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
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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-riboseylation.

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-riboseylation of DNA-binding proteins (Udea, 1990; Lautier et al., 1993; Lindahl et al., 1995; D’Amours et al., 1999). Activated PARP is auto-polyADP-ribosylated (Desma-rais et al., 1991; Satoh et al., 1994; Kim et al., 1997). PolyADP-riboseylation is terminated by the release of extensively polyADP-riboseylated (negatively charged) PARP molecules from DNA (Satoh et al., 1994). ADP-ribose polymers are then immediately 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; Ksiazid 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-riboseylation modulates their activity, influencing DNA replication (Cesarone et al., 1990), transcription (Mesterernst et al., 1997, D’Amours et al., 1999), and repair (Satoh and Lindahl, 1992; Satoh et al., 1994). A citrullinated PARP is auto-polyADP-riboseylated (Desmara-rais et al., 1991; Satoh et al., 1994; Kim et al., 1997). PolyADP-riboseylation is terminated by the release of extensively polyADP-riboseylated (negatively charged) PARP molecules from DNA (Satoh et al., 1994). ADP-ribose polymers are then immediately 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).

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1 Abbreviations used in this paper: BrdUrd, 5-bromodeoxyuridine; IP3, inositol 1,4,5-trisphosphate; PARP, poly(ADP-ribose) polymerase.
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 genamin. Plating density was 10^5 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 nuclei were homogenized on ice in isotonic 0.32 M sucrose containing PM SF (0.1 mM) and a glass/cell 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 electronmicrograph in Fig. 8a).

Recording of Membrane Potential during Depolarizing Stimulation

Cultured cortical neurons were depolarized by raising the extracellular [Na]Cl (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 was measured by the permeant-labeled cation, tetraphenyl-phosphonium ([TTP]; 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 Heps, 1 M gATP, 1 CaCl_2, 2 BAPTA (pH 7.2) and 310 mMqsm. Bath solution contained (mM): 130 NaCl, 5 KCl, 30 Glucose, 25 Heps, 1 M gC12, 2 CaCl_2 (pH 7.4) and 300 mMqsm.

Immunoprecipitation

PolyA DP-ribosylated proteins were immunoprecipitated from nuclear protein extracts by monoclonal antibody directed against ADP-ribosylation (10H). Cells in the nuclei of cortical neurons using the Hirt procedure (Hirt, 1967). The migration of equivalent amounts of DNA was analyzed by UV illumination.

Selective Extraction of Fragmented DNA from Nuclei

Fragmented DNA was selectively extracted from prefixed nuclei in high molarity phosphate–citrate buffer (Dzarnikiewicze and Julian, 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 [3H]IP_3 by IP_3

Samples (20 μl) of crude nuclei (1.5 mg protein/ml) were incubated (10 min, 4°C) with [3H]IP_3 (200 pmol/20 μl sample) in the solution used for [3H]PolyA DP-ribosylation. Crude nuclei were then rapidly washed under

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) and treated with PBS containing 0.1% Tween 20 and exposed to the secondary antibody (dilution 1:500) for 3 h at room temperature. A DP-riboside polymers bound to the nuclear proteins were visualized by the FITC-conjugated affinity pure goat anti–mouse IgG as secondary antibody, using a fluorescence confocal inverted microscope (ZEISS LSM 410).
Materials were from Sigma-Aldrich. and secondary antibodies were from Santa Cruz Biotechnology. Other clonal 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) (pH 8.0). The amount of [3H]IP, bound to the crude nuclei was assayed by counting their β emission in scintillation fluid. Nonspecific binding of [3H]IP, was determined in the presence of 10 μM IP3.

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 8.3), 1 DTT, 20 KCl, 10 MgCl2, 0.5 EDTA, 20 μg/ml BSA, and (as substrate) 250 ng of pUC-C9, a supercoiled DNA plasmid (Promega). A fiter 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 I 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) and postfixed with 1% OsO4 and 1.5% potassium ferricyanide in PBS at 4°C for 2 h. The samples were examined under a jeol j em-100CX electron microscope.

