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Calcium and Osmotic Regulation of the Na^+/H^+ Exchanger in Neonatal Ventricular Myocytes

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A. N. Moor, R. Murtazina and L. Fliegel. Calcium and Osmotic Regulation of the Na^+/H^+ exchanger in Neonatal Ventricular Myocytes. *Journal of Molecular and Cellular Cardiology* (2000) **32**, 925–936. Intracellular pH regulation in primary cultures of neonatal cardiac myocytes has been characterized. Myocytes were exposed to hyperosmolar solutions to examine the effects on pH regulation by the Na^+/H^+ exchanger. Exposure to 100 mM NaCl, sorbitol, N-methyl-D-glucamine, or choline chloride all caused significant increases in steady state pH_i in myocytes. Omission of extracellular calcium or administration of calmodulin antagonists reduced the osmotic activation of the exchanger. The myosin light-chain inhibitor ML-7 completely blocked osmotic activation of the exchanger suggesting that myosin light-chain kinase is involved in osmotic activation of the exchanger in the myocardium. The calmodulin-dependent protein kinase II inhibitor KN-93 inhibited the rate of recovery from an acute acid load as did trifluoperazine (TFP) and the calmodulin blocker W7, [N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide]. Addition of the calcium ionophore ionomycin caused a large increase in resting pH_i in isolated myocytes. However, this effect was largely resistant to HMA (5-(N,N-hexamethylene)-amiloride) indicating that an alternative mechanism of pH_i regulation is responsible. The results demonstrate that the Na^+/H^+ exchanger of the neonatal myocardium is responsive to calcium and osmotically responsive pathways and that myosin light-chain kinase is a key protein involved in mediating the osmotic response.

Key Words: intracellular pH; calmodulin; osmotic stress; cardiomyocytes; pH regulation.

Introduction

The Na^+/H^+ exchanger is an integral membrane glycoprotein that functions to exchange one intracellular proton for one extracellular sodium in response to intracellular acidification. Several isoforms of the Na^+/H^+ exchanger exist which have been designated NHE1 to NHE6 for isoforms one to six. NHE1 was the first cloned[1] and is the most widespread isoform with the other isoforms having a more restricted distribution. It is more sensitive to amiloride and its derivatives than the other isoforms.[2–4] In the myocardium the NHE1 isoform is the only plasma membrane isoform present in significant amounts in the sarcolemma[5–10] and it has recently been localized to the intercalated disk and t-tubule regions of cardiomyocytes.[11] Other pH regulatory proteins also contribute to removal of protons or their equivalent from the myocardium. However, the Na^+/H^+ exchanger is the major pH regulatory protein, especially under conditions of acute acidosis which occur during ischemia.[12–14]

In the heart, the Na^+/H^+ exchanger is involved in tissue injury which occurs during myocardial ischemia and reperfusion. During ischemia intracellular pH (pH_i) drops and protons accumulate.

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Abbreviations used: BCECF-AM, 2′,7′bis(2-Carboxylethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; CaMKinaseII, calmodulin-dependent protein kinase II; HMA, 5-(N,N-hexamethylene)-amiloride; MAP-kinase, mitogen activated protein kinase; TFP, trifluoperazine; W7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

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Na^+/H^+ exchanger activity during ischemia and reperfusion causes removal of the intracellular protons and results in accumulation of intracellular Na^+. The increased levels of intracellular Na^+ may cause reduced extrusion of Ca^{2+} by the Na^+/Ca^{2+} exchanger or Ca^{2+} entry may also occur by this bi-directional exchanger.[15–18] Excess Ca^{2+} may therefore accumulate and is believed to cause detrimental effects including cell necrosis, contracture and arrhythmias. Amiloride derivatives are successful in blocking this cycle of damage to the myocardium. In addition, a new class of anti-ischemic guanidinium derivative compounds has proven useful for this purpose including Hoe 694 and Hoe 642 (cariporide).[15–17]

The human NHE1 protein has been cloned from the myocardium.[6] The deduced NHE1 protein is 815 amino acids and is thought to consist of a 500 amino acid membrane domain with 12 integral membrane segments and a long intracellular carboxyl-terminus of about 315 amino acids.[3] Regulation of the Na^+/H^+ exchanger in the myocardium has not been well studied. However, in other tissues it has been shown that the cytoplasmic domain is responsible for regulation of NHE1 activity.[4–18] Growth factors can act to shift the set point of the exchanger such that it is more active in a more alkaline pH range.[19] Distal regions of this domain are involved in regulation by phosphorylation. It has been shown that phosphorylation by MAP kinase[20,21] and CaM Kinase II[22] may be involved in regulation of the Na^+/H^+ exchanger in the myocardium.

While the distal region of the exchanger is involved in regulation by phosphorylation, more proximal regions are involved in regulation which is phosphorylation independent.[23,24] In this regard it has been shown that calcium, calmodulin and osmotic regulation are important regulators of the exchanger in several tissues.[25–27] In addition, upon exposure to hyperosmotic solutions, the Na^+/H^+ exchanger responds with rapid increases in activity that results in cytosolic alkalinization. This is part of a regulatory volume increase in cells which compensates for skrinkage induced by external hyperosmolarity.[23]

