Gap Junctional Conductance between Pairs of Ventricular Myocytes Is Modulated Synergistically by H^+ and Ca^{++}

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ABSTRACT Gap junctional conductance (g_j) between cardiac ventricular myocyte pairs is rapidly, substantially, and reversibly reduced by sarcoplasmic acidification with CO_2 when extracellular calcium activity is near physiological levels (1.0 mM $CaCl_2$ added; 470 μM Ca^{++}). Intracellular calcium concentration (Ca_i), measured by fura-2 fluorescence in cell suspensions, was 148 ± 39 nM (± SEM, $n = 6$) and intracellular pH (pH_i), measured with intracellular ion-selective microelectrodes, was 7.05 ± 0.02 ($n = 5$) in cell pair preparations bathed in medium equilibrated with air. Ca_i increased to 515 ± 12 nM ($n = 6$) and pH_i decreased to 5.9–6.0 in medium equilibrated with 100% CO_2.

In air-equilibrated low-calcium medium (no added $CaCl_2$; 2–5 μM Ca^{++}), Ca_i was 61 ± 9 nM ($n = 13$) at pH_i 7.1. Ca_i increased to only 243 ± 42 nM ($n = 9$) at pH_i 6.0 in CO_2-equilibrated low-calcium medium. Junctional conductance, in most cell pairs, was not substantially reduced by acidification to pH_i 5.9–6.0 in low-calcium medium. Cell pairs could still be electrically uncoupled reversibly by the addition of 100 μM octanol, an agent which does not significantly affect Ca_i.

In low-calcium low-sodium medium (choline substitution for all but 13 mM sodium), acidification with CO_2 increased Ca_i to 425 ± 35 nM ($n = 11$) at pH_i 5.9–6.0 and g_j was reduced to near zero. Junctional conductance could also be reduced to near zero at pH_i 6.0 in low-calcium medium containing the calcium ionophore, A23187. The addition of the calcium ionophore did not uncouple cell pairs in the absence of acidification. In contrast, acidification did not substantially reduce g_j when intracellular calcium was low. Increasing intracellular calcium did not appreciably reduce g_j at pH_i 7.0. These results suggest that, although other factors may play a role, H^+ and Ca^{++} act synergistically to decrease g_j.

INTRODUCTION

Gap junctions, large highly conductive hydrophilic channels, provide a direct intercellular diffusion pathway between heart cells. Gap junctional conductance (g_j) reflects the intercellular permeability to K^+ and other small ions. Changes in g_j may

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be the result of differences in the number of junctional channels available for diffusion or differences in permeability at the level of single channels (Verselis et al., 1986). While the direct physiological effects of changes in gap junctional permeability are not yet established in heart, one possible role may be to limit propagation of damage when a section of the heart is injured.

Healing over, a compensatory mechanism following injury, was attributed in part to sealing of the cells at the intercalated disk (Weidmann, 1952), implying closure of the gap junctions. Extracellular calcium is required for healing over (Délèze, 1970). DeMello (1975) showed that intracellular injections of calcium reversibly abolished cell-to-cell coupling in Purkinje fiber preparations and established a role for intracellular Ca^{++} in gating of the gap junction channel. An increase in intracellular calcium from 100 to 4,000–8,000 nM brought about morphological changes in gap junction plaques between sheep Purkinje fibers and also uncoupled the cells electrically (Dahl and Isenberg, 1980).

The role of intracellular pH (pH_i) in modulating g_j in heart tissue has also been described. Early reports indicated that longitudinal resistance in Purkinje fibers was increased by 30% when pH_i was reduced from 7.4 to 6.8 (Reber and Weingart, 1982). We reported previously that g_j, measured between pairs of adult rat ventricular myocytes, was reduced by intracellular acidification with CO_2 (White et al., 1985).

Junctional conductance becomes independent of pH_i in ventricular myocyte pairs superfused with low-calcium medium (White et al., 1986). Dye coupling between neonatal cardiac cells is reduced only when intracellular concentration of both H^+ and Ca^{++} are elevated (Burt, 1987). However, in the absence of acidification, pairs of ventricular myocytes remain coupled when intracellular calcium (Ca_i) may have risen to ~1,000 nM (White et al., 1985; Maurer and Weingart, 1987). We now report simultaneous measurement of g_j between cells of paired ventricular myocytes and measurement of pH_i in the same cells using intracellular microelectrodes. We also measured intracellular calcium in heart cells in suspension with fura-2 fluorescence in order to define the dependence of g_j on both calcium and pH. We show that acidification, which increases Ca_i from 148 to 515 nM, strongly reduces g_j. In contrast, acidification does not substantially reduce g_j when Ca_i is low. Increasing Ca_i does not appreciably reduce g_j at pH_i 7.0. These data indicate that intracellular calcium ions and hydrogen ions act synergistically to reduce g_j. A brief report of these findings has appeared (White et al., 1987).

METHODS

Dissociation Procedure

The dissociation of adult rat heart into functionally intact, calcium-tolerant myocyte pairs requires that cell pairs be freed from the extracellular connective tissue framework and blood capillaries without significant disruption of the cell-to-cell connections between individual cell pairs. Heart cells were prepared by a modification of the procedure of Wittenberg et al. (1986, 1988). Adult male rats were heparinized, painlessly killed, and the heart was removed rapidly. Retrograde aortic perfusion was begun immediately with HEPES (*N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; pK_a = 7.5) buffered minimal essential medium (MEM) containing (in millimolar): 117 NaCl, 5.7 KCl, 4.4 $NaHCO_3$, 1.5 NaH_2PO_4, 1.7 $MgCl_2$, 21.1

