Effect of Acidosis on Intracellular pH and Calcium Concentration in the Newborn and Adult Rabbit Myocardium

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This study investigated developmental changes in the effect of acidosis on intracellular pH (pH$_i$) and [Ca]$_i$ in the isolated heart and isolated myocyte preparations. The whole heart or myocytes of newborn (5–7 days old) and adult rabbits were loaded with the fluorescent pH indicator 2',7''-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) or calcium indicator fura-2. Left ventricular pressure in the isolated heart preparation and the magnitude of cell contraction in the single-cell preparation were monitored. The heart and single cell were illuminated with excitation lights (340 and 380 nm, respectively, for fura-2 and 438 and 490 nm for BCECF). The intensity of fluorescence from the ventricular surface or from the cell was detected. [Ca]$_i$ was estimated from the following ratio: fluorescence at 505 nm during excitation at 340 nm/fluorescence at 505 nm during excitation at 380 nm. pH$_i$ was estimated from the following ratio: fluorescence at 530 nm during excitation at 490 nm/fluorescence at 530 nm during excitation at 438 nm. In the newborn, depression of contractile function during respiratory acidosis or metabolic acidosis was less than in the adult. Diastolic and systolic [Ca]$_i$ increased during respiratory acidosis in both the newborn and adult, and the net changes in [Ca]$_i$ were similar in the two age groups. During respiratory or metabolic acidosis, pH$_i$ decreased, but the decrease in the newborn was significantly less than in the adult. These data suggest that the greater resistance of the newborn myocardium to acidosis is due to the smaller change of pH$_i$ in this age group and not due to the difference in [Ca]$_i$ alteration. (Circulation Research 1990;67:111–123)

Acidosis decreases myocardial contractile function.1,2 Decreased sensitivity of myofibrils to calcium3,4 and modification of the function of the sarcoplasmic reticulum that regulates [Ca]$_i$5 have been postulated to be the mechanisms of the altered mechanical function during acidosis.

Our earlier studies6,7 showed that in the isolated rabbit heart preparation the effect of acidosis on contractile function in the premature myocardium was less than in the mature myocardium. The reasons for the developmental changes in the sensitivity to acidosis remain undetermined. We also speculated that intracellular buffer capacity may be greater in the premature myocardium.6 Solaro and colleagues8,9 showed that in the rat the sensitivity of contractile proteins to acidosis was less in the premature myocardium. To understand more clearly the mechanisms of the age-related difference in the negative inotropism of acidosis, the effect of acidosis on intracellular pH (pH$_i$) should be compared in the premature and mature myocardium. However, pH$_i$ has not yet been measured in the premature mammalian myocardium.

Although [Ca]$_i$ has a direct effect on myocardial contractile function, little is known about developmental changes in [Ca]$_i$ and the effect of acidosis on [Ca]$_i$. This study was designed to investigate developmental changes in the effect of acidosis on pH$_i$ and [Ca]$_i$ using the new fluorescent dyes 2',7''-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) and fura-2 in the isolated heart preparation and isolated myocytes of newborn and adult rabbits.

Materials and Methods

Experiments were performed in either the isolated, arterially perfused ventricular preparation or...
single myocytes isolated from the ventricle. Each preparation has advantages and disadvantages. For the experiments using fluorescent dyes, fluorescence observed in the ventricular preparation may come from cells other than myocytes; therefore, the single-cell preparation may be more suitable. However, the effect of the isolation procedure on cellular integrity may be different in the newborn and adult, and this may cause age-related differences in the behavior of the isolated cells to various interventions. Furthermore, in our apparatus for the study of single cells, it was not possible to change extracellular pH rapidly. Therefore, we used two kinds of preparations and compared the data obtained in each preparation.

Newborn (5–7 days old) and adult (6 months old) New Zealand White rabbits were used. The animals were heparinized (15 units/100 g body wt i.v.) and killed by a sharp blow to the skull. The heart was then excised from the chest cavity and used immediately for the study.

**Solutions**

The control Krebs-Henseleit solution contained (mM) NaCl 118, KCl 6, CaCl₂ 1.5, glucose 6, MgCl₂ 1, NaHCO₃ 24, and NaH₂PO₄ 0.436. The control solution was equilibrated with 95% O₂-5% CO₂, yielding a final pH of 7.38–7.42. Respiratory acidosis was produced by equilibration with 80% O₂-20% CO₂, and the perfusate pH was 6.80±0.01. Metabolic acidosis was produced by decreasing NaHCO₃ to 6 mM (the solution was gassed with 95% O₂-5% CO₂), and pH of the perfusate was 6.78±0.02. In the metabolic acidosis solution, NaHCO₃ was replaced with NaCl, and the total sodium concentration was kept to 142 mM.