It is clear that calcium and osmotic regulation of the exchanger are important regulators of the Na^+/H^+ exchanger in other tissues. Also, that calcium plays an important role in the injury to the myocardium that occurs during ischemia and reperfusion (see above). To further our understanding of pH_i regulation in the neonatal myocardium, we examined pH_i regulation in primary cultures of isolated neonatal cardiomyocytes. The newborn myocardium is vulnerable to the tissue injury which occurs during myocardial ischemia and reperfusion and is mediated by the Na^+/H^+ exchanger.[28,29] It has been suggested that the newborn myocardium is more vulnerable than the adult to ischemic injury.[30–32] While it clear that the newborn myocardium expresses the NHE1 isoform of the protein, it is known that regulation of expression of the protein varies from the adult. In the newborn heart the expression of the exchanger is greatly elevated.[33] We therefore examined calcium, calmodulin and osmotic regulation of the activity of the Na^+/H^+ exchanger in the neonatal myocardium. The results show that calcium and calmodulin play important roles in regulation of the exchanger in the young cardiomyocyte. The newborn Na^+/H^+ exchanger of cardiomyocytes is also very responsive to osmotic stress. The results suggest that activation by calcium/calmodulin or osmotic stress might further exacerbate the role the exchanger plays in damage to the myocardium during ischemia and reperfusion. Our results are the first detailed examination of the role of calcium/calmodulin myosin light-chain kinase and osmotic regulation of the Na^+/H^+ exchanger in the neonatal myocardium.

Materials and Methods

Materials

Collagenase Type 2 was obtained from Worthington Biochemical Corporation (Lakewood, NJ). BCECF-AM was purchased through Molecular Probes (Eugene, OR). TFP, W7, nigericin and ionomycin were obtained from Sigma (St. Louis, MO). HMA (5-(N, N-hexamethylene)-amiloride) and PD123319 were from Research Biochemicals International (Natick, MA). KN-93 and ML-7 were purchased from Calbiochem (La Jolla, CA). All other chemicals were of analytical grade and were purchased from Fisher Scientific (Ottawa, ON), Sigma (St. Louis, MO) or BDH (Toronto, ON).

Primary cultures of neonatal myocytes

Primary myocyte cultures were prepared from neonatal Sprague Dawley rats as described previously.[34] Briefly, hearts were removed from 4–6 day-old rats under aseptic conditions and ventricles minced to small size. The tissue was digested with a series of

treatments with 0.1% collagenase at 37°C. Dissociated cells were incubated in Corning T-25 culture flasks at 37°C in a humidified atmosphere (5% CO_2, 95% air) for 20 minutes. During this time nonmyocytes (fibroblasts, endothelial cells and smooth muscle cells) attach and the majority of myocytes remain in suspension. Subsequently, myocytes were removed and plated onto glass coverslips for pH_i measurements. Myocytes were maintained for 4–5 days in medium containing Dulbecco's modified Eagle's medium (DMEM/F12) supplemented with 10% fetal bovine serum (FBS), 10 μg/ml transferrin, 10 μg/ml insulin, 10 ng/ml selenium, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mg/ml bovine serum albumin (BSA), 5 μg/ml linoleic acid, 3 mM pyruvic acid, 0.1 mM minimum essential medium (MEM) non-essential amino acids, 10% MEM vitamin, 0.1 mM bromodeoxyuridine, 100 μm L-ascorbic acid and 30 mM HEPES, pH 7.1. Myocytes were serum-starved overnight prior to all experiments.

Cell culture and transfections

AP-1 cells[35] are derived from wild-type Chinese hamster ovary (CHO) cells that have lost their endogenous Na^+/H^+ exchanger activity. Cells were grown as described earlier.[36] Briefly, cells were maintained in alpha-MEM medium supplemented with 10% FBS and 25 mM pH 7.4, and incubated in a humidified atmosphere with 5% CO_2, 95% air at 37°C. The plasmid pYN4+ encodes for the intact mammalian NHE1 protein. It was used for stable expression of the NHE1 gene in mammalian cells, as we have described earlier.[36] Stably transfected AP-1 cells were used to characterize osmotic regulation of the intact exchanger in this cell line. Cells were subcultured onto glass coverslips for measuring pH_i.

Measurement of pH_i

pH_i regulation by the Na^+/H^+ exchanger was examined in isolated neonatal myocytes and AP-1 cells. Myocytes or AP-1 cells were grown on glass coverslips and the acetoxy methyl ester of 2′-7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM) was used to measure pH_i. Cells on coverslips were placed into a holder device and inserted into a 1×1 cm fluorescence cuvette at 37°C and bathed in a normal buffer containing 137 mM sodium chloride, 1.6 mM magnesium sulfate, 5.4 mM potassium chloride, 1.2 mM sodium dihydrogen orthophosphate, 20 mM HEPES, 15 mM D-glucose and 1.0 mM calcium chloride pH 7.1. For experiments performed with calcium-free buffer, calcium chloride was omitted from the above buffer. BCECF-AM (1 μg/ml) was added for 5 minutes, after which coverslips were bathed in fresh buffer.[37] Intracellular pH was measured using the dual excitation single emission ratio technique using a temperature-controlled Shimadzu RF5000 spectrofluorophotometer. Excitation wavelengths were at 452 and 503 nm with the emission wavelength at 524 nm. This allowed measurement of pH_i that was independent of cell concentration and loading.[38] A calibration curve for intracellular dye was generated using the K^+/nigericin method as previously described.[36,38] Steady-state pH_i and rates of recovery from acid load were measured following an acid challenge using the ammonium chloride prepulse method as previously described.[39] The initial pH prior to acid loading was usually 7.0–7.1. To produce an acid load, ammonium chloride (20–30 mM) was added for 4 minutes followed by a pH recovery that was obtained by transferring the cells to normal buffer containing 137 mM NaCl after a brief exposure to a Na^+-free buffer (approximately 30 s). For measuring initial rate of recovery from an acid load the initial changes in pH_i were examined during the first 10–20 s after NaCl addition.

