Actions of pertussis toxin on the inhibitory effects of dopamine and somatostatin on prolactin and growth hormone release from ovine anterior pituitary cells

R. S. Boyd, K. P. Ray and M. Wallis

Biochemistry Laboratory, School of Biological Sciences, University of Sussex, Falmer, Brighton, Sussex BN1 9QG

(K. P. Ray is now at Department of Cell Biology, Glaxo Group Research Ltd, Greenford, Middlesex UB6 0HE)

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ABSTRACT

Forskolin and the phorbol ester 12-O-tetradecanoylphorbol 13-acetate stimulate prolactin and GH release from ovine anterior pituitary cells cultured in vitro. Dopamine and somatostatin inhibit release of prolactin and GH respectively, after stimulation by these agents, but without effects on intracellular cyclic AMP concentrations. In each case the inhibitory effects were reversed by pretreatment of cells with pertussis toxin, in a dose-related fashion (1–100 ng/ml), again without affecting cyclic AMP levels. The results suggest that the inhibitory effects of dopamine and somatostatin in this system are mediated by one or more pertussis toxin-sensitive G proteins, and that these act by a mechanism which does not involve inhibition of adenylate cyclase.


INTRODUCTION

Dopamine and somatostatin inhibit prolactin and growth hormone (GH) release respectively from anterior pituitary cells. The mechanism of their action is not yet clearly defined. A correlation has been established in some species between an inhibition of hormone release and a fall in intracellular concentrations of cyclic AMP or inhibition of adenylate cyclase (Barnes, Brown, Gard et al. 1978; Onali, Schwartz & Costa, 1981; McDonald, Sibley, Kilpatrick & Caron, 1984; Spada, Vallar & Giannattasio, 1984; Law, Ray & Wallis, 1985). However, the work of a number of authors indicates that a major component of the inhibitory actions of dopamine (Ray & Wallis, 1982a; Delbeke, Scammell, Martinez-Campos & Dannies, 1986; Lafond, Ducharme & Collu, 1986; Ray, Gomm, Law et al. 1986a; Ray, Hart & Wallis, 1986b) and somatostatin (Koch, Dorflinger & Schonbrunn, 1985; Law et al. 1985; Sheppard, Moor & Kraicer, 1985; Ray et al. 1986a, b) is cyclic AMP independent.

A decrease in the intracellular concentration of free calcium has been postulated to be involved in the transducing mechanisms of these inhibitory agents (e.g. Thorner, Hackett, Murad & MacLeod, 1980; Ray & Wallis, 1982b; Merritt & Brown, 1984). Studies employing the calcium indicators Quin-2 and Fura-2 (Schofield, 1983; Koch et al. 1985; Schlegel, Wuarin, Zbaren et al. 1985; Hart, Ray & Wallis, 1986; Malgaroli, Vallar, Elahi et al. 1987) and 45Ca2+ (Schofield & Bicknell, 1978; Schrey, Clark & Franks, 1986) have confirmed this idea. Whether this effect is associated with changes in the intracellular concentration of inositol trisphosphate is not clear and is the subject of some controversy (Brown, Baird, Quilliam et al. 1985; Canonico, Jarvis, Judd & MacLeod, 1986; Enjalbert, Sladeczek, Guillon et al. 1986; Journot, Homberger, Pantaloni et al. 1987). Studies employing the divalent cation ionophore A23187 suggest, however, that both dopamine and somatostatin have Ca2+-independent actions (Bicknell & Schofield, 1976; Tam & Dannies, 1980; Kraicer & Spence, 1981; Ray & Wallis, 1982b). In addition, both dopamine and somatostatin can inhibit phorbol ester-increased hormone release, presumably acting directly at or distal to the level of protein kinase C (Koike, Judd, Yasumoto & MacLeod, 1985; Ray et al. 1986b).

