Evidence for Endogenous ADP-ribosylation of GTP-binding Proteins in Neuronal Cell Nucleus

POSSIBLE INDUCTION BY MEMBRANE DEPOLARIZATION

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GTP-binding protein(s) recognized by antibodies against the α-subunits of Go- and Gα-proteins were detected in crude nuclei isolated from rat brain stem and cortex. Immunohistochemical staining indicated that in the cortex these proteins are perinuclear, or are embedded in the nuclear membrane. Evidence is presented for an endogenous ADP-ribosylation of these proteins, which competes with their PTX-catalyzed ADP-ribosylation. The endogenous reaction has the characteristics of nonenzymatic ADP-ribosylation of cysteine residues, known to involve NAD-glycohydrolase activity. In vitro experiments showed that the α-subunit of Gα-proteins in the cell membrane also acts as a substrate of this endogenous ADP-ribosylation. The in situ effect of membrane depolarization on the nuclear GTP-binding proteins may be attributable to their depolarization-induced endogenous ADP-ribosylation, suggesting a novel signaling mechanism in neuronal cells in the central nervous system.

NAD is the substrate for enzymes that catalyze ADP-ribosylation, i.e. cleavage of the bond between nicotinamide and ribose and the transfer of ADP-ribose to nucleophilic acceptors (1). ADP-ribosylation represents a mechanism for post-translational modification of proteins. Among the known acceptors for ADP-ribose are GTP-binding proteins in cell membranes (2), ADP-ribosyltransferases that catalyze ADP-ribosylation of arginine residues in GTP-binding proteins (similar to cholera toxin) (2) have been detected in eukaryotic cells (3). An endogenous ADP-ribosylation of cysteine residues of membrane G-proteins (similar to pertussis toxin (PTX) (1)-catalyzed ADP-ribosylation) (2) has been suggested to occur in erythrocytes (4, 5).

In this report we present evidence for an endogenous ADP-ribosylation of cysteine residues in GTP-binding protein(s) in the nuclei of cells in rat brain stem and cortex. These proteins also act as substrates of PTX-catalyzed ADP-ribosylation and react with antibodies against the α-subunits of Gα- and Gβ-proteins (Gαi- and Gαo-proteins). Unlike these membrane G-proteins, however, the nuclear GTP-binding proteins are apparently modified by a nonenzymatic ADP-ribosylation of cysteine residues, which competes with their PTX-catalyzed ADP-ribosylation. Also, unlike membrane G-proteins, the nuclear GTP-binding proteins are not extractable by detergents, nor are they activated by membrane depolarization (6–9). The evidence presented here is consistent with a depolarization-induced ADP-ribosylation of these nuclear GTP-binding proteins, suggesting a novel signaling mechanism in neuronal cells in the central nervous system.

MATERIALS AND METHODS

Reagents—Nicotinamide adenine dinucleotide (grade I), adenosine triphosphate (grade I), GDPβS, GDPγS, tetrodotoxin, dithiothreitol (DTT), azidoaniline, sodium nitroprusside, 3-aminobenzamide (3-AB), and thymine were all purchased from Sigma. PTX and the A-protomer of PTX (ADP-ribosyltransferase) were purchased from List Biological Laboratories. (adenylate-32P) Nicotinamide adenine dinucleotide, di(triethyl-ammonium) salt ([32P]NAD) (1000 Ci/mmol), [32P]GTP (800 Ci/mmol), and (batrachotoxinin-B) (3H-BTX-B) (50 Ci/mmol) were purchased from DuPont NEN. [3H]NAD (57 Ci/mmol) was purchased from Amersham Corp. Antibodies against the C terminus of Go- and Gαi-proteins were kindly supplied by Dr. Milligan, Glasgow University, UK. Antibodies against the N terminus of Gαo-proteins (GQ2) and α-common antibodies against the binding sites of GTP in Go- and Gαo-proteins (EC2) were purchased from DuPont. The neurotoxin batrachotoxin (BTX) was kindly supplied by Dr. J. W. Daly, NIH, Bethesda, MD.

Brain Tissue Preparation—Adult male rats of the CD strain were maintained as described previously (10). Brain stem and cortical regions were obtained from 2-3-month-old rats. Crude nuclei were isolated from pooled rat brain stems or cortex according to a previously described procedure (11), with slight modifications. The tissues were homogenized in 0.32 M sucrose with a glass/Teflon homogenizer, and the homogenate was centrifuged at 900 x g for 15 min at 4 °C. The resulting pellet was resuspended twice in hypotonic solution (50 mM Tris-HCl, pH 7.4) and centrifuged at 900 x g for 10 min at 4 °C. The pellet was resuspended in the same solution and centrifuged at 12,000 x g for 15 min at 4 °C. The final pellet was designated as crude nuclei (11). In a separate procedure, crude nuclei were isolated on a sucrose gradient (12). Membranes were prepared from synaptoneurosomes as described elsewhere (13).