HEPES, 11.7 glucose, amino acids and vitamins (#57-627; Hazleton Biologics, Inc., Aberdeen, MD). We added 2 mM L-glutamine and 10 mM taurine; the pH was adjusted to 7.2 with NaOH. All solutions were prepared with ASTM type I water produced by treating house distilled water with a commercial mixed bed ion exchanger followed by a charcoal filter and finally a Millipore filter. This solution was 285 mosmol, and the free calcium activity was 2–5 μM as measured with a Möller calcium ion-selective electrode. This solution is defined as low-calcium MEM. For the perfusion steps, we added 28 μM $CaCl_2$ to MEM to give a final calcium activity of 13 μM. After blood washout, the perfusion medium was supplemented with 0.1% collagenase (Worthington type II; batches are selected for a high yield of viable myocyte pairs). This solution was recirculated at 7 ml/min for 25 min. All perfusion solutions were maintained at 32°C and gassed with a water-saturated 85% O_2/15% N_2 gas mixture. The heart was removed from the perfusion apparatus and cut into 8–10 chunks in incubation medium containing 0.1% collagenase. The composition of incubation medium was the same as MEM (above) with the addition of 0.5 mM $CaCl_2$ and 0.5% dialyzed bovine serum albumin (BSA, Fraction V). The suspension was gently swirled in Erlenmeyer flasks at 32°C by a wrist action shaker. Cells, dissociated into the supernatant, were separated from the tissue by decanting, washed by low-speed centrifugation (34 *g*) and resuspended in incubation medium. Incubation of the tissue suspension with collagenase was repeated at least two more times. The cell suspension was highly enriched in paired cells. Cells separated from the tissue suspension were washed to complete the washout of collagenase and some subcellular debris. The combined, washed cells were centrifuged through isotonic Percoll to separate intact cells and cell pairs from tissue debris and rounded cells. Cells were resuspended in MEM containing 1.0 mM $CaCl_2$ (Ca-MEM; calcium activity was 470 μM), and maintained in this medium at room temperature. Since the isolated, perfused heart continues to beat when perfused with this medium, the calcium activity in this medium is considered to be physiological. Just before recording, cells were resuspended in medium without BSA.

The yield with this procedure was 4.6 ± 0.8 × 10^6 cells per heart (±SEM; n = 7), of which 67 ± 7% (n = 5) were rectangular. 15–20% of the rectangular cells were paired end-to-end. Intracellular ATP and phosphocreatine concentration were 32.2 ± 4.3 and 41.6 ± 5.7 nmol/mg rectangular cell protein (n = 5), respectively.

Measurement of pH_i and g_j

One member of a cell pair was impaled with a pH-sensitive microelectrode and a voltage-recording microelectrode. The pH-sensitive microelectrodes were made by silanizing single-barrel microelectrode glass after pulling to an optimal tip configuration with a Kopf (Tujunga, CA) electrode puller. 5% trimethylchlorosilane in ultra-pure CCl_4 was used for silanization. The tip of the pH microelectrode was filled with H^+-selective resin (CH-9470; Fluka Chemie AG, FRG) and the rest of the electrode was backfilled with 100 mM citrate buffer (pH 6) in a solution of 100 mM NaCl. A silver wire plated with silver chloride was inserted into the back of the electrode to complete the electrical connection to the electrode, and the back of the electrode was sealed with wax. The voltage-recording electrode was a conventional microelectrode filled with 150–300 mM CsCl. The pH microelectrodes were calibrated by measuring the voltage difference between the pH electrode and the voltage electrode in buffered MEM solutions of known pH between 6.0 and 7.2. The membrane potential (V_m) recorded in the cell was subtracted from the summed (pH + V_m) voltage of the pH microelectrode to give pH. A silver chloride–plated silver wire in an agar bridge containing a solution of CsCl at the same concentration as that used in the voltage recording electrode was placed in the bath to complete the circuit.

The other cell of the cell pair was impaled with a conventional microelectrode to pass current and record voltage. Each cell of a cell pair was either voltage clamped or current

clamped with independent discontinuous clamp circuits. pH_i membrane voltages, and currents in a pair of cardiac myocytes were measured with these three electrodes. The advantage of using cell pairs as opposed to strips of tissue or cell clumps is that junctional and nonjunctional conductances are unambiguously and independently assessed. Accordingly, measurements of junctional conductance are not influenced by experimentally induced changes in nonjunctional conductance. For experiments in which substantial uncoupling was observed, g_j was calculated using the pitee transform (Bennett, 1966) on data recorded from current-clamped cell pairs. In other experiments where small changes in g_j were difficult to detect when using double current-clamped cells and the pitee transform (coupling ratio is near 1), the current-clamp/voltage-clamp paradigm (Wittenberg et al., 1986) was used to measure g_j and to detect small changes in g_j in closely coupled cells. One cell of the pair was current clamped and the other cell was voltage clamped. When current was passed in the current-clamped cell, junctional current was measured as an incremental current in the voltage-clamped cell whose sign was opposite to the one passed in the current-clamped cell. The transjunctional voltage is the difference voltage between the voltage-clamped cell and the current-clamped cell. Junctional conductance is the ratio of junctional current to transjunctional voltage.

Measurement of Ca_i by Fura-2

We have adapted published methods (Grynkiewicz et al., 1985) to measure Ca_i at different values of pH_i with the fluorescent calcium indicator fura-2 in populations of heart cells in suspension. The calcium calibration procedure requires three steps that must be repeated at both pH 6.0 and 7.0: (*a*) calibration of the calcium ion-selective electrode; (*b*) determination of the calcium dissociation constant (K_d) of ethyleneglycol-bis-(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA); and (*c*) calibration of fura-2 fluorescence with Ca-EGTA buffers. Standard solutions of known calcium concentration and pH were used to calibrate the Möller calcium ion-selective electrode. A Nernstian relation was observed in pH-buffered solutions of $CaCl_2$ from 10^{-5} to 10^{-3} M at pH 6.0 and 7.0. Calibration curves constructed in this manner give the calcium activity, which is assumed to equal calcium concentration because the activity coefficient (γ) for calcium is near 1 in these low ionic strength solutions. In physiological solutions, free calcium ion activity is depressed by other salts and is less than the total concentration of $CaCl_2$ added to the medium (Moore and Ross, 1965). We found γ in MEM to be 0.47. Ca_i activity, on the other hand, could not be calculated since we did not know the γ of the intracellular milieu. We determined the dissociation constant of the EGTA-calcium chelate at pH 6.0 and 7.0 according to the method of Bers (1982), using the calibrated calcium electrode, calcium concentrations near 1 μM, and 1.0 mM EGTA in solutions buffered with 10 mM HEPES at pH 7.0 and 10 mM MES (2-[*N*-morpholino]ethanesulfonic acid; $pK_a = 6.1$) at pH 6.0. The free calcium ion activity of a Ca-EGTA mixture may be calculated from the calcium concentration and K_{dEGTA}, at either pH. These values agree with those obtained with the Möller electrode.

