Relative Roles of Ca\(^{2+}\)-Dependent and
Ca\(^{2+}\)-Independent Mechanisms in Hypoxic
Contractile Dysfunction

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Contractile function is known to be impaired during hypoxia or metabolic inhibition, but the relative importance of activator Ca\(^{2+}\) deficiency compared with the accumulation of depressant metabolites remains controversial. To distinguish between these possibilities, we used nuclear magnetic resonance (NMR) spectroscopy to measure the most likely mediators—intracellular [Ca\(^{2+}\)]\(_i\), inorganic phosphate concentration ([Pi]), and pH—before and during hypoxia in perfused ferret hearts. Ca\(^{2+}\) transients were quantified by gated fluorine-19 NMR spectroscopy. Left ventricular developed pressure decreased to steady-state levels approximately 60% of control values after 20 minutes of hypoxic perfusion (induced by equilibrating the perfusate with 10% O\(_2\)-90% N\(_2\)). With hypoxia, phosphorus NMR revealed an increase in [Pi] and a mild intracellular acidosis. Both [Pi] and intracellular pH correlate well with the extent of decline of developed pressure in each heart, but multiple regression analysis points to the changes in [Pi] as the dominant influence. In contrast, [Ca\(^{2+}\)]\(_i\), at end diastole was not influenced by hypoxia, whereas the peak systolic values were paradoxically increased. The ratio of Ca\(^{2+}\) transient amplitude in hypoxia to that in control had no correlation with percent of developed pressure. These findings indicate that contractile failure during relatively mild, steady-state hypoxia is not due to a critical failure of any of the mechanisms that regulate cytoplasmic activator Ca\(^{2+}\). Instead, the accumulation of Pi (and to a lesser degree, H\(^+\)) mediates hypoxic contractile dysfunction. (Circulation 1990;82:528–535)

Exposure of heart muscle to hypoxia or metabolic inhibition depresses the muscle’s ability to generate contractile force. Although this phenomenon has long been recognized,\(^1,2\) its cellular basis remains controversial. Two general classes of mechanisms have been proposed. Much attention has been focused on the possibility that failure of excitation is primary,\(^3–5\) with a consequent decline in the cytoplasmic Ca\(^{2+}\) that is available to initiate contraction. The activation of normally quiescent ATP-sensitive potassium channels features prominently in this scheme.\(^4–6\) Alternatively, intracellular accumulation of depressant metabolites rather than an excitation-related fall in cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) has been viewed as primary. Inorganic phosphate (Pi) and protons are known to reduce the ability of the myofilaments to generate force in response to Ca\(^{2+}\), and both accumulate when respiration is inhibited.\(^2,7–12\) In this view, contractile dysfunction occurs at the most distal steps of excitation-contraction coupling, with no requirement for a fall in cytoplasmic Ca\(^{2+}\) transients.

Despite the recent explosion of information in this area, experiments have investigated one possible mechanism at a time, and results have often appeared to be contradictory. Allen and Orchard\(^13\) reported that moderate hypoxia does not blunt Ca\(^{2+}\) transients, whereas Morgan and coworkers\(^14,15\) have observed a gradual decrease of systolic [Ca\(^{2+}\)]\(_i\) that occurs hand-in-hand with the onset of contractile depression. Measurements of [Ca\(^{2+}\)]\(_i\) in isolated myocytes during metabolic inhibition also defy unification. In chick ventricular cells, the early phase of contractile failure induced by cyanide and deoxyglucose is not accompanied by a decline in Ca\(^{2+}\) transients.\(^16,17\) Contrary to observations in rat cells,\(^18\) Many studies that have focused on Pi and pH measurements rather than on [Ca\(^{2+}\)]\(_i\) have documented profound changes that are usually in the correct

See p 652

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direction\textsuperscript{7,19,20} to explain the depression of contraction (however, see Allen et al\textsuperscript{21} and Eisner et al\textsuperscript{18}). The variability in the various experimental approaches and in the resulting data makes it difficult to reach firm conclusions regarding the relative roles of Ca\textsuperscript{2+}-dependent and -independent mechanisms.