Fura-2, fura-2-AM, BCECF, and BCECF-AM were purchased from Molecular Probes, Junction City, Ore. Dimethylsulfoxide (DMSO) was purchased from Eastman Kodak, Rochester, N.Y. Fura-2, fura-2-AM, BCECF, and BCECF-AM were dissolved in DMSO to 1 mM, and these stock solutions were stored at −80°C. Nigericin (Calbiochem, La Jolla, Calif.) dissolved to 10 mM in dimethylformamide : ethanol (3:1) and ionomycin (Calbiochem) dissolved to 1 mM in DMSO were also stored at −80°C. Cremophor EL (Sigma Chemical, St. Louis), a noncytotoxic detergent, was diluted to 25% with H₂O and stored at room temperature. Emulgen 810, a nonfluorescent detergent, was a gift from Kao, Tokyo, Japan. Triton X-100 was purchased from Sigma Chemical. Digitonin was purchased from Dojin, Kumamoto, Japan.

**Ventricular Preparation**

In the ventricular preparation, the aorta was cannulated with a polyethylene cannula and perfused with oxygenated perfusate at a constant perfusion rate of 5 ml/g tissue/min with a roller pump. The atrial wall was cut open, and a suture was placed in the atrioventricular nodal region to block atrioventricular conduction. The heart was stimulated at 40 beats/min, and its temperature was maintained at 27°C. A latex balloon was placed into the left ventricle via the left atrium. A fluid-filled polyethylene cannula was attached to the intraventricular balloon, and left ventricular pressure was monitored using a pressure transducer and an amplifier (San-ei Sokki, Tokyo, Japan). The volume of water in the balloon was adjusted using a syringe connected to the side arm of the cannula to obtain left ventricular resting pressure of 6–8 mm Hg in both the newborn and adult. The heart preparation was placed in a tissue bath that was mounted on the stage of an inverted microscope (model TMD, Nikon, Tokyo, Japan). By use of micromanipulators connected to the intra-aortic and intraventricular cannulas, the position of the heart was adjusted so that only a small portion of the ventricular surface (approximately 3–7 mm in diameter) touched the cover glass lightly. When the heart contracted, the heart moved upward, but the observed area of the cardiac surface (left ventricular free wall) was still touching the glass coverslip. To minimize the effect of cardiac movement, a ×10 objective (Fluor, Nikon), rather than an objective with higher magnification, was used.

**Dye loading in the ventricular preparation.** Fura-2-AM and BCECF-AM are acetoxyethyl esters of fura-2 and BCECF, respectively, and are membrane permeable. Once fura-2-AM and BCECF-AM enter the cell, the ester is hydrolyzed, and the free form of fura-2 or BCECF does not leak out of the cell easily. Initially, the heart was perfused with a control solution for 20 minutes to allow for stabilization of the mechanical function. The fluorescent dye was dissolved in a perfusion solution using a modified method of Poenie et al: a mixture was made of 200 μl of 1 mM fura-2-AM or BCECF-AM in DMSO, 50 μl of 25% wt/wt cremophor EL, and 1,500 μl fetal calf serum. The mixture was added to 80 ml oxygenated Krebs-Henseleit solution and mixed well. The flask containing this solution was then placed in the water bath of an ultrasonicator (Hitachi, Tokyo, Japan) and sonicated for 20 minutes. The heart was then perfused with the solution containing 2.5 μM fura-2-AM or 2.5 μM BCECF-AM for 20 minutes. In the adult heart, the perfusion solution containing fura-2-AM or BCECF-AM was recirculated. After 20 minutes of perfusion with the solution containing the fluorescent dye, the heart was perfused for 60 minutes with dye-free Krebs-Henseleit solution to wash out extracellular fura-2-AM or BCECF-AM. Ventricular pressure decreased to about 60% of the control value after 20 minutes of perfusion with a solution containing fura-2-AM or BCECF-AM, but the mechanical function returned to the control level after washout of the dye.

**Single-Cell Preparation**

The heart was excised from the chest cavity and perfused via the aorta with oxygenated calcium-free Krebs-Henseleit solution for 3 minutes (newborn) or 5 minutes (adult) at a flow rate of 5 ml/g/min. The heart was then perfused with calcium-free Krebs-Henseleit...
solution containing collagenase (500 units/mg, 50 mg/100 ml) (Yakult, Tokyo, Japan) and hyaluronidase (295 units/mg, 50 mg/100 ml) (type I-S, Sigma) for 3–10 minutes at 37°C until it became flaccid. After perfusion for 3 minutes with calcium-free Krebs-Henseleit solution that did not contain enzymes, the left ventricular muscle was minced with fine scissors and suspended in a calcium-free N-(2-hydroxyethyl)piperazine-N'- (2-ethanesulfonic acid) (HEPES) buffer solution containing (mM) NaCl 142, MgCl₂ 1, KCl 5, HEPES 5, and glucose 6, pH 7.4. The cells were dispersed from the small pieces by mechanical agitation and were filtered through a mesh to remove tissue debris. The cell suspension was centrifuged for 3 minutes at 100 rpm, and the supernatant was discarded. The cell pellet was suspended in a HEPES solution containing 0.1 mM calcium and 2.5 μM fura-2-AM or 2.5 μM BCECF-AM. After 7 minutes, the suspension was centrifuged for 3 minutes at 100 rpm at room temperature. The resulting supernatant was discarded, and the pellet was suspended in a HEPES solution containing 0.3 mM calcium. An aliquot of the cell suspension was placed in the tissue bath, which was mounted on the microscope stage. After the cells settled to the bottom of the tissue bath, they were superfused with oxygenated Krebs-Henseleit solution containing 1.5 mM calcium (the same solutions used in the ventricular preparation) at a flow rate of 1 ml/min using roller pumps. At 1.5 mM extracellular calcium, 50–70% of the adult cells and 20–50% of the newborn cells exhibited a rodlike configuration (Figure 1), and the rest were round. Gas-impermeable tubes (Irika Kogyo, Tokyo, Japan) were used in the roller pumps. In addition, the tissue bath (10 mm × 30 mm, 2 mm in depth) was sealed with plastic wrap using silicon grease, and the same gas mixture that was used into bubble the solution in the reservoir was infused into the surface of the solution in the tissue bath. The pH of the solution in the tissue bath was monitored continuously using a flat-type pH probe (Orion, Cambridge, Mass.). The pH of the control solution in the tissue bath was 7.42±0.02, and that of the respiratory acidosis solution was 6.82±0.02 (0.02 units higher than the pH values in the reservoir). The temperature of the tissue bath was monitored continuously and maintained at 27°C. After changing from one solution to another, it took 5–7 minutes for the extracellular pH to reach the new steady state. The cells were stimulated electrically at 40 beats/min, and fluorescence from the single rod-shaped cell was observed using a ×40 objective (Fluor, Nikon).

