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Role of Na^+/Ca^{2+} Exchanger in Preventing Na^+ Overload and Hepatocyte Injury: Opposite Effects of Extracellular and Intracellular Ca^{2+} Chelation

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We have previously shown that an increase of intracellular Na^+ occurs in isolated rat hepatocytes undergoing ATP depletion and that Na^+ accumulation is associated with an uncontrolled influx of Ca^{2+} through the activation in reverse mode of the Na^+/Ca^{2+} exchanger. In the present study we have investigated the relationship between alterations of Na^+ and Ca^{2+} homeostasis and hepatocyte killing using treatments which differentially chelate extracellular or intracellular Ca^{2+}. Chelation of extracellular Ca^{2+} by ethylene glycol bis-(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA) potentiated Na^+ overload and cell killing induced in isolated rat hepatocytes by hypoxia or menadione. Similar effects were also observed when Na^+ accumulation was induced by the combined addition of Na^+ ionophore monensin and the inhibition of plasma membrane Na^+/K^+ ATPase by ouabain. Conversely, the use of the intracellular Ca^{2+} chelator EGTA acetoxymethyl ester (EGTA/AM) reduced Na^+ overload and hepatocyte death induced by hypoxia or cell treatment with menadione or monensin plus ouabain. The effects of EGTA/AM were reverted in the presence of bepridil, an inhibitor of Na^+/Ca^{2+} exchanger. Altogether these results indicated that differential chelation of intracellular or extracellular Ca^{2+} influences in opposite ways hepatocyte killing due to ATP depletion by modulating intracellular Na^+ levels through the reversed activity of the Na^+/Ca^{2+} exchanger. © 1997 Academic Press

Previous studies from our laboratory have shown that the exposure of isolated rat hepatocytes to hypoxia, mitochondrial toxins or oxidative stress, treatments that all induce ATP depletion, produced an irreversible increase of intracellular Na^+ content (1,2). Na^+ accumulation is due to the inhibition of the activity of the Na^+/K^+ ATPase as consequence of ATP depletion and to the concomitant activation of the Na^+/H^+ exchanger and of the Na^+/HCO_3^- cotrasporter in response of the intracellular acidification (2). Na^+ overload appears to be critical for the development of cell damage and hepatocyte incubation in a Na^+-free medium prevents the killing of ATP depleted cells (1,2).

We have observed that among the effects of Na^+ accumulation, the activation in the reverse mode of the Na^+/Ca^{2+} exchanger, significantly contributed to Ca^{2+} increase in isolated hepatocytes undergoing ATP depletion (3). It is well known that elevation of cytosolic Ca^{2+} levels is associated to the development of irreversible hepatocyte injury and the activation of Ca^{2+}-dependent proteases, phospholypases and endonucleases has been proposed as possible mechanism in causing cell damage (4). However, in many instances chelation of extracellular Ca^{2+} or cell incubation in a Ca^{2+}-free medium has failed to prevent hepatocyte killing (5-9). Since the activity in the reverse mode of Na^+/Ca^{2+} exchanger can significantly contribute to Na^+ extrusion (10), we have investigated whether modulation of the exchanger activity by chelation of intracellular or extracellular Ca^{2+} with, respectively, ethylene glycol bis-(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA) or of its cell permeable acetoxymethyl ester (EGTA/AM) might influence hepatocyte Na^+ overload and Na^+-mediated cell damage.

MATERIALS AND METHODS

Chemicals and animals. Collagenase (Type I), ouabain, N-(2-hydroxyethyl)-piperazine-N′-(2-ethanesulfonic acid) (HEPES), monensin, ethylene glycol bis-(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA) and 2-methyl-1,4-naphtoquinone (menadione) were purchased from Sigma (St Louis, MO, USA). Ethylene glycol bis-(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid-acetoxymethyl ester (EGTA/AM) was from Calbiochem (St Diego, CA, USA). Percoll was supplied by Pharmacia (Upsala, Sweden). All the other chemicals were of analitical grade and were purchased from Merch (Darmstad, Germany). Male Wistar rats (180-250 g weight) were obtained from

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Halman-Nossan (Corezzana, Italy) and allowed free access to water and food.

