ATP Dependence of Calcium Uptake by the Na-Ca Exchanger of Adult Heart Cells

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The ATP dependence of the Na-Ca exchanger was investigated in isolated adult rat heart cells to evaluate the extent to which ATP depletion after a period of ischemia plus reperfusion in whole hearts could limit calcium uptake by Na-Ca exchange. A standard state for measurement of Na-Ca exchange activity that could be used with cells depleted of ATP to different degrees was defined. This was a state of zero sarcolemmal gradient for sodium, potassium, and pH and was achieved by incubation of the cells for 5 minutes with EDTA, EGTA, ouabain, and nigericin. Heterogeneity of cell ATP levels was minimized by using a protocol of total ATP depletion by incubation under conditions similar to ischemia, followed by reoxygenation to give partial restoration of ATP levels. No ATP was regenerated when cells were reoxygenated in the presence of rotenone, and such cells showed a very low rate of calcium uptake. Without rotenone, cells showed an almost complete restoration of Na-Ca exchange activity, in spite of a restoration of ATP levels to only one third of control values. Thus, the dependence of calcium uptake on ATP was highly nonlinear under these conditions. The calculated $K_a$ for ATP was no more than 10% of normal ATP levels. We conclude that ATP depletion after ischemia plus reperfusion is unlikely to limit the rate of calcium uptake through Na-Ca exchange in the whole heart if at least one quarter of the ATP is restored.

In addition, we measured the apparent ATP dependence of calcium uptake by Na-Ca exchange in cells under conditions in which we previously had concluded that cell ATP distributions were very heterogeneous: when cells undergo contracture during incubation with oligomycin and without glucose. A linear relation between calcium uptake rate and ATP was observed at all ATP levels. This can be understood if cells in contracture that are incubated with oligomycin cannot take up calcium because of low ATP, whereas rod-shaped cells are able to retain a full uptake capability. This result further supports our conclusion that the ATP level declines catastrophically to near zero in these oligomycin-incubated cells just before contracture. (Circulation Research 1992;71:210–217)

KEY WORDS • Na-Ca exchange • ATP • ischemia • calcium overload

Failure of hearts to recover function after a period of ischemia is correlated with an increased uptake of calcium from the perfusate, most likely via Na-Ca exchange. We have found that the exchanger in heart is inhibited by ATP depletion, as is the exchanger in squid axon. Since heart tissue undergoing ischemia suffers a loss of ATP followed by limited regeneration of ATP on reperfusion, this raises the question of whether ATP depletion can limit calcium uptake by Na-Ca exchange on reoxygenation.

One way to address this question would be to measure the magnitude of Na-Ca exchange currents in perfused single cells or giant excised patches at different levels of ATP. Such methods have the advantage of affording almost complete control of the environment at both sides of the membrane. One problem with such measurements, however, is that the intrusion necessary to achieve such control comes at the price of potentially altering the properties of labile control mechanisms. There is already evidence that the control mechanisms of the Na-Ca exchanger are easily altered. Although sarcolemmal vesicles show considerable Na-Ca exchange activity and some control by both calcium and ATP has been demonstrated, single cells show an extremely high affinity for the action of calcium at the intracellular regulatory site, whereas excised patches show an affinity for calcium that is very much lower at this site. The value found with perfused single cells would lead one to expect that the site would be occupied at resting levels of intracellular calcium and hence to expect that the exchanger would always be active. On the other hand, using intact cells, we have found evidence that the exchanger is almost inactive in cells at rest and becomes switched on by intracellular calcium when the cells are stimulated to beat. These results suggest that the affinity for calcium at this site is itself under regulation and that the regulation is altered under conditions of internal perfusion. It is entirely possible that the regulation by ATP is similarly vulnerable. For this reason it is valuable to examine the question of the ATP concentration dependence of the exchanger, if possible, in cells that are intact. We do this here, using isolated adult rat heart cells depleted of ATP to various degrees by incubation under conditions we have previously used that simulate some of the conditions experienced by heart cells in vivo during
ischemia: Cells were incubated in anoxic medium without glucose in a glass tube, such that the cells formed a pellet. These cells experience deprivation of both oxygen and glycolytic substrate and a degree of acidosis.

