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

R. L. WHITE, J. E. DOELLER, V. K. VERSELIS, and B. A. WITTENBERG

From the Department of Neuroscience and the Department of Physiology and Biophysics Albert Einstein College of Medicine, Bronx, New York

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 \(\mu\)M Ca\(^{++}\)). Intracellular calcium concentration (Ca\(_i\)), measured by fura-2 fluorescence in cell suspensions, was 148 \pm 39 nM (\(\pm\) SEM, \(n = 6\)) and intracellular pH (pH\(_i\)), measured with intracellular ion-selective microelectrodes, was 7.05 \pm 0.02 (\(n = 5\)) in cell pair preparations bathed in medium equilibrated with air. Ca\(_i\) increased to 515 \pm 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 \(\mu\)M Ca\(^{++}\)), Ca\(_i\) was 61 \pm 9 nM (\(n = 13\)) at pH 7.1. Ca\(_i\) increased to only 243 \pm 42 nM (\(n = 9\)) at pH 6.0 in CO\(_2\)-equilibrated low-calcium medium. Junctional conductance, in most cell pairs, was not substantially reduced by acidification to pH 5.9-6.0 in low-calcium medium. Cell pairs could still be electrically uncoupled reversibly by the addition of 100 \(\mu\)M 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 \pm 35 nM (\(n = 11\)) at pH 5.9-6.0 and \(g_j\) was reduced to near zero. Junctional conductance could also be reduced to near zero at pH 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 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...
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 (Délieze, 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 Ca²⁺ 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 (pHᵢ) in modulating gj in heart tissue has also been described. Early reports indicated that longitudinal resistance in Purkinje fibers was increased by 30% when pHᵢ was reduced from 7.4 to 6.8 (Reber and Weingart, 1982). We reported previously that gj, measured between pairs of adult rat ventricular myocytes, was reduced by intracellular acidification with CO₂ (White et al., 1985).

Junctional conductance becomes independent of pHᵢ 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 Ca²⁺ are elevated (Burt, 1987). However, in the absence of acidification, pairs of ventricular myocytes remain coupled when intracellular calcium (Caᵢ) may have risen to ~1,000 nM (White et al., 1985; Maurer and Weingart, 1987). We now report simultaneous measurement of gj between cells of paired ventricular myocytes and measurement of pHᵢ 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 gj on both calcium and pH. We show that acidification, which increases Caᵢ from 148 to 515 nM, strongly reduces gj. In contrast, acidification does not substantially reduce gj when Caᵢ is low. Increasing Caᵢ does not appreciably reduce gj at pHᵢ 7.0. These data indicate that intracellular calcium ions and hydrogen ions act synergistically to reduce gj. 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; pKₑ₅ = 7.5) buffered minimal essential medium (MEM) containing (in millimolar): 117 NaCl, 5.7 KCl, 4.4 NaHCO₃, 1.5 NaH₂PO₄, 1.7 MgCl₂, 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 CaCl₂ 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% O₂/15% N₂ 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 CaCl₂ 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 CaCl₂ (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 × 10⁶ 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 pHi and gj**

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 CCl₄ 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 pHi. 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

---

**Note:** The text is a continuation from the previous page, discussing the methods and procedures used in a study on calcium and pH effects on junctional conductance in heart.
clamped with independent discontinuous clamp circuits. pH, 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 $Ca_{ij}$ by Fura-2**

We have adapted published methods (Grynkiewicz et al., 1985) to measure $Ca_{ij}$ at different values of pH, 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) $N,N',N''$-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 $10^{-9}$ to $10^{-5}$ 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 ($\gamma$) 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 $\gamma$ in MEM to be 0.47. $Ca_{ij}$ activity, on the other hand, could not be calculated since we did not know the $\gamma$ 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 $K_{d,EGTA}$ at either pH. These values agree with those obtained with the Möller electrode.

The dissociation constant, $K_{d,EGTA}$, 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 $Ca_{ij}$, cells were loaded with fura-2 by incubation with the membrane-permeant fura-2 acetoxyethyl ester. The cells were extensively washed immediately before calcium measurements to remove extracelluar 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$_2$PO$_4$, 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 CaCl₂ or no CaCl₂ 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% CO₂ 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.

