Purification, Characterization, and Localization of an ADP-ribosylactin Hydrolase That Uses ADP-ribosylated Actin from Rat Brains as a Substrate*

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Mammalian ADP-ribosylation is poorly understood. An ADP-ribosylprotein hydrolase that acted on ADP-ribosylated actin was purified from rat brain. The molecular weight of this enzyme was 62,000 as determined by SDS-polyacrylamide gel electrophoresis and gel filtration. Enzyme activity with ADP-ribosylated actin as a substrate was inhibited by NAD, ATP, ADP, and GDP-ribose, but not by AMP. Mg2+ increased Vmax. Purified ADP-ribosylactin hydrolase catalyzed the hydrolysis of ADP-ribosylated subunits Gα, Gβ, and Gγ and elongation factor-2. After de-ADP-ribosylation by the purified ADP-ribosylactin hydrolase, the proteins were re-ADP-ribosylated by brain mono-ADP-ribosyltransferases and bacterial toxins. The actin that was de-modified by ADP-ribosylactin hydrolase could form actin filaments. Two kinds of monoclonal antibodies against ADP-ribosylactin hydrolase were prepared and characterized. In an immunohistochemical study, the plasma membranes and cytoplasmic regions of the nerve cells in the rat brain were immunoreactive. In subcellular fractionation of the brains, most of the ADP-ribosylactin hydrolase activity was found in the cytosol and synaptosome fractions. When the synaptosomes were treated with a hypotonic solution, ADP-ribosylactin hydrolase activity was found in the supernatant. Our findings suggest that brain ADP-ribosylactin hydrolase has the important function of polymerizing actin for signal transduction in the cytosol of nerve cells and synaptosomes.

ADP-ribosylation is one kind of covalent modification of cellular proteins. It involves the transfer of ADP-ribose from NAD to various acceptor proteins. Cholera, pertussis, diphtheria, and botulinum toxins have ADP-ribosyltransferase activity for specific acceptor proteins such as the heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins),1 cytoskeletal actin, microtubules, and elongation factor-2 (1–4). Cytoskeletal actin is the most abundant protein in nonmuscle cells and is important in exocytosis, endocytosis, locomotion, cell division, and cytoplasmic streaming (5, 6). Actin in nerve cells and is important in exocytosis, endocytosis, locomotion, cell division, and cytoplasmic streaming (5, 6). Actin in nerve cells and is important in exocytosis, endocytosis, locomotion, cell division, and cytoplasmic streaming (5, 6). Actin in nerve cells and is important in exocytosis, endocytosis, locomotion, cell division, and cytoplasmic streaming (5, 6). Actin in nerve cells and is important in exocytosis, endocytosis, locomotion, cell division, and cytoplasmic streaming (5, 6). Actin in nerve cells and is important in exocytosis, endocytosis, locomotion, cell division, and cytoplasmic streaming (5, 6). Actin in nerve cells and is important in exocytosis, endocytosis, locomotion, cell division, and cytoplasmic streaming (5, 6). Actin in nerve cells and is important in exocytosis, endocytosis, locomotion, cell division, and cytoplasmic streaming (5, 6). Actin in nerve cells and is important in exocytosis, endocytosis, locomotion, cell division, and cytoplasmic streaming (5, 6).

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There are few reports on ADP-ribosylation in animals. NAD:arginine ADP-ribosyltransferases have been purified from turkey erythrocytes and chicken livers (10, 11), and human erythrocytes have an ADP-ribosyltransferase that acts on Gα (12). ADP-ribosyltransferase activity has been found in rabbit muscles and canine hearts (13, 14) and has been cloned from other animals (15). Four ADP-ribosyltransferases that use cytoplasmic actin as a substrate have been purified from rat brains and adrenal medullae. The modified actin does not form actin filaments after the addition of Mg2+ (16, 17). Therefore, it seems that the ADP-ribosylation of actin can break an actin barrier for the movement of secretory granules in exocytotic cells. Little is known about the de-ADP-ribosylation of modified proteins. An ADP-riboseprotein hydrolase that hydrolyzes ADP-ribosylated arginine has been purified from turkey erythrocytes (18). The immunoreactivity of certain proteins was identified by the use of a rat ADP-ribosylhydrolase antibody found in the most abundance in the brain, spleen, and testis (19). ADP-ribosearginine hydrolases from rats, humans, and mice have been cloned, and their sequences have been found to be similar (20). It is not known whether ADP-ribosylated histone, G proteins, and cytoskeletal proteins are hydrolyzed in animals by their own enzymes or not. If actin polymerization is regulated by ADP-ribosylation, then ADP-ribosylactin hydrolase can probably be found in rat brains. Actin filaments must be destabilized before catecholamine secretion, and so stabilization by phallolidin reduces catecholamine release (21). Therefore, ADP-ribosylation may help regulate exocytosis through actin polymerization and depolymerization. The de-modification of ADP-ribosylated proteins might be needed to destroy the actin barrier. Consequently, the characterization and location of ADP-ribosylactin hydrolase in the brain are of interest. Here, we describe the purification and characterization of ADP-ribosylactin hydrolase from rat brain and report on its location.

EXPERIMENTAL PROCEDURES

Materials—[adenosine-32P]NAD (800 Ci/mmol), [adenosine-ultrahigh specific activity]NAD (400 mCi/mmol), and [125I]labeled Bolton-Hunter reagent (2200 Ci/mmol) were purchased from NEN Life Science Products. NAD and ATP were from the Oriental Yeast Co. Cholera toxin, AMP, ADP, and ADP-ribose were from Sigma. Diphtheria toxin was from Calbiochem. Pertussis toxin was a gift from Kaken Pharmaceutical Co. Botulinum C2 toxin was a gift from Dr. Shunji Kozaki (Laboratory of Public Health, Osaka Prefecture University). A neuron-specific enolase monoclonal antibody was purchased from InRo Biometek. The other reagents used were analytical grade.

