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A GUANINE NUCLEOTIDE-DEPENDENT REGULATORY PROTEIN COUPLES SUBSTANCE P RECEPTORS TO PHOSPHOLIPASE C IN RAT PAROTID GLAND

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SUMMARY. Electrically permeabilized cells of rat parotid gland, prelabelled with [^{3}H]-inositol, synthesized [^{3}H]-inositol phosphates (IP_3 and IP_2) when stimulated with α_1-adrenergic, muscarinic-cholinergic, and substance P receptor-agonists. Non-hydrolyzable analogues of GTP (GTPγS and GppNHp) also stimulated [^{3}H]-IP_3 formation by permeabilized cells and they potentiated the stimulation by receptor-agonists. These effects of guanine nucleotides occurred only with GTP analogues and only in permeabilized cells indicating an intracellular site of action. NaF stimulated [^{3}H]-IP_3 accumulation, an effect that was not entirely attributable to the ability of F^- to inhibit (1,4,5)IP_3 degradation. These results suggest that a guanine nucleotide-dependent regulatory protein couples Ca^{2+}-mobilizing receptors to phospholipase C in parotid gland.

Extensive studies of receptor-regulation of adenylate cyclase have established that G proteins couple receptors to stimulation or inhibition of adenylate cyclase (1,2), and thereby control formation of the intracellular messenger, cyclic AMP. Another signalling pathway involves receptors that couple to phospholipase C leading to hydrolysis of phosphatidylinositol bisphosphate with the formation of the intracellular messengers, IP_3 and diacylglycerol (3). Diacylglycerol activates protein kinase C (4) and (1,4,5)IP_3 mobilizes intracellular Ca^{2+} (5,6). Early indications that Ca^{2+}-mobilizing receptors also associate with a G protein were provided when the affinity of several of these receptors was found to be modulated by guanine nucleotides (7,8), and when stable analogues of GTP were shown to activate

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Abbreviations: G protein, guanine nucleotide-dependent regulatory protein; GDPβS, guanosine-5'-O-(2-thiodiphosphate); GppNHp, guanylylimidodiphosphate; GTPγS, guanosine-5'-O-(3-thiotriphosphate); IP, inositol phosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate.

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phospholipase C (9,10). However, until very recently, there has been no direct evidence that a G protein couples receptors to phospholipase C. Here we report a synergistic stimulation of phospholipase C by stable analogues of GTP and Ca^{2+}-mobilizing receptor-agonists in permeabilized parotid cells. This result lends further support to the contention that Ca^{2+}-mobilizing receptors couple to phospholipase C through a G protein (11,12,13).

MATERIALS AND METHODS

Collagenase, guanine nucleotides and substance P were obtained from Boehringer Mannheim. [^{3}H]-Inositol (10-30 Ci/mmol) was from American Radiolabeled Chemicals, Inc. (St. Louis, MO). All other reagents were from Sigma.

Acinar cells were prepared from parotid glands of male Sprague-Dawley rats (150-200 g) using the method previously described for preparation of pancreatic acinar cells (13). Intact cells (20 mg cell protein/ml) were incubated for 3-4 h at 37°C in a modified Krebs-Henseleit medium (13) containing [^{3}H]-inositol (100 μCi/ml). Cells were washed and resuspended in the Krebs-Henseleit medium for studies of intact cells, or the cytosolic-type medium for studies of permeabilized cells. The cytosolic-type 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, $CaCl_2$, 0.435 mM (free [Ca^{2+}] = 140 nM); ATP, 1.5 mM; creatine phosphate, 5 mM; creatine kinase, 5 units/ml; bovine serum albumin, 2%; soyabean trypsin inhibitor, 0.01 %; pH 7.2 at 37°C; the gas phase was 5% CO_2 and 95% O_2 (13,14). Cells were permeabilized by exposure to an intense electric field (10 exposures to a field strength of 2 kV/cm; time constant 100 μs), after which more than 99% of cells were permeable to trypan blue and remained permeable throughout subsequent experiments. Immediately after permeabilization, 2,4-dinitrophenol (0.5 mM), oligomycin (10 μM) and antimycin (10 μM) were added, and the cells were incubated for 5 min prior to use in experiments. This protocol ensures that the observed responses are entirely those of permeabilized cells since in the presence of these mitochondrial inhibitors, receptor-agonists are unable to stimulate formation of [^{3}H]-inositol phosphates by intact cells, whereas the responses of permeabilized cells are unaffected by mitochondrial inhibitors, but are entirely dependent on exogenous ATP (Results not shown). Incubations (in duplicate) were initiated by addition of 250 μl aliquots of permeabilized cells to the test substances in 250 μl of the cytosolic-type medium with the final free [Ca^{2+}] buffered at 140 nM (14) and the final cell density at 1 mg cell protein/ml. Incubations were terminated and inositol phosphates separated as previously described (13).