The dissociation constant, $K_{dfura\text{-}2}$, was calculated using the method of Tsien et al. (1982) from fura-2 fluorescence at pH 6.0 and 7.0. Fura-2–loaded heart cells were suspended in HEPES-MES–buffered standard calcium solutions. The cell membranes were made permeable with digitonin and the fluorescence spectrum was measured as described below.

To measure Ca_i, cells were loaded with fura-2 by incubation with the membrane-permeant fura-2 acetoxymethyl ester. The cells were extensively washed immediately before calcium measurements to remove extracellular dye and fluorescent MEM components. To eliminate fluorescence from the bathing medium, cells were resuspended in a balanced salt medium (BSM) containing (in millimolar): 117 NaCl, 5.7 KCl, 4.4 $NaHCO_3$, 1.5 NaH_2PO_4, 1.7 $MgCl_2$, 21.1 HEPES, 11.7 glucose, 2 L-glutamine, 10 taurine; pH was adjusted to 7.2 with NaOH.

The calcium activity coefficient was unchanged from MEM so either 1.0 mM $CaCl_2$ or no $CaCl_2$ was added as was appropriate for the experiment. The fluorescence excitation spectrum of a stirred heart cell suspension was recorded from 300 to 400 nm with emission at 510 nm in a Perkin-Elmer 650-40 fluorescence spectrophotometer. After acquiring a spectrum in air-equilibrated BSM, cells were centrifuged and resuspended in fresh BSM equilibrated with 100% CO_2 in the liquid and gas phases, and another spectrum was recorded. Fluorescence excitation spectra of unlabeled cells (autofluorescence) were recorded in the same manner and subtracted to obtain R (see below). The concentration of fura-2 in the extracellular fluid was sufficiently low so that it did not affect the fluorescence spectrum. The addition of Mn^{++} did not immediately change the fluorescence spectrum.

Ca_i was calculated from the ratio (R) of fluorescent light intensities at (excitation) 340 and 380 nm:

$$Ca_i = (R - R_{min})/(R_{max} - R) \cdot S_f/S_b \cdot K_{dfura\text{-}2}$$

Labeled cells were treated with digitonin (50 μg/mg protein) in the presence of 1–2 mM $CaCl_2$ to liberate fura-2 from the cells and to record the spectrum of fully calcium-bound fura-2 to calculate R_{max}. Cells were then treated with 15–30 mM EGTA to record the spectrum of the fully calcium-free fura-2 to calculate R_{min}. S_f/S_b is the fluorescence ratio of the fully bound to the fully free fura-2 at (excitation) 380 nm. All of these constants were determined with an aliquot of cells suspended in BSM equilibrated with air and with a separate aliquot suspended in BSM equilibrated with 100% CO_2. Table I lists experimentally determined fura-2 constants used to calculate Ca_i in suspensions of cells enriched in myocyte pairs exposed to MEM equilibrated with air or with CO_2.

TABLE I

The Effect of pH on Fura-2 Constants

	pH 7		pH 6	
R_{max}	21.6 ± 2.3	$n = 6$	11.2 ± 0.9	$n = 18$
R_{min}	0.87 ± 0.10	$n = 6$	0.97 ± 0.08	$n = 7$
Sf/Sb	6.9 ± 1.0	$n = 6$	6.9 ± 1.0	$n = 6$
$K_{d\,fura}$ (*nM*)	473 ± 56	$n = 3$	1,270 ± 169	$n = 3$

±SEM, n = number of hearts.

RESULTS

The Effects of CO_2 on pH_i and g_j

pH_i and g_j were monitored continuously with intracellular microelectrodes impaled in individual cell pairs. Heart cell pairs were bathed in air-equilibrated MEM. To study the effects of CO_2, MEM equilibrated with 100% CO_2 was rapidly superfused over the cells. CO_2 was rapidly removed by superfusion with air-equilibrated MEM to study the reversibility of CO_2-induced effects.

Physiological extracellular calcium concentration. pH_i in air-equilibrated MEM containing 1 mM $CaCl_2$ (Ca-MEM) was 7.05 ± 0.02 (±SEM, $n = 5$). This value is in agreement with that measured in these cells with ^{31}P NMR and 6-carboxyfluorescein absorbance (Wittenberg et al., 1988). pH_i rapidly declined to 6.2 within 8–12 s after the initial application of CO_2 and continued to decrease slowly to a final value of

