^{19}F Nuclear Magnetic Resonance Studies of Free Calcium in Heart Cells

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ABSTRACT ^{19}F nuclear magnetic resonance is used in conjunction with 5,5′-difluoro-1,2-bis(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (5FBapta), a fluorinated calcium chelator, to report steady-state intracellular free calcium levels ($[Ca^{2+}]_i$) in populations of resting, quiescent, isolated adult heart cells. ^{31}P nuclear magnetic resonance shows that 5FBapta-loaded cells maintain normal intracellular high-energy phosphates, pH, and free Mg^{2+}. The intracellular free calcium concentration of well perfused, isolated heart cells is 61 ± 5 nM, measured with 5FBapta, which has a dissociation constant (K_d) for calcium chelation of 500 nM. A similar value is obtained with Quin-MF, another fluorinated calcium chelator with K_d and maximum calcium sensitivity at 80 nM. We find that the steady-state level of intracellular free calcium is increased by decreased extracellular sodium concentration, omission of extracellular magnesium, decreased extracellular pH, hyperglycemia, and upon treatment with lead acetate. Further, extracellular ATP caused a large transient increase in $[Ca^{2+}]_i$. Thus, while heart cells maintain a very low level of intracellular free Ca^{2+}, acute alterations in extracellular environment can cause derangement of calcium homeostasis, resulting in measurable increases in $[Ca^{2+}]_i$.

INTRODUCTION

^{31}P nuclear magnetic resonance (NMR) of perfused hearts has been used in various laboratories to study cardiac bioenergetics for many years (Katz et al., 1989; Kingsley-Hickman et al., 1987). We recently reported a ^{31}P NMR investigation of isolated heart cells, which provide a good system for studying the cellular basis of cardiac function (Gupta and Wittenberg, 1991). We were able to maintain heart cells well oxygenated, at constant pH, and supplied with essential nutrients in the NMR tube during the long periods of time necessary for the performance of NMR experiments. The cells were obtained in high yields by dissociation of adult rat heart into functionally intact, calcium-tolerant myocytes, which were free from extracellular connective tissue, blood capillaries, and junctional and non-junctional connections. In view of the importance of intracellular free calcium ions ($[Ca^{2+}]_i$) in the maintenance of ion gradients, in the generation of cardiac action potentials, and in excitation-contraction coupling, we have now utilized the ^{19}F NMR technique in combination with the membrane impermeant fluorinated intracellular calcium chelator 5,5′-difluoro-1,2-bis(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (5FBapta) to measure intracellular free Ca^{2+} of heart cells (Smith et al., 1983). While fluorescent indicators such as Fura-2 and Indo-1 offer greater sensitivity, permitting measurements of $[Ca^{2+}]_i$ in single cells, it is often desirable to measure average properties of a population of cells. An advantage of the NMR method is that it offers the possibility of using ^{31}P NMR to monitor high-energy phosphates, pH_i, and $[Mg^{2+}]_i$ in the same population of cells used for $[Ca^{2+}]_i$ measurements. In addition, NMR allows examination of reversibility of measured effects and a study of longer term effects that require incubation for an hour or more in a well superfused preparation, such as the effects of lead and glucose reported in this paper. Furthermore, the NMR technique is also applicable to perfused intact organs, allowing a comparison of resting heart cells with perfused hearts using the same technique.

In this article, we have established the value of $[Ca^{2+}]_i$ in well perfused resting heart cells. Further, we present a survey of the effects of several interventions on the $[Ca^{2+}]_i$ of heart cells. The effects of extracellular pH, no flow ischemia, and replacement of Na^+ by choline were studied to demonstrate that the response of isolated cells measured by ^{19}F NMR is similar to that detected by other techniques. We report the effects of hyperglycemia and lead acetate on intracellular free calcium in the resting myocytes. Recently the level of extracellular Mg^{2+} has been shown to modulate basal tone and contractile function of smooth muscle cells, presumably by affecting cellular calcium homeostasis (Altura et al., 1992). We have therefore also investigated the effect of extracellular Mg^{2+} on intracellular free Ca^{2+} of isolated heart cells.

MATERIALS AND METHODS

Preparation of isolated heart cells

Heart cells were prepared by the procedure of Gupta and Wittenberg (1991). The three-step procedure involves perfusion with collagenase (Worthington Type II, Worthington Biochemical Corp., Freehold, NJ) in a low calcium medium, mechanical dissociation of cells from the tissue matrix in 1.0 mM calcium chloride-containing medium, followed by separation of intact cells with isosmotic Percoll. Adult male Wistar rats (400–500 g) were heparinized and decapitated in accordance with institutional guidelines. Two hearts were rapidly removed and retrograde aortic perfusion was begun with a modified HEPES-buffered minimal essential medium (MEM) (Eagle-Joklik's modified MEM, H57–435, Hazelton Biologics, Inc., Lenexa, KS) containing: 117 mM NaCl, 5.7 mM KCl, 4.4 mM $NaHCO_3$, 1.5 mM NaH_2PO_4, 1.7 mM

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$MgCl_2$, 21.1 mM Hepes, 11.7 mM glucose, amino acids and vitamins with added 10 mM taurine, 2 mM glutamine, and 0.01 mM $CaCl_2$; pH was adjusted to 7.2 with NaOH. (Taurine ($C_2H_7NO_3S$, Sigma, St. Louis, MO), a normal constituent of heart, is added because we find it preserves cell membrane integrity during the isolation.) This solution was 285 mOsM, and the free calcium activity measured with a Moeller (Zurich, Switzerland) calcium ion selective electrode was low (5 μM). After blood and calcium washout, 30 ml of freshly oxygenated Hepes-MEM containing collagenase was recirculated through the heart for 25 min at 7 ml/min. and 32°C. Final collagenase activity was ~200 units/ml, and trypsin activity was 1–3 units/ml (manufacturer's assay). After collagenase dissociation the hearts were cut into 8–10 pieces and digested at 32°C with shaking in incubation medium (MEM, supplemented with 1.0 mM $CaCl_2$ and 0.5% bovine serum albumin (BSA) fraction V) containing 0.1% collagenase. After being washed, the dissociated cells were purified with Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden). Cells were suspended in isotonic Percoll diluted with Hepes-incubation medium to a final concentration of 0.2–0.4 $\times$ 10^6 cells/ml and 41% Percoll. Ten-milliliter portions of the Percoll cell suspension in 15-ml Corex centrifuge tubes were centrifuged for 5 min at room temperature and 34 *g*. Intact rectangular cells sediment to the bottom of the tube and damaged and rounded cells form a distinct layer at the surface. The intact cell pellet was washed in incubation medium to remove Percoll and dispersed in incubation medium at a concentration of 0.5 $\times$ 10^6 cells/ml. Cells were stored at 30°C in incubation medium.

After Percoll purification, the final yield from each heart was 8–16 $\times$ 10^6 cells, of which 80–90% were rectangular and functionally intact (sarcomere length 2.0 μm; cells contract specifically and synchronously in response to electrical stimulation, ~15 V/mm, 6-ms duration).

Loading of heart cells into fiber bundles for NMR measurements

We previously developed a method for oxygenation of heart cells loaded inside a bundle of hollow fibers by superfusion in a 10-mm outer diameter NMR sample tube in a VXR-500 multinuclear NMR spectrometer (Gupta and Wittenberg, 1991; Wittenberg et al., 1988). A thick slurry of heart cells (~25% cells or ~6 $\times$ 10^6 cells/ml) was drawn up into the fiber bores of a 6-mm diameter bundle of hollow cellulose acetate dialysis fibers (180-μm internal diameter) (Baxter Healthcare Corporation, Deerfield, IL), of 3.5–4.0 cm length, by gentle suction. After the fiber bundle was filled with heart cell slurry, it was sealed at both ends with cyanoacrylate glue (Krazy Glue), rinsed with medium, and inserted into a 10-mm diameter NMR tube, which contained incubation medium without BSA (cell perfusion medium). The fiber bundle diameter was chosen so that it could fit snugly in the vertical position, without restraint, in the NMR tube. The total time needed for loading the fiber bundle with isolated myocytes was ~5 min, and perfusion with well-oxygenated medium at 15 ml/min using an LKB pump was begun immediately after loading. Inlet and outlet tubes from the perfusion pump were securely fastened inside the NMR tube. Medium was recirculated, but the 300-ml reservoir was refurbished with fresh medium every 90–120 min. Although the flow of perfusate around the fibers reduced cell sedimentation, there was some visible settling of cells during the time course of NMR measurements. The sample tube was positioned in the NMR probe such that the cells were contained within the sensitive volume of the radiofrequency coil. A reservoir containing about 300 ml of cell perfusion medium was gassed with humidified 95% O_2, 5% CO_2 mixture. The medium used for perfusion of the dialysis fibers in the NMR tube (cell perfusion medium) was Hepes-MEM supplemented with 3 mM $CaCl_2$ and 2 mM pyruvate; sodium bicarbonate concentration was 9.4 mM, and unless otherwise specified, medium pH (pH_o) was maintained at 7.2. Phosphate was omitted from the medium when ^{31}P NMR experiments were carried out in order to locate intracellular P_i resonance. Hepes was omitted from the medium, and the bicarbonate concentration was changed in experiments designed to study the effects of varying pH. $MgCl_2$ was omitted from the medium when experiments were designed to test the effects of varying extracellular [Mg^{2+}]. We have demonstrated rapid exchange of substrates across the fiber walls by measuring an equilibration time of 5 min for the Na^+ shift reagent dysprosium tripolyphosphate (Gupta and Gupta, 1982). The myocyte ATP level (30 nmol/mg protein) and phosphocreatine (PCr) level (41 nmol/mg protein) were measured on separate samples by high-pressure liquid chromatography. The ^{31}P resonances of high-energy phosphates were unchanged when the heart cells were superfused inside hollow fibers in the NMR spectrometer for up to 6 h (Gupta and Wittenberg, 1991; Wittenberg et al., 1988).