In the present study, nuclear magnetic resonance (NMR) spectroscopy was used to measure the various candidate mediators ([Ca\textsuperscript{2+}], Pi, and pH) before and during steady-state hypoxic contractile depression. Ca\textsuperscript{2+} transients in perfused ferret hearts are quantified with gated fluorine-19 NMR spectroscopy.\textsuperscript{22} This method for measuring [Ca\textsuperscript{2+}], has the unique advantage of also enabling the detection of intracellular pH (pH\textsubscript{i}) and Pi in the same hearts.\textsuperscript{23-25} Our results indicate that the functional deterioration during relatively mild, steady-state hypoxia can be explained by Pi and H\textsuperscript{+} accumulation, not by a decline in Ca\textsuperscript{2+} transients.

Methods

The experimental preparation has been described in detail elsewhere.\textsuperscript{22-26} After male ferrets 11–14 weeks old were anesthetized and heparinized, hearts (n=8) were excised and Langendorff-perfused with 100% O\textsubscript{2}-bubbled solution of the following composition (mM): 108 NaCl, 5 KCl, 1 MgCl\textsubscript{2}, 5 HEPES, 2 CaCl\textsubscript{2}, 20 Na acetate, and 10 glucose. pH was adjusted to 7.40 at 30°C by titration with NaOH. Coronary flow rate was adjusted so that the initial coronary pressure measured at the aortic cannula equaled 80 mm Hg; flow was kept constant throughout the experiment. A thin latex balloon tied to the end of a polyethylene tube was inserted into the left ventricle and connected to a transducer to measure isovolumic pressure. The balloon was filled with 1 mM 6-fluoro-tryptophan (6F-Trp) as a standard for the 19F NMR measurements. The balloon volume was set to achieve an initial end-diastolic pressure of 10–20 mm Hg and kept isovolumic throughout the experiment. Each heart was paced at 1.0–1.2 Hz via an agar wick electrode saturated with 3 M KCl.

After data were obtained under control conditions, the perfusate was switched to hypoxic solution (equilibrated with 10% O\textsubscript{2}-90% N\textsubscript{2}). When cardiac function reached a new, depressed steady state, the appropriate measurements were repeated.

Measurement of Ca\textsuperscript{2+} Transients in Perfused Hearts

Seven hearts were lowered into the bore of a superconducting magnet (8.5 T) connected to a Bruker AM-360 NMR spectrometer (Bruker Instruments, Billerica, Mass.) operated in the pulsed Fourier transform mode. The Ca\textsuperscript{2+} indicator 5F-BAPTA [the 5,5'-difluoro derivative of 1,2-bis(o-aminophenoxo)ethane-N,N,N',N'-tetraacetic acid]\textsuperscript{27,28} was loaded into the hearts by perfusion with its tetra-acetoxymethyl ester derivative (15 μM) for 15–20 minutes.\textsuperscript{22,26} Afterwards, the perfusate calcium concentration ([Ca\textsubscript{2+}]) was raised to 8 mM to antagonize partially the negative inotropic effect of the calcium buffering introduced by 5F-BAPTA. The methods used for resolving the changes in [Ca\textsuperscript{2+}], during the cardiac cycle (i.e., the Ca\textsuperscript{2+} transients) with gated 19F NMR spectroscopy have been described by Marban et al.\textsuperscript{22} The excitatory radiofrequency pulses and subsequent NMR data acquisition were gated according to a programmable delay from the time of the pacing stimulus. One pulse was applied during each cycle to make the total interpulse delay approximately 800–1,000 msec. At each point in the cardiac cycle, 480–600 consecutive gated scans were averaged to achieve an acceptable signal-to-noise ratio. All chemical shifts were referenced to the 6F-Trp signal, assigned to 0 parts per million (ppm). [Ca\textsuperscript{2+}], is calculated according to the equation [Ca\textsuperscript{2+}]=K\textsubscript{d}⋅[B]/[F], where K\textsubscript{d} is the dissociation constant of Ca-5F-BAPTA. [B] and [F] represent the concentrations of the indicator bound to

\begin{figure}[h]
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\caption{Time course of functional depression during hypoxia in a heart not loaded with the Ca\textsuperscript{2+} indicator 5F-BAPTA. Heart was perfused with Tyrode's solution (2 mM CaCl\textsubscript{2}) bubbled with 100% O\textsubscript{2} at 30°C. Subsequently, perfusate was switched to hypoxic Tyrode's solution equilibrated with 10% O\textsubscript{2}-90% N\textsubscript{2} at time 0. LVP, left ventricular pressure.}
\end{figure}
calcium and free in the cytoplasm, respectively, and are proportional to the areas under characteristic peaks in the $^{19}$F spectra. We have used the $K_d$ of 285 nM previously measured at 30°C in EGTA-buffered solutions to calibrate our signals.26

