Modification by pertussis toxin of the responses of bovine anterior pituitary cells to acetylcholine and dopamine: effects on hormone secretion and $^{86}$Rb efflux

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ABSTRACT

Acetylcholine is known to stimulate the secretion of growth hormone and prolactin and the efflux of $^{86}$Rb from bovine anterior pituitary cells; dopamine prevents the stimulation of $^{86}$Rb efflux and of prolactin but not growth hormone secretion. The sensitivity of these responses to pertussis toxin has been determined.

Treatment of bovine anterior pituitary cells in primary culture with pertussis toxin (18 h, 100 ng/ml) did not modify the stimulation of prolactin secretion by acetylcholine, but prevented its inhibition by dopamine. In lactotrophs, dopamine but not acetylcholine receptors are therefore coupled to secretion through a pertussis toxin substrate. The stimulation of $^{86}$Rb efflux by acetylcholine was also unaffected by pertussis toxin and, again, its inhibition by dopamine was prevented.

Treatment of the cells with pertussis toxin enhanced the secretion of growth hormone in response to acetylcholine. Nitrendepine (1 μmol/l) prevented the cholinergic stimulation of growth hormone but not prolactin secretion from these cells. Acetylcholine increased the cytoplasmic calcium concentration and this rise was enhanced by treatment of the cells with pertussis toxin. Nitrendepine partially inhibited the rise in calcium caused by acetylcholine, and prevented the enhancement of the rise following pertussis toxin treatment.

Cholinergic stimulation of growth hormone therefore depends on calcium entry through nitrendepine-sensitive channels, whereas stimulation of prolactin secretion does not, and in somatotrophs a pertussis toxin substrate may limit calcium entry through these channels. These different sensitivities of somatotrophs and lactotrophs to pertussis toxin and nitrendepine may reflect differences in the properties of the predominant calcium currents in the two cell types. J. Endocr. (1988) 116, 393–401

INTRODUCTION

Anterior pituitary cells from many species possess muscarinic acetylcholine receptors (Burt & Taylor, 1980; Mukherjee, Snyder & McCann, 1980). Interestingly, the effects of acetylcholine on pituitary hormone secretion are different in normal bovine pituitary cells and in neoplastic rodent tumour cell lines. Whereas it stimulates secretion of growth hormone (Young, Bicknell & Schofield, 1979) and prolactin (Bicknell & Chapman, 1983) from bovine pituitary slices and cells, acetylcholine is inhibitory in rodent pituitary tumour cell lines, decreasing secretion of adrenocorticotropic hormone from mouse AtT-20 cells (Heisler, Larose & Morisset, 1983) and of prolactin and growth hormone from rat GH3 cells (Onali, Eva, Oliannes et al. 1983). The difference in response suggests that activation of muscarinic receptors is coupled to different intracellular effector systems in the two pituitary preparations.

Two effector systems are frequently associated with the activation of muscarinic cholinergic receptors, the inhibition of adenylate cyclase and the activation of a phosphoinositide-specific phospholipase C. In the heart, the processes which couple the muscarinic receptor to these two effector systems can be distinguished on the basis of their sensitivity to pertussis toxin, which ADP-ribosylates and inactivates the guanine-nucleotide binding proteins $N_\text{i}$ and $N_\text{o}$ (Liang, Hellmick, Neer & Galper, 1986). Pertussis toxin prevents the cholinergic inhibition of adenylate cyclase without affecting the cholinergic activation of phosphoinositide metabolism (Hepler & Harden, 1986).
Cholinergic inhibition of secretion from rodent pituitary tumour cell lines is associated with inhibition of adenylate cyclase (Wojcikiewicz, Dobson, Irons et al. 1984) and a decrease in cytoplasmic calcium concentrations (Schlegel, Wuarin, Zharan et al. 1985). Pretreatment of rodent pituitary tumour cell lines with pertussis toxin prevents all three inhibitory responses (Wojcikiewicz et al. 1984; Schlegel et al. 1985). Cholinergic stimulation of secretion from the bovine pituitary gland is associated with increased phosphatidylinositol labelling, increased efflux of $^{45}$Ca and $^{86}$Rb, and increased cyclic GMP concentrations (Young et al. 1979; Schofield & Saith, 1981) but the effects of pertussis toxin on these responses are not known. However, the effects of dopamine in normal pituitary cells do resemble those of acetylcholine in the tumour cell lines. Dopamine inhibits prolactin secretion and adenylate cyclase in normal pituitary cells, effects which are again prevented by pertussis toxin (Cronin, Myers, Macleod & Hewlett, 1983), and decreases cytoplasmic calcium concentration in bovine pituitary cells (Schofield, 1983). We have therefore compared the effects of pertussis toxin on the responses of bovine anterior pituitary cells to acetylcholine and dopamine.

