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DEPENDENCE OF JUNCTIONAL CONDUCTANCE ON PROTON, CALCIUM AND MAGNESIUM IONS IN CARDIAC PAIRED CELLS OF GUINEA-PIG

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SUMMARY

1. The dependence of gap junctional conductance on the intracellular concentrations pf H^+, Ca^{2+} and Mg^{2+} was studied in paired myocytes dissociated enzymatically from guinea-pig ventricle. To apply an internal solution buffered to specific H^+, Ca^{2+} or Mg^{2+} concentration directly to one aspect of the gap junction, the non-junctional membrane of one of the pair was mechanically ruptured. The junctional conductance was measured by clamping the membrane potential of the other cell using a two-pipette voltage-clamp method.

2. The conductance of the non-junctional membrane was kept low in comparison with that of the junctional membrane ($< 1/50$) by replacing both external and internal K^+ with Cs^+.

3. The current–voltage (I–V) relation of the junctional conductance was linear over the potential range examined (from -100 to $+100$ mV). No voltage or time dependence was detected.

4. The conductance of the gap junction between the paired cells ranged from 90 to 3900 nS with a peak distribution at 1000 nS.

5. The effect of H^+ was examined over the pH range 7·4–5·4, while keeping the free-Ca^{2+} concentration at zero, or pCa 6·3 or 7·0 using 2–10 mM-EGTA. The junctional conductance was almost constant from pH 7·4 to 6·5 and decreased in a dose-dependent manner with further acidification. There was no difference in the pH–conductance relationships at various Ca^{2+} concentrations. The Hill coefficient was approximately 2·4 and the half-maximum concentration (pK'_H) was 6·1.

6. The closing effect of Ca^{2+} on the gap junction channel was examined over the concentration range from pCa 7 to 5, while keeping the pH at 7·4, 7·0 or 6·5. At each pH, increasing Ca^{2+} decreased the junctional conductance with similar Hill coefficients of about 3·4. The pCa–conductance relationship shifted toward a higher Ca^{2+} concentration range as the pH was lowered (pK'_{Ca} = 6·6, 6·4 and 5·6, at pH 7·4, 7·0 and 6·5, respectively).

7. Increasing Mg^{2+} also caused a fall in the junctional conductance over the pMg range 3·0–2·0 with a pK'_{Mg} of 2·5 (3·2 mM), and a Hill coefficient of 3·0.

8. These results suggest that there are two respective binding sites for divalent cations and H^+, and that the gap junctional conductance is regulated reversibly by

the ligand–receptor reactions. Comparing the threshold concentrations of Ca^{2+} and H^+ for electrical uncoupling, it was concluded that Ca^{2+} plays a more important role in regulating the gap junctional conductance of cardiac cells under physiological conditions.

INTRODUCTION

Cardiac gap junctions provide a low-resistance pathway for the local current between adjacent cells and thus facilitate the spread of the action potential over the entire heart. The conductance of the gap junction, however, is not constant and is affected by various ions, which exist in the cell interior under physiological conditions. This variation of the junctional conductance was originally examined by measuring the exponential decay of tonic potential along strands of cardiac preparations (for review, see Weingart, 1981; De Mello, 1982). These experiments showed that the space constant decreased when cardiac cells were treated by procedures which were supposed to raise intracellular Ca^{2+} concentration (De Mello, 1975, 1976; Weingart, 1977) or to induce intracellular acidosis (De Mello, 1980; Reber & Weingart, 1982). In other tissues, an increase in cytosolic Mg^{2+} has also been reported to cause cell uncoupling (Oliveira-Castro & Loewenstein, 1971; Peracchia & Peracchia, 1980). In spite of these observations, however, because of the scarcity of quantitative data on the independent effects of each ion, it is still not clear to what extent the individual ions are involved in the regulation of gap junctional conductance in cardiac cells.

In intact cells, concentrations of cytosolic Ca^{2+} and H^+ are interdependent. Thereby, an increase of intracellular Ca^{2+} lowers the intracellular pH (Meech & Thomas, 1977) and, conversely, acidosis may raise intracellular Ca^{2+} (Lea & Ashley, 1978; Rose & Rick, 1978). Thus, for examining the independent effects of individual ions, it is necessary to keep the concentration of the other ions constant. In multicellular preparations, this has been virtually impossible.