^{31}P NMR measurements

^{31}P NMR spectra were obtained with a Varian VXR500 spectrometer at 202.4 MHz (10-mm outside diameter NMR tube) as previously described (Gupta and Wittenberg, 1991). The experimental parameters were: 28-μs 90° radiofrequency pulses; 1.5-s recycle time; 1000 transients; 30 Hz line broadening, and a spectral width of 10 KHz (data size, 16K). The spectral baseline was flattened by using standard Varian software to achieve multiple first-order curve-fitting of baseline regions containing no resonances.

The chemical shifts of the ^{31}P resonances of intracellular ATP were used to calculate the concentration of intracellular free Mg^{2+}. It has been shown (Gupta et al., 1978; Gupta and Moore, 1980; Gupta and Yushok, 1980) that the chemical shift difference between the α- and β-phosphoryl group resonances of ATP, $\delta_{\alpha\beta}$, along with a knowledge of the apparent K_d of MgATP under intracellular ionic conditions can be used to determine the concentration of free Mg^{2+} ([Mg^{2+}]$_i$) from the following equation:

$$[Mg^{2+}]_i = K_d^{MgATP}(\phi^{-1} - 1)$$

where $\phi = (\delta_{\alpha\beta}^{obs} - \delta_{\alpha\beta}^{MgATP})/(\delta_{\alpha\beta}^{ATP} - \delta_{\alpha\beta}^{MgATP})$. The $\delta_{\alpha\beta}^{obs}$ is the value of $\delta_{\alpha\beta}$ for the myocyte ATP; $\delta_{\alpha\beta}^{ATP}$ and $\delta_{\alpha\beta}^{MgATP}$ are the values of $\delta_{\alpha\beta}$ in noncellular ATP and MgATP solutions under simulated intracellular ionic conditions. At the magnetic field strength of 11.8 Telsa, 30°C, pH 7.2 (202.5 MHz ^{31}P NMR frequency), $\delta_{\alpha\beta}^{ATP}$ = 2190 Hz and $\delta_{\alpha\beta}^{MgATP}$ = 1700 Hz. We used a K_d = 50 μM at 30°C and pH 7.2 for the calculation of intracellular free Mg^{2+}.

^{31}P NMR was also used to measure intracellular pH, pH_i, in heart cells from the pH-dependent chemical shift of the intracellular inorganic phosphate resonance relative to that of the pH-independent (over the physiological pH range) ^{31}P resonance of PCr as previously described (Cingolani et al., 1990; Gupta and Wittenberg, 1991; Wittenberg et al., 1988). In P_i-free medium it was easy to identify intracellular P_i resonance from its chemical shift and pH dependence.

NMR measurements of intracellular free [Ca^{2+}]

Free Ca^{2+} concentrations were measured on a Varian VXR500 spectrometer using ^{19}F NMR at a frequency of 470 MHz and a spectral width of 20,000 Hz; 4000 scans were collected. For acquisition of ^{19}F spectra a recycle time of 0.4 s was found to be adequate since the ratio of the areas of the Ca5FBapta and the 5FBapta resonances was unchanged upon increasing the recycle time to 0.8 or 1.6 s. The intracellular indicator 5FBapta (a calcium-sensitive probe) was loaded into the cells using its membrane-permeant acetoxymethyl ester derivative. The intracellular free Ca^{2+} concentration was determined from the areas under the Ca^{2+}-bound 5FBapta (A_{bound}) and the free 5FBapta (A_{free}) resonances using the following equation:

$$[Ca^{2+}]_i = K_d(A_{bound}/A_{free}).$$

The K_d at 30°C for Ca5FBapta was previously determined in our laboratory to be 500 nM (Schanne et al., 1989a). It was determined under the following conditions: KCl (135 mM), $MgCl_2$ (0.5 mM), $K_4$5FBapta (0.385 mM), 2-(*N*-morpholino)propanesulfonic acid buffer (10 mM), pH 7.1, 30°C and varying amounts of $CaCl_2$. Free Ca^{2+} concentrations were measured by using a Ca^{2+}-selective ion electrode (Orion) that was calibrated with Ca-EDTA standards at 30°C. The intensities of Ca5FBapta and 5FBapta resonances, which are proportional to their concentrations, were measured using the areas under their respective ^{19}F peaks. The electrode-determined free Ca^{2+} concentration was plotted versus the ratio of the bound/free 5FBapta, yielding the K_d as the slope.

To obtain sufficient loading of 5FBapta into the intracellular compartment, heart cells (~20 million) were incubated in 160 ml incubation medium supplemented with 2 mM pyruvate and 10 μM acetoxymethyl ester of

5FBapta (5FBapta-AM; Molecular Probes, Inc, Eugene, OR) at 37°C for 40 to 60 min after Percoll purification. At the end of the incubation, just before NMR measurements, the cells were washed twice to remove any extracellular 5FBapta and resuspended in a volume of fresh incubation medium equal to one-half the volume of the heart cell pellet (total volume of heart cell slurry about 1–1.5 ml). For studies of intracellular free Ca^{2+} during hyperglycemia or during extracellular cation changes, several duplicate spectra were first obtained under control perfusion conditions and then glucose concentration or cations in the perfusion medium were changed and ^{19}F spectra were obtained again until a stable $[Ca^{2+}]_i$ was reached. Each determination is usually the average of four measurements in several million intact heart cells. To examine reversibility of the effects, the perfusion constituents were brought back to normal levels, and spectra were rerun until a stable $[Ca^{2+}]_i$ reading was obtained. Thus, each measurement is preceded and followed by an internal control.

Statistical analysis

Data from multiple measurements are presented as mean ± SE, and the probability p that values of $[Ca^{2+}]_i$ following interventions are significantly different from control is determined with the two-tailed paired t test. Data are considered significantly different when $p \leq 0.05$.

RESULTS

Characterization of 5FBapta-loaded heart cells

About 60–67% of the 5FBapta-loaded cells, initially 67–73% rectangular, maintained rectangular shape measured microscopically in individual fibers at the end of the experiment. The PCr/ATP ratio, pH_i, and $[Mg^{2+}]_i$ have previously been reported in isolated heart cells not loaded with 5FBapta (Gupta and Wittenberg, 1991). Here we show that these parameters were quite comparable in 5FBapta-loaded cells sampled by ^{31}P NMR: $pH_i = 6.97 \pm 0.06$; $[Mg^{2+}]_i = 0.5 \pm 0.1$ mM (mean ± SEM; $n = 4$); and PCr/ATP was essentially unchanged. A typical ^{31}P NMR spectrum with good preservation of phosphocreatine and ATP levels and low inorganic phosphate is shown in Fig. 1. Phosphocreatine and ATP levels were measured in 5FBapta-loaded cells by high-pressure liquid chromatography as previously described (Gupta and Wittenberg, 1991). Heart cells loaded with 0, 5, 10, and 20 μM 5FBapta-AM were 70, 73, 71, and 67% rectangular, respectively, at the end of a 1-h incubation period. The PCr/ATP ratio, a sensitive indicator for maintenance of high-energy phosphate levels, was 1.28 ± 0.04 ($n = 5$) after loading with 5FBapta compared with 1.38 ± 0.05 in cells incubated without 5FBapta. However, 5FBapta-loaded cells did not contract upon electrical stimulation up to 60 V/mm, 6 ms duration, even when tested at high concentrations of extracellular calcium (3–8 mM). Heart cells incubated without 5FBapta contract and relengthen specifically and synchronously in response to electrical stimulation. When the cells were loaded using 2 μM 5FBapta-AM (one-tenth the usual concentration), some contractility of the cells was observed, but the intracellular accumulation of 5FBapta was not sufficient under these conditions to allow observation of ^{19}F NMR signals. Cells incubated with 0.05 μM 5FBapta-AM responded specifically to electrical stimulation at 5 mM extracellular calcium. In perfused heart studies, it has previously been reported that 5FBapta lowers the contractility of the heart, as the contractile function of the heart is dependent on the ability of intracellular free calcium to increase rapidly in response to external stimulation. Our observations suggest