The proton flux was calculated for all experiments involving rate of recovery from an acid load as we have described earlier using buffering capacity.[40] Buffering capacity was determined at various pH_i by varying amounts of NH_4Cl. Proton flux (J_{H^+}) produced due to the Na^+/H^+ exchanger after acid loading, was calculated from the buffer capacity at mid point pH × delta pH/delta time.[40]

Statistics

Analysis of results of pH_i measurements was carried out using a Mann-Whitney U test. Values presented are mean ± SEM with at least four and up to eight experiments being done in every case; *P*-values <0.05 were considered statistically significant.

Results

The isolated ventricular myocytes were initially characterized by testing their activity in the presence or absence of various agonists known to stimulate Na^+/H^+ exchanger activity. The effects on the rate of recovery (protein efflux per minute) were

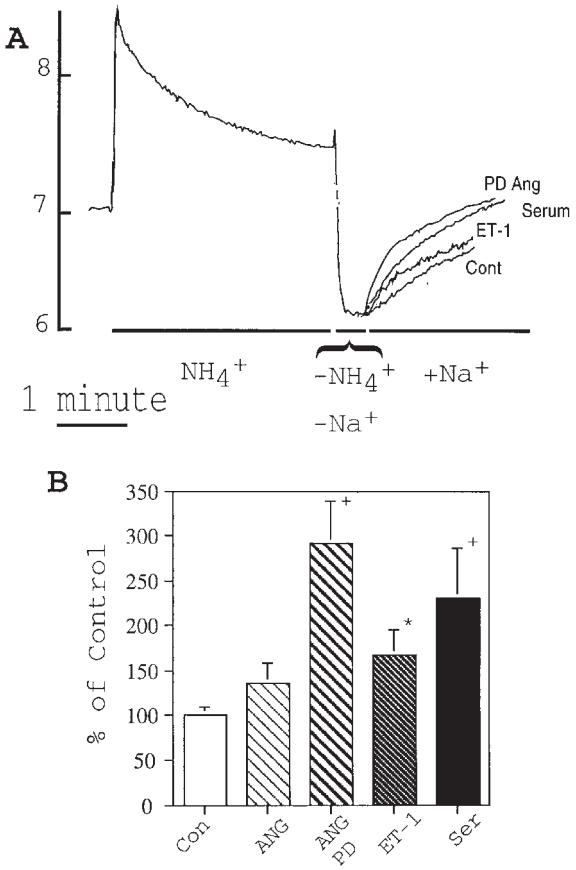

Figure 1 Recovery from an acute acid load by neonatal ventricular myocytes stimulated with agents that activate Na^+/H^+ exchanger activity. Ammonium chloride prepulse was used to induce acute acidosis (NH_4^+) for the time period indicated by the solid line. After ammonium chloride, cells were subjected to a brief period in Na^+-free buffer ($-Na^+$) in the absence of NH_4^+ ($-NH_4^+$). The pH_i recovery was then measured in the presence of 135 mM NaCl ($+Na^+$). (A) Representative experiments of intracellular pH recovery of myocytes stimulated with; angiotensin II (100 nM) in the presence of PD123319 (PDAng), serum (10%), endothelin (ET-1) (0.5 nM) or left unstimulated as controls (Cont). Stimulatory agents were added to the NH_4^+ containing buffer, the Na^+-free buffer and the recovery buffer containing Na^+. (B) Bar graph summarizing the results of pH_i recovery from an acute acid load. Results are mean $\pm$ SE of 8–10 experiments. (+) indicates signficantly different from control values at $P<0.005$ or $P<0.05$ (*).

examined from an ammonium chloride induced acid load as described in the "Material and Methods". Angiotensin II in the presence of the angiotensin II receptor subtype 2 (AT_2) antagonist (PD123319), endothelin (ET-1) and serum were used to activate the exchanger. Figure 1(A) shows examples of the effects on the rate of recovery from an acute acid load. The tracing for angiotensin II was not included as it was not significantly different from the controls [see Fig. 1(B)]. All experiments were compared to the absolute control value of proton efflux which was 1.129 ± 0.102 mM/min and was set at 100%. The mean acidification of controls and experimentals after ammonium chloride was within 0.1 pH units of each other and was $6.3 \pm .03$ for the controls. Angiotensin II (100 nM) by itself had no significant effect on protein efflux (136% of control value), while angiotensin II (100 nM) in the presence of the AT_2 receptor antagonist PD123319 significantly increased proton efflux (291%). Serum (10%) and ET-1 (0.5 nM) also increased proton efflux (229% and 167%). Figure 1(B) summarizes the effects of the agonists on the rate of recovery from an acid load. The data confirmed the viability of the neonatal ventricular myocytes used in this study and their sensitivity to stimulation by physiological agonists.

The authors[20] and others[41] have previously shown that in CHO and other cell types the Na^+/H^+ exchanger can be activated by hypertonic stimulation (100 mM NaCl). To ensure that we have a responsive system for stimulation and monitoring of cells we duplicated this effect using 2 agents, NaCl (100 mM) and the sugar sorbitol (100 mM). AP1 cells that were stably transfected with the Na^+/H^+ exchanger were exposed to a brief ammonium chloride prepulse and a 5 min recovery from an acid load as described in "Materials and Methods". The effects on steady-state pH_i were then measured after the addition of either NaCl or sorbitol. Figure 2(A) shows examples of the effects obtained during measurements of steady-state pH_i. NaCl and sorbitol both caused marked elevations of intracellular pH indicating activation of the Na^+/H^+ exchanger. Fig. 2(B) shows a summary of the effects. The absolute value of the change in pH_i for adding 100 mM NaCl was 0.2239 ± 0.0356 which was set at 100% in the figure. After 5 min of NaCl stimulation the pH_i rose a further 27% of this value. Control, unstimulated cells changed less than 5% of the stimulated value for both the 2 and 5-min time points. The addition of sorbitol significantly increased steady-state pH_i values at both time points to values of 51% and 72% of the amount of stimulation of the 2-min NaCl value. HMA (10 μM) eliminated the effects of both sorbitol and NaCl (not shown). Therefore, both agents create a hypertonic environment for the AP-1 cells and this stimulated Na^+/H^+ exchanger activity and raised pH_i through the activity of this protein.

