Reduced Nicotinamide Adenine Dinucleotide-Selective Stimulation of Inositol 1,4,5-Trisphosphate Receptors Mediates Hypoxic Mobilization of Calcium

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To evaluate the relationship of inositol 1,4,5-trisphosphate (IP₃) receptor-mediated signal transduction and cellular energy dynamics, we have characterized effects of nucleotides on IP₃ receptor (IP₃R)-mediated calcium (Ca²⁺) flux in purified IP₃ receptors reconstituted in lipid vesicles (IP₃RV) and examined hypoxia-induced augmentation of intracellular Ca²⁺ in intact cells. Reduced nicotinamide adenine dinucleotide (NADH) increases IP₃-mediated Ca²⁺ flux in IP₃RV. This effect is highly specific for NADH. Hypoxia elicited by brief exposure of nerve growth factor-differentiated PC12 cells or cerebellar Purkinje cells to cyanide elicits rapid increases in internal [Ca²⁺], which derives from IP₃-sensitive stores. Blockade of this effect by

2-deoxyglucose and inhibition of glyceraldehyde-3-phosphate dehydrogenase implicates enhanced glycolytic production of NADH in the Ca²⁺ stimulation. Internal [Ca²⁺] is markedly and specifically increased by direct intracellular injection of NADH, and this effect is blocked by heparin, further implicating IP₃R stores. These findings indicate that direct regulation of IP₃R by NADH is responsible for elevated cytoplasmic [Ca²⁺] occurring in the earliest phase of hypoxia. This link of IP₃R activity with cellular energy dynamics may be relevant to both hypoxic damage and metabolic regulation of IP₃ signaling processes.

Key words: Ca²⁺; NADH; hypoxia; signal transduction; inositol-1,4,5-trisphosphate receptor; metabolism

The role of the inositol 1,4,5-trisphosphate receptor (IP₃R) in mobilizing calcium from internal stores in response to receptor-coupled signal transduction is well established (Berridge, 1993). The resultant rise in cytosolic calcium (Ca²⁺) serves as an intracellular second messenger activating diverse cellular processes. Recent evidence suggests that IP₃Rs are responsive to alterations in cellular energy dynamics, and, conversely, that Ca²⁺ released by IP₃R activation modulates mitochondrial function. Rizzuto et al. (1993) showed that IP₃ can release Ca²⁺ in close proximity to mitochondria, markedly elevating mitochondrial Ca²⁺ levels. Although it was initially believed that mitochondria merely buffer Ca²⁺ (Becker et al., 1980), recent studies indicate that Ca²⁺ is an effector of mitochondrial function, acting at multiple sites to increase mitochondrial respiration and thereby stimulate ATP formation (McCormack et al., 1990). Mitochondrial function alters in response to mobilization of Ca²⁺ from IP₃-sensitive stores in numerous cell types (Duchen et al., 1993; Loew et al., 1994; Sheu and Jou, 1994). Taken together, these studies provide a potential link between IP₃R-mediated signal transduction and energy production.

Conversely, metabolites generated during energy production may influence IP₃R-mediated Ca²⁺ dynamics. ATP stimulates Ca²⁺ flux from purified IP₃R reconstituted in lipid vesicles (IP₃RV) (Ferris et al., 1990), from IP₃R incorporated into lipid bilayers (Bezprozvanny and Ehrlich, 1993), and from IP₃-sensitive Ca²⁺ stores in permeabilized cells (Iino, 1991). The stimulation by ATP is selective for adenine nucleotides and is much greater with ATP than with ADP or AMP (Ferris et al., 1990).

ATP is not the only indicator of the energy status of a cell. Depletion of cellular energy reserves leads to the accumulation of cytoplasmic reduced nicotinamide adenine dinucleotide (NADH) (Veech et al., 1970). This may be particularly relevant in pathological conditions such as hypoxia. Hypoxia decreases mitochondrial respiration by inhibiting the terminal step in the electron transport chain. Perturbations in cellular energy production in hypoxia provoke a rapid rise in Ca²⁺ in most cells, which if sustained may lead to cell death (Schanne et al., 1979; Siesjo, 1981; Cervos-Navarro and Diemer, 1991). A substantial component of this Ca²⁺ derives from intracellular stores (Krnjevic and Xu, 1989; Biscoe and Duchen, 1990; Duchen et al., 1990; Silver and Erecinska, 1990; Dubinsky and Rothman, 1991; Hasham et al., 1994; Latha et al., 1994). Recently it was shown that in CA1 pyramidal neurons maintained in a hippocampal slice preparation, the induction of anoxia resulted in an intracellular Ca²⁺ release, predominantly from an IP₃-sensitive store (Belousov et al., 1995). The mechanisms that mediated this release, however, remained elusive. Hypoxic insults also increase cytoplasmic NADH levels as a result of enhanced glycolysis (Duffy et al., 1972; Ozaki et al., 1988; Yager et al., 1991; Behar, 1993).

We now demonstrate that NADH selectively stimulates the release of Ca²⁺ mediated by IP₃R both in purified reconstituted

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Table 1. ATP and NADH increase Ca²⁺ flux of IP₃RV in the presence of a fixed concentration of IP₃

[IP ₃] (nM)	[ATP] (μM)	[NADH] (μM)	Stimulation of Ca ²⁺ flux (%)
10	50	0	39 ± 3
10	0	300	38 ± 5
40	50	0	30 ± 2
40	0	300	29 ± 4

IP₃RVs were preincubated for 30 sec in the presence of ⁴⁵Ca²⁺ and IP₃ at the concentrations indicated. After this incubation, ATP or NADH was added with a sufficient amount of IP₃ to maintain [IP₃] at the indicated value. Flux was allowed to proceed for an additional 30 sec, followed by processing of the vesicles to determine the total amount of Ca²⁺ flux obtained. Data are expressed as the percentage stimulation of specific Ca²⁺ flux seen in the presence of each nucleotide relative to the specific flux obtained in their absence. Data are presented as mean ± SEM, *n* = 2. This experiment was repeated three times with the same results.

receptor preparations and in intact cells. Hypoxic mobilization of Ca²⁺ reflects NADH stimulation of IP₃R activity.

MATERIALS AND METHODS

Materials. [³H]IP₃ and ⁴⁵Ca²⁺ were obtained from Du Pont-NEN (Boston, MA), D-myo-I(1,4,5)P₃, hexapotassium salt from LC Laboratories (Woburn, MA), and concanavalin A-sepharose from Pharmacia LKB Biotechnology (Piscataway, NJ). Phospholipids for reconstitution were obtained from Avanti Polar Lipids (Birmingham, AL), and Fura-2 from Molecular Probes (Eugene, OR). Koningic acid was the generous gift of Akira Endo. All other reagents were from Sigma (St. Louis, MO).

