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Metabolic Inhibition Alters Subcellular Calcium Release Patterns in Rat Ventricular Myocytes
Implications for Defective Excitation-Contraction Coupling During Cardiac Ischemia and Failure

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Abstract—Metabolic inhibition (MI) contributes to contractile failure during cardiac ischemia and systolic heart failure, in part due to decreased excitation-contraction (E-C) coupling gain. To investigate the underlying mechanism, we studied subcellular Ca\(^{2+}\) release patterns in whole cell patch clamped rat ventricular myocytes using two-dimensional high-speed laser scanning confocal microscopy. In cells loaded with the Ca\(^{2+}\) buffer EGTA (5 mmol/L) and the fluorescent Ca\(^{2+}\)-indicator fluo-3 (1 mmol/L), depolarization from −40 to 0 mV elicited a striped pattern of Ca\(^{2+}\) release. This pattern represents the simultaneous activation of multiple Ca\(^{2+}\) release sites along transverse-tubules. During inhibition of both oxidative and glycolytic metabolism using carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, 50 mmol/L) and 2-deoxyglucose (2-DG, 10 mmol/L), there was a decrease in inward Ca\(^{2+}\) current (I\(_{c}\)), the spatially averaged Ca\(^{2+}\) transient, and E-C coupling gain, but no reduction in sarcoplasmic reticulum Ca\(^{2+}\) content. The striped pattern of subcellular Ca\(^{2+}\) release became fractured, or disappeared altogether, corresponding to a marked decrease in the area of the cell exhibiting organized Ca\(^{2+}\) release. There was no significant change in the intensity or kinetics of local Ca\(^{2+}\) release. The mechanism is not fully explained by dephosphorylation of L-type Ca\(^{2+}\) channels, because a similar degree of I\(_{c}\)”rundown” in control cells did NOT result in fracturing of the Ca\(^{2+}\) release pattern. We conclude that metabolic inhibition interferes with E-C coupling by (1) reducing trigger Ca\(^{2+}\), and (2) directly inhibiting sarcoplasmic reticulum Ca\(^{2+}\) release site open probability. (Circ Res. 2005;96:551-557.)

Key Words: excitation-contraction coupling ■ metabolic inhibition ■ heart ■ ischemia ■ calcium

Cardiac ischemia is associated with a rapid loss of contractile amplitude, followed by inexcitability and ultimately contracture. Systolic heart failure is also characterized by contractile dysfunction. Defective excitation-contraction coupling at the single myocyte level has been implicated as a root cause of contractile dysfunction associated with both myocardial ischemia and systolic heart failure. However, the cellular basis of failed E-C coupling during ischemia and systolic heart failure is not completely understood.

Ca\(^{2+}\) transients are believed to be the summation of many microscopic Ca\(^{2+}\) release events triggered by one or more unitary Ca\(^{2+}\) currents.\(^1\) It is possible to study these microscopic release events in single myocytes using rapid (240 to 480 hz) 2-dimensional (2D) laser scanning confocal microscopy (LSCM). This novel technique has revealed that depolarization leads to the recruitment of couplons and therefore Ca\(^{2+}\) release from distinct spark sites along the transverse tubules of the myocyte, eliciting a fused pattern of stripes that run along the z-lines.\(^2\)

Depressed metabolism is a prominent feature of ischemia\(^3\) and systolic heart failure.\(^4\) In isolated patch-clamped ventricular myocytes, where application of metabolic inhibitors can reproduce the metabolic stress of ischemia and systolic heart failure, we have previously observed an unexpectedly large reduction in the amplitude of the Ca\(^{2+}\) transient during a voltage clamp, which is out of proportion to the decrease in I\(_{c}\). We also observed, and which is not due to depleted sarcoplasmic reticulum (SR) Ca\(^{2+}\) stores.\(^5\) This indicates a reduction in E-C coupling gain, a convenient measure of the efficiency of E-C coupling, and which is defined as the rate of Ca\(^{2+}\) release divided by the amplitude of the Ca\(^{2+}\) current.\(^6\) The reasons for this reduction in gain are uncertain.

Because reduced gain may be an important contributor to contractile failure during ischemia and systolic heart failure, we sought to explain the mechanism that underlies reduced gain using rapid 2D LSCM to evaluate the response of local Ca\(^{2+}\) release events to depolarization in whole-cell patch-clamped rat ventricular myocytes exposed to metabolic in-
hibitors. We found that metabolic inhibition fractures the Ca\(^{2+}\) release pattern, indicating reduced ability of the macroscopic Ca\(^{2+}\) current to recruit local release sites. Ca\(^{2+}\) flux through surviving release sites was unaffected.

