Effects of Intracellular Acidosis on $[\text{Ca}^{2+}]_i$
Transients, Transsarcolemmal Ca$^{2+}$ Fluxes, and Contraction in Ventricular Myocytes

Osami Kohmoto, Kenneth W. Spitzer, Matthew A. Movsesian, and William H. Barry

We examined the effects of intracellular acidosis produced by washout of NH$_4$Cl on $[\text{Ca}^{2+}]_i$ transients (indo-1 fluorescence), cell contraction (video motion detector), and $^{45}$Ca and $^{24}$Na fluxes in cultured chick embryo ventricular myocytes. Exposure of cells to 10 mM NH$_4$Cl produced intracellular alkalosis (pH 7.6), and subsequent washout resulted in a transient acidosis (pH 6.5). Exposure to 10 mM NH$_4$Cl slightly decreased $[\text{Ca}^{2+}]_i$, transients but increased the amplitude of cell contraction. Subsequent washout of NH$_4$Cl initially increased diastolic $[\text{Ca}^{2+}]_i$ and decreased the peak positive and negative $d[\text{Ca}^{2+}]_i/dt$, while the amplitude of cell contraction was markedly decreased. Subsequently, peak systolic $[\text{Ca}^{2+}]_i$ increased with partial recovery of contraction. A similar increase in $[\text{Ca}^{2+}]_i$ and decrease in contraction after washout of NH$_4$Cl was observed in single paced adult guinea pig ventricular cells. Acidosis decreased $^{45}$Ca uptake by sarcoplasmic reticulum vesicles isolated from chick embryo ventricle. However, the $[\text{Ca}^{2+}]_i$ increase caused by intracellular acidosis was also observed in the presence of 10 mM caffeine, suggesting that altered sarcoplasmic reticulum handling of calcium is not the only mechanism involved. Intracellular acidosis only slightly increased total $^{24}$Na uptake under these conditions, an effect resulting from the combination of a stimulation of amiloride-sensitive sodium influx (Na$^+$-H$^+$ exchange) and inhibition of sodium influx via Na$^+$-Ca$^{2+}$ exchange, manifested by a significant decrease in $^{45}$Ca efflux. Further support for a lack of involvement of an increased $[\text{Na}^+]_i$ in the observed increase in $[\text{Ca}^{2+}]_i$ during acidosis was provided by the observation that intracellular acidosis could produce an increase in $[\text{Ca}^{2+}]_i$ in low-sodium, nominal 0-calcium extracellular solution, an experimental condition that minimizes the possible effects of Na$^+$-H$^+$ exchange and Na$^+$-Ca$^{2+}$ exchange. We conclude that the $[\text{Ca}^{2+}]_i$ increase caused by intracellular acidosis in cultured ventricular cells is primarily due to changes in $[\text{Ca}^{2+}]_i$, buffering and $[\text{Ca}^{2+}]_i$, extrusion, rather than to an increase in transsarcolemmal calcium influx. Intracellular acidosis also markedly decreases the sensitivity of the contractile elements to $[\text{Ca}^{2+}]_i$, in cultured chick embryonic and adult guinea pig ventricular myocytes. (Circulation Research 1990;66:622–632)

The depressant effect of acidosis on the force of cardiac muscle contraction has been long recognized$^{1,2}$ and appears to result almost entirely from a reduction in intracellular pH (pHi) and not a reduction in extracellular pH.$^3$ Much of this inhibitory action is caused by a direct effect of H$^+$ on the Ca$^{2+}$ sensitivity of the contractile proteins.$^4,5$ Recent work has indicated that intracellular acidosis also modifies cardiac [Ca$^{2+}$]$_i$ levels, thereby altering the availability of Ca$^{2+}$ to the myofilaments. For example, intracellular acidosis increases resting [Ca$^{2+}$]$_i$ measured with calcium-sensitive microelectrodes in cardiac Purkinje strands.$^6$ In contracting papillary muscles, intracellular acidosis produced by increasing CO$_2$ concentration or by the application and subsequent removal of NH$_4$Cl results in increased [Ca$^{2+}$]$_i$, transients measured with aequorin.$^7-9$ However, pHi-induced changes in resting or diastolic [Ca$^{2+}$]$_i$, are not detectable with aequorin unless [Ca$^{2+}$]$_i$ is increased above normal or the sodium pump is inhibited.$^7-9$ In contracting cultured chick ventricular cells, NH$_4$Cl removal has also been reported to increase both intracellular calcium con-
tent, measured with atomic absorption spectrophotometry, and time-averaged [Ca\(^{2+}\)]\(_i\), estimated from fura-2 fluorescence. However, neither of these two techniques provides selective detection of changes in both diastolic and systolic [Ca\(^{2+}\)], levels in contracting heart cells.

Improved resolution of pH\(_i\) effects on [Ca\(^{2+}\)], transients has recently been reported by Lee et al\(^{12}\) with intact rabbit hearts loaded intracellularly with the Ca\(^{2+}\) fluorescent dye indo-1. These authors found that acidosis produced by elevated CO\(_2\) increases both systolic and diastolic [Ca\(^{2+}\)]. However, the mechanisms by which changes in pH\(_i\) affect [Ca\(^{2+}\)], and the relative effects of pH\(_i\) on diastolic and systolic [Ca\(^{2+}\)], have not been completely elucidated. Several schemes have been proposed to account for pH\(_i\)-induced changes in [Ca\(^{2+}\)]. For example, intracellular acidosis could lead to H\(^+\) efflux and Na\(^+\) influx via Na\(^+\)-H\(^+\) exchange in cultured ventricular cells. An increased sodium influx via Na\(^+\)-H\(^+\) exchange may augment calcium influx or slow calcium efflux by modulation of Na\(^+\)-Ca\(^{2+}\) exchange. Recent work by Kim and Smith\(^{11}\) has suggested that this is the primary mechanism by which [Ca\(^{2+}\)], is increased by decreased pH\(_i\). However, Allen et al\(^{13}\) found that, in an aequorin-loaded ferret ventricular muscle, CO\(_2\)-induced acidification increased [Ca\(^{2+}\)], even in the absence of Na\(_o\), thus questioning the involvement of Na\(^+\)-H\(^+\) and Na\(^+\)-Ca\(^{2+}\) exchange in this phenomenon.

