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Sodium Nitroprusside Prevents Chemical Hypoxia-Induced Cell Death Through Iron Ions Stimulating the Activity of the Na^+–Ca^{2+} Exchanger in C6 Glioma Cells

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Abstract: In C6 glioma cells exposed to chemical hypoxia, an increase of extracellular lactate dehydrogenase (LDH) activity, cell death, and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) occurred. Sodium nitroprusside (SNP), a nitric oxide donor and an iron-containing molecule, reduced chemical hypoxia-induced LDH release and cell death. These effects were counteracted by bepridil and by 5-(*N*-4-chlorobenzyl)-2′,4′-dimethylbenzamil (CB-DMB), two specific inhibitors of the Na^+–Ca^{2+} exchanger. SNP also increased the activity of the Na^+–Ca^{2+} exchanger as a Na^+ efflux pathway, stimulated by Na^+-free conditions and evaluated by monitoring $[Ca^{2+}]_i$ in single cells. In addition, SNP produced a further increase of chemical hypoxia-elicited $[Ca^{2+}]_i$ elevation, and this effect was blocked by bepridil. Chemical hypoxia-evoked cell death and LDH release were counteracted by the ferricyanide moiety of the SNP molecule, $K_3Fe(CN)_6$, and by ferric chloride ($FeCl_3$), and this effect was counteracted by CB-DMB. In addition, the iron ion chelator deferoxamine reversed the protective effect exerted by SNP on cell injury. Collectively, these findings suggest that the protective effect of SNP on C6 glioma cells exposed to chemical hypoxia is due to the activation of the Na^+–Ca^{2+} exchanger operating as a Na^+ efflux–Ca^{2+} influx pathway induced by iron present in the SNP molecule. **Key Words:** Na^+–Ca^{2+} exchanger—Sodium nitroprusside—Iron—Chemical hypoxia—C6 glioma—Cell survival.
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The Na^+–Ca^{2+} exchanger is a bidirectional pathway that couples the extrusion of Ca^{2+} to the entrance of Na^+ into the cell, or vice versa (Sanchez-Armass and Blaustein, 1987; Taglialatela et al., 1990). Under chemical hypoxia, a condition in which intracellular Ca^{2+} and Na^+ homeostasis is altered (Siesjö et al., 1989), the activation of the Na^+–Ca^{2+} exchanger, when it is operating as a Na^+ extrusion–Ca^{2+} influx pathway, prevents cell damage in C6 glioma cells (Amoroso et al., 1997) and reduces aspartate release from the hippocampus (Amoroso et al., 1993). It has been recently reported that sodium nitroprusside (SNP), a nitric oxide (NO)-generating compound, possesses the ability to activate the Na^+–Ca^{2+} exchanger in cultured rat astrocytes (Asano et al., 1995). On the other hand, the SNP molecule contains iron, besides the NO group. This metal ion, mediating the transfer of electrons between the cellular redox compounds and the appropriate disulfide-thiol groups of the Na^+–Ca^{2+} exchanger molecule, can produce a stimulation of the antiporter activity (Reeves et al., 1986). Therefore, it appeared of interest to investigate (a) whether SNP could prevent chemical hypoxia-induced C6 glioma cell death, (b) whether this neuroprotective action was due to a stimulation of the Na^+–Ca^{2+} exchanger activity, and (c) whether these effects were due to the NO donor property or to iron ions present in the SNP molecule.

To this aim, C6 glioma cells, in which the activation of the Na^+–Ca^{2+} exchanger as a Na^+ efflux–Ca^{2+} influx pathway reduces cell injury induced by chemical hypoxia (Amoroso et al., 1997), were used. These cells, which are able to express the same Na^+–Ca^{2+} exchanger isoform (NCX_1) of astrocytes (He et al., 1998) and allow the study of the direct effect of chemical hypoxia without any concomitant interference by glutamate receptor activation (Kato et al., 1984), were exposed to chemical hypoxia obtained by adding oligomycin plus 2-deoxyglucose (2-DG) to a glucose-free medium (Lehninger, 1975; Schmid-Antomarchi et al., 1987, 1990; De Weille et al., 1989; Amoroso et al., 1990, 1993, 1997).

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Abbreviations used: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CB-DMB, 5-(*N*-4-chlorobenzyl)-2′,4′-dimethylbenzamil; DCFH, 2′,7′-dichlorofluorescin; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DFX, deferoxamine; 2-DG, 2-deoxyglucose; FDA, fluorescein diacetate; LDH, lactate dehydrogenase; NO, nitric oxide; NO_x, NO_2^- + NO_3^-; PI, propidium iodide; SIN-1, 3-(*N*-morpholino)sydnonimine; SNP, sodium nitroprusside.

MATERIALS AND METHODS

Cell culture

C6 glioma cells (purchased from the American Type Culture Collection) were cultured as monolayers in polystyrene dishes (100 mm in diameter) and grown in Dulbecco's modified Eagle's medium (ICN Laboratories, Milan, Italy) containing 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT, U.S.A.), 100 IU of penicillin/ml, and 100 μg of streptomycin/ml (ICN). Cells were cultured in a humidified incubator at 37°C in a 5% CO_2 atmosphere. The medium was changed every 2 days. Each experiment was performed with cells from passages 45 to 65 in 12-well plates (Falcon). The wells were precoated with 30 μg/ml poly-L-lysine (Sigma, Italy) to improve cell adhesion.

Chemical hypoxia

Chemical hypoxia was reproduced by adding 5 μg/ml oligomycin plus 2 m*M* 2-DG in glucose-free medium to the cells. This combination produces a fall in cell ATP content (Schmid-Antomarchi et al., 1987; De Weille et al., 1989; Amoroso et al., 1990) because oligomycin inhibits oxidative phosphorylation (Lehninger, 1975; Dubinski and Rothman, 1991), whereas 2-DG causes inhibition at the first step of the glycolytic pathway, that is, at the reaction catalyzed by hexokinase (Devlin, 1982). The composition of the medium was 145 m*M* NaCl, 5 m*M* KCl, 1.2 m*M* $MgSO_4$, 10 m*M* HEPES, 1.5 m*M* $CaCl_2$, and 0.2% bovine serum albumin, pH adjusted to 7.4 with 1 *M* Tris (standard buffer).