**Materials and Methods**

Enzymatically isolated ventricular myocytes were obtained from adult Sprague-Dawley rats as described previously, and studied with the whole-cell patch clamp technique. The Cs\(^{+}\)-based pipette solution contained 5 mmol/L EGTA and 1 mmol/L fluo-3 to limit the diffusion distance of free Ca\(^{2+}\) to less than 50 nm without reducing SR Ca\(^{2+}\) release. Under these high buffering conditions, spatial and temporal resolutions are determined primarily by the less mobile Ca\(^{2+}\)-fluor-3 complex.\(^9\) Such an approach has been used successfully to more effectively restrict fluorescence increases to Ca\(^{2+}\) release sites,\(^2,10,11\) thus improving the precision of Ca\(^{2+}\) localization with confocal imaging. Song et al\(^{12}\) have used a similar strategy to map of the active release sites in both the whole-cell patch technique. The Cs\(^{+}\)-based pipette solution contained 5 mmol/L EGTA and 1 mmol/L fluo-3 to limit the diffusion distance of free Ca\(^{2+}\) to less than 50 nm without reducing SR Ca\(^{2+}\) release. Under these high buffering conditions, spatial and temporal resolutions are determined primarily by the less mobile Ca\(^{2+}\)-fluor-3 complex.\(^9\) Such an approach has been used successfully to more effectively restrict fluorescence increases to Ca\(^{2+}\) release sites,\(^2,10,11\) thus improving the precision of Ca\(^{2+}\) localization with confocal imaging. Song et al\(^{12}\) have used a similar strategy to

Cells were imaged in 2D at high speed (240 frames per second) using a Noran Odyssey XL rapid 2D LSCM (Noran Instruments, Middleton, WI).\(^3\) We applied strong depolarizations to 0 mV, where Ca\(^{2+}\) spark recruitment is high (and where sparks tend to fuse), because this is more physiological than weaker depolarizations to negative potentials where Ca\(^{2+}\) release is not uniform. For image analysis, we found it impractical to apply the classical Ca\(^{2+}\) spark detection algorithms, which had been created for line-scan images of infrequent spontaneous sparks, to our 2D image stacks of evoked Ca\(^{2+}\) release. We therefore developed a semiautomated method of detecting and analyzing the characteristics of localized Ca\(^{2+}\) release patterns in 2D during strong depolarizations, so that we could avoid using Ca\(^{2+}\) channel blockers, reduced extracellular Ca\(^{2+}\), or signal averaging.

Z-lines were visually identified by the striped pattern of localized increases in fluorescence during depolarization.\(^2\) We then set a threshold that maximized detection of fluorescence along the z-lines on release, while minimizing detection of fluorescence in the “inter-z” space. Pixels exceeding the threshold were used to create a map of the active release sites in both the x and y dimensions, which was applied to the entire image stack. The average fluorescence intensity of mapped pixels was then obtained for each image in the stack collected during the depolarization to determine the time course of local changes in Ca\(^{2+}\) concentration. The total number of mapped pixels was used to calculate the “release area.” This operation was performed individually for each voltage clamp depolarization analyzed. The investigator determining the threshold was blinded to the condition (control, rundown, metabolic inhibition).

This method minimizes problems caused by changes in baseline fluorescence level due to progressive dye loading, and real effects of metabolic inhibition and rundown on diastolic Ca\(^{2+}\) levels. The averaged Ca\(^{2+}\) release site transients analyzed in this fashion were similar to those obtained by examining rarely observed unfused individual Ca\(^{2+}\) sparks.

For additional details, please refer to the expanded Materials and Methods section in the online data supplement available at http://circres.ahajournals.org.