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

\[ \text{Ca}_i = \frac{(R - R_{\text{min}})/(R_{\text{max}} - R) \cdot S_f/S_b \cdot K_{\text{fura2}}}{\text{Sf/Sb}} \]

Labeled cells were treated with digitonin (50 µg/mg protein) in the presence of 1-2 mM CaCl₂ to liberate fura-2 from the cells and to record the spectrum of fully calcium-bound fura-2 to calculate R_{\text{max}}. Cells were then treated with 15-30 mM EGTA to record the spectrum of the fully calcium-free fura-2 to calculate R_{\text{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% CO₂. Table I lists experimentally determined fura-2 constants used to calculate Caᵢ in suspensions of cells enriched in myocyte pairs exposed to MEM equilibrated with air or with CO₂.

| \( R_{\text{max}} \) | \( R_{\text{min}} \) | \( S_f/S_b \) | \( K_{\text{fura2}} \) (nM) |
|----------------------|----------------------|-----------------|-------------------------|
| 21.6 ± 2.3           | 0.87 ± 0.10          | 6.9 ± 1.0       | 475 ± 56                |
| \( n = 6 \)          | \( n = 6 \)          | \( n = 6 \)     | \( n = 6 \)             |
| 11.2 ± 0.9           | 0.97 ± 0.08          | 6.9 ± 1.0       | 1,270 ± 109             |
| \( n = 18 \)         | \( n = 7 \)          | \( n = 6 \)     | \( n = 3 \)             |

±SEM, \( n \) = number of hearts.

### RESULTS

#### The Effects of CO₂ on \( \text{pH}_i \) and \( g_j \)

\( \text{pH}_i \) 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 CO₂, MEM equilibrated with 100% CO₂ was rapidly superfused over the cells. CO₂ was rapidly removed by superfusion with air-equilibrated MEM to study the reversibility of CO₂-induced effects.

**Physiological extracellular calcium concentration.** \( \text{pH}_i \) in air-equilibrated MEM containing 1 mM CaCl₂ (Ca-MEM) was 7.05 ± 0.02 (±SEM, \( n = 5 \)). This value is in agreement with that measured in these cells with \(^{31}\text{P}\) NMR and 6-carboxyfluorescein absorbance (Wittenberg et al., 1988). \( \text{pH}_i \) rapidly declined to 6.2 within 8–12 s after the initial application of CO₂ 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. 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% \( \text{CO}_2 \) was applied 56 s before the beginning of the record shown and pH decreased to 6.2. The cells began to uncouple 100 s after the application of \( \text{CO}_2 \) (~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 \( \text{CO}_2 \) application (arrow). \( g_j \) decreased from 0.1 \( \mu \text{S} \) at pH 7.0 to near zero (0.002 \( \mu \text{S} \)) 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 \( \mu \text{S} \) 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 \( \text{CO}_2 \)-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 CO₂ was washed out, pHᵢ 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 gᵢ and its sensitivity of CO₂. Junctional conductance was reduced to 2 ± 1% of control (n = 6) in CO₂-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, gᵢ of only four cell pairs was resistant to acidification in Ca-MEM.) During the initial acidification, the decrease in pHᵢ precedes the reduction in gᵢ with a variable time course. In a steady-state experiment, pHᵢ of several cell pairs was held at a fixed level for ~3 min by adjusting the superfusion rate of CO₂-equilibrated Ca-MEM. At pHᵢ 6.7, gᵢ was reduced in seconds to 80% of the value recorded at pHᵢ 6.9 and remained steady. Acidification to pHᵢ 6.6 reduced gᵢ to 62% of that recorded at pHᵢ 6.9. gᵢ was reduced to near zero at pHᵢ 6.2.

gᵢ and pHᵢ recovered to their preacidification values after CO₂ was washed out. During alkalinization, in contrast to the initial acidification, pHᵢ and gᵢ changed concomitantly and were similar to steady-state measurements. A plot of gᵢ vs. pHᵢ in a representative cell pair during recovery shows a steep relation (Fig. 2, closed symbols).