Purification of ADP-ribosylactin Hydrolase from Rat Brain—Adult male Wistar rats (body weight, 250–300 g) were anesthetized with ether and killed. About 60 g of isolated brain (wet weight) was washed with cold saline. The isolated brains were homogenized in 4 volumes of "advertisement" in accordance with 18 U.S.C. Section 1734 solely to cause this fact.

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1 The abbreviations used are: G proteins, heterotrimeric guanine nucleotide-binding regulatory proteins; HPLC, high-pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis.
20 mM potassium phosphate (pH 7.5) that contained 0.2 mM dithiothreitol. The purification is summarized in Table I. The homogenate was centrifuged at 80,000 x g for 30 min at 4 °C. The supernatant (~1200 mg of protein) was applied to a CM-Sepharose column (2.5 x 20 cm) equilibrated with homogenization buffer. The column was washed with 200 ml of the same buffer. The unabsorbed fraction (~1100 mg) was collected and loaded onto a DEAE-Sepharose column (2.5 x 20 cm) equilibrated with homogenization buffer. Elution was done with a linear gradient of NaCl from 0 to 0.7 M. The fractions that contained ADP-ribosylactin hydrolase activity with [32P]ADP-ribosylated actin as a substrate were collected. NaCl was added to the fractions that contained ADP-ribosylactin hydrolase activity to a final concentration of 1.5 M. The fractions that contained ADP-ribosylactin hydrolase activity were collected and concentrated with a Mini-module apparatus. Actin (0.2 mg/ml) was incubated with 1.0 mM ADP-ribose for 30 min at 4 °C. The supernatant (500 μl) was applied to a Bio-Gel P-2 column equilibrated with 20 mM potassium phosphate buffer (pH 7.5) containing 2 mM dithiothreitol. The fraction containing the radiolabeled ADP-ribosylated actin (0.74–0.92 mol of ADP-ribose/mol of actin, 10,000 cpm/μg of protein) was used as the substrate for ADP-ribosylactin hydrolase. In some experiments, botulinum C2 toxin or nonenzymatically ADP-ribosylated actin was prepared as described elsewhere (22, 23); the excess radiolabeled NAD or ADP-ribose was removed by gel filtration on a TSK 3000SW or Bio-Gel P-2 column as described above, and the fraction containing ADP-ribosylated actin was used as the substrate. For the nonenzymatic preparation of ADP-ribosylated actin, [32P]ADP-ribose was produced by the incubation of 0.1 mM [32P]NAD with NADase from Neurospora crassa (Sigma) for 20 min at 35 °C. The NADase was inactivated by boiling for 3 min. [32P]ADP-ribose was separated by HPLC as described below. The purified [32P]ADP-ribose was concentrated with an evaporator. Actin (0.2 mg/ml) was incubated with 1.0 mM [32P]ADP-ribose and 10 mM diethiothreitol in 25 mM triethanolamine HCl (pH 7.5) for 30 min at 30 °C. After incubation, the reaction mixture was kept on ice for 2 days to allow the enzyme activity to be completely lost. The excess [32P]NAD or [14C]NAD in the reaction mixture was then removed with a Bio-Gel P-2 column equilibrated with 20 mM potassium phosphate buffer (pH 7.5) containing 2 mM dithiothreitol. The fraction containing the radiolabeled ADP-ribosylated actin (0.74–0.92 mol of ADP-ribose/mol of actin, 10,000 cpm/μg of protein) was used as the substrate for ADP-ribosylactin hydrolase. In some experiments, botulinum C2 toxin or nonenzymatically ADP-ribosylated actin was prepared as described elsewhere (22, 23); the excess radiolabeled NAD or ADP-ribose was removed by gel filtration on a TSK 3000SW or Bio-Gel P-2 column as described above, and the fraction containing ADP-ribosylated actin was used as the substrate. For the nonenzymatic preparation of ADP-ribosylated actin, [32P]ADP-ribose was produced by the incubation of 0.1 mM [32P]NAD with NADase from Neurospora crassa (Sigma) for 20 min at 35 °C. The NADase was inactivated by boiling for 3 min. [32P]ADP-ribose was separated by HPLC as described below. The purified [32P]ADP-ribose was concentrated with an evaporator. Actin (0.2 mg/ml) was incubated with 1.0 mM [32P]ADP-ribose and 10 mM diethiothreitol in 25 mM triethanolamine HCl (pH 7.5) for 30 min at 30 °C. After incubation, the reaction mixture was kept on ice for 2 days to allow the enzyme activity to be completely lost. The excess [32P]NAD or [14C]NAD in the reaction mixture was then removed with a Bio-Gel P-2 column equilibrated with 20 mM potassium phosphate buffer (pH 7.5) containing 2 mM dithiothreitol. The fraction containing the radiolabeled ADP-ribosylated actin (0.74–0.92 mol of ADP-ribose/mol of actin, 10,000 cpm/μg of protein) was used as the substrate for ADP-ribosylactin hydrolase.

