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Botulinum ADP-ribosyltransferase C3 but not botulinum neurotoxins C1 and D ADP-ribosylates low molecular mass GTP-binding proteins

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Botulinum ADP-ribosyltransferase C3 modified 21–24 kDa proteins in a guanine nucleotide-dependent manner similar to that described for botulinum neurotoxin C1 and D. Whereas GTP and GTPγS stimulated C3-catalyzed ADP-ribosylation in the absence of Mg^{2+}, in the presence of added Mg^{2+} ADP-ribosylation was impaired by GTPγS. C3 was about 1000-fold more potent than botulinum C1 neurotoxin in ADP-ribosylation of the 21–24 kDa protein(s) in human platelet membranes. Antibodies raised against C3 blocked ADP-ribosylation of the 21–24 kDa protein by C3 and neurotoxin C1 but neither cross reacted with neurotoxin C1 immunoblots nor neutralized the toxicity of neurotoxin C1 in mice. The data indicate that the ADP-ribosylation of low molecular mass GTP-binding proteins in various eukaryotic cells is not caused by botulinum neurotoxins but is due to the action of botulinum ADP-ribosyltransferase C3. The weak enzymatic activities described for botulinum neurotoxins appear to be due to the contamination of C1 and D preparations with ADP-ribosyltransferase C3.

ADP-ribosylation; Botulinum ADP-ribosyltransferase C3; Botulinum-neurotoxin C1; Botulinum neurotoxin D; GTP-binding protein

1. INTRODUCTION

Various strains of *Clostridium botulinum* produce at least eight different toxins which belong to the most potent biological active substances so far known. Seven of the eight toxins (A, B, C1, D, E, F, G) are neurotoxic and block the neurotransmitter release at cholinergic but also at various other synapses by a still unknown mechanism [1]. The binary botulinum C2 toxin is not neurotoxic but cytotoxic and acts by ADP-ribosylating non-muscle actin of the target cell thereby severely reducing the ability of actin to polymerize [2].

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Recently it has been reported that also botulinum neurotoxins C1 and D possess ADP-ribosyltransferase activity [3–5]. Both toxins reportedly ADP-ribosylate 21–26 kDa GTP-binding proteins supposedly involved in exocytosis [3–5].

Previously we described the purification of an additional botulinum ADP-ribosyltransferase C3 from the culture filtrates of *C. botulinum* type C [6]. The enzyme has a molecular mass of about 25 kDa and is, thus, distinct from botulinum neurotoxins (150 kDa) and the binary botulinum C2 toxin (50 and 100 kDa, component I and II, respectively) also produced by *C. botulinum* type C [1,2,6]. The substrate of botulinum ADP-ribosyltransferase C3 appears to be 21–24 kDa protein(s) found in membranes of human platelets, chicken embryo fibroblasts, PC12-cells,

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neuroblastoma × glioma hybrid cells, S49 lymphoma cells and various other cell types [6,7]. Since C3-catalyzed ADP-ribosylation of the eukaryotic protein substrate is regulated by guanine nucleotide [7] as reported also for neurotoxins C1 and D [4,5] and since all these agents are produced by *C. botulinum* type C we studied whether they are structurally and immunologically related. From these studies we conclude that the ADP-ribosylation of low molecular mass GTP-binding proteins in various eukaryotic cells is not caused by botulinum neurotoxins C1 and D but by botulinum ADP-ribosyltransferase C3.

2. MATERIALS AND METHODS

2.1. *Materials*

Botulinum ADP-ribosyltransferase C3 was purified as described [6] from culture filtrate of *C. botulinum* type C strain 4/12 kindly donated by Dr Semler (Gießen, FRG). Botulinum C1 neurotoxin was purified from *C. botulinum* type C strain C 250 (donated by Dr G. Sakaguchi, Osaka, Japan) according to the method of Kurazono et al. [8] and was kindly donated by Dr J. Frevert (Frankfurt, FRG). All nucleotides used were obtained from Boehringer (Mannheim, FRG). [^{32}P]NAD was prepared as described by Cassel and Pfeuffer [9].

2.2. *Preparation of human platelet membranes*

Human platelet membranes were isolated as described [10], lysed by freezing and thawing in a hypotonic buffer containing 10 mM triethanolamine-HCl (pH 7.4) and 5 mM EDTA. Crude platelet membranes were obtained by centrifugation of the lysate for 15 min at 30000 × *g*. The pellet was washed twice with hypotonic buffer then used for the ADP-ribosylation assay.