Two problems complicate the assessment of the ATP dependence of Na-Ca exchange under these conditions. The first is that we previously found evidence that the decline in ATP measurable under these conditions reflects not only an early synchronous decline in all cells but also a later asynchronous decline, that is, a very sudden near-total loss of ATP that in each cell is accompanied by contracture.

Either in tissue or in cell suspensions the onset of contracture occurs at different times in different cells, probably coincident with the exhaustion of endogenous glycogen stores. Consequently, ATP measurements made on tissue or cell suspensions with some cells in contracture and others not in contracture are likely to reflect a very heterogeneous distribution of ATP levels from cell to cell. Under these conditions, although typical of tissue subjected to ischemia, the true relation between intracellular ATP concentration and Na-Ca exchange activity cannot be defined.

We have found that we can minimize this difficulty by first allowing all cells to undergo contracture, thus reducing ATP levels in all cells to near zero. On reoxygenation, a certain amount of ATP is regenerated, presumably in all cells, although some degree of heterogeneity may remain.

The second difficulty of measuring the ATP dependence of calcium uptake by Na-Ca exchange in cells depleted of ATP under these conditions is that such calcium uptake will be strongly affected by the transmembrane gradients of sodium and calcium, by the membrane potential, and by both intracellular and extracellular pH. The incubation conditions used will affect all of these parameters.

To address this problem we have defined standard conditions for the measurement of calcium uptake by Na-Ca exchange that are attainable both by cells with normal ATP and by ATP-depleted cells. The standard conditions were complete equilibration of sodium, potassium, and pH gradients and, hence, zero membrane potential. This condition, which strongly favors calcium influx when calcium is restored, was achieved by a 5-minute incubation of the cells with EDTA, EGTA, and nigericin in the presence of ouabain.

Materials and Methods

Cell Isolation

Heart cells were isolated from female retired breeder rats according to our original method, as recently modified. The modification used was condition 5 in Table 2 of Reference 17: The perfusion buffers contained 25 mM HEPES, adjusted to pH 7.4 with NaOH, in place of bicarbonate, plus basal Eagle medium amino acids. Calcium (1 mM) was restored to the recirculating perfusate 15 minutes after enzyme addition. This method gave a high yield of cells with a high percentage (74.3±6.0%) of rod-shaped cells in the presence of 1 mM calcium.

Experimental Medium

Cells were suspended (four 2-ml aliquots of ~8 mg protein/ml) in a medium containing (mM) NaCl 118, KCl 4.8, HEPES 25, KH2PO4 1.2, MgSO4 1.2, and CaCl2 1.0, adjusted to pH 7.4 with NaOH. Suspensions were maintained aerobic by equilibration with air in a shaking incubator at 37°C.

Fura-2 Labeling

Cells in experimental medium were labeled with fura-2 by incubation with fura-2 AM (10 µg/ml) for 20 minutes at 37°C. The cells were washed and resuspended in experimental medium.

86Rb Labeling

Cells in experimental medium were labeled with 86Rb by aerobic incubation with 86Rb (0.6 µCi/ml) for 40 minutes at 37°C before subjecting them to the experimental conditions (see “Results”). 86Rb uptake was expressed as potassium equivalents, that is, as though the 86Rb behaved just like labeled K.

