Feedback Inhibition of Sodium/Calcium Exchange by Mitochondrial Calcium Accumulation*

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Chinese hamster ovary cells expressing the bovine cardiac Na⁺/Ca²⁺ exchanger were subjected to two periods of 5 and 3 min, respectively, during which the extracellular Na⁺ concentration ([Na⁺]o) was reduced to 20 mM; these intervals were separated by a 5-min recovery period at 140 mM Na⁺. The cytosolic Ca²⁺ concentration ([Ca²⁺]i) increased during both intervals due to Na⁺-dependent Ca²⁺ influx by the exchanger. However, the peak rise in [Ca²⁺]i during the second interval was only 26% of the first. The reduced rise in [Ca²⁺]i was due to an inhibition of Na⁺/Ca²⁺ exchange activity rather than increased Ca²⁺ sequestration since the influx of Na⁺, which is not sequestered by internal organelles, was also inhibited by a prior interval of Ca²⁺ influx. Mitochondria accumulated Ca²⁺ during the first interval of reduced [Na⁺]o, as determined by an increase in fluorescence of the Ca²⁺-indicating dye rhod-2, which preferentially labels mitochondria. Agents that blocked mitochondrial Ca²⁺ accumulation (uncouplers, nocardazole) eliminated the observed inhibition of exchange activity during the second period of low [Na⁺]o. Conversely, diltiazem, an inhibitor of the mitochondrial Na⁺/Ca²⁺ exchanger, increased mitochondrial Ca²⁺ accumulation and also increased the inhibition of exchange activity. We conclude that Na⁺/Ca²⁺ exchange activity is regulated by a feedback inhibition process linked to mitochondrial Ca²⁺ accumulation.

The Na⁺/Ca²⁺ exchanger is an electrogenic, high capacity transporter found in the plasma membrane of many cell types. The exchanger is the primary mechanism for Ca²⁺ efflux in cardiac myocytes, and its activity therefore plays a central role in regulating the force of cardiac muscle contraction (reviewed in Ref. 1). The stoichiometry of the exchange process is generally considered to be three Na⁺ ions/Ca²⁺ (2), although a higher stoichiometry has been proposed recently (3). The exchanger can transport Ca²⁺ in either direction across the plasma membrane, and a reduction in the extracellular Na⁺ concentration ([Na⁺]o) leads to a net influx of Ca²⁺ in cells expressing the exchanger. Regulation of exchange activity through positive modulation by phosphatidylinositol 4,5-bisphosphate or cytosolic Ca²⁺ has been characterized in electrophysiological experiments with excised membrane patches (4, 5). Results with transfected cells, cardiac myocytes, and squid giant axons have suggested that exchange activity may also be regulated by protein kinases (6–9) and by the actin cytoskeleton (10). The importance of these modes of regulation in intact cells under physiological conditions is uncertain, however (see Ref. 11).

Our laboratory has been investigating the regulation of Na⁺/Ca²⁺ exchange activity in stably transfected Chinese hamster ovary (CHO) cells expressing the bovine cardiac Na⁺/Ca²⁺ exchanger. The results of our studies suggest that the above processes are of limited importance in modulating exchange activity under physiological conditions. Thus, Na⁺/Ca²⁺ exchange activity in these cells is nearly fully activated by cytosolic Ca²⁺ under resting conditions (11), and it is not affected by variations in phosphatidylinositol 4,5-bisphosphate that are likely to occur physiologically (12) or by activation or inhibition of protein kinases (10). Although exchange activity is inhibited by agents that disturb cytoskeletal integrity in these cells (10, 13), the relevance of these findings to more physiological conditions is unclear.

In this report, we describe a two-pulse protocol in which Ca²⁺ influx during a "conditioning" interval in a low [Na⁺]o medium leads to inhibition of exchange activity during a subsequent "test" interval. The data show that mitochondria accumulate Ca²⁺ during the conditioning interval of Ca²⁺ influx and that this process is essential for the subsequent inhibition of exchange activity. These findings reveal a previously unsuspected link between mitochondria and the regulation of Na⁺/Ca²⁺ exchange activity.

EXPERIMENTAL PROCEDURES

Cells—CHO cells expressing Na⁺/Ca²⁺ exchange activity (CK1.4 cells) were prepared by transfecting the cells (CCL 61, American Type Culture Collection) with the expression vector pCNAI/Neo (Invitrogen, Carlsbad, CA) containing a cDNA insert coding for the bovine cardiac Na⁺/Ca²⁺ exchanger (14). The cells were grown in Iscove’s modified Dulbecco’s medium containing 10% fetal calf serum and antibiotics as described (14).

