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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(Received 11 September 1984/Accepted 20 September 1984)

Pertussis toxin (PT) caused the ADP-ribosylation of a M_r -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 M_r -41 000 protein is modified in intact GH₃ cells, and that this protein (a component of the putative N_i 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 N_s (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, N_i , 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 N_i unit, since, in addition to catalysing the ADP-ribosylation of a M_r -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 N_i unit is a M_r -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 M_r -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.;

Abbreviations used: SDS, sodium dodecyl sulphate; PT, pertussis toxin = islet-activating protein as described by Ui and collaborators (Yajima *et al.*, 1978; Katada & Ui, 1979, 1980, 1981, 1982); CT, cholera toxin; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

[α - 32 P]NAD $^{+}$ (700 Ci/mmol) was from New England Nuclear, Boston, MA, U.S.A.; Na 125 I 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 GH $_3$ 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 KH $_2$ PO $_4$ /8.1 mM Na $_2$ HPO $_4$, pH 7.2, scraped from the plates in 137 mM NaCl/5.36 mM KCl/1.1 mM KH $_2$ PO $_4$ /1.08 mM Na $_2$ HPO $_4$, 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 (3×10^7 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, 4×5 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 μ g of protein was obtained from each confluent plate (1.5×10^7 cells). ADP-ribosylation of GH $_3$ 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. 32 P in protein bands, assessed by autoradiography, was used as an index of incorporation of ADP-ribosyl groups. SDS-7 gel marker proteins (Sigma) were used as M_r 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, GH $_3$ cells (2×10^5) 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 μ l of fresh culture medium to which was added, at the appropriate time, 10 μ l 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 μ M-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 M_r 41 000 protein in GH $_3$ -cell plasma-membrane preparations (Fig. 1, lanes 5 and 6) that is distinct from the proteins ADP-ribosylated by CT (Fig. 1,

Fig. 1. Autoradiograph showing incorporation of [32 P]-ADP-ribose into GH $_3$ -cell plasma membranes

Membranes (approx. 200 μ g of protein) were incubated with 2 μ Ci of [α - 32 P]NAD $^{+}$ and pre-activated CT (20 μ g/ml) and PT (10 μ g/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 - 32 P]NAD $^{+}$ in the absence of membranes, to demonstrate that no auto-ADP-ribosylation occurred. The mean ($\pm$ S.D.) M_r values of proteins radiolabelled in the presence of PT and CT in three similar independent experiments are: PT, M_r 40 700 $\pm$ 300; CT, M_r 43 700 $\pm$ 300, 48 200 $\pm$ 800 and 50 300 $\pm$ 600.

Fig. 2. Autoradiograph showing the effect of pretreatment of cultured GH₃ cells with PT: inhibition of PT-induced incorporation of [³²P]ADP-ribose into plasma membranes

Cultured GH₃ cells were exposed to PT (50 ng/ml) for the times stated, and plasma membranes were prepared as described in the Materials and methods section. Membranes (approx. 200 µg of protein) were incubated with pre-activated PT (10 µg/ml) and an autoradiograph of the electrophoresed samples was prepared as described in Fig. 1. The results shown are from one of three independent experiments with similar results.

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 α -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 α -subunit of N_s (Northup *et al.*, 1980). Thus, by these and other criteria (Guild & Drummond, 1983; Brown *et al.*, 1984), GH₃ cells contain both N_s 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 (50 ng/ml) before membrane preparation. Pretreatment of GH₃ cells with PT for increasing lengths of time caused a gradual decrease in subsequent PT-

Fig. 3. Effects of PT on cultured GH₃ cells

(a) Blockage of PT-induced incorporation of [³²P]ADP-ribose into a M_r -41000 protein. Cultured GH₃ cells were exposed to PT (50 ng/ml) for the times stated as described in the Materials and methods section. Membranes were prepared and were incubated with [³²P]NAD⁺ in the presence (●) or absence (○) of pre-activated PT (10 µg/ml) as described in Fig. 1. Radioactivity in the M_r -39000-43000 region of the gel was quantified by liquid-scintillation counting of NCS-solubilized (90% NCS at 50°C for 2h) fragments. Normalized data for three (with PT; mean $\pm$ s.e.m.) and two (without PT; mean $\pm$ range) separate determinations are shown. (b) Reversal of muscarinic inhibition of prolactin secretion. Monolayers of GH₃ cells were preincubated with PT (50 ng/ml) for the times stated, and incubated in the presence (■) or absence (□) of oxotremorine (10 µM) as described in the Materials and methods section. Data points are for three (with oxotremorine; mean $\pm$ s.e.m.) and two (without oxotremorine; mean $\pm$ range) separate determinations.

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 M_r -41000 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 N_i 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 neurohormones on anterior-pituitary-hormone release (Wojcikiewicz *et al.*, 1984; Cronin *et al.*, 1983a,b).

We thank Dr. Lutz Birnbaumer, Baylor College of Medicine, Houston, TX, U.S.A., for valuable discussions. We are grateful to the National Pituitary Program, National Institutes of Health, U.S.A., for the reagents for the assay of prolactin, and to Ms. W. J. Pontefract for preparation of the manuscript. R. J. H. W. is the recipient of an M.R.C. studentship.

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