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Pharmacological evidence that the activation of the Na^+-Ca^{2+} exchanger protects C6 glioma cells during chemical hypoxia

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1 In C6 glioma cells exposed to chemical hypoxia a massive release of lactate dehydrogenase (LDH) occurred at 3 and 6 h, coupled with an increased number of propidium-iodide positive dead cells.

2 Extracellular Na^+ removal, which activates the Na^+-Ca^{2+} exchanger as a Na^+ efflux pathway and prevents Na^+ entrance, significantly reduced LDH release and the number of propidium iodide positive C6 cells.

3 During chemical hypoxia, in the presence of extracellular Na^+ ions, a progressive increase of $[Ca^{2+}]_i$ occurred; in the absence of extracellular Na^+ ions $[Ca^{2+}]_i$ was enhanced to a greater extent.

4 The blockade of the Na^+-Ca^{2+} exchanger by the amiloride derivative 5-(N-4-chlorobenzyl)-2′,4′-dimethylbenzamil (CB-DMB), lanthanum (La^{3+}) and the Ca^{2+} chelator EGTA, completely reverted the protective effect exerted by the removal of Na^+ ions on C6 glioma cells exposed to chemical hypoxia.

5 The inhibition of the Na^+-Ca^{2+} antiporter enhanced chemical hypoxia-induced LDH release when C6 glioma cells were incubated in the presence of physiological concentrations of extracellular Na^+ ions (145 mM), suggesting that the blockade of the Na^+-Ca^{2+} antiporter during chemical hypoxia can lead to increased cell damage.

6 Collectively, these results suggest that activation of the Na^+-Ca^{2+} exchanger protects C6 glioma cells exposed to chemical hypoxia, whereas its pharmacological blockade can exacerbate cellular injury.

Keywords: Na^+-Ca^{2+} exchanger; amiloride analogue; chemical hypoxia; C6 glioma cells

Introduction

The mechanisms leading to cell swelling and injury in glial cells involve a perturbation of intracellular Na^+ and Ca^{2+} homeostasis (McCarthy & O'Neill, 1992; Staub *et al*., 1993; Goldberg & Choi, 1993; O'Connor & Kimelberg, 1993). Since the intracellular concentration of both cations in astrocytes depends mainly on the activity of the plasma membrane Na^+-Ca^{2+} exchanger (Goldman *et al*., 1994), which couples the uphill extrusion of Ca^{2+} ions to the entrance of Na^+ ions into the cells (Taglialatela *et al*., 1990), it was of interest to investigate whether the activation or the pharmacological inhibition of the Na^+ -Ca^{2+} exchanger may interfere with cell injury induced by chemical hypoxia.

To this aim, C6 glioma cells, a cellular line which has been extensively used as a model for cerebral glia (Erecinska *et al*., 1993) and allows 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 (Schmid-Antomarchi *et al*., 1987; De Weille *et al*., 1989; Amoroso *et al*., 1990; 1993; Schmid-Antomarchi *et al*., 1990) in experimental conditions of activation or pharmacological inhibition of the Na^+-Ca^{2+} exchanger activity.

Methods

Cell culture

C6 glioma cells (purchased from American Type Culture Collection) were cultured as monolayers, in polystyrene dishes (100 mm) and grown in Dulbecco's Modified Eagle's Medium (DMEM) (ICN Laboratories, Milan, Italy) containing 10% heat inactivated foetal calf serum (Hyclone, Utah, U.S.A.), 100 iu penicillin ml^{-1}, 100 μg of streptomycin ml^{-1} (ICN). Cells were cultured in a humidified incubator at 37°C in 5% CO_2 atmosphere. The medium was changed every two days. Each experiment was performed with cells from passages 45–65 in 6 multiwell plates (Falcon). To improve cell adhesion the wells were precoated with 30 μg ml^{-1} poly-L-lysine (Sigma, Italy).

Chemical hypoxia

Chemical hypoxia was reproduced by adding to the cells 5 μg ml^{-1} oligomycin plus 2 mM 2-DG in glucose-free medium. This combination produces a fall in cell adenosine 5′-triphosphate (ATP) content (Schmid-Antomarchi *et al*., 1987; De Weille *et al*., 1989; Amoroso *et al*., 1990) since oligomycin inhibits oxidative phosphorylation (Lehninger, 1975; Dubinski & Rothman, 1991) whereas 2-DG causes inhibition at the first step of glycolytic pathway, that is, at the reaction catalyzed by hexokinase (Devlin, 1982). The composition of the medium was in mM: NaCl 145, KCl 5, $MgSO_4$ 1.2, HEPES 10, $CaCl_2$ 1.5 and BSA 0.2%, pH adjusted to 7.4 with Tris 1 M (standard buffer). When a Na^+-free medium was used, 145 mM NaCl was replaced with 145 mM choline chloride.