**Figure 1.** Rod-shaped single cells in the adult (panel A) and newborn (panel B) rabbit myocardium. The shape of the adult cell was rectangular, whereas that of the newborn cell was more cylindrical. Calibration bars, 20 μm.
Mechanical function of single cells. The optical system to monitor cell motion was similar to that described by Peeters et al. The cells were illuminated using the standard microscope light source (12-V lamp) passed through a 750-nm sharp-cut filter. Twenty percent of the red light illuminating the cell was reflected by a prism and delivered to a low light level Newvicon television camera (model NC-70X, Dage-MTI, Michigan City, Ind.). The image of the cell was monitored by the television camera, and the television camera output was connected to a video motion analyzer (Perceptoscope C3161, Hamamatsu Photonics, Hamamatsu, Japan). The cell length was measured by the video motion detector every 32 msec and recorded on a strip-chart recorder (San-ei Sokki). The amplitude of cell shortening was calculated as follows: (cell length at diastole−cell length at end systole)/cell length at diastole×100.

Fluorescence Measurements

Excitation lights of 340 and 380 nm wavelength (for fura-2) or 439 and 490 nm (for BCECF) were obtained alternatively using a xenon lamp (450 W), two monochrometers, and a chopper (model CM2, SPEX, Edison, N.J.). The light was directed from the fluorometer to the microscope and illuminated the heart preparation through a glass coverslip mounted on the bottom of the tissue bath. The emitted light passed through a 505-nm (for fura-2) or 530-nm (for BCECF) band-pass filter, and its intensity was measured by a photomultiplier (model R928, Hamamatsu Photonics). The signal was recorded and analyzed using an IBM PC/AT–based data collection system (model DM3000CM, SPEX). In the present experiment, the intensity of fura-2 fluorescence at each excitation wavelength was observed for 5 msec at 20-msec intervals: both fluorescence intensity at 340-nm excitation and that at 380-nm excitation were measured at 20-msec intervals. Since pH did not change with cardiac contraction, BCECF fluorescence was measured for 1 second at 5-second intervals. In all experiments, the autofluorescence intensity was measured in the heart before fura-2 or BCECF loading, and the data were stored in a hard disk of the computer. At the end of the experiment, the autofluorescence value was subtracted from the value obtained after the dye-loading. The fura-2 fluorescence ratio was then calculated as follows using the computer: ratio=(fluorescence at 340 nm−autofluorescence at 340 nm)/(fluorescence at 380 nm−autofluorescence at 380 nm). The BCECF fluorescence ratio (490/439 nm) was calculated in a similar fashion. Acidosis did not significantly alter the autofluorescence at the fura-2 or BCECF excitation wavelength. In the experiment using single cells, autofluorescence was measured on myocytes from the same batch that were not loaded with the dye.

Calibration of BCECF fluorescence ratio. The relation between the fluorescence ratio and pH was determined using single cells of the newborn and the adult. Myocytes were loaded with BCECF-AM and washed 3 times to remove extracellular BCECF-AM. Aliquots of the myocytes were suspended in pH calibration solutions with various pH values. The calibration solution contained 10 μM nigericin, 30 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), 1 mM EGTA, 3 mM MgCl₂, 133 mM KCl, 11 mM NaCl, and pH was adjusted to 7.4, 7.1, 6.8, and 6.5 with 0.5N KOH. After the cells were allowed to equilibrate with extracellular pH for 30 minutes, fluorescence from single cells was measured in a manner similar to that described above. Variation among the ratios measured in the cells at each pH was small, and the value in the newborn was similar to that in the adult (Figure 2A). Therefore, the in vivo calibration curve was used to estimate pH.

