Pertussis toxin does not inhibit muscarinic-receptor-mediated phosphoinositide hydrolysis or calcium mobilization

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Pertussis toxin was used to examine the role of the inhibitory guanine nucleotide regulatory protein, N_i , in muscarinic-receptor-mediated stimulation of phosphoinositide turnover and calcium mobilization. In cultured chick heart cells, pertussis-toxin treatment inhibited muscarinic-receptor-mediated attenuation of isoprenaline-stimulated cyclic AMP accumulation. This finding is consistent with the proposal that pertussis toxin blocks the capacity of N_i to couple muscarinic receptors to adenylate cyclase. In contrast, treatment of chick heart cells or 1321N1 human astrocytoma cells with pertussis toxin did not block muscarinic-receptor-mediated stimulation of phosphoinositide hydrolysis, as measured by [3 H]inositol phosphate accumulation in the presence of Li^+ . Pertussis-toxin treatment also had little effect on basal and muscarinic-receptor-stimulated phosphatidylinositol synthesis, as measured by the incorporation of [3 H]inositol into phosphatidylinositol. Activation of muscarinic receptors also enhances the rate of unidirectional $^{45}Ca^{2+}$ efflux in 1321N1 cells; this response, like phosphoinositide hydrolysis, was not prevented by pertussis-toxin treatment. Our data suggest that muscarinic receptors are not coupled to phosphoinositide hydrolysis or calcium mobilization through N_i .

Insight into the molecular basis of hormonal regulation of adenylate cyclase was advanced by the discovery of a pair of guanine nucleotide regulatory proteins that couple agonist-occupied receptors to the catalytic component of adenylate cyclase. Stimulatory hormone receptors catalyse the exchange of GTP for GDP on the regulatory protein, N_s , which in turn interacts with the catalytic protein to form the active enzymic species (Ross & Gilman, 1980; Rodbell, 1980; Gilman, 1984). Receptor-mediated inhibition of adenylate cyclase involves the coupling function of another protein, N_i , which shares a number of structural and functional similarities with N_s . A toxin

purified from *Bordetella pertussis* (pertussis toxin) catalyses the ADP-ribosylation of the 41 000- M_r α -subunit of N_i (Kurose *et al.*, 1983; Gilman, 1984; Katada *et al.*, 1984). This action correlates with the capacity of pertussis toxin to block agonist-mediated inhibition of adenylate cyclase (Katada & Ui, 1980; Hazeki & Ui, 1981; Kurose *et al.*, 1983; Gilman, 1984; Katada *et al.*, 1984).

Several hormones that inhibit adenylate cyclase also increase the turnover of membrane phosphoinositides and cause calcium mobilization (Michell, 1975; Berridge, 1981; Brown & Brown, 1984). In contrast with the adenylate cyclase system, little is known about the molecular mechanisms by which receptor occupation leads to these other responses. An important question is whether a guanine nucleotide regulatory protein is involved in hormonal regulation of phosphoinositide turnover. There is indirect evidence in support of this possibility: guanine nucleotides regulate agonist affinity for receptors that are apparently coupled to phosphoinositide hydrolysis and calcium mobilization (Snively & Insel, 1982; Koo *et*

Abbreviations used: InsP, inositol phosphate; PtdIns, phosphatidylinositol; N_i , the guanine nucleotide regulatory protein that couples inhibitory receptors to adenylate cyclase; N_s , the guanine nucleotide regulatory protein that couples stimulatory receptors to adenylate cyclase.

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al., 1983; Boyer *et al.*, 1984; Evans *et al.*, 1985), and guanine nucleotides affect calcium mobilization and diacylglycerol formation in permeabilized cells (Gomperts, 1983; Haslam & Davidson, 1984). Additionally, pertussis toxin has been proposed to act as a calcium ionophore (Katada & Ui, 1979) and more recently shown to block calcium mobilization and related responses induced by chemotactic factors in neutrophils (Okajima & Ui, 1984; Molski *et al.*, 1984; Bokoch & Gilman, 1984). N_i has also been implicated in hormonal regulation of phosphoinositide turnover by Moreno *et al.* (1983), who reported that treatment of rats with pertussis toxin inhibits α_1 -adrenergic-receptor-stimulated phosphoinositide synthesis in adipocytes, as measured by [32 P]P $_i$ incorporation into phosphatidic acid and PtdIns.