The same type of stimulation was then used to examine if primary cultures of neonatal cardiac myocytes exhibited similar responsiveness to hypertonic stimulation. A variety of different methods of

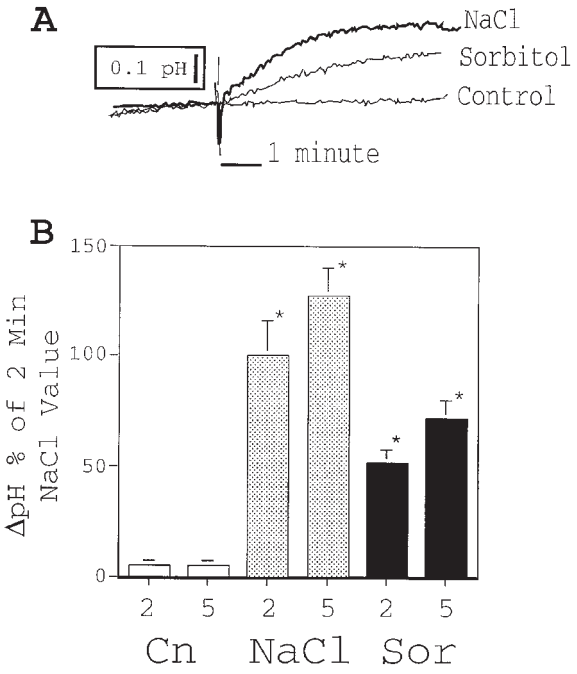

Figure 2 Effect of osmotic stress on steady-state pH_i of AP-1 cells stably transfected with NHE1. After the addition of either NaCl (100 mM) or sorbitol (100 mM) the changes from resting pH_i were measured for 5 minutes and results were summarized at 2 and 5 min intervals. (A) Examples of typical effects of addition of NaCl or sorbitol on resting pH_i. Results are mean ± SE of 3–4 experiments. (*) indicates significantly different from control values at $P<0.05$.

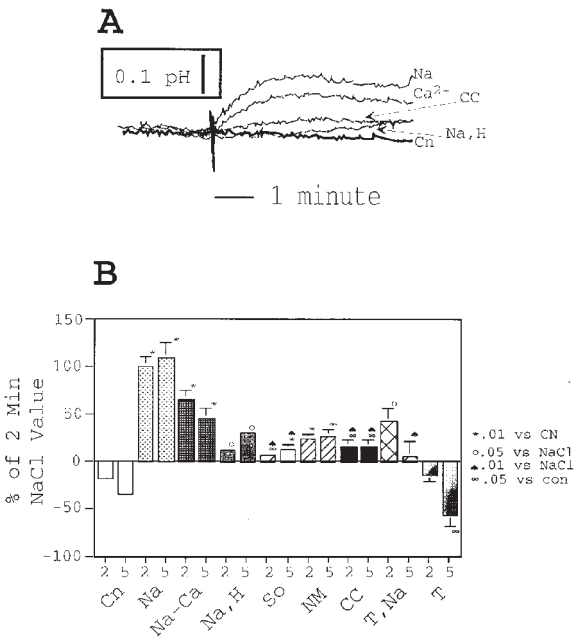

Figure 3 Effect of osmotic stress on steady-state pH_i of neonatal ventricular myocytes. After the addition of either NaCl (100 mM), sorbitol (100 mM), N-methyl-D-glucamine (100 mM) or choline chloride (100 mM) the changes from resting pH_i were measured for 5 min and results were summarized at 2 and 5 min intervals. (A) Examples of typical effects of NaCl (Na), NaCl in calcium-free buffer (Na-Ca^{2+}), choline chloride (CC) and NaCl in the presence of HMA (10 μM) (Na, H) on steady-state pH_i. (B) Bar graph summarizing the effects of the above agents including sorbitol (So), N-methyl-D-glucamine (NM), NaCl in the presence of TFP (60 μM) (T, Na) and TFP alone (T). Results are mean ± SE of 4–8 experiments. Where not shown SE was too small to be displayed.

