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Pertussis toxin distinguishes between muscarinic receptor-mediated inhibition of adenylate cyclase and stimulation of phosphoinositide hydrolysis in Flow 9000 cells

William W.Y. Lo and John Hughes

Parke-Davis Research Unit, Addenbrookes Hospital Site, Hills Road, Cambridge CB2 2QB, England

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Pretreatment of human embryonic pituitary tumour cells (Flow 9000) with pertussis toxin significantly reduced carbachol-mediated inhibition of isoprenaline and prostaglandin E_2 stimulation of cyclic AMP formation. This is in accord with an action on the inhibitory G_i-protein by pertussis toxin. In contrast, pertussis toxin-pretreatment had no effect on either muscarinic agonist or GTP[S] (a non-hydrolysable GTP analogue) stimulation of [^{3}H]inositol phosphate production in intact and permeabilized [^{3}H]inositol-prelabelled Flow 9000 cells, respectively. These results suggest that muscarinic receptors are linked to the inhibition of adenylate cyclase and the stimulation of phosphoinositidase C via two different G-proteins in Flow 9000 cells.

G-protein; Muscarinic receptor; Pertussis toxin; Inositol phosphate; cyclic AMP; (Flow 9000 cell)

1. INTRODUCTION

Activation of muscarinic cholinergic receptors leads to several distinct intracellular biochemical changes. These include changes in intracellular cyclic AMP (cAMP) and cyclic GMP (cGMP) content, calcium mobilization, and increased breakdown of inositol phospholipid [1]. Recent evidence has suggested that changes in intracellular calcium and cGMP levels may be secondary to increased hydrolysis of polyphosphoinositides (PI). Indeed, inositol 1,4,5-trisphosphate (1,4,5-IP_3) has been shown to release intracellularly sequestered calcium from a non-mitochondrial store, presumably endoplasmic reticulum [2]. Moreover, alteration of guanylate cyclase-mediated cGMP production may be related to increased intracellular calcium levels [3].

It is now generally accepted that a family of guanine nucleotide-binding proteins (G-proteins) is involved in the coupling of a variety of receptors to their second messenger-generating systems including adenylate cyclase and the PI-hydrolysing enzyme, phosphoinositidase C (PIC) [4]. While it is generally accepted that there are two G-proteins (G_s and G_i) responsible for the coupling of stimulatory and inhibitory receptors to the activation and inhibition of adenylate cyclase, the identity of the G-protein involved in receptor-PIC coupling is still uncertain. Recent evidence has implicated that there are at least two different G-proteins participated in receptor-linked PI metabolism [5]. These G-proteins are classified by

Correspondence address: W.W.Y. Lo, Parke-Davis Research Unit, Addenbrookes Hospital Site, Hills Road, Cambridge CB2 2QB, England

Abbreviations: G-proteins, guanine nucleotide-binding proteins; G_i, inhibitory G-protein; [^{3}H]IP, total [^{3}H]inositol phosphates; PI, polyphosphoinositides; GTP[S], guanosine 5′-*O*-(thiotriphosphate)

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their different susceptibilities to pertussis toxin. The first one is a substrate of neither pertussis toxin nor cholera toxin and is present in a great variety of tissues [6]. The p21 G-protein encoded by *ras* oncogenes is a candidate of this category [7]. On the other hand, in other cell types such as mast cells, neutrophils and neuroblastoma cells, the G-protein responsible for coupling receptors to PIC is pertussis toxin-sensitive and therefore can be either G_i, G_o (G-protein first identified in bovine brain but has no known function [8]) or a G_i/G_o-like protein [9,10]. We have been able to show that muscarinic agonists interact with both adenylate cyclase and PI metabolism in Flow 9000 cells and in the present study we have attempted to identify the G-protein(s) that couple these two signalling pathways.