Hepatocyte preparation and incubation. Isolated rat hepatocytes were prepared by liver perfusion with collagenase as previously described (2). For the experiments hepatocytes (10^6/mL) were suspended in Krebs-Henseleit-HEPES buffer containing 118 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, 1.3 mmol/L $CaCl_2$, 25 mmol/L $NaHCO_3$ and 20 mmol/L HEPES at pH 7.4 and were incubated at 37° C in 50 mL glass bottles under continous fluxing of a 5% CO_2-95% O_2 mixture. When indicated, a Na^+ free Krebs-Henseleit-HEPES buffer containing 118 mmol/L choline chloride instead of NaCl and 25 mmol/L $KHCO_3$ replacing $NaHCO_3$ was employed. Hepatocytes maintained under hypoxic condition were incubated under continuous fluxing of 5% CO_2-95% N_2. Menadione was dissolved in dimethylsulfoxide and aliquots of 1 μL/mL were added to the hepatocyte suspension to obtain a final concentration of 50 μmol/L. The same amount of dimethylsulfoxide was added to control cells. Monensin and ouabain were solubilized in the incubation medium and added to the final concentration of 10 μmol/L and 1 mmol/L, respectively. In some experiments hepatocytes were loaded with EGTA by 15 min preincubation in the presence of 25 μmol/L EGTA-AM. Cell viability was estimated by microscope-counting the hepatocytes excluding Trypan blue. In our hands this procedure gave results comparable to the measurement of lactate dehydrogenase (LDH) release.

Intracellular Na^+ measurements. Aliquots of cell suspensions were layered on the top of 3 mL 37.5% Percoll solution (d = 1.06) in 0.25 M sucrose and spun 1 min at 1,000 g on a beanch-top centrifuge in order to remove the incubation medium and dead cells. The cell pellet was extracted with 0.5 mL of 0.8 N perchloric acid. Na^+ was measured using a Varian AA-1475 atomic absorption spectrophotometer in aliquots of the protein-free acidic supernatant diluted 200 times with distilled water and the values were corrected for the protein content of each pellet, estimated by the Lowry method as modified by Peterson (11).

Cytosolic Ca^{2+} measurements. Cytosolic free Ca^{2+} concentration was measured using the fluorescent indicator dye Fura-2. Briefly isolated hepatocytes (3×10^6 cells/mL) were loaded with Fura-2/AM by 15 min incubation in a modified Krebs-Henseleit medium containing 10 mmol/L glucose, 2% bovine serum albumin, 20 mmol/L HEPES pH 7.4 and 4 μmol/L Fura-2/AM. Cells were then washed, resuspended in the same medium and further incubated at 25° C to allow complete de-esterification of Fura-2/AM. Fluorescence was measured with a computer-assisted Perkin-Elmer LS-5B fluorometer. Excitation wavelenght was alternatively positioned at 340 and 380 nm; emission wavelenght was set at 509 nm. The fluorescence ratio signal was measured and stored every excitation signal (3 sec). Calibration values for maximum and minimum fluorescence ratio were obtained by permeabilisation of hepatocytes with digitonin (10 μg/mL) followed by addition of 5 mmol/L EGTA and Tris to adjust the final pH at 8.

Statistical analysis. Statistical analysis for multiple comparisons was performed by one-way ANOVA test with Bonferroni's corrections for multiple comparisons. A p value less than 0.05 was considered significant. Distribution normality of the groups considered was preliminary evaluated by the Shapiro-Wilk test and obtained w values did not reject H_0 at the level of $p < 0.05$.

RESULTS AND DISCUSSION

Hepatocytes maintained under hypoxic conditions or undergoing oxidative stress following the treatment with the redox-cycling quinone, menadione (50 μmol/L), showed a progressive increase of intracellular Na^+

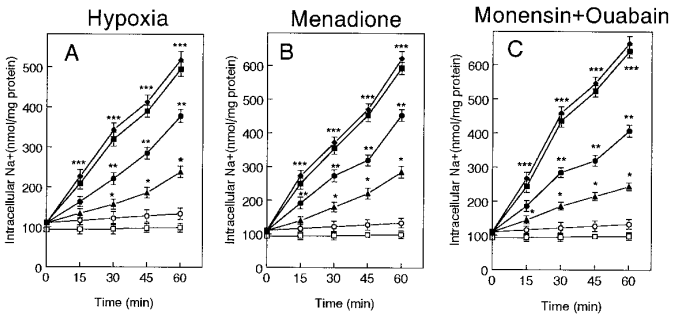

FIG. 1. Changes of Na^+ intracellular levels in isolated rat hepatocytes incubated under hypoxic conditions (A) or treated with 50 μmol/L menadione (B) or with 10 μmol/L monensin plus 1 mmol/L ouabain (C). The symbols represent: control hepatocytes incubated without further treatments (○); hepatocytes exposed to hypoxia, menadione or monensin plus ouabain in Na^+-containing medium (●); hepatocytes exposed to the same treatments as above but incubated in a Na^+-free Krebs-Henseilt-HEPES buffer (□); hepatocytes incubated under hypoxic conditions, with menadione or monensin plus ouabain in the presence of 3 mmol/L EGTA (♦), 25 μmol/L EGTA-AM (▲) or with EGTA-AM and 50 μmol/L bepridil (■). No effect on intracellular Na^+ concentration was observed after 60 min incubation of hepatocytes with 50 μmol/L bepridil alone (not shown). Results are means of 4-5 experiments ± S.D. Difference statistically significant (p values ranging from < 0.05 to < 0.001) versus: * untreated controls or hepatocytes exposed to hypoxia or treated with menadione or monensin plus ouabain; ** hepatocytes pretreated with EGTA/AM; *** hepatocytes exposed to hypoxia or treated with menadione or monensin plus ouabain.