hypertonic stimulation were tested, and examined the effect of removal of calcium from the buffer and the effect of inhibiting calmodulin, the calcium binding protein associated with the C-terminus of the exchanger.[42] The effects on steady-state pH_i were then measured as described in the "Materials and Methods" after the addition of either NaCl (100 mM), sorbitol (100 mM), N-methyl-D-glucamine (100 mM), or choline chloride (100 mM). Figure 3(A) is a typical example of physiologic tracings obtained during measurements of steady-state pH_i. The data were also summarized and compared to initial pH_i readings (ΔpH) after measurement at 2 and 5 min after treatment [Fig. 3(B)]. The addition of NaCl significantly increased steady-state pH_i. At 2-min after addition the increase was 0.1042 ± 0.0099 and this value was set at 100%. The changes for controls were −20 and −40% at the 2 and 5 min time points respectively. The reason for this slight decline in resting pH_i is not yet clear at this time but was quite reproducible. In the absence of calcium, the addition of NaCl still significantly increased steady-state pH_i at both time points 73% and 51% compared to the 2-min NaCl value in the presence of calcium. However the response to NaCl was lower compared to the response achieved in the presence of calcium. Pre-incubating the cells with HMA (10.0 μM) significantly blocked the response to the additional NaCl [Fig. 3(B)] however there was still a slight increase in intracellular pH. Steady-state pH_i values, with the addition of either sorbitol (7% and 13%), N-methyl-D-glucamine (26% and 29%) or choline chloride (16% and 16%), were all significantly increased when compared to control unstimulated cells. However, the responses to these three agents were significantly lower compared to the response to NaCl. Increasing the concentration of sorbitol to 200 mM instead of 100 mM, did not appreciably increase its effectiveness (not shown). HMA (10 μM) completely blocked the stimulatory effect of N-methyl-D-glucamine (not shown). Finally, when the calmodulin inhibitor TFP (60.0 μM) was used the response to NaCl was significantly decreased at both time points (47% and 5%) compared to NaCl alone. When TFP alone was

used at the 2 minute time point, steady-state pH_i was similar to controls (−16%). However, a decrease in steady-state pH_i was observed at the 5 min time point (−65%) compared to controls. Thus, TFP had an effect on steady-state pH_i and had a major effect on the response of the Na^+/H^+ exchanger to an osmotic stress.

The Na^+/H^+ exchanger has been previously shown to bind the calcium binding protein calmodulin.[25] In addition, our earlier evidence has suggested that CaM dependent protein kinase II may be involved in regulation of the protein.[22] Thus, we examined the regulation of the Na^+/H^+ exchanger activity by calmodulin in the presence of two inhibitors of calmodulin, TFP (60 μM) and W7 (60 μM). All of the experiments were performed in the presence of external calcium chloride (1.0 mM). The effects of TFP and W7 were compared on the rate of recovery (protein efflux per min) from an ammonium chloride induced acid load as described above. The mean acidification of the groups was within 0.13 pH units of each other and that of the control was 6.2 ± 0.06. Figure 4(A) is a typical example of physiologic tracings obtained during measurements of the rate of recovery from acid load. Both calmodulin antagonists TFP and W7 significantly reduced the rate of recovery from acid load compared to controls (Fig. 4). The CaM-dependent protein kinase inhibitor KN-93 (3.0 μM) had no effect on the rate of recovery at low concentrations, while a higher concentration of KN-93 (10.0 μM) significantly reduced the rate of recovery compared to controls. Figure 4(B) is a bar graph summarizing the effects of the above inhibitors on the rate of recovery from an acid load. Results are represented as a percentage of all the control proton efflux per minute data and all inhibitors were compared with the activity of controls done on the same preparation of cells on the same day.

ML-7 is an inhibitor of myosin light-chain kinase. It was used to examine the role of this kinase in activation of the Na^+/H^+ exchanger in this tissue. When cells were pretreated with this inhibitor it eliminated the stimulatory effect of NaCl almost completely (Fig. 5). ML-7 alone did not decrease the resting pH_i of the cells (not shown).

To further investigate the role that calcium plays in regulating Na^+/H^+ exchanger activity, ionomycin, a calcium ionophore, was added and the effects on steady-state pH_i were measured as described above. Ionomycin (1.0 μM) was added in the presence or absence of external calcium chloride (1.0 mM) for 5 min. The potent inhibitor of the Na^+/H^+ exchanger, HMA (10.0 μM), was used prior to the addition of ionomycin (5.0 μM) to see if

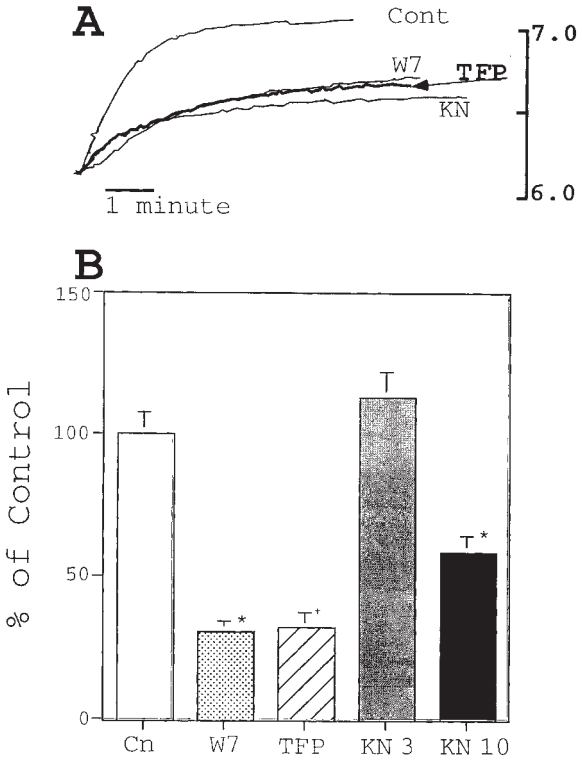

Figure 4 Recovery from an acute acid load by neonatal rat ventricular myocytes treated with inhibitors of calmodulin and calmodulin-dependent protein kinase II. Ammonium Chloride prepulse and stimulatory agents were added similar to as described in Figure 1. (A) Intracellular pH recovery of untreated myocytes (Cont), pretreated for 30 minutes with either W7 or TFP (60 μM), and pretreated for 30 minutes with KN-93 (10 μM). (B) Bar graph summarizing the results of pH_i recovery from acute acid load. Results are mean ± SE of 6 experiments. (+) indicates significantly different from control values at $P<0.005$ or $P<0.05$ (*).

