ADP-ribosylation of a Mr 21,000 Membrane Protein by Type D Botulinum Toxin*

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When crude membrane fraction from bovine adrenal gland was incubated with type D botulinum toxin in the presence of NAD, a membrane protein with a molecular weight of 21,000 was specifically ADP-ribosylated. This ADP-ribosylation occurred dependent on the dose of the toxin and was abolished by prior boiling. ADP-ribose transfer to the membrane protein was significantly suppressed when arginine and L-arginine methyl ester were included in the reaction mixture. Dithiothreitol stimulated this ADP-ribosylation about 3-fold. Incubation of membrane fractions from mouse brain and pancreas with this toxin also resulted in ADP-ribosylation of a protein of the same molecular weight. These results suggested that type D botulinum toxin catalyzed transfer of an ADP-ribose moiety of NAD to the specific membrane protein common to secretory cells.

Botulinum toxins are potent neurotoxins which act at pre-synaptic terminals of cholinergic as well as other neurons and block the release of neurotransmitters (1). Although the exact mechanism of this inhibition has not yet been clarified, a three-step model has been proposed to account for their actions (2). This model consists of the binding step in which the toxins bind to receptors on the plasma membrane of target cells, the internalization step in which they enter the cells possibly via receptor-mediated endocytosis, and the poisoning step in which they damage release function of the cells. This model has been critically examined in recent years. Among the three steps, the former two steps have been proved in several tissues using various types of botulinum toxins (8, 9), but the mechanism of intracellular poisoning remains to be elucidated. A hypothesis that botulinum neurotoxin is an enzyme has been proposed based on several findings (2). They include the remarkable potency and long duration of action which are difficult to explain by a nonenzymatic mechanism. The hypothesis is also based on many similarities of botulinum toxin in structure and origin to diphtheria toxin which is an enzyme with ADP-ribosyltransferase activity (2, 7). In this study we have examined enzyme activity of type D botulinum toxin on the homogenate of bovine adrenal gland, since adrenal medullary cells are sensitive to this type of botulinum toxin (8). We provide evidence that type D toxin is really an enzyme with ADP-ribosyltransferase activity.

**EXPERIMENTAL PROCEDURES**

Materials—Type D botulinum toxin (2.0 × 10⁶ mice LD₅₀/mg of protein) was obtained from Wako Chemicals, Osaka, Japan. The toxin was purified according to the method of Miyazaki et al. (9) and the preparation was apparently homogeneous on native polyacrylamide gel electrophoresis. On SDS-polyacrylamide gel electrophoresis the preparation showed four protein bands at molecular weights of 110,000, 100,000, 48,000, and 28,000. The protein was isolated from this progenitor toxin by DEAE-Sephadex chromatography as described (9, 10). Cholera toxin was from the Chemo-Sero Therapeutic Research Institute, Kumamoto, Japan. [adenine-¹⁴C]NAD (281 mCi/mmol) and [carbonyl-¹⁴C]NAD (53 mCi/mmol) were purchased from Amersham International. [¹⁴C]NAD (14.9 Ci/mmol) and enhancer for autoradiography (ENHANCE) were from New England Nuclear. [ribose-⁻¹⁴C]NAD (200 mCi/mmol) was a generous gift from Dr. K. Ueda, Kyoto University Faculty of Medicine. ATP, GTP, NAD, nicotinamide, thymidine, and dithiothreitol were from Boehringer Mannheim. Agmatine sulfate, L-arginine methyl ester, and L-lysine methyl ester were from Sigma, L-arginine, L-lysine, and L-citrulline were from Kyowa Hakko Ltd., Tokyo. Trypsin and snake venom phosphodiesterase were from Worthington and the latter enzyme was purified according to the method of Oka et al. (11).

