A Fast Signal–induced Activation of Poly(ADP-ribose) Polymerase: A Novel Downstream Target of Phospholipase C

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Abstract. We present the first evidence for a fast activation of the nuclear protein poly(ADP-ribose) polymerase (PARP) by signals evoked in the cell membrane, constituting a novel mode of signaling to the cell nucleus. PARP, an abundant, highly conserved, chromatin-bound protein found only in eukaryotes, exclusively catalyzes polyADP-ribosylation of DNA-binding proteins, thereby modulating their activity. Activation of PARP, reportedly induced by formation of DNA breaks, is involved in DNA transcription, replication, and repair. Our findings demonstrate an alternative mechanism: a fast activation of PARP, evoked by inositol 1,4,5,-trisphosphate–Ca^{2+} mobilization, that does not involve DNA breaks. These findings identify PARP as a novel downstream target of phospholipase C, and unveil a novel fast signal–induced modification of DNA-binding proteins by polyADP-ribosylation.

Key words: poly(ADP-ribose) polymerase • calcium signaling • inositol 1,4,5-trisphosphate • electrical stimulation • brain neurons

Introduction

Membrane depolarization influences neuronal development (Oppenheim, 1991; Spitzer, 1991) and prevents apoptotic cell death of neurons in cultures deprived of growth factors (Brenneman et al., 1990; Franklin and Johnson, 1992; D'Mello et al., 1993; Galli et al., 1995) by a poorly understood mechanism. These phenomena prompted us to examine the possible effect of membrane depolarization on the activity of the nuclear protein poly(ADP-ribose) polymerase (PARP).[1] PARP is an abundant and highly conserved chromatin bound protein (113 kD), found only in eukaryotes, which catalyzes exclusively polyADP-ribosylation of DNA-binding proteins (Udea, 1990; Lautier et al., 1993; Lindahl et al., 1995; D'Amours et al., 1999). Reportedly, PARP, activated by binding to free DNA-endings, acts as an ADP-ribose transferase, adding ADP-ribose to carboxyls of aspartic and glutamic residues. This reaction proceeds by a short-lived ($t_{1/2} = 1$ min) polymerization of ADP-riboses, i.e., polyADP-ribosylation (Kup-

per et al., 1990; Satoh and Lindahl, 1992; Satoh et al., 1994). Activated PARP is auto-polyADP-ribosylated (Desmarais et al., 1991; Satoh et al., 1994; Kim et al., 1997). PolyADP-ribosylation is terminated by the release of extensively polyADP-ribosylated (negatively charged) PARP molecules from DNA (Satoh et al., 1994). ADP-ribose polymers are then instantaneously subjected to partial degradation by polyADP-ribose–glycohydrolase, and completely degraded by a relatively slow process (20–30 min; Satoh et al., 1994; Lin et al., 1997).

Known substrates of PARP include topoisomerase I (Ferro and Olivera, 1984; Kasid et al., 1989), RNA-polymerase II (Hanawalt et al., 1994, Li Oei et al., 1998), DNA polymerases (Simbulan et al., 1993), transcription factors (Rawling and Alvarez-Gonzalez, 1997; Li Oei et al., 1998), histones (Boulikas, 1990; D'Amours et al., 1999), high mobility group proteins (Tsai et al., 1992; D'Amours et al., 1999), p53 (Li Oei et al., 1998), and DNA-dependent kinase (Ruscetti et al., 1998). PolyADP-ribosylation modulates their activity, influencing DNA replication (Cesarone et al., 1990), transcription (Meisterernst et al., 1997, D'Amours et al., 1999), and repair (Satoh and Lindahl, 1992; Lazebnik et al., 1994; Nicholson et al., 1995; Schreiber et al., 1995; Martinou, 1996; Trucco et al., 1998).

The findings presented here demonstrate a fast signal–induced activation of PARP in brain cortical neurons, mediated by inositol 1,4,5,-trisphosphate (IP_3)–induced Ca^{2+} mobilization, which does not involve DNA damage. Thus, PARP acts as a downstream target of phospholipase C.

Materials and Methods

Primary Culture of Rat Brain Cortical Neurons

Primary culture of rat brain cortical neurons was prepared from 18–19-d-old embryos of Sprague Dawley rats. Brain cortex was dissociated mechanically and plated in MEM (Biological Industries), containing 8% horse serum, 8% FCS, 0.6% glucose, 2 mM glutamine, and 15 μg/ml gentamicin. Plating density was 10^6 cells per 35-mm-diameter Nunc plates, precoated with 50 μg/ml poly-L-lysine. Glial cell proliferation was blocked by the addition of 20 μg/ml 5-fluoro-2-deoxyuridine and 50 μg/ml uridine on the third day after plating. Experiments were performed on the fifth and sixth days. Neurons survived in these cultures for 15–18 d.

Crude Nuclei

Crude nuclei were isolated from lysed brain cortical neurons (Cohen-Armon et al., 1996). Cultured cortical neurons were homogenized on ice in isotonic 0.32 M sucrose containing PMSF (0.1 mM), using a glass/glass homogenizer, and were centrifuged at 900 g for 10 min at 4°C. Cells in the resulting pellet were lysed in hypotonic solution (50 mM Tris-Cl, pH 7.4) and centrifuged as described above. This procedure was repeated in 0.32 M sucrose (900 g for 10 min at 4°C) and in 50 mM Tris-Cl, pH 7.4 (12,000 g for 10 min, 4°C). The resulting pellet contained isolated crude nuclei (see electromicrograph in Fig. 8 a).

Recording of Membrane Potential during Depolarizing Stimulation

Cultured cortical neurons were depolarized by raising the extracellular [K^+] from 4.7 mM to 60 mM (high-[K^+]) in the absence of extracellular Ca^{2+}. The added KCl always replaced NaCl, thus preserving the physiological osmolarity and ionic strength of the original solutions (Cohen-Armon and Sokolovsky, 1991). Changes in the resting potential of the cultured neurons were measured by the accumulation of the permeant-labeled cation, tetraphenyl-phosphonium ([^{3}H]TPP$^+$; Cohen-Armon and Sokolovsky, 1991). Alternatively, cortical neurons were depolarized by pulsed electrical stimulation, using a pulse generator (Gruss Medical Instruments) and Pt electrodes installed in 2 ml/plate of either MEM or bath solution (defined below). There was no direct contact between neurons and stimulating electrodes (bath-stimulation). Membrane potential was recorded in individual neurons during stimulation by the patch-clamp technique, using the "whole cell" configuration in the current-clamp mode (Hamill et al., 1981), with Axopatch amplifier 200A and pCLAMP6.0 software (Axon Instruments, Inc.). Signals were filtered at 2 kHz (-3dB point) and digitized at a rate of 50 kHz. The solution in the patch pipette contained (mM): 146 KCl, 5 NaCl, 10 Hepes, 1 MgATP, 1 CaCl$_2$, 2 BAPTA (pH 7.2) and 310 mOsm. Bath solution contained (mM): 130 NaCl, 5 KCl, 30 Glucose, 25 Hepes, 1 MgCl$_2$, 2 CaCl$_2$ (pH 7.4) and 300 mOsm.

Immunoprecipitation

PolyADP-ribosylated proteins were immunoprecipitated from nuclear protein extracts by monoclonal antibody directed against ADP-ribose polymers containing >10 ADP-riboses (10H; Lamarre et al., 1988; Shah et al., 1995) (see Materials). PARP was immunoprecipitated from the nuclear protein extracts by an affinity-purified goat polyclonal antibody raised against amino acids 1–20 at the NH$_2$ terminus of human PARP (N-20; see Materials). For immunoprecipitation, nuclear proteins ($\sim$400 μg protein/sample) were extracted during incubation of crude nuclei (30 min, 4°C) with 50 μl buffered solution containing 500 mM NaCl, 1.5 mM MgCl$_2$, 10 mM Tris-Cl (pH 7.4). Samples were then centrifuged (10,000 g, 5 min) and the supernatants were diluted in buffered solution containing 1.5 mM MgCl$_2$ and 10 mM Tris-Cl. Nuclear proteins were exposed in this solution (overnight, 4°C) to the first antibody (dilution 1:20). Proteins bound to the antibody were precipitated during overnight incubation with protein G–conjugated agarose beads at 4°C, and then extracted from the beads after several washes with PBS by boiling for 2 min in sample buffer.

In Situ Immunofluorescent Labeling of PolyADP-ribosylated Proteins in Cultured Cortical Neurons

Tissue cultures were prepared on coverslips. Monoclonal 10H antibody (dilution 1:10) was introduced into rapidly fixed neurons (fixed for 10 min in ice-cold methanol/acetone 1:1, vol/vol). After overnight incubation with the first antibody at 4°C, neurons were washed with PBS containing 0.1% Tween 20 and exposed to the secondary antibody (dilution 1:500) for 3 h at room temperature. ADP-ribose polymers bound to the nuclear proteins were visualized by the FITC-conjugated affinity pure goat anti–mouse IgG$_3\kappa$ secondary antibody, using a fluorescence confocal inverted microscope (ZEISS LSM 410).

[^{32}P]PolyADP-ribosylation of Proteins in Isolated Crude Nuclei

Unless indicated otherwise, crude nuclei isolated from cortical neurons were incubated for 1–5 min with [^{32}P]NAD (1,000 Ci/mmol; 1μ Ci/sample) and 2.3 mM MgATP at 37°C in a solution containing (mM): 0.045 EDTA, 60 Tris-Cl, 1 MgCl$_2$, and 0.8 DTT (pH 7.4). Deionized water contained 25–30 nM Ca^{2+} (determined by atomic absorbtion). [^{32}P]Poly-ADP-ribosylation was terminated by high salt extraction of the nuclear proteins (500 mM NaCl, 10 mM Tris-Cl [pH 7.4], 4°C, 30 min). [^{32}P]polyADP-ribosylated PARP was immunoprecipitated from the nuclear proteins extracts, subjected to SDS-PAGE, electroblotted, and autoradiographed. [^{32}P]polyADP-ribosylation was quantified by densitometry.

Thymidine Incorporation into DNA during Stimulation

Cultured neurons were incubated with [^{3}H]thymidine (1 μCi/ml) for 1 h before stimulation. 4 h after stimulation, neurons were lysed and harvested onto filters (GF/C, Whatman). The tritium β emission of incorporated [^{3}H]thymidine was counted in scintillation mixture (Friedberg et al., 1995).

Incorporation of modified thymidine, 5-bromodeoxyuridine (BrdUrd) was measured by immunolabeling with anti-BrdUrd monoclonal antibody IU-4 (Caltag Laboratories). BrdUrd (50 μM) was added to cultured neurons 1 h before stimulation. 6 h after stimulation, the neurons were fixed and treated with RNase A. A limited DNA denaturation was performed to allow access of anti-BrdUrd antibody into the DNA (Selden and Dolbeare, 1994). Immunolabeled neurons were then incubated with 5 μg/ml propidium iodide, which intercalates into native DNA (Selden and Dolbeare, 1994). The amount of incorporated BrdUrd labeled by FITC-conjugated secondary antibody (green fluorescence), indicating DNA synthesis, and the amount of intercalated propidium iodide (red fluorescence), indicating the amount of double stranded DNA, were measured by flow cytometry (FACSort machine operated by CellQest software; Becton Dickinson).