In the studies described here pertussis toxin was utilized to evaluate further the role of N_i in muscarinic-receptor-mediated phosphoinositide hydrolysis. Our findings demonstrate that pertussis toxin does not block muscarinic-receptor-mediated stimulation of phosphoinositide hydrolysis, phosphatidylinositol synthesis or calcium mobilization. It is concluded that muscarinic receptors do not couple to phosphoinositide hydrolysis and calcium mobilization through N_i .

Experimental

Heart cells were prepared from 13-day chick embryos as described previously (Brown & Brown, 1984). Cells were suspended in Dulbecco's Eagle medium containing 5% (v/v) foetal calf serum and 1% (v/v) Fungi-Bact (Irvine Scientific) and cultured for 24 h in 35 mm culture dishes in an atmosphere of air/CO $_2$ (9:1). Astrocytoma cells were grown as described previously (Meeker & Harden, 1982; Masters *et al.*, 1984). Pertussis toxin was purified as described by Hughes *et al.* (1984) and added to the culture medium 24 h before assay of cyclic AMP formation, phosphoinositide hydrolysis, PtdIns synthesis or unidirectional 45 Ca $^{2+}$ efflux. All assays were carried out at 37°C in buffer composed of 118 mM-NaCl, 4.7 mM-KCl, 1.8 mM-CaCl $_2$, 1.2 mM-MgSO $_4$, 1.2 mM-KH $_2$ PO $_4$, 10 mM-glucose and 20 mM-Hepes, pH 7.4. The buffer used in experiments with astrocytoma cells was the same except that it contained 3.0 mM-CaCl $_2$ and 0.5 mM-Na $_2$ EDTA.

For cyclic AMP assays, cultured heart cells were treated with carbachol and isoprenaline for 2 min, the incubation was terminated by the addition of 10% (w/v) trichloroacetic acid, and cyclic AMP was purified and assayed by the competitive protein-binding assay as described previously (Brown, 1979).

Cellular phosphoinositides were labelled by

incubating cells in growth medium with [3 H]inositol (1 μ Ci/ml) for 24 h. Phosphoinositide hydrolysis was monitored by measuring the accumulation of [3 H]InsP in the presence of 10 mM-Li $^+$, an inhibitor of the phosphatase that converts InsP into inositol (Berridge *et al.*, 1982). Phospholipids were extracted and the [3 H]InsP in the aqueous phase was separated from [3 H]inositol by anion-exchange chromatography by a modification (Masters *et al.*, 1984) of the method described by Berridge *et al.* (1982).

In experiments in which PtdIns synthesis was studied, cells were washed free of growth medium and labelled with 4 μ Ci of [3 H]inositol/ml for 60 min. Vehicle or carbachol was then added for various times, and the 3 H incorporated into PtdIns was analysed as described previously (Masters *et al.*, 1984).

For unidirectional 45 Ca $^{2+}$ -efflux assays, cell monolayers were labelled to isotopic equilibrium by incubation with growth medium containing 5 μ Ci of 45 CaCl $_2$ /ml for 18–20 h. The efflux assay was initiated and terminated as described by Masters *et al.* (1984). The rate constant was calculated from the zero-time cellular 45 Ca $^{2+}$ and the cellular 45 Ca $^{2+}$ present 0.5 min after the addition of carbachol or vehicle. At this time the unidirectional 45 Ca $^{2+}$ efflux rate approximates to a first-order exponential with a single kinetic component (Masters *et al.*, 1984).

Results

Cyclic AMP formation in chick heart cells is stimulated by the β -adrenergic-receptor agonist isoprenaline. Isoprenaline-stimulated cyclic AMP formation is inhibited by approx. 70% in the presence of the cholinergic agonist carbachol (Brown & Brown, 1984; see also Fig. 1). The capacity of carbachol to inhibit cyclic AMP formation was lost after pertussis-toxin treatment (Fig. 1). This effect of pertussis toxin was concentration-dependent and was maximal at 100 ng/ml. Pertussis toxin also caused a small increase in isoprenaline-stimulated cyclic AMP formation (Fig. 1).