Simultaneous Recording of Rhod-2 Fluorescence

Isolated crude nuclei were loaded with the Ca2+ indicator rhod-2/AM (4.5 μM, 30 min incubation, 25°C, at dark), washed, and attached to poly-l-lysine-coated coverslips. Ca2+-induced fluorescence 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 570 nm) and postfixed with 1% OsO4 and 1.5% potassium ferricyanide in PBS at 4°C for 2 h. The samples were examined under a jeol j em-100CX electron microscope.

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 DNAase I (RNase free; D 7291, Sigma-A Idrich) in buffered solution containing 20 mM M2+, 10% glycerol, 10 mM Tris-Cl, and 1 mM EDTA (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.

Results

Membrane Depolarization Induces PolyADP-ribo lysation 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; K asid 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 Methods and Materials). Immunolabeling of ADP-ribosylated proteins indicated an increased polyADP-ribo lysylation of proteins in the nuclei of depolarized neurons, relative to that in nuclei of unstimulated neurons (Fig. 1 a). Moreover, in situ polyADP-ribosylated PARP was immunoprecipitated by 10H antibody from nuclear extracts of depolarized or electrically stimulated neurons, indicating its enhanced polyADP-ribosylation during depolarization (Fig. 1 b). A significantly higher polyADP-ribosylation of PARP was observed in nuclei of neurons pretreated with H2O2, 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 [32P]polyADP-ribosylation in Their Isolated Nuclei (“back [32P]polyADP-ribosylation”). Despite evidence indicating an enhanced polyADP-ribosylation of PARP in depolarized neurons (Fig. 1 a and b), the [32P]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 [32P]polyADP-ribosylation of PARP in those nuclei (Fig. 2 a). Furthermore, the dose-dependent effect of added NAD on [32P]polyADP-ribosylation of PARP indicated that the ratio between the concentrations of NAD and [32P]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 (102–103 higher than the concentration of [32P]NAD, 10–8 M).

The possibility that depolarization renders PARP inactive or refractory to [32P]polyADP-ribosylation in the isolated nuclei was also excluded, since PARP was similarly activated by agents inducing formation of DNA breaks (H2O2 and DNAse I) in unstimulated or depolarized neurons (Fig. 2 b). However, although PARP was extensively [32P]polyADP-ribosylated in nuclei subjected to a mild DNA fragmentation by DNAse I (see Fig. 6 d) during [32P]polyADP-ribosylation (Fig. 2 b, lanes 3 and 6), PARP....

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was scarcely \[^{32}\text{P}]\text{polyADP-ribosylated} in nuclei treated with DNAse I before \[^{32}\text{P}]\text{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 \(\text{H}_2\text{O}_2\) (Fig. 2 b, lanes 2 and 5). PolyADP-ribosylation of PARP is suppressed in the presence of \(\text{H}_2\text{O}_2\) (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}\text{P}]\text{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}\text{P}]\text{phosphorylation} (back-\[^{32}\text{P}]\text{phosphorylation}).

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

Thus, for DNA-bound PARP undergoing [\textsuperscript{32}P]polyA DP-ribosylation in the isolated nuclei, the more extensive the PARP endogenous polyA DP-ribosylation, the lower its measured [\textsuperscript{32}P]polyA DP-ribosylation. This is illustrated in Fig. 3, based on the schematic presentation of polyA DP-ribosylation by Satoh et al. (1994). The low extent of PARP [\textsuperscript{32}P]polyA DP-ribosylation in nuclei isolated from depolarized neurons (Fig. 1 c) is in accordance with its high endogenous polyA DP-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-[\textsuperscript{32}P]polyA DP-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). [\textsuperscript{32}P]polyA DP-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-[\textsuperscript{32}P]polyA DP-ribosylation of PARP in their isolated nuclei (Fig. 4 b). This was consistent with the directly assayed enhanced polyA DP-ribosylation of PARP in depolarized neurons (Fig. 1, a and b). The effect of depolarization on PARP ac-
Activity 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 topoisomerase 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 Dobbe, 1994) into DNA (see Materials and Methods).

