A Phosphorus-31 Nuclear Magnetic Resonance Study of the Metabolic, Contractile, and Ionic Consequences of Induced Calcium Alterations in the Isovolumic Rat Heart

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SUMMARY. Isolated adult rat hearts perfused in an isovolumic mode were used to study the effects of sodium-potassium pump inhibition and sodium-calcium exchange alterations on the tissue content of adenosine triphosphate, phosphocreatine, inorganic phosphate, and intracellular pH, all measured by phosphorus-31 nuclear magnetic resonance spectroscopy. Rates of oxygen consumption, contractile function, and the cell contents of calcium, sodium, and potassium also were determined. The inhibition of sodium-potassium adenosine triphosphatase, either by the reduction in perfusate potassium from 5.9 to 1 millimolar or less, or by the addition of $10^{-4}$ molar ouabain, transiently increased systolic pressure. This was followed by a decrease in systolic pressure, an increase in diastolic pressure, and eventual inexcitability. This contractile profile was accompanied by a persistent increase in oxygen consumption, a monotonic decline in cellular adenosine triphosphate and phosphocreatine content, the development of marked intracellular acidosis, a gain in cell sodium and calcium content, and a reduction in cell potassium. Quite similar metabolic changes were also observed when cell calcium was increased after a reduction in perfusate sodium. These metabolic and contractile effects could be prevented or reversed by decreasing perfusate calcium. The results emphasize the profound role of calcium in modulating cell oxygen consumption, energy balance, pH, excitability, and force production. These data are discussed in light of changes in the myocardial energy supply/demand balance, as well as from the viewpoint of the known competition between mechanisms for mitochondrial calcium transport vs. high-energy phosphate production. (Circ Res 58: 539-551, 1986)

THE MAINTENANCE of a transmembrane Na⁺ gradient, critical for the function and viability of excitable cells, is accomplished by the energy dependent Na⁺-K⁺ pump. When this pump is inhibited in the myocardial cell, either by cardiac glycosides or by extracellular ionic changes, initial cellular Na⁺ and Ca²⁺ increases are associated with enhanced contractile function. If allowed to progress, further Na⁺ and Ca²⁺ loading leads to compromised contractile function, increased diastolic tone, marked alterations in energy production and utilization, cellular acidosis, arrhythmias, conduction disturbances, and irreversible ultrastructural changes (for reviews, see Lee and Klaus, 1971; Glitsch, 1979).

The magnitude of these alterations depends on the tissue and on the extent and duration of Na⁺-K⁺ pump inhibition (Eisner and Lederer, 1979), and can also be graded by changing the extracellular milieu. Recent studies have employed ion-selective microelectrodes to delineate the electrophysiological, ionic, and tonic force changes that result from Na⁺-K⁺ pump inhibition. These have been performed in preparations with an abnormally low metabolic demand, e.g., quiescent cardiac Purkinje tissue (Led-erer and Tsien, 1976; Ellis and Deitmer, 1978; Deit-mer and Ellis, 1980; Bers and Ellis, 1982; Eisner et al., 1983; Vaughan-Jones et al., 1983). In other studies, metabolic parameters following Na,K-ATPase inhibition have been measured, but without reference to changes in O₂ consumption, cell ionic content, pH, or contractile function (Finkelstein and Bodansky, 1948; Wood et al., 1972; Gervais et al., 1978; Matthews et al., 1982). Thus, the relationships between these multiple parameters observed during Na⁺-K⁺ pump inhibition have not been concurrently evaluated in the intact myocardium.

In the present study we have measured the effects of both Na⁺-K⁺ pump inhibition and Na⁺-Ca²⁺ exchange alterations on contractile function and O₂ consumption while using 31P nuclear magnetic resonance (31P NMR) to monitor the high-energy phosphate content and intracellular pH continuously in an intact perfused isovolumic heart preparation. The metabolic and contractile changes were then correlated to alterations in the cellular ionic content as determined by atomic absorption. In this way, the complex relationships among these factors could be more comprehensively evaluated.
Methods

Perfused Isovolumic Heart Preparation

Because of their high sensitivity to calcium alterations, their long-standing use as a heart metabolic model, and the ease by which they can be assessed by 31P NMR, rat hearts were used as the investigational model in these studies. Adult male Wistar rats weighing 500–600 g were heparinized and anesthetized with sodium pentobarbital. The heart was rapidly excised and the aorta cannulated for Langendorff perfusion at 36°C using a constant flow of 15 ml/min with the following perfusate composition (in mm): NaCl, 118; NaHCO3, 25; KCl, 5.9; MgSO4, 1.2; CaCl2, 2.5; glucose, 16; and mannitol, 1.12. The perfusate was saturated with 95% O2 + 5% CO2 (pH = 7.35). Each heart was paced just above threshold voltage at a rate slightly higher than its intrinsic frequency via a catheter filled with agar-KCl (3 m) positioned in the right ventricle and secured to the pulmonary artery. A latex balloon was introduced into the vented left ventricle and connected to a Statham P23db transducer. Left ventricular systolic and diastolic pressures were monitored continuously on a brush recorder. The balloon was progressively inflated until a peak in developed pressure was observed. Under these conditions, the end-diastolic pressure was 8–10 mm Hg.