Preparation of Slices—Rat brain cortex and brain stem were depolarized by exposure to high concentrations of K+ in Ca2+-free Krebs-Henseleit buffer solution through an exchange of Na+ for K+ (7). In most experiments, membrane depolarization was achieved by an increase in K+ from 4.7 to 50 mM. Cell membranes were also depolarized by inward Na+ current induced by prolonged activation of the voltage-dependent Na+ channel in the presence of BTX (14).

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1 The abbreviations used are: PTX, pertussis toxin; 3-AB, 3-amino-benzamide; BTX, batrachotoxin; BTX-B, batrachotoxinin-B; DTT, dithiothreitol; Gαi, α-subunit of G-protein; Gαo, α-subunit of G-protein; GDPβS, guanosine 5′-O-(3-thiotriphosphate); GDPγS, guanosine 5′-O-(2-thiodiphosphate); PARP, poly(ADP-ribose)-polymerase; PAGE, polyacrylamide gel electrophoresis; TTX, tetrodotoxin.

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by the A-protomer of PTX (2). Antibody specificity was determined by omitting the antibody name, CA). The peroxidase activity was visualized with diaminobenzidine.

Antibodies against the C terminus of Gα-proteins (kindly donated by Dr. G. Milligan) were used as specific markers (see below).

**RESULTS**

**Characteristics of GTP-binding Proteins in Crude Nuclei of Cells in Brain Stem and Cortex**—Crude nuclei prepared from rat brain stem and cortical slices were found to contain proteins that are specifically photolabeled with [azido-32P]GTP (Fig. 1A). Like Gαo- and Gαi-proteins in the cell membrane (20), these nuclear proteins (39–40 kDa on SDS-PAGE) acted as substrates for PTX-catalyzed ADP-ribosylation (Fig. 1B). The presence of these GTP-binding proteins in the crude nuclei could not be attributed to cytosolic contamination, as only traces of these proteins were found in cytosolic fractions of homogenates prepared from brain stem or cortex (Fig. 1B). In addition, the possibility that they are embedded in membrane residues in the crude nuclear fraction was ruled out by using specific markers (see below).

The GTP-binding nuclear proteins reacted with antibodies against both Gαo- and Gαi-proteins in the cell membrane (19) (Fig. 1C). Immunolabeling in crude nuclei revealed, however, that these nuclear proteins were not extractable by treatment with detergents that extract Gαo- and Gαi-proteins from the cell membrane (6) (Fig. 1C). Moreover, the PTX-catalyzed ADP-ribosylation of these nuclear proteins was even enhanced following detergent treatment, as if the proteins had now become

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**Endogenous ADP-ribosylation of Nuclear GTP-binding Proteins**

[32P]ADP-ribosylation of proteins (200 µg/50 µl) was catalyzed by the A-protomer of PTX (2 µg/ml) in the presence of 9–10 pmol/ml [32P]NAD (1 µCi) in hypotonic buffered solution containing 2 mM ATP, 50 mM Tris-HCl, pH 7.4, 0.5 mM MgCl₂, 0.05 mM EDTA, and 20 mM DTT, according to a previously described procedure (7). In the absence of the PTX A-protomer, by incubation of the nuclei for 30 min at 37°C in the above solution in which the DTT concentration was changed to 0.5 mM, or by incubation in Krebs-Henseleit buffer, pH 7.4, containing 0.5 mM DTT and 10 pmol/ml [32P]NAD. Membranes were [32P]ADP-labeled, in the absence of the PTX A-protomer, by incubation with crude nuclei and [32P]NAD (50 pmol/ml) at 37°C for 30 min in the same hypotonic buffered solution as that used for the PTX-independent reaction in the crude nuclei, but with the addition of the protease inhibitors aprotinin (5 units/ml), pepstatin A (5 µg/ml), and phenylmethylsulfonyl fluoride (1 mM). The labeled nuclei or membranes were pelleted, resuspended in sample buffer (17), and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (7.5 or 10% polyacrylamide). The dried gels were autoradiographed (usually for 24 h at ~7°C). Densitometry was performed with a laser densitometer (LKB Broma Ultrascan II).

**Immunostaining**—Gels were electroblotted onto nitrocellulose paper overnight at 10°C and a current constant of 150 mA, as described elsewhere (18). Nitrocellulose strips were exposed to specific antibodies against the N terminus of the α-subunit of Gα-protein (19) or α-common antibodies (20), or antibodies against the C terminus of αo- and αi-proteins (19). Protein bands with bound antibodies were detected by binding of peroxidase-conjugated second antibody.

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**toaffinity Labeling with [azido-32P]GTP—[azido-32P]GTP was synthesized by incubation of [32P]GTP with azidoamine and purified by ion-pairing chromatography (21). Crude nuclei in samples containing approximately 200 µg of protein were UV-irradiated (300 nm, 350 W) for 5 min on ice in the presence of [azido-32P]GTP (0.3–0.6 µM). GTP-binding proteins were photolabeled by this procedure as described previously (22).