Purified and reconstituted IP₃Rs. IP₃Rs were purified from adult male Sprague–Dawley rat cerebellum and reconstituted into lipid vesicles as described (Ferris et al., 1989), by a two-step affinity-chromatography procedure using sequential heparin-agarose and concanavalin A-sepharose columns. After purification to apparent homogeneity, detergent-solubilized receptor protein was mixed with sonicated lipids, and the mixture was dialyzed to effect detergent removal and vesicle formation.

Stock ATP, pyridine nucleotide, sodium lactate, and sodium pyruvate solutions were prepared fresh in NKT buffer (50 mM NaCl, 50 mM KCl, and 20 mM Tris-Cl, pH 7.4), and the pH was adjusted to 7.4 with NaOH when necessary. Sodium cyanide solutions, also prepared fresh in NKT buffer, were adjusted to pH 7.4 with HCl.

Reconstituted proteoliposomes were assayed for IP₃-stimulated ⁴⁵Ca²⁺ flux as described (Ferris et al., 1989). After preincubation under various conditions, vesicles were incubated in the presence of 2 μCi ⁴⁵Ca²⁺ with or without IP₃. Unless otherwise indicated (Table 1), all Ca²⁺ flux incubations were for 15 sec at room temperature. The flux reaction was stopped by the addition of excess buffer containing unlabeled divalent cations and heparin. The vesicle/buffer mixture was passed over a cation-exchange column (Dowex 50W, Sigma), and the intravesicular ⁴⁵Ca²⁺ content was measured by scintillation spectrometry. Specific Ca²⁺ flux was calculated as the total flux seen with IP₃ (± nucleotides) minus the blank flux obtained in the absence of IP₃. Lactate, pyruvate, cyanide, and any of the nucleotides that were used did not affect the blank. [³H]IP₃-binding was assayed by precipitation of IP₃RV with polyethylene glycol, using gamma globulin as carrier protein, as described (Ferris et al., 1989).

Ca²⁺ imaging. PC12 cells were grown on polylysine-coated glass coverslips and differentiated with 50 ng/ml nerve growth factor (NGF) for 4–6 d before use. During Ca²⁺-imaging experiments, PC12 cells and embryonic mouse Purkinje cells (Linden et al., 1991) were bathed in a solution that contained NaCl (140 mM), KCl (5 mM), EGTA (0.2 mM), MgCl₂ (0.8 mM), HEPES (10 mM), glucose (10 mM), tetrodotoxin (0.5 μM), and picrotoxin (0.1 mM), adjusted to pH 7.35 with NaOH. This solution flowed at a rate of ~0.5 ml/min through a bath of 1.0 ml volume. Fura-2 dye was introduced via a whole-cell patch pipette. Patch pipettes were pulled from N51A glass and polished on a microforge to yield a resistance of 3–6 MΩ. They contained KCl (100 mM), HEPES (10 mM), and Fura-2, K₂-salt (10 mM), adjusted to pH 7.1 with KOH. For loading, a gigohm seal was formed under voltage-clamp conditions, and a command potential of −70 mV was applied. After membrane rupture, loading of the cell with dye was monitored by fluorescence at 384 nm, as described below. When sufficient dye perfusion had occurred in PC12

cells, the patch electrode was slowly retracted axially to allow the cell membrane to reseal. The cells were then allowed to equilibrate for 20 min before stimulation. Purkinje cells were loaded with a solution containing KCl (140 mM), HEPES (10 mM), and Fura-2, K₂-salt (200 μM) and then stimulated with the loading electrode attached after dye perfusion.

Imaging of intracellular free Ca²⁺ was accomplished by measuring the background-corrected fluorescence ratio at 337 and 384 nm excitation, using a cooled CCD camera system. Illumination at 337 nm was provided by a pulsed nitrogen laser operating at 20 Hz, and 384 nm was provided by a separate nitrogen-pumped laser using the dye Exalite 384 (Laser Science, Woburn, MA). This light was passed through a quartz fiberoptic Y-conduit to the epifluorescence train of a Zeiss Axiovert 100 equipped with a 40 × 1.3 NA oil-immersion objective. Emission at 505 nm was passed through a dichroic mirror and was focused on the chip (Thompson 7883) of a slow-scan, cooled CCD camera (Photometrics CH250, Tucson, AZ). Exposure times were 400 msec per single wavelength image. Digitized images were acquired to disk using custom software (kindly provided by Dr. D. W. Tank, AT&T Bell Labs). Free Ca²⁺ concentration was derived from the F337/F384 ratio measurements, using the standard relationship outlined by Grynkiewicz et al. (1985), incorporating a viscosity correction factor of 0.85, the latter derived from comparisons between calibrations made in internal saline and those made in the cytoplasm of ionomycin-permeabilized Purkinje cells. Background fluorescence in unloaded cells was <1% of the resting signal at either excitation wavelength.

For stimulating PC12 cells, pressure-ejection pipettes (1–2 μm tip diameter) were filled with various solutions and were positioned ~100 μm away from the cell soma. Ejection was accomplished by applying pressure pulses of 4–6 psi. This configuration allowed us to apply significant concentrations of test solutions to single cells or, occasionally, to several cells positioned closely together. To produce a transient oxidative stress, external saline supplemented with NaCN (2 mM) was ejected for 10 sec. To activate ryanodine receptor-gated Ca²⁺ stores, external saline with NaCl reduced to 130 mM and supplemented with caffeine (20 mM) was ejected for 10 sec. Heparin (100 μg/ml), de-N-sulfated heparin (100 μg/ml), ruthenium red (10 μM), and konigic acid (50 μM) were included in the internal saline used for Fura-2 loading. Thapsigargin (1 μM), U73122 (5 μM), or iodoacetate (500 μM) were applied in the bath starting immediately after Fura-2 loading, as was an external saline in which glucose (10 mM) was replaced with 2-deoxyglucose (10 mM).

In one set of experiments (see Fig. 6), PC12 cells were loaded with Fura-2/AM, washed, and then perfused internally with various nucleotides dissolved in a solution containing KCl (140 mM) and HEPES (10 mM). These compounds were β-NADH, Na₂ salt, β-reduced nicotinamide adenine dinucleotide phosphate (β-NADPH) Na₂-salt, and ATP, K₂ salt.