MATERIALS AND METHODS

Materials

DNase I (Type 11), soya bean trypsin inhibitor (Type 1S), Triton X-100, thyrotrophin-releasing hormone (TRH) and bovine serum albumin were obtained from Sigma Chemical Co. Ltd, Poole, Dorset. Ultroser G, Sephadex G-10, and Pertcoll were purchased from Pharmacia, Milton Keynes, Bucks. Collagenase (Clostridium histolyticum) was from BCL, Lewes, East Sussex. Quin-2 tetra-acetoxymethyl ester (Quin-2/AM) was purchased from Lancaster Synthesis Ltd, Morecambe, Lancs. Pertussis toxin was from Microbial Technology Laboratory (PHLS-CAMR), Porton Down, Salisbury, Wilts. MEM-d-VAL medium for cell culture was obtained from GIBCO Ltd, Paisley, Strathclyde. Radiochemicals were obtained from Amersham International plc, Bucks. Other chemicals were obtained from BDH Ltd, Poole, Dorset.

Cell dispersion procedure

Pituitary glands were removed from heifers (non-lactating females, 16–24 months old) and placed in sterile dispersion buffer at 37 °C. The composition of the dispersion buffer was: 137 mmol NaCl/l, 5 mmol KCl/l, 5 mmol NaHCO$_3$/l, 2.5 mmol NaH$_2$PO$_4$/l, 25 mmol Hepes/l (pH 7.3), 5-6 mmol glucose/l, 0.5 g albumin/l, 0.1 g streptomycin/l and 0.064 g penicillin/l. The glands were sliced, washed, and incubated with shaking for 20 min at 37 °C in 20 ml dispersion buffer containing DNase 1 (0.01 g/l). They were then washed and incubated with shaking for 90 min at 37 °C in 45 ml dispersion buffer containing collagenase (0.25 g/l) and soya bean trypsin inhibitor (0.11 g/l). The cells were dispersed by drawing the slices through plastic pipette tips and undispersed material was removed by filtration through nylon gauze. They were collected by centrifugation, resuspended in isotonic dispersion buffer containing Percoll (40%, v/v) and centrifuged for 10 min at 250 g. This procedure removes damaged cells and some gonadotrophs; lactotrophs and somatotrophs are recovered in the pellet. The yields averaged over 25 preparations were 57 × 10$^6$ cells per g wet weight pituitary before the Percoll separation of which 21.5 × 10$^6$ cells per g wet weight were recovered in the pellet after Percoll, with a viability normally about 95%.

Cell culture and pertussis toxin treatment

The anterior pituitary cells were maintained for up to 6 days on bacteriological grade Petri dishes, at a concentration of 5 × 10$^6$ cells per dish, in MEM-d-VAL supplemented with non-essential amino acids and with 5% (v/v) fetal calf serum, 1% (v/v) Ultroser G, 2 mmol glutamine/l, 0.068 g penicillin/l, 0.1 g streptomycin/l and 2.5 g fungizone/l. Where appropriate, pertussis toxin (final concentration 100 ng/ml) was added to Petri dishes 18–24 h before the experiment.

