Role of Changes in [Ca\(^{2+}\)]\(_i\) in Energy Deprivation Contracture

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Mechanisms of energy deprivation contracture were investigated in cultured chick embryo ventricular cells. In the presence of zero-extracellular-Na\(^+\) (choline chloride substitution)-nominal-zero-Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\) ~ 5 μM), exposure of ventricular cells to 1 mM cyanide (CN) and 20 mM 2-deoxyglucose (2-DG)-zero-glucose solution resulted in the development of a contracture (video motion detector) in 5.9 ± 0.5 minutes. Early after contracture development, the resupply of extracellular Na\(^+\), in the continued presence of CN + 2-DG, resulted in a rapid partial relaxation (t\(_p\) = 1.9 ± 0.3 seconds), associated with an increase in Ca\(^{2+}\) efflux, presumably due to transsarcolemmal Ca\(^{2+}\) extrusion due to Na\(^-\)-Ca\(^{2+}\) exchange. Resupply of glucose and removal of CN + 2-DG, in the continued absence of Na\(^+\), resulted in an initially slower (t\(_p\) = 11.6 ± 2.5 seconds), but more complete relaxation of contracture, which was not associated with increased Ca\(^{2+}\) efflux. Pretreatment with 20 mM caffeine delayed the onset of contracture (9.2 ± 1.1 minutes) and resulted in a contracture that could not be released by resupply of external Na\(^+\) only. Studies using the fluorescent Ca\(^{2+}\) probe indo1 demonstrated that in zero-Na\(^-\)-zero-Ca\(^{2+}\) solutions, contracture due to CN + 2-DG was associated with an initial rise in [Ca\(^{2+}\)], but that this did not account for all of contracture force development. In cells exposed to CN + 2-DG in the presence of normal extracellular Na\(^+\) and Ca\(^{2+}\) concentrations, a small rise in [Ca\(^{2+}\)], was associated with initial contracture development, consistently preceding the development of a larger accelerated contracture presumably due to ATP depletion. We conclude that an early component of ATP depletion contracture is due to an increase in [Ca\(^{2+}\)]. The rate of this increase in [Ca\(^{2+}\)], depends to some extent on the loading of internal stores of Ca\(^{2+}\), particularly sarcoplasmic reticulum. Elevation of [Ca\(^{2+}\)], may promote subsequent rigor by hastening ATP depletion by activation of Ca\(^{2+}\)-ATPases.

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When the production of high-energy phosphates by myocardium is impaired by ischemia, hypoxia, or metabolic inhibition, development of contractile force initially declines. With continued lack of adenosine triphosphate (ATP) synthesis, the myocardium begins to develop an increase in resting force, which could be due to a rise in cytosolic free calcium ion concentration ([Ca\(^{2+}\)]\(_i\)) promoting actin-myosin interaction via Ca\(^{2+}\) binding to the troponin-tropomyosin complex and/or due to a fall in ATP concentration, producing rigor due to failure of actin-myosin cross-bridge dissociation.

A number of investigators have examined this issue. Lewis et al concluded from studies of elastic and viscous components of total myocardial stiffness that hypoxic contracture is due not only to a rise in intracellular Ca\(^{2+}\) but also to an increased stiffness due to ATP depletion. Holubaesch et al measured myocardial heat production during hypoxic and potassium chloride contracture. They concluded that hypoxic contracture may not be due to an elevation in [Ca\(^{2+}\)], but instead to rigor-like actin-myosin bonds. This conclusion is supported by Allen and Orchard, who found no significant increase in aequorin luminescence in ferret papillary muscle during contracture induced by glycogen depletion and exposure to cyanide. In addition, Cobbold and Bourne noted that cyanide plus 2-deoxyglucose produced contracture of isolated rat ventricular myocytes within 10 minutes, before an increase in aequorin luminescence was noted.