**Results**

**Ca\(^{2+}\) Release Patterns and Area During Metabolic Inhibition**

We exposed patch-clamped myocytes to a low concentration of carbonyl cyanide-p-trifluoromethoxyphenyldrazone (FCCP, 50 nmol/L), to limit oxidative metabolism, in conjunction with a standard concentration of 2-deoxyglucose (2-DG, 10 mmol/L), to inhibit glycolytic metabolism, and depolarized the cells at 1-minute intervals from a holding potential of −40 mV to a test potential of 0 mV. Each test pulse was preceded by six 100 ms conditioning pulses from −40 to 0 mV at 1 Hz. We simultaneously recorded 2-dimensional fluo-3 fluorescence confocal images and whole-cell current. Figure 1A displays normalized (F/F\(_0\)) images, release area maps, and membrane currents from a representative cell under control conditions, and after IC\(_o\) had decreased by 20% during metabolic inhibition. The displayed images were selected from their respective image stacks at the onset of Ca\(^{2+}\) release. The control image was obtained 10 minutes after forming the whole-cell patch, which was long enough to dialyze the cytoplasm with the fluo-3/EGTA internal solution to steady state. Note the striped appearance of the Ca\(^{2+}\) release pattern under control conditions, consistent with previous studies;\(^2\) and which is thought to represent numerous adjacent discrete Ca\(^{2+}\) sparks that fuse together when activated simultaneously. After metabolic inhibition sufficient to reduce IC\(_o\) by 20%, the Ca\(^{2+}\) release pattern fractured. In rare instances, particularly when the release pattern was slightly fractured at the outset, there was no organized Ca\(^{2+}\) release at all after exposure to metabolic inhibitors.

Because it is not practical to quantify the number of individual Ca\(^{2+}\) release sites during a full depolarization, we measured a surrogate: the area of the cell exhibiting coordinated Ca\(^{2+}\) release on depolarization. Release area maps were prepared as described in the Methods. The release area maps for the myocyte shown in Figure 1A are presented adjacent to the corresponding normalized image. On average, we found that metabolic inhibition sufficient to decrease IC\(_o\) by 20% caused a 75±7% decrease in the area of organized Ca\(^{2+}\) release (Figure 1C; P<0.05, n=7). The decrease in release area observed with metabolic inhibition is consistent with a reduction in the number of organized release sites, and the availability of couplons.\(^13\) Why, therefore, in the presence of metabolic inhibitors do SR release sites cease to function? There are two likely possibilities. The first is that the opening of ryanodine receptors is directly inhibited by metabolic inhibition. The second is that the Ca\(^{2+}\) trigger, i.e., L-type Ca\(^{2+}\) channel opening, is partially inhibited. It is possible, of course, that both these processes may explain the loss of release sites.

**Ca\(^{2+}\) Release Patterns and Area During Rundown**

To determine whether the disintegration of the Ca\(^{2+}\) release pattern and the corresponding decrease in Ca\(^{2+}\) release area were simple consequences of reduced macroscopic IC\(_o\), we took advantage of the fact that Ca\(^{2+}\) channels reduce their open probability over time in patch-clamped myocytes, which causes rundown of the macroscopic Ca\(^{2+}\) current,\(^14-16\) despite the presence of Mg\(^{2+}\), EGTA, and cAMP in the patch pipette. We therefore examined the Ca\(^{2+}\) release patterns during IC\(_o\) rundown. Control cells were depolarized and imaged as described earlier, but in the absence of metabolic inhibitors. The subcellular Ca\(^{2+}\) images, release area maps, and currents
from a representative myocytes are shown in Figure 1B. As in Figure 1A, the normalized control depolarization image was taken 10 minutes after forming the whole-cell patch, and again shows the typical striped pattern of Ca^{2+}/H11001 release. 

$I_{\text{Ca}}$ and subcellular fluorescence were monitored during depolarizations at 1-minute intervals, until the current had decreased by 20% due to rundown. This was the same extent of reduced macroscopic Ca^{2+} current as during metabolic inhibition. However, in contrast to metabolic inhibition, we found that repeated depolarizations to 0 mV reproducibly activated the same Ca^{2+} release sites and caused relatively small changes in the Ca^{2+} release pattern, as shown in the normalized rundown image in Figure 1B. This impression was confirmed by the corresponding Ca^{2+} release area maps adjacent to the normalized images, and by the summary (Figure 1C, n=7).

These findings remain consistent with the general conclusions that we stated.