Isotope loading and perfusion

The procedures for loading and perfusion of the cells have been described previously (Saith, Bicknell & Schofield, 1984). In brief, the experiments were performed in a bicarbonate-buffered salt solution equilibrated with O$_2$:CO$_2$ (95:5, v/v). The cells (10$^7$/ml) were incubated for 150 min in either $^{45}$Ca (60 µCi/ml containing no added calcium) or $^{86}$Rb (50 µCi/ml in medium containing 1 mmol CaCl$_2$/l). At the end of the incubation the cells were diluted with 10 volumes incubation buffer, recovered by centrifugation and resuspended in 1 ml incubation buffer.

Aliquots (10$^6$ cells) were added to each of eight columns (2 cm × 0.4 cm internal diameter) containing 75 mg Sephadex G-10 pre-swollen in incubation buffer. The columns were transferred to a cabinet at 37 °C, incubation buffer was pumped through them at a flow rate of 0.19 ml/min, and 2-min fractions were collected. The first 20 fractions were discarded. At the end of the experiment the cells remaining on the columns were lysed in 2.0 ml Triton X-100 (2.5%, v/v) containing EGTA (5 mmol/l, pH 8.3).
The $^{45}$Ca or $^{86}$Rb contents of the effluents were determined by scintillation and Cerenkov counting respectively. The radioactivity in each 2-min fraction was expressed as a percentage of the total counts on the column at the beginning of that 2-min period. Growth hormone and prolactin concentrations were determined by radioimmunoassay as previously described (Schofield & Saith, 1981), except that the bound hormone was precipitated using polyethylene-glycol 6000 (10%, pH 7.5) in the presence of bovine serum (1:25, v/v) and recovered by centrifugation (2000 g for 20 min). In each experiment duplicate columns were exposed to each set of conditions. Each experiment was repeated at least once.

**Fluorescent measurements**

EGTA (final concentration 2 mmol/l) was added to each Petri dish and the cells were incubated for 10 min and then recovered by centrifugation. They were resuspended ($8 \times 10^6$ cells/ml) in a buffered-saline solution (composition: 129 mmol NaCl/l, 4.7 mmol KCl/l, 2.7 mmol NaH$_2$PO$_4$/l, 4.7 mmol NaHCO$_3$/l, 1 mmol CaCl$_2$/l, 1.2 mmol MgCl$_2$/l, 11.3 mmol Hepes/l (pH 7.4), 5.2 mmol glucose/l, 0.5 g albumin/l) containing Quin-2/AM (20 µmol/l), gassed with O$_2$, and incubated at 37°C for 30 min. The cells were then centrifuged, resuspended ($8 \times 10^6$ cells/ml) in the buffered-saline solution, and stored at room temperature. For fluorescence measurements an aliquot containing $4 \times 10^6$ cells was centrifuged and resuspended in a plastic cuvette in 3 ml buffered-saline solution. The cuvette was examined (excitation 340 nm, emission 492 nm) in a fluorimeter (SLM Instruments, Urbana, IL, U.S.A.) at 37°C and stirred magnetically; the fluorescence was averaged on a 2 s time-base. Each cuvette was calibrated using digitonin and EGTA (Schofield, 1983), and the fluorescence replotted as calcium concentration.

**RESULTS**

**Hormone secretion**

Exposure of bovine pituitary cells to pertussis toxin (100 ng/ml for 18 h) enhanced the secretion of growth hormone in response to acetylcholine (25 µmol/l) but did not greatly affect the secretion of prolactin (Fig. 1). The stimulatory effects of acetylcholine on secretion are therefore affected by a pertussis toxin substrate in somatotrophs but not in lactotrophs. Nitrenepine (1 µmol/l) prevented the stimulation of growth hormone but not prolactin secretion from cells treated with pertussis toxin (Fig. 2), a further difference between the cholinergic responses in the two cell types.