Materials and Solutions—Na-PSS contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM glucose, and 20 mM Mops, adjusted to pH 7.4 (37 °C) with Tris. K-PSS had the same composition as Na-PSS, except that NaCl was replaced with KCl (140 mM total concentration). Na-PSS was diluted 7-fold with K-PSS to yield 20:120 Na/K-PSS. Fura-2/AM, rhod-2/AM, and pluronic F-127 were purchased from Molecular Probes, Inc. (Eugene, OR). All other biochemicals were purchased from either Sigma or Calbiochem. Oligomycin was a mixture of oligomycin A, B, and C; an average molecular weight of 792 was assumed for computing concentrations.

Two-pulse Protocol—CK1.4 cells were grown on coverslips to 70–80% confluency and incubated for 40 min at room temperature in Na-PSS plus 1% bovine serum albumin containing 3 μM fura-2/AM, 0.25 mM sulfinpyrazone (to retard transport of fura-2 out of the cells), and
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**RESULTS**

**Two-pulse Protocol for Na\(^+\)/Ca\(^{2+}\) Exchange**—In cells expressing Na\(^+\)/Ca\(^{2+}\) exchange activity, a reduction in [Na\(^+\)]\(_o\) leads to an increase in [Ca\(^{2+}\)]\(_i\), due to Na\(^+\)-dependent Ca\(^{2+}\) influx. For the data shown in Fig. 1, two sequential intervals of low [Na\(^+\)]\(_o\), (20 mM), separated by a 5-min recovery period in physiological [Na\(^+\)]\(_o\), (140 mM), were applied to fura-2-loaded CHO cells expressing the bovine cardiac Na\(^+\)/Ca\(^{2+}\) exchanger. Ca\(^{2+}\) (1 mM) was present in all solutions. During the first (conditioning) interval in 20 mM Na\(^+\)\(_o\), the 334:380 nm excitation ratio for fura-2 rose rapidly from a resting value of 0.44 to a peak value of 1.8 and then declined; the ratios corresponded to approximate [Ca\(^{2+}\)]\(_i\) of 80 and 620 nM, respectively. [Ca\(^{2+}\)]\(_i\) rapidly returned to resting values when [Na\(^+\)]\(_o\) was restored to 140 mM. After the second (test) interval in 20 mM Na\(^+\)\(_o\), the increase in the 334:380 nm ratio was 26 ± 3% (n = 29) of that observed during the conditioning pulse (peak [Ca\(^{2+}\)]\(_i\), ~210 nM).

Fig. 2A depicts the effects of varying the duration of the conditioning pulse on the rise in [Ca\(^{2+}\)]\(_i\), during the test pulse. A conditioning pulse of 1 min (trace a) had no effect on the Ca\(^{2+}\) influx during the test pulse. After a 3-min conditioning pulse (trace b), the peak increase in the fura-2 ratio during the test pulse was reduced to 54 ± 6% of the conditioning peak. For 5- and 7-min conditioning pulses (traces c and d, respectively), the test peaks were 37 ± 7 and 41 ± 7% of the conditioning peak, respectively. Thus, a conditioning interval of at least 3 min was required to inhibit the rise in [Ca\(^{2+}\)]\(_i\), during the test pulse. Note that for each time interval, the peak [Ca\(^{2+}\)]\(_i\) attained during the test interval was nearly identical to the [Ca\(^{2+}\)]\(_i\) at the end of the declining phase of the conditioning pulse. In other experiments (data not shown), we varied the duration of the recovery interval between the conditioning and test pulses. Exchange activity remained inhibited following a recovery period of 10 min, but after 30 min of recovery, the rise in [Ca\(^{2+}\)]\(_i\), during the test interval returned to normal.

As shown in Fig. 3 (boldface trace), when Ca\(^{2+}\) was omitted from the medium during the conditioning interval in 20 mM Na\(^+\)\(_o\), the rise in [Ca\(^{2+}\)]\(_i\), during the subsequent test interval was not inhibited. The slight rise in [Ca\(^{2+}\)]\(_i\), during the conditioning interval probably reflected small amounts of residual Ca\(^{2+}\) in the medium. We conclude that Ca\(^{2+}\) influx during the conditioning interval was required to inhibit the rise in [Ca\(^{2+}\)]\(_i\), during the test interval and that the inhibition was not simply a consequence of lowering [Na\(^+\)]\(_o\), to 20 mM.