2. MATERIALS AND METHODS

Flow 9000 cells (passsage 17–26) were purchased from Flow Laboratories (Herts). Cells were cultured in Ham's F10 medium supplemented with sera and antibiotics [11]. Agonist-induced total [^{3}H]inositol phosphate ([^{3}H]IP) accumulation in the presence of 10 mM LiCl was used as an index of PI turnover. Methods for [^{3}H]inositol prelabelling and agonist stimulation of the cells as well as extraction and quantification of [^{3}H]IP were as described in [11]. Pertussis toxin was routinely added only after the cells had been labelled with [^{3}H]inositol to isotopic equilibrium (48 h) for 24 h. For cAMP determination, cells were incubated with different drugs in Krebs-Ringer bicarbonate buffer (supplemented with glucose) for 15–30 min. Reaction was terminated by aspirating the incubation medium followed by adding 1 ml ice-cold 6% trichloroacetic acid. Cells were then scraped from cultured wells and were then extracted with 2 vols water-saturated ether solution (three times) to remove the acid. cAMP concentrations were determined by radioimmunoassay using a ^{125}I-cAMP-RIA kit obtained from New England Nuclear. Carbachol, acetylcholine hydrochloride, prostaglandin E_2 (PGE_2) and isoprenaline were obtained from Sigma. GTP[S] was purchased from Calbiochem. Pertussis toxin was obtained from Lists Biologicals (Campbell, CA). Cell culture medium, sera and antibiotics were purchased from either Gibco or Flow Laboratories. All other chemicals were from Fisons.

All experiments, unless otherwise stated, have been performed at least three times. Results presented are means ± SE of three or more experiments. Statistical analyses were conducted by Student's *t*-test.

3. RESULTS AND DISCUSSION

Initial experiments established that, even in the absence of any phosphodiesterase inhibitor, isoprenaline or PGE_2 both significantly increased cAMP formation in intact Flow 9000 cells (fig.1). Propranolol (10 nM), but not phentolamine (10 nM), completely blocked the stimulatory action of isoprenaline on cAMP formation indicating that the response of isoprenaline was mediated via β-adrenergic receptors (not shown).

Carbachol (0.1–100 μM) had no effect on basal cAMP level (fig.1). However, it was able to inhibit the increase in cAMP levels elicited by either isoprenaline or PGE_2 (fig.2). Carbachol (1 mM) caused 65 and 75% inhibition of the isoprenaline- and PGE_2-mediated responses, respectively. The

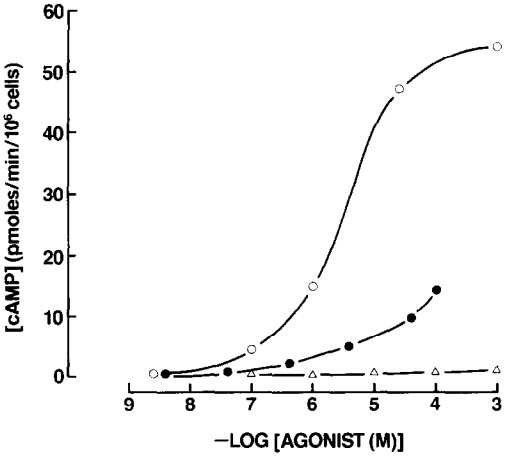

Fig.1. Dose-dependent effects of (○) isoprenaline, (●) PGE_2 and (△) carbachol on cAMP production in Flow 9000 cells. Flow 9000 cells were incubated with different agonists of various concentrations for 15 min. The reaction was terminated and cAMP extracted and quantified as described in the text. Results presented are means of 4 independent experiments in triplicate. SE is within 10% in each data point.