Intracellular calcium measurements

Just before the experiment, C6 glioma cells were detached by gently streaming the culture medium on the surface of the monolayer, centrifuged and resuspended in 1 ml standard buffer plus 10 mM glucose. The cells (2×10^6 ml^{-1}) were then incubated with 5 μM Fura 2-AM 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. $[Ca^{2+}]_i$ was

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measured in a 2 ml suspension of C6 glioma cells (5×10^5 ml^{-1}) 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 340 and 380 nm (bandpass 5 nm) with the emission at 510 nm (bandpass 5 nm). $[Ca^{2+}]_i$ was determined according to the equation of Grynkiewicz *et al*. (1985).

Determination of LDH

Cultured C6 glioma cells (10^6 ml^{-1} 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 12000 g for 5 min in a microfuge. The supernatant was used for lactate dehydrogenase (LDH) determination by spectrophotometric assay (Gay *et al*., 1968).

Intravital staining of the culture

After the experimental procedures, C6 glioma cells (3×10^5–5×10^5 ml^{-1} per well) were washed with the standard buffer and stained for 3 min at 22°C with a solution containing 36 μM fluorescein diacetate (FDA) (Sigma, Italy) and 7 μM propidium iodide (PI) (Calbiochem., San Diego, CA, U.S.A.). The stained cells were examined immediately with a standard epi-illumination fluorescence microscope. FDA, a non polar 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 which, by interacting with nuclear DNA, yields a bright red fluorescence (Manev *et al*., 1990).

Drugs

Oligomycin, gadolinium (Gd^{3+}), lanthanum (La^{3+}), EGTA, and 2-DG were obtained from Sigma, Italy. The amiloride derivatives were synthesized and supplied by Dr Cragoe (Nacogdoches, TX, U.S.A.). Darodipine was obtained through the courtesy of Dr Flavio Franch, Sandoz (Milan, Italy). All other reagents were obtained from commercial sources and were of the highest purity available. Oligomycin was dissolved in ethanol (stock solution 5 mg ml^{-1}); the amiloride derivatives were dissolved in dimethyl sulphoxide (DMSO; stock solution 10 mM). Gd^{3+}, La^{3+}, EGTA and 2-DG were diluted in water. FDA was dissolved in acetone and PI was diluted in water; both were protected from light. Darodipine, protected from the light, was diluted in DMSO. The final concentration of DMSO in the experimental medium was always less than 1%.

Statistics

Data were analysed by one way analysis of variance (ANOVA) followed by the Newmann-Keul's test.

Results

Effect of extracellular Na^+ ion removal on LDH release and on cellular death evoked by chemical hypoxia

C6 glioma cells were exposed to chemical hypoxia by adding 5 μg ml^{-1} oligomycin plus 2 mM 2-DG in glucose-free medium. This treatment induced a time-dependent increase of LDH release, an index of cellular injury (Choi *et al*., 1988) which reached the highest value at 6 h (Figure 1). After the same period of exposure to oligomycin and 2-DG approximately 60% of C6 cells died as revealed by the staining with PI (Figure 2a). The removal of extracellular Na^+ ions, replaced with equimolar concentrations of choline chloride, significantly reduced at 3 (60%) and 6 h (70%) LDH release induced by chemical hypoxia (Figure 1) as well as the number of PI positive C6 cells (Figure 2b).

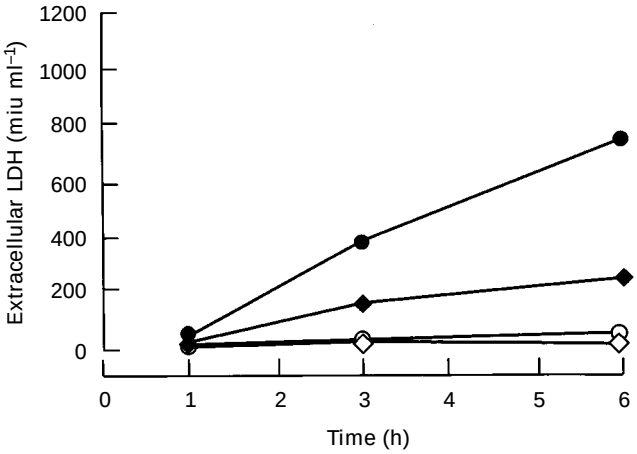

Figure 1 Effect of chemical hypoxia on LDH release in the presence and absence of extracellular Na^+ ions. C6 glioma cells were exposed to chemical hypoxia for a 6 h period. Samples for LDH determination were taken at 1, 3 and 6 h after the beginning of the experiments. Each point represents the mean of 30 values obtained in 5 independent experiments. The means of the values obtained at 3 and 6 h after chemical hypoxia treatment, in the absence of extracellular Na^+ ions (◆), were significantly different ($P<0.01$) from those observed in the presence of extracellular Na^+ ions (●). (◇) and (○) Represent LDH values obtained in the absence and presence of extracellular Na^+ ions, respectively, in C6 glioma cells not exposed to chemical hypoxia.