Excitation spectra (emission at 530 nm) of BCECF loaded into the ventricular preparation may be different from that of the dye in the solution. Therefore, calibration was performed using the dye extracted from the muscle by homogenization. The muscle loaded with BCECF was homogenized with 10 vol (muscle weight in grams times 10 ml) of a solution containing (mM) TES 30, EGTA 1, MgCl₂ 3, KCl 133, and NaCl 11. The pH of the homogenate was then adjusted to 7.4, 7.1, 6.8, and 6.5 with 0.5N KOH or 0.5N HCl, and the fluorescence of an aliquot of the homogenate was measured. The homogenate of the muscle not loaded with BCECF was used to measure autofluorescence. The autofluorescence intensity, however, was not significantly different from the background counts that were measured using H₂O. The BCECF fluorescence ratio of the muscle homogenate at various pH values was slightly different from that obtained in the calibration solution, but the value in the newborn was similar to that in the adult (Figure 2B). The pH in the ventricular preparation was estimated using the calibration curve obtained with the homogenate. If the in vivo calibration curve was used to estimate pH, the pH value would have been approximately 0.1 unit higher in both the newborn and adult.

It might be more desirable to construct an in vivo calibration curve in the ventricular preparation. Therefore, in a preliminary experiment, the ventricular preparation loaded with BCECF was perfused with solutions containing 10 μM nigericin, 30 mM TES, 1 mM EGTA, 3 mM MgCl₂, 133 mM KCl, 11 mM NaCl (pH 7.4, 7.1, 6.8, and 6.5 with 0.5N KOH), and the fluorescence from the ventricular surface was measured. The duration of perfusion was 20 minutes at each pH value. The fluorescence intensity reached a new steady state within 10 minutes. The in vivo calibration curve constructed by nigericin perfusion in the ventricular preparation was similar to that obtained using the muscle homogenate in the two age groups. However, this calibration procedure required a large amount of nigericin, especially in the adult. Therefore, calibration using the muscle homogenate was routinely performed.

Calibration of fura-2 fluorescence ratio. Initially, attempts to construct an in vivo calibration curve of...
fura-2 fluorescence were made. The myocytes were loaded with fura-2-AM as described above, and extracellular fura-2-AM was removed by centrifugation (100 rpm for 2 minutes). Myocytes were washed three times, and an aliquot of the cell suspension was added to the calibration solutions. The calibration solution contained (mM) TES 30, EGTA 10, MgCl₂ 3, KCl 115, and NaCl 11 and various amounts of CaCl₂ (free calcium ranging from 1 mM to 2 mM) and 6 μM ionomycin (pH 7.1 with KOH). A desired calcium concentration in the medium was obtained using an EGTA buffer system. The 2 mM calcium solution did not contain EGTA. After the cells were allowed to equilibrate with extracellular calcium for 30 minutes, an aliquot of the cell suspension was placed on the glass coverslip, and fluorescence from single cells was measured at each calcium concentration. Fura-2 (5 μM), rather than fura-2-loaded cells, was added to the calibration solution, and an in vitro calibration curve was also constructed. Although the fluorescence ratio in each cell was variable (Figure 2C), the peak value obtained at each calcium concentration was similar in the two age groups. At pCa 2.7 (2 mM calcium), the ratios in the cells were less

**Figure 2.** Panel A: Graph showing in vitro and in vivo calibration of 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) fluorescence ratio. In vivo calibration was performed in single cells loaded with BCECF in the presence of 5 μM nigericin. The fluorescence ratio was measured in 10 single cells at each pH in both newborn and adult rabbit myocardium; values are mean±SEM. The in vivo calibration curve was similar in the newborn and adult but slightly different from the in vitro calibration curve. Panel B: Graph showing fluorescence ratio of the homogenate of the muscle loaded with BCECF-AM. In vitro calibration using solutions containing BCECF-AM is also shown. The fluorescence ratio of the homogenate was similar in the newborn and adult but slightly different from that of the calibration solution. Values are mean±SEM of 11 experiments. Panel C: Scatterplots showing in vitro and in vivo calibration of fura-2 fluorescence ratio. In vivo calibration was performed in single cells of the newborn and adult in the presence of 6 μM ionomycin. At pCa 2.7 (2 mM calcium), the ratio values obtained in the cells were similar in the two age groups and approached the value obtained in the solution.
variable and approached the value in the solution. Furthermore, the ratio obtained in the newborn cells at pCa 2.7 was similar to the value in the adult cells. In additional experiments, 2% Triton X-100 or 2% Emulgen 810, a nonfluorescent detergent, was added to the cell suspension to lyse the cell membrane, and a calibration curve was constructed using the supernatant. The curve was identical to the in vitro calibration curve. These data suggest that the difference between the in vivo and in vitro calibration results from the inadequate effect of ionomycin to increase membrane permeability to calcium rather than from calcium-insensitive forms of fura-2. The reason for the inadequacy of the ionomycin effect is not clear, but Cheung et al. and Wier et al. also reported similar technical difficulties with ionomycin. From these data, we decided to use an in vitro calibration curve to estimate \([Ca_i]\) in the newborn and adult single-cell preparation.