On the other hand, Dahl and Isenberg noted that dinitrophenol produced within 2–6 minutes a significant increase in [Ca\(^{2+}\)], measured with a Ca\(^{2+}\) micro-electrode. In addition, Murphy et al reported that exposure to iodo-acetate and an inhibitor of mitochondrial electron transport produced a rise in [Ca\(^{2+}\)], in cultured chick embryo ventricular cells measured with the fluorescent Ca\(^{2+}\) indicator quin2 within 4–6 minutes. Snowdowne et al reported that hypoxia induced an increase in aequorin luminescence in dissociated myocytes within 4–5 minutes.

We have approached this issue by studying the development and relief of contracture in cultured chick embryo ventricular cells exposed to metabolic inhibitors of ATP production during conditions in which Ca\(^{2+}\) influx and efflux across the sarcolemma are limited and controlled. In addition, we have utilized the newly developed fluorescent Ca\(^{2+}\) indicator, indo1, to determine coincident changes in [Ca\(^{2+}\)]. Our results indicate that increases in [Ca\(^{2+}\)], contribute to contracture development early after metabolic inhibition.

Materials and Methods

Tissue Culture

Layer cultures of contracting chick embryo ventricular cells were prepared as described previously.

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Ventricles from 10-day-old embryos were minced and placed in Ca\textsuperscript{2+}-Mg\textsuperscript{2+}-free Hanks' solution. The tissue was trypsinized in 10 ml 0.025% trypsin in calcium-magnesium-free Hanks' solution at 37° C for four cycles of 7 minutes each. The supernatant suspensions containing dissociated cells were placed in cold trypsin inhibitor medium and centrifuged at 2,000 rpm for 10 minutes. The supernatant was discarded, and the cells were resuspended in culture medium consisting of 6% heat-inactivated fetal calf serum, 40% medium 199 (Gibco Laboratories, Grand Island, N.Y.), 0.1% penicillin-streptomycin antibiotic solution, and 54% balanced salt solution containing (in mM): NaCl 116, NaH\textsubscript{2}PO\textsubscript{4} 1.0, MgSO\textsubscript{4} 0.8, KCl 1.18, NaCO\textsubscript{3} 26.2, and glucose 5. The cell suspension was diluted to 4 x 10\textsuperscript{3} cells/ml and placed in plastic petri dishes containing 25-mm circular glass coverslips. Cultures were incubated in a 5% CO\textsubscript{2} atmosphere for 3 days at 37° C. All studies were performed on cells after 3 days of culture.

**Measurement of Cell Motion**

A glass coverslip with an attached cell layer was placed in a chamber in which a small segment of the culture was continuously superfused with a layer of fluid 0.5 mm deep. The chamber was placed on the stage of a Nikon Diaphot inverted phase-contrast microscope (S&M Microscopes, Colorado Springs, Colo.), enclosed in a Lucite box heated to 37° C. The inlet to the perfusion chamber was connected by a manifold and polyethylene tubing to five Sage syringe pumps (Orion Research Inc., Cambridge, Mass.) so that a culture segment could be sequentially superfused with different media. At a flow rate of 2 ml/min, the medium bathing a cell in the center of the superfused segment could be exchanged with a time constant of 2 seconds.

The optical apparatus was supported by an air table (Barry Wright Corp., Watertown, Mass.) to damp vibration, and the cells were magnified using a 40 x objective. Plastic microspheres 2-3 μm in diameter (3M, New Brighton, Mass.) were added to the cultures on day 2 of culture; they became attached to the cell surfaces and provided an improved image for contraction measurement. The image was monitored with a low-light-level TV camera (Dage 650 SSK, Dage-MTI Inc., Michigan City, Ind.) attached to the microscope observation port. Motion along a selected raster line segment was quantitated as previously described. The video signal was processed using a Processor 604, a Sync Stripper 302-2, and a Video Motion Analyzer 633 (Colorado Video, Boulder, Colo.). The analog output of the motion analyzer was recorded as an analog signal on a strip-chart recorder (custom built) and on magnetic tape (model 3968A, Hewlett-Packard Co., Palo Alto, Calif.).