EDTA Treatment

Cells were treated with EDTA, EGTA, ouabain, and nigericin to equilibrate sodium, potassium, and proton gradients across the sarcolemma. EDTA facilitated rapid equilibration of monovalent ions across the sarcolemma through calcium channels. We previously found that EDTA treatment of heart cells resulted in a rapid thallium efflux and sodium uptake that was verapamil sensitive. EGTA was included to maintain near-zero levels of calcium on restoration of normal magnesium levels; otherwise, micromolar levels of free calcium are released by displacement from EDTA. Ouabain prevented the reestablishment of gradients by inhibition of the sodium pump. Nigericin ensured clamping of the intracellular pH to the extracellular pH value via the zero K gradient and K-H exchange. Cell suspensions under each of the experimental conditions (see “Results”) were centrifuged. The supernatant (1.9 ml) was removed and replaced by 5.9 ml O2-saturated medium similar to the experimental medium but containing no magnesium or calcium and containing 0.2 mM EGTA, 0.2 mM EDTA, and 1 mM ouabain. For cells incubated with rotenone or oligomycin (see text), the medium also contained these agents. Nigericin (4 µM) was added, the cells were incubated for 5 minutes, and then magnesium (1.2 mM) plus ruthenium red (5 µM) were added. The ruthenium red was used to prevent adventitious calcium uptake by the mitochondria of cells with a ruptured sarcolemma. The suspension was divided into two 3-ml aliquots for simultaneous measurement of calcium uptake by fura-2 fluorescence (aliquot A) and by 45Ca (aliquot B).

22Na, 14C]Sucrose, and 14C-DMO Uptake

Cells were resuspended in EDTA medium (as mentioned above) containing 2 mM carrier sucrose plus tritiated water (1 µCi/ml) and also containing [14C]sucrose. 22Na (0.1 µCi/ml), or 5,5-dimethyl-2-4-oxazolidinedione ([14C]DMO, 0.1 µCi/ml) at time zero. The DMO samples also contained 0.1 mM unlabeled carrier DMO. DMO uptake was used to measure intracellular pH. At the times shown, aliquots (0.5 ml) of cell
suspension were removed and centrifuged through bromododecane (0.5 ml) into perchloric acid (0.1 ml). Radioactivity in aliquots of the perchloric acid and of the supernatant was measured in a liquid scintillation counter. The pellet ratio ($R_p$) of $^{14}$C to tritium or $^{22}$Na to tritium was then expressed as a percentage of the supernatant ratio ($R_I$). Thus

$$\% \text{Permeation} = (R_p/R_I) \times 100$$

Intracellular pH (pH$_I$) values were calculated from percent permeation values for DMO (%DMO) and sucrose (%suc) by the following formula:

$$pH_I = pK - \log_{10} ((100 - \% \text{suc})/ [(1 + R)\% \text{DMO} - \% \text{suc}) - R(100 - \% \text{suc})])$$

where R is $10^{(pK-\text{pH})}$, p$K$ is the pK for DMO dissociation (6.13), and pH is the extracellular pH.

**ATP Measurement**

Thirty seconds after the addition of ruthenium red, an aliquot (0.2 ml) was removed from aliquot B (see “EDTA Treatment") and mixed with an equal volume of 16% perchloric acid on ice for ATP analysis by high-performance liquid chromatography.

**$^{45}$Ca Uptake**

One-minute after the addition of ruthenium red, calcium (1.4 mM) with $^{45}$Ca was added to aliquot B, along with $^3$H$_2$O. Aliquots (0.5 ml) were removed at time intervals and centrifuged, as previously described. 20

**Intracellular Calcium Measurement With Fura-2**

**Cells in suspension.** Aliquot A (see “EDTA Treatment" above) was placed into a cuvette that was in a water-jacketed holder maintained at 37°C, which was in a fluorescence spectrophotometer (model CM111, SPEX Industries Inc., Edison, N.J.). The cell suspension was stirred from above. Fura-2 fluorescence was measured at excitation wavelengths of 340 and 358.3 nm and an emission wavelength of 505 nm. We found 358.3 nm to be the isosbestic wavelength in our system. Integration time was 1 second; the time increment was 3 seconds. One minute after the addition of ruthenium red, calcium (1.4 mM) was added; after 1.5 minutes, digitonin (17 $\mu$g/ml) was added. Fluorescence was expressed as a ratio (R) of that excited at 340 nm to that excited at 358.3 nm. $R_{max}$ was taken as the ratio before calcium addition. $R_{max}$ was taken as the ratio after digitonin addition. Fluorescence from extracellular dye was taken into account by also measuring $R_{max}$, the initial fast rise in ratio on calcium addition, attributable to saturation with calcium of dye outside the intact cells. The value of $R_{max}$ was evident by inspection even from the tracings with the fastest rates of calcium influx as a point of inflection. Intracellular calcium concentration ([Ca$_I$]) was then calculated from the following equation:

