Effects of Acidosis on Resting Cytosolic and Mitochondrial Ca²⁺ in Mammalian Myocardium

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ABSTRACT   Acidosis increases resting cytosolic [Ca²⁺], (Ca$_i$) of myocardial preparations; however, neither the Ca²⁺ sources for the increase in Ca$_i$ nor the effect of acidosis on mitochondrial free [Ca²⁺], (Ca$_m$) have been characterized. In this study cytosolic pH (pH$_i$) was monitored in adult rat left ventricular myocytes loaded with the acetoxymethyl ester (AM form) of SNARF-1. A stable decrease in the pH$_i$ of 0.52 ± 0.05 U (n = 16) was obtained by switching from a bicarbonate buffer equilibrated with 5% CO₂ to a buffer equilibrated with 20% CO₂. Electrical stimulation at either 0.5 or 1.5 Hz had no effect on pH$_i$ in 5% CO₂, nor did it affect the magnitude of pH$_i$ decrease in response to hypercarbic acidosis. Ca$_i$ was measured in myocytes loaded with indo-1/free acid and Ca$_m$ was monitored in cells loaded with indo-1/AM after quenching cytosolic indo-1 fluorescence with MnCl₂. In quiescent intact myocytes bathed in 1.5 mM [Ca²⁺], hypercarbia increased Ca$_i$ from 130 ± 5 to 221 ± 13 nM. However, when acidosis was effected in electrically stimulated myocytes, diastolic Ca$_i$ increased more than resting Ca$_i$ in quiescent myocytes, and during pacing at 1.5 Hz diastolic Ca$_i$ was higher (285 ± 17 nM) than at 0.5 Hz (245 ± 18 nM; P < 0.05). The magnitude of Ca$_i$ increase in quiescent myocytes was not affected either by sarcoplasmic reticulum (SR) Ca²⁺ depletion with ryanodine or by SR Ca²⁺ depletion and concomitant superfusion with a Ca²⁺-free buffer. In unstimulated intact myocytes hypercarbia increased Ca$_m$ from 95 ± 12 to 147 ± 19 nM and this response was not modified either by ryanodine and a Ca²⁺-free buffer or by 50 μM ruthenium red in order to block the mitochondrial uniporter. In mitochondrial suspensions loaded either with BCECF/AM or indo-1/AM, acidosis produced by lactic acid addition decreased both intra- and extramitochondrial pH and increased Ca$_m$. Studies of mitochondrial suspensions bathed in indo-1/free acid–containing solution showed an increase in extramitochondrial Ca²⁺ after the addition of lactic acid. Thus, in quiescent myocytes, cytoplasmic and intramitochondrial buffers, rather than transsarcolemmal Ca²⁺ influx or SR Ca²⁺ release, are the likely Ca²⁺ sources for the increase in Ca$_i$ and Ca$_m$, respectively;

additionally, Ca^{2+} efflux from the mitochondria may contribute to the raise in Ca_i. In contrast, in response to acidosis, diastolic Ca_i in electrically stimulated myocytes increases more than resting Ca_i in quiescent cells; this suggests that during pacing, net cell Ca^{2+} gain contributes to enhance diastolic Ca_i.

INTRODUCTION

It has been documented that a decrease in extracellular pH (pH_o) can profoundly modify myocardial function. Acidosis decreases transsarcolemmal Ca^{2+} influx (I_{Ca}) via L-type Ca^{2+} channels (Irisawa and Sato, 1986; Kaibara and Kameyama, 1988; Krafte and Kass, 1988) and also affects Na^+/Ca^{2+} exchange (Philipson, Bersohn, and Nishimoto, 1985; Earm and Irisawa, 1986), Na^+/H^+ exchange (Lazdunski, Frelin, and Vigne, 1985), and the Na^+/K^+ ATPase (Sperelakis and Lee, 1971). Additionally, a decrease in cytosolic pH (pH_i) affects Ca^{2+} uptake and release from the sarcoplasmic reticulum (SR) (Fabiato and Fabiato, 1978; Orchard, Houser, Kort, Bahinski, Capogrossi, and Lakatta, 1987) and it also diminishes Ca^{2+} binding to the sarcolemmal sites (Langer, 1985), to troponin C (Blanchard and Solaro, 1984; Solaro, El-Saleh, and Kentish, 1989), and possibly also to other Ca^{2+} buffer sites (for review see Orchard and Kentish, 1990).

In intact myocardial preparations hypercarbic acidosis causes an initial decrease in twitch amplitude followed by a secondary increase in contractility (Fry and Poole-Wilson, 1981; Allen and Orchard, 1983; Cingolani, Koretsune, and Marban, 1990). The initial phase is associated with a diminished myofilament sensitivity to Ca^{2+} while the recovery in contraction is accompanied by an enhanced amplitude of the cytosolic $[Ca^{2+}]$ (Ca_i^{2+}) transient (Allen and Orchard, 1983; Kurihara, Tanaka, Hongo, Suda, Okazaki, and Saeki, 1991) and an increase in diastolic Ca_i (Bers and Ellis, 1982; Allen, Eisner, and Orchard, 1984). The increased Ca_i transient amplitude appears secondary to an enhanced SR Ca^{2+} release and the rise in diastolic Ca_i may serve to augment SR Ca^{2+} loading (Orchard et al., 1987). It is still unclear why diastolic Ca_i should increase during acidosis, but several possibilities may be considered. (a) A decrease in pH_i may activate Na^+/H^+ exchange and the associated rise in cytosolic $[Na^+]$ (Na_i) would then modulate Na^+/Ca^{2+} exchange and enhance Ca_i (Bountra and Vaughan-Jones, 1989). (b) Ca^{2+} may be released from the SR into the cytosol (Orchard et al., 1987) and in the absence of significant Ca^{2+} reuptake the SR would be depleted of Ca^{2+}. However, if this were the case one would expect a diminution rather than an increase in the Ca_i transient amplitude triggered by electrical stimulation. (c) Ca_i may increase because of decreased Ca^{2+} affinity for cytosolic buffer sites. (d) An increased extramitochondrial $[H^+]$ would be expected to enhance H^+ uptake into the mitochondrion via Na^+/H^+ exchange. This may increase the Na^+ gradient across the mitochondrial membrane, lead to Na^+ influx and Ca^{2+} efflux from the mitochondria via Na^+/Ca^{2+} exchange (Gunter and Pfeiffer, 1990), and result in an increase in Ca_i and a decrease in intramitochondrial free $[Ca^{2+}]$ (Ca_m). (e) Alternatively, Ca_m may increase because of decreased affinity of Ca^{2+} for intramitochondrial buffer sites and be released into the cytosol.

The purpose of this study was to elucidate the effects of acidosis on resting and diastolic Ca_i and on Ca_m in isolated myocytes and mitochondria from the adult rat heart.

Preliminary reports of this work have appeared in abstract form (Gambassi, Hansford, Sollott, and Capogrossi, 1991a; Gambassi, Sollott, Spurgeon, and Capogrossi, 1991b).

METHODS

Myocyte Isolation Procedure

Left ventricular myocytes were isolated from the hearts of 2–4-mo-old male Wistar rats with minor modification of a technique previously described (Capogrossi, Kort, Spurgeon, and Lakatta, 1986a). Briefly, after anesthesia of the rat with an intraperitoneal injection of sodium pentobarbital (30 mg/kg), the heart was rapidly removed and retrogradely perfused with 25 ml of a nonrecirculating, nominally Ca^{2+}-free buffer of the following composition (mM): 116.4 NaCl, 26.2 $NaHCO_3$, 10.1 NaH_2PO_4, 5.4 KCl, 0.8 $MgSO_4$, and 5.5 d-glucose. This medium was maintained at 36 ± 1°C and was continuously gassed with 95% O_2 and 5% CO_2 to keep pH at ~7.40. The perfusate was then switched to a similar solution to which 1 mg/ml collagenase, 0.04 mg/ml protease, and 1 mg/ml bovine serum albumin had been added. Perfusion with the enzyme-containing solution lasted ~20 min.

Cell Length and Ca_i Measurements in Myocytes

At the end of the dissociation procedure, when the tissue consistency abruptly changed as a result of enzyme digestion, the left ventricular apex was excised and mechanically dissociated in a HEPES-buffered solution containing 1 mM indo-1 pentapotassium salt, 250 μM bathing [Ca^{2+}] (Ca_o), and physiologic concentrations of other electrolytes at pH 7.40 and 23°C (Sollott, Ziman, and Lakatta, 1992). Approximately 10–15 min were allowed for indo-1 to diffuse into the cells, possibly via the momentarily permeable gap junctions, before their complete sealing after the dissociation procedure. Indo-1, as well as other fluorescent Ca^{2+} indicators, are usually loaded into cells via their acetoxymethylester derivative (AM form), which is membrane permeant. The loading procedure that utilizes the membrane-permeant form of indo-1 leads to significant compartmentalization of the indicator into the mitochondria as well as into the cytosol. In myocardial cells mitochondria comprise ~35% of the intracellular volume and compartmentalization of the ester derivative of indo-1 prevents any meaningful calibration of the probe (Spurgeon, Stern, Baartz, Raffaeli, Hansford, Talo, Lakatta, and Capogrossi, 1990). In contrast, loading of indo-1 free acid with the technique outlined above is not associated with compartmentalization of the indicator into the mitochondria, and absolute values of Ca_i can be derived from the indo-1 fluorescence ratio (Sollott et al., 1992).

After loading, the cells were resuspended in a bicarbonate-buffered solution containing 1.5 mM Ca_o and equilibrated with 5% CO_2. Subsequently the cells were transferred to a Lucite chamber with a glass coverslip bottom. This chamber was placed on the stage of a modified inverted microscope (model IM-35; Carl Zeiss, Inc., Thornwood, NY) where Ca_i and length were monitored simultaneously as previously described (Spurgeon et al., 1990). Indo-1 fluorescence was excited by epi-illumination with 10-μs flashes of 350 ± 5 nm light. Paired photomultipliers collected indo-1 emission by simultaneously measuring spectral windows of 391–434 and 457–507 nm selected by bandpass interference filters. The ratio of indo-1 emission at the two wavelengths was monitored using a pair of fast integrator sample-and-hold circuits under the control of a VAX 11/730 computer. Ca_i was calculated by the 410/490-nm ratio of emitted indo-1 fluorescence according to an in vivo calibration curve (Spurgeon et al., 1990). Intrinsic autofluorescence of non–indo-1-loaded myocytes before and during acidosis was recorded on each experimental day. These values were subtracted from the fluorescence signals obtained on the same day in indo-1-loaded cells in order to reliably calculate Ca_i.

