Ca\textsuperscript{2+} Transients in Perfused Hearts Revealed by Gated \textsuperscript{19}F NMR Spectroscopy

E. Marban, M. Kitakaze, V.P. Chacko, and M.M. Pike

Gated acquisition of \textsuperscript{19}F nuclear magnetic resonance spectra from perfused ferret hearts loaded with the fluorinated Ca\textsuperscript{2+} indicator \textsuperscript{5,5'}-\textsuperscript{F}-BAPTA allows direct quantitation of the cyclical changes in the intracellular free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) that underlie contraction in intact hearts. [Ca\textsuperscript{2+}], increased from approximately 200 nM in diastole to ~1 \mu M or higher in early systole. Although the \textsuperscript{19}F spectra that report [Ca\textsuperscript{2+}] changed dramatically and reproducibly during the cardiac cycle, no changes were detectable in gated phosphorus spectra. We exploited the ability to control the coronary arterial flow of our hearts to investigate the mechanism of the fall in contractility that results from a decrease in perfusion even when the flow suffices to sustain normal high energy phosphate concentrations. Under these conditions, the amplitude of Ca\textsuperscript{2+} transients falls markedly along with the decline in pressure. This down-regulation of Ca\textsuperscript{2+} transients constitutes a novel protective mechanism that minimizes energy demand during low-flow ischemia. (Circulation Research 1988;63:673-678)

The application of various techniques to measure intracellular free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) in isolated heart muscle and in single cardiac cells has led to considerable conceptual advances regarding excitation-contraction coupling and other Ca\textsuperscript{2+}-mediated processes (see Blinks et al\textsuperscript{1} for review). Nevertheless, major gaps remain in our understanding of the regulation of [Ca\textsuperscript{2+}] in hearts perfused via the normal arterial circulation. Almost all measurements of [Ca\textsuperscript{2+}] are restricted to superfused muscle or enzymatically dissociated cells whose responses cannot be assumed to resemble those of the native tissue. In particular, the effects of alterations in coronary flow can only be crudely mimicked in isolated muscle or cells. Nuclear magnetic resonance (NMR) spectroscopy can detect signals from fluorinated Ca\textsuperscript{2+} indicators\textsuperscript{2,3} and has already yielded useful measurements of time-averaged [Ca\textsuperscript{2+}], in perfused hearts,\textsuperscript{4,5} but the lack of time resolution has restricted further progress.

We report here the use of gated \textsuperscript{19}F NMR to obtain time-resolved measurements of [Ca\textsuperscript{2+}] during the cardiac cycle in perfused ferret hearts. This technique is exploited to investigate the interrelation of Ca\textsuperscript{2+} regulation and energy metabolism during normal and decreased coronary flow. Two preliminary reports have appeared.\textsuperscript{6,7}

Materials and Methods

Perfused ferret hearts were placed in a Bruker AM-360 NMR spectrometer (8.46 Tesla; Billerica, Massachusetts) for the simultaneous measurement of NMR spectra and isovolumic left ventricular pressure.\textsuperscript{4} The perfusate, equilibrated with 100% O\textsubscript{2}, contained (mM) NaCl 108, KCl 5, MgCl\textsubscript{2} 1, HEPES 5 (pH adjusted to 7.4 with NaOH), Na acetate 20, and glucose 10. Hearts were paced at 0.9-1.6 Hz and maintained at a temperature of 30° C. Loading with the Ca\textsuperscript{2+} indicator \textsuperscript{5,5'}-difluoro derivative of 1,2-bis(o-aminophenoxy)ethane-N,N',N'',N'''-tetraacetic acid (BAPTA), was achieved by addition of the cell-permeant acetoxymethyl ester form (15 \mu M; lot 7B, Molecular Probes, Eugene, Oregon) to the perfusate for 30-60 minutes. The anion transport inhibitor probenecid (1 mM; Sigma, St. Louis, Missouri) was added to the perfusate after loading with \textsuperscript{5F}-BAPTA to minimize extrusion of the indicator from cells;\textsuperscript{8} this resulted in stable intracellular [\textsuperscript{5F}-BAPTA] (estimated as in Marban et al\textsuperscript{4}) for two or more hours under our experimental conditions.