**Phosphorus NMR Measurements**

After the control measurements of calcium transients, phosphorus-31 NMR spectra were obtained in the same hearts with methods similar to those described previously.24 The amounts of Pi, phosphocreatine (PCr), and ATP in the myocardium were determined by planimetry of the areas under individual peaks using a digitizer. The tissue contents of Pi, PCr, and ATP were expressed as percent of the area under the ATP peak during the control period in each individual experiment.2 $pH_i$ was estimated from the chemical shift of Pi measured relative to the resonance of PCr.

**Statistical Analysis**

Pooled data are given as mean±SEM. Statistical analysis was performed by paired t test, with $p$ of less than 0.05 considered significant. Linear regression analysis, including multiple methods, was performed with least-squares optimization.29

**Results**

**Steady-State Contractile Depression During Hypoxia**

The fact that NMR does not yield real-time measurements prompted us to investigate steady-state hypoxic conditions. The continuous record of isovolumic left ventricular pressure in Figure 1 illustrates our experimental strategy. Low-grade hypoxia was produced by switching to perfusate equilibrated with 10\% O$_2$ at time 0; this led to a stable depression of contractile pressure. Steady state was attained after approximately 15 minutes, with developed pressure reaching 34\% of control levels in this heart.

The time course of developed pressure during hypoxia is similar to that from each of the seven hearts that had been loaded with 5F-BAPTA and studied in 8 mM [Ca]$_{ox}$, although the extent of the decrease in developed pressure was somewhat less in these experiments (45–67\%; mean, 60±4\% of control developed pressure).
Metabolic Changes During Hypoxia

Figure 2 shows typical $^{31}$P NMR spectra in control and during hypoxia. At baseline, PCR and ATP are present in abundance, whereas the Pi peak is quite small. This spectrum is in quite typical of normal, well-oxygenated myocardium. During hypoxia, the PCr peak decreases moderately, with a concomitant increase in the Pi resonance. The Pi shift position also reports a mild intracellular acidosis (pH$_i$ 7.08 versus 7.23 in control). [ATP] remains stable at this low level of hypoxia.

Pooled data (Figure 3) reveal that the changes evident in the single experiment in Figure 2 are representative of all the hearts—[Pi] increases (p<0.001), [PCR] decreases (p<0.01), and mild acidosis develops (p<0.02), but [ATP] does not change significantly during hypoxia. These observations, particularly the increase in [Pi] and the decrease in pH$_i$, are certainly consistent with the notion that the accumulation of metabolites is primary, but the possibility of a coexistent decrease in Ca$^{2+}$ transients is not excluded. This possibility is explored in the next section.

Calcium Transients During Hypoxia

Figure 4 shows typical gated $^{19}$F NMR spectra taken at end diastole and peak systole during control and hypoxia. In the control diastolic spectrum, the area under the free peak (~2 ppm) is visibly smaller than that under the bound peak (~8 ppm), signifying that diastolic [Ca$^{2+}$]$_i$ is low. During systole, the bound peak increases substantially while the free peak decreases, reflecting a sizable rise in [Ca$^{2+}$]$_i$. Gated spectra from the same heart during hypoxia again show a striking difference between the two extremes of the cardiac cycle, with the ratio of [B] to [F] much greater during systole than at end diastole. If a side-to-side comparison of the spectra is made, little difference in diastole occurs; in systole, the predominance of the bound peak is, if anything, accentuated during hypoxia. Thus, inspection of the raw signals gives no reason to expect that the availability of activator Ca$^{2+}$ is decreased during hypoxia.

