Pertussis toxin blocks the inhibitory effect of muscarinic cholinergic agonists on cyclic AMP accumulation and prolactin secretion in GH₃ anterior-pituitary tumour cells

Barry L. BROWN,* Richard J. H. WOJCIKIEWICZ,* Pauline R. M. DOBSON,*
Andrew ROBINSON† and Laurence I. IRONS†

*Department of Human Metabolism and Clinical Biochemistry, University of Sheffield Medical School,
Beech Hill Road, Sheffield S10 2RX, and

†Pathogenic Microbes Research Laboratory, Public Health Laboratory Service,
Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wilts. SP4 0JG, U.K.

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The inhibition of prolactin secretion and cyclic AMP accumulation in GH₃ cells by muscarinic agonists was blocked by preincubation of the cells with pertussis toxin (islet-activating protein). There was a lag of approx. 80 min in the onset of the effect on secretion. These results suggest that muscarinic agonists decrease prolactin secretion by inhibiting adenylate cyclase activity.

Several lines of evidence indicate that both normal and tumour anterior-pituitary cells possess receptors for muscarinic cholinergic agonists (Schaeffer & Hsueh, 1980; Burt & Taylor, 1980; Rudnick & Dannies, 1981; Heisler *et al.*, 1983). The mechanism of action of these agents in these cells is unclear, although effects on phospholipid metabolism (Young *et al.*, 1979) and on cyclic AMP (Heisler *et al.*, 1983) have been reported. The fact that muscarinic agonists stimulate growth-hormone (somatotropin) release (Young *et al.*, 1979), yet inhibit prolactin secretion (Rudnick & Dannies, 1981), from normal pituitary cells complicates elucidation of their effects in this tissue. We have observed that cholinergic agonists inhibit both prolactin secretion and cyclic AMP accumulation in GH₃ pituitary-tumour cells (Wojcikiewicz *et al.*, 1984a,b); a similar finding has also been reported by Onali *et al.* (1983). Furthermore, the stimulatory effects of VIP on both parameters are also blunted by these agents. These effects appear to be via muscarinic receptors, since atropine, but not tubocurarine, blocks the inhibition (Wojcikiewicz *et al.*, 1984a,b).

It has been shown that the effects of inhibitory hormones on adenylate cyclase activity are mediated by a guanine-nucleotide- and Mg²⁺-dependent regulatory protein. This protein is often designated N_i (Hildebrandt *et al.*, 1983; Codina *et al.*, 1983),

and its α -subunit is a substrate for the ADP-ribosylation activity of a protein exotoxin from *Bordetella pertussis*. This toxin also has the ability to block the effect of agents which inhibit adenylate cyclase activity (Katada & Ui, 1980, 1981; Codina *et al.*, 1983; Cronin *et al.*, 1983a,b). In order to further our understanding of the mode of action of muscarinic agonists, and indeed of inhibitory mechanisms in general in GH₃ cells, we have investigated the effect of PT on both cyclic AMP accumulation and prolactin secretion.

Materials and methods

The materials for culture and incubation of GH₃ cells {Ham's F10 medium, Dulbecco's modification of Eagle's medium with 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer, penicillin/streptomycin, foetal-calf serum and trypsin/EDTA} were all from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. Radiochemicals were obtained from Amersham International, Amersham, Bucks., U.K. Sigma Chemicals, Poole, Dorset, U.K., provided the bovine serum albumin, oxotremorine, acetylcholine chloride, carbamoylcholine chloride (carbachol) and cyclic AMP. VIP was purchased from Merseyside Laboratories, St. Helens, Lancashire, U.K.

Bordetella pertussis exotoxin (PT, lymphocytosis-promoting factor, histamine-sensitizing factor, islet-activating protein) was prepared from *B. pertussis*, strain Wellcome 28, as described pre-

Abbreviations used: VIP, vasoactive intestinal polypeptide; PT, pertussis toxin.

viously (Robinson & Irons, 1983). The materials for the radioimmunoassay of rat prolactin were kindly given by the National Institutes of Health, Bethesda, MD, U.S.A. The antiserum for the radioimmunoassay of cyclic AMP was generously given by Dr. Alton Steiner, Houston, TX, U.S.A.

GH₃ cells (passage nos. 80–100) were cultured routinely in Ham's F10 medium containing 10% foetal-calf serum and penicillin/streptomycin (100units/ml) in Petri dishes (9cm diam.). When required for experiments, the cells were released by incubation with 0.05% trypsin/0.02% EDTA and either were incubated in microbiological Petri dishes with Cytodex beads (Pharmacia) or were re-plated into 24-well multiwell dishes at 10⁵ cells/well in supplemented Ham's F10 medium (1 ml).