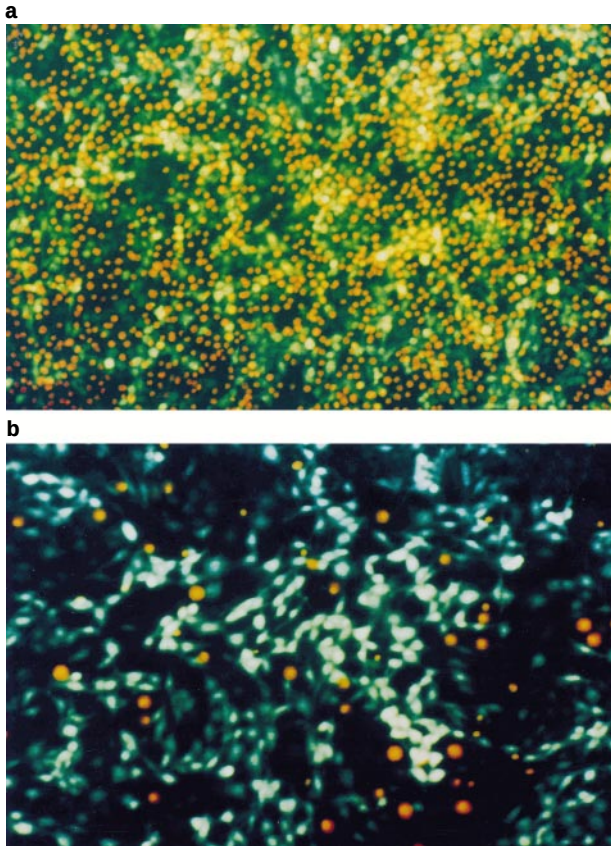

Figure 2 Effect of chemical hypoxia on cell death in the presence and absence of extracellular Na^+ ions. C6 glioma cells were subjected to chemical hypoxia for a 6 h period in the presence (a) and absence (b) of extracellular Na^+ ions. At the end of the experiments the cells were washed twice and then exposed for 5 min to a new incubation medium containing 36 μM FDA and 7 μM PI. The intravital staining yields green yellow fluorescence for vital cells and red fluorescence for dead cells. The drawing is representative of at least 7 independent experiments. For experimental details see Methods. (a) Original magnification 130×. (b) Original magnification 270×.

Effect of darodipine and Gd^{3+} on chemical hypoxia-induced LDH release

The organic Ca^{2+} entry blocker darodipine, in a concentration (10 μM) which largely blocks voltage-dependent calcium channels (VDCC) (Gaggi *et al*., 1995), did not prevent LDH release induced by chemical hypoxia (data not shown). The same lack of effect was observed in the presence of 300 μM Gd^{3+}, a lanthanide compound that prevents Ca^{2+} entrance through L, N and T subtype VDCC without interfering with the plasma membrane Na^+-Ca^{2+} exchanger (Canzoniero *et al*., 1993) (data not shown).

La^{3+} and EGTA enhanced cell injury induced by chemical hypoxia and reversion of the protection exerted by Na^+ removal on cell damage

La^{3+}, an inorganic compound that, besides blocking VDCC also inhibits the plasma membrane Na^+-Ca^{2+} exchanger (Sanchez-Armass & Blaustein, 1987), induced an enhancement of LDH release induced by chemical hypoxia (Figure 3a). Interestingly, the chelator of extracellular Ca^{2+} ions, EGTA (2 mM), did not counteract but rather reinforced LDH release elicited by chemical hypoxia (Figure 3b). In addition, both agents were able to reverse the reduction of LDH release produced by the removal of Na^+ ions from the extracellular medium, an ionic condition which activates the Na^+-Ca^{2+} exchanger as a Na^+ extrusion pathway (Sanchez-Armass & Blaustein, 1987; Taglialatela *et al*., 1988; 1990) (Figure 4a and b).

