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ADP-RIBOSYLATION OF BRAIN SYNAPTOSOMAL PROTEINS CORRELATES WITH ADENYLATE CYCLASE ACTIVATION

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ADP-ribosylation of the adenylate cyclase G/F regulatory subunit by cholera toxin is a major tool for the study of this enzyme. Investigation of the brain enzyme has been hampered up to now by the failure to demonstrate cholera toxin-dependent ADP-ribosylation of membrane-bound proteins. Synaptosomes prepared by flotation from fresh brains homogenized in the presence of protease inhibitors yielded membranes of which several proteins could be ADP-ribosylated by the toxin. The same membranes subjected to mild proteolysis could not be ADP-ribosylated. Adenylate cyclase activation and ADP-ribosylation were simultaneous processes. The major labeled species was of 47,000 M_r. It was solubilized by Lubrol-PX, together with other labeled polypeptides. As analyzed on sucrose gradients, the 47,000 M_r protein was found both in the 3S region, and in the adenylate cyclase containing fraction (9.1S).

Cholera toxin exerts its effects on eucaryotic cells through the activation of adenylate cyclase (1-3). The enzyme stimulation results from the ADP-ribosylation of the G/F regulatory subunit, as shown by reconstitution experiments (4-6). ADP-ribosylation is thus a very useful tool to study adenylate cyclase, since it provides a way to covalently label one of its many components with a radioactive group. Up to now, such a tool was lacking with respect to the brain enzyme, since ADP-ribosylation of brain membrane proteins by cholera toxin could not, despite numerous attempts, be demonstrated (3, 7). In this study, we show that synaptosomal membranes can be ADP-ribosylated, provided that they are protected from proteolysis. Such a modification occurrs concomitantly with adenylate cyclase activation. Furthermore, we show that a 47,000 M_r labeled protein comigrates in sucrose gradients with the Lubrol solubilized adenylate cyclase activity.

EXPERIMENTAL

Synaptosomes were prepared by flotation from fresh rat brains homogenized in the presence of protease inhibitors : 0.3 mM PMSF, 10 µM benzethonium chloride, 1 mg pepstatin and 1 mg kallicrein-trypsin-inhibitor

ABBREVIATIONS

PMSF, phenylmethyl-sulfonyl fluoride ; TEA, triethanolamine ; DTT, dithiothreitol ; SDS, sodium dodecyl sulfate.

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per gm brain tissue according to (8). They were lysed by incubation under magnetic stirring for 1 1/2 hour in a cold (4°) 4 mM TEA buffer pH 8.1 containing 2 mM DTT, 1 mM EDTA and 0.2 mM ATP. Membranes were centrifuged and resuspended in 50 mM TEA buffer pH 8.1.

The ADP-ribosylation medium was adapted from (9) and contained, in a final volume of 200 µl, 10 mM TEA buffer pH 8, 100 µg of synaptosomal membrane proteins, 10 µg of freshly activated cholera toxin, 15 x 10^6 cpm of (α-^{32}P) NAD (50 Ci/mMole, New England Nuclear), 300 µM or 3 µM NAD, 5 mM $MgCl_2$, 10 mM ADP-ribose, 5 mM nicotinamide, 50 mM sodium phosphate pH 7.5, 0.1 mM ATP and 1 mM GTP. A nucleotide regenerating system composed of 20 mM phosphocreatine, 130 units/ml creatine kinase and 200 units/ml myokinase, was added, or else was replaced by the sequential addition of 4 µl of TEA buffer containing 50 mM GTP and 5 mM ATP, every two minutes. After 20 minutes at 32°5, the samples were centrifuged for 2 minutes at 178,000 g in a Beckman airfuge. The pellet was resuspended in 30 µl of 0.1 mM GTP in 50 mM TEA pH 8. 10 µl were assayed for adenylate cyclase ; 20 µl were solubilized with 1 % SDS, 1 % EDTA and 1 % β-mercaptoethanol in 50 mM Tris-HCl buffer pH 8.8, and subjected to electrophoresis.

In some cases, the membranes were solubilized with Lubrol-PX (8). 0.5 ml Lubrol supernatants (5 mg membrane proteins) were layered on 12.2 ml linear 2-4.5 % sucrose gradients containing 50 mM TEA buffer pH 8.1, 2 mM DTT, 1 mM EDTA, 1 mM $MgCl_2$ and 0.05 % Lubrol-PX. They were centrifuged in a Beckman rotor SW 41 at 4° for 12 hours at 286,000 g. 350 µl fractions were collected.