Hormonal actions on adenylate cyclase, and prob-
ably several other transducing mechanisms, are thought to involve interaction of hormone–receptor complexes with guanine nucleotide-binding proteins (G proteins) (Gilman, 1987). The stimulatory (Gs) and inhibitory (Gi) G proteins involved in regulation of adenylate cyclase have been well characterized, but those which mediate the actions of hormones on other effector mechanisms are less well understood. Work in several laboratories has shown that the inhibitory effects of dopamine (Cronin, Myers, MacLeod & Hewlett, 1983a), somatostatin (Cronin, Rogol, Myers & Hewlett, 1983b; Koch et al. 1985; Yajima, Akita & Saito, 1986) and muscarinic cholinergic agonists (Brown, Wojcikiewicz, Dobson et al. 1984) on stimulated or basal GH and prolactin secretion can be inhibited by pretreatment of cells with pertussis toxin, which is known to ADP-ribosylate and to block the coupling of receptors with Gi and another G protein, Go, of unknown function (Gilman, 1987). Such effects of pertussis toxin on pituitary cells appear to involve reversal of inhibition of adenylate cyclase and reversal of lowering of the intracellular Ca2+ concentration, implying involvement of G proteins in both of these processes (Cronin et al. 1983b; Koch et al. 1985; Lewis, Weight & Liuni, 1986; Yajima et al. 1986; Malgaroli et al. 1987). Work on rat and monkey anterior pituitary glands has demonstrated the presence of three pertussis toxin-sensitive G proteins of M, approximately 39000 (probably Go), 40000 and 41000 (probably Gi) (Zysk, Pobiner, Hewlett et al. 1986; Journot et al. 1987). Doublets have also been observed in a number of other tissues (e.g. Rapięjko, Northup, Evans et al. 1986).

We have previously established a cultured ovine anterior pituitary cell system in which marked inhibitory effects of dopamine and somatostatin on GH and prolactin secretion are not accompanied by substantial changes in cyclic AMP concentrations (Ray & Wallis, 1982a; Law et al. 1985; Ray et al. 1986a, b). This provides a useful model for study of non-cyclic AMP-associated transducing mechanisms. Here we have utilized pertussis toxin to investigate the role of G proteins in the transduction of the inhibitory effects of dopamine and somatostatin on 12-O-tetradecanoylphorbol 13-acetate (TPA) and forskolin-induced increases in hormone release. The diterpene forskolin is an activator of adenylate cyclase (Seamon & Daly, 1986) and the phorbol ester TPA is an activator of protein kinase C (Nishizuka, 1984); their use enables the normal hormone-receptor activation of these enzymes to be by-passed, ensuring that any inhibitory effects observed with dopamine or somatostatin do not reflect simple reversal of Gs activation.


MATERIALS AND METHODS

Materials

Synthetic somatostatin (1–14), TPA, hyaluronidase (type 1–S) and dopamine were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Forskolin was obtained from Calbiochem, San Diego, CA, U.S.A. Ovine prolactin (NIAMDD-oPRL-14) and ovine growth hormone (NIH-GH-S9) were gifts from Dr A. E. Wilhelmi, Dr A. F. Parlow and the National Hormone and Pituitary Program of the NIADDK, NIH, Bethesda, MD, U.S.A. Collagenase (Clostridium histolyticum) and deoxyribonuclease I (grade II from bovine pancreas) were from BCL, Lewes, East Sussex, U.K. All media and sera for cell culture were obtained from Gibco BRL, Paisley, Strathclyde, U.K. Bordetella pertussis toxin was a gift from Dr L. Irons, PHLS Centre for Applied Microbiology and Research, Porton Down, Wilts, U.K.

