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Clostridium botulinum type C produces a novel ADP-ribosyltransferase distinct from botulinum C2 toxin

Klaus Aktories, Ulrich Weller and Gursharan S. Chhatwal*

Rudolf-Buchheim-Institut für Pharmakologie and **Institut für Bakteriologie und Immunologie der Justus-Liebig-Universität, Frankfurter Str. 107, D-6300 Gießen, FRG*

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The culture medium of certain strains of *Clostridium botulinum* type C contains two separable ADP-ribosyltransferases. Besides the ADP-ribosylation of actin due to botulinum C2 I toxin, a second microbial enzyme causes the mono-ADP-ribosylation of a eukaryotic protein with a molecular mass of about 20 kDa found in platelets, neuroblastoma × glioma hybrid cells, S49 lymphoma cells, chick embryo fibroblasts and sperm. The eukaryotic substrate is inactivated by heating and trypsin treatment. In contrast, the novel ADP-ribosyltransferase, which can be separated by DEAE-Sephadex chromatography, is largely resistant in the short term to trypsin digestion.

ADP-ribosyltransferase; Botulinum C2 toxin; (*Clostridium botulinum*)

1. INTRODUCTION

ADP-ribosylation of regulatory proteins is an important pathophysiological mechanism by which bacterial toxins such as cholera toxin, pertussis toxin and diphtheria toxin affect eukaryotic cell functions [1–3]. Botulinum C2 toxin, produced by certain strains of *Clostridium botulinum* type C, is another bacterial toxin, which possesses ADP-ribosyltransferase activity [4–6]. Recently, we have shown that the enzymatically active component I of botulinum C2 toxin ADP-ribosylates non-muscle actin [5,6], leading to drastic changes in the functional properties of actin. In the present communication we report that in addition to botulinum C2 toxin certain strains of *Clostridium botulinum* type C produce another ADP-ribosyltransferase, whose eukaryotic substrate is clearly distinct from actin, the target of botulinum C2 toxin.

Correspondence address: K. Aktories, Rudolf-Buchheim-Institut für Pharmakologie, Justus-Liebig-Universität, Frankfurter Str. 107, D-6300 Gießen, FRG

2. MATERIALS AND METHODS

2.1. *Materials*

All chemicals were of analytical grade and were obtained from sources already described elsewhere [6]. *Clostridium botulinum* type C strain 486c was donated by Dr Sebald, Pasteur Institut, Paris. The culture medium consisted of 3% peptone from meat (tryptic digest), 2% yeast extract, 0.5% glucose, and 0.1% cysteine hydrochloride (all from Merck, Darmstadt, FRG). Before autoclaving the pH of the medium was adjusted to pH 7.2. The cultures were grown under anaerobic condition using a BBL gas-pack system. After incubation for 4 days at 37°C, the cell-free culture supernatant was obtained by centrifugation and subsequent filtration through 0.45 μm Millipore filter. Botulinum C2 toxin was purified according to Ohishi et al. [7].

2.2. *ADP-ribosylation assay*

ADP-ribosylation of eukaryotic proteins was performed essentially as described [6]. Briefly, the ADP-ribosylation medium contained 10 mM

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thymidine, 0.5 mM $MgCl_2$, 0.5 mM ATP, 0.1–1 μM [^{32}P]NAD (about 0.5–1 μCi/tube) and 50 mM triethanolamine-HCl, pH 7.5. The incubation was performed for the indicated time at 37°C in a total volume of 100 μl. The protein concentration was 20–100 μg/tube. The concentration of botulinum C2 toxin was 1 μg/ml. The amount of added bacterial culture medium or purified ADP-ribosyltransferase preparation was as indicated. Determination of the ADP-ribosyltransferase activity was performed by stopping the reaction with 400 μl SDS (2%, w/v), BSA (1 mg/ml) and precipitation of the proteins with 500 μl 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 analysis of retained radioactivity.