The NMR methods differed from our previous descriptions\textsuperscript{1} in that the excitatory radio-frequency pulses and the subsequent NMR data acquisition were gated according to a programmable delay from the time of the pacing stimulus. Two pacemakers (S Series, Grass Instruments, Quincy, Massachusetts)
were used for gating. The first, which set the overall cycle length, supplied a synchronization signal to the pulse programmer controlling the spectrometer and to a second pacemaker that stimulated the heart with no delay. For gated NMR data acquisition, a delay relative to the pacemaker signal was included in the pulse sequence so that the radio-frequency pulse could be imposed at any time during the cardiac cycle. One pulse was applied during each cycle; hence, the total interpulse delay approximated 1 second (the exact value depending on the pacing rate). At each point in the cardiac cycle, 100–1,200 consecutive gated scans were acquired to achieve an acceptable signal-to-noise ratio. A family of $^1$H (or $^3$P) spectra was acquired throughout the cardiac cycle by sampling at various delay settings. For fluorine, the $^1$H decoupler coil of the 25 mm broad-band probe was tuned to the $^1$H resonance frequency (338.8 MHz). After appropriate phasing of the spectra, a Bruker polynomial baseline subtraction routine (fitted by eye) was used to remove a broad baseline roll derived from $^1$H in the probe construction materials. Signal-to-noise ratio was improved by exponential multiplication of the frequency induction delay (120–200 Hz). This introduced no noticeable distortion of line shape. Phosphorus spectra were acquired with the broad-band Helmholtz coil tuned to the $^3$P resonance frequency (145.8 MHz) and application of 22 $\mu$s excitatory radio-frequency pulses (34°). The frequency induction delays were accumulated during a 0.34-second acquisition period with 4K data points at a spectral width of ~6 kHz. At the repetition intervals determined by the pacing rates used in this study, the $^3$P spectra were partially saturated; the appropriate correction factors, calculated from Equation 17 in Becker et al assuming the spin lattice relaxation time ($T_1$) values in Kusukoku et al, are given in the figure legends. A family of gated $^1$H spectra was generally obtained first, after which a family of gated phosphorus spectra was acquired in the same heart.

$$[\text{Ca}^{2+}] = K_d \cdot \frac{[B]}{[F]}$$

is calculated according to the equation, where $[B]$ and $[F]$ represent the concentrations of the indicator bound to calcium and free in the cytoplasm, respectively; these in turn are proportional to the areas under characteristic peaks in the $^1$H spectra (see below and Smith et al). We have used the $K_d$ of 285 nM previously measured at 30°C in EGTA-buffered solutions to calibrate our signals.

**Results**

Figure 1 shows $^1$H (middle row) and $^3$P (bottom) NMR spectra obtained at the two times during the cardiac cycle indicated on the pressure record (top): a, 10 msec before the stimulus, and b, 75 msec after the stimulus. The $^1$H spectra show two peaks, one at ~2 ppm corresponding to free 5F-BAPTA (F) and the other at ~8 ppm corresponding to 5F-BAPTA bound to calcium (B). [Ca$^{2+}$], is proportional to the ratio of the areas under the two peaks ($[B]/[F]$). The peak at 0 ppm arises from an aqueous solution of 6-F-tryptophan (1 mM), which filled the intraventricular balloon and served as a standard. At time a, just before the pacing stimulus, the bound peak is smaller than the free peak ([B]/[F] = 0.62), corresponding to a diastolic [Ca$^{2+}$] of 177 nM. Just 75 msec after the stimulus (b), however, the spectrum changes dramatically: the bound peak now exceeds the free ([B]/[F] = 2.9), yielding a [Ca$^{2+}$] of nearly 1 μM. Another diastolic spectrum obtained afterwards (not shown) was superimposable upon that in a.

