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BOTULINUM TOXIN D ADP-RIBOSYLATES A 22-24 KDa MEMBRANE PROTEIN IN PLATELETS AND HL-60 CELLS THAT IS DISTINCT FROM $p21^{N\text{-}RAS}$

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SUMMARY: Botulinum toxin D ADP-ribosylates a 22-24 KDa protein in platelets, GH3 and HL-60 cells, and a mouse T-cell line CTLL. In platelet homogenates the protein is localized to the membrane fraction, and ADP-ribosylation can also be produced in saponin-permeabilized and intact cells. In the latter, the toxin also potentiates secretion caused by a variety of agonists. In platelets and HL-60 cells the toxin substrate is shown, by use of anti-ras monoclonal antibody, to be distinct from the ras family of proteins. This toxin substrate may represent an additional class of proteins involved in stimulus-response coupling. © 1988 Academic Press, Inc.

The neurotoxic effects of a variety of botulinum toxins have been known for quite some time, but the ADP-ribosyltransferase activity in some of these toxins has only recently been appreciated (1-5). Botulinum toxin C2 ADP-ribosylates non-muscle actin thereby impairing its ability to polymerize in vitro (1). Botulinum toxin D (BTX-D), on the other hand, ADP-ribosylates a novel, low molecular weight protein of about 22 KDa in bovine adrenal membranes and membranes of mouse brain and pancreas (6). Since BTX-D inhibits exocytosis in adrenal chromaffin cells (7) and microinjection of human ras oncogene product (p21) results in degranulation of mast cells (8) it has been suspected that BTX-D substrate may be related to the ras family of oncogene products (9) and involved in stimulus-response coupling. The possibility that BTX-D substrate is yet another transducer protein (like the

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G-proteins that are ADP-ribosylated by cholera and pertussis toxins) has already been entertained (9).

In this study we have used human platelets and HL-60 cells as test systems to address the following issues: (a) is the 22-24 KDa botulinum toxin D substrate related to ras proteins, and (b) does the toxin affect platelet function?

METHODS AND MATERIALS

Preparation of Washed Platelets and Cytosol:
Human platelets purchased from the Connecticut Red Cross Blood Center were washed as previously described (10) and suspended at $4x10^9$ platelets/ml in medium containing (in mM) 123 NaCl, 12 sodium citrate, 5.5 KCl, 9 sodium bicarbonate, 1 $MgCl_2$, 5.5 dextrose, 10 Tris HCl and 2 HEPES, pH 7.3. One to 2.0 ml portions of platelet suspension kept on ice for 30 min were sonicated (Branson Sonifier, setting 4) 3 times for 5s each at 2-min intervals. Leupeptin (10 μM) and pepstatin (10 μM) were added and the sonicate was centrifuged at 100,000 x g for 30 min at 4°C in a Beckman TL100 ultracentrifuge to obtain the supernatant designated as cytosol.

Preparation of Membranes.
Platelets were pelleted at 750 x g at 4°C and suspended in 10 ml ice-cold hypotonic medium (10 mM triethanolamine HCl, 5 mM EDTA, 10 μM leupeptin, 10 μM pepstatin; pH 7.4). After 30-45 min on ice the platelets were homogenized by 20 strokes in a stainless steel Dounce homogenizer with a tight fitting pestle. Unbroken cells were removed by centrifugation at 3000 x g for 10 min and the supernatant was centrifuged at 39,000 x g for 30 min. The membrane pellet was washed twice and then resuspended to 1-2 mg protein/ml in ADP-ribosylation medium : 50 mM triethanolamine HCl, 1 mM EDTA, 10 mM thymidine, 5 mM $MgCl_2$, 0.5 mM ATP, final pH 7.4. Membranes from HL-60, GH3 and CTLL cells were prepared and treated in essentially the same way.

Treatment of Intact Platelets with Botulinum Toxin D.
Botulinum toxin D (Wako Chemicals, Dallas,TX), activated with 10 mM DTT for 10 min at 30°C, was added to 10^9/ml washed platelets at a final concentration of 80 μg/ml. After 1 h at 30°C the platelets were diluted 60-fold with ice-cold hypotonic medium and allowed to stand on ice for 30-45 min. The swollen platelets were then used for preparing membranes, as described above.