2.3. *Analysis of the labelled proteins*

After the indicated incubation time the ADP-ribosylation reaction was stopped by the addition of 1 ml trichloroacetic acid (20%, w/v). The resultant pellet was washed with ether, dissolved in 50 μl electrophoresis buffer and analyzed by SDS-polyacrylamide gel electrophoresis according to Laemmli [8]. Gels were stained, destained and subjected to autoradiography.

2.4. *Trypsin treatment*

For trypsin treatment purified ADP-ribosyltransferase (about 2 μg of protein) or platelet membranes (about 50 μg of protein) were incubated in the presence of 0.1 mg/ml trypsin for 10 min at 37°C. The reaction was stopped by the addition of soybean trypsin inhibitor (1 mg/ml final concentration). For control conditions the ADP-ribosyltransferase or platelet membranes were incubated in the presence of both trypsin and soybean trypsin inhibitor before carrying out the ADP-ribosylation reaction.

2.5. *Purification of the second ADP-ribosyltransferase*

50 ml of culture medium was precipitated by the addition of 19 g $(NH_4)_2SO_4$. After 12 h the formed precipitate was collected by centrifugation and dissolved in 20 ml of 100 mM Tris-HCl, pH 7.5. The solution was dialyzed againsst 50 mM Tris-HCl, pH 7.5 and applied to a DEAE-Sephadex column (1.5 × 5 cm). The column was washed with 50 ml of the same buffer and eluted with 20 ml of 50 mM Tris-HCl (pH 7.5) containing 300 or 400 mM NaCl. The nonbound fraction contained the ADP-ribosyltransferase, which labelled the 20 kDa protein, the eluate contained the botulinum C2 toxin. The concentrated nonbound fraction of the DEAE-column was injected into a Waters HPLC, composed of a UK6 injector, a 6000 A pump and a Pye Unicam absorbance detector (LC-UV, 280 nm) in 200 mM phosphate buffer (pH 7) at a flow rate of 0.5 ml/min. Proteins were separated on a Zorbax Du Pont 9, 4 × 250 mm, GF-250. The fraction size was 210 μl.

3. RESULTS AND DISCUSSION

In lysates of human platelet membranes and with [^{32}P]NAD, filtrates of culture medium of *Clostridium botulinum* type C strain 486c labelled two different proteins with relative molecular masses of about 43 and 20 kDa, respectively (fig.1,I). The labelled 43 kDa protein was actin ADP-ribosylated by botulinum C2 toxin [5,6], which is produced by this strain of *Clostridium botulinum*. The labelling of the 20 kDa substrate was also caused by an ADP-ribosylation and not by mere phosphorylation, because the labelling was readily abolished in the presence of 100 μM unlabelled NAD but not with 0.5 mM ATP, which was always present in the reaction mixture (fig.1,II). Labelling of the 20 kDa substrate was a time-dependent process and reached a maximum after an incubation time of 20 min (fig.2). Similarly, as found for the ADP-ribosylation of actin by botulinum C2 toxin [6], the labelling of the 20 kDa substrate was apparently due to a mono-ADP-ribosylation. This conclusion can be drawn from the finding that the total amount of label increased with time, but was not accompanied by a concomitant increase in the apparent molecular weight of the labelled substrate. The proteinaceous nature of the 20 kDa substrate was shown by studying the effects of trypsin treatment of crude platelet membranes on the ADP-ribosylation reaction. When platelet membranes were pretreated with trypsin (100 μg/ml), the labelling of the 20 kDa substrate was completely abolished (fig.2). Also heating of platelet membranes for 10 min at 95°C prevented the subsequent ADP-ribosylation of the 20 kDa