$$[Ca_I] = -\frac{K_d(R - R_{min}) + [Ca]_o + [Ca]_o(R_{ex} - R_{min})}{\{R_{max} - R\} + [Ca]_o - K_d(R_{ex} - R_{min})}$$

where $K_d$ is the dissociation constant of fura-2 for calcium (a value of 224 nm was used), and [Ca], is the concentration of extracellular calcium, here 1 mM. This equation is valid only when the second excitation wavelength is isosbestic. Note that no subtraction of autofluorescence is required, although this is only true as long as changes in autofluorescence between low and high calcium are negligible compared with changes in dye fluorescence. This condition is achieved under the dye-loading conditions used here.

**Single cells.** Fura-2–loaded cells in experimental medium were attached to laminin-coated (4 $\mu$g/cm$^2$) quartz coverslips by incubation for 10 minutes at 25°C. The coverslips were incorporated into a perfusion chamber, where the cells were perfused with experimental medium at 37°C without calcium but with EGTA (0.2 mM) and exposed to either 1) no incubation or 2) a 60-minute incubation with experimental medium plus rotenone (3 $\mu$M). During this time the cell with rotenone was observed to undergo contracture. For cells under both conditions, chamber perfusate was then switched to the EGTA-EDTA-nigericin-ouabain medium used for the EDTA treatment. We were concerned that delivery of nigericin to the cells in the chamber could be problematic, since nigericin is hydrophobic and could stick to tubing. However, preliminary experiments with cells loaded with the pH-sensitive dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) showed that perfun with medium containing nigericin after ATP depletion was effective at equilibrating intracellular and extracellular pH within 5 minutes (data not shown). After 5 minutes of superfusion of the fura-2–loaded cells with EGTA-EDTA-nigericin-ouabain medium, the perfusate was switched to an identical medium with magnesium (1.2 mM) and ruthenium red (5 $\mu$M) added, and fluorescence measurement was begun. Excitation wavelengths of 340 and 380 nm were used. The system used a Nikon Diaphot fluorescence microscope with a 510/20-nm barrier filter on emitted light and a Nikon CF fluor $\times 40$ objective. After 1 minute, the perfusate was switched to an identical medium with 1.4 mM calcium added. Thus, the protocols were very similar to those of conditions 2 and 4 for the cells in suspension (see “Results”). The perfusate was finally switched to an identical medium plus ionomycin (3 $\mu$M) 1.5 minutes after calcium addition. [Ca$^2+$] concentration was calculated from the following formula:\n
$$[Ca] = \frac{K_d(R_{min} - R_{min}) - [Ca]_o}{\{R_{max} - R\} - K_d(R_{ex} - R_{min})}$$

where

$$\beta = \frac{\beta(R_{max} - R_{min}) + (R_{max} - R_{min})}{\beta(R_{min} - R_{min}) + (R_{max} - R_{min})}$$

and

$$\beta = S_{22}/S_{21}$$

As is conventional, 22 $S_{22}$ and $S_{21}$ are constants of proportionality between fluorescence and dye concentration for pure dye with calcium bound (b) or free (f) at excitation wavelength 2 (380 nm), $R_{max}$ is the ratio of fluorescence excited at 340 nm to that excited at 380 nm for calcium-saturated pure dye, and $R_{min}$ is the same ratio for dye in the absence of calcium. These values were measured on pure fura-2 in our chamber without cells. $R_{min}$ was taken as the 340/380 ratio from the dye-loaded cell before perfusion with calcium, and $R_{max}$
FIGURE 1. Graphs showing equilibration of sodium and rubidium resulting from EDTA treatment. isc, Ischemia; reox, reoxygenation; rot, rotenone. The incubation conditions corresponding to each symbol are described in the text; the EDTA treatment is described in “Materials and Methods.” Panel A: 22Na and 14C/sucrose. Panel B: 86Rb. Data shown are mean values from three experiments.

was taken as the ratio after perfusion with calcium and ionomycin. This calibration also does not require the subtraction of fluorescence contributions from autofluorescence or calcium-insensitive dye.

