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ADP-ribosylation of specific membrane proteins in pheochromocytoma and primary-cultured brain cells by botulinum neurotoxins type C and D

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Type C_1 and D toxins produced by *Clostridium botulinum* caused ADP-ribosylation of a protein of 24 kDa in membrane preparations of rat clonal pheochromocytoma cells (PC12) and of proteins of 25 and 26 kDa in neuron-rich culture of fetal rat brain cells. The ADP-ribosylation reaction was dependent on the presence of $MgCl_2$, GTP and GTPγS. The results obtained suggested that the ADP-ribosylation reaction is responsible for the development of the biological activity of the botulinum neurotoxins and that the target of this reaction may be novel GTP-binding proteins localized on cell membranes.

ADP-ribosylation; Botulinum neurotoxin; GTP-binding protein

1. INTRODUCTION

The ADP-ribosylation reaction has been shown to play important roles in pathological changes induced by various bacterial toxins on eukaryotic cell [1-5]. Extensive analysis of this reaction revealed the presence of specific nucleotide-binding proteins as the substrates which are involved in the mechanisms of important cellular functions: Diphtheria toxin catalyses the ADP-ribosylation of elongation factor-2, which results in inhibition of protein synthesis [1]. Cholera toxin and pertussis toxins ADP-ribosylate GTP-binding proteins, G_s, G_i (regulatory components in adenylate cyclase system) [2,3], and G_t (regulatory component of photoreception system) [4]. Recently, botulinum binary toxin (C_2 toxin) was found to ADP-ribosylate non-muscle actin [5]. These observations encouraged us to examine whether botulinum neurotoxins have the ability to catalyse ADP-ribosylation.

Botulinum neurotoxins which are classified into seven immunologically distinct types (A, B, C_1, D, E, F, G) have been shown to inhibit irreversibly the spontaneous and evoked release of acetylcholine at peripheral synapses [6,7]. More recently, botulinum neurotoxin type C_1 was shown to possess neuron-specific cytotoxic activity in cultures of fetal rat brain cells [8]. Although the binding properties of botulinum neurotoxins to synaptosomal membranes have been reported [9], the intracellular mechanism of the actions of botulinum neurotoxin remains unknown. Here, we describe the ability of botulinum neurotoxin type C_1 and D to ADP-ribosylate specific proteins of 24-26 kDa in membrane preparations of cells of neural origin. The dependence of the ADP-ribosylation reaction on $MgCl_2$ and guanine nucleotide suggests the possibility that the 24-26 kDa proteins are novel GTP-binding proteins.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Dulbecco's modified Eagles medium, fetal calf serum and horse serum were purchased from Gibco (NY). [*adenylate*-^{32}P]Nicotinamide adenine dinucleotide (NAD) (spec. act. 990 Ci/mmol) was purchased from New England Nuclear. Other chemicals used were of reagent grade.

2.2. *Toxins*

Botulinum type C_1 and D toxins were purified from culture media of *C. botulinum* type C strain Stockholm and type D strain South Africa as described in [10]. Botulinum type A toxin was purchased from Wako (Tokyo) and purified as in [11]. Botulinum type C progenitor toxins (C-L and C-M from strain CB19), D progenitor toxins (D-L and from strain CB16) [12] and purified botulinum C_2 were kindly donated by Dr I. Ohishi (Osaka).

2.3. *Cell culture*

Clonal rat pheochromocytoma cells (PC12) were cultured as described [13] in Dulbecco's modified Eagles medium supplemented with 5% fetal calf serum, 5% horse serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Dissociated cells of embryonic (16th day) brain of Wistar rats were prepared and cultured as in [8] using the same medium as for PC12 cells. The brain cells were treated with 10 μM cytosine β-D-arabinofuranoside from the 4th day in culture for 24 h to suppress multiplication of glial cells and harvested on the 8th day in culture.

2.4. *Preparation of cell homogenates and membranes*

Pelleted cells (500–1000 μg protein) were lysed by addition of 500 μl of 10 mM Na-P_i, pH 7.4, and sonicated for 30 s at 0°C to obtain cell homogenates for 90 min at 100 000 × g. The cell membranes were washed by repeating the centrifugation and suspended in 10 mM Na-P_i solution (pH 7.4) containing 20 mM KCl and 1 mM phenylmethylsulfonyl fluoride.

