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Muscarinic cholinergic stimulation of inositol phosphate production in cultured embryonic chick atrial cells

Evidence for a role of two guanine-nucleotide-binding proteins

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These studies demonstrate a novel mechanism for the coupling of the muscarinic receptor to phospholipase C activity in embryonic chick atrial cells. In monolayer cultures of atrial cells from hearts of embryonic chicks at 14 days *in ovo*, carbamylcholine stimulated the sequential appearance of InsP_3, InsP_2 and InsP_1 with an EC_{50} (concn. causing 50% of maximal stimulation) of 30 μM. In the presence of 15 mM-Li, a 5 min exposure to carbamylcholine (0.1 mM) increased InsP_3 levels to a maximum of 47 ± 12% over basal, InsP_2 to 108 ± 13% over basal and InsP_1 to 42 ± 5% over basal. This effect was blocked by 5 μM-atropine. Incubation of these cells with pertussis toxin (15 h; 0.5 ng/ml) inhibited carbamylcholine-stimulated InsP_3, InsP_2 and InsP_1 formation by 42 ± 7%, 30 ± 3% and 48 ± 7% respectively. The IC_{50} (concn. causing 50% inhibition) for pertussis toxin inhibition of all three inositol phosphates was 0.01 ng/ml, with a half-time of 6 h at 0.5 ng/ml. This partial sensitivity to pertussis toxin was not due to incomplete ADP-ribosylation of the guanine-nucleotide-binding protein (G-protein), since autoradiography of polyacrylamide gels of cell homogenates incubated with [^{32}P]NAD^+ in the presence of pertussis toxin demonstrated that incubation of cells with 0.5 ng of pertussis toxin/ml for 15 h resulted in complete ADP-ribosylation of pertussis toxin substrates by endogenous NAD^+. In cells permeabilized with saponin (10 μg/ml), 0.1 mM-GTP[S] (guanosine 5′-[γ-thio]triphosphate) stimulated InsP_1 by 102 ± 15% (mean ± S.E.M., $n = 4$), InsP_2 by 421 ± 67% and InsP_3 by 124 ± 33% above basal. Incubation of cells for 15 h with 0.5 ng of pertussis toxin/ml decreased GTP[S]-stimulated InsP_1 production in saponin-treated cells by 30 ± 10% ($n = 3$), InsP_2 production by 45 ± 7% ($n = 4$) and InsP_3 production by 49 ± 6% ($n = 4$). These data demonstrate that in embryonic chick atrial cells at least two independent G-proteins, a pertussis toxin-sensitive G-protein and a pertussis toxin-insensitive G-protein, play a role in coupling muscarinic agonist binding to phospholipase C activation and to inositol phosphate production.

INTRODUCTION

In many cell types, activation of phospholipase C by receptor occupancy has been shown to be mediated via guanine-nucleotide-binding proteins (G-proteins) [1–3], some which are sensitive and others which are insensitive to pertussis toxin. In plasma membranes from rat liver [4] and permeabilized GH_3 cells [5], non-hydrolysable analogues of GTP stimulate InsP_3 production. In these cells, pertussis toxin has no effect on agonist-stimulated InsP_3 production [4–6], suggesting that a pertussis toxin-insensitive G-protein, termed G_p, may couple the receptor to phospholipase C [7]. In other cell types, including neutrophils and mast cells, pretreatment with pertussis toxin results in nearly complete inhibition of agonist-stimulated InsP_3 formation [8–11], implying a role for the pertussis toxin substrates (G_i or G_o) in agonist–response coupling in these cells. Some cells, such as rat hepatocytes, appear to contain both pertussis toxin-sensitive and pertussis toxin-insensitive pathways for coupling of receptors to phospholipase C. Most of these involve two different receptors coupled to phospholipase C via separate G-proteins. Angiotensin II-stimulated phosphoinositide metabolism in rat hepatocytes is unaffected by pertussis toxin [12], whereas epidermal growth factor stimulation of InsP_3 formation in these cells is abolished by pertussis toxin pretreatment [13]. Few examples exist of a single class of receptors coupled to an effector via two independent G-proteins.