**Data Presentation**

All experiments were done at least three times. Data in figures are either from single representative experiments or are pooled from several experiments, as described in the legends.

**Results**

In an attempt to minimize the heterogeneity of cell ATP content, as discussed, our first strategy for ATP depletion was to deplete ATP levels in all cells to near zero with a period of anoxic incubation in medium without glucose and then to restore a certain level of ATP by reoxygenation.

Cells in experimental medium were incubated under four conditions: 1) ischemia plus reoxygenation, 2) ischemia plus reoxygenation plus rotenone, 3) control at end, and 4) control at start.

For condition 1 (ischemia plus reoxygenation), a 2-ml aliquot in experimental medium was equilibrated with N2 and placed in a sealed glass tube. After incubation at 37°C for 60 minutes, the cells were resuspended by tube inversion and then subjected to EDTA treatment.

For condition 2 (ischemia plus reoxygenation plus rotenone), a 2-ml aliquot was equilibrated with N2 and rotenone (3 μM) also was added. The cells were placed in a sealed glass tube at 37°C for 60 minutes and then resuspended by tube inversion before EDTA treatment. The purpose of the rotenone was to prevent ATP resynthesis by oxidative phosphorylation on reoxygenation.

For condition 3 (control at end), a 2-ml aliquot was incubated aerobically at 37°C for 60 minutes in a shaking incubator before EDTA treatment.

For condition 4 (control at start), a 2-ml aliquot was subjected to EDTA treatment at time zero, when the other aliquots began their incubation.

After EDTA treatment calcium uptake rates were measured by fura-2 and by 45Ca. However, it was first necessary to establish the efficacy of the EDTA treatment: Did this treatment put the cells in a uniform state of zero gradient, regardless of prior treatment history? The efficacy of the EDTA treatment was evaluated from its effect on the uptake of 22Na and the efflux of 86Rb. It can be seen from Figure 1A that 22Na uptake was essentially complete within 5 minutes of exposure to the EDTA medium and from Figure 1B that 86Rb efflux was also essentially complete, for all four of the experimental conditions. There was no effect of the EDTA treatment on sucrose exclusion (Figure 1A), although there could have been a small loss of sucrose-impermeable space caused by the ischemic incubation conditions (Figure 1A). The ischemic samples began with less 86Rb than the aerobic samples because of 86Rb efflux during the ischemic incubation. Measurements with DMO indicated that intracellular pH values had also completely equilibrated with extracellular pH after 3 minutes of the EDTA treatment. Intracellular pH values after 3 minutes were 7.462±0.015 for ischemia plus reoxygenation, 7.511±0.040 for ischemia plus reoxygenation plus rotenone, 7.490±0.061 for control at end, and 7.449±0.026 for control at start. These values were not significantly different from each other, by analysis of variance, and were unchanged after 6 minutes of incubation in the EDTA medium. It is apparent, however, that these values are all slightly above the extracellular pH value of 7.4. This is most likely an artifactual difference caused by a small degree of DMO binding to the cells. In summary, these results show that for each condition the EDTA treatment puts them into the same standard state with respect to intracellular and extracellular concentrations of sodium, potassium, and pH, for the measurement of calcium uptake by Na-Ca exchange.