ADP-Ribosylation of Proteins:
ADP-ribosylation was carried out with [^{32}P]NAD (New England Nuclear, 800 Ci/mmol): 50 μCi/ml (1 μM) [^{32}P]NAD plus 80 μg/ml of preactivated botulinum toxin. After incubation at 37°C for 1 h the reaction was terminated by an equal volume of ice-cold 20% trichloroacetic acid. The precipitate was washed with ether and analyzed essentially as described by Laemmli (11) on 7-17% polyacrylamide gradient gels containing SDS. The ADP-ribosylated bands were detected by autoradiography.

Biosynthetic Labeling of HL-60 Cells.
HL-60 cells (6 x 10^7) were washed three times with methionine-free modified Eagle's medium (MEM) and cultured at 1 x 10^7/ml in

the same medium containing 15% fetal calf serum and 300 μCi [^{35}S]-methionine (ICN Radiochemicals) for 5 h at 37°C. The cells were then washed with ice cold MEM containing methionine before solubilizing in 0.5% SDS prior to immunoprecipitation.

Immunoprecipitation of p21 Using Monoclonal Antibody ras-11-pan. [^{35}S]-methionine labelled HL-60 cells or ADP-ribosylated platelet and HL-60 membranes were solubilized in 250 μl of 0.5% SDS at room temperature for 10 min. The SDS was diluted to 0.1% with buffer A [150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 1 mM PMSF]. Samples were centrifuged at 13,000 x g for 5 min and the detergent soluble supernatant was used for immunoprecipitation. 40 μl of mouse monoclonal antibody ras-11-pan (Du Pont, NEI-704) or 5 μg of mouse IgG_{2b} (Sigma Chemical Co.) were added to 550 μl of soluble fraction and incubated overnight at 4°C with constant mixing. Samples were then mixed for 3 hours with 20 μg of affinity purified goat anti-mouse IgG heavy and light chain specific antibody (Organon Teknika) followed by 150 μl of 50% Protein A-Sepharose (Pharmacia P-L Biochemicals) in PBS. After 3 hours the samples were centrifuged and Protein A-Sepharose washed 3 times with buffer A containing 0.1% SDS and twice with PBS. The proteins bound to Protein A-Sepharose were eluted with Laemmli sample buffer (containing 2% SDS, 5% mercaptoethanol) or 0.2 M glycine-HCl, pH 2.9 containing 25 mM N-octylglucoside, then immediately adjusted to pH 7.2 with Tris and analyzed by SDS-PAGE.

RESULTS AND DISCUSSION

Incubation of saponin-permeabilized platelets (12) with BTX-D and [^{32}P]NAD caused ADP-ribosylation of a 22-24 KDa protein in a dose-dependent manner (Fig. 1A). The substrate is localized in the crude membrane fraction with virtually no ADP-ribosylation in the cytosol (Fig. 1B). A similar protein was also labelled in membrane preparations of HL-60 cells (kindly provided by Dr. F. Rickles, UCONN Hlth. Ctr.), GH3 cells and a mouse interleukin-2-dependent cytotoxic T cell line (CTLL cells, kindly provided by Dr. B. Woda, Univ. Mass. Med. Ctr, Worcester, MA) (not shown).

The apparent molecular weight (22 KDa) of the polypeptide ADP-ribosylated by BTX-D suggested that it might be the 21 KDa ras proto-oncogene product ($p21^{N\text{-}ras}$). It has been reported that BTX-D does not ADP-ribosylate purified human N- and Ha-ras (15). Nevertheless, we chose to investigate this possibility directly in our systems by using a monoclonal antibody (Ras-11-Pan) which immunoprecipitates all three members of the ras oncogene family

