Inhibition of formation of cyclic AMP and cyclic GMP by vasopressin in smooth-muscle cells is insensitive to pertussis toxin

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Pretreatment of A-10 cells with pertussis toxin had no effect on [arginine]vasopressin-mediated inhibition of cyclic nucleotide accumulation. Pretreatment of the cells with the same concentration of pertussis toxin produced 90–95 % inhibition of [³²P]ADP ribosylation in membranes, suggesting that these cells possess pertussis-toxin substrate and that the toxin enters the cells to reach its site of action. The functional integrity of the pertussis-toxin substrate in these cells is confirmed by the observation that in these cell membranes increasing concentrations of GTP inhibited basal, forskolin- and NaF-stimulated adenylate cyclase activities, and this inhibition was abolished when the cells were pretreated with pertussis toxin. In addition, thrombin-mediated inhibition of isoprenaline-stimulated cyclic AMP accumulation was also inhibited by pertussis-toxin pretreatment of the cells. These data suggest that, unlike thrombin, [arginine]vasopressin-induced inhibitory effects on cyclic nucleotide accumulation in smooth-muscle cells are not mediated by pertussis-toxin substrate.

INTRODUCTION

Two guanine nucleotide-binding regulatory proteins, G_s and G_i, mediate stimulation and inhibition of adenylate cyclase activities respectively [1–9]. These proteins contain α , β and γ subunits, and specific toxins are used to identify the α subunits of these proteins [1–9]. Cholera toxin, which ADP-ribosylates G_s [10], has no effect on receptor-mediated inhibition of adenylate cyclase [11,12]. Thus muscarinic, α_2 and other inhibitory receptors regulate adenylate cyclase activity through a guanine nucleotide-binding protein that is distinct from G_s. This inhibitory protein can be identified by its susceptibility to modification by pertussis toxin. Pertussis-toxin-catalysed ADP-ribosylation inhibits the ability of G_i to mediate receptor-induced inhibition of adenylate cyclase. In addition to the inhibition of adenylate cyclase, pertussis-toxin substrate has been implicated to modulate a host of receptor-mediated events such as secretion, increase in intracellular free Ca²⁺, chemotaxis, superoxide production, cyclic AMP and cyclic GMP phosphodiesterases, liberation of arachidonic acid from phospholipids and degradation of inositol phospholipids [13–22]. Rat aortic smooth-muscle cells (A-10; American Type Culture Collection CRL 1476) display vasopressin receptors of V₁ subtype [23], β -adrenergic receptors [24] and ANF receptors [25]. Activation of the vasopressin receptors results in (a) stimulation of inositol phosphate accumulation and Ca²⁺ efflux [26], (b) inhibition of isoprenaline- and forskolin-stimulated cyclic AMP accumulation [27] and (c) inhibition of ANF-stimulated cyclic GMP accumulation [25]. All the above responses display similar dose-dependency, antagonist potency and selectivity, suggesting that they are mediated by the same receptors. These vasopressin receptors mediate their responses

through a guanine nucleotide-binding protein, since the binding of vasopressin to these receptors is modulated by guanine nucleotides [28,29]. We have reported that the inhibition of accumulation of cyclic nucleotides induced by AVP ([8-arginine]vasopressin) is not mediated by activation of phosphodiesterase since the inhibition was observed even in the presence of 3-isobutyl-1-methylxanthine, a potent phosphodiesterase inhibitor [25,27]. Since pertussis toxin has been reported to abolish the inhibitory effect of α_2 , muscarinic and other cyclase-coupled inhibitory receptors, we have used this as a tool to delineate the mechanism of AVP-mediated inhibition of cyclic nucleotide accumulation.

MATERIALS AND METHODS

Materials

AVP was from Bachem, Torrance, CA, U.S.A.; 3-isobutyl-1-methylxanthine, ATP, cyclic AMP, GTP, pyruvate kinase, phosphoenolpyruvate, imidazole, thrombin, ANF-II and (–)-isoprenaline (isoproterenol) were from Sigma. Cyclic [³H]AMP and radioimmunoassay kits for cyclic AMP and cyclic GMP were from New England Nuclear, Boston, MA, U.S.A. AG 1X8 resin (formate form; 100–200 mesh) was from Bio-Rad, Richmond, CA, U.S.A. Pertussis toxin was obtained from List Biological Laboratories, Campbell, CA, U.S.A. All other chemicals were of highest reagent grade available.