Low extracellular calcium concentration. Cell pairs were bathed in air-equilibrated, low-calcium MEM (no added CaCl₂; the residual calcium activity was 2–5 μM) and then impaled with voltage-recording and pH-sensitive microelectrodes. CO₂-equilibrated, low-calcium MEM was superfused over the cells and subsequent recovery in the same medium was studied after CO₂ washout. Octanol (100 μM; an agent known to uncouple myocyte pairs; White et al., 1985) was added during CO₂ exposure to determine whether gap junctions could still be closed at low calcium and low pHᵢ.

Resting pHᵢ was 7.13 and did not change after incubation in low-calcium MEM. Subsequent superfusion with 100% CO₂ resulted in acidification of the sarcoplasm (Fig. 1 B; lowest trace). gᵢ was 80 ± 10% (n = 12) of control in low-calcium MEM, even when cell pairs were held at pHᵢ 5.9–6.1 for several minutes. (In a larger series of 30 cell pairs, gᵢ of only three cell pairs was substantially and reversibly reduced to 2% of control.)

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

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

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 (V₁₂; 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 gᵢ from 1.45 to 1.32 μS) as pH decreased from 7.1 to 5.9. No further change in gᵢ was observed during the next 8 min of sustained acidification.
near zero.) Fig. 1 B is an illustration of a typical record of \( pHi \), membrane voltages, and currents in a current-clamped/voltage-clamped cell pair bathed in low-calcium MEM. Intracellular \( pHi \), measured in cell 2, was 7.1 before the addition of \( \text{CO}_2 \) and reached \( \sim 5.9 \) by the end of the record shown (150 s after application of \( \text{CO}_2 \)). Constant current pulses applied to cell 1 showed that acidification by \( \text{CO}_2 \) resulted in a small increase in transjunctional voltage \( (V_{ij}) \) and a small decrease in junctional current \( (I_j) \). \( g_j \) in this cell pair was reduced by 10% from 1.45 \( \mu \)S at \( pHi 7.1 \) to 1.32 \( \mu \)S at \( pHi 5.9 \). \( pHi \) 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 \( pHi \) was first decreased and then increased. Fig. 2 (open symbols) is a plot of normalized \( g_j \) as a function of \( pHi \) in one such cell pair during recovery. \( g_j \) remained constant at 1.2 \( \mu \)S from \( pHi 5.9 \) to 7.04.

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 \( \mu \)M octanol to acidification-resistant cell pairs in the presence of \( \text{CO}_2 \)-equilibrated low-calcium MEM (\( pHi 6.0 \); Fig. 3).

Treatments that increase \( Ca_{ec} \). 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 \( \text{CO}_2 \)-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 CaCl₂). This treatment had no detectable effect on gj. When cells were acidified with CO₂ in this medium, gj 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, the calcium ionophore A23187 (2 μM) and 100 μM CaCl₂ was added to low-calcium MEM. After gj between paired cells was

![Figure 3](image-url)

**Figure 3.** The effect of octanol on gj in a cell pair acidified in low-calcium MEM. gj was measured in double current-clamped cell pairs after soaking in low-calcium MEM for 30 min. Cells were acidified with 100% CO₂ and gj was found to be acidification-resistant. While the cells were acidified, CO₂-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 gj 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 CO₂-equilibrated low-calcium MEM and the cells were recoupled in the presence of CO₂. 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 CaCl₂. 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, CO₂ was applied and gj 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 CaCl₂ and 2 μM A23187. gj 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 aliquot 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$_2$.
Calcium and pH Effects on Junctional Conductance in Heart