All experiments were conducted at ambient room temperature.

RESULTS

NADH stimulates Ca²⁺ release from purified and reconstituted IP₃R

In IP₃RV, 50 μM ATP maximally stimulates Ca²⁺ flux (Fig. 1A). At higher concentrations of ATP, stimulation of Ca²⁺ flux diminishes so that at 1 mM ATP, Ca²⁺ flux is close to control values. NADH also stimulates Ca²⁺ flux with maximal effects at 300 μM and half-maximal stimulation at 30 μM. NADH (300 μM) increases Ca²⁺ flux at all [IP₃], resembling the effects of ATP (50 μM) (Fig. 1B). Neither NADH nor ATP augment Ca²⁺ flux in the absence of IP₃ (data not shown). NADH and ATP seem to act at the same site, as their effects are not additive (Fig. 1C). Scatchard analysis of [³H]IP₃ binding to IP₃RV reveals no change in either *K_d* or *B_{max}* in the presence of 50 μM ATP or 300 μM NADH [*K_d* = 20 ± 4.1 nM (mean ± SEM, *n* = 5), 20 ± 1.6 nM, 19 ± 3.5 nM, and *B_{max}* = 2.0 ± 0.2 nM, 2.1 ± 0.1 nM, and 2.0 ± 0.2 nM under control conditions, with ATP or with NADH, respectively].

The enhancement of Ca²⁺ flux by NADH is strikingly selective (Fig. 2). Although 10 μM NADH stimulates Ca²⁺ flux by >30%, a 30-fold higher concentration of NAD is required to attain the same effect. Maximal stimulation of Ca²⁺ flux by NAD is only 20% of that elicited by NADH. NADP and NADPH fail to

Figure 1. Stimulation of purified IP₃R activity by NADH and ATP. *A*, Concentration–response relationships for the effects of NADH and ATP on IP₃-mediated Ca²⁺ flux. Ca²⁺ flux was determined for IP₃RVs incubated with various concentrations of NADH (Δ) or ATP (○) in the presence of 40 nM IP₃. Data are presented as a percentage of the specific Ca²⁺ flux obtained in the presence of 40 nM IP₃ alone (calculated as described in Materials and Methods). Error bars show SEM, *n* = 2. Points without error bars represent data in which SEM was less than the size of the plot symbol. This experiment was repeated four times with separate batches of IP₃RVs yielding the same pattern of concentration–response. For each batch, the response was highly reproducible, as exemplified by the data shown in the figure. Between batches, however, the magnitude of the stimulation varied, giving a maximal stimulation by NADH and ATP that ranged from 170% and 190% (expressed as percentage control using 40 nM IP₃ alone) to 280% and 330%, respectively. *B*, Effects of NADH and ATP on IP₃ concentration–response. The Ca²⁺ flux of IP₃RVs was determined in the absence of nucleotides (control, □) or in the presence of 300 μM NADH (●) or 50 μM ATP (○), *n* = 2. This experiment was repeated three times with the same results. *C*, Effects of NADH and ATP on IP₃-mediated Ca²⁺ flux are not additive. Data are presented as the percentage stimulation of specific Ca²⁺ flux obtained in the presence of 100 nM IP₃ alone (*n* = 2). This experiment was repeated five times with the same results.

stimulate Ca²⁺ flux at any concentration. Instead, they exert an inhibitory effect, with NADPH being more potent and producing a larger maximal effect than NADP.

Basal endogenous IP₃ concentrations are in the micromolar range (Bredt et al., 1989), which are sufficient to cause maximal IP₃ enhancement of Ca²⁺ flux as assessed *in vitro* (Fig. 1*B*). Assuming a roughly similar potency *in vivo*, this suggests that there are inhibitory influences on the IP₃R responsiveness in the

Figure 2. Specificity of the effects of NADH on IP₃-mediated Ca²⁺ flux. Ca²⁺ flux was determined for IP₃RVs incubated with various concentrations of pyridine nucleotides in the presence of 40 nM IP₃. Data are presented as the percentage stimulation of specific Ca²⁺ flux obtained in the presence of 40 nM IP₃ alone (*n* = 2). This experiment was repeated four times with the same results.

cell. Moreover, at some intracellular loci, Ca²⁺ release by IP₃R may not be determined by changes in IP₃ concentration, which is present in abundance, but rather by an alternate stimulatory mechanism, such as alterations in levels of ATP or NADH. To approximate this situation, we preincubated IP₃ and ⁴⁵Ca²⁺ with IP₃RV for 30 sec and then added ATP or NADH (Table 1). Because we do not know the *in vivo* mechanisms that presumably inhibit IP₃R responsiveness but we do know that IP₃R is not maximally stimulated by basal levels of IP₃, we preincubated with [IP₃] that would produce a submaximal response *in vitro*. The design of this experiment is complicated by the fact that exposure of IP₃RV to various concentrations of IP₃ results in a biphasic stimulation of Ca²⁺ flux, which is rapid for the first 10 sec and then slows (Ferris et al., 1992). This is a reflection of the quantal nature of IP₃R activation, a property intrinsic to the purified receptor. Nevertheless, under the conditions employed in Table 1, both ATP and NADH still provide substantial increases in Ca²⁺ flux in the continued presence of a fixed concentration of IP₃ and Ca²⁺. The absolute magnitude of the stimulation achieved under these conditions is less than that achieved in Figure 1, suggesting that the nucleotides exert more dramatic effects on the fast phase of Ca²⁺ flux from IP₃RV. The results depicted in Table 1 demonstrate that changes in cytoplasmic concentrations of ATP or NADH could result in Ca²⁺ efflux from IP₃R stores in the absence of increases in IP₃.