In the heart, agonist-induced phosphoinositide metabolism has been demonstrated only in cultured embryonic chick ventricular cells. Muscarinic stimulation of total inositol phosphates in these ventricular cells appears to be mediated by a pertussis toxin-insensitive G-protein [14,15]. However, muscarinic receptors are not coupled to the same physiological responses in atrium and ventricle. Thus the guanine nucleotide-dependent coupling of muscarinic receptors to an acetylcholine-sensitive K^+ channel can be demonstrated in atrium [16,17] but not in ventricle [18]. Little information is currently available concerning the activation of the inositol phosphate second messenger pathway in atrial cells. In the present study, we demonstrate that, in contrast with the situation in ventricular cells, muscarinic stimulation of cultured embryonic chick atrial cells results in the accumulation of InsP_1, InsP_2 and InsP_3 which is partially sensitive to pertussis toxin. This suggests that at least two G-proteins, one pertussis toxin-sensitive and one pertussis toxin-insensitive, couple muscarinic receptors to the activation of phospholipase C in chick atrial cells.

MATERIALS AND METHODS

myo-[^{3}H]Inositol (12.8 Ci/mmol) was obtained from New England Nuclear, Boston, MA, U.S.A.; oxotremorine, atropine, saponin and carbamylcholine were obtained from Sigma

Abbreviations used: G-protein, guanine-nucleotide-binding protein; GTP[S], guanosine S′-[γ-thio]triphosphate; CMH, chloroform/methanol/HCl (1:2:0.05, by vol.); EC_{50}, concn. causing 50% of maximal stimulation.

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Chemical Co., St. Louis, MO, U.S.A.; Dowex AG1X8 resin was obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A.; and guanosine 5'-[γ-thio]triphosphate (GTP[S]), creatinine phosphate and creatinine kinase were obtained from Boehringer Mannheim, Indianapolis, IN, U.S.A. Chloroform (American Chemical Society-certified), h.p.l.c.-grade methanol and HCl were from Fisher Scientific, Fairlawn, NJ, U.S.A. Hepes was from CalBiochem, San Diego, CA, U.S.A,; Medium M199 was obtained from Gibco, Grand Island, NY, U.S.A.; pertussis toxin was obtained from List Biochemicals, Campbell, CA, U.S.A.; and Hionic-fluor was from Packard, Downers Grove, IL, U.S.A. Embryonated chick eggs were obtained from Spafas, Greenwich, CT, U.S.A.

Culture of embryonic chick atrial cells

Atria of hearts from embryonic chicks at 14 days *in ovo* were cultured by a modification of the method of DeHaan [19], as described previously [20].

Assay of inositol phosphates

Inositol phosphates were determined by a modification of the method of Berridge *et al.* [21]. Cultured embryonic atrial cells from hearts of chicks at 14 days *in ovo* were incubated for 3 days with 2.5 μCi of *myo*-[^{3}H]inositol/ml. On the third day, cells were washed with a buffer containing 25 mM-Hepes, pH 7.4, 20% (w/v) Medium M199, 0.9 mM-NaH_2PO_4, 5 mM-dextrose, 0.8 mM-$MgSO_4$, 1.8 mM-$CaCl_2$, 4.4 mM-KCl, 117 mM-NaCl and 15 mM-LiCl. Cells were equilibrated with buffer for 11 min, followed by incubation for various times in fresh buffer with or without 0.1 mM-carbamylcholine. The buffer was removed, 1 ml of chloroform/methanol/HCl (1:2:0.05, by vol.) (CMH) was added, and the sample was transferred to glass centrifuge tubes and combined with a second 0.5 ml CMH rinse. After addition of 0.4 ml of chloroform and 0.7 ml of water to each centrifuge tube, the mixture was vortex-mixed and spun at 700 *g* for 10 min at 10 °C. The chloroform layer was discarded and the aqueous layer was rinsed with 0.8 ml of chloroform. The sample was then transferred to a polystyrene tube, evaporated for 1 h under N_2 and diluted with 2 ml of water, and the pH was adjusted to 7.0 with 10 M-KOH.

Chromatography of inositol phosphates

Sodium tetraborate solution (10 mM, 5 ml) and 2 ml of water was added to each sample. The samples were then loaded on to a 0.5 ml anion-exchange column (AG1X8 resin, formate form) and InsP_1, InsP_2 and InsP_3 were eluted sequentially into glass scintillation vials with 2 × 4 ml portions of each of 180 mM-ammonium formate/5 mM-sodium tetraborate; 400 mM-ammonium formate/100 mM-formic acid; and 1 M-ammonium formate/100 mM-formic acid. To each sample 17 ml of Hionic-fluor was added, and radioactivity was determined on a Beckman scintillation counter. Stimulation of inositol phosphates in c.p.m. by carbamylcholine or other agonists is expressed as percentage stimulation of inositol phosphates above basal levels. Columns were calibrated using known amounts of *myo*-[^{3}H]inositol, [^{3}H]InsP_1, [^{3}H]InsP_2 and [^{3}H]InsP_3. Recovery was greater than 95% for each isotope, and separation was essentially complete with these conditions.