3P Nuclear Magnetic Resonance Methods

The heart was positioned in the 25-mm probe of the superconducting magnet of a Bruker WH 180 spectrometer interfaced with a Nicolet 1080 computer (field 4.2 Tesla; phosphorus frequency = 72.89 MHz; proton decoupled). Fourier transform spectra were obtained with a pulse duration of 25 μsec (45°) with a pulse interval of 2 seconds; the acquisition time was 2, 5, or 10 minutes. Data were accumulated with a 4K data table at a 3,000 Hz spectral width. Under these conditions, spectral saturation was presumed to be minimal since, at this field, the Ti relaxation time for phosphocreatine (PCr) was approximately 2.72 seconds in perfused rabbit hearts (Flaherty et al., 1982).

NMR Measurements

The peak areas observed at characteristic frequencies for PCr, inorganic phosphate (Pi), and adenosine triphosphate (ATP) were integrated with a Hewlett-Packard 9810 digitizer. To correct for baseline abnormalities, the normal spectral peaks for Pi, PCr, and ATP (line broadening, 10 Hz) were extrapolated to a smooth spectrum obtained with a line broadening of 300 Hz. In some experiments, the stability of the signal was assessed by measuring the peak area of a concentrated phosphate solution introduced in a closed microsphere in the intraventricular latex balloon. Under these conditions, no spectral differences were noted. Intracellular pH (pHi) was measured from the chemical shift of the Pi peak relative to PCr peak, according to the following equation:

\[
pH_i = pK - \log \frac{\delta_0 - \delta_B}{\delta_A - \delta_0}
\]

with \(pK = 6.90; \delta A = 3.29 \text{ ppm}; \delta B = 5.085 \text{ ppm}; \) and \(\delta_0\) the observed chemical shift in ppm of the Pi peak measured relative to the PCr peak (Jacobus et al., 1982).

Biochemical Determinations

The tissue contents of Pi, PCr, and ATP were measured by direct chemical analysis of freeze-clamped hearts which were then extracted in 7% perchloric acid (PCA). Pi was immediately measured in the acid extract by a colorimetric method (Berenson and Chain, 1938). Extracts were neutralized, and ATP and PCr were measured by an enzymatic determination (Estabrook and Maitra, 1962). Protein was quantified by the biuret method. Metabolite contents are expressed as nmol/mg protein.

To compare the NMR spectrum from an isolated perfused heart with its PCA extract, a subset of hearts was rapidly frozen after control NMR spectra were obtained, stored in liquid-nitrogen, and extracted with 7% PCA. A 15-ml sample containing added D2O and EGTA was analyzed for 5.5 hours at 8°C (2,000 pulses).

Oxygen Consumption

In a separate series of hearts not perfused in the NMR spectrometer, the pulmonary artery was cannulated and the O2 content was monitored continuously with a thermostated PO2 electrode (Radiometer pHM 72) calibrated with perfusate at the experimental temperature and flow. Control experiments performed in closed chamber measuring the O2 concentration of the total effluent showed that the pulmonary artery PO2 measurement appropriately reflected the total venous PO2. Perfusion arterial PO2 was measured at the beginning and end of the experiments; the two did not differ.

Ionic Contents

The perfusate for the hearts employed in the ionic determinations contained 1 mm potassium cobaltic EDTA (KCo-EDTA), prepared in pure crystalline form by the method of Dwyer et al. (1935), as an extracellular marker (Bridge et al., 1982). Because 1.0 mm potassium EDTA was utilized in these studies, Kc could not be reduced to less than 1.0 mm.

After the protocol was completed, wet and dry heart weights were determined. The dried tissue was completely digested with HNO3 and PCA. The total tissue contents of Ca++, Co++, Na+, and K+ were then determined by atomic absorption. The extracellular space (ECS) was estimated using the following equation:

\[
ECS = \frac{\text{Co total}}{[\text{Co}][\text{H}_2\text{O}]}
\]

where CoTOTAL = total tissue cobalt, [Co] = perfuse cobalt concentration, and H2O = total tissue water. Cell ionic contents were then calculated as follows:

\[
I_c = I_T - (ECS)(H_2O)(I_p)
\]

with Ic = cell ion content, IT = total tissue ion content, and Ip = concentration of ion in the perfusate. Cell ionic contents are expressed as μmol/g dry weight.

Experimental Protocols

A 30-minute equilibration period followed inflation of the balloon, and NMR spectra were collected during the last 15 minutes. To ensure uniform hearts, only those meeting the following criteria were retained: (1) intrinsic heart rate >150 beats/min, (2) heart rate × developed pressure (systolic-diastolic) >3 × 104, (3) diastolic pressure <13 mm Hg, (4) ratio of systolic to diastolic pressure >15, and (5) stable ATP and PCr peaks as determined by NMR.
To determine the metabolic and functional stability of hearts meeting these criteria, we perfused six hearts under control conditions for 90 minutes. NMR spectra were acquired initially, and at the end of the perfusion. Under these conditions, hearts were both mechanically and metabolically stable, heart rate was 206 ± 6 beats/min, and the average developed pressure was 193 ± 16 mm Hg. These parameters decreased by less than 5 ± 1%, while PCr and ATP decreased by 8 ± 4% and 10 ± 4%, respectively.

Metabolic and mechanical parameters were then measured in the following series of experiments.