**Ca\textsuperscript{2+} Efflux**

For these measurements, isotopic tracer techniques were employed as described. Cells were labelled to equilibrium for 2 hours in \textsuperscript{43}Ca. Individual coverslips were then washed for 15 seconds at 37° C to remove the extracellular tracer. They were then immersed for 5 seconds serially in seven 2-ml volumes of efflux solution at 37° C. Thus, efflux was measured over a 35-second period. \textsuperscript{43}Ca counts were determined in each 2-ml volume. Then we measured the total \textsuperscript{43}Ca remaining in the cells on each individual coverslip after the efflux was measured. Next, we calculated the fractional efflux for each 5-second period as the fraction of total \textsuperscript{43}Ca counts per minute lost during the entire efflux period and counts per minute remaining in the cells after completion of the 35-second efflux.

**Measurement of Changes in [Ca\textsuperscript{2+}]**

Changes in [Ca\textsuperscript{2+}], were detected using the new fluorescent probe, indo1,12,13 Cells were exposed for 15 minutes to 10 μM of the acetoxyethyl ester form (indo1AM). This is a permeable compound that diffuses into the cell, where the ester groups are cleaved by intracellular esterases, leaving the indo1 molecule free to bind Ca\textsuperscript{2+}. This results in an initial depression of contractility to about 50% of control. However, after cells are washed free of dye for 1 hour, contractile motion amplitude returns to control levels.

Cells were subsequently placed in a superfusion chamber on the stage of a Nikon inverted microscope. A high-pressure Hg-arc lamp was used as the excitation light source because it provides an intense peak at 360 nm. Further selection of this peak was made with narrow-bandwidth (5 nm) interference filters (Ditric, Hudson, Mass.). The excitation beam was chopped at 360 Hz and focused on the cultured heart cells via epifluorescence optics by a 40 x objective lens. Chopping the excitation beam decreases dye bleaching and allows lock-in amplifier processing, which minimized drift. These features are useful for the relatively prolonged measurements used in these experiments. The fluorescent light was collected by the objective lens and divided with a beam splitter to permit simultaneous measurement of intensity at both 410-nm and 480-nm wavelengths using two separate Hamamatsu Model 1869 AH photomultiplier tubes. Baseline autofluorescence of cells not loaded with dye was low and was adjusted to zero with DC offset.

The image of the cells was obtained by 12 V DC illumination via the standard microscope condenser, passed through a 700-nm bandpass filter. This wavelength was long enough to not interfere with the fluorescence detection at 410 and 480 nm and was matched to the spectral sensitivity of the video camera, which was used to detect cell motion via the video motion detector system described above. Fluorescence was measured from the entire 40 x objective field (400 μm diameter), while motion was detected from a single cell within the center of the field. The fluorescence intensity at 410 nm increases, and the intensity at 480 nm decreases, with increases in [Ca\textsuperscript{2+}]. Therefore, the ratio of 410:480 nm intensities was used as an indicator of [Ca\textsuperscript{2+}], and this analog signal was filtered (25 Hz low pass) and recorded on magnetic tape and a strip chart recorder. More details of [Ca\textsuperscript{2+}] measurement using this system may be found in Peeters et al.13
Solutions

All experiments were done using HEPES-buffered solutions (pH 7.35) as described previously. For zero-extracellular-Na+ experiments, choline chloride was substituted for sodium chloride on a molar basis. In nominally zero-extracellular-Ca2+ experiments, no Ca2+ was added to the solutions, and the total Ca2+ concentration ranged from 1 to 5 μM, as measured with a Ca2+-sensitive electrode or by atomic absorption spectroscopy. Unless otherwise specified, the Ca2+ concentration in all other experiments was 1.8 mM.

Statistical Analysis

Paired or nonpaired Student's *t* tests were used in comparing two groups. For multiple comparisons, a one-way analysis of variance with repeated measures was used.