**Two-dimensional Gel Electrophoresis**—Samples containing 200 µg of protein were applied to isoelectric focusing gels (first dimension) containing 1.8% Ampholines, pH 5–8, and 0.3% Ampholines, pH 3–10. Isoelectric focusing followed by SDS-PAGE (second dimension) was performed according to Ferro-Luzzi and Niki (23). In the second dimension, gels containing the electrofocused proteins were applied on 10% polyacrylamide slab gels and subjected to SDS-PAGE at room temperature. These gels were then dried and autoradiographed.

**Immunohistochemistry**—Brain cortex was cut into small pieces (5 × 5 mm²) and fixed with freshly prepared paraformaldehyde (4%) in phosphate-buffered saline for 20 h at 4°C. The tissue was then washed in phosphate-buffered saline, dehydrated, and embedded in paraﬁn. Polyclonal antibodies against the C terminus of Gαo-proteins (kindly donated by Dr. G. Milligan) were localized by a standard immunoperoxidase method (24). Parafﬁn sections (4 µm thick) were mounted on slides that were precoated in 1 x HCl and coated with poly-L-lysine. After drying overnight at 37°C the sections were dewaxed, treated with 0.3% H₂O₂ for 30 min, and incu-

bated with 3% goat serum in phosphate-buffered saline for 20 h at 4°C or for 1 h at 37°C. The antibody was detected by the use of goat anti-rabbit peroxidase conjugated antibody, or alternatively by the use of biotinylated anti-rabbit IgG and avidin-biotin complex peroxidase (ABC; Vector Labs, Burlingame, CA). The peroxidase activity was visualized with diaminobenzidine. Antibody speciﬁcity was determined by omitting the antibody against the C terminus of Gαo-proteins. All tissue sections were counterstained with hematoxylin.

[32P]ADP-ribose (40,000 cpm/µmol) was prepared from [32P]NAD according to the protocol of McDonald et al. (1).
more available as substrates for ADP-ribosyltransferase (PTX A-protomer) (Fig. 1D). The possibility that these proteins are embedded in the nuclear membrane was examined by treatment with citric acid (1%), which separates the nuclear membrane from the inner matrix (25). Compared with controls, the treated nuclei exhibited only faint labeling with antibodies against the C terminus of Gαi-proteins (Fig. 1C). Traces of the [32P]ADP-ribosylated proteins detected in the nuclear extracts were attributed to extraction of these proteins by the citric acid treatment.

The possibility that these proteins are embedded in the nuclear membrane was supported by the results of immunohistochemical staining. Post-embedding labeling of paraffin sections of brain cortex (see “Materials and Methods”) with antibodies against the C terminus of Gαi-proteins and the N terminus of Gαo-proteins resulted in intense antibody binding to the cell nuclei (which were counterstained by hematoxylin), with a halo of dark staining at the nuclear borders that was clearly distinguishable from the immunolabeling of the cytoplasm (Fig. 2). Some of the cell nuclei in the section were not labeled (Fig. 2, A and B), and no labeling was observed in control samples in which the anti-Gαi or anti-Gαo-antibody was omitted (Fig. 2C). The specific immunolabeling supports the presence of these proteins in the crude nuclei (Figs. 1C and 2).

Poly(ADP-ribose)-polymerase (PARP) activity was used as a marker of the nuclear fraction (26) (see Figs. 1, 7, and 8). Significant contamination of the crude nuclei by cell membranes during preparation was excluded by the use of [3H]BTX-B (a labeled derivative of the lipophilic neurotoxin, batrachotoxin), which binds specifically to voltage-dependent Na⁺-channels in the cell membrane (15, 27) (Fig. 3). No such binding was detected in crude nuclei isolated from slices of brain stem or brain cortex, while binding of [3H]BTX-B to membranes prepared from these brain regions was similar to that observed previously in membranes prepared from brain synaptoneurosomes (15). Moreover, in agreement with our previous findings, the specific binding of [3H]BTX-B in membranes was stimulated by muscarinic agonists (10, 15), indicating a possible interaction between muscarinic receptors and voltage-dependent Na⁺-channels in the cell membrane (8, 10, 15); this effect was not observed in the crude nuclear fraction (Fig. 3). These differences between BTX-B binding in cell membranes and in the crude nuclei were attributed to the lack of voltage-dependent Na⁺-channels and muscarinic receptors and hence of membrane residues in the crude nuclei.