Kameyama (1983) first examined paired ventricular cells, obtained by treating the heart with collagenase, and found that the conductance of the gap junction was well maintained (ranging from 0·3 to 11 MΩ) in these cells. Using similar coupled cells, H. Irisawa & I. Imanaga (personal communication) recently observed that when fluorescent dyes of molecular weight up to 860 daltons were injected into one of the pair, the dyes diffused into the other across the gap junction. These findings clearly suggest that such paired cells are suitable for electrophysiological studies of the gap junction.

In the present study, we developed a method for exposing one aspect of the gap junction to the bath solution. By breaking the non-junctional membrane of one of the paired cells, different concentrations of H^+, Ca^{2+} and Mg^{2+} could be applied directly to the gap junction. The conductance of the gap junction was measured by clamping the other intact cell using two giga-sealed patch pipettes, whereby the interference of series resistance was eliminated and accurate measurements of the junctional conductance were achieved. These methods allowed us to quantitatively analyse the direct effects of H^+, Ca^{2+} and Mg^{2+} on junctional conductance independently. The interactions between H^+ and Ca^{2+} in the regulation of junctional conductance were also studied.

METHODS

Preparation. The technique of preparing dissociated cells using collagenase has been described elsewhere (Taniguchi, Kokubun, Noma & Irisawa, 1981; Kameyama, 1983). In brief, a guinea-pig heart was treated with collagenase and stored in high-K^+ low-Cl^- stock solution (Isenberg & Klöckner, 1982) at 4 °C. Before each experiment a small piece of tissue was dissected out from the left ventricular wall and gently shaken in a recording chamber (0·5 cm^3) to dissociate the cells. The paired cells were discriminated from single cells by the presence of contact regions between apposed cells as observed using an inverted microscope (Nikon, TMD, Tokyo, Japan) as has been shown by Kameyama (1983, Pl. 1; see also Pl. 1 *A* in the present study). When paired cells were deliberately injured at the end of the experiments, they went into contracture and became two separate rounded cells, giving verification that they had originally been paired cells.

Experimental protocol. The whole-cell voltage-clamp technique using a giga-sealed patch electrode (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) was modified and applied to one of the paired cells. In the present paper we designate the cell with the applied patch electrode as the clamped cell, and the other as the partner cell. After clamping the membrane potential, the bath solution was switched from the control Tyrode solution (1·8 mM-Ca^{2+}) to a low-Ca^{2+} internal solution and then a part of the surface membrane of the partner cell was disrupted. This procedure allowed bath solutions to affect one cytoplasmic aspect of the gap junction facing the partner cell. Under such conditions the measured current flowed from the clamped cell mostly through the gap junction into the partner cell whose interior was short circuited to earth.

The equivalent circuit after the disruption of the membrane of the partner cell is illustrated in Fig. 1. Here, the input conductance (G_i) is given by,

$$G_i = G_{m,1} + G_j \cdot (G_{m,2} + G_s)/(G_j + G_{m,2} + G_s), \quad (1)$$

where G_j is the conductance of the gap junction, G_s the conductance through the hole made in the partner cell, and $G_{m,1}$ and $G_{m,2}$ the respective non-junctional membrane conductance of the clamped cell and the partner cell. If G_s is much greater than G_j and $G_{m,2}$, G_j is given by,

$$G_j = G_i - G_{m,1}. \quad (2)$$

The value of $G_{m,1}$ was approximated as a half of the conductance of the total non-junctional membrane ($G_{m,1} + G_{m,2}$) measured before perforating the partner cell.

During the experiment, the interior of the clamped cell remained clear and the striation pattern remained regular, while the striation of the ruptured partner cell became blurred and faint with time (Pl. 1*B*), indicating that the intracellular medium of the partner cell was replaced effectively with the bath solution. The intracellular medium of the clamped cell may be equilibrated with the pipette solution contained in the patch electrode and partly with the bath solution through the gap junction.