**Inhibition of Na\(^+\)/Ca\(^{2+}\) Exchange Activity**—The decrease in the peak [Ca\(^{2+}\)]\(_i\), during the test interval could be due to either a reduced influx of Ca\(^{2+}\), i.e. an inhibition of Na\(^+\)/Ca\(^{2+}\) exchange activity, or an increase in the sequestration of cytosolic Ca\(^{2+}\) by intracellular organelles. The data in Fig. 4 demonstrate that the first alternative is correct, i.e. exchange activity is inhibited during the test pulse. For this experiment, we used Ba\(^{2+}\) as a Ca\(^{2+}\) surrogate during the test pulse. Ba\(^{2+}\) is transported by the Na\(^+\)/Ca\(^{2+}\) exchanger, but it is not sequestered by
Fig. 3. Ca\(^{2+}\) dependence of inhibition. The standard two-pulse protocol was applied to fura-2-loaded cells as indicated by the bar below the trace. For the trace shown by the broken line without error bars, 1 mM CaCl\(_2\) was present in all solutions; for the boldface trace, Ca\(^{2+}\) was omitted from the low [Na\(^+\)]\(_m\), medium during the conditioning interval, as shown by the bar above the trace. Results are the means ± S.E. (for every fourth data point) of seven coverslips; for the control trace, cells from the same batch of coverslips were used (n = 4).

Fig. 4. Ba\(^{2+}\) influx during test interval. The standard two-pulse protocol was applied to fura-2-loaded cells as indicated by the bar below the trace, with the exception that 2 mM BaCl\(_2\) was substituted for CaCl\(_2\) during the second (test) interval in 20:120 Na/K-PSS. Inset, 20:120 Na/K-PSS and 2 mM Ba\(^{2+}\) were applied to the cells (arrow) without a prior conditioning interval of Ca\(^{2+}\) influx. Excitation wavelengths were at 350 and 390 nm for this experiment. (n = 6; for inset, n = 3).

the endoplasmic reticulum and is only poorly, if at all, taken up by mitochondria in these cells (17). As shown in Fig. 4 (inset), Ba\(^{2+}\) entered cells rapidly when [Na\(^+\)]\(_o\) was reduced to 20 mM (arrow) in the presence of 2 mM Ba\(^{2+}\); this Ba\(^{2+}\) concentration was chosen to produce a rise in the fura-2 ratio that was roughly equivalent to that seen with 1 mM Ca\(^{2+}\). For Fig. 4 (main panel), a standard conditioning interval in 20 mM Na\(^+\), plus 1 mM Ca\(^{2+}\), was applied to the cells. This was followed by a 5-min recovery period in normal Na-PSS before [Na\(^+\)]\(_o\) was again reduced to 20 mM, this time in the presence of 2 mM Ba\(^{2+}\).

As shown, Ba\(^{2+}\) influx during the test pulse was nearly completely blocked. We conclude that the reduced rise in [Ca\(^{2+}\)]\(_i\) during the test pulse for the experiments shown in Figs. 1 and 2 was due to an inhibition of Na\(^+\)/Ca\(^{2+}\) exchange activity rather than increased Ca\(^{2+}\) sequestration.

Mitochondrial Ca\(^{2+}\) Accumulation during the Ca\(^{2+}\) Influx Mode of Na\(^+\)/Ca\(^{2+}\) Exchange—We have shown previously that in transfected cells that had been loaded with high concentrations of cytosolic Na\(^+\) using gramicidin (a channel-forming antibiotic) or ouabain (a Na\(^+\)/K\(^-\)ATPase inhibitor), reducing [Na\(^+\)]\(_o\) led to large increases in [Ca\(^{2+}\)]\(_i\), and an extensive accumulation of Ca\(^{2+}\) in the mitochondrial compartment (18). To examine whether mitochondria also accumulated Ca\(^{2+}\) in the absence of cytosolic Na\(^+\) loading, we labeled cells with the Ca\(^{2+}\)-sensitive dye rhod-2. This indicator preferentially accumulates within mitochondria because the positively charged, unhydrolyzed form of the dye equilibrates with the negative mitochondrial membrane potential during dye loading (19). Fig. 5A shows rhod-2-labeled cells following exposure to 20 mM Na\(^+\), in the presence of 1 mM Ca\(^{2+}\). Rhod-2 fluorescence was intense in the perinuclear region of the cell and showed a particulate pattern suggestive of mitochondria. Prior to reducing [Na\(^+\)]\(_o\), rhod-2 fluorescence was much lower in intensity and more generally distributed throughout the cell (data not shown, but see below). To demonstrate that the increased rhod-2 fluorescence was in fact associated with mitochondria, the cells in this experiment were also labeled with the selective mitochondrial Ca\(^{2+}\) indicator MitoTracker Green (Fig. 5B). Fig. 5C is the superposition of the two images in A and B. The yellow color denotes areas where rhod-2 and MitoTracker Green coincide and demonstrates a nearly complete correspondence between the rhod-2 and MitoTracker labeling. The results confirm the conclusion of Rutter et al. (20) that rhod-2 preferentially labels mitochondria in CHO cells and further demonstrate that the mitochondria accumulate Ca\(^{2+}\) under low [Na\(^+\)]\(_o\) conditions in the transfected cells. No mitochondrial Ca\(^{2+}\) accumulation was detected when the low [Na\(^+\)]\(_o\) medium was applied to vector-transfected CHO cells, which do not exhibit Na\(^+\)/Ca\(^{2+}\) exchange activity (data not shown).