Intracellular calcium measurements

Just before the experiment, C6 glioma cells were detached by gently streaming the culture medium on the surface of the monolayer, then centrifuged, and resuspended in 1 ml of standard buffer plus 10 m*M* glucose. The cells (2×10^6/ml) were then incubated with 5 μM fura 2-AM (Molecular Probes, Eugene, OR, U.S.A.) for 45 min at 37°C. After the loading period, the medium was diluted with 2 volumes of the same balanced salt solution, incubated at 37°C, and then washed twice before the experiment was performed. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured in a 2-ml suspension of C6 glioma cells (2×10^6/ml) at 37°C in a quartz cuvette equipped with a magnetic stirrer bar. Fura-2 fluorescence was monitored in a Perkin-Elmer model 50 LS B spectrophotofluorimeter. The excitation wavelengths were of 340 and 380 nm (bandpass 5 nm) with emission at 510 nm (bandpass 5 nm). $[Ca^{2+}]_i$ was determined according to the equation of Grynkiewicz et al. (1985). When experiments in single cells were performed, C6 glioma cells were grown on coverslips and loaded with 5 μM fura-2. At the end of loading, the coverslip was mounted in a perfusion chamber (Medical System Co., Greenvale, NY, U.S.A.) on an inverted Nikon Diaphot fluorescence microscope. The cells were superfused continuously throughout the experiment with standard buffer using a peristaltic pump (Gilson, France) and a microtube, positioned with a macromanipulator (Narishige, Japan) on the cells under observation. The perfusion medium was removed continuously from the superfusion chamber by suction using a microaspirator (Medical System Co.) connected with a vacuum pump (Hoefer, San Francisco, CA, U.S.A.). All drugs used were introduced into the superfusion line using an injection loop and a two-way valve (Thomson, Springfield, VA, U.S.A.). A 100-W xenon lamp (Osram, Germany) with a computer-operated filter wheel bearing two different interference filters (340 and 380 nm) illuminated the microscopic field with UV light alternatively at wavelengths of 340 and 380 nm, with an interval of 500 ms between lighting at 340 and 380 nm. The interval between each pair of lightings and the next was chosen according to the experimental protocol. Emitted light was passed through a 400-nm dichroic mirror, filtered at 510 nm, and collected by a CCD camera (Photonic Science, Robertsbridge, East Sussex, U.K.) connected with a light amplifier (Applied Imaging Ltd., Dukesway Gateshead, U.K.). Images were digitized and analyzed with a Magiscan image processor (Applied Imaging Ltd.). The Tardis software (Applied Imaging Ltd.) calculated $[Ca^{2+}]_i$ corresponding to each pair of images, from the ratio between the intensity of the light emitted when the cells were lighted at 340 and 380 nm, as previously described by Cataldi et al. (1996).

Determination of lactate dehydrogenase (LDH) activity

Cultured C6 glioma cells (2×10^6/ml per well) were washed twice with standard buffer and incubated for the desired time at 37°C with experimental solutions. After the treatment, the incubation medium was removed and centrifuged at 12,000 *g* for 5 min in a microfuge. The supernatant was used for LDH activity determination by spectrophotometric assay (Gay et al., 1968).

Intravital staining of the culture

After the experimental procedures, C6 glioma cells (5×10^5/ml per well) were washed with standard buffer and stained for 3 min at 22°C with a solution containing 36 μM fluorescein diacetate (FDA; Sigma) and 7 μM propidium iodide (PI) (Calbiochem, San Diego, CA, U.S.A.). The stained cells were examined immediately with a standard epiillumination fluorescence microscope. FDA, a nonpolar ester, crosses the cell membrane and is hydrolyzed by intracellular esterases to produce a green–yellow fluorescence. Cell injury curtails FDA staining and allows cell permeation with PI, a polar compound, that, by interacting with nuclear DNA, yields a bright red fluorescence (Manev et al., 1990; Amoroso et al., 1997).

NO_2^- + NO_3^- (NO_x) assay

The amount of NO_x present in the medium was determined according to the method of Thomsen et al. (1990). After NO_3^- to NO_2^- was reduced using acid-washed cadmium powder (Aldrich, Milan), NO_2^- was quantified using a microplate assay method based on the Griess reaction.

Measurement of free radical production

Intracellular free radical production was measured with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes), as previously described by Amoroso et al. (1999). DCFH-DA is a stable, nonfluorescent molecule that readily crosses the cell membrane and is hydrolyzed by intracellular esterases to the nonfluorescent probe 2′,7′-dichlorofluorescin (DCFH), which is rapidly oxidized in the presence of peroxides to the highly fluorescent 2′,7′-dichlorofluorescein (Bass et al., 1983). Cells (2×10^6/ml) were loaded with 1 μM DCFH-DA for 30 min at 37°C. After loading, the cells were washed twice, and the fluorescence was recorded at 495 nm E_x and 530 nm E_m (bandpass 2.5 nm).

Drugs and chemicals

Oligomycin, 2-DG, and bepridil were obtained from Sigma, Italy. The amiloride derivative 5-(*N*-4-chlorobenzyl)-2′,4′-dimethylbenzamil (CB-DMB) was synthesized and supplied by Dr. E. J. Cragoe, Jr. (Nagodoches, TX, U.S.A.). Oligomycin was dissolved in ethanol (stock solution of 5 mg/ml). SNP, $K_3Fe(CN)_6$, and $FeCl_3$ were dissolved in water and protected from light. The amiloride derivative was dissolved in dimethyl

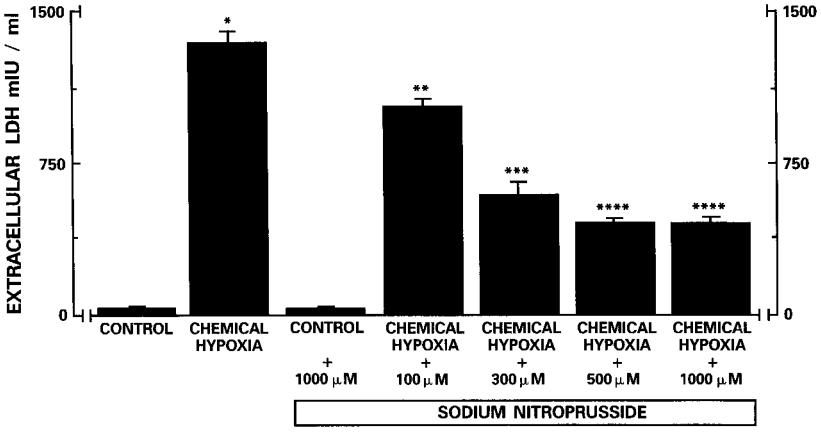

FIG. 1. Effect of SNP on LDH release induced by chemical hypoxia. C6 glioma cells were exposed to chemical hypoxia conditions for 6 h, in the presence or in the absence of increasing concentrations of SNP. Samples for LDH determination were taken 6 h after the beginning of the experiments. Data are mean ± SE (bars) values of 18 determinations obtained in three independent experiments. In each experiment n = 6 for each experimental group. $^{*}p < 0.01$ versus all experimental groups, $^{**}p < 0.01$ versus chemical hypoxia, $^{***}p < 0.01$ versus chemical hypoxia + 100 μM SNP, $^{****}p < 0.01$ versus chemical hypoxia + 300 μM SNP.

sulfoxide (stock solution of 10 m*M*), whereas bepridil was dissolved in a mixture of acetone and water (stock solution of 25 m*M*). 2-DG was diluted in water. FDA was dissolved in acetone, and PI was diluted in water; both were protected from light. DCFH-DA was dissolved in ethanol (stock solution of 5 m*M*). The final concentration of ethanol and dimethyl sulfoxide in the experimental medium was always <0.1 and 1%, respectively. Deferoxamine (DFX) was dissolved in water.

Statistics

Data were analyzed by one-way ANOVA followed by the Newman–Keuls test.