Cell length was measured simultaneously by edge tracking, using red light (650–750 nm) to form a bright-field image of the cell which was projected onto a photodiode array (1024 SAQ/RC 1024 LNA Starlight; EG & G Reticon, Sunnyvale, CA) with a 5-ms scan rate.

In each experiment myocytes were continuously superfused and studied either at rest or during electrical stimulation with field electrodes that delivered pulses of 2–4 ms in duration.

Mitochondrial Ca^{2+} Measurements in Myocytes

The technique to measure mitochondrial free $[Ca^{2+}]$ in living single rat cardiac myocytes has been described in detail elsewhere (Miyata, Silverman, Sollott, Lakatta, Stern, and Hansford, 1991). Briefly, myocytes were loaded with 25 μM indo-1/AM according to a standard technique (Spurgeon et al., 1990). Subsequently an aliquot of myocytes was placed in the chamber on the stage of the microscope used for Ca_i measurements and cytosolic indo-1 fluorescence was quenched by continuously superfusing the cells with a buffer containing 200 μM $MnCl_2$. Complete loss of cytosolic indo-1 fluorescence was assessed by the presence of an electrically stimulated contraction in the absence of an indo-1 fluorescence transient. The contractile parameters were not affected by treatment with $MnCl_2$. After complete quenching of cytosolic fraction the remaining emitted fluorescence was due mainly to the mitochondrial indo-1 pool and was used to monitor Ca_m.

pH_i Measurements in Myocytes

The fluorescent pH probe carboxy-seminaphthorhodafluor-1 (SNARF-1) was used to monitor pH_i according to a technique recently described (Blank, Silverman, Chung, Hogue, Stern, Hansford, Lakatta, and Capogrossi, 1992). Isolated myocytes bathed in bicarbonate buffer were loaded by a 7-min exposure to 10 μM SNARF-1/AM and fluorescence was measured with a modification of the same optical system used for indo-1. SNARF-1 fluorescence was excited at 530 ± 5 nm. Emitted SNARF-1 light at 590.5 ± 5.5 and 640.5 ± 6.0 nm represent, respectively, the H^+-bound and H^+-free forms of the indicator. We have previously shown that loading of cardiac myocytes with SNARF-1/AM is not associated with significant compartmentalization of this indicator into the mitochondria and the emitted SNARF-1 fluorescence ratio (590/640 nm) is calibrated as absolute pH_i values (Blank et al., 1992).

Experimental Protocols with Myocytes

Experiments with single cardiac cells were implemented in a bicarbonate buffer of the following composition unless indicated otherwise (mM): 116.4 NaCl, 5.4 KCl, 1.6 $MgSO_4$, 26.2 $NaHCO_3$, 1.2 NaH_2PO_4, 5.6 d-glucose, and 1.5 $CaCl_2$. After equilibration with 95% O_2 and 5% CO_2 buffer pH (pH_o) was 7.36 ± 0.05. Acidosis was obtained by switching to a similar perfusate equilibrated with 80% O_2 and 20% CO_2, which on average decreased pH_o to 6.82 ± 0.04 (n = 4). Superfusion with the acidotic buffer lasted 10 min and the decrease in pH_i remained stable, without any evidence of spontaneous recovery for as long as the myocytes were exposed to the superfusate equilibrated with 20% CO_2 (Fig. 1). A similar stable decrease in pH_i was also observed in experiments in which acidosis with 20% CO_2 lasted up to 20 min (not shown). The effect of a change in pH_o on pH_i achieved steady state within 3–4 min of exposure to the new buffer (Fig. 1) and a complete turnover of the perfusate in the Lucite chamber required ∼60 s (not shown). In addition, there was no effect of electrical stimulation up to 1.5 Hz on pH_i either in 5 or 20% CO_2 (Fig. 1 and Table I).

Changes in Ca_i and Ca_m were calculated from the increase in $[Ca^{2+}]$ measured after 10 min of acidosis vs. control before exposure to the buffer equilibrated with 20% CO_2. This approach was taken since both cytosolic and mitochondrial baseline Ca^{2+} values in control before and after acidosis were not different.
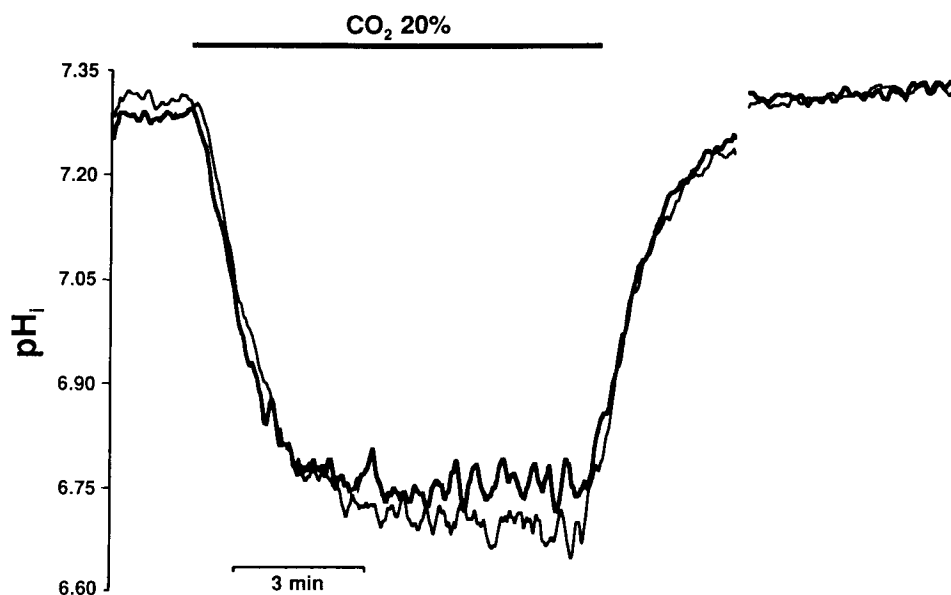
CO$_2$ 20%

FIGURE 1. Representative example of the effect of acidosis achieved by switching from a bicarbonate-buffered solution equilibrated with 95% O_2 and 5% CO_2 to one gassed with 80% O_2 and 20% CO_2 on pH$_i$ of an adult rat cardiac myocytes loaded with SNARF-1. Upon exposure to the buffer equilibrated with 20% CO_2, pH$_i$ decreases and achieves steady state within 4 min. Thereafter pH$_i$ remains stable and exhibits no evidence of recovery. Upon returning to the perfusate gassed with 5% CO_2, pH$_i$ recovers to its control value without exhibiting an overshoot. The same myocyte was studied at rest (*bold*) and during electrical stimulation at 1.5 Hz. It is apparent that electrical pacing did not significantly modify pH$_i$ either in 5 or 20% CO_2, nor did it modify the rates of acidification upon exposure to 20% CO_2 or pH$_i$ recovery on return to control buffer.

All experiments were implemented at 23°C because a more rapid loss of indo-1 from the cell at 37°C has been previously reported (Spurgeon et al., 1990).

pH and Ca^{2+} Measurements in Mitochondrial Suspensions

Cardiac mitochondria were prepared and were loaded with indo-1 by exposure to indo-1/AM at room temperature, followed by washing, as described in detail elsewhere (Moreno-Sanchez and Hansford, 1988). For measurement of intramitochondrial pH, 2,7 bis carboxyethyl-5-(6)-carboxyfluorescein (BCECF/AM) was substituted for indo-1/AM (Davis, Altschuld, Jung, and Brierley, 1987). Experiments with isolated mitochondria used a medium comprising 0.12 M

TABLE I

	Rest $n = 7$	0.5 Hz $n = 3$	1.5 Hz $n = 3$
CO$_2$ 5%	7.26 ± 0.03	7.26 ± 0.05	7.27 ± 0.05
CO$_2$ 20%	6.74 ± 0.03	6.76 ± 0.05	6. 72 ± 0.05

Average data on the effect of hypercarbic acidosis on pH$_i$ of rat myocytes studied either at rest or during electrical stimulation at 0.5 or 1.5 Hz.

KCl, 20 mM K HEPES, 10 mM NaCl, 1 mM $MgCl_2$, 5 mM L-glutamate, 5 mM L-malate, and 5 mM KH_2PO_4, pH 7.2, unless otherwise indicated: the temperature was 25°C. Lactic acid addition was chosen for these experiments as the protocol used in the cell studies, that is, the increase of CO_2 from 5 to 20% of the gas phase did not give a rapid acidification of the content of a 3-ml spectrofluorometer cuvette. Lactic acid was thought to be preferable to HCl in that it rapidly penetrates the mitochondrial inner membrane in the uncharged form, thereby acidifying the matrix, as does increase in CO_2 tension. Fluorescence was measured using a Deltascan spectrofluorometer (Photon Technology, Inc., Princeton, NJ), using excitation at 350 nm and emission at 400 and 473 nm for indo-1; for BCECF, excitation was at 450 and 505 nm and emission was measured at 550 nm.

Estimation of Ca_m was based on determination of R_{min} for indo-1 in vitro in mitochondria exposed to 0.2 mM EGTA in the presence of the ionophore 4-Br A23187 (1 μM), followed by generation of R_{max} with 0.35 mM $CaCl_2$. Subtraction of the intrinsic fluorescence of a mitochondrial suspension, not loaded with indo-1 but exposed to otherwise identical manipulations, was carried out before obtaining the ratio of the fluorescence at 400/473 nm. Ca_m was calculated from the expression:

$$Ca_m = K_d \times \frac{R - R_{min}}{R_{max} - R}$$

This simplified relation can be used as one of the wavelengths (473 nm) is isosbestic.

When the effect of mitochondrial Ca^{2+} transport upon the free Ca^{2+} of the surrounding medium was to be studied, indo-1/free acid was added to 2.5 μM to a suspension of mitochondria (1 mg protein/ml) in the medium detailed above. In some experiments Mg^{2+} and Na^+ were omitted as indicated. Fluorescence emission at 400/473 nm was measured as described above, and calibration was achieved by reference to R_{min} and R_{max} values obtained with 0.5 mM EGTA and 0.6 mM $CaCl_2$, added sequentially. Under these conditions autofluorescence of the mitochondria was trivial relative to that of indo-1 and was not subtracted.

