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PERTUSSIS TOXIN INHIBITS α_2-ADRENOCEPTOR-MEDIATED INHIBITION OF ADENYLATE CYCLASE WITHOUT AFFECTING MUSCARINIC REGULATION OF $[Ca^{2+}]_i$ OR INOSITOL PHOSPHATE GENERATION IN SH-SY5Y HUMAN NEUROBLASTOMA CELLS*

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Abstract—The present study reports the differential effects of pertussis toxin on muscarinic regulation of intracellular Ca^{2+} and inositol phosphate generation and α_2-adrenoceptor-mediated inhibition of cAMP formation in SH-SY5Y human neuroblastoma cells. Carbachol caused a biphasic increase in intracellular Ca^{2+} (release of internal stores and Ca^{2+} entry) and a dose-dependent increase in inositol phosphate formation. Pertussis toxin pretreatment did not affect either of these components of the signal transduction pathway but did completely reverse the α_2-adrenoceptor-mediated inhibition of forskolin-stimulated cAMP formation. These data indicate that muscarinic regulation of inositol phosphate generation occurs via a pertussis toxin-insensitive G-protein and that Ca^{2+} entry in these cells may not occur via a G-protein.

Heterotrimeric guanine nucleotide binding proteins (G-proteins) can be distinguished in part by their sensitivity to pertussis toxin. For example, whereas receptor-mediated stimulation of adenylate cyclase is insensitive to pertussis toxin, inhibition of this enzyme or receptor-mediated regulation of K^+ channels is pertussis toxin sensitive in all systems examined [1, 2]. In complete contrast, receptor stimulated phospholipase-C has been shown to be pertussis toxin-sensitive in some systems but not in others [3, 4] probably indicating mediation by different G-proteins. We have recently become interested in this idea since there is evidence that muscarinic receptors present on the human neuroblastoma SH-SY5Y cell mediate phosphoinositide metabolism in a pertussis toxin-sensitive manner [5]. Furthermore since we have established that muscarinic agonists can induce Ca^{2+} entry across the plasma membrane at lower concentrations than required to release intracellular stores [6] we wondered whether pertussis toxin may display different sensitivity to these components of signalling. Finally the recent identification of α_2-adrenoceptors on SH-SY5Y/SK-N-SH cells negatively linked to adenylate cyclase [7, 8] provided a useful comparison of an established pertussis toxin-sensitive response.

MATERIALS AND METHODS

Sources of reagents. Reagents of analytical grade and double distilled water were used throughout. The chemicals and their sources were as follows; minimum essential medium, trypsin/EDTA, foetal calf serum, glutamine, penicillin/streptomycin, fungizone and 175-cm^2 tissue culture flasks were from Gibco (Paisley, U.K.). Fura 2/am (pentaacetoxymethylester) was from either Calbiochem (La Jolla, CA, U.S.A.) or the Sigma Chemical Co. (Poole, U.K.). Carbachol, pertussis toxin, adrenaline, phentolamine, IBMX, forskolin, yohimbine and Triton X-100 were from the Sigma Chemical Co. Freon (1,1,2-trichloro-1,2,2-trifluoroethane) was from Fisons Scientific (Loughborough, U.K.) and tri-n-octylamine was from Aldrich Chemicals (Gillingham, U.K.). *myo*-[2-^{3}H(N)]Inositol (15.1 Ci/mmol), [*methyl*-^{3}H]rauwolscine (82.2 Ci/mmol) and [2,8-^{3}H]cAMP (30–50 Ci/mmol) were from N.E.N. (Stevenage, U.K.). All other reagents were from BDH (Poole, U.K.).

Cell culture. SH-SY5Y human neuroblastoma stock cultures (a kind gift from Dr J. Biedler, Sloan-Kettering Institute, NY, U.S.A.) were routinely maintained in minimum essential medium supplemented with 2 mmol/L L-glutamine, 100 I.U./mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL fungizone and 10% fetal calf serum. Cultures were seeded into 175-cm^2 tissue culture flasks containing 30 mL of supplemented medium and maintained at 37° in 5% CO_2/humidified air. All experimental work reported here was performed with passages 67–92. Pertussis toxin pretreatment was for 20–24 hr.