**Incubation of Membrane Fractions with Botulinum Toxin**—Crude membrane fractions were prepared from bovine adrenal gland, mouse brain, and pancreas as follows. Tissues were suspended in 1 volume of 0.25 M sucrose containing 3.3 mM CaCl₂ and washed once. Washed tissues were then suspended in 3 volumes of the same solution and homogenized by a Potter-Elvehjem homogenizer. The crude homogenate was centrifuged at 1,300 × g for 10 min. The supernatant was filtered through six layers of cheese cloth and centrifuged at 16,000 × g for 30 min. The pellet was suspended in half of the original volume of 0.25 M sucrose containing 3.3 mM CaCl₂ by the use of a loosely fitted Potter-Elvehjem homogenizer and was used as a crude membrane fraction. Membrane fractions (100 µg of protein) were incubated in 200 µl of the reaction mixture containing 100 mM Tri-HCl (pH 7.6), 10 µM [⁴⁺¹⁴C]NAD (5 µCi), 10 mM thymidine, and toxin. When [¹⁴C]NAD was added instead of [³²P]NAD, 150 µM either [adenine-¹⁴C]NAD, [carbonyl-¹⁴C]NAD, or [ribose-⁴⁻¹⁴C]NAD (1.5 µCi), and 10 mM dithiothreitol were added to the reaction mixtures. After incubation for 45 min at 30 °C, 20 µl of 100% (w/v) trichloroacetic acid was added. Proteins were precipitated by centrifugation, solubilized in 2% SDS, and subjected to SDS-polyacrylamide gel electrophoresis.

SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (12). Autoradiography of the dried gel was carried out as previously described (13). Quantification of the incorporated radioactivity was carried out by exciting radioactive bands from the gel and measuring ³²P contents in a Triton/toluene scintillator.

Release of [¹⁴C]nicotinamide from [carbonyl-¹⁴C]NAD was assayed as described by Moss and Vaughan (14). Briefly, the assay mixture (200 µl) contained 100 mM Tris-HCl (pH 7.6), 10 mM dithiothreitol, 19 µM [carbonyl-¹⁴C]NAD (100 cpm/µmol), 75 µM either agmatine sulfate or L-arginine methyl ester, and 15 µg either type D toxin or cholera toxin. After the reaction was carried out at 30 °C for 90 min, the mixture was chilled and applied to the Dowex 1 column (formate form, 1 ml of bed volume). Released [¹⁴C]nicotinamide was eluted with 0.25 M sucrose containing 3.3 mM CaCl₂ by the use of a loosely fitted Potter-Elvehjem homogenizer and was used as a crude membrane fraction. Membrane fractions (100 µg of protein) were incubated in 200 µl of the reaction mixture containing 100 mM Tris-HCl (pH 7.6), 10 µM [⃒⁺⁶⁻¹⁴C]NAD (5 µCi), 10 mM thymidine, and toxin. When [¹⁴C]NAD was used instead of [³²P]NAD, 150 µM either [adenine-¹⁴C]NAD, [carbonyl-¹⁴C]NAD, or [ribose-⁴⁻¹⁴C]NAD (1.5 µCi), and 10 mM dithiothreitol were added to the reaction mixtures. After incubation for 45 min at 30 °C, 20 µl of 100% (w/v) trichloroacetic acid was added. Proteins were precipitated by centrifugation, solubilized in 2% SDS, and subjected to SDS-polyacrylamide gel electrophoresis.