Single Strand DNA Breaks Examined by Alkaline Gel Electrophoresis

This method provides a sensitive and rapid method for direct quantitation of breaks in DNA single strands (Sutherland et al., 1999). DNA was isolated from the nuclei of cortical neurons using the Hirt procedure (Hirt, 1967). The migration of equivalent amounts of DNA was analyzed by electrophoresis on 1% alkaline agarose gel (Sutherland et al., 1999). DNA was stained with ethidium bromide (1 μg/ml) and photographed under UV illumination.

Selective Extraction of Fragmented DNA from Nuclei

Fragmented DNA was selectively extracted from prefixed nuclei in high molarity phosphate–citrate buffer (Darzynkiewicz and Juan, 1999). High molecular weight DNA and DNA attached to the nuclear matrix resisted extraction, but fragmented DNA was extracted from the nuclei and identified on agarose gel by staining with ethidium bromide (1 μg/ml).

Displacement of Bound [^{3}H]IP$_3$ by IP$_3$

Samples (20 μl) of crude nuclei (1.5 mg protein/ ml) were incubated (10 min, 4°C) with [^{3}H]IP$_3$ (200 pmol/20 μl sample) in the solution used for [^{32}P]polyADP-ribosylation. Crude nuclei were then rapidly washed under

pressure on Whatman GF/B glass-fiber filters, with ice-cold solution containing 25 mM Tris-Cl, 5 mM NaHCO$_3$ and 1 mM EDTA, pH 8.0 (Challiss et al., 1990). The amount of [^{3}H]IP$_3$ bound to the crude nuclei was assayed by counting their β emission in scintillation fluid. Nonspecific binding of [^{3}H]IP$_3$ was determined in the presence of 10 μM IP$_3$.

Topoisomerase I Activity

Topoisomerase I activity was measured in nuclear protein extracts as described previously (Liu and Miller, 1984). Extracted nuclear proteins (0.1 μg/sample) were added to a reaction mixture containing, at a final volume of 25 μl (mM): 20 Tris-Cl (pH 8.1), 1 DTT, 20 KCl, 10 MgCl$_2$, 0.5 EDTA, 20 μg/ml BSA, and (as substrate) 250 ng of pUC-19, a supercoiled DNA plasmid (Promega). After incubation at 37°C for 30 min, the reaction was terminated by the addition of 5 μl of buffer containing: 50 mM EDTA (pH 8.0), 1% SDS, 15% glycerol, and 0.05% bromophenol blue. The reaction products were analyzed by electrophoresis on 1% agarose gel. Under these experimental conditions topoisomerase II is not activated (Liu and Miller, 1984).

Electron Microscopy

Nuclei isolated from cultured brain cortical neurons were fixed with glutaraldehyde/paraformaldehyde (3:1%) in Krebs-Henseleit buffer (pH 7.4) containing 30% BSA. They were then washed at 4°C with 0.1 M PBS (pH 7.4) and postfixed with 1% OsO$_4$ and 1.5% potassium ferricyanide in PBS at 4°C for 2 h. The samples were examined under a Jeol Jem-100CX electron microscope.

Simultaneous Recording of Rhod-2 Fluorescence

Isolated crude nuclei were loaded with the Ca^{2+} indicator rhod-2/AM (4.5 μM, 30 min incubation, 25°C, at dark), washed, and attached to poly-L-lysine–coated coverslips. Ca^{2+}-induced fluorescent signal of rhod-2 (excitation, 540 nm; emission, >570 nm) was collected through appropriate filters above 520 nm and monitored by confocal inverted microscope (ZEISS LSM 410), equipped with a 25 mW krypton–argon laser (488- and 568-nm lines) and 10 mW He–Ne laser (633-nm line). A 40× NA/1.2 C-apochromat water-immersion lens (Axiovert 135 M, ZEISS) was used for imaging.

DNAse I Activity in Nuclei Isolated from Cultured Neurons

DNAse I activity in nuclei isolated from cultured neurons was assayed according to the procedure described by Boulikas (1990). Nuclei were incubated with DNAse I (RNAse-free; D 7291, Sigma-Aldrich) in buffered solution containing 20 mM Mn^{2+}, 10% glycerol, 10 mM Tris-Cl, and 1 mM DTT (pH 7.4). The reaction was terminated by the addition of 25 mM EDTA (pH 8.0). Fragmented DNA was examined by gel agarose electrophoresis.

Materials

[Adenylate-^{32}P]nicotinamide-adeninindinucleotide, di(tri-ethyl-ammonium) salt ([^{32}P]NAD) (1,000 Ci/mmol) was purchased from DuPont or from Amersham Pharmacia Biotech. D-myo-[^{3}H]Inositol 1,4,5,-trisphosphate, potassium salt ([^{3}H]IP$_3$) (20–60 Ci/mmol) was from Amersham Pharmacia Biotech. [Methyl-^{3}H]thymidine 5′-triphosphate, tetrasodium salt (70–90 Ci/mmol) and [phenyl-^{3}H]tetraphenyl phosphonium bromide ([^{3}H]TPP$^+$) (35 Ci/mmol) were from DuPont. IP$_3$ (hexapotassium salt) was from BIO-MOL. Ethylenediamine-tetraacetic acid (EDTA) and ethyleneglycol-bis(β-amino-ethyl) N,N,N′,N′-tetraacetic acid (EGTA) were from Merck. D(−)-2-amino-5-phosphovaleric acid (APV) was from Cambridge Research Biochemicals. Ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl) ester (BAPTA AM) and rhod-2 AM were from Molecular Probes. (+)-MK-801 hydrogen maleate was from Biotrend. The polyclonal anti–human PARP antibody Vic-5 and monoclonal antibody 10H, directed against ADP-ribose polymers, were kind gifts from Dr. Sugimura, Tokyo Cancer Center, Japan. Anti–human PARP antibody (N-20) and secondary antibodies were from Santa Cruz Biotechnology. Other materials were from Sigma-Aldrich.

Results

Membrane Depolarization Induces PolyADP-ribosylation of Nuclear Proteins in Rat Cortical Neurons

We examined the effect of membrane depolarization on PARP activity in rat brain cortical neurons. Enhanced activity of PARP in depolarized neurons was indicated by: in situ immunolabeling of polyADP-ribosylated proteins; auto-polyADP-ribosylation of PARP; and inhibition of topoisomerase I activity (Ferro and Olivera, 1984; Kasid et al., 1989).

In Situ Immunolabeling of PolyADP-ribosylated Nuclear Proteins by Antibody Directed against ADP-ribose Polymers. PolyADP-ribosylated proteins were immunolabeled in situ by monoclonal antibody directed against ADP-ribose polymers (10H; Shah et al., 1995) in neurons, permeabilized by a rapid fixing procedure (see Materials and Methods). Immunolabeling of ADP-ribose polymers indicated an increased polyADP-ribosylation of proteins in the nuclei of depolarized neurons, relative to that in nuclei of unstimulated neurons (Fig. 1 a). Moreover, in situ poly-ADP-ribosylated PARP was immunoprecipitated by 10H antibody from nuclear extracts of depolarized or electrically stimulated neurons, indicating its enhanced poly-ADP-ribosylation during depolarization (Fig. 1 b). A significantly higher polyADP-ribosylation of PARP was observed in nuclei of neurons pretreated by H$_2$O$_2$, an agent producing DNA breaks (Dizdaroglu, 1992; de Murcia et al., 1994; Fig. 1 b). PARP in nuclei of unstimulated neurons was not immunoprecipitated by 10H antibody (Fig. 1 b).

The Extent of In Situ PolyADP-ribosylation of PARP in Cortical Neurons, Determined by its Subsequent [^{32}P]poly-ADP-ribosylation in Their Isolated Nuclei ("back [^{32}P]poly-ADP-ribosylation"). Despite evidence indicating an enhanced polyADP-ribosylation of PARP in depolarized neurons (Fig. 1, a and b), the [^{32}P]polyADP-ribosylation of PARP in their isolated nuclei was significantly lower than that in nuclei isolated from unstimulated neurons (Fig. 1 c). This could not be explained by NAD depletion in nuclei isolated from depolarized neurons; increasing the extranuclear concentration of NAD (which permeates the nuclear membrane) did not enhance the [^{32}P]polyADP-ribosylation of PARP in those nuclei (Fig. 2 a). Furthermore, the dose-dependent effect of added NAD on [^{32}P]poly-ADP-ribosylation of PARP indicated that the ratio between the concentrations of NAD and [^{32}P]NAD in nuclei of depolarized and unstimulated neurons was similarly altered by adding NAD (Fig. 2 a), indicating a similar concentration of endogenous NAD in both preparations (10^3–10^4 higher than the concentration of [^{32}P]NAD, 10^{-8} M).

