Vol. 203, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1994 Pages 93-98

The Role of Na^+/H^+ Exchange and the Na^+/K^+ Pump in the Regulation of $[Na^+]i$ during Metabolic Inhibition in Guinea Pig Myocytes

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Received July 11, 1994

Summary : To investigate the mechanisms of Na^+ loading during metabolic inhibition (MI), $[Na^+]i$ and pHi were measured in quiescent guinea pig myocytes using fluorescent probes, sodium-binding benzofuran isophthalate and 2,7,bis(carboxyethyl)-5,6-carboxyfluorescein. When myocytes were exposed to MI (3.3mM amobarbital and 5μM carbonyl cyanide *m*-chlorophenylhydrazone, without glucose) for 20 min, $[Na^+]i$ increased from 8.3±0.7 mM to 17.7±1.3 mM ($p<0.01$) and pHi decreased from 7.22±0.03 to 7.00±0.04 ($p<0.05$). The inhibition of Na^+-H^+ exchange by hexamethylene amiloride (HMA) significantly attenuated the increase in $[Na^+]i$ during MI (9.3±0.9 mM ; $p<0.01$ vs MI without HMA). When a K^+-free solution was perfused to inhibit the Na^+/K^+ pump in the presence of HMA, there was an immediate increase in $[Na^+]i$ during MI. Perfusion of a K^+-free solution after 10 min of MI caused no change in the rate of the increase in $[Na^+]i$. We concluded that 1) Na^+/H^+ exchange was an important mechanism for Na^+ elevation during MI, and 2) the Na^+/K^+ pump was functional during the early phase of MI, but was inhibited 10 min after MI in this model.

An excessive accumulation of intracellular Ca^{2+} has been implicated in the pathogenesis of irreversible cell injury during ischemia/reperfusion (1, 2) or hypoxia/reoxygenation (3, 4). The sarcolemmal Na^+-Ca^{2+} exchange has been considered, at least in part, as a route for Ca^{2+} entry into the cell (1, 2, 4, 5). This concept requires that intracellular Na^+ concentration ($[Na^+]i$) increases during the hypoxic or ischemic period, or upon reperfusion. We and other investigators have demonstrated that $[Na^+]i$ increased during hypoxia or ischemia (6, 7, 8). We have also shown that $[Na^+]i$ in quiescent myocytes was determined by the balance between Na^+ influx via Na^+/H^+ exchange and Na^+ extrusion via the Na^+/K^+ pump during normoxic conditions (9). However, the contribution of Na^+/H^+ exchange and the Na^+/K^+ pump in the regulation of $[Na^+]i$ during hypoxia or ischemia remains incompletely resolved. In this study, we used isolated myocytes exposed to metabolic inhibition (MI), and measured $[Na^+]i$ and pHi by the use of ion-sensitive fluorescent probes, sodium-binding benzofuran isophthalate (SBFI) (10) and 2,7, bis (carboxyethyl)-5,6-carboxyfluorescein (BCECF) (3). We have previously reported that these fluorescent indicators were nondestructive and fast responding probes for measuring $[Na^+]i$ and pHi in isolated myocytes (3, 10). The aims of this study were 1) to

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measure the changes in $[Na^+]i$ and pHi during MI, and 2) to assess the role of Na^+/H^+ exchange and the Na^+/K^+ pump in the regulation of $[Na^+]i$ during MI.

