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EVIDENCE FOR A SODIUM-DEPENDENT CALCIUM INFLUX IN ISOLATED RAT HEPATOCYTES UNDERGOING ATP DEPLETION

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ATP depletion caused by menadione and triethyllead in isolated hepatocytes is associated with intracellular acidosis and a sustained increase in intracellular Na^+ and Ca^{2+} concentrations. Removal of Na^+ from the incubation medium as well as the inclusion of EGTA largely prevented the increase in cytosolic Ca^{2+}, thus indicating that Ca^{2+} was mobilized from the extracellular medium in response to Na^+ load. To further validate these findings, hepatocytes were incubated with a combination of sodium propionate and ouabain in order to induce intracellular acidosis and inhibit Na^+ extrusion. This treatment promoted a marked increase in intracellular Na^+ and Ca^{2+} concentrations that was prevented by omission of Na^+ from the incubation medium as well as by agents that inhibited cellular Na^+ influx. These data indicate that following Na^+ load, Ca^{2+} can be accumulated in hepatocytes via a Na^+/Ca^{2+} antiporter operating on a reverse mode.

Impairement of cellular Ca^{2+} homeostasis associated with an uncontrolled and persistent elevation of cytosolic Ca^{2+} levels is a common feature in cell injury caused by many toxins, oxidative stress and hypoxia (1-3). The inhibition of Ca^{2+} translocase activities of the plasma membrane and the endoplasmic reticulum, often combined with the release of Ca^{2+} from the mitochondria, has been demonstrated to be involved in causing the increase of cytosolic free Ca^{2+}observed in the various toxic conditions (1). Recently, Gasbarrini and coworkers using hepatocytes, loaded with the Ca^{2+} indicator aequorin and made hypoxic, reported that the increase in cytosolic free Ca^{2+} caused by anoxia occured in two distinct phases (4). The initial phase was mainly dependent from the release of the ion from the intracellular stores, while the late and more intense rise of Ca^{2+} was of

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extracellular origin and was efficiently prevented by the omission of Ca^{2+} from the incubation medium (4).

The possibility that in some condition the activation of Ca^{2+} channels might contribute in causing influx of Ca^{2+} from the extracellular spaces has been raised (1,5). However, the secondary increase of cytosolic Ca^{2+} occuring during anoxia was not prevented by Ca^{2+} channel blockers (6). Haigen and colleagues have reported that in isolated cardiac myocytes the prevention of intracellular Na^{+} rise by the omission of Na^{+} from the incubation medium markedly reduced the increase in cellular Ca^{2+} consequent to oxygen deprivation (7). Thus, they postulated that the elevation of intracellular Na^{+} might trigger Ca^{2+} influx through myocyte plasma membrane by activating a Na^{+}/Ca^{2+} exchanger operating in a reverse mode (7).

On the light of the controversy about the presence and the function of a Na^{+}/Ca^{2+} exchanger in hepatocyte plasma membrane (8-10), we have investigated whether a Na^{+}-dependent Ca^{2+} influx might occur in isolated rat hepatocytes intoxicated with the redox-cycling quinone menadione and triethyllead ($PbEt_3$) two compounds that are known to deplete intracellular ATP and to impair Ca^{2+} homeostasis (11-13).

Materials and Methods

Collagenase (Type I), ouabain, N-(2-hydroxyethyl)-piperazine-N'-(2-ethanesulfonic acid) (HEPES), FURA-2/AM, sodium propionate, 2-methyl-1,4-naphtoquinone (menadione), amiloride, 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene (DIDS) and digitonin were purchased from Sigma (St Louis, MO, USA). Triethyllead ($PbEt_3$) was supplied by Alpha Products (Darmstad, Germany). All the other chemicals were of analitical grade and were purchased from Merch (Darmstad, Germany). Male Wistar rats (180-250 g weigth) were obtained from Nossan (Corezzana, Italy) and allowed free access to water and food.