1. The Na⁺-K⁺ pump was inhibited by a reduction of perfusate K⁺ (K₀) to 1.0 mM or less—i.e., a range demonstrated previously to result in Na⁺-K⁺ inhibition (Glitsch 1979)—or by the addition of 10⁻⁴ M ouabain for 22 minutes. This was followed by 30 minutes of perfusion in normal K₀ or in the absence of glycoside.

2. Perfusate Na⁺ (Na₀) was reduced to 25 mM (with an equimolar substitution by Li⁺ or K⁺) for 22 minutes followed by 30 minutes in normal Na₀.

3. Perfusate Ca++ (Ca₀) was reduced to 0.08 mM either before or after Na⁺-K⁺ inhibition or Na₀ reduction. Biochemical determinations of ATP, PCr and Pi, or ionic contents were made after 22 minutes of low K₀ or low Na₀ perfusion, or control perfusion. Finally, in companion studies, rates of O₂ consumption were measured during various experimental perturbations and recovery.

Statistical Analysis

Values were expressed as mean ± SEM. Standard error bars are shown on all figures, except when the bars are smaller than the indicated data points or when metabolic data for individual hearts are presented (Figs. 3 and 11); t-test or analysis of variance was performed when appropriate.

Results

Effects of Na,K-ATPase Inhibition

Figure 1 shows a typical mechanical record and a series of NMR spectra obtained at various times during a reduction of K₀ from 5.9 to 0 mM. The duration of this exposure was 22 minutes, followed by 30 minutes of normal K₀. Systolic pressure increased transiently in the absence of an increase in diastolic pressure; then, systolic pressure decreased and diastolic pressure increased until arrest occurred. Prior to the cessation of systole, spontaneous arrhythmias (not resolved at the recording speed shown in Fig. 1) were seen in most hearts. During the period of high systolic pressure, coronary perfusion pressure increased markedly, in some cases to 200 mm Hg after 22 minutes of K₀ zero perfusion. The contractile alterations induced by the K₀-free perfusion were associated with a rapid decrease in PCr and ATP, a rise in sugar phosphates and Pi, an acidic shift of Pi, and the appearance of a second peak in the Pi region. It is noteworthy that the decrease in high-energy phosphates began during the initial period when contractility was increased. Quite similar changes in coronary perfusion pressure and tissue metabolite content were seen in a series of hearts when the intraventricular balloon was deflated during the period of low K₀ perfusion. After reintroduction of normal K₀, PCr, Pi, and mechanical function partially recovered and the second Pi peak was no longer observed.

To determine whether this second peak represented a unique phosphorylated compound, or a fraction of Pi in a more acidic environment, NMR spectra of whole hearts were compared to their PCA extracts. If a new phosphorylated intermediate was the source of the second peak, it should also be detectable in the extract sample. However, while the heart spectrum of the K₀-free perfused heart exhibited two Pi peaks, the spectrum of the PCA extract from this same heart showed only one Pi peak. These data suggest that the two peaks observed in the K₀-free perfused heart were Pi in two different pH environments. Therefore, in all experiments in which two Pi peaks were noted, the area reported for Pi was the sum of the two peaks.

The extent to which the metabolic and contractile changes occurred could be graded by altering the
extent of $K_0$ reduction. Figure 2 illustrates the average effects of a reduction of $K_0$ to 0.8 mM (Fig. 2A) or to 0 mM (Fig 2B). In $K_0$ of 0.8 mM, the enhancement of contractility persisted long enough so that NMR measurements could be made during the initial positive inotropic state. After 5 minutes of $K_0 = 0.8$ mM, systolic pressure was 132 ± 9% of control, whereas diastolic pressure was unchanged. Under these conditions, PCr was 79 ± 3% of control, Pi was 154 ± 12% of control, while pH$_i$ was 7.15 ± 0.02 vs. 7.19 ± 0.02 in control. When $K_0$ was reduced to 0 mM (Fig. 2B), systolic activity was abolished and diastolic pressure quickly rose after only 2.5 minutes. After 22 minutes of $K_0$-free perfusion, pH decreased to 7.20 ± 0.01 to 7.06 ± 0.03 or 6.36 ± 0.10 (second peak), while PCr, ATP, and P, were 24%, 33%, and 473% of their respective control values. In both cases of $K_0$ reduction, PCr was the first compound to decrease and the sum of the phosphorylated compounds did not vary.

The biochemical changes observed by NMR were confirmed by direct chemical analysis of the metabolites. After the acquisition of the NMR spectrum at 22 minutes of $K_0$-free perfusion, hearts were removed from the spectrometer and frozen with clamps precooled in liquid nitrogen. The tissue then was extracted with 7% PCA, and the metabolite content was determined by direct chemical analysis. Table 1 shows that the relative changes in the chemical measurements agreed quite closely with the peak area differences observed by NMR.

$Na^+\cdot K^+$ inhibition with ouabain had similar effects as reductions in $K_0$. The only exception was that the rise in diastolic pressure was more variable with ouabain. Therefore, two examples of ouabain at $10^{-4}$ M are shown in Figure 3. In both cases, after 1 minute of ouabain exposure, systolic pressure was 132 ± 9% control, PCr was 92 ± 2.0% control while ATP, pH$_i$, and diastolic pressures were unchanged.