The possibility that depolarization renders PARP inactive or refractory to [^{32}P]polyADP-ribosylation in the isolated nuclei was also excluded, since PARP was similarly activated by agents inducing formation of DNA breaks (H$_2$O$_2$ and DNAse I) in unstimulated or depolarized neurons (Fig. 2 b). However, although PARP was extensively [^{32}P]polyADP-ribosylated in nuclei subjected to a mild DNA fragmentation by DNAse I (see Fig. 6 d) during [^{32}P]polyADP-ribosylation (Fig. 2 b, lanes 3 and 6), PARP
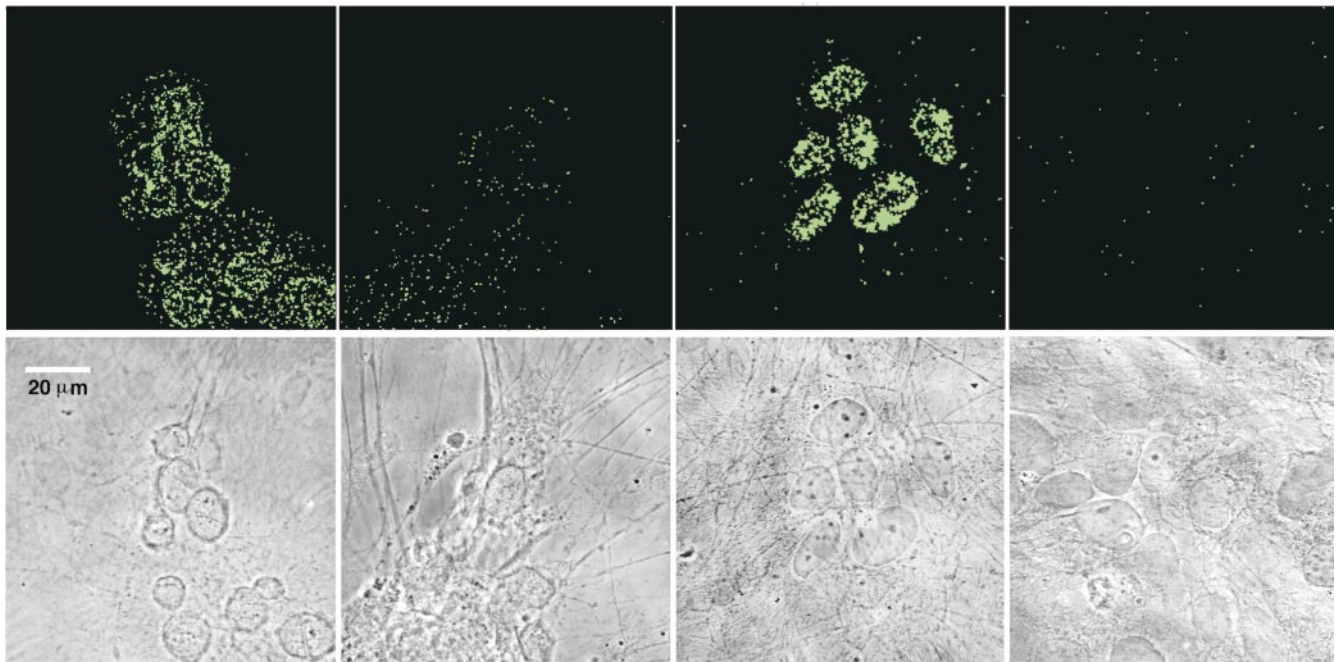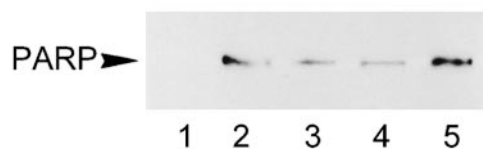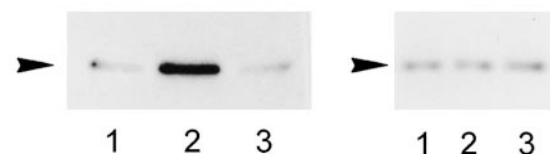
Figure 1. Membrane depolarization induces polyADP-ribosylation of nuclear proteins in brain cortical neurons. (a) PolyADP-ribosylated proteins in the nuclei of prefixed cultured rat cortical neurons were immunolabeled in situ with monoclonal antibody directed against ADP-ribose polymers (10H; see Materials and Methods). Confocal images of neurons, labeled with fluorescein-conjugated secondary antibody (top), were also visualized in transmitted light (bottom). The four frames, from left to right, show neurons depolarized by high-[K$^+$] for 5 min; unstimulated neurons; neurons pretreated with H$_2$O$_2$ (1 mM, 10 min); and depolarized neurons labeled only with the secondary antibody ($n = 4$). (b) Western blots of polyADP-ribosylated PARP immunoprecipitated by 10H antibody from nuclei of unstimulated (lane 1) and depolarized (lanes 2–4) cortical neurons. Neurons were depolarized by high-[K$^+$] (lane 2), or stimulated by a 2-min train of repetitive (100 Hz) 30-volt, 0.1 ms pulses (lane 3), or by a 10-min train of repetitive (10 Hz) 30-volt, 0.1 ms pulses (lane 4). (Lane 5) Neurons pretreated with H$_2$O$_2$. Immunoprecipitated PARP was immunolabeled by anti-PARP, Vic-5 antibody ($n = 6$). (c, left) Autoradiograms presenting [^{32}P]polyADP-ribosylated PARP (5 min, 37°C) in isolated nuclei of unstimulated neurons (lane 2) and depolarized neurons (high-[K$^+$]; lane 1, stimulated by a 2-min train of repetitive [100 Hz] 30-volt, 0.1 ms pulses; lane 3). [^{32}P]polyADP-ribosylated PARP was immunoprecipitated from the nuclear protein extracts by N-20 antibody (see Materials and Methods), subjected to SDS-PAGE, autoradiographed, electroblotted (Western blot), and immunolabeled (on right) by anti-PARP, Vic-5 antibody ($n = 6$).

was scarcely [^{32}P]polyADP-ribosylated in nuclei treated with DNAse I before [^{32}P]polyADP-ribosylation (Fig. 2 b, lanes 8 and 10; see Fig. 6 c). As elaborated below, this effect could be attributed to an endogenous polyADP-ribosylation of PARP, evoked by DNA-nicks formation (D'Amours et al., 1999) but prevented in the presence of H$_2$O$_2$ (Fig. 2 b, lanes 2 and 5). PolyADP-ribosylation of PARP is suppressed in the presence of H$_2$O$_2$ (data not shown), apparently due to the destruction of its zinc-fingers by this oxidizing agent (Wu et al., 1996; Park et al., 1999).

The observations described in Fig. 2 b led us to suggest a sensitive method for determining changes in the activity of PARP in intact cells by measuring the extent of its [^{32}P]polyADP-ribosylation in their isolated nuclei. The concept underlying this method was first introduced by Nestler and Greengard (1980) for assaying in situ phosphorylation of proteins by measuring their in vitro [^{32}P] phosphorylation (back-[^{32}P]phosphorylation).

PolyADP-ribosylation of PARP in intact cells can be assayed by its [^{32}P]polyADP-ribosylation in their isolated

a

Unstimulated neurons

[32P]ADP

[NAD] (μM) 0. .01 .05 .1 .5 1. 5. 10. 50. 100. 500.

PARP ▶

Anti-PARP

▶

1 2 3 4 5 6 7 8 9 10 11

Depolarized neurons

[32P]ADP

[NAD] (μM) 0. .01 .05 .1 .5 1. 5. 10. 50. 100. 500.

PARP ▶

Anti-PARP

▶

1 2 3 4 5 6 7 8 9 10 11

b

[32P]ADP
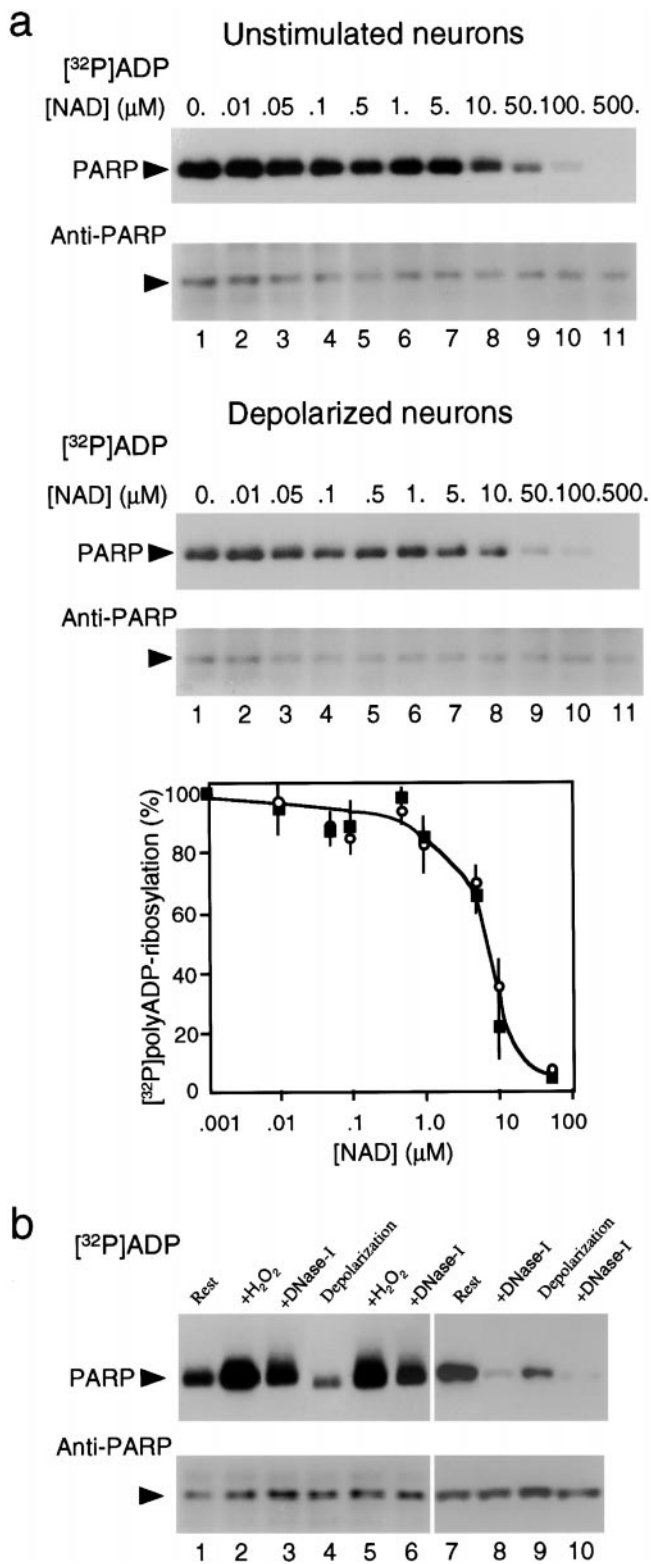
PARP ▶

Anti-PARP

▶

1 2 3 4 5 6 7 8 9 10

Figure 2. [32P]polyADP-ribosylation of PARP in nuclei isolated from unstimulated and depolarized neurons. (a) [32P]polyADP-ribosylation of PARP (2 min, 37°C) in crude nuclei isolated from unstimulated (top) and depolarized (bottom) neurons, in the presence of increasing concentrations of added NAD. Nuclear protein extracts were analyzed by SDS-PAGE and electroblotted. [32P]polyADP-ribosylated PARP in the nuclear protein extracts of both preparations was autoradiographed and immunolabeled by N-20 antibody. The curve presents average values ± SD

nuclei (back-[32P]polyADP-ribosylation), since PARP activity is preserved in the isolated nuclei, and only DNA-bound PARP is polyADP-ribosylated (Satoh et al., 1994). Extensively polyADP-ribosylated PARP is released from DNA, and its ADP-ribose polymers are immediately subjected to a partial degradation (Satoh et al., 1994). PARP carrying partially degraded ADP-ribose polymers is not rebound to DNA (Udea, 1990; Satoh et al., 1994; Lindahl et al., 1995). Since a complete degradation of ADP-ribose polymers, which would enable PARP de novo binding to DNA, is very slow relative to the time course of its [32P]polyADP-ribosylation (see Materials and Methods; Satoh et al., 1994), extensively polyADP-ribosylated PARP in situ may not undergo further [32P]polyADP-ribosylation. Thus, although PARP was extensively [32P]poly-ADP-ribosylated during DNA-nicks formation by DNAse I, it was scarcely [32P]polyADP-ribosylated in nuclei pre-treated with DNAse I before [32P]polyADP-ribosylation (Fig. 2, compare lanes 3 and 6 with lanes 8 and 10).