As a check against possible artifacts, we kept the gating parameters unchanged and acquired the $^3$P spectra shown in the bottom row of Figure 1. There is no clear difference between a and b; both show the normal pattern of phosphorus NMR spectra in well-oxygenated hearts, with prominent phospho-
FIGURE 2. Stack plot of $^1$H NMR spectra in one heart at different times during the cardiac cycle, with the spectra staggered proportional to the time at which they were acquired with respect to the pacing stimulus. For each spectrum, 600–752 scans were accumulated. Preparation MF76; pacing rate 1.00 Hz; 8 mM [Ca$^{2+}$].

creatinine and ATP peaks. The finding of cyclical changes in one set of nuclei but not another in the same heart indicates that the changes in the $^1$H spectra do not represent artifacts related to contraction. Indeed, we expect [Ca$^{2+}$] to vary by as much as an order of magnitude during the cardiac cycle; on the other hand, cyclical changes in phosphorus metabolites have been sought but not found in ferret hearts, and even in rat hearts at high work loads amount at most to changes of $\pm 20\%$. By obtaining $^1$H spectra at several times during the cardiac cycle, we can map out the change in [Ca$^{2+}$], during each contraction (the Ca$^{2+}$ transient). Figure 2 shows a family of $^1$H NMR spectra stacked according to the time delay from the pacing stimulus. In the first spectrum, acquired just before stimulation, the free peak at 2 ppm slightly surpasses the bound peak (8 ppm). After the stimulus at time 0, the bound peak increases rapidly and reaches a maximum within 100 msec, while the free peak reaches its nadir. Thereafter, the signals return toward baseline. Such data form the basis for the Ca$^{2+}$ transients shown in Figures 3 and 4. Figure 3A shows the Ca$^{2+}$ transient from a heart perfused with 2 mM [Ca$^{2+}$], along with the corresponding record of ventricular pressure. [Ca$^{2+}$] rises sharply from just under 200 nM in diastole to a maximum of $\sim 1 \mu$M. The Ca$^{2+}$ transient peaks at 75 msec, well before the corresponding peak in contractile pressure but close to the time at which the rate of pressure development (dP/dt; not shown) is greatest. A similar concordance between peak [Ca$^{2+}$] and the maximal rate of force generation (dF/dt) was previously found using the Ca$^{2+}$ indicator aequorin in ferret papillary muscles. The absolute values for diastolic and systolic [Ca$^{2+}$] are also quite similar, although the rate of decay of the transients is definitely slower with $5F$-BAPTA (see "Discussion").

Because of calcium buffering by intracellular $5F$-BAPTA, twitch pressure after loading with the indicator is relatively low in 2 mM [Ca$^{2+}$]. To approximate more closely the pressures achieved during physiological excitation-contraction coupling, we routinely raised [Ca$^{2+}$] to 8 mM, which in this example augmented the developed pressure to 68 mm Hg (Figure 3B, top row). In association with the increase in pressure, systolic [Ca$^{2+}$] increased dramatically, peaking at approximately 2,400 nM. Nevertheless, diastolic [Ca$^{2+}$], remained virtually unchanged, and the overall shape of the Ca$^{2+}$ transient was similar at both [Ca$^{2+}$] values. The results shown in Figure 3 point out the ambiguities that can be introduced by studying only time-averaged [Ca$^{2+}$]. Although we had previously found an increase in time-averaged [Ca$^{2+}$], after raising [Ca$^{2+}$] from 2 to 8 mM, we could not be certain until now that the increase in [Ca$^{2+}$] occurred selectively during systole. For all 16 hearts studied in 8 mM [Ca$^{2+}$], the Ca$^{2+}$ transient varied from a low of $202 \pm 22$ nM (mean $\pm SEM$) in diastole to a peak of $1,660 \pm 260$ nM in systole. The values for systole, being further from the $K_d$ than those for diastole, are more susceptible to misestimation due to noise. Despite this fact, most of the variability was among

FIGURE 3. Effects of increasing extracellular calcium concentration on developed pressure (upper row) and Ca$^{2+}$ transients (lower row). Panel A: 2 mM [Ca$^{2+}$]; each point was calculated from a gated spectrum obtained over 183–1,061 scans. Ca$^{2+}$ transients in this and subsequent figures were fit (solid line) by eye to the sum of cosine and rising exponential functions, which predominate during the rising phase, and a declining single exponential which prevails after the peak of the transient. Panel B: 8 mM [Ca$^{2+}$], with each point based on a spectrum acquired over 600–1,059 scans. Preparation MF62 paced at 1.18 Hz.
The factors: phosphocreatine, 0.69; ATP, 0.90; inorganic phosphate, 0.73. Preparation MF64 paced at 1.35 Hz in 8 peaks are attenuated by saturation by the following.