Intracellular acidosis has been reported to reduce the uptake of calcium by sarcoplasmic reticulum (SR). In addition, acidosis would be expected to decrease calcium extrusion via Na\(^+\)-Ca\(^{2+}\) exchange. Both of these effects would be expected to decrease the rate of decline in the [Ca\(^{2+}\)], transient during intracellular acidosis, as reported by Orchard\(^{8}\) and Lee et al.\(^{12}\) However, Kim and Smith\(^{11}\) reported no change in Ca\(^{2+}\) efflux in cultured heart cells during intracellular acidosis produced by washout of NH\(_4\)Cl; they also reported an effect of caffeine but not of ryanodine\(^{11}\) on the increase in [Ca\(^{2+}\)], produced by intracellular acidosis.

Changes in pH\(_i\) may also affect calcium homeostasis within mitochondria and binding of Ca\(^{2+}\) to sarcosomal phospholipids and proteins. Indeed, studies by Vaughan-Jones et al\(^{11}\) have suggested that competition between Ca\(^{2+}\) and H\(^+\) at intracellular binding sites occurs and that this may account for the interaction between pH and [Ca\(^{2+}\)], in myocardium, independent of an alteration of Na\(^+\)-H\(^+\) or Na\(^+\)-Ca\(^{2+}\) exchange. In the present work, we have used improved methodologies for measurement of phasic changes in [Ca\(^{2+}\)], with indo-1\(^{22}\) during rapid changes in pH\(_i\) produced by abrupt exposure to and washout of NH\(_4\)Cl. We have attempted to reexamine the role of different mechanisms, described above, in contributing to the increase in [Ca\(^{2+}\)], produced by a decrease in pH, in ventricular myocytes.

**Materials and Methods**

**Ventricular Myocyte Culture**

Layer cultures of spontaneously contracting ventricular myocardial cells were prepared from 10-day-old chick embryos with modification of previously described techniques. Ventricles were minced and placed in Ca\(^{2+}\)-Mg\(^{2+}\)-free Hanks’ solution with 80 units/ml collagenase at 37°C for four cycles of 7 minutes each. The supernatant was discarded, and the cells were resuspended in culture medium consisting of 6% heat-inactivated fetal calf serum, 40% medium 199 (GIBCO Laboratories, Grand Island, New York), 0.1% penicillin-streptomycin antibiotic solution, and 54% balanced salt solution containing (mM) NaCl 116, NaH\(_2\)PO\(_4\) 1.0, MgSO\(_4\) 0.8, NaHCO\(_3\) 26.2, and glucose 5. The cell suspension was diluted to 4 \times 10^5 cells/ml and placed in plastic Petri dishes containing glass coverslips. Cultures were incubated in a 5% CO\(_2\)-95% air atmosphere for 3 days at 37°C. All studies were performed on cells after 3 days of culture.

**Dissociation of Adult Ventricular Myocytes**

Hearts were removed from guinea pigs (200–300 g) anesthetized with sodium pentobarbital (40 mg/kg i.p.). The heart was immediately attached to an aortic cannula, which provided continuous retrograde coronary artery perfusion at 37°C by gravity at 60 cm H\(_2\)O. The heart was first perfused with nominally calcium-free solution for 3–5 minutes, immediately followed by 15 minutes of recirculating perfusion with the same solution containing 1 mg/ml collagenase (class II, Worthington Biochemical, Freehold, New Jersey). Both cell isolation solutions contained (mM) NaCl 126.0, KCl 4.4, MgCl\(_2\) 5.0, NaHCO\(_3\) 18.0, NaH\(_2\)PO\(_4\) 0.33, and glucose 11.0 and were gassed with 5% CO\(_2\)-95% O\(_2\) (pH 7.30). The heart was detached from the cannula, and the ventricles were cut into small pieces and then gently shaken for about 5 minutes at 37°C in the enzyme solution to disperse the cells.

**Cell Bathing Solutions**

The control superfusate for cultured chick ventricular cells was a HEPES-buffered normal Tyrode’s solution (NT) containing (mM) NaCl 137.0, KCl 3.7, MgCl\(_2\) 0.5, CaCl\(_2\) 1.8, glucose 5.6, and HEPES (free acid) 4.0 titrated to pH 7.35 with 2.1 mM NaOH. In low-sodium, nominal 0-calcium solution, NaCl was replaced by choline chloride, and CaCl\(_2\) was not added. The only sodium in this solution was that required for titration of HEPES (2.1 mM). Various test solutions contained 10 mM NH\(_4\)Cl or 10 mM caffeine added to the HEPES-buffered physiological solution described above.

In isolated adult myocyte experiments, the HEPES-buffered NT contained (mM) NaCl 140.0, KCl 4.4, CaCl\(_2\) 2.7, MgCl\(_2\) 1.0, glucose 11.0, and HEPES (free acid) 12.0 titrated to pH 7.35 with 6.4 mM NaOH.

**Simultaneous Measurement of [Ca\(^{2+}\)],** and **Cell Motion**

[Ca\(^{2+}\)] was measured with Ca\(^{2+}\) fluorescent dye indo-1, as described by Peeters et al. To prepare the indo-1 AM (indo-1 acetoxymethy ester; Calbiochem, La Jolla, California), the method of duBell et
al was used. First, 10 ml fetal calf serum was mixed with 234 μl of 25% Pluronic F127 (wt/wt in dimethyl sulfoxide; Performance Chemicals, Parsippany, New Jersey) and sonicated. Then, 1,000 μl fresh 1 mM indo-1 AM in dimethyl sulfoxide (DMSO) was added to 9 ml of the fetal calf serum–Pluronic F127 mixture, sonicated to mix, and divided into fifty 200-μl aliquots, which were stored frozen at −20°C in light-proof containers. This “loading stock” of 100 μM indo-1 AM was diluted in culture medium or physiological buffer to give appropriate indo-1 AM concentrations for cell loading.