**Evidence for Endogenous ADP-ribosylation of GTP-binding Proteins in Crude Nuclei**—GTP-binding proteins (39–40 kDa) in crude nuclei prepared from brain stem or cortical slices, that act as substrates for [32P]ADP-ribosylation catalyzed by the PTX-A protomer, were [32P]ADP-labeled by incubation of the nuclei with [32P]NAD even in the absence of the PTX A-protomer (Fig. 4). These proteins were labeled in nuclei incubated at 37°C for 30 min, either in the hypotonic buffered solution...
used for their PTX-catalyzed ADP-ribosylation, in which DTT concentration was reduced to 0.5 mM, or in Krebs-Henseleit buffer, pH 7.4, containing 0.5 mM DTT. PTX-catalyzed $^{[32P]}$ADP-ribosylation of GTP-binding proteins in crude nuclei that had been preincubated under the above experimental conditions in the absence of $^{[32P]}$NAD and PTX A-protomer was inhibited by 62 ± 5% ($n = 10$) (Fig. 4, lane 2), suggesting that an endogenous reaction of NAD with these nuclear proteins during preincubation may have interfered with their subsequent PTX-catalyzed ADP-ribosylation. The PTX-independent $^{[32P]}$ADP-labeling of the nuclear proteins in the presence of $^{[32P]}$NAD (Fig. 4, lane 3) was temperature-dependent (inactive at 4°C and optimal at 37°C), suggesting the possible involvement of an enzymatic process. In addition, this labeling was inhibited by 40 ± 5% ($n = 3$) in the presence of GTPγS (100 μM) (Fig. 4, lane 8), reminiscent of the effect of GTPγS on ADP-ribosylation of membrane G$_i$- and G$_o$-proteins, which are better substrates for PTX-catalyzed ADP-ribosylation in their nonactivated than in their activated state (28). However, labeling of the nuclear G-proteins in control preparations was not enhanced by GDPβS (100 μM) ($n = 3$) (Fig. 4, lane 7). Changes in $^{[32P]}$ADP-labeling of the nuclear proteins were quantified by scanning analysis (see “Materials and Methods”).

Inhibition of PTX-catalyzed ADP-ribosylation of the 39–40-kDa proteins in preincubated brain stem crude nuclei was also detected by two-dimensional gel electrophoresis. Crude nuclear proteins were $^{[32P]}$ADP-ribosylated by PTX A-protomer after preincubation of the cell nuclei under the experimental conditions employed for their PTX-independent ADP-ribosylation, but in the absence of $^{[32P]}$NAD (i.e. 37°C, 30 min, Krebs-Henseleit buffer containing 0.5 mM DTT) (Fig. 5A). Subsequent PTX-catalyzed $^{[32P]}$ADP-ribosylation of the 39–40-kDa proteins, which reacted with antibodies against the C terminus of G$_i$-proteins, was inhibited in the preincubated nuclei (Fig. 5A). This inhibition may be attributable to an endogenous ADP-ribosylation of these proteins, as indicated by their PTX-independent reaction with $^{[32P]}$NAD (Fig. 4). Since ADP-ribosylation of the 39–40-kDa proteins would decrease their isoelectric pH, the possibility that these proteins act as substrates of an endogenous ADP-ribosylation was further examined by

**Fig. 4. Evidence for endogenous ADP-ribosylation of GTP-binding proteins in the crude nuclear preparation.** Proteins in the crude nuclei were $^{[32P]}$ADP-labeled in the presence of $^{[32P]}$NAD and in the presence (lanes 1, 2, and 9) or in the absence (lanes 3, 7, and 8) of the PTX A-protomer. Lanes 1 and 9, PTX-catalyzed ADP-ribosylation of nuclear proteins. Lane 2, subsequent PTX-catalyzed $^{[32P]}$ADP-ribosylation of these proteins in crude nuclei that were preincubated for 30 min at 37°C in Krebs-Henseleit buffer, pH 7.4, containing 0.5 mM DTT. Lanes 3, 7, and 8, proteins in the crude nuclei were $^{[32P]}$ADP-labeled by incubation for 30 min at 37°C with $^{[32P]}$NAD in Krebs-Henseleit buffer, pH 7.4, containing 0.5 mM DTT, in the absence of PTX A-protomer. Lane 3, control; lane 7, in the presence of 100 μM GDPβS; lane 8, in the presence of 100 μM GTPγS. The autoradiograph shows the labeled proteins separated by SDS-PAGE (10% polyacrylamide). Lanes 4–6 present immunostaining of the blotted $^{[32P]}$ADP-ribosylated proteins (autoradiographed in lanes 1–3) with antibodies against the C terminus of G$_i$-proteins ($n = 3$). Each lane contained 200 μg of protein (41).
comparing their isoelectric pH in crude nuclei before and after incubation under conditions enabling their PTX-independent \(^{32}\)P-ADP-labeling on exposure to \(^{32}\)P-NAD. Measurement of the mobility shifts of these nuclear proteins on two-dimensional gel electrophoresis revealed a shift toward acidic pH, detected by immunolabeling of the blotted proteins with antibodies against Go\(_{o}\)-proteins following either their PTX-catalyzed ADP-ribosylation or their incubation under conditions inducing competition with their PTX-catalyzed ADP-ribosylation. The isoelectric pH of the blotted 39–40-kDa proteins in untreated nuclei was less acidic by approximately 0.5 pH units (n = 3) (Fig. 5B).