Thus, for DNA-bound PARP undergoing [32P]polyADP-ribosylation in the isolated nuclei, the more extensive the PARP endogenous polyADP-ribosylation, the lower its measured [32P]poly-ADP-ribosylation. This is illustrated in Fig. 3, based on the schematic presentation of poly-ADP-ribosylation by Satoh et al. (1994). The low extent of PARP [32P]polyADP-ribosylation in nuclei isolated from depolarized neurons (Fig. 1 c) is in accordance with its high endogenous polyADP-ribosylation (Figs. 1, a and b, and 3).

Since NAD does not permeate cell membranes, we determined the activity of PARP in intact neurons by measuring the extent of its back-[32P]polyADP-ribosylation in their isolated nuclei. Changes in PARP activity during electrical stimulation were examined by this method.

Cortical neurons in culture were stimulated by pulsed electrical stimuli (see Materials and Methods). Evoked action potentials and postsynaptic potentials were recorded in individual neurons during stimulation by using the patch-clamp whole cell configuration (see Materials and Methods; Hamill et al., 1981) (Fig. 4 a). Immediately after stimulation, neurons were lysed and their nuclei were isolated (see Materials and Methods). [32P]polyADP-ribosylation was conducted in the isolated nuclei (see Materials and Methods). Generally, a continuous electrical activity in the cortical neurons resulted in a low back-[32P]poly-ADP-ribosylation of PARP in their isolated nuclei (Fig. 4 b). This was consistent with the directly assayed enhanced polyADP-ribosylation of PARP in depolarized neurons (Fig. 1, a and b). The effect of depolarization on PARP ac-

of the depletion (%) in PARP [32P]polyADP-ribosylation in nuclei isolated from unstimulated (○) and depolarized (●) neurons due to increasing extranuclear NAD concentration (n = 6). (b) [32P]polyADP-ribosylation of PARP (2 min, 37°C) was conducted in nuclei isolated from unstimulated (lanes 1–3, 7, 8) and depolarized (lanes 4–6, 9, 10) neurons. (Lanes 2 and 5) Neurons treated by H$_2$O$_2$ (1 mM, 10 min, 25°C). (Lanes 3 and 6) Nuclei treated with DNAse I (20 μg/ml, 2 min, 37°C) during [32P]poly-ADP-ribosylation. (Lanes 8 and 10) Nuclei treated with DNase I (80 μg/ml, 2 min, 37°C) before [32P]polyADP-ribosylation (n = 4).

depolarization   **resting potential**

in-situ polyADP-ribosylation

Back polyADP-ribosylation

[^{32}P]polyADP-ribosylation in isolated nuclei
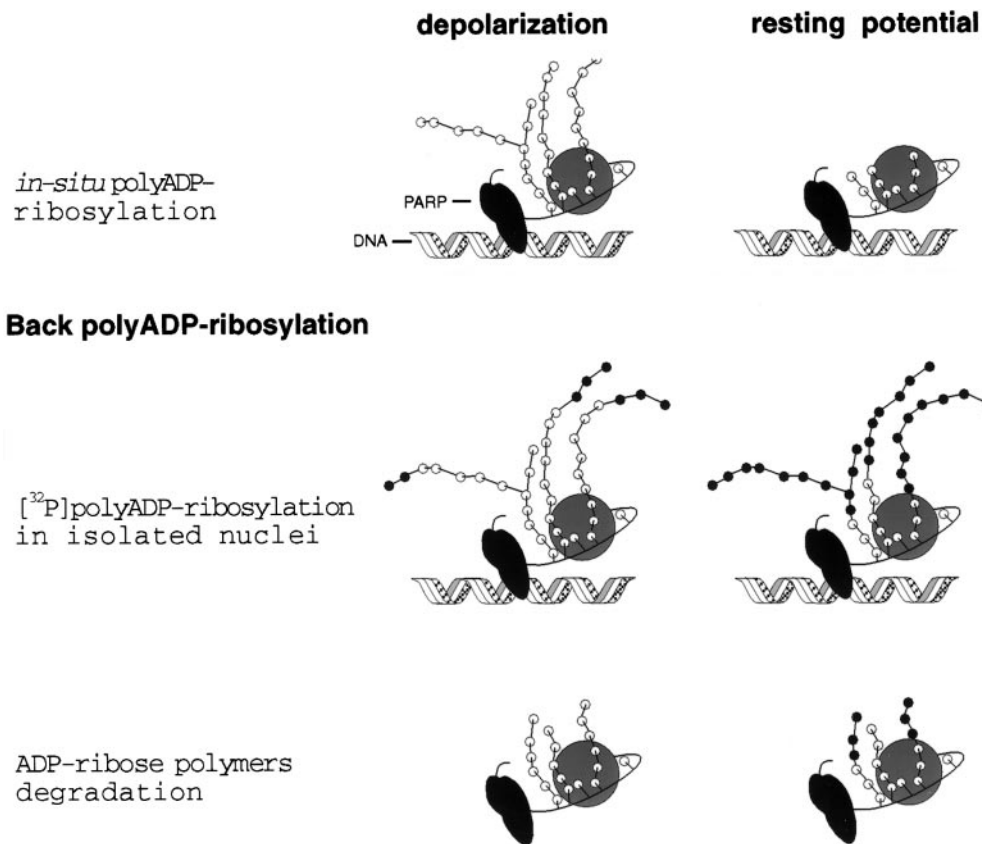
ADP-ribose polymers degradation

Figure 3. A schematic illustration of the concept underlying back polyADP-ribosylation. The extent of polyADP-ribosylation of PARP in intact cells reflected in the [^{32}P]polyADP-ribosylation of PARP in their isolated nuclei. ○, ADP-ribose; ●, [^{32}P]ADP-ribose.

tivity was reversed by repolarization (Fig. 4 b); the more effective the stimulation, the longer the repolarization period required for reversal (Fig. 4 b). Stimulated neurons preserved their resting potential (Fig. 4 a), evidence that they were not damaged by the depolarizing stimulations. Also, depolarized neurons survived in their cultures for 10 d after stimulation, similar to the survival period of unstimulated neurons.

Inhibition of Topoisomerase I Activity in Depolarized Neurons Due to polyADP-ribosylation. The activation of PARP in depolarized neurons was further examined by measuring the activity of topoisomerase I, a known substrate of PARP (Ferro et al., 1983) inhibited by polyADP-ribosylation (Ferro and Olivera, 1984; Kasid et al., 1989). Topoisomerase I catalyzes the relaxation of supercoiled DNA, initiating DNA transcription and replication in eukaryotes (Wang, 1996). We therefore used the relaxation of a supercoiled DNA-plasmid (related inversely to its mobility in gel agarose electrophoresis; Liu and Miller, 1984) to assay topoisomerase I activity. A lower mobility indicated plasmid relaxation and, by inference, the activation of topoisomerase I.

We examined the effect of membrane depolarization on both activity and polyADP-ribosylation of topoisomerase I. Incubation of the supercoiled DNA-plasmid with proteins extracted from nuclei of depolarized neurons resulted in a significantly reduced topoisomerase I activity (Fig. 5 a, lanes 7–11), as compared with its activity in protein extracts of unstimulated or repolarized neurons (Fig. 5 a, lanes 3–6 and 12). Moreover, inhibition of topoisom-

erase I activity in depolarized neurons was prevented by suppression of PARP activity with 3-aminobenzamide (3-AB; Udea, 1990) (Fig. 5 a, lanes 8 and 10). This result was in line with polyADP-ribosylation of topoisomerase I in the depolarized neurons (Fig. 5 b), thereby indicating that topoisomerase I is inhibited in depolarized neurons by polyADP-ribosylation.

No Evidence of DNA Breaks Formation in Depolarized Neurons

Since PARP activation is reportedly induced by binding to free DNA endings in nicked DNA (Menissier-de Murcia et al., 1989; Kupper et al., 1990; Satoh and Lindahl, 1992), we examined the possibility that membrane depolarization induces polyADP-ribosylation of PARP due to the formation of DNA breaks.

Depolarizing stimulation induced a transient polyADP-ribosylation of PARP, which disappeared as the resting potential was restored (Fig. 4 b). Therefore, we used methods suitable for detecting DNA repair in intact neurons during this transient effect. Induction of DNA breaks should be reflected in an increased DNA repair in depolarized neurons (Friedberg et al., 1995). We therefore examined DNA synthesis in the stimulated neurons by measuring the incorporation of thymidine (Friedberg et al., 1995) or the thymidine analogue BrdUrd (Selden and Dolbeare, 1994) into DNA (see Materials and Methods).

[^{3}H]Thymidine was incorporated only in nicked DNA of neurons pretreated with H_2O_2 (Fig. 6 a). There was no sig-
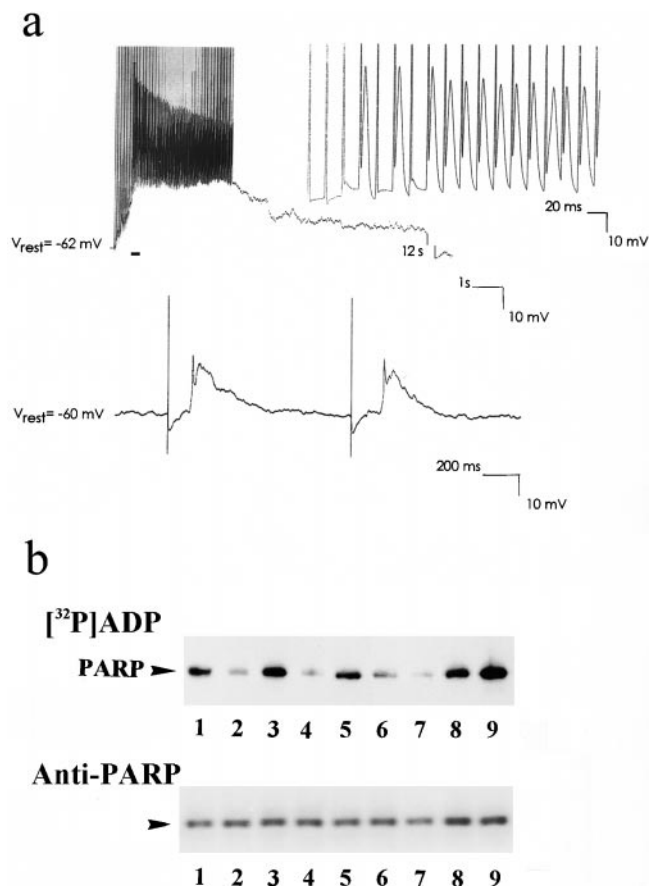
Figure 4. Electrical activity inducing PARP activation in brain cortical neurons. (a) Evoked action potentials and synaptic potentials, recorded by the patch-clamp whole cell technique in the current clamp mode in cultured cortical neurons during electrical stimulation ($n = 4$). (Top) stimulation inducing polyADP-ribosylation of nuclear proteins (see b): 2-s repetitive (100 Hz) 30-volt, 0.1 ms pulses. Evoked action potentials, underlined by a bar, are presented on an expanded time-base to the right. The break in the trace represents a gap of 12 s. (Bottom) A stimulation which did not induce polyADP-ribosylation: 2-s repetitive (1 Hz) 30-volt, 0.1 ms pulses. (b) (Top) Autoradiograms of [^{32}P]polyADP-ribosylated PARP in crude nuclei isolated from cortical neurons pretreated as follows: lane 1, unstimulated neurons; lanes 3, 5, 7, and 9, neurons repolarized for 20 min after the following depolarizations (respectively): high-[K$^+$] for 5 min (lane 2), a 10-min train of repetitive (10 Hz) 30-volt, 0.1 ms pulses (lane 4), a 2-min train of repetitive (100 Hz) 30-volt, 0.1 ms pulses (lane 6), and repetitive (100 Hz) 30-volt, 0.1 ms pulses, applied for 2 s every minute for 10 min (lane 8). PARP was immunoprecipitated from the nuclear protein extracts by N-20 antibody, subjected to SDS-PAGE, autoradiographed, and electroblotted (Western blot). (Bottom) Immunolabeling of [^{32}P]polyADP-ribosylated PARP by anti-PARP Vic-5 antibody in the immunoprecipitates ($n = 8$).