Coverslips of cultured chick ventricular cells were incubated at 37°C in culture media containing 5 μM indo-1 AM for 15 minutes and then washed in indo-1-free solution for 30 minutes. After dye loading, a coverslip was placed in the flow-through cell chamber and continuously superfused with NT. Approximately 2–3 seconds was required to exchange the solution in the cell chamber, which was equipped with a clear glass bottom and mounted on the stage of an inverted microscope (Nikon Diaphot, Tokyo, Japan). Solution temperature in the cell chamber was 37°C.

Dissociated adult ventricular myocytes were loaded with indo-1 with a modification of the above method. A 1.0-ml aliquot of cell-containing enzyme solution (described in previous section) was introduced into the cell chamber. Cells rapidly settled on the chamber bottom, which was treated with 1 μg/ml laminin (Collaborative Research, Bedford, Massachusetts) to improve cell adhesion. The enzyme-free, calcium-free solution was then continuously directed through the chamber for 1–2 minutes, followed by the same solution containing 2.7 mM calcium. Superfusion with HEPES-buffered NT was begun approximately 15 minutes later. After about 30 minutes in NT, the cells were incubated in NT containing 10 μM indo-1 AM for approximately 30 minutes. After loading, superfusion with NT was begun to remove extracellular dye.

The instrumentation used for fluorescence measurement has been described. Briefly, the system uses a high-pressure mercury-arc lamp for the excitation source because it provides an intense emission peak at 360 nm. Further selection of this peak was made with narrow-bandwidth interference filters at 360 Hz, and the specimen was illuminated via epifluorescence optics using a Fluor ×40 objective lens (Nikon). The fluorescent light was collected by the objective lens and divided with a beam splitter to permit simultaneous measurement of both 410 and 480 nm wavelengths by use of two separate photomultiplier tubes. The ratio of emitted fluorescence (410/480 nm) was obtained on-line through an analog divider circuit. Previous calibration studies in cultured chick ventricular myocytes have shown that under control conditions end-diastolic [Ca2+]i averages 328 nM and peak-systolic [Ca2+]i averages 813 nM. In the present work, we have not calibrated the [Ca2+]i levels and have used the 410/480 fluorescence ratio as an indicator of changes in [Ca2+]i. When measuring [Ca2+]i or pH in guinea pig myocytes, an adjustable rectangular window was used to restrict the optical image to the cell of interest, thereby minimizing background fluorescence from other cells and debris.

The image of the cells was obtained by illumination via the standard microscope light source passed through a 700-nm bandpass filter. This wavelength was long enough not to interfere with the fluorescence detection at 410 and 480 nm. Cell motion in cultured cells was detected with a video motion analyzer (Colorado Video, Boulder, Colorado). Cell-length changes in guinea pig myocytes were measured with a video length detector. Although the chick monolayers were spontaneously contracting, none of the guinea pig cells used in this study displayed spontaneous contractions.

In some experiments, electrical field stimulation was used to pace guinea pig and chick myocytes. Constant current pulses (3–8-msec duration) were delivered via a glass capillary tube (70-μm tip diameter) filled with NT and positioned approximately 0.5 mm from the cell. The other electrode was an isolated silver wire (0.13-mm diameter) positioned 1 mm from the tip of the capillary tube.

**Intracellular pH Measurement**

pH was measured using the pH-sensitive fluorescent dye BCECF [2’,7’-bis(2-carboxethyl)-5(6)-carboxylfluorescein]. A stock solution of BCECF AM (BCECF acetoxymethyl ester, Calbiochem) was prepared by dissolving 1 mg dye in 1 ml DMSO. This was dispersed into 20-μl vials and stored at −25°C in the dark. Cells were equilibrated for 40–60 minutes in NT containing 20 μM BCECF AM stock solution. After dye loading, NT was continuously directed through the bath to remove extracellular dye. To measure pH, with BCECF, our fluorescence system was modified to provide dual excitation with rotating interference filters. Cells were sequentially excited at 500 and 440 nm (frequency, 180 Hz) while recording fluorescence emission at 530 nm. The ratio of emission intensity (500/440) served as the measure of pH. At the end of the experiment, the emission ratio was calibrated in situ as described by Thomas et al by exposing cells to solutions of varying pH. Each solution contained 11 mM of the K+-H+ exchange ionophore, nigericin (Calbiochem), and (mM) HEPES 12.0 (titrated with 1 M KOH), K+ 140.0 (KCl adjusted to keep K+ constant), MgCl2 1.0, and glucose 11.0.

**Ion Fluxes**

Na+ and Ca2+ fluxes were measured using modifications of previously described techniques. Briefly, for 24Na or 45Ca uptake experiments, coverslips of cells were immersed in 24Na or 45Ca containing media (37°C, 5 μCi/ml) for 30 and 60 seconds. Coverslips of cells were then washed in ice-cold physiological solution for 30 seconds, and the cells were scraped off the coverslip and dissolved in sodium dodecyl
sulfate–sodium borate solution. $^{24}$Na or $^{45}$Ca counts per sample were measured on a liquid scintillation spectrometer, and calculated sodium or calcium contents were normalized relative to milligrams of cell protein.

For $^{45}$Ca efflux measurements, cells were labeled to equilibrium with $^{45}$Ca (5 μCi/ml) for 2 hours. Individual coverslips were then washed for 30 seconds at 37°C to remove extracellular tracer. They were then immersed for 5 seconds serially in seven 2-ml volumes of efflux solution at 37°C. Thus, efflux was measured over a 35-second period. $^{45}$Ca counts were determined in each 2-ml volume, and the total $^{45}$Ca remaining in the cells on each individual coverslip after the total efflux was measured. The fractional efflux for each 5-second period was then calculated as the percent of total $^{45}$Ca counts present in the cells at the beginning of that 5-second period lost during the subsequent 5 seconds. This method has been described in detail previously.23

**Preparation of Cardiac Sarcoplasmic Reticulum Vesicles**

The procedure was modified from a method previously used to prepare SR vesicles from canine left ventricle.29 Tissue from ventricles excised from 18-day chick embryos was homogenized in 5 vol of 0.29 M sucrose, 3 mM Na₂HPO₄, 1 mM dithiothreitol, and 10 mM 3-(4-morpholino)propanesulfonic acid (MOPS) at pH 7.0 and 4°C (sucrose buffer), to which was added 2 mM EGTA, 0.1 mM pepstatin and 0.8 mM phenylmethylsulfonyl fluoride. After two 10-minute sedimentations at 8,000 rpm (5,000g) in a Beckman JA-20 rotor (Beckman Instruments, Fullerton, California), the supernatant was sedimented for 60 minutes at 19,000 rpm (30,000g) in a Beckman 55.2 Ti rotor. The resulting pellet was resuspended by hand homogenization (glass-glass) in 10 ml of 0.6 M KCl, 3 mM Na₂HPO₄, 1 mM dithiothreitol, and 10 mM MOPS at pH 6.8 and 4°C. After resedimentation for 40 minutes at 37,000 rpm (124,000g) in a Beckman 55.2 Ti rotor, the pellet was suspended in sucrose buffer and stored at −80°C. The procedure yielded 1.8–2.0 mg SR protein30 per gram of starting material.