Chemical hypoxia mobilizes Ca²⁺ from IP₃-sensitive intracellular stores via NADH production

The strikingly selective stimulation of IP₃-mediated Ca²⁺ flux by NADH suggests a physiological role. To explore this possibility, we sought a system in which NADH levels increase in response to physiological perturbation. Cytoplasmic stores of NADH, the pool that would be exposed to IP₃R, increase substantially during hypoxia (Duffy et al., 1972; Ozaki et al., 1988; Yager, 1991; Lai and Behar, 1993). Moreover, increases in Ca²⁺ after the induc-

Figure 3. Ca^{2+} mobilization evoked by a brief hypoxic stimulus in PC12 cells as measured using Fura-2 microfluorimetry. *A*, The most common form of response in which successive pulses of cyanide (2 mM, 10 sec) give rise to decremental mobilizations. Each horizontal row is a Ca^{2+} map of the same cell before, during, and after a cyanide stimulus. Images within the rectangular box begin with the cyanide pulse and proceed at 5 sec intervals. Total elapsed time in seconds is indicated. The arrow represents the position of the cyanide-containing micropressure pipette. Scale bar, 10 μm . *B*, A less common form of response in which the Ca^{2+} levels evoked by the first pulse do not return to baseline and in which the second pulse then gives rise to a persistent activation. In this case, the interval between images depicted in the horizontal box is 4 sec. Scale bar, 20 μm .

tion of hypoxia occur in both neuronal and non-neuronal cells (Krnjevic and Xu, 1989; Biscoe and Duchen, 1990; Duchen et al., 1990; Silver and Erecinska, 1990; Dubinsky and Rothman, 1991; Hasham et al., 1994; Latha et al., 1994). Rapid application of cyanide provides an efficient, quantifiable, and reproducible means of inducing cellular hypoxia (Albaum, 1946). Cyanide has no direct effect on the IP₃-mediated Ca^{2+} flux of purified IP₃RV [0.1 mM, 1 mM, or 10 mM sodium cyanide in the presence of 1 μM IP₃ results in specific Ca^{2+} flux that is $94 \pm 3\%$ (mean $\pm$ SEM, $n = 3$), $99 \pm 0.4\%$, or $100 \pm 4\%$, respectively, of flux obtained in the absence of cyanide]. PC12 cells adopt a neuronal phenotype upon treatment with NGF and have been used often in studies of cyanide influences on cellular metabolism (Maduh et al., 1991; Latha et al., 1994). We measured intracellular Ca^{2+} in differentiated PC12 cells by ratiometric imaging of Fura-2. These experiments were conducted in Ca^{2+} -free 0.2 mM EGTA external saline so that increases in Ca^{2+} could be attributed to mobilization from internal stores rather than influx.

Micropressure application of sodium cyanide (2 mM) for 10 sec elicits a rapid increase in intracellular Ca^{2+} (Fig. 3*A*). The increase in Ca^{2+} begins at the side of the cell closest to the micropressure pipette, is apparent within seconds of cyanide exposure, and becomes maximal within 15 sec. At peak response, Ca^{2+} occurs in a gradient across the cell, with highest levels, ~ 400 nM, at the side closest to the micropressure pipette and with lowest levels, ~ 200 nM, at the opposite pole. Ca^{2+} decreases

rapidly from peak levels during the ensuing 60 sec, reaching basal values within 3 min of the peak Ca^{2+} response. In cells that possess neurites (Fig. 3*B*), the pattern of Ca^{2+} enhancement is similar, with the Ca^{2+} wave beginning at the side of the cell closest to the micropressure pipette. Increases in Ca^{2+} occur first in neurites and then spread to the soma. Typically, subsequent applications of cyanide elicit a similar pattern of Ca^{2+} release, but with lower peak levels after each successive application (Fig. 3*A*, 4*A*).

Ten of 16 cells displayed a pattern of Ca^{2+} response to cyanide, as depicted in Figures 3*A* and 4*A*, whereas two cells showed a continual increase in Ca^{2+} in response to a single dose of cyanide, which did not plateau or recover, and one cell showed no Ca^{2+} response to repeated cyanide pulses. In the remaining three cells, a different pattern was evident (Figs. 3*B*, 4*B*). In these cells, after the initial peak, Ca^{2+} levels failed to return to baseline but recovered to below half-peak values. A subsequent administration of cyanide provoked an increase in Ca^{2+} to levels comparable to those that followed the initial cyanide stimulus. This second Ca^{2+} response, however, did not recover; levels remained at or above peak values for the duration of the reporting period. These variations in the temporal pattern of Ca^{2+} response to hypoxia did not correlate with cellular morphology. For example, one of the three cells that did not fully recover after an initial cyanide pulse lacked neurites, and 5 of the 10 cells that showed complete recovery had neurites.

Figure 4. The time course of Ca^{2+} mobilization in PC12 cells responding to a brief hypoxic stimulus. *A*, Representation of the peak Ca^{2+} level at each time point for the cell depicted in Figure 3*A*. *B*, The same representation for the cell depicted in Figure 3*B*. In each case, cyanide pulses are indicated by horizontal bars. Those points that have corresponding images in Figure 3 are plotted with a filled circle, whereas points without corresponding images in Figure 3 are plotted with a filled diamond.

The magnitude of Ca^{2+} responses in PC12 cells was assessed by measuring peak Ca^{2+} levels during three consecutive 10 sec exposures to 2 mM sodium cyanide and subtracting peak prestimulation Ca^{2+} levels. Under these conditions, cyanide increases peak Ca^{2+} levels by 204 ± 33 nM (mean $\pm$ SEM, $n = 13$) above prestimulation levels of 114 ± 7 nM.

Similar but more dramatic results were obtained with embryonic mouse Purkinje cells maintained in a dispersed culture (Fig. 5*B*). In a Ca^{2+} -free 0.2 mM EGTA external saline solution, micropressure application of sodium cyanide (2 mM, 10 sec) to the Purkinje cells elicits a rapid increase in peak Ca^{2+} during three successive trials of 4483 ± 1523 nM (mean $\pm$ SEM, $n = 9$). Prestimulation Ca^{2+} levels in these cells were 110 ± 10 nM. Because the linear reporting range for Fura-2 extends only to ~ 1000 nM, the Ca^{2+} achieved in this case should be considered