RESULTS AND DISCUSSION

In permeabilized parotid cells, [^{3}H]-inositol phosphate formation was stimulated by a maximal concentration of substance P (10 nM) in the presence of GTPγS (1 μM) (Fig. 1). There were substantial increases in the amounts of [^{3}H]-IP_3 and [^{3}H]-IP_2, but no detectable increase in [^{3}H]-IP. The rate of accumulation of [^{3}H]-IP_3 and [^{3}H]-IP_2 began to decline within 10 min, but we have not established whether this results from degradation of the inositol

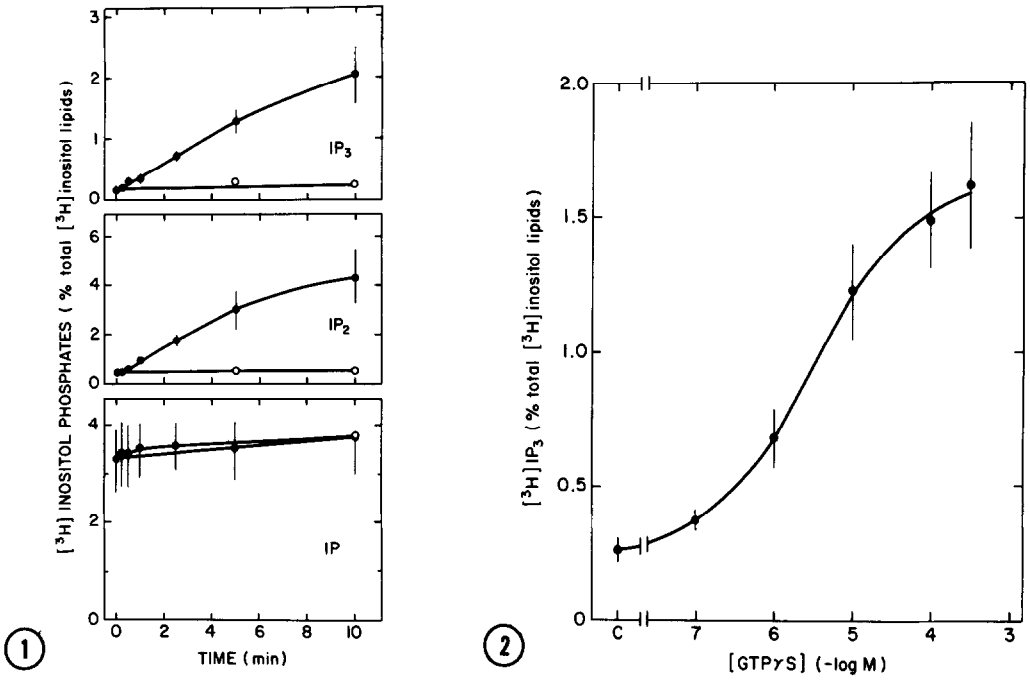

FIGURE 1. Stimulation of [^{3}H]-inositol phosphate formation by substance P and GTPγS in permeabilized parotid cells.
Permeabilized cells in a cytosolic-type medium were incubated in the absence (o) or presence (•) of substance P (10 nM) and GTPγS (1 μM), and the formation of [^{3}H]-IP$_3$, [^{3}H]-IP$_2$ and [^{3}H]-IP was measured. [^{3}H]-Inositol phosphates are shown as percentages of total [^{3}H]-inositol lipids (means ± SE of 4 independent experiments).