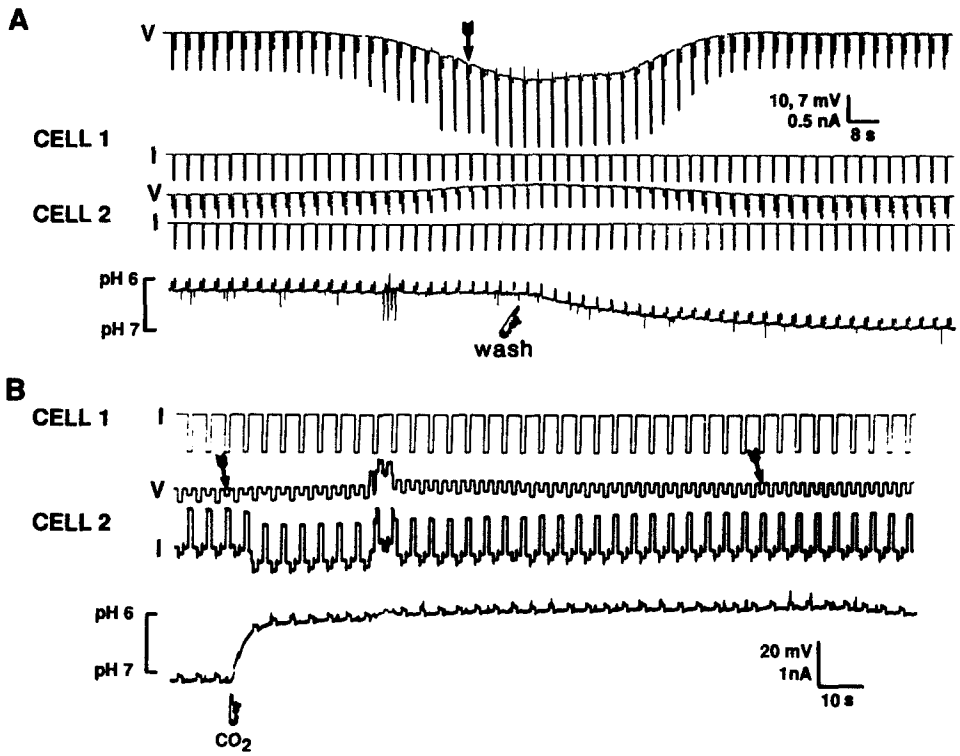

FIGURE 1. Junctional conductance between ventricular myocytes. (*A*) Double current-clamp record of junctional and nonjunctional currents and voltages in a pair of myocytes bathed in Ca-MEM. The top two traces are voltage and current, respectively, in cell 1, the next two traces are voltage and current, respectively, in cell 2, and the bottom trace is pH_i. This cell pair was first incubated in low-calcium MEM and g_j was found to be acidification-resistant. After this calcium depletion, Ca-MEM equilibrated with 100% CO_2 was applied 56 s before the beginning of the record shown and pH_i decreased to 6.2. The cells began to uncouple 100 s after the application of CO_2 (~44 s after the beginning of the record shown) as indicated by an increase in input voltage. The transfer voltages were clearly reduced by 144 s after CO_2 application (*arrow*). g_j decreased from 0.1 μS at pH 7.0 to near zero (0.002 μS) at pH 6.4. Cells were superfused with air-equilibrated Ca-MEM at the point indicated by the hand and g_j recovered concomitantly with sarcoplasmic alkalinization. Nonjunctional conductances were 0.02–0.04 and 0.18 μS for cell 1 and cell 2, respectively. Calibrations: 10 mV for the voltage in cell 1 and 7 mV for the voltage in cell 2. (*B*) Current clamp-voltage clamp record of junctional and nonjunctional currents and voltages in a pair of myocytes bathed in low-calcium MEM. Cell pairs were transferred from Ca-MEM. After 30 min in low-calcium MEM, a cell pair was impaled with recording and pH-measuring microelectrodes. The top two traces are current and voltage, respectively, in cell 1 (this cell was current clamped). The third trace from the top is the current record from cell 2 (this cell was voltage clamped). Cell 2 was given 10-mV hyperpolarizing voltage steps from a holding potential near −50 mV (voltage trace not shown). The pH-sensitive microelectrode was in cell 2 and resting sarcoplasmic pH was 7.1 for this cell pair. Superfusion with CO_2-equilibrated low-calcium MEM at the point indicated by the hand resulted in an initial fast acidification of the sarcoplasm to about pH 6.3. The sarcoplasmic pH reached 5.9 by the end of the record shown here. Changes in g_j

6.1. After CO_2 was washed out, pH_i recovered slowly to about its initial value (see Fig. 1 *A*, *lowest trace*).

Junctional conductances in cell pairs bathed in air-equilibrated Ca-MEM varied between 0.1 and 2 μS, but was constant for at least 15–20 min in any one cell pair after a short (1 min) stabilization period after impalement. There was no correlation between the magnitude of g_j and its sensitivity of CO_2. Junctional conductance was reduced to 2 ± 1%[1] of control (n = 6) in CO_2-equilibrated MEM and cell pairs were electrically uncoupled (shown in Fig. 1 *A* as a reduction in the amplitude of the transfer voltages and an increase in the input resistance). (In a larger series of 28 cell pairs, g_j of only four cell pairs was resistant to acidification in Ca-MEM.) During the initial acidification, the decrease in pH_i precedes the reduction in g_j with a variable time course. In a steady-state experiment, pH_i of several cell pairs was held at a fixed level for ~3 min by adjusting the superfusion rate of CO_2-equilibrated Ca-MEM. At pH_i 6.7, g_j was reduced in seconds to 80% of the value recorded at pH_i 6.9 and remained steady. Acidification to pH_i 6.6 reduced g_j to 62% of that recorded at pH_i 6.9. g_j was reduced to near zero at pH_i 6.2.

g_j and pH_i recovered to their preacidification values after CO_2 was washed out. During alkalinization, in contrast to the initial acidification, pH_i and g_j changed concomitantly and were similar to steady-state measurements. A plot of g_j vs. pH_i in a representative cell pair during recovery shows a steep relation (Fig. 2, *closed symbols*).

Low extracellular calcium concentration. Cell pairs were soaked for 30 min in air-equilibrated low-calcium MEM (no added $CaCl_2$; the residual calcium activity was 2–5 μM) and then impaled with voltage-recording and pH-sensitive microelectrodes. CO_2-equilibrated low-calcium MEM was superfused over the cells and subsequent recovery in the same medium was studied after CO_2 washout. Octanol (100 μM; an agent known to uncouple myocyte pairs; White et al., 1985) was added during CO_2 exposure to determine whether gap junctions could still be closed at low calcium and low pH_i.

Resting pH_i was 7.13 and did not change after incubation in low-calcium MEM. Subsequent superfusion with 100% CO_2 resulted in acidification of the sarcoplasm (Fig. 1 *B*; *lowest trace*). g_j was 80 ± 10% (n = 12) of control in low-calcium MEM, even when cell pairs were held at pH_i 5.9–6.1 for several minutes. (In a larger series of 30 cell pairs, g_j of only three cell pairs was substantially and reversibly reduced to

[1] A junctional conductance of 2% of control is not different from zero since 2% represents the residual noise in our system. Hereafter, we describe g_j as abolished when measured values drop to near 2% of control.