Assay of inositol phosphates in permeabilized cells

Cultured atrial cells from chicks at 14 days *in ovo* were incubated for 3 days with 2.5 μCi of *myo*-[^{3}H]inositol/ml. On day 3 of culture, the cells were permeabilized by a modification of the method of Jones *et al.* [14] and assayed for inositol phosphate production as described. Specifically, growth medium was removed and the cells were incubated for 11 min in 1 ml of a buffer containing 20 mM-Hepes, 1 mM-KH_2PO_4, 1 mM-EGTA, 4 mM-$MgCl_2$, 110 mM-KCl, 10 mM-NaCl, 3 mM-Na_2ATP, 8 mM-creatinine phosphate and creatinine kinase (6 μg/ml) at pH 7.0. Saponin was added to give a final concentration of 10 μg/ml and the incubation was continued for 5 min. Cells were then washed twice with 750 μl portions of fresh buffer and incubated for 20 min with 750 μl of buffer containing 10 mM-LiCl with or without 0.1 mM-GTP[S]. The incubation buffer was then transferred to glass centrifuge tubes. Cells were rinsed with 1 ml of CMH, which was mixed with the original incubation buffer. Each dish was further rinsed with 0.5 ml of CMH which was added to the tube. The aqueous layer was then transferred to a polystyrene tube and evaporated for 1 h under N_2 and the inositol phosphates were separated as described above.

ADP-ribosylation with pertussis toxin

Embryonic atrial cells from hearts of chicks at 14 days *in ovo* were cultured as described above. On day 2 of culture, pertussis toxin was added to the culture to yield a final concentration of 0.5 ng/ml. After a 15 h incubation, cells were washed with buffer containing 50 mM-Tris/HCl, 1 mM-EDTA, 2.4 mM-EGTA, 0.2 M-sucrose, 1 mM-dithiothreitol, 75 mM-NaCl and leupeptin, soybean and lima bean trypsin inhibitors (each at 0.5 mg/ml) at pH 7.4, scraped from the plate in a small volume of buffer, frozen and thawed twice, and homogenized in a Dounce homogenizer. The ability of pertussis toxin to catalyse the incorporation of [^{32}P]ADP-ribose from [^{32}P]NAD^+ into proteins migrating with molecular masses of 39 and 41 kDa on polyacrylamide gels of cell homogenates was determined as described previously [22]. Protein was determined by the method of Lowry *et al.* [23].

Statistical analysis

Data from dose–response and time course studies were analysed by analysis of variance. Student's *t*-tests were used as indicated in the text or Figure legends.

RESULTS

Time course of the production of inositol phosphates in embryonic atrial cells from chicks at 14 days *in ovo*

To determine whether carbamylcholine is capable of stimulating the production of inositol phosphates in cultured embryonic chick atrial cells, monolayer cultures were assayed for inositol phosphates after various times of exposure to 0.1 mM-carbamylcholine (Fig. 1). InsP_3 production plateaued at 1 min but InsP_2 and InsP_1 continued to increase, with InsP_2 demonstrating a more rapid increase over time. Data from six experiments demonstrated that after a 5 min incubation with carbamylcholine, the increase in each of the three inositol phosphates was statistically significant. Furthermore, when the mean percentage increase in each inositol phosphate was determined, levels of InsP_1 increased by 42 ± 5% (means ± S.E.M., $n = 6$), levels of InsP_2 increased by 108 ± 13% and levels of InsP_3 increased by 47 ± 12% above basal (Fig. 1). These data suggest that carbamylcholine stimulates the production of InsP_3, which is then sequentially metabolized to InsP_2 and InsP_1. Incubation of cells in the presence of 5 μM-atropine plus 0.1 mM-carbamylcholine resulted in complete inhibition of the increases in all forms of inositol phosphate (results not shown).