Enzyme Assay—The standard assay for ADP-ribosylactin hydrolase activity was as follows. The substrate was prepared by the in vitro reaction at 37 °C for 30 min of 0.2 μg/ml purified ADP-ribosyltransferase II (16), 42 μM monomeric cytoplasmic actin, and 0.2 mM [32P]NAD or [14C]NAD in 50 mM imidazole HCl (pH 8.0) containing 10% propylene glycol and 2 mM mercaptoethanol. After incubation, the reaction mixture was kept on ice for 2 days to allow the enzyme activity to be completely lost. The excess [32P]NAD or [14C]NAD in the reaction mixture was then removed with a Bio-Gel P-2 column equilibrated with 20 mM potassium phosphate buffer (pH 7.5) containing 2 mM dithiothreitol. The fraction containing the radiolabeled ADP-ribosylated actin (0.74–0.92 mol of ADP-ribose/mol of actin, 10,000 cpm/μg of protein) was used as the substrate for ADP-ribosylactin hydrolase. In some experiments, botulinum C2 toxin or nonenzymatically ADP-ribosylated actin was prepared as described elsewhere (22, 23); the excess radiolabeled NAD or ADP-ribose was removed by gel filtration on a TSK 3000SW or Bio-Gel P-2 column as described above, and the fraction containing ADP-ribosylated actin was used as the substrate. For the nonenzymatic preparation of ADP-ribosylated actin, [32P]ADP-ribose was produced by the incubation of 0.1 mM [32P]NAD with NADase from Neurospora crassa (Sigma) for 20 min at 35 °C. The NADase was inactivated by boiling for 3 min. [32P]ADP-ribose was separated by HPLC as described below. The purified [32P]ADP-ribose was concentrated with an evaporator. Actin (0.2 mg/ml) was incubated with 1.0 mM [32P]ADP-ribose and 10 mM diethiothreitol in 25 mM triethanolamine HCl (pH 7.5) for 30 min at 30 °C. After incubation, the reaction mixture was kept on ice for 2 days to allow the enzyme activity to be completely lost. The excess [32P]NAD or [14C]NAD in the reaction mixture was then removed with a Bio-Gel P-2 column equilibrated with 20 mM potassium phosphate buffer (pH 7.5). The fraction containing radiolabeled actin (1.72–2.05 mol of ADP-ribose/mol of actin, 12,500 cpm/μg) was concentrated with an Amicon 10 microconcentrator and used as the substrate. In a check of the bonding of cysteine with ADP-ribose, a portion of nonenzymatically ADP-ribosylated actin was incubated with 0.5 mM NaCl or 0.5 mM NH₄OH (pH 7.5) for 2 h or with 1 mM HgCl₂ for 30 min at 37 °C as previously reported (17). After the incubation, actin was precipitated by the addition of trichloroacetic acid to a final concentra-
tion of 10%. The precipitated actin was examined by SDS-PAGE and autoradiography. Other proteins (the α-subunits of the G proteins Gα, Gβ, and Gγ and elongation factor-2) were ADP-ribosylated with ADP-ribosyltransferase II from rat brain or with cholera, pertussis, or diphtheria toxin as previously reported (1, 2, 16, 24). The excess [32P]NAD and toxin were removed with two connected TSK 3000SW columns as described above. ADP-ribosylated actin, one of the G proteins, or elongation factor-2 (0.4 mg/ml) was incubated with purified ADP-ribosylactin hydrolase (5 μg/ml) in 20 mM potassium phosphate buffer (pH 7.5) with or without 2 mM dithiothreitol at 30 °C for 30 min. During or after incubation, the radioactivity that remained in the actin or the other proteins was measured by instant TLC by the method of Huang and Robinson (25). To check for ADP-ribosylactin hydrolase activity, we examined the products of the reaction using HPLC as follows. The reaction mixture was denatured by the addition of an equal volume of 10% perchloric acid to a final concentration of 5%. After removal of the precipitate, the pH of the supernatant was adjusted to 6.0 with KOH. The resulting precipitate was removed by centrifugation, and the supernatant was assayed by HPLC. Analysis was done with a C18 column (4.6 × 150 mm; G.L. Sciences Co.). After injection of the sample, 20 mM potassium phosphate buffer (pH 6.0) was supplied at a flow rate of 0.8 ml/min for 5 min, after which the concentration of acetonitrile was increased linearly from 0 to 55% during the next 15 min. The column effluent was monitored at 280 nm with a UV detector and a β-ray detector (A-120, Packard Japan), and the radiolabeled ADP-ribose was detected.

For separation of ADP-ribosylated and unmodified actin from the reaction mixture, the incubation mixture was assayed by HPLC. Analysis was done with a C18 column (6.0 × 150 mm, 5-μm particles, 3-μm pores; G.L. Sciences Co.) equilibrated with 10 mM potassium phosphate buffer (pH 6.5) and 10% acetonitrile. After injection of the sample, the equilibrium buffer was supplied continuously for 6 min at a flow rate of 1.0 ml/min, after which the concentration of acetonitrile was increased linearly up to 70% during the next 15 min. The column effluent was monitored at 280 nm with a UV detector. The effluent was fractionated. The fractions were further analyzed by SDS-PAGE and autoradiography.

Further confirmation of the product in question as ADP-ribose was undertaken in two additional experiments. After the reaction of ADP-ribosylactin hydrolase and ADP-ribosylated actin was stopped by the addition of 9 volumes of ethanol, the supernatant was freeze-dried. The sample was dissolved in 100 μl of water. A portion of the solution was examined by HPLC with a C18 or Bio-Rex 70 column. The remaining solution was mixed with 2 mM MgCl₂, 2 mM CaCl₂, and 0.1 mM ZnCl₂. The mixture was treated with venom phosphodiesterase (100 units/ml; Boehringer Mannheim) at 37 °C for 1 h. The radiolabeled ADP-ribose, AMP, and adenosine generated were analyzed by HPLC with a C18 or Bio-Rex 70 column, a UV monitor at 260 nm, and a β-ray detector (A-120, Packard Japan), and the radiolabeled ADP-ribose was detected.

