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Botulinum toxin-induced ADP-ribosylation and inhibition of exocytosis are unrelated events

V. Adam-Vizi, S. Rosener*, K. Aktories* and D.E. Knight

MRC Secretory Mechanisms Group, Division of Biomedical Sciences, King's College, London WC2R 2LS, England and **Rudolf-Buchheim-Institut für Pharmacologie, Justus-Liebig-Universitat, D-6300 Gießen, FRG*

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The hypothesis that inhibition of secretion by botulinum neurotoxin type D occurs by an intracellular process involving ADP-ribosylation has been directly tested by measuring both the extent of inhibition of secretion and of ADP-ribosylation in the same cells. Although the inhibitory effect of unpurified toxin closely parallels intracellular ribosylation, the two events are clearly unrelated, as using purified D and C3 toxins together with their antibodies, each of these events can be either stimulated or inhibited independently of each other.

Botulinum toxin; ADP-ribosylation; Exocytosis; Catecholamine; (Adrenal medulla)

1. INTRODUCTION

The mechanism by which botulinum neurotoxins inhibit exocytosis is unclear [1] but in the case of the adrenal medullary cell they seem to act at or near the site of exocytosis itself, rather than inhibiting the $[Ca^{2+}]_i$ transient normally associated with secretion [2,3]. One popular hypothesis is that the neurotoxins are internalised by the cell, and secretion blocked when a small protein fragment is revealed [4–6]. Other toxins, such as cholera and diphtheria, express their potencies through the ADP-ribosylase transferase activity of such a small fragment. As there are marked structural similarities between these toxins and botulinum toxins, the idea has arisen that perhaps botulinum toxins might inhibit secretion by a similar method, i.e. one in which intracellular processing of the toxin reveals a small protein fragment which ADP-ribosylates a key protein associated with exocytosis. Although two neurotoxins (types C1 and D) have been reported to ADP-ribosylate a low molecular mass protein in the homogenate of adrenal medullary tissue [7–9], other evidence suggests that the ADP-ribosyltransferase activity is not associated with the whole neurotoxin but rather to a small protein 'C3' [10–12] present with, but perhaps unrelated to, the larger whole neurotoxin [14–16]. By exposing electropermeabilised chromaffin cells to both toxins and labelled NAD we have been able to directly measure the extents of secretion and the associated levels of ADP-ribosylation. This paper therefore describes a series of experiments which address the following questions: (i) Is the ribosylation pattern triggered by toxin D the same as that triggered by toxin C3? (ii) Is the inhibitory effect on secretion of toxin D mimicked by toxin C3? (iii) Using purified toxins and their antibodies, can the botulinum toxin type D-induced events of ribosylation and inhibition of secretion be separated? Our results clearly show that botulinum toxin-induced ADP-ribosylation and inhibition of secretion are distinct and unrelated events, and as such argue against a common underlying mechanism of action for both botulinum neurotoxin and cholera/diphtheria toxins.

Correspondence address: V. Adam-Vizi, MRC Secretory Mechanisms Group, Division of Biomedical Sciences, King's College, London WC2R 2LS, England

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2. MATERIALS AND METHODS

Three types of botulinum toxin were used, (a) unpurified toxin type D (gift from E. Schantz, Wisconsin, USA), (b) purified toxin type C3 [10,12,14], and purified toxin type D (purified [17] and provided by Wako Co., Japan). Antibody to purified D neurotoxin was obtained from the National Institute for Biological Standards and Control, London, England, and the antibody to purified C3 toxin prepared as described previously [14].

Bovine chromaffin cells were isolated and maintained in culture for 24 h as described previously [2] before being suspended at 0°C in a potassium glutamate based medium [18] containing 150 mM potassium glutamate, 4 mM Mg acetate, 2 mM MgATP, 20 mM Pipes, 18 mM thymidine, 0.1 mM GTP, 0.4 mM EGTA, pH 6.6, and 12 μM [^{32}P]NAD (10 μCi/ml). The cells were incubated at 0°C for 15 min with various concentrations of toxin before being rendered leaky by exposure to brief intense electric fields [18] (10 exposures of 2 kV/cm, τ 200 μs). After a further 10 min at 0°C followed by 15 min at 37°C the Ca-dependent secretory response and the corresponding extent of ADP-ribosylation were determined. Secretion was measured by the amount of catecholamine secreted over a further 15 min period in response to raising the Ca^{2+} level from 0.01 μM to 10 μM using Ca-EGTA buffers [18]. The extent of ribosylation was determined by precipitating the cell proteins in 12.5% trichloroacetic acid (TCA), centrifuging at 0°C for 5 min at 12000 × g, washing the pellet in ethanol and ether, solubilising in Laemmli's sample buffer and then electrophoresing on an SDS-polyacrylamide gel [19] or on a 2D gel [20]. The extent of ribosylation was quantified from densitometer traces of autoradiograms.