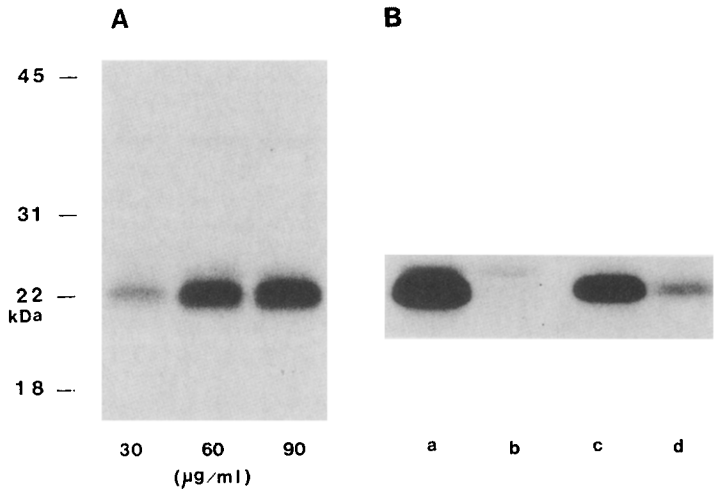

Figure 1. ADP-Ribosylation by Botulinum Toxin D. (A) Platelets (10^9/ml) permeabilized with saponin (15 μg/ml) were incubated with 30, 60 or 90 μg/ml toxin for 60 min at 37°C and [^{32}P]ADP-ribosylated protein identified after SDS-PAGE by radioautography. (B) Platelet sonicate (a) or cytosol (b) were treated with 80 μg/ml botulinum toxin D (BTX-D) and [^{32}P]NAD and analyzed as above. Intact platelets were incubated for 1 h at 30°C in the absence (c) or presence (d) of 80 μg/ml BTX-D before preparing membranes. The membrane fractions were then incubated with toxin and radioactive NAD to permit ADP-ribosylation.

(Ha-, Ki- and N-ras) and detects both the normal and oncogenic forms of ras p21 (13). HL-60 cells, which contain $p21^{N\text{-}ras}$ (14), were used as a positive control. The antibody (Ras-11-pan) did immunoprecipitate the 21 KDa ras protein from [^{35}S]methionine-labelled HL-60 cells (Fig. 2C). In contrast, the antibody failed to immunoprecipitate the [^{32}P]ADP-ribosylated protein from platelet or HL-60 membranes that were treated with BTX-D plus [^{32}P]-NAD (Fig. 2A,B). We conclude from this data that the toxin substrates in platelets and HL-60 cells are distinct from the ras proteins. This is in agreement with studies in rat brain cortex and bovine adrenal medulla (15) in which rabbit polyclonal antibody against $p21^{N\text{-}ras}$ failed to immunoprecipitate toxin substrate.

Apart from Gs and Gi there are other platelet GTP-binding proteins in the molecular weight range as the botulinum toxin D

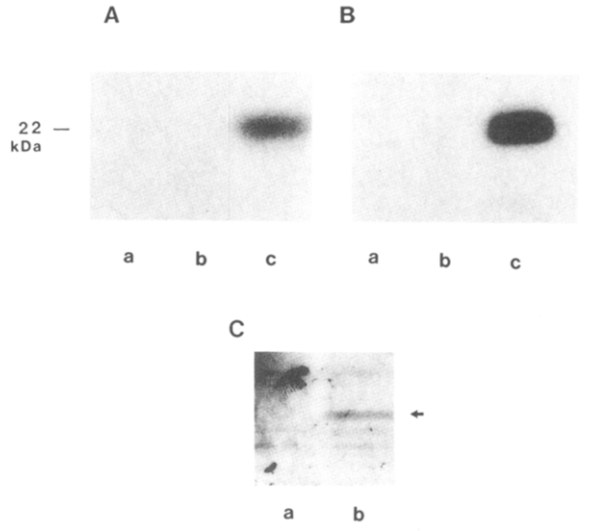

Figure 2. Immunoprecipitation with mouse anti-ras antibody. Detergent solublized fractions from ADP-ribosylated platelet (A), HL-60 cell (B) or [^{35}S]methionine labelled HL-60 cell (C) membranes were immunoprecipitated with mouse anti-ras antibody (a) or mouse IgG2b (b). The immunoprecipitates were analyzed by SDS gel electrophoresis followed by radioautograph. Lane (c) shows radioautograph of 0.1 of the amount of ADP-ribosylated membranes used for immunoprecipitation. The arrow in (C) shows position of immunoprecipitated N-ras from HL-60 cells.