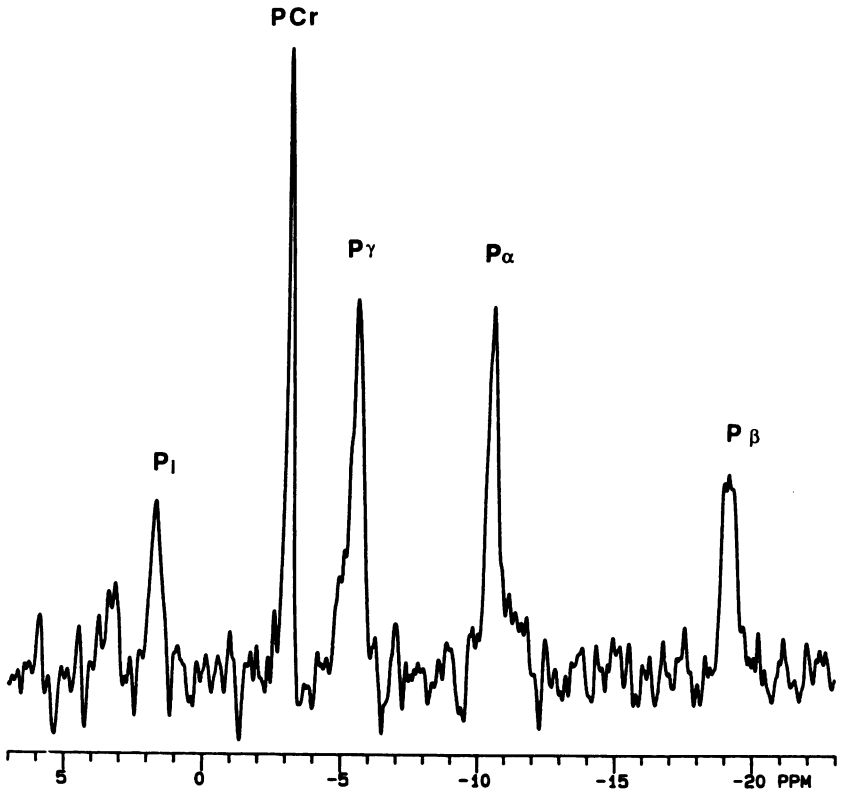

FIGURE 1 ^{31}P NMR spectrum of isolated heart cells loaded with 5FBapta. Cells were perfused with phosphate-free HEPES-MEM containing in addition 2 mM adenosine, 1 mM pyruvate, 1 mM $CaCl_2$, and 9.4 mM HCO_3, gassed with 95% O_2, 5% CO_2. Perfusate flow rate was 10 ml/min. NMR instrumental parameters were: transients, 1000; recycle time, 1.5 s; and acquisition time, 0.8 s. P_α, P_β, and P_γ are the resonances of the α, β, and γ phosphates of ATP, respectively. P_i is the resonance of intracellular inorganic phosphate, and its chemical shift yielded $pH_i = 7.13$.

that the decreased contractility of heart cells is probably due to the buffering effect of intracellular 5FBapta which, although it may not affect the steady-state levels, alters the time course and magnitude of transient free calcium changes in response to external stimulation.

Intracellular free [Ca^{2+}] of resting cardiac myocytes

Fig. 2 (left) shows typical ^{19}F NMR spectra obtained from 5FBapta-loaded heart cells. The Ca5FBapta and 5FBapta resonances are labeled. The relative intensities of these resonances remained stable for several hours, although the absolute intensities decreased slowly with time due to gradual loss of the 5FBapta probe from the intracellular environment. This loss was greater at 37°C than at 30°C. Therefore, all reported measurements were conducted at 30°C.

Assuming a K_d of 500 nM at pH 7.1, 30°C, for the 5FBapta-Ca^{2+} complex, we obtain a value of 60 nM for intracellular free calcium, $[Ca^{2+}]_i$, in untreated control cells. An average of 11 such measurements gave $[Ca^{2+}]_i$ of 61 ± 5 nM. These values are somewhat lower than those reported by us using fluorescent probes (Doeller and Wittenberg, 1990). Therefore, to further verify the low values of intracellular free calcium measured using 5FBapta, we used a different fluorinated Ca^{2+} chelator, Quin-MF (Levy et al., 1987), which can also be introduced into the cells using its acetoxymethyl ester derivative. Quin-MF binds Ca^{2+} ~6–7 times more tightly than 5FBapta; K_d for the Quin-MF-Ca^{2+} complex is 80 nM (Levy et al., 1987). The right panel in Fig. 2 shows ^{19}F NMR spectra recorded for heart cells loaded with Quin-MF. The resonances of Ca-Quin-MF and Quin-MF are labeled and their integrated areas yielded a value for intracellular free calcium of 65 nM, which is similar to that calculated using 5FBapta. The similarity of free [Ca^{2+}] values obtained using 5FBapta and Quin-MF, the two probes with very different affinity for calcium, served to validate the ^{19}F NMR method for measuring free calcium.

Role of Na^+:Ca^{2+} exchange and norepinephrine in the maintenance of myocyte $[Ca^{2+}]_i$

To examine the role of Na^+:Ca^{2+} exchange in the maintenance of intracellular free Ca^{2+} in these cells, we investigated the effect of partially replacing extracellular NaCl by isotonic choline chloride. Intracellular free Ca^{2+} rose significantly (up to 5-fold, $n = 5$) and reached a value of 322 ± 42 nM after a 100-min exposure to choline-containing low Na^+ (16 mM Na^+, 124 mM choline) perfusion medium. The effect of choline on intracellular free Ca^{2+} was largely reversible, and the free calcium returned to 76 ± 17 nM upon reperfusion with normal, Na^+-containing medium, compared to initial values of 64 ± 4 nM. A typical experiment is shown in Fig. 3. The magnitude of the increase in free

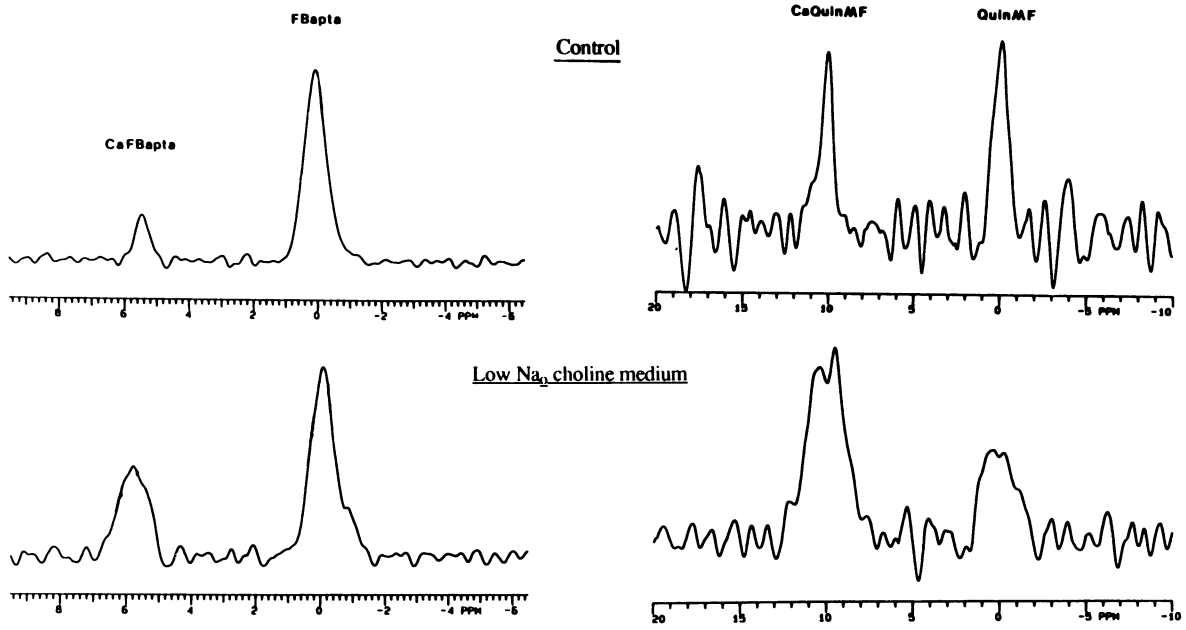

FIGURE 2 Comparison of ^{19}F spectra of heart cells loaded with 5FBapta (*left side*) and Quin-MF (*right side*) with and without choline replacement of $[Na^+]_o$. Perfusion medium was Hepes-MEM with 9.4 mM HCO_3, supplemented with 2 mM adenosine, 1 mM pyruvate, and 1 mM $CaCl_2$. Perfusate flow rate was 15 ml/min and pulse recycle time was 0.5 s; 4000 transients were time-averaged. Cells were loaded with Quin-MF using the same procedure described for loading with 5FBapta, but an additional 20 μM Quin-MF acetoxymethyl ester was used in the cell perfusion medium to obtain adequate loading. Comparable values of $[Ca^{2+}]_i$ were obtained with both indicators although K_d is 500 nM for Ca5FBapta and 80 nM for CaQuin-MF. Note the differences in the relative areas of free and bound peaks observed with the two probes. Top trace: control cells, $[Ca^{2+}]_i$ = 60 nM, determined with 5FBapta, and 68 nM, determined with Quin-MF. Bottom trace: cells in choline medium (when Na_o was decreased to 6 mM), $[Ca^{2+}]_i$ = 274 nM measured with 5FBapta and 240 nM measured with Quin-MF.