**Calcium Current and Transients During Metabolic Inhibition and Rundown**

If the similar decline in macroscopic $I_{\text{Ca}}$ during metabolic inhibition and rundown is due to similar changes in the microscopic properties of the currents, then the additional fractionation of the Ca^{2+} release pattern during metabolic inhibition is probably due to loss of functioning couplons as a result of a decline in available ryanodine receptors (RyRs). At a whole-cell level, a reduction in couplons would result in a reduction in the global Ca^{2+} transient that is out of proportion to the reduction in $I_{\text{Ca}}$. Indeed, we have demonstrated a disproportionate reduction in the Ca^{2+} transient in guinea pig ventricular myocytes exposed to metabolic inhibitors. To confirm that this also occurs in rat cells, we reanalyzed the 2D confocal images obtained during metabolic
inhibition to examine the relationship between whole-cell $I_{\text{Ca}}$ and the whole-cell (spatially averaged) Ca\textsuperscript{2+} transient. After 5.6±0.8 minutes of metabolic inhibition, there was a 20% reduction in $I_{\text{Ca}}$ (from 3.69±0.31 to 2.87±0.31 nA, n=7; $P<0.001$, Figure 1A) and a 39±4% reduction in the amplitude of the spatially averaged Ca\textsuperscript{2+} transient (Figure 1D). We then compared the Ca\textsuperscript{2+} transient during rundown. A 20% reduction in $I_{\text{Ca}}$ during rundown for 4.9±1.1 minutes ($I_{\text{Ca}}$ reduced from 3.04±0.41 to 2.41±0.35 nA, n=7; $P<0.001$, Figure 1B) caused only a 23±6% (n=7) reduction in the Ca\textsuperscript{2+} transient (Figure 1E). Thus the extent of Ca\textsuperscript{2+} released during metabolic inhibition is reduced well beyond what one would expect in response to a simple reduction in the amplitude of $I_{\text{Ca}}$, recapitulating our previous findings in guinea pig using fura-2 as the Ca\textsuperscript{2+} indicator.5 Similarly, we also found that there was a significantly greater decline in the maximum rate of rise of the averaged whole-cell Ca\textsuperscript{2+} transient ($\text{dF}/\text{dt}_{\text{max}}$) during metabolic inhibition compared with rundown (61±9% versus 33±4% decline, respectively, $P<0.05$, n=7 each group), despite similar reductions in $I_{\text{Ca}}$. The $\text{dF}/\text{dt}_{\text{max}}$ is a more appropriate measure of release flux than amplitude of the Ca\textsuperscript{2+} transient when computing E-C coupling gain. When expressed as a gain function (gain=$\text{dF}/\text{dt}_{\text{max}}/I_{\text{Ca}}$), we found that rundown was associated with only a 14±6% decrease in gain, whereas metabolic inhibition was associated with a 49±11% decrease ($P<0.05$ for rundown versus metabolic inhibition). These results are consistent with a significantly greater reduction in SR Ca\textsuperscript{2+} release flux during metabolic inhibition than during control rundown. There are two possible explanations for these findings. First, although rundown and metabolic inhibition produce similar changes in magnitude of $I_{\text{Ca}}$ with a similar time course, the microscopic properties of the two currents may differ fundamentally and in a way that affects triggering to a greater extent in the case of the current recorded during metabolic inhibition. Alternatively, the microscopic and macroscopic properties of the currents during metabolic inhibition and rundown are identical, in which case metabolic inhibition affects some other aspect of the release mechanism, for example the open probability of RyRs. We will discuss these possibilities later (see Discussion).

**Characteristics of Ca\textsuperscript{2+} Release Site Transients**

We also considered the possibility that the reduced macroscopic $\text{dF}/\text{dt}_{\text{max}}$ of the Ca\textsuperscript{2+} transient produced by metabolic inhibition was caused by reduced Ca\textsuperscript{2+} flux through individual Ca\textsuperscript{2+} release sites. Although we cannot easily assess individual release site (spark) Ca\textsuperscript{2+} flux during a depolarization to 0 mV, we can measure the flux averaged across all active release sites. We therefore measured the average intensity and kinetics of fluo-3 fluorescence from all the active release sites during metabolic inhibition and during rundown. Peak average release site fluorescence intensity declined slightly for both metabolic inhibition (33±4%) and rundown (21±7%), but the extent of decline was similar for both groups ($P=0.19$ metabolic inhibition versus rundown). Metabolic inhibition and rundown cells also exhibited equivalent reductions in the maximum rate of rise ($\text{dF}/\text{dt}_{\text{max}}$) of averaged Ca\textsuperscript{2+} release site transients (31±8% for metabolic inhibition and 22±15% for rundown, $P=0.64$), as shown in Figure 2A. Because Ca\textsuperscript{2+} flux across release sites is related to the time derivative of the rising phase of the Ca\textsuperscript{2+} transient, or proportional to the fluorescence transient under highly Ca\textsuperscript{2+} buffered conditions,10,12 our results suggest that the flux of Ca\textsuperscript{2+} across release sites is not significantly altered by metabolic inhibition compared with rundown. Finally, the time to peak of the release transient was unaffected by either metabolic inhibition or rundown (data not shown).