Cell culture and growth conditions

Rat thoracic aortic smooth-muscle cells (A-10) were obtained from the American Type Culture Collection and cultured in Dulbecco's Modified Eagle's Medium plus 20 % (v/v) fetal-calf serum in air/CO₂ (19:1) at

Abbreviations used: ANF, atrial natriuretic factor; AVP, [8-arginine]vasopressin; G-proteins, guanine nucleotide-binding regulatory proteins (G_s, stimulatory; G_i, inhibitory).

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37 °C. Before experiments were initiated, the cells were subcloned twice by limiting dilution. The experiments were carried out with cells passaged for no more than 4 months. Culture flasks (Falcon T-150) or wells (35 mm diameter) were inoculated with 1.5×10^6 or 75000 cells respectively and cultured in 20 ml or 1 ml of medium respectively. Experiments were carried out after 3 days unless otherwise indicated.

Pertussis-toxin treatment and measurement of cyclic nucleotides

Culture wells were washed once with 1 ml of Dulbecco's phosphate-buffered saline containing 10 mM-MgCl₂, 0.1 % glucose and 0.2 % bovine serum albumin, and preincubated without or with the indicated concentrations of pertussis toxin for the indicated times. After preincubation, the cells were washed with 2×1 ml of Dulbecco's phosphate-buffered saline. Subsequently, 1 ml of Dulbecco's phosphate-buffered saline containing 0.5 mM-isobutylmethylxanthine was added to each well, followed by addition of AVP. The reaction was started by adding 10 μ l of 0.1 mM-($-$)-isoprenaline or 10 μ l of 0.1 mM-forskolin (for cyclic AMP) or 10 μ l of 0.1 mM-ANF (for cyclic GMP) and continued for 10 min at 37 °C. The incubation was stopped by adding 100 μ l of ice-cold 100 % trichloroacetic acid to each well. Cyclic nucleotides were extracted and assayed by radioimmunoassay as previously described [25,27].

Adenylate cyclase assay

Assays were performed as described in ref. [28]. The final incubation mixture (100 μ l) contained 30 mM-Tris/HCl, pH 7.5, 2.0 mM-MgCl₂, 0.6 mM-EDTA, 100 mM-sucrose, 0.2 mM-dithiothreitol, 0.12 mM-ATP, 1 μ Ci of [α -³²P]ATP, 0.1 mM-cyclic AMP, 2.8 mM-phosphoenolpyruvate, 5.2 μ g of pyruvate kinase/ml, 10 μ g of myokinase/ml and indicated concentrations of GTP in the absence or presence of 0.1 mM-forskolin or 10 mM-NaF. The reaction was initiated by the addition of 20 μ l of membrane suspension (30–60 μ g of protein) and continued for 20 min at 30 °C. The incubation was terminated by the addition of 1.0 ml of ice-cold stop solution, containing 0.4 mM-ATP, 0.3 mM-cyclic AMP and cyclic [³H]AMP (15000–20000 c.p.m.). Cyclic AMP was isolated as described by Salomon *et al.* [30].

Pertussis-toxin-mediated ADP-ribosylation

This was done by the procedure of Rich *et al.* [31], with slight modification. Membranes were incubated in a final volume of 200 μ l (~ 1 mg/ml) for 20 min at 30 °C in the presence of 150 mM-sodium phosphate buffer, pH 7.5, 100 mM-thymidine, 0.5 mM-ATP, 0.05 mM-GTP and 10 μ M-[³²P]NAD⁺ (5000 c.p.m./pmol) in the absence or presence of activated pertussis toxin (1 μ g/ml). After the incubation, the membranes were diluted to 5 ml with ice-cold 25 mM-Hepes/NaOH, pH 8.0, and centrifuged at 40000 *g* for 30 min. The supernatant was discarded and the pellet resuspended in 50 μ l of 25 mM-Hepes/NaOH, pH 8.0. This was subjected to electrophoresis on 10 % polyacrylamide gels in the presence of SDS as described in ref. [32].

Activation of the toxin

The toxin (0.5 mg/ml) was incubated for 20 min at 30 °C in 100 mM-sodium phosphate buffer, pH 7.0, in the presence of 20 mM-dithiothreitol.