When fura-2–loaded cells were exposed to the EDTA treatment (control at start) and then calcium uptake was measured, the cells showed a high rate of calcium uptake either by fluorescence (Figure 2A, open circles) or by 45Ca (Figure 2B, open circles). Cells incubated aerobically for 1 hour and then exposed to the EDTA treatment (control at end) showed a similar high rate of calcium uptake by either method (Figure 2, filled cir-
When cells were incubated for 1 hour under the conditions of simulated ischemia, they all underwent contracture, as determined by visual inspection of aliquots of resuspended cells by light microscopy, whether or not rotenone was included in the incubation medium. This was consistent with our previous observations. Subsequent exposure of the control or rotenone-treated cells to reoxygenation also resulted in reoxygenation of the cells, although in the sample with rotenone ATP resynthesis was prevented (Figure 2C, filled triangles). When calcium uptake was measured on these cells, the reoxygenated cells without rotenone showed an almost complete recovery of the initial rate of calcium uptake (Figures 2A and 2B, open triangles), even though they had recovered only one third of their ATP (Figure 2C, open triangles), whereas the cells with rotenone showed a strong inhibition of calcium uptake (Figures 2A and 2B, filled triangles). The apparent rate of rise of Ca, observed under the latter conditions (256±15 nM/min, n=3 experiments like those represented by the filled triangles in Figure 2A) is probably an overestimate that was caused by the finite rate of cell breakage while the cells were stirred in the cuvette. This possibility was suggested to us by the observation that the amount of fluorescence that was rapidly quenched on addition of manganese increased with the time of stirring (data not shown). Therefore, we undertook similar measurements on single cells, where the influence of cell breakage could be eliminated.

In experiments with single cells, it was difficult to reproduce all of the conditions of simulated ischemia that we used with the cell suspensions. However, the control condition and the treatment with rotenone should leave single cells in a state similar to that achieved by similar treatment of cells in suspension, especially after normalization to the standard state by the EDTA treatment. In experiments with single cells depleted of ATP by incubation with rotenone (see “Materials and Methods” for details), much lower rates of calcium entry were observed (Figure 3): the rate of rise of intracellular calcium in such cells was only 10.3±4.6 nM/min for the first 2.5 minutes after calcium addition for three different experiments like that shown in Figure 3.

Since the above evidence indicated that the initial rate of entry of calcium into ATP-depleted cells was very low, even for cells with calcium entry favored by the standard state, values for the initial rate of 45Ca uptake were taken as 45Ca uptake at 1 minute minus the linearly extrapolated time-zero uptake with ischemia plus rotenone. These values were plotted against cell ATP values, and the data were fit to the Michaelis-Menten equation for each preparation. As a good approximation, the fit was forced through zero, although this does not imply that the rate of calcium entry in the absence of ATP is actually zero. The best fit
maximum rate of calcium uptake ($V_{\text{max}}$) values for each preparation were 6.82, 4.54, and 6.72 nmol·min$^{-1}$·mg$^{-1}$. Each experimental value was then expressed as a fraction of its own best fit (i.e., $V/V_{\text{max}}$) and plotted against ATP (Figure 2C). The best fit line is also shown in Figure 2C. The best fit aggregate $K_m$ for ATP was 1.96 nmol/mg. This value could err on the high side, since all the data that contributed to the fit were within 27% of $V_{\text{max}}$. Thus, ATP depletion of 90% results in, at most, a 50% reduction in the rate of Na-Ca exchange. When a sucrose-impermeable space of 60% (Figure 1A) and 2.45 μl/mg total pellet water were used, $K_m$ for ATP was calculated to be 1.3 mM.