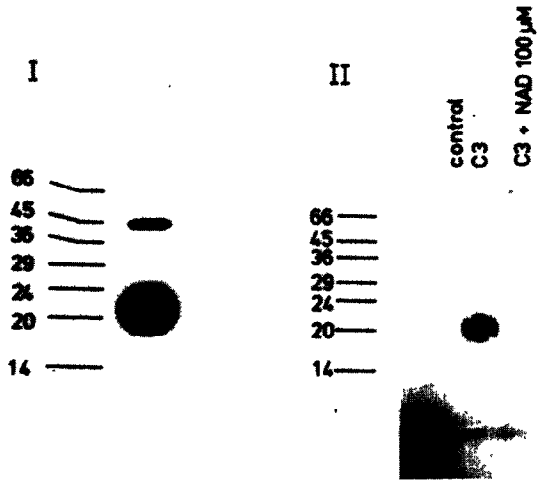

Fig.1. (I) ADP-ribosylation of crude human platelet membranes by clostridia culture medium. Crude human platelet membranes (80 μg of protein) were incubated with [^{32}P]NAD (0.1 μM, about 0.5 μCi) and 10 μl of *Clostridium botulinum* culture filtrate for 30 min at 37°C as described in section 2. The autoradiogram of the SDS-polyacrylamide gel of the labelled proteins is shown. The M_r markers were bovine serum albumin (M_r 66000), ovalbumin (M_r 45000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36000), carbonic anhydrase (M_r 29000), trypsinogen (M_r 24000), soybean trypsin inhibitor (M_r 20000), c-lactalbumin (M_r 14200). (II) Effects of NAD on the ADP-ribosylation of the 20 kDa substrate in neuroblastoma × glioma hybrid cell membranes. Crude neuroblastoma × glioma hybrid cell membranes (25 μg of protein) were incubated with 0.1 μM [^{32}P]NAD (0.1 μM, about 1 μCi), without (control) and with 3 μl of purified botulinum ADP-ribosyltransferase preparation (C3, DEAE-column flowthrough, 0.5 μg of protein) in the absence and presence of NAD (100 μM) for 30 min at 37°C. Thereafter polyacrylamide gel electrophoresis of the platelet proteins and autoradiography (shown) of the labelled proteins were performed.

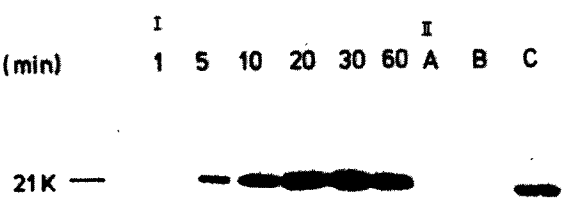

Fig.2. ADP-ribosylation of the 20 kDa protein in platelet membranes by the botulinum ADP-ribosyltransferase. (I) Time course of the ADP-ribosylation of the 20 kDa protein. Crude human platelet membranes were incubated with [^{32}P]NAD and 2 μl of purified botulinum ADP-ribosyltransferase (DEAE-column flowthrough, 0.3 μg protein) for the indicated times. (II) Effects of trypsin treatment and heating of platelet membranes on the subsequent ADP-ribosylation of the 20 kDa protein. Crude platelet membranes were heated for 10 min at 95°C (A) or treated with trypsin (B, 100 μg/ml) or with trypsin plus trypsin-inhibitor as control (C, 1 mg/ml). Thereafter the ADP-ribosylation of the 20 kDa platelet membrane protein was performed with [^{32}P]NAD (0.1 μM, 1 μCi) and 2 μl of the purified botulinum ADP-ribosyltransferase.

