells (Merritt & Rubin, 1985). Dinitrophenol (0.5 mM), oligomycin (10 μM) and antimycin (10 μM) were included in all incubations to inhibit mitochondrial metabolism; ATP was maintained by the regenerating system. Incubations (37 °C) were terminated by addition of 1 ml of cold 4.5% (v/v) $HClO_4$, the pH of the supernatant was corrected to 8–9, and inositol phosphates were separated by anion exchange chromatography (Berridge *et al.*, 1983). In some experiments, these [^{3}H]IP_3 fractions were further separated into [^{3}H](1,3,4)IP_3 and [^{3}H](1,4,5)IP_3 by h.p.l.c. (Burgess *et al.*, 1985). For each experiment, total lipids were extracted (Weiss & Putney, 1981) and [^{3}H]inositol phosphates were then expressed as a percentage of total [^{3}H]inositol lipids. To measure [^{3}H]PIP_2, [^{3}H]inositol lipids were separated by t.l.c. (Weiss & Putney, 1981), detected by fluorography, and the spots corresponding to [^{3}H]PIP_2 were scraped and counted for radioactivity.

ADP-ribosylation of α_i

The methods were modified from those of Hildebrandt *et al.* (1983). Intact cells (5 mg of cell protein/ml) were incubated for 2 or 4 h with pertussis toxin (0.2–20 μg/ml), then washed, resuspended in the Ca^{2+}-free cytosolic-type medium, and permeabilized. They were resuspended (1 mg of cell protein/ml) such that the final composition of the incubation medium was: thymidine, 15 mM; ATP, 1.5 mM; phosphocreatine, 5 mM; creatine kinase, 5 units/ml; GTP, 0.1 mM; EDTA, 1 mM; BSA, 0.03%; oligomycin, 10 μM; antimycin, 10 μM; Tris, 25 mM (pH 7.0 at 32 °C). Pertussis toxin, freshly activated by treatment for 30 min at 37 °C with dithiothreitol (25 mM), was included at a final concentration of 20 μg/ml; control incubations included dithiothreitol at the same concentration. Reactions (at 32 °C) in a final volume of 45 μl were initiated by addition of [α-^{32}P]NAD^+ (final concn. 10 μM and sp. radioactivity about 30 Ci/mmol) and terminated by addition of trichloroacetic acid. Proteins were separated by SDS/polyacrylamide-gel electrophoresis (8–12% gradient of acrylamide) (Laemmli, 1970), and phosphoproteins detected by autoradiography. Protein bands were excised from the gel, dissolved in NaOH, and their ^{32}P radioactivity was measured by liquid-scintillation counting.

RESULTS

Responses of permeabilized cells

In electrically permeabilized rat pancreatic acinar cells, caerulein (Fig. 1) or carbachol stimulated formation of [^{3}H]inositol phosphates. Permeabilized cells produced less [^{3}H]IP_3 than did intact cells, so it was important to establish that the responses were entirely those of permeabilized cels and not of residual intact cells. After rapid centrifugation of stimulated cells, $96 \pm 4\%$ ($n = 3$) of [^{3}H]IP_3 was recovered in the supernatant from permeabilized cells and $90 \pm 2\%$ ($n = 3$) in the pellet from intact cells. In the presence of mitochondrial inhibitors, permeabilized cells responded only when ATP and a regenerating system were provided; intact cells, dependent on endogenous ATP, never responded in the presence of mitochondrial inhibitors. Therefore, in all experiments with permeabilized cells, mitochondrial inhibitors were included to eliminate responses from residual intact cells. The decreased responsiveness of permeabilized cells was not due to loss of PIP_2, since [^{3}H]PIP_2 concentrations were maintained for up to 3 h after permeabilization, and, at the time when most of our measures of IP_3 were made, [^{3}H]PIP_2 was $0.84 \pm 0.12\%$ ($n = 6$) of total [^{3}H]inositol lipids, compared with $0.71 \pm 0.08\%$ ($n = 7$) in intact cells.