The relative changes can be assessed more readily by comparing the values for [Ca$^{2+}$]$_i$ derived from these and other gated spectra before and during hypoxia. Such transients are plotted as a function of the time from the pacing stimulus in Figure 4C. During control, [Ca$^{2+}$]$_i$ increased from 0.16 μM before the stimulus to a peak of 0.70 μM in early systole. The plot of the Ca$^{2+}$ transient during hypoxia confirms our suspicion that [Ca$^{2+}$]$_i$ has increased, particularly during systole, when [Ca$^{2+}$]$_i$ reaches values as high as 0.82 μM.

The paradoxical increases in systolic [Ca$^{2+}$]$_i$, and, as a corollary, in the amplitude of the Ca$^{2+}$ transient were observed quite consistently. Figure 4 summarizes the data: The diastolic [Ca$^{2+}$]$_i$ levels are equivalent (0.18±0.03 μM in control versus 0.17±0.03 μM in hypoxic hearts), whereas the [Ca$^{2+}$]$_i$ at peak systole did increase (0.78±0.07 μM in control versus 1.11±0.18 μM in hypoxic hearts, p<0.01). Concomitantly, the amplitude of Ca$^{2+}$ transients in hypoxic hearts (0.94±0.16 μM) was significantly greater than in control hearts (0.59±0.06 μM, p<0.01).

Relative Roles of Pi, pH, and [Ca$^{2+}$]$_i$

Taken together, our observations seem most consistent with the idea that changes in Pi and pH$_i$ play critical roles in the functional deterioration during hypoxia. To test this idea directly, we plotted the developed pressure during hypoxia, normalized relative to control levels, as a function of Pi or pH$_i$ in each heart. Figure 6A shows the correlation between [Pi] and percent developed pressure, whereas Figure 6B shows the simultaneous correspondence with pH$_i$. As [Pi] increases or pH$_i$ decreases, developed pressure falls. Both metabolic factors are correlated with the decline of developed pressure at a high level of significance ([Pi]: r=-0.906, p<0.02; pH$_i$: r=0.884, p<0.02). If we check the relative weight of these factors by multiple regression analysis, [Pi] contributes more than 80% to the ratio, whereas pH$_i$ does not achieve a significant level. These data strongly suggest that the functional decline during hypoxia is derived from these metabolic changes, particularly the accumulation of Pi.

In contrast to the excellent correlation between the metabolic changes and percent developed pressure, Figure 6C shows the scattergram produced by plotting the changes in Ca$^{2+}$ transient amplitude com-
FIGURE 4. Changes in $^{19}$F nuclear magnetic resonance (NMR) spectra during the cardiac cycle observed before and after hypoxia. Panel A: $^{19}$F NMR spectra at end diastole in control and during hypoxia. Peaks for bound (8 PPM [parts per million]) and free (2 PPM) 5F-BAPTA show no significant changes between the two conditions. Panel B: $^{19}$F NMR spectra at peak systole in control and during hypoxia. Panel C: Calcium transients in control and during hypoxia, calculated from families of gated spectra including those shown in panels A and B. $[Ca^{2+}]_i$, intracellular $[Ca^{2+}]$.

Discussion

Technical Limitations of 5F-BAPTA–NMR Method

In this study, we took full advantage of the unique ability of NMR spectroscopy to quantify not only $[Ca^{2+}]_i$, but also phosphorus-containing metabolites and pH in the same hearts. The major disadvantages of the technique arise from calcium buffering and the need for signal averaging. The fact that 5F-BAPTA has a $K_d$ for $Ca^{2+}$ within the physiological range of $[Ca^{2+}]_i$ makes it sensitive for quantifying both systolic and diastolic values, but the inherent insensitivity of NMR necessitates loading with the indicator to cytoplasmic concentrations of 0.1–0.2 mM.26 Such loading adds substantially to the already high cytoplasmic concentration of high-affinity $Ca^{2+}$ buffers. Accordingly, force development often remains depressed despite the elevation of perfusate [Ca]. It is thus particularly important that each heart served as its own control in this study; the functional consequences of hypoxia could be expressed relative to the prehypoxic values, with equal 5F-BAPTA loading.
Metabolic Inhibition was significantly increased in each heart at end diastole and peak systole before (left, CONTROL) and during hypoxia (right). Data from each individual heart in the two phases of each experiment are connected; mean ± SEM values are also shown. End-diastolic \([Ca^{2+}]_i\) did not change; in contrast, \([Ca^{2+}]_i\) at peak systole was significantly increased during hypoxia (p<0.01).