protein (fig.2). The labelling of the 20 kDa protein was also found in membrane preparations of neuroblastoma × glioma hybrid cells, S49 lymphoma cells, chick embryo fibroblasts and sperm (not shown). In order to clarify whether the 20 kDa substrate was also a target of botulinum C2 toxin or whether a different ADP-ribosyltransferase was involved, we purified the ADP-ribosyltransferases found in the culture medium. The extract of an ammonium sulfate precipitation of the culture medium was able to ADP-ribosylate both substrates (not shown). However, further purification by means of DEAE-Sephadex clearly separated the two ADP-ribosyltransferase activities. The DEAE-Sephadex column flowthrough contained the enzyme for ADP-ribosylation of the 20 kDa substrate, whereas the activity for ADP-ribosylation of the 43 kDa substrate was retained on the DEAE column (fig.3,I). The labelling of the 43 kDa protein was evidently due to botulinum C2 toxin, which is known to adsorb to DEAE under the conditions used [7]. This notion was substantiated by the finding that the DEAE column eluate but not the flowthrough was able to ADP-ribosylate isolated platelet G-actin (not shown). SDS-polyacrylamide gel electrophoresis of the DEAE column flowthrough showed one major protein band with a molecular mass of about 24 kDa (fig.3,II). In order to purify further the ADP-ribosyltransferase involved in the ADP-ribosylation of the 20 kDa protein, we concentrated the unadsorbed fraction of the DEAE column and applied it to gel filtration on a GF 250 column. As shown in fig.4 the en-

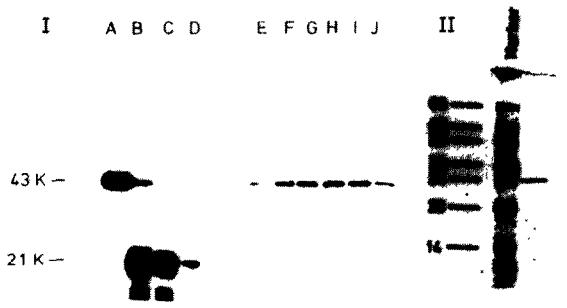

Fig.3. (I) DEAE-Sephadex chromatography of botulinum C2 toxin and of a second botulinum ADP-ribosyltransferase. DEAE-Sephadex chromatography was performed according to the procedure described in section 2. For determination of the ADP-ribosyltransferase activity 1 μg/ml purified botulinum C2 toxin (A), 10 μl of the culture medium filtrate (B), 10 μl of the DEAE-Sephadex column flowthrough (C), 10 μl of the column wash (D, 50 mM Tris-HCl, pH 7.5) and 10 μl of the fractions eluted with 50 mM Tris-HCl and 300 mM NaCl (E–J) were incubated with platelet lysate (30 μg of protein) and [^{32}P]NAD (0.2 μM, about 0.5 μCi) for 30 min at 37°C. The autoradiogram of the labelled platelet lysate proteins after SDS gel electrophoresis is shown. (II) SDS-polyacrylamide gel analysis of the purified botulinum ADP-ribosyltransferase C3. The botulinum ADP-ribosyltransferase responsible for labelling of the 20 kDa eukaryotic membrane protein was purified as described in section 2. An SDS-polyacrylamide gel of the concentrated DEAE-Sephadex column flowthrough (about 10 μg of protein) is shown.

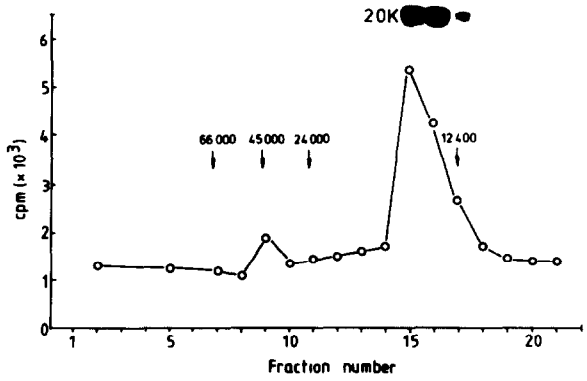

Fig.4. ADP-ribosylation of a 20 kDa platelet membrane protein by the botulinum ADP-ribosyltransferase C3 purified by HPLC. The concentrated DEAE-column breakthrough was applied to gel filtration by HPLC. The ADP-ribosyltransferase activity of each fraction was determined with [^{32}P]NAD (0.1 μM, about 0.3 μCi) and crude platelet membranes (50 μg of proteins) by the filtration method as described in section 2. The labelled proteins were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (shown).