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failure reported in some patients with coronary artery disease ("hibernating myocardium"

lar pressure (top row) and the Ca2+ transient (middle row) during normal and decreased coronary perfusion. Panel A: Records obtained with coronary flow rate set at 18 ml/min. Panel B: Data during the steady-state effect of decreasing the flow rate to 10 ml/min. The spectra used to calculate the Ca2+ transients in this experiment each consisted of 263-594 scans. For the 31P spectra, the peaks are attenuated by saturation by the following factors: phosphocreatine, 0.69; ATP, 0.90; inorganic phosphate, 0.73. Preparation MF64 paced at 1.35 Hz in 8 mM [Ca2+].

hearts, the amplitude of the Ca2+ transient being quite reproducible in any given heart when [Ca2+]o, pacing rate, and flow were kept unchanged.

Our experimental preparation is uniquely well-suited to address questions related to coronary flow and to energy metabolism, since we can manipulate the flow rate at will while measuring [Ca2+]i, high energy phosphate concentrations, and intracellular pH (from 31P NMR spectra). We have exploited these capabilities of our system to investigate the mechanism of the fall in myocardial force generation during reduced coronary perfusion. As coronary flow rate is decreased, contractile pressure can fall by 50% or more with only minimal changes in high-energy phosphate concentrations. Unlike the contractile failure of hypoxia or severe ischemia that accompanies the intracellular accumulation of inorganic phosphate, the decrease in contractile force during a moderate reduction of arterial flow occurs without appreciable inorganic phosphate buildup. Contractile dysfunction during low-flow ischemia presumably underlies the reversible pump failure reported in some patients with coronary artery disease ("hibernating myocardium"

We investigated this problem in the experiment illustrated in Figure 4. Panel A shows left ventricular pressure (top row) and the Ca2+ transient (middle row) during perfusion at a coronary flow rate of 18 ml/min (at which level coronary perfusion pressure was 80 mm Hg). The corresponding 31P NMR spectrum (A, bottom row) has a normal appearance, with an intracellular pH of 7.07. Flow was then decreased until developed pressure reached a new steady state at 40% of control (B, top row; new flow rate = 10 ml/min). Under these conditions, the Ca2+ transient (B, middle row) was markedly attenuated, but the 31P NMR spectrum (bottom row) showed only minor changes. Intracellular pH did fall to 6.97 in B, but this mild degree of acidosis can itself account for less than half of the observed decrease in pressure. By comparison to experiments such as that in Figure 3 in which pressure was altered by changing [Ca2+]o, the decline in the amplitude of the Ca2+ transient during low flow appears quite sufficient to explain the decrease in force generation. The finding of a decrease in systolic [Ca2+]i, during low-flow ischemia with little observable alteration of energy metabolism was confirmed in two other experiments.

Discussion
The use of gated acquisition techniques has allowed us to overcome the lack of time resolution that has restricted previous estimates of [Ca2+]i in perfused hearts using NMR. This conclusion is based on consideration of the various factors which determine the time resolution: the response time of the indicator (i.e., its association and dissociation rate constants for calcium binding); the duration of the radio-frequency pulse; the time constant (T2*) of the frequency induction delays; and the time required for electrical activation of the heart. Smith et al.