Figure 5. Pharmacological characterization of the hypoxia-induced Ca^{2+} mobilization. Ca^{2+} imaging was performed on PC12 cells or cerebellar Purkinje cells placed in Ca^{2+} -free saline. *A*, Ca^{2+} mobilization from PC12 cells was produced by micropressure ejection of either 2 mM NaCN for 10 sec or 20 mM Caffeine for 15 sec. Data are presented as the difference in the peak Ca^{2+} levels obtained during three pulses versus the peak Ca^{2+} levels present immediately before the stimulation. Ca^{2+} levels were permitted to return to baseline between each of the three pulses. Drugs were either bath-applied after the retraction of the patch pipette used to load Fura-2 into the cells or co-loaded with Fura-2 (see Materials and Methods). The drugs tested were as follows: 1 μM thapsigargin (Tg, bath-applied), 10 μM ruthenium red (RR, co-loaded), 100 $\mu\text{g}/\text{ml}$ Heparin (co-loaded), 100 $\mu\text{g}/\text{ml}$ de-N-sulfated heparin (DNS Heparin, co-loaded), 5 μM U73122 (bath-applied), 10 mM 2-deoxyglucose (2DG, bath-applied), 0.5 mM iodoacetate (IAA, bath-applied), or 50 μM Koningic Acid (co-loaded). Data are presented as the mean $\pm$ SEM, $n = 5$, with the following exceptions: $n = 13$ for control conditions using NaCN stimulation and $n = 4$ for Tg and control and heparin conditions using caffeine stimulation. Peak Ca^{2+} levels present immediately before stimulation were as follows: control 114 ± 7 nM (mean $\pm$ SEM, n as indicated above), Tg 120 ± 14 nM, RR 124 ± 16 nM, Heparin 120 ± 20 nM, DNS Heparin 108 ± 8 nM, U73122 133 ± 4 nM, IAA 145 ± 3 nM, Koningic Acid 105 ± 8 nM. *B*, Purkinje cells were perfused with Fura-2 with or without either 100 $\mu\text{g}/\text{ml}$ heparin or 50 μM koningic acid. The Ca^{2+} mobilization was produced by micropressure application of 2 mM NaCN for 10 sec. The response in the control condition extended into the nonlinear reporting range for Fura-2 and hence should be interpreted as being >1000 nM. Data are presented in *A*, with $n = 9$, 6, and 5 for control, heparin, and koningic acid, respectively.

>1000 nM but not explicitly quantifiable. This release took the same general spatial form as that seen in PC12 cells in terms of sensitivity of processes and progression of the response from a site nearest the cyanide-containing pipette. Purkinje cells show a complete recovery of Ca^{2+} after applications of cyanide (data not shown).

We next investigated the potential sources of Ca^{2+} released in response to cyanide stimulation. Because all experiments were conducted in a Ca^{2+} -free medium, hypoxia-induced Ca^{2+} mobilization derives from internal compartments rather than from influx across the plasma membrane. Consistent with this observation, cyanide usually elicits lower peak Ca^{2+} levels after each successive application (Fig. 4*A*), indicating depletion of an intracellular Ca^{2+} store (Murphy and Miller, 1988). Three potential

Figure 6. NADH mobilizes intracellular Ca²⁺ via IP₃R activation. *A*, PC12 cells were loaded with Fura-2/AM, and after washout of unincorporated dye, cells were internally perfused using a pipette containing either no drug ($n = 5$), 4 mM ATP ($n = 6$), 100 μ M NADPH ($n = 6$), or 100 μ M NADH ($n = 7$). Values represent the peak [Ca²⁺]_i achieved during a 4–8 min monitoring period. *B*, To determine whether the Ca²⁺ mobilization produced by internal perfusion with NADH was mediated by IP₃R, a protocol was used in which cells were loaded with a pipette containing heparin (100 μ g/ml, $n = 6$) or de-*N*-sulfated heparin (100 μ g/ml, $n = 4$) and Fura-2, K_s salt. After removal of this pipette, and a brief recovery period, cells were repatched with a separate pipette containing NADH (100 μ M).

sources are the ryanodine- or IP₃-sensitive endoplasmic reticular stores [both of which have been characterized in PC12 and Purkinje cells (Zacchetti et al., 1991; Reber et al., 1993)], as well as the mitochondria. Thapsigargin inhibits the Ca²⁺-ATPase of the endoplasmic reticulum, selectively depleting Ca²⁺ from both IP₃- and ryanodine-sensitive stores (Thastrup et al., 1990). Bath application of thapsigargin to PC12 cells abolishes the cyanide-induced release of Ca²⁺ (Fig. 5*A*), thereby ruling out mitochondria as a potential source. Ruthenium red at high nanomolar to low micromolar concentrations blocks release of Ca²⁺ by ryanodine receptors, leaving IP₃R functionally intact (Ma, 1993). Intracellular perfusion with 10 μ M ruthenium red results in Ca²⁺ mobilization by cyanide that is indistinguishable from control conditions (Fig. 5*A*), suggesting that ryanodine receptors are not stimulated under these hypoxic conditions.

Heparin competitively inhibits the binding of IP₃ to its receptor, thereby blocking IP₃-induced Ca²⁺ release (Ghosh, 1988). Intracellular perfusion with heparin (100 μ g/ml) abolishes cyanide-induced Ca²⁺ release from both PC12 (Fig. 5*A*) and Purkinje cells (Fig. 5*B*). De-*N*-sulfated heparin, which is structurally similar to heparin but does not inhibit IP₃R activity (Yamamoto et al., 1990; Thorn and Petersen, 1993), fails to inhibit cyanide-induced mobilization of Ca²⁺ from both PC12 and Purkinje cells (Figs. 5*A*, 6*B*), suggesting that the effects of heparin are specific. Caffeine is a selective agonist of ryanodine receptors with an EC₅₀ of 13 mM in

PC12 cells (Barry and Cheek, 1994). Micropressure application of caffeine (20 mM, 10 sec) produces an increase of Ca²⁺ comparable in magnitude to that elicited by cyanide (Fig. 5*A*). Heparin (100 μ g/ml) perfusion of cells does not affect the increase in Ca²⁺ evoked by caffeine (Fig. 5*A*), indicating specificity for the inhibition of cyanide-induced Ca²⁺ release by heparin. Taken together, these data suggest that cyanide-mediated hypoxia selectively mobilizes Ca²⁺ from IP₃R stores.

Cyanide might mobilize Ca²⁺ by stimulating phospholipase C to produce IP₃, although decreases in the levels of IP₃ occur after hypoxia *in situ* (Kapuscinski, 1993; Matsumoto, 1994) and cyanide treatment of cells in culture (Kiang and Smallridge, 1994). We evaluated effects of U73122, a selective phospholipase-C inhibitor with an IC₅₀ of 200 nM in neuronal cells that abolishes IP₃ production at 1 μ M (Jin et al., 1994). Treatment with 5 μ M U73122 fails to block cyanide-induced elevation of Ca²⁺ (Fig. 5*A*), suggesting that the cyanide effect does not require IP₃ synthesis.

How might hypoxia stimulate Ca²⁺ release from IP₃-sensitive stores? Hypoxia is manifested initially as an inhibition of mitochondrial metabolism, whereas IP₃Rs reside in the cytoplasm. One of the early cytoplasmic compensatory responses to hypoxia is a stimulation of glycolysis, the Pasteur effect, which provides a means for energy production under anaerobic conditions (Krebs, 1972). This phenomena has been characterized in numerous contexts, including hypoxic human brains (Meyer et al., 1969) and neuronal cell cultures exposed to inhibitors of mitochondrial respiration (Pauwels et al., 1985). We decided to block glycolysis to ascertain whether this response to hypoxia plays a role in the stimulation of IP₃R activity.