To investigate whether lactotrophs had been affected by the toxin, we examined whether pertussis toxin treatment modified the inhibition of acetylcholine responses by dopamine (Cronin et al. 1983). As previously reported (Saith, Bicknell & Schofield, 1982), in control cells dopamine (10 µmol/l) completely inhibited the secretion of prolactin in response to acetylcholine (25 µmol/l, Fig. 3a), without affecting growth hormone secretion (data not shown). However, in cells pretreated with pertussis toxin the inhibitory effect of dopamine was largely prevented (Fig. 3c).

**Potassium permeability**

Pertussis toxin was also used to compare the mechanisms by which acetylcholine and dopamine affect the potassium permeability of the cells as shown by $^{86}$Rb efflux. As previously reported (Saith et al. 1982), acetylcholine increased $^{86}$Rb efflux and dopamine prevented this efflux response (Fig. 3b). The effects of pertussis toxin on this $^{86}$Rb efflux were similar to those on prolactin secretion. In the cells exposed to pertussis toxin, acetylcholine still caused $^{86}$Rb efflux, but dopamine no longer inhibited that increase (Fig. 3d).

**Cytoplasmic calcium concentration**

The possible participation of pertussis toxin substrates in the regulation of cytoplasmic calcium concentrations was investigated in cells containing Quin-2/AM. As previously reported (Schofield, 1983), dopamine decreased the cytoplasmic calcium in the cells and partially inhibited the response to TRH (Fig. 4a,c). Treatment of the cells with pertussis toxin prevented both of the effects of dopamine, but the response to TRH itself was not affected (Fig. 4b,d). Dopamine therefore affected the cytoplasmic calcium concentration through a pertussis toxin substrate.

As previously reported (Wood & Schofield, 1986) acetylcholine increased the cytoplasmic calcium concentration in bovine pituitary cells (Fig. 5). The rise in calcium concentration caused by acetylcholine was slow but sustained at agonist concentrations below 1 µmol/l and was faster and transient at higher agonist concentrations: the maximum rise was seen at acetylcholine concentrations above 5 µmol/l (Fig. 5). In cells treated with pertussis toxin the basal calcium concentration was the same as in control cells but the rise in calcium caused by acetylcholine was larger, the enhancement being most marked at low agonist concentrations (Fig. 5).

The calcium channel blocker nitrendepine was used to determine whether pertussis toxin enhanced calcium entry through voltage-gated channels. In control cells, the rise in calcium caused by acetylcholine at
Effect of pertussis toxin on the cholinergic stimulation of (a) prolactin and (b) growth hormone secretion from bovine pituitary cells. The cells were maintained in culture for 3 days, the last 18 h being in the presence (○) or absence (●) of pertussis toxin (100 ng/ml). Values are means ± S.E.M. for 12 experiments. Acetylcholine (25 µmol/l) was present from the arrow.

0.2 µmol/l was almost prevented by nitrendipine (1 µmol/l). However, the rise caused by 25 µmol acetylcholine/l was only partially inhibited (Fig. 6) and higher concentrations of nitrendipine did not produce more complete inhibition (data not shown). Nitrendipine (1 µmol/l) completely prevented the enhancement by pertussis toxin of the calcium rise seen at 0.2 µmol acetylcholine/l (Fig. 7).

Changes in cytoplasmic calcium could also reflect mobilization of internally stored calcium. The effects of nitrendipine and pertussis toxin were therefore examined on acetylcholine-induced 45Ca efflux, which reflects, at least in part, the mobilization of internal calcium. If pertussis toxin affected calcium mobilization, it would be expected to modify 45Ca efflux elicited from preloaded cells by acetylcholine. However, treatment of the cells with pertussis toxin did not modify efflux of 45Ca in response to acetylcholine (Fig. 8a). In fact the basal rate of efflux was slightly higher from pertussis toxin-treated cells and this difference persisted when acetylcholine was added (Fig. 8a). This difference in efflux between control and pertussis toxin-treated cells was related to the activity of voltage-gated calcium channels since it was eliminated by treatment with nitrendipine (Fig. 8b).