The possibility that cysteine residues in the 39–40-kDa proteins are acceptors for endogenous ADP-ribosylation in the crude nuclei was examined by the use of cell membrane Go\(_{o}\) and Go\(_{i}\)-proteins as substrates (29). Membranes prepared from brain stem or cortical synaptoneurosomes (7) were incubated for 30 min at 37°C with crude nuclei prepared from these brain regions, in the presence of protease inhibitors, in buffered hypotonic solution containing \(^{32}\)P-NAD (10 pmol, 1000 Ci/ mmol), 0.5 mm DTT, 0.5 mm MgCl\(_2\), 0.05 mm EDTA, 2 mm ATP, 20 mm Tris-HCl, pH 7.4. The crude nuclei in this mixture had been preincubated for 30 min at 37°C in the absence of \(^{32}\)P-NAD in order to prevent subsequent \(^{32}\)P-ADP-labeling of the 39–40-kDa nuclear proteins (see Figs. 4 and 5). After incubation, the labeled membranes were separated from the mixture by the following procedure. The incubated mixture was centrifuged (30,000 × g, 15 min), and the pellet was resuspended in isotonic sucrose (0.32 M) 1:50 (v/v) and centrifuged (800 × g, 15 min). The pellet, which contained the crude nuclei, was removed. A protein band (100 kDa), which includes poly-(ADP-ribosylated) proteins (see Figs. 1B, 7, and 8), was used as a marker for the crude nuclear fraction (Figs. 6A). The supernatant was centrifuged (800 × g, 15 min), the pellet was removed, six volumes of 20 mm Tris, pH 7.4, were added to the supernatant, and the mixture was centrifuged (30,000 × g, 15 min). The resulting pellet contained cell membranes (12). Proteins in the last pelleted fraction were separated by SDS-PAGE and blotted. Proteins (39 kDa) that were specifically immunolabeled with antibodies against the N terminus of Go\(_{o}\)-proteins had also been \(^{32}\)P-ADP-labeled during incubation with the crude nuclei and \(^{32}\)P-NAD (Fig. 6). No \(^{32}\)P-ADP-labeling of membrane proteins was observed following their incubation with \(^{32}\)P-NAD in the absence of crude nuclei (Fig. 6A). The \(^{32}\)P-ADP-labeling of the 39-kDa membrane protein was susceptible to proteases (see “Materials and Methods”) and to cleavage of thioglycoside bonds by merciric ions (4), suggesting that an endogenous reaction in the crude nuclei that competes with PTX-catalyzed ADP-ribosylation (Figs. 4 and 5) might have catalyzed ADP-ribosylation of cysteine residues in membrane Go\(_{o}\)-proteins. For further verification, the products of the PTX-independent reaction, i.e. the \(^{32}\)P-ADP-labeled proteins in membranes prepared from brain cortex, were analyzed by two-dimensional gel electrophoresis, blotted, and subjected to immunolabeling with antibodies against the N terminus of Go\(_{o}\)-proteins (Fig. 6B). Proteins (39 kDa) in membranes preincubated with crude nuclei and \(^{32}\)P-NAD by the above procedure were found to be both \(^{32}\)P-ADP-labeled and immunolabeled with antibodies against Go\(_{o}\)-proteins, suggesting that \(^{32}\)P-ADP-labeling of the membrane Go\(_{o}\)-proteins had occurred during their incubation with the crude nuclei in the presence of \(^{32}\)P-NAD.

**Characteristics of the PTX-independent Reaction of the 39–40-kDa Proteins with NAD in the Crude Nuclei**—In order to determine whether the PTX-independent reaction of the proteins with NAD is their ADP-ribosylation, we examined the
characteristics of the PTX-independent [32P]ADP-labeling of the 39–40-kDa crude nuclear GTP-binding proteins that also act as substrates for PTX-catalyzed ADP-ribosylation. First we examined the possibility that their PTX-independent [32P]ADP-labeling might be a result of reactions other than ADP-ribosylation. A nitric oxide-induced reaction of nicotinamide with cysteine residues of the GTP-binding proteins was excluded by the negative results obtained following the protocol of McDonald and Moss (4) for NO-induced binding of nicotinamide-labeled NAD to cysteine residues in the cytosolic enzyme gyceraldehyde-3-phosphate dehydrogenase.