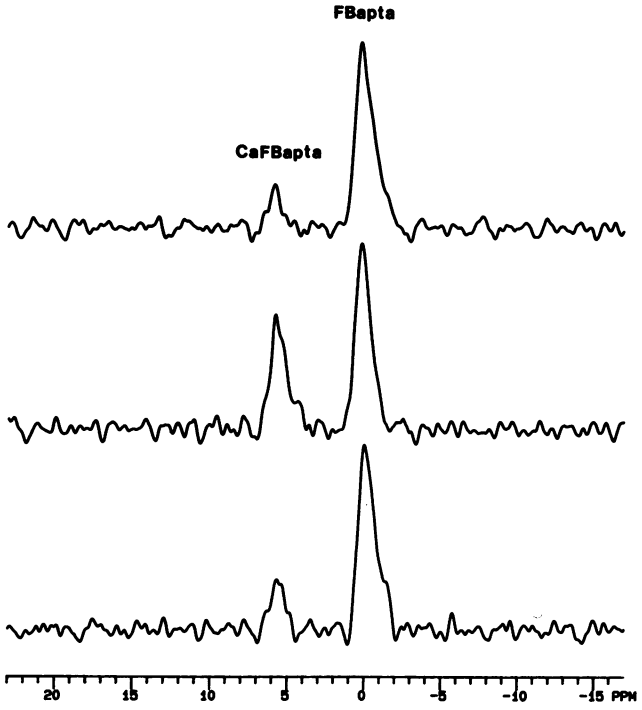

FIGURE 3 ^{19}F spectra showing reversible effect of Na_o replacement by choline on intracellular free Ca^{2+} in heart cells. Perfusate flow rate was 15 ml/min; cell perfusion medium contained 5 mM pyruvate and 3 mM calcium. In the choline medium $[Na]_o$ was at 16 mM. After a change to choline medium, 3 runs of 4000 transients each were required (about 100 min) before $[Ca^{2+}]_i$ reached its new steady state. When choline medium was replaced by MEM, the $[Ca^{2+}]_i$ dropped again toward the control value. Top trace: control perfusion medium, $[Ca^{2+}]_i$ = 67 nM; middle trace: choline medium, $[Ca^{2+}]_i$ = 316 nM; bottom trace: return to control perfusion medium, $[Ca^{2+}]_i$ reversed to 104 nM.

calcium (about 5-fold) in low Na^+ medium is similar to that reported previously using fluorescent probes (Doeller and Wittenberg, 1990), although the reversibility of the effect could be demonstrated only by NMR. Using ^{31}P NMR, we showed that during choline perfusion when intracellular free Ca^{2+} was elevated, the pH_i remained essentially unchanged at 6.97 ± 0.08. Thus, with adequate buffering there was no detectable intracellular acidification associated with the 5-fold increase of intracellular free Ca^{2+}. In the same group of cells, we also measured $[Mg^{2+}]_i$ = 0.4 ± 0.1 mM. There was no detectable change in the $[Mg^{2+}]_i$ when cells were superfused with choline-containing, low-sodium medium.

The increase in intracellular free calcium in low extracellular Na^+ (Na_o) medium was not observed when extracellular calcium was omitted from the medium. This confirms that the increase is due to entry of calcium from the extracellular medium and not merely to its release from intracellular stores.

Norepinephrine, an inotropic agent, did not affect the steady-state $[Ca^{2+}]_i$ in well-perfused resting heart cells. These data seem to suggest that $[Ca^{2+}]_i$ homeostasis is not affected by norepinephrine in healthy resting heart cells. However, in heart cells perfused with low Na_o, choline-containing medium, the observed increase in $[Ca^{2+}]_i$ in the presence of norepinephrine was much greater ($[Ca^{2+}]_i$ = 570 nM) than that observed in the choline medium without norepinephrine ($[Ca^{2+}]_i$ = 320 nM).

Effects of extracellular pH, ATP and no flow ischemia on $[Ca^{2+}]_i$

Consistent with previous studies of others, using a different technique (Orchard et al., 1987), the NMR-measured $[Ca^{2+}]_i$ showed an increase when the cells were acidified by perfusion with a low pH (pH_o = 6.5) medium. Acidification of the medium was achieved by lowering the bicarbonate content. It is well known that intracellular pH changes in the same direction as extracellular pH. However, when pH_o was increased, no significant change in free calcium was observed (up to pH_o as high as 7.9). Representative spectra are presented in Fig. 4. $[Ca^{2+}]_i$ remained within the range from 68 to 88 nM (78 ± 5 nM; n = 6) when the pH_o was changed from 7.9 to 7.15, but increased 2-fold upon further acidification to 6.5 ($[Ca^{2+}]_i$ = 151 ± 19; n = 5, p = 0.02 versus control) (Table 1).

The effect of extracellular ATP on intracellular free calcium was investigated to determine if the NMR technique was sensitive enough to detect a transient increase in free

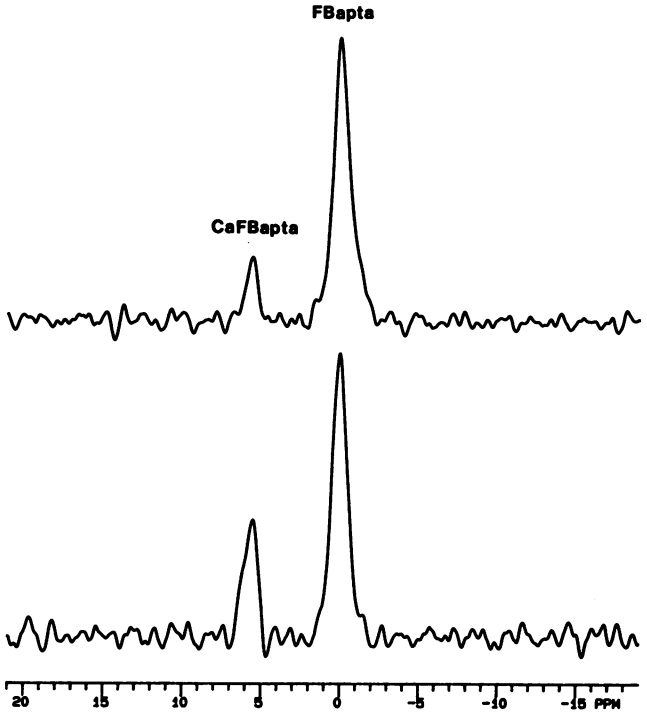

FIGURE 4 ^{19}F NMR spectra showing the effect of pH on intracellular free Ca^{2+}. Cell perfusion medium contained no Hepes, variable bicarbonate as stated below, 2 mM pyruvate, and 3 mM $CaCl_2$ and was gassed with 5% CO_2, 95%O_2. Other conditions were as described in the legend to Fig. 3. There was no measurable change in $[Ca^{2+}]_i$ as pH_o was varied from 7.2 to 7.9. However, at pH values below 7, there was an increase in $[Ca^{2+}]_i$. Upper trace: pH_o = 7.9, the medium contained 108 mM $NaHCO_3$, $[Ca^{2+}]_i$ = 68 nM. Bottom trace: pH_o = 6.5, the medium contained 2.9 mM $NaHCO_3$, $[Ca^{2+}]_i$ = 170 nM.

TABLE 1 Effect of extracellular pH on intracellular free calcium

pH_o	$[Ca^{2+}]_i$ (nM)
7.9	68 ± 5
7.2	61 ± 5
6.5	151 ± 19

calcium that has been reported previously using other methods (Bjornsson et al., 1989; De Young and Scarpa, 1991; Puceat et al., 1991). Addition of 1 mM ATP to the perfusing medium resulted in a transient rise in intracellular free calcium, which reached a maximum within 40 min. Fig. 5 shows a typical spectrum obtained in the presence of 1 mM ATP. As is clear from quick inspection of the data, the Ca5FBapta peak is much larger in ATP-treated heart cells than in control cells. We estimate that the free calcium concentration becomes transiently as high as 540 nM upon treatment with ATP, a 9-fold increase over the control value near 60 nM. At an ATP concentration of 0.25 mM, a reversible, significant, but smaller (2.5-fold) transient increase in $[Ca^{2+}]_i$ to 148 ± 12 nM was observed.