Effect of Changes in pH on the K_d of Indo-1

The K_d of indo-1 for Ca^{2+} is known to be pH dependent and it increases as pH is decreased from 7.4 to 5.5 (Lattanzio, 1990). In contrast, pH changes between 7.4 and 8.0 have only a minor effect on the K_d of indo-1. We used recently published K_d values for indo-1 to calculate absolute [Ca^{2+}] values at different pH_i and at 22°C (Lattanzio, 1990). Thus, K_d's for indo-1 of 252 and 329 nM were used to calculate Ca_i during superfusion with the buffers gassed with 5 and 20% CO_2, respectively, and a value of 352 nM was used in determining extramitochondrial Ca^{2+} after addition of lactic acid (pH 6.69). Intramitochondrial pH is significantly higher than pH_i and, in response to the experimental protocols that we used to examine the effect of acidosis, mitochondrial pH shifted within a range that was not expected to have a significant effect on the K_d for indo-1. Thus only one K_d of 252 nM was used to calculate Ca_m under control conditions and in response to acidosis. Additionally, all experiments reported in this study were implemented at 23°C, and no correction was required for the temperature dependence of the dissociation constant for indo-1.

Statistical Analysis

Data have been calculated as mean ± SEM. The effect of acidosis in each experimental condition has been compared with paired student's t test. Ca_i values in 5% CO_2 across the different experimental protocols were compared by a one-way analysis of variance, with post hoc assessment by the Bonferroni test when appropriate. The same procedure has been followed for Ca_i values in 20% CO_2. A value of $P < 0.05$ was taken as statistically significant.

RESULTS

Effect of Acidosis on Ca_i in Unstimulated Myocytes

Fig. 2 shows the effect of acidosis on Ca_i in a representative myocyte studied in the absence of electrical stimulation in 1.5 mM Ca_o. It is apparent that the effect of a decrease in pH_i is to enhance Ca_i. In 12 myocytes studied with this protocol Ca_i increased from 130 ± 5 to 221 ± 13 nM with an average ΔCa_i from control of 91 ± 8 nM ($P < 0.01$). The Ca^{2+} source for the increase in Ca_i observed under these experimental conditions is undefined and the influx of Ca^{2+} from the extracellular space and/or release from the SR are potential mechanisms for this response.

The contribution of SR Ca^{2+} release to the increase in Ca_i in unstimulated myocytes was examined by repeating the experiment depicted in Fig. 2 after SR Ca^{2+} depletion with ryanodine (Hansford and Lakatta, 1987). For these studies SR Ca^{2+}

CO₂ 20%

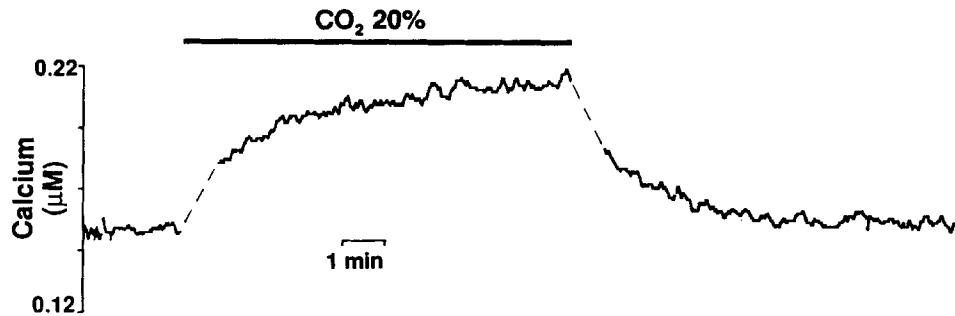Calcium (μM)

0.22

0.12

1 min

FIGURE 2. Representative example of the effect of acidosis to increase Ca_i in the absence of electrical stimulation. The dotted line indicates the time when pH_i is changing, and because of the pH dependence of the K_d of indo-1, fluorescence cannot be reliably calibrated as Ca_i. Once pH_i has achieved steady state, the appropriate K_d is used to calculate Ca_i (see Methods). In this myocyte the effect of acidosis on Ca_i was examined during superfusion with a buffer containing 1.5 mM Ca_o. Hypercarbic acidosis caused a progressive increase in Ca_i which was completely reversible upon wash-out.

depletion was achieved by exposing myocytes bathed in control buffer with 1.5 mM Ca_o to 5 μM ryanodine. Twitch and Ca_i transient amplitudes were used to monitor the extent of SR Ca^{2+} depletion in response to ryanodine. Approximately 10 min of superfusion with ryanodine was required to achieve a stable decrease in twitch and Ca_i transient during rested-state contractions to ~10% of control. At this stage electrical stimulation was interrupted and, in the continuing presence of ryanodine, myocytes were exposed to a bicarbonate-buffered solution gassed with 20% CO_2. Under these conditions Ca_i increased from 140 ± 11 to 234 ± 21 nM ($n = 5$) with an average ΔCa_i from control of 94 ± 15 nM. This was comparable to the increase in Ca_i observed in myocytes not exposed to ryanodine and studied as in Fig. 2.

Additional experiments were aimed at determining whether transsarcolemmal Ca^{2+} influx contributes to the increase in Ca_i due to acidosis. For these studies myocytes were exposed to ryanodine as described above and subsequently superfused

with a buffer without added Ca_o, with 0.1 mM EGTA (Ca^{2+}-free buffer), and with ryanodine. Depletion of cell Ca^{2+} due to superfusion with the Ca^{2+}-free buffer was minimized by examining the effect of acidosis on Ca_i within 3–4 min after the complete exchange of the buffer in the chamber with the Ca^{2+}-free solution containing ryanodine. It is noteworthy that under these conditions the change in pH_o achieved by increasing CO_2 is not expected to alter Ca_o significantly, owing to the large excess of EGTA over contaminating Ca^{2+}. The representative example in Fig. 3 shows that under conditions that prevent Ca^{2+} influx from the extracellular space and Ca^{2+} release from the SR, the effect of acidosis on Ca_i was comparable to that observed in control (compare Fig. 3 with Fig. 2). In 11 myocytes studied with this protocol Ca_i increased in response to acidosis from 115 ± 5 to 198 ± 14 nM with an average ΔCa_i of 83 ± 6 nM. The increase in Ca_i due to acidosis was not significantly different between the two groups (ΔCa_i in 1.5 mM $Ca_o = 91 \pm 8$ nM vs. ΔCa_i in Ca^{2+}-free and ryanodine $= 83 \pm 6$ nM, NS).
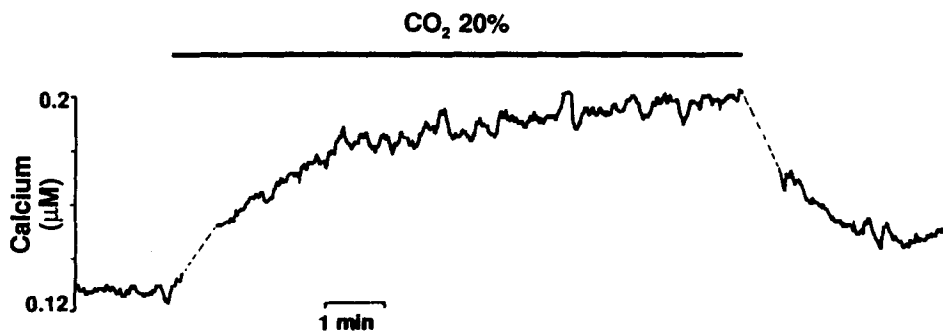

CO₂ 20%

FIGURE 3. Representative example of the effect of acidosis on Ca_i during superfusion with a Ca^{2+}-free buffer containing 0.1 mM EGTA and 5 μM ryanodine (see text). Under these conditions neither Ca^{2+} release from the SR nor Ca^{2+} influx from the extracellular space is expected to contribute to the increase in Ca_i. The experimental protocol is as in Fig. 2. Exposure to the superfusate gassed with 20% CO_2 caused an increase in Ca_i comparable to that achieved in 1.5 mM Ca_o and in the absence of ryanodine (Fig. 2).

These results suggest that neither transsarcolemmal Ca^{2+} influx nor net SR Ca^{2+} release contributes significantly to the effect of hypercarbic acidosis to increase Ca_i in unstimulated myocytes.

Two additional mechanisms need to be considered to account for the increase in Ca_i due to acidosis in the absence of electrical stimulation: a decrease in pH_i may trigger Ca^{2+} release from the mitochondria into the cytosol and/or Ca^{2+} ions may be displaced from cytosolic buffer sites by competition with H^+ ions.

Effect of Acidosis on Ca_m in Unstimulated Myocytes

Changes in pH_i may have significant effects on mitochondrial Ca^{2+} homeostasis. In cardiac mitochondria the uptake of H^+ ions via Na^+/H^+ exchange will affect the Na^+ gradient across the mitochondrial membrane and favor Ca^{2+} extrusion via Na^+/Ca^{2+} exchange. However, acidosis also increases Ca_i and modulates the function of both

the Ca^{2+} uniporter and Na^+/Ca^{2+} exchange in the mitochondrial membrane. In addition, CO_2 is membrane permeant; hypercarbic acidosis is not expected to be limited to the cytosol but will also decrease mitochondrial pH. Thus, it is difficult to predict how acidosis will affect Ca_m. Using a novel technique that allows the continuous monitoring of Ca_m (Miyata et al., 1991) we found that acidosis obtained by superfusion with a bicarbonate buffer gassed with 20% CO_2 and containing 1.5 mM Ca_o enhanced Ca_m (Fig. 4). In six myocytes studied with this protocol Ca_m increased from 95 ± 12 to 147 ± 19 nM with an average ΔCa_m from control of 52 ± 13 nM ($P < 0.01$). We subsequently examined whether this response to acidosis differed after SR Ca^{2+} depletion with 5 μM ryanodine and during superfusion with a Ca^2-free buffer. The effect of acidosis on Ca_m persisted and the magnitude of this effect was comparable to that in a buffer with 1.5 mM Ca_o and without ryanodine (compare Figs. 4 and 5). The average increase in Ca_m was from 103 ± 4 to 153 ± 8 nM ($\Delta Ca_m = 50 ± 8$ nM; $n = 4$; $P < 0.01$).
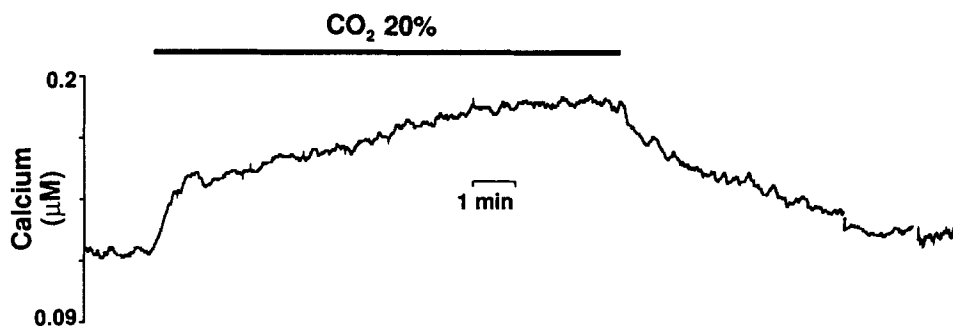

FIGURE 4. Representative example of the effect of acidosis to increase Ca_m in the absence of electrical stimulation. Myocytes were loaded with indo-1/AM and cytosolic fluorescence was quenched with $MnCl_2$ as described in Methods. This myocyte was bathed in a bicarbonate buffer with 1.5 mM Ca_o and responded to acidosis with 20% CO_2 with a progressive increase in Ca_m which was fully reversible with wash-out.