**Ca²⁺ Uptake by Isolated Sarcoplasmic Reticulum**

The procedure was modified from a previously published method.29 Vesicles prepared as described were suspended at 0.18 mg/ml in 0.12 M KCl, 1.0 mM EGTA, 5.0 mM oxalic acid, and 20 mM MOPS at pH 7.05 and 37°C (uptake buffer). $^{45}$CaCl₂ was added as appropriate to yield a free [Ca²⁺] of 0.2 to 0.8 μM at a free [Mg²⁺] of 0.4 mM according to Fabiato's method.31 Ca²⁺ uptake was initiated by addition of 5.0 mM ATP. Aliquots were removed at 1–2-minute intervals over a 4- or 8-minute course throughout which uptake was linear. Aliquots were filtered and washed three times with 2.0 ml of 0.12 M KCl/2.0 mM EGTA under vacuum through 25-mm mixed cellulose ester disks of pore size 0.22 μm (Millipore, Bedford, Massachusetts). $^{45}$Ca adherent to filter disks (i.e., taken up by the vesicles) was determined by scintillation spectrometry. Uptake was normalized relative to vesicle protein, and Ca²⁺ uptake rates were calculated by linear regression.

**Statistical Analysis**

Data were expressed as mean±SEM. Unpaired Student's t-test or analysis of variance was used in comparing different groups.

**Results**

**Effects of Changes of pH, on [Ca²⁺], Transients and Cell Contraction**

The first experiments were designed to investigate the effects of changing pH on [Ca²⁺], transients and cell contraction under conditions in which the pH of the superfusing solution (extracellular pHₐ) was held constant at pH 7.35. This was achieved by brief external application and then removal of NH₄Cl, which produces reproducible changes in pHᵢ in a variety of cell types,32 including cultured chick ventricular cells.17

To limit the magnitude and duration of the intracellular acidosis, in all experiments we used a 2-minute exposure to 10 mM NH₄Cl. Figure 1 shows the effects of exposure to and washout of NH₄Cl on pHᵢ in cultured chick embryo ventricular cells and a resting adult guinea pig ventricular cell. The application of NH₄Cl initially produced an intracellular alkalosis because the highly permeant NH₃ crosses the membrane more quickly than does NH₄⁺.33 Once inside the cell, NH₃ combines with H⁺ to form NH₄⁺, thus raising pHᵢ. However, during continued exposure to NH₄Cl, the initial alkalosis is not maintained, and pHᵢ gradually declines as NH₄⁺ enters the cell. A possible contribution of HCO₃⁻ efflux to this secondary decline in pHᵢ seems unlikely since the experiments were conducted in HEPES-buffered solutions containing no added CO₂ or HCO₃⁻. The subsequent abrupt removal of external NH₄Cl produces a
marked intracellular acidosis that occurs because NH$_4^+$ ions that have accumulated intracellularly dissociate to form NH$_3$, which rapidly leaves the cell resulting in intracellular retention of H$.^4$ The subsequent increase in pH occurs against the inwardly directed electrochemical gradient for H$.^4$ Since these experiments were performed in the absence of HCO$_3^-$ or CO$_2$, this acid-load recovery is probably mediated by Na$^+$/H$^+$ exchange.$^{10,35,36}$

Examples of the effects of pH$_i$ changes produced by exposure to and washout of NH$_4$Cl on [Ca$^{2+}$], transients and simultaneous cell contractions are shown in Figures 2 and 3. Alkalosis (exposure to 10 mM NH$_4$Cl) increased the amplitude of cell contraction but slightly decreased both the peak and the diastolic level of [Ca$^{2+}$]. Intracellular acidosis decreased the amplitude of cell contraction but increased both end-diastolic and peak-systolic [Ca$^{2+}$]. Similar results were obtained from six different cultures. The sensitivity of indo-1 to [Ca$^{2+}$] is not changed significantly in these pH ranges.$^{12}$ Thus, it is evident that intracellular acidosis decreases the Ca$^{2+}$ sensitivity of contractile elements.

We also studied the effects of pH$_i$ changes on [Ca$^{2+}$], and cell length in adult guinea pig ventricular myocytes. In Figure 3A, the cell was paced at 0.2 Hz. Exposure to 10 mM NH$_4$Cl (causing intracellular alkalosis) decreased [Ca$^{2+}$], but increased both end-diastolic and peak-systolic cell shortening. Subsequent washout of NH$_4$Cl caused an increase in [Ca$^{2+}$], but decreased cell shortening (Figure 3A). In Figure 3B, the same cell was not paced and was quiescent. Exposure to NH$_4$Cl decreased resting [Ca$^{2+}$], but decreased resting cell length. Washout of NH$_4$Cl increased resting [Ca$^{2+}$], but increased resting cell length. Similar results were obtained in 13 other cells. These results indicate that intracellular acidosis causes an increase in [Ca$^{2+}$], as well as a decrease in the sensitivity of contractile elements to Ca$^{2+}$ both in cultured embryonic ventricular cells and in adult isolated ventricular myocytes.