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Details of the purification are given under “Experimental Procedures.” Values are typical values from the five experiments.

| Step                              | Total protein | Total activity | Specific activity | Yield | Purification |
|-----------------------------------|---------------|----------------|------------------|-------|--------------|
| 80,000 × g supernatant            | 1220          | 9.72           | 0.008            | 1     | -fold        |
| CM-Sephadex                      | 1120          | 24.0           | 0.021            | 100   | 1            |
| DEAE-Sephadex                    | 216           | 20.3           | 0.094            | 84.6  | 3            |
| Butyl-Toyopearl                  | 1.02          | 15.6           | 15.3             | 65.0  | 1910         |
| Sephadex C-75                    | 0.54          | 14.3           | 26.5             | 59.6  | 3310         |

FIG. 3. Elution profile of ADP-ribosylated actin by HPLC. A, elution profile of actin ADP-ribosylated in vitro. Actin was ADP-ribosylated by rat brain mono-ADP-ribosyltransferase II (0.76 mol of ADP-ribose/mol of actin). a, SDS-PAGE of the sample in A after elution. Bottom, autoradiograph of the sample in a. B, elution profile of actin (0.51 mol of ADP-ribose/mol of actin) de-ADP-ribosylated by purified ADP-ribosylactin hydrolase. ADP-ribosylated actin was incubated with purified ADP-ribosylactin hydrolase in 20 mM potassium phosphate buffer (pH 7.5) containing 2 mM dithiothreitol with 2 mM Mg²⁺ at 30 °C for 30 min. b, SDS-PAGE of the sample in B after elution. Bottom, autoradiograph of the sample in b. The molecular mass markers are the same as described for Fig. 2C. Closed triangles, ADP-ribosylated actin; open triangles, unmodified actin.
incubated with an enzyme or a bacterial toxin and [32P]NAD again. The rate of re-ADP-ribosylation was assayed by the method described above.

**Immunohistochemical Experiments**—We prepared IgG from monoclonal antibodies against rat brain ADP-ribosylactin hydrolase by the method of Goding (28). In a test of what region of rat brain ADP-ribosylactin hydrolase was recognized by the IgG, purified rat brain antibody to rat brain ADP-ribosylactin hydrolase was loaded onto an HPLC column, a large single peak was identified as [32P]ADP-ribose by HPLC (data not shown). When the peak fractions containing ADP-ribose were monitored with a beta-image analyzer, its angle of inclination was 25°. A stainless steel ball was placed on the meniscus of the sample. The dropping velocity of the ball from the meniscus to the end tube was measured.

SDS-PAGE was done by the method of Laemmli (39). The protein on the gel was stained with silver or Coomassie Brilliant Blue. The gel was dried and exposed to x-ray film. Some of the gel was electroblotted on a nitrocellulose membrane, and the membrane was treated with monoclonal antibodies. Immunocomplexes were detected with diaminobenzidine. The protein was assayed by the method of Bradford (40) or Lowry et al. (41).

**RESULTS**

**Purification of ADP-ribosylactin Hydrolase**—After chromatography on CM-Sepharose, the unabsorbed fraction did not contain ADP-ribosyltransferase activity when monomeric cytosolic actin was the substrate (data not shown). The unabsorbed fraction had two peaks of hydrolytic activity for ADP-ribosylated actin as seen by DEAE-Sepharose column chromatography (Fig. 1A). The product in peak 1 (fractions 22–24) was identified as radiolabeled 5'-AMP, and that in peak 2 (fractions 29–35) was identified as radiolabeled ADP-ribose (Fig. 1B). Therefore, peak 1 was phosphodiesterase activity, and peak 2 was ADP-ribosehydrolyase activity. The peak 2 fraction gave a single peak as determined by butyl-Toyopearl column chromatography (Fig. 2A). The product of the enzyme reaction was identified by HPLC as radiolabeled ADP-ribose (data not shown). When the peak fractions containing ADP-ribosylactin hydrolase activity were chromatographed on a Sephadex G-75 column, a single peak of absorbance at 280 nm was found (Fig. 2B). The product of the enzyme reaction of the single peak was identified as [32P]ADP-ribose by HPLC (data not shown). The molecular weight of the purified ADP-ribosylactin hydrolase was estimated to be 62,000 from the results of gel filtration. SDS-PAGE of the purified enzyme showed it to be homogeneous and to have a molecular weight of 62,000; one major band of protein was detected on the gel by silver staining (Fig. 2C). When actin ADP-ribosylated by purified ADP-ribosyltransferase II was loaded onto an HPLC column, a large amount of ADP-ribosylated actin was eluted separately from a small amount of unmodified actin (Fig. 3A). When a mixture of
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In panel A, actin was ADP-ribosylated by botulinum C2 toxin and [32P]NAD. In panel B, actin was ADP-ribosylated by botulinum C2 toxin and [14C]NAD. In panel C, actin was ADP-ribosylated by a nonenzymatic reaction and [32P]ADP-ribose. Values are the means ± S.D. of three experiments.

| Product                  | Treatment                        | ADP-ribosylactin hydrolase<sup>a</sup> | Phosphodiesterase<sup>b</sup> | Phosphodiesterase + alkaline phosphatase<sup>c</sup> |
|--------------------------|----------------------------------|--------------------------------------|-------------------------------|---------------------------------------------------|
|                          |                                   | mol product/mol actin                |                               |                                                   |
| A. [32P]ADP-ribose<sup>d</sup> | 0.77 ± 0.04 (100%)               | 0.13 ± 0.03 (16.8%)                 | 0.11 ± 0.01 (14.2%)           |
| 5′-[32P]AMP<sup>d</sup>    | ND<sup>e</sup>                   | 0.59 ± 0.05 (76.6%)                 | 0.19 ± 0.02 (24.7%)           |
| Adenosine<sup>e</sup>     | ND<sup>e</sup>                   | ND                                   | 0.39 ± 0.04 (50.6%)           |
| Phosphoribose<sup>e</sup> | ND<sup>e</sup>                   | 0.61 ± 0.05 (79.2%)                 | 0.57 ± 0.01 (74.0%)           |
| Ribose<sup>e</sup>        | ND<sup>e</sup>                   | ND                                   | ND                            |
| [32P]Phosphate<sup>e</sup> | ND<sup>e</sup>                   | ND                                   | ND                            |
| B. [14C]ADP-ribose<sup>d</sup> | 0.79 ± 0.02 (100%)               | 0.11 ± 0.04 (10.9%)                 | 0.08 ± 0.01 (9.2%)            |
| 5′-[14C]AMP<sup>d</sup>    | ND<sup>e</sup>                   | 0.63 ± 0.04 (79.7%)                 | 0.21 ± 0.03 (26.6%)           |
| [14C]Adenosine<sup>e</sup> | ND<sup>e</sup>                   | ND                                   | 0.37 ± 0.02 (46.8%)           |
| Phosphoribose<sup>e</sup> | ND<sup>e</sup>                   | 0.64 ± 0.03 (81.0%)                 | 0.69 ± 0.06 (87.3%)           |
| Ribose<sup>e</sup>        | ND<sup>e</sup>                   | ND                                   | ND                            |
| Phosphate                 | ND<sup>e</sup>                   | ND                                   | ND                            |
| C. [32P]ADP-ribose<sup>d</sup> | 1.29 ± 0.12 (100%)               | 0.27 ± 0.03 (20.9%)                 | 0.17 ± 0.02 (13.2%)           |
| 5′-[32P]AMP<sup>d</sup>    | ND<sup>e</sup>                   | 1.05 ± 0.04 (81.4%)                 | 0.43 ± 0.04 (33.3%)           |
| Adenosine<sup>e</sup>     | ND<sup>e</sup>                   | ND                                   | 0.57 ± 0.05 (44.2%)           |
| Phosphoribose<sup>e</sup> | ND<sup>e</sup>                   | 1.01 ± 0.05 (78.3%)                 | 1.06 ± 0.07 (82.2%)           |
| Ribose<sup>e</sup>        | ND<sup>e</sup>                   | ND                                   | ND                            |
| [32P]Phosphate<sup>e</sup> | ND<sup>e</sup>                   | ND                                   | 0.53 ± 0.06 (41.1%)           |