Methods

Ovine anterior pituitary cells were prepared and incubated as described by Ray & Wallis (1982a). Cells were dispersed from the anterior lobes of pituitary glands taken from lambs (4–12 months of age), using digestion with collagenase (3 mg/ml), hyaluronidase (1 mg/ml) and deoxyribonuclease (0.25 mg/ml). The dispersed cells were pipetted into plastic tissue-culture grade Petri dishes (0.6–1.0 x 10⁶ cells/dish) and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 44 mm NaHCO₃, streptomycin sulphate (100 μg/ml) and penicillin (35 μg/ml), supplemented with 5% fetal calf serum and 10% horse serum. All cell cultures were incubated at 37°C in a humidified atmosphere (air/CO₂; 19:1) for 72–96 h before use in experiments.

Secretion experiments using monolayer cultures of pituitary cells (including use of antioxidant buffer for experiments where effects of dopamine were studied) were performed as described by Ray & Wallis (1982a). However, the cells were incubated in serum-free DMEM with or without pertussis toxin (0.1–100 mg/ml) for 5 h before experimental incubations of 30 min with test substances. At the end of the experimental incubation, media were removed and stored frozen before hormone assay, and cell monolayers were extracted with 5% (w/v) trichloroacetic acid for 2 h at 4°C before estimation of cyclic AMP. The extracts containing cyclic AMP were treated with water-saturated diethyl ether (3 x 5 ml) to remove the trichloroacetic acid. Cyclic AMP was determined by radioimmunoassay as described by Brooker, Harper, Terasaki & Moylan (1979).

Radioimmunoassay procedures for both ovine GH and ovine prolactin were based on methods de-
scribed previously (Ray & Wallis, 1982a). Hormones were labelled with $^{125}$I by the Iodogen method.

Results are expressed as means ± S.E.M., and the statistical significance of differences between means was tested using Student's t-test. All experiments were repeated at least three times, using different cell preparations and various concentrations of pertussis toxin, with similar results.

RESULTS

Effects of pertussis toxin are usually studied after a prolonged exposure of cells to the toxin. Pretreatment periods of 18–24 h are frequently used, though in many cases the effects of the toxin may be seen after a shorter pretreatment (e.g. Brown et al. 1984; Thomas & Hoffman, 1986). In the present study maximal effects of pertussis toxin on hormonal secretion from ovine pituitary cells were observed after a 5-h pretreatment and this period was used routinely. Similar effects to those described here were found for cells pretreated with pertussis toxin for periods between 3 and 24 h.

We have previously studied time-courses for the effects of forskolin, TPA, dopamine and somatostatin on prolactin and GH secretion (Ray & Wallis, 1982a; Ray et al. 1986a, b). In all cases substantial effects were seen after 30 min of incubation, and this time was chosen for the studies described here.

Pretreatment of ovine pituitary cells with pertussis toxin (0.1–100 ng/ml) for 5 h did not significantly alter basal, forskolin-stimulated or TPA-stimulated GH or prolactin release. It also had no effect on the basal or stimulated intracellular concentration of cyclic AMP.

As we have observed previously (Ray et al. 1986a), forskolin (10 μM) markedly stimulated GH secretion and intracellular cyclic AMP concentrations (Fig. 1). The stimulation of GH secretion was completely reversed by somatostatin (100 nM) but cyclic AMP levels were not altered by the peptide. Pretreatment of cells with pertussis toxin prevented the inhibitory effects of somatostatin on GH release in a dose-related fashion, a significant effect (*P < 0.01) being seen with $1 \text{ng}$ pertussis toxin/ml and complete reversal with $100 \text{ng}$/ml (Fig. 1). Pertussis toxin pretreatment did not alter cyclic AMP levels in the presence of somatostatin.