(Fig. 1A, B). Na^+ accumulation was associated with irreversible cell damage and preventing the increase of intracellular Na^+ by hepatocyte incubation in a Na^+-free medium (Fig. 1A, B) greatly reduced cell killing (Fig. 2A, B). Similar effects were also evident in undamaged cells in which the influx of extracellular Na^+ and the impairement of the Na^+/K^+ ATPase consequent to ATP depletion were mimiked by the simultaneous treatment with the Na^+ ionophore monensin (10 μmol/L) and by inhibiting plasma membrane Na^+/K^+ ATPase with ouabain (1 mmol/L) (Figs. 1C, 2C). The increase in intracellular Na^+ caused by anoxia or by the treatment with menadione or with monensin plus ouabain was associated to a rise of cytosolic Ca^{2+} levels that was completely prevented by chelation of extracellular Ca^{2+} with 3 mmol/L EGTA or by hepatocyte incubation in a Na^+-free buffer (Table 1).

Hepatocytes death induced by menadione, hypoxia or monensin plus ouabain was not prevented by extracellular Ca^{2+} chelation, but, in the presence of EGTA, cell killing appeared to be rather potentiated (Fig. 2). On the other hand, in hepatocytes preloaded with membrane permeant EGTA-acetoxymethylester (EGTA/AM) cell death induced by the above treatments was significantly delayed (Fig. 2). Interestingly, chelation of extracellular Ca^{2+} with EGTA significantly increased the Na^+ load of hepatocytes exposed to menadione, hypoxia and monensin plus ouabain, whereas che-

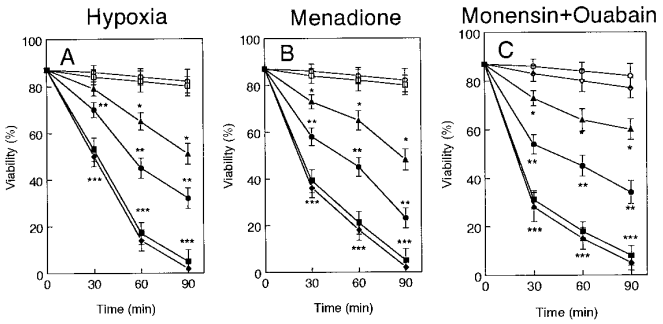

FIG. 2. Killing of isolated rat hepatocytes incubated under hypoxic conditions (A) or treated with 50 μmol/L menadione (B) or with 10 μmol/L monensin plus 1 mmol/L ouabain (C). The symbols represent: control hepatocytes incubated without further treatments (○); hepatocytes exposed to hypoxia, menadione or monensin plus ouabain in Na^+-containing medium (●); hepatocytes exposed to the same treatments as above but incubated in a Na^+-free Krebs-Henseilt-HEPES buffer (□); hepatocytes incubated under hypoxic conditions, with menadione or monensin plus ouabain in the presence of 3 mmol/L EGTA (♦), 25 μmol/L EGTA-AM (▲) or with EGTA-AM and 50 μmol/L bepridil (■). No effect on intracellular Na^+ concentration was observed after 60 min incubation of hepatocytes with 50 μmol/L bepridil alone (not shown). Results are means of 4-5 experiments ± S.D. Difference statistically significant (p values ranging from $<$ 0.05 to $<$ 0.001) versus: * untreated controls or hepatocytes exposed to hypoxia or treated with menadione or monensin plus ouabain; ** hepatocytes pretreated with EGTA/AM; *** hepatocytes exposed to hypoxia or treated with menadione or monensin plus ouabain.

lation of intracellular Ca^{2+} with EGTA/AM, reduced Na^+ accumulation (Fig. 1).