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are reflected by changes in transjunctional voltage and/or junctional current. The incremental junctional current (the upward deflections in the third trace) in response to applied current in cell 1 was not significantly affected. Transjunctional voltage (V_{j1}; two examples are indicated by the arrows in the second trace) showed an increase as a result of an increase in the input resistance of cell 1 (reflecting a slight reduction in g_j from 1.45 to 1.32 μS) as pH decreased from 7.1 to 5.9. No further change in g_j was observed during the next 8 min of sustained acidification.

near zero.) Fig. 1 *B* is an illustration of a typical record of pH_i, membrane voltages, and currents in a current-clamped/voltage-clamped cell pair bathed in low-calcium MEM. Intracellular pH, measured in cell 2, was 7.1 before the addition of CO_2 and reached ~5.9 by the end of the record shown (150 s after application of CO_2). Constant current pulses applied to cell 1 showed that acidification by CO_2 resulted in a small increase in transjunctional voltage (V_{11}) and a small decrease in junctional current (I_j). g_j in this cell pair was reduced by 10% from 1.45 μS at pH 7.1 to 1.32 μS at pH 5.9. pH_i and g_j recovered to near initial values when the cell pair was superfused with air-equilibrated low-calcium MEM.

g_j between paired cells bathed in low-calcium MEM remained constant as pH_i was first decreased and then increased. Fig. 2 (*open symbols*) is a plot of normalized g_j as a function of pH_i in one such cell pair during recovery. g_j remained constant at 1.2 μS from pH 5.9 to 7.04.

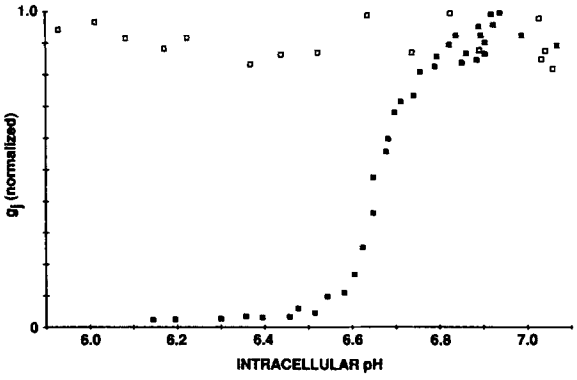

FIGURE 2. Normalized junctional conductance as a function of pH_i in heart cell pairs bathed in Ca-MEM or low-calcium MEM. The pH_i-g_j relation illustrated was plotted from data obtained during the washout of CO_2 as pH_i recovered from pH 6 to 7. Filled symbols illustrate data collected from a cell pair bathed in Ca-MEM (same cell pair as in Fig. 1 *A*). Open symbols illustrate data collected from a cell pair bathed in low-calcium MEM. g_j becomes independent of pH_i between 5.9 and 7.1 in this medium.

Acidification-resistant cell pairs could not be uncoupled even with prolonged acidification (>15–20 min) sufficient to cause membrane blebbing of the cells and irreversible damage. However, g_j was reversibly abolished by the addition of 100 μM octanol to acidification-resistant cell pairs in the presence of CO_2-equilibrated low-calcium MEM (pH_i 6.0; Fig. 3).

Treatments that increase Ca_i. To test the effect of increased extracellular calcium on cell pairs made acidification-resistant in low-calcium MEM, these cell pairs were superfused with air-equilibrated Ca-MEM. During superfusion, g_j remained the same or decreased slightly to a new stable value. After 5–15 min, air-equilibrated Ca-MEM was replaced with CO_2-equilibrated Ca-MEM and g_j was abolished (n = 10). Junctional conductance returned to its former value when air-equilibrated Ca-MEM was applied (see Fig. 1 *A*).

To study the effect of increased Ca_i (see calcium measurements below), extracellular sodium was decreased. After g_j between paired cells was shown to be acidifica-

tion-resistant in low-calcium MEM, the extracellular sodium was reduced by superfusing with air-equilibrated low-sodium low-calcium MEM (choline chloride was substituted for all but 13 mM sodium, and without added $CaCl_2$). This treatment had no detectable effect on g_j. When cells were acidified with CO_2 in this medium, g_j was rapidly and reversibly reduced to near zero ($n = 6$). Fig. 4 illustrates a typical experiment of this sort.

To study the effect of increased Ca_i the calcium ionophore A23187 (2 μM) and 100 μM $CaCl_2$ was added to low-calcium MEM. After g_j between paired cells was