3. RESULTS AND DISCUSSION

Botulinum toxin type D inhibits both basal and evoked secretion from cultured bovine adrenal medullary cells [2,21]. The incubation time and doses needed to inhibit catecholamine secretion however are relatively high compared to that needed to block acetylcholine release at the neuromuscular junction. For example 5 μg of unpurified D toxin takes several days to block catecholamine secretion from cultured chromaffin cells whereas 0.5 ng is sufficient to inhibit acetylcholine release at the neuromuscular junction within 24 h. The long incubation times are likely to reflect the inaccessibility of the toxin to the interior of the cell due to the effective barrier of the plasma membrane. The toxin is far more effective, however (operating within minutes rather

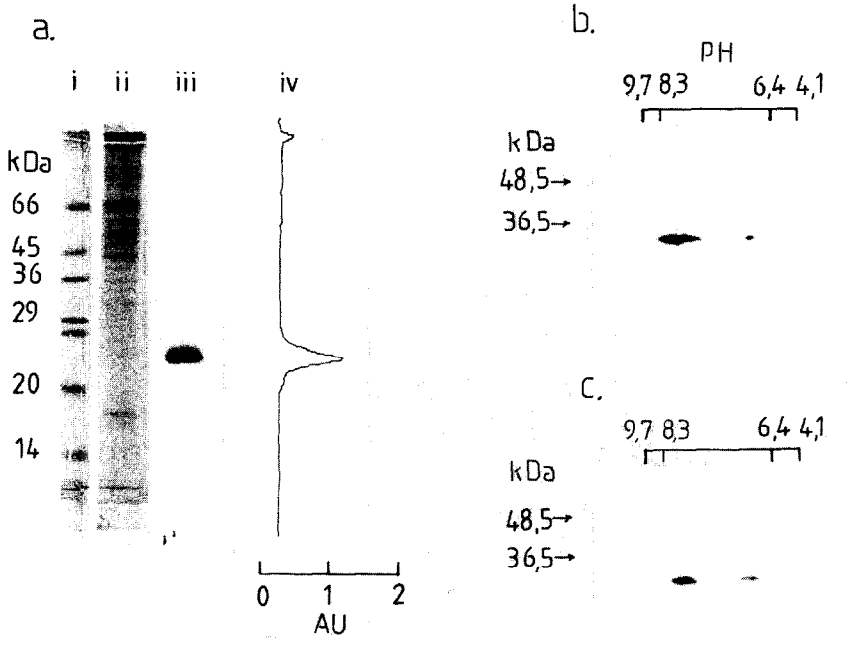

Fig.1. (a) Unpurified botulinum toxin type D exposed to leaky chromaffin cells ADP-ribosylates a clearly visible protein close to 24 kDa. (i) Molecular mass standards, (ii) Coomassie blue stained gel, (iii) ^{32}P-labelled autoradiogram, (iv) densitometer trace of autoradiogram. (b,c) Autoradiograms showing that the intracellular protein, ADP-ribosylated by purified toxin C3, comigrates in 2D electrophoresis with that ADP-ribosylated by impure botulinum toxin type D. Electropermeabilised cells were incubated with either 6 μg/ml of impure type D neurotoxin (a,c), or with 0.5 μg/ml of purified toxin C3 (b).

than days), when exposed to chromaffin cells in which the plasma membrane has been rendered 'leaky' by electric fields and the cells subsequently triggered to secrete by a micromolar Ca^{2+} challenge [18,22]. As this technique 'peppers' the secretory cell with holes of 4 nm effective diameter [18], [^{32}P]NAD can also be introduced into the cytosol allowing the extent of ADP-ribosylation to be measured alongside Ca^{2+}-dependent secretion. Such experiments show that when leaky cells are incubated with unpurified toxin D, both Ca^{2+}-dependent secretion is inhibited and an intracellular protein with an approximate molecular mass of 24 kDa (P24) is ADP-ribosylated [23]. Fig.1a shows this clear ribosylation pattern seen in toxin-treated electropermeabilised cells. The toxin dose response curves, incubation times, and temperature dependences for both the ribosylation of P24 and inhibition of secretion in the same leaky cells are very similar. For example, when leaky cells are exposed at 37°C to 5 μg of unpurified toxin D, half-maximal ribosylation and inhibition of secretion occurs within 4 min, whereas at 20°C 11 min of incubation are needed for both, and at 0°C the toxin is virtually without effect for over half an hour.

In order to test if the ribosylation event is associated with the neurotoxin or with a contaminant, such as C3 toxin, or what its relationship is to the inhibitory process, electropermeabilised chromaffin cells were exposed to purified toxins C3 and D. C3 toxin ADP-ribosylates a 24 kDa intracellular protein in leaky chromaffin cells that comigrates on 2D gel electrophoresis with the protein ribosylated by impure toxin D (fig.1b,c). The sites of ribosylation on this protein are probably the same as the extent of ribosylation induced by saturating levels of C3 when no further enhancement is obtained by the addition of botulinum toxin. Fig.2 shows the effect on secretion and ribosylation of introducing C3 toxin and purified toxins into the leaky cells. Although C3 fully ADP-ribosylates P24, secretion remains largely unaffected. Purified toxin D however, when presented to the leaky cells, fully inhibits secretion but shows little ribosylase activity. The activity associated with pure neurotoxin is less than 0.1% of that induced by an equivalent weight of the unpurified toxin D.