Effect of pH change on fura-2 fluorescence was also determined using in vitro calibration solutions with free calcium concentrations of 1 nM and 2 mM. At each calcium concentration, pH was adjusted to 6.5, 7.1, and 7.7, and fluorescence was measured. There was no significant effect of pH change on fura-2 fluorescence.

Fura-2 may be incorporated into intracellular organelles, such as sarcoplasmic reticulum and mitochondria, and this dye compartmentation may interfere with the cytosolic calcium estimation. In a preliminary experiment, the degree of the dye compartmentation was assessed using digitonin, which disrupts the sarcolemma but not the mitochondrial or sarcoplasmic reticulum membrane. The fura-2-loaded single cells were superfused with calcium-free Krebs-Henseleit solution containing digitonin (50 \(\mu\)M), and the fluorescence intensity (excitation at 360 nm) was measured. After 5 minutes of digitonin perfusion, fura-2-associated fluorescence intensity decreased to a level that was not significantly different from the autofluorescence intensity in the two age groups. This suggests that most of the intracellular fura-2 was in the cytosol in both the newborn and the adult.

Because manipulation of \([Ca_i]\) using ionomycin was technically difficult, the in vivo calibration could not be performed in the whole-heart preparation. Since intensity of autofluorescence at 340- and 380-nm excitation was significant and varied depending on the metabolic conditions, calibration of the fluorescence ratio using muscle homogenate could not be performed. Therefore, the absolute value of \([Ca_i]\) was not estimated in the ventricular preparation.

**Intracellular Buffering Power Estimation**

Intracellular buffering power was calculated according to the method of Roos and Boron as follows: buffering power = net change in \([HCO_3^-]\)/net change in pH during acidosis. \([HCO_3^-]\) was calculated as follows: \([HCO_3^-] = s \cdot P_{CO_2} \cdot 10^{pK-s}\), where \(s\) and \(pK\) are the CO₂ solubility and dissociation constant, respectively. The \(s\) and \(pK\) values in the newborn and adult rabbit myocardium have not been measured directly and were assumed to be 0.0391 mM/l/mm Hg and 6.17, respectively, at 27°C. Cellular Pco₂ was assumed to be equal to the extracellular Pco₂, and extracellular Pco₂ was measured directly using a blood-gas analyzer (Radiometer, Copenhagen, Denmark).

**Experimental Protocol**

In both the ventricular preparation and the single-cell preparation, 60 minutes was allowed for stabilization of mechanical function. In the ventricular preparation, the heart was perfused with an acidic (either respiratory or metabolic) solution for 10 or 30 minutes and then perfused with a control solution for 30 minutes.

In the single-cell preparation, only the effect of respiratory acidosis was examined. Since the solutions in the tissue bath could not be exchanged rapidly, the respiratory acidosis solution was perfused for 20–30 minutes, and mechanical function and fluorescence were recorded at least 10 minutes after the extracellular pH reached the new steady state.

**Statistical Analysis**

Results were expressed as mean±SEM. Statistical significance of difference between group means was determined using Student’s t test. Statistical significance of response of \([Ca_i]\) and pH to acidosis was analyzed using the paired t test and a repeated-measures analysis of variance. Percent changes were compared using a nonparametric method (Wilcoxon’s rank-sum test). The probability was considered to be significant at \(p<0.05\).

**Results**

**Ventricular Preparation**

**Mechanical function.** Left ventricular contractile function was measured in the isolated isovolumic heart preparation, and baseline mechanical function was similar to the data of Parrish et al. In brief, under our experimental conditions in which left ventricular diastolic pressure was set at 6–8 mm Hg in both the newborn and the adult, left ventricular developed pressure in the newborn (19±3 mm Hg, \(n=20\)) was significantly less than in the adult (74±3 mm Hg, \(n=16\)).

The effect of acidosis on mechanical function was similar to the previously reported data. In the newborn, left ventricular developed pressure decreased transiently to 59±6% of the control value and recovered to 92±7% at 10 minutes into respiratory acidosis (\(n=6\), Figure 2). In the adult, left ventricular developed pressure decreased transiently to 27±3% and recovered to 48±4% at 10 minutes into respiratory acidosis (\(n=6\), Figure 2). The negative inotropic effect of respiratory acidosis in the newborn was significantly less than in the adult. On returning to the control solution, left ventricular developed pressure
FIGURE 3. Recordings showing effect of respiratory acidosis on intracellular pH (pHi) and left ventricular (LV) pressure in the adult (panel A) and newborn (panel B) rabbit ventricular preparation. On induction of respiratory acidosis, both pHi and LV pressure decreased immediately. LV pressure recovered significantly after a transient depression, but pHi showed minimal recovery. In the newborn, LV pressure at the new steady state was not significantly different from the control level. Depression of pHi in the newborn was less than in the adult. On returning to the control solution, an overshoot of LV pressure was observed. pHi returned to the control level with minimal overshoot.

increased to a level greater than control and then returned to the control level gradually.