Accumulation of [^{3}H]IP_3 in response to caerulein (100 nM) was linear for at least 10 min in permeabilized cells (Fig. 1), whereas the rate declined within 5 min in intact cells (Rubin, 1984). LiCl (10 mM) potentiated caerulein-stimulated [^{3}H]IP_3 accumulation in intact cells (Rubin, 1984), but had no effect in permeabilized cells (results not shown). We believe that these differences may result from diffusion of IP_3 from permeabilized cells to the medium, where it is no longer readily accessible to Li^+-sensitive degradative enzymes.

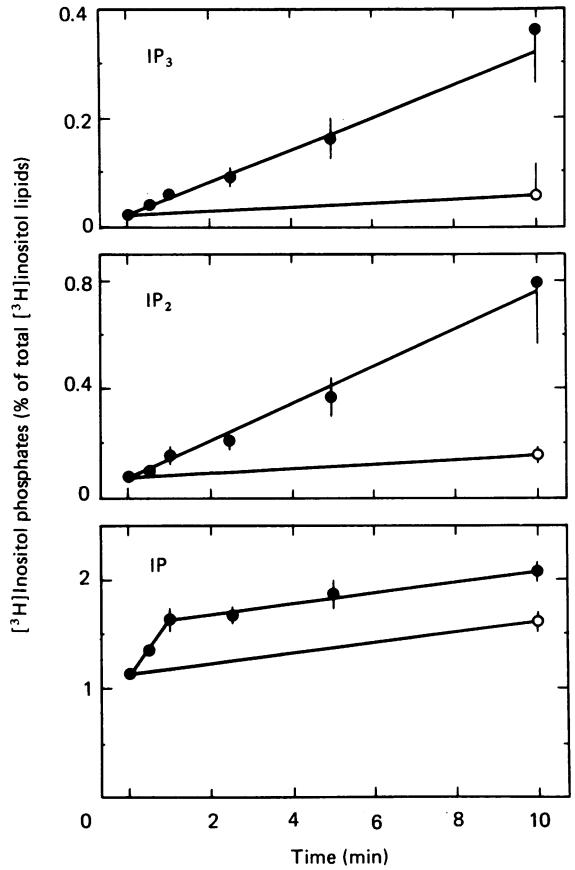

Fig. 1. Stimulation of [^{3}H]inositol phosphate accumulation by caerulein in permeabilized pancreatic acinar cells

Permeabilized cells in a cytosolic-type medium, which included GTP (10 μM), were incubated in the presence (●) or absence (○) of caerulein (100 nM), and the accumulation of [^{3}H]IP_3, [^{3}H]IP_2 and [^{3}H]IP was measured as percentages of total [^{3}H]inositol lipids (means ± S.E.M. for five independent experiments).

Effects of guanine nucleotides

All incubations in these and subsequent experiments were for 6 min, since [^{3}H]IP_3 accumulation was linear for at least 10 min (Fig. 1). In permeabilized cells, responses to agonists were not dependent on exogenous GTP. This effect was apparently not due to its rapid hydrolysis (Cockcroft & Gomperts, 1985), since, even at a concentration of 1 mM, aded GTP had no effect (see Fig. 5). This suggests that sufficient GTP, from cells and contamination of reagents (Glossmann *et al.*, 1974), may be present to support an agonist-induced response. To overcome possible variability in the extent of this contamination, unless otherwise stated GTP (10 μM) was routinely included in all experiments with permeabilized cells.

In permeabilized cells, the stable analogues of GTP, GTP[S] (10 μM) and p[NH]ppG (10 μM), markedly potentiated accumulation of [^{3}H]IP_3 in response to carbachol or caerulein (Fig. 2). Fig. 3 shows the concentration-dependent stimulation of [^{3}H]IP_3 accumulation by GTP[S] and p[NH]ppG, alone or in combination with caerulein (1 nM) or carbachol (100 μM). Preliminary analyses of the isomers of [^{3}H]IP_3 have shown that the ratio of [^{3}H](1,4,5)IP_3 to [^{3}H](1,3,4)IP_3 formed is the same whether the permeabilized cells are stimulated with GTP[S] (100 μM) or caerulein (100 nM) (Table 1).