**Preservation of SR Ca\textsuperscript{2+} Store During Metabolic Inhibition**

SR Ca\textsuperscript{2+} load has been shown by some groups to affect Ca\textsuperscript{2+} spark frequency and E-C coupling gain.18 A reduction in SR Ca\textsuperscript{2+} load could reduce the amplitude of the Ca\textsuperscript{2+} transient in response to $I_{\text{Ca}}$ and could also reduce the flux across the Ca\textsuperscript{2+} release sites. To assess SR Ca\textsuperscript{2+} stores, we applied 5 mmol/L caffeine to the bath surrounding the myocyte for 500 ms. This caused a Ca\textsuperscript{2+} transient whose amplitude reflects the SR Ca\textsuperscript{2+} store. Similar to our previous work,5 caffeine-induced transients (Figure 2B) were not significantly altered by application of metabolic inhibitors or by rundown (Figure 2C, n=6 each group, $P=0.46$). Thus, the effects of metabolic inhibition on Ca\textsuperscript{2+} release could not be attributed to a reduced SR Ca\textsuperscript{2+} load. It is also notable that RyRs are still capable of responding to caffeine during metabolic inhibition.

**Discussion**

Previously, we reported that combined inhibition of oxidative and glycolytic metabolism interfered with the ability of the macroscopic Ca\textsuperscript{2+} current to trigger Ca\textsuperscript{2+} release from a fully loaded SR in patch-clamped guinea pig ventricular myocytes. The reasons for the reduction in E-C coupling gain were unknown, but we postulated a fundamental defect in the response of RyRs to triggering by Ca\textsuperscript{2+} entering through Ca\textsuperscript{2+} channels.

The present study extends our previous work by demonstrating that combined inhibition of oxidative and glycolytic metabolism causes a marked disintegration of the Ca\textsuperscript{2+} release pattern and a reduced Ca\textsuperscript{2+} release area in ventricular myocytes. This is due to a reduction in the number of Ca\textsuperscript{2+} release sites (sparks) recruited by the Ca\textsuperscript{2+} current during voltage clamp depolarization. The reduced number of release sites cannot be simply attributed to the 20% decline in the macroscopic Ca\textsuperscript{2+} current, because a similar decline in $I_{\text{Ca}}$ during rundown had little effect on the Ca\textsuperscript{2+} release pattern. Furthermore, neither metabolic inhibition nor rundown had a differential effect on the maximum rate of rise of the averaged release site fluorescence transients. Thus, the decrease in macroscopic E-C coupling gain caused by combined inhibition of oxidative and glycolytic metabolism is due to a decrease in release site recruitment by $I_{\text{Ca}}$, rather than a reduction in release site flux or intensity. How can we explain the reduced availability of couplons during metabolic inhibition compared with rundown, despite similar changes in the macroscopic Ca\textsuperscript{2+} current?

**Ca\textsuperscript{2+} Current and SR Ca\textsuperscript{2+} Release During Metabolic Inhibition**

There are three possible explanations for the reduction of couplons during metabolic inhibition. First, if microscopic
Rundown is caused by dephosphorylation of Ca\(^{2+}\) channels, which increases Mode 0 gating, the probability that a channel fails to open during depolarization (P_{null}), and thus the number of blank sweeps during single channel recording.\(^{14-16}\) There is now evidence that regardless of whether or not a number of L-type Ca\(^{2+}\) channels are required to trigger SR Ca\(^{2+}\) release, a cluster of L-type Ca\(^{2+}\) channels is required to insure adequate coupling fidelity with RyRs.\(^{21}\) Thus, if we assume for simplicity that every opening of an L-type Ca\(^{2+}\) channel triggers a Ca\(^{2+}\) release event, then the probability of generating a spark (P_{s}) is 1-(P_{null})^{N} where N equals the number of L-type Ca\(^{2+}\) channels. Therefore, as P_{null} increases during rundown, P_{s} should decline and produce fractionation in the pattern of Ca\(^{2+}\) release. However, rundown does not produce significant fractionation of the pattern. The most likely explanation for this preserved Ca\(^{2+}\) release is that clusters of L-type Ca\(^{2+}\) channels arranged in a couplon (ie, N) are sufficiently large to compensate for any increase there might be in P_{null} due to rundown. It is entirely plausible that metabolic inhibition, like rundown, also leads to dephosphorylation of L-type Ca\(^{2+}\) channels. If the mechanisms and time course of Ca\(^{2+}\) current reduction are the same for both rundown and metabolic inhibition, then the fractionation of the Ca\(^{2+}\) release pattern during the latter is most likely caused by a direct effect of metabolic inhibition on RyRs.