In chick heart cells and 1321N1 astrocytoma cells, muscarinic agonists stimulate phosphoinositide hydrolysis, which results in the formation of inositol tris-, bis- and mono-phosphates (Masters *et al.*, 1985). When cells are treated with Li $^+$, the inositol phosphates formed by phosphoinositide hydrolysis accumulate as InsP. In chick heart cells, pertussis-toxin treatment did not decrease either basal or carbachol-stimulated InsP formation under conditions in which the cyclic AMP response was fully inhibited (Fig. 2). In 1321N1 cells 100 ng of pertussis toxin/ml fully

Table 1. *Pertussis toxin does not inhibit carbachol-stimulated InsP formation or $^{45}\text{Ca}^{2+}$ efflux in 1321N1 astrocytoma cells*

The accumulation of [^3H]InsP was measured 10 min after the addition of LiCl (basal) or LiCl and 100 μM -carbachol (carbachol) to normal cells or to cells treated with pertussis toxin. The basal rate of unidirectional $^{45}\text{Ca}^{2+}$ efflux and that stimulated by 100 μM -carbachol were determined as described in the Experimental section. The zero-time $^{45}\text{Ca}^{2+}$ was 4518 ± 104 c.p.m. in normal cultures, 3853 ± 58 c.p.m. in cultures treated with 10 ng of pertussis toxin/ml, and 3685 ± 102 c.p.m. in cultures treated with 100 ng of pertussis toxin/ml. The zero-time $^{45}\text{Ca}^{2+}$ in pertussis-toxin-treated cultures was significantly different from that in normal cultures ($P < 0.05$) when effects were tested by one-way analysis of variance and the Dunnett test. All values given are means $\pm$ S.E.M. for four determinations.

	[^3H]InsP (c.p.m.)			$^{45}\text{Ca}^{2+}$ efflux rate (min^{-1})		
	Basal	+ Carbachol	Increase	Basal	+ Carbachol	Increase
Control	392 ± 2	1243 ± 29	842	0.19 ± 0.06	1.22 ± 0.05	1.03
Pertussis toxin (10 ng/ml)	362 ± 10	1181 ± 40	819	0.19 ± 0.07	1.06 ± 0.04	0.87
Pertussis toxin (100 ng/ml)	354 ± 10	1185 ± 13	831	0.20 ± 0.07	1.13 ± 0.07	0.93

Fig. 1. *Pertussis-toxin treatment blocks muscarinic inhibition of isoprenaline-stimulated cyclic AMP accumulation*

Cultures of chick heart cells were treated for 24 h with various concentrations of pertussis toxin. Isobutylmethylxanthine (100 μM) was added to the cells 20 min before the addition of 3 μM -isoprenaline (○) or 3 μM -isoprenaline plus 10 μM -carbachol (●). The basal cyclic AMP concentration (not shown) was 5.3 pmol/mg of protein. Values are means $\pm$ S.E.M. for four determinations.

Fig. 2. *Pertussis-toxin treatment does not affect stimulation of InsP formation by carbachol in chick heart cells*

Cultures of chick heart cells were treated for 24 h with various concentrations of pertussis toxin. The accumulation of [^3H]InsP was measured 30 min after the addition of LiCl (basal, ○) or LiCl and 100 μM -carbachol (●). Values are means $\pm$ S.E.M. for four determinations.

ADP-ribosylates the α -subunit of N_i and functionally inactivates N_i (Evans *et al.*, 1985; Hughes *et al.*, 1984). This concentration of toxin had no effect on either basal or carbachol-stimulated [^3H]InsP accumulation in 1321N1 cells (Table 1). Thus pertussis toxin does not block the muscarinic-receptor-mediated increase in phosphoinositide hydrolysis in either cell type.

To determine if PtdIns synthesis was inhibited by pertussis toxin, the incorporation of [^3H]inositol into PtdIns was examined. Carbachol stimulated [^3H]inositol incorporation into PtdIns in control and pertussis-toxin-treated cells (Fig. 3). A very small but significant ($P < 0.05$) decrease in basal and carbachol-stimulated [^3H]inositol incorporation was evident in pertussis-toxin-treated cells, associated with a small (approx. 20%) but signifi-

cant decrease in cellular free [^3H]inositol (c.p.m.).

We have previously shown that carbachol stimulates unidirectional $^{45}\text{Ca}^{2+}$ efflux from 1321N1 cells (Masters *et al.*, 1984, 1985). Neither the control nor the carbachol-stimulated rate of $^{45}\text{Ca}^{2+}$ efflux from astrocytoma cells was affected by pertussis-toxin treatment (Table 1). Both concentrations of pertussis toxin tested caused a slight decrease in the zero-time cellular $^{45}\text{Ca}^{2+}$ (see the legend to Table 1).