[^{3}H]IP_3 accumulation in intact cells, in the absence or presence of caerulein (1 nM), was unaffected by p[NH]ppG (10–300 μM), GTP[S] (10–300 μM) or GTP (10–1000 μM) (results not shown), indicating an intracellular site of action of guanine nucleotides in permeabilized cells. These effects were specific for the stable analogues of GTP, since GDP, GDP[S] and GMP (each at 300 μM) did not affect [^{3}H]IP_3 accumulation in permeabilized cells in the presence or absence of caerulein (100 nM) (results not shown). In the presence of atropine (1 mM), responses to guanine nucleotides were unaffected, but their synergistic interactions with carbachol were abolished (Fig. 4); responses to caerulein, however, were unimpaired. Some guanine nucleotides are supplied as Li^+ salts, but Li^+ itself had no effect in permeabilized cells (results not shown).

Although the responses of permeabilized cells were not dependent on added GTP, the effects of p[NH]ppG and GTP[S] were inhibited in a concentration-dependent manner by GTP (Fig. 5). These inhibitory effects of GTP are unlikely to be a consequence of its hydrolysis to GDP, since the synergistic interaction of GTP[S] with caerulein was more effectively inhibited by GTP (1 mM; 92% inhibition) than by GDP (1 mM; 68% inhibition). In the presence of ATP and a regenerating system, it is likely that GDP is rapidly phosphorylated to GTP; this may then explain the lesser inhibitory effect of added GDP. In support of this suggestion is the finding that GDP (300 μM) did not affect the response to caerulein (100 nM) in the presence of only GTP (results not shown). These results suggest that sufficient contaminating GTP may be present to support an agonist-induced response, and that stable analogues of GTP, by virtue of their generally greater efficacy in activating G proteins (Codina *et al.*, 1984; Smigel *et al.*, 1984), may displace this endogenous GTP and potentiate the response to agonists.

Effects of pertussis and cholera toxins

In permeabilized pancreatic acinar cells, activated pertussis toxin (20 μg/ml) stimulated [^{32}P]ADP-ribosylation of a protein of molecular mass 41 kDa (Fig. 6). By analogy with many other tissues (Katada & Ui, 1982), we believe this protein to be the α_i subunit of the guanine nucleotide-binding protein, G_i. Preincubation of intact cells with pertussis toxin decreased [^{32}P]ADP-ribosylation of α_i when the cells were subsequently permeabilized and incubated with activated toxin (Fig. 6). With this protocol, we were able to demonstrate that treatment of intact cells with pertussis toxin (20 μg/ml) for 4 h caused ADP-ribosylation of $63 \pm 6\%$ ($n = 3$) of α_i. More extensive ADP-ribosylation of α_i was impracticable, since the concentration of pertussis toxin used was maximal and more prolonged incubation impaired cell viability.

Intact cells, preincubated with pertussis toxin (20 μg/ml for 4 h), were permeabilized, and [^{3}H]IP_3 accumulation in response to GTP[S] with or without agonists was examined. These responses were unaffected by pretreatment with pertussis toxin (Table 2). Similarly, pretreatment of intact acinar cells with cholera toxin (20 μg/ml for 4 h), a protocol that produces maximal effects on adenylate cyclase in pancreas (Gardner & Rottman, 1979), did not affect responses of these cells, after permeabilization, to agonists or GTP[S] (Table 2).