Results

The importance of [Ca2+]i in development of energy dependent contracture may depend on the degree of Ca2+-loading of the intracellular stores and the effectiveness of Ca2+-extrusion. To examine these issues, we initially performed the experiment shown in Figure 1. First, cells were abruptly superfused with zero-Na+-nominal-zero-Ca2+ solution. This produces a transient contracture, followed by oscillatory mechanical activity typical of Ca2+ overload of SR. This contracture is probably due to Ca2+ entry into the cell from the interstitial space via Na+-Ca2+ exchange, as external [Na+] initially declines more rapidly than [Ca2+]. The cell then relaxes over a period of 1-2 minutes, during which time Ca2+ is extruded from the cell by an external Na+-independent mechanism, presumably the Ca2+-ATPase pump in the sarcolemma. At this point, the cell is relaxed, yet the SR is well loaded with Ca2+. Ca2+ entry into the cell is minimized by the presence of nominal-zero-[Ca2+] in the extracellular fluid, and Ca2+ extrusion is slowed by virtue of a lack of extracellular Na+ to drive Ca2+ extrusion via Na+-Ca2+ exchange.

Metabolic inhibition of ATP production was then produced by exposure of the cell to 1 mM NaCN, (CN) and 20 mM 2-deoxyglucose, (2-DG)-zero glucose solution. Within 2 minutes (Figure 1), the oscillatory mechanical activity disappeared, and a contracture began to develop. Resupply of glucose and washout of CN and 2-DG, while maintaining zero-Na+-nominal-zero-Ca2+ superfusion (Figure 1, 3rd vertical arrow), resulted in complete relaxation of the contracture and reoccurrence of mechanical oscillations. This demonstrates that reversible energy deprivation contracture occurs readily under these conditions.

To determine if this contracture is because of an increase in [Ca2+], or ATP depletion, we performed the protocol shown in Figure 2. In this experiment, the cell was exposed to zero-Na+-nominal-zero-Ca2+ solution, and then CN + 2-DG as before. However, when contracture subsequently developed, instead of washing out metabolic inhibitors and resupplying glucose, we simply resupplied extracellular Na+, abruptly reactivating Ca2+ extrusion via the Na+-Ca2+ exchange mechanism. This produced a rapid partial relaxation (3rd vertical arrow). Subsequent return to control solution produced more complete relaxation. Another example of this phenomenon is shown in Figure 3. In this experiment, the relaxation produced by restoration of extracellular Na+ was less complete, and in fact, after an initial relaxation, the cell started to redevelop contracture, which was relieved after resupply of glucose and washout of CN and 2-DG (3rd vertical arrow). This is consistent with our observation (not illustrated) that if the cell were allowed to remain in CN + 2-DG for several minutes after contracture initially developed, even partial relaxation on resupply of Na+ did not occur.

As shown in Table 1, the *t* 1/2 of the initial relaxation of contracture was more rapid when Na+-Ca2+ exchange was activated than when metabolic inhibitors were removed but the cell was kept in zero Na+. To examine changes in Ca2+ efflux under these two
circumstances, we performed the experiments shown in Figure 4. Cells labelled with 45Ca were exposed to zero-Na\textsuperscript{+}-nominal-zero-Ca\textsuperscript{2+}. 4Ca and CN + 2-DG for 6 minutes. After washing for 15 seconds at 37°C in unlabelled solution to remove extracellular tracer, 4Ca fractional efflux was measured at 5-second intervals. “Control solution” is represented by open circles, in which cells were maintained in zero-Na\textsuperscript{+}-zero-Ca\textsuperscript{2+}, CN + 2-DG for the entire efflux period. In cells indicated by the squares, efflux for the first 5 seconds was in zero-Na\textsuperscript{+}-zero-Ca\textsuperscript{2+}, CN + 2-DG solution. However, after the first 5 seconds, as indicated by the vertical arrow, cells were exposed to normal-Na\textsuperscript{+}-zero-Ca\textsuperscript{2+}, CN + 2-DG solution. A marked increase in 4Ca efflux occurred within 5 seconds, consistent with activation of rapid Ca\textsuperscript{2+} extrusion via the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger. In contrast, resupply of glucose and washout of metabolic inhibitors (solid circles) did not increase Ca\textsuperscript{2+} efflux if external Na\textsuperscript{+} was not resupplied. This suggests that reuptake of Ca\textsuperscript{2+} by intracellular stores, possibly sarcoplasmic reticulum (SR), rather than Ca\textsuperscript{2+} extrusion from the cell via a reactivated sarcolemmal Ca\textsuperscript{2+}-ATPase pump,\textsuperscript{11} could be involved in the initial relaxation produced by relief of metabolic inhibition. Alternatively, relaxation could be due to a rise in ATP and relaxation of rigor.