Incubation of C3 toxin with its antibody

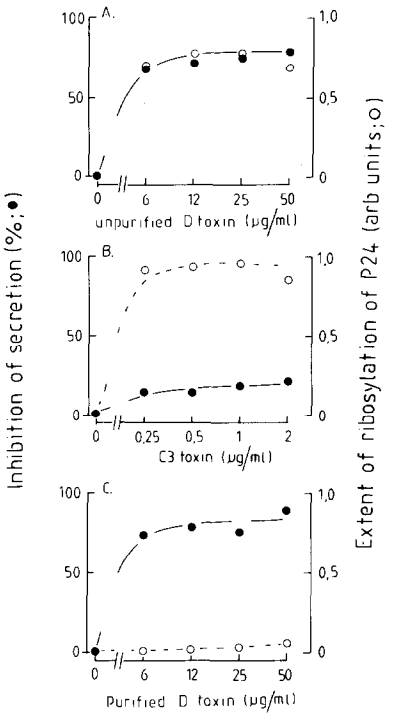

Fig.2. Effect of botulinum toxins on the Ca-dependent secretory response and on the extent of ADP-ribosylation in leaky chromaffin cells. The left-hand ordinate shows the extent of inhibition of Ca-dependent secretion (•) and the right-hand ordinate of the extent of ribosylation in the cells (○). Cells exposed to (A) impure D toxin, (B) C3 toxin, and (C) purified toxin D. The amount secreted by cells not exposed to toxins was 16% of the total cellular catecholamine content.

neutralises its ADP-ribosyltransferase activity [14]. Fig.3 shows that this antibody also neutralises the ADP-ribosyltransferase activity of unpurified toxin D, but leaves unaltered the inhibitory effect on secretion of the toxin. Conversely the inhibitory effect of this unpurified toxin can be removed, but its ADP-ribosyltransferase activity left unaltered, by incubating with an antibody raised to purified toxin D. These data clearly separate the ribosylation and inhibitory steps triggered by botulinum toxin.

Further evidence dissociating these two steps comes from the finding that the presence of C3 toxin, or the absence of NAD in the medium bathing the leaky cell do not alter the potency of the neurotoxin to block secretion. Furthermore treatment of toxin D with dithiothreitol, an agent which might be expected to release an active fragment [5,6], does not seem to alter the potency of

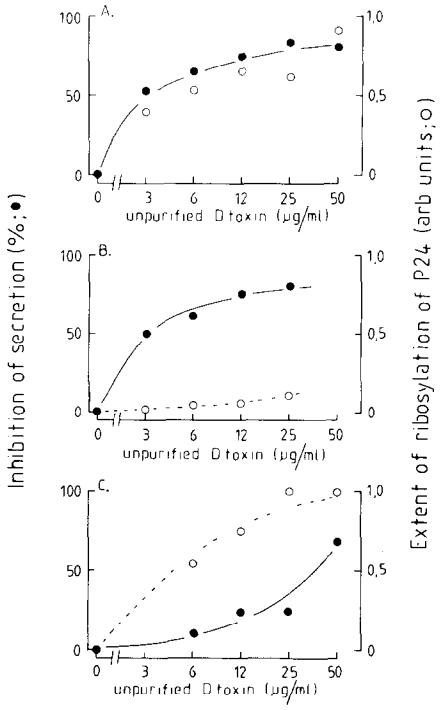

Fig.3. The ribosyltransferase component, but not the inhibitory component, of the impure neurotoxin D can be blocked by antibody to toxin C3, whereas the inhibitory effect, but not the ribosyltransferase activity can be removed by the antibody to the purified neurotoxin D. (A) The inhibitory effect and extent of ribosylation in cells exposed to the impure neurotoxin type D. (B) cells exposed to the impure toxin D preincubated for 15 min at 0°C with antibody to toxin C3. The amount of antibody used was sufficient to neutralise the ribosyltransferase activity of 1 μg of C3. (C) Cells exposed to the impure toxin D preincubated as above with 5 IU of antibody to purified neurotoxin D (from the National Institute for Biological Standards and Control, London, England). The catecholamine secreted by cells not exposed to toxins was 14% of the cellular content.

the toxin to block secretion. Finally if ADP-ribosylation were involved in the mechanism of botulinum poisoning, then residual ADP-ribosyltransferase activity might be expected to be present in the lysates of cultured chromaffin cells in which exocytosis had been blocked by botulinum toxins. Cells poisoned by purified botulinum toxin type D (or A) however, do not possess such ribosyltransferase activity (see also [24]). Cells poisoned with unpurified D toxin do possess this activity, but as it can be neutralised by C3 antibodies, it is not attributed to the neurotoxic component of the toxin.

Taken together these data argue strongly against a role of ADP-ribosylation in the mechanism by which botulinum toxins inhibit secretion.

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