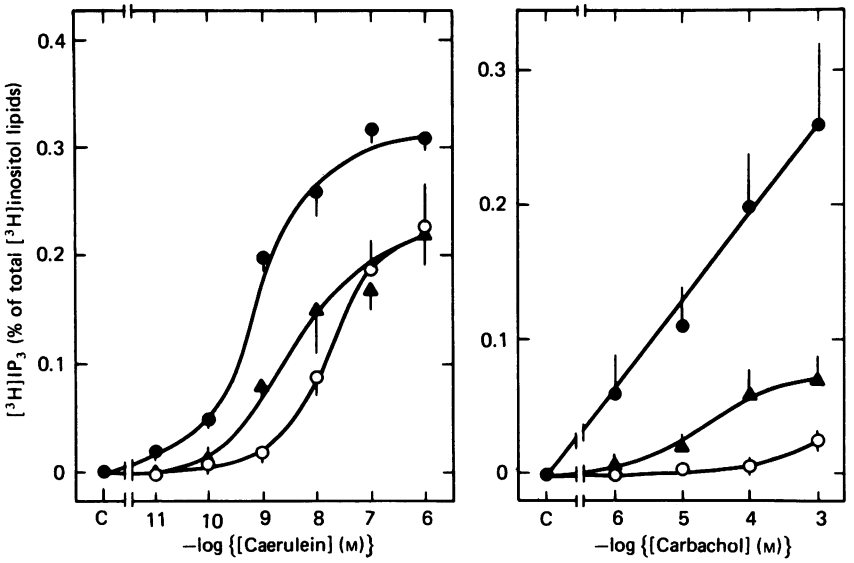

Fig. 2. Concentration–response relationships between receptor agonists and [^{3}H]IP_3 accumulation in the presence of guanine nucleotides

Permeabilized cells were incubated for 6 min in the presence of GTP (10 μM; ○) or with GTP replaced by p[NH]ppG (10 μM; ▲) or GTP[S] (10 μM; ●). The concentration-dependence of [^{3}H]IP_3 accumulation in response to caerulein or carbachol is shown after subtraction of the stimulatory effects of guanine nucleotide alone. The effects of guanine nucleotides alone were as follows: GTP, 0.05±0.01; p[NH]ppG, 0.07±0.01; GTP[S], 0.12±0.02. Results are shown as means±S.E.M. for four independent experiments. C, control (no agonist).

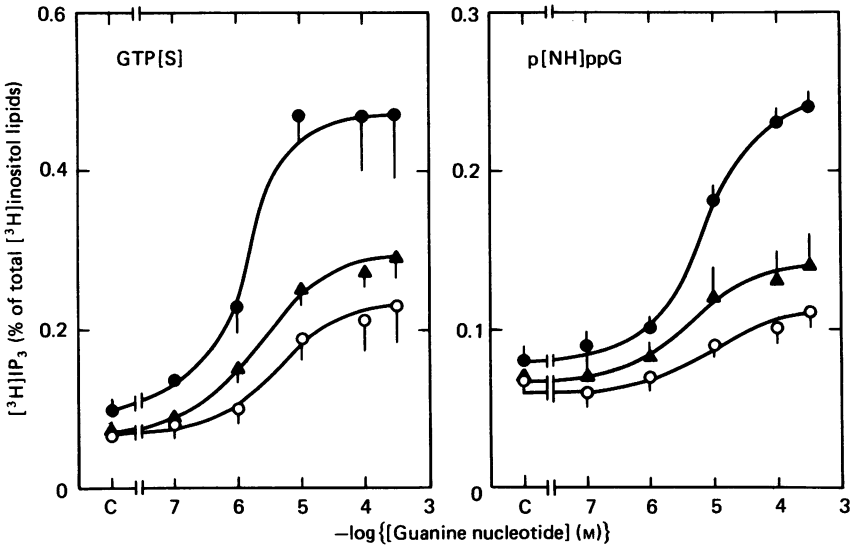

Fig. 3. Concentration–response relationships between stable guanine nucleotides and [^{3}H]IP_3 accumulation in the presence of receptor agonists

Permeabilized cells were incubated for 6 min in the presence of submaximal concentrations of caerulein (1 nM; ●) or carbachol (100 μM; ▲) or in the absence of agonist (○). The concentrations of GTP[S] or p[NH]ppG were varied between 0 and 300 μM. Accumulation of [^{3}H]IP_3 is shown as the means±S.E.M. for three independent experiments.