ionomycin would have any effects on steady-state pH_i after the Na^+/H^+ exchanger had been blocked. Figure 6(A) is a typical example of the results and Figure 6(B) summarizes the data obtained during measurements of steady-state pH_i. Ionomycin (1.0 μM) in the presence of external calcium, significantly increased steady-state pH_i at the 5-min time points (0.2818 ± 0.0179) which was designated as 100% for comparison purposes. At 2 min of stimulation with ionomycin the stimulation was about half the 5-min value. The changes in unstimulated controls (in the presence of calcium) were −7 and −15% respectively. Another set of controls (tested in the absence of calcium) was also measured and was not significantly different from controls tested in the presence of calcium (−7 and −16%). In the absence of calcium, it was notable that ionomycin did not affect steady-state pH_i (−10 and 10% at the 2- and 5-min time points). A

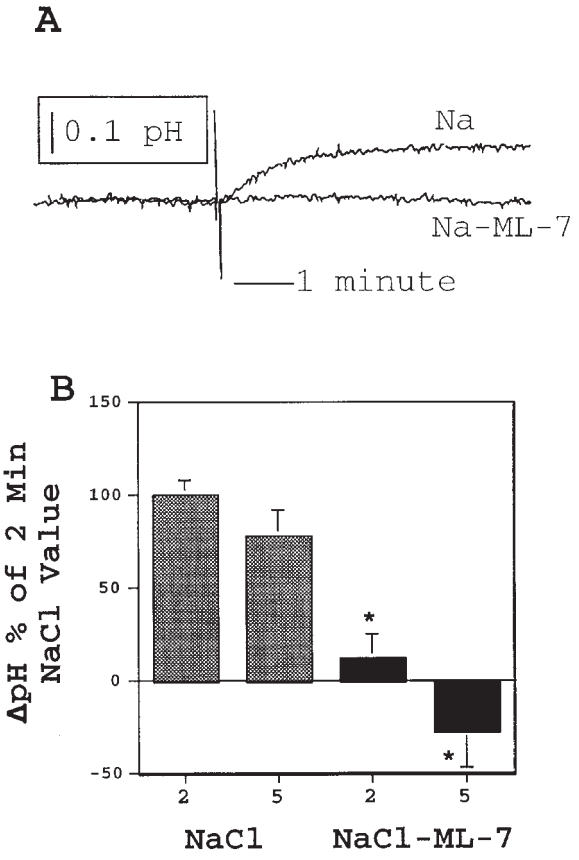

Figure 5 Effect of the myosin light-chain kinase inhibitor ML-7 on the response of neonatal rat ventricular myocytes to osmotic stress. Cells were preincubated with 100 μM ML-7 for seven minutes prior to the addition of 100 mM NaCl. NaCl was added and pH_i measured and summarized as described in Figure 3. (A) Examples of the effect of addition of ML-7 on steady-state pH_i. Top tracing NaCl alone. Bottom tracing NaCl added after preincubation with ML-7. (B) Bar graph summarizing the effects. (*) indicates significantly different from control values at $P<0.01$.

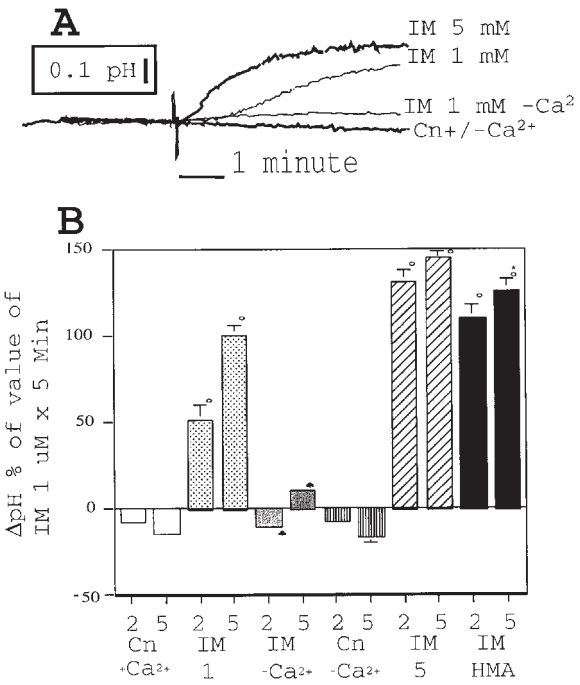

Figure 6 Effect of calcium on steady-state pH_i of neonatal rat cardiac myocytes. The changes from resting pH_i were measured for 5 min after the addition of ionomycin (1.0 μM or 5.0 μM), in the presence and absence of calcium chloride. The results were summarized at 2 and 5 minute intervals. (A) Examples of effects of the addition of ionomycin (IM 5 μM and IM 1 μM) in the presence of calcium chloride and ionomycin (IM 1 μM $-Ca^{2+}$) in the absence of calcium chloride on steady-state pH_i. Controls (in the presence and absence of calcium chloride) are represented by the bottom tracing (Cn $\pm Ca^{2+}$. (B) Bar graph summarizing the effects of the above agents including HMA-inhibited Na^+/H^+ exchanger in the presence of 5.0 μM ionomycin (IM HMA). Cn-Ca^{2+}, controls in the absence of external calcium. Results are mean $\pm$ SE of 4–5 experiments. (○) indicates significantly different from control values at $P<0.01$. (♠) indicates significantly different from ionomycin (1.0 μM) (in the presence of calcium chloride) values at $P<0.05$). (*) indicates significantly different from ionomycin (5.0 μM) (in the absence of HMA) value at $P<0.05$.

higher dose of ionomycin (5.0 μM) also significantly increased steady-state pH_i (131% and 145%) at both time points compared to controls in the presence of calcium. Blocking the Na^+/H^+ exchanger with HMA (10.0 μM) only slightly decreased the effect of 5.0 μM ionomycin (111% and 126% at 2 and 5 min respectively), significantly reducing the alkalinizing effect at the 5 min time point.