The results presented so far show that acidosis increases $[Ca^{2+}]$ both in the cytosol and in the mitochondria and that, in quiescent cells, neither Ca^{2+} influx from the extracellular space nor release from the SR contribute substantially to the increase in Ca_i and Ca_m. An unanswered question is whether during acidosis there is a net Ca^{2+} loss from the mitochondria into the cytosol or whether the increase in Ca_i contributes to enhance Ca_m. This question was addressed in additional experiments with ruthenium red which, in both isolated hearts (McCormack and England, 1983) and enzymatically dissociated cardiac myocytes (Hansford, 1985), has been shown to block activation of the Ca^{2+}-dependent intramitochondrial enzyme pyruvate dehydrogenase. In addition, a recent study with isolated myocardial cells has shown that ruthenium red is highly effective in preventing mitochondrial Ca^{2+} influx under conditions of marked cytosolic Ca^{2+} overload (Miyata et al., 1991). Fig. 6 shows the effect of acidosis with 20% CO_2 on the Ca_m of a representative myocyte that had been pretreated with 50 μM ruthenium red for 30 min before and throughout the course
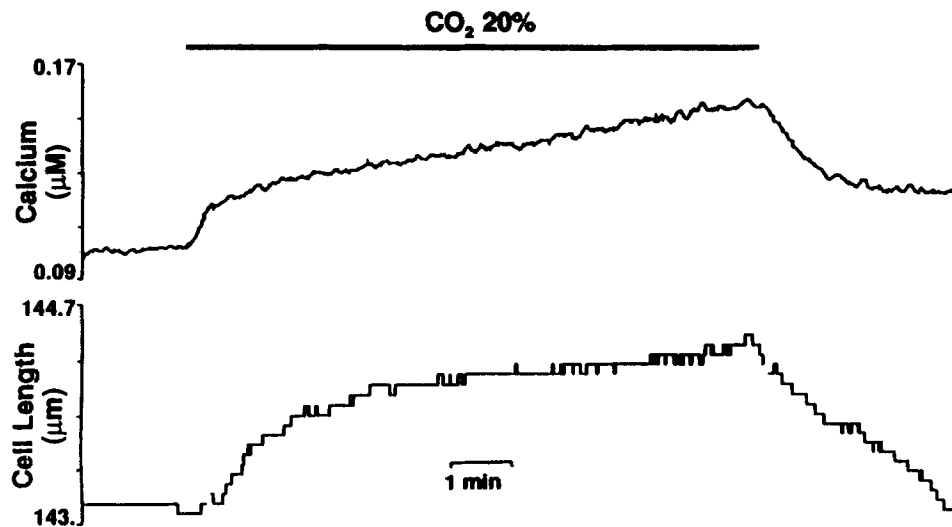
FIGURE 5.   Representative example of the effect of acidosis on Ca_m during superfusion with a Ca^{2+}-free buffer containing 0.1 mM EGTA and 5 μM ryanodine (see text). The paradigm is as in Fig. 4. Under these conditions the effect of acidosis on Ca_m was comparable to that observed in 1.5 mM Ca_o and depicted in Fig. 4.

of the experiment. Under these conditions Ca_m increased and the average effect was comparable to that achieved in the absence of ruthenium red (cf. Fig. 4 and also see average results in Fig. 7 A).

The results suggest that under our experimental conditions Ca^{2+} influx from the cytosol does not contribute to increase Ca_m, and that Ca^{2+} displacement from intramitochondrial buffer sites by H^+ is the only mechanism for the increase in Ca_m. Fig. 7 B depicts the difference between Ca_i and Ca_m in Ca^{2+}-free buffer with ryanodine and in 1.5 mM Ca_o. In 5% CO_2, Ca_i was higher than Ca_m; in response to acidosis Ca_i increased more than Ca_m regardless of whether the experiments were implemented in a Ca_o-free buffer containing ryanodine or in 1.5 mM Ca_o. This
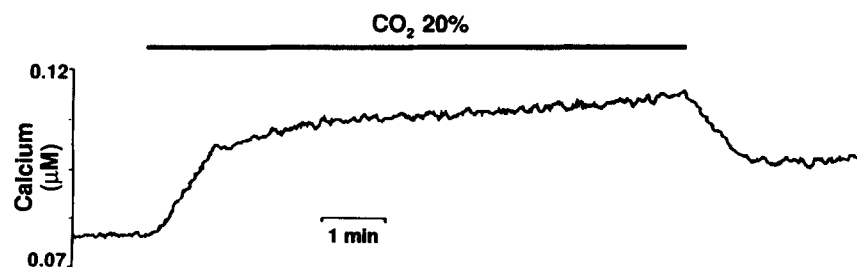

FIGURE 6.   Representative example of the effect of acidosis to increase Ca_m in the presence of ruthenium red. This myocyte was exposed to 50 μM ruthenium red for 30 min before the onset of acidosis and throughout the exposure to 20% CO_2 (see text). Conditions were otherwise similar to those used for the experiment depicted in Fig. 4. The increase in Ca_m in response to acidosis was comparable to that achieved in the absence of ruthenium red (cf. Fig. 4).

suggests that during acidosis cytosolic Ca^{2+} buffers may release more Ca^{2+} than mitochondrial Ca^{2+} binding sites. Alternatively, Ca^{2+} may be lost from the mitochondria via Na^+/Ca^{2+} or H^+/Ca^{2+} exchange, owing to changes in mitochondrial membrane Na^+ or H^+ gradients, and contribute to an increase in Ca_i. This possibility and additional evidence on the effect of acidosis on Ca_m were examined in experiments with mitochondrial suspensions which will be described below.
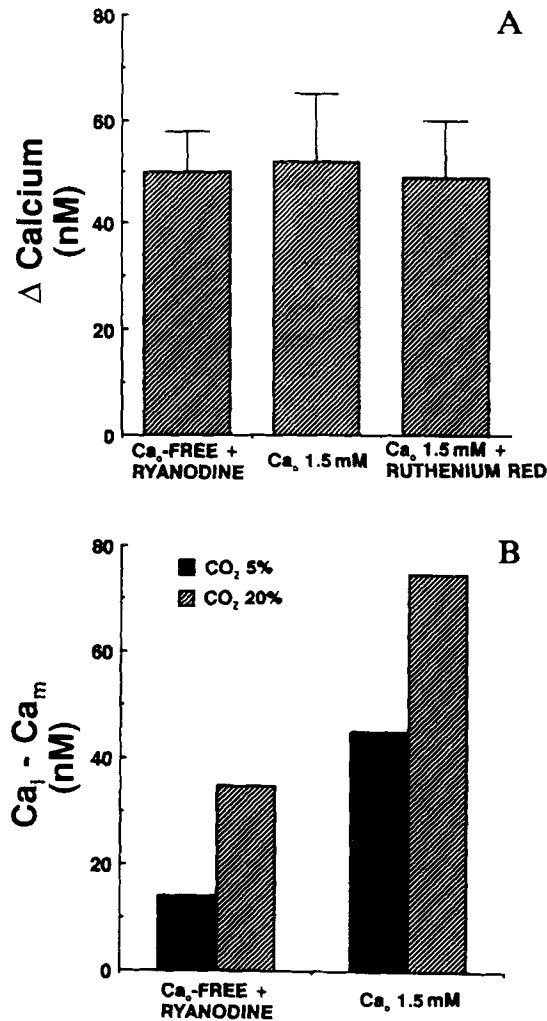

FIGURE 7. Average results on the effect of hypercarbic acidosis on Ca_m and on the difference between Ca_i and Ca_m of myocytes studied in the absence of electrical stimulation. (A) Effect of acidosis on ΔCa_m of myocytes in Ca_o-free buffer with ryanodine, in 1.5 mM Ca_o, and in 1.5 mM Ca_o plus 50 μM ruthenium red (see text). The Δ's indicate that the average increase in Ca_m due to hypercarbic acidosis was similar between groups. (B) Bars represent the difference $Ca_i - Ca_m$ for myocytes superfused with 1.5 mM Ca_o or Ca-free buffer with ryanodine, equilibrated with 5% (*filled bars*) or 20% CO_2 (*hatched bars*). Ca_i was higher than Ca_m and the difference was enhanced during acidosis (see text).

Effect of Acidosis on Isolated Mitochondria

Experiments with isolated mitochondria loaded with BCECF/AM revealed that addition of lactic acid results in a rapid fall in intramitochondrial pH (Fig. 8, *top*). There was a corresponding rise in Ca_m which was shown in parallel experiments using other aliquots of the same mitochondrial suspension, but was loaded with the

fluorescent Ca^{2+} indicator indo-1/AM (Fig. 8, *bottom*). In response to the above protocol Ca_m increased in all mitochondrial suspensions studied ($n = 4$) to $183 \pm 29\%$ of control value (control Ca_m was 123 ± 27 nM, $P < 0.05$). The decrease in pH achieved with this protocol occurred more rapidly than that obtained in intact myocytes by changing superfusate (compare Fig. 8, *top*, with Fig. 1) and this is the likely cause for the more rapid increase in Ca_m observed under these conditions (compare Fig. 8, *bottom*, with Figs. 4 and 5).
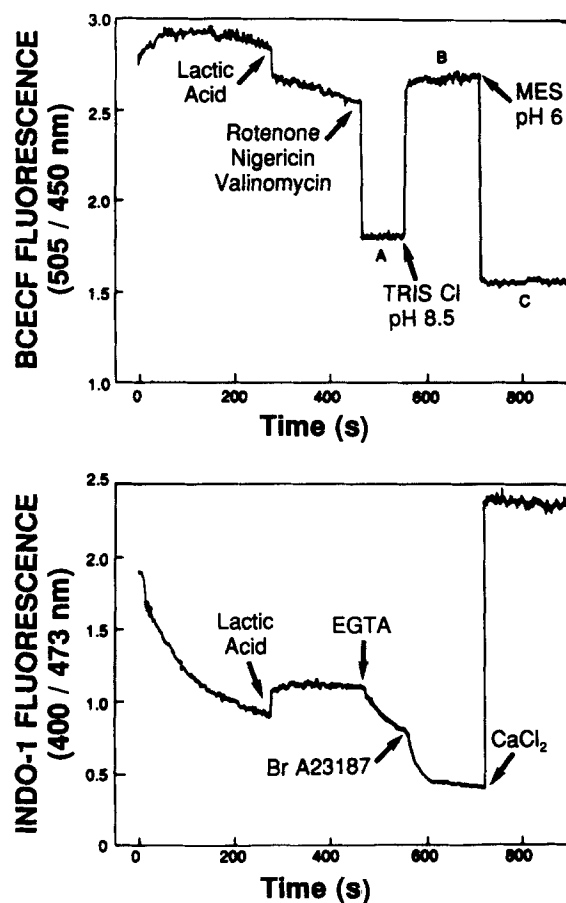