Inhibition of glycolysis by 100 μM iodoacetate had little effect on $[Ca^{2+}]_i$, the level staying within the range from 67 to 86 nM. This may not be surprising since in the presence of pyruvate, as was the case here, it is well known that oxidative phosphorylation is able to maintain full ATP and phosphocreatine reserves without glycolysis. This has been demonstrated in beating hearts (Kingsley-Hickman et al., 1987) as well as in isolated heart cells (Gupta and Wittenberg, 1991). However, when oxidative phosphorylation was subsequently blocked with oligomycin (9 μM), accompanied by shut off of oxygen flow, there was a marked increase in $[Ca^{2+}]_i$ from 86 to 357 nM, presumably due to ATP depletion. Subsequent stoppage of flow to give no flow ischemia rapidly caused an irreversible increase in $[Ca^{2+}]_i$ to a value >4 μM. These data are similar to those obtained by others on intact hearts (Steenbergen et al., 1990) and show that calcium homeostasis is lost when ATP synthesis is inhibited.

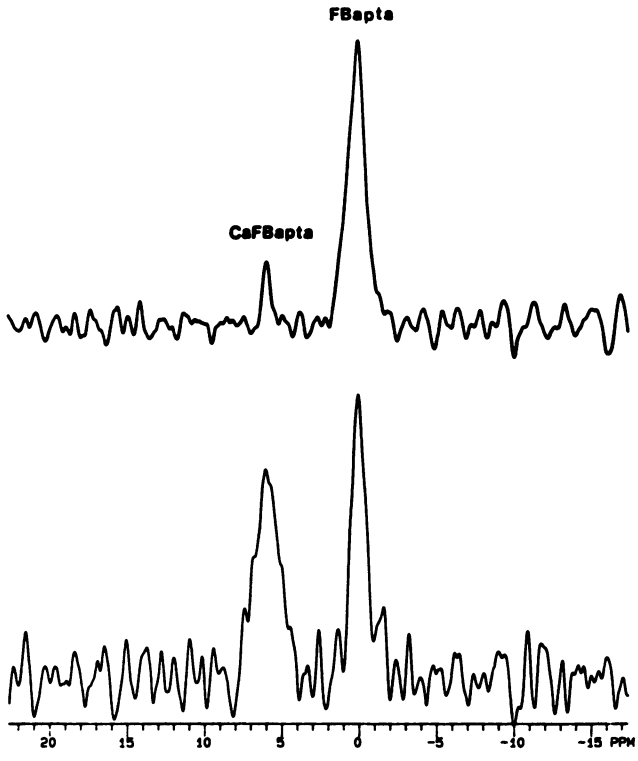

FIGURE 5 ^{19}F NMR spectra showing the effect of extracellular ATP on intracellular free $[Ca^{2+}]$. Conditions were as described in the legend to Fig. 3; cell perfusion medium contained 2 mM pyruvate. The addition of 1 mM ATP to the perfusion medium gave a transient reversible increase in $[Ca^{2+}]_i$, which was maximal at 40 min. Upper trace: control ^{19}F spectrum, $[Ca^{2+}]_i$ = 50 nM; lower trace: spectrum obtained with 1 mM ATP at 40 min in the perfusion medium, $[Ca^{2+}]_i$ = 541 nM.

Effect of myoglobin inactivation on intracellular free $[Ca^{2+}]$

We have previously shown that 1–2 mM $NaNO_2$ converts intracellular myoglobin to the inactive high-spin ferric form, and decreases heart cell phosphocreatine levels within 1 h (Doeller and Wittenberg, 1991; Gupta and Wittenberg, 1991; Wittenberg and Wittenberg, 1989), an effect that can be reversed by removal of nitrite. To investigate the possibility that decreased phosphorylation potential may affect calcium homeostasis, we studied the effect of myoglobin inactivation on $[Ca^{2+}]_i$. When intracellular myoglobin was inactivated with nitrite, we found, in several different preparations, the steady-state $[Ca^{2+}]_i$ after 60 min to be similar to control values. This confirms our earlier finding with Fura-2, where the absolute values were somewhat higher, that there was no consistent effect of myoglobin inactivation on $[Ca^{2+}]_i$ (Doeller and Wittenberg, 1990). The present experiments show that there is no specific effect of intracellular oxymyoglobin or of decreased phosphocreatine levels on calcium homeostasis in well-oxygenated heart cells as long as ATP levels are maintained.

Effects of extracellular lead, and Mg^{2+} on intracellular free $[Ca^{2+}]$

Effects of extracellular lead on $[Ca^{2+}]_i$ were investigated since lead exposure and toxicity remain a serious health concern, and cardiovascular effects of lead toxicity may lead to hypertension. Lead has been shown to interfere with cellular calcium homeostasis and to cause an increase in free calcium in cultured brain and bone cells (Schanne et al., 1989a, b). Whether similar effects occur in cells of the cardiovascular system has not been known previously. We therefore examined the effect of adding 10–20 μM lead acetate to the perfusion medium on the $[Ca^{2+}]_i$ in heart cells. The presence of lead in the perfusion medium consistently increased the steady-state level of $[Ca^{2+}]_i$ about 2-fold within a period of 2 h. Thus, $[Ca^{2+}]_i$ increased to 172 ± 36 nM (n = 4, p = 0.05) in the presence of 10–20 μM lead acetate. Typical ^{19}F

NMR spectra obtained with Pb^{2+}-treated and untreated cells are shown in Fig. 6. The elevation in $[Ca^{2+}]_i$ could not be reversed rapidly (within 2 h) by removal of lead acetate from the perfusion medium, presumably because some of the lead entered the cytoplasm, as has been shown previously in cultured osteoblastic bone cells (Schanne et al., 1989a). The irreversibility of the Pb effect may be only apparent, since the off rates for Pb binding to the target sites could be so slow that we would not see Pb come off in our experiments. In any case, the data clearly demonstrate that lead interferes with the regulation of intracellular free calcium in heart cells.

The effects of extracellular Mg^{2+} on $[Ca^{2+}]_i$ were determined when extracellular Mg^{2+} ($[Mg^{2+}]_o$) was varied from <10 μM to 10 mM, with spectra measured at <10 μM, 0.6 mM, 1.7 mM, and 10 mM $MgCl_2$ (Table 2). The only reproducible effect on intracellular free $[Ca^{2+}]$ was observed when extracellular magnesium was omitted ($[Mg^{2+}]_o$ <10 μM; Fig. 7). Under these conditions there was a significant increase in $[Ca^{2+}]_i$ to 132 ± 11 nM (versus 61 ± 5 in Mg^{2+}-containing control cells).

Effect of extracellular Na^+ on intracellular free $[Mg^{2+}]$

A possible effect of extracellular Na^+ on intracellular free Mg^{2+} was investigated by ^{31}P NMR as described under "Materials and Methods." In Ca^{2+}-free medium ($[Ca^{2+}]_o<10$ μM), there was no detectable change in the separation of α and β peaks of ATP upon perfusion with low Na_o, choline medium. Thus, perfusion of the heart cells in fiber bundles with calcium-free Na^+-containing medium gave an α and β difference of 1765 ± 10 Hz, corresponding to intracellular free $[Mg^{2+}]$ of 327 ± 50 μM, similar to the α and β separation of 1771 ± 10 Hz obtained in calcium-free choline medium which yielded a $[Mg^{2+}]_i$ of 295 ± 50 μM. The pH_i ranged from 7.14 to 7.07 in Ca^{2+}-free media. These values of $[Mg^{2+}]_i$ are somewhat lower than those measured in calcium-containing media (510 ± 40 μM; Gupta and Wittenberg, 1991). This may be due to loss of intracellular Ca^{2+} in Ca^{2+}-free medium (Cheung et al., 1982; Doeller and Wittenberg, 1990), which would result in binding of intracellular Mg^{2+} to Ca^{2+} binding sites, thereby decreasing $[Mg^{2+}]_i$. However, our data, showing the absence of an effect of Na^+_o removal on $[Mg^{2+}]_i$, clearly argue against the presence of an active Na^+:Mg^{2+} exchange in resting heart cells.