FIGURE 2. Effect of GTPγS on [^{3}H]-IP$_3$ formation.
Permeabilized cells were incubated for 6 min in the presence of various concentrations of GTPγS. [^{3}H]-IP$_3$ accumulation is shown as means ± SE of 5 independent experiments.

phosphates, desensitization of the substance P receptor, or degradation of substance P. In all subsequent experiments, incubations were for 6 min.

The stable analogue of GTP, GTPγS, caused a concentration-dependent stimulation of [^{3}H]-IP$_3$ formation in permeabilized cells (Fig. 2). Stimulation was also observed with GppNHp (Results not shown); but other guanine nucleotides, GDP, GDPβS and GMP (each at 300 μM) were without effect. Substance P, in the absence of added guanine nucleotides, significantly stimulated [^{3}H]-IP$_3$ formation by permeabilized cells, although the response was relatively small (Fig. 3). In the presence of GTP (10 μM), the response to substance P was increased, although the effect was not statistically

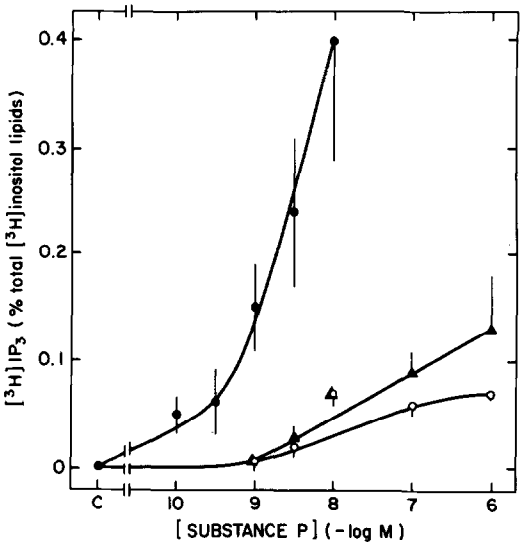

FIGURE 3 Concentration-response relationships between substance P and $[^3H]$-IP_3 accumulation in the presence of guanine nucleotides. Permeabilized cells were incubated for 6 min in the absence of guanine nucleotides (o), or in the presence of either GTP (10 μM; ▲), or GTPγS (1 μM; ●). $[^3H]$-IP_3 accumulation in response to substance P is shown after subtraction of the values in its absence. In the absence of substance P, the levels of $[^3H]$-IP_3 were: in the absence of guanine nucleotides, 0.15 ± 0.02; in the presence of 10 μM GTP, 0.18 ± 0.02; and in the presence of 1 μM GTPγS, 0.59 ± 0.14. Results are means ± SE of at least 4 independent experiments.

significant (2 way analysis of variance). The response to substance P was, however, significantly potentiated by the addition of GTPγS (1 μM). Essentially similar results were obtained with carbachol (muscarinic receptor) or epinephrine (α_1-receptor), although their synergism with guanine nucleotides was not statistically significant (Table 1). In intact cells, none of the guanine nucleotides tested (GTP, GTPγS, GppNHp, GMP), at concentrations of up to 100 μM, had any effect on $[^3H]$-IP_3 formation, nor were there any synergistic interactions with receptor-agonists (Results not shown).†

†Although low concentrations of guanine nucleotides (≤ 100 μM) had no effect on phospholipase C activity in intact parotid cells, higher concentrations (300 μM) of GTPγS or GTP, but not GppNHp, stimulated $[^3H]$-IP_3 formation (2 fold increase with guanine nucleotides, cf. a 10 fold increase with a maximal concentration of carbachol. Results not shown). This result, together with the absence of any synergistic interaction between receptor-agonists and these guanine nucleotides on intact cells, suggests a non-specific effect of GTPγS and GTP (or a contaminant of these reagents) that is unrelated to their effects on a G protein.