FIGURE 8. Measurement of intramitochondrial pH and Ca_m of a suspension of cardiac mitochondria. (*Top*) Mitochondria were loaded with BCECF/AM and incubated in a standard medium (see Methods) supplemented with 1 mg/ml bovine serum albumin and 0.1 mM EGTA. At the points indicated by the arrows, the following additions were made: 2.5 mM lactic acid, 1 μM rotenone, 1 μM nigericin, and 1 μM valinomycin. Then Tris Cl was added to increase buffer pH (20 mM; pH 8.5), and finally K MES was added to decrease buffer pH (80 mM; pH 6). Fluorescence was excited at 505 and 450 nm and the ratio 505/450 is presented. Upon addition of lactic acid intramitochondrial pH fell from 8.0 to 7.77. (*Bottom*) Mitochondria were loaded with indo-1/AM and the incubation medium was the same as in the upper panel. Where indicated, the following additions were made: 2.5 mM lactic acid, 0.2 mM EGTA, 1 μM 4 Br A23187, and 0.35 mM $CaCl_2$. Fluorescence emission at 400 and 473 nm was measured, and the ratio 400/473 is presented. Upon addition of lactic acid there was an increase in Ca_m from 90 to 140 nM.

In the experiments described above we have used 0.1 mM EGTA to control extramitochondrial Ca^{2+} concentrations. EGTA is an effective Ca^{2+} buffer in the 0.1–1 μM range but its affinity for Ca^{2+} is modified by changes in pH. To conclusively rule out the possibility that a rise in medium $[Ca^{2+}]$, due to a release of Ca^{2+} from EGTA at the more acid pH, was causing the mitochondria to take more Ca^{2+}, we have conducted additional experiments using higher concentrations of EGTA (i.e., 1 mM instead of 0.1 mM). The results of these experiments suggest that

even in the presence of negligible extramitochondrial free Ca^{2+} (in control and in acidosis), an increase in Ca_m upon lactic acid addition is present and of comparable magnitude to the one observed with 0.1 mM EGTA (not shown). Other potential artifacts that could cause a rise in medium $[Ca^{2+}]$ upon a fall in pH (e.g., change of Ca^{2+} binding to phosphate in the medium) have been checked with experiments in the absence of mitochondria.

In an attempt to identify the Ca^{2+} source responsible for the increase in Ca_m in isolated mitochondria, indo-1/free acid was used as an indicator of extramitochondrial free $[Ca^{2+}]$. These experiments showed that the addition of lactic acid (5 mM) always enhanced extramitochondrial $[Ca^{2+}]$ and the average increase was 166 ± 26 nM ($137 \pm 6\%$ of baseline value; baseline extramitochondrial $[Ca^{2+}]$ was 453 ± 21 nM; $n = 3$; $P < 0.005$). In additional experiments extramitochondrial Na^+ and Mg^{2+} were removed in order to facilitate mitochondrial Ca^{2+} uptake vs. release and bring extramitochondrial $[Ca^{2+}]$ into a lower range. Under these conditions the effect of acidosis to enhance extramitochondrial $[Ca^{2+}]$ was retained and the average increase was 43 ± 4 nM ($139 \pm 4\%$ of baseline value; baseline extramitochondrial $[Ca^{2+}]$ was 107 ± 5 nM; $n = 3$; $P < 0.05$). It is noteworthy that baseline extramitochondrial $[Ca^{2+}]$ before lactic acid addition ranged between 95 and 495 nM depending on whether Na^+ and Mg^{2+} were present. However, acidosis always enhanced Ca^{2+} efflux from the mitochondria and the increase in extramitochondrial $[Ca^{2+}]$ was not blunted when control extramitochondrial $[Ca^{2+}]$ was >200 nM, i.e., within the range achieved in the cytosol in intact myocytes during hypercarbia. Similar responses were observed upon HCl addition (2.5 or 5 mM) regardless of whether Na^+ and Mg^{2+} ions were present or absent (not shown).

Thus, the origin of the rise in Ca_m upon acidification is not a flux of Ca^{2+} into the mitochondria; rather, we infer that a raised Ca_m, probably a consequence of competition between H^+ and Ca^{2+} for intramitochondrial binding sites, leads to Ca^{2+} flux out of the mitochondria.

Effect of Acidosis on Diastolic Ca_i in Electrically Stimulated Myocytes

The results presented so far indicate that in unstimulated myocytes acidosis increases Ca_i, probably because of decreased binding to cytoplasmic buffers and release from the mitochondria, while neither SR Ca^{2+} release nor transsarcolemmal Ca^{2+} influx contribute to such a rise in Ca_i. During an action potential, cell Ca^{2+} homeostasis is affected by pH-sensitive mechanisms that are modulated by membrane depolarization, e.g., I_{Ca}, Na^+/Ca^{2+} exchange, and Na^+/K^+ ATPase. We examined whether the increase in Ca_i due to acidosis is enhanced during electrical stimulation at either 0.5 or 1.5 Hz. It is noteworthy that under our experimental conditions pH_i in 5 or 20% CO_2 was not affected by pacing either at 0.5 or 1.5 Hz (Fig. 1 and Table I).

Fig. 9 shows the effect of acidosis with 20% CO_2 on the twitch and Ca_i of an isolated myocyte paced at 0.5 Hz. The lower tracings were obtained at the times indicated by the letters below the continuous length record and show, on an expanded time scale, the twitch and the simultaneously recorded Ca_i transient in control and at different times during acidosis. In response to hypercarbia this cell exhibited an initial decrease in contraction amplitude without any significant change in peak Ca_i. This was followed by a slow recovery in twitch amplitude which was associated with an

increase in Ca_i, which finally achieved a steady state slightly above control. Diastolic cell length increased throughout the period of acidosis in spite of a higher diastolic Ca_i, a finding consistent with the known effect of acidosis to decrease myofilament sensitivity to Ca^{2+}. Upon returning to the buffer equilibrated with 5% CO_2 there was a transient overshoot in the amplitude of the contraction, which then decayed toward its preacidosis baseline, and diastolic cell length shortened to its control value.
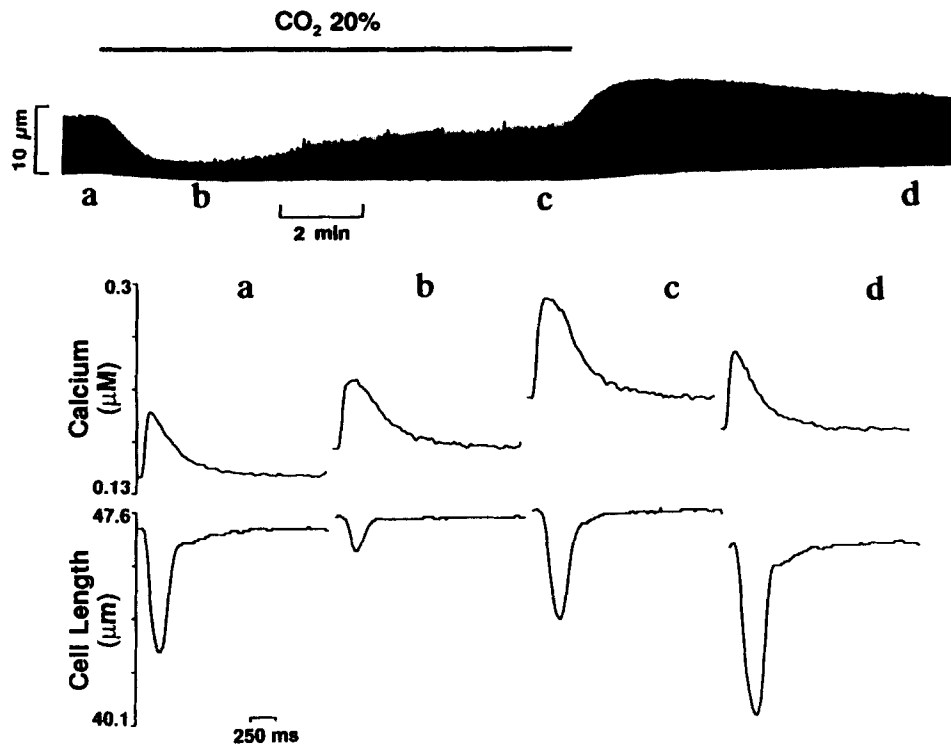

FIGURE 9.   Representative example of the effect of acidosis with 20% CO_2 on the simultaneously recorded contraction and Ca_i of a rat myocyte stimulated at 0.5 Hz in 1.5 mM Ca_o. The lower tracings of Ca_i and length are on an expanded scale and were obtained at the times indicated by the letters below the continuous length record; control (a), onset of acidosis (b), after 10 min exposure to the buffer equilibrated with 20% CO_2 (c), and return to control (d). In response to acidosis, diastolic length increased while twitch amplitude exhibited a rapid decrease followed by a slow recovery. In 20% CO_2 both systolic and diastolic Ca_i were higher than in control (see text).