Materials and Methods

Calcium tolerant myocytes were isolated from female guinea pigs (400-600 g) by the method previously described (11). The cells were loaded with the acetoxymethyl ester (AM) of 5μM SBFI or 0.5μM BCECF (Molecular Probe Inc.), at room temperature for 30min. A small portion of SBFI or BCECF loaded cells placed in a Perspex bath was mounted on the stage of Nikon TMD inverted microscope. The cells were perfused with a modified Krebs solution (mM) : NaCl 113.1; KCl 4.6 ; $CaCl_2$ 2.45 ; $MgCl_2$ 1.2 ; NaH_2PO_4 3.4 ; $NaHCO_3$ 21.9 ; glucose 10 ; equilibrated with 95% O_2-5% CO_2 (pH 7.4), at room temperature. The cells were illuminated by a transmitted illuminator or ultraviolet (UV) light, via an epifluorecence illuminator from a 100W xenon lamp equipped with an interference filter. Fluorescences of SBFI or BCECF loaded cells were imaged using a Nikon fluor (x20 objective). Images were obtained using a silicon intensified target camera (Hamamatsu Photonics) with the output digitized to a resolution of 512 x 512 pixels by an image processor, ARGUS (Hamamatsu Photonics). The excitation wavelengths were 340 and 380nm for SBFI, and 490 and 450nm for BCECF. All filters had half bandwidths of 10nm. After passing the filters, the exciting light was reflected by dichroic mirrors suitable for each dye (400nm half-pass wavelength for SBFI and 510nm for BCECF). The fluorescent signal was obtained with emission wavelengths at 520nm for SBFI or at 505-560nm for BCECF after background subtraction. Images of fluorescence ratios were obtained by dividing, pixel by pixel, the 340nm image by the 380nm image (SBFI), and by dividing the 490nm image by the 450nm image (BCECF). *In vivo* calibration was conducted using gramicidin (10μM) for SBFI fluorescence, and nigericin (10μM) for BCECF fluorescence, according to the methods previously reported (3,10). For MI, the perfusate contained 3.3mM amobarbital (amytal) and 5μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (12) in the absence of glucose. Results are expressed as means±SE for the indicated number of myocytes obtained from at least 3 guinea pigs. Paired t test and one- or two-way analysis of variance (ANOVA) were used for statistical analyses, and the probability was considered significant at $p<0.05$.

Results

Changes in $[Na^+]i$ and pHi during MI with or without glucose

Fig. 1A illustrates the changes in $[Na^+]i$ during MI with or without glucose. Perfusion of glucose-free MI for 20 min increased $[Na^+]i$ from 8.3±0.7 mM to 17.7±1.3 mM ($p<0.01$). When myocytes were exposed to MI in the precence of 10mM glucose, $[Na^+]i$ showed a small but significant increase from 8.2±0.8 mM to 9.3±0.8 mM ($p<0.05$), and this increase was significantly lower than that of MI without glucose ($p<0.01$ by ANOVA).

Fig. 1B shows the changes in pHi during 20 min of MI in the presence or absence of glucose. When glucose was absent, pHi decreased from 7.22±0.03 to 7.00±0.04 ($p<0.05$) after 10 min, and there were no further changes during the following 10 min. When glucose was present, pHi decreased from 7.26±0.05 to 7.18±0.05 ($p<0.05$) after 20 min of MI, and this change was less severe than that of MI without glucose ($p<0.05$ by ANOVA).

Effects of hexamethylene amiloride (HMA) on the changes in $[Na^+]i$ during MI

Fig. 2 demonstrates the changes in $[Na^+]i$ during MI with or without HMA, a specific blocker of Na^+-H^+ exchange (13). $[Na^+]i$ decreased from 8.2±0.8 mM to 7.2±0.9 mM (n.s.) after 20 min perfusion of 1μM HMA, and there was no change in $[Na^+]i$ following a further 20 min perfusion. When cells were exposed to MI in the continued presence of 1μM HMA for 20