DISCUSSION

The 'Ca^{2+}-mobilizing' agonists, carbachol and caerulein, stimulate [^{3}H]IP_3 accumulation in electrically permeabilized rat pancreatic acinar cells. Other workers have reported that carbachol and cholecystokinin stimulate [^{3}H]IP_3 accumulation in pancreatic acinar cells permeabilized by washing in a nominally Ca^{2+}-free medium, but in that study the responses were not convincingly shown to be entirely those of permeabilized cells (Streb *et al.*, 1985). We have established a protocol that allows the responses of electrically permeabilized cells to be examined free of any contribution from intact cells. With such a preparation, the intracellular environment can be manipulated and the effects of such changes on receptor-mediated events investigated.

With the free [Ca^{2+}] buffered at the value found in unstimulated pancreatic acinar cells, agonists stimulate

Table 1. Isomers of [^{3}H]IP_3 formed in permeabilized cells in response to caerulein or GTP[S]

[^{3}H]IP_3 produced by permeabilized cells in response to caerulein or GTP[S] was further separated into [^{3}H](1,3,4)IP_3 and [^{3}H](1,4,5)IP_3 by h.p.l.c. The amount of [^{3}H](1,4,5)IP_3 produced is expressed as a percentage of the total [^{3}H]IP_3. Results are shown as the range of two determinations for the 10 s incubations and the range of three determinations for the 6 min incubations.

	[^{3}H](1,4,5)IP_3 (% of total [^{3}H]IP_3)	
Incubation time...	10 s	6 min
Caerulein (100 nM)	23–28	66–69
GTP[S] (100 μM)	22–31	64–71

[^{3}H]IP_3 accumulation in permeabilized cells, indicating that the increase in cytosolic Ca^{2+} that normally accompanies receptor activation is not necessary for stimulation of IP_3 formation. This result and similar observations by Streb *et al.* (1985) are entirely consistent with the proposal that IP_3 formation is the cause, rather than the consequence, of the increase in cytosolic Ca^{2+} concentration; a proposal lent further support by studies of the effects of (1,4,5)IP_3 on intracellular Ca^{2+} pools (Berridge & Irvine, 1984), and from the kinetics of IP_3 formation compared with Ca^{2+}-mediated cellular responses (Berridge *et al.*, 1984).

Accumulating evidence suggests considerable similarities between guanine nucleotide effects on receptor regulation of adenylate cyclase and phospholipase C.

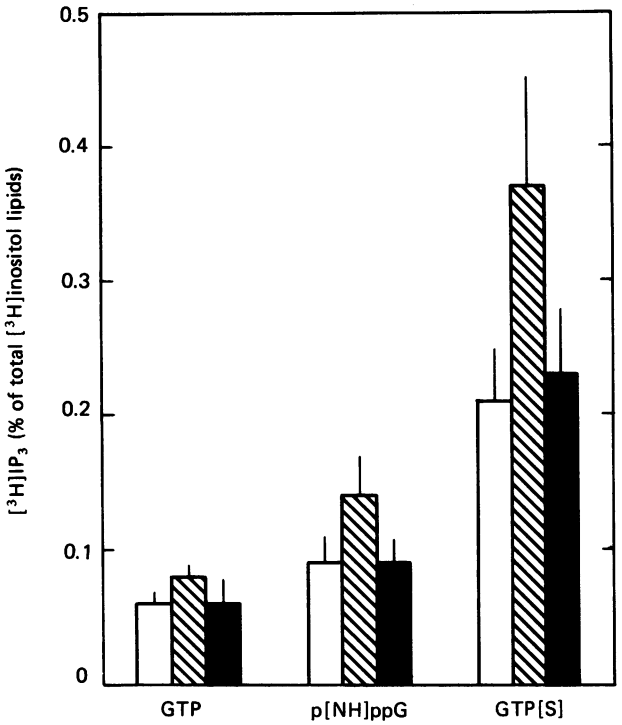

Fig. 4. Effects of atropine on carbachol- and guanine nucleotide-stimulated [^{3}H]IP_3 accumulation in permeabilized cells