In these experiments, diastolic [Ca$^{2+}$], went up before an increase in systolic [Ca$^{2+}$], was observed. This suggested that intracellular acidosis may increase [Ca$^{2+}$], initially through an impairment of diastolic calcium extrusion or reuptake by SR. Support for this hypothesis is provided by Figure 4, which shows an example of the effects of intracellular acidosis on [Ca$^{2+}$], transients, d[Ca$^{2+}$]/dt, and motion in cultured cells that were paced to maintain a constant contraction rate. As can be seen, there was an initial decrease in peak negative d[Ca$^{2+}$]/dt preceding the increase in peak [Ca$^{2+}$]. Figure 5 summarizes these changes in [Ca$^{2+}$], and d[Ca$^{2+}$]/dt in four paced cultured preparations after NH$_4$Cl removal. The increase in diastolic [Ca$^{2+}$], and decrease in peak negative d[Ca$^{2+}$]/dt preceded the increase in peak-systolic [Ca$^{2+}$]. As shown in Figures 4 and 5, intracellular acidosis also initially decreased both the magnitude of the Ca$^{2+}$ transient (peak-systolic minus end-diastolic [Ca$^{2+}$]) and the

**FIGURE 2.** Tracings showing effect of exposure to and washout of 10 mM NH$_4$Cl on [Ca$^{2+}$], transients (ratio of 410/480 nm indo-1 fluorescence, upper tracings) and motion (lower tracings) in a layer culture of spontaneously contracting chick embryo ventricular cells. In this and subsequent figures, the time base was initially briefly increased ×10 to display individual [Ca$^{2+}$], transients and motion. Panel A: Example showing that exposure to NH$_4$Cl (and the resulting intracellular alkalosis) causes a reduction in both end-diastolic and peak-systolic [Ca$^{2+}$], yet peak-systolic position in the motion signal shifts upward, consistent with an increase in sensitivity of the contractile elements to [Ca$^{2+}$]. In the right part of panel A, the effects of NH$_4$Cl washout are shown. During intracellular acidosis induced by washout of NH$_4$Cl, there was a rapid initial increase in end-diastolic [Ca$^{2+}$], followed by an increase in peak-systolic [Ca$^{2+}$]. There was a downward shift in diastolic and peak-systolic cell position, indicating a decrease in sensitivity of the contractile elements to [Ca$^{2+}$]. Panel B: Another example of the effects of NH$_4$Cl washout on [Ca$^{2+}$], and motion in cultured ventricular myocytes. In this example, there was almost complete inhibition of cell motion during intracellular acidosis in spite of a marked increase in diastolic and peak systolic [Ca$^{2+}$]. In these spontaneously contracting cells, there was frequently transient slowing of the rate of beating during both exposure to and washout of NH$_4$Cl.
Factors Causing an Increase in \([\text{Ca}^{2+}]_i\), During Intracellular Acidosis

There are several mechanisms that could contribute to the initial increase in diastolic \([\text{Ca}^{2+}]_i\), elicited by intracellular acidosis. First, it is possible that a fall in pH decreases \(\text{Ca}^{2+}\) uptake by SR. To test this hypothesis, we determined the effects of acidosis on \(\text{Ca}^{2+}\) uptake by SR vesicles isolated from embryonic chick ventricular myocardium (Table 1). Over the range of \([\text{Ca}^{2+}]_i\) values developed in these cells during a normal \([\text{Ca}^{2+}]_i\), transient, there was a marked inhibition of \(\text{Ca}^{2+}\) uptake as pH was reduced from 7.05 to 6.60. This could contribute to the decrease in peak negative \(d[\text{Ca}^{2+}]_i/dt\) shown in Figures 4 and 5. However, it is unlikely that the \([\text{Ca}^{2+}]_i\) increase caused by intracellular acidosis is due solely to an effect on \(\text{Ca}^{2+}\) uptake by SR, because, as shown in Figure 6, if \(\text{NH}_4\text{Cl}\) washout is performed in the presence of caffeine, an increase in \([\text{Ca}^{2+}]_i\) still occurs. With exposure to caffeine, there was a transient increase in both diastolic and systolic \([\text{Ca}^{2+}]_i\), and then the amplitude of \([\text{Ca}^{2+}]_i\) transients decreased, although smaller \([\text{Ca}^{2+}]_i\) transients continued. During exposure to \(\text{NH}_4\text{Cl}\), there was a significant decrease in \([\text{Ca}^{2+}]_i\), and subsequent washout of \(\text{NH}_4\text{Cl}\) increased \([\text{Ca}^{2+}]_i\), at least as much as under control conditions (e.g., see Figure 2).

These experiments suggested that, although diminished SR \(\text{Ca}^{2+}\) uptake during intracellular acidosis may have contributed to the observed increase in \([\text{Ca}^{2+}]_i\), and decrease in peak negative \(d[\text{Ca}^{2+}]_i/dt\), other mechanisms must also be involved. Intracellular acidosis may directly inhibit \(\text{Na}^-\text{Ca}^{2+}\) exchange,
the primary transsarcolemmal Ca\textsuperscript{2+} extrusion system in these cells.\textsuperscript{23} In addition, intracellular acidosis could lead to H\textsuperscript{+} efflux and Na\textsuperscript{+} influx via Na\textsuperscript{+}-H\textsuperscript{+} exchange in cultured ventricular cells.\textsuperscript{10} This increased sodium influx via Na\textsuperscript{+}-H\textsuperscript{+} exchange may result in an increase in Na\textsubscript{o} and, thus, also increase [Ca\textsuperscript{2+}], via an alteration of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange.\textsuperscript{17}

To examine these possibilities, we first sought to demonstrate Na\textsuperscript{+}-H\textsuperscript{+} exchange in ventricular myocytes during recovery from acidosis. Cultured chick ventricular myocytes were exposed to 10 mM NH\textsubscript{4}Cl in low-sodium, nominal 0-calcium solution (Figure 7). Exposure to low-sodium, nominal 0-calcium solution decreased pH\textsubscript{i}, probably due to Na\textsuperscript{+}-H\textsuperscript{+} exchange inhibition and a transient increase in [Ca\textsuperscript{2+}]. Exposure to NH\textsubscript{4}Cl increased pH\textsubscript{i} and subsequent washout of NH\textsubscript{4}Cl caused intracellular acidosis. These effects were similar to those caused by NH\textsubscript{4}Cl exposure and washout in normal sodium, normal calcium solution. However, in low-sodium, 0-calcium solution, there was slower rate of pH\textsubscript{i} recovery after NH\textsubscript{4}Cl washout. To show this more clearly, normal Na\textsubscript{o} was abruptly resupplied 60 seconds after the second washout of NH\textsubscript{4}Cl in the same cell. As shown in Figure 7, intracellular pH recovery was markedly accelerated by resupply of Na\textsubscript{o}. These results indicate that Na\textsuperscript{+}-H\textsuperscript{+} exchange does contrib-