Metabolic acidosis caused a transient increase in left ventricular developed pressure and then a gradual decrease. In the newborn, left ventricular developed pressure at 30 minutes into metabolic acidosis was 96±2% of the control value (n=5), which was greater than in the adult (80±5%, n=5).

pHi in the whole heart. Under control conditions, pHi in the newborn (7.03±0.04, n=6) was not significantly different from that in the adult (7.10±0.09, n=6). In both the newborn and adult, respiratory acidosis caused a rapid decline in pHi, which was followed by a slight but significant recovery (Figure 3). pHi during acidosis in the newborn was significantly higher than in the adult (Figure 4). The net decrease of pHi during 10 minutes of respiratory acidosis in the newborn (0.21±0.04 pH unit) was significantly less than in the adult (0.46±0.04 pH unit). The present finding of pHi changes during acidosis in the adult was similar to the data obtained using pH-sensitive microelectrodes.32

During metabolic acidosis, pHi increased initially and then decreased gradually in both the newborn and adult (Figure 5). The reason for the initial increase in pHi is not clear, but a similar finding was reported by Ellis and Thomas.32 After 30 minutes into metabolic acidosis, the pHi in the newborn was significantly higher than in the adult (Figure 6). The net decrease in pHi during 30 minutes of metabolic acidosis in the newborn (0.08±0.01 pH unit, n=5) was less than in the adult (0.32±0.03 pH unit, n=5).

Ca2+ in the whole heart. Fluorescence obtained at 340-nm excitation increased and that obtained at 380-nm excitation decreased with cardiac contraction. Autofluorescence, which is fluorescence without fura-2, was less than 25% of the fura-2 fluorescence and did not change with cardiac contraction. Under control conditions, fluorescence ratios in the adult were greater than in the newborn (Figure 7). In both the newborn and adult, the diastolic and systolic [Ca2+] increased gradually during acidosis and reached the steady state after 5 minutes into acidosis (Figure 7). On returning to the control solution, the fluorescence ratios during diastole and during systole were still increased and returned to the control level gradually.

Single-Cell Preparation

Mechanical function. In the single-cell preparation, cell length and width in the newborn were less than in the adult (Figure 1). Under control conditions, cell shortening in the newborn was less than in the adult (Table 1). During respiratory acidosis, contractility did not change significantly in the newborn, but it decreased to 69% of the control value in the adult. The negative inotropic effect of respiratory acidosis in the newborn was less than in the adult (Table 1). In the single-cell preparation, transient depression fol-
Figure 5. Recordings showing effect of metabolic acidosis on intracellular pH (pHi) and left ventricular (LV) pressure in the adult (panel A) and newborn (panel B) rabbit ventricular preparation. On induction of metabolic acidosis, both pHi and LV pressure increased transiently and then decreased gradually. In the newborn, decreases in pHi and LV pressure during acidosis were minimal.

Followed by a recovery of mechanical function was not observed, probably because solution exchange was not as rapid as that in the ventricular preparation.

pHi in single cells. Under control conditions, pHi in the single-cell preparation (Table 2) was similar to that in the ventricular preparation. Changes in extracellular pH and pHi during acidosis are shown in Figure 8. Since the solutions could not be exchanged rapidly and since the time course of the extracellular pH change was variable in each experiment, only pHi at a steady state was used for data analysis. The net decrease in pHi during respiratory acidosis in the newborn (0.2 pH unit) was significantly less than in the adult (0.32 pH unit, Table 2). Intracellular buffering power, which was calculated by using the values in Table 2, in the adult (41 ± 6 mM/pH) was significantly less than in the newborn (82 ± 14 mM/pH).

[Ca2+]i in single cells. Under control conditions, [Ca2+]i estimated by using the in vitro calibration curve in the newborn cell was less than in the adult (Table 3). In both the newborn and adult, the diastolic and systolic [Ca2+]i increased gradually during acidosis and reached a new steady state (Figure 9). Net changes in diastolic [Ca2+]i, systolic [Ca2+]i, and the magnitude of calcium transient in the newborn were not significantly different from those in the adult (Table 3).

Discussion

The present study measuring left ventricular pressure confirmed the data in our previous study,6 which showed that the contractile function of the premature myocardium is more resistant to acidosis. The present study also showed that the age-related difference in the effect of acidosis on contractile function, pHi, and [Ca2+]i observed in the ventricular preparation was directionally similar to that observed in the single-cell preparation. This suggests that isolated single cells retain the age-related properties observed in the intact muscle. Although relative depression of mechanical function during respiratory acidosis in the adult single cells (69% of control) was less than in the ventricular preparation (48% of control), this may result from the difference in mechanical loading conditions in the two preparations. In addition, the net decrease in pHi in the adult single-cell preparation (0.32 pH unit) was less than in the ventricular preparation (0.46 pH unit), and this may be partly responsible for the difference in mechanical function during acidosis. Since only a limited number of cells were used and data were taken only from rod-shaped cells, there may be a bias in the data obtained in the single-cell preparation.