To examine further the role of the SR in the development of contracture under these conditions, we performed the experiments shown in Figure 5. In this protocol, the cells were exposed to 20 mM caffeine prior to exposure to zero-Na\textsuperscript{+}-zero-Ca\textsuperscript{2+}, and then CN + 2-DG. Caffeine releases Ca\textsuperscript{2+} from SR in this preparation, producing a negative inotropic effect\textsuperscript{13} and a decrease in calcium content.\textsuperscript{11} As shown in Figure 5, after a typical caffeine effect, exposure to zero-Na\textsuperscript{+}-zero-Ca\textsuperscript{2+} produced a transient contracture, but no mechanical oscillatory activity was noted, consistent with the effects of caffeine on SR Ca\textsuperscript{2+} cycling.\textsuperscript{16} Subsequent exposure to CN + 2-DG produced a contracture, but under these conditions, resupply of external Na\textsuperscript{+} produced no relaxation. However, washout of caffeine and inhibitors allowed relaxation to occur. As shown in Table 2, pretreatment with caffeine prolonged the time required to develop contracture as well as resulting in a contracture that was not sensitive to activation of Ca\textsuperscript{2+} extrusion via Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange.

The above results suggested that in the presence of zero-Na\textsuperscript{+}-zero-Ca\textsuperscript{2+} solution, the initial phase of contracture was associated with a rise in [Ca\textsuperscript{2+}], but a more prolonged duration of contracture or depletion of the SR of Ca\textsuperscript{2+} resulted in contracture that was not Ca\textsuperscript{2+}-dependent. To examine this issue more directly, changes in [Ca\textsuperscript{2+}], were measured under similar conditions using indol. An example is shown in Figure 6, in which the ratio of fluorescence at 410:480 nm, representing [Ca\textsuperscript{2+}], and cell motion were recorded simultaneously. Initial exposure to zero-Na\textsuperscript{+}-zero-Ca\textsuperscript{2+} resulted in a transient contracture and a rise in [Ca\textsuperscript{2+}], to levels slightly higher than are seen during a normal twitch. (Studies with Ca\textsuperscript{2+}-buffered solutions and the nonfluorescent Ca\textsuperscript{2+} ionophore Bromo-A23187\textsuperscript{19} have indicated that the normal systolic level of [Ca\textsuperscript{2+}]) in indol-loaded cells reached during a twitch is 813 ± 72 nM, mean ± SEM, n = 8). Subsequently, relaxation occurred with a decline in [Ca\textsuperscript{2+}], to near the normal diastolic level (328 ± 32 nM). Exposure to CN and 2-DG then resulted in development of a contracture, which was associated with a rise in free [Ca\textsuperscript{2+}], to a level well above normal diastolic [Ca\textsuperscript{2+}], but well below what would be predicted based on the relative
Table 1. Effects of Resupply of Na\(^+\) vs. Washout of Metabolic Inhibitors on Relaxation

<table>
<thead>
<tr>
<th>Group</th>
<th>Time to contracture (minutes)</th>
<th>Relaxation (t_{1/2}) (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Contracture induced in zero-Na(^+)-zero-Ca(^{2+}), CN + 2-DG. Relaxation induced by resupply of Na(^+) ((n=11, \text{mean } \pm \text{ SEM})).</td>
<td>6.0 ± 0.6</td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td>2. Contracture induced in zero-Na(^+)-zero-Ca(^{2+}), CN + 2-DG. Relaxation induced by washout of CN + 2-DG and resupply of glucose ((n=8)).</td>
<td>5.9 ± 0.9</td>
<td>8.9 ± 2.0</td>
</tr>
<tr>
<td>3. Contracture induced in normal Na(^+) and Ca(^{2+}), CN + 2-DG. Relaxation induced by washout of CN + 2-DG and resupply of glucose ((n=5)).</td>
<td>7.2 ± 1.0</td>
<td>6.1 ± 1.6</td>
</tr>
</tbody>
</table>