<sup>a</sup> Each kind of ADP-ribosylated actin (0.4 mg/ml) was incubated with 5 μg/ml purified ADP-ribosylactin hydrolase at 30 °C for 30 min.

<sup>b</sup> After incubation with ADP-ribosylactin hydrolase and ADP-ribosylated actin, the incubation mixture was denatured by the addition of 9 volumes of ethanol. The supernatant was freeze-dried, and the sample was dissolved in 100 μl of water. The sample was incubated with 100 units/ml each phosphodiesterase and alkaline phosphatase at 37 °C for 1 h. After incubation, the reaction mixture was centrifuged. A portion of the supernatant was assayed by HPLC. After freeze-drying, the remaining supernatant was spotted on a plate for high-resolution TLC and analyzed.

<sup>c</sup> After incubation with ADP-ribosylactin hydrolase and ADP-ribosylated actin, the compounds released from the incubation mixture were treated with ethanol as described above. The sample was incubated with 100 units/ml each phosphodiesterase and alkaline phosphatase at 37 °C for 1 h. The products were assayed by HPLC and high-resolution TLC as described above.

<sup>d</sup> Material was assayed by HPLC.

<sup>e</sup> ND, not detected; —, not examined.

<sup>f</sup> Material was assayed by high-resolution TLC. A laser densitometric analyzer linearly detected a ribose from 0.1 to 10 μg after spraying aniline/diphenylamine/phosphoric acid reagent.

<sup>g</sup> Material was extracted by the addition of silicotungstic solution. The radioactivity was measured by a scintillation counter.

ADP-ribosylated actin and purified ADP-ribosylactin hydrolase was loaded onto an HPLC column, a large amount of unmodified actin was eluted separately from a small amount of ADP-ribosylated actin (Fig. 3B). After fractionation of the mixture by HPLC, the radioactivity that corresponded to the ADP-ribosylated actin had decreased, and SDS-PAGE did not show protein degradation (Fig. 3, a and b). Comparison of the elution profile with that of a standard mixture of nucleotides showed that the retention times of the products from the reaction mixture of purified ADP-ribosylactin hydrolase and ADP-ribosylated actin were the same as the retention time of free ADP-ribose (Fig. 1). The area of the product peak increased with increasing incubation time, suggesting that the formation of the product was time-dependent. The purification of brain ADP-ribosylactin hydrolase is summarized in Table I. The enzyme was purified 3310-fold in terms of its specific activity with ADP-ribosylated actin as the substrate as compared with the supernatant of the brain homogenate.
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To confirm that ADP-ribose was produced from \(^{32}\)P- or \(^{14}\)C]ADP-ribosylated actin by the reaction of purified ADP-ribosylactin hydrolase, we assayed the supernatant of the reaction mixture by HPLC after the end of this reaction was assayed. Radiolabeled ADP-ribose was eluted. When the supernatant was incubated with phosphodiesterase, radiolabeled ADP-ribose and 5'-AMP were eluted, and nonradiolabeled phosphoribose, radiolabeled ADP-ribose, and radiolabeled 5'-AMP were found by high-resolution TLC. When the supernatant of the reaction mixture after the end of the reaction with \(^{32}\)P- or \(^{14}\)C]ADP-ribosylated actin was incubated with both phosphodiesterase and alkaline phosphatase, \(^{32}\)P- or \(^{14}\)C]ADP-ribose, 5'-[\(^{32}\)P]- or 5'-[\(^{14}\)C]AMP, and \(^{14}\)C]adenosine were eluted from the HPLC column. Inorganic phosphate labeled with \(^{32}\)P was detected in an assay. Nonradiolabeled ADP-ribosylactin hydrolase, almost all of the radioactivity disappeared (Fig. 5). The radiolabeled compound in the reaction mixture was identified as ADP-ribose by HPLC (data not shown). The reaction was completed in 20 min. When actin ADP-ribosylated by botulinum C2 toxin or a nonenzymatic reaction was incubated with ADP-ribosylactin hydrolase, almost all of its radioactivity was lost. After de-ADP-ribosylated actin was further incubated with C2 toxin and \(^{32}\)P]NAD or in the presence of \(^{32}\)P]ADP-ribose without toxin, the actin was found to be radioactive. In a nonenzymatic reaction, the radioactivity was stable during treatment with NaCl or NH₄OH, but not after treatment with HgCl₂ (data not shown). There were no differences in the distribution of the peptide and in the radioactivity after digestion by trypsin (Fig. 6a). Comparison with these two kinds of ADP-ribosylated actin showed that a peptide with a molecular weight of 20,000 (indicated by the arrow in Fig. 6b) had strong radioactivity in the nonenzymatic reaction. Autoradiography after SDS-PAGE showed that the second nonenzymatic \(^{32}\)P]ADP-ribose was degraded by the addition of HgCl₂ (data not shown).