**DISCUSSION**

Acetylcholine stimulates the release of growth hormone (Young et al. 1979) and prolactin (Bicknell & Chapman, 1983) from bovine anterior pituitary cells. It also increases the cytoplasmic calcium concentration in the cells and this rise in calcium is partially inhibited by nitrendipine (Wood & Schofield, 1986). Thus acetylcholine increases entry of external calcium through voltage-gated channels that are nitrendipine-sensitive, into at least one of the cell types present in the dispersed cell population. It is difficult to assign this effect to a particular cell type unambiguously, but the data reported here that nitrendipine inhibited the stimulation by acetylcholine of the secretion of growth hormone but not prolactin suggest that channels sensitive to nitrendipine are more important in somatotrophs than lactotrophs.

This is consistent with electrophysiological studies which show that the voltage-sensitive calcium current...
FIGURE 3. Effect of pertussis toxin on the ability of dopamine to prevent the cholinergic stimulation of (a and c) prolactin secretion and (b and d) $^{86}$Rb efflux in bovine anterior pituitary cells. Pituitary cells were maintained in culture for 4 days, the last 18 h being in (a and b) the absence or (c and d) the presence of pertussis toxin (100 ng/ml). Values are means of duplicate observations in a representative perfusion in the absence (•) or presence from the first arrow (○) of dopamine (10 μmol/l). Acetylcholine (25 μmol/l) was present from the second arrow. The experiment was performed four times.

FIGURE 4. Effect of pertussis toxin on the ability of dopamine to decrease the cytoplasmic calcium concentration in bovine anterior pituitary cells. Cells from a single preparation were maintained in culture for 3 days, the last 18 h being in (a and c) the absence or (b and d) the presence of pertussis toxin (100 ng/ml). In a and b dopamine (10 μmol/l) was added at the first and TRH (0.1 μmol/l) at the second arrow; in c and d TRH (0.1 μmol/l) was added at the first and dopamine (10 μmol/l) at the second arrow.
in anterior pituitary cells is composed of two components with different characteristics (Armstrong & Matteson, 1985; Lingle, Sombati & Freeman, 1986). In normal rat lactotrophs, identified immunologically, the predominant component of the calcium current is the transient current which would be expected to be nitrendepine-insensitive (Lingle et al. 1986; DeRiemer & Sakmann, 1987). The observation that prolactin secretion is insensitive to inhibition by nitrendepine suggests that insufficient numbers of the second calcium channel, sensitive to the dihydropyridine, are present to affect the cytoplasmic calcium concentration in lactotrophs. Acetylcholine stimulation of prolactin secretion would be insensitive to nitrendepine if it resulted from phospholipase C activation and the consequent release of internally stored calcium and activation of protein kinase C. This could be the mechanism of acetylcholine action in the bovine lactotroph since it stimulates efflux of $^{45}$Ca and increases phosphatidylinositol turnover (Young et al. 1979) and the concentration of inositol triphosphate (Wood & Schofield, 1986). Alternatively, acetylcholine could stimulate calcium entry through a channel not sensitive to nitrendepine.

The mechanism by which acetylcholine stimulates the nitrendepine-sensitive channel in somatotrophs is not clear. We have previously shown that removal of external sodium prevents muscarinic stimulation of growth hormone but not prolactin secretion from the bovine cells (Saith et al. 1984). It is therefore possible that acetylcholine depolarizes somatotrophs by increasing their sodium permeability through either a selective sodium channel or a non-selective cation conductance similar to that reported in rabbit.

**FIGURE 5.** Effect of pertussis toxin on the ability of acetylcholine at a range of concentrations to increase the cytoplasmic free $\text{Ca}^{2+}$ concentration in bovine pituitary cells. Cells from a single preparation were maintained in culture for 3 days, the last 18 h being in the absence (left-hand panels) or presence (right-hand panels) of pertussis toxin (100 ng/ml). Acetylcholine at the concentrations indicated was added at the arrows.