If identical reductions in macroscopic Ca\(^{2+}\) currents generate different release patterns without any effect on RyRs, then we must explain the difference in triggering by differences in microscopic properties of the two currents that do not influence the macroscopic amplitude. The metabolic sensitivity of I_{Ca} is well known,\(^{5,19,20}\) and ATP generated by glycolysis may have particular regulatory properties on the Ca\(^{2+}\) channel.\(^{19}\) We have already suggested that metabolic inhibition, like rundown, may lead to dephosphorylation of I_{Ca}, and an increase in P_{null}. However, to explain the different pattern of Ca\(^{2+}\) release, we would have to postulate that the effect of metabolic inhibition on L-type Ca\(^{2+}\) channel phosphorylation is more extensive than that which occurs during rundown. The effect, therefore, on triggering would be greater resulting in the observed fractionation. However, in that case, we would not expect the macroscopic currents to be of similar amplitude. To maintain similarity in the macroscopic currents, we would have to postulate an increase in channel conductance to compensate for the increase in P_{null}. However, an increase in channel conductance seems highly unlikely during metabolic inhibition.

If the primary effect of metabolic inhibition on L-type Ca\(^{2+}\) channels is not reduced P_{s} and increased P_{null} due to dephosphorylation, as it appears to be with rundown, then the most likely alternative is reduced single channel current due to an increase in subconductance states. Subconductances have been described previously in L-type Ca\(^{2+}\) channels,\(^{21}\) although an association with metabolic inhibition has not to our knowledge been previously reported. Because the probability of evoking a Ca\(^{2+}\) spark depends in part on the amplitude of the unitary L-type Ca\(^{2+}\) current,\(^{22,23}\) the development of subconductance states could affect Ca\(^{2+}\) release (although why Ca\(^{2+}\) release should show a greater dependence on channel conductance versus channel number is uncertain). Again, the existence of subconductance states during metabolic inhibition would require some compensating change in the microscopic properties of Ca\(^{2+}\) currents during rundown.
those currents so that the macroscopic currents are the same during rundown and metabolic inhibition. These issues clearly require further investigations of microscopic currents, which are beyond the scope of this study.

We cannot absolutely exclude the possibility that the high concentrations of EGTA and fluo-3 in the pipette solution interfered with alterations in E-C coupling introduced by metabolic inhibition. However, because the conditions were similar for both metabolic inhibition and rundown, this seems an unlikely explanation for the differential response of the Ca\(^{2+}\) release pattern to these two conditions.

**Role of RyR Activity**

RyR openings are required to generate Ca\(^{2+}\) sparks. Both rundown and metabolic inhibition caused a 20% reduction in trigger Ca\(^{2+}\), but only metabolic inhibition resulted in a reduction in Ca\(^{2+}\) spark activation. The most straightforward explanation for the marked reduction in Ca\(^{2+}\) release site probability is a decrease in the Ca\(^{2+}\) dependence of RyR P\(_o\). This makes sense on theoretical grounds, because the open probability of RyRs is ATP dependent.\(^{24}\) Elevations in Mg\(^{2+}\) and H\(^+\) may also contribute to inhibition of Ca\(^{2+}\) release from RyRs during metabolic inhibition and ischemia\(^{25–29}\) if these ions escape control by patch electrode dialysis. Our results are also consistent with those of Overend et al,\(^{30}\) who demonstrated that metabolic inhibition with cyanide and 2-DG reduces the tendency of Ca\(^{2+}\) challenged myocytes to develop spontaneous Ca\(^{2+}\) wave activity and eliminates spontaneous spark activity at diastolic potentials. However, our experiments indicate that some RyRs can still be activated by I\(_{Ca}\), as well as caffeine, at least at an early stage of metabolic inhibition before the onset of rigor.

**Role of SR Ca\(^{2+}\) Load and NCX Activity**

Spark probability is influenced not only by RyR behavior, but also by SR Ca\(^{2+}\) load\(^{18}\) and sodium-calcium exchange (NCX).\(^{7}\) Consistent with our prior studies,\(^{5}\) we found no evidence for any significant change in the SR Ca\(^{2+}\) store due to metabolic inhibition (Figure 2B and 2C). Preservation of SR Ca\(^{2+}\) during metabolic inhibition and hypoxia has been observed by other investigators.\(^{30,31}\) An intact SR excludes changes in spark probability due to alterations in SR Ca\(^{2+}\). The intact SR Ca\(^{2+}\) load also accounts for the preserved kinetics of Ca\(^{2+}\) release from surviving release sites. The paradox of maintained SR Ca\(^{2+}\) load during metabolic stress is most readily explained by a decrease in the fraction of SR Ca\(^{2+}\) released during depolarization, owing to defective E-C coupling.\(^{30,32}\) This allows for an energy-compromised SERCA to maintain the SR Ca\(^{2+}\) load. Reduced uptake of Ca\(^{2+}\) by mitochondria during application of FCCP might also support increased uptake by the SR.