Figure 5. Suppressed activity of topoisomerase I in depolarized neurons. (a) Mobility in gel agarose of supercoiled plasmid (pUC-19) before (lanes 1 and 2) and after (lanes 3–12) incubation with nuclear proteins of unstimulated (lanes 3–6) or depolarized (lanes 7–11) cortical neurons, neurons treated with the PARP inhibitor, 3-AB (0.5 and 1.0 mM: lanes 4 and 5, respectively), neurons depolarized for 5 min by high-[K$^+$] (lanes 7 and 8), neurons depolarized by 2-min train of repetitive (100 Hz) 30-volt, 0.1 ms pulses (lanes 9 and 10), neurons pretreated with 0.5 mM 3-AB (lanes 8 and 10), and neurons depolarized by 10-min train of repetitive (10 Hz) 30-volt 0.1 ms pulses (lanes 11 and 12) and repolarized for 20 min (lane 12). Each lane contained 100 ng protein ($n = 5$). (b) Topoisomerase I (110 kD) immunolabeled with anti–human topoisomerase I polyclonal antibody in Western blots (lanes 1 and 2) of [^{32}P]polyADP-ribosylated nuclear proteins (5 min, 37°C), separated by SDS-PAGE and autoradiographed (lanes 3 and 4). Proteins were extracted from nuclei of unstimulated (lanes 1 and 3) and high-[K$^+$] depolarized (lanes 2 and 4) cortical neurons. Each lane contained 200 μg protein ($n = 3$).

nificant incorporation of [^{3}H]thymidine or BrdUrd into DNA of depolarized or unstimulated neurons (Fig. 6, a and b, respectively).

Lack of DNA breaks in the depolarized neurons was further confirmed by two sensitive methods for DNA breaks detection: (i) alkaline gel electrophoresis of DNA, for detecting breaks in single stranded DNA (Sutherland et al., 1999) and (ii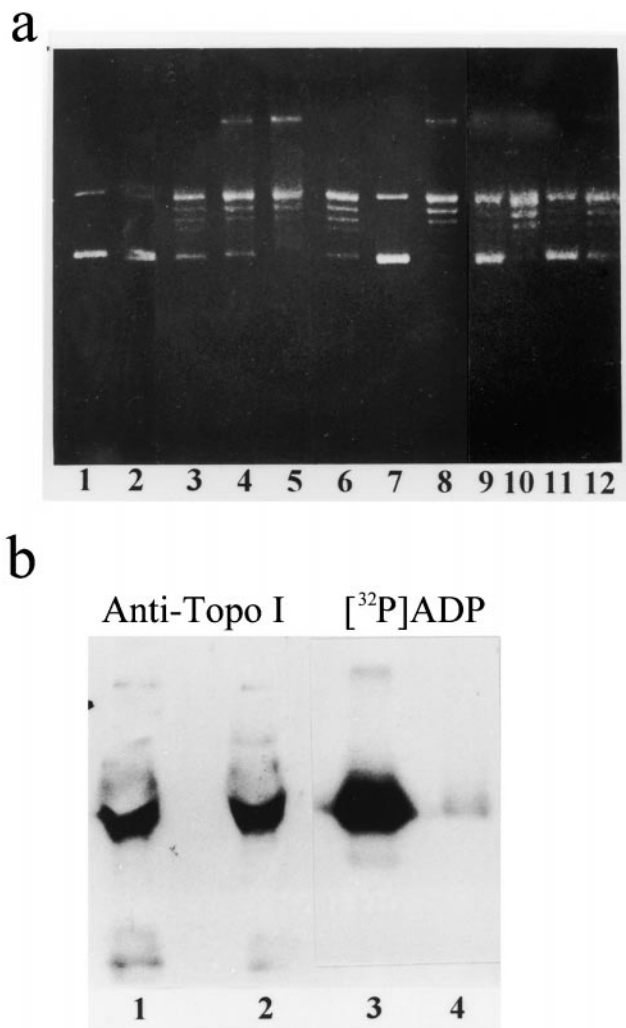) selective extraction of fragmented DNA from isolated nuclei (Darzynkiewicz and Juan, 1999) (see Materials and Methods).

The results depicted in Fig. 6 c show no evidence of breaks in single DNA strands of depolarized or unstimulated neurons. Moreover, there was no evidence of DNA fragmentation in either unstimulated or depolarized neu-
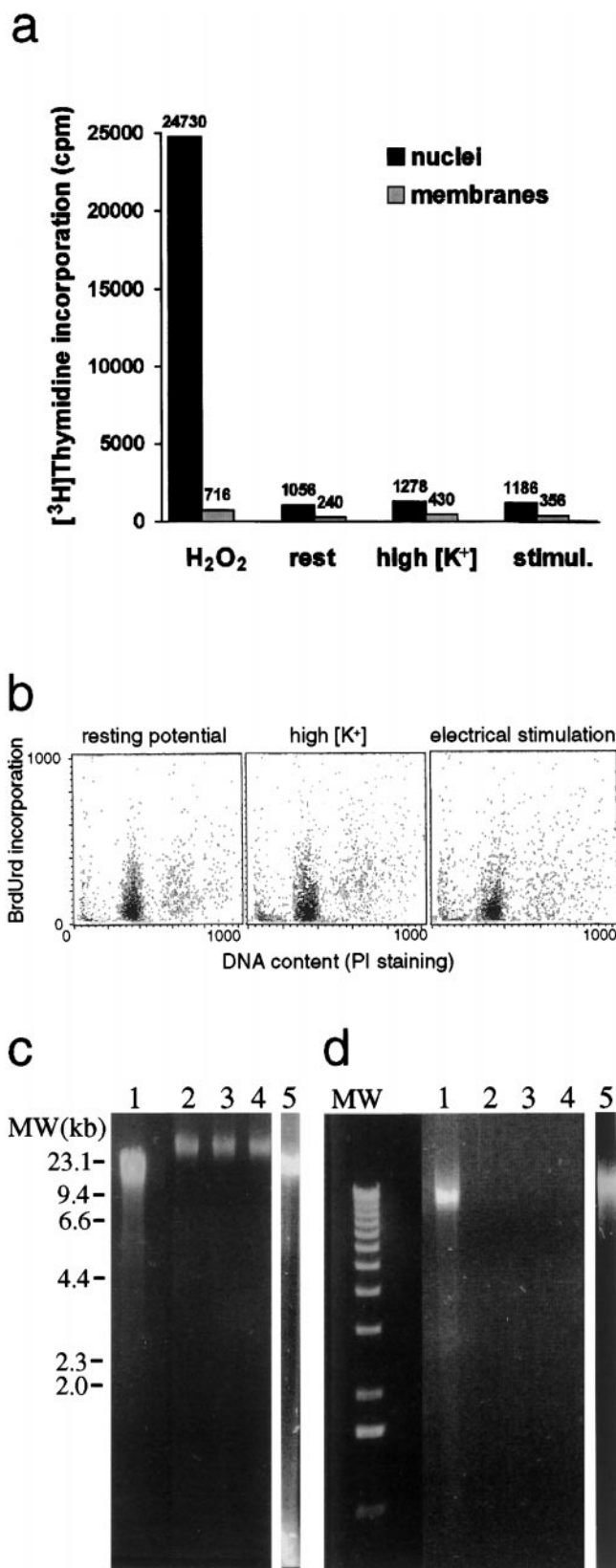
a

b

c   d

Figure 6. DNA repair examined in depolarized and unstimulated cortical neurons. (a) [³H]Thymidine incorporation in the DNA of cortical neurons pretreated with H₂O₂, unstimulated neurons (rest), high-[K⁺]-depolarized neurons (5 min), and electrically stimulated neurons (2-min train of repetitive [100 Hz] 30-volt,

rons (Fig. 6 d). Nicked DNA single strands or fragmented DNA were extracted only from neurons pretreated by H₂O₂ or from nuclei pretreated with DNAse I (Fig. 6, c and d). These results strongly suggest that the enhanced polyADP-ribosylation of PARP in depolarized neurons is not derived from the formation of DNA breaks.

Lack of breaks in the DNA of depolarized neurons (Fig. 6) is consistent with the lack of NAD consumption in the depolarized neurons (Fig. 2 a) (Satoh and Lindahl, 1992).

Evidence Associating Activation of PARP with IP₃-mobilized Ca²⁺

Extranuclear Ca²⁺ Promotes Activation of PARP. An increased intracellular Ca²⁺ concentration ([Ca²⁺]) is measured in neurons during membrane depolarization (Al-Mohanna et al., 1994). We therefore examined the possibility that Ca²⁺ is a mediator of depolarization-induced PARP activation. The effect of extranuclear [Ca²⁺] on [³²P]polyADP-ribosylation of nuclear proteins was examined in isolated nuclei of cortical neurons in the presence of ATP (Methods). Nuclei were exposed to increasing [Ca²⁺], added before or after the addition of [³²P] NAD, which initiates [³²P]polyADP-ribosylation.

Increasing the extranuclear [Ca²⁺] during [³²P]poly-ADP-ribosylation enhanced, by a dose-dependent manner, the [³²P]polyADP-ribosylation of PARP (Fig. 7, a and b). The effect of Ca²⁺ on polyADP-ribosylation was very fast. It was therefore identified better at 25°C (rather than at 37°C; Fig. 7 a, lanes 1–6). Accordingly, when Ca²⁺ was added to the nuclei before [³²P]polyADP-ribosylation, the [³²P]polyADP-ribosylation of PARP decreased in a dose-dependent manner by increasing extranuclear [Ca²⁺] (Fig.