Discussion

The NHE1 isoform of the Na^+/H^+ exchanger is well known to regulate intracellular pH in the mammalian myocardium. In other tissues it has been shown that exchanger activity is stimulated by osmotic activation.[23] Calmodulin has also been shown to be important in regulation of the activity of the protein and is likely to act at least partially independent of the site of osmotic activation.[23, 25–27] It is known that during ischemic injury, the myocardium is subjected to increases in intracellular calcium.[15–17] Also osmotic changes occur in pathological states such as ischemia, septic shock and diabetic coma. During myocardial ischemia, metabolites can accumulate extracellularly causing a hyperosmotic extracellular milieu. This hyperosmotic milieu may be rapidly replaced by normosmotic blood upon reperfusion causing rapid changes is osmolality in the surrounding medium.[43]

It was of particular interest to examine this form of regulation in the neonatal mycardium. The neonatal heart varies in many clinically important ways from the adult heart. When compared to the adult myocardium there is a large difference in substrate use and in the effects of ischemia, hypoxia and acidosis. The fetal and newborn hearts are more resistant to hypoxia than the adult myocardium.[44,45] However in the ischemic heart the situation is reversed. The newborn myocardium is more vulnerable than the adult to ischemic injury.[30–32] Different effects of acidosis on the neonatal *v* adult heart have also been well documented in models such as the rat ventricle.[46,47] In addition differences in regulation of expression of the Na^+/H^+ exchanger between the neonatal myocardium and the adult are well documented.[33] We therefore investigated osmotic, calcium/calmodulin and protein kinase mediated regulation of the exchanger in primary cultures of neonatal cardiac myocytes.

Initially the responsiveness of the primary cultures of isolated myocytes was characterized. Both ET-1 and serum stimulated the exchanger causing a rise in the rate of recovery from an acid load. Angiotensin alone had a slight insignificant stimulatory effect however, in the presence of the AT_2 receptor antagonist PD123319, angiotensin greatly increased proton efflux (Fig. 1). Similar results have been reported earlier in isolated rat ventricular myocytes[48] and in macula densa cells of the kidney cortical thick ascending limb.[49] Since our cells were very responsive to both acid load and hormonal regulation, it was clear that the Na^+/H^+ exchanger was present and functioning normally in these cells.

We next examined the effect of osmotic stimulation of CHO cells which had the endogenous exchanger deleted and were transfected with the NHE1 isoform of the Na^+/H^+ exchanger. There was a marked rise in intracellular pH with addition of NaCl (Fig. 2). These results were similar to what we[20] and others[23,50] have reported earlier. Sorbitol also caused a significant rise in pH_i, however this was smaller than that caused by NaCl. The reason for the difference is not certain, however it could be due to the increased concentration of Na^+ on the external side of the membrane. Since the driving force of the exchanger is the Na^+ ion concentration gradient the increased Na^+ gradient might affect the activity of the Na^+/H^+ exchanger. The effects were specific since they were inhibited by HMA. Another possible explanation for the larger effect of increased extracellular Na^+ is that is causes activation of the Na^+/H^+ exchanger. In this regard it has recently[51] been shown that increased Na^+ influx can activate the Na^+/H^+ exchanger. Increased extracellular Na^+ could result in increased intracellular Na^+ and could therefore cause activation of the exchanger, accounting for the greater osmotic effect of NaCl compared to sorbitol and other osmolytes. Further studies are necessary to confirm if this possible mechanism is involved.

Primary cultures of neonatal cardiac myocytes were then tested to see if they were also responsive to hyperosmotic stimulation. It was found that the addition of NaCl resulted in a marked stimulation of activity of the exchanger. Previously, it has been demonstrated that the adult myocardium is responsive to osmotic stimulation.[52,53] This data demonstrated for the first time that the neonatal myocardium is also responsive to osmotic stress. Though the osmotic effect has been demonstrated earlier with externally applied sucrose,[52,53] we characterized it further by examining the effect of different forms of stimulation. In addition to NaCl, N-methyl-D-glucamine and choline chloride also significantly increased intracellular pH although similar to the AP1 cells. However we found that the effect of N-methyl-D-glucamine and choline chloride was quite reduced in comparison to NaCl. HMA eliminated most of the stimulatory effect of NaCl. HMA also eliminated the smaller stimulatory effect of sorbitol, N-methyl-D-glucamine and choline chloride (not shown) suggesting that these effects were due to the Na^+/H^+ exchanger. Higher concentrations of sorbitol could not mimic the effect of NaCl suggesting that this was not simply a difference in osmotic strength. The reason for the more significant effect of Na^+ likely is due to either the greater Na^+ gradient or the effect of increased Na^+ influx as described above.[51] Taken together, the effects of NaCl, sorbitol, N-methyl-D-glucamine and choline chloride suggest that a variety of different osmotic stimuli can activate the Na^+/H^+ exchanger. They confirm that the effects are due to osmotic effects of the media and not due to specific effects of sugar or of NaCl addition to the media.