Measurement of intracellular Ca^{2+}. Confluent 6–7 day cultures of SH-SY5Y cells were harvested from the tissue culture flasks into Krebs buffer of the following composition (mmol/L): Na^+ (143.3), K^+ (5.9), Mg^{2+} (1.2), Ca^{2+} (1.3), Cl^- (128.3), $H_2PO_4^-$ (2.2), HCO_3^- (24.9), SO_4^{2-} (1.2) and glucose (10). After two washes in fresh Krebs buffer the suspension was incubated for 45 min at 37° with 5 μmol/L Fura 2/am. At the end of this "loading"

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period the cell suspension was washed three times in Krebs buffer and resuspended in an appropriate volume (approx. 18–24 mL/flask) of fresh buffer. The stock of loaded cells was maintained at room temperature until use.

Intracellular Ca^{2+} was measured in 3 mL suspensions of Fura 2-loaded SH-SY5Y cells at 37° in polypropylene cuvettes containing a stirrer bar. Fura 2 fluorescence was monitored in a Perkin-Elmer LS5B spectrofluorimeter. The excitation wavelengths were 340 and 380 nm with emission at 509 nm. The time taken to drive between 340/380 nm excitation intensities was 3.8 sec. Intracellular Ca^{2+} was calculated from the ratio of fluorescence at 340/380 nm excitation wavelengths according to Grynkiewicz *et al.* [9], where R_{max} and R_{min} were determined using Triton X-100 (0.1%) and EGTA (3 mmol/L), respectively.

Measurement of [^{3}H]*inositol phosphate accumulation.* SH-SY5Y cells were harvested and resuspended in Krebs–Henseleit pH 7.4 supplemented with 4 μCi [^{3}H]inositol (previously cleaned through a small Dowex chloride 100–200 mesh column) and incubated at 37° for 1 hr to allow incorporation of ^{3}H into phosphoinositides. Prelabelled cells (300 μL) were then challenged with carbachol (10^{-3}–10^{-7} M) in the presence of 5 mM Li^+ for a further 30 min. The reaction was terminated by addition of 300 μL ice-cold trichloroacetic acid. Total [^{3}H]inositol phosphates were extracted with Freon/octylamine and separated by Dowex chromatography (Chloride Form 100–200 mesh) as described previously [10].

α_2-Adrenoceptor binding. The binding of [^{3}H]rauwolscine (0.05–3.50 μM) to whole cell suspensions was assessed at 37° for 30 min in 250 μL volumes using 300–400 μg protein per incubation. Bound and free ligand were separated by vacuum filtration onto Whatman GF/B filters and washed with 2 × 4 mL aliquots of Krebs–Henseleit buffer (+0.1% ascorbic acid). Non-specific binding was defined in the presence of 10 μM phentolamine [11].

cAMP determination. SH-SY5Y cells (400–600 μg) were incubated in a total volume 330 μL Krebs–Henseleit buffer containing IBMX (1 mM), forskolin (10 μM), adrenaline (1 or 10 μM) and yohimbine (1 μM) in various combinations. Incubations were performed at 37° for 10 min. The reaction was terminated by addition of 20 μL 10.0 M HCl, 20 μL 10.0 M NaOH and 180 μL 1 mM Tris (pH 7.5). cAMP concentration of supernatants was determined using [^{3}H]cAMP and a bovine adrenal binding protein according to the method of Brown *et al.* [12].

Data analysis. Data are expressed as either mean ± SE with the number of determinations in parentheses or shown as a typical experiment of at least three. EC_{50} values (half maximum stimulation) were obtained by computer assisted curve fitting using ALLFIT [13]. Where appropriate statistical significance was assessed using Student's *t*-test and considered significant when $P < 0.05$.

RESULTS

Muscarinic receptors

Carbachol (1 and 0.1 mM) caused a marked biphasic elevation of intracellular Ca^{2+} rising (in the case of 1 mM) from a resting 55 nM to a peak of 505 nM 11–12 sec after carbachol addition. $[Ca^{2+}]_i$ then declined to a steady 144 nM above basal which could be maintained for at least 6 min (Fig. 1, Table 1). Pertussis toxin pretreatment 100 ng/mL 24 hr (or 10 ng/mL 24 hr, data not shown) failed to influence either the peak or plateau phase $[Ca^{2+}]_i$ (Fig. 1, Table 1).