2.3. *ADP-ribosylation assay*

ADP-ribosylation was carried out essentially as described [6]. The reaction medium contained 10 mM thymidine, 0.5 mM ATP, 1 mM EDTA, 0.5 μM [^{32}P]NAD (0.3–1 μCi), the indicated concentrations of C3 and 50 mM triethanolamine-HCl (pH 7.4) in a total volume of 100 μl. Protein concentration was 50–200 μg/tube. After incubation for 15 min at 37°C the reaction was stopped by adding 1 ml trichloroacetic acid (20%, w/v). The formed pellet was washed with ether and dissolved in 50 μl electrophoresis buffer. SDS-gel electrophoresis was performed according to Laemmli [11]. Gels were stained with Coomassie blue, destained and subjected to autoradiography for 48 h. Alternatively, after the incubation time of 15 min the reactions were stopped by the addition of 400 μl of a solution containing SDS (2%, w/v), bovine serum albumin (1 mg/ml) and precipitation of the proteins with 500 μl of trichloroacetic acid (30%, w/v). Proteins were collected onto nitrocellulose filters. The filters were washed 10 times with 2 ml of 6% trichloroacetic acid and placed in scintillation fluid for counting of retained radioactivity.

2.4. *Immunoblotting*

Immunoblotting was performed according to Towbin et al. [12] with rabbit anti-C3 serum (1:100), peroxidase-coupled swine IgG to rabbit

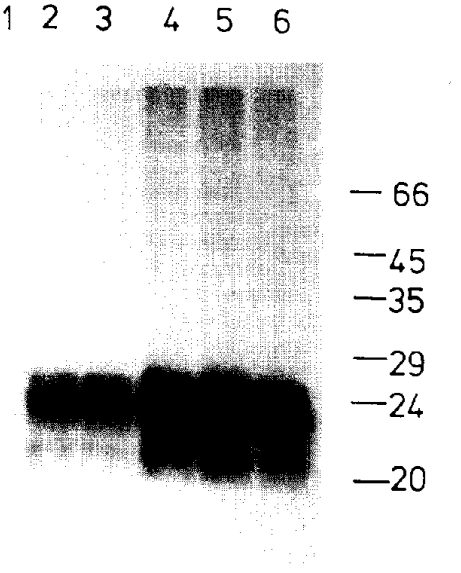

Fig.1. ADP-ribosylation of human platelet proteins by botulinum ADP-ribosyltransferase C3. SDS-polyacrylamide (15%) gel analysis of radioactive products resulting from ADP-ribosylation of human platelet membrane proteins by C3 (35 ng) without added $MgCl_2$ (lanes 1–3) and with $MgCl_2$ (5 mM, lanes 4–6) in the absence (lanes 1,4) and presence of GTP (100 μM, lanes 2,5) and GTPγS (100 μM, lanes 3,6).

Table 1

ADP-ribosylation of the 21 kDa platelet protein by botulinum ADP-ribosyltransferase C3 and botulinum neurotoxin C1

ADP-ribosyltransferase	Activity (pmol ADP-ribose/mg protein per 5 min)
C3 (ng/100 μl)	
0.4	0.45
4.	2.6
40	8.4
C1 (ng/100 μl)	
70	0.085
700	0.54

Human platelet membrane proteins were ADP-ribosylated with the indicate concentrations of C3 or C1 for 30 min as described in section 2 with the exception that 1 mM $MgCl_2$ were presented and EDTA was omitted. The radioactivity of the labelled proteins was determined by the filter method. The data are means of duplicate observations. The amount of ADP-ribose is expressed per mg of platelet membrane protein

IgG (Dakopatts) as second antibody and 4-chloro-1-naphthol and H_2O_2 as peroxidase substrate.

3. RESULTS AND DISCUSSION

It has been reported that botulinum neurotoxin C1 and D possess ADP-ribosyltransferase activities [3–5] which were speculated to be involved in toxin inhibition of exocytosis processes. Both toxins reportedly ADP-ribosylate identical 21–26 kDa protein substrates in pheochromocytoma and primary-cultured mouse brain or rat brain preparations [3–5]. ADP-ribosylation by these neurotoxins was dependent on magnesium ions and guanine nucleotides [4,5]. As with botulinum neurotoxins, magnesium ions and guanine nucleotides regulated C3-catalyzed ADP-ribosylation of 21–24 kDa platelet proteins. Fig.1 shows that in the absence of added $MgCl_2$ and with 1 mM EDTA, GTP (100 μM) and GTPγS (100 μM) increased the C3-induced labelling of 21–24 kDa protein(s). GMP and ATPγS did not show any effects (not shown). In the presence of $MgCl_2$, however, GTPγS impaired the ADP-ribosylation. In com-