A recent study has demonstrated that Na^+/Ca^{2+} exchanger is expressed in several tissues including the liver (12-14). Lidofsky and coworkers have shown that Na^+/Ca^{2+} antiporter does not contribute to the regulation of hepatocyte cytosolic Ca^{2+} under resting conditions or in response to Ca^{2+}-mobilizing hormones (14). Nonetheless, sustained increases of, respectively, intracellular Na^+ and Ca^{2+} levels have been shown to activate in opposite directions hepatocyte Na^+/Ca^{2+} exchanger (3,15). Eisner and Lederer have proposed that the direction of Na^+ and Ca^{2+} fluxes across Na^+/Ca^{2+} exchanger is influenced by several factors including the plasma membrane potential and the intracellular and extracellular concentrations of either Na^+ and Ca^{2+} (16). In this connection it is likely that chelation of extracellular Ca^{2+} with EGTA would inhibit Na^+ extrusion by the reversed activity of the Na^+/Ca^{2+} exchanger, thus resulting in a further increase of intracellular Na^+ load. On the other hand, titration of intracellular Ca^{2+} with EGTA/AM should promote the activity of the Na^+/Ca^{2+} exchanger by steadily keeping the at low level free cytosolic Ca^{2+} and thus favouring a persistent efflux of intracellular Na^+ in exchange with extracellular Ca^{2+}. Consistently, we have observed that in hepatocytes exposed to menadione, hypoxia and monensin plus ouabain inhibition of Na^+/Ca^{2+} exchanger by bepridil (50 μmol/L)(17) abolished the protective effect on Na^+ accumulation exerted by intracellular Ca^{2+} chelation with EGTA/AM (Fig. 1). Bepridil addition also reverted the protection by EGTA/AM on hepatocyte killing caused by menadione, hypoxia and monensin plus ouabain (Fig. 2).

Increase of intracellular Ca^{2+} can contribute to the impairement of mitochondrial functions associated to hepatocyte poisoning with several toxins (4,18). Furthermore, Pastorino and coworkers have recently shown that chelation of intracellular Ca^{2+} with BAPTA-AM prevented the killing of rat hepatocytes by anoxia or rotenone and that this effect was related to the inhibition of Ca^{2+}-mediated induction mitochondrial permeability transition (19). We have previously reported that Na^+ overload is associated with hepatocyte swelling, suggesting that osmotic stress might be critical for hepatocyte injury caused by ATP depletion (1). The results here presented indicate that, beside the effects on mitochondrial functions (7,18,19), chelation

TABLE 1

Cytosolic Ca^{2+} Concentrations in Isolated Rat Hepatocytes Exposed to Hypoxia or Treated with Menadione or Monensin Plus Ouabain: Effect of EGTA Addition or of Omission of Extracellular Na^+

	Cytosolic Ca^{2+} (μmol/L)		
	Complete medium	Complete medium + EGTA	Na^+-free medium
Control	0.123 ± 0.045	0.107 ± 0.039	0.111 ± 0.065
Hypoxia	0.492 ± 0.089*	0.115 ± 0.078	0.131 ± 0.092
Menadione	1.645 ± 0.098*	0.127 ± 0.085	0.142 ± 0.103
Monensin+ouabain	1.895 ± 0.108*	0.131 ± 0.083	0.148 ± 0.105

Note. Isolated rat hepatocytes (10^6 cell/mL) were loaded with FURA-2 by 15 min preincubation with 4 μmo/L FURA-2AM and then incubated 45 min under 95% O_2-5%CO_2 atmosphere without further additions or in presence of 50 μmol/L menadione or 10 μmol/L monensin plus 1 mmol/L ouabain. Hypoxic conditions were obtained by fluxing the cells with 95% N_2–5%CO_2. When indicated 3 mmol/L EGTA was also added to the incubation medium. For the experiments performed in the absence of extracellular Na^+, NaCl and $NaHCO_3$ in the medium were replaced by equimolar amount of choline chloride and $KHCO_3$, respectively. The results are means of 3-6 experiments ± S.D. Statistical significance: * $p < 0.001$ versus controls or hepatocytes exposed to hypoxia or undergoing treatments with menadione or monensin plus ouabain in the presence of EGTA or in absence of extracellular Na^+.

of intracellular Ca^{2+} can reduce cell killing by favouring Na^+ extrusion through the Na^+/Ca^{2+} exchanger. Thus, the reversed activation of Na^+/Ca^{2+} exchanger in hepatocyte plasma membrane can be envisaged to play an important role in regulating intracellular Na^+ levels following the impairment of plasma membrane Na^+/K^+ ATPase caused by ATP depletion. Moreover, these results suggest that potentiation of Na^+ overload consequent to the impairment of Ca^{2+}-mediated Na^+ extrusion might explain the lack of protection on the cytotoxicity of several compounds observed following chelation of extracellular Ca^{2+} or cell incubation in a Ca^{2+}-free medium (5-9).

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