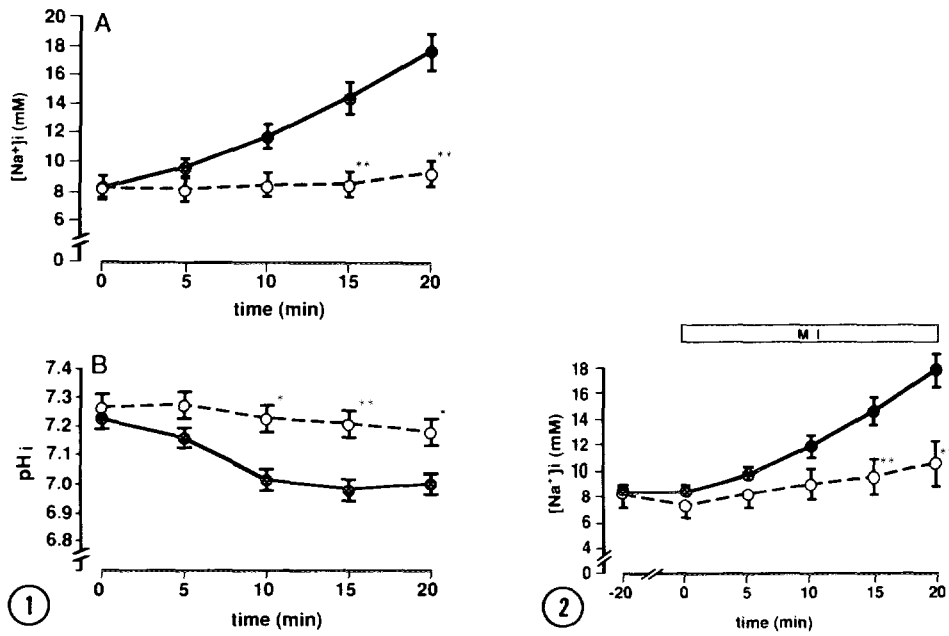

Fig. 1. Effects of MI on [Na⁺]i and pHi during metabolic inhibition (MI). Myocytes were incubated with metabolic inhibitors, 3.3 mM amobarbital and 5 μM CCCP. A: The values of [Na⁺]i in the absence of glucose (●; n=22) and in the presence of glucose (O; n=15). B : The values of pHi in the absence of glucose (●; n=28) and in the presence of glucose (O; n=17). Values are means±SE. *p<0.05, **p<0.01vs MI without glucose by two-way ANOVA.

Fig. 2. Effects of HMA on the changes in [Na⁺]i during MI. The values of [Na⁺]i during MI with HMA (O; n=13) and without HMA (●; n=22). In the HMA group, HMA (1μM) was present from 20 min before MI and during the entire period of MI. MI was applied at 0 min and the values at 20 min before the exposure of MI (-20 min) were included. Values are means±SE. **p<0.01 vs MI without HMA by two-way ANOVA.

min, $[Na^+]i$ increased to 9.3±0.9 mM, which was significantly lower than that of MI without HMA ($p<0.01$ by ANOVA). These results suggested that Na^+-H^+ exchange played an important role in the increase of $[Na^+]i$ during MI.

Effects of the reactivation of Na^+/K^+ pump on the changes in $[Na^+]i$ during MI

Fig. 3 shows the changes in $[Na^+]i$ when myocytes were exposed to a K^+-free solution, which would inhibit the Na^+/K^+ pump. To prevent immediate Ca^{2+} loading of myocytes exposed to a K^+-free solution (11), Ca^{2+} was removed and 1mM ethylene glycol-bis(ß-aminoethyl ether)-N,N,N',N',-tetraacetic acid (EGTA) was added from the beginning of MI. We have previously shown that there was no significant difference in the increase of $[Na^+]i$ during K^+-free perfusion with or without Ca^{2+} (11). $[Na^+]i$ increased from 6.7±0.2 mM to 25.9±1.4 mM ($p<0.01$) during normoxic perfusion, and from 6.7±0.5 mM to 33.3±2.7 mM ($p<0.01$) during MI. The readmission of 5mM K^+ ($5K^+$), which reactivates the Na^+/K^+ pump, could partly reverse $[Na^+]i$ during normoxic perfusion, but could not reverse $[Na^+]i$ during MI. It was suggested that the Na^+/K^+ pump was significantly inhibited after 10 min of MI.