TABLE 2 Effect of extracellular magnesium concentration on intracellular free calcium

$[Mg^{2+}]_o$ (mM)	$[Ca^{2+}]_i$ (nM)
0	187 ± 10
0.6	64 ± 10
1.7	61 ± 5
10	57 ± 12

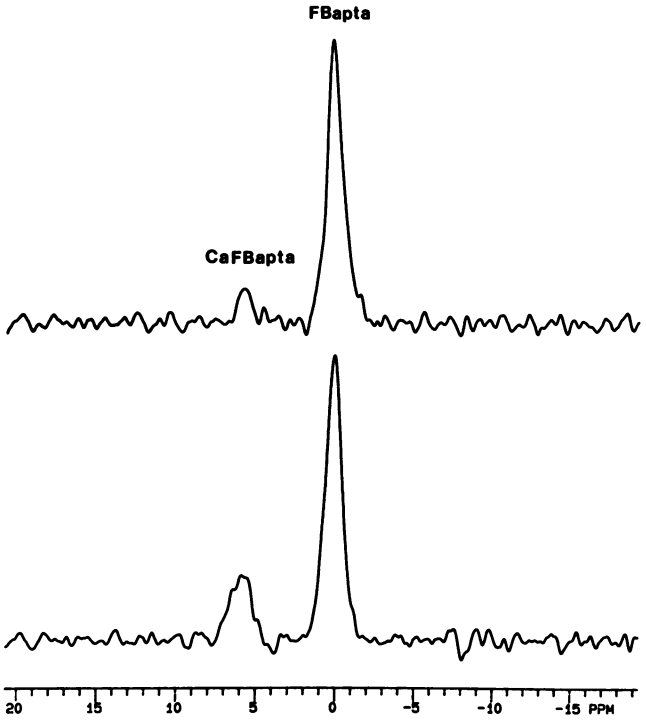

FIGURE 7 ^{19}F spectra showing the effect of extracellular Mg^{2+} on intracellular free $[Ca^{2+}]$ in heart cells. Conditions were as described in the legend to Fig. 5. The removal of extracellular Mg^{2+} caused a significant increase in $[Ca^{2+}]_i$. Upper trace: control heart cells, $[Ca^{2+}]_i$ = 54 nM. Bottom trace: cells perfused with Mg-free ($[Mg^{2+}]_o$ ~10 μM) medium for 2 h, $[Ca^{2+}]_i$ = 135 nM.

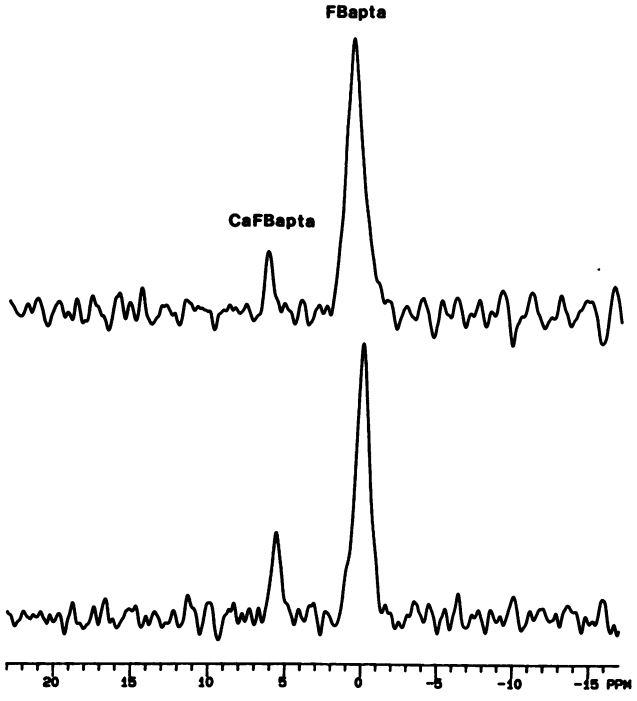

FIGURE 6 ^{19}F NMR spectra showing the effect of lead acetate on intracellular free $[Ca^{2+}]$ in heart cells. Perfusion conditions were as described in the legend to Fig. 5. The addition of lead acetate caused a significant increase in $[Ca^{2+}]_i$. Upper trace: control cells, $[Ca^{2+}]_i$ = 45 nM; bottom trace: cells perfused with 10 μM PbAc for 2 h, $[Ca^{2+}]_i$ = 101 nM.

Effect of elevated extracellular glucose on intracellular free [Ca^{2+}]

The effect of hyperglycemia was investigated since hyperglycemia-induced alteration of $[Ca^{2+}]_i$ might play a role in diabetic cardiomyopathy. An increase in intracellular Na^+ during hyperglycemia has been reported in renal tubules (Dowd and Gupta, 1993). However, no published reports yet exist showing an effect of hyperglycemia on cytosolic free calcium in heart cells, although such effects have been described in pancreatic β cells (Arkhammar et al., 1989). We find that, in isolated heart cells, increasing the extracellular glucose concentration from 5.4 to 25 mM reversibly increased $[Ca^{2+}]_i$ nearly 2-fold (Fig. 8). Cytosolic free calcium, initially 67 nM, increased significantly to 119 ± 11 nM in the presence of 25 mM glucose.

DISCUSSION

NMR measurement of intracellular free Ca^{2+}

The ^{19}F NMR measurement of $[Ca^{2+}]_i$ of isolated heart cells using 5FBapta that we describe here complements methods using fluorescent calcium-sensitive probes (Table 3). The present technique permits the measurement of the average calcium levels of a large population of cells, well superfused with oxygenated medium supplied over short diffusion distances. Cells retained functional integrity measured by phosphocreatine levels for up to 6 h so that the reversibility of interventions could readily be monitored. We further showed that neither phosphocreatine and P_i levels nor pH_i were detectably changed by 5FBapta loading of heart cells. These properties are sensitive criteria for the maintenance of functional cellular integrity. We have utilized NMR techniques to study the effects of low Na_o on $[Ca^{2+}]_i$ as well as pH_i and $[Mg^{2+}]_i$ and showed that neither pH_i nor $[Mg^{2+}]_i$ was detectably changed by decreasing the Na^+ free energy gradient, although $[Ca^{2+}]_i$ was significantly increased. An advantage of the ^{19}F NMR method is that $[Ca^{2+}]_i$ can be monitored in the same cell preparation, in which pH_i, $[Mg^{2+}]_i$, and high-energy phosphates are measured by ^{31}P NMR.

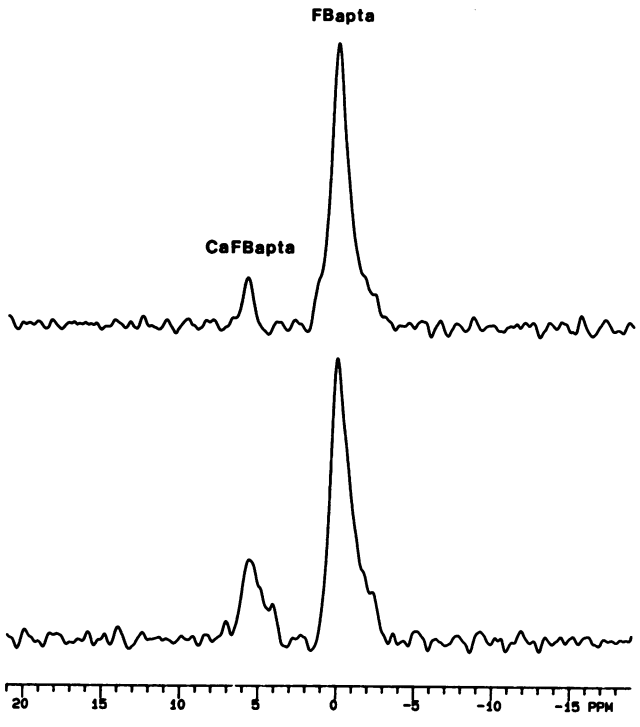

FIGURE 8 ^{19}F NMR spectra showing the effect of hyperglycemia on intracellular free $[Ca^{2+}]$. Conditions were as described in the legend to Fig. 5; control perfusion medium contained 5.4 mM glucose. Changing the glucose concentration in the perfusion medium to 25 mM caused an increase in steady-state $[Ca^{2+}]_i$, which was reversed by changing the perfusate glucose concentration back to 5.4 mM. Upper trace: control heart cells perfused with 5.4 mM glucose medium, $[Ca^{2+}]_i$ = 45 nM; bottom trace: cells perfused with medium containing 25 mM glucose for 2.5 h, $[Ca^{2+}]_i$ = 147 nM.

The 5FBapta-loaded cells were observed to be about 67–73% rectangular initially in individual dialysis fibers and, after several hours of NMR measurements, 60–67% retained their rectangular shape. Rounded cells did not load with 5FBapta. We have found that ischemic cells in low Na^+ medium all round up, and the ^{19}F NMR signal decreases to undetectable levels. Round heart cells therefore appear permeable to the probe and do not contribute to the measured ^{19}F NMR signal. It should be noted that rounded heart cells are in calcium overload, and any contribution from rounded cells, even when present, would have tended to give higher values of $[Ca^{2+}]_i$ and could not explain the lower values of $[Ca^{2+}]_i$ measured here relative to those measured by us previously using Fura-2. That, in our NMR experiments, we were measuring a population of healthy cells which remained in good shape over several hours of superfusion was also indicated by the constancy and low value of measured $[Ca^{2+}]_i$.