2-Deoxyglucose (2DG), an analog of glucose, is taken up by cells and phosphorylated by hexokinase in the same manner as glucose. Unlike glucose 6-phosphate, however, phosphorylated deoxyglucose cannot be metabolized further (Siesjo, 1978). Thus, 2DG is a highly specific inhibitor of glycolysis (Bachelard, 1972). Substitution of 2DG for glucose abolishes cyanide-induced Ca²⁺ release (Fig. 5*A*). An alternative method for interrupting glycolysis involves inhibiting glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which functions to generate NADH during the cytoplasmic catabolism of glucose. Iodoacetate is an irreversible GAPDH inhibitor, reacting with the sulfhydryl group of an essential cysteine residue at the catalytic site of the enzyme (Malhotra et al., 1993). Koningic (or heptelidic) acid, a sesquiterpene antibiotic, acts similarly to inhibit GAPDH, but is considerably more potent and selective (Kato et al., 1992). Bath application of iodoacetate to PC12 cells (500 μ M) or perfusion of either PC12 cells or Purkinje cells with koniginic acid (50 μ M) eliminates cyanide-induced mobilization of Ca²⁺ (Fig. 5*A*, *B*). Thus, stimulation of glycolysis is necessary to mediate the effects of hypoxia on IP₃R activity.

Which product of glycolysis might account for the cyanide-induced Ca²⁺ release? Glycolysis leads to the net accumulation of NADH, ATP, and pyruvate, which is converted to lactate under hypoxic conditions. Pyruvate has no direct effect on the IP₃-mediated Ca²⁺ flux of purified IP₃RV [0.1 mM, 1 mM, or 10 mM sodium pyruvate in the presence of 1 μ M IP₃ results in specific Ca²⁺ flux that is 98 $\pm$ 3% (mean $\pm$ SEM, $n = 3$), 99 $\pm$ 2%, or 93 $\pm$ 2% of flux obtained in the absence of pyruvate, respectively]. Physiologically relevant concentrations of lactate also have no effect on Ca²⁺ flux of IP₃RV [0.1 mM, 1 mM, or 10 mM sodium lactate in the presence of 1 μ M IP₃ results in specific Ca²⁺ flux that is 99 $\pm$ 0.4% (mean $\pm$ SEM, $n = 3$), 100 $\pm$ 2%, or 97 $\pm$ 3%, respectively, of flux obtained in the absence of lactate].

Both NADH and ATP can stimulate activity of IP₃RV, although they differ in their concentration-response profiles. To examine directly the effects of these nucleotides on intact cells, we loaded PC12 cells with Fura-2/AM and then perfused them internally with test nucleotides (Fig. 6A). NADH (100 μ M) produces a dramatic increase in Ca²⁺_i, increasing peak Ca²⁺_i levels by almost 1200 nM, resulting in approximately sixfold greater enhancement than observed in PC12 cells treated with cyanide. The increase in Ca²⁺_i elicited by NADH perfusion of PC12 cells continues into the nonlinear reporting range for Fura-2, so that the absolute increase in Ca²⁺ attained may be substantially greater than 1200 nM. NADPH (100 μ M) does not mobilize Ca²⁺, demonstrating that the Ca²⁺ response to NADH is specific and not attributable to indirect effects on the cytoplasmic redox potential. Additionally, internal perfusion with ATP (4 mM) has no influence on Ca²⁺_i, suggesting that glycolytically derived ATP does not mediate IP₃R stimulation during hypoxia. Loading of the cells with heparin (but not de-*N*-sulfated heparin) before internal perfusion with NADH blocks the subsequent Ca²⁺ release, further implicating IP₃R stores as the source of the mobilized Ca²⁺ (Fig. 6B).

DISCUSSION

The main findings of this study are that NADH selectively increases IP₃R-mediated Ca²⁺ flux in purified and reconstituted receptor preparations, and that this effect seems to mediate hypoxia-induced Ca²⁺ release in intact cells. The demonstration here that IP₃Rs are metabolically stimulated in the absence of increases in [IP₃] represents the first demonstration of physiological receptor activation in the absence of phosphoinositide hydrolysis. This mode of regulation has important implications for both normal and pathological cellular regulation.

Characteristics of the stimulation of IP₃R by NADH

NADH and ATP stimulate the Ca²⁺ channel activity of purified and reconstituted IP₃R at all [IP₃], and they seem to be acting at the same site because their effects are not additive. Because neither nucleotide affects the *K*_d or *B*_{max} of IP₃R-binding, alterations in affinity of the receptor for its ligand or recruitment of inactive receptor-binding sites do not account for NADH and ATP actions. Instead, NADH and ATP presumably increase the efficiency of coupling between ligand binding and channel opening, consistent with the localization of all suspected nucleotide-binding sites between the IP₃-binding site and the Ca²⁺ channel domain of IP₃R (Ferris and Snyder, 1992).

The activation of IP₃R-mediated Ca²⁺ flux by NADH is strikingly selective: NADH is four times more potent than NAD and produces a fivefold greater maximal effect. NADH is a dinucleotide composed of nicotinamide and adenine nucleotide moieties. Selectivity for NADH over NAD demonstrates that IP₃Rs recognize the nicotinamide moiety, where the hydride residue that differentiates the two species resides, in addition to the adenine nucleotide moiety recognized in ATP. Surprisingly, NADP and NADPH inhibit IP₃-mediated Ca²⁺ flux, with NADPH displaying the greatest inhibitory potency and efficacy. What might account for this inhibition? A recently discovered IP₃R agonist called adenophostin A, the core structure of which contains an adenine nucleoside analog with a 2'-phosphate, potentially competes with [³H]IP₃ for binding to the IP₃R (*K*_i = 0.18 nM). Removal of the 2'-phosphate results in a 1000-fold reduction in binding affinity to the IP₃R (Takahashi et al., 1994). Because NADPH/NADP differ from NADH/NAD only by the presence of a 2'-phosphate on the ribose ring of their adenine nucleotide moiety, the inhibitory

effects of NADPH/NADP may reflect competition with IP₃ for binding to IP₃R. Consistent with this proposal, increasing concentrations of IP₃ overcome the inhibitory effects of NADPH and NADP on IP₃-mediated Ca²⁺ flux (data not shown).