These initial results were quite similar to those reported for a lower drug concentration which did not induce a rise in diastolic pressure (Matthews et al., 1982). However, at the concentration of ouabain used in the present study, the hearts progressed into contracture, i.e., increased diastolic pressure accompanied by decreased systolic pressure. In Figure 3A, at the onset of contracture, the rate of high-energy phosphate depletion was accelerated, and the appearance of the second Pi peak (acidotic) was noted. In Figure 3B, the onset of contracture occurred at a later time, and, likewise, the metabolic changes were delayed.

The metabolic changes seen in these hearts (Figs. 2 and 3) are quite similar to those observed in ischemia when $O_2$ delivery is reduced. In a separate series of hearts, $O_2$ consumption and contractile performance were measured during $K_0$-free perfusion (Fig. 4). Note that shortly after the reduction of $K_0$, a significant increase in $O_2$ consumption occurred, and that this persisted throughout the period of $K_0$-free perfusion. Thus, the metabolic depletion associated with $Na^+\cdot K^+$ pump inhibition, acidosis, and increased diastolic pressure (Figs. 1–3), are accompanied by an increased rather than a decreased...

<table>
<thead>
<tr>
<th>Table 1: Determination of Metabolites by Biochemical and NMR Methods in Control and $K_0$-free Perfused Hearts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 5)</td>
</tr>
<tr>
<td>PCA extract in nmol/mg protein</td>
</tr>
<tr>
<td>NMR (% control)</td>
</tr>
<tr>
<td>$K_0$-free (22 minutes) (n = 5)</td>
</tr>
<tr>
<td>PCA extract in nmol/mg protein</td>
</tr>
<tr>
<td>NMR (% control)</td>
</tr>
</tbody>
</table>
rate of O₂ consumption. If the metabolic changes reported above reflected ischemia, decreased O₂ consumption would have been expected. Therefore, these results (Fig. 4) suggest that other intracellular events may be responsible for the observed metabolic alterations.

Effects of Reduced Extracellular Sodium

It is well known that Na,K-ATPase inhibition leads to a rise in intracellular Na⁺. To test whether increased Na⁺ per se induced the observed metabolic, ionic, and contractile alterations, the effect of lowering Na₀ was examined. This is a perturbation known to induce a decrease in cell Na⁺ content, thereby causing a rise in cell Ca++ via Na⁺-Ca++ exchange. Figure 5 shows the changes in the NMR spectra and contractile performance following a reduction in Na₀ from 142 to 25 mM with Li⁺ substituted for reduced Na⁺. Note that qualitatively similar changes as in K₀-free perfusion are initially seen. A transient increase in contractility is followed by asystole, an increase in diastolic pressure, a decrease in PCr and ATP, a rise in Pᵢ, and a decrease in pH₁. However, the splitting of the Pᵢ peak previously observed did not occur under these conditions. The average metabolic effects of Na₀ reductions in five hearts is shown in Figure 6A. In low Na₀-Li⁺ substituted perfusate, systolic pressure initially increased from control and then decreased in conjunction with the increased diastolic pressure. It is important to note, however, that in the continued presence of low Na₀, a transient recovery in systolic and diastolic
pressures was observed prior to cessation of contraction. This was unlike the case in low \( K_o \). Additionally, Figure 6A illustrates that during the continued presence of reduced \( Na^+ \), there was a partial recovery of \( PCr \), \( P_i \), and \( pH_i \). Upon reintroduction of normal \( Na_o \), there was full recovery of \( PCr \), \( P_i \), \( pH_i \), and diastolic and systolic pressure, although the ATP content remained depleted.

Several laboratories have reported that when \( K^+ \) is substituted for \( Na^+ \), a marked cell depolarization occurs which causes \( Ca^{++} \) loading via \( Na^+-Ca^{++} \) exchange and slow inward current. Under these conditions, (Fig. 6B), systolic activity was immediately abolished without a transient increase in systolic pressure, and diastolic pressure rose to 155 ± 18 mm Hg within 1 minute. At the first measurement, \( PCr \) rapidly dropped to 40% of control, while ATP depletion was unchanged. No recovery of \( PCr \) was observed during low \( Na^+ \), \( K^+ \)-substituted perfusion, as was seen in the low \( Na^+ \)-\( Li^+ \) perfusion. The metabolic profiles in Figure 6, A and B, illustrate that the depletion of the high-energy phosphates is not a consequence of the transient increase in developed pressure observed at the onset of \( Ca^{++} \) loading and before the rise in diastolic pressure.

The oxygen consumption during low \( Na^+ \) also differed with the substituting cation (Fig. 7). In the case of \( Li^+ \) substitution, the rate of oxygen consumption transiently increased and then decreased when the hearts became asystolic. Whereas the secondary decline in \( O_2 \) consumption may be attributed in part to the absence of systole, the \( O_2 \) consumption rate after plateau was higher than basal oxygen consumption rates observed when hearts were arrested with 0.08 mM \( Ca^{++} \) (see below). Furthermore, in Figure 7B, a condition in which the delay of asystole during low \( Na^+ \) does not confound the issue, the rate of oxygen consumption remained elevated throughout the entire low \( Na^+ \) phase. Note also that, on returning to normal \( Na^+ \) perfusion, oxygen consumption increased with the early resumption of systole (Fig. 7A), but initially decreases in Figure 7B, where the resumption of systole was delayed.