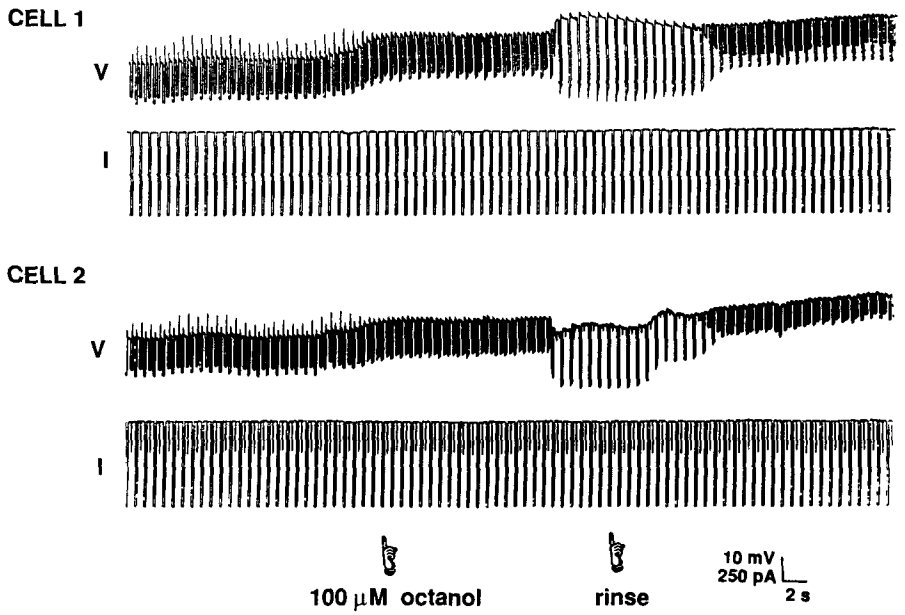

FIGURE 3. The effect of octanol on g_j in a cell pair acidified in low-calcium MEM. g_j was measured in double current-clamped cell pairs after soaking in low-calcium MEM for 30 min. Cells were acidified with 100% CO_2 and g_j was found to be acidification-resistant. While the cells were acidified, CO_2-equilibrated low-calcium MEM containing 100 μM octanol was superfused over the cells at the point indicated by the first hand. This treatment rapidly reduced g_j from 0.15 to 0.001 μS. Nonjunctional conductances were unchanged by octanol treatment; the values were 0.02–0.03 and 0.03–0.04 μS for cell 1 and cell 2, respectively. Octanol was washed out with CO_2-equilibrated low-calcium MEM and the cells were recoupled in the presence of CO_2. Note the rapid decline and subsequent reappearance of the transfer voltages.

shown to be acidification-resistant in low-calcium MEM, cell pairs were superfused with air-equilibrated MEM containing 100 μM $CaCl_2$. Sarcomere length was 1.93 ± 0.01 μm (±SEM; $n = 5$) in this medium, which is the same as that measured in cells bathed in Ca-MEM. After 30 min, CO_2 was applied and g_j was reduced slightly to 75% of control ($n = 2$), a change not significantly different from that observed in low-calcium MEM. The bathing solution was changed to air-equilibrated MEM containing 100 μM $CaCl_2$ and 2 μM A23187. g_j was unaffected by this treatment. The sarcomere length decreased to 1.85 ± 0.01 μm ($n = 8$) in this medium, indicating an

increase in Ca_i (Doeller, J. E., and B. A. Wittenberg, manuscript submitted for publication). When cells were superfused with this medium equilibrated with 100% CO_2, g_j was substantially but reversibly reduced to near zero by this treatment ($n = 2$; Fig. 5). This is a substantial increase in pH sensitivity over that observed without A23187.

The Effects of CO_2 on Intracellular Calcium

Intracellular calcium was measured by fura-2 fluorescence in heart cells in suspension. Control values of Ca_i were measured in air-equilibrated MEM. The same ali-

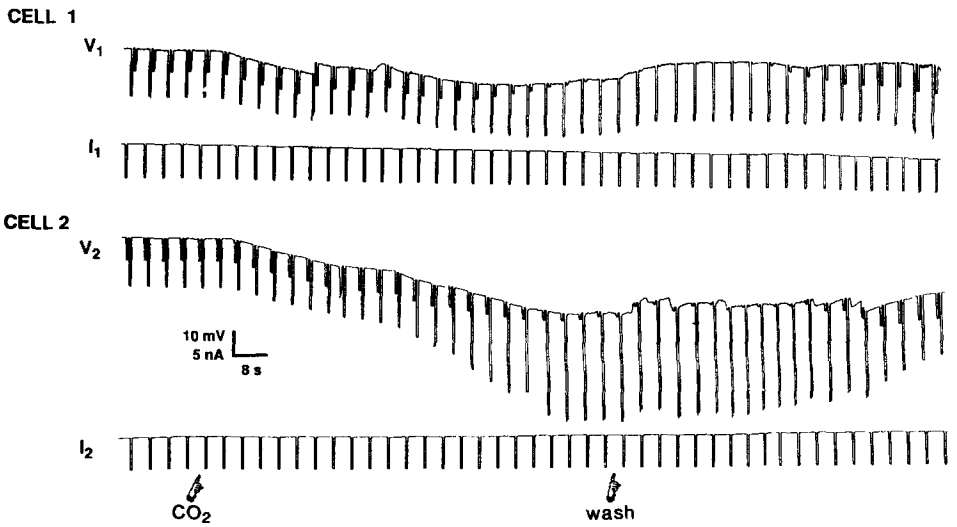

FIGURE 4. The effects of low-sodium low-calcium MEM on pH sensitivity of g_j. The top two traces are voltage and current in cell 1 and the next two traces are voltage and current in cell 2; pH_i was not recorded in this experiment. Cells were soaked in low-calcium medium with sodium for 20 min. Transient acidification established that g_j; was not detectably changed from 0.1 μS. Junctional conductance was determined by solving the pitee transform in double current clamp. Air-equilibrated low-sodium low-calcium MEM was subsequently superfused for 15 min. The superfusion was switched to CO_2-equilibrated low-sodium low-calcium MEM (indicated by the first hand). g_j was reversibly reduced to near zero (0.004 μS). The cells recoupled when air-equilibrated low-sodium low-calcium MEM was washed in (indicated by the other hand). Nonjunctional conductances were 0.12–0.15 and 0.11–0.07 μS in cell 1 and cell 2, respectively.

quot of cells was subsequently resuspended in CO_2-equilibrated MEM and calcium was measured. Results are reported in Fig. 6.

Physiological calcium concentration. Ca_i in cells suspended in air-equilibrated Ca-MEM was 148 ± 39 nM (±SEM, n = 6). Ca_i in cells bathed in CO_2-equilibrated Ca-MEM was significantly increased ($P < 0.05$) to 515 ± 12 nM ($n = 6$). As a consequence of the extensive manipulations required, Ca_i measurements were recorded 5 min after the initial acidification. To demonstrate that the effects of CO_2 were not the consequence of anoxia, cells were exposed to MEM equilibrated with 24% CO_2