Fig. 10 A shows a representative cell that was exposed to acidosis during stimulation at 1.5 Hz. In Fig. 10 B diastolic Ca_i during pacing at 1.5 Hz is plotted with resting Ca_i in the same cell studied in the absence of electrical stimulation. Under both conditions there was a significant increase in diastolic Ca_i which was more marked during pacing than at rest. This result is confirmed by the average data shown in Fig. 11. During superfusion with the control buffer Ca_i was 130 ± 5 nM at
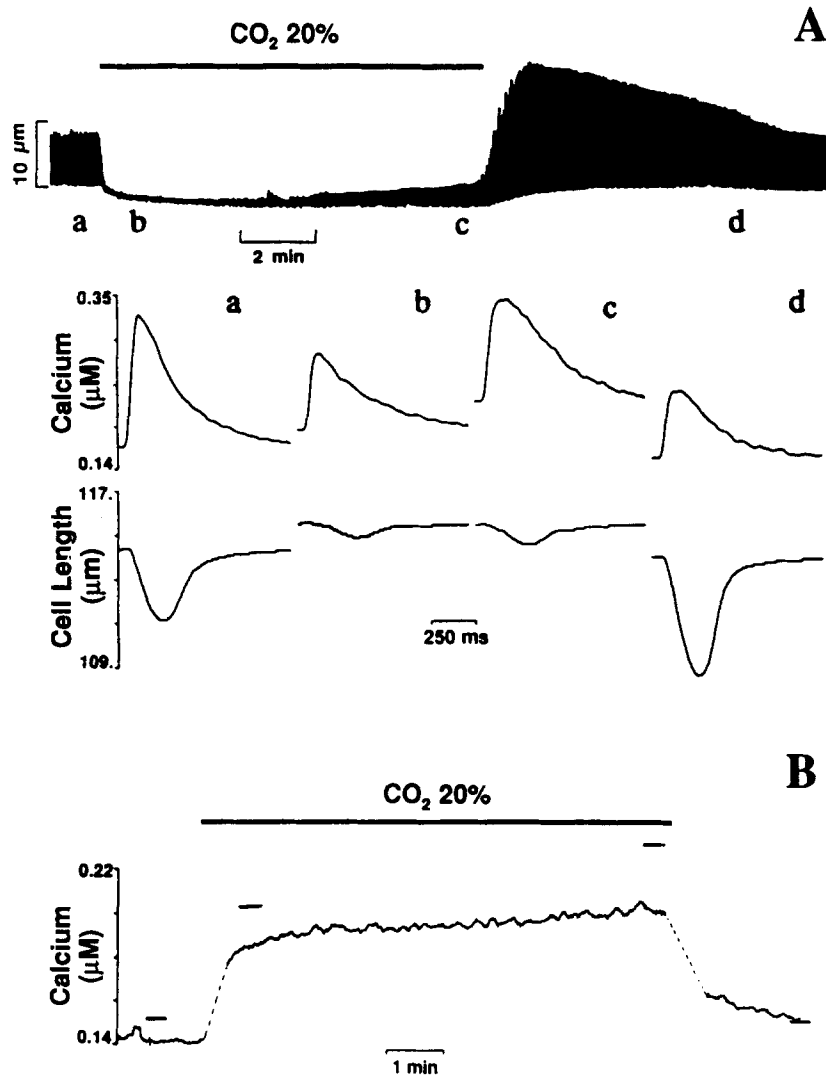
FIGURE 10.   Representative example of the effect of acidosis with 20% CO_2 on the simultaneously recorded contraction and Ca_i of a rat myocyte paced at 1.5 Hz. The same myocyte was also studied in the absence of electrical stimulation. Ca_o was 1.5 mM. (*A*) The lower tracings of Ca_i and length are on an expanded scale and were obtained at the times indicated by the letters below the continuous length record; control (*a*), onset of acidosis (*b*), after 10 min exposure to the buffer equilibrated with 20% CO_2 (*c*), and return to control (*d*). In response to acidosis, diastolic length increased while twitch amplitude exhibited a rapid decrease followed by a slow and incomplete recovery. After 10 min exposure to 20% CO_2 both systolic and diastolic Ca_i were higher than in control (see text). (*B*) The effect of acidosis on Ca_i is shown in the same representative myocardial cell examined at rest. The black bars above the Ca_i tracing indicate diastolic Ca_i during stimulation at 1.5 Hz (from *A*). In 5% CO_2 resting Ca_i was slightly higher during electrical pacing than in the unstimulated state. Moreover, the increase in diastolic Ca_i due to acidosis for 10 min was more pronounced during stimulation than at rest.

rest; diastolic Ca_i increased to 135 ± 14 nM during stimulation at 0.5 Hz (NS) and to 154 ± 4 nM during stimulation at 1.5 Hz (NS when compared either to diastolic Ca_i either at rest or at 0.5 Hz). A different result was obtained when the effect of pacing on diastolic Ca_i was examined at the end of a 10-min period of acidosis. Under these conditions Ca_i was 221 ± 13 nM at rest and it increased to 245 ± 18 nM at 0.5 Hz and to 285 ± 17 nM during stimulation at 1.5 Hz ($P < 0.05$ when compared to diastolic Ca_i at rest or at 0.5 Hz).

Thus, during superfusion with the control buffer there was no significant effect of electrical stimulation on diastolic Ca_i up to 1.5 Hz; in contrast, there was a stimulation frequency-dependent enhancement of the effect of acidosis to raise diastolic Ca_i.
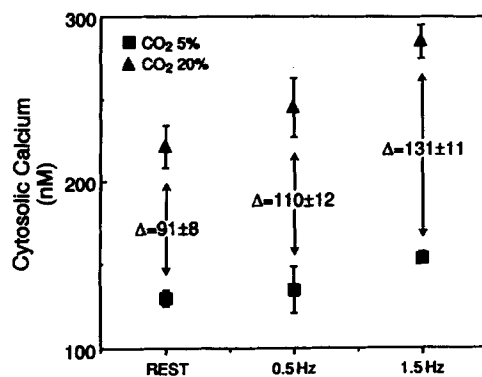

FIGURE 11. Average results of the effect of electrical stimulation at 0.5 Hz ($n = 5$) and 1.5 Hz ($n = 11$) on diastolic Ca_i during superfusion with the control buffer and after 10 min of hypercarbic acidosis. Ca_i values for unstimulated myocytes ($n = 12$) are also shown. In 5% CO_2 electrical stimulation at 0.5 and 1.5 Hz did not enhance diastolic Ca_i over the Ca_i level at rest. In 20% CO_2 electrical stimulation increased diastolic Ca_i above Ca_i at rest and the increase was more pronounced during stimulation at 1.5 Hz than at 0.5 Hz (see text).

DISCUSSION

The results presented in this study show that, in myocardial cells, hypercarbic acidosis causes a stable decrease in pH_i and enhances both Ca_i and Ca_m. In resting cells the increase in Ca_i and Ca_m is unaffected by Ca^{2+} influx from the extracellular space and release from the SR, while the experiments with isolated mitochondria indicate that during acidosis Ca^{2+} is released from these organelles. In electrically stimulated myocytes, the effect of acidosis on diastolic Ca_i is more pronounced than in unstimulated myocytes. This suggests that during electrical pacing acidosis causes a net cell Ca^{2+} gain from the extracellular space.

The present findings regarding the effect of hypercarbic acidosis on pH_i are in agreement with those of other investigators who did not observe a pH_i recovery in the continuing presence of the hypercarbic solution (Ellis and Thomas, 1976; Pressler, 1989; Mohabir, Lee, Kurtz, and Clusin, 1991; Harrison, Frampton, McCall, Boyett, and Orchard, 1992). However, a partial pH_i recovery during continuing superfusion with a hypercarbic solution has also been reported (Bountra, Powell, and Vaughan-Jones, 1990; Cingolani et al., 1990) and the reason for these different results remains to be elucidated.

The effect of acidosis on diastolic Ca_i was initially observed in Purkinje fibers, using

Ca^{2+} sensitive electrodes, during NH_4Cl pulses (Bers and Ellis, 1982). Subsequent studies were implemented with multicellular preparations injected with the Ca^{2+}-sensitive photoprotein aequorin in order to identify the magnitude and source of the increase in Ca_i. However, aequorin is poorly sensitive to changes in $[Ca^{2+}]$ as low as it may be found in quiescent myocardial preparations. For this reason no effect of acidosis on diastolic Ca_i was initially detected (Allen and Orchard, 1983) unless Ca_i was markedly increased by exposure to strophanthidin, removal of bathing $[Na^+]$ (Allen et al., 1984), or raising Ca_o to 5 mM (Orchard, 1987). Under these conditions, mammalian myocardial preparations will manifest asynchronous spontaneous SR Ca^{2+} oscillations (Orchard et al., 1983; Wier, Kort, Stern, Lakatta, and Marban, 1983; Capogrossi et al., 1986a; Capogrossi, Suarez-Isla, and Lakatta, 1986b; Capogrossi, Stern, Spurgeon, and Lakatta, 1988) and because of the nonlinearity of the relationship between aequorin light and $[Ca^{2+}]$ the assessment of true Ca_i will be prevented (Orchard, 1987).

Other studies have examined the effect of acidosis on cell Ca^{2+} homeostasis in spontaneously beating cultures of chick embryo heart cells (Kim and Smith, 1987, 1988; Kim, Cragoe, and Smith, 1987; Kohmoto, Spitzer, Movsesian, and Barry, 1990). It is difficult to compare some of those with the present results because of species differences, the use of multicellular preparations rather than single cells, the use of NH_4Cl prepulses to decrease pH_i, and because in some studies cells were allowed to beat spontaneously. Nevertheless, in one study (Kohmoto et al., 1990) spontaneous cell beating was abolished by superfusion with a low-Na^+ buffer without added Ca^{2+} and SR Ca^{2+} depletion was achieved by simultaneous exposure to caffeine. Under those conditions, acidosis after NH_4Cl washout was still associated with an increase in Ca_i, a result qualitatively similar to that depicted in Fig. 3 of this study.

Despite a large body of experimental work, the mechanisms responsible for the effect of a sustained decrease in pH_o and pH_i to enhance diastolic Ca_i are still largely unknown. Additionally, the effect of acidosis on Ca_m in intact cells has not been investigated.