**Ionic Alterations**

Changes in intracellular calcium and other ions occur during \( Na,K \)-ATPase inhibition, or following reductions of \( Na^+ \). To assess the magnitude of these changes under our conditions, we measured the cell ionic contents using the Co-EDTA method of Bridge et al. (1982), and compared these results to NMR spectral data taken at the end of the intervention. The data of Table 2 clearly show almost a 4-fold increase in cellular \( Na^+ \) and \( Ca^{++} \) during low \( K^+ \) perfusion. In contrast, during low \( Na^+ \) perfusion, cellular \( Na^+ \) was reduced, while intracellular calcium still increased 2-fold. Note also that while diastolic pressure was increased from control after 22 minutes of \( Na^+ \) reduction, the level to which the diastolic pressure increased was several-fold less than that seen when \( K_o \) was reduced. The relationships among these ionic, metabolic, and functional changes are shown in Figure 8, where the data are plotted as a function of cellular \( Ca^{++} \) content. The data for the metabolic, ionic, and functional changes versus cell \( Ca^{++} \), strongly suggest that graded increases in cell \( Ca^{++} \) may be related to the observed metabolic abnormalities.

**Effects of Reduced \( Ca_o \)**

Two further protocols were designed to test this hypothesis. The extracellular \( Ca^{++} \) concentration (\( Ca_o \)) was reduced, either before the changes in \( Na_o \) or \( K_o \), or 10 minutes after the monovalent cation changes. The effect of a reduction of \( Ca_o \) from 2.5 to 0.08 mM prior to a reduction in \( K_o \) is shown in Figure 9. A \( Ca_o \) of 0.08 mM \( Ca^{++} \) was chosen because preliminary experiments showed that this was low enough to abolish systolic tension, while not so low as to induce "Ca paradox" (Crevey et al., 1978). Figure 9 shows typical NMR spectra for a control heart, a heart perfused for 15 minutes in \( K_o \)-free medium at normal \( Ca_o \), and a heart perfused at a \( Ca_o \) of 0.08 mM for 5 minutes prior to 15 minutes with \( K_o \)-free perfusate. Note that, in reduced \( Ca_o \), a normal metabolic profile persists after reduction in \( K_o \). Figure
TABLE 2
The Effects of 22 Minutes of $K_\text{o}$ Reduction (1.0 mM) and $Na_\text{o}$ Reduction (25 mM + Lithium Substitution) on Cell Ions, Metabolites, and Diastolic Pressure

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control $(n = 5)$</th>
<th>$K_\text{o} = 1.0$ mM $(n = 5)$</th>
<th>$Na_\text{o} = 25$ mM $(n = 5)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell potassium (µmol/g dry)</td>
<td>373.76 ± 7.84</td>
<td>178.43 ± 11.98</td>
<td>208.61 ± 9.00</td>
</tr>
<tr>
<td>Cell sodium (µmol/g dry)</td>
<td>59.90 ± 9.91</td>
<td>198.91 ± 17.43</td>
<td>44.21 ± 3.97</td>
</tr>
<tr>
<td>Cell calcium (µmol/g dry)</td>
<td>6.21 ± 1.36</td>
<td>26.54 ± 4.07</td>
<td>12.20 ± 1.31</td>
</tr>
<tr>
<td>Cell sodium-potassium</td>
<td>0.16 ± 0.02</td>
<td>1.11 ± 0.18</td>
<td>0.21 ± 0.02</td>
</tr>
<tr>
<td>Cell H$_2$O (ml/g wet wt)</td>
<td>0.31 ± 0.03</td>
<td>0.22 ± 0.02</td>
<td>0.38 ± 0.03</td>
</tr>
<tr>
<td>PCr (% control)</td>
<td>100%</td>
<td>23% ± 5</td>
<td>49% ± 8</td>
</tr>
<tr>
<td>ATP (% control)</td>
<td>100%</td>
<td>16% ± 17</td>
<td>28% ± 4</td>
</tr>
<tr>
<td>Pi (% control)</td>
<td>100%</td>
<td>211 ± 16.4</td>
<td>182 ± 11</td>
</tr>
<tr>
<td>pH</td>
<td>7.20 ± 0.01</td>
<td>7.04 ± 0.07</td>
<td>7.00 ± 0.04</td>
</tr>
<tr>
<td>Diastolic pressure (mm Hg)</td>
<td>7.20 ± 0.80</td>
<td>134.80 ± 9.71</td>
<td>82.80 ± 8.66</td>
</tr>
</tbody>
</table>

10 illustrates the changes in the metabolic and contractile parameters induced by the reduction of $Ca_\text{o}$ from 2.5 to 0.08 mM (Fig. 10A) and by a similar reduction in $Ca_\text{o}$ followed by removal of $K_\text{o}$ (Fig. 10B), or by a reduction of $Na_\text{o}$ to 25 mM (Fig. 10C). In Figure 10A, a reduction of $Ca_\text{o}$ by itself abolished systolic pressure and resulted in a 25% increase in PCr, a depletion in Pi, and no significant change in ATP. pH increased significantly from 7.17 ± 0.02 to 7.28 ± 0.05 after 20 minutes and was 7.37 ± 0.06 after 50 minutes of low $Ca_\text{o}$ perfusion (not shown in Fig. 10A). In addition, the rate of oxygen consumption markedly decreased during the first minute from 166 ± 5 to 65 ± 3 µmol O$_2$/min per g wet weight and remained stable thereafter. In Figure 10, B and C, there appears to be a reduction in the high-energy phosphate starting at the time of reduced $K_\text{o}$ or $Na_\text{o}$. This suggests that a $Ca_\text{o}$ of 0.08 mM is still sufficient for some $Na^+-Ca^{++}$ exchange to occur.