Although we have shown that NCX can alter spark probability,\(^{7}\) we did not observe any effect of combined oxidative and glycolytic inhibition on NCX activity in patch clamped guinea pig ventricular myocytes.\(^{5}\) However, it is known that NCX is regulated by ATP, likely via PIP\(_2\),\(^{33}\) and reverse-mode NCX may be sensitive to hypoxia.\(^{34}\) Whether NCX is less sensitive than other E-C coupling proteins to changes in ATP levels, or whether other counter-regulatory events take place during met-

**Implications for E-C Coupling**

Our explanation for the effect of metabolic inhibition and rundown on E-C coupling is consistent with the idea that a coupling not only contains numerous RyRs, but also a cluster of L-type Ca\(^{2+}\) channels as well.\(^{11}\) A cluster of L-type Ca\(^{2+}\) channels would provide a safety factor for E-C coupling. It has been proposed that synchronization of release sites can affect the whole-cell Ca\(^{2+}\) transient and the Ca\(^{2+}\) myofilament interaction.\(^{12}\) We did not observe any loss of synchronization of Ca\(^{2+}\) release during metabolic inhibition, although asynchronous Ca\(^{2+}\) release has been described in a model of postinfarction cardiac failure.\(^{35}\) It is unlikely that the high Ca\(^{2+}\) buffering we used obscured loss of synchronization, because Song et al\(^{12}\) used a similar buffering strategy to study Ca\(^{2+}\) release site synchronization.

**Implications for Clinical Conditions Characterized by Metabolic Stress, eg, Ischemia, Reperfusion, and Failure**

To study E-C coupling in detail, it is essential to use isolated heart cells. This means that we must simulate the metabolic stress of ischemia and heart failure by applying metabolic inhibitors. Metabolic stress is but one aspect of the complex milieu of ischemia and heart failure. Our conclusions and their relevance to ischemic heart disease and systolic heart failure must therefore be appropriately guarded.

The effects of metabolic inhibition on Ca\(^{2+}\) release occurred before major reductions in I\(_{Ca}\), or the development of rigor or contracture. This suggests that metabolic stress could contribute to the early contractile dysfunction of ischemia, currently attributed to mechanisms such as the “garden-hose effect,”\(^{76}\) and shortening of the action potential due to activation of I\(_{KATP}\).\(^{31}\) The development of defective E-C coupling before other major abnormalities typically associated with ATP depletion also reinforces the importance of this aspect of Ca\(^{2+}\) regulation to control of contractile force in systolic heart failure.

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MATERIALS AND METHODS

Cell Isolation

Enzymatically isolated ventricular myocytes were obtained from adult Sprague-Dawley rats as described previously.\(^1\) Briefly, adult rats (200-300 g) were anesthetized with sodium pentobarbitone, their hearts removed and perfused via retrograde aortic perfusion with a solution containing collagenase (2 mg/ml Type II collagenase, GIBCO BRL, Life Technologies, Gaithersburg, MD, cat# 17101) and protease (0.166 mg/ml Type XIV Protease, SIGMA, St. Louis, MO, cat# P-5147), according to the method of Mitra and Morad.\(^2\) The cells were stored in a modified Na\(^+\)-Tyrodes solution, containing (in mmol/L): 136 NaCl, 5.4 KCl, 10 HEPES, 1 MgCl\(_2\), 1.8 CaCl\(_2\), 10 Glucose, pH 7.4 with NaOH (temperature 20-22\(^\circ\)C).