In the single-cell preparation, baseline shortening fraction in the newborn was less than in the adult. This finding is consistent with the data of Nassar et al,33 who showed that shortening of sarcomere length in the 3-week-old rabbit was less than in the adult. The decreased contractility in the premature myocardium may be partly due to the relatively scarce and disorganized myofilaments.33–35

The greater resistance of the contractile function of the newborn myocardium to acidosis may be due to the age-related difference in 1) the intracellular buffer capacity, 2) sensitivity of contractile proteins to acidosis, and/or 3) sensitivity of the regulatory system of [Ca2+]i to acidosis. The present study showed that changes in pHi during acidosis were less in the newborn than in the adult (Figure 4, Table 3) and
that the intracellular buffer capacity in the newborn (82 mM) was greater than in the adult (41 mM). The mechanism for the greater buffer capacity in the newborn is not clear, but greater sarcoplasmic Na-H exchange activity and/or greater sarcoplasmic surface/volume ratio may be partly responsible.

Solaro et al.\textsuperscript{8,9} showed that in the newborn dog and rat the sensitivity of contractile proteins to acidosis was less than in the adult. However, Nakanishi and colleagues showed that myofibrillar ATPase activity decreased at low pH similarly in the newborn and adult rabbit. Species difference may exist in the sensitivity of contractile proteins to acidosis, but at least in the rabbit we think it is unlikely that the age-related difference in the sensitivity of contractile proteins to acidosis is responsible for the difference in the negative inotropism of acidosis. Thus, the present study indicates that the greater resistance of the contractile function in the premature myocardium to acidosis may be at least partly due to the greater intracellular buffer capacity.

Previous studies using adult hearts (guinea pig, rat, rabbit, and ferret) have shown that [Ca\textsubscript{i}] increases during acidosis. Although the mechanism of increased [Ca\textsubscript{i}] during acidosis is not clear, it may be a result of decreased calcium sequestration by intracellular organelles such as the sarcoplasmic reticulum.\textsuperscript{5} Solaro et al.\textsuperscript{8} used aequorin as a calcium

**TABLE 1. Effect of Respiratory Acidosis on Mechanical Function in the Single-Cell Preparation From Myocytes of Adult and Newborn Rabbits**

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<td>(n)</td>
<td>(% of resting length)</td>
<td>(% of resting length)</td>
<td>(% of control)</td>
</tr>
<tr>
<td>Adult</td>
<td>27</td>
<td>119±4</td>
<td>10.3±0.4</td>
<td>7.0±0.5</td>
</tr>
<tr>
<td>Newborn</td>
<td>26</td>
<td>77±4*</td>
<td>6.6±0.2*</td>
<td>6.4±0.2</td>
</tr>
</tbody>
</table>

Values are mean±SEM.
All measurements were performed at least 10 minutes after pH in the tissue bath reached the new steady state.

*Significantly different from the adult value.
TABLE 2. Effect of Respiratory Acidosis on Intracellular pH in the Single-Cell Preparation From Myocytes of Adult and Newborn Rabbits

<table>
<thead>
<tr>
<th></th>
<th>pH&lt;sub&gt;i&lt;/sub&gt;</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Control</td>
<td>Acidosis</td>
</tr>
<tr>
<td>Adult</td>
<td>15</td>
<td>7.01±0.04</td>
<td>6.69±0.03</td>
</tr>
<tr>
<td>Newborn</td>
<td>13</td>
<td>6.88±0.04*</td>
<td>6.66±0.03</td>
</tr>
</tbody>
</table>

Values are mean±SEM. pH<sub>i</sub>, intracellular pH; ΔpH<sub>i</sub>, net decrease in pH<sub>i</sub> during respiratory acidosis. Values are in pH unit. All measurements were performed at least 10 minutes after pH in the tissue bath reached the new steady state.

*Significantly different from the adult value.

indicator and showed that in the adult rat recovery of mechanical function after a transient depression during acidosis was associated with increases in the magnitude of calcium transient. They also showed that in the newborn rat contractile function deteriorated gradually during acidosis and that this deterioration was associated with small decreases in the magnitude of calcium transient.

In contrast to the data in the rat, contractile function depressed transiently and then recovered during acidosis in both the newborn and adult rabbit. In addition, significant increases in diastolic and systolic [Ca], were observed in the two age groups (Figure 7). It is most likely that the recovery of mechanical function is due to small recovery of pH<sub>i</sub> (Figure 4) and to increased [Ca], during acidosis. In the present study, the net recovery of left ventricular pressure during acidosis in the newborn (33±8% of control) was not significantly different from that in the adult (21±4%). This finding is consistent with the similar recovery of pH<sub>i</sub> and similar increases in [Ca]<sub>i</sub> in the two age groups (Table 3). Therefore, it is unlikely that the difference in contractile function

![Figure 8. Recordings showing effect of respiratory acidosis on intracellular pH (pH<sub>i</sub>) and pH in the tissue bath (pH<sub>b</sub>) in the adult (panel A) and newborn (panel B) rabbit myocyte preparation. The rate of exchange of the perfusate was variable in each experiment, but the change in pH<sub>i</sub> was directionally similar to that observed in the ventricular preparation. Depression of pH<sub>i</sub> in the newborn was less than in the adult.](http://circres.ahajournals.org/doi/10.1161/01.RES.67.1.120)
During acidosis is explained by the changes in \([\text{Ca}]_i\) in the rabbit.