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Release of [¹⁴C]nicotinamide from [carbonyl-¹⁴C]NAD was assayed as described by Moss and Vaughan (14). Briefly, the assay mixture (200 µl) contained 100 mM Tris-HCl (pH 7.6), 10 mM dithiothreitol, 19 µM [carbonyl-¹⁴C]NAD (100 cpm/µmol), 75 µM either agmatine sulfate or L-arginine methyl ester, and 15 µg either type D toxin or cholera toxin. After the reaction was carried out at 30 °C for 90 min, the mixture was chilled and applied to the Dowex 1 column (formate form, 1 ml of bed volume). Released [¹⁴C]nicotinamide was eluted with 0.25 M sucrose containing 3.3 mM CaCl₂ by the use of a loosely fitted Potter-Elvehjem homogenizer and was used as a crude membrane fraction. Membrane fractions (100 µg of protein) were incubated in 200 µl of the reaction mixture containing 100 mM Tris-HCl (pH 7.6), 10 µM [⃒⁺⁶⁻¹⁴C]NAD (5 µCi), 10 mM thymidine, and toxin. When [¹⁴C]NAD was used instead of [³²P]NAD, 150 µM either [adenine-¹⁴C]NAD, [carbonyl-¹⁴C]NAD, or [ribose-⁴⁻¹⁴C]NAD (1.5 µCi), and 10 mM dithiothreitol were added to the reaction mixtures. After incubation for 45 min at 30 °C, 20 µl of 100% (w/v) trichloroacetic acid was added. Proteins were precipitated by centrifugation, solubilized in 2% SDS, and subjected to SDS-polyacrylamide gel electrophoresis.

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1. The abbreviations used are: LD₅₀, median lethal dose; SDS, sodium dodecyl sulfate.
with 2.7 ml of 20 mM formic acid, and the radioactivity in the 2-ml eluate was determined.

Product Analyses—Trypsin digestion of the radioactive product was carried out as follows. Acid-insoluble precipitates of radioactive products were rinsed with diethyl ether and dissolved in 100 μl of 0.1 M NaOH. The pH of the solution was adjusted to pH 8.0 by adding 50 μl of 0.5 M Tris-HCl (pH 7.0). Trypsin (10 μg) was added to the solution, and the mixture was incubated at 37 °C for 2 h. After incubation, 15 μl of 100% trichloroacetic acid was added, and the mixture was centrifuged to separate acid-soluble and acid-insoluble radioactivities.

Digestion of products with phosphodiesterase and paper and Dowex 1 column chromatographies of the digested products were carried out as described (13, 15). The gel corresponding to a radioactive band at M(21,000) was excised out, and the radioactive product was extracted from the gel by the method of Ferro and Olivera (16). Phosphodiesterase digestion was carried out by incubating the product with 2 μl of phosphodiesterase in 25 mM Tris-HCl (pH 9.0), 10 mM MgCl₂, and 1 mM AMP at 37 °C for 6 h. A digested sample containing 3,000 cpm of 32P was applied to a Dowex 1 column (formate form, 0.7 cm) together with unlabeled AMP (0.12 μmol) and ADP-ribose (0.2 μmol). The column was eluted with a linear gradient of 0 to 6 M formic acid (total volume, 100 ml). Fractions (1 ml) were taken and the radioactivity and absorbance at 260 nm were determined.

RESULTS

Crude membrane fraction from bovine adrenal gland was incubated with type D botulinum toxin in the presence of [α-32P]NAD. After incubation, radioactive incorporation into the acid-insoluble fraction was analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. As shown in Fig. 1A, an intense radioactive band was observed at a M(21,000) in the presence of the toxin in the reaction mixture (lanes 1 and 2). When the labeled membrane was treated with trypsin and then centrifuged, more than 99% of the radioactivity was recovered in the supernatant, confirming that the radioactivity was incorporated into protein. The addition of 10 mM dithiothreitol significantly enhanced this labeling (lane 3), whereas agmatine or L-arginine methyl ester, known inhibitors of NAD:arginine ADP-ribosyltransferase (17, 18), significantly attenuated the labeling (lanes 4 and 5). The botulinum toxin also catalyzed labeling of the membrane protein of the same molecular weight in tissues other than bovine adrenal gland. As shown in Fig. 1B, a M(21,000) protein of membranes from mouse brain and pancreas was labeled by this toxin (lanes 2 and 4). The incorporation of 32P into the M(21,000) protein increased with incubation time; it increased linearly in 10 min and reached plateau at 45 min. It was also dependent on the amounts of the toxin and the membranes in the reaction mixture; the incorporation increased with the toxin up to 20 μg of protein and with the membranes up to 100 μg of protein. Prior boiling of the toxin completely abolished this labeling.