Table 1. Effects of pH Change on \textsuperscript{46}Ca Uptake by Sarcoplasmic Reticulum

<table>
<thead>
<tr>
<th>pH</th>
<th>200 nM Ca\textsuperscript{2+}</th>
<th>500 nM Ca\textsuperscript{2+}</th>
<th>800 nM Ca\textsuperscript{2+}</th>
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<td>7.05</td>
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<td>111.4±2.3</td>
<td>162.9±4.1</td>
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<td>6.80</td>
<td>21.7±1.1</td>
<td>71.4±3.0</td>
<td>120.5±6.4</td>
</tr>
<tr>
<td>6.60</td>
<td>3.0±0.1</td>
<td>18.4±1.3</td>
<td>34.9±1.0</td>
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Values are mean±SEM; n=4.

To determine further if Na\textsuperscript{+}-H\textsuperscript{+} exchange is stimulated during intracellular acidosis, we studied \textsuperscript{24}Na uptake in cultured cells during NH\textsubscript{4}Cl washout in the presence and absence of amiloride (Table 2). Relative to control in NT, acidosis did not increase \textsuperscript{24}Na uptake by 30 seconds and only slightly increased \textsuperscript{24}Na uptake by 60 seconds. Amiloride-sensitive \textsuperscript{24}Na uptake was also measured to estimate that component of sodium uptake resulting from Na\textsuperscript{+}-H\textsuperscript{+} exchange. As shown in Table 2, although amiloride did not decrease sodium influx relative to control, during intracellular acidosis there was a significant increase in the amiloride-sensitive component of...
TABLE 2. Effects of Acidosis and Amiloride on \(^{24}\text{Na}\) Uptake

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<thead>
<tr>
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<th>(^{24}\text{Na}) uptake (nmol/mg protein)</th>
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<tr>
<td></td>
<td>30 sec</td>
</tr>
<tr>
<td>Control</td>
<td>31.9±1.8</td>
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<tr>
<td>Amiloride (1 mM)</td>
<td>31.0±1.5*</td>
</tr>
<tr>
<td>Acidosis</td>
<td>32.7±1.6*</td>
</tr>
<tr>
<td>Acidosis + amiloride</td>
<td>26.8±1.5†</td>
</tr>
</tbody>
</table>

Values are mean±SEM; \(n=8-10\).

\(^*p<0.05\) vs. control.

\(^†p<0.05\) vs. control.

Sodium uptake. These results suggested that although the amiloride-sensitive component of rapid sodium uptake (presumably reflecting \(\text{Na}^+-\text{H}^+\) exchange) was increased during acidosis, net rapid sodium uptake was not altered. This might occur if acidosis increased sodium uptake via \(\text{Na}^+-\text{H}^+\) exchange but decreased sodium influx by \(\text{Na}^+-\text{Ca}^{2+}\) exchange. To examine this possibility, fractional efflux of \(^{45}\text{Ca}\) was measured at 5-second intervals in cultured cells in \(\text{NH}_4\text{Cl}\) and in cells in which \(\text{NH}_4\text{Cl}\) was quickly removed (Figure 8). Fractional efflux of \(^{45}\text{Ca}\) was significantly diminished by \(\text{NH}_4\text{Cl}\) washout, in spite of the increase in [\(\text{Ca}^{2+}\)], that occurs under these conditions (Figure 2). This finding is consistent with a decrease in \(\text{Na}^+-\text{H}^+\)-dependent calcium efflux via \(\text{Na}^+-\text{Ca}^{2+}\) exchange in spontaneously contracting cells, due to a direct inhibition of \(\text{Na}^+-\text{Ca}^{2+}\) exchange by intracellular acidosis.

We also studied the effects of pH changes on \(^{45}\text{Ca}\) uptake (Table 3). To produce intracellular alkalosis, cells were preincubated in normal HEPES-buffered physiological solution for 2 minutes and then immersed in uptake solution containing 10 mM \(\text{NH}_4\text{Cl}\). Under these conditions, \(^{45}\text{Ca}\) uptake was significantly decreased compared with control. Intracellular acidosis was produced by first preincubating cells in 10 mM \(\text{NH}_4\text{Cl}\); then \(^{45}\text{Ca}\) uptake was measured after abruptly immersing cells in solution without \(\text{NH}_4\text{Cl}\). Abrupt production of intracellular acidosis did not significantly increase \(^{45}\text{Ca}\) uptake compared with that during alkalosis, and \(^{45}\text{Ca}\) uptake remained significantly decreased as compared with control uptake. This attenuated calcium uptake may reflect partial inhibition of inward calcium current and/or calcium entry via \(\text{Na}^+-\text{Ca}^{2+}\) exchange.

These results, taken together, suggest that augmented \(\text{Ca}^{2+}\) influx via \(\text{Na}^+-\text{Ca}^{2+}\) exchange, produced by an increased [\(\text{Na}^+\)], does not contribute significantly to the increase in [\(\text{Ca}^{2+}\)], occurring during acidosis under these experimental conditions. Additional support for this view is provided by Figure 9, which shows effects of \(\text{NH}_4\text{Cl}\) washout on [\(\text{Ca}^{2+}\)] in low-sodium, nominal 0-calcium solution. If an increase in [\(\text{Ca}^{2+}\)] during intracellular acidosis was caused primarily by alteration of \(\text{Na}^+-\text{Ca}^{2+}\) exchange via an increase in \(\text{Na}^+\) mediated by \(\text{Na}^+-\text{H}^+\) exchange, this increase should not be observed in low-sodium, nominal 0-calcium solution. In low-sodium, 0-calcium solution, \(\text{Na}^+-\text{H}^+\) exchange is attenuated during acidosis. Furthermore, in nominal 0-\(\text{Ca}^{2+}\) solution, any increase in [\(\text{Na}^+\)], that might occur due to residual \(\text{Na}^+-\text{H}^+\) exchange or acidosis-induced inhibition of the sodium pump could not produce \(\text{Ca}^{2+}\) influx via \(\text{Na}^+-\text{Ca}^{2+}\) exchange. As shown in