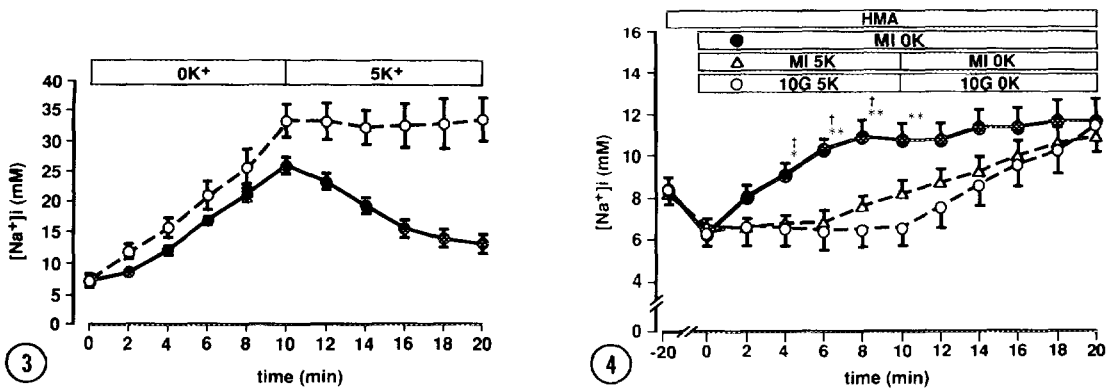

Fig. 3. Efects of the reactivation of the Na^+/K^+ pump on the changes in $[Na^+]i$ during MI. The values of $[Na^+]i$ when myocytes were perfused for 10 min with the solution in which K^+ and Ca^{2+} were simultaneously omitted during normoxic perfusion (●; n=19) and during MI (O; n=28). Five mM K^+ was reintroduced (5 K^+ in the figure) to reactivate the Na^+/K^+ pump. Values are means±SE.

Fig. 4. Effects of a K^+-free solution on the changes in $[Na^+]i$. After 20 min preincubation with HMA (1μM), myocytes were exposed to $5K^+$ for 10 min and the solution was switched to a K^+-free solution for further 10 min in the normoxic condition (O; n=16) or during MI (Δ; n=25). In another group, myocytes were perfused with a K^+-free solution for 20 min during MI (●; n=14). MI was applied at 0 min and the values at 20 min before the exposure of MI (-20 min) were included. Values are means±SE. *$p<0.05$, **$p<0.01$ vs (O) by one-way ANOVA. †$p<0.05$ vs (Δ) by one-way ANOVA.

Effects of Na^+/K^+ pump inhibition on the changes in $[Na^+]i$ during MI

Although HMA prevented the rise of $[Na^+]i$ significantly, there was still a small but steady increase in $[Na^+]i$ during MI and the activity of the Na^+/K^+ pump was significantly inhibited after 10 min of MI. We examined whether the decrease in the extrusion of Na^+ via the Na^+/K^+ pump contributed to the rise of $[Na^+]i$ during MI. The MI solution containing 5 mM K^+ was switched to a K^+-free solution in the presence of 1μM HMA, and Ca^{2+} was omitted and 1 mM EGTA was added to the solution. This could emphasize the effect of the inhibition of the Na^+/K^+ pump on $[Na^+]i$ by excluding the contribution of Na^+-H^+ exchange. During normoxic perfusion, there was no increase in $[Na^+]i$ when myocytes were incubated with $5K^+$ for 10 min. The switch to a K^+-free solution increased $[Na^+]i$ from 6.4±0.8 mM to 11.4±1.4 mM ($p<0.01$) during the following 10 min (Fig. 4; open circle). This K^+ removal produced different results during MI. During the initial perfusion of the $5K^+$ solution, $[Na^+]i$ was kept constant until 6 min, but began to increase from 8 min and was 8.1±0.7 mM at 10 min. The switch to a K^+-free solution after 10 min, however, did not affect the rate of the increase in $[Na^+]i$ (open triangle). When a K^+-free solution was perfused from the beginning of MI, $[Na^+]i$ began to increase immediately and reached 11.6±1.2 mM at 20 min (closed circle). There were no differences in the maximum values of $[Na^+]i$ at 20 min among the three groups.

Discussion

The role of Na^+-H^+ exchange during MI

There was an increase in $[Na^+]i$ during MI in this study, and a similar finding has been reported in other models of hypoxia/ischemia (4, 7, 14). Three possible pathways for Na^+ in the sarcolemma include, 1) the voltage dependent Na^+ channel, 2) Na^+/H^+ exchange, and 3) the Na^+/K^+ pump. In this model, the contribution of the Na^+ channel to the increase in $[Na^+]i$ is unlikely, since experiments were performed in quiescent myocytes, and we have demonstrated that 10 μM TTX did not change the increase in $[Na^+]i$ during MI in unstimulated myocytes (6).