[3H]Thymidine was incorporated only in nicked DNA of neurons pretreated with H2O2 (Fig. 6 a). There was no sig-
significant incorporation of $[3H]$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-
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]polyADP-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. 7 a, lanes 1–6). 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) A 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).
7 a, lanes 7–12), indicating a decreased back-[32P] 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 (Harding 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. 7c). However, neither depletion of extracellular Ca\(^{2+}\) during high-[K\(^{+}\)]-induced depolarization (see Materials and Methods; Figs. 1, 4b, 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 (Ollivera et al., 1994) nor by blocking of Ca\(^{2+}\) influx, evoked by stimulation of NMDA-glutamate receptors (Sharkey et al., 1996) (Fig. 7c). 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 Ollivera, 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. 8a; see Materials and Methods) were isolated from brain cortical neurons and loaded with the permeant fluorescent Ca\(^{2+}\) indicator rhod-2 AM (Maina et al., 1999) 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; Figs. 8b–d). Ca\(^{2+}\) was markedly increased in the nucleoplasm of nuclei isolated from depolarized neurons (Fig. 8b), in line with the transient increase in nuclear [Ca\(^{2+}\)] in depolarized neurons (Al-Mohanna et al., 1994; Harding 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. 8d); 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 P3 (1–2 nM; Fig. 8c). In the presence of ATP, Ca\(^{2+}\) was also moderately released into the nucleoplasm when extranuclear [Ca\(^{2+}\)] was elevated (Fig. 8d). CaDP-ribose (5–20 mM), 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 results are consistent with the above observation that additional release of Ca\(^{2+}\), induced by ATP, is required for Ca\(^{2+}\) entry into the nucleoplasm.
Figure 8. Ca\(^{2+}\) mobilization in crude nuclei isolated from brain cortical neurons. (a) Electronmicrograph 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 mM) to crude nuclei of unstimulated neurons in the presence or absence of 5 mM caffeine, or to nuclei of neurons pretreated by 3 mM 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\textsuperscript{2+} permeates the nuclear membrane mainly via Ca-A TPase–induced Ca\textsuperscript{2+} accumulation in IP\textsubscript{3}-gated perinuclear stores (Gerashenko et al., 1995; H ennager et al., 1995; M alviya and R ogue, 1998) and with evidence indicating phosphatidylinositol signaling in the nuclei (Boronenkov et al., 1998).

The release of Ca\textsuperscript{2+} into the nucleoplasm was prevented by caffeine, added to the crude nuclei at concentrations suppressing IP\textsubscript{3}-induced Ca\textsuperscript{2+} mobilization (EHrlich et al., 1994) (Fig. 8c). Release of Ca\textsuperscript{2+} into the nucleoplasm was also prevented in nuclei isolated from neurons pretreated by thapsigargin that inhibits Ca-A TPase activity (Takemura et al., 1989), thereby preventing Ca\textsuperscript{2+} accumulation in the perinuclear stores (M alviya and R ogue, 1998) (Fig. 8c).

**A Fast Activation of PARP by IP\textsubscript{3} in Isolated Nuclei of Cortical Neurons.** We next examined the possibility that PARP is polyADP-ribosylated by IP\textsubscript{3}-induced Ca\textsuperscript{2+} mobilization. [\textsuperscript{32}P]polyADP-ribosylation of PARP was examined in the presence of IP\textsubscript{3} added to nuclei isolated from unstimulated neurons. E DTA was omitted from the incubation solution (see Materials and Methods), to avoid chelation of free Ca\textsuperscript{2+}. In addition, [\textsuperscript{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\textsubscript{3} was added after the addition of [\textsuperscript{32}P]NAD. [\textsuperscript{32}P]polyADP-ribosylated proteins were extracted 1 min after the addition of IP\textsubscript{3}. IP\textsubscript{3} at concentrations of 50 nM to 5 \mu M enhanced the [\textsuperscript{32}P]polyADP-ribosylation of PARP in a dose-dependent manner. Maximal 10-fold enhancement was measured with a half maximal effect in IP\textsubscript{3} (Takemura et al., 1989), thereby preventing Ca\textsuperscript{2+} mobilization. [\textsuperscript{32}P]polyADP-ribosylation of PARP was exclusively. IP\textsubscript{3}-induced [\textsuperscript{32}P]poly-ADP-ribosylation of PARP was completely suppressed in nuclei isolated from neurons pretreated by thapsigargin that inhibits Ca-A TPase activity (Takemura et al., 1989), thereby preventing Ca\textsuperscript{2+} accumulation in the perinuclear stores (M alviya and R ogue, 1998) (Fig. 8c).