When elongation factor-2 ADP-ribosylated by diphtheria toxin was incubated with ADP-ribosylactin hydrolase, there was no radioactivity. The de-ADP-ribosylated elongation factor-2 was radioactive again after incubation with the toxin. There were no differences in the distribution of peptides on SDS-PAGE and in radioactivity between the first and the re-ADP-ribosylated elongation factor-2 that had been treated with diphtheria toxin (Fig. 7a). After incubation with ADP-ribosylactin hydrolase, the G proteins that had been ADP-ribosylated by ADP-ribosyltransferase II, cholera toxin, or pertussis toxin were re-ADP-ribosylated by a toxin or an enzyme (Fig. 7b).

**Table IV**

| Substrate | No addition | Dithiothreitol (2 mM) | Mg²⁺ (2 mM) | Dithiothreitol and Mg²⁺ (2 mM each) |
|-----------|-------------|----------------------|-------------|-----------------------------------|
| Arg-actin | 0.17 ± 0.01 | 1.73 ± 0.21          | 2.34 ± 0.15 | 33.7 ± 4.2                        |
| Cys-actin | 0.15 ± 0.03 | 2.71 ± 0.30          | 1.65 ± 0.23 | 33.0 ± 2.8                        |

*Actin ADP-ribosylated by rat brain ADP-ribosyltransferase II. ADP-ribosylated by a nonenzymatic reaction. To confirm that the cysteine of actin were modified, a portion of the preparation was incubated with NaCl, NH₄OH, or HgCl₂ as described under “Experimental Procedures.”

*Fig. 5. A, SDS-PAGE of the incubation mixture of purified ADP-ribosylactin hydrolase and actin ADP-ribosylated by rat brain mono-ADP-ribosyltransferase II (ADP-T II), botulinum C2 toxin (C2), or a nonenzymatic reaction (Non-Enz.). Each kind of ADP-ribosylated actin (0.4 mg/ml) was incubated with 5 μg/ml purified ADP-ribosylactin hydrolase in 20 mM potassium phosphate buffer (pH 7.5) containing 2 mM dithiothreitol and MgCl₂ for 30 min at 30 °C. The molecular mass markers are the same as described for Fig. 2C, d.f., dye front. B, autoradiography of A. 42 kDa indicates actin. Ten micrograms of protein was loaded onto each lane.*

**FIG. 5.** A, SDS-PAGE of the incubation mixture of purified ADP-ribosylactin hydrolase and actin ADP-ribosylated by rat brain mono-ADP-ribosyltransferase II (ADP-T II), botulinum C2 toxin (C2), or a nonenzymatic reaction (Non-Enz.). Each kind of ADP-ribosylated actin (0.4 mg/ml) was incubated with 5 μg/ml purified ADP-ribosylactin hydrolase in 20 mM potassium phosphate buffer (pH 7.5) containing 2 mM dithiothreitol and MgCl₂ for 30 min at 30 °C. The molecular mass markers are the same as described for Fig. 2C, d.f., dye front. B, autoradiography of A. 42 kDa indicates actin. Ten micrograms of protein was loaded onto each lane.

**Characteristics of ADP-ribosylactin Hydrolase**—The optimum pH was 7.5 for ADP-ribosylactin hydrolase with ADP-ribosylated actin as the substrate. The Kₘ and Vₘₐₓ for ADP-ribosylated actin, calculated from Lineweaver-Burk plots, were 6.7 ± 0.4 μM and 6.8 ± 0.3 nmol/min (mean ± S.D.; five preparations), respectively. Of the possible inhibitors examined, ADP-ribose caused more inhibition than NAD, ATP, or ADP, and AMP caused no inhibition (Table III). ADP-ribosylactin hydrolase activity was maximum with 2 mM Mg²⁺. When Mg²⁺ was added to the reaction mixture to a final concentration of 2 mM, the Vₘₐₓ of ADP-ribosylactin hydrolase for ADP-ribosylated actin increased to 10.0 ± 0.4 nmol/min. ADP-ribosylactin hydrolase had maximum activity in the presence of dithiothreitol without Mg²⁺, cleaved nonenzymatically ADP-ribosylated actin (Table IV).

**ADP-ribosylation Cycle with Various Proteins**—When actin ADP-ribosylated by rat brain ADP-ribosyltransferase II, botulinum C2 toxin, or a nonenzymatic reaction was incubated with purified ADP-ribosylactin hydrolase, almost all of the radioactivity of the actin disappeared (Fig. 5).
where synapses were abundant as well as in the cytoplasm of immunocomplexes were seen to be on plasma membranes (Fig. 10, I). In Brain—When nonspecific IgG was used to treat rat

IgG had no effect on ADP-ribosylactin hydrolase activity at a high IgG concentration. Nonspecific antibody 9E7. Monoclonal antibodies 8F12 and 7B2 inhibited half of ADP-ribosylated actin was completely inhibited by the addition of 9E7. Immunobeads conjugated with 9E7, 8F12, or 7B2 precipitated iodinated rat brain ADP-ribosylactin hydrolase. Immunobeads conjugated with nonspecific IgG did not precipitate iodinated rat brain ADP-ribosylactin hydrolase. At each step, a portion of the actin was boiled and treated with trypsin as described above. The arrow indicates a band of radioactive peptide containing cysteine not seen in A. All proteins in the reaction mixture were first ADP-ribosylated by toxin or a nonenzymatic reaction. After incubation, the protein was loaded onto a TSK 300SW column. The ADP-ribosylated protein fraction was concentrated, and the protein was incubated with ADP-ribosylactin hydrolase. The de-ADP-ribosylated protein was again loaded onto a TSK 300SW column. The protein was concentrated and incubated with a toxin or ADP-ribose again. Ten micrograms of protein was loaded onto each lane. The molecular mass markers are ovalbumin (42.7 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

Mg$^{2+}$ was added to the actin that was removed ADP-ribose by ADP-ribosylactin hydrolase, the viscosity of the actin preparation increased. After de-ADP-ribosylation, the actin was separated by gel filtration. When this actin was again incubated with botulinum C2 toxin and NAD, the actin was re-ADP-ribosylated by C2 toxin, treated with ADP-ribosylactin hydrolase, and further ADP-ribosylated with [32P]ADP-ribose. At each step, a portion of the actin was removed by gel filtration (TSK 300SW) as described in the legend of Fig. 5.