0.1 ms pulses). Numbers above columns indicate the average values (SD < 10%) of tritium β emission (cpm) from [³H]thymidine incorporated into DNA (black) and from nonspecifically bound [³H]thymidine to neuronal cells membranes (gray) (*n* = 5). (b) Incorporation of BrdUrd into the DNA of cortical neurons, detected by flow cytometry (see Materials and Methods). Neurons were either unstimulated or depolarized by high-[K⁺], or by a 10-min train of repetitive (10 Hz) 30-volt, 0.1 ms pulses. Incorporated BrdUrd was detected by immunolabeling with anti-BrdUrd monoclonal antibody (IU-4) (Methods) and visualized by FITC-conjugated secondary antibody. The content of double stranded DNA in the preparations was indicated by propidium iodide (PI) intercalation (*n* = 3). (c) Alkaline gel electrophoresis of DNA extracted from nuclei of neurons pretreated with H₂O₂ (1 mM, 10 min; lane 1) or from unstimulated neurons (lane 2), neurons depolarized for 5 min by high-[K⁺] (lane 3) or stimulated by a 10-min train of repetitive (10 Hz) 30-volt, 0.1 ms pulses (lane 4), and from nuclei treated for 2 min at 37°C with 80 μg/ml DNAse I (lane 5). DNA was stained with ethidium bromide (1 μg/ml) in alkaline gel agarose (1%) and photographed under UV illumination (*n* = 3). (d) A selective extraction of fragmented DNA from nuclei of neurons pretreated with H₂O₂ (1 mM, 10 min; lane 1) or DNAse I (20 μg/ml, 2 min, 37°C; lane 5). DNA fragments were not extracted from unstimulated neurons (lane 2) or from neurons depolarized by high-[K⁺] (lane 3) or by electrical stimulation (10-min train of repetitive [10 Hz] 30-volt, 0.1 ms pulses; lane 4). DNA fragments were stained with ethidium bromide (1 μg/ml) in 1% gel agarose and photographed under UV illumination. Markers on left: 1-kb DNA ladder (0.5–10 kb) (*n* = 3).
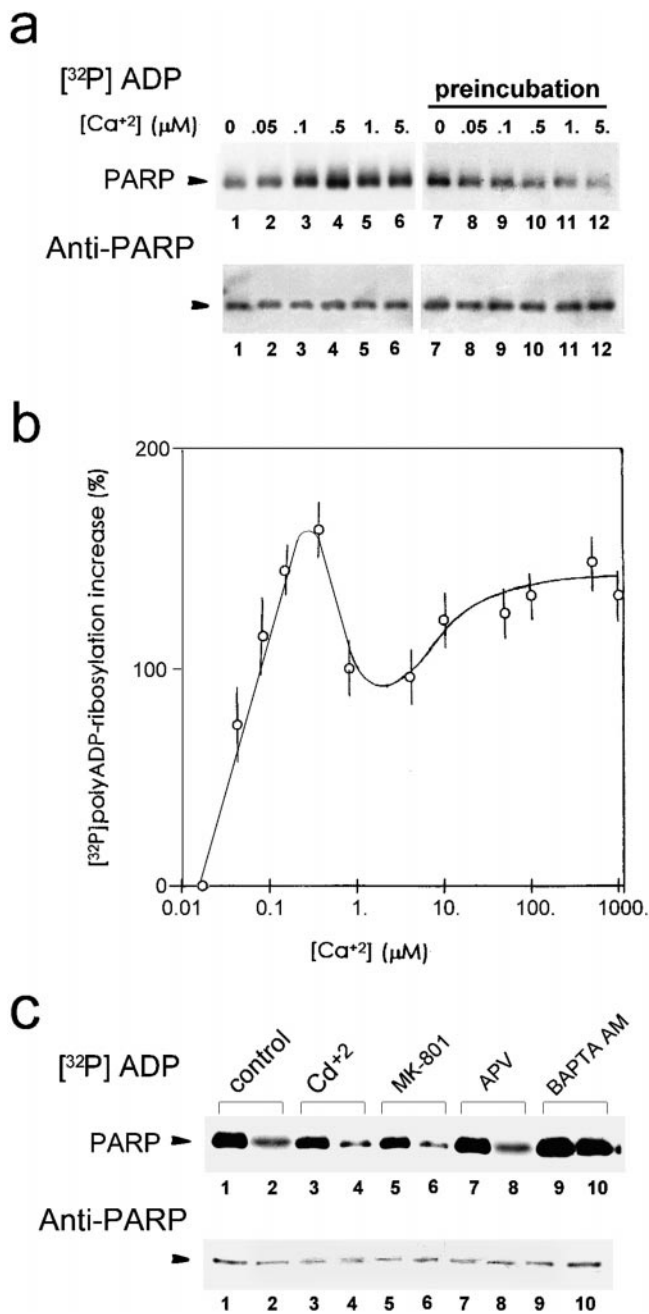
Figure 7. Extra-nuclear Ca^{2+} promotes polyADP-ribosylation of PARP. (a) PolyADP-ribosylation of PARP in crude nuclei isolated from unstimulated neurons, exposed to increasing $[Ca^{2+}]$, 1 min before (preincubation) or during $[^{32}P]$polyADP-ribosylation (2 min, 25°C). $[^{32}P]$polyADP-ribosylated PARP was immunoprecipitated, electroblotted (Western blot), autoradiographed, and immunolabeled by Vic-5 antibody. (b) The curve shows the average increase ($\pm$SD) in $[^{32}P]$polyADP-ribosylation of PARP (measured by densitometry) versus the increase in extranuclear $[Ca^{2+}]$, relative to PARP $[^{32}P]$polyADP-ribosylation in extranuclear $[Ca^{2+}]$ = 25 nM (see Materials and Methods) ($n = 9$). (c) Back-$[^{32}P]$polyADP-ribosylation of PARP, in nuclei of depolarized or unstimulated neurons treated with Ca^{2+} influx blockers and the permeant chelator BAPTA AM. (Top) Autoradiograms of $[^{32}P]$polyADP-ribosylated PARP in crude nuclei of unstimulated neurons (lanes 1, 3, 5, 7, and 9), and neurons depolarized by a 10-min train of repetitive (10 Hz) 30-volt, 0.1 ms pulses (lanes 2, 4, 6, 8, and 10). Cultured neurons were preincubated for

7 a, lanes 7–12), indicating a decreased back-$[^{32}P]$poly-ADP-ribosylation of the activated PARP (see Figs. 1 and 3).

The stimulatory effect of extra-nuclear $[Ca^{2+}]$ on PARP activity was further examined in depolarized neurons, loaded with the permeant Ca^{2+}-chelator, BAPTA AM (Hardingham et al., 1997; Al-Mohanna et al., 1994). Capture of intracellular Ca^{2+} by BAPTA AM completely abolished the increase in polyADP-ribosylation of PARP in depolarized neurons (Fig. 7 c). However, neither depletion of extracellular Ca^{2+} during high-$[K^+]$–induced depolarization (see Materials and Methods; Figs. 1, 4 b, and 5) nor blocking Ca^{2+} influx prevented the polyADP-ribosylation of PARP in depolarized neurons. Its polyADP-ribosylation was neither suppressed by blocking of voltage-dependent Ca^{2+} channels (Olivera et al., 1994) nor by blocking of Ca^{2+} influx, evoked by stimulation of NMDA-glutamate receptors (Sharkey et al., 1996) (Fig. 7 c). These findings strongly suggest that PARP activation in depolarized neurons is mediated by Ca^{2+} release from intracellular stores (Zacchetti et al., 1991; Ehrlich et al., 1994; Ehrlich, 1995). It should be noted that in vitro conducted polyADP-ribosylation of PARP is similarly enhanced by Mg^{2+} (10 mM; Ferro and Olivera, 1982).

Ca^{2+} Release into the Nucleoplasm in Isolated Nuclei of Cortical Neurons. We next examined the possibility that Ca^{2+}, mobilized from intracellular stores, is released into the nucleoplasm. Crude nuclei (Fig. 8 a; see Materials and Methods) were isolated from brain cortical neurons and loaded with the permeant fluorescent Ca^{2+} indicator rhod-2 AM (Minta et al., 1989) in the absence of extranuclear Ca^{2+} (see Materials and Methods). Capture of Ca^{2+} by rhod-2 was visualized by confocal microscopy (see Materials and Methods; Fig. 8, b–d). $[Ca^{2+}]$ was markedly increased in the nucleoplasm of nuclei isolated from depolarized neurons (Fig. 8 b), in line with the transient increase in nuclear $[Ca^{2+}]$ in depolarized neurons (Al-Mohanna et al., 1994; Hardingham et al., 1997).

An increase of extranuclear $[Ca^{2+}]$ did not induce Ca^{2+} release into the nucleoplasm unless ATP (2.5 mM) was added (Fig. 8 d); extranuclear Ca^{2+}, in its physiological concentration range, was instantaneously accumulated in perinuclear compartments by adding ATP (Fig. 8, c and d). Under these experimental conditions, Ca^{2+} was instantaneously released into the nucleoplasm by the addition of IP_3 (1–2 μM; Fig. 8 c). In the presence of ATP, Ca^{2+} was also moderately released into the nucleoplasm when extranuclear $[Ca^{2+}]$ was elevated (Fig. 8 d). cADP-ribose (5–20 μM), reportedly inducing Ca^{2+}-dependent Ca^{2+} release from perinuclear stores (Gerasimenko et al., 1995), had a very small effect on Ca^{2+} release into the nucleoplasm under these experimental conditions (data not shown). These