The crude cytosolic fraction was the supernatant obtained by centrifuging rat brain homogenate 1 hour at 100,000 g. It was in some cases boiled for two minutes or treated by 0.3 mg/ml trypsin for 1 hour at 30°.

0.1 % SDS, 1.5 mm slab gels contained 10 % acrylamide for the running gel and 4 % acrylamide for the stacking gel. Electrophoresis was performed for 3 hours at 35 mA in 0.1 % SDS, 25 mM Tris-HCl buffered at pH 8.3 with glycine (10). Gels were stained with 0.1 % Coomassie Blue, dried under vacuum and exposed to Kodak X-Omat AR5 films for 2-10 days, at -20°C.

The adenylate cyclase assay contained, in a final volume of 300 µl, 10 µl of the enzyme sample, 0.2 mM (α-^{32}P) ATP (3 x 10^5 cpm, New England Nuclear), 0.5 mM unlabeled cAMP, 5 mM DTT, 10 mM $MgCl_2$, 0.2 mM GTP, 20 mM creatine phosphate, 70 units/ml creatine kinase. The enzyme activities were determined after 3 and 6 minutes at 30°, according to (11).

Membranes were sometimes treated at 30° with trypsin (18 µg/ml, 10 µg trypsin for 1 mg membrane protein) at pH 8.0 for 1 to 4 minutes, after which soybean trypsin inhibitor was added (3 µg per µg trypsin). Control samples were treated with a mixture of preincubated trypsin and trypsin inhibitor. Trypsin digestion was performed either on membranes before ADP-ribosylation, or on the ADP-ribosylated, Lubrol-soluble proteins.

Proteins were quantified according to (12).

RESULTS

Optimal conditions for activation of adenylate cyclase by cholera toxin. A maximal, 2.5 to 3.5 fold stimulation of adenylate cyclase by cholera toxin was obtained with a ratio of 7 to 10 µg of activated toxin for 100 µg membrane proteins. Adenylate cyclase was fully stimulated within 20 minutes at 32°5, and the activation was stable for at least 1h30. It was NAD-dependent (Figure 1). The ADP-ribosylation medium routinely included 0.1 mM ATP and 1 mM GTP. However, the omission of either GTP or ATP did *not* prevent adenyl cyclase activation by cholera toxin ; the omission of both nucleotide-triphosphates decreased the activation by cholera toxin by 40 % (Table I).

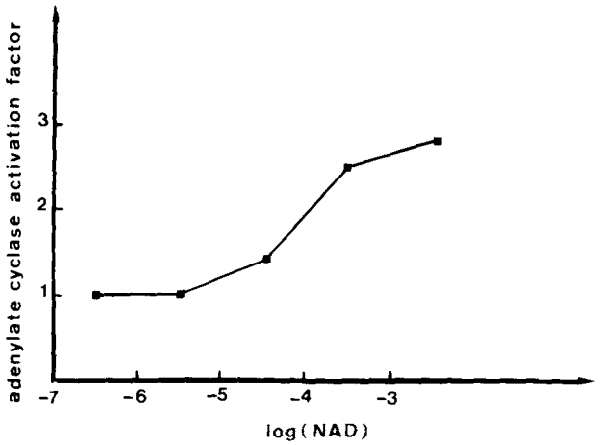

Figure 1. Effect of NAD concentration on adenylate cyclase activation by cholera toxin.

Addition of 1 mM GDP, instead of GTP, to the medium containing ATP, did *not* change the enzyme activation by cholera toxin (Table I).

ADP-ribosylation of synaptosomal membrane proteins. Several membrane proteins were labeled when incubated in the presence of (^{32}P) NAD (Fig. 2B, lanes 1 to 4). The labeling was strictly toxin-dependent (Fig. 2B, lane 5) and occurred simultaneously with adenylate cyclase activation. It was

TABLE I

Effects of nucleotide-triphosphates and EGTA on adenylate cyclase activation by cholera toxin (CT).

Incubation medium*	Adenylate cyclase specific activity pmoles cAMP/mg/min.		CT activation factor
	- CT	+ CT	
+ 1 mM GTP and 0.1 mM ATP	102	265	2.6
+ 0.1 mM ATP	110	286	2.6
+ 1 mM GTP	108	270	2.6
No nucleotide triphosphate	90	160	1.8
+ 1 mM GDP and 0.1 mM ATP	115	300	2.6

*Incubations were performed as described in Material and Methods, with 300 µM NAD and no labeled NAD, and without a nucleotide regenerating system.