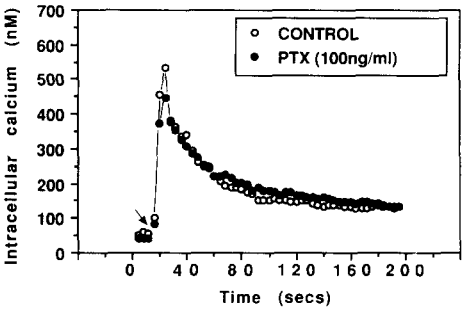

Fig. 1. Effect of pertussis toxin (PTX) on carbachol stimulated $[Ca^{2+}]_i$ levels in SH-SY5Y human neuroblastoma cells. Cells were preincubated with 100 ng/mL pertussis toxin for 24 hr. Data are from a single experiment typical of three others. Carbachol addition is indicated by the arrow.

Carbachol induces a dose-related increase in total [^{3}H]inositol phosphate formation, maximum stimulation of some 7-fold occurring in response to 1 mM carbachol. Preincubation with 100 ng/mL PTX for 24 hr also failed to significantly affect the dose–response relationship to carbachol (Fig. 2). The EC_{50} values for carbachol stimulated inositol phosphate formation were 30.8 ± 6.3 μM in the absence and 42.7 ± 16.2 μM in the presence of 100 ng/mL pertussis toxin.

α_2 Adrenoceptors

As a positive control for the effects of pertussis toxin we examined the effects of this toxin on α_2-adrenoceptor-mediated inhibition of cAMP formation previously reported in SK-N-SH cells [8]. SH-SY5Y cells express relatively low density of α_2-adrenoceptor binding sites with k_D of 0.54 ± 0.08 nM and B_{max} of 16.0 ± 1.4 fmol/mg protein (Fig. 3). Non-specific binding at the radioligand k_D accounted for some 47% of total binding. Forskolin (10 μM) increased [cAMP] from basal of 17.7 ± 1.3 to 1477 ± 121.9 pmol/mg protein/10 min (83-fold), this increase was reversed by 1 and 10 μM adrenaline by 61.3 and 68.7%, respectively. Co-incubation with yohimbine (1 μM) blocked this reversal of cAMP formation, indicating the involvement of an α_2-adrenoceptor (Fig. 4). More importantly, the α_2-inhibition of cAMP formation was completely reversed by pertussis toxin pretreatment (100 ng/mL 20–24 hr) (Fig. 4), demonstrating the ability of this toxin to inhibit the G_i linkage of α_2-adrenoceptors to adenylate cyclase in these cells.

DISCUSSION

The human neuroblastoma cell SH-SY5Y expresses a homogeneous M_3 muscarinic receptor

Table 1. The effect of pertussis toxin (PTX) on carbachol stimulated $[Ca^{2+}]_i$ levels in SH-SY5Y human neuroblastoma cells

[Carbachol] (mol/L)	[PTX] (ng/mL)	$[Ca^{2+}]_i$ (nmol/L)		
		Basal	Peak	Plateau
10^{-4}	0	75.1 ± 9.7	445.7 ± 23.2*	176.5 ± 26.7*
10^{-4}	100	62.7 ± 9.9	426.0 ± 61.5*	192.2 ± 11.4*
10^{-3}	0	54.5 ± 8.1	505.2 ± 19.5*	144.8 ± 6.8*
10^{-3}	100	64.1 ± 15.8	477.3 ± 62.1*	208.0 ± 39.7*

Cells were preincubated in 100 ng/mL pertussis toxin for 24 hr.
Data are mean ± SE (N = 3–4).
* $P < 0.05$, significantly increased above basal.