Isolated rat hepatocytes were prepared by liver perfusion with collagenase as previously described (5). In all the preparation hepatocyte viability, as estimated by measuring the percentage of cells excluding Trypan blue, was ranging from 87% to 92%. For the experiments hepatocytes (10^6/ml) were suspended in Krebs-Henseleit medium containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 25 mM $NaHCO_3$ and 20 mM N HEPES and were incubated at pH 7.4 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 medium containing 118 mM choline chloride was employed instead of NaCl and 25 mM $KHCO_3$ replaced $NaHCO_3$.

Cytosolic free Ca^{2+} concentration was measured using the fluorescent indicator dye Fura-2. Briefly isolated hepatocytes (3 x 10^6 cells/ml) were loaded with 4 μM Fura-2/AM in a modified Krebs-Henseleit medium containing 10 mM glucose, 2% bovine serum albumin and 20 mM Hepes, pH 7.4 for 15 min. 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 fluorimeter. 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 mM EGTA and Tris to adjust the final pH at 8.

Intracellular Na^{+} content was measured by atomic absorbtion using a Varian AA-1475 atomic absorption spectrophotometer. Aliquots of cell suspensions were layered on

the top of a Percoll (Pharmacia, Upsala, Sweden) solution (3 ml, d = 1.06) in 0.25 M sucrose and spun 1 min at 1000 RPM on a beanch-top centrifuge in order to remove the incubation medium and damaged or dead cells. After centrifugation the Percoll solution was rapidly removed by aspiration and cell pellet was extracted with 0.5 ml of 0.8 N perchloric acid. Na^+ was measured in aliquots of the protein-free acidic supernatant diluted 200 times with distilled water and the values corrected for the protein content of each pellet.

Protein concentration was estimated by the Lowry method as modified by Peterson (14).

Staristical analysis was performed with one-way ANOVA, with Bonferroni's correction for multiple comparisons.

Results and Discussion

The incubation of isolated rat hepatocytes with either 50 μM menadione or triethyllead ($PbEt_3$) causes a sustained elevation of cytosolic free Ca^{2+} (Fig. 1B).

The measurement of intracellular Na^+ reveals that, concomitantly with the rise of Ca^{2+}, Na^+ content is significantly increased in hepatocytes intoxicated with menadione and $PbEt_3$ and after 30 min of treatment Na^+ levels are about 3 and 7 fold higher than untreated controls (Fig. 1A). The increase in intracellular Na^+ is completely prevented when isolated hepatocytes are incubated with the two compounds in a medium where Na^+ is replaced by an equimolar amount of choline chloride (Fig. 1A), indicating that Na^+ increase is due to an influx of the ion from the extracellular space.

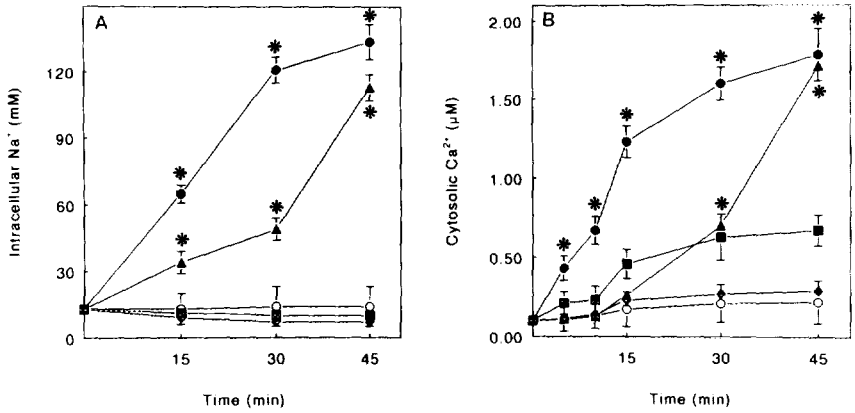

Figure 1. Increase in intracellular Na^+ (panel A) and cytosolic Ca^{2+} (panel B) concentrations in isolated hepatocytes intoxicated with menadione or $PbEt_3$ and effects of Na^+ omission.
Liver cells loaded with Fura 2/AM were used to measure cytosolic Ca^{2+}, while intracellular Na^+ was measured by atomic absorption. Rat hepatocytes (10^6 cells/ml) were incubated at 37° C with or without 50 μM menadione or $PbEt_3$ in a Krebs-Henseleit buffer containing 114 NaCl and 25 mM $NaHCO_3$ or equivalent concentrations of choline chloride and $KHCO_3$. The symbols represent untreated control cells maintained in the presence of Na^+ (○); menadione-treated cells incubated in the presence (▲) or in the absence (◆) of NaCl; $PbEt_3$-treated cells incubated in the presence (●) or in the absence (■) of NaCl. The results are means of three different experiments ± S.D.
* : statistically different vs controls or cells treated with either menadione or $PbEt_3$ and incubated in the absence of Na^+ (p values ranging from $p<0.05$ and $p<0.001$).