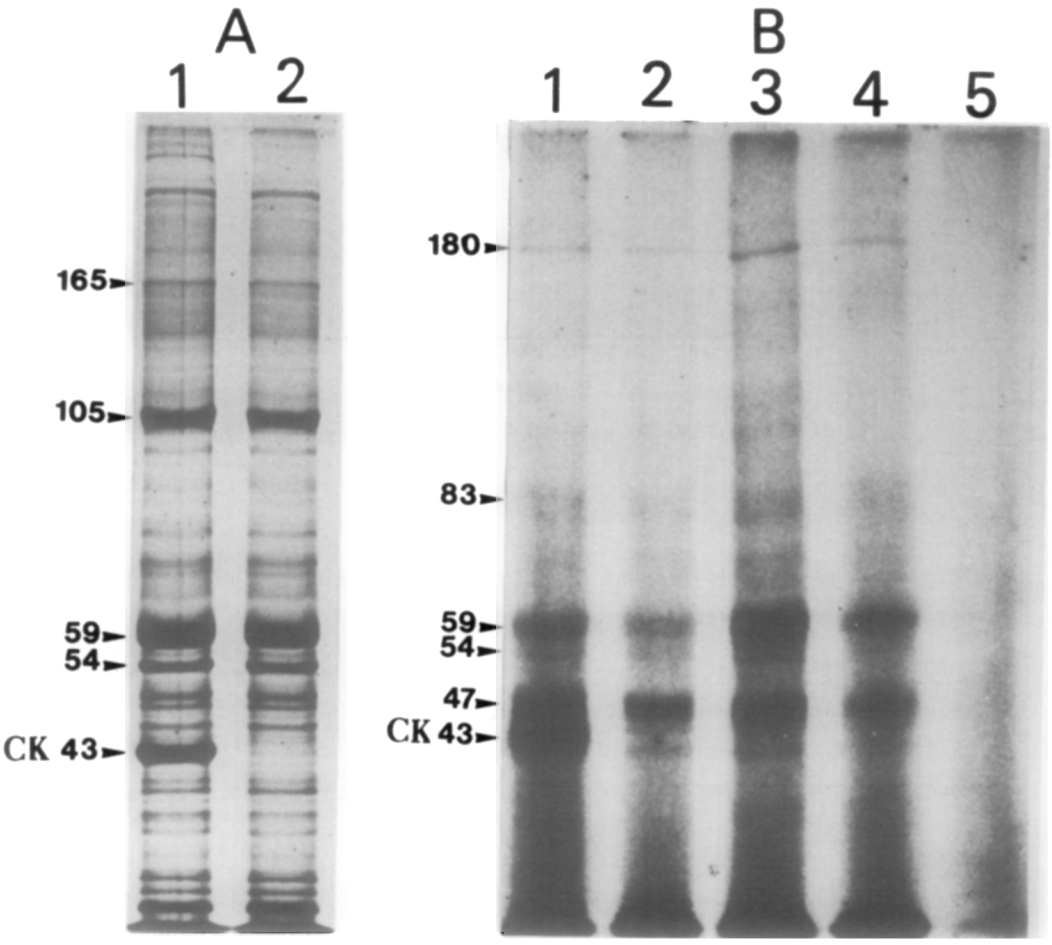

Figure 2. SDS gel electrophoresis of synaptosomal membrane proteins incubated in an ADP-ribosylation medium. 100 µg membrane proteins were incubated with or without a nucleotide triphosphate regenerating system comprising creatine-kinase, and with 300 µM (alpha^{32}P) NAD. A : Coomassie blue stained gel. Lane 1 : sample incubated in the presence of creatine-kinase (CK) ; lane 2 : sample incubated without creatine-kinase. B : autoradiogram of the same gel. The medium included : lane 1 : cholera toxin and creatine-kinase (CK) ; lane 2 : cholera toxin, no nucleotide triphosphate regenerating system ; lane 3 : as lane 2, + 28 µg protein of dialysed cytosol ; lane 4 : as lane 2, + 0.1 mM forskolin ; lane 5 : as lane 1, without cholera toxin. Numbers : molecular weight x 10^{-3}. (Markers were myosin, 200,000 M_r, β-galactosidase, 115,000 M_r, phosphorylase B, 97,000 M_r, catalase, 60,000 M_r and aldolase, 40,000 M_r).

already detected at NAD concentrations too low to produce a significant increase in adenylate cyclase activity (compare Fig. 1 and 3A). Since a component of the regenerating system, creatine-kinase (43,000 M_r) was ADP-ribosylated by cholera toxin and stuck to the membranes (Fig. 2A and B, compare lanes 1 and 2), the nucleotide regenerating system was routinely