Effect of the amiloride analogue, 5-(N-4-chlorobenzyl)-2′,4′-dimethylbenzamil (CB-DMB), a specific inhibitor of the Na^+-Ca^{2+} exchanger, on chemical hypoxia-induced LDH release and on the protective effect exerted by Na^+ removal on cellular injury

CB-DMB (3 μM), a specific inhibitor of the Na^+-Ca^{2+} exchanger (Andreeva *et al*., 1991), when added for 6 h to C6 glioma cells incubated in a standard medium containing

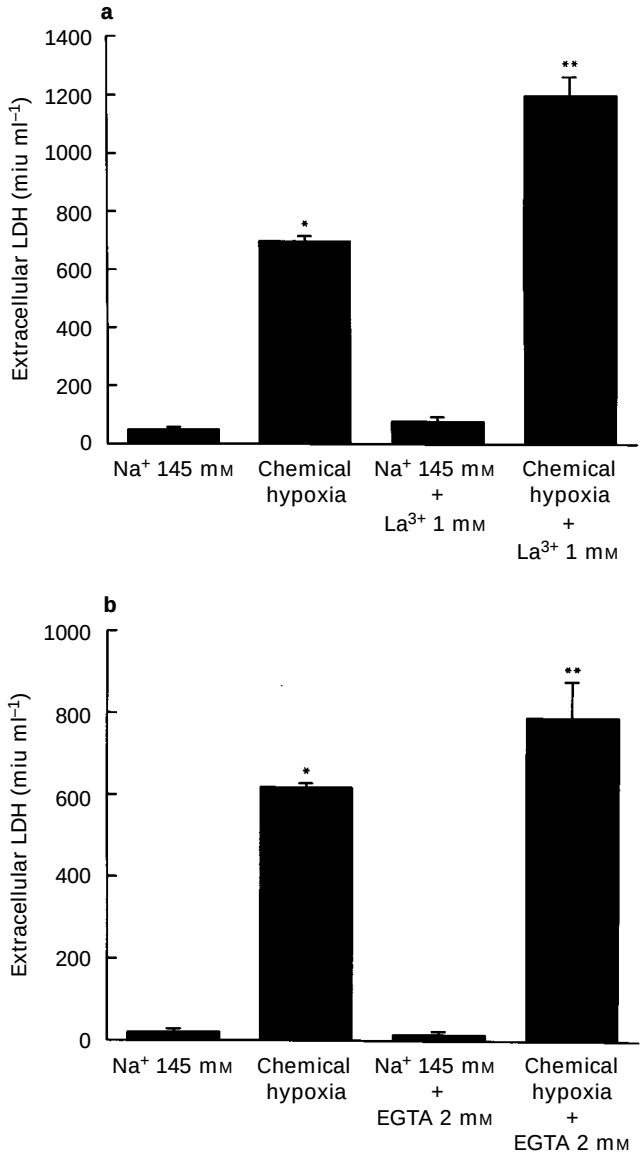

Figure 3 Effect of La^{3+} and EGTA on cell damage induced by chemical hypoxia in the presence of extracellular Na^+ ions. La^{3+} (a) and EGTA (b) were added to the incubation medium for a 6 h period in the presence of extracellular Na^+ ions. Each column represents the mean of 18 values obtained in 3 independent experiments. *$P<0.01$ versus 145 mM Na^+ group; **$P<0.01$ versus chemical hypoxia group.