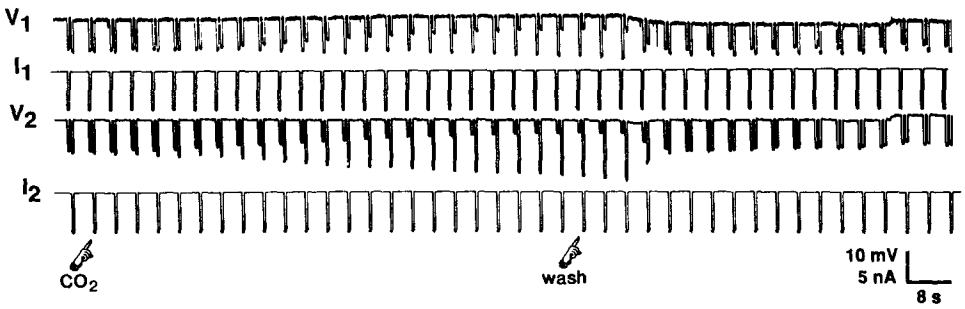

FIGURE 5. The effects of the calcium ionophore A23187 on pH sensitivity of g_j. Currents and voltages are as in Fig. 4. Cells were bathed in MEM containing 100 μM $CaCl_2$ and 2 μM A23187. g_j was 1.45 μS and remained unaffected. 100% CO_2 was applied at the point indicated by the first hand. CO_2 was washed out at the point indicated by the other hand. g_j was substantially but reversibly reduced to 0.07 μS by this treatment. Nonjunctional conductances remained 0.2 and 0.13 μS after the addition of A23187.

and 76% O_2. In one experiment, intracellular pH (as measured with pH-sensitive microelectrodes in individual cell pairs) was 6.3 and intracellular calcium (measured in cells in suspension) increased to 535 nM.

Low extracellular calcium concentration. Ca_i measured in cells suspended in air-equilibrated low-calcium MEM was 61 ± 13 nM (n = 9). In CO_2-equilibrated low-calcium MEM, Ca_i was increased significantly ($P < 0.05$) to 243 ± 42 nM (n = 9), a value significantly lower ($P < 0.02$) than that seen in CO_2-equilibrated Ca-MEM (Fig. 6). After the addition of 100 μM octanol to cells incubated in air-equilibrated low-calcium MEM, Ca_i was 94 ± 17 nM (n = 3), a value not significantly greater ($P > 0.05$) than 70 ± 17 nM obtained with the paired controls before the addition

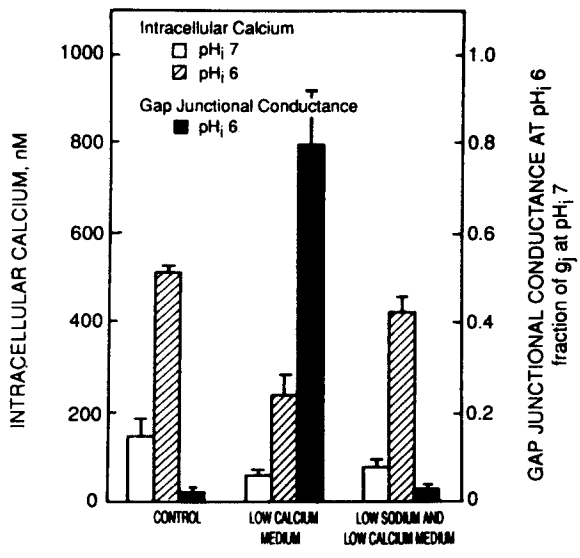

FIGURE 6. The effect of acidification on Ca_i and g_j. Separate aliquots of cells were loaded with fura-2 and bathed in Ca-MEM or low-calcium MEM. A third aliquot of cells which had been soaked in low-calcium MEM for 30 min was subsequently bathed in low-sodium low-calcium MEM (13 mM sodium). Bars illustrate mean values of Ca_i collected from cells bathed in air-equilibrated media (*plain bars*) and then CO_2-equilibrated media (*shaded bars*). The solid black bars show g_j's measured in cell pairs during exposure to CO_2 expressed as a fraction of that measured in medium equilibrated with air.

of octanol. Sarcomere length of cells bathed in air-equilibrated low-calcium medium was 1.93 ± 0.01 μm (±SEM; $n = 5$), a value not significantly different ($P > 0.05$) from sarcomere length of control cells (1.89 ± 0.02 μm; $n = 3$) in air-equilibrated Ca-MEM. Sarcomere length was not significantly affected ($P > 0.05$) by the addition of octanol (1.92 ± 0.01 μm; $n = 5$). Therefore Ca_i was decreased by lowering extracellular calcium and octanol did not increase Ca_i.

Increased intracellular calcium. Cells were treated with a paradigm comparable to that used during measurements of g_j. Cells were first soaked in air-equilibrated low-calcium MEM (with 117 mM sodium) for 30 min. To reduce extracellular sodium, the cells were bathed in air-equilibrated low-sodium low-calcium MEM. Measured Ca_i was 80 ± 17 nM ($n = 13$). This value increased significantly ($P < 0.02$) to 425 ± 35 nM ($n = 11$) in CO_2-equilibrated low-sodium low-calcium MEM. This value was significantly larger ($P < 0.05$) than Ca_i measured in CO_2-equilibrated low-calcium MEM containing 117 mM NaCl (see Fig. 6).

DISCUSSION

We measured g_j and pH_i simultaneously in the same ventricular myocyte cell pairs. Ca_i was measured in ventricular myocytes in suspensions. We found that acidification of ventricular myocytes bathed in Ca-MEM, by CO_2 concentrations as low as 24% (76% O_2) substantially increases Ca_i. This effect was previously reported by others (Lea and Ashley, 1978; Orchard et al., 1987). Under these experimental conditions, we show that g_j is remarkably dependent on pH_i. Incubation of ventricular myocytes in low-calcium medium causes a large reduction in Ca_i and sharply reduces the increase in Ca_i when cells are acidified to pH 6. g_j between ventricular myocytes is not substantially reduced by acidification with CO_2 when Ca_i is low. These results indicate that intracellular Ca^{++} and intracellular H^+ are both required to uncouple ventricular myocytes. Similar results were reported by Burt (1987) who showed that intercellular spread of dye between adjacent rat embryonic myocytes was blocked when cells were treated with both calcium ionophore and acidification. We show evidence that the action of Ca^{++} in increasing the sensitivity of g_j to acidification is intracellular since raising intracellular Ca^{++} (by removing most of the extracellular sodium or by the addition of the calcium ionophore A23187) is effective.