For the perfusion experiments, the cells were cultured on Cytodex beads for 72h and were perfused with medium (Krebs–Ringer bicarbonate, pH 7.6, containing 0.5% bovine serum albumin + 0.02% glucose) as described previously (Schrey *et al.*, 1977). After perfusion for 1h, to enable the system to stabilize, the cells were exposed to medium containing oxotremorine (1 μ M), followed in some experiments by PT (50ng/ml). Fractions of the eluate were collected and assayed for prolactin content by radioimmunoassay.

For the static incubations, the toxin was added

Fig. 1. Time course of oxotremorine-induced inhibition of prolactin secretion from GH₃ cells on Cytodex and its blockade by PT (50ng/ml)

Cells were perfused as indicated in the text. PT was included in the perfusion medium from 90 to 280min. Cells perfused with medium alone for up to 300min maintained a relatively constant rate of hormone output.

to each well, at the concentrations shown, after the cells had been in culture for 48h. Then 24h later the medium was aspirated and the cell layer washed three times with serum-free Hepes-buffered Dulbecco's modification of Eagle's medium (containing 0.5% bovine serum albumin). The cells were preincubated in this medium for 30min, which was then removed and replaced by the same medium containing the drugs and/or hormones. After the appropriate incubation time (see the Results and discussion section), the medium was withdrawn and stored at -20°C for determination of prolactin content. Ethanol (65%, v/v; 4°C) was added to the cell layer, and the plates were then maintained at -20°C for at least 16h. The cell layer in each well was scraped and removed together with a wash (65% ethanol) into tubes. The protein was pelleted by centrifugation (1000g), and the supernatant was removed and evaporated to dryness. The extracts were redissolved in water and assayed for cyclic AMP by radioimmunoassay after acetylation (Harper & Brooker, 1975). Triplicate wells were used for each treatment, and experiments were performed at least three times. Significances were determined by analysis of variance.

Results and discussion

Perfusion of GH₃ cells on Cytodex beads showed that the inhibitory effect of oxotremorine on prolactin secretion was blocked by PT (Fig. 1). Reversal of inhibition (at a dose of 50ng of PT/ml) was not apparent until 80min after addition of toxin, but was maximal at 3h. The time lag before onset of blockade may be due to the time taken for the toxin to enter the cell and interact with cellular components.

As we had observed that inhibition by oxotremorine of cyclic AMP accumulation was maximal at 5min, we determined the effect of muscarinic agonists on both cyclic AMP and prolactin, after preincubation of the cells with PT (24h), at this time. Fig. 2 shows that the inhibitory effect of oxotremorine (1 μ M) on basal cyclic AMP concentration was blocked by preincubation with PT, the blockade being virtually complete at 0.05ng of PT/ml. It is also clear that cholinergic inhibition of VIP-stimulated cyclic AMP accumulation was also blocked by PT. Similar blockade of inhibition induced by acetylcholine (1 μ M) and carbachol (1 μ M) was observed at this incubation time (results not shown). The effect on basal and VIP-stimulated prolactin secretion was, however, small and generally insignificant at this short incubation time. Therefore, a further series of experiments were performed, this time with an incubation time of 15min (Fig. 3 and Table 1). It is apparent that

Fig. 2. Histogram depicting the effect of preincubation of GH₃ cells with a range of doses of PT on cyclic AMP accumulation in response to VIP, oxotremorine and VIP plus oxotremorine

The incubation time was 5 min. Results, expressed as means + s.d., are from triplicate incubations in one experiment representative of at least three. Significances were assessed by analysis of variance: * $P < 0.001$, ** $P < 0.0001$ (as compared with own control, i.e. without PT). The concentrations of PT (ng/ml) were: A = 0; B = 0.05; C = 0.5; D = 5.0; E = 50.

Fig. 3. Effect of preincubation of GH₃ cells with PT on prolactin secretion in response to VIP, oxotremorine and VIP plus oxotremorine

The incubation time was 15 min. The results are expressed as means ± s.d. for triplicate incubations and are from one experiment representative of at least three similar results. Significances: * $P < 0.001$, ** $P < 0.0001$ (compared with own control, i.e. without PT). The concentrations of PT used were as in the legend to Fig. 2.

preincubation with a range of doses of PT resulted in blockade of the inhibition of prolactin secretion. Table 1 shows the effect of preincubation with 0.5 ng of PT/ml on the cyclic AMP accumulation

and prolactin secretion at 15 min with all three agonists. Preincubation with PT also blocked the inhibitory effects of a supra-maximal dose (1 mM) of oxotremorine (results not shown). Although

Table 1. *Effect of oxotremorine, acetylcholine and carbachol on basal and VIP-stimulated cyclic AMP accumulation and prolactin secretion with and without preincubation (24 h) with PT (0.5 ng/ml)*

The incubation time was 15 min, and the volume was 1 ml. Significances were assessed for each pair (i.e. with and without PT) by analysis of variance: ^a $P < 0.001$; ^b $P < 0.0001$. Results (means $\pm$ s.d.; $n =$ three wells) are representative of at least three experiments with similar results.