These findings indicated that increases in [Ca\(^{2+}\)]\(_i\) account for a component of contracture after exposure to CN and 2-DG. However, it may be questioned whether changes that occur in cells superfused with zero-Na\(^+\)-zero-Ca\(^{2+}\) during metabolic inhibition are analogous to or comparable to the effects of metabolic inhibition in the presence of a normal extracellular ionic milieu. To address this question, the experiment shown in Figure 8 was performed, in which a culture superfused with normal medium was abruptly exposed to CN and 2-DG. Initially, after exposure to CN and 2-DG, diastolic [Ca\(^{2+}\)]\(_i\) increased slightly for 20-30 seconds, then fell to below diastolic levels as the cell stopped contracting and relaxed fully. Based on the previously mentioned calibration studies, we estimate that when the cell fully relaxed, the [Ca\(^{2+}\)]\(_i\) was approximately 200 nM. There then occurred a rise in [Ca\(^{2+}\)]\(_i\) to above normal diastolic levels. This rise in [Ca\(^{2+}\)]\(_i\), an approximate doubling in 3 minutes, was associated with a gradual upward shift in the cell position. We believe this corresponds to the "initial contracture" previously described under similar conditions, and it appears to be Ca\(^{2+}\)-dependent. There then occurred an accelerated contracture development, which was not associated with a corresponding rise in [Ca\(^{2+}\)]\(_i\). On resupply of control solution, the cell shown in Figure 8 relaxed, and the level of [Ca\(^{2+}\)]\(_i\) returned to diastolic levels. If metabolic inhibition of ATP production was sustained after contracture de-

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Effects of resupply of Na\(^+\) versus washout of metabolic inhibitors on Ca\(^{2+}\) efflux. Cells were labelled with "Ca for two hours in normal medium and for 1 minute in zero-Na\(^+\)-nominal-zero-Ca\(^{2+}\) solution (see text). Fractional efflux was then measured in washed cells in zero-Na\(^+\)-zero-Ca\(^{2+}\), CN + 2-DG solution (○), and in cells in zero-Na\(^+\)-zero-Ca\(^{2+}\), CN + 2-DG for the first 5 seconds, but then exposed (vertical arrow) to normal [Na\(^+\)] CN + 2-DG solution (○) or zero-Na\(^+\)-zero-Ca\(^{2+}\), glucose solution (•). Resupply of Na\(^+\) increased Ca\(^{2+}\) efflux within 5 seconds \((p<0.001)\), whereas resupply of glucose and washout of metabolic inhibitors, without resupply of Na\(^+\), had no effect on Ca\(^{2+}\) efflux. Points indicate the mean ± SEM, \(n=12\).
Development, a continuing rise in \([\text{Ca}^{2+}]\), occurred. An example is shown in Figure 9. In the seven similar experiments, time required for \([\text{Ca}^{2+}]\), to exceed normal peak systolic levels was 10.9 ± 2.0 minutes (mean ± SEM).