[32P]Poly(ADP-ribosylation) of the 39–40-kDa proteins was excluded by the use of specific inhibitors of PARP, which apparently catalyzes poly(ADP-ribosylation) of 100-kDa proteins present in crude nuclei prepared from brain stem or cortex (26) (Fig. 7). The presence of poly(ADP-ribosylated) PARP in the 100-kDa protein band was indicated by immunolabeling with antibodies against PARP (not shown). ADP-ribosylation of the 39–40-kDa proteins in the presence of PTX was completed within 2 min, while maximal ADP-ribosylation of the 100-kDa proteins was reached only after 30 min (26) (Fig. 7A). The [32P]ADP-labeling of the 39–40-kDa nuclear proteins was not inhibited by specific inhibitors of PARP (26) (Fig. 7B). Thus, 3-aminobenzamide (3-AB) (10 μM-1 mM), a potent inhibitor of PARP, did not affect either the PTX-catalyzed [32P]ADP-ribosylation or the PTX-independent [32P]ADP-labeling of the 39–40-kDa proteins, whereas it did inhibit poly(ADP-ribosylation) of the 100-kDa proteins (30) (Fig. 7B). Thymine (up to 1 mM), another potent inhibitor of PARP (26), also had no effect on the PTX-independent [32P]ADP-labeling of the 39–40-kDa nuclear proteins while inhibiting the poly(ADP-ribosylation) of the 100-kDa proteins (Fig. 7B). ADP-ribosylation of the 39–40-kDa proteins was significantly inhibited (20–40%, n = 3) by higher concentrations of either 3-AB or thymine (2–10 mM). In addition, PTX-independent [32P]ADP-labeling of the 39–40-kDa nuclear proteins was resistant to hydroxylamine-induced cleavage of ADP-ribose-arginine bonds (31, 32), while poly-(ADP-ribosylation) of the 100-kDa proteins was not resistant to this treatment (26) (Fig. 7C). These findings may exclude both poly(ADP-ribosylation) (26, 31) and arginine-mono-ADP-ribosylation (32, 33) of the 39–40-kDa proteins.

Both the PTX-independent [32P]ADP-labeling and the PTX-catalyzed ADP-ribosylation of the 39–40-kDa crude nuclear proteins were eliminated as a result of the cleavage of thioglycoside bonds by mercuric ions (4) (Fig. 7D). Thioglycoside bonds are reportedly formed as a result of PTX-catalyzed ADP-ribosylation of cysteine residues in membrane Goα- and Goαi-proteins (29). These observations may therefore indicate the formation of thioglycoside bonds in the PTX-independent [32P]ADP-labeling of the 39–40-kDa nuclear proteins, suggesting an endogenous ADP-ribosylation of cysteine residues in these proteins in the crude nuclei.

The product of a nonenzymatic ADP-ribosylation of cysteine residues in these nuclear proteins by free ADP-ribose, produced by NAD-glycohydrolase-catalyzed cleavage of NAD to ADP-ribose and nicotinamide (1, 5), would be cleaved by mercuric ions, similarly to cleavage of the product of ADP-ribosylation by PTX-ADP-ribosyltransferase (5). We therefore further characterized the PTX-independent ADP-ribosylation of the 39–40-kDa proteins by examining the effect of free ADP-ribose on their [32P]ADP-ribosylation. ADP-ribose inhibited the PTX-independent [32P]ADP-ribosylation of these proteins (Fig. 8A); 50% inhibition was achieved in the presence of 2 ± 0.5 μM ADP-ribose (n = 3). These results may suggest that ADP-ribose is a donor in the PTX-independent ADP-ribosylation of these proteins, unlike in their ADP-ribosylation catalyzed by ADP-ribosyltransferase (PTX A-protomer) activity (where 50% inhibition was achieved by ADP-ribose at 100 ± 10 μM (n = 3)), or in the poly(ADP-ribosylation) of the 100-kDa proteins (31) (Fig. 8, A and B). This suggestion is supported by labeling of the 39–40-kDa proteins with [32P]ADP-ribose (140,000 cpm/pmol) prepared from [32P]NAD (1), under the experimental conditions used for their PTX-independent ADP-ribosylation (Fig. 8C). These findings may suggest that the PTX-independent ADP-ribosylation of the 39–40-kDa proteins (Figs. 1, 4, 5, 7, and 8) occurs via nonenzymatic ADP-ribosylation of their cysteine residues (1, 5, 34).

Effect of Membrane Depolarization on the ADP-ribosylation of Nuclear GTP-binding Proteins—A possible role for the endogenous ADP-ribosylation of GTP-binding proteins in the crude nuclei may be inferred from the effect of membrane depolarization on their ADP-ribosylation (Figs. 9 and 10). Membrane depolarization of cells in rat brain stem and cortical slices was induced either by their exposure to high-[K+] Krebs-Henseleit buffer (7) or by prolonged activation of voltage-dependent Na+ -channels induced by BTX (14, 35). In the in situ effect of membrane depolarization on the 39–40-kDa nuclear GTP-binding proteins could not be directly detected in this preparation by the technique used in synaptosomess to examine its in situ effect on membrane...
G-proteins (7, 8). We therefore used the subsequent PTX-catalyzed $[^{32}P]$ADP-ribosylation and [azido-$^{32}P$]GTP labeling of the GTP-binding nuclear proteins as probes for detecting the in situ effect of membrane depolarization on these proteins. This procedure has been found useful in the study of PTX-sensitive G-proteins in the cell membrane (7–10). PTX-catalyzed $[^{32}P]$ADP-ribosylation of the 39–40-kDa proteins in crude nuclei prepared from depolarized cells in brain stem or cortical slices was 60 ± 7% less efficient (n = 10) than that occurring in crude nuclei prepared from brain stem or cortical slices at resting potential (Fig. 9, A and C). Similar inhibition (78 ± 5%, n = 5) of their subsequent PTX-independent $[^{32}P]$ADP-ribosylation was observed in crude nuclei prepared from depolarized cells, while no significant effect was observed either in their subsequent labeling with [azido-$^{32}P$]GTP (Fig. 9B) or in their immunolabeling with α-common antibodies (36). On the basis of previous results (8, 9) these findings are not consistent with a depolarization-induced activation of the GTP-binding proteins (see also Fig. 4).