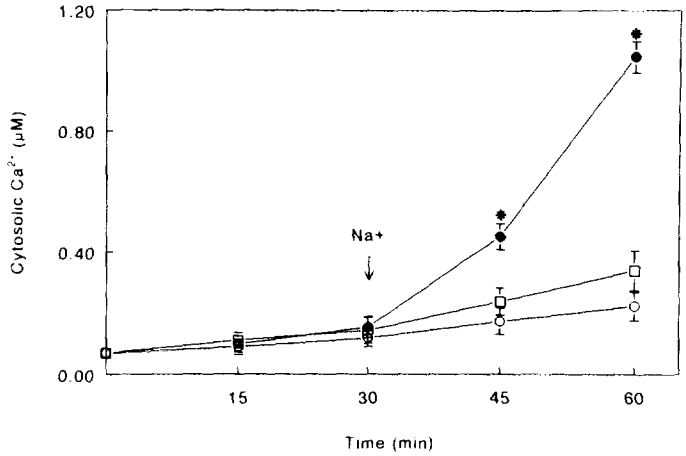

Figure 2. Extracellular origin of Ca^{2+} mobilized by Na^+ load in hepatocytes intoxicated with menadione.
Rat hepatocytes (10^6 cells/ml) loaded with Fura 2/AM were incubated at 37° C with (●) or without (○) 50 μM menadione in Na^+-free medium. After 30 min, the incubation medium was removed and cell pellets were resuspended in Na^+-containing medium supplemented with 1.3 mM $CaCl_2$ and further incubated 30 min in the presence (●) or in the absence of 5 mM EGTA (□). The results are means of three different experiments ± S.D.
* : statistically different vs controls or cells treated with menadione in the presence of EGTA (p values ranging from $p<0.05$ and $p<0.001$).

The omission of Na^+ from the incubation medium strongly decreases the elevation of cytosolic Ca^{2+} induced by menadione and $PbEt_3$ (Fig. 1A). The causal association between the elevation of cytosolic Ca^{2+} and the influx of Na^+ is supported by the observation that transfering menadione-intoxicated hepatocytes from a Na^+-free buffer to a medium containing 140 mM NaCl causes a rapid increase in cell Ca^{2+} (Fig. 2). This effect is completely prevented when 5 mM EGTA is added to the medium containing NaCl (Fig. 2). On the other hand, the incubation of hepatocytes with 10 μM verapamil and nifedipine does not affect the rise of cytosolic Ca^{2+} induced by menadione in Na^+-supplemented cells (not shown). Thus, a Na^+-dependent Ca^{2+} influx occurres in isolated hepatocyte intoxicated with menadione or $PbEt_3$ and contributes to the massive elevation of cytosolic Ca^{2+} levels caused by the two compounds.

At variance with other well characterized Ca^{2+} transporting systems in cellular membranes Na^+/Ca^{2+} antiporter can mediate either Ca^{2+} influx or efflux depending on prevaling electrochemical gradients (15). Na^+/Ca^{2+} antiporter plays an important role in the regulation of cytosolic Ca^{2+} levels in many excitable tissues, but its role in non-exitable cells is still unclear (15). The presence of Na^+-dependent Ca^{2+} transport has been confirmed in hepatocyte (8,9), however, Lidofsky and coworkers have shown that, Na^+/Ca^{2+} antiporter does not contribute to the physiological regulation of cytosolic Ca^{2+} levels in hepatocytes (10). Recently, Na^+/Ca^{2+} Haigney and colleagues have calculated that in anoxic cardiac myocytes the exchanger Na^+/Ca^{2+} activity would

reverse, favoring Ca^{2+} entry rather than extrusion, when cytosolic Na^+ would exceed 17 mM at a plasma membrane potential of -80 mV (6). Studies in anoxic hepatocytes have demonstrate that oxygen deprivation increased by 2-3 fold cytosolic Na^+ level (4,16). A similar effect has been observed in the present study following hepatocyte intoxication with menadione and $PbEt_3$. Thus, the levels of Na^+ observed in hepatocytes following anoxia or the intoxication with menadione or $PbEt_3$ are sufficient to promote the activity of Na^+/Ca^{2+} exchanger in the reversed mode.