zymatic activity, which causes ADP-ribosylation of the 21 kDa substrate, eluted in a fraction corresponding with a molecular mass of about 12–20 kDa. When the fractions containing the enzymatic activity were further analyzed by SDS gel electrophoresis, a protein with a relative molecular mass of 24 kDa was detected by means of silver staining. Thus, this ADP-ribosyltransferase, for which we propose the term 'C3', is clearly distinct from botulinum C2 toxin, whose molecular mass is about 50 kDa [8]. The relative molecular mass of the ADP-ribosyltransferase C3 was slightly larger than that of the eukaryotic substrate determined by means of SDS gel electrophoresis. Fig.5 shows that the labelling of the substrate by C3 largely increased on addition of increasing amounts of platelet membranes to the reaction mixture. In contrast, labelling of the 20 kDa substrate was not further enhanced by increasing the concentration of the microbial ADP-ribosyltransferase from 2 up to 20 μg/ml (not shown). These findings indicate that a eukaryotic substrate in platelet membranes but not the microbial enzyme itself was ADP-ribosylated. This notion was further supported by the finding that in contrast to the 20 kDa substrate protein, the ADP-ribosyltransferase C3 was largely stable against trypsin treatment (not shown).

Taken together, the culture medium of *Clostridium botulinum* type C contains two ADP-ribosyltranferases. One enzyme is botulinum C2 toxin, which ADP-ribosylates non-muscle G-actin [5,6]. The second ADP-ribosyltransferase, which is clearly distinct from botulinum C2 toxin, has a relative molecular mass of about 24 kDa on SDS gel and ADP-ribosylates a eukaryotic protein substrate of about 20 kDa. We propose the term C3 for this ADP-ribosyltransferase to distinguish this newly found enzyme from botulinum neurotoxin C1 and botulinum C2 toxin, which are also produced by this strain of *Clostridium botulinum*. In order to obtain more insights into the role of this newly found bacterial ADP-ribosyltransferase we are now studying the nature and function of the 20 kDa eukaryotic substrate.

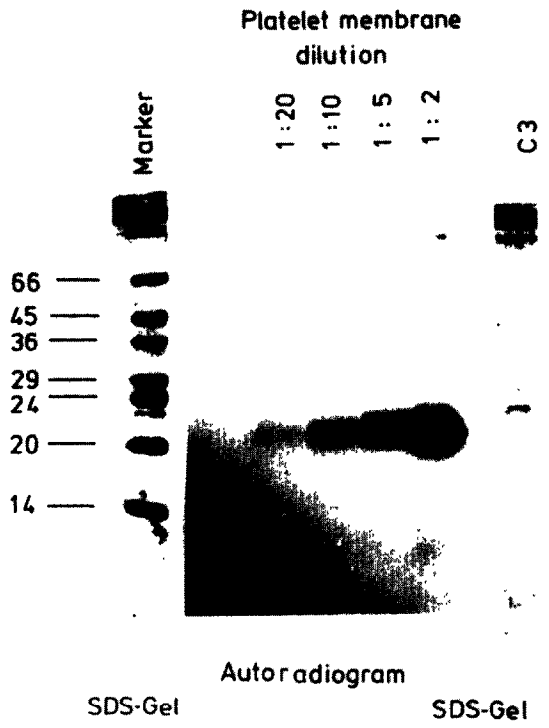

Fig.5. ADP-ribosylation of the 20 kDa protein in platelet membranes by purified botulinum ADP-ribosyltransferase C3. Various dilutions of crude platelet membranes (dilution 1:2 refers to 80 μg of protein) were incubated with 3 μl of purified botulinum ADP-ribosyltransferase C3 (DEAE-flowthrough, 0.5 μg of protein) in the presence of [^{32}P]NAD (0.1 μM, about 0.5 μCi) for 1 h at 37°C. Thereafter the labelled proteins were analyzed by SDS-polyacrylamide gel electrophoresis and by autoradiography (shown). Additionally the SDS-polyacrylamide gel analysis of the purified botulinum ADP-ribosyltransferase (C3) is shown.

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