RESULTS

Effect of SNP on LDH release and FDA-PI-positive staining evoked by chemical hypoxia

C6 glioma cells were exposed to chemical hypoxia by adding 5 μg/ml oligomycin plus 2 m*M* 2-DG in a glucose-free medium for 6 h. This experimental condition induced an increase of LDH release (Fig. 1), an index of cellular injury (Choi et al., 1988). After 6 h of chemical hypoxia, ~70% of C6 cells died as revealed by PI staining (Fig. 2c). SNP significantly counteracted in a dose-dependent manner (100, 300, and 500 μM) LDH release induced by chemical hypoxia (Fig. 1), as well as the number of PI-positive C6 cells (300 μM SNP; Fig. 2d). The LDH release inhibition obtained with the highest concentrations of SNP (500–1,000 μM) reached ~70%.

Effect of SNP on Na^+–Ca^{2+} exchanger activity evaluated as Ca^{2+} influx during chemical hypoxia in the absence and in the presence of an inhibitor of the Na^+–Ca^{2+} exchanger, bepridil

Because it has been previously demonstrated that the activation of the Na^+–Ca^{2+} exchanger when it operates as a Na^+ efflux–Ca^{2+} influx pathway prevents chemical hypoxia-induced cell damage (Amoroso et al., 1997), the effect of SNP on $[Ca^{2+}]_i$ elevation as an index of the activity of the antiporter in the reverse mode of operation was evaluated. A progressive increase of $[Ca^{2+}]_i$ occurred when C6 glioma cells were exposed to chemical hypoxia (Fig. 3). SNP (300 μM) added to the incubation medium produced a further elevation of chemical hypoxia-elicited $[Ca^{2+}]_i$ increase (Fig. 3). This SNP effect was counteracted by the simultaneous presence in the incubation medium of the Na^+–Ca^{2+} exchanger inhibitor bepridil (10 μM) (Stys et al., 1991, 1992; Waxman et al., 1991; Kiedrowski et al., 1994). Both SNP and bepridil did not induce any $[Ca^{2+}]_i$ increase in the absence of chemical hypoxia (data not shown).

Effect of SNP on Na^+–Ca^{2+} exchanger activity stimulated by extracellular Na^+ removal and evaluated as Ca^{2+} influx in single cells, in the absence and in the presence of an inhibitor of the Na^+–Ca^{2+} exchanger, bepridil

The extracellular Na^+ removal, an experimental condition widely used to stimulate the activity of the Na^+–Ca^{2+} exchanger as a Ca^{2+} influx pathway (Sanchez-Armass and Blaustein, 1987; Taglialatela et al., 1990; Amoroso et al., 1997), induced an increase of single-cell monitored $[Ca^{2+}]_i$ that was reinforced by SNP (300 μM). Bepridil (10 μM) completely prevented the increase of $[Ca^{2+}]_i$ elicited by both extracellular Na^+ removal and SNP plus Na^+-free medium (Fig. 4).

Effect of two Na^+–Ca^{2+} exchanger inhibitors, bepridil and CB-DMB, an amiloride derivative, on the protective effect exerted by SNP on chemical hypoxia-induced cell damage

Bepridil and CB-DMB, another specific inhibitor of the Na^+–Ca^{2+} exchanger activity (Andreeva et al., 1991; Wacholtz et al., 1993; Amoroso et al., 1997), in a dose-dependent manner (1, 3, and 10 μM) reversed the protective effect exerted by SNP (300 μM) on cell injury elicited by chemical hypoxia evaluated as LDH release (Fig. 5). At the highest concentration tested (10 μM), bepridil and CB-DMB did not exert any toxic effect on LDH release (control = 17.0 ± 1.5 mIU/ml, control plus 10 μM bepridil = 18 ± 1 mIU/ml; control = 22 ± 1 mIU/ml, control plus 10 μM CB-DMB = 20 ± 2 mIU/ml). In addition, bepridil (Fig. 2b) and CB-DMB (data not shown) did not interfere with cell survival in nonhypoxic control conditions, whereas they counteracted the protective effect of SNP (Fig. 2e and f).