An interesting novel finding was that omission of $CaCl_2$ from the external medium reduced the effect of addition of NaCl, however a major part of the response was still evident (Fig. 3). This result shows that external calcium is not absolutely necessary for osmotic stimulation of the Na^+/H^+ exchanger. Thus, while it is known that calcium can affect regulation of the Na^+/H^+ exchanger,[25–27] clearly osmotic regulation of the exchanger can occur in the absence of high concentrations of external calcium.

It was found that the calmodulin antagonist trifluoperazine also reduced, but did not eliminate

the effect of the addition of NaCl. It should be noted however that trifluoperazine also reduced the resting pH_i of isolated myocytes when applied without the above stimulation. It seems apparent that osmotic activation of the exchanger still occurs in the presence of calmodulin inhibition. One possible explanation is that the calmodulin inhibitor prevents calmodulin from blocking the effects of the autoinhibitory domain of the exchanger[23,25–27] thereby reducing the apparent osmotic activation of the exchanger. Overall this result and the effects of omission of external calcium, suggest that Ca^{2+}/calmodulin are not the key direct mediators of osmotic activation of the exchanger in the neonatal myocardium.

We then examined if calmodulin played a significant role in activity of the exchanger during an acute acid load. Both W7 and trifluoperazine significantly reduced the rate of recovery from an acute acid load (Fig. 4). These results were in agreement with an earlier report that also showed that calmodulin antagonists decrease the activity of the Na^+/H^+ exchanger.[54] A novel finding was that the CaM Kinase II inhibitor KN-93 (10 μM) inhibited the exchanger mediated recovery from an acid load. A low concentration of KN-93 did not inhibit the exchanger however the amount of KN-93 required for inhibition of activity (Fig. 4) was similar to that used in a number of other recent studies.[55–57] Earlier we have suggested that phosphorylation of the Na^+/H^+ exchanger could be mediated by CaM dependent protein kinase II[22]. These results suggest that phosphorylation by CaM-dependent protein kinase II is also important in activity of the exchanger in the myocardium.

Another protein kinase, myosin light-chain kinase, has earlier been suggested to be involved in mediating the effects of cell shrinkage in rat astrocytes.[58] We therefore examined if this protein kinase could also be involved in mediated the osmotic activation of the exchanger in neonatal cardiac myocytes. The results (Fig. 5) showed that ML-7 completely blocked the effect of addition of external sodium. Similar experiments also showed that ML-7 also blocked the effect of addition of sorbitol (not shown). These results suggested that as with astrocytes, myosin light-chain kinase plays a critical role in osmotic activation of the exchanger. The involvement of myosin light-chain kinase is consistent with the effects we noted of calcium omission and calmodulin antagonists. It was found that partial blockage of osmotic activation was caused by calmodulin antagonists and by omission of calcium from the extracellular medium. Since binding of a Ca^{2+}-calmodulin complex activates myosin light-chain kinase the partial inhibition obtained by these treatments may be mediated through effects on myosin light-chain kinase. Overall these results suggest that myosin light-chain kinase may be a crucial mediator of osmotic activation of the exchanger in the myocardium. It is unlikely that myosin light-chain kinase directly phosphorylates the Na^+/H^+ exchanger protein since no evidence for this has ever been reported. In addition activation of the antiporter by osmotic stimulation has been shown to occur without direct phosphorylation of the protein.[59] One possibility is that other accessory or regulatory proteins are involved and that they are regulated by that myosin light-chain kinase, however their identity is not yet known.

While it is clear that the exchanger can be activated by osmotic stimulation, it was evident that increasing calcium entry (by use of ionomycin) had only a small stimulatory effect on Na^+/H^+ exchanger activity. Ionomycin did elevate intracellular pH markedly (Fig. 6), however the effect was only partially reduced by HMA indicating the exchanger was only partially involved. It is therefore likely that calcium is normally not limiting in amount for the osmotic activation of the exchanger. An interesting aspect of our study was the finding that treatment of neonatal cardiac myocytes with the calcium ionophore ionomycin resulted in an HMA-resistant increase in intracellular pH. It is unclear at the present time what is the mechanism of this rise in pH_i. Presuming that the elevation of intracellular pH is not due to some change in metabolism, another ion transporter may mediate the effect. Since the medium used for pH_i measurement was essentially bicarbonate free, it is unlikely that bicarbonate-based transport is involved. One possible candidate is lactate/H^+ symport. It is conceivable that ionomycin causes calcium overload of the mitochondria leading to uncoupling. This could lead to excess lactate production followed by cotransport with protons[60] however, further investigations are necessary to confirm this suggestion.

Overall the results show that osmotic stimulation of the Na^+/H^+ exchanger is functionally active in the neonatal myocardium. It has been demonstrated for the first time that myosin light-chain kinase is involved in osmotic activation of the exchanger in the myocardium and that Ca^{2+}/calmodulin dependent protein kinase plays a significant role in recovery from an acute acid load. In addition while it is clear that calcium and calmodulin play an important role in activity of the Na^+/H^+ exchanger in some tissues under specific

conditions[25–27] it seems that in the myocardium endogenous levels of calcium are normally high enough such that further additional calcium is only slightly stimulatory. Omission of calcium from the external medium and the calmodulin antagonists may reduce the osmotic response through myosin-light chain kinase. While it is clear that in pathological states elevated intracellular calcium and accumulation of extracellular metabolites could occur, our results suggest that osmotic stimulation of the exchanger could be more of a significant activator of the exchanger than increased intracellular calcium. Future experiments can determine if these mechanisms of activation of the Na^+/H^+ exchanger contribute *in vivo* to the tissue injury which occurs during myocardial ischemia and reperfusion.

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