**Figure 8.** Graph showing effect of \(\text{NH}_4\text{Cl}\) washout on \(^{45}\text{Ca}\) efflux in cultured ventricular cells. Fractional efflux of \(^{45}\text{Ca}\) was measured at 5-second intervals in cells in \(\text{NH}_4\text{Cl}\) (open squares) and in cells in which \(\text{NH}_4\text{Cl}\) was removed after the first 10 seconds (vertical arrow, closed diamonds). \(^{45}\text{Ca}\) efflux was significantly diminished within 5 seconds after \(\text{NH}_4\text{Cl}\) washout, in spite of the increase in [\(\text{Ca}^{2+}\)], that occurs under these conditions (Figure 2). \(^*p<0.01\).

**Figure 9.** Tracing showing effects of intracellular alkalosis and acidosis on [\(\text{Ca}^{2+}\)], in chick ventricular cells exposed to low-sodium, nominal 0-calcium solution. A slight fall in [\(\text{Ca}^{2+}\)], occurred during alkalosis, and an increase in [\(\text{Ca}^{2+}\)], was elicited by intracellular acidosis, comparable with that in normal-sodium, normal-calcium solution (see Figures 2 and 3).
Acidosis decreases exposed calcium caused a 10-fold decrease in [Ca^{2+}], and then [Ca^{2+}] returned to normal diastolic levels in 2 minutes. Alkalosis produced by NH4Cl caused an abrupt increase in [Ca^{2+}], and subsequent washout of NH4Cl caused a transient increase in [Ca^{2+}], which was comparable with that produced in normal sodium solution (compare with Figure 2).

To exclude a possible effect of H^+ on SR uptake of Ca^{2+} under these conditions, we performed the experiments illustrated in Figure 10. In this culture, after superfusion with low-sodium, nominal 0-calcium solution, Ca^{2+} was released from the SR by abrupt exposure to caffeine, before exposure to and washout of NH4Cl. Even when the SR was disabled by the presence of 10 mM caffeine, in the presence of low-sodium, nominal 0-calcium solution, exposure to NH4Cl caused a decrease in [Ca^{2+}], and NH4Cl washout caused [Ca^{2+}] to increase. This result suggests that a significant component of the acidosis-induced increase in [Ca^{2+}], results from Ca^{2+} displacement from intracellular buffers and/or organelles other than SR.

**Discussion**

**Ca^{2+} Sensitivity of Cardiac Muscle During Intracellular Acidosis**

Studies of skinned ventricular muscle fibers have revealed that acidosis markedly decreases myofilament sensitivity to Ca^{2+}; this decrease produces a right shift in the tension-pCa curve. Subsequent work demonstrated that the sensitivity of cardiac myofilaments to acidosis results in part from a decrease in the affinity of troponin C for calcium. Allen and Orchard confirmed this effect in intact ventricular myocardium by showing that a decrease in twitch tension during intracellular acidosis was not associated with a decrease in systolic [Ca^{2+}].

Allen and Orchard confirmed this effect in intact ventricular myocardium by showing that a decrease in twitch tension during intracellular acidosis was not associated with a decrease in systolic [Ca^{2+}], transients measured with aequorin. The newly developed [Ca^{2+}], indicator, indo-1, has some advantages over aequorin. Indo-1 is sensitive to diastolic levels of [Ca^{2+}], and relatively insensitive to pH changes or changes in [Mg^{2+}]. The present work with indo-1 provides further confirmation of the marked effect of H^+ on Ca^{2+} sensitivity of contractile elements in intact myocardium, by simultaneous measurement of [Ca^{2+}] and cell contraction during intracellular acidosis in both cultured chick embryo ventricular myocytes and adult guinea pig ventricular cells. As shown in Figures 2 and 3, during intracellular acidosis, the magnitude of the [Ca^{2+}] transient is increased while the magnitude of cell contraction is decreased.

**The Mechanism of [Ca^{2+}] Increase During Intracellular Acidosis**

Bers and Ellis reported that intracellular acidosis induced by NH4Cl removal increases resting [Ca^{2+}], measured with Ca^{2+}-sensitive microelectrodes, in cardiac Purkinje strands. They also found a close temporal relation between the changes in [Ca^{2+}], and pH (pH microelectrode). Our results also provide evidence for a rapid effect of increased [H^+] on diastolic [Ca^{2+}]. There are several possible mechanisms for a diastolic or resting [Ca^{2+}] increase during intracellular acidosis, and one that has been frequently considered is alteration of transsarcomemmal Ca^{2+} flux mediated by Na^+-Ca^{2+} exchange.