2.5. *Assay of ADP-ribosylation*

ADP-ribosylation was carried out in a reaction mixture containing 10 mM thymidine, 2 mM EDTA, 2 mM dithiothreitol, 2 μM [^{32}P]NAD (0.5–1 μCi/tube) and 20 mM Hepes-NaOH (pH 7.4). Protein amounts of toxin and cell sample used are indicated in the figure legends. The incubation was performed at 37°C in a total volume of 40 μl. After the indicated time the reaction was terminated by the addition of 20 μl of 1% SDS and boiling for 2 min.

2.6. *SDS-polyacrylamide gel electrophoresis*

SDS-polyacrylamide gel electrophoresis (12% gel) was performed according to Laemmli [14]. The following molecular mass markers were used: β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.2 kDa). Samples were alkylated with *N*-ethylmaleimide prior to electrophoresis as described in [15]. Gels were stained with Coomassie brillant blue R-250 and subjected to autoradiography.

3. RESULTS AND DISCUSSION

Homogenates of PC12 cells were incubated with [^{32}P]NAD and 4 mM $MgCl_2$ in the presence of various types of botulinum neurotoxins and botulinum C_2 toxin to examine their ability to catalyse ADP-ribosylation. As shown in fig.1, type C_1, D and type C progenitor toxin (C-M) specifically labeled a single protein band of 24 kDa. Type A toxin which is the most potent botulinum neurotoxin failed to label this protein band. Botulinum C_2 toxin which has been shown to ADP-ribosylate nonmuscle actin of 45 kDa also failed to label the band of 24 kDa. Therefore, the possibility that the labeling of the 24 kDa band is due to C_2 toxin contaminating in the samples of the neurotoxins was excluded. Type D toxin-catalyzed ADP-ribosylation of the 24 kDa protein was also found in the membrane preparation of PC12 cells (fig.2, lanes 4,5) indicating that this protein substrate is localized on the membrane. When a membrane preparation of neuron-rich fetal rat brain cells was incubated with [^{32}P]NAD in the presence of type D toxin, radioactivities were incorporated into two protein bands with closely related molecular masses, 25 and 26 kDa (fig.2, lanes 6,7) slightly above the band of PC12 cells with similar intensities. Type D toxin-catalyzed ADP-

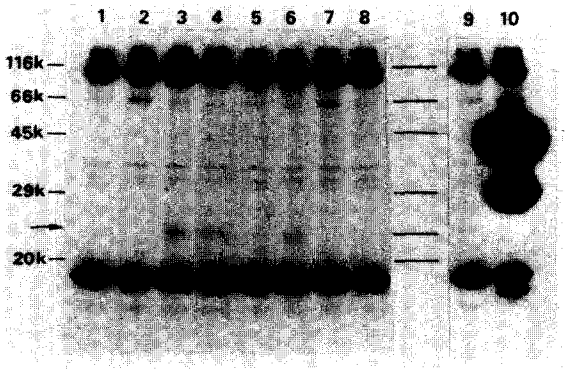

Fig.1. ADP-ribosylation of homogenates of PC12 cells by several botulinum toxins. PC12 cell homogenates (30 μg protein) were incubated without (lanes 1,9) or with 0.1 μg botulinum type A toxin (lane 2), type C_1 toxin (lane 3), type D toxin (lane 4), type C-L progenitor toxin (lane 5), type C-M progenitor toxin (lane 6), type D-L progenitor toxin (lane 7), type D-M progenitor toxin (lane 8), and botulinum type C_2 toxin (lane 10) in the presence of [^{32}P]NAD (990 Ci/mmol, about 1 μCi) and 4 mM $MgCl_2$ for 30 min at 37°C as described in section 2. The autoradiogram of the SDS-polyacrylamide gel of the labeled proteins is shown. The arrow denotes the position of the ADP-risobylation substrate of 24 kDa.