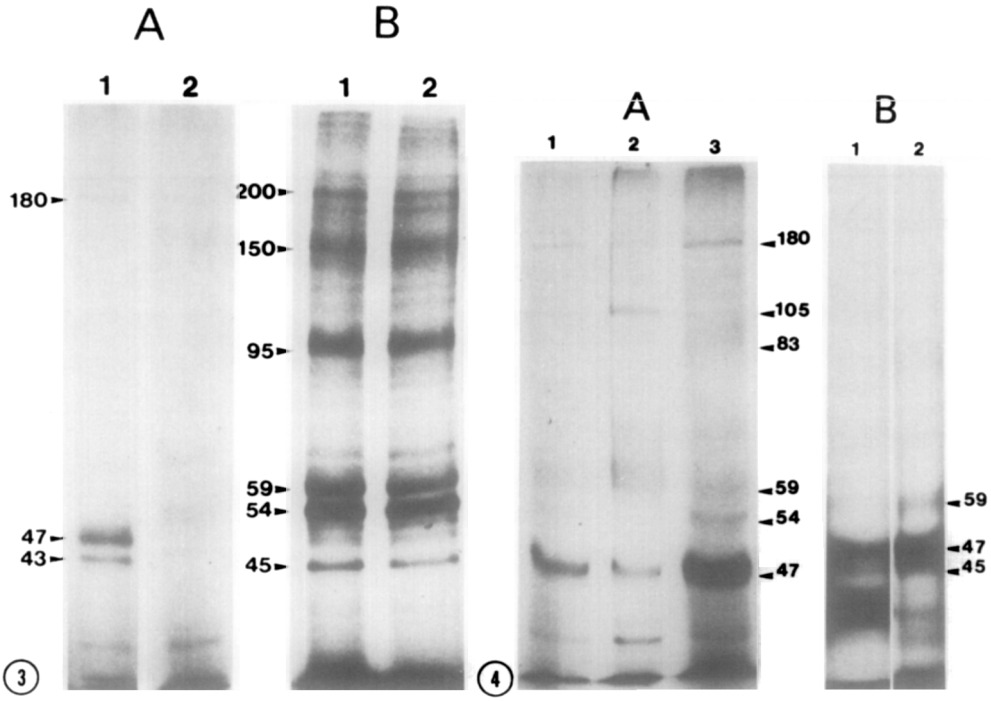

Figure 3. Autoradiograms of synaptosomal membranes incubated in the presence of (^{32}P) NAD in (A), in the presence of (γ^{32}P) ATP in (B) : A. The ADP-ribosylation medium contained 15 x 10^6 cpm (^{32}P) NAD and : lane 1, 3 µM NAD ; lane 2, 300 µM NAD. (Same gel, same exposure time). B. The "ADP-ribosylation medium" contained 15 x 10^6 cpm (γ^{32}P) ATP, instead of (^{32}P) NAD, and 300 µM NAD. Lane 1 : with cholera toxin ; lane 2 : without cholera toxin.

Figure 4. Autoradiograms of ADP-ribosylated, Lubrol solubilized membrane proteins.
A. Membranes were homogenized with Lubrol, lane 1. Resuspended pellet, lane 2. Supernatant, lane 3.
B. The Lubrol supernatant was fractionated on a sucrose gradient and the fractions were TCA-precipitated. Lane 1 : fraction of the gradient containing 3S proteins. Lane 2 : adenylate cyclase activity containing fraction (9.1S).

replaced by the sequential addition of ATP and GTP. Using this procedure, only membrane-bound proteins were labeled (Fig. 2B, lanes 2 to 4). There were two major labeled bands of 59,000 and 47,000 M_r, and four other well-defined bands of 180,000, 83,000, 54,000 and 43,000 M_r. A few other bands were slightly labeled, at 105,000 M_r and in the low molecular weight range.

This pattern was stictly identical from one batch of membranes to another. The addition of 0.1 mM forskolin, a potent adenylate cyclase activator (13), in the ADP-ribosylation medium did not modify the pattern or specific radioactivity of the labeled bands (Fig. 2B, lane 4).

If (γ-^{32}P) ATP was substituted for (^{32}P) NAD, a cholera toxin *independent* phosphorylation occurred, giving a pattern of labeled proteins very different from the ADP-ribosylated protein pattern (Fig. 3B, lanes 1 and 2).

TABLE II. Effect of cytosol on adenylate cyclase activation by cholera toxin (CT).