There was a rapidly developing acidosis during MI. When myocytes were exposed to MI in the presence of glucose, the fall of pHi was less than that during MI without glucose. Although the exact mechanism for the difference is unknown, it has been reported that acidosis produced by MI depends on the balance between phosphocreatine hydrolysis (consuming protons) and the break-down of ATP (releasing protons) (14). The more rapid break-down of ATP during MI in the absence of glucose could, therefore, account for the larger decline of pHi. Since extracellular pH was constant (=7.4), the trans-sarcolemmal H^+ gradient would have stimulated Na^+/H^+ exchange (15) and have resulted in Na^+ loading. Our results show that the inhibition of Na^+/H^+ exchange by HMA prevented the increase of $[Na^+]i$, and therefore support the idea that Na^+/H^+ exchange is one of the main pathways for Na^+ loading during MI. The larger increase in $[Na^+]i$ during MI in the absence of glucose could be due to the more active Na^+/H^+ exchange induced by a larger decrease in pHi.

The inhibition of the Na^+/K^+ pump during MI

The inhibition of the Na^+/K^+ pump could be another contributing factor to the larger increase in $[Na^+]i$ during MI without glucose. When a K^+-free solution was perfused in the presence of HMA from the beginning of MI, $[Na^+]i$ increased soon after the exposure to MI. In contrast, there was no increase in $[Na^+]i$ during the initial 6 min when myocytes were exposed to MI with $5K^+$. The most possible explanation for the lower $[Na^+]i$ during MI with $5K^+$ is that the Na^+/K^+ pump was active in this period. $[Na^+]i$ began to increase from 8 min after the exposure to MI with $5K^+$. It has been reported that when rat myocytes were incubated with 3mM amytal and 2μM CCCP, ATPi fell dramatically and reached minimum by 5 min (12). Assuming that normoxic cellular ATPi was 8 mM (5), and that ATPi fell to approximately 2.5% of the normoxic level after MI (12), the calculated ATPi level would be 0.2 mM. This value is comparable to the reported Michaelis constant for the Na^+/K^+ pump (0.1mM) (16). It is, therefore, possible that ATPi depletion during MI could cause the time-dependent inhibition of the Na^+/K^+ pump, resulting in a gradual increase in $[Na^+]i$. We can not exclude the possibility that the Na^+/K^+ pump was inhibited by the decline of pHi, since the Na^+/K^+ pump activity has been reported to be dependent on pH (17). However, Deitmer and Ellis showed that altering the pHo (and thereby pHi) from 8.4 to 6.4 did not affect the activity of the Na^+/K^+ pump in sheep Purkinje fibers (18).

The activity of the Na^+/K^+ pump during ischemia/hypoxia has been extensively investigated using an enzymatic method (19), histochemical assay and immunocytochemical

method (20), *in vivo* uptake of ouabain (21), and ion-sensitive microelectrodes (8, 22, 23). Previous studies have demonstrated the incomplete inhibition of the Na^+/K^+ pump during hypoxia/ischemia in sheep Purkinje fibers (8), guinea pig papillary muscles (22), and canine ventricular muscles (23). In our experimental condition, the reactivation of the Na^+/K^+ pump did not reverse the elevated $[Na^+]i$, and the inhibition of the Na^+/K^+ pump caused no change on the rate of rise in $[Na^+]i$ after 10 min of MI. It is likely that the Na^+/K^+ pump had been already inhibited by 10 min after MI. Accumulation of K^+ in the intercellular clefts is known to occur during hypoxia/ischemia in multicellular preparations (22, 23), which could partly activate the residual Na^+/K^+ pump. Therefore, the conflicting results could be explained by the difference in experimental models.

In conclusion, this study suggests that 1) $[Na^+]i$ increased substantially and pHi decreased during MI. The changes in $[Na^+]i$ and pHi were dependent on the availability of substrate. 2) Na^+-H^+ exchange was identified as an important mechanism for Na^+ accumulation during MI. 3) the Na^+/K^+ pump was functional during the early phase of MI, but was inhibited by 10 min after MI in this model.

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