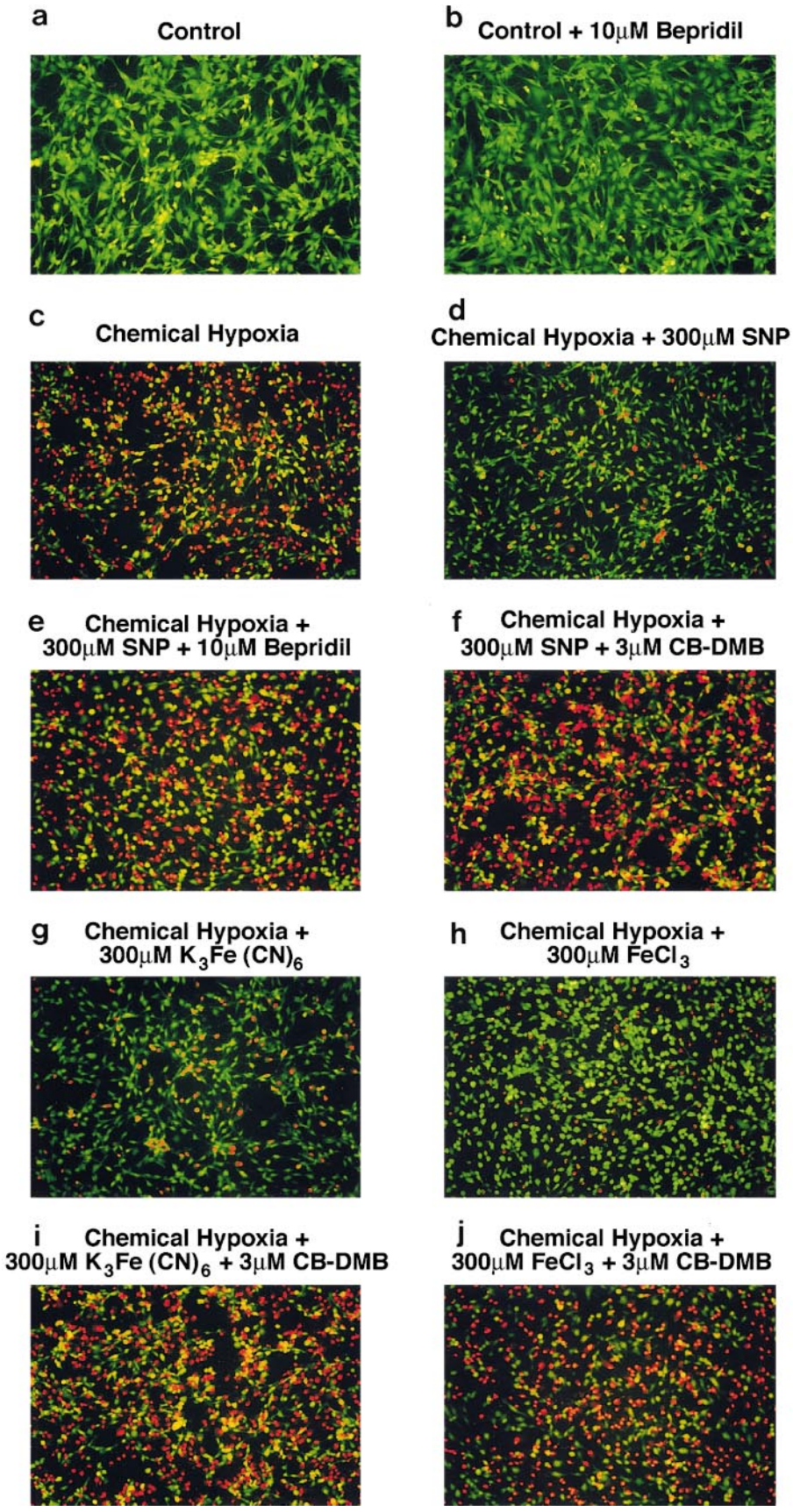

FIG. 2. Protective effect of SNP, $K_3Fe(CN)_6$, and $FeCl_3$ on chemical hypoxia-induced cell death and its reversal by two Na^+–Ca^{2+} exchanger inhibitors, bepridil and CB-DMB. C6 glioma cells were incubated for a 6-h period in a standard buffer (**a**) or in a standard buffer plus 10 μM bepridil (**b**) or subjected to chemical hypoxia (**c**) in the presence of 300 μM SNP (**d**), 300 μM $K_3Fe(CN)_6$ (**g**), or 300 μM $FeCl_3$ (**h**). **e** and **f:** C6 glioma cells exposed to chemical hypoxia in the presence of 300 μM SNP plus 10 μM bepridil or 300 μM SNP plus 3 μM CB-DMB, respectively. **i** and **j:** C6 glioma cells exposed to chemical hypoxia in the presence of 300 μM $K_3Fe(CN)_6$ plus 3 μM CB-DMB or 300 μM $FeCl_3$ plus 3 μM CB-DMB, respectively. Each micrograph is representative of at least four independent experiments. For experimental details, see Materials and Methods. Original magnification $\times$103.

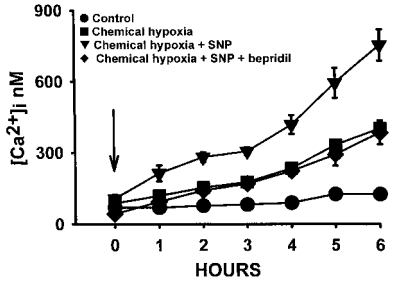

FIG. 3. $[Ca^{2+}]_i$ in fura-2-loaded C6 glioma cells exposed to standard Krebs–Ringer's bicarbonate buffer (●), chemical hypoxia (■), chemical hypoxia plus SNP (▼), and chemical hypoxia plus SNP plus bepridil (◆). C6 glioma cells were exposed to the different experimental conditions for 6 h. Data are mean ± SE (bars) values of three independent experiments (n = 3 for each experimental group). SNP and bepridil incubated for 6 h in a standard medium did not modify basal $[Ca^{2+}]_i$ (data not shown). The arrow shows the minute at which each experiment started.

Effect of tetrodotoxin, an inhibitor of Na^+ influx through voltage-sensitive Na^+ channels, on LDH release induced by chemical hypoxia

Tetrodotoxin added to the incubation medium in a concentration of 10 μM, a concentration known to inhibit Na^+ channels in glial cells (Sontheimer et al., 1994), caused a 30% reduction of LDH release induced by chemical hypoxia (data not shown).

Effect of SNP on NO production during chemical hypoxia

During chemical hypoxia, NO production, measured as NO_x levels, was not different from that detected in nonhypoxic conditions, thus suggesting that a stimulation of NO synthase activity does not occur during chemical hypoxia and that NO is not involved in the mechanisms leading to C6 glioma cell death. SNP at 300 μM, as expected, induced a sixfold increase of NO_x levels (Table 1).

Effect of 3-(*N*-morpholino)sydnonimine (SIN-1) and 8-bromo-cyclic GMP on LDH release evoked by chemical hypoxia

SIN-1 (300–1,000 μM), another NO donor like SNP but chemically different (Feelisch and Noack, 1987), and 8-bromo-cyclic GMP (1,000 μM), a cyclic GMP analogue that is used in cell culture because it offers the advantages of being able to cross the cell membrane and has a longer lifetime than cyclic GMP itself, which is known to be the final effector of NO effects (Dawson and Dawson, 1996), were unable to modify LDH release induced by chemical hypoxia at the respective concentrations known to release effectively NO and to mimic NO stimulation of guanylate cyclase (Kankaanranta et al., 1997) (data not shown).

Effect of two iron salts, $K_3Fe(CN)_6$ and $FeCl_3$, on LDH release and FDA-PI-positive staining induced by chemical hypoxia

Both $K_3Fe(CN)_6$, the part of the SNP molecule lacking the NO group, and $FeCl_3$, another compound containing iron ions in the trivalent (oxidized) form as it occurs in the SNP molecule, at a concentration of 300 μM caused an ~65% prevention of chemical hypoxia-induced LDH release (Fig. 6). This reduction was of the same extent as that produced by 300 μM SNP. In addition, $K_3Fe(CN)_6$ and $FeCl_3$ significantly reduced the number of dead cells induced by chemical hypoxia, as revealed by PI staining (Fig. 2g and h).

Effect of the amiloride analogue CB-DMB, a specific inhibitor of the Na^+–Ca^{2+} exchanger, on the protective effect exerted by $K_3Fe(CN)_6$ and $FeCl_3$ on cellular injury

CB-DMB, a specific inhibitor of the Na^+–Ca^{2+} exchanger that does not affect C6 glioma cell viability in nonhypoxic conditions (Amoroso et al., 1997), when added to cells during the whole period of chemical hypoxia at 3 μM concentration, completely counteracted the reduction of LDH release induced by $K_3Fe(CN)_6$ and $FeCl_3$ (Fig. 7). In accordance with the LDH release results, the experiments performed with FDA-PI staining showed that the increase of cell survival produced by $K_3Fe(CN)_6$ and $FeCl_3$ was completely blocked by the simultaneous presence of the Na^+–Ca^{2+} exchanger inhibitor CB-DMB (3 μM; Fig. 2i and j).

Effect of DFX, an iron ion chelator, on the protective effect exerted by SNP on chemical hypoxia-induced cell death

To evaluate whether the SNP effects were dependent on iron ions present in its molecule, the well-known iron ion chelator DFX (1 mM) (Starke and Farber, 1985) was used. This iron chelator completely counteracted the reduction of cell injury produced by SNP (Fig. 8).

Effect of SNP, $K_3Fe(CN)_6$, and $FeCl_3$ on free radical production

Because it has been reported that addition of iron ions in the incubation medium can induce cell injury through free radical production (Mattson and Mark, 1996), the effect of SNP, $K_3Fe(CN)_6$, and $FeCl_3$ on 2′,7′-dichlorofluorescein fluorescence intensity was evaluated in C6

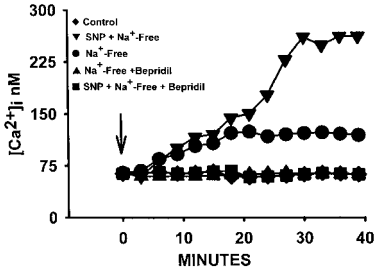

FIG. 4. Effect of SNP, in the absence and in the presence of bepridil, on Na^+–Ca^{2+} exchanger activity stimulated by extracellular Na^+ removal, evaluated as Ca^{2+} influx in single cells. Single C6 glioma cells were exposed to a standard medium (◆), Na^+-free medium in the absence (●) or in the presence (▲) of 10 μM bepridil, or Na^+-free medium plus 300 μM SNP in the absence (▼) or in the presence (■) of 10 μM bepridil. Data are mean ± SE (bars) values of 30 cells obtained in five independent experiments. In each experiment n = 6 for each experimental group.