The phorbol ester TPA (100 nM) also stimulated GH secretion markedly and, again, the effect was largely reversed by somatostatin (Fig. 2) as observed previously (Ray et al. 1986b). TPA treatment led to a small increase in cyclic AMP levels (*P < 0.01) which was not affected by somatostatin. We have observed and discussed this stimulatory effect of TPA on cyclic AMP levels in this system previously (Ray et al. 1986b) and others have described similar effects in other systems (e.g. Brostrom, Brostrom, Brotman & Green, 1983; Cronin & Canonicco, 1985; Quilliam, Dobson & Brown, 1985; Cronin, Summers, Sortino

FIGURE 1. Effects of pertussis toxin (PT) treatment on the inhibition of forskolin-stimulated (a) GH secretion and (b) cellular cyclic AMP concentrations by somatostatin. Cultured ovine anterior pituitary cells were pretreated for 5 h with the concentration of PT shown and then incubated for 30 min with forskolin (10 μM) with or without somatostatin (100 nM) as indicated. Concentrations of GH in the medium and cyclic AMP in cell extracts were measured by radioimmunoassay. Values shown are means ± S.E.M. of three observations. Pretreatment with PT reversed the inhibitory effects of somatostatin on forskolin-stimulated GH secretion (*P < 0.01, **P < 0.001; Student's t-test).
& Hewlett, 1986). A possible explanation is that TPA-activated protein kinase C leads to phosphorylation and activation of adenylate cyclase or a protein involved in activation of this enzyme. Pretreatment of cells with pertussis toxin led to a dose-related reversal of the inhibitory effects of somatostatin on GH secretion, a slight but not significant effect being seen with 1 ng/ml and almost complete reversal of the inhibition at 100 ng/ml. Pertussis toxin pretreatment had no significant effect on intracellular cyclic AMP concentrations in the presence of TPA and somatostatin.

Forskolin (10 μM) treatment of ovine pituitary cells gave a modest stimulation of prolactin secretion, as has been observed previously (Ray et al. 1986a). The stimulation was reversed completely by dopamine (100 nM) which inhibited prolactin secretion to about 40% of the control level without altering the much increased cyclic AMP concentration. Pretreatment of cells with pertussis toxin blocked the inhibitory effect of dopamine on forskolin-stimulated prolactin secretion, in a dose-related fashion, although only with the maximal dose of pertussis toxin used (100 ng/ml) was the prolactin secretion greater than the basal level (Fig. 3). Cyclic AMP levels in the presence of forskolin and dopamine were not affected by pertussis toxin pretreatment.

TPA (100 nM) also stimulated prolactin secretion (two- to threefold) and the effect was reversed by dopamine. In contrast to the inhibition of forskolin-stimulated prolactin secretion, inhibition in the presence of TPA and dopamine was not significantly below control levels. Dopamine had no effect on the slight increase (P < 0.01) of cyclic AMP levels seen in the presence of TPA. Pretreatment of cells with pertussis toxin blocked the inhibitory effect of dopamine on TPA-stimulated prolactin secretion, again in a dose-related fashion (Fig. 4); 10 ng pertussis toxin/ml gave a significant effect (P < 0.01) and 100 ng/ml completely reversed the inhibition.

**DISCUSSION**

These studies confirm earlier work indicating that in cultured ovine pituitary cells both dopamine and somatostatin can inhibit hormonal secretion without decreasing increased cyclic AMP levels (Law et al. 1985; Ray et al. 1986a). Although we have examined only effects after a 30-min incubation, our previous studies have established a similar discrepancy between effects on hormone secretion and cyclic AMP levels over a range of incubation times. This contrasts with results obtained using rat pituitary cells (Barnes et al. 1978; Law et al. 1985). Under some circumstances, however, dopamine can reduce the basal cyclic AMP level in ovine pituitary cells (Ray & Wallis, 1982a).

The results presented here suggest that one or more pertussis toxin-sensitive G proteins mediate the inhibitory actions of dopamine and somatostatin on GH and prolactin secretion from ovine anterior pituitary cells, without effects on cyclic AMP levels. G proteins that are known to be pertussis toxinn...
Effects of pertussis toxin (PT) treatment on the inhibition of forskolin-stimulated (a) prolactin secretion and (b) cellular cyclic AMP concentrations by dopamine. Cultured ovine anterior pituitary cells were pretreated for 5 h with the concentration of PT shown and then incubated for 30 min with forskolin (10 μM) with or without dopamine (100 nM) as indicated. Concentrations of prolactin in the medium and cyclic AMP in cell extracts were measured by radioimmunoassay. Values shown are means ± S.E.M. of three observations. Pretreatment with PT blocked the inhibitory effect of dopamine on forskolin-stimulated prolactin secretion (*P < 0.01; **P < 0.001; Student’s t-test).