Effect of Acidosis on Ca_i in Unstimulated Myocytes

Different Ca^{2+} sources could account for the effect of acidosis to enhance Ca_i in unstimulated myocardial cells: (a) the extracellular space, (b) the sarcoplasmic reticulum, (c) the mitochondria, and (d) Ca^{2+} buffer sites which may include the sarcolemma and troponin C. Our results indicate that an increase in Ca_i in unstimulated cells still occurs in the absence of transsarcolemmal Ca^{2+} influx and after SR Ca^{2+} depletion, and the magnitude of such increase is similar to that in myocytes not exposed to ryanodine and bathed in 1.5 mM Ca_o. This suggests that in the absence of electrical stimulation neither the SR nor the extracellular space contributes to the increase in Ca_i. This conclusion differs from that of a prior study which examined the effect of acidosis during NH_4Cl washout on Ca_i of chick ventricular cells (Kim and Smith, 1988) and showed that Ca^{2+} influx from the extracellular space appeared to contribute to the increase in Ca_i. A possible explanation for this discrepancy lies in the different methods used to cause acidosis. NH_4Cl washout is expected to decrease pH_i without affecting pH_o, thus creating a

gradient for protons across the sarcolemma. Under these conditions pH_i recovery occurs via Na^+/H^+ exchange (Piwnica-Worms, Jacob, Horres, and Lieberman, 1985; Liu, Piwnica-Worms, and Lieberman, 1990) and, if the experiments are conducted in a bicarbonate-buffered solution, also via extracellular Na^+ (Na_o)-dependent Cl^-/HCO_3^--exchange (Liu et al., 1990). Both mechanisms increase Na_i and will cause cell Ca^{2+} loading via Na^+/Ca^{2+} exchange. Thus, transsarcolemmal Ca^{2+} influx in response to acidosis with an NH_4Cl prepulse would be expected to occur regardless of whether the myocyte is quiescent or electrically stimulated. We examined the effect of hypercarbic acidosis and in some experiments with isolated mitochondrial suspensions we also used lactic acid. Both CO_2 and lactic acid readily cross biologic membranes and are expected to decrease pH_o, pH_i, and intramitochondrial pH. It is questionable whether under these conditions Na^+/H^+ exchange and Na_o-dependent Cl^-/HCO_3^- exchange are activated and increase cell Na^+ loading. Furthermore, extracellular H^+ inactivation of Na^+/H^+ exchange has been demonstrated in Purkinje fibres (Vaughan-Jones and Wu, 1990). Thus, either both Na_o-dependent acid-extruding mechanisms are inactivated or some degree of cell Na^+ loading occurs without overcoming the effect of acidosis to inhibit Na^+/Ca^{2+} exchange (Philipson et al., 1985; Earm and Irisawa, 1986).

Hence, by exclusion our data indicate that the acidosis-induced increase in Ca_i in unstimulated cells is probably due to $Ca^{2+}-H^+$ competition in the cytosol. Furthermore, mitochondrial Ca^{2+} release may also enhance Ca_i. It is noteworthy that a sustained rather than a transient increase in Ca_i is not contradictory to this interpretation. In fact, the sustained increase in Ca_i observed during acidosis is probably due to progressive inhibition of the mechanisms responsible for Ca^{2+} extrusion from the cytosol in the continued presence of Ca^{2+} displacing phenomena. This leads to the conclusion that during acidosis there is a new set-point for Ca_i which is higher than in the control condition.

Effect of Acidosis on Ca_m

We examined the effect of acidosis on mitochondrial Ca^{2+} homeostasis both in intact myocytes and in mitochondrial suspensions. Our results show that Ca_m increases in response to acidosis due to hypercarbia or to lactic acid. In myocytes, the increase in Ca_m was of similar magnitude under three different experimental conditions: (a) when the cells were in 1.5 mM Ca_o, (b) when the superfusate was Ca^{2+} free and the SR had been Ca^{2+}-depleted with ryanodine, and (c) when the experiment was conducted in the presence of 50 μM ruthenium red, a blocker of the mitochondrial Ca^{2+} uniport (Figs. 6 and 7 A). Our result suggests that Ca^{2+} influx does not contribute to the increase in Ca_m when pH is decreased both inside and outside the mitochondria. Thus, decreased Ca^{2+} binding to intramitochondrial buffer sites is the likely mechanism for the increase in Ca_m caused by acidosis.

To determine whether Ca^{2+} efflux from the mitochondria occurs during acidosis we used mitochondrial suspensions and extramitochondrial $[Ca^{2+}]$ was monitored with indo-1 free acid. Under these conditions lactic acid addition caused mitochondrial Ca^{2+} release and, in agreement with a prior study (Fry, Powell, Twist, and Ward, 1984), we obtained a similar result with HCl addition (not shown). However, since HCl is poorly membrane permeant, it would be expected to increase the H^+ gradient

across the mitochondrial membrane; this will favor H^+ uptake into the mitochondria in exchange for Na^+ and lead to mitochondrial Ca^{2+} release via Na^+/Ca^{2+} exchange. In contrast, addition of lactic acid decreases pH both inside and outside the mitochondria and may not significantly modify the H^+ gradient. Under these conditions the increase in Ca$_m$, rather than Na^+/H^+ exchange, will be expected to drive mitochondrial Ca^{2+} release. Therefore, regardless of the mechanism involved, it appears likely that mitochondrial Ca^{2+} efflux contributes to the increase in Ca$_i$ during acidosis in quiescent myocytes.

As can be seen from Figs. 2 and 3, the rise in Ca$_i$ with acidosis comprises an initial fast component and a second sustained phase. It could be argued that if the mitochondria were to contribute to the increase in Ca$_i$, the release would be only transient and then dissipate, the mitochondria being a large but finite store of Ca^{2+}. It is hypothesized that the kinetics of Ca^{2+} efflux from the mitochondria, after its rather abrupt rise, are probably nonlinearly related to the absolute Ca$_i$ level and to the difference between Ca$_i$ and Ca$_m$. While the initial rate of Ca$_m$ efflux is probably very rapid (as shown by our data), owing both to a large (relative to the mitochondrial free Ca^{2+}) initial transmitochondrial Ca^{2+} gradient and to the accentuated Na^+/Ca^{2+} exchange driving force resulting from mitochondrial proton influx, the Ca$_m$ source is indeed finite and probably soon runs down. Thus, we propose that the secondary, slow Ca$_i$ accumulation phase in these experimental conditions is probably explained, at least in part, by a long "tail" in the dissipation of the acidosis-induced Ca$_m$ elevation. This, in turn, is due both to the increased Ca$_i$ set-point level discussed above and to the stabilization of the pH-induced flux changes.

For the aforementioned reasons it is not surprising that the changes in $[Ca^{2+}]$ do not occur with the same time course as the change in pH$_i$.

Effect of Acidosis on Diastolic Ca$_i$ in Electrically Stimulated Myocytes

The mechanisms responsible for cell Ca^{2+} homeostasis during electrical stimulation differ from those operative in quiescent cells. With each action potential Ca^{2+} enters the cell via I_{Ca} and Na/Ca^{2+} exchange. I_{Ca} is the trigger for SR Ca^{2+} release and Ca^{2+} entering the cell via I_{Ca} and Na^+/Ca^{2+} exchange also repletes the SR. Ca^{2+} released into the cytosol with each action potential is subsequently reaccumulated into the SR or extruded into the extracellular space via Na^+/Ca^{2+} exchange and the sarcolemmal Ca^{2+} pump. In addition, the increase in intracellular $[Na^+]$ that occurs during electrical stimulation (Harrison et al., 1992) will modulate cell Ca^{2+} loading. Acidosis affects the mechanisms responsible for Ca^{2+} entry into the cytosol and Ca^{2+} extrusion from the cytosol. Thus, the enhanced SR content (Harrison et al., 1992) and Ca$_i$ transient amplitude (Figs. 9 and 10) observed during acidosis occur despite inhibition of SR Ca^{2+} uptake (Fabiato and Fabiato, 1978) and a diminished I_{Ca}, i.e., a suboptimal trigger for Ca^{2+}-induced release of Ca^{2+} (Fabiato, 1985). Our results show that during electrical stimulation the increase in diastolic Ca$_i$ caused by acidosis is enhanced by comparison with unstimulated myocytes, and the magnitude of the increase is more marked when cells are paced at 1.5 Hz rather than at 0.5 Hz. This indicates that a net cell Ca^{2+} gain has occurred. In contrast, under control conditions, stimulation up to 1.5 Hz has no significant effect on diastolic Ca$_i$ (Fig. 11).

Thus, with each action potential, less Ca^{2+} may be entering the cell during acidosis

than in control conditions. However, the effect of acidosis to inhibit Ca^{2+} extrusion from the cytosol may be predominant and enhance Ca_i in conjunction with Ca^{2+} displacement from cytosolic buffers and mitochondrial Ca^{2+} release. In addition, the increase in diastolic Ca_i is more pronounced during stimulation at 1.5 Hz than at 0.5 Hz. The likely mechanisms for this effect are the enhanced Ca^{2+} and Na^+ entry and the shortened time for Ca_i extrusion at the higher stimulation frequency.

Conclusions

While the physiologic relevance of our findings remains to be determined, the increase in diastolic Ca_i is the likely cause for the increase in Ca_i transient amplitude and for the recovery of the contraction that occurs during acidosis while the increase in Ca_m may augment the activity of mitochondrial Ca^{2+}-dependent dehydrogenases which catalyze oxidation of carbohydrates and fatty acids and enhance ATP production.

REFERENCES

Allen, D. G., D. A. Eisner, and C. H. Orchard. 1984. Factors influencing free intracellular calcium concentration in quiescent ferret ventricular muscle. *Journal of Physiology.* 350:615–630.

Allen, D. G., and C. H. Orchard. 1983. The effects of changes of pH on intracellular calcium transients in mammalian cardiac muscle. *Journal of Physiology.* 335:555–567.

Bers, D. M., and D. Ellis. 1982. Intracellular calcium and sodium activity in sheep heart Purkinje fibers: effects of changes of external sodium and intracellular pH. *Pflügers Archiv.* 393:171–178.

Blanchard, E. M., and R. J. Solaro. 1984. Inhibition of the activation and troponin calcium binding of dog cardiac myofibrils by acidic pH. *Circulation Research.* 55:382–391.

Blank, P. S., H. S. Silverman, O. Y. Chung, B. A. Hogue, M. D. Stern, R. G. Hansford, E. G. Lakatta, and M. C. Capogrossi. 1992. Cytosolic pH measurements in single cardiac myocytes using carboxy-seminapthorhodafluor-1. *American Journal of Physiology.* 263:H276–H284.

Bountra, C., T. Powell, and R. D. Vaughan-Jones. 1990. Comparison of intracellular pH transients in single ventricular myocytes and isolated ventricular muscle of guinea-pig. *Journal of Physiology.* 424:343–365.

Bountra, C., and R. D. Vaughan-Jones. 1989. Effect of intracellular and extracellular pH on contraction in isolated mammalian cardiac muscle. *Journal of Physiology.* 418:163–187.

Capogrossi, M. C., A. A. Kort, H. A. Spurgeon, and E. G. Lakatta. 1986a. Single adult rabbit and rat cardiac myocytes retain the Ca^{2+}- and species-dependent systolic and diastolic contractile properties of the intact muscle. *Journal of General Physiology.* 88:589–613.

Capogrossi, M. C., M. D. Stern, H. A. Spurgeon, and E. G. Lakatta. 1988. Spontaneous Ca^{2+} release from the sarcoplasmic reticulum limits Ca^{2+}-dependent twitch potentiation in individual cardiac myocytes: a mechanism for maximum inotropy in the myocardium. *Journal of General Physiology.* 91:133–155.