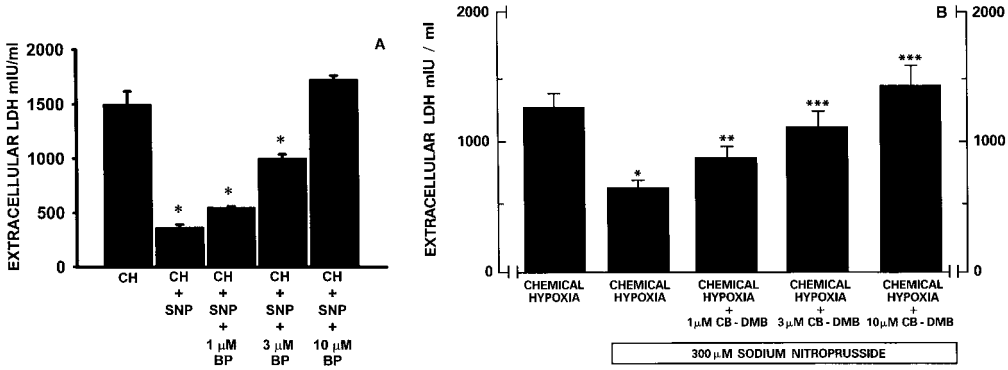

FIG. 5. Effect of increasing concentrations of bepridil (BP) and CB-DMB on LDH release reduction induced by SNP during chemical hypoxia (CH). C6 glioma cells were subjected to CH in the simultaneous presence of 300 μM SNP and of increasing concentrations of (**A**) BP or (**B**) CB-DMB for a 6-h period. Data are mean ± SE (bars) values of 18 determinations obtained in three independent experiments. In each experiment n = 6 for each experimental group. In A, $^{*}p < 0.01$ versus all the other groups. In B, $^{*}p < 0.01$ versus CH, $^{**}p < 0.01$ versus CH + 300 μM SNP, $^{***}p < 0.01$ versus CH + 300 μM SNP + 1 μM CB-DMB.

glioma cells. SNP, $K_3Fe(CN)_6$, and $FeCl_3$ added to the incubation medium at the same concentration (300 μM) that exerted a protective action on C6 glioma cells during chemical hypoxia did not cause any increase of DCFH-DA fluorescence intensity (data not shown).

DISCUSSION

It has been recently demonstrated that in C6 glioma cells during chemical hypoxia, the Na^+–Ca^{2+} exchanger can reverse its mode of operation, extruding Na^+ and allowing the entry of Ca^{2+} (Amoroso et al., 1997). This mode of operation exerts a protective effect on cell damage induced by chemical hypoxia (Amoroso et al., 1997). The results of the present study showed that SNP significantly reduced cellular injury elicited in C6 glioma cells by chemical hypoxia. This protective effect seems to be due to the activation of the Na^+–Ca^{2+} exchanger as a Na^+ efflux–Ca^{2+} influx pathway. In fact, SNP was able to enhance the activity of the Na^+–Ca^{2+} exchanger, evaluated as the $[Ca^{2+}]_i$ increase induced by chemical hypoxia. A further support of this hypothesis on the effects of SNP was the ability of the Na^+–Ca^{2+} exchanger inhibitor bepridil (Stys et al., 1991, 1992; Waxman et al., 1991; Kiedrowski et al., 1994) to counteract the SNP-induced $[Ca^{2+}]_i$ increase. In accordance with these results, bepridil was also able to counteract the protective effect exerted by SNP on chemical hypoxia-induced cell damage evaluated as LDH release and by the number of PI-positive C6 cells. The same reversal of the protective effect induced by SNP on cell damage was obtained by using CB-DMB, another inhibitor of the Na^+–Ca^{2+} exchanger (Andreeva et al., 1991; Wacholtz et al., 1993; Amoroso et al., 1997). Because bepridil and CB-DMB have been reported to also block Ca^{2+} (Kaczorowski et al., 1989; Sharikabad et al., 1997) and Na^+ channels (Kaczorowski et al., 1989), there is the possibility that the counteraction by these drugs of the protective effect exerted by SNP might be due to the blockade of Na^+ and/or Ca^{2+} channels and not of the antiporter. However, this possibility can be completely ruled out because the blockade of Na^+ channels by tetrodotoxin attenuated rather than reinforced chemical hypoxia-induced cell injury. Furthermore, it was previously demonstrated that the blockade of the Ca^{2+} channels induced by organic and inorganic agents does not exert any effect on cell death during chemical hypoxia in C6 glioma cells (Amoroso et al., 1997). Therefore, all these results strongly suggest that SNP exerts its protective action during chemical hypoxia through the activation of the Na^+–Ca^{2+} exchanger, as the Na^+ efflux–Ca^{2+} influx pathway. This hypothesis was further supported by the results showing that in single C6 glioma cells the stimulation of the Na^+–Ca^{2+} exchanger activity as a Na^+ efflux–Ca^{2+} entry pathway, obtained by removing extracellular Na^+ (Sanchez-Armass and Blaustein, 1987; Taglialatela et al., 1990; Amoroso et al., 1993), was reinforced by SNP addition and that this effect was completely prevented by the exchanger inhibitor bepridil. On the other hand, extracellular Na^+ removal, a condition that increases $[Ca^{2+}]_i$ by promoting its influx from the extracellular space coupled to intracellular Na^+ extrusion, is able to protect the same cell line from chemical hypoxia, and this effect is blocked by a specific inhibitor of the Na^+–Ca^{2+} exchanger, CB-DMB (Amoroso et al., 1997).

TABLE 1. *Effect of SNP on NO production during chemical hypoxia*

Treatment	NO_x (μM)
Control (n = 10)	6.5 ± 1.0
Chemical hypoxia (n = 10)	5.7 ± 1.1
Control + 300 μM SNP (n = 10)	39.0 ± 3.5[a]
Chemical hypoxia + 300 μM SNP (n = 10)	37.3 ± 3.2[a]

NO_x concentrations were measured in the medium after a 6-h period during which cells were incubated in normoxic or hypoxic conditions, both in the presence and in the absence of 300 μM SNP.

[a] $p < 0.01$ versus control and chemical hypoxia groups.