5FBapta is thought to report cytosolic free $[Ca^{2+}]_i$ since cytosolic esterases rapidly cleave the ester linkages of the membrane-permeant 5FBapta-AM, leaving the charged anion, which should not readily permeate the membranes of intracellular organelles. In superfused brain slices, <1% of the deesterified 5FBapta was found in mitochondria while the balance of deesterified 5FBapta was found in the cytosolic fraction (Badar-Goffer et al., 1990). Marban et al. (1990) separated cytosolic and mitochondrial fractions from homogenates of hearts loaded with 5FBapta and found that most of the 5FBapta (about 0.3 mM) was in the cytosolic fraction. Not enough ^{19}F was detected in the mitochondrial fraction to measure the 5FBapta concentration accurately by NMR. When the mitochondria were lysed with detergent, the concentration of released 5FBapta was estimated to be ≤3% of that in the cytosolic fraction. This is in contrast to the situation with the fluorescent calcium indicators Fura-2 and Indo-1, where concerns have been expressed relating to incomplete deesterification and compartmentalization of the indicator into mitochondria (Doeller and Wittenberg, 1990), although this problem may be obviated by injecting the membrane-impermeable form of the dye directly into the cytosol (Sollott et al., 1992).

TABLE 3 Intracellular free calcium of resting myocytes reported using fluorescent indicators

$[Ca^{2+}]_i$ (nM)	Sample	Indicator	K_d (nM)	Reference
69 ± 7	Single cells	Indo-1	250	Puceat et al. (1991)
65 ± 5	Cell suspension	Fura-2	224*	Bjornsson et al. (1989)
77 ± 3	Single cells	Fura-2	224*	Grouselle et al. (1991)
82 ± 8	Single cells	Fura-2	224*	Hayashi et al. (1992)
92 ± 35	Cell suspension	Fura-2	224*	De Young and Scarpa (1991)
134 ± 43	Single cells	Fura-2	320	Wier et al. (1987)
136 ± 6	Single cells	Indo-1		Sollott et al. (1992)
155 ± 7	Cell suspension	Fura-2	435	Doeller and Wittenberg (1990)

* K_d was not directly determined, but assumed to be 224 nM (Grynkiewicz et al., 1985).

Free calcium in cardiac myocytes

$[Ca^{2+}]_i$ of resting heart cells is regulated to maintain a steady-state level of about 60 nM in the face of millimolar calcium concentrations in the extracellular environment. The steady-state calcium level is the balance between calcium extrusion by ATP requiring pumps (sarcolemmal ATPase, sarcoplasmic reticulum calcium uptake) and Na^+:Ca^{2+} exchange and by calcium influx through the sarcolemma. This is demonstrated here using the NMR technique, which shows that steady-state $[Ca^{2+}]_i$ is markedly increased when ATP supply is inadequate during ischemia, when Na^+:Ca^{2+} exchange is blocked by decreased $[Na^+]_o$, and when calcium influx is increased by decreased extracellular pH or added extracellular ATP. Our results show that partial reduction of ATP supply by inhibition of glycolysis with iodoacetate, or inhibition of myoglobin function, is not sufficient to affect calcium homeostasis in resting heart cells. Specific changes in the composition of the ambient medium increase the steady-state intracellular concentration of free calcium. Thus, deficiency of $[Mg^{2+}]_o$, addition of lead acetate, and hyperglycemia all lead to increased $[Ca^{2+}]_i$.

The intracellular free Ca^{2+} value of 61 ± 5 nM for the perfused heart cells is similar to that previously reported for cultured vascular smooth muscle cells (Altura et al., 1992) and in several other reports on isolated heart cells using fluorescent probes (Table 3). Our earlier experiments (Doeller and Wittenberg, 1990), as well as those of a few other investigators (e.g., Wier et al., 1987) with Fura-2, however, gave significantly higher values of $[Ca^{2+}]_i$ in isolated heart cells, of about 155 ± 7 nM (Table 3). In these studies, the K_d for Ca^{2+}-Fura determined in situ (Doeller and Wittenberg, 1990; Wier et al., 1987) was 50 to 100% higher than that determined in model solutions (Grynkiewicz et al., 1985) (Table 3). Using Indo-1, $[Ca^{2+}]_i$ values from 69 ± 7 to 136 ± 6 nM have been reported in isolated single cells (Puceat et al., 1991; Sollott et al., 1992). The variation in reported Indo-1 values may be due to differences in the distribution of the dye within intracellular compartments. While differences in K_d may, in part, account for the discrepancy in measured values using Fura-2, it is possible that our own earlier values using this indicator may be higher due to the necessity to work in the absence of BSA for fluorescent studies or to the fact that readings had to be taken in nonperfused cells. In the present study, heart cells, maintained in BSA medium, are adequately perfused continuously during the calcium measurements and are well supplied with oxygen and nutrients. Higher values of $[Ca^{2+}]_i$ could also result from transient acidification, leakage of probe from the cells into the calcium-containing medium, damage to cell membranes causing cell beating, or acute death of a small fraction of cells. In the present experiments, we have minimized these sources of error by high perfusion rates, adequate pH buffering of intracellular and extracellular environments, maintenance of BSA in the medium surrounding the cells, and adequate equilibration of cells until stable $[Ca^{2+}]_i$ values were achieved.

By examining ^{19}F spectra during perfusion of an intact heart with 5FBapta-AM, Marban et al. (1990) found that the free and Ca-bound 5FBapta peaks appeared simultaneously in the expected intensity ratio. There was no evidence for the presence of partially deesterified 5FBapta, which would enhance the intensity of the free peak relative to the calcium-bound peak since the ester and partially deesterified products do not have as high an affinity for Ca^{2+} as does the tetra anion. Thus, it would appear that the values of myocyte $[Ca^{2+}]_i$ reported in this paper are free from the above complications. The gradual decrease in the ^{19}F intensity of Ca5FBapta and 5FBapta peaks with time presumably reflects loss of the indicator from the cells, which is estimated to escape from rectangular cells with a time constant of about 6 h at 30°C. We suggest that the low levels of $[Ca^{2+}]_i$ reported here reflect the basal value of healthy, well-perfused, resting heart cells.

The reported value of $[Ca^{2+}]_i$ depends crucially on the value of K_d. The K_d of Ca5FBapta has been a subject of controversy in the literature, with reported values ranging from 308 to 710 nM (Kirschenlohr et al., 1988; Levy et al., 1987; Marban et al., 1990; Smith et al., 1983). In their first paper, Smith et al. (1983) reported a K_d for Ca5FBapta of 710 nM at 37°C in the presence of 1 mM Mg^{2+} using citrate buffer to calibrate free calcium in the solutions used. Measurements using the fluorescent indicator Fura-2 have led to the report of a K_d of 635 nM at 37°C (Levy et al., 1987), but it has been pointed out that the K_d for the Ca-Fura-2 complex itself is somewhat uncertain (Marban et al., 1990). In sharp disagreement with the above, Marban et al. (1990) have reported a K_d of 308 ± 26 nM at 30°C and pH 7.2, using a computer program to calculate the free calcium concentrations in their calibrating solutions from the analytically determined absolute K_d values for the reactions of EGTA with

Ca^{2+} and H^+ at the appropriate temperature and ionic strength. To resolve these controversies and to eliminate complications due to possible formation of ternary complexes involving Ca5FBapta and citrate, Fura-2, or EGTA, we have previously remeasured K_d using a well-calibrated calcium-selective electrode to directly measure free calcium ions and the ^{19}F NMR to measure the calcium-bound to free 5FBapta concentration ratio in the same solutions (see "Materials and Methods"). We thus obtained a K_d of 554 ± 18 nM at 22°C and a K_d of 492 ± 33 nM at 37°C and pH 7.2 (Jelicks and Gupta, 1990). The same approach yielded a similar K_d of 500 ± 20 nM at 30°C (Schanne et al., 1989a). In agreement with these values, a K_d of 540 nM at 30°C and pH 7.1 has been measured by Kirschenlohr et al. (1988) using ultraviolet spectroscopy. Thus, it appears that the controversy is finally resolved and that a K_d value in the range from 492 to 554 nM would be widely acceptable.

Good agreement between the values of $[Ca^{2+}]_i$ reported with 5FBapta and Quin-MF, two indicators with very different affinity for calcium (see Fig. 2), suggests that the ratios of K_d values for Ca5FBapta and Ca-Quin-MF used in this work are reasonably accurate. This further strengthens our confidence in the K_d value of 500 nM for Ca5FBapta at 30°C.

The steady-state level of $[Ca^{2+}]_i$ in resting heart cells (about 61 nM) is much lower than that measured in beating whole heart using the same ^{19}F NMR technique. This is presumably in part because in the beating whole heart the measurement reflects a nonlinear average over the entire cardiac cycle. However, even gated measurements of $[Ca^{2+}]_i$ reveal a minimum of 400 nM at any point in the cardiac cycle (Kirschenlohr et al., 1988). Thus, the beating whole heart appears to maintain a much higher steady-state level of $[Ca^{2+}]_i$ than resting isolated heart cells.