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jejunum smooth muscle (Benham et al. 1985). Alternatively acetylcholine could depolarize the cells by decreasing their potassium permeability (Sims, Singer & Walsh, 1985; Brown, Gähwiler, Marsh, Selyanko, 1986). If so, the observation that cholinergic stimulation actually increases $^{86}$Rb efflux from bovine pituitary cells would require explanation and this is further discussed below.
The data reported here show that the response of normal bovine somatrophs to acetylcholine is limited by a GTP-binding protein sensitive to pertussis toxin. The step affected by pertussis toxin is apparently calcium entry through channels sensitive to nitrendipine since, in the presence of the dihydropyridine, pertussis toxin failed to enhance the rise in calcium concentration and growth hormone secretion caused by acetylcholine. It is interesting that in rodent pituitary tumour cell lines acetylcholine not only inhibits hormone secretion and adenylyl cyclase (Heisler et al. 1983; Onali et al. 1983) but also decreases the cytoplasmic calcium concentration (Schlegel et al. 1985; Heisler, 1985), effects which are also prevented by pertussis toxin (Brown & Brown, 1984; Heisler, 1985; Schlegel et al. 1985). However, acetylcholine does not act in the same way in normal bovine cells and rodent pituitary tumour cells, since it does not stimulate secretion from the tumour cells even after they have been treated with pertussis toxin. It therefore appears to have an additional action in the normal bovine somatroph which leads to increased calcium entry.

The mechanism by which pertussis toxin enhanced the entry of calcium into somatrophs merits brief discussion. It implies that calcium entry is inhibited by a GTP-binding protein. This protein could be activated by acetylcholine itself or be maintained in the active state by a substance released from another cell type in the mixed cell population, and it could affect the calcium channels themselves directly or affect them indirectly by hyperpolarizing the cells. Receptor-mediated inhibition of calcium channels involving the pertussis toxin substrate Nₐ has been reported (Henschler, Rosenthal, Trautwein & Schultz, 1987), and acetylcholine activates potassium channels and 86Rb efflux through a pertussis toxin substrate in atrial cells (Martin, Hunter & Nathanson, 1985; Pfaffinger, Martin, Hunter et al. 1985; Logothetis, Kurachi, Galper et al. 1987; Yatani, Codina, Brown & Birnbaumer, 1987).

The latter possibility appears unlikely. Although the observation that acetylcholine stimulates 86Rb efflux from pituitary cells suggests an action similar to that on the heart, the absence of any effects of pertussis toxin on the efflux implies that different mechanisms are involved in the two tissues. Moreover, in the bovine pituitary, 86Rb efflux appears to be associated with prolactin rather than growth hormone secretion. Thus dopamine prevented both the acetylcholine-induced 86Rb efflux and prolactin secretion, without modifying growth hormone secretion (Saith et al. 1982) and, as reported here, pertussis toxin treatment overcame the inhibitory effects of dopamine on both efflux and prolactin secretion. This is consistent with the suggestion that the 86Rb efflux comes from lactotrophs, and is not related to the control of growth hormone secretion from somatrophs.

The effects of dopamine on bovine anterior pituitary cells are apparently mediated by a GTP-binding protein sensitive to pertussis toxin. Dopamine has been shown to hyperpolarize human prolactinoma cells by increasing their potassium permeability (Israel, Jaquet & Vincent, 1985) and recently a GTP-binding protein has been implicated in the activation of K⁺ channels by dopamine in aplysia (Sasaki & Sato, 1987). Inhibition of 86Rb efflux from bovine anterior pituitary cells by dopamine is therefore surprising. A possible explanation would be that dopamine, acting through a pertussis toxin substrate, hyperpolarizes the cells by activating a K⁺ channel which 86Rb cannot penetrate. The hyperpolarization could prevent activation by acetylcholine of the channel through which 86Rb moves, provided the latter was voltage-sensitive.

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REFERENCES