Discussion

Pertussis toxin ADP-ribosylates N_i and blocks the inhibition of adenylate cyclase mediated through receptors that couple to this protein (Katada & Ui, 1980; Hazeki & Ui, 1981; Kurose *et al.*, 1983; Gilman, 1984; Katada *et al.*, 1984). Fain and co-workers have reported that pertussis toxin

Fig. 3. Pertussis-toxin effects on $[^3\text{H}]$ inositol incorporation into PtdIns

The radioactivity in PtdIns in control (○, △) or carbachol (100 μM)-treated cells (●, ▲) cultured in the absence (○, ●) or presence (△, ▲) of 100 ng of pertussis toxin/ml is shown. The effect of pertussis toxin on $[^3\text{H}]$ PtdIns was compared with controls by two-way analysis of variance.

blocks hormonally stimulated PtdIns synthesis in adipocytes from rats treated with this toxin (Moreno *et al.*, 1983). This led to the speculation that N_i might also couple receptors to the effector(s) involved in phosphoinositide metabolism.

The initiating event in hormone-stimulated PtdIns turnover is the hydrolysis of phosphoinositides, and this in turn is followed by an increase in the synthesis of phosphatidic acid and PtdIns (Berridge, 1981; Michell & Kirk, 1981). The studies described here demonstrate that pertussis-toxin treatment does not block the hydrolysis of phosphoinositides stimulated through muscarinic receptors in either chick heart or 1321N1 cells. Furthermore, pertussis-toxin treatment has little effect on carbachol-stimulated phosphoinositide synthesis, assessed by measuring the incorporation of $[^3\text{H}]$ inositol into PtdIns. This latter observation suggests that the inhibitory effect of pertussis toxin on PtdIns synthesis *in vivo* (Moreno *et al.*, 1983) does not result from a direct effect of the toxin on phosphoinositide synthesis.

The failure of pertussis toxin to block muscarinic-receptor-mediated stimulation of phosphoinositide hydrolysis in chick heart cells is instructive because the same concentrations of pertussis toxin antagonize muscarinic inhibition of cyclic AMP formation. This latter finding corroborates that of Hazeki & Ui (1981) on rat heart cells and indicates that pertussis toxin also affects N_i in intact chick heart cells. These data also indicate that the concentration range of pertussis toxin is appropriate for uncoupling the muscarinic receptor from N_i . Preliminary experiments measuring $[^{32}\text{P}]\text{ADP-ribosylation}$ of heart cell membranes

indicate that a pertussis-toxin substrate of the same size as the α -subunit of N_i is present in the chick heart cells and is fully ribosylated by treatment with 100 ng of pertussis toxin/ml.

1321N1 cells contain a 41 000- M_r pertussis-toxin substrate which has been shown to be ribosylated at the concentrations of toxin used here (Hughes *et al.*, 1984). This protein appears to represent functional N_i because guanine nucleotides inhibit forskolin-activated adenylate cyclase in membranes from these cells, and pertussis-toxin treatment disrupts this function (Hughes *et al.*, 1984). Moreover, an adenosine receptor that couples through N_i to inhibit adenylate cyclase has recently been shown to be present in these cells (A. R. Hughes & T. K. Harden, unpublished work). Although N_i can apparently associate with the adenylate cyclase in 1321N1 cells, it does not couple the muscarinic receptor to adenylate cyclase inhibition (Meeker & Harden, 1982). Several studies have used pertussis toxin to demonstrate that N_i mediates hormone-induced calcium mobilization in neutrophils (Molski *et al.*, 1984; Okajima & Ui, 1984; Bokoch *et al.*, 1984). In contrast, our finding that pertussis toxin does not affect muscarinic-receptor-stimulated $[^3\text{H}]\text{InsP}$ formation or unidirectional $^{45}\text{Ca}^{2+}$ efflux indicates that the N_i present in 1321N1 cells does not couple the muscarinic receptor to either phosphoinositide hydrolysis or calcium mobilization.

Although it does not appear that N_i couples muscarinic receptors to the effectors for phosphoinositide turnover and calcium mobilization, these responses may be mediated through a guanine nucleotide regulatory protein whose function is not inhibited by pertussis toxin. Muscarinic receptors in membranes from 1321N1 cells express GTP-sensitive high-affinity binding of carbachol, which is not affected by pertussis toxin (Evans *et al.*, 1985). In addition, the extent of guanine nucleotide-sensitive high-affinity binding of cholinergic agonists corresponds with the relative efficacy of these agonists for stimulation of phosphoinositide hydrolysis and Ca^{2+} mobilization (Hepler *et al.*, 1984). Thus there is evidence for a muscarinic-receptor-guanine nucleotide regulatory protein interaction in these cells, but, on the basis of the effect of pertussis toxin on binding and on the functional responses to cholinergic agonists examined here, this protein does not appear to be N_i . The identity of this putative protein and its possible role in regulation of phosphoinositide and calcium metabolism are subjects of considerable interest.

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