However, these changes are much less rapid and severe than the metabolic transitions noted in the presence of normal calcium (see Figs. 2B and 6A). In another series of experiments, perfusate $Ca^{++}$ was reduced after the onset of the metabolic and contractile abnormalities induced by reduced $Na_\text{o}$ or $K_\text{o}$ (Fig. 11). An immediate striking reversal of the metabolic and contractile abnormalities was observed at the time of $Ca^{++}$ reduction. These results strongly suggest a change in cell calcium as a mechanism for the observed metabolic and contractile alterations.

**Discussion**

The data presented in this communication emphasize the role of the $Na^+-K^+$ pump and cell $Na^+$ in maintaining the cell calcium gradient. They further underscore the importance of cell $Ca^{++}$ in the regulation of myocardial metabolism and force production. A reduction of cell $Ca^{++}$ content and systolic work due to a reduction in $Ca_\text{o}$ resulted in an increase in PCr and pH and a decrease in Pi (Fig. 10A) accompanied by a reduction in O$_2$ consumption. Conversely, the initial gain in cell $Ca^{++}$ induced by $Na^+-K^+$ pump inhibition or reduced $Na_\text{o}$ caused an increase in contractility and O$_2$ consumption, whereas PCr declined. The extent of these tissue averaged metabolic and contractile changes appeared to be graded to the average ultimate $Ca^{++}$ gain, regardless of whether an increase or reduction in $Na_\text{o}$ occurred (Table 2; Fig. 8). Figure 8 also depicts the inverse relations observed between the cell $Ca^{++}$ load and the reduction in ATP and PCr and the direct relations between cell $Ca^{++}$ and diastolic pressure. These data suggest that the role of $Ca^{++}$ as a modulator of myocardial metabolism and function under normal conditions and during $Ca^{++}$ overload forms a continuum, albeit a nonlinear one. This interpretation is not inconsistent with the current view that under physiological conditions, variations of the resting free calcium concentrations could affect the rates of mitochondrial respiration (Hansford and Castro, 1982). Under normal conditions, how-
ever, such calcium transport would be much too slow, and would have a much too low calcium affinity for modifying the cytosolic free calcium concentration during beat-to-beat calcium regulation. However, under pathological conditions (extremes such as seen in this work), it is possible that mitochondria accumulate a large amount of calcium, see below. As little as a doubling of cell Ca\(^{++}\) was associated with depletion of ATP and PCr. This metabolic influence of Ca\(^{++}\) is further emphasized by Figures 9–11 which demonstrate that a decrease in Cao not only prevents but also reverses the consequences of Na,K-ATPase inhibition of Na\(^{+}\)-Ca\(^{++}\) exchange alterations.

In the present study, a reduction of K\(_o\) increased cell sodium, reduced cell potassium, and markedly increased the sodium:potassium ratio, i.e., caused Na\(^{+}\)-K\(^{+}\) pump inhibition (Table 2). Previous studies have demonstrated that cell Ca\(^{++}\) content and Cai increase via Na\(^{+}\)-Ca\(^{++}\) exchange when either Na\(_o\) is elevated due to Na\(^{+}\)-K\(^{+}\) pump inhibition or when Na\(_o\) is reduced (Thomas, 1960; Marban et al., 1980; Lee et al., 1980b; Bers and Ellis, 1982; Sheu and Fozzard, 1982; Allen et al., 1983; Bridge and Bassingthwaite, 1983; Orchard et al., 1983; Vaughan-Jones et al., Wier et al., 1983). Thus, the relationship between cell Ca\(^{++}\) and Na\(^{+}\) may be expected from previous theoretical and experimental data regarding the Na\(^{+}\)-Ca\(^{++}\) exchange system (Reuter and Seitz, 1960, Marban et al., 1980; Mullins, 1981). It is noteworthy that a reduction of Na\(_o\), when substituted by lithium, caused a modest reduction in cell Na\(^{+}\) but also substantially reduced cell K\(^{+}\) so that the sodium:potassium ratio increased by 30%. The

![Figure 9](image_url)

**Figure 9.** The effects of a prior reduction of perfusate Ca\(_o\), Cai on the contractile and metabolic alterations induced by K\(_o\) = 0 perfusion. Successive spectra (top to bottom) of a control heart, a heart after 15 minutes of K\(_o\) reduction from 5.9 to 0 m\(\text{M}\), and a heart after 15 minutes of K\(_o\) reduction to 0 m\(\text{M}\) when Cao had been reduced from 2.5 m\(\text{M}\) to 0.08 m\(\text{M}\) prior to and during low K\(^{+}\) perfusion.