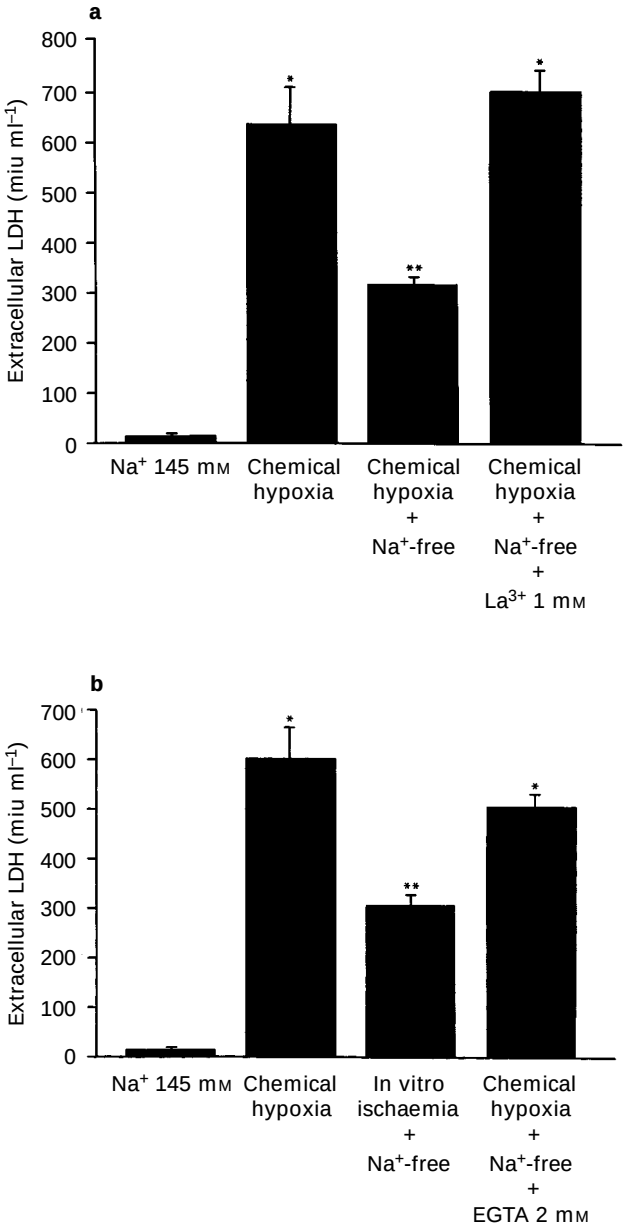

Figure 4 Effect of La^{3+} and EGTA on cell injury induced by chemical hypoxia in the absence of extracellular Na^+ ions. For experimental details see legend of Figure 3. *$P<0.01$ versus 145 mM Na^+ groups; **$P<0.01$ versus chemical hypoxia and chemical hypoxia + Na^+-free + La^{3+} or EGTA groups.

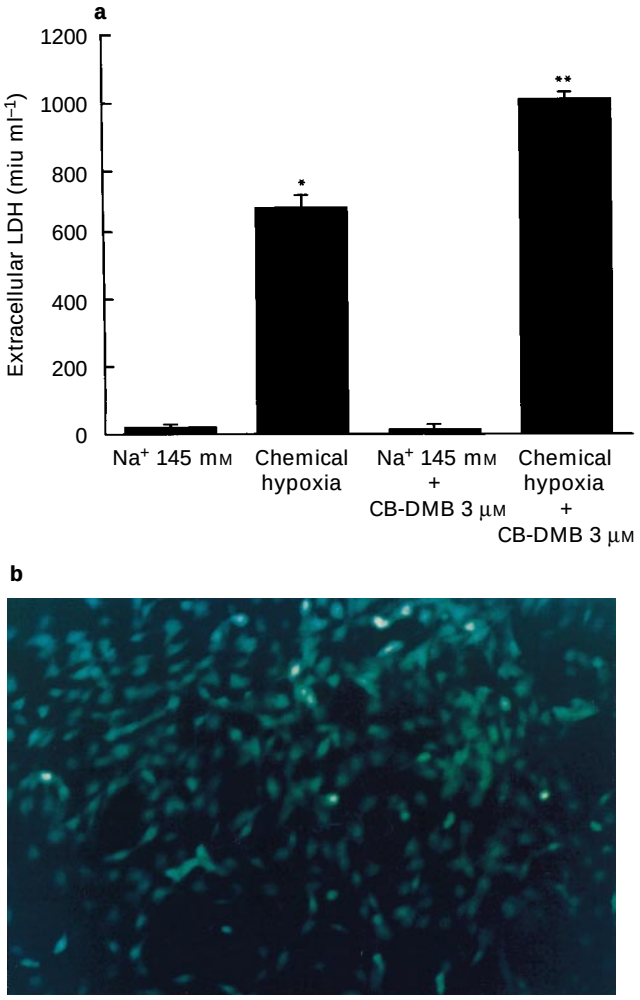

Figure 5 Effect of the Na^+-Ca^{2+} exchanger inhibitor CB-DMB on LDH release and on cellular death. C6 glioma cells were subjected to a 6 h chemical hypoxia period in the presence and absence of 3 μM CB-DMB. (a) The effects of CB-DMB on LDH release both in chemical hypoxia and non chemical hypoxia conditions. Each column represents the mean of 18 values obtained in 3 independent experiments. (b) The effects on cellular death in non chemical hypoxia conditions. Original magnification 270×. *$P<0.01$ versus 145 mM Na^+ group; **$P<0.01$ versus chemical hypoxia group.