From the perspective of cellular energy dynamics, NADH and ATP are the two most important cellular metabolites. IP₃RV responsiveness to both NADH and ATP suggests that under physiological conditions, IP₃R activity may be linked to changes in the energy status of the cell. Several characteristics of the stimulation of IP₃R by nucleotides demonstrates that the receptor is ideally suited to respond to such changes. First, the stimulatory effects of NADH and ATP on IP₃R activity do not desensitize (data not shown), suggesting that IP₃R can respond continuously to changes in the cytoplasmic levels of these nucleotides. Second, in the continued presence of a fixed concentration of IP₃, which approximates intracellular conditions, addition of NADH or ATP stimulates IP₃R activity (Table 1). This suggests that changing levels of these nucleotides could alter Ca²⁺_i in the absence of increases in [IP₃].

Hypoxia mobilizes Ca²⁺ from intracellular stores

There are several interesting aspects to the spatiotemporal pattern of cyanide-induced Ca²⁺ mobilization. First, the response is strongly directional. It originates on the side of the cell nearest the cyanide-containing micropressure pipette and usually spreads across the cell in a decremental manner, resulting in the temporary establishment of a gradient of Ca²⁺_i. In some cases, this gradient is transient and Ca²⁺ concentrations become equally elevated across the cell. Second, in cells bearing neurites, Ca²⁺ responses typically originate in the neurites and then invade the soma. This spatial pattern of response is superimposed on the pipette gradient such that in these cells, the earliest and most dramatic responses are seen in neurites close to the pipette.

A recent report has characterized the Ca²⁺ waves in NGF-differentiated PC12 cells that are elicited by bath application of bradykinin, a phospholipase C-coupled receptor, in a Ca²⁺-free medium (Lorenzon et al., 1995). These Ca²⁺ waves, which result from activation of IP₃-sensitive Ca²⁺ stores, are strikingly similar to the waves elicited by treating NGF-differentiated PC12 cells with sodium cyanide (Fig. 3B). In the case of both bradykinin and cyanide, (1) Ca²⁺ waves originate in the neurites and subsequently invade the soma, (2) the mode of transmission of the waves is tide propagation, where there is no decline in Ca²⁺ levels behind the leading edge, and (3) the time course of the evolution of the waves, from high Ca²⁺ levels in some neurites to invasion of the soma and the rest of the cell, transpires over ~1 min.

Although similar qualitatively, the Ca²⁺ response to hypoxia in PC12 cells and Purkinje cells differed greatly in magnitude. In fact, because the peak Ca²⁺_i response of Purkinje cells to hypoxia shown in Figure 5B falls into the nonlinear reporting range for Fura-2, it is likely that the difference in the responses of the two cells shown in Figure 5 are underestimated. Purkinje cells possess one of the highest concentrations of IP₃R of any cell yet tested (20 pmol/mg), as assessed by [³H]IP₃-binding analysis (Worley et al., 1987). Thus, one potential source of the different Ca²⁺ responses of PC12 cells and Purkinje cells to hypoxia relates to the size of their respective stores. An alternative source of this discrepancy relates to the different methods used with each cell type. After the PC12 cells were loaded with Fura-2, the electrode was retracted, and the cells were allowed to reseal and recover for 20 min before cyanide application. Because of their fragility, Purkinje cells were challenged with cyanide after perfusion with Fura-2, with the

loading electrodes still attached. Because of the prolonged perfusion by the loading electrode, the Purkinje cells may have lost some cytoplasmic constituents that aided in buffering Ca²⁺_i. Substantial portions of cytoplasmic constituents were retained in these cells, however, as evidenced by the fact that koniginic acid blocked the cyanide-induced Ca²⁺ response, demonstrating that a functional glycolytic pathway was maintained in the cells that responded.

Hypoxia leads to enhanced glycolytic formation of NADH, which stimulates IP₃R to release Ca²⁺

We identified IP₃R activation as the source of the Ca²⁺ mobilized by hypoxia (Fig. 5A). Moreover, it seems that hypoxia-induced Ca²⁺ mobilization from IP₃R stores takes place in the absence of increases in [IP₃]. How then is hypoxia linked to IP₃R activation? As a consequence of the metabolic coupling between mitochondrial respiration and glucose metabolism, hypoxia stimulates glycolysis in various tissues and organisms (Krebs, 1972). Inhibition of glycolysis blocks hypoxia-induced mobilization of Ca²⁺ (Fig. 5A). Glycolysis provides a crucial source of energy under hypoxic circumstances, especially in neurons (Siesjo, 1978). If hypoxia caused mobilization of Ca²⁺ as a result of a passive leak generated by energy depletion, inhibition of glycolysis would be expected to exacerbate this process. The finding that glycolytic flux is necessary to facilitate hypoxia-induced Ca²⁺ release highlights the active nature of the stimulus for hypoxic mobilization of IP₃-sensitive Ca²⁺ stores. In addition, anaerobic glycolysis acidifies the cytoplasm (Cervos-Navarro and Diemer, 1991), and IP₃Rs are known to be inhibited by decreasing pH levels (Worley et al., 1987). The activation of Ca²⁺ release from IP₃R stores under these conditions again underscores the existence of an active signalling process.

Glycolysis leads to the net accumulation of NADH, ATP, and pyruvate, which can be converted to lactate under anaerobic conditions. Unlike NADH and ATP, neither pyruvate nor lactate has any effect on Ca²⁺ flux of IP₃RV. ATP maximally stimulates IP₃R-mediated Ca²⁺ flux at 50 μM, with decreasing effects at higher [ATP] (Fig. 1A). Because tissue cytoplasmic [ATP] ranges from 2 to 8 mM, with values of 2.6 mM measured in rat brain (Veech et al., 1979), declines in normal ATP levels should stimulate Ca²⁺ release by IP₃R. This suggests that increases in ATP as a result of glycolytic flux would not stimulate IP₃R until cells became dramatically energy-depleted (e.g., to <2% of normal levels in the brain). A previous report on PC12 cells exposed to 10 mM cyanide for 2.5 min showed a 30% reduction in ATP (Maduh et al., 1991). Under the conditions used in our experiments, 2 mM cyanide for 10 sec, it is highly unlikely that cellular ATP levels would be significantly affected. Moreover, mice exposed to profound hypoxia (close to the threshold for survival) for up to 30 min did not show changes in brain levels of ATP, primarily as a result of increased glycolysis (Duffy et al., 1972). To test the potential role of ATP directly, we perfused PC12 cells with supraphysiological levels of ATP and found no increase in Ca²⁺_i (Fig. 6A). It seems, therefore, that ATP generated during glycolysis does not mediate hypoxic stimulation of IP₃R activity.