Table 1. Effect of pertussis-toxin pretreatment on AVP-mediated responses in A-10 cells

A-10 cells were pretreated with and without pertussis toxin (PT; 1 μ g/ml) overnight, and the cyclic nucleotides were measured as explained in the Materials and methods section. The data presented are means $\pm$ S.E.M. of values obtained from three separate wells from one experiment which is representative of three experiments. *Not significantly different ($P > 0.05$) from values obtained in the absence of PT (control cells).

Condition	Cyclic AMP (pmol/assay)	
	Control cells	PT-treated cells
Basal	14 $\pm$ 3	12 $\pm$ 2
1 μ M-Isoprenaline	152 $\pm$ 8	153 $\pm$ 7
1 μ M-Isoprenaline + 10 nM-AVP	120 $\pm$ 1	122 $\pm$ 1*
1 μ M-Forskolin	129 $\pm$ 1	105 $\pm$ 11
1 μ M-Forskolin + 10 nM-AVP	91 $\pm$ 2	70 $\pm$ 10*

Condition	Cyclic GMP (fmol/assay)	
	Control cells	PT-treated cells
Basal	8 $\pm$ 2	9 $\pm$ 1
1 μ M-ANF	276 $\pm$ 14	339 $\pm$ 30
1 μ M-ANF + 10 nM-AVP	164 $\pm$ 14	176 $\pm$ 24*

Presentation of data

Each experiment was repeated at least twice. All values represent means $\pm$ S.E.M. for triplicate cultures from a representative experiment. Although absolute values varied, similar results were obtained in additional experiments. Wherever S.E.M. values are not given, the error bars were smaller than the symbols.

RESULTS

Addition of AVP to A-10 cells resulted in inhibition of isoprenaline- and forskolin-induced cyclic AMP accumulation and of ANF-induced cyclic GMP accumulation (Table 1). Pretreatment of the cells with pertussis toxin (1 μ g/ml) for up to 16 h did not significantly inhibit AVP-mediated inhibitory responses (Table 1). We have previously demonstrated that these cells have pertussis-toxin-sensitive G-protein, but AVP-mediated accumulation of inositol phosphates was not significantly inhibited by pertussis-toxin treatment. These data suggest one of the following possibilities: (a) the toxin cannot enter the cells to its site of action; or (b) the toxin-sensitive substrate may not be functional; or (c) AVP-mediated inhibitory responses are not mediated by a pertussis-toxin-sensitive G-protein.

As shown in Fig. 1, preincubation of the cells with pertussis toxin (1 μ g/ml) overnight and subsequent ADP-ribosylation of the membranes with the pertussis

Fig. 1. Pretreatment of A-10 cells with increasing concentrations of pertussis toxin dose-dependently inhibits [32 P]ADP-ribose incorporation in the membranes

A-10 cells were pretreated without and with increasing concentrations of pertussis toxin overnight. The cells were washed, and membranes were prepared and subjected to ADP-ribosylation with [32 P]NAD $^{+}$ and pertussis toxin, SDS/polyacrylamide-gel electrophoresis and autoradiography. Lanes: 1, no toxin; 2, control cell membranes; 3–6, pertussis-toxin-pretreated cell membranes (3, 1 ng/ml; 4, 10 ng/ml; 5, 100 ng/ml; 6, 1 μ g/ml). Peak areas were measured by densitometry scanning. M_r markers were: albumin, 67000; ovalbumin, 43000; carbonic anhydrase, 30000.

toxin and [32 P]NAD $^{+}$ produced 90–95% inhibition of the [32 P]ADP-ribosylation in membranes from cells that are treated with pertussis toxin (Fig. 1, lane 6). These data suggest that the toxin can penetrate the cells to its site of action and ADP-ribosylate the protein with cellular NAD $^{+}$, resulting in the inhibition of transfer of [32 P]ADP-ribose from exogenous [32 P]NAD $^{+}$.