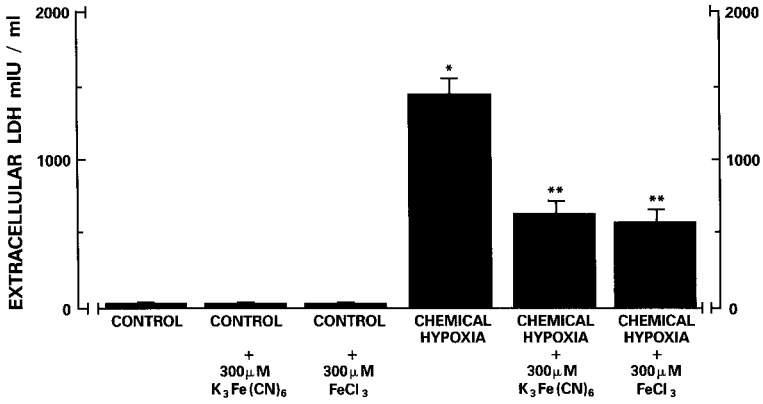

FIG. 6. Effect of the iron salts $K_3Fe(CN)_6$ and $FeCl_3$ on chemical hypoxia-induced LDH release. C6 glioma cells were subjected to chemical hypoxia for a 6-h period in the absence or in the presence of 300 μM $K_3Fe(CN)_6$ or 300 μM $FeCl_3$. Data are mean ± SE (bars) values of 24 determinations obtained in four independent experiments. In each experiment n = 6 for each experimental group. *$p < 0.01$ versus all the other groups, **$p < 0.01$ versus chemical hypoxia group.

The previous (Amoroso et al., 1997) and the present results demonstrate that the activation of the Na^+–Ca^{2+} exchanger as a Na^+ efflux–Ca^{2+} entry pathway is protective against chemical hypoxia in C6 glioma cells even if a $[Ca^{2+}]_i$ increase occurs, suggesting that in this type of cells and in this kind of hypoxia, the $[Ca^{2+}]_i$ increase is not involved in the mechanisms leading to cell death. In line with this hypothesis, in a glial cell line Jurkowitz-Alexander et al. (1992) demonstrated that cell death is independent of the $[Ca^{2+}]_i$ increase during chemical hypoxia. Also, in neurons during chemical hypoxia it has been shown that although a large increase of $[Ca^{2+}]_i$ occurs, this event does not result in cell death (Dubinski and Rothman, 1991). On the other hand, the further possibility that the $[Ca^{2+}]_i$ increase might be protective cannot be sustained because when Na^+ ions are present in the extracellular space during chemical hypoxia, even if there is a $[Ca^{2+}]_i$ increase, large percentages of C6 cells die (Amoroso et. al., 1997; present study). Therefore, the protective effect exerted by the activation of the Na^+–Ca^{2+} exchanger, when it is operating as a Na^+ efflux–Ca^{2+} entry pathway, might be dependent on Na^+ extrusion rather than on $[Ca^{2+}]_i$ increase.

The activation of the Na^+–Ca^{2+} exchanger induced by SNP has already been described in normoxic astrocytes by Asano et al. (1995). These authors suggested that this activation may be due to NO generated by the SNP molecule. However, in our chemical hypoxia model, the activation of the Na^+–Ca^{2+} exchanger does not seem to be dependent on NO release by the SNP molecule. In fact, SIN-1, another NO donor (Feelisch and Noack, 1987), and 8-bromo-cyclic GMP, a cyclic GMP analogue that is used in cell culture because it offers the advantages of being able to cross the cell membrane and having a longer lifetime than cyclic GMP itself, which is known to be the final effector of NO effects (Dawson and Dawson, 1996), were unable to prevent LDH release induced by chemical hypoxia. Another issue that deserves to be emphasized is that although C6 glioma cells possess both constitutive (Barna et al., 1996) and inducible (Feinstein et al., 1994) NO synthase, NO generation does not seem to be involved in chemical hypoxia-induced C6 cell injury because NO levels were found in the present article to be similar both in nonhypoxic and in hypoxic conditions. Even when intracellular NO levels were markedly increased by SNP, no cellular injury occurred, as otherwise observed in hippocampal neurons (Loiacono and Beart, 1992). A possible explanation for this different effect may be that neurons are sensitive to the toxic effect of NO, whereas C6 glioma cells are resistant. In this regard C6 glioma cells resemble micro-

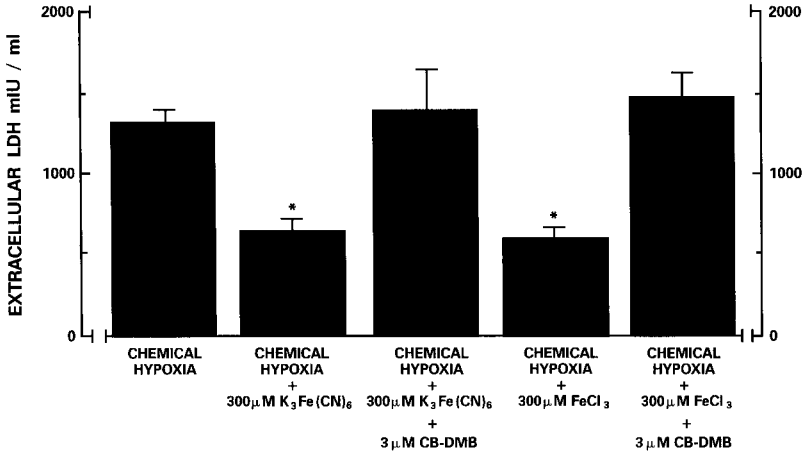

FIG. 7. Effect of CB-DMB on LDH release reduction induced by $K_3Fe(CN)_6$ and $FeCl_3$ during chemical hypoxia. C6 glioma cells were subjected to chemical hypoxia in the simultaneous presence of $K_3Fe(CN)_6$ or $FeCl_3$ and 3 μM CB-DMB for a 6-h period. Data are mean ± SE (bars) values of 18 determinations obtained in three independent experiments. In each experiment n = 6 for each experimental group. *$p < 0.01$ versus chemical hypoxia, chemical hypoxia + 300 μM $K_3Fe(CN)_6$ + 3 μM CB-DMB, and chemical hypoxia + 300 μM $FeCl_3$ + 3 μM CB-DMB groups, respectively.

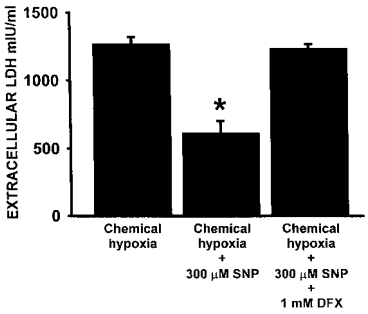

FIG. 8. Effect of DFX on the protective effect exerted by SNP on chemical hypoxia-induced cell death. C6 glioma cells were exposed for 6 h to chemical hypoxia in the presence and in the absence of SNP or SNP plus DFX. Data are mean ± SE (bars) values of 16 determinations obtained in four independent experiments. In each experiment n = 4 for each experimental group. *$p < 0.01$ versus all other groups.

glia, which have been shown to be resistant to NO-induced damage (Mitrovic et al., 1994).