The concentration–response relationship for NADH, which is normally present in low micromolar concentrations in the cytoplasm (Sies, 1982), suggests that increases in NADH derived from glycolysis would stimulate IP₃R activity (Fig. 2). Increases in PC12 cell cytoplasmic NADH levels are sufficient to dramatically mobilize Ca²⁺ from intracellular stores (Fig. 6A). Inhibition of the effects of NADH by heparin, but not de-N-sulfated heparin,

further implicates IP₃R in this Ca²⁺-release process (Fig. 6B). Taken together, our data suggest that NADH generated as a result of enhanced glycolytic flux under hypoxic conditions leads to a stimulation of IP₃R activity resulting in increases in Ca²⁺_i.

Functional significance of hypoxia-induced activation of IP₃R

In Purkinje cells, stacked endoplasmic reticulum cisternae highly enriched in IP₃R lie closely apposed to mitochondria (Mignery et al., 1989). In HeLa cells, IP₃R activation releases Ca²⁺ in close proximity to mitochondria, markedly elevating mitochondrial matrix Ca²⁺ levels (Rizzuto et al., 1993). This matrix Ca²⁺ enhances the production of ATP through oxidative phosphorylation, presumably by stimulating diverse processes that include NAD-linked dehydrogenase, ATP synthase and adenine nucleotide transporter activities, and electron transport (McCormack et al., 1990; Gunter et al., 1994). In this way, activation of IP₃R activity by glycolytic NADH could represent an attempt to stimulate energy production during hypoxic inhibition of mitochondrial respiration. This augmentation of mitochondrial function may also provide energy to fuel the diverse cellular processes stimulated by physiological signal transduction involving IP₃.

Some CNS neurons, such as cortical and hippocampal CA1 pyramidal neurons, become hyperpolarized during hypoxia. This decreases their probability of firing and results in an inhibition of excitatory synaptic transmission, perhaps to conserve energy and avoid excitotoxicity (Somjen et al., 1993). The hyperpolarization results from stimulation of Ca²⁺-activated potassium channels, which seem to be responding predominantly to Ca²⁺ released by IP₃R (Leblond and Krnjevic, 1989; Latha et al., 1994; Belousov et al., 1995). Thus, in addition to augmenting energy production, hypoxic stimulation of IP₃R activity may lead to energy preservation in some cells.

Pathological significance of hypoxia-induced IP₃R activation

Our studies and a recent study using N₂ aeration to induce hypoxia in hippocampal slices (Belousov et al., 1995) implicate IP₃R in Ca²⁺ released by hypoxia. Ca²⁺ influx may also contribute to hypoxia-induced increases in Ca²⁺_i, because placing cells in a Ca²⁺-free solution resulted in a partial decrease in the levels of Ca²⁺_i that were obtained (Krnjevic and Xu, 1989; Biscoe and Duchon, 1990; Duchon et al., 1990; Hasham et al., 1994; Latha et al., 1994). Numerous examples exist in which mobilization of Ca²⁺ from IP₃-sensitive stores subsequently leads to Ca²⁺ influx (Irvine, 1992; Fasolato et al., 1994).

Some reports indicate that hypoxia-induced increases in Ca²⁺_i are a relatively late phenomenon that occurs minutes to hours after the initiation of hypoxia (Arnould et al., 1992; Kiang and Smallridge, 1994; Ray et al., 1994). These reports suggest that increases in Ca²⁺_i result primarily from Ca²⁺ influx (Arnould et al., 1992; Kiang and Smallridge, 1994) or inhibition of the endoplasmic reticular Ca²⁺ pump (Ray et al., 1994). These experiments, however, were performed in the absence of glucose (Arnould et al., 1992; Kiang and Smallridge, 1994) or in the presence of 2DG (Ray et al., 1994), conditions that prevent the glycolytic formation of NADH. This experimental paradigm, in which hypoxia is induced while glycolysis is inhibited, is believed to model conditions found after the interruption of blood supply in ischemic hypoxia. Under physiological conditions, however, the brain maintains a glycogen reserve that becomes rapidly depleted after the cessation of blood flow (Siesjo, 1978). Increases in tissue

lactate concentrations occur after the induction of ischemic hypoxia in animals, indicating an increase in anaerobic glycolysis (Palmer et al., 1990). Thus, although the induction of hypoxia in the absence of glucose may adequately reflect the metabolic conditions to which neurons are exposed relatively late in ischemic hypoxia, they do not seem to reflect the conditions encountered during the early moments after the cessation of O₂ supply. It is during this initial period that we have demonstrated activation of IP₃R activity by hypoxia.

The precise mechanisms by which hypoxia results in cell death and certain cells such as neurons are rendered particularly vulnerable to hypoxic injury are not known in detail. It is widely believed, however, that Ca²⁺ plays a central role in these processes, perhaps serving as the trigger for a series of reactions that culminate in cellular demise (Schanne et al., 1979; Siesjö, 1981). The current model for hypoxia-mediated increases in Ca²⁺, maintains that reduced O₂ levels lead to depletion of energy stores and result in an inability to sequester Ca²⁺ (Somjen et al., 1993). Irreversible neuronal injury can occur in the absence of ATP depletion (Cervos-Navarro and Diemer, 1991), however, and our work demonstrates that hypoxia mobilizes Ca²⁺ in an active process that does not involve inhibition of Ca²⁺ sequestration. Interestingly, the distribution of neurons in the brain that are most vulnerable to hypoxic injury (Siesjö, 1981; Kuroiwa and Okeda, 1994) corresponds with regions of greatest IP₃R density (Worley et al., 1989; Nakanishi et al., 1991; Sharp et al., 1993; Suburo et al., 1993), such as cerebellar Purkinje cells, pyramidal neurons in the CA1 region of the hippocampus, layers III, V, and VI of the cortex, and small- and medium-sized striatal neurons.

We have used purified and reconstituted IP₃R, NGF-treated PC12 cells, and cerebellar Purkinje neurons to demonstrate that within seconds after the induction of hypoxia, Ca²⁺ is mobilized from intracellular stores as the result of NADH-mediated stimulation of IP₃R channel activity. Further studies should help elucidate whether this Ca²⁺ acts as a messenger, perhaps serving to help regulate metabolism, or as a trigger of subsequent deleterious events.

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