Concentration dependence of carbamylcholine-stimulated production of inositol phosphates

The concentration-dependence of carbamylcholine-stimulated

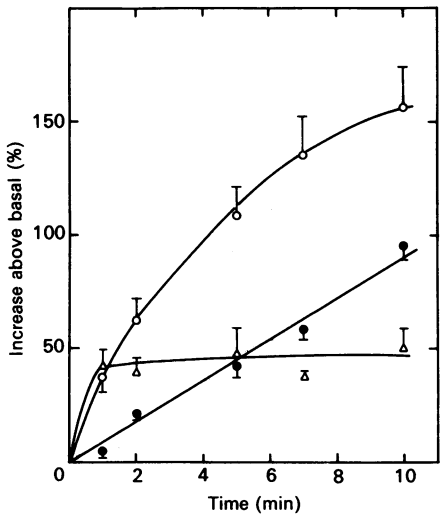

Fig. 1. Time course for carbamylcholine stimulation of inositol phosphate production in cultured embryonic chick atrial cells

Atrial cells from embryonic chicks at 14 days *in ovo* were cultured as described in the Materials and methods section. Cells were incubated with 0.1 mM-carbamylcholine for the times indicated and InsP_1 (●), InsP_2 (○) and InsP_3 (△) were determined as described. Data are presented as percentage increases above basal; means ± S.E.M. for six experiments performed in triplicate. Basal c.p.m. values ($n = 6$) were: InsP_1, 7536 ± 502; InsP_2, 1190 ± 120; InsP_3, 1347 ± 100.

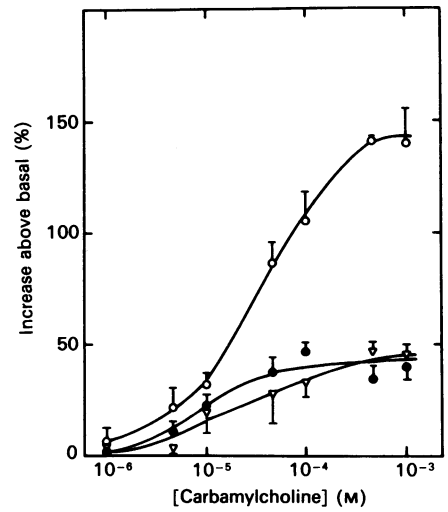

Fig. 2. Concentration–response curves for carbamylcholine stimulation of inositol phosphate production in cultured chick atrial cells

Atrial cells from embryonic chicks 14 days *in ovo* were cultured as described in the Materials and methods section. The cells were washed and incubated for 5 min in the presence of the indicated concentrations of carbamylcholine, and levels of InsP_1 (●), InsP_2 (○) and InsP_3 (△) were determined as described. Data are presented as percentage increase above basal; means ± S.E.M. for three experiments performed in triplicate.

inositol phosphate production was determined after 5 min of stimulation. Maximal stimulation occurred at a concentration of 0.5 mM-carbamylcholine for each of the inositol phosphates measured (Fig. 2). The EC_{50} (concn. causing 50% of maximal stimulation) for production of each of the inositol phosphates was approx. 30 μM. Oxotremorine had no significant effect on inositol phosphate levels at any concentration studied (results not shown). This agonist specificity is consistent with data

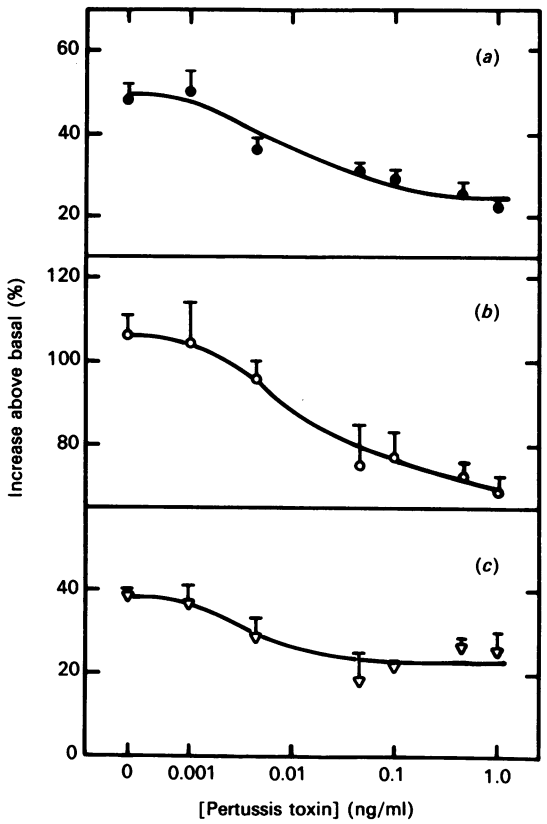

Fig. 3. Effect of pertussis toxin on inositol phosphate production in cultured embryonic chick atrial cells

Atrial cells from embryonic chicks at 14 days *in ovo* were cultured as described in the Materials and methods section. On day 2 of culture, 15 h before challenge with carbamylcholine, the indicated concentrations of pertussis toxin were added to the culture medium. After incubation with pertussis toxin, the cells were washed and exposed to 0.1 mM-carbamylcholine for 5 min, and the levels of inositol phosphates were determined as described. Data are presented as percentage increase above basal; means ± S.E.M. for six experiments performed in triplicate. (*a*) InsP_1; (*b*) InsP_2; (*c*) InsP_3.

presented by Brown & Brown [24] in cultured chick ventricular cells.