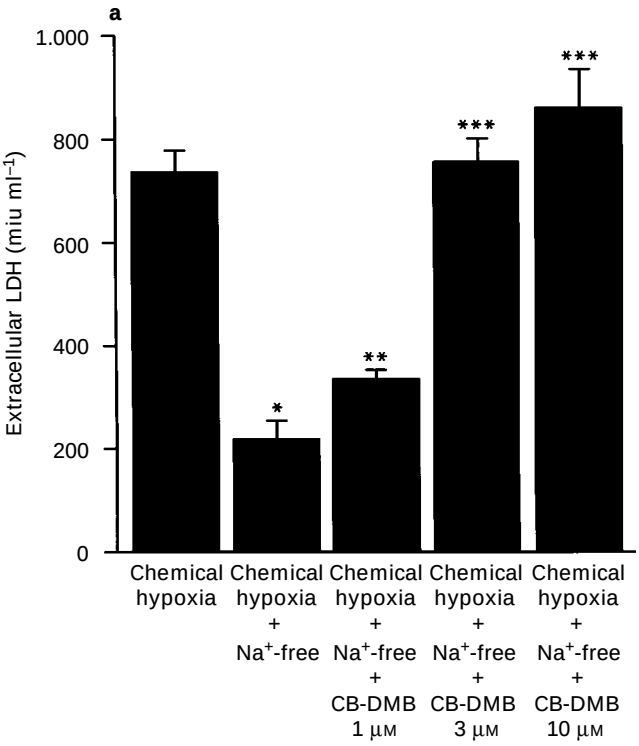

Figure 6 Effect of increasing concentrations of CB-DMB on LDH release and cellular death reduction produced by extracellular Na^+ removal. (a) The effects on LDH release of different concentrations of CB-DMB, added for a 6 h chemical hypoxia period. (b) The effect of 3 μM CB-DMB on cellular death. Each column in (a) represents the mean of 15 values. (b) Original magnification 270×. *$P<0.01$ versus chemical hypoxia group; **$P<0.01$ versus chemical hypoxia and chemical hypoxia + Na^+-free groups; ***$P<0.01$ versus chemical hypoxia + Na^+-free and chemical hypoxia + Na^+-free + 1 μM CB-DMB groups.

145 mM Na^+ and not exposed to chemical hypoxia, did not cause any increase of LDH release (Figure 5a). However, when the Na^+-Ca^{2+} exchanger inhibitor was added during the whole chemical hypoxia period, it reinforced LDH release (Figure 5a). Furthermore, the Na^+-Ca^{2+} inhibitor, in a concentration-dependent manner, reverted the reduction of LDH release produced by the removal of extracellular Na^+ ions (Figure 6a). Accordingly, during chemical hypoxia in a Na^+-free medium the Na^+-Ca^{2+} exchanger inhibitor increased the number of PI positive cells as compared to that observed in the absence of the inhibitor (Figure 6b), whereas in non chemical hypoxia conditions it did not exert any toxic effect (Figure 5b).

Effect of 5-(N,N-hexamethylene) amiloride (HMA), a specific inhibitor of the Na^+-H^+ antiporter, on LDH release induced by chemical hypoxia in the presence and absence of extracellular Na^+ ions

HMA, another amiloride derivative, which is devoid of any Na^+-Ca^{2+} exchanger inhibitory activity whereas it specifically blocks the Na^+-H^+ antiporter (Simchowitz & Cragoe, 1986), was unable to modify LDH release induced by chemical hypoxia, either in the presence or absence of Na^+ ions (Figure 7).

Effect of chemical hypoxia on $[Ca^{2+}]_i$ in C6 glioma cells in the presence and absence of extracellular Na^+ ions

When C6 glioma cells were exposed to chemical hypoxia, in the presence of extracellular Na^+ ions a progressive increase of $[Ca^{2+}]_i$ occurred. This increase of $[Ca^{2+}]_i$ started almost immediately and reached a plateau at the 3rd hour. In C6 glioma cells exposed to chemical hypoxia in the absence of extracellular Na^+ ions, a condition which is known to activate the Na^+-Ca^{2+} antiporter as a Na^+ efflux-Ca^{2+} influx pathway, a greater increase of $[Ca^{2+}]_i$ occurred (Figure 8). $[Ca^{2+}]_i$ rose before the start of the chemical hypoxia as a consequence of the activation of the Na^+-Ca^{2+} exchanger which occurs in Na^+-free conditions.