**Characterization of Monoclonal Antibodies to ADP-ribosylactin Hydrolase—** Immunobeads conjugated with nonspecific IgG did not precipitate iodinated rat brain ADP-ribosylactin hydrolase. Immunobeads conjugated with 9E7, 8F12, or 7B2 precipitated the radiolabeled enzyme in a dose-dependent way (Fig. 9A). The activity of purified ADP-ribosylactin hydrolase toward ADP-ribosylated actin was completely inhibited by the addition of 9E7. Monoclonal antibodies 8F12 and 7B2 inhibited half of the enzyme activity at a high IgG concentration. Nonspecific IgG had no effect on ADP-ribosylactin hydrolase activity (Fig. 9B).

**Immunohistochemical Findings of ADP-ribosylactin Hydrolase in Brain—** When nonspecific IgG was used to treat rat brain slices, immunoreactive materials were not found (Fig. 10A). Use of monoclonal antibody 9E7 or 8F12 gave immunocomplexes in the nerve cell layers of the rat brain hippocampus (Fig. 10, B and C). When observed at higher magnification, the immunocomplexes were seen to be on plasma membranes where synapses were abundant as well as in the cytoplasm of the nerve cells (Fig. 10D).

**Subcellular Distribution of ADP-ribosylactin Hydrolase—** We examined radiolabeled ADP-ribose produced from an incubation mixture that contained radiolabeled ADP-ribosylated actin and a portion of subcellular fractions by HPLC. The cytosol, mitochondria, and synaptosome fractions contain high ADP-ribosylactin hydrolase activity. The specific activity of the ADP-ribosylactin hydrolase in the synaptosome fraction was higher than that in the other fractions. Some fractions had a high level of enzyme activities used as markers (Table V). After treatment of synaptosomes with a hypotonic and alkaline solution, there was much ADP-ribosylactin hydrolase activity in the soluble fraction, but the membrane fraction of the synaptosomes did not have much of this activity after such treatment (Table VI). On immunoblotting of the subcellular fraction, the synaptosome and cytosol fractions contained the 62-kDa protein that had reacted with monoclonal antibody 9E7 against purified ADP-ribosylactin hydrolase. The soluble fraction of synaptosomes had the same 62-kDa protein that had reacted with monoclonal antibody 9E7; this protein was not found in the membrane fraction of synaptosomes. The brain-specific enolase was found in the cytosol fraction and in the soluble fraction of synaptosomes (Fig. 11).

**DISCUSSION**

ADP-ribosylprotein hydrolase with ADP-ribosylarginine as the substrate has been purified from rat brain and turkey erythrocytes (19, 42). This enzyme activity with histone as the substrate has been found in human neutrophils (43). When
ADP-ribosylprotein hydrolase was purified from rat brain, phosphorylase did not contaminate the final preparation of ADP-ribosylactin hydrolase. There was no proteolytic digestion that yielded ADP-ribose during the de-ADP-ribosylation reaction with the purified ADP-ribosylactin hydrolase. Rat brain ADP-ribosylactin hydrolase is different in molecular weight (62,000) and optimum pH (7.5) from turkey erythrocyte ADP-ribosylarginine hydrolase (40,000 and pH 7.0). The difference in the substrates may account for the difference in the optimum pH. At any rate, the differences are not species-related; rat brain ADP-ribosylarginine hydrolases have been cloned in *Escherichia coli* (20).

The mechanism of the de-ADP-ribosylation reaction is not understood in detail. It is of interest that ADP-ribosylactin hydrolase cleaved ADP-ribose from various mono-ADP-ribosylated proteins. The purified ADP-ribosylactin hydrolase produced only radiolabeled ADP-ribose, so ADP-ribose inhibited enzyme activity more than ATP or ADP, and purified ADP-ribosylactin hydrolase did not produce phosphoribose from ADP-ribosylated actin. Therefore, ADP-ribosylactin hydrolase purified from rat brain recognized the ADP-ribose moiety in ADP-ribosylated proteins. Furthermore, the ADP-ribosylactin hydrolase we purified may have thioglucohydrolase activity that cleaves ADP-ribose from the cysteine residue of a protein by pertussis toxin or by a nonenzymatic reaction because we did not find 5'-AMP, adenosine, phosphoribose, or ribose other than ADP-ribose after the reaction of ADP-ribosylactin hydrolase and ADP-riboisoprotein. ADP-ribosylactin hydrolase and ADP-ribose may form an ADP-ribose-enzyme complex, and the enzyme may catalyze the hydrolytic cleavage of the ADP-ribose-protein linkage. The addition of Mg²⁺ and dithiothreitol to the enzyme reaction mixture increased ADP-ribosylactin hydrolase activity. This finding may indicate that Mg²⁺ forms a complex with ADP-ribose in the catalytic region of the enzyme during the hydrolysis of various ADP-ribosylated proteins. With dithiothreitol, the enzyme catalytic region may contain SH, and a cysteine in the ADP-ribosylated protein may be needed to maintain the usual conformation during hydrolysis. C–N and C–S bonds in the various ADP-ribosylated proteins...
ADP-ribosylactin Hydrolase in Rat Brain