![Figure 10](image_url)

**Figure 10.** The effects of a reduction of Cao to 0.08 m\(\text{M}\) by itself (panel a), and either before K\(_o\) removal (panel b), or Na\(^{+}\) reduction with Li\(^{+}\) substitution (panel c). n = 4 for each panel.
general role of cell Ca++ in altering metabolism and diastolic tone is further emphasized by the differences between the various protocols of Ca++ loading. The initial increase in myoplasmic [Ca++] due to a Ca++ influx linked to the sodium gradient, partially dissipates with time, possibly due to the drop in cell [Na+] (Ellis, 1977; Marban et al., 1980; Lee et al., 1980a; Bers and Ellis, 1982). Similarly, in the present study a partial recovery occurred in diastolic pressure, PCr content (Fig. 6A) and oxygen consumption (Fig. 7a). It has been reported that, with time, a sustained Ca++ gain occurs when the Na+-K+ pump is inactive (Lee et al., 1980a; Bers and Ellis, 1982; Allen et al., 1983; Vaughan-Jones et al., 1983; Wier et al., 1983) or when Na+ exchange is altered (Fig. 3). Under these conditions, we noted profound reductions in systolic pressure, PCr, ATP, and pH, an increase in diastolic pressure, and an absence of systolic activity, all occurring while O2 consumption remained elevated.

Origins of High-Energy Phosphate Depletion

Under our experimental conditions, NMR spectroscopy measures the time- and tissue-averaged content of the high-energy phosphates and intracellular pH. These reflect the important net balance between rates of energy production and utilization. Therefore, reductions in ATP and phosphocreatine accompanied by increased Pi, could occur from either increased demand, decreased production, or both. Calcium can increase energy turnover by several mechanisms. Recent observations in isolated cardiac muscle demonstrated that during Ca++ loading, spontaneous Ca++ oscillations occur in the myoplasm (Glitsch and Pott, 1975; Lederer and Tsien, 1976; Lakatta and Lappe, 1981; Orchard, 1983; Stern et al., 1983; Wier et al., 1983; Kort and Lakatta, 1984; Walford et al., 1984). The frequency of these oscillations varies with the Ca++ load, resulting in a Ca++-dependent tone (Stern et al., 1983). Other recent studies have documented spontaneous oscillations in the intact perfused rat heart (Stern, personal communication). These oscillations increase in frequency as cell Ca++ increases, and are abolished by ryanodine, a drug that prevents sarcolemmal release of Ca++. In addition, ryanodine reduced the Ca++-dependent decline in energy phosphates in the Li+-substituted perfusate (Renlund et al., personal communication). Increased high-energy phosphate utilization may result from an increased frequency of Ca++-induced oscillations observed in the absence of organized systole. In response, continuous ATP-dependent Ca++ transport by the sarcolemmal reticulum and cyclic changes in ATP utilization by the myofilaments would occur. In low Na+ medium (Li+ substitution), the oscillation frequency initially increases to a peak level, but then decreases as the myoplasmic Ca++ (and cell) declines with time. Thus, the changes in developed pressure and PCr prior to asystole (Fig. 6A), along with the decline in O2 consumption in low Na+, (following an initial increase, Fig. 7) might be related to an eventual reduction in average myoplasmic Ca++ and in the frequency of oscillations. However, this is not totally clear, since asystole eventually occurred even in low Na+ with Li+ substitutes. In contrast, in a low Na+ medium containing K+ (118 mm), depolarization results in a sustained Ca++ load (Mullins et al., 1983). In this situation, decreased ATP and PCr with increased Pi, oxygen consumption, and diastolic pressure occur (Fig. 6B). Similar changes were also observed when the Na,K-ATPase was inhibited by low K+ (Fig. 2) or ouabain (Fig. 3). Under these conditions, the Ca++ load is also sustained (Lee et al., 1980; Bers and Ellis, 1982; Bridge and Bassingthwaighte, 1983) and the increased frequency of Ca++ oscillations is persistent (Orchard et al., 1983; Stern et al., 1983; Kort and Lakatta, 1984b; Walford et al., 1984).

High-energy phosphate depletion could also result from enhanced ATP hydrolysis by the myofilament ATPase (Ventura-Clapier and Vassort, 1981). This would be induced by a combined steady increase in myoplasmic [Ca++] with oscillations occurring above this level. A high rate of myofilament ATP utilization, characteristic of increased crossbridge cycling, and the development of an active type of diastolic tone is associated with enhanced...
oxygen consumption and heat production (Ventura-Clapier et al., 1982). Marked ATP depletion may lead to another form of enhanced diastolic tone, that due to the formation of rigor bonds. This interaction produces tone when ATP deficiency occurs, but does not require calcium (Weber and Murray, 1973). It is important to note that, in the presence of metabolic poisons, rigor can occur when there is still substantial tissue ATP (Hearse et al., 1977). In low Na+ high K+ medium, both types of diastolic tone have been postulated on the basis of time-dependent changes in viscoelastic properties (Ventura-Clapier and Vassort, 1981). Thus, two calcium-dependent factors could contribute to the increase in diastolic tone observed in the present study: increased cross-bridge cycling, which is directly related to Ca++ dynamics, and the formation of rigor complexes, which are indirectly related to Ca++ overload when ATP utilization outstrips energy supply.