For Fig. 6, the standard two-pulse protocol was applied to rhod-2-labeled cells, and fluorescence was monitored in mitochondrion-rich areas of the cells (see “Experimental Procedures”). Rhod-2 fluorescence increased ~5-fold when [Na\(^+\)]\(_o\) was reduced to 20 mM in the presence of 1 mM Ca\(^{2+}\). Restoration of 140 mM Na\(^+\) evoked a fall in fluorescence intensity back to initial levels. The changes in rhod-2 fluorescence were much slower than the corresponding changes in the fura-2 signal (compare with Fig. 1), indicating that the rhod-2 measurements did not simply reflect changes in [Ca\(^{2+}\)]\(_i\). The slow decline in rhod-2 fluorescence upon restoration of 140 mM Na\(^+\) is consistent with the relatively slow rate of Ca\(^{2+}\) efflux from mitochondria (19). Upon re-exposure to 20 mM Na\(^+\), rhod-2 fluorescence did not increase, consistent with a reduced Ca\(^{2+}\) influx during the test pulse. When Ca\(^{2+}\) influx was stimulated with ionomycin (2 \(\mu\)M) during the test pulse, there was a sharp increase in mitochondrial rhod-2 fluorescence (data not shown), indicating that the mitochondria were still responsive to an increase in [Ca\(^{2+}\)]\(_i\). From these results, taken together with the Ba\(^{2+}\) influx measurements (Fig. 4), we conclude that the reduced rise in [Ca\(^{2+}\)]\(_i\) during the test interval was due to an inhibition of Na\(^+\)/Ca\(^{2+}\) exchange activity and could not be attributed to an increase in organelar Ca\(^{2+}\) sequestration.

Inhibition of Exchange Activity Is Dependent upon Mitochondrial Ca\(^{2+}\) Accumulation—The results presented below indicate that the accumulation of Ca\(^{2+}\) by mitochondria during the conditioning pulse is an essential factor in mediating the subsequent inhibition of exchange activity during the test pulse. Fig. 7A shows that the inhibition of Ca\(^{2+}\) influx during the test pulse was eliminated when the mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP; 2 mM) and the F$_{0}$F$_{1}$-ATPase inhibitor oligomycin (3.2 \(\mu\)M) were both present during the two-pulse protocol. (Oligomycin was added to reduce ATP hydrolysis by the F$_{0}$F$_{1}$-ATPase; similar results were obtained in the presence of uncoupler alone.) In the presence of CCCP (Fig. 7A, C/O trace), the peak ratio during the conditioning pulse was similar to that for untreated controls, but the subsequent decline in [Ca\(^{2+}\)]\(_i\), following the peak of the conditioning pulse was eliminated. Following addition of 140 mM Na\(^+\), [Ca\(^{2+}\)] fell to values that were somewhat higher than for untreated controls, perhaps reflecting the loss of mitochondrial Ca\(^{2+}\)-sequestering activity. The peak increase in the fura-2 ratio during the test interval was 110 ± 9% of that during the
completely blocked mitochondrial Ca$^{2+}$ loading among different coverslips. As expected, the inhibitors variability primarily reflects differences in the extent of dye coverslips in cellular rhod-2 fluorescence. Presumably, this images.

The image labeled Composite is a superposition of the two individual wavelength images.