Effects of pertussis toxin (PT) treatment on the inhibition of TPA-stimulated (a) prolactin secretion and (b) cellular cyclic AMP concentrations by dopamine. Cultured ovine anterior pituitary cells were pretreated for 5 h with the concentration of PT shown and then incubated for 30 min with TPA (100 nM) with or without dopamine (100 nM) as indicated. Concentrations of prolactin in the medium and cyclic AMP in cell extracts were measured by radioimmunoassay. Values shown are means ± S.E.M. of three observations. Pretreatment with PT blocked the inhibitory effect of dopamine on TPA-stimulated prolactin secretion (*P < 0.01; **P < 0.001; Student’s t-test).

Sensitive include Gi and Go (Gilman, 1987), and it is possible that either (or both) of these, and/or possibly an as yet unidentified protein, mediate the inhibitory effects of dopamine and somatostatin. In the rat anterior pituitary, structural and immunological studies indicate that the D-2 dopamine receptor is coupled mainly to Go or an unusual form of Gi (Senogles, Benovic, Amlaiky et al. 1987); it is now clear that several different forms of Gi occur (Beals, Wilson & Perlmutter, 1987; Gilman, 1987; Murphy, Eide, Goldsmith et al. 1987). The situations in rat and ovine pituitaries differ, however, in that in the former dopamine and somatostatin act, at least partly, by inhibiting adenylate cyclase. Pertussis-sensitive G proteins have been implicated in the lowering of intracellular free Ca2+ by dopamine and somatostatin in rat pituitary cells (Koch et al. 1985; Schlegel et al. 1985; Malgaroli et al. 1987), and direct
actions on ion channels may be involved (Lewis et al. 1986; Dunlap, Holz & Rane, 1987). Whether the G protein-mediated effects of dopamine and somatostatin observed here can be explained primarily by effects on Ca\(^{2+}\) concentration is not clear. It is possible that lowering of Ca\(^{2+}\) levels can override the stimulatory effects of cyclic AMP or TPA. However, dopamine can inhibit prolactin secretion in the presence of fairly high concentrations of the Ca\(^{2+}\) ionophore A23187 (Ray & Wallis, 1982b) under which circumstance modulation of Ca\(^{2+}\) concentration seems unlikely. It is possible that a mechanism involving interaction of G protein and calmodulin could act to prevent the actions of Ca\(^{2+}\) on secretion (Katada, Kusakabe, Oinuma & Ui, 1987).

Finally, it should be stressed that the ovine pituitary cell preparation used here is heterogeneous. Lactotrophs and somatotrophs make up the bulk of the cells present (approximately 66 and 24\% respectively of the cells present; Gomm, Ray & Wallis, 1987) but the presence of other cell types complicates the picture. Furthermore, it is likely that both the lactotroph and somatotroph populations are themselves heterogeneous (Walker & Farquhar, 1980; Luque, Munoz de Toro, Smith & Neill, 1986; Gomm et al. 1987). The possibility cannot be excluded that a small sub-population of the lactotrophs present is responsible for most of the secretory responses observed, in which case changes in cyclic AMP occurring within this sub-population could be mediating the actions of dopamine but pass undetected because cyclic AMP levels are unchanged in the majority of lactotrophs. A similar argument could apply to regulation of secretory responses from somatotrophs. If this was the case the argument that blockade of the effects of dopamine and somatostatin by pertussis toxin pretreatment of cells occurs without effects on G-protein(s) linked to adenylate cyclase would not be valid.

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