Because the SNP molecule contains iron that is released in ionic form on disintegration (Butler and Glidewell, 1987), there is the possibility that the protective effect exerted by SNP on C6 cell death could be due to iron ions. In fact, evidence has been provided that this metal ion, by mediating the thiol-disulfide interchange of the Na^+–Ca^{2+} exchanger molecule, induces its activation (Reeves et al., 1986), and therefore, in this way, it may protect C6 glioma cells during chemical hypoxia. The results showing that both $K_3Fe(CN)_6$, the portion of the SNP molecule containing iron, and $FeCl_3$ reduced LDH release and the number of PI-positive C6 cells evoked by chemical hypoxia seem to confirm that the protective role of SNP is mediated by iron ions. This hypothesis is strongly supported by the findings showing that the protective effect of SNP on chemical hypoxia-induced cell death was completely prevented by DFX, a well-known iron ion chelator (Starke and Farber, 1985). On the other hand, evidence that SNP can act through iron ions and not through a NO–cyclic GMP-dependent mechanism has already been proposed in other cell types (Kiedrowski et al., 1992).

The hypothesis that $K_3Fe(CN)_6$ and $FeCl_3$, like SNP, are neuroprotective through an activation of the Na^+–Ca^{2+} exchanger is supported by the results of the experiments performed with the antiporter inhibitor CB-DMB. In fact, this compound counteracted the protective effect of $K_3Fe(CN)_6$ and $FeCl_3$ as assessed as LDH release and by the number of dead cells during chemical hypoxia.

The protective effect exerted by ionic iron in C6 glioma cells exposed to chemical hypoxia seems to be in contrast with findings obtained in neurons, where it has been reported that it can cause severe damage by promoting protein and lipid oxidation by virtue of its ability to induce hydroxyl radical production from hydrogen peroxide (Mattson and Mark, 1996). In fact, exposure of cultured rat hippocampal and human neocortical neurons to iron results in hydroxyl radical production and neuronal injury (Zhang et al., 1996). However, in C6 glioma cells this mechanism can be ruled out because iron did not cause any increase of free radical production. A possible explanation for the failure to see enhanced free radical formation in C6 glioma cells may be the transformed nature of these cells. It is also possible that glioma cells possess antioxidant reserves greater than neurons. The lack of production of free radicals following iron exposure may be the reason why iron did not exert any toxic effect. In contrast, iron ions, by stimulating the Na^+–Ca^{2+} exchanger activity in chemical hypoxic conditions, exerted a protective effect.

In conclusion, the present results suggest that SNP may reduce cell injury induced by chemical hypoxia through a stimulation of the Na^+–Ca^{2+} exchanger activity, confirming the protective role played by this antiporter during chemical hypoxia in C6 glioma cells (Amoroso et al., 1997). The final effector of this effect seems to be iron which, by modifying the conformation of the Na^+–Ca^{2+} exchanger carrier from a less active (reduced) to a more active (oxidized) form (Reeves et al., 1986), increases the activity of the antiporter.

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REFERENCES

Amoroso S., Schmid-Antomarchi H., Fosset M., and Lazdunski M. (1990) Glucose, sulfonylureas, and neurotransmitter release: role of ATP-sensitive K^+ channels. *Science* **247,** 852–854.

Amoroso S., Sensi S., Di Renzo G. F., and Annunziato L. (1993) Inhibition of the Na^+–Ca^{2+} exchanger enhances anoxia and glucopenia-induced ^{3}H-aspartate release in hippocampal slices. *J. Pharmacol. Exp. Ther.* **264,** 515–520.

Amoroso S., De Maio M., Russo G. M., Catalano A., Bassi A., Montagnani S., Di Renzo G. F., and Annunziato L. (1997) Pharmacological evidence that the activation of the Na^+–Ca^{2+} exchanger protects C6 glioma cells during chemical hypoxia. *Br. J. Pharmacol.* **121,** 303–309.

Amoroso S., Gioielli A., Cataldi M., Di Renzo G. F., and Annunziato L. (1999) In the neuronal cell line SH-SY5Y, oxidative stress-induced free radical overproduction causes cell death without any participation of intracellular Ca^{2+} increase. *Biochim. Biophys. Acta* **1452,** 151–160.

Andreeva N., Khodorov B., Stelmashook E., Cragoe E. J. Jr., and Victorov I. (1991) Inhibition of Na^+–Ca^{2+} exchanger enhances delayed neuronal death elicited by glutamate in cerebellar granule cell cultures. *Brain Res.* **548,** 322–325.

Asano S., Matsuda T., Takuma K., Kim H. S., Sato T., Nishikawa T., and Baba A. (1995) Nitroprusside and cyclic GMP stimulate Na^+–Ca^{2+} exchange activity in neuronal preparations and cultured rat astrocytes. *J. Neurochem.* **64,** 2437–2441.

Barna M., Komatsu T., and Reiss C. S. (1996) Activation of type III nitric oxide synthase in astrocytes following a neurotropic viral infection. *Virology* **223,** 331–343.

Bass D. A., Parce J. W., Dechatet L. R., Szejda P., Seeds M., and Thomas M. C. (1983) Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. *J. Immunol.* **130,** 1910–1917.

Butler A. R. and Glidewell C. (1987) Recent chemical studies of sodium nitroprusside relevant to its hypotensive action. *Chem. Soc. Rev.* **16,** 361–380.

Cataldi M., Taglialatela M., Guerriero S., Amoroso S., Lombardi G., Di Renzo G. F., and Annunziato L. (1996) Protein-tyrosine kinases activate while protein-tyrosine phosphatases inhibit L-type calcium channel activity in pituitary GH3 cells. *J. Biol. Chem.* **271,** 9441–9446.

Choi D. W., Koh J., and Peters S. (1988) Pharmacology of glutamate neurotoxicity in cortical cell culture: attenuation by NMDA antagonists. *J. Neurosci.* **8,** 185–196.

Dawson T. M. and Dawson V. L. (1996) Nitric oxide synthase: role as a transmitter/mediator in the brain and endocrine system. *Annu. Rev. Med.* **47,** 219–227.

Devlin T. M. (1982) *Textbook of Biochemistry with Clinical Correlations.* John Wiley & Sons, New York.

De Weille J. R., Schmid-Antomarchi H., Fosset M., and Lazdunski M. (1989) Regulation of ATP-sensitive K^+ channels in insulinoma cells: activation by somatostatin and protein kinase C and the role of cAMP. *Proc. Natl. Acad. Sci. USA* **86,** 2971–2975.

Dubinski J. M. and Rothman S. M. (1991) Intracellular calcium concentrations during chemical hypoxia and excitotoxic neuronal injury. *J. Neurosci.* **11,** 2545–2551.

Feelisch M. and Noack E. A. (1987) Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. *Eur. J. Pharmacol.* **139,** 19–30.

Feinstein D. L., Galea E., Roberts R., Berquist H., Wang H., and Reis D. J. (1994) Induction of nitric oxide synthase in rat C6 glioma cells. *J. Neurochem.* **62,** 315–321.

Gay R. J., McComb R. B., and Bowers G. N. (1968) Optimum reaction conditions for human lactate dehydrogenase isoenzymes as they affect total lactate dehydrogenase activity. *Clin. Chem.* **14,** 740–747.

Grynkiewicz G., Poenie M., and Tsien R. Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **264,** 3440–3450.