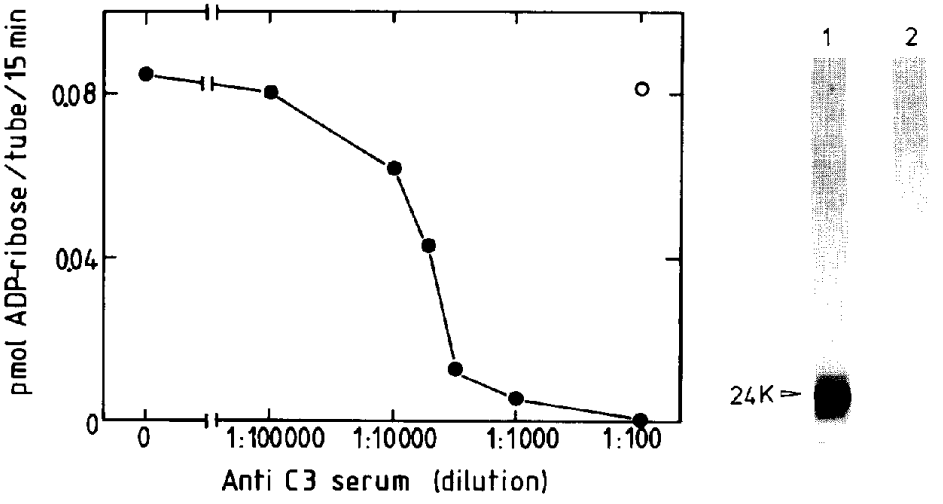

Fig.2. Inhibition of the C3-catalyzed ADP-ribosylation by anti-C3 antibodies. Influence of increasing concentrations of anti-C3 serum on the C3-induced ADP-ribosylation. (Left) C3 (15 ng) was incubated without and with the indicated dilutions of anti-C3 serum (•) or non-immune serum (○) for 30 min at 4°C. Thereafter the ADP-ribosylation of platelet proteins was started with the anti-C3 serum mixture in a medium as described above with the exception that 2 mM $MgCl_2$ was present and EDTA was omitted. The radioactivity of labelled proteins was measured by the filter method as described. (Right) Autoradiogram of SDS-polyacrylamide (11%) gel analysis of platelet proteins ADP-ribosylated as described above with non-immune serum (lane 1) and anti-C3 serum (lane 2) at a dilution of 1:100.

paring the ADP-ribosyltransferase activities of botulinum neurotoxin C1 and C3, we observed that C3 was about 3 orders of magnitude more potent than purified neurotoxin C1 in labelling the 21–24 kDa substrate(s) in platelet membranes (table 1) or rat brain tissue preparations (not shown). Notably, the C1 preparation used was extremely toxic in mice (LD_{50}) about 15 pg/mouse, i.p.) while C3 was not toxic even up to 1 μg/mouse. In order to exclude the possibility that C3 is an active fragment of botulinum C1 toxin, which was not released under the conditions used, we raised antibodies against purified botulinum ADP-ribosyltransferase C3. The antibodies recognized the antigen at a titer of 1:100000. As shown in fig.2 antiserum but not preimmune serum blocked the ADP-ribosyltransferase activity of C3 indicating the specificity of the antibodies. In contrast to C3, this antiserum failed to crossreact with botulinum neurotoxin C1 indicating that there was apparently no structural relation between C3 and C1 (fig.3). Furthermore, the anti-C3 serum did not affect the toxicity of the neurotoxin in mice studied by means of time-to-death determinations (not shown). On the other hand, the anti-C3 antibodies blocked the weak labelling of the 21 kDa protein caused by the botulinum neurotoxin C1 preparation (fig.4). In contrast ADP-ribosylation of actin by botulinum C2 toxin was not impaired by anti-C3 antibodies indicating that the ADP-ribosylation reaction was not unspecifically blocked by the antibodies used (not shown). Thus, our findings strongly argue against the notion that botulinum neurotoxin C1 or an active fragment of