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 CaCl₂ and 2 μM A23187. $g_j$ was 1.45 μS and remained unaffected. 100% CO₂ was applied at the point indicated by the first hand. CO₂ 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% O₂. 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.** $C_a$, measured in cells suspended in air-equilibrated low-calcium MEM was 61 ± 13 nM ($n = 9$). In CO₂-equilibrated low-calcium MEM, $C_a$ was increased significantly ($P < 0.05$) to 243 ± 42 nM ($n = 9$), a value significantly lower ($P < 0.02$) than that seen in CO₂-equilibrated Ca-MEM (Fig. 6). After the addition of 100 μM octanol to cells incubated in air-equilibrated low-calcium MEM, $C_a$ 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 $C_a$ 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 $C_a$ collected from cells bathed in air-equilibrated media *plain bars* and then CO₂-equilibrated media *shaded bars*. The solid black bars show $g_j$'s measured in cell pairs during exposure to CO₂ 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 pHi 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_i-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 \( \text{Ca}_i \) nor \( \text{pH}_i \) 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 \( \text{H}^+ \) might not equilibrate with the extracellular medium despite the presence of a large electrical conductance (mediated presumably by \( \text{K}^+ \); Byerly and Moody, 1986). The effect of \( \text{pH} \) 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 \( \text{Ca}_i \) was very different. Under physiological conditions, at an extracellular \( \text{pH} \) of 7.2, and a calcium concentration of 1 mM, the isolated heart beats. We find that under these conditions, \( \text{Ca}_i \) equals 150 nM and \( \text{pH}_i \) is 7.05. Our experiment with A23187 shows that an increase in \( \text{Ca}_i \) at \( \text{pH} \) 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 \( \text{Ca}_i \) level under these conditions is much greater than 1 \( \mu \text{M} \) (Wier et al., 1987). We did not detect a decrease in \( g_j \) at measured \( \text{Ca}_i \) up to \( \sim 250 \) nM at \( \text{pH} \) 6. We cannot exclude the possibility that at \( \text{pH}_i \) lower than 5.9, \( g_j \) would have diminished. It should be noted that at \( \text{pH}_i \) 6, we measured a large increase in \( \text{Ca}_i \) 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 \( \text{Ca}^{++} \) at a single site. When we use our measured values for \( \text{pH}_i \) and \( \text{Ca}_i \) in medium with 1.0 mM \( \text{CaCl}_2 \) and 100% \( \text{CO}_2 \) together with Noma and Tsuboi's values for \( \text{Ca}^{++} \) and \( \text{H}^+ \) 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 \( \text{pH} \) of the perfusate was low (Arellano et al., 1986). However, \( g_j \) was substantially reduced when the \( \text{pH}_i \) of the unperfused side was reduced. Arellano et al. (1986, 1988) suggest that \( \text{H}^+ \) 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 \( \text{H}^+\text{-Ca}^{++} \) 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 \( \text{pH}_i \) 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 \( \text{pH}_i \) 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 \( \text{CO}_2 \) immediately after exposure to low-calcium MEM. The latency of uncoupling was greatly reduced when \( \text{CO}_2 \)-equilibrated Ca-MEM was applied a second time. These results suggest that the rate of increase in \( \text{Ca}_i \) 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 $g_j$.

We thank Ms. Jacqueline Lopez-Cepero and Mr. Joseph Zavilowitz for technical assistance.

This work was supported in part by National Institutes of Health grants HL-33655 (R. L. White), HL-19299, and HL-40998 (B. A. Wittenberg), and NS-07512 (V. K. Verselis). J. E. Doeller is a Fellow of the American Heart Association, New York Heart Affiliate.

Original version received 12 January 1989 and accepted version received 27 December 1989.

References

Arellano, R. O., F. Ramon, A. Rivera, and G. A. Zampighi. 1986. Lowering of pH does not directly affect the junctional resistance of crayfish segmented axons. Journal of Membrane Biology. 94:293–299.

Arellano, R. O., F. Ramon, A. Rivera, and G. A. Zampighi. 1988. Calmodulin acts as an intermediary for the effects of calcium ions on gap junctions from crayfish lateral axons. Journal of Membrane Physiology. 101:119–131.

Bennett, M. V. L. 1966. Physiology of electronic junctions. Annals of the New York Academy of Sciences. 137:509–539.

Bers, D. M. 1982. A simple method for the accurate determination of free [Ca] in Ca-EGTA solutions. American Journal of Physiology. 242(Cell Physiology 11):C404–408.

Burt, J. M. 1987. Block of intracellular communication: interaction of intracellular H$^+$ and Ca$^{++}$. American Journal of Physiology. 253(Cell Physiology 22):C607–C612.