substrate (16). ADP-ribosylation of Gi (the pertussis toxin substrate) is greatly influenced by agents like thrombin, PMA and prostaglandin D_2, probably due to their effects on G-protein oligomer dissociation (10). To test the effect of various agents on BTX-D substrate, ADP-ribosylation was carried out in membranes prepared from platelets that were incubated with thrombin, epinephrine, arachidonic acid, prostaglandin D_2, dibutyryl cyclic AMP, A23187 or phorbol diester. As expected (10), thrombin (1U/ml) caused a dramatic decrease in ADP-ribosylation of 41 KDa pertussis toxin substrate, but ADP-ribosylation mediated by botulinum toxin D was not altered appreciably by any of the agents listed (not shown).

Unlike pertussis toxin (17), botulinum toxin D is apparently able to enter platelets and act on its substrate as shown in figure 1B. Preincubation of intact platelets with 80 ug/ml of

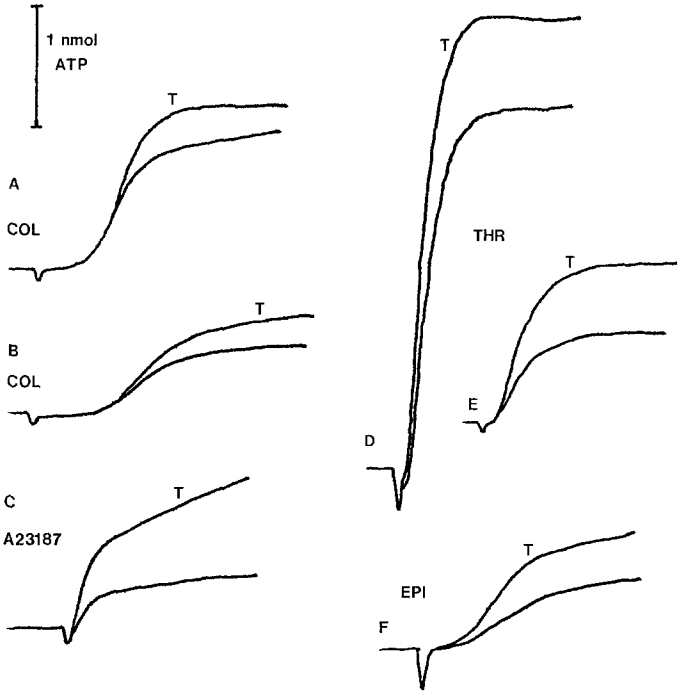

Figure 3. Effect of BTX-D on Platelet Secretion. Intact platelets were incubated with 80 μg/ml BTX-D activated with dithiothreitol (T) or with dithiothreitol (1.25 mM) alone for 60 min at 30°C. $2x10^8$/ml platelets were then stimulated with various agonists: A - collagen 4 μg/ml; B - collagen 2 μg/ml; C -A23187 0.5 μM; D - thrombin 1 U/ml; E - thrombin 0.25 U/ml; E - epinephrine 100 μM. ATP secretion was measured (19) with Chronolume reagent (luciferin, luciferase; Chronolog Corp.) in a Lumiaggregometer (Chronolog Corp.).

toxin for 1 h at 30°C (fig. 1B,d) substantially reduces (63%) subsequent ADP-ribosylation of 22 KDa protein compared to membranes from untreated cells (fig. 1B,c). Under the same conditions secretion due to thrombin, collagen, epinephrine or A23187 was potentiated (Fig.3). A similar action was recently reported in human neutrophils (18). The toxin's stimulatory effect on secretion in these two cell types clearly differs from the inhibition of exocytosis observed in bovine medullary cells (7). The ability of BTX-D to potentiate secretion in platelets suggests a potential role for the substrate protein in stimulus-response coupling.

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