The depolarization-induced effect on the subsequent PTX-catalyzed ADP-ribosylation of these proteins could be reversed by membrane repolarization (Fig. 9C). In addition, the depolarization induced effect did not occur following in situ PTX-catalyzed ADP-ribosylation (7) (Fig. 9A), suggesting the involvement of PTX-sensitive G-proteins in the effect of depolarization on the nuclear GTP-binding proteins.

Similarly, BTX-induced membrane depolarization evoked marked inhibition (65 ± 5%, n = 3) of the subsequent PTX-catalyzed $[^{32}P]$ADP-ribosylation of the 39–40-kDa crude nuclear proteins in brain stem and cortex (Fig. 9D). The effect of BTX has been attributed to membrane depolarization induced by the delayed inactivation of inward Na+ current (10, 35), and was indeed antagonized by blocking of the Na+ current with tetrodotoxin, a specific blocker of the voltage-dependent Na+ channel (10) (Fig. 9D). Exposure of cells in brain stem and cortical slices to BTX or high K+ apparently did not change the amount of 39–40-kDa GTP-binding nuclear proteins, as estimated from immunolabeling with α-common antibodies (36) (Fig. 9D). This may exclude a possible transfer of these proteins from the cell nucleus in response to membrane depolarization or inward Na+ current.

Since neither the exposure of crude nuclei (rather than cells) to high K+ nor their treatment with BTX affected the ADP-ribosylation of these nuclear proteins, the effect of these agents, when applied on cells, was attributed to their known induction of membrane depolarization (7, 8, 15).

Fig. 10 presents the effect of membrane depolarization induced by high extracellular K+ on the isoelectric pH (pl) of the 39–40-kDa proteins in crude nuclei prepared from depolarized cells in cortical slices. Slices were exposed for 10 min to Krebs-Henseleit buffer containing 4.7 mM K+ (lanes 1 and 2) or 50 mM K+ (lanes 3 and 4) in Krebs-Henseleit buffer and then exposed at 37°C for 10 min to Krebs-Henseleit buffer containing 4.7 mM K+ (lanes 1 and 3) or 50 mM K+ (lanes 2 and 4). Proteins in the crude nuclei prepared from these slices were $[^{32}P]$ADP-ribosylated by PTX A-protomer, subjected to SDS-PAGE analysis and autoradiographed (n = 10). Each lane contained 200 μg of protein (41). B. Effect of high K+-induced membrane depolarization on PTX-independent $[^{32}P]$ADP-labeling of GTP-binding proteins in crude nuclei isolated from brain stem slices. Slices were incubated for 10 min at 37°C in Krebs-Henseleit buffer containing 4.7 mM K+ (lanes 1 and 3) or 50 mM K+ (lanes 2 and 4). Proteins in the crude nuclei isolated from these slices were either $[^{32}P]$ADP-ribosylated in the absence of PTX A-protomer (lanes 1 and 2) or photolabeled with [azido-$^{32}P$]GTP (lanes 3 and 4). Autoradiograms of labeled proteins separated by SDS-PAGE analysis are presented (n = 5). Each lane contained 200 μg of protein. 

C. Reversibility of the depolarization-induced effect on the 39–40-kDa nuclear proteins as indicated by their subsequent PTX-catalyzed $[^{32}P]$ADP-ribosylation. Brain stem slices were incubated for 10 min at 37°C in Krebs-Henseleit buffer containing 4.7 mM K+ (lane 1) and then exposed to Krebs-Henseleit buffer containing 50 mM K+ at 37°C (lane 2). Slices incubated for 10 min in the 50 mM K+ buffer (lane 3) were repolarized by reclosure for 10 min to the 4.7 mM K+ buffer (lane 4). Proteins in the crude nuclei prepared from these slices were $[^{32}P]$ADP-ribosylated by PTX A-protomer, subjected to SDS-PAGE analysis, and autoradiographed (n = 3). Each lane contained 200 μg of protein. 

D. Effect of membrane depolarization, induced by inward Na+ current, on the subsequent PTX-catalyzed $[^{32}P]$ADP-ribosylation of proteins in the crude nuclei. Brain stem slices (untreated, lane 1) were pretreated with 1 μM BTX (37°C, 30 min) (lane 2) or with 1 μM BTX and 1 μM tetrodotoxin (lane 3). Proteins in the crude nuclei prepared from these slices were $[^{32}P]$ADP-ribosylated by PTX A-protomer in the presence of [α-P]NAD and analyzed by SDS-PAGE. Blotted proteins (Western blots) were autoradiographed and immunostained with α-common antibodies (lanes 4–6) (n = 3). Each lane contained 200 μg of protein.
DISCUSSION

This study provides evidence for the presence of GTP-binding proteins (39–40 kDa) in cell nuclei in the rat brain stem and cortex (apparently perinuclear proteins) that are recognized by antibodies against the α-subunits of membrane G_{α}- and G_{βγ}-proteins (Figs. 1 and 2). Proteins immunoblotted with antibodies against these membrane proteins have been previously been detected in perinuclear structures of PC12 cells (37). Nuclear proteins (40 kDa) that act as substrates for PTX-catalyzed ADP-ribosylation and react with antibodies against membrane G_{α}_{i}-proteins have been detected in cell nuclei isolated from rat liver (38).