Sensitivity of the production of inositol phosphates to pertussis toxin

To determine whether muscarinic stimulation of inositol phosphate production in chick atrial cells is coupled to phospholipase C via a pertussis toxin substrate, cells were incubated for 15 h in the presence of concentrations of pertussis toxin ranging from 0.001 to 1.0 ng/ml, washed and exposed to 0.1 mM-carbamylcholine for 5 min, and stimulation of inositol phosphates was determined as described in the Materials and methods section. The inhibition of muscarinic stimulation of inositol phosphates was maximal at 0.5 ng of pertussis toxin/ml, resulting in a 48 ± 7% (mean ± S.E.M., $n = 6$) decrease in the response of InsP_1 to carbamylcholine, a 30 ± 3% decrease in the response of InsP_2 and a 42 ± 7% decrease in the response of InsP_3 (Fig. 3). This inhibition of inositol phosphate production by pertussis toxin was significant by analysis of variance ($P < 10^{-7}$ for InsP_1, $P < 0.0003$ for InsP_2 and $P < 0.017$ for InsP_3). The EC_{50} for pertussis toxin inhibition of InsP_1, InsP_2 and InsP_3 was identical for all three inositol phosphates, at 0.01 ng/ml. Pretreatment with pertussis toxin had no significant effect on basal levels of any of the inositol phosphates studied.

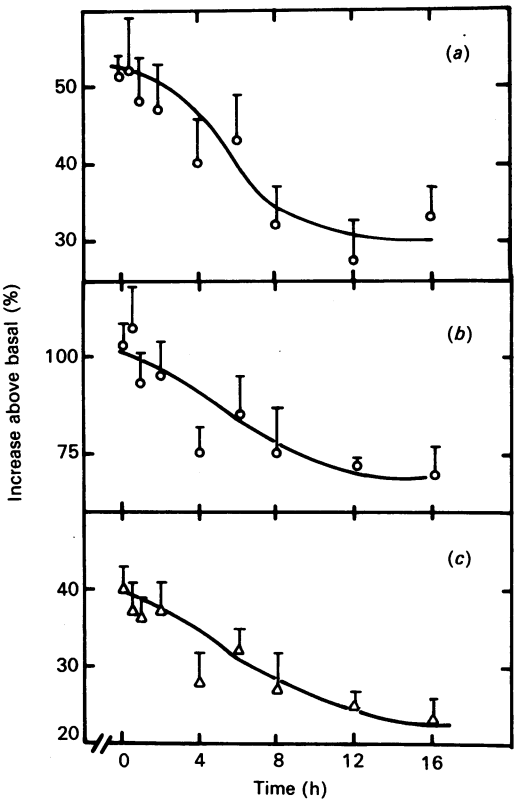

Fig. 4. Time course for pertussis toxin inhibition of inositol phosphate production

These experiments were carried out as in Fig. 3, except that cells were incubated for the times indicated in the presence of 0.5 ng of pertussis toxin/ml. Data are presented as percentage increase above basal; means ± S.E.M. for seven experiments performed in triplicate. (*a*) InsP_1; (*b*) InsP_2; (*c*) InsP_3.

The time course for pertussis toxin inhibition was measured using 0.5 ng of pertussis toxin/ml. At this concentration, pertussis toxin inhibition of inositol phosphate production was maximal at 8 h, with a half-time of 6 h for all three inositol phosphates measured (Fig. 4). These changes in inositol phosphate production in the presence of pertussis toxin were significant by analysis of variance ($P < 0.02$ for InsP_1, $P < 0.021$ for InsP_2 and $P < 0.01$ for InsP_3).