The cell metabolic profile induced by Ca++ loading—namely, the depletion of high-energy phosphate and intracellular acidosis—is, at first glance, very similar to the changes observed as a result of reduced oxygen delivery (Jacobus et al., 1977). However, during ischemia caused by reductions in flow or oxygen delivery, rates of oxygen consumption are decreased below control. Therefore, the present observations of increased rates of oxygen consumption under all conditions of Ca++ loading (Figs. 4 and 7) suggest that ATP depletion is not simply a consequence of oxygen deprivation. At least two well-defined calcium-dependent mitochondrial activities could lead to enhanced O2 consumption with reduced ATP synthesis. The first is the direct, energy-linked Ca++ uptake, which occurs in the presence of a permeant anion such as phosphate. The second mechanism is mitochondrial Na+–induced Ca++ exchange (for a review, see Nicholls and Akerman, 1982). With respect to the first mechanism, the high cell content of P, present during the Ca++ overload period could foster Ca++ entry into mitochondria (Lehninger et al., 1967). It has been shown in both liver and heart mitochondria (Rossi and Lehninger, 1964; Vercesi et al., 1978) that phosphate-dependent Ca++ transport can take priority over oxidative phosphorylation. As a result, net calcium accumulation occurs, i.e., massive calcium loading (Fig. 12A). Under conditions of Na+-K+ pump inhibition, it has recently been shown that 45% of the accumulated Ca++ was localized in the mitochondria (Murphy et al., 1983). With respect to the second mechanism, mitochondrial Na+-induced Ca++ efflux has been defined by a number of investigators in heart, brain, and skeletal muscle mitochondria (Fig. 12B). According to this mechanism, matrix Ca++ is released during Na+ influx. Na+ then leaves the mitochondrial matrix in exchange for H+ via the Na+-H+ antiport system. As a result, the entering H+ combines with matrix OH−, dissipating the transmembrane proton-motive force, the driving energy for numerous mitochondrial reactions. Therefore, Na+-induced Ca++ cycling occurs in direct competition with ATP production, since these processes are driven by the same component of the proton motive force produced by electron transport. The uniport carrier for Ca++ and the adenine nucleotide translocase are both energized by the delta-psi component (Fig. 12C). Since respiratory energy is limited, an enhancement of Ca++ translocation by either mechanism would result in reduced ADP transport into the matrix. Thus, oxidative phosphorylation would be directly compromised. Under normal conditions, sodium-induced Ca++ exchange accounts for only a small rate of oxygen consumption (Carafoli et al., 1974). The degree of enhancement of either calcium-linked mitochondrial transport mechanism requires further study, as does a detailed evaluation of these exchange mechanisms under pathological conditions in the myocardium.
Intracellular Acidosis

It has been reported that increased Ca++ may be related to cellular acidois (Aickin and Thomas, 1977; Meech and Thomas, 1977; Ahmed and Connor, 1980; Deitmer and Ellis, 1980; Vaughan-Jones, 1983); this is also seen in the present experiments. It is noteworthy that the magnitude of acidois now shown is higher than previously reported. These differences might result from the modest ATP requirements in a resting Purkinje fiber as compared to the energy consumed by an isovolumic beating heart. In resting preparations, a reduction of Na+ to zero produced only a slight increase in Ca, induced a small inconsistent contracture, and did not significantly affect pH (Marban et al., 1980; Deitmer and Ellis, 1980), whereas, in the isovolumic beating heart a reduction of Na+ to 25 mM induced a significant acidois (Fig. 6A) and a persistent rise in diastolic pressure. Under the isovolumic conditions employed in the present study, myofibrils would be stretched near the maximum of their length-tension curve and, thus, be more sensitive to calcium (Fabiato and Fabiato, 1978). In addition, the myoplasmic Ca++ transients associated with contraction are greater compared to un-stretched preparations (Allen and Kunihara, 1982). Furthermore, the intracellular activities for Na+ and Ca++ increase with stimulation of the preparation (Lado et al., 1982; Cohen et al., 1982).

The absence of a correlation at the end of 22 minutes of Ca+++ load between pH, and any of the contractile, ionic, or metabolic alterations (Fig. 8) suggest multiple origins for the acidois: exchange or competition of H+ with other ions and the production of H+ by metabolic pathways. Competition or exchange between H+ and Ca++ could occur at the sarcolemma, the sarcoplasmic reticulum, the myofilaments, the mitochondria, and at other intracellular buffering sites. Mitochondria exchange protons for calcium ions with a variable stoichiometry which depends on the presence of phosphate, other permeant anions, adenine nucleotides, and Mg++. There is also some evidence for a modulation of myocardial pH by a sarcolemmal Na+-H+ exchanger (Deitmer and Ellis, 1980; Prelin et al., 1984), although the results in Figure 11 suggest that the change in cell pH is not dependent on the change in the cell Na+ gradient, but rather on the change in cell Ca++. In a Ca++-loaded heart, the net production of protons could result from increased cross-bridge cycling when ATP synthesis was impaired and phosphocreatine content decreased. Although oxidative phosphorylation and PCr hydrolysis both result in the net generation of OH−, under metabolically restricted conditions net acidification would occur since ATP hydrolysis produces H+.

In summary, the results presented in this paper illustrate the diversity of metabolic events associated with calcium overload in the isovolumic heart and the rapid rate at which these changes are expressed. At the onset of Ca++ gain, an apparent energy supply/demand imbalance was noted. Likewise, the transition to low Ca+ (Fig. 11) induced an almost immediate metabolic recovery. Together, these data clearly illustrate the close relationships between global energy metabolism and the tissue calcium load.

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