Whole-cell voltage clamp using two giga-sealed patch electrodes. It was a prerequisite to eliminate any ohmic potential drop across the series resistance in the electrode tip from measurements of the clamped potential. Initially we employed a single electrode voltage clamp and adopted the electronic circuit for the series resistance compensation in the amplifier (Matsuda & Noma, 1984). We found, however, that the tip resistance of the electrode varied during the experiment, most probably due to cellular materials plugging the pipette during suction. Since the magnitude of the tip resistance was very similar to that of the junctional resistance and because they were connected in series, even a slight variation in tip resistance interfered with accurate measurement of the junctional conductance. To overcome this difficulty we developed a voltage-clamp method using two giga-sealed patch electrodes.

Two patch pipettes were each fixed on respective paired miniature manipulators (Narishige, HMT 3, Tokyo, Japan) and their tips were positioned at the same level under the microscope (Nikon, IM2, Tokyo, Japan). The whole miniature manipulator assembly was mounted onto another large Leitz manipulator, which was used to apply the electrodes to the cell. When their tips were attached to the cell surface, negative pressure of 20–40 cmH_2O was applied simultaneously to the interior of the two pipettes. During this period, the switch (s) between the current-feeding electrode (El_I) and the voltage-sensing one (El_V) in the diagram shown in Fig. 1 was closed, and the increase in total sealing resistance at the tips of the two electrodes was monitored as usually done in the conventional patch-clamp technique (Hamill *et al.* 1981). After establishing the giga-seal, the feed-back gain was lowered temporarily to prevent the switching transients from breaking the

giga-seal and the switch (s) was opened. The frequency response was improved by increasing the positive feed-back gain through a small capacitor connecting the current signal to the inputs (A_2). The membrane potential (V_m) was monitored through a voltage follower (A_1, BB3523), and the command potential was applied to the input of a negative feed-back amplifier (A_3). The current signal (I_m) was obtained at the input of the current-clamp circuit (A_4 and A_5). When the ramp-clamp method was employed, triangular command potentials of 2·5 s duration were applied every 6 s.

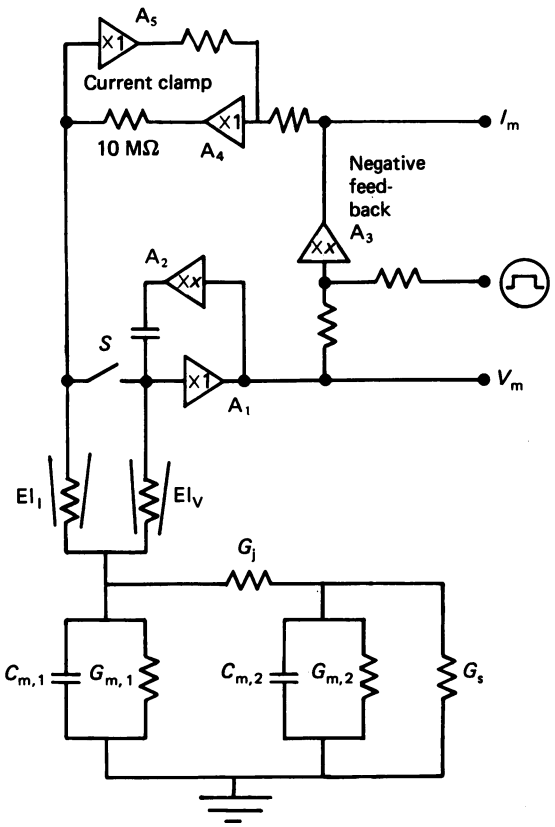

Fig. 1. Diagram of the voltage-clamp circuit (upper part) and the equivalent circuit of the open-cell system (lower part). See text for most of the notations. $C_{m,1}$ and $G_{m,1}$ are the membrane capacitance and conductance of the clamped cell, and $C_{m,2}$ and $G_{m,2}$ those of the partner cell.