For example, Na^+-H^+ exchange occurs in cultured chick ventricular cells, and Piwnica-Worms et al. reported that intracellular acidosis induced by exposure to and washout of 20 mM NH4Cl caused a marked increase in intracellular sodium content and a rise in intracellular calcium content. Kim and Smith have reported a lag between the fall in pH, induced by 20 mM NH4Cl washout and the subsequent rise in [Ca^{2+}]. In addition, they found that inhibition of Na^+-H^+ exchange by ethylisopropylamiloride completely eliminated this slowly developing [Ca^{2+}] increase during intracellular acidosis and suggested that sodium loading via Na^+-H^+ exchange during intracellular acidosis is solely responsible for the [Ca^{2+}] increase by changing the Na^+-Ca^{2+} exchange equilibrium. Although differences in the degree of intracellular acidosis produced and methods for [Ca^{2+}], detection could account for these findings, our results suggest that other mechanisms must be considered as a cause of the increase in [Ca^{2+}], produced by intracellular acidosis. At the lower NH4Cl concentration (10 mM) used in our studies, we were able to achieve an increase in [Ca^{2+}], with acidosis in the absence of significant stimulation of net Na^+ influx (Table 1). Na^+-H^+ exchange (amiloride-sensitive sodium uptake) was activated by acidosis, but amiloride-insensitive sodium influx (in part Na^+-Ca^{2+} exchange) was decreased by acidosis (Table 2). Thus, net sodium influx was not increased under these experimental conditions. We also observed an increase in [Ca^{2+}] on NH4Cl washout in low-sodium, 0-calcium solution (Figures 6 and 7) that was comparable in magnitude with that occurring in normal sodium solution.
With a rapidly exchanging superfusion system and indo-1 as a Ca\(^{2+}\)-indicator, we also observed a much more rapid [Ca\(^{2+}\)] increase after washout of NH\(_4\)Cl than reported by Kim and Smith.\(^{11}\) Furthermore, this [Ca\(^{2+}\)] increase was associated with a decrease in peak negative d[Ca\(^{2+}\)]/dt and an initial greater increase in end-diastolic [Ca\(^{2+}\)], than in peak-systolic [Ca\(^{2+}\)], suggesting that Ca\(^{2+}\) uptake and/or extrusion mechanisms were rapidly impaired by NH\(_4\)Cl washout. Both Ca\(^{2+}\) extrusion by Na\(^+\)-Ca\(^{2+}\) exchange and SR Ca\(^{2+}\) uptake contribute to the rapid fall in [Ca\(^{2+}\)], during myocyte relaxation,\(^{44}\) and thus, alteration of either could account in part for the rise in diastolic [Ca\(^{2+}\)], and decline in peak negative d[Ca\(^{2+}\)]/dt during intracellular acidosis. Our finding of an inhibitory effect of acidosis on SR vesicle Ca\(^{2+}\) uptake is consistent with earlier work\(^{4,14}\) and is evidence for a possible alteration of SR function during acidosis. Our \(^{40}\)Ca efflux results also indicate that Ca\(^{2+}\) extrusion via Na\(^+\)-Ca\(^{2+}\) exchange is inhibited. However, we observed a [Ca\(^{2+}\)] increase in caffeine-treated cells during intracellular acidosis both in low and normal Na\(_0\). This suggests that neither compromised SR Ca\(^{2+}\) uptake (Table 1) nor impaired Ca\(^{2+}\) extrusion via Na\(^+\)-Ca\(^{2+}\) exchange can completely account for the [Ca\(^{2+}\)] increase during acidosis. Since transsarcolemmal calcium influx is not increased by NH\(_4\)Cl washout (Table 3), we conclude that Ca\(^{2+}\)-H\(^{+}\) exchange at intracellular buffer sites other than SR is involved in the [Ca\(^{2+}\)] increase produced by intracellular acidification.

It is important to note, however, that alterations in SR function by intracellular acidosis may affect the time course of changes in [Ca\(^{2+}\)], induced by NH\(_4\)Cl washout. For example, Orchard\(^{8}\) has reported the effects of intracellular acidosis produced by NH\(_4\)Cl washout on tension and [Ca\(^{2+}\)], transients (measured with aequorin) in ferret right ventricular papillary muscle. In this intact tissue preparation, on washout of NH\(_4\)Cl, there was a decrease in twitch tension with initially no change on average in peak systolic [Ca\(^{2+}\)]. There was a slowing in the decline of the [Ca\(^{2+}\)] transient initially, followed by a marked increase in the peak of the aequorin [Ca\(^{2+}\)], transient and partial recovery of tension development. In the presence of caffeine (10 mM), there was a more rapid increase in systolic [Ca\(^{2+}\)], on NH\(_4\)Cl washout and an attenuation of contractile tension recovery. Orchard suggested that intracellular acidosis has two effects: reduction in Ca\(^{2+}\) binding within the cell and reduction in Ca\(^{2+}\) release from the SR. Under control conditions, the former would tend to increase the size of the [Ca\(^{2+}\)], transient and the latter to decrease it, resulting in an initial lack of change in the peak [Ca\(^{2+}\)]. In caffeine, however, an effect on the binding of Ca\(^{2+}\) predominates, resulting in a more rapid increase in the peak of the [Ca\(^{2+}\)], transient.

Although our experiments were performed in a different preparation and used a shorter exposure time to a lower NH\(_4\)Cl concentration, our results are consistent with this interpretation. First, aequorin cannot detect changes in diastolic [Ca\(^{2+}\)], in the ferret papillary muscle preparations.\(^{8}\) Thus, the initial increase in diastolic [Ca\(^{2+}\)], preceding the increase in peak systolic [Ca\(^{2+}\)], we have detected could have been missed in the aequorin measurements. The relative insensitivity of “time-averaged” [Ca\(^{2+}\)], measured with fura-2 by Kim and Smith\(^{11}\) may also account for their failure to detect an increase in [Ca\(^{2+}\)], early after washout of NH\(_4\)Cl in cultured ventricular myocytes. Our findings of the influence of caffeine on the effects of NH\(_4\)Cl washout are also consistent with the observations of Orchard.\(^{8}\) That is, the increase in peak systolic [Ca\(^{2+}\)], on washout of NH\(_4\)Cl was somewhat more rapid in the presence of caffeine (compare Figures 2 and 4 with Figure 6). That this could reflect a decrease in Ca\(^{2+}\) release from SR is supported by the observed decline in peak d[Ca\(^{2+}\)]/dt early after NH\(_4\)Cl washout (Figures 4 and 5). Our results, discussed above, and the findings of Orchard\(^{8}\) and Lee et al\(^{12}\) of a slowing in fall of the [Ca\(^{2+}\)], transient are also consistent with an effect of intracellular acidosis on SR Ca\(^{2+}\) uptake.

We conclude that the effects of intracellular acidosis on [Ca\(^{2+}\)], are multifaceted and, under the experimental conditions employed in the present study, probably include impairment of calcium extrusion via Na\(^+\)-Ca\(^{2+}\) exchange, impairment of calcium uptake by sarcoplasmic reticulum, and competition between H\(^{+}\) and Ca\(^{2+}\) for intracellular calcium–binding sites.

Acknowledgments

We gratefully acknowledge Bruce M. Steadman and Kent B. Moore for designing and constructing many of the electronic and mechanical devices required for fluorescence measurements, signal processing, and solution switching.

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KEY WORDS  •  cardiac myocytes  •  contraction  •  intracellular calcium  •  intracellular pH
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O Kohmoto, K W Spitzer, M A Movsesian and W H Barry

doi: 10.1161/01.RES.66.3.622

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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