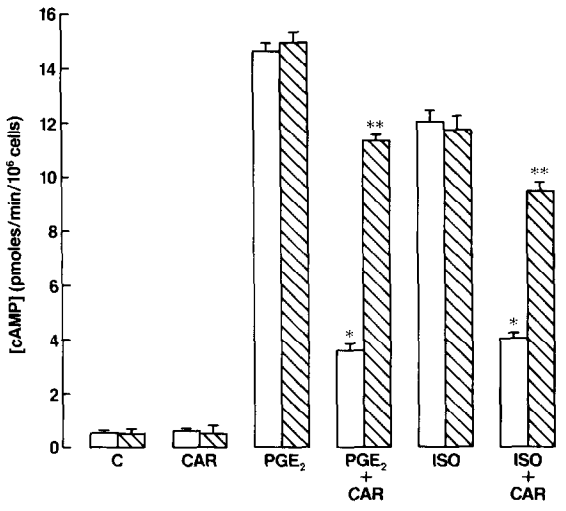

Fig.2. Effects of pertussis toxin pretreatment on carbachol inhibition of agonist-induced cAMP formation. Control (open bar) and pertussis toxin-pretreated (hatched bar) cells were incubated with carbachol (CAR, 1 mM) and/or isoprenaline (ISO, 1 μM) or PGE_2 (40 μM) for 30 min. Data represent means ± SE of 3 separate experiments in duplicate. * $P < 0.01$ vs agonist stimulation in control cells. ** $P < 0.02$ vs carbachol plus isoprenaline or PGE_2 in control cells.

inhibitory action of carbachol was completely blocked by atropine (1 μM).

Pretreatment of Flow 9000 cells with pertussis toxin (50 μg/ml, 24 h) affected neither basal nor agonist-stimulated cAMP levels. However, pertussis toxin treatment significantly reduced the carbachol inhibition of both isoprenaline and PGE_2 stimulation of cAMP formation (fig.2). This is in accord with an action on the inhibitory G_i-protein by pertussis toxin. Pertussis toxin is known to ADP-ribosylate the α-subunit of G_i-protein and by doing so prevents the activation of G_i by GTP [12]. Concomitantly, the coupling of hormone receptors to G_i is also interrupted [13].

Recently, we have shown that in [^{3}H]inositol-prelabelled Flow 9000 cells, muscarinic agonists stimulate a rapid and transient increase in the formation of inositol phosphates [14]. When these cells were permeabilized by saponin treatment, non-hydrolysable GTP analogues (GTP[S] and GppNHp) were able to activate inositol phosphate production in a time- and dose-dependent manner [15]. Moreover, these GTP analogues significantly potentiated the responses of cholecystokinin octapeptide and muscarinic agonists on PI hydrolysis [15]. These data indicate that receptor-mediated PI turnover is mediated via a G-protein in Flow 9000 cells. In the present study, pretreatment of both intact and saponin-permeabilized [^{3}H]inositol-labelled Flow 9000 cells with pertussis toxin (under conditions enough to modify the function of G_i) had no effect on either muscarinic agonists or GTP[S] stimulation of [^{3}H]IP accumulation (table 1). These results imply that the G-protein involved in the coupling of the muscarinic receptor to PIC is not a substrate of pertussis toxin. In other systems such as cardiac myocytes and astrocytoma cells, a similar observation has been made in that the G-protein coupling to PI metabolism is not pertussis toxin-sensitive [16,17].

In conclusion, this study shows that activation of muscarinic receptors is linked to both inhibition of adenylate cyclase activity and stimulation of PI hydrolysis in human embryonic pituitary clonal cells Flow 9000. Moreover, these two muscarinic

Table 1

Effects of pertussis toxin pretreatment on PI hydrolysis in Flow 9000 cells

	[^{3}H]IP (dpm/10^6 cells)	
	Control	Pertussis toxin-treated
Intact cells		
Basal	7440 ± 593	6363 ± 882
Carbachol (25 μM)	32550 ± 3906	33921 ± 1696
Acetylcholine (25 μM)	52749 ± 2109	49106 ± 3240
Saponin-permeabilized cells		
Basal	5857 ± 351	5996 ± 479
GTP[S] (100 μM)	10659 ± 426	10573 ± 539

[^{3}H]Inositol-labelled Flow 9000 cells were pretreated with pertussis toxin (50 μg/ml) or an equal amount of culture medium for 24 h. Cells were then washed thoroughly and then divided into two slots. The first slot of cells (intact) was challenged with muscarinic agonists for 30 min. The second slot was treated with saponin (50 μg/ml) for 15 min and subsequently exposed to GTP[S] for 30 min. Total [^{3}H]IP were extracted as described in section 2. Data are means ± SE of 5 separate experiments

receptor-mediated second messenger pathways can be distinguished by pertussis toxin. Experiments are in progress to determine whether one homogeneous class of muscarinic receptors or different receptor subtypes [18] are involved in the activation of these signalling mechanisms.

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