Preparation of the open-cell system. The cell membrane of the partner cell was ruptured by gently crushing the tip of a glass capillary against the cell on the glass bottom of the recording chamber. The glass capillary used for the disruption was the same shape as the patch electrodes. The tip of the pipette was broken during this procedure so that its diameter became larger than 10 μm. The shunt resistance through the ruptured membrane was estimated by rupturing the cell membrane of a single cell. After the rupture of the membrane the input resistance decreased to less than 0·1 MΩ. The plate-like shape of the ventricular cell (typically $130 \times 30 \times 6$ μm) facilitated the procedure of perforation. A similar technique was used to make a hole in a non-junctional surface membrane of insect salivary gland cells by Oliveira-Castro & Loewenstein (1971).

Solutions. The compositions of Tyrode and internal solutions are listed in Table 1. The internal solution filling the pipette (pipette solution) had essentially the same composition as the internal test solution used to superfuse the perforated cell and was mainly composed of Cs aspartate. Both solutions contained adenosine 5′-triphosphate (ATP, dipotassium salt, Sigma) as an energy source

and tetraethylammonium chloride (TEA Cl) to block the potassium channels. Phosphocreatine (CrP, dipotassium salt, Sigma) was added to the pipette solution as a reservoir of high-energy phosphate bonds.

When examining the effects of H^+, Ca^{2+} or Mg^{2+} on the gap junction, internal test solutions I, II and III shown in Table 1 were employed, respectively. To obtain the desired concentrations of free Ca^{2+} and Mg^{2+}, the amounts of $CaCl_2$ and $MgCl_2$ were varied at a fixed concentration of glycoletherdiaminetetraacetic acid (EGTA). The apparent stability constants at a given pH for EGTA–Ca, EGTA–Mg, ATP–Ca and ATP–Mg were calculated using a computer program which

TABLE 1. Composition of solutions (mM)

Tyrode solution

NaCl	NaH_2PO_4	KCl	$CaCl_2$	$MgCl_2$	HEPES
136·5	0·3	5·4	1·8	0·5	5

Internal solution

	Cs aspartate	TEA Cl	ATP	CrP	$CaCl_2$	$MgCl_2$	HEPES	EGTA
Pipette solution	100	20	5	5	(−)	3	5	2–5
Test solution								
I	100	20	2 or 5	(−)	(−) or (+)*	3	5	2–10
II	100	20	2 or 5	(−)	varied	3	5	1–10
III	100	20	5	(−)	(−)	varied	5	5

The pH of solutions was adjusted with CsOH or NaOH. The pH of pipette solution, Tyrode solution and solution III was adjusted to 7·4. In solutions I and II, pH was varied according to experimental protocols (for details, see text).

* In some experiments, pCa of solution I was adjusted to 7·0 or 6·8 by adding $CaCl_2$.

was essentially the same as that developed by Fabiato & Fabiato (1979). The absolute stability constants for ATP were the same as used by Fabiato & Fabiato (1979) and those for EGTA were the values from Schwarzenbach, Senn & Anderegg (1957) cited by Blinks, Wier, Hess & Prendergast (1982). According to the suggestion by Tsien & Rink (1980), the concentration of H^+ was given as $[H^+] = a_H/0{\cdot}78$, where a_H is activity of H^+ as measured with a pH meter. The total amount of Ca^{2+} contaminating the internal solution was measured using a conventional Ca^{2+} electrode (Orion Research, model 811, New York, U.S.A.) before adding EGTA. The concentration was about 1 μM and was included in the calculation of free Ca^{2+}.

The intracellular organelles in the ruptured cell might provide another source of Ca^{2+} contamination. In skinned cardiac fibres, when an EGTA concentration of less than 1 mM is used, movements of Ca^{2+} out of and into the sarcoplasmic reticulum cannot be buffered (Fabiato & Fabiato, 1975). To avoid the possibility that the Ca^{2+}-induced release of Ca^{2+} might mediate the response of the gap junction at low concentrations near pCa 6·7, we used EGTA concentrations higher than 1 mM, mostly 10 mM. The threshold concentration of free Ca^{2+} for the closing effect of the gap junction did not differ irrespective of whether 1 or 10 mM-total EGTA was used.