Extent of ADP-ribosylation of substrate by endogenous NAD^+ in intact atrial cells incubated with pertussis toxin

One possible explanation for the partial sensitivity of inositol phosphate production to pertussis toxin was that, at the concentrations of pertussis toxin studied and the times examined, ADP-ribosylation was incomplete. In order to rule out this possibility, embryonic chick atrial cell cultures were incubated for 15 h with 0.5 ng of pertussis toxin/ml and homogenates were assayed for remaining pertussis toxin substrate in the presence of [^{32}P]NAD^+ as described in the Materials and methods section. Under these conditions, cells demonstrated complete loss of both the 39 and 41 kDa bands (Fig. 5). Addition of cholate (0.01–1 %) to homogenates of chick atrial cells pretreated with pertussis toxin had no effect on the level of incorporation of [^{32}P]NAD^+, suggesting that pertussis toxin substrates were not sequestered in these homogenates. Addition of Lubrol (0.01 %) to homogenates of chick atrial cells pretreated with pertussis toxin demonstrated that less than 1 % of α_{39} and α_{41} (39 and 41 kDa α subunits of G-protein) could be ribosylated.

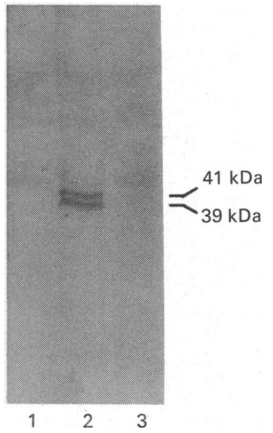

Fig. 5. Effect of pertussis toxin pretreatment on ADP-ribosylation of homogenates of embryonic chick atrial cells

Cultured embryonic chick atrial cells were incubated under control conditions or with 0.5 ng of pertussis toxin/ml for 15 h as described in the legend to Fig. 3. Cells were washed, harvested and homogenized as described in the Materials and methods section and incubated in the presence of pertussis toxin and [^{32}P]NAD^+ for 30 min. The sample was solubilized and electrophoresed on a polyacrylamide gel followed by autoradiography. Each lane contains 60 μg of protein, as determined by the method of Lowry *et al.* [23]. Molecular masses (kDa) were determined by comparison with the migration of standards run with each gel. A representative autoradiogram is depicted. Lane 1, control cells incubated with [^{32}P]NAD^+ in the absence of pertussis toxin; lane 2, control cells incubated with [^{32}P]NAD^+ in the presence of pertussis toxin; lane 3, cells incubated for 15 h with 0.5 ng of pertussis toxin/ml and subsequently incubated with [^{32}P]NAD^+ in the presence of pertussis toxin.

Effects of GTP[S] on the production of inositol phosphates in permeabilized embryonic chick atrial cell cultures

To determine whether a pertussis toxin-insensitive G-protein is coupled to phospholipase C in embryonic chick atrial cells, cells were permeabilized with saponin and stimulated with GTP[S] in the presence and absence of pertussis toxin. Incubation of atrial cells with GTP[S] in the absence of saponin did not cause a significant elevation in the levels of inositol polyphosphates (results not shown). In contrast, a 5 min incubation with 10 μg of saponin/ml resulted in a 102 ± 15 % ($n = 4$) increase in InsP_1, a 421 ± 67 % increase in InsP_2 and a 124 ± 33 % increase in InsP_3 when cells were exposed to 0.1 mM-GTP[S]. At this concentration of saponin, membranes of 90 % of the cells appeared to be intact. Higher concentrations (20–75 μg/ml) caused a disruption of cell morphology. Carbamylcholine had no effect on inositol phosphate production in saponin-permeabilized cells, even in the presence of GTP[S], suggesting that saponin treatment uncoupled the receptor from phospholipase C activation.

A separate set of experiments performed to determine the sensitivity of GTP[S]-stimulated inositol phosphate production to pertussis toxin is summarized in Table 1. A representative experiment carried out in triplicate (column 1) demonstrated that GTP[S] increased in InsP_1 levels by 84 % (calculated as the GTP[S]-stimulated level minus the basal level divided by the basal level) in control cells compared with 51 % in pertussis toxin-treated cells, increased InsP_2 by 450 % in control cells compared with 27 % in pertussis-toxin treated cells, and increased InsP_3 by 80 % in control cells compared with 28 % in pertussis toxin-treated cells. The mean of three experiments similar to those in Table 1 demonstrated that preincubation with pertussis toxin decreased GTP[S]-stimulated InsP_1 production by 30 ± 10 %. The mean of four experiments similar to that in Table

Table 1. Inhibition by pertussis toxin of GTP[S]-stimulated inositol phosphate production in permeabilized atrial cells from chicks at 14 days *in ovo*

Permeabilized cells were exposed to 10 μg of saponin/ml for 5 min as described in the Materials and methods section, and cells were rinsed and incubated for 20 min with fresh buffer containing 10 mM-LiCl with or without 0.1 mM-GTP[S]. Cells were exposed or not to 0.5 ng of pertussis toxin/ml for 15 h as described in the Materials and methods section. Data are expressed as means ± S.E.M. for samples assayed in triplicate and are typical of four such experiments.