By way of contrast with the above results, we have also investigated the apparent ATP dependence of calcium uptake by Na-Ca exchange in cells depleted of ATP under conditions in which we expect the distribution of ATP among the cells to be the most heterogeneous. Aliquots of cells in experimental medium were incubated in tubes at 37°C in the presence of oligomycin (40 μM) for 10, 20, 30, 40, or 60 minutes. After incubation, each aliquot was resuspended and subjected to EDTA treatment. Measurements of ATP and $^{45}$Ca uptake were done as before; in addition, a small aliquot of cell suspension was fixed with glutaraldehyde, and a slide was made for subsequent determination of the percentage of rod-shaped cells by light microscopy. We have previously found that under these conditions cells undergo contracture very suddenly, so that the percentage of cells with a normal sarcomere length of >1.8 μm, which we term rod-shaped, is easily determined. As before, we found a bell-shaped dependence of the percentage of rod-shaped cells with the time of incubation, and ATP content declined in a parallel fashion. Moreover, the amount of calcium taken up after 1 minute under our standard condition declined in a similar way with the time of incubation. Consequently, when calcium uptake was plotted against the percentage of rod-shaped cells (Figure 4A) or against ATP (Figure 4B), a linear correlation was observed ($r=0.966$ for Figure 4A, and $r=0.965$ for Figure 4B). Figure 4B clearly contrasts with the ATP dependence of calcium uptake observed for cells reoxygenated after ischemic incubation without oligomycin or rotenone (Figure 2C). The positive intercept for calcium uptake at zero ATP in Figures 4A and 4B corresponds mostly to the amount of extracellular calcium bound rather than a cellular rate of calcium uptake (e.g., compare with Figure 2B, filled triangles).

**Discussion**

We reported previously that calcium influx was inhibited by 82% in ATP-depleted cells, as measured from rates of $^{45}$Ca uptake. This result was qualified by some uncertainties that made that value a lower limit. We noted then that the residual rate of calcium influx was further inhibited by as much as 90% between minutes 1 and 12 after calcium addition. We now think it is probable that the latter rate is closer to the true rate of entry, that is, about 2% of the rate of calcium entry into cells undepleted of ATP (Figure 6, Table 1, and text of Reference 2). The apparently higher initial rate reported in that experiment could have arisen from an artifact of the baseline measurement. The major reason

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**Figure 3.** Graph showing inhibition by ATP depletion of rise in Ca, on calcium restoration after EDTA treatment, measured on single cells. isc, Ischemia; reox, reoxygenation; rot, rotenone. See "Materials and Methods" for experimental details. Data are from a single experiment.

**Figure 4.** Graphs showing calcium uptake after 1 minute by Na-Ca exchange in cells that were incubated with oligomycin and had a heterogeneous distribution of ATP. See text for experimental design. Panel A: Dependence on percentage of rod-shaped cells. Panel B: Dependence on ATP. Data are from three experiments.
for believing that no initial burst of calcium entry occurs is that the data from single cell fluorescence in the present study shows that there is none (Figure 3). This measurement is free of interference from extracellular dye, and any initial burst of calcium uptake, if present, would be clearly visible.

The fluorescence measurements on cell suspensions in our previous study and also in this study are complicated by a contribution from a finite rate of breakage of cells, which, in the case of the ATP depleted cells, results in a much overestimated rate of calcium entry (Figure 2A). Only the single-cell measurement is free of this artifact, and this measurement shows the rate of calcium entry to be very strongly inhibited with ATP depletion (Figure 3).

In our previous study, we made no attempt to neutralize the acidosis that occurs on ATP depletion. Since acidosis is a well-known inhibitor of the Na-Ca exchanger, part of the inhibition we observed with ATP depletion could have come from this source. In the present study, on the other hand, we applied normalized conditions that removed acidosis as a factor. Under these conditions, ATP depletion still resulted in a very strong inhibition of calcium influx by Na-Ca exchange.

An apparently inhibited rate of calcium uptake could in theory arise from an enhanced rate of calcium efflux. This explanation is excluded by the observation that in ATP-depleted cells C\textsubscript{a} rises extremely slowly (Figure 3). Since the conditions favor a massive calcium influx through the exchanger, if it were active, the appearance of a minimal rate of rise of C\textsubscript{a} would require a huge rate of calcium efflux. It is difficult to imagine how calcium efflux through the calcium pump could be so stimulated under these conditions, since the pump requires ATP to function. Given that this result under these conditions does show inhibition of calcium influx, it then becomes superfluous to postulate the existence of enhanced efflux as an explanation for the conditions in which a less complete slowing of calcium accumulation is observed.