10 min at 25°C with 1 mM $CdCl_2$ (lanes 3 and 4) or with antagonists of NMDA-glutamate receptors, MK-80l (20 μM; lanes 5 and 6) and APV (500 μM; lanes 7 and 8), or preincubated (30 min, 25°C) with the permeant Ca^{2+} chelator, BAPTA AM (50 μM; lanes 9 and 10). PARP was immunoprecipitated from the nuclear protein extracts of these neurons by N-20 antibody, subjected to SDS-PAGE, electroblotted (Western blot), and autoradiographed. (Bottom) Immunolabeling of $[^{32}P]$-polyADP-ribosylated PARP by Vic-5 antibody in the immunoprecipitates ($n = 6$).
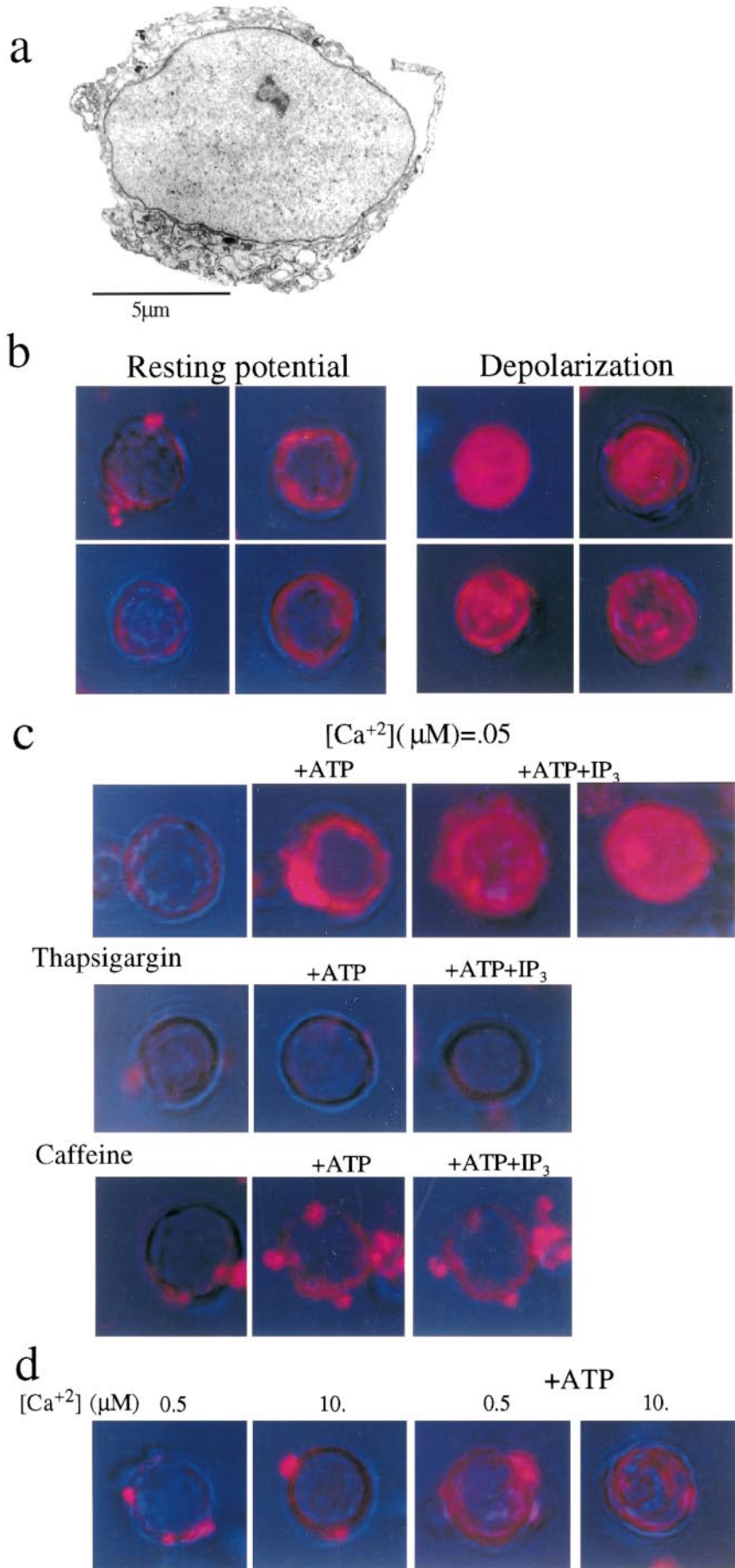
Figure 8. Ca^{2+} mobilization in crude nuclei isolated from brain cortical neurons. (a) Electromicrograph of a crude nucleus isolated from lysed brain cortical neuron (Materials and Methods). (b–d) Confocal microscopy showing Ca^{2+} redistribution in crude nuclei of cortical neurons as indicated by changes in the fluorescence of rhod-2 AM (Materials and Methods). (b) Ca^{2+} detected in the nucleoplasm of depolarized (high-[K$^+$] depolarization, 5 min) and unstimulated neurons. (c) Ca^{2+} redistribution, visualized instantaneously during application of ATP (2.5 mM) and IP$_3$ (1 μM) to crude nuclei of unstimulated neurons in the presence or absence of 5 mM caffeine, or to nuclei of neurons pretreated by 3 μM thapsigargin (10 min, 37°C). (d) Ca^{2+} redistribution in crude nuclei, evoked by increased extranuclear [Ca^{2+}] in the presence or absence of ATP (2.5 mM).

findings are consistent with a growing body of evidence indicating that extranuclear Ca^{2+} permeates the nuclear membrane mainly via Ca-ATPase–induced Ca^{2+} accumulation in IP_3-gated perinuclear stores (Gerasimenko et al., 1995; Hennager et al., 1995; Malviya and Rogue, 1998) and with evidence indicating phosphatidylinositol signaling in the nucleus (Boronenkov et al., 1998).

The release of Ca^{2+} into the nucleoplasm was prevented by caffeine, added to the crude nuclei at concentrations suppressing IP_3-induced Ca^{2+} mobilization (Ehrlich et al., 1994) (Fig. 8 c). Release of Ca^{2+} into the nucleoplasm was also prevented in nuclei isolated from neurons pretreated by thapsigargin that inhibits Ca-ATPase activity (Takemura et al., 1989), thereby preventing Ca^{2+} accumulation in the perinuclear stores (Malviya and Rogue, 1998) (Fig. 8 c).

A Fast Activation of PARP by IP_3 in Isolated Nuclei of Cortical Neurons. We next examined the possibility that PARP is polyADP-ribosylated by IP_3-induced Ca^{2+} mobilization. [^{32}P]polyADP-ribosylation of PARP was examined in the presence of IP_3 added to nuclei isolated from unstimulated neurons. EDTA was omitted from the incubation solution (see Materials and Methods), to avoid chelation of free Ca^{2+}. In addition, [^{32}P]polyADP-ribosylation was carried out at 25°C to enable detection of fast changes in the activity of PARP. For the same reason, IP_3 was added after the addition of [^{32}P]NAD. [^{32}P]polyADP-ribosylated proteins were extracted 1 min after the addition of IP_3. IP_3 (at concentrations of 50 nM to 5 μM) enhanced the [^{32}P]polyADP-ribosylation of PARP in a dose-dependent manner. Maximal 10-fold enhancement was measured with a half maximal effect induced by 100 ± 30 nM IP_3 (Fig. 9 a). At the same concentration range, IP_3 displaced specifically bound [^{3}H]IP_3 from its receptors in the crude nuclei ($IC_{50} = 30 \pm 5$ nM; Fig. 9 b).

Agents suppressing IP_3-induced Ca^{2+}-mobilization also suppressed IP_3-induced PARP activation; addition of the Ca^{2+}-chelator, BAPTA AM, to the isolated nuclei completely suppressed the IP_3-induced [^{32}P]polyADP-ribosylation of PARP (Fig. 9 c, lanes 6–8). Addition of caffeine (3–5 mM; Ehrlich et al., 1994; Ehrlich, 1995) had a similar effect (Fig. 9 c, lanes 12–14). Heparin (100 mg/ml, Grade 1-A; Sigma-Aldrich) (Ehrlich et al., 1994) acted similarly to caffeine (data not shown). Neither BAPTA nor caffeine prevented the basal [^{32}P]polyADP-ribosylation of PARP in the absence of IP_3 (Fig. 9 c, lanes 6 and 9–11, respectively). IP_3-induced [^{32}P]poly-ADP-ribosylation of PARP was also suppressed in nuclei isolated from neurons pretreated by thapsigargin (Fig. 9 c, lanes 15 and 16). IP_3-gated Ca^{2+} stores turn "leaky" by interaction with FK-506 (Cameron et al., 1995; Mikoshiba, 1997; Mackrill, 1999). We therefore examined the effect of FK-506 on [^{32}P]polyADP-ribosylation of PARP.

Addition of FK-506 (1 μM) altered the dose-dependent effect of IP_3 on [^{32}P]polyADP-ribosylation of PARP; the concentration of IP_3 required for enhancement of [^{32}P]polyADP-ribosylation in the presence of FK-506 was 10 times lower than that required in untreated nuclei (Fig. 9 d). Hence, the fast polyADP-ribosylation of PARP by IP_3 (Fig. 9, a and b), and its modulation by agents affecting IP_3-gated Ca^{2+} release (Figs. 8 c and 9, c and d), strongly

suggest that PARP in the isolated nuclei was activated via IP_3-induced Ca^{2+} mobilization.

IP_3-induced [^{32}P]polyADP-ribosylation of PARP in isolated nuclei was neither affected by the addition of calmodulin (10–20 μM; Mackrill, 1999; data not shown), nor by preventing Ca-calmodulin binding to CAM-kinase II in the presence of saturating amounts (1.5 μM) of the Ca-calmodulin binding peptide on CAM-kinase II (Payne et al., 1988; data not shown). cADP-ribose (5–20 μM) did not alter the basal [^{32}P]poly-ADP-ribosylation of PARP in the crude nuclei (data not shown).

Discussion

The results of this study indicate a fast activation of PARP by electrical activity in brain cortical neurons. This is directly demonstrated by in situ immunolabeling of polyADP-ribosylated proteins in depolarized neurons (Fig. 1, a and b) and, indirectly, by inhibition of topoisomerase I activity due to polyADP-ribosylation in depolarized neurons (Fig. 5). PARP activation was quantified by the extent of its back-[^{32}P]polyADP-ribosylation in isolated nuclei of depolarized neurons (Figs. 1 c and 4 b). These findings constitute the first evidence for a fast activation of PARP by physiological signals in the cell membrane.

High-[K^+]–induced membrane depolarization promoted polyADP-ribosylation of nuclear proteins in the absence of extracellular Ca^{2+} (Figs. 1, 4 b, and 5). Findings indicating that PARP is activated by intracellular Ca^{2+} mobilization in the depolarized neurons include: a fast dose-dependent activation of PARP by extranuclear [Ca^{2+}] (Fig. 7, a and b), independent of extracellular Ca^{2+} influx (Fig. 7 c); and a fast dose-dependent PARP activation by physiological concentrations of IP_3 (Fig. 9, a and b), modulated by agents affecting IP_3-induced Ca^{2+} mobilization (Fig. 9, c and d).

IP_3-induced Ca^{2+} release into the nucleoplasm (Fig. 8 c) may underlie the depolarization-induced activation of PARP (Figs. 1 and 4 b). This is supported by data indicating Ca^{2+} release into the nucleoplasm of depolarized cortical neurons (Fig. 8 b; Al-Mohanna et al., 1994; Hardingham et al., 1997) and IP_3-induced Ca^{2+} release into the nucleoplasm of cortical neurons (Fig. 8 c), also reported in other cell types (Malviya and Rogue, 1998).

An enhanced IP_3 production has been measured in depolarized neurons (Gusovsky et al., 1986). It may be attributed to an accelerated phosphoinositide turnover (Gusovsky et al., 1986; Gurwitz and Sokolovsky, 1987), as well as to the stimulation of receptor tyrosine kinases (Castren et al., 1992; Huang et al., 1999), or activation of trimeric G-proteins (Banno et al., 1987; Berridge and Irvine, 1989; Exton, 1990; Sierro et al., 1992; Anis et al., 1999).