Effect of Na^+:Ca^{2+} exchange and norepinephrine on $[Ca^{2+}]_i$

In a low Na^+ medium, the Na^+:Ca^{2+} exchanger is unable to extrude Na^+, since it is driven by the Na^+ free energy gradient. In the absence of Na^+ extrusion by Na^+:Ca^{2+} exchange, the burden of calcium removal is placed on the transsarcolemmal Ca-ATPase, the activity of which alone is apparently not sufficient to maintain low intracellular free calcium, even in these resting cells. Norepinephrine does not increase $[Ca^{2+}]_i$ in resting heart cells, presumably because norepinephrine, upon binding to sarcolemmal receptors, does not enhance calcium entry through L-type channels in the absence of membrane depolarization. Low Na^+ perturbation (replacement of much of the NaCl by choline chloride in the extracellular medium) probably depolarizes the cells sufficiently to cause phosphorylated L-type calcium channels to open and permit calcium entry. Such membrane depolarization by choline substitution has been observed in the T-tubule moiety of isolated triad vesicles (Ikemoto et al., 1992). Membrane depolarization is also observed in guinea pig cardiac myocytes, where the resting potential of cells, initially −60 mV, decreased to −20 mV after withdrawal of both external Na^+ and K^+ (Le Grand et al., 1990). The increase in $[Ca^{2+}]_i$ induced by norepinephrine in low Na^+ medium would then be explained by further enhancement of the open probability of the L-type calcium channels. The effects of norepinephrine on $[Ca^{2+}]_i$ in the isolated resting heart cells must be different from those in the beating whole heart, where depolarization and Ca^{2+} entry occur with each beat, and a much higher level of steady-state average $[Ca^{2+}]_i$, about 550 nM, is maintained (Steenbergen et al., 1990).

Effect of extracellular ATP and pH on $[Ca^{2+}]_i$

The present study shows a rapid transient rise in intracellular free $[Ca^{2+}]$ upon treatment with extracellular ATP. Others (Bjornsson et al., 1989; De Young and Scarpa, 1991; Puceat et al., 1991) have shown a similar but smaller effect of ATP (up to 100% increase over basal) on rat myocytes and have suggested that the increase is due to activation of purinoreceptors associated with voltage-activated cation channels, the opening of which causes rapid entry of Ca^{2+} into the cells (Bjornsson et al., 1989). A similar response observed in single cardiac myocytes has been attributed to initial intracellular acidification after activation of sarcolemmal receptors by ATP (Puceat et al., 1991). The effect of ATP on myocyte $[Ca^{2+}]_i$ was transient and the free calcium level returned close to control values (about 100 nM) about 1.5 h after ATP treatment. The rapid onset of $[Ca^{2+}]_i$ increase in these calcium-buffered cells would support the suggestion that the ATP response can be attributed to opening of voltage-activated Ca^{2+} channels, which would cause rapid entry of calcium.

The increase in $[Ca^{2+}]_i$ with decreasing pH observed here, and reported previously (Orchard et al., 1987; White et al., 1990) may arise from a displacement of intracellular calcium ions activating a nonspecific membrane conductance to cause depolarization and increased calcium influx (Puceat et al., 1991).

Effect of extracellular Mg^{2+} on intracellular free $[Ca^{2+}]$

Over the years, data have accumulated in the literature to indicate that the level of extracellular Mg^{2+} can alter myogenic tone and contractile response of smooth muscle cells in response to various external stimuli by affecting calcium homeostasis (Altura et al., 1992). Magnesium supplementation has recently been reported to have a beneficial effect on the recovery of myocardium from ischemia and to reduce the death rate from heart attacks (Woods et al., 1992). We therefore investigated the acute effects of severe deficiency of $[Mg^{2+}]_o$ on $[Ca^{2+}]_i$. Our data clearly indicate that removing magnesium from the perfusion medium rapidly induces a consistent, sizable increase in $[Ca^{2+}]_i$ from 54 to 135 nM (see Fig. 8), an increase of more than 100%. However, smaller decreases in $[Mg^{2+}]_o$ did not produce measurable increases in $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ of heart cells in Mg^{2+}-deficient medium may arise from an increase in permeability of the cell membrane to Ca^{2+}.

The intensity of ^{19}F signals was consistently reduced in all heart cells subjected to low $[Mg^{2+}]_o$ medium due to rounding up of cells and subsequent loss of 5FBapta. This effect of $[Mg^{2+}]_o$ on cell shape may be due to an increase in $[Ca^{2+}]_i$ beyond the level needed for maintenance of cell viability. It is possible that extracellular Mg^{2+} supplementation may limit Ca^{2+} entry and decrease cell morbidity and thereby may improve recovery of ischemic myocardium following heart attacks.

Effect of glucose on intracellular free $[Ca^{2+}]$

We have found a 100% increase in $[Ca^{2+}]_i$ with 25 mM glucose that was nearly reversible when the glucose concentration was brought back to 5 mM. The effect is not due to osmolarity changes since the osmolarity of the perfusate was maintained constant by addition of water as needed. This unavoidably resulted in about a 10% decrease in extracellular $[Na^+]$. However, separate experiments indicated no effect of this slight decrease in $[Na^+]_o$ on $[Ca^{2+}]_i$.

Na^+:Ca^{2+} exchange and sarcolemmal Ca^{2+}-ATPase are responsible for the maintenance of low $[Ca^{2+}]_i$ in heart cells. Unlike the case of kidney cells, which possess a Na:glucose cotransporter and where hyperglycemia increases intracellular Na^+ (Dowd and Gupta, 1993), there is no evidence for an increase in intracellular Na^+ in whole heart (L. A. Jelicks and R. K. Gupta, unpublished data). Thus, it appears more likely that the activity of Ca^{2+}-ATPase is altered during hyperglycemia. Indeed, the activity of Ca^{2+}-ATPase has been found to be decreased after in vivo or in vitro glucose administration in red blood cells (Davis et al., 1985). However, we cannot exclude the possibility that the increase is due to a greater influx of calcium through voltage-activated calcium channels in the presence of high glucose, as found previously in pancreatic β cells (Arkhammar et al., 1989). High glucose has been reported to increase protein kinase C activity (Ayo et al., 1991), which could be due in part to an increase in $[Ca^{2+}]_i$, similar to the one observed here. The increased $[Ca^{2+}]_i$ during hyperglycemia also provides a basis for understanding the frequent clinical association of diabetes and hypertension, since smooth muscle tone is dependent on intracellular free calcium. Increased $[Ca^{2+}]_i$ in vascular smooth muscle has previously been associated with hypertension (Jelicks and Gupta, 1990), and a recent study reported that a high glucose diet alone can significantly increase blood pressure in rats (Reaven and Ho, 1991). Our findings reveal that glucose itself may be a factor contributing to an abnormal cellular calcium ion homeostasis in heart muscle. If this effect also occurs in vascular smooth muscle, it may account for the increased risk of hypertension in hyperglycemic states such as non-insulin-dependent diabetes mellitus. Altered Ca^{2+} homeostasis during hyperglycemia might also in part contribute to insulin resistance in non-insulin-dependent diabetes (Drazin et al., 1987; Levy et al., 1989). It has been shown that in hyperglycemic spontaneously beating as well as paced diabetic rat hearts, there is a decrease in heart rate and rate-pressure product, an effect that is partially reversed upon increasing glucose oxidation (Nicholl et al., 1991). Thus hyperglycemia may play a role in diabetic cardiomyopathy as well.

Effect of lead on intracellular free calcium

Lead poisoning remains a serious health concern, despite governmental regulations on lead content in gasoline and in foods and water. Even low level lead toxicity has been associated with disturbances in cardiovascular function, and lead has been implicated in the etiology of hypertension. Although the exact mechanism of lead toxicity on the cardiovascular system is not known, lead appears to interfere with the hormonal regulation of the cardiovascular system (Giridhar and Isom, 1990). Since lead has been shown to increase $[Ca^{2+}]_i$ in cultured brain and bone cells (Schanne et al., 1989a, b), it occurred to us that cardiovascular effects of lead might also be associated with an increase in $[Ca^{2+}]_i$ in cells of the cardiovascular system. The present study confirmed this by showing a sizable (100%) increase in $[Ca^{2+}]_i$ after acute (2 hr) treatment of heart cells with 10–20 μM lead acetate.

There are several reports showing a significant correlation between level of lead intoxication and blood pressure in humans (Pirkle et al., 1985). Studies of animals fed lead acetate in drinking water have suggested a causal association between lead intoxication and hypertension (Webb et al., 1981). A significant increase in $[Ca^{2+}]_i$ of heart muscle cells with lead treatment observed in the present study provides a possible mechanism for lead-induced hypertension, since a similar increase in $[Ca^{2+}]_i$ in vascular smooth muscle cells would cause increased tension development and vasoconstriction leading to hypertension.

The $[Ca^{2+}]_i$ reported here with 5FBapta mainly describes the cytosolic steady-state value of functionally intact, well-perfused heart cells. We have measured the effects of various interventions on calcium homeostasis in well-perfused, quiescent cells. The ^{19}F NMR technique is useful for the measurement of changes in steady-state intracellular $[Ca^{2+}]_i$ under a variety of conditions over a period of hours.

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