Since calcium uptake under the normalized conditions is taken as a measure of Na-Ca exchange activity, it is important to know the extent of calcium uptake through other pathways under these conditions. The most likely alternative route of entry is through calcium channels. Also, the exchanger is activated by C\textsubscript{a}, and calcium entry through calcium channels can be important for this activation process, although less so the stronger the driving force is for calcium entry through the exchanger. We have previously investigated the verapamil sensitivity of calcium uptake by cells loaded with sodium by incubation with ouabain. We found that verapamil (2 \mu M) inhibited initial rates of \textsuperscript{45}Ca uptake by 12.6% on average (Table 1 of Reference 25). We concluded then that calcium channels were not contributing much to the calcium uptake rate under those conditions. Similar measurements on cells in our standard state used here showed that \textsuperscript{45}Ca uptake after 1 minute was inhibited no more than this amount by up to 10 \mu M verapamil (data not shown). Thus, although inhibition of calcium channels by ATP depletion could contribute to the inhibition of calcium uptake under our standard conditions, it does not appear to be a major factor.

From our results, it is clear that the ATP dependence of calcium uptake by Na-Ca exchange depends critically on the method used to alter the ATP content of the cell suspension. However, the results we found do fit nicely into the concept of ATP depletion that we have previously developed, without any special pleading. According to this concept, cells exposed to simulated ischemia without glucose undergo a small initial synchronous drop in ATP when oxidative phosphorylation is interrupted, but they remain rod shaped, and ATP levels remain stable. Subsequently, as glycogen stores are used up by glycolysis, cells one by one undergo a catastrophic loss of ATP, resulting in a rigor-induced contracture. The inferred levels of ATP in such cells are extremely low, since rigor is inhibited by levels of ATP >0.1 mM. Thus, at a point in time when half of the originally rod-shaped cells have undergone contracture, half of the cells would contain essentially normal levels of ATP, and the other half would contain virtually none at all. The ATP values measured on the suspension would, of course, be half the value measured before the onset of contracture. We have previously found that essentially complete depletion of ATP results in almost total inhibition of the Na-Ca exchanger in the presence of extracellular Na, and our present data further support this conclusion (Figures 2A, 2B, and 3). Thus, we explain the linear correlation between calcium uptake and ATP under the conditions of Figure 4 by attributing the calcium uptake only to the cells that, before calcium addition, were rod shaped. The ATP level in the contracted cells must be too low to activate the exchanger. Under the conditions of Figure 2, on the other hand, all cells have undergone ATP depletion and contracture and have been allowed to resynthesize some ATP on reoxygenation. Thus, all cells are able to participate in calcium uptake to the extent that the exchanger is reactivated. Hence, this condition most truthfully reveals the ATP dependence of the exchanger.

It is clear from Figure 2C that the level of ATP required to reactivate the exchanger is not very great. This result has a further implication. The lower the ATP requirement truly is, the more remarkable it is that the relation in Figure 4B is linear, and the more our conclusion is supported that the contracted cells under these conditions contain virtually no ATP.

What are the implications of these results for calcium uptake by the myocardium on reflow after ischemia? We have noted previously the similarities between isolated cells and whole hearts, in terms of the biphasic decline in ATP seen and also the timing and heterogeneity of contracture. It is entirely possible that cells in ischemic hearts undergo the kind of heterogeneous decline in ATP that we have inferred from experiments done in the presence of oligomycin (Figure 4). Under these conditions, in cells where ATP has declined to near zero, calcium influx through the exchanger could be limited by ATP depletion. Even so, over the course of time, calcium influx through the exchanger could still be significant, and it could still be the main pathway for calcium entry. Furthermore, even a tiny supply of oxygen, such as is common with ischemia, could have a strong effect on the low rate of exchange activity, given the sensitivity of the exchanger to ATP. On reperfusion after ischemia, there will certainly be resynthesis of some significant level of ATP. From our results in Figure 2 we would conclude that calcium influx through
the Na-Ca exchanger is hardly limited at all when only one quarter of the original ATP is present. Hence, we conclude that calcium uptake through the Na-Ca exchanger is unlikely to be much limited by ATP in hearts reperfused after a period of ischemia, as long as they are able to restore ATP to 25% of the original level.

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