The relationship between pertussis-toxin-induced ADP-ribosylation of a plasma-membrane protein and reversal of muscarinic inhibition of prolactin secretion in GH3 cells

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Pertussis toxin (PT) caused the ADP-ribosylation of a Mr-41 000 protein in GH3-cell plasma-membrane preparations. This effect, and muscarinic inhibition of prolactin release, were reversed at similar rates by pretreatment of intact cells with PT. These results suggest that the Mr-41 000 protein is modified in intact GH3 cells, and that this protein (a component of the putative Nt unit of adenylate cyclase) is involved in the expression of muscarinic inhibition.

It is well known that adenylate cyclase is subject to stimulatory and inhibitory control [Murad et al. (1962); see Rodbell (1980) and Limbird (1981) for reviews]. In recent years, it has become apparent that the action of stimulatory agonists on adenylate cyclase activity is mediated by a guanine-nucleotide-binding protein, termed Nt (Rodbell et al., 1971; Kimura et al., 1976; Pfeuffer, 1977; Northup et al., 1980). Evidence also exists (Cooper, 1982; Jakobs et al., 1983; Hildebrandt et al., 1983) supporting a role for a similar protein, Nt, in the mechanism of action of inhibitory agonists. The discovery and characterization of PT (Yajima et al., 1978; Katada & Ui, 1979, 1980, 1981, 1982; Hazeki & Ui, 1981; Kurose et al., 1983), a protein exotoxin from Bordetella pertussis, has greatly facilitated investigation of the putative Nt unit, since, in addition to catalysing the ADPribosylation of a Mr-41 000 guanine-nucleotide-dependent plasma-membrane protein (Katada & Ui, 1982; Kurose et al., 1983), PT also blocks the action of inhibitory agonists on adenylate cyclase activity (Katada & Ui, 1981; Kurose et al., 1983). More recent evidence (Bokoch et al., 1984; Katada et al., 1984a,b,c) strongly suggests that the Nt unit is a Mr-41 000/35 000 dimeric protein, and that reversal by PT of agonist-induced decreases in adenylate cyclase activity is due to an inhibition of the dissociation of the two subunits (Katada et al., 1984b).

In a previous study using GH3 rat anterior-pituitary tumour cells (Brown et al., 1984), we have demonstrated that the muscarinic inhibition of intracellular cyclic AMP accumulation and hormone release (Wojcikiewicz et al., 1984) is reversed by pretreatment with PT. Similar results have been obtained for inhibition of normal anterior-pituitary cells by dopamine and somatostatin (Cronin et al., 1983a,b). These data have been taken as evidence supporting a role for adenylate cyclase inhibition in the mechanism of action of these agents. In this study we have investigated the temporal nature of the effects of PT in anterior-pituitary cells, and show that the rate at which PT causes modification of a Mr-41 000 protein in intact GH3 cells corresponds closely to the rate at which muscarinic inhibition of prolactin secretion is reversed.

Materials and methods

The materials and methods for culture of GH3 cells in this laboratory (passage nos. 100–120) have been described elsewhere (Wojcikiewicz et al., 1984). Bovine serum albumin (fraction V), oxotremorine, CT, acrylamide, dithiothreitol, thymidine, ATP and GTP were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K.;
[α-32P]NAD+ (700 Ci/mmol) was from New England Nuclear, Boston, MA, U.S.A.; Na125I and NCS tissue solubilizer were from Amersham International, Amersham, Bucks., U.K.; and all other reagents were from BDH Chemicals, Poole, Dorset, U.K. PT was prepared from Bordetella pertussis (strain Wellcome 28) as described previously (Robinson & Irons, 1983).

In experiments concerned with the effects of PT pretreatment of cells in culture on subsequent ADP-ribosylation, the medium from confluent dishes of GH3 cells (3-day cultures) was replaced with 4.9 ml of fresh Ham's F10 culture medium. To this was added, at the appropriate time, 0.1 ml of a concentrated solution of PT, giving a final concentration of 50 ng of PT/ml. The cell monolayers were then washed twice at room temperature with 137 mM NaCl/2.7 mM KCl/1.5 mM KH2PO4/8.1 mM Na2HPO4, pH 7.2, scraped from the plates in 137 mM NaCl/5.36 mM KCl/1.1 mM KH2PO4/1.08 mM Na2HPO4, pH 7.2, and plasma membranes were prepared by a modification of the method of Ross et al. (1977). Briefly, the cells were centrifuged at 200 g for 5 min and the pellet was resuspended (3 x 10^7 cells/ml) in 150 mM NaCl/20 mM sodium Hepes/1 mM EDTA, pH 7.4 at 0°C. The cells were disrupted with a Polytron homogenizer (setting 6, 4 x 5 s bursts) and centrifuged at 900 g for 5 min at 0°C. The supernatant was then centrifuged at 43,000 g for 20 min at 4°C and the pellet, a crude plasma-membrane fraction, resuspended in 20 mM sodium Hepes/1 mM EDTA/10% (w/w) sucrose/1 mM dithiothreitol, pH 8, and stored at -70°C. Approx. 200 μg of protein was obtained from each confluent plate (1.5 x 10^7 cells). ADP-ribosylation of GH3 plasma membranes with CT and PT (pre-activated by treatment with 20 mM dithiothreitol for 45 min at 32°C) and subsequent SDS/polyacrylamide-gel electrophoresis were performed under the conditions described previously (Hindebrandt et al., 1983; Rudolph & Krueger, 1979). Gels were stained with Coomassie Brilliant Blue R and dried with a heated vacuum gel drier. 32P in protein bands, assessed by autoradiography, was used as an index of incorporation of ADP-riboyl groups. SDS-7 gel marker proteins (Sigma) were used as M, markers. Protein concentration was determined (Lowry et al., 1951) with bovine serum albumin as a standard.