have estimated that 5F-BAPTA will respond within 10 msec to changes in [Ca2+]i, of the magnitude we observe. Given their published dissociation rate constant for Ca-5F-BAPTA and our value of 285 nM for the Kd at 30°C, we estimate that the response to a step change in [Ca2+]i from 10^-7M to 10^-5M (or vice versa) will be >90% complete in ~5 msec. The radio-frequency pulse itself is negligibly short (70-90 μsec), but the frequency induction delays which report the chemical environment of the 19F nuclei decays with a T2* of approximately 10 msec and consequently very steep changes in [Ca2+]i would be averaged out over this time interval. Finally, the activation of the ventricles occurs over less than 10 msec from separate measurements of the extracellular electrogram in paced ferret hearts. All these potential sources of error are on the order of 10 msec or less, and only the pulse duration and the T1* are clearly additive. Conservatively, we estimate the time resolution of our measurements to be 10-20 msec, which is ≈2% of our usual cycle length. Because of the relatively low concentration of 19F in the heart, several minutes of signal averaging (≈100 pulses) are still required for each gated spectrum to exhibit a satisfactory signal-to-noise ratio.
ratio, but this only restricts the measurements of \( \text{Ca}^{2+} \) transients to steady-state experiments.

Although aequorin and 5F-BAPTA are both nonlinear \( \text{Ca}^{2+} \) indicators, the likely errors each would introduce in estimating [\( \text{Ca}^{2+} \)], are complementary, since their [\( \text{Ca}^{2+} \)]-response functions have opposite convexities.\(^4\) The general agreement between our absolute values for diastolic and systolic [\( \text{Ca}^{2+} \)], and those deduced from aequorin luminescence is therefore reassuring for both methods. If anything, the values for [\( \text{Ca}^{2+} \)], from 5F-BAPTA tend to be higher than might have been expected given the degree of calcium buffering introduced by the indicator.\(^4\) Even though the effect of buffering on the amplitude of the Ca\(^{2+}\) transient can be antagonized by increasing [\( \text{Ca} \)], (Figure 3), the rate with which [\( \text{Ca}^{2+} \)], returns to baseline may well remain delayed as a consequence of calcium buffering. Such an effect could explain the apparent slowing in the decay of the 5F-BAPTA \( \text{Ca}^{2+} \) transients relative to those deduced from aequorin\(^5,16\); alternatively, one indicator or the other may misrepresent the time course of changing [\( \text{Ca}^{2+} \)]. Simultaneous measurements with both types of indicators (calcium buffers and Ca\(^{2+}\)-activated photoproteins) will be required to distinguish between these possibilities.

Signals related to [\( \text{Ca}^{2+} \)], in perfused hearts can also be obtained from the fluorescence produced by Ca\(^{2+}\) indicators chemically similar to 5F-BAPTA.\(^1,2\) The strengths and limitations of this approach are, in many respects, complementary to those of 5F-BAPTA and NMR spectroscopy.\(^4\) The use of indo 1 in particular offers good time resolution even without signal averaging, at intracellular concentrations that produce little calcium buffering.\(^22\) These favorable properties allow indo 1 to track moment-to-moment changes in Ca\(^{2+}\) transients. Nevertheless, the technique is subject to several limitations that the investigators are careful to acknowledge. Fluorescence reports not only the reaction of the indicator with Ca\(^{2+}\), but also binding to heavy metals and fluorescence arising from endogenous compounds and Ca\(^{2+}\)-insensitive forms. Movement artifacts can be minimized but not totally eliminated. Finally, only localized epicardial regions of the heart are readily accessible. The NMR approach, while requiring signal averaging and careful compensation for calcium buffering, offers the unique opportunity to sample a number of nuclei in rapid alternation. This has enabled us not only to measure the fluorinated Ca\(^{2+}\) indicator but also endogenous high-energy phosphates in the same heart. No other approach, even in isolated muscle or single cells, offers the exceptional opportunity to study [\( \text{Ca}^{2+} \)], and energy metabolism simultaneously.

The observation that Ca\(^{2+}\) transients are decreased during low-flow ischemia suggests a novel negative feedback process whereby energy demand can be reduced in ischemia, at the expense of a substantial decrease in myocardial force generation. Since [\( \text{Ca}^{2+} \)], rises much less during each beat, the energy required for the cycling of intracellular Ca\(^{2+}\) and the activation of the contractile proteins (which can account for more than 80% of myocardial oxygen consumption\(^23,24\)) will be substantially diminished.

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