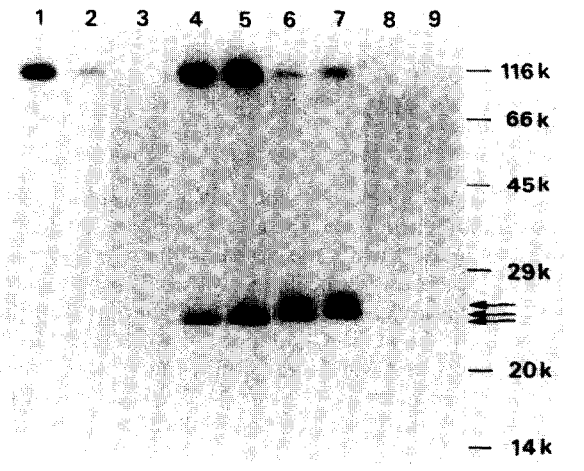

Fig.2. Comparison of molecular masses of ADP-ribosylation substrates in membranes from different cell sources of rat. Membrane preparations of PC12 cells (70 μg protein; lanes 1,4,5), neuron-rich fetal brain cells (50 μg protein; lanes 2,6,7) and C6 cells (50 μg protein; lanes 3,8,9) were incubated without (lanes 1–3) or with 0.5 μg botulinum type D toxin (lanes 4–9) in the presence of 2 μM [^{32}P]NAD (about 2 μCi) and 4 mM $MgCl_2$ for 30 min at 37°C. The reaction mixtures corresponding to lanes 5, 7 and 9 contained 20 μM GTP.

ribosylation of a membrane preparation of C6 glioma cells was also found in the protein band of 24 kDa (fig.2, lanes 8,9), but the intensity of the incorporated radioactivities was very weak.

When PC12 membranes were incubated with [^{32}P]NAD in the presence of type D toxin for various times, labeling of the 24 kDa protein was observed at incubation time longer than 20 min (fig.3, lanes 1–5). Type D toxin-catalyzed ADP-ribosylation of the 24 kDa protein was abolished by monoclonal antibody designated as CA12 which recognizes the heavy chain of type C_1 and D toxins and inhibits the binding of these toxins to synaptosomal membrane [9,16]. Together with the knowledge on other bacterial toxins which bind to plasma membranes by their heavy chain and release the activated light chain possessing ADP-ribosyltransferase activity toward intracellular targets, it is anticipated that the light chain of botulinum neurotoxin has a similar enzymatic function. The above results also suggest that the botulinum neurotoxin types C_1 and D which bind to neuronal membrane by their heavy chain undergo an activation process corresponding to the lag time required for the exertion of the ADP-ribosyltransferase activity by their light chain.

The effects of divalent cations and nucleotides on the type D toxin-catalyzed ADP-ribosyltransferase activity in membrane preparations of PC12 (fig.4A) and fetal brain cells (fig.4B) were examined. In the absence of both $MgCl_2$ and nucleotides, no ADP-ribosyltransferase activity was found in both membrane preparations (fig.4, lanes 3). Addition of 4 mM $MgCl_2$ alone greatly stimulated the toxin-catalyzed ADP-ribosylation of the 24 kDa protein in membranes of PC12 (fig.4A, lane 7) and 25, 26 kDa doublet proteins in fetal brain cells (fig.4B, lane 7). $CaCl_2$ at 4 mM also stimulated the ADP-ribosylation (fig.3, lane 6). However, the combination of 4 mM $MgCl_2$ and 4 mM $CaCl_2$ rather abolished the ADP-ribosylation (fig.3, lane 7). Although the addition of 20 μM ATP had no effect (fig.4, lanes 4), 20 μM of either GTP or GTPγS, (a nonhydrolyzable

Fig.3. Time course of the toxin-catalyzed ADP-ribosylation reaction and the effect of monoclonal anti-toxin-antibody. Membrane preparations of PC12 cells (120 μg protein) were incubated with 0.6 μg botulinum type D toxin and 2 μM [^{32}P]NAD (about 1 μCi) in the presence of 4 mM $MgCl_2$ (lanes 1-5,8), 4 mM $CaCl_2$ (lane 6), or 4 mM $MgCl_2$ and 4 mM $CaCl_2$ (lane 7) for 5 (lane 1), 10 (lane 2), 20 (lane 3), 30 (lanes 4,6-8) or 60 min (lane 5) at 37°C. The arrow indicates the position of 24 kDa.

analog of GTP) alone stimulated the ADP-ribosylation of 24-26 kDa substrate (fig.4, lanes 5,6) to a lesser extent than that by 4 mM $MgCl_2$. In contrast, in the presence of 4 mM $MgCl_2$, GTPγS greatly diminished the ADP-ribosylation of 24-26 kDa substrates (fig.4, lanes 10), although ATP and GTP had little effect on the ADP-ribosylation in the presence of $MgCl_2$ (fig.4, lanes 8,9). The ADP-ribosylation catalyzed by type C_1 toxin also showed a similar dependence on $MgCl_2$ and guanine nucleotides (not shown).