		[^{3}H]Inositol phosphate (c.p.m.)	
Inositol phosphate	GTP[S]	Control	Pertussis toxin exposure
InsP_1	−	6790 ± 434	7143 ± 346
	+	12508 ± 55	10806 ± 774
InsP_2	−	776 ± 40	762 ± 50
	+	4320 ± 613	2795 ± 162
InsP_3	−	839 ± 37	895 ± 39
	+	1504 ± 97	1143 ± 28

1 demonstrated that pretreatment with pertussis toxin decreased GTP[S]-stimulated InsP_2 production by 45 ± 7% and InsP_3 production by 49 ± 6%. The magnitude of this partial inhibition of GTP[S]-stimulated inositol phosphate production is similar to that observed for pertussis toxin inhibition of carbamylcholine-stimulated inositol phosphate production. Pertussis toxin had no effect on basal levels of InsP_1, InsP_2 and InsP_3 (Table 1). The finding that GTP[S] stimulation of all three inositol phosphates decreases following incubation with pertussis toxin in the presence of Li^+ makes it unlikely that a decrease in InsP_3 stimulation in pertussis toxin-treated cells is due to increased inositol phosphatase activity. These data demonstrate that a pertussis toxin-insensitive GTP[S]-stimulated phospholipase C activity is present in cultured chick atrial cells, and support the conclusion that the pertussis toxin-insensitive fraction of carbamylcholine-stimulated inositol phosphate formation is coupled via a G-protein.

DISCUSSION

Data presented here demonstrate that muscarinic cholinergic stimulation of cultured chick atrial cells causes a significant increase in the levels of inositol phosphates. The time course of InsP_3 production is rapid, reaching a plateau by 1 min, and the relative time course of appearance of each of the three inositol phosphates is consistent with the existence of a precursor–product relationship between InsP_3, InsP_2 and InsP_1.

The carbamylcholine-stimulated production of inositol phosphates was concentration-dependent, with an EC_{50} value of 30 μM. This value is similar to the value of 20–30 μM for total inositol phosphate production reported by Brown & Brown [24] in suspensions of chick ventricular cells. The finding that oxotremorine had no significant effect on the levels of InsP_1, InsP_2 and InsP_3 at any concentration studied is also consistent with data presented by Brown & Brown [24], which demonstrated that oxotremorine had little effect on total inositol phosphate production in ventricular cells.

In contrast with the findings of Masters *et al.* [15] that pertussis toxin had no effect on total carbamylcholine-induced inositol phosphate formation in 24 h cultures of embryonic chick ventricular cells, in cultures of chick atrial cells carbamylcholine stimulation of inositol phosphate production was inhibited by 40–50% by pertussis toxin. One interpretation of this incomplete inhibition of inositol phosphate production is that it may represent an incomplete ADP-ribosylation of pertussis toxin substrate. However, pertussis toxin-catalysed ADP-ribosylation of cells incubated with 0.5 ng of pertussis toxin/ml for 15 h demonstrated that pertussis toxin substrates were completely ADP-ribosylated by endogenous NAD^+. Furthermore, incubation of chick heart cells with similar concentrations of pertussis toxin has been shown to completely abolish other muscarinic-receptor stimulated functions in the embryonic chick heart. Thus incubation of cultured chick heart cells completely inhibited muscarinic stimulation of acetylcholine-sensitive K^+ channels [16], and in homogenates of embryonic chick hearts it completely inhibited muscarinic receptor-mediated inhibition of isoprenaline-stimulated adenylate cyclase activity [25]. These observations taken together suggest that muscarinic receptors are coupled to phospholipase C by two pathways, one which is pertussis toxin-sensitive and another which is pertussis toxin-insensitive.