Table 1

Effects of carbachol, epinephrine and GTPγS on [^{3}H]-IP_3 formation by permeabilized cells

	[^{3}H]-IP_3 (% total [^{3}H]-inositol lipids)	
	Control	GTPγS 1 μM
Control	0.15 ± 0.02	0.67 ± 0.14
Carbachol (100 μM)	0.18 ± 0.02	1.03 ± 0.19
Carbachol (1 mM)	0.20 ± 0.01	1.16 ± 0.24
Epinephrine (10 μM)	0.20 ± 0.04	0.81 ± 0.18
Epinephrine (100 μM)	0.19 ± 0.03	0.91 ± 0.19

Cells were incubated in the presence of carbachol, or epinephrine with propranolol (10 μM), in the presence or absence of GTPγS (1 μM), and [^{3}H]-IP_3 accumulation was measured after 6 min. Results are means ± SE of 5 independent experiments.

Stimulation of [^{3}H]-IP_3 formation by stable analogues of GTP in permeabilized, but not intact cells, suggests that guanine nucleotides, acting at an intracellular site, can activate a G protein which then stimulates phospholipase C. The synergistic stimulation of [^{3}H]-IP_3 formation by Ca^{2+}-mobilizing agonists and GTPγS demonstrates an interaction between these receptors, a G protein and phospholipase C. We therefore conclude that a G protein couples substance P, and probably muscarinic-cholinergic and α_1-adrenergic receptors to phospholipase C activation in rat parotid gland.

AlF_4^- has been reported to promote dissociation of the α from the βγ subunits of the G proteins involved in receptor-regulation of adenylate cyclase (G_s and G_i) and of transducin (15,16). In permeabilized parotid cells, NaF caused a concentration-dependent increase in [^{3}H]-IP_3 accumulation (Fig. 4), this effect was specific for NaF since neither NaBr nor NaI had any effect (Results not shown). No precautions were taken to avoid etching of Al^{3+} from glass incubation tubes, it is therefore likely that whenever incubations included F^-, sufficient Al^{3+} was etched from the glass to allow formation of the active AlF_4^- species (15). The (1,4,5)IP_3 5-phosphatase that degrades IP_3 to IP_2 is inhibited by F^- with a K_i of about 3 mM (17); the effects of F^- on IP_3 accumulation (Fig. 4) could therefore be equally well explained by F^-

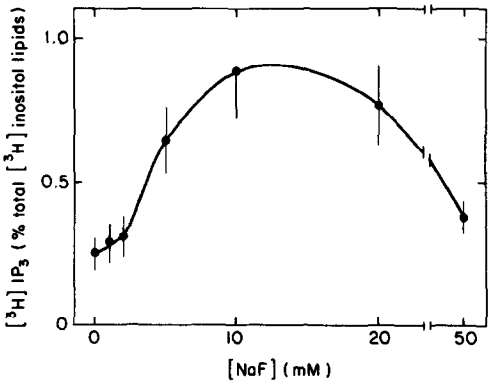

FIGURE 4. Effect of NaF on [^{3}H]-IP$_3$ accumulation in permeabilized cells. Permeabilized cells were incubated for 6 min with various concentrations of NaF. [^{3}H]-IP$_3$ accumulations are shown as the means ± SE of 7 independent experiments.

inhibition of IP$_3$ breakdown or by stimulation of its formation. In permeabilized parotid cells, F^- stimulated [^{3}H]-IP$_3$ accumulation when cells were incubated with a concentration of 2,3 bisphosphoglyceric acid (10 mM) that substantially inhibited breakdown of IP$_3$ to IP$_2$ (Results not shown). Although we cannot discount an effect of F^- on IP$_3$ breakdown in permeabilized parotid cells, its effects must, at least in part, result from stimulation of IP$_3$ formation. Similarly, in hepatocytes AlF_4^- stimulates phospholipase C activity (18).

In conclusion, by analogy with the effect of F^- on G_s, G_i and transducin, the effects of F^- on IP$_3$ formation suggest that dissociation of a G protein may regulate phospholipase C activity. The synergistic stimulation of IP$_3$ formation by stable analogues of GTP and receptor-agonists, suggest that a G protein couples substance P and probably α_1-adrenergic and muscarinic-cholinergic receptors to phospholipase C in rat parotid gland.

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