Incorporation of radioactivity into the M(21,000) protein was also observed when [adenine-14C]NAD and [ribose-14C] NAD were used instead of [α-32P]NAD. However, no labeling was found with [carboxyl-14C]NAD (Fig. 2). To identify the radioactive product, the 32P-labeled product was extracted from the gel and treated with snake venom phosphodiesterase. When the digested products were analyzed by Dowex 1 column chromatography, more than 95% of the radioactivity was recovered as [32P]5′-AMP. A similar finding was also obtained by paper chromatography; about 93% of the total radioactivity was comigrated with authentic 5′-AMP at the Rf of 0.54 (data not shown). Thus, these results taken together strongly suggested that type D botulinum toxin catalyzed mono ADP-ribosylation of the specific membrane protein of M(21,000).

In order to confirm that the enzyme activity was associated with the toxic component of the progenitor toxin, we carried out DEAE-Sephadex column chromatography of the toxin to isolate the 7 S toxic component (Fig. 3). As described previously (9), the toxin was eluted in two protein peaks on this chromatography; the first and second peaks were the toxic and nontoxic components, respectively. On SDS-polyacryl-
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Fig. 2. Identification of the product. A, polyacrylamide gel analysis of bovine adrenal membranes incubated with type D botulinum toxin and $[^{14}C]$NAD labeled at various positions. Incubation of the membranes with the toxin and $[^{14}C]$NAD was carried out as described under “Experimental Procedures.” After electrophoresis, the gel was treated with EN3HANCE and subjected to fluorography. Lane 1, [carboxyl-$^{14}$C]NAD; lane 2, [adenine-$^{14}$C]NAD; lane 3, [ribose-$^{14}$C]NAD; B, Dowex 1 column chromatography of phosphodiesterase-digested radioactive products. Radioactive products bound to $M_r 21,000$ protein were extracted from the gel and digested with phosphodiesterase as described (13). The digested products were analyzed by Dowex 1 column chromatography as described under “Experimental Procedures.” Arrows denote the elution positions of carrier markers; AMP, 5'-AMP; ADPR, ADP-ribose. O---O, absorbance at 260 nm; ••••, radioactivity.

Fig. 3. DEAE-Sephadex column chromatography of the type D progenitor toxin. The toxin (600 pg) in 10 mM potassium phosphate (pH 7.8) was applied to a column of DEAE-Sephadex (0.9 x 11.1 cm) equilibrated with the same buffer. Elution was performed with the linear gradient from 0 to 0.3 M NaCl in the buffer in a total volume of 15 ml. Fractions (0.5 ml) were collected, and protein contents (●) and ADP-ribosyltransferase activity on bovine adrenal membrane in the presence of 10 mM dithiothreitol (○) were assayed.

Effects of various compounds which affect ADP-ribosylating activity of other bacterial toxins such as cholera toxin and pertussis toxin (17–23) were examined on this reaction. As shown in Table I, dithiothreitol stimulated the activity of the toxin about 3-fold. Small stimulation was also observed with GTP and ATP (about 1.5-fold). Among various amino acid analogues, L-arginine, L-arginine methyl ester, and agmatine could inhibit the enzyme activity. Inhibition by agmatine was observed in a concentration-dependent manner over 2 mM and was complete at 75 mM. Since these compounds work as alternative acceptors of the ADP-ribose moiety in the cholera toxin-catalyzed reaction (14), we measured the releases of $[^{14}$C]nicotinamide from [carboxyl-$^{14}$C]NAD in the presence of 75 mM agmatine or arginine methyl ester by type D toxin and compared them with those by cholera toxin. The radioactivity released in the absence of toxins was 1,893 cpm. Cholera toxin