In a number of systems, pertussis toxin has been shown to uncouple G-proteins from interaction with the receptor without an effect on GTP or fluoride activation of the effector. Smith *et al.* [26] demonstrated that pertussis toxin uncoupled fMetLeuPhe binding from PtdIns(4,5)P_2 hydrolysis, but had no effect on GTP[S]-stimulated PtdIns(4,5)P_2 hydrolysis. Only a few reports have suggested that pertussis toxin uncouples the G-protein from the effector. Ichiro & Hsiu-Hsiung [27] demonstrated that pertussis toxin inhibited fluoride-stimulated thromboxane release, and both thrombin and fluoride inhibition of adenylate cyclase activity were inhibited by treatment of Chinese hamster cells with pertussis toxin. In the studies reported here, GTP[S] stimulation of inositol phosphate production was partially sensitive to pertussis toxin, suggesting that pertussis toxin not only uncoupled the muscarinic receptor from phospholipase C activity, but also uncoupled the G-protein from phospholipase C activity.

The data presented here suggest that in atrial cells, muscarinic receptors may be coupled to phospholipase C activation via at least two G-proteins: a pertussis toxin-sensitive G-protein, either G_o or one of the members of the family of G_i proteins [28], and a pertussis toxin-insensitive G-protein, i.e. G_p. The mechanism by which multiple G-proteins might couple muscarinic receptors to phospholipase C activity is complicated by the observation that muscarinic receptors exist in at least five isoforms [29–31] and that phospholipase C exists in as many as five isoforms [32,33]. Transfection studies have suggested the M_1 and M_4 forms of the muscarinic receptor give a large stimulation of phospholipase C activity and actually stimulate adenylate cyclase activity [29]. The M_2 and M_3 forms of the muscarinic receptor demonstrate significantly lower efficiency of coupling to phospholipase C activity and an inhibition of adenylate cyclase activity [29]. Phospholipase C isoforms may also be coupled to different physiological responses in the cell [33,34]. Thus it is possible that several subtypes of the muscarinic receptor and phospholipase C may be involved in mediating a muscarinic response in these cells; one subtype might be coupled via a pertussis toxin-sensitive G-protein and another via a pertussis toxin-insensitive G-protein. However, it appears from the data presented here that at least two G-proteins are involved in coupling these receptors to inositol phosphate production.

Coupling of muscarinic receptors to phospholipase C activity via a pertussis toxin-insensitive pathway has been well established. Helper & Harden [35] demonstrated that, although muscarinic stimulation of InsP_3 production in membranes of human astrocytoma cells was insensitive to pertussis toxin, guanine nucleotides stimulated InsP_3 release in these membranes.

Several studies have demonstrated that, in a given cell type, a specific class of receptors may be coupled to phospholipase C via a pertussis toxin-insensitive pathway and another class of receptors may be coupled to phospholipase C via a pertussis toxin-sensitive pathway [12,13]. In the platelet, fluoride stimulates $InsP_3$ production, arachidonic acid release and thromboxane formation, but only fluoride-stimulated thromboxane formation was sensitive to pertussis toxin, suggesting that within the same cell different enzymic processes may be coupled via independent G-proteins [36]. Although many different receptors have been shown to exist in multiple isoforms, the finding that a single receptor isoform or two separate isoforms might be coupled to inositol phosphate production via both pertussis toxin-sensitive and pertussis toxin-insensitive G-proteins is relatively unique. A partial pertussis toxin sensitivity of α-adrenergic-stimulated total inositol phosphate production in cultured rat myocytes [37] and of ATP-stimulated $InsP_3$ production in rat hepatocytes [38] has been demonstrated. However it was unclear whether the pertussis toxin-insensitive $InsP_3$ production in these cells was coupled to the receptor via G_p. More recently, Dubyak *et al.* [39] demonstrated that purinergic receptors stimulated the breakdown of inositol phospholipids via a partially pertussis toxin-sensitive pathway in cells in which fluoride mediated an increase in cytosolic Ca^{2+} release from ATP-sensitive Ca^{2+} pools. Whether fluoride-sensitive phospholipase C activity was pertussis toxin-sensitive in those studies was not clear. Transfection studies with muscarinic receptor isoforms have suggested that different subtypes of muscarinic receptors may couple to effectors via separate G-proteins [40].

Data presented here support the conclusion that two separate G-proteins are involved in coupling muscarinic agonist binding to stimulation of phospholipase C activity. The transduction of information from a given receptor to an effector via two separate G-proteins in the same cell suggests that each pathway may regulate an independent physiological function. The possibility of multiple interactions among the different receptor subtypes and different G-protein and phospholipase C isoforms suggests that the regulation of the relative levels of a given G-protein, receptor or phospholipase C isoform might control the extent of a given cellular response. The developing embryonic chick heart may represent a system in which changes in the relative levels of a given G-protein, receptor or effector might be related to important alterations in physiological responses.

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