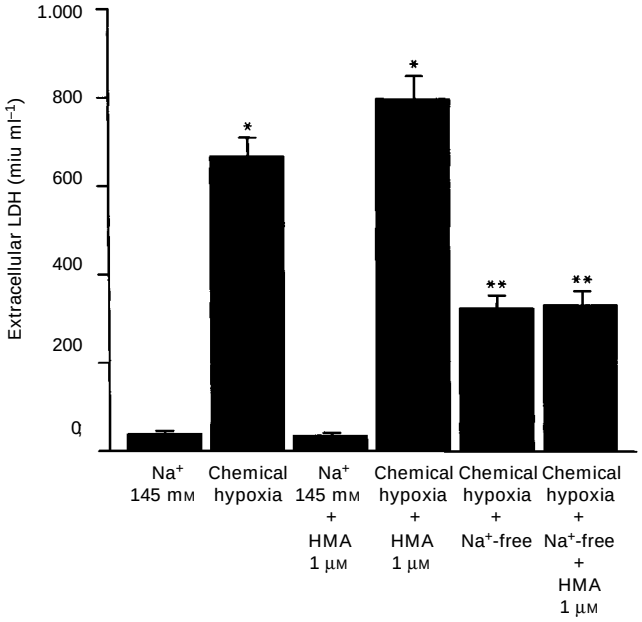

Figure 7 Effect of the Na^+-H^+ antiporter inhibitor HMA on LDH release induced by chemical hypoxia both in the presence and absence of extracellular Na^+ ions. HMA was added to the incubation medium during chemical hypoxia for a 6 h period. Each column represents the mean of 15 values obtained in 3 independent experiments. $*P<0.01$ versus 145 mM Na^+ and 145 mM $Na^+ + 1$ μM HMA groups; $**P<0.01$ versus chemical hypoxia and chemical hypoxia + 1 μM HMA groups.

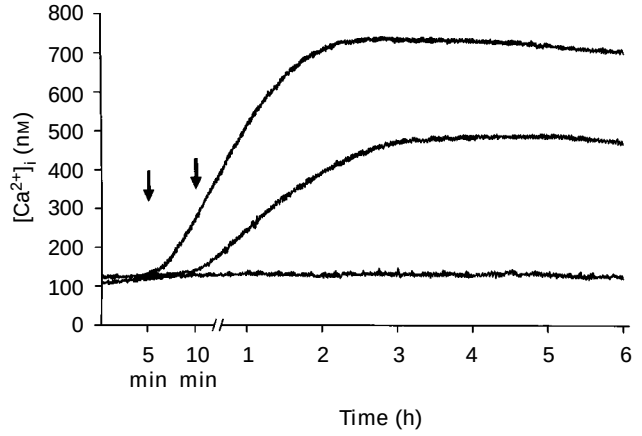

Figure 8 $[Ca^{2+}]_i$ in Fura-2 loaded C6 glioma cells exposed to 145 mM Na^+, 145 mM Na^+ plus chemical hypoxia and Na^+-free plus chemical hypoxia. The lowest trace represents the pattern of $[Ca^{2+}]_i$ in C6 glioma cells exposed to standard buffer. The middle and the upper traces show the effects of exposure to chemical hypoxia in the presence and absence of 145 mM Na^+ ions, respectively. Each trace is representative of 5 similar traces. Each single experiment was performed on 1×10^6 cells. Only for the trace which refers to experiments performed in Na^+-free conditions, the first arrow indicates the minute at which standard buffer was replaced with Na^+-free medium. The second arrow shows the minute at which C6 glioma cells were exposed to chemical hypoxia.

Discussion

The results of the present study show that the removal of Na^+ ions from the extracellular medium, a condition which is known to activate the Na^+-Ca^{2+} exchanger as a Na^+ ion efflux (Sanchez-Armass & Blaustein, 1987; Taglialatela *et al.*, 1988; 1990), attenuated cellular injury elicited in C6 glioma cells by chemical hypoxia.

Evidence that the Na^+-Ca^{2+} exchanger, when it is operating as a Na^+ efflux pathway, may exert a protective role in chemical hypoxia conditions comes from the experiments performed with the extracellular Ca^{2+} chelator EGTA. In fact, EGTA was able to counteract completely the LDH decrease produced by the removal of Na^+ ions from the extracellular medium. A possible explanation for these findings is that the presence of EGTA inhibits the Na^+-Ca^{2+} exchanger in as much as it chelates extracellular Ca^{2+} ions that cannot be exchanged with intracellular Na^+. That the inhibition of the Na^+-Ca^{2+} exchanger system, when it is operating as a Na^+ efflux-Ca^{2+} ion entry pathway, may be responsible for the reversion of the protective effect exerted by the Na^+ removal, was also supported by the results of experiments with La^{3+}. In fact, this trivalent lanthanide, which besides blocking VDCC (Taglialatela *et al.*, 1988) effectively inhibits the activity of the Na^+-Ca^{2+} exchanger (Sanchez-Armass & Blaustein, 1987), counteracted the reduction of LDH release occurring in conditions in which there was an absence of Na^+ ions in the extracellular space. On the other hand, these effects of EGTA and La^{3+} cannot be attributed to their ability to block Ca^{2+} entry through Ca^{2+} channels since blockers of L, N and T calcium channel subtypes, darodipine and Gd^{3+} (Canzoniero *et al.*, 1993) failed to exert any effect on cellular injury.