The need for signal averaging limits the quantification of \(Ca^{2+}\) transients to steady-state conditions. Thus, we have restricted our investigation to relatively mild hypoxia, in which contractile function reaches a depressed but steady level. The relative roles of \(Ca^{2+}\)-dependent and -independent mechanisms may differ in severe hypoxia or complete anoxia, when metabolic deterioration is rapid and progressive.2-6,7

Possible Mechanisms of Increase in \([Ca^{2+}]_i\) During Hypoxia

The observed fall in pH must be considered as one possible mechanism causing an increase in \(Ca^{2+}\) transient amplitude during hypoxia. Acidosis itself leads to an increase in the amplitude of \(Ca^{2+}\) transients.30 Although the direct effect of acidosis on the sarcoplasmic reticulum (SR) is inhibitory in heart muscle,31,32 the increase in \([Ca^{2+}]_i\) attributable to the operation of Na\(^+\)-H\(^+\) exchange33,34 in parallel with Na\(^+\)-Ca\(^{2+}\) exchange35-37 and the displacement of \(Ca^{2+}\) from binding sites by H\(^+\)38,39 promote \(Ca^{2+}\) loading that might overcome the direct inhibitory effect of acidosis on the SR. The fact that there is no change in diastolic \([Ca^{2+}]_i\), but there is an increase in systolic \([Ca^{2+}]_i\), may reflect enhanced \(Ca^{2+}\) loading of the SR, so that more \(Ca^{2+}\) is available to be released during each beat (but not so much that the capacity for diastolic \(Ca^{2+}\) reuptake by the SR is exceeded). The increase in \(Ca^{2+}\) transient amplitude during hypoxia would be expected to offset, at least partially, the depressant effect of Pi and H\(^+\) on the myofilaments (compare Lee and Allen,40 Figure 1).

Comparison With Previous Work on Hypoxia or Metabolic Inhibition

Our findings indicate that mild hypoxic contractile dysfunction is mediated by the accumulation of metabolites (mainly, Pi and, to a lesser degree, H\(^+\)). These results are consistent with our previous work7-19 and that of others.2-20 Kusuoka et al7 investigated the relative roles of Pi and H\(^+\) in early contractile failure during hypoxia; an inverse correlation was observed between [Pi] and maximal \(Ca^{2+}\)-activated pressure (MCAP) during tetani, but pH\(_i\) showed no significant correlation with MCAP. Mild acidosis (pH\(_i\)>6.8) was associated with a less than 20% depression of MCAP, but much more depression was observed at lower pH\(_i\) (see also Marban and Kusuoka19). The unique advantage of MCAP as an index of contractility is its independence from intracellular \(Ca^{2+}\), but studies measuring only twitch contractions during relatively mild hypoxia have reached similar conclusions.2-20

Figure 5. Paired data showing intracellular \(Ca^{2+}\) ([\(Ca^{2+}\)]\(_i\)) in each heart at end diastole and peak systole before (left, CONTROL) and during hypoxia (right). Data from each individual heart in the two phases of each experiment are connected; mean ± SEM values are also shown. End-diastolic \([Ca^{2+}]_i\) did not change; in contrast, \([Ca^{2+}]_i\) at peak systole was significantly increased during hypoxia (p<0.01).

Figure 6. Relations between developed pressure (DP) and inorganic phosphorus ([Pi], A), intracellular pH (pH\(_i\), B), or ratio of \(Ca^{2+}\) transient amplitude (hypoxia-to-control ratio, C). The DPs were normalized by those in control period (mean, 23±3 mm Hg). One heart was excluded from A and B (but not C) because of spontaneous ventricular fibrillation that began just before the hypoxic \(^{31}P\) nuclear magnetic resonance spectrum could be acquired.
The mechanism of functional depression by Pi appears to involve a direct inhibition of crossbridge cycling. From the perspective of the 