The functional integrity of the toxin-sensitive protein was tested by the following experiments. A-10 cell membrane adenylate cyclase activity was markedly stimulated by forskolin and NaF. Addition of increasing concentrations of GTP inhibited forskolin- and NaF-stimulated as well as basal adenylate cyclase activities in a dose-dependent fashion (Fig. 2), suggesting that concentrations higher than 10 μ M-GTP can activate G_i , resulting in the inhibitory effect. When the cells were pretreated with pertussis toxin (1 μ g/ml) overnight and then analysed for basal, NaF- and forskolin-stimulated membrane adenylate cyclase activities in the presence of increasing concentrations of GTP, it was observed that the GTP-induced inhibitory effect was abolished in pertussis-toxin-pretreated cell membranes, and this depended on the concentration of pertussis toxin used in the pretreatment, as shown in Table 2. At concentrations of 100 ng and 1 μ g of pertussis toxin/ml, the inhibition was completely abolished and stimulation became

Fig. 2. GTP-induced inhibition of adenylate cyclase activities was abolished by pretreatment of the cells with pertussis toxin

A-10 cells were pretreated without ($\circ$, $\square$, $\triangle$) and with ($\bullet$, $\blacksquare$, $\blacktriangle$) pertussis toxin (1 μ g/ml) overnight. The cells were washed and membranes were prepared and assayed for basal ($\circ$, $\bullet$), forskolin ($\square$, $\blacksquare$) and NaF ($\triangle$, $\blacktriangle$)-stimulated adenylate cyclase activities. The data presented are means $\pm$ S.E.M. for triplicate determinations from one experiment which is representative of four similar experiments.

apparent (Table 2). This reversal of GTP-mediated inhibition compares very well with the loss of incorporation of [32 P]ADP-ribose into the M_r -39000 band in membranes prepared from cells pretreated with increasing concentrations of pertussis toxin (Fig. 1). These data suggest that the pertussis-toxin substrate is functional and that it mediated GTP-dependent inhibition of adenylate cyclase activities. Under identical experimental conditions AVP did not inhibit basal, forskolin- or NaF-stimulated adenylate cyclase activities (Table 3), suggesting that AVP-induced inhibition of cyclic AMP production was not mediated by G_i . The functional integrity of G_i in these cells was further strengthened by the observation that thrombin-mediated inhibition of cyclic AMP accumulation was inhibited by pertussis-toxin pretreatment of the cells (Table 4).

DISCUSSION

Pretreatment of different cell types with pertussis toxin produces inhibition of agonist-induced responses [13–22], and in all cases an involvement of G_i protein with those responses has been implicated. Pertussis toxin modifies and inactivates G_i , the guanine nucleotide-binding regulatory protein that is involved in the receptor-coupled inhibition of adenylate cyclase activities. Exposure of rat aortic smooth-muscle cells to AVP results

Table 2. Effect of 100 μ M-GTP on forskolin- and NaF-stimulated adenylate cyclase activity in control and pertussis-toxin-pretreated A-10 cell membranes

A-10 cells were pretreated in the absence or presence of increasing concentrations of pertussis toxin (PT) overnight, and membranes were prepared and assayed for forskolin- and NaF-stimulated adenylate cyclase activities in the presence of 100 μ M-GTP: (–) and (+) represent inhibition and stimulation respectively. The data presented are means $\pm$ S.E.M. of triplicate values from one experiment which is representative of four similar experiments.

Condition	Adenylate cyclase activity	
	Forskolin-stimulated % change	NaF-stimulated % change
Control membranes	-20 ± 3.8	-34 ± 2.5
PT (1 ng/ml)	-19 ± 1.8	-31 ± 2.8
PT (10 ng/ml)	-5 ± 0.9	-14 ± 0.9
PT (100 ng/ml)	-2 ± 0.2	-2 ± 0.2
PT (1 μ g/ml)	$+22 \pm 1.5$	$+40 \pm 3.8$

Table 3. Vasopressin effect on adenylate cyclase activity

Membranes were prepared from A-10 cells and assayed for basal, forskolin (100 μ M)- and NaF (10 mM)-stimulated adenylate cyclase activities in the absence and presence of 1 μ M-AVP. The data obtained in the absence and presence of AVP were not significantly different, and represent means $\pm$ S.E.M. of triplicate determinations from one experiment which is representative of two similar experiments.