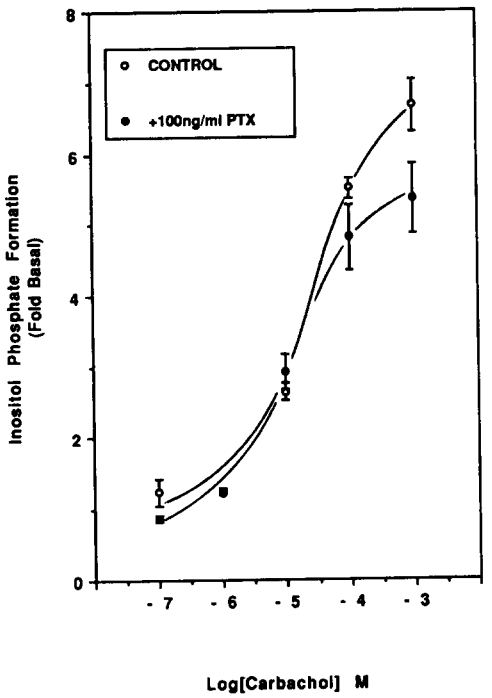

Fig. 2. Carbachol causes a dose-related increase in [^{3}H]inositol phosphate production in the absence and presence of pertussis toxin (PTX). Cells were prelabelled with [^{3}H]*myo*-inositol and stimulated in the presence of 5 mM Li^+. In pertussis toxin treated (100 ng/mL) cells the toxin was added 24 hr prior to experiments. Data are means ± SE (N = 3).

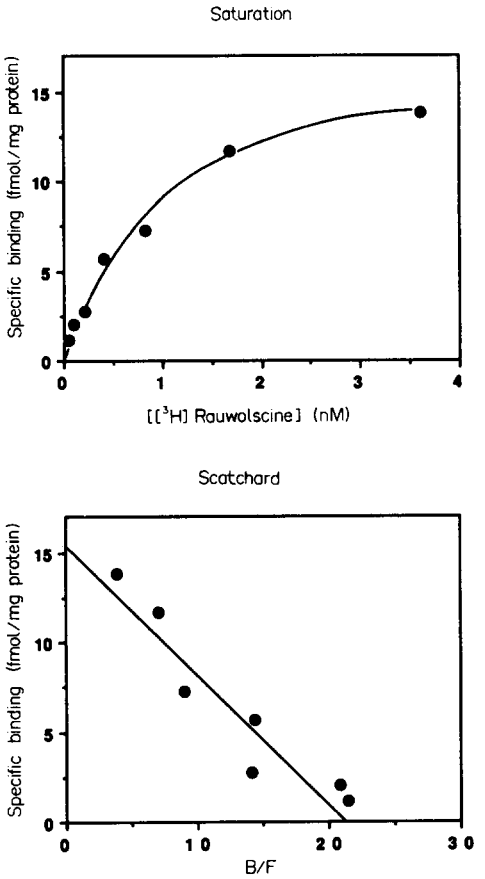

Fig. 3. [^{3}H]Rauwolscine binding to intact SH-SY5Y human neuroblastoma cells. Studies were performed at 37° in 250 μL volumes for 30 min. Non-specific binding was defined in the presence of 10 μM phentolamine. Data are from a single experiment typical of two others.

population that is linked to phosphoinositide metabolism [6, 14]. Activation of such cell surface sites leads to characteristic changes in Ca^{2+} signalling dependent on release of intracellular stores and the entry of Ca^{2+} across the plasma membrane, through a non-voltage-sensitive channel [6, 15, 16]. Whether these events are related is not known, but the ability of low concentrations of full muscarinic agonists and most concentrations of partial agonists to stimulate Ca^{2+}-influx without significant elevation of $Ins(1,4,5)P_3$ and release of intracellular Ca^{2+} could indicate a receptor-operated channel for Ca^{2+} [6]. In the present experiments, we have examined whether pertussis toxin-induced inactivation of G-proteins influenced any components of this complex signalling response to muscarinic agonists.

The results of these experiments have failed to reveal any inhibitory effects of the toxin on phosphoinositide metabolism or any aspect of Ca^{2+} signalling. These data strongly suggest that the muscarinic M_3 receptor present on SH-SY5Y cell surface [14] which can stimulate polyphosphoinositide metabolism in a GTP-dependent manner