All solutions were adjusted to a given pH with 5 mM-HEPES buffer. The pH of the pipette solution, Tyrode solution and solution III was 7·4. Test solution I was adjusted to various pH values after adding Ca^{2+} to the solution just before each experiment. Although the buffering capacity of HEPES is known to be poor at low pH values, the preparation was continuously perfused at a rate of 1–3 ml/min during the recording. The pH of solution II will be described for individual experiments in the Results section. All experiments were carried out at 34–35 °C. The numerical data will be expressed as mean ± S.D. (n, number of experiments).

RESULTS

Input conductance measured before and after disruption of the membrane

When the conductance of the gap junction is measured in the open-cell system as represented in the equivalent circuit in Fig. 1, the conductance of the non-junctional membrane of the clamped cell should be kept low in comparison with the junctional conductance. This was examined in every experiment by measuring the conductance before and after replacing the Tyrode solution with the Cs^+-rich, low-Ca^{2+} internal solution. In Tyrode solution, depolarizing clamp pulses from the holding potential of −43 mV induced activation of the Ca^{2+} current (Fig. 2) and at hyperpolarizing potentials a large conductance due to the inward rectifier K^+ channels was observed.

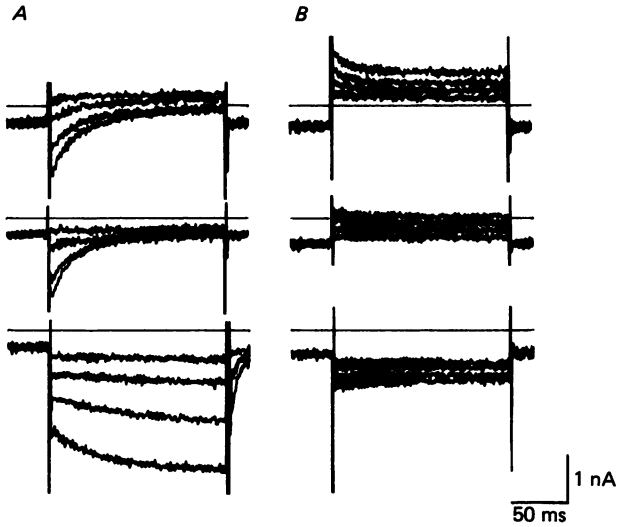

Fig. 2. Voltage-clamp records of paired ventricular cells obtained in normal Tyrode solution (*A*) and several minutes after replacing the bath solution with low-Ca^{2+} Cs^+-rich internal solution (*B*). The distance between the two giga-sealed pipettes was about 10 μm. The Na^+ channels were inactivated by setting the holding potential to −43 mV (corrected for the junction potential of −13 mV). Horizontal lines indicate zero current level. The clamp pulses were given in 10 mV steps and the currents during pulses from 7 to 37 mV (top), from −33 to −3 mV (middle), and from −53 to −83 mV (bottom row) were superimposed. Note that the Ca^{2+} current on depolarization and the inward rectifier current on hyperpolarization disappeared on switching the Tyrode solution to internal solution, leaving almost ohmic leak conductance. With the Cs^+-rich pipette solution in *A*, the inward rectifier K^+ current shows a time-dependent increase during hyperpolarization (Matsuda & Noma, 1984).

Only in a few paired cells obvious escape of the membrane potential occurred due to weak coupling of the pair during the activation of the Ca^{2+} current. When the bath solution was switched from the Tyrode solution to the internal solution containing 1 mM-EGTA, the conductance generated by the voltage-dependent channels disappeared, leaving an almost linear and stable current–voltage (*I–V*) relationship (Fig. 2*B* and open circles in Fig. 3). The average slope conductance of fifty-seven experiments was 14 ± 15 nS at around −40 mV. When the membrane leak conductance increased noticeably with time in the low-Ca^{2+} internal solution, the cells were

discarded. The relatively high input resistance compared to that of the gap junction of less than 1 MΩ (see Figs. 3 and 5) facilitated the measurements of the gap junction conductance after perforation of the partner cell membrane.

Fig. 3 shows *I–V* relationships measured before and after the disruption of the membrane of the partner cell. The slope conductance quickly increased from 13 to