Byerly, L., and W. J. Moody. 1986. Membrane currents of the internally perfused neurones of the snail Lymnaea stagnalis, at low intracellular pH. Journal of Physiology. 376:477–491.

Dahl, G., and G. Isenberg. 1980. Decoupling of heart muscle cells: correlation with increased cytosplasmic calcium activity and with changes of nexus ultrastructure. Journal of Membrane Biology. 53:65–75.
Delèze, J. 1970. The recovery of resting potential and input resistance in sheep heart injured by knife or laser. Journal of Physiology. 208:547–562.

DeMello, W. C. 1975. Effect of intracellular injection of calcium and strontium on cell communication in heart. Journal of Physiology. 250:231–245.

Gryniewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca indicators with greatly improved fluorescent properties. Journal of Biological Chemistry. 260:3440–3450.

Lea, T. J., and C. C. Ashley. 1978. Increase in free Ca** in muscle after exposure to CO2. Nature. 257:236–238.

Maurer, P., and R. Weingart. 1987. Cell pairs isolated from adult guinea pig and rat hearts: effects of [Ca**], on nexal membrane resistance. Pfliigers Archiv Gesamte Physiologie. 409:394–402.

Moore, E. W., and J. W. Ross. 1965. NaCl and CaCl2 activity coefficients in mixed aqueous solutions. Journal of Applied Physiology. 20:1332–1336.

Noma, A., and N. Tsuboi. 1987. Dependence of junctional conductance on proton, calcium, and magnesium ions in paired cardiac cells of guinea pig. Journal of Physiology. 382:193–211.

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** release in rat myocardium. Journal of General Physiology. 90:145–165.

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

Reber, W., and R. Weingart. 1982. Ungulate Purkinje fibers: the influence of intracellular pH on the cell-to-cell coupling. Journal of Physiology. 328:87–104.

Tsien, R. Y., T. Pozzan, and T. L. Rink. 1982. Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored by a new, intracellular trapped fluorescent indicator. Journal of Cell Biology. 94:325–334.

Verselis, V. K., R. L. White, D. C. Spray, and M. V. L. Bennett. 1986. Gap junctional conductance and permeability are linearly related. Science. 234:461–464.

Verselis, V. K., R. L. White, D. C. Spray, J. Zavilowitz, and M. V. L. Bennett. 1987. Induced asymmetry of gating in amphibian blastomeres. Journal of Cell Biology. 105:509a. (Abstr.)

Weidmann, S. 1952. The electrical constants of Purkinje fibers. Journal of Physiology. 118:348–360.

White, R. L., D. C. Spray, A. C. Campos deCarvalho, B. A. Wittenberg, and M. V. L. Bennett. 1985. Some electrical and pharmacological properties of gap junctions between adult ventricular myocytes. American Journal of Physiology. 249:C447–C455.

White, R. L., V. K. Verselis, J. Doeller, B. A. Wittenberg, and M. V. L. Bennett. 1987. Acid sensitivity of ventricular myocytes is modulated by calcium ions. Journal of Cell Biology. 105:507a. (Abstr.)

White, R. L., V. K. Verselis, D. C. Spray, M. V. L. Bennett, and B. A. Wittenberg. 1986. Acidification-resistant junctional conductance between some pairs of ventricular myocytes from rat. Journal of Cell Biology. 105:363a. (Abstr.)

Wier, W. G., M. B. Cannell, J. R. Berlin, E. Marban, and W. J. Lederer. 1987. Cellular and subcellular heterogeneity of (Ca) in single heart cells revealed by fura-2. Science. 235:325–328.

Wittenberg, B. A., J. E. Doeller, R. K. Gupta, and R. L. White. 1988. Measurement of sarcoplasmic permeability and intracellular pH, free magnesium, and high energy phosphates of isolated heart cells. In Biology of Isolated Myocytes. T. K. Borg, R. S. Decker, W. Clark, editors. Elsevier.

Wittenberg, B. A., R. L. White, R. D. Ginsberg, D. C. Spray. 1986. Effect of calcium on the dissociation of the mature rat heart into individual and paired myocytes: electrical properties of cell pairs. Circulation Research. 59:143–150.