Discussion

Our results indicate that myocardial cells that are well \(\text{Ca}^{2+}\)-loaded by virtue of a rapid beating rate, and especially if \(\text{Ca}^{2+}\) extrusion is impaired by virtue of removal of extracellular \(\text{Na}^{+}\), respond to metabolic inhibition of ATP production with a sufficient increase in \([\text{Ca}^{2+}]\), to result in an increased resting tension. This conclusion is based on both the observation that resupply of extracellular \(\text{Na}^{+}\) without relief of metabolic inhibition produces partial relaxation (coincident with activation of \(\text{Ca}^{2+}\) efflux via \(\text{Na}^{+}\)-\(\text{Ca}^{2+}\) exchange) and direct measurement of changes in \([\text{Ca}^{2+}]\), using the fluorescent \(\text{Ca}^{2+}\) indicator, indol. These results are at variance with the finding of Eisner et al. who failed to note an increase in aequorin luminescence in metabolically inhibited ferret papillary muscles during development of contracture, even in the absence of extracellular \(\text{Na}^{+}\). There are several possible explanations for this discrepancy. Clusin has proposed that perhaps aequorin measurements are unreliable under these conditions, with aequorin luminescence being more sensitive to \(\text{Ca}^{2+}\) released from SR than uniformly distributed \(\text{Ca}^{2+}\). Another possible limitation of aequorin is that it is relatively insensitive...
to diastolic levels of [Ca\(^{2+}\)], and may be influenced by
to changes in Mg\(^{2+}\) concentration,\(^7\) which are unknown.
In addition, the cultured cells we studied were main-
tained at 37\(^\circ\) C and beat at physiologic rates. Ca\(^{2+}\) may
be rapidly lost from SR with cessation or slowing of
stimulation.\(^9\) Since the caffeine results reported here
suggest that SR Ca\(^{2+}\) can influence the magnitude of the
[Ca\(^{2+}\)], increase during metabolic inhibition, variation
in Ca\(^{2+}\)-loading could also account for some of the
differences noted in various studies referenced in the
introduction.

In agreement with reports by Allen and Orchard\(^3\) and
Chapman,\(^21\) our findings in cells bathed in normal
medium indicate that a completely developed energy
depprivation contracture cannot be accounted for solely
on the basis of an increase in [Ca\(^{2+}\)]. However, we do
find in cultured heart cells a definite increase in [Ca\(^{2+}\)],
to above normal end-diastolic levels, associated with a
relatively small "initial" contracture preceding a
larger Ca\(^{2+}\)-independent contracture. Based on prelim-
inary measurements of mechanical properties of rat
heart trabeculae during hypoxia, Bucx et al\(^{22}\) also have
reported that a Ca\(^{2+}\)-dependent phase of contracture
may precede ATP-depletion rigor. That this initial
increase in [Ca\(^{2+}\)], may be important is suggested by the
observations, summarized by Nayler et al,\(^1\) that a delay,
attenuation, or even prevention of contracture during
hypoxia can be achieved when Ca\(^{2+}\) is removed from
the extracellular fluid before the onset of contracture.
Also consistent with this hypothesis is our observation\(^7\)
that treatment of cultured ventricular cells with La\(^3+\)
(which inhibits Ca\(^{2+}\) influx) after metabolic inhibition
but before the onset of contracture can prolong the time
required for contracture development. It may be that an
increase in [Ca\(^{2+}\)], in the early phase of energy
depprivation, due to release of Ca\(^{2+}\) from intracellular
stores and/or Ca\(^{2+}\) influx across the sarcolemma, may
activate Ca\(^{2+}\)-sensitive ATPases. This could produce a
more rapid and progressive decline in ATP concen-
tration, thus contributing to the eventual development
of a rigor-type contracture. It should be noted that in
hyperpermeabilized cells, a low ATP concentration

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FIGURE 7. These tracings show motion
and the ratio of 410/480 nm fluorescence
in a culture subjected to the same protocol
as for Figure 6, except that at the third
vertical arrow, normal extracellular [Na]
was restored, while metabolic inhibition
with CN and 2-DG was continued. Note that
resupply of sodium induced only a partial
relaxation of contracture in spite of an
abrupt decrease in [Ca\(^{2+}\)], to below normal
diastolic levels.

FIGURE 8. This recording shows
changes in cell motion (upper trace)
and [Ca\(^{2+}\)], (ratio of 410:480 nm
fluorescence, lower trace) in a cul-
tured cell exposed to CN + 2-DG
(zero glucose) in the presence of nor-
mal extracellular Na\(^+\) and Ca\(^{2+}\)
concentrations. Exposure to CN + 2-
DG produced a negative inotropic
effect, associated with an increase in
diastolic [Ca\(^{2+}\)],, followed by cessa-
tion of contraction and more com-
plete relaxation, with a decline in
[Ca\(^{2+}\)], to below normal diastolic
levels. Subsequently, there occurred
a gradual increase in [Ca\(^{2+}\)], to
above normal diastolic levels, asso-
ciated with an initial contracture.
The subsequent accelerated phase of
contracture was not associated with
marked changes in [Ca\(^{2+}\)]. On
return to control solution containing
glucose and no metabolic inhibitors,
relaxation occurred, associated with
a fall in [Ca\(^{2+}\)], back to diastolic
levels.
may also result in increased sensitivity of the contractile elements to Ca^{2+}. This factor could also contribute to the magnitude of the accelerated contracture we have observed.