Permeabilized cells were incubated with carbachol (1 mM), with or without atropine (1 mM), in the presence of guanine nucleotides (each at 10 μM), and [^{3}H]IP_3 accumulation was measured after 6 min. The effects of carbachol were blocked by atropine. Results are means ± S.E.M. for three independent experiments. Key: □, control; ▨, carbachol; ■, carbachol + atropine.

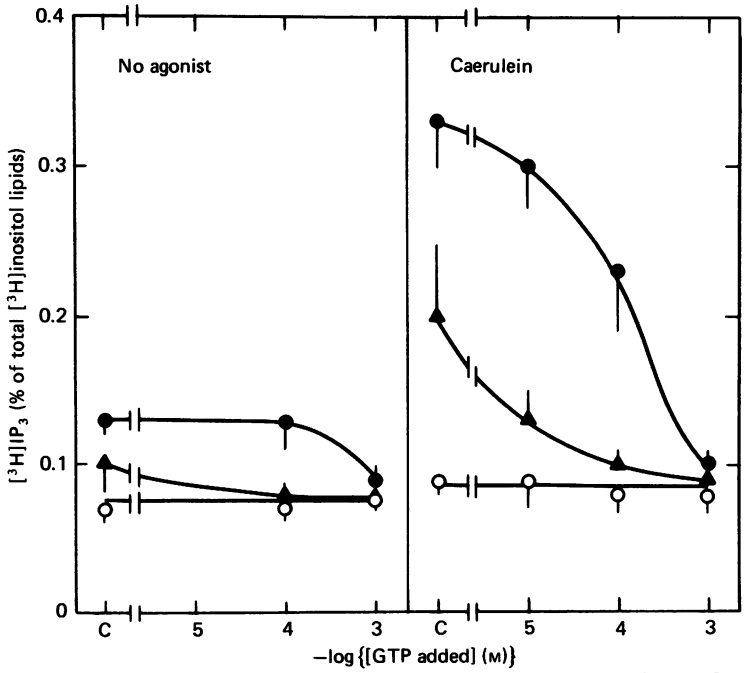

Fig. 5. Concentration-dependent inhibition, by exogenous GTP, of the effects of stable guanine nucleotides on [^{3}H]IP_3 accumulation in permeabilized acinar cells

Permeabilized cells were incubated for 6 min in the absence or presence of caerulein (1 nM). The concentration of added GTP was varied between 0 and 1000 μM in the presence of GTP[S] (10 μM; ●), p[NH]ppG (10 μM; ▲) or without further additions (○). Total [^{3}H]IP_3 accumulations are shown as means ± S.E.M. for three independent experiments.

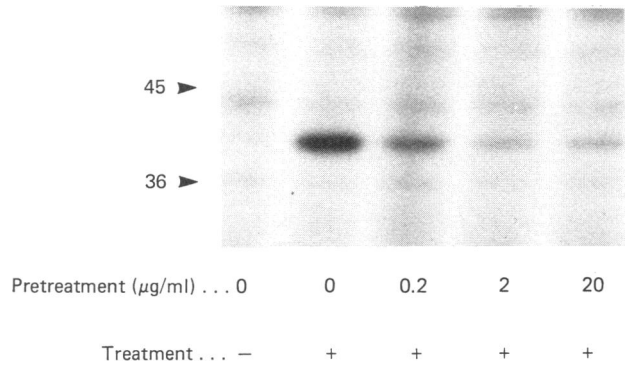

Fig. 6. Autoradiograph showing the effects of pertussis toxin on [^{32}P]ADP-ribose incorporation into a 41 kDa protein of pancreatic acinar cells