The fast polyADP-ribosylation of PARP by IP_3 in the isolated nuclei (Fig. 9) is compatible with the time course of Ca^{2+} release from IP_3-gated stores (Ferris and Snyder, 1992). Moreover, IP_3 stimulated polyADP-ribosylation in the isolated nuclei of cortical neurons (Fig. 9 b) at concentrations compatible with the affinity of IP_3-receptors IP_3-R1 and IP_3-R2 (Mignery et al., 1992; Miyakawa et al., 1999), identified in the brain (Mignery et al., 1992; Ross et al., 1992). IP_3-gated Ca^{2+} stores have been identified in the
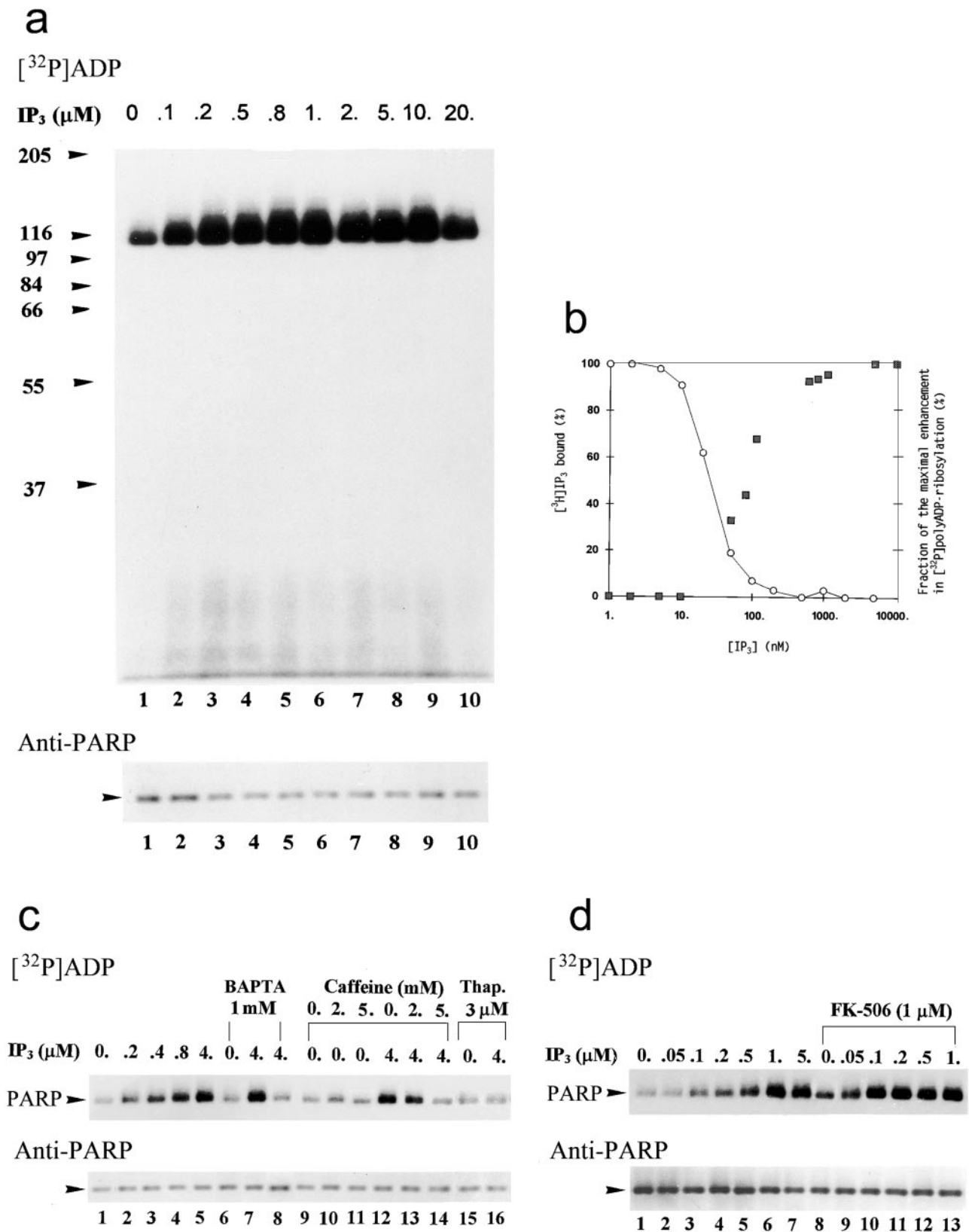
a

[^{32}P]ADP

IP$_3$ (μM)   0   .1   .2   .5   .8   1.   2.   5.   10.   20.

205 ►

116 ►
97 ►
84 ►
66 ►

55 ►

37 ►

1 2 3 4 5 6 7 8 9 10

Anti-PARP

◄

1 2 3 4 5 6 7 8 9 10

b

c

[^{32}P]ADP

					BAPTA 1 mM		Caffeine (mM)						Thap. 3 μM	
IP$_3$ (μM)	0.	.2	.4	.8	4.	0.	4.	4.	0.	0.	0.	4.	4.	4.	0.	4.

PARP ►

Anti-PARP

◄

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

d

[^{32}P]ADP

FK-506 (1 μM)

IP$_3$ (μM)   0.   .05   .1   .2   .5   1.   5.   0.   .05   .1   .2   .5   1.

PARP ►

Anti-PARP

◄

1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 9. IP$_3$ induces a fast [^{32}P]polyADP-ribosylation of PARP in crude nuclei of brain cortical neurons. (a) Autoradiograms of [^{32}P]polyADP-ribosylated PARP in crude nuclei of unstimulated brain cortical neurons in the absence (lane 1) or presence of IP$_3$ at the indicated concentrations (lanes 2–10). [^{32}P]polyADP-ribosylation (2 min, 25°C) was terminated 1 min after the addition of IP$_3$. Nuclear proteins were extracted, separated by SDS-PAGE, and electroblotted (Western blot). PARP was immunolabeled by N-20 antibody (*n* = 7). (b) Left ordinate shows displacement of bound [^{3}H]IP$_3$ by IP$_3$ in crude nuclei of cortical neurons (○). Maximal specific binding of [^{3}H]IP$_3$ (10.5 nM) was 18,500–20,500 cpm/mg protein. Nonspecific binding of [^{3}H]IP$_3$ (~60,000 cpm/mg protein) was determined in the

inner nuclear membrane (Nicotera et al., 1990; Gerasimenko et al., 1995; Malviya and Rogue, 1998). Phosphatidylinositol signaling pathways have been identified in the nuclei of several cell types (Boronenkov et al., 1998).

The enhanced activity of PARP in depolarized neurons was independent of extracellular $[Ca^{2+}]$ (see Materials and Methods; Figs. 1, 4 b, and 5) and resisted Ca^{2+} influx blockers, including agents suppressing NMDA-induced Ca^{2+} influx (Fig. 7 c). We therefore consider it unlikely that PARP is activated in depolarized neurons by DNA damage, caused by nitric oxide formation (Zhang et al., 1994; Shah et al., 1996). It is also unlikely that the fast signal–induced activation of PARP was mediated by Ca^{2+}-induced activation of endonucleases, producing DNA breaks (Arends et al., 1990). The activation of Ca,Mg-endonuclease would require extranuclear Ca^{2+} concentrations 100–1,000-fold higher (Peitsch et al., 1993; Peitsch et al., 1994) than those inducing PARP activation (Fig. 7, a and b). Endonuclease activity at $[Ca^{2+}] <1$ μM has a much slower time course (>30 min; Jones et al., 1989). Accordingly, DNA breaks or NAD depletion (Satoh and Lindahl, 1992) were not detected in the depolarized cortical neurons (Figs. 6 and 2 a, respectively).

A fast signal–induced PARP activation via IP_3-induced Ca^{2+} mobilization constitutes a novel mode of signaling to the cell nucleus: PARP, being a downstream target of phospholipase C, modulates by polyADP-ribosylation the activity of nuclear proteins in response to signals promoting phosphoinositides turnover and phosphatidyl-inositol 4,5-bisphosphate (PIP_2) hydrolysis (Berridge and Irvine, 1989; Fruman et al., 1998; Toker, 1998). A fast modification of transcription factors by polyADP-ribosylation (Li Oei et al., 1998) during electrical activity in brain cortical neurons may associate depolarization-induced polyADP-ribosylation with "memory storage" (Kandel, 1997).

The role of PARP in DNA repair and transcription (Satoh and Lindahl, 1992; Oliver et al., 1998; Trucco et al., 1998) may underlie the effect of depolarization in protecting growth factor–deprived neurons from apoptotic cell death (D'Mello et al., 1993; Galli et al., 1995). This mechanism suggests a crucial influence of neuronal activity in preserving the viability of brain cortical neurons, thereby implementing the rule of "use it or lose it."

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presence of 10 μM IP_3. The data show the amount of specifically bound [³H]IP_3 (as a percentage of its maximal specific binding), determined as the mean of three experiments performed in triplicates, varying by $<15\%$. Right ordinate shows enhancement in [³²P]polyADP-ribosylation of PARP (measured by densitometry; see Materials and Methods) by IP_3 (■). Values are means of seven experiments, expressed for each experiment as a percentage of the maximal enhancement in [³²P]polyADP-ribosylation of PARP. (c, top) Autoradiograms of [³²P]polyADP-ribosylated PARP in crude nuclei (2 min at 25°C) in the absence (lanes 1, 6, 9–11, and 15) or presence (lanes 2–5, 7, 8, 12–14, and 16) of IP_3, BAPTA (lanes 6 and 8), or caffeine (lanes 10, 11, 13, and 14), and in crude nuclei of neurons pretreated with thapsigargin (10 min, 37°C, lanes 15 and 16). Preincubation with BAPTA or caffeine lasted 5 min at 25°C. [³²P]polyADP-ribosylated PARP was immunoprecipitated from nuclear proteins extracts by N-20 antibody, subjected to SDS-PAGE, electroblotted (Western blot), autoradiographed, and immunolabeled (bottom) with anti-PARP, Vic-5 antibody ($n = 3$). (d, top) Autoradiograms of [³²P]polyADP-ribosylated PARP (2 min at 25°C) in crude nuclei of cortical neurons in the absence (lanes 1 and 8) and presence (lanes 2–7 and 9–13) of IP_3 or FK-506 (lanes 8–13). Since FK-506 was dissolved in ethanol, all the samples contained 0.03% ethanol. [³²P]polyADP-ribosylated PARP was immunoprecipitated from nuclear protein extracts by N-20 antibody, subjected to SDS-PAGE, electroblotted (Western blots), autoradiographed, and immunolabeled (bottom) with anti-PARP Vic-5 antibody ($n = 3$).

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