The possibility that contamination of the nuclear fraction by membranes was responsible for our observations was excluded by immunohistochemical staining of nuclei (Fig. 2) and by the lack of cell membrane markers in the crude nuclear preparation (Fig. 3).

Unlike GTP-binding proteins in the cell membrane (3), the nuclear proteins apparently act as substrates for an endogenous ADP-ribosylation (Figs. 4–8). They also differ from membrane G-proteins in their response to membrane depolarization: ADP-ribosylation of G_{α}_{i}- and G_{α}_{o}-proteins in membranes prepared from depolarized brain synaptoneurosomes was similar to that in membranes prepared from synaptoneurosomes at resting potential, unless the synaptoneurosomes were subjected to PTX-catalyzed ADP-ribosylation during depolarization (Fig. 11) (8, 9). This may be attributable to the lack of endogenous ADP-ribosylation of membrane G_{α}_{i}- and G_{α}_{o}-proteins in these preparations (3). When brain stem synaptoneurosomes were treated with PTX during membrane depolarization, PTX-catalyzed ADP-ribosylation of these proteins was inhibited (Fig. 11) (8, 9), apparently as a result of uncoupling of the α-subunit from the βγ-subunits in PTX-sensitive G-proteins (28) in response to their depolarization induced activation (8, 9). As a consequence, their subsequent PTX-catalyzed [32P]ADP-ribosylation was enhanced (by 60 ± 10%, n = 10) (8, 9). In contrast, according to the present results the subsequent PTX-catalyzed [32P]ADP-ribosylation of the 39–40-kDa nuclear GTP-binding proteins in depolarized cells was inhibited by 60–80% (n = 10) (Figs. 9 and 11). This effect may be attributable to a depolarization-induced endogenous ADP-ribosylation of cysteine residues in these proteins, which would prevent these sites from undergoing subsequent PTX-catalyzed [32P]ADP-ribosylation (Figs. 9 and 10). This suggestion is supported by evidence indicating an endogenous ADP-ribosylation of the 39–40-kDa GTP-binding proteins in crude nuclei (Figs. 4–8).

This activity apparently catalyzes the in vivo formation of thiglycoside bonds (29) in these nuclear proteins in the presence of NAD (Figs. 7 and 8), as well as the in vitro ADP-ribosylation of G_{α}_{o}-proteins in membranes incubated with the
crude nuclei and [\textsuperscript{32}P]NAD under experimental conditions that enable the PTX-independent [\textsuperscript{32}P]ADP-ribosylation of the nuclear proteins (Fig. 6).

Our findings ruled out poly(ADP-ribosylation) of the 39–40-kDa nuclear proteins (31), as well as NO-stimulated covalent binding of NAD to cysteine residues in these proteins (4) (Fig. 7). Since ADP-ribosine is apparently a donor in the PTX-independent ADP-ribosylation of these proteins (Fig. 8), their endogenous ADP-ribosylation appears to involve NAD-glycohydrolase activity followed by nonenzymatic ADP-ribosylation of cysteine residues by free ADP-ribose (5) (Figs. 7 and 8). In view of the cytosolic concentration of free ADP-ribose (39), this reaction could conceivably occur under physiological conditions. However, although the findings are consistent with non-enzymatic ADP-ribosylation of cysteine residues in the 39–40-kDa proteins (Figs. 9 and 10), the possibility of an enzymatic ADP-ribosylation of these proteins by free ADP-ribose is not excluded.

Since the PTX-independent ADP-ribosylation of the 39–40-kDa GTP-binding proteins with [\textsuperscript{32}P]NAD has characteristics in common with their ADP-ribosylation catalyzed by PTX-ADP-ribsytransferase (Figs. 5–7), inhibition of the subsequent PTX-catalyzed [\textsuperscript{32}P]ADP-ribosylation of these proteins in crude nuclei prepared from depolarized cells (Figs. 9–11) may be attributable to their depolarization-induced endogenous ADP-ribosylation. In view of the need for a reducing agent (DTT) in this reaction, recent evidence for a depolarization-induced increase in the concentration of the reduced form of glutathione in brain cortex (40) may be consistent with the proposed depolarization-induced ADP-ribosylation of these proteins (Figs. 9 and 10).

A depolarization-induced modification of these perinuclear GTP-binding proteins by ADP-ribosylation may represent a novel signaling mechanism in the cascade of signal transduction events in neuronal cells.

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