GTPγS-dependent inhibition of bacterial toxin-catalyzed ADP-ribosylation is a property characteristic to the GTP-binding proteins, G_s and G_i [17]. Therefore, it is strongly suggested that the substrate for the type C_1 and D toxin-catalyzed ADP-ribosylation is a GTP-binding protein itself or a component involved in the GTP-dependent regulatory system. The difference in molecular mass of botulinum neurotoxin-substrate between PC12 cells (24 kDa) and neuron-rich fetal rat brain cells (25 and 26 kDa) suggests that this protein exists in different tissues as different subtypes. Furthermore, the incorporation of radioactivity from [^{32}P]NAD with much higher intensity into PC12 cells and neuron-rich brain cells than in C6 glioma cells suggests that the 24-26 kDa protein substrates are involved in secretion and transmitter release. Recently, it was reported that a 21 kDa membrane protein of bovine adrenal grand serves as the substrate for the botulinum type D toxin-catalyzed ADP-ribosylation [18]. However, the characteristics of this 21 kDa protein as a GTP-binding protein are not yet known and its molecular mass is lower than that which we found in the present study. Recently, a novel GTP-binding protein of 24 kDa has been purified to homogeneity from pig brain membranes (Katada, T., personal communication). A collaborative work to examine whether the purified 24 kDa GTP-binding protein serves as the substrate for the ADP-ribosyltransferase activity of botulinum neurotoxin is currently underway.

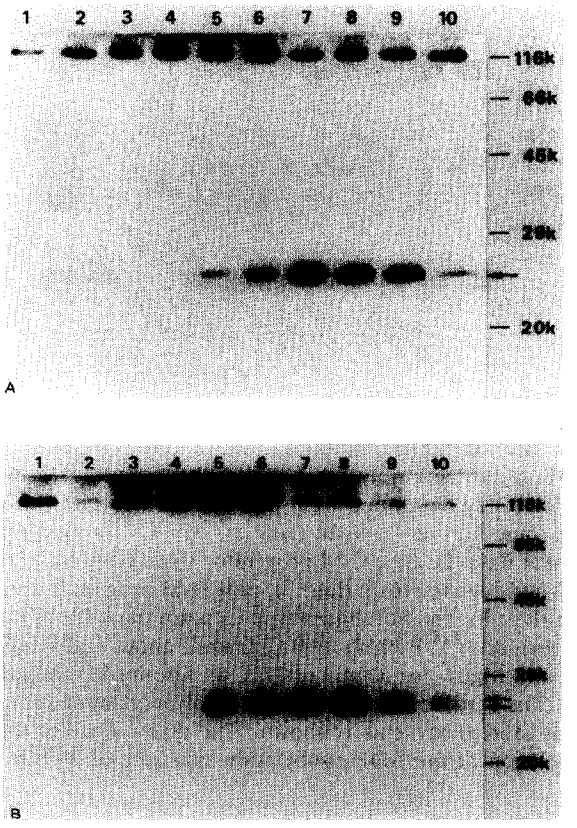

Fig.4. Effects of $MgCl_2$ and nucleotides on the ADP-ribosylation of the 24-26 kDa substrates. Membrane preparations of PC12 cells (A, 260 μg protein) or neuron-rich culture of fetal rat brain cells (B, 240 μg protein) were incubated without (lanes 1,2) or with 0.5 μg botulinum type D toxin (lanes 3-10) in the absence (lanes 1,3-6) or presence of 4 mM $MgCl_2$ (lanes 2,7-10) for 30 min at 37°C. The reaction mixtures contained the following nucleotides; 20 μM ATP (lanes 3,7), 20 μM GTP (lanes 4,8), 20 μM GTPγS (lanes 5,10). The reaction mixtures also contained 2 μM [^{32}P]NAD (about 1 μCi).

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