Intrasyaptosomal distribution of ADP-ribosylactin hydrolase activity

TABLE V
Subcellular distribution of ADP-ribosylactin hydrolase in rat brain

| Fraction   | ADP-ribosylactin hydrolase | Succinate dehydrogenase | NADH-cytochrome c reductase | Enolase |
|------------|----------------------------|-------------------------|-----------------------------|---------|
|            | Total activity | Specific activity | Total activity | Specific activity | Total activity | Specific activity | Total activity | Specific activity |
| Cytosol    | 110.0 ± 12.0 | 0.4 ± 0.0 | 226.7 ± 12.7 | 0.7 ± 0.0 | 137.5 ± 11.6 | 0.5 ± 0.0 | 1367 ± 175 | 5.0 ± 0.2 |
| Mitochondria | 133.0 ± 31.0 | 2.7 ± 0.1 | 113.8 ± 17.4 | 2.3 ± 0.3 | 3.5 ± 0.3 | 0.1 ± 0.0 | 511 ± 12 | 10.4 ± 0.2 |
| Synaptosomes | 156.7 ± 12.0 | 6.4 ± 0.1 | 38.1 ± 4.7 | 1.6 ± 0.2 | 5.6 ± 0.6 | 0.2 ± 0.0 | 435 ± 30 | 18.1 ± 1.3 |

Values are the means ± S.D. of five experiments.

a ER, endoplasmic reticulum.

FIG. 11. SDS-PAGE and immunoblotting of brain cytosol and synaptosomes. After centrifugation of the rat brain homogenate at 1000 × g for 10 min, the supernatant was layered on top of a sucrose gradient (0.85, 1.0, and 1.2 M). After centrifugation at 92,500 × g for 2 h, the cytosol fraction was found in the top layer. The synaptosome fraction was found in the boundary between the 1.0 and 1.2 M sucrose layers. The synaptosome fraction was further treated with a hypotonic solution and centrifuged at 150,000 × g for 1 h. The precipitate was used for the enzyme assay.

Intrasyaptosomal distribution of ADP-ribosylactin hydrolase activity

TABLE VI

| Fraction | ADP-ribosylactin hydrolase | Succinate dehydrogenase | Enolase |
|----------|----------------------------|-------------------------|---------|
|          | Total activity | Specific activity | Total activity | Specific activity | Total activity | Specific activity |
| Synaptosome | 156.7 ± 12.0 | 6.4 ± 0.1 | 38.1 ± 4.7 | 1.6 ± 0.2 | 435 ± 30 | 18.1 ± 1.3 |
| Membrane | 20.1 ± 4.0 | 1.1 ± 0.0 | 27.6 ± 1.8 | 1.4 ± 0.0 | 17 ± 2 | 0.1 ± 0.0 |

Values are the means ± S.D. of five experiments.

a Synaptosomes were treated with a hypotonic solution and centrifuged at 150,000 × g for 1 h. The supernatant was used for the enzyme assay.

FIG. 11. SDS-PAGE and immunoblotting of brain cytosol and synaptosomes. After centrifugation of the rat brain homogenate at 1000 × g for 10 min, the supernatant was layered on top of a sucrose gradient (0.85, 1.0, and 1.2 M). After centrifugation at 92,500 × g for 2 h, the cytosol fraction was found in the top layer. The synaptosome fraction was found in the boundary between the 1.0 and 1.2 M sucrose layers. The synaptosome fraction was further treated with a hypotonic solution and centrifuged at 150,000 × g for 1 h. The precipitate was used for the enzyme assay.

Intrasynaptosomal distribution of ADP-ribosylactin hydrolase activity

Probably are cleaved by nucleophilic substitution of the enzyme.

ADP-ribosylated actin interacts with fast-growing barbed ends of actin filaments in the same way that capping proteins do, and ADP-ribosylation inhibits the polymerization of cytoplasmic actin (44). The ADP-ribosylation of arginine 177 increases the exchange rate from ADP to ATP with actin monomers, and actin-catalyzed ATP-hydrolysis is inhibited by ADP-ribosylation (17, 45). Low concentrations of Mg²⁺ cause actin polymerization in vitro. ATP is hydrolyzed during filament polymerization; therefore, the de-ADP-ribosylation of actin may restore actin’s ability to hydrolyze ATP. When actin has a cysteine that is bound with ADP-ribose by a nonenzymatic reaction, it cannot polymerize with filaments after treatment with phalloidin (23). Rat brain ADP-ribosylactin hydrolase must be important to form actin filaments because actin-actin polymerizing ability is lost during ADP-ribosylation. ADP-ribosylactin hydrolase seems to regulate actin-actin polymerization in nonmuscle cells. Nonenzymatically ADP-ribosylated actin has been found in human neutrophils (23). Consequently, ADP-ribosylated actin may be part of the actin pool in cells. The distribution of radioactivity in digested peptides was similar after the first and second ADP-ribosylations, so the proteins that were de-ADP-ribosylated by ADP-ribosylactin hydrolyase were re-ADP-ribosylated at the same site. This finding indicates that ADP-ribosylactin hydrolase is important in the ADP-ribosylation cycle in animals, as already proposed by Moss et al. (18). Most actin filaments are on the innermost side of plasma membranes, and they may be essential for nerve cell functioning as well as for cell configuration. Synaptic terminals are attached to the plasma membranes of nerve cells in the brain, and they transmit a signal through exocytosis of a neurotransmitter. In synaptic terminals, actin is on the innermost side of plasma membranes, where it acts as a barrier that prevents the movement of synaptic vesicles that contain neurotransmitters (46). The finding of ADP-ribosylactin hydrolase in the synaptosomal cytosolic fraction may mean that the enzyme is involved in actin filament formation because de-ADP-ribosylated actin could form actin filaments after the addition of Mg²⁺. ADP-ribosylactin hydrolase purified from rat brain de-ADP-ribosylated heterotrimeric GTP-binding proteins. Perhaps the enzyme regulates such protein functions.

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