Further support for the idea that the Na^+-Ca^{2+} exchanger, when it operates as a Na^+ extruding system, may protect C6 cells from chemical hypoxia injury, derives also from the experiments performed with the amiloride derivative CB-DMB. This specific inhibitor of the Na^+Ca^{2+} exchanger (Andreeva *et al.*, 1991), which is devoid of cytotoxic effects on cells in normal conditions, completely reverted the protective effect of extracellular Na^+ removal on LDH release and cellular death elicited by chemical hypoxia. The specificity of the effect of CB-DMB on the Na^+Ca^{2+} exchanger activity was further supported by the lack of any effect on chemical hypoxia-induced LDH release, either in the presence or absence of extracellular Na^+ ions, of HMA, a compound which, although possessing a pirazine ring like CB-DMB, is devoid of any inhibitory property on the Na^+Ca^{2+} exchanger (Simchowitz & Cragoe, 1986). In addition, since HMA has, at a concentration of 1 μM, Na^+-H^+ antiporter inhibitory properties (Simchowitz & Cragoe, 1986), its failure to interfere with LDH release would suggest that the Na^+-H^+ antiporter is not involved in the mechanisms leading to glial injury during chemical hypoxia. All these results suggest that the Na^+-Ca^{2+} exchanger, when it is operating as a Na^+ efflux pathway, may exert a partial protective effect during chemical hypoxia when extracellular Na^+ is removed. By contrast when C6 glioma cells were exposed to chemical hypoxia, in the presence of physiological concentrations of Na^+ ions, the blockade of the Na^+-Ca^{2+} exchanger activity by EGTA, La^{3+} and CB-DMB, enhanced chemical hypoxia-induced LDH release. A possible explanation of these results is that the blockade of the Na^+-K^+ ATPase elicited by the fall in ATP concentrations (Macknight & Leaf, 1977; Kempski *et al.*, 1992) results in a loss of K^+ ions and a gain of Na^+ ions into the cells. This increase of intracellular Na^+ ion concentrations may induce the Na^+-Ca^{2+} exchanger to reverse its mode of operation, extruding Na^+ ions (Amoroso *et al.*, 1993). Therefore, the inhibition of Na^+ ion extrusion caused by the blockade of the Na^+-Ca^{2+} exchanger by EGTA, La^{3+} and CB-DMB could produce a worsening of cell injury, as demonstrated by the increase of LDH release.

Another finding that should be considered is that during chemical hypoxia, a progressive increase of $[Ca^{2+}]_i$ occurred and that the activation of the Na^+-Ca^{2+} exchanger as a Na^+ efflux-Ca^{2+} influx pathway, obtained by extracellular Na^+ ion removal, resulted in a further enhancement of $[Ca^{2+}]_i$ increase. These results suggest that in C6 glioma cells under chemical hypoxia, Ca^{2+} ion entry does not have a relevant role in the induction of cell injury as it has been proposed to do in neurones during ischaemic conditions (Choi, 1988; Farooqui &

Horrocks, 1991). In fact, in C6 glial cells exposed to chemical hypoxia, $[Ca^{2+}]_i$ increased to a greater extent when the cells were protected by extracellular Na^+ ion removal. In line with our findings Jurkowits-Alexander *et al*. (1992) demonstrated that cellular swelling, bleb formation and death are independent of Ca^{2+} entry during chemical hypoxia in a glial cell line. On the other hand, in neurones, under conditions of chemical hypoxia, an enormous sustained magnitude of $[Ca^{2+}]_i$ has also been found not to result in an increase of cell death (Dubinski & Rothman 1991). Therefore, the increase in $[Ca^{2+}]_i$ does not appear to be involved in cellular injury induced by chemical hypoxia in C6 glial cells. The apparent discrepancy between our results and those showing that in neurones the increase in $[Ca^{2+}]_i$ is toxic (Choi, 1988; Farooqui & Horrocks, 1991) may be due to the difference in the experimental models. In fact an increase in $[Ca^{2+}]_i$ seems to be involved in neurotoxicity induced by NMDA receptor overactivation, whereas C6 glioma cells are almost insensitive to glutamate since only concentrations of the excitatory amino acid in the millimolar range (4 mM) are able to elicit cellular damage (Kato *et al*., 1984). Furthermore the hypothesis that an increase in $[Ca^{2+}]_i$ may produce cell injury has recently been questioned (Harman & Maxwell, 1995).

In conclusion, the protective effect played by the activation of Na^+-Ca^{2+} exchanger, when it is operating as a Na^+ efflux-Ca^{2+} influx pathway, together with the exacerbation of cellular injury by its pharmacological blockade, seems to suggest that the extrusion of Na^+ ions may play a relevant role in the survival of C6 glial cells exposed to chemical hypoxia.

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