Several mechanisms might account for the elevation of intracellular Na^+ caused by anoxia or toxic injury. First of all, ATP depletion consequent to the collapse of mitochondrial membrane potential (3,10,12) can affect the activity of Na^+/K^+ translocase in the plasma membranes. Furthermore, direct impairement of the enzyme activity caused by $PbEt_3$ (17) and menadione (Carini et al. unpublished result) can worsen the effect of ATP loss. In addition, it should be considered that the impairement of mitochondrial functions causes a decrease in intracellular pH (18) which, in turn, triggers the activation of acid buffering mechanisms involving Na^+/H^+ exchanger and of Na-HCO_3^- cotransporter (19,20). Preliminary experiments in our laboratory have demonstrated that the inhibition of Na^+/H^+ exchanger and Na-HCO_3^- cotransporter prevented the influx of Na^+ caused by menadione intoxication. The importance of Na^+ influx, due to the lowering intracellular pH, in triggering Ca^{2+} entrance also in the absence of cellular injury is demonstrated by experiments where acidosis was stimulated by the addition of 5 mM sodium propionate in the presence of 1 mM ouabain to block Na^+/K^+ translocase (Fig.

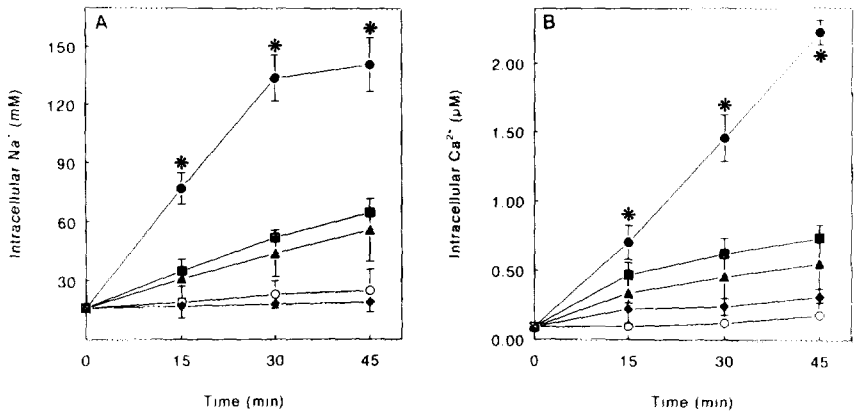

Figure 3. Changes of intracellular Na^+ and Ca^{2+} concentrations in isolated hepatocytes undergoing cytosolic acidification.
Isolated rat hepatocytes (10^6 cells/ml) were incubated in the absence (○) or in the presence of 5 mM sodium propionate and 1 mM ouabain (●) and with propionate and ouabain plus 1 mM DIDS (■) or 1 mM amiloride (▲). Similar experiments were also performed using propionate/ouabain-treated cells incubated in a Na^+-free medium (◆). Intracellular Na^+ was measured by atomic absorbtion (panel A), while cytosolic free Ca^{2+} was evaluated in Fura-2/AM loaded hepatocytes (panel B). The results are means of three different experiments ± S.D.
* : statistically different vs controls or propinate-ouabain-treated cells incubated in the absence of Na^+ or with, respectively, DIDS and amiloride (p values ranging from $p<0.05$ and $p<0.001$).