Intact cells were pretreated for 4 h with pertussis toxin at 0, 0.2, 2 or 20 μg/ml. The cells were then permeabilized and incubated for 90 min with [α-^{32}P]NAD^+ and activated pertussis toxin at 0 μg/ml (−) or 20 μg/ml (+). Proteins were separated by SDS/polyacrylamide-gel electrophoresis and radiolabelled proteins were detected by autoradiography. Preincubation of intact cells with pertussis toxin decreased the amount of [^{32}P]ADP-ribose subsequently incorporated into the 41 kDa protein. The positions of ovalbumin (45 kDa) and glyceraldehyde-3-phosphate dehydrogenase (36 kDa) are indicated.

Guanine nucleotides regulate the agonist affinities of many 'Ca^{2+}-mobilizing' receptors, including muscarinic (Evans *et al.*, 1985), α_1-adrenergic (Goodhardt *et al.*, 1982) and chemotactic peptide (Snyderman *et al.*, 1984). More direct evidence for guanine nucleotide involvement in phospholipase C regulation has been provided by studies of broken-cell preparations. Stable analogues of GTP activate phospholipase C in neutrophil (Cockcroft & Gomperts, 1985) and hepatocyte (Wallace & Fain, 1985) plasma membranes, and in permeabilized platelets (Haslam & Davidson, 1984). From each of these studies, a G protein has been proposed to regulate phospholipase C, but receptor regulation of that G protein has not been established.

In permeabilized pancreatic acinar cells, GTP[S] and p[NH]ppG appear to displace endogenous GTP from an intracellular G protein, stimulate [^{3}H]IP_3 accumulation, and potentiate the effects of caerulein and carbachol. This synergism demonstrates an interaction between receptors, a G protein and phospholipase C. The ratio of [^{3}H](1,4,5)IP_3 to [^{3}H](1,3,4)IP_3 produced by permeabilized cells is similar whether the cells are stimulated by GTP[S], acting at the G protein, or by caerulein, acting at its receptor. This result lends further support to the suggestion that agonists and guanine nucleotides stimulate phospholipase C through a shared G protein. Isolated membranes of blowfly salivary glands produce IP_3 in response to 5-hydroxytryptamine, but only in the presence of GTP; and the response is potentiated by GTP[S] or p[NH]ppG (Litosch *et al.*, 1985). These results from blowfly salivary gland and our results from exocrine pancreas provide direct evidence for an interaction between 'Ca^{2+}-mobilizing' receptors, a G protein and phospholipase C.

In mast cells and neutrophils, the G protein appears to be G_i, since the effects of guanine nucleotides (Nakamura & Ui, 1984) and chemotactic factors (Bradford & Rubin, 1985) are inhibited by pretreatment with pertussis toxin. By contrast, in astrocytoma and chick heart cells, activation of phospholipase C by muscarinic agonists is insensitive to pertussis toxin (Masters *et al.*, 1985), and guanine nucleotide effects on receptor affinity for muscarinic agonists in astrocytoma cells are also insensitive to pertussis toxin (Evans *et al.*, 1985). In these latter cell types, available evidence implies that coupling between muscarinic receptors and cellular responses does not involve G_i, but no evidence directly implicating G proteins in coupling receptors to phospholipase C is available.