Since the depression of pH during acidosis was greater in the adult, one may expect the greater change in \([\text{Ca}]_i\) in this age group. The reason for the similar changes in \([\text{Ca}]_i\) in the two age groups is not clear, but the effect of intracellular acidosis on the function of the sarcoplasmic reticulum is complex. At low values of pH, calcium uptake and release from the sarcoplasmic reticulum may be decreased. However, an increased diastolic \([\text{Ca}]_i\) caused by acidosis may result in the greater calcium-induced calcium release from the sarcoplasmic reticulum. Thus, it is difficult to estimate the magnitude of \([\text{Ca}]_i\) change from the size of the pH change. Of course, further studies to determine the effect of various degrees of intracellular acidosis on the function of the sarcoplasmic reticulum should be performed.

On returning to the control solution, an overshoot of mechanical function was observed (Figure 7). Since \([\text{Ca}]_i\) was still increased at the time of the overshoot, increased \([\text{Ca}]_i\) and restored sensitivity of contractile proteins to calcium may have caused the overshoot.

In the present study, both diastolic and systolic \([\text{Ca}]_i\) in the adult was higher than in the newborn (Table 3). Nakanishi and Jarmakani showed previously in the isolated rabbit heart preparation that the extracellular calcium–tension curve in the newborn shifted to the right compared with that in the adult. Since the sensitivity of contractile proteins to calcium was similar in the newborn and adult, they speculated that \([\text{Ca}]_i\) in the newborn may be lower than in the adult. The present data were in agreement with this speculation. Since the present study did not measure calcium flux across the sarcolemma directly and since \([\text{Ca}]_i\) is dependent on this parameter, the precise reasons for the age-related difference in \([\text{Ca}]_i\) remain unclear.

One may argue that the lower \([\text{Ca}]_i\) in the newborn may be responsible for the greater tolerance of contractile function to acidosis. However, in our previous study, the effect of the reduction of pH from 7.1 to 6.5 on the myofibrillar ATPase activity was greater at the lower calcium concentrations. Although the biochemical data may not be directly applied to the intact muscle, the data suggest that the negative inotropic effect of acidosis will be greater if \([\text{Ca}]_i\) is lower. Therefore, it is unlikely that lower \([\text{Ca}]_i\)

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**TABLE 3. Effect of Respiratory Acidosis on \([\text{Ca}]_i\) in the Single-Cell Preparation From Myocytes of Adult and Newborn Rabbits**

<table>
<thead>
<tr>
<th></th>
<th>Newborn</th>
<th></th>
<th></th>
<th>Adult</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diastole (nM)</td>
<td>Systole (nM)</td>
<td>(\text{Ca} \text{ transient} (nM))</td>
<td>Diastole</td>
<td>Systole</td>
<td>(\text{Ca} \text{ transient} (nM))</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>54±13*</td>
<td>229±11*</td>
<td>175±18</td>
<td>169±22</td>
<td>366±41</td>
</tr>
<tr>
<td>Acidosis</td>
<td>6</td>
<td>88±14†</td>
<td>272±12†</td>
<td>184±17</td>
<td>208±28†</td>
<td>442±50†</td>
</tr>
<tr>
<td>Net change</td>
<td>+33±6</td>
<td>+43±11</td>
<td>+10±11</td>
<td>+39±13</td>
<td>+76±29†</td>
<td>+38±21</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

- \(\text{Ca transient, systolic} \ [\text{Ca}]_i \text{ minus diastolic} \ [\text{Ca}]_i; \text{net change,} \ [\text{Ca}]_i \text{ value minus control value. All measurements were performed at least 10 minutes after pH in the tissue bath reached the new steady state.}\)

- *Significantly different from the adult value.
- †Significantly different from the control value.

---

**FIGURE 9.** Recordings showing effect of respiratory acidosis on cell contraction and fura-2 fluorescence in the adult (panel A) and newborn (panel B) rabbit myocyte preparation. Note that the two parameters were not recorded on the same recording paper (paper speed of 25 mm in both parameters). Contractility of the cell was depressed during acidosis in the adult, but not in the newborn. During acidosis, \([\text{Ca}]_i\) was significantly higher than the control level in both the newborn and adult.
is responsible for the greater tolerance to acidosis in the newborn rabbit.

Finally, it must be noted that determination of the absolute value of \([\text{Ca}^+]\), is problematic. Although we used 6 \(\mu\text{M}\) ionomycin in the in vivo calibration, which is a higher concentration than that used by other investigators, the fluorescence ratio in the cell was still variable (Figure 2), suggesting the inadequacy of the ionomycin effect to increase the cell membrane permeability to calcium. In a preliminary study, ionomycin concentrations higher than 6 \(\mu\text{M}\) resulted in loss of fura-2 from the cell. Since it was unlikely that the difference between the in vivo calibration and in vitro calibration curve (Figure 2) was caused by calcium-insensitive forms of fura-2, we used an in vitro calibration curve and obtained \([\text{Ca}^+]\) values similar to those reported by other investigators.

In summary, the present data suggest that the greater resistance of contractile function in the premature myocardium is at least in part due to the greater buffer capacity and not due to the difference in the \([\text{Ca}^+]\) alteration.

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• intracellular calcium
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