We report that g_j in rat ventricular myocyte pairs increases with increasing pH_i from 6 to 7 in Ca-MEM. The pH_i-g_j relation for ventricular myocytes is much steeper and shifted in an acidic direction compared with that reported for pH_i-longitudinal resistance in a multicellular Purkinje fiber preparation (Reber and Weingart, 1982).

Noma and Tsuboi (1987) reported measurements of g_j between pairs of guinea pig ventricular myocytes. In their preparation, the membrane of one cell of a coupled pair was made permeable by smashing that cell with a glass capillary in reduced calcium medium. Changes in extracellular pH and/or calcium were assumed to equilibrate with the contents of the broken cell thus the interventions were unilaterally imposed from the broken cell side of the junction. It is possible that their pH-g_j relationship is shifted in an acidic direction since it was recently shown that when pairs of *Rana* blastomeres were unilaterally acidified, the pH_i-g_j relation measured in the acidified cell was shifted in the acidic direction compared with the relation

measured during bilateral acidification (Verselis et al., 1987). Neither Ca_i nor pH_i of either cell in the "broken cell" preparation of Noma and Tsuboi (1987) was directly measured. In the broken cell preparation, highly buffered intracellular ions such as calcium or H^+ might not equilibrate with the extracellular medium despite the presence of a large electrical conductance (mediated presumably by K^+; Byerly and Moody, 1986). The effect of pH on g_j in the broken cell preparation was qualitatively the same as we report here, i.e., g_j was reduced by acidification. However, the dependence of g_j on Ca_i was very different. Under physiological conditions, at an extracellular pH of 7.2, and a calcium concentration of 1 mM, the isolated heart beats. We find that under these conditions, Ca_i equals 150 nM and pH_i is 7.05. Our experiment with A23187 shows that an increase in Ca_i at pH 7 is not sufficient to reduce g_j (Fig. 5). Even when we overload cells with calcium sufficient to cause hypercontraction, g_j is not reduced (White et al., 1985). The Ca_i level under these conditions is much greater than 1 μM (Wier et al., 1987). We did not detect a decrease in g_j at measured Ca_i up to ~250 nM at pH 6. We cannot exclude the possibility that at pH_i lower than 5.9, g_j would have diminished. It should be noted that at pH_i 6.0, we measured a large increase in Ca_i from 150 to 500 nM. If this increase occurred in the experiments of Noma and Tsuboi (1987), their methods would not have detected it. Noma and Tsuboi (1987) interpreted their results as simple competitive binding between protons and Ca^{++} at a single site. When we use our measured values for pH_i and Ca_i in medium with 1.0 mM $CaCl_2$ and 100% CO_2 together with Noma and Tsuboi's values for Ca^{++} and H^+ binding constants, their model predicts that g_j would be reduced by <0.01% by acidification. This prediction is contrary to our findings, and our data does not fit their model.

An interesting possibility in any internal dialysis experiment is the washing out of important cytoplasmic constituents. Unilateral internal perfusion did not reduce g_j between crayfish lateral axons when the pH of the perfusate was low (Arellano et al., 1986). However, g_j was substantially reduced when the pH_i of the unperfused side was reduced. Arellano et al. (1986, 1988) suggest that H^+ acts through an intermediary that is washed out or inactivated by perfusion. It is not known what effects on g_j between heart cells might be induced by unilateral perfusion or ionic changes. Whether H^+-Ca^{++} synergism acts via an intermediary has not been addressed.

Repetitive acidification in Ca-MEM uncoupled the cells with progressively less delay and the change in g_j tracked the change in pH_i more closely. A similar result was reported in Purkinje fibers (Pressler, 1989). The change in g_j was always in the same direction as the change in pH_i below 6.8. The time delay of the reduction in g_j was longest for the first acidification when we applied Ca-MEM equilibrated with CO_2 immediately after exposure to low-calcium MEM. The latency of uncoupling was greatly reduced when CO_2-equilibrated Ca-MEM was applied a second time. These results suggest that the rate of increase in Ca_i upon acidification is slower than the rate at which we can acidify the cell pair (8–10 s). There is also the possibility that intermediate reaction steps or protein structural changes must be completed before calcium and pH can reduce g_j after exposure to low calcium. We find that in the presence of intracellular fura-2, the latency of the reduction in g_j upon acidification is increased by several minutes (unpublished observation). This suggests that fura-2 may bind calcium until its buffering power is overwhelmed. This would increase the time it takes calcium to build up to a critical level. We conclude

that fura-2 may dampen calcium transients during slow inward currents and our steady-state calcium values may be minimal values. Our measurements of g_j and pH_i involved the impalement of two microelectrodes in one cell of a coupled pair. While the initial response of the cells was often a brief contraction, any ion leakage about the electrodes is either buffered or pumped out since we are able to maintain rectangular cells at normal sarcomere lengths for periods of up to 2 h. Sarcomere lengths sensitivity report Ca_i at constant pH_i and intracellular ATP. We find significant shortening when Ca_i is increased to 250 nM (Doeller, J. E., and B. A. Wittenberg, manuscript submitted for publication).

To summarize, g_j in ventricular myocyte pairs superfused with medium containing 1.0 mM $CaCl_2$ is strongly dependent on pH_i and decreases substantially between pH_i 6.8 and 6.4. g_j in pairs of ventricular myocytes bathed in medium containing no added $CaCl_2$ (low-calcium medium) is relatively independent of pH_i as low as 5.9. g_j could be reversibly abolished by octanol in acidified ventricular myocyte pairs bathed in low-calcium medium. g_j between myocyte pairs in low-calcium medium becomes pH dependent when Ca_i is increased by exposing the cells to low-sodium low-calcium medium. Increasing sarcoplasmic calcium with a calcium ionophore also increases the pH sensitivity of g_j. Measurement of Ca_i by fura-2 fluorescence in heart cells in suspension show that Ca_i is substantially increased by acidification with 24–100% CO_2. Ca_i is reduced compared with control by incubation in low-calcium medium both when pH_i is 7.0 and when pH_i is 6.0. We conclude that calcium and hydrogen ions act synergistically to reduce g_j.

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