Pancreatic acinar cells contain both G_i and G_s (Lambert *et al.*, 1985), but accumulation of [^{3}H]IP_3 in response to agonists or guanine nucleotides is unaffected by pretreatment of the cells with pertussis or cholera toxins under conditions that produce substantial covalent modification of both G_i and G_s (Table 2). In these experiments, where only 63% of α_i was modified, it is possible that the remaining unmodified G_i could still be sufficient to allow coupling of receptors to phospholipase C. The failure of this regimen to inhibit agonist activation of IP_3 production therefore does not unequivocally rule out a role for G_i in this pathway. However, when IP_3 production in permeabilized cells is activated by a

Table 2. Effects of pertussis or cholera toxins on [^{3}H]IP_3 accumulation in permeabilized cells

Pretreatment of intact cells with pertussis or cholera toxins, each at 20 μg/ml, for 4 h did not affect the responses of subsequently permeabilized cells, incubated for 6 min with GTP[S], carbachol (results not shown) or caerulein ($P > 0.05$, Student's t test). Results are means ± S.E.M. for four independent experiments for control and pertussis-toxin-treated cells, and ranges from two independent experiments for the cholera-toxin treatment.

	[^{3}H]IP_3 (% of total [^{3}H]inositol lipids)		
Treatment...	Control	Pertussis toxin	Cholera toxin
GTP (10 μM)	0.10 ± 0.03	0.10 ± 0.03	0.09–0.11
GTP[S] (10 μM)	0.23 ± 0.03	0.25 ± 0.05	0.26–0.30
GTP[S] (300 μM)	0.27 ± 0.03	0.28 ± 0.05	0.31–0.31
Caerulein (1 nM) + GTP (10 μM)	0.11 ± 0.02	0.11 ± 0.03	0.10–0.12
Caerulein (100 nM) + GTP (10 μM)	0.18 ± 0.03	0.17 ± 0.02	0.18–0.21
Caerulein (1 nM) + GTP[S] (10 μM)	0.38 ± 0.04	0.36 ± 0.05	0.43–0.52
Caerulein (100 nM) + GTP[S] (10 μM)	0.49 ± 0.03	0.46 ± 0.02	0.51–0.55

submaximal or limiting concentration of guanine nucleotide, then the concentration of G-protein should also become limiting. Under these conditions, a 63% decrease in functional G_i should be reflected as a similarly substantial decrease in IP_3 formation. Since pertussis toxin did not inhibit the response to a submaximal concentration of GTP[S] (Table 2), it is unlikely that G_i is involved in coupling receptors to phospholipase C in exocrine pancreas.

Other workers have adopted a different approach. Receptor-mediated inhibition of adenylate cyclase probably requires that $\beta\gamma$ subunits, formed by dissociation of α_i from G_i, associate with α_s and thereby relieve its stimulation of adenylate cyclase (Smigel *et al.*, 1984). Such a model predicts that, relative to α_s, large amounts of free $\beta\gamma$ subunits must become available to inhibit adenylate cyclase effectively. Thus inhibitory control of adenylate cyclase may be more susceptible to pertussis toxin, which prevents dissociation of α_i from $\beta\gamma$, than is any process controlled by direct interaction with α_i. It has been suggested (Masters *et al.*, 1985) that in chick heart G_i does not couple muscarinic receptors to phospholipase C, since, under conditions where pertussis toxin prevented coupling of these receptors to inhibition of adenylate cyclase, their coupling with phospholipase C was unaffected. However, it remains possible that, under these conditions, sufficient α_i was modified to prevent receptor regulation of adenylate cyclase, but enough α_i may remain unmodified to allow receptor regulation of phospholipase C. We believe that this potential problem may be overcome by using limiting concentrations of GTP[S] in permeabilized cells to assess more directly the amount of functional G protein that couples receptors to phospholipase C.

We have provided evidence suggesting that a G protein couples 'Ca^{2+}-mobilizing' receptors to phospholipase C in exocrine pancreas, and that this G protein is probably neither G_i nor G_s. This presents a striking parallel with receptor regulation of adenylate cyclase, yet allows independent control of each of these signalling pathways.

We thank Monica Chapman for her technical assistance. This work was supported by grants AM 28029 and DE 05764 from the National Institutes of Health.

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Received 25 October 1985/17 December 1985; accepted 20 January 1986