[Ca2+]r-tension relation, contractile dysfunction due to Pi accumulation is mediated primarily by a decrease of MCAP, not by a change in myofilament sensitivity (at least in intact hearts19). Depression of MCAP points to a decrease in the maximal number of force-generating crossbridges. In some direct studies of the contractile proteins in skinned preparations, additional effects of Pi on the [Ca2+]r-tension relation have been found. Kentish9 and Godt and Nosek41 reported that Pi not only depresses maximal force but also induces a shift in myofilament Ca2+ sensitivity, suggesting a decrease in the Ca2+-binding affinity of the myofibrils. On the other hand, Solaro et al32 reported no shift in sensitivity. Nevertheless, there is general agreement that Pi exerts a potent depressant effect on contraction at the level of the myofilaments.

Measurements of [Ca2+]r have produced less of a consensus. Allen and Orchard13 found that when glycolysis was able to continue, anoxia was not associated with any systematic changes in the amplitude of Ca2+ transients in ferret papillary muscles micro-injected with aequorin. Our results are consistent with these observations despite the very different technical limitations of aequorin and 5F-BAPTA as Ca2+ indicators.26 During anoxia in glucose-free medium, Allshire et al43 also found no significant changes in [Ca2+]r in rat myocytes injected with aequorin, although these cells were not stimulated to contract. More recently, Lee and Allen40 noted that the behavior of [Ca2+]r in hypoxia could be quite variable: Ca2+ transients during respiratory inhibition reached 163% to 55% of control values, although the mean amplitude remained unchanged. As a possible source of the variability, these investigators recognized that repeated exposures to anoxia reduce glycogen concentration in the muscle and thus inhibit lactate production; the depletion of glycogen would tend to attenuate acidosis, thereby blunting the rise in Ca2+ transients that occurs when glycogen is plentiful. This study concluded that the response of the Ca2+ transients to anoxia is dependent on the metabolic integrity of the muscle.

In contrast to the findings of Allen and co-workers,13,40 MacKinnon et al14 reported that anoxia in the presence of glucose always caused a gradual decline in the amplitude of the Ca2+ transients in ferret papillary muscles chemically loaded with aequorin. Recently, Kihara and同事15 made similar observations using a modification of same procedure to load aequorin into perfused hearts. We cannot determine the exact origin of the discrepancy, but Lee and Allen40 have argued that the rate of glycolysis may have been changed or that the glycogen reserves may have been depleted by the chemical loading procedure. This method involves exposing the muscle to very low Ca2+ concentrations for periods long enough to allow large molecules of photoprotein to permeate cell membranes. Although the contractile performance of such chemically loaded preparations is reassuringly robust,14,15 changes in the metabolic condition of the muscle have not been conclusively excluded.

The use of a simple analogue of hypoxia (cyanide) to inhibit oxidative phosphorylation in isolated ventricular cells has also produced variable results. In the work of Eisner et al,18 such metabolic inhibition did not consistently decrease the force of contraction (in eight of 12 cases, contraction was actually increased). The observed changes of contractile amplitude were paralleled by changes in Ca2+ transients. When deoxyglucose is added to CN−, there is general agreement that contractile failure occurs rapidly.16-18 Unfortunately, the contribution of Ca2+ transients to this failure varies among myocardial cell types and species. In adult rat ventricular cells,18 a decline of Ca2+ transients, presumably secondary to action potential failure,4,5 has been found, whereas Ca2+ transients continue unimpaired during the onset of contractile dysfunction in chick ventricular cell monolayers.16,17 Rat myocytes might be expected to be unusually susceptible to failure of excitation during hypoxia. They are known to possess a rich complement of ATP-sensitive potassium channels4; because the action potential is much more brief to begin with in rat than in other species,44 any given tip of the balance in favor of repolarization would be expected to suppress excitability disproportionately in the rat. This peculiar property should prompt caution in extrapolating results from research in rat cells directly to other species. Nevertheless, the discrepancy with our results may simply reflect a difference in the severity of hypoxia; the contractile depression we observe is mild, with function remaining stable for at least 60 minutes. In more severe respiratory blockade, action potentials are depressed within 10 minutes,45 even in species other than rat. A total loss of excitability, as would be expected to occur during severe hypoxia, would obviously lead to a fall in the cytosolic Ca2+ concentration regardless of the species.

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