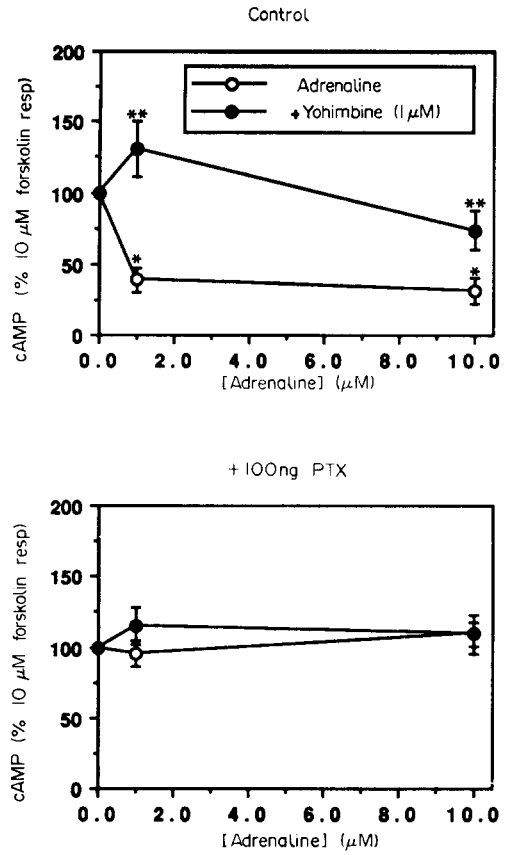

Fig. 4. Pertussis toxin (PTX) pretreatment (100 ng/mL, 24 hr) reverses α_2-adrenoceptor mediated inhibition of forskolin-stimulated cAMP formation in SH-SY5Y human neuroblastoma cells. SH-SY5Y cells were incubated in 330 μL volumes containing IBMX (1 mM), forskolin (10 μM), adrenaline (1 or 10 μM) and yohimbine (1 μM) in various combinations. Incubations were performed at 37° for 10 min. [cAMP] was measured using bovine adrenal binding protein. Data are mean ± SE (N = 3). Basal and forskolin-stimulated [cAMP] were 17.7 and 1477.7 pmol/mg protein/10 min, respectively. Forskolin-stimulated [cAMP] was normalized to 100%. *P < 0.05 reduced compared with forskolin alone. **P < 0.05 increased compared with adrenaline.

[17] almost certainly involves a PTX-insensitive G-protein. These data would be consistent with several reports indicating that muscarinic receptors including transfected cloned M_1 and M_3 subtypes can stimulate phosphoinositide metabolism in a pertussis toxin-insensitive manner (see Ref. 18). The nature of this G-protein is still to be established but a potential candidate may be a protein like G_z that is pertussis toxin-insensitive [19].

Our data do however, contrast with those of Mei *et al.* [5] who demonstrated inhibitory effects of heroic concentrations of pertussis toxin (1 and 10 μg/mL) on muscarinic receptor-stimulated phosphoinositide metabolism. The possibility remains that since, in contrast to our cells, the SH-SY5Y used by Mei *et al.* [5] expresses a different muscarinic receptor subtype [20] different transmembrane signalling mechanisms could operate. However, it seems more likely that non-specific effects of this toxin should be considered. Indeed in CHO cells transfected with muscarinic receptor, 100 ng/mL pertussis toxin was sufficient to ADP ribosylate all detectable pertussis toxin substrate [21]. Furthermore, the present studies have confirmed the existence of α_2-adrenoceptors on SH-SY5Y cells [8], and have shown a pertussis toxin sensitive linkage through G_i to adenylate cyclase, previously demonstrated in the parent SK-N-SH cell [7]. The inhibition of forskolin-stimulated cAMP production by pertussis toxin confirms the integrity of the batch used.

With the confident assumptions that pertussis toxin would be expected to have inactivated all sensitive G-proteins, the present data also suggests that muscarinic receptor stimulation of Ca^{2+}-entry in SH-SY5Y cells may not involve a G-protein linkage. There is increasing evidence that receptors may regulate K^+ and voltage-sensitive Ca^{2+} channels via a pertussis toxin sensitive G-protein [22]. Recent reports implicate a G_s linkage to cardiac voltage-sensitive L-channels [23] and this could still remain a possibility here although muscarinic receptor linked Ca^{2+} entry in SH-SY5Y cells is not voltage-sensitive [15, 16]. Alternatively, the present data would be consistent with a channel regulated by inositol polyphosphates (see Ref. 24) or other messengers linked to muscarinic receptors via a pertussis toxin-insensitive G-protein.

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