There was a progressive rise in [Ca^{2+}], in these cells with continued metabolic inhibition after development of contracture (Figure 9). This finding is consistent with the results of Snowdowne et al., who found during hypoxia a progressive reversible increase in [Ca^{2+}], in isolated ventricular myocytes loaded with aequorin. Preliminary studies in our laboratory have shown that cultured myocytes can recover completely their contractile activity and potassium transport capacity within 24 hours after metabolic inhibition not exceeding 30 to 45 minutes duration. These findings indicate that biologically significant [Ca^{2+}] increases can occur during reversible ATP depletion.

Although our discussion has focused on effects of metabolic inhibition on motion and [Ca^{2+}], after cells have stopped beating, the initial effects of CN and 2-DG are also of interest. As shown in Figures 8 and 9, within 30 seconds of abrupt exposure to CN and 2-DG, there was a slight increase in [Ca^{2+}], associated with a transient upward shift of the end diastolic cell position. These transient changes in diastolic position and [Ca^{2+}], are not related to rate changes and resemble in part the effects of abrupt exposure to caffeine (Figure 5, and Rasmussen et al.). Paulus et al. have demonstrated that abrupt exposure to caffeine augments an increase in stiffness that occurs in the left ventricle during pacing-induced ("demand") ischemia. The combination of moderate ATP depletion with continued rapid beating of cultured heart cells, present within 30 seconds of exposure to CN and 2-DG, may be analogous to "demand" ischemia in the intact heart. If so, our findings suggest that an increase in diastolic [Ca^{2+}], possibly due to release of Ca^{2+} from SR, could indeed account for a component of the decrease in diastolic ventricular compliance noted during pacing-induced ischemia. In addition, we note that a decline in amplitude of contractile motion early after metabolic inhibition can occur without a corresponding decrease in peak systolic [Ca^{2+}], (Figures 8 and 9). This is consistent with a direct depression of Ca^{2+}-dependent contractile responses during ATP depletion as suggested by Allen and Orchard.

In these cultured cells, there is also substantial inhibition of sarcolemmal Na^{+}-K^{+} ATPase manifest by a reduction in "K uptake, gain in Na", and loss of cellular K after 5 to 10 minutes of metabolic inhibition. Previous studies have demonstrated that 5 to 10 minutes of exposure to CN + 2-DG in these cells produces a decline in bulk cellular adenosine triphosphate (ATP) content to about 40-50% of control values, or to 10-15 nmol/mg protein. Given a cell water content of approximately 7 μl/mg protein, this indicates that the ATP concentration at the time rigor and Na pump inhibition due to ATP depletion develop is 1.5-2.0 mM. This ATP concentration is higher than that required for in vitro function of the Na^{+}-K^{+} ATPase (K_m~0.5 mM) or for ATP-dependent dissociation of actin-myosin (K_m~1 mM; Taylor). A comparable discrepancy in [ATP] requirement for in vitro and in vivo function of the Na^{+}-K^{+} ATPase in renal tubular cells has been noted by Soltoff and Mandel. In their studies, the K_m for Na^{+}-K^{+} ATPase in membrane fragments was ~0.5 mM ATP, whereas in intact cells a 50% reduction in normal active K transport rate was observed at 2 mM ATP. These discrepancies could be due to cellular factors that decrease the apparent affinity of ATP binding to critical molecules or to a nonuniform distribution of ATP-producing and consuming systems. There may also be substantial stores of ATP within the myocardial cell that are not accessible to cytoplasmic and sarcolemmal sites.

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References


**Key Words** • Ca$^{2+}$ • contracture • ATP depletion • indo1
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