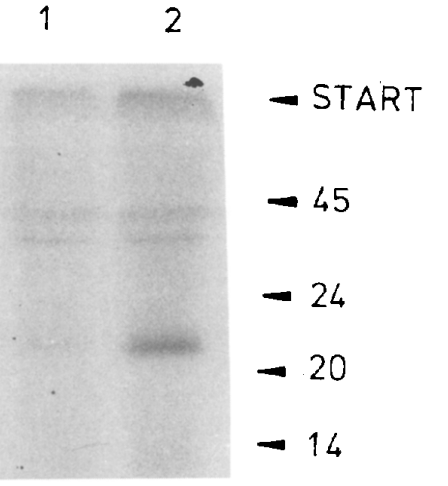

Fig.4. Impairment of botulinum neurotoxin C1-induced ADP-ribosylation of platelet proteins by anti-C3 antibodies. SDS-polyacrylamide (15%) gel analysis of platelet proteins labelled by botulinum neurotoxin C1 (1.4 μg) pretreated with non-immune serum (lane 1) and anti-C3 serum (lane 3) for 1 h at room temperature. ADP-ribosylation was performed as described with the exception that $MgCl_2$ was 2 mM and EDTA was omitted.

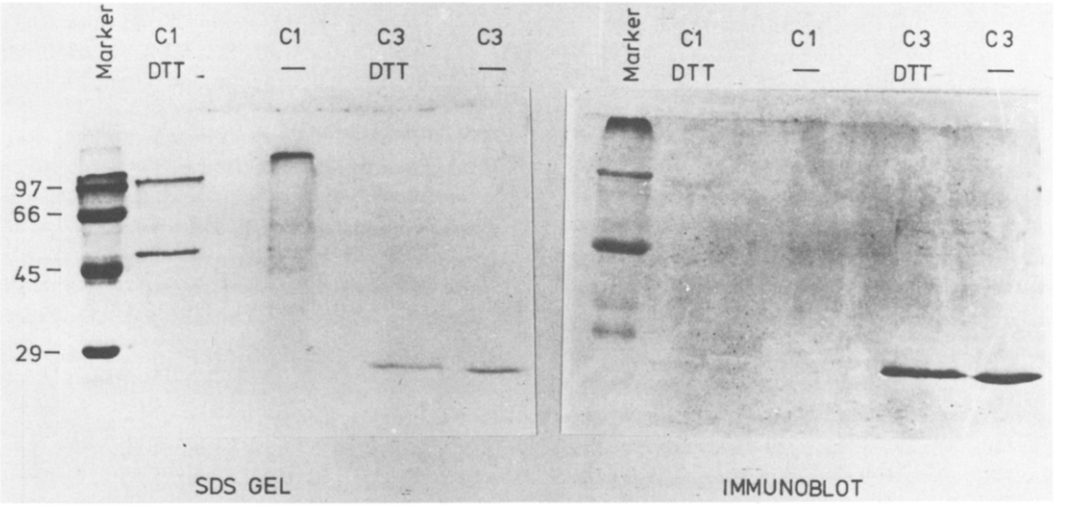

Fig.3. SDS gel and immunoblot of ADP-ribosyltransferase C3 and botulinum neurotoxin C1. C1 (1.4 μg) and C3 (1.25 μg) were heated without and with dithiothreitol (DTT, 10 mM) and analyzed by SDS-polyacrylamide gel electrophoresis (SDS gel) and thereafter immunoblotting was performed as described.

the neurotoxin ADP-ribosylates the 21–24 kDa substrate(s). Taking into consideration the rather high concentration (2–100 μg/ml) of neurotoxins used in earlier reports [4–6], our data lead to the hypothesis that the ADP-ribosyltransferase activities of botulinum neurotoxins C1 and D are merely due to a contamination by C3.

The nature of the low molecular mass GTP-binding protein, modified by C3 is not known at present. Several GTP-binding proteins with molecular masses of 20–30 kDa have been identified recently. Among these are various ras proteins supposed involved in cell differentiation or growth [13]. We have tested H-ras and elongation factor Tu, which shows high homology with ras but could not find any labelling of these proteins. Other ras proteins, the ADP-ribosylating factor (ARF) [14], which is necessary for cholera toxin action on G_s and the G_p protein [15] purified from placenta tissue with still unknown functions remain to be tested. Furthermore, it has recently been shown that apparently 4 different low molecular mass GTP-binding proteins can be identified by [α-^{32}P]GTP binding in platelets [16]. Considering the importance of cholera toxin and pertussis toxin for studying the function and role of GTP-binding proteins involved in adenylate cyclase regulation one can expect that the novel ADP-ribosyltransferase C3 will also be very useful to distinguish and characterize these low molecular mass GTP-binding proteins. Our data, however, lend no support to the hypothesis that the inhibitory effects on neurotransmitter release by botulinum neurotoxins C1 and D are based on ADP-ribosylation of GTP-binding proteins.

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