Conditions	Adenylate cyclase activity (pmol of cyclic AMP/mg of protein)		
	Basal	Forskolin	NaF
No additions	75 ± 7.5	663 ± 37	198 ± 2
AVP	68 ± 6.0	564 ± 49	188 ± 14

in (a) stimulation of inositol phosphate formation and Ca^{2+} efflux, (b) inhibition of isoprenaline- and forskolin-induced cyclic AMP accumulation and (c) inhibition of ANF-induced cyclic GMP accumulation. These AVP-mediated responses are mediated by a G-protein [29,33]. In order to identify the mechanism of AVP-induced inhibition of cyclic nucleotide accumulation, we used pertussis toxin as a tool. When the cells are pretreated with pertussis toxin and tested for AVP-mediated effects, it was observed that AVP-induced inositol phosphate accumulation was marginally inhibited [26], whereas the inhibition of cyclic nucleotide accumulation was unaffected. This is in contrast with what has been observed for α_2 -adrenergic-receptor-mediated inhibition of adenylate cyclase activity. Since we did not see any effect of pertussis toxin on AVP-mediated responses, it was important to demonstrate that these cells possess

Table 4. Effect of pertussis-toxin pretreatment on thrombin-mediated effect in A-10 cells

A-10 cells were pretreated with and without pertussis toxin (PT; 1 μ g/ml) for 3 h, and cyclic AMP was measured as explained in the Materials and methods section. The data presented are means $\pm$ S.E.M. of values obtained from four separate wells from one experiment which is representative of two experiments. *Significantly different from value obtained with 1 μ M-isoprenaline alone ($P < 0.05$). **Not significantly different from value obtained with 1 μ M-isoprenaline alone ($P > 0.05$).

Condition	Cyclic AMP (pmol/assay)	
	Control cells	PT-treated cells
Basal	16 ± 0.8	14 ± 0.6
1 μ M-Isoprenaline	176 ± 9.2	172 ± 14
1 μ M-Isoprenaline + thrombin (5 μ g/ml)	$124 \pm 9.6^*$	$164 \pm 17^{**}$

pertussis-toxin substrate, that it is functional and that the toxin can penetrate the cell to its site of action.

The data in the present paper demonstrate that this is indeed the case. This is supported by the following findings. (1) Treatment of the membranes with pertussis toxin and [32 P]NAD $^+$ resulted in incorporation of [32 P]ADP-ribose into a M_r -39000 peptide. (2) Pretreatment of the cells with pertussis toxin inhibited 32 P incorporation into the M_r -39000 peptide by 90–95% when membranes from toxin-pretreated cells were used for [32 P]ADP-ribosylation. (3) Increasing concentrations of GTP inhibited NaF- and forskolin-stimulated as well as basal adenylate cyclase activities. (4) Pretreatment of cells with pertussis toxin dose-dependently inhibited the GTP-mediated inhibitory effect on adenylate cyclase activities, and this dose-dependency correlated well with the loss of incorporation of [32 P]ADP-ribose into M_r -39000 peptide. (5) Finally, thrombin-mediated inhibition of isoprenaline-stimulated cyclic AMP accumulation was inhibited by pertussis-toxin pretreatment of the cells. These data suggest that these cells have a functional pertussis-toxin substrate and that AVP-induced inhibition of cyclic nucleotide accumulation is not mediated by pertussis-toxin-sensitive G-protein(s).

The lack of pertussis-toxin sensitivity differentiates these V_1 receptors from the classical adenylate cyclase-coupled inhibitory receptors. AVP did not inhibit basal, forskolin- and NaF-stimulated adenylate cyclase activities under conditions where G_i -mediated inhibition of adenylate cyclase activity could be demonstrated. We believe that AVP-mediated inhibition of cyclic nucleotide accumulation is secondary to its stimulation of inositol phosphates and diacylglycerol formation, a function that involves a G-protein that is not a substrate for pertussis toxin [26]. This is supported by the observation that tumour-promoter phorbol esters which directly activate protein kinase C also inhibit cyclic nucleotide accumulation [29]. Similarly NaF, which stimulates formation of inositol phosphates and diacylglycerol in these cells, also inhibits cyclic nucleotide accumulation (P. Nambi, N. Aiyar, M. Whitman & S. T. Crooke, unpublished work). It is possible that, when protein

kinase C is activated either by exogenously added phorbol esters or by endogenously formed diacylglycerol (in response to AVP or NaF), it might phosphorylate some key regulatory protein(s) in the cyclic nucleotide pathway, resulting in an attenuated response. Protein kinase C-mediated phosphorylation of α_1 - and β -adrenergic receptors resulted in an attenuated response mediated by these receptors [34–36]. Further work is required to identify these phosphorylated proteins.

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