3A, 3B). Propionate is protonated in the extracellular space and permeates the plasma membrane as propionic acid which dissociates again in the cytosolic compartment decreasing intracellular pH (21). As shown in Figure 3A, the exposure of hepatocytes to sodium propionate and ouabain increases by about 10 fold intracellular Na^+ content and this effect is associated with a marked elevation of cytosolic Ca^{2+}. Conversely, minor changes of cell Ca^{2+} are observed when hepatocytes are incubated with sodium propionate and ouabain in a Na^+-free medium or when 1 mM amiloride or 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene (DIDS) are used to inhibit, respectively, plasma membrane Na^+/H^+ exchanger or Na^+-HCO_3^- cotransporter (Fig. 3B). Taken together these results suggest that during hepatocyte anoxia or following the intoxicaton with compounts that interefere with ATP production, a massive elevation of intracellular Na^+ might favor the reversed activation of a normaly inactive Na^+/Ca^{2+} antiporter in an attempt to counteract the elevation of Na^+. In these conditions, the failure of the mechanisms controlling Ca^{2+} homeostasis can result in a sustained and prolonged elevation of cytosolic free Ca^{2+} that might be involved in the pathogenesis of irreversible cell injury (1).

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References

1. Nicotera, P., Bellomo, G., and Orrenius, S. (1992) Annu.Rev.Pharmacol.Toxicol. 32, 449-470.

2. Farber, J.L. (1990) Environm. Health Perspectives, 84, 107-111.

3. Kehrer, J.P., Jones, D.P., Lemasters, J.J., Farber, J.L., and Jaeschke, H., (1990) Toxicol. Appl. Pharmacol. 106, 165-178.

4. Gasbarrini, A., Borle, A.B., Farghali, H., Francavilla, A., and Van Thiel, D. (1992) J. Biol. Chem. 267,6654-6663.

5. Albano, E., Bellomo, G., Parola, M., Carini, R., and Dianzani, M.U. (1991) Biochim. Biophys. Acta 1091, 310-316.

6. Gasbarrini, A., Borle, A.B., and Van Thiel, D. (1993) Biochim. Biophys. Acta, 1177, 1-7.

7. Haigen, M.C.P., Miyata, H., Lakatta, E.G., Stern, M.D., and Silverman, H.S. (1992) Circul. Res. 71, 547-557.

8. Shanne, F.A.X., and Moore, L. (1986) J. Biol. Chem. 261, 9886-9889.

9. Kofuji, P., Hadley, R.W., Kieval, R.S., Lederer, W.J., and Schulze, D.H. (1992) Am. J. Physiol. 263, C1241-C1249.

10. Lidofsky, S.D., Xie, M.H., and Scharschmidt, B.F. (1990) Am. J. Physiol. 259, G56-G61.

11.Redelgeld, F.A.M., Moison, R.M.W., Barentsen, H.M., Koster, A.S. and Noordhoek, J. (1990) Arch. Biochem. Biophys. 280, 130-136.

12. Bellomo, G., Fulceri, R., Albano, E., Gamberucci, A., Pompella, A., Parola, M., and A. Benedetti (1991) Cell Calcium, 12, 369-377.

13. Albano, E., Bellomo, G., Benedetti, A., Carini, R., Fuceri, R., Gamberucci, A., Parola, M., and Comporti, M. (1994) Chem-Biol. Interacts. 90, 59-72.

14. Peterson, G.L. (1977) Anal. Biochem. 83, 346-356.

15. Carafoli, E. (1987) Annu. Rev. Biochem. 56 395-433.

16. Berger, M.L., Reynolds, R.C., Hagler, H.K, Bellotto, D., Parsons, D., Mulligan, K.J., Buja, M. (1989) Hepatol. 9, 219-228.

17. Munter, K., Athanasiou, M. and Stournaras, C.(1989) Biochem. Pharmacol., 38, 3941-3945.

18. Gore, G.L., Nieminen, A.L., Fleishman, K.A., Dawson, T.L., Herman, B., and Leemaster, J.J. (1988) Am. J. Physiol. 255, C315-C322.

19. Gleeson, O., Smith, N.D., and Boyer, J.L. (1989) J. Clin. Invest. 84, 312-321.

20. Anwer, M.S., and Nolan, K. (1988) Hepatol. 8, 728-734.

21. Hoffmann, E.K, and Simonsen, L.O. (1989) Physiol. Rev. 1989; 69:315-382.