Capogrossi, M. C., B. A. Suarez-Isla, and E. G. Lakatta. 1986b. The interaction of electrically stimulated twitches and spontaneous contractile waves in single cardiac myocytes. *Journal of General Physiology.* 88:615–633.

Cingolani, H. E., Y. Koretsune, and E. Marban. 1990. Recovery of contractility and pH_i during respiratory acidosis in ferret hearts: role of Na^+/H^+ exchange. *American Journal of Physiology.* 259:H843–H848.

Davis, M. H., R. A. Altschuld, D. W. Jung, and G. P. Brierley. 1987. Estimation of intramitochondrial Ca and pH by FURA-2 and 2,7 biscarboxyethyl-5 (6)-carboxyfluorescein (BCECF) fluorescence. *Biochemical and Biophysical Research Communications.* 149:40–45.

Earm, Y. E., and H. Irisawa. 1986. Effects of pH on the Na-Ca exchange current in single ventricular cells of the guinea pig. *Japanese Heart Journal.* 27(Suppl.):153–158.

Ellis, D., and R. C. Thomas. 1976. Microelectrode measurement of the intracellular pH of mammalian heart cells. *Nature.* 262:224–225.

Fabiato, A. 1985. Use of aequorin for the appraisal of the hypothesis of the release of calcium from the sarcoplasmic reticulum induced by a change of pH in skinned cardiac cells. *Cell Calcium.* 6:95–108.

Fabiato, A., and F. Fabiato. 1978. Effects of pH on the myofilaments and the sarcoplasmic reticulum of skinned cells from cardiac and skeletal muscles. *Journal of Physiology.* 276:233–255.

Fry, C. H., and P. A. Poole-Wilson. 1981. Effects of acid-base changes on excitation-contraction coupling in guinea-pig and rabbit cardiac ventricular muscle. *Journal of Physiology.* 313:141–160.

Fry, C. H., T. Powell, V. W. Twist, and J. P. T. Ward. 1984. The effects of sodium, hydrogen and magnesium ions on mitochondrial calcium sequestration in adult rat ventricular myocytes. *Proceedings of the Royal Society of London Series B Biological Sciences.* 223:239–254.

Gambassi, G., R. G. Hansford, S. J. Sollott, and M. C. Capogrossi. 1991a. Acidosis increases cytosolic and mitochondrial Ca^{2+} in cardiac myocytes. *Biophysical Journal.* 59:238a. (Abstr.)

Gambassi, G., S. J. Sollott, H. A. Spurgeon, and M. C. Capogrossi. 1991b. Transsarcolemmal Ca^{2+} influx increases cytosolic Ca^{2+} during acidosis in cardiac myocytes. *Circulation.* 84:II-29.

Gunter, T. E., and D. R. Pfeiffer. 1990. Mechanisms by which mitochondria transport calcium. *American Journal of Physiology.* 258:C755–C786.

Hansford, R. G. 1985. Relation between mitochondrial calcium transport and control of energy metabolism. *Reviews of Physiology, Biochemistry and Pharmacology.* 102:1–72.

Hansford, R. G., and E. G. Lakatta. 1987. Ryanodine releases Ca^{2+} from sarcoplasmic reticulum in calcium-tolerant rat cardiac myocytes. *Journal of Physiology.* 390:453–467.

Harrison, S. M., J. E. Frampton, E. McCall, M. R. Boyett, and C. H. Orchard. 1992. Contraction and intracellular Ca^{2+}, Na^+, and H^+ during acidosis in rat ventricular myocytes. *American Journal of Physiology.* 262:C348–C357.

Irisawa, H., and R. Sato. 1986. Intra- and extracellular actions of proton on the calcium current of isolated guinea pig ventricular cells. *Circulation Research.* 59:348–355.

Kaibara, M., and M. Kameyama. 1988. Inhibition of the calcium channel by intracellular protons in single ventricular myocytes of the guinea-pig. *Journal of Physiology.* 403:621–640.

Kim, D., E. J. Cragoe, Jr., and T. W. Smith. 1987. Relations among sodium pump inhibition, Na-H exchange activities, and Ca-H interaction in cultured chick heart cells. *Circulation Research.* 60:185–193.

Kim, D., and T. W. Smith. 1987. Altered Ca fluxes and contractile state during pH changes in cultured heart cells. *American Journal of Physiology.* 253:C137–C146.

Kim, D., and T. W. Smith. 1988. Cellular mechanisms underlying calcium-proton interactions in cultured chick ventricular cells. *Journal of Physiology.* 398:391–410.

Kohmoto, O., K. W. Spitzer, M. A. Movsesian, and W. H. Barry. 1990. Effects of intracellular acidosis on $[Ca^{2+}]_i$ transients, transsarcolemmal Ca^{2+} fluxes, and contraction in ventricular myocytes. *Circulation Research.* 66:622–632.

Krafte, D. S., and R. S. Kass. 1988. Hydrogen ion modulation of Ca channel current in cardiac ventricular cells. *Journal of General Physiology.* 91:641–657.

Kurihara, S., E. Tanaka, K. Hongo, N. Suda, O. Okazaki, and Y. Saeki. 1991. Effects of intracellular acidification on Ca^{2+} transients and contraction in mammalian cardiac muscles. *In* The Diabetic Heart. M. Nagano and N. S. Dhalla, editors. Raven Press, New York. 515–522.

Langer, G. A. 1985. The effect of pH on cellular and membrane calcium binding and contraction of myocardium: a possible role for sarcolemmal phospholipid in EC coupling. *Circulation Research.* 571:374–382.

Lattanzio, F. A., Jr. 1990. The effects of pH and temperature on fluorescent calcium indicators as determined with Chelex-100 and EDTA buffer systems. *Biochemical and Biophysical Research Communications.* 171:102–108.

Lazdunski, M., C. Frelin, and P. Vigne. 1985. The sodium/hydrogen exchange system in cardiac cells: its biochemical and pharmacological properties and its role in regulating internal concentrations of sodium and internal pH. *Journal of Molecular and Cellular Cardiology.* 17:1029–1042.

Lee, H.-C., R. Mohabir, N. Smith, M. R. Franz, and W. T. Clusin. 1988. Effect of ischemia on calcium-dependent fluorescence transients in rabbit hearts containing Indo 1: correlation with monophasic action potentials and contraction. *Circulation.* 78:1047–1059.

Liu, S., D. Piwnica-Worms, and M. Lieberman. 1990. Intracellular pH regulation in cultured embryonic chick heart cells. Na^+-dependent Cl^-/HCO_3^- exchange. *Journal of General Physiology.* 96:1247–1269.

McCormack, J. G., and P. J. England. 1983. Ruthenium red inhibits the activation of pyruvate dehydrogenase caused by positive inotropic agents in the perfused rat heart. *Biochemical Journal.* 214:581–585.

Miyata, H., H. S. Silverman, S. J. Sollott, E. G. Lakatta, M. D. Stern, and R. G. Hansford. 1991. Measurement of mitochondrial free Ca^{2+} concentration in living single rat cardiac myocytes. *American Journal of Physiology.* 261:H1123–H1134.

Mohabir, R., H.-C. Lee, R. W. Kurtz, and W. T. Clusin. 1991. Effects of ischemia and hypercarbic acidosis on myocyte calcium transients, contraction, and pH_i in perfused rabbit hearts. *Circulation Research.* 69:1525–1537.

Moreno-Sanchez, R., and R. G. Hansford. 1988. Dependence of cardiac mitochondrial pyruvate dehydrogenase activity on intramitochondrial free Ca^{2+} concentration. *Biochemical Journal.* 256:403–412.

Orchard, C. H. 1987. The role of the sarcoplasmic reticulum in the response of ferret and rat heart muscle to acidosis. *Journal of Physiology.* 384:431–449.

Orchard, C. H., D. A. Eisner, and D. G. Allen. 1983. Oscillations of intracellular Ca^{2+} in mammalian cardiac muscle. *Nature.* 304:735–738.

Orchard, C. H., S. R. Houser, A. A., Kort, A. Bahinski, M. C. Capogrossi, and E. G. Lakatta. 1987. Acidosis facilitates spontaneous sarcoplasmic reticulum Ca^{2+} release in rat myocardium. *Journal of General Physiology.* 90:145–165.

Orchard, C. H., and J. C. Kentish. 1990. Effects of changes of pH on the contractile function of cardiac muscle. *American Journal of Physiology.* 258:H967–H981.

Philipson, K. D., M. M. Bersohn, and A. Y. Nishimoto. 1985. Effects of pH on Na^+/Ca^{2+} exchange in canine cardiac sarcolemmal vesicles. *Circulation Research.* 50:287–293.

Piwnica-Worms, D., R. Jacob, C. R. Horres, and M. Lieberman. 1985. Na/H exchange in cultured chick heart cells. pH_i regulation. *Journal of General Physiology.* 85:43–64.

Pressler, M. L. 1989. Intracellular pH and cell-to-cell transmission in sheep Purkinje fibers. *Biophysical Journal.* 55:53–65.

Solaro, R. J., S. C. El-Saleh, and J. C. Kentish. 1989. Ca^{2+}, pH and the regulation of cardiac myofilament force and ATPase activity. *Molecular and Cellular Biochemistry.* 89:163–167.

Sollott, S. J., B. D. Ziman, and E. G. Lakatta. 1992. A novel technique to load indo-1 "free acid" into single adult cardiac myocytes, to assess cytosolic Ca^{2+}. *American Journal of Physiology.* 262:H1941–H1949.

Sperelakis, N., and E. C. Lee. 1971. Characterization of Na$^+$/K$^+$-ATPase isolated from embryonic chick hearts and cultured chick heart cells. *Biochimica et Biophysica Acta.* 233:562–579.

Spurgeon, H. A., M. D. Stern, G. Baartz, S. Raffaeli, R. G. Hansford, A. Talo, E. G. Lakatta, and M. C. Capogrossi. 1990. Simultaneous measurements of Ca^{2+}, contraction and potential in cardiac myocytes. *American Journal of Physiology.* 258:H574–H586.

Vaughan-Jones, R. D., and M.-L. Wu. 1990. Extracellular H$^+$ inactivation of Na$^+$-H$^+$ exchange in the sheep cardiac Purkinje fibre. *Journal of Physiology.* 428:441–466.

Wier, W. G., A. A. Kort, M. D. Stern, E. G. Lakatta, and E. Marban. 1983. Cellular calcium fluctuations in mammalian heart: direct evidence from noise analysis of aequorin signals in Purkinje fibers. *Proceedings of the National Academy of Sciences, USA.* 80:7367–7371.