| Additions | ADP-ribosylation of $M_r 21,000$ protein (pmol/mg membranes) |
|-----------|--------------------------------------------------------------|
| Toxin (-) | 0                                                            |
| Toxin     |                                                              |
| 20 µg     | 1.10                                                          |
| 20 µg + 10 mM dithiothreitol | 3.42                          |
| 20 µg + 0.75 mM GTP | 1.54                          |
| 20 µg + 0.75 mM ATP | 1.71                          |
| 20 µg + 75 mM L-lysine | 0.90                          |
| 20 µg + 75 mM L-lysine methyl ester | 0.93                        |
| 20 µg + 75 mM L-arginine methyl ester | 1.18                       |
| 20 µg + 75 mM NaCl | 1.20                          |
| 20 µg + 75 mM KCl | 1.06                          |
enhanced the release to 2,300 cpm in the absence of compounds and to 18,601 and 17,687 cpm in the presence of arginine and arginine methyl ester, respectively. In contrast, no significant enhancement of the release was observed with the type D toxin. The released radioactivities were 1,895 cpm with the toxin only and 1,899 and 2,108 cpm in the presence of arginine and arginine methyl ester, respectively. Enhanced release was not observed at lower concentrations (5, 10, 20, and 50 mM) of arginine or arginine methyl ester, either.

**DISCUSSION**

Although accumulating evidence has suggested an enzyme nature of botulinum neurotoxins (1, 2), there has been no report to identify any enzyme activity in this species of toxin. In this communication, we have demonstrated for the first time that type D botulinum neurotoxin has an enzyme activity and ADP-ribosylates a M21,000 membrane protein in bovine adrenal gland. This modification reaction was stimulated by dithiothreitol and inhibited by arginine and arginine methyl ester. Since botulinum toxins consist of light and heavy chains bound by an interchain disulfide bond and toxic activity resides solely in the light chain (1, 2), the dithiothreitol effect is probably due to cleavage of this disulfide bond and release of active light chain molecules. Arginine and arginine methyl ester are known as inhibitors of an NADarginine ADP-ribosyltransferase such as cholera toxin (17, 18); however, unlike cholera toxin, the type D toxin did not show significant release of [14C]nicotinamide from [carbonyl-14C]NAD on incubation with these compounds. This result suggests that both arginine and arginine methyl ester work as pure inhibitors and not as alternative acceptors of the enzyme. Thus, type D botulinum toxin is somewhat different in catalytic properties from cholera toxin (14, 19).

We found that the ADP-ribosyltransferase activity was associated with the toxic component of the progenitor toxin. It is, therefore, most likely that the ADP-ribosylation occurs in situ in poisoned cells and alters cell function. Protein substrate for ADP-ribosylation by type D toxin was a M21,000 membrane protein. There has been no report on ADP-ribosylation of this protein by other bacterial toxins and mammalian ADP-ribosyltransferases. We have also shown that this protein is present not only in adrenal gland but also in other tissues such as brain and pancreas. Recently Knight et al. (8), using bovine adrenal medullary cells, showed that type D botulinum toxin inhibited the release of catecholamine downstream from Ca\(^{2+}\) entry into cells. These results strongly indicate that the M21,000 protein identified here is directly involved in the exocytosis process in general. Since almost all substrate proteins for bacterial toxin-catalyzed ADP-ribosylation thus far characterized are GTP-binding proteins, it is probable that this M21,000 protein is also a GTP-binding protein. Recently, a GTP-binding protein with a molecular weight of 21,000 (G\(_M\)) was purified from human placental membranes (24). Kahn and Gilman (25) also reported recently that the protein cofactor necessary for ADP-ribosylation of G\(_M\) is a GTP-binding protein of similar molecular weight (25). Whether the substrate protein for type D toxin is identical with such a protein should be rigorously examined, as should whether or not ADP-ribosyltransferase is a common property of all botulinum neurotoxins. We could not demonstrate such activity in type A botulinum toxin under the same conditions as for type D toxin.\(^2\) Whether this is due to the lack of such activity in type A toxin or to suboptimal experimental conditions for type A toxin should be clarified in the future.

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\(^2\) Y. Ohashi and S. Narumiya, unpublished observation.