Conditions	PT	Cyclic AMP (pmol/well)	Prolactin (ng/ml)
Control	—	1.20 $\pm$ 0.20	11.7 $\pm$ 0.9
	+	0.99 $\pm$ 0.06	12.8 $\pm$ 1.1
Oxotremorine	—	0.79 $\pm$ 0.07	9.8 $\pm$ 1.4
	+	1.07 $\pm$ 0.08 ^a	15.8 $\pm$ 1.2 ^a
Acetylcholine	—	0.71 $\pm$ 0.04	7.5 $\pm$ 1.9
	+	1.08 $\pm$ 0.09 ^a	13.0 $\pm$ 1.3 ^a
Carbachol	—	0.96 $\pm$ 0.07	9.1 $\pm$ 1.2
	+	1.13 $\pm$ 0.30	15.9 $\pm$ 2.1 ^a
VIP	—	3.40 $\pm$ 0.55	20.9 $\pm$ 3.4
	+	3.42 $\pm$ 0.04	23.1 $\pm$ 1.4
VIP + oxotremorine	—	1.09 $\pm$ 0.32	7.5 $\pm$ 0.4
	+	3.35 $\pm$ 0.30 ^b	17.7 $\pm$ 2.7 ^b
VIP + acetylcholine	—	1.03 $\pm$ 0.14	10.0 $\pm$ 2.1
	+	2.90 $\pm$ 0.39 ^b	24.9 $\pm$ 0.6 ^b
VIP + carbachol	—	1.74 $\pm$ 0.05	15.0 $\pm$ 1.8
	+	3.27 $\pm$ 0.29 ^b	21.5 $\pm$ 3.6 ^a

enhancement by PT of stimulation has been observed, notably with prostaglandin E₁ (Cronin *et al.*, 1983c), our studies indicate that PT alone had no effect on basal or VIP-stimulated cyclic AMP accumulation or prolactin secretion.

Thus preincubation of GH₃ cells under basal conditions with the toxin blocked the inhibitory effect of oxotremorine, acetylcholine and carbachol not only on prolactin secretion but also on intracellular cyclic AMP concentrations. Moreover, PT virtually abolished the muscarinic inhibition of VIP-stimulated prolactin secretion and cyclic AMP accumulation. Indeed, the blockade was more apparent in the presence of VIP than in its absence. PT (or islet-activating protein) has been shown to cause an increase in insulin secretion by blocking the α -adrenergic-mediated inhibition of secretion (Katada & Ui, 1980). More recently, these observations have been extended to other tissues, and it is now clear that PT blocks the effects of agents which inhibit adenylate cyclase activity (Katada & Ui, 1980, 1981; Codina *et al.*, 1983; Cronin *et al.*, 1983a; Jakobs *et al.*, 1983). Indeed, there have been two reports of such effects in the normal anterior pituitary with dopamine (Cronin *et al.*, 1983b) and with somatostatin (Cronin *et al.*, 1983a). The present data support the notion that the mode of action of muscarinic inhibition is to inhibit adenylate cyclase activity in

GH₃ cells. On the basis that pertussis toxin acts by interaction with the inhibitory nucleotide regulatory subunit (N_i) of adenylate cyclase, these results would also indicate that GH₃ cells possess functional N_i subunits. These effects are, to our knowledge, the first reported indication that such mechanisms are operative in GH₃ tumour cells, although a similar effect has been observed with another pituitary tumour (i.e. 235-1; Cronin *et al.*, 1983c).

Although it seems that Ca²⁺ is an important mediator of the effects of modulators of prolactin secretion, e.g. thyrotropin-releasing hormone (Gershengorn, 1982) and possibly also dopamine (Thorner *et al.*, 1980; Ray & Wallis, 1982; Merritt & Brown, 1984), it is clear that changes in cyclic AMP concentration within the cell can influence the secretory process. These results indicate that lowering intracellular cyclic AMP is associated with a decreased secretory rate.

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