High-[K\textsuperscript{+}]-induced membrane depolarization promoted polyADP-ribosylation of nuclear proteins in the absence of extracellular Ca\textsuperscript{2+} (Figs. 1, 4b, and 5). Findings indicating that PARP is activated by intracellular Ca\textsuperscript{2+} mobilization in the depolarized neurons include: a fast dose-dependent activation of PARP by extranuclear Ca\textsuperscript{2+} (Figs. 7a, 7b, and 7c), independent of extracellular Ca\textsuperscript{2+} influx (Fig. 7c); and a fast dose-dependent PARP activation by physiological concentrations of IP\textsubscript{3} (Figs. 9a, b, and d), independently of extracellular Ca\textsuperscript{2+} influx (Fig. 7c).

**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-[\textsuperscript{32}P]polyADP-ribosylation in isolated nuclei of depolarized neurons (Figs. 1c and 4b). These findings constitute the first evidence for a fast activation of PARP by physiological signals in the cell membrane.
Figure 9. IP₃ induces a fast [³²P]polyADP-ribosylation of PARP in crude nuclei of brain cortical neurons. (a) Autoradiograms of [³²P]polyADP-ribosylated PARP in crude nuclei of unstimulated brain cortical neurons in the absence (lane 1) or presence of IP₃ at the indicated concentrations (lanes 2–10). [³²P]polyADP-ribosylation (2 min, 25°C) was terminated 1 min after the addition of IP₃. 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 [³H]IP₃ by IP₃ in crude nuclei of cortical neurons (○). Maximal specific binding of [³H]IP₃ (10.5 nM) was 18,500–20,500 cpm/mg protein. Nonspecific binding of [³H]IP₃ (~60,000 cpm/mg protein) was determined in the
inner nuclear membrane (Nicotera et al., 1990; Gerassimenko et al., 1995; M aliya and R ogue, 1998). Phosphatidylino- 
sitol signaling pathways have been identified in the 
nuclei of several cell types (B ronenkov et al., 1998).

The enhanced activity of PARP in depolarized neurons 
was independent of extracellular [Ca2+] (see M etods and 
M etods; Figs. 1, 4 b, and 5) and resisted Ca2+ influx 
blocks, including agents suppressing NM D A-induced 
Ca2+ influx (F i g. 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 Ca2+-induced activation of endonucleases, producing 
DNA breaks (A rends et al., 1990). The activation of 
Ca,M g-endonuclease would require extracellular Ca2+ 
concentrations 100–1,000-fold higher (Peitsch et al., 1993; 
Peitsch et al., 1994) than those inducing PARP activation 
(F i g. 7, a and b). Endonuclease activity at [Ca2+] < 1 μM 
has a much slower time course (> 30 min; Jones et al., 
1989). A corollary, DNA breaks or N AD depletion (S a- 

toh and L indahl, 1992) were not detected in the depolar- 
ized cortical neurons (F i g. 6 and 2 a, respectively).

A fast signal–induced PARP activation via IP 3-induced 
Ca2+ mobilization constitutes a novel mode of signaling 
to the cell nucleus: PARP, being a downstream target of 
phospholipase C, modulates by polyAD P-ribosylation the 
activity of nuclear proteins in response to signals promot- 
ing phosphoinositides turnover and phosphatidylinositol 
4,5-bisphosphate (PIP2) hydrolysis (Berridge and Irvine, 
1989; Fruman et al., 1998; Toker, 1998). A fast modifica- 
tion of transcription factors by polyAD P-ribosylation (Li 
1989; Fruman et al., 1998; Toker, 1998). A fast modifica- 
tion of transcription factors by polyAD P-ribosylation (Li 
Oei et al., 1998) during electrical activity in brain cortical 
neurons may associate depolarization-induced polyAD P- 
ribosylation with "memory storage" (Kandel, 1997).

The role of PARP in DNA repair and transcription (Sa- 
toh and L indahl, 1992; O liver et al., 1998; Trucco et al., 
1998) may underlie the effect of depolarization in protect- 
ing growth factor-deprived neurons from apoptotic cell 
d eath (D ’Me llo et al., 1993; Galli et al., 1995). This mecha- 
nism 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." We would 
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