Patch Clamp

Patch electrodes were pulled from borosilicate glass (World Precision Instruments, Sarasota, FL, TW150F-3) on a Sutter P-87 horizontal puller (Sutter Instruments, Novato, CA). The electrodes had a tip diameter of 2-3 \(\mu\)m, and a resistance of 2-4 M\(\Omega\) after gentle fire-polishing and when filled with an internal solution containing (in mmol/L): 125 CsCl, 5 Na-pyruvate, 5 glutathione, 5 EGTA, 1 MgCl\(_2\), 1 KH\(_2\)PO\(_4\), 1 KADP, 0.1 cAMP, 10 TEACl, 15 HEPES, 1 Fluo-3, pH 7.4. The concentrations of EGTA and the Ca\(^{2+}\) indicator Fluo-3 we used have been calculated to limit the diffusion distance of free Ca\(^{2+}\) to less than 50 nm\(^3\) without reducing SR Ca\(^{2+}\) release. Under these high buffering conditions, spatial and temporal resolutions are determined primarily by the less mobile Ca\(^{2+}\)-fluo-3 complex.\(^4\) Such an approach has been used successfully to
more effectively restrict fluorescence increases to Ca\(^{2+}\) release sites,\(^5,6\) thus improving the precision of Ca\(^{2+}\) localization with confocal imaging. Song et al.\(^7\) have used a similar strategy to measure SR Ca\(^{2+}\) flux \(J_{SR}\) at release site microdomains. Pyruvate, PO\(_4\), and ADP were added to the pipette solution as substrates for endogenous synthesis of ATP, which avoids difficulty in interpreting effects of metabolic inhibition in the presence of exogenous ATP.\(^8\) Glutathione was included as an antioxidant to reduce the phototoxicity common to laser scanning.

Cells were depolarized for 100 ms from a holding potential of −40 mV to 0 mV using an Axopatch 200A patch clamp amplifier (Axon Instruments, Foster City, CA) and a Digidata 1200 (Axon Instruments) data acquisition system controlled by pClamp 6 software (Axon Instruments). A rapid solution exchanger was used to perfuse the myocyte under study and to exchange solutions with a halftime of <100 ms. Under control conditions, myocytes were perfused with a K\(^+\)-free Tyrodes solution containing (mmol/L): 136 NaCl, 5.4 CsCl, 1 MgCl\(_2\), 1.8 CaCl\(_2\), 0.33 NaH\(_2\)PO\(_4\), 10 HEPES, 10 glucose, pH 7.4. Metabolic inhibition was achieved by switching to a similar solution, except with 50 nmol/L carbonyl cyanide-\(p\)-trifluoromethoxyphenylhydrazone (FCCP) added and 10 mmol/L 2-deoxyglucose (2-DG) replacing glucose. SR Ca\(^{2+}\) load was assessed where indicated by applying a rapid pulse of caffeine using the solution exchanger, as described previously.\(^1\)

**Rapid 2-Dimensional Confocal Ca\(^{2+}\) Imaging**

Cells were loaded with the Ca\(^{2+}\) indicator fluo-3 via the patch pipette (see above) and then imaged using a Noran Odyssey XL rapid 2D LSCM (Noran Instruments,
Middleton, WI) attached to a Zeiss Axiovert TV100 inverted microscope fitted with a 40X water immersion objective lens (Zeiss C-Apochromat 40/1.2 W Corr). The excitation wavelength of the Argon-Krypton laser was set to 488 nm, and fluorescence emission wavelengths greater than 510 nm were detected by the photomultiplier of the confocal system. Image acquisition was synchronized with patch clamp protocols using a Silicon Graphics Indy (Silicon Graphics, Inc., Mountain View, CA) workstation to trigger the patch clamp computer. Synchronization was verified by flashing a light emitting diode. 80 sequential images were collected at 240 Hz (4 ms per frame). The size of each two-dimensional frame was 232x98 pixels and the pixel size was 0.19 x 0.19 µm. To reduce photobleaching of the indicator as well as phototoxicity to the cells, the laser was shuttered electronically and triggered to open only during the 320 ms acquisition period. Fluorescence signals (F) were normalized by dividing them by the average fluorescence intensity at rest (F₀). This procedure removes the contribution of nonuniformities in dye concentration within the confocal plane and gives a signal that can be directly related to intracellular Ca²⁺ concentration.⁹

**Gain Function**

E-C coupling consists of Ca²⁺ release triggered by L-type Ca²⁺ current. The existence of various substances and structures that bind intracellular Ca²⁺ cause a significant delay between the onset of the trigger and the subsequent release that it evokes. It is nevertheless convenient to characterize this process with a gain function, which is usually calculated as the rate of SR Ca²⁺ release divided by the amplitude of the L-type Ca²⁺ current. This measure of the efficiency of E-C coupling is, for example,
sensitive to the redundancy in the quantity of trigger Ca\(^{2+}\) and to coupling fidelity, which is determined by the number of ryanodine receptors gated by the unitary Ca\(^{2+}\) current. In this paper, we use the gain function to characterize E-C coupling.

**References**