He S., Ruknudin A., Bambrick L. L., Lederer W. J., and Schulze D. H. (1998) Isoform-specific regulation of the Na^+–Ca^{2+} exchanger in rat astrocytes and neurons by PKA. *J. Neurosci.* **18,** 4833–4841.

Jurkowitz-Alexander M. S., Altschuld R. A., Hohl C. M., Johnson J. D., McDonald J. S., Simmons T. D., and Horrocks L. A. (1992) Cell swelling, blebbing, and death are dependent on ATP depletion and independent of calcium during chemical hypoxia in a glial cell line (ROC-1). *J. Neurochem.* **59,** 344–352.

Kaczorowski G. J., Slaughter R. S., King V. F., and Garcia M. L. (1989) Inhibitors of sodium–calcium exchanger: identification and development of probes of transport activity. *Biochim. Biophys. Acta* **988,** 287–302.

Kankaanranta H., Knowles R. G., Vuorinen P., Kosonen O., Holm P., and Moilanen E. (1997) 3-Morpholino-sydnonimine-induced suppression of human neutrophil degranulation is not mediated by cyclic GMP, nitric oxide or peroxynitrite: inhibition of the increase in intracellular free calcium concentration by *N*-morpholinoiminoacetonitrile, a metabolite of 3-morpholino-sydnonimine. *Mol. Pharmacol.* **51,** 882–888.

Kato S., Higashida H., Higuchi Y., Hatakenaka S., and Negishi K. (1984) Sensitive and insensitive states of cultured glioma cells to glutamate damage. *Brain Res.* **303,** 365–373.

Kiedrowski L., Costa E., and Wrobleski J. T. (1992) Sodium nitroprusside inhibits *N*-methyl-D-aspartate-evoked calcium influx via a nitric oxide- and cGMP-independent mechanism. *Mol. Pharmacol.* **41,** 779–784.

Kiedrowski L., Brooker G., Costa E., and Wrobleski J. T. (1994) Glutamate impairs calcium extrusion while reducing sodium gradient. *Neuron* **12,** 295–300.

Lehninger A. L. (1975) *Biochemistry,* 2nd edit. Worth, New York.

Loiacono R. E. and Beart P. M. (1992) Hippocampal lesions induced by microinjection of the nitric oxide donor nitroprusside. *Eur. J. Pharmacol.* **216,** 331–333.

Manev H., Favaron M., Vicini S., Guidotti A., and Costa E. (1990) Glutamate-induced neuronal death in primary cultures of cerebellar granule cells: protection by synthetic derivatives of endogenous sphingolipids. *J. Pharmacol. Exp. Ther.* **252,** 419–427.

Mattson M. P. and Mark R. J. (1996) Excitotoxicity and excitoprotection in vitro, in *Advances in Neurology, Vol. 71: Cellular and Molecular Mechanisms of Ischemic Brain Damage* (Siesjö B. K. and Wieloch T., eds), pp. 1–30. Lippincott–Raven Publishers, Philadelphia.

Mitrovic B., Ignarro L. J., Montestruque S., Smoll A., and Merrill J. E. (1994) Nitric oxide as a potential pathological mechanism in demyelination: its differential effects on primary glial cells in vitro. *Neuroscience* **61,** 575–585.

Reeves J. P., Bailey C. A., and Hale C. C. (1986) Redox modification of the sodium–calcium exchange activity in cardiac sarcolemmal vesicles. *J. Biol. Chem.* **261,** 4948–4955.

Sanchez-Armass S. and Blaustein M. P. (1987) Role of sodium–calcium exchange in regulation of intracellular calcium in nerve terminals. *Am. J. Physiol.* **252,** C595–C603.

Schmid-Antomarchi H., De Weille J. R., Fosset M., and Lazdunski M. (1987) The receptor for antidiabetic sulfonylureas controls the activity of the ATP-modulated K^+ channels in insulin-secreting cells. *J. Biol. Chem.* **262,** 15840–15844.

Schmid-Antomarchi H., Amoroso S., Fosset M., and Lazdunski M. (1990) K^+ channel openers activate brain sulfonylurea-sensitive K^+ channels and block neurosecretion. *Proc. Natl. Acad. Sci. USA* **87,** 3489–3492.

Sharikabad M. N., Cragoe E. J. Jr., and Brors O. (1997) Inhibition by *N*-(4-chlorobenzyl)-2′,4′-dimethylbenzamil of Na^+–Ca^{2+} exchange and L-type Ca^{2+} channels in isolated cardiomyocytes. *Pharmacol. Toxicol.* **80,** 57–61.

Siesjö B., Bengtsson F., Grampp W., and Theander S. (1989) Calcium, excitotoxins and neuronal death in the brain. *Ann. NY Acad. Sci.* **568,** 234–251.

Sontheimer H., Fernandez-Marques E., Ullrich N., Pappas C. A., and Waxman S. G. (1994) Astrocyte Na^+ channels are required for maintenance of Na^+–K^+ ATPase activity. *J. Neurosci.* **14,** 2464–2475.

Starke P. E. and Farber J. L. (1985) Ferric ion and superoxide ions are required for the killing of cultured hepatocytes by hydrogen peroxide: evidence for the participation of hydroxyl radicals formed by an iron-catalyzed Haber–Weiss reaction. *J. Biol. Chem.* **260,** 10099–10103.

Stys P. K., Waxman S. G., and Ransom B. R. (1991) Na^+–Ca^{2+} exchanger mediates Ca^{2+} influx during anoxia in mammalian central nervous system white matter. *Ann. Neurol.* **30,** 375–380.

Stys P. K., Waxman S. G., and Ransom B. R. (1992) Ionic mechanisms of anoxic injury in mammalian CNS white matter: role of Na^+ channels and Na^+–Ca^{2+} exchanger. *J. Neurosci.* **12,** 430–439.

Taglialatela M., Di Renzo G. F., and Annunziato L. (1990) Na^+–Ca^{2+} exchange activity in central nerve endings. I. Ionic conditions that discriminate $^{45}Ca^{2+}$ uptake through the exchanger from that occurring through voltage-operated Ca^{2+} channels. *Mol. Pharmacol.* **38,** 385–392.

Thomsen L. L., Ching L. M., and Baguley B. C. (1990) Evidence for the production of nitric oxide by activated macrophages treated with the antitumor agents 8-acetic acid and xanthenone-4-acetic acid. *Cancer Res.* **50,** 6966–6970.

Wacholtz M. C., Cragoe E. J. Jr., and Lipski P. E. (1993) Delineation of the role of a Na^+–Ca^{2+} exchanger in regulating intracellular Ca^{2+} in T cells. *Cell. Immunol.* **147,** 95–109.

Waxman S. G., Ransom B. R., and Stys P. K. (1991) Non-synaptic mechanism of Ca^{2+}-mediated injury in CNS white matter. *Trends Neurosci.* **14,** 461–468.

Zhang Y., Tatsuno T., Carney J., and Mattson M. P. (1996) FGF, NGF and IGFs protect hippocampal neurons against iron-induced degeneration. *J. Cereb. Blood Flow Metab.* **13,** 378–388.