The absence of exchange inhibition in the presence of the uncouplers was not due to a reduction in mitochondrial produced ATP since the normal inhibition of Ca$^{2+}$ influx during the test pulse was not affected by oligomycin alone (data not shown).

The data in Fig. 7B show a similar experiment carried out in the presence of CCCP/oligomycin, but in this case, 2 mM Ba$^{2+}$ was substituted for Ca$^{2+}$ during the test interval (arrow). In contrast to the results for untreated cells shown previously (Fig. 4), a robust influx of Ba$^{2+}$ was observed in the inhibitor-treated cells. The data in Fig. 7C (C/O trace) depict rhod-2 fluorescence in the presence of CCCP and oligomycin. The initial value of rhod-2 fluorescence appeared to be higher than that for the untreated cells, but this difference was not significant ($p > 0.2$) and reflected the large variations among different coverslips in cellular rhod-2 fluorescence. Presumably, this variability primarily reflects differences in the extent of dye loading among different coverslips. As expected, the inhibitors completely blocked mitochondrial Ca$^{2+}$ accumulation during the conditioning pulse. Oligomycin alone did not block mitochondrial Ca$^{2+}$ accumulation (data not shown). These data confirm that the rhod-2 fluorescence measurements reflect mitochondrial rather than cytosolic [Ca$^{2+}$] since the rise in [Ca$^{2+}$] was unaffected by CCCP. The control traces represent rhod-2 fluorescence for cells from the same batch of coverslips in the absence of inhibitors ($n = 4$).

The mitochondrial Ca$^{2+}$ content is regulated by the balance between Ca$^{2+}$ uptake and efflux mechanisms. Previous results with permeabilized CHO cells demonstrated that the mitochondria in these cells possess a Na$^+$/Ca$^{2+}$ exchange mechanism for Ca$^{2+}$ efflux (18). The mitochondrial Na$^+$/Ca$^{2+}$ exchanger is different from the plasma membrane exchanger and is inhibited by agents such as diltiazem, clonazapam, and CGP-37157 that do not affect the plasma membrane exchanger (21, 22).

When the two-pulse protocol was carried out in the presence of diltiazem (100 μM), we observed a more pronounced inhibition of Ca$^{2+}$ influx during the test pulse than for control cells (Fig. 8A). Diltiazem had no significant effect on Ca$^{2+}$ influx during the conditioning pulse. Changes in fluorescence of rhod-2-labeled cells in the presence of diltiazem are shown in Fig. 8B. Both the initial level and the peak values of rhod-2 fluorescence during the conditioning interval were higher for diltiazem-
treated cells than for control cells (p = 0.05 and 0.005, respectively). Whether this reflects overexpression variability or a true increase in mitochondrial Ca\(^{2+}\) content with diltiazem is difficult to assess with certainty. More important, the results clearly demonstrate that the efflux of Ca\(^{2+}\) from mitochondria was reduced when 140 mM Na\(^+\) was restored in the presence of diltiazem compared with untreated controls. Mitochondrial [Ca\(^{2+}\)]\(_i\) remained elevated in diltiazem-treated cells throughout the recovery period in 140 mM Na\(^+\) (Fig. 8), in contrast to untreated cells (Fig. 8, Control trace; and Fig. 6), where mitochondrial [Ca\(^{2+}\)]\(_i\) decreased to initial values. These results are consistent with inhibition of the mitochondrial Na\(^+\)/Ca\(^{2+}\) exchange by diltiazem. We attempted to use the more selective mitochondrial exchange inhibitor CGP-37157 for these studies, but observed no effect either on [Ca\(^{2+}\)]\(_i\) during the two-pulse protocol or on mitochondrial Ca\(^{2+}\) uptake or efflux (data not shown). The lack of effect of CGP-37157 in these cells might be due to poor cellular permeability or intracellular binding. In permeabilized cells, we found that this compound was highly effective in blocking Na\(^+\)-dependent mitochondrial Ca\(^{2+}\) efflux (18).