For the study of the effects of PT on hormone release, GH3 cells (2 x 10^5) were subcultured into 24-well multiwell plates (Falcon) in 1 ml of Ham's F10 culture medium. After 3 days in culture, the medium was withdrawn and replaced with 190 μl of fresh culture medium to which was added, at the appropriate time, 10 μl of concentrated PT to give a final concentration of 50 ng/ml. After removal of this medium and two washes, the monolayers were incubated with or without 10 μM oxotremorine in 1 ml of serum-free Ham's F10 culture medium containing 0.5% bovine serum albumin for 30 min. Media were collected and stored at -20°C until radioimmunoassay with prolactin reference preparation no. 3 (National Pituitary Program, N.I.H., U.S.A.) as a standard.

Results and discussion

PT catalysed the ADP-ribosylation of a Mr 41,000 protein in GH3-cell plasma-membrane preparations (Fig. 1, lanes 5 and 6) that is distinct from the proteins ADP-ribosylated by CT (Fig. 1, lane 1).

Fig. 1. Autoradiograph showing incorporation of [32P]-ADP-ribose into GH3-cell plasma membranes
Membranes (approx. 200 μg of protein) were incubated with 2 μCi of [α-32P]NAD+ and pre-activated CT (20 μg/ml) and PT (10 μg/ml) as described in the Materials and methods section. A portion of each sample after reaction (lanes 3–6) and protein markers (lane 2) were electrophoresed in a SDS/10% polyacrylamide slab gel and an autoradiograph was prepared by using XAR-5 film (Kodak) and a 3-day exposure time. In lane 1, CT and PT were incubated with [α-32P]NAD+ in the absence of membranes, to demonstrate that no auto-ADP-ribosylation occurred. The mean (± S.D.) M, values of proteins radiolabelled in the presence of PT and CT in three similar independent experiments are: PT, M, 40,700 ± 300; CT, M, 43,700 ± 300, 48,200 ± 800 and 50,300 ± 600.
lanes 4 and 6). The $M_r$-41000 protein that is labelled in these and other membranes (Katada & Ui, 1982; Kurose et al., 1983; Aktories et al., 1983) is thought to be associated with the $\alpha$-subunit of $N_i$ (Codina et al., 1983; Katada et al., 1984a), whereas the proteins labelled in the presence of CT ($M_r$ 44000, 49000 and 51000) are thought to be associated with the $\alpha$-subunit of $N_i$ (Northup et al., 1980). Thus, by these and other criteria (Guild & Drummond, 1983; Brown et al., 1984), GH3 cells contain both $N_i$ and $N_i$.

In order to establish whether PT can cause ADP-ribosylation of the $M_r$-41000 peptide in cultured GH3 cells, where effects on secretion and cyclic AMP accumulation have been observed (Brown et al., 1984), a series of experiments were carried out on plasma membranes prepared from cells that had been preincubated with PT (50ng/ml) before membrane preparation. Pretreatment of GH3 cells with PT for increasing lengths of time caused a gradual decrease in subsequent PT-induced radiolabelling of the $M_r$-41000 protein (Fig. 2, lanes 3–9).

These changes in radioactivity were quantified by liquid-scintillation counting of the radiolabelled areas (Fig. 3a). Loss of labelling began to occur...
after a time lag of approx. 60 min and was virtually complete within 3 h. It seems likely, therefore, that pretreatment of intact cells with PT causes a modification of the M410000 peptide (possibly owing to covalent addition of ADP-ribosyl groups) that decreases the number of sites available for subsequent radiolabelling. A time lag preceding the effects of PT in intact cells has been reported elsewhere (Katada & Ui, 1980; Hazeki & Ui, 1981; Brown et al., 1984), and probably reflects the complexity of the interaction of PT with plasma membranes (Moss et al., 1983; Katada et al., 1983).

In a previous study, we demonstrated that inhibition of prolactin release from GH3 cells by oxotremorine, a muscarinic agonist, was blocked by pretreatment of the cells with PT (Brown et al., 1984). Here we have determined the time course of this effect (Fig. 3b). Reversal of oxotremorine-induced inhibition began after a lag of approx. 1 h and was complete within 4 h. These data compare well with that obtained for perfused GH3 cells (Brown et al., 1984), where a time lag of approx. 80 min was observed.

Preincubation of anterior-pituitary cells with PT for 24 h does not affect basal hormone release (Cronin et al., 1983a,b; Brown et al., 1984). However, in the present study, where the cells were exposed to PT for shorter periods of time, basal prolactin release was stimulated (Fig. 3b). It is possible that PT has stimulatory effects on basal hormone release that are evident for only a few hours after exposure to the toxin.

Fig. 3 shows that there is good agreement between the rate at which the putative Nt unit is modified in intact cells and the rate at which muscarinic inhibition of hormone release is blocked. This strengthens the argument that there is a causal relationship between these two events and that agonist-induced inhibition of adenylate cyclase is involved in the action of certain neuropeptides on anterior-pituitary-hormone release (Wojcikiewicz et al., 1984; Cronin et al., 1983a,b).

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References