Incubation medium*	Adenylate cyclase specific activity pmoles/cAMP/mg/min.	CT activation factor
Minus CT	150	-
Minus CT + 10 mM NaF	400	2.7
+ CT	528	3.5
+ CT + 10 µg cytosolic protein	630	4.2
+ CT + 50 µg cytosolic protein	750	5
+ CT + 10 µg 0-50 % $(NH_4)_2SO_4$-precipitated cytosolic protein	535	3.6
+ CT + 10 µg 50-100 % $(NH_4)_2$ SO_4-precipitated cytosolic protein	638	4.25
+ CT + 28 µg dialysed cytosolic protein	750	5
+ CT + 50 µg boiled cytosolic protein	500	3.3
+ CT + 28 µg trypsin-treated cytosolic protein	813	5.4

*Incubation were performed as described in "Material and Methods", in the presence of 300 µM (^{32}P)-NAD. The same results were obtained with the nucleotide regenerating system or by sequential addition of ATP and GTP.

Effect of cytosol. The addition of brain high speed supernatant to synaptosomal membranes enhanced their ADP-ribosylation (Fig. 2B, lane 3). Cytosolic factor was not required for adenylate cyclase activation by cholera toxin, but slightly enhanced it (Table II). The factor had no endogenous adenylate cyclase activity. It could be precipitated by ammonium sulfate and was not dialysed from a 3,500 molecular weight cut off dialysis bag. It was inactivated by boiling but was, however, fully trypsin-resistant (Table II).

Solubilization of ADP-ribosylated membrane proteins. In our solubilization conditions, roughly 50 % of the membrane proteins, and close to 100 % of the adenylate cyclase activity present in the solubilized sample were found in the supernatant (8). The ADP-ribosylated membrane proteins (Fig. 4A, lane 1) partitioned in the Lubrol-insoluble pellet (Fig. 4A, lane

2) and in the supernatant (Fig. 4A, lane 3). In the supernatant, the major labeled polypeptide was of 47,000 M_r. Other labeled proteins were mainly found in the supernatant, of 180,000, 59,000, 54,000 M_r, and a few lower M_r proteins. About a fifth of the 47,000 M_r polypeptide originally contained in the solubilized sample was found in the Lubrol-insoluble pellet, as well as 105,000, 59,000 and lower M_r proteins.

The labeled supernatants were analyzed by sucrose gradient centrifugation. The adenylate cyclase activity was found in a single peak of 9.1S (d'Alayer, J., Berthillier, G., and Monneron, A., submitted). This fraction contained a prominent ADP-ribosylated protein band of 47,000 M_r, together with two more slightly labeled bands of 59,000 and 45,000 M_r (Fig. 4B, lane 2). The 3S region of the sucrose gradient, devoid of adenylate cyclase activity, also contained the 47,000 M_r polypeptide, together with smaller M_r labeled proteins (Fig. 4B, lane 1).

Proteolysis. When synaptosomal membranes were subjected to a very limited proteolysis, adenylate cyclase activity was enhanced in a first step (d'Alayer, J., Berthillier, G., and Monneron, A. subm.). Under such conditions, membranes could no longer be ADP-ribosylated, whereas membranes treated by a mixture of trypsin and trypsin-inhibitor were ADP-ribosylated as described above. Conversely, when ADP-ribosylated membranes were solubilized, and the supernatant subjected to mild proteolysis, all the labeled bands described above disappeared from the autoradiograms, giving rise to lower molecular weight ADP-ribosylated products. This occurred within one minute, before any decrease in adenylate cyclase activity was noticed.

DISCUSSION

The use of fresh material, of protease inhibitors during the preparative process, and the immediate membrane flotation devised to get rid of the lysosomes, were the simple procedures which allowed us to detect for the first time brain membrane protein ADP-ribosylation. Indeed, the cholera toxin targets proved to be very sensitive to trypsin digestion, and it is likely that they were quickly degraded in unprotected samples by endogenous proteases, whether lysosomal or membrane-bound.

Cholera toxin activation of brain adenylate cyclase and ADP-ribosylation of membrane-bound proteins were concomitant. Among the several modified synaptosomal proteins, the 47,000 M_r polypeptide was the major labeled species. Its apparent molecular weight is close to that of the purified adenylate cyclase G/F regulatory subunit from erythrocytes or liver (4-6). The 47,000 M_r protein was solubilized by Lubrol along with the adenylate cyclase activity, and stayed partly associated with it upon sucrose gradient centrifugation. Purification of these ADP-ribosylated components, which is

needed to assess their biological role by complementation assays, is in progress.

This study also shows that a cytosolic macromolecular factor is *not* required for ADP-ribosylation of brain membranes by cholera toxin, although it enhances it. In some tissues, cytosolic factors are absolutely required. This discrepancy may pertain to true tissular differences. Alternatively, the distribution of such factors either in the membrane, or in the cytosol, may also be influenced by tissue processing, or by the occurrence of proteolysis.

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