An additional correlation between mitochondrial Ca\(^{2+}\) accumulation and inhibition of exchange activity was provided, surprisingly, by nocodazole, an agent that depolymerizes microtubules. Fig. 9A compares the results of the two-pulse protocol for control cells and for cells that had been pretreated with 17 mM nocodazole for 15–20 min prior to beginning the experiment; nocodazole was also included in the medium during the two-pulse protocol. Nocodazole had no effect on the peak [Ca\(^{2+}\)]\(_i\), and slightly reduced the post-peak decline in [Ca\(^{2+}\)]\(_i\) during the conditioning pulse. However, nocodazole practically eliminated the inhibition of Ca\(^{2+}\) influx during the test pulse; the peak [Ca\(^{2+}\)]\(_i\) during the test interval was 84 ± 6% of the conditioning peak. Fig. 9B shows that the fluorescence intensity of rhod-2 did not increase when the cells were exposed to 20 mM Na\(^+\). Since the rise in [Ca\(^{2+}\)]\(_i\) was only slightly affected (Fig. 9A), we conclude that the nocodazole treatment blocked the accumulation of Ca\(^{2+}\) by mitochondria during the conditioning interval. As a positive control for these data, we found that the addition of ionomycin to the nocodazole-treated cells during the conditioning interval produced the expected rise in rhod-2 fluorescence (data not shown). This indicates that the nocodazole treatment did not interfere with rhod-2 localization or its ability to respond to increased [Ca\(^{2+}\)]\(_i\).

In summary, the results demonstrate that the inhibition of exchange activity during the test interval was dependent upon mitochondrial Ca\(^{2+}\) accumulation during the conditioning interval. Agents that blocked mitochondrial Ca\(^{2+}\) accumulation (CCCP, nocodazole) also blocked the inhibition of exchange activity, whereas an agent that blocked mitochondrial Ca\(^{2+}\) efflux (diltiazem) enhanced the inhibition of exchange activity.

**DISCUSSION**

Phosphatidylinositol 4,5-bisphosphate, cytosolic Ca\(^{2+}\), protein phosphorylation, and the actin cytoskeleton have each been suggested as regulators of the cardiac Na\(^+\)/Ca\(^{2+}\) exchanger (see the Introduction). The evidence supporting their involvement stemmed from studies that either utilized subcellular preparations, i.e. excised membrane patches, or involved the addition of exogenous inhibitors or other agents. Thus far, there has been no clear demonstration that any of these processes are important regulators of exchange activity under physiological conditions. The experimental design in the present studies involved a simple two-pulse protocol for activating exchange activity and did not require the addition of exogenous agents or the experimental alteration of normal cytosolic concentrations of Na\(^+\) or Ca\(^{2+}\). Our results show that Na\(^+\)/Ca\(^{2+}\) exchange activity in transfected CHO cells is regulated by a previously unsuspected process linked to mitochondrial Ca\(^{2+}\) accumulation. These findings provide the first evidence for a distinct regulatory pathway that modulates Na\(^+\)/Ca\(^{2+}\) exchange activity in intact cells.

The central finding of these studies is that a conditioning interval of Ca\(^{2+}\) influx by Na\(^+\)/Ca\(^{2+}\) exchange produced an inhibition of exchange activity during a subsequent test interval (Figs. 1 and 2). Ba\(^{2+}\) influx was also inhibited during the test interval (Fig. 4). Since Ba\(^{2+}\) is not efficiently transported by organellar or plasma membrane Ca\(^{2+}\)-ATPases (17), we concluded that the reduced rise in [Ca\(^{2+}\)]\(_i\) during the test interval reflected an inhibition of exchange activity itself rather than an increased sequestration or efflux of Ca\(^{2+}\). This conclusion is consistent with the absence of mitochondrial Ca\(^{2+}\) accumulation during the test interval (Fig. 6).

The inhibition of exchange activity was eliminated (or potentiated) by agents that prevented (or promoted) mitochondrial Ca\(^{2+}\) accumulation. Thus, CCCP (Fig. 7) and nocodazole (Fig. 9) each blocked mitochondrial Ca\(^{2+}\) accumulation during the conditioning interval and eliminated the inhibition of exchange activity during the test interval. On the other hand, diltiazem, a blocker of the mitochondrial Na\(^+\)/Ca\(^{2+}\) exchanger (21, 22), inhibited mitochondrial Ca\(^{2+}\) efflux during the recovery interval and enhanced the inhibition of exchange activity during the test interval (Fig. 8). These data suggest that the increase in the mitochondrial Ca\(^{2+}\) content led to the development of an inhibitory signal that reduced Na\(^+\)/Ca\(^{2+}\) exchange activity.

It seems unlikely that these effects were simply due to a reduction in [Na\(^+\)]\(_i\), resulting from the exposure to 20 mM Na\(^+\) during the conditioning pulse. We were unable to detect changes in [Na\(^+\)]\(_i\) during the two-pulse protocol in cells loaded with the Na\(^+\) indicator sodium-binding benzofuran iso-
phthale (data not shown). However, it is possible that this method is not sufficiently sensitive to detect small reductions in [Na⁺], that could inhibit exchange activity. Reducing [Na⁺]o to 20 mM in the absence of Ca²⁺ did not reduce exchange activity during a subsequent test pulse (Fig. 3), indicating that Ca²⁺ entry was required to inhibit exchange activity. Finally, nocodazole, CCCP, and diltiazem would not be expected to alter [Na⁺], in ways that would be compatible with the results obtained. Thus, a trivial explanation in terms of reduced [Na⁺], seems unlikely, although we cannot rule out a more complex mechanism in which alterations in [Na⁺], are somehow linked to mitochondrial Ca²⁺ accumulation.

The nature of the inhibitory signal generated during mitochondrial Ca²⁺ accumulation is unknown. Since an increase in Ca²⁺ within the mitochondrial matrix activates several different dehydrogenases (19, 23), it is possible that increased production of an end product of mitochondrial respiration provides a signal for inhibiting exchange activity. However, the mitochondrial production of ATP seems unimportant since oligomycin, an inhibitor of the FₑFₒ-ATPase, did not block inhibition of exchange activity. Mitochondrial production of reactive oxygen species (24), nitric oxide (25, 26), and glutamate (27) has been invoked as regulatory effectors in some cells, and these possibilities deserve further investigation.

The gradual development of exchange inhibition over a 1–5 min period (Fig. 2) suggests that time is required to generate the signal that inhibits exchange activity. This possibility is consistent with the time course of the changes in [Ca²⁺], that occurred during the initial conditioning interval in low [Na⁺]. When 20 mM Na⁺ was first applied to the cells, [Ca²⁺], increased to a peak value and subsequently declined. During the following test interval, the maximal rise in [Ca²⁺], was approximated equal to the final value attained during the declining phase of the conditioning interval (Figs. 1 and 2). In the presence of CCCP + oligomycin, the post-peak decline in [Ca²⁺], during the conditioning interval was absent, suggesting that the decline reflected the developing inhibition of exchange activity. For diltiazem-treated cells, the decline in [Ca²⁺], after the peak was similar to that for control cells (Fig. 5B), indicating that exchange inhibition did not develop more rapidly, despite a possible increase in mitochondrial Ca²⁺ content. However, a possibility of inhibition exchange activity during the test pulse was more pronounced than for the control cells (Fig. 5A). We suggest that this reflects the continued development of the inhibition process due to the retention of high levels of mitochondrial Ca²⁺ during the recovery period in diltiazem-treated cells (Fig. 5B).

The results with nocodazole are complex and incompletely understood. The data suggest that microtubular structure exerts an important influence on mitochondrial function. Mitochondria are attached to microtubules and utilize these structures for intracellular movement (28–30). Following treatment with nocodazole, the mitochondria stained intensely with the potential-sensitive dye tetramethylrhodamine ethyl ester (data not shown), indicating that the reduced Ca²⁺ accumulation was not a consequence of a reduced membrane potential. Perhaps microtubular disruption altered the location or the local environment of the mitochondria in a way that interfered with their ability to accumulate Ca²⁺ during the conditioning interval. This might reflect an alteration in the local [Ca²⁺], in the vicinity of the mitochondria or a regulatory alteration in the activity of the mitochondrial Ca²⁺ uptake mechanism. This issue is currently under investigation.

What are the physiological implications of the link between Na⁺/Ca²⁺ exchange activity and mitochondrial Ca²⁺ accumulation? In cardiac myocytes, the mitochondrial Ca²⁺ concentration increases markedly with contractile frequency, particularly in the presence of β-adrenergic stimulation (31–33). An associated inhibition of Na⁺/Ca²⁺ exchange activity might therefore make a significant contribution to the positive force-frequency relationship observed in many species. Under pathological conditions, the mitochondrial interchanger interaction could in some circumstances form a deleterious positive feedback system that contributes to Ca²⁺ overload. Alternatively, in cells with elevated [Na⁺], Ca²⁺ accumulation in the mitochondria might act to limit further Ca²⁺ entry by inhibiting the Ca²⁺ influx mode of the exchanger. These considerations are entirely speculative at present, and additional experimentation is clearly needed to assess the physiological importance of this novel regulatory pathway.

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REFERENCES

1. Blaustein, M. P., and Lederer, W. J. (1999) Physiol. Rev. 79, 763–854
2. Reeves, J. P., and Hale, C. C. (1984) J. Biol. Chem. 259, 7733–7739
3. Fujoka, Y., Komeda, M., and Matsuoka, S. (2000) J. Physiol. (Lond.) 523, 339–351
4. Higlmann, D. W., and Ball, R. (1996) Science 273, 856–859
5. Higlmann, D. W., Collins, A., and Matsuoka, S. (1992) J. Gen. Physiol. 100, 933–961
6. DiPolo, R., and Beauge, L. (1999) Biochim. Biophys. Acta 1422, 57–71
7. Iwamoto, T., Pan, Y., Wakabayashi, S., Imagawa, T., Yamanaka, H. I., and Shigekawa, M. (1996) J. Biol. Chem. 271, 13609–13615
8. Shigekawa, M., Iwamoto, T., and Wakabayashi, S. (1996) Ann. N. Y. Acad. Sci. 789, 249–257
9. He, S., Rukunadin, A., Bambrick, L. L., Lederer, W. J., and Schulze, D. H. (1998) J. Neurosci. 18, 4833–4841
10. Condrescu, M., Gardner, J. P., Chernaya, G., Aceto, J. F., Kroups, C., and Reeves, J. P. (1995) J. Biol. Chem. 270, 9137–9146
11. Reeves, J. P. (1998) J. Bioenerg. Biomembr. 30, 151–160
12. Reeves, J. P., Condrescu, M., and Fang, Y. (1998) Internet Association for Biomedical Sciences 99: 5th Internet World Congress on Biomedical Sciences at McMaster University, http://www.mcmaster.ca/inabis98/lytton/revees0755/index.html
13. Condrescu, M., Hantash, B. M., Fang, Y., and Reeves, J. P. (1999) J. Biol. Chem. 274, 35279–35282
14. Pijuan, V., Zhuang, Y., Smith, L., Kroupis, C., Condrescu, M., Aceto, J. F., Reeves, J. P., and Smith, J. B. (1993) Am. J. Physiol. 264, C1066–C1074
15. Schilling, W. P., Rajan, L., and Strobl-Jager, E. (1989) J. Biol. Chem. 264, 12838–12846
16. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3420–3450
17. Condrescu, M., Chernaya, G., Kalaria, V., and Reeves, J. P. (1997) J. Gen. Physiol. 109, 41–51
18. Condrescu, M., Opon, K., and Reeves, J. P. (1999) Biophys. J. 76, A253 (abstr.)
19. Hajneczky, G., Robb-Gaspers, L. D., Seitz, M. B., and Thomas, A. P. (1995) Cell 82, 415–424
20. Rutter, G. A., Burnett, P., Rizzuto, R., Brini, M., Murgia, M., Pozzan, T., Tavare, J. M., and Denton, R. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5489–5494
21. Cox, D. A., and Matlib, M. A. (1993) Trends Pharmacol. Sci. 14, 408–413
22. Cox, D. A., Conforti, L., Sperelakis, N., and Matlib, M. A. (1993) J. Cardiovasc. Pharmacol. 21, 585–599
23. Hansford, R. G. (1991) J. Bioenerg. Biomembr. 23, 823–834
24. Chakraborti, T., Das, S., Mondal, M., Roychoudhury, S., and Chakraborti, S. (1999) Cell Signal. 11, 77–85
25. Ghafourifar, P., and Richter, C. (1997) FEBS Lett. 418, 291–296
26. Ghafourifar, P., Schenk, U., Klein, S. D., and Richter, C. (1999) J. Biol. Chem. 274, 13118–13128
27. Maechler, P., and Wollheim, C. B. (1999) Nature 402, 685–689
28. Bereiter-Hahn, J., and Voith, M. (1994) Microsc. Res. Tech. 27, 198–219
29. Summerhayes, I. C., Wong, D., and Chen, L. B. (1983) J. Cell Sci. 61, 87–105
30. Soltys, R. J., and Gupta, R. S. (1992) Biochem. Cell Biol. 70, 1174–1186
31. Miyata, H., Silverman, H. S., Sollott, S. J., Lakatta, E. G., Stern, M. D., and Hansford, R. G. (1999) Am. J. Physiol. 261, H1123–H1134
32. Ohbata, H., Chacon, E., Tesfai, S. A., Harper, J. S., Herman, B., and Lemasters, J. J. (1998) J. Bioenerg. Biomembr. 30, 207–222
33. Di Lisa, F., Gambassi, G., Spurgeon, H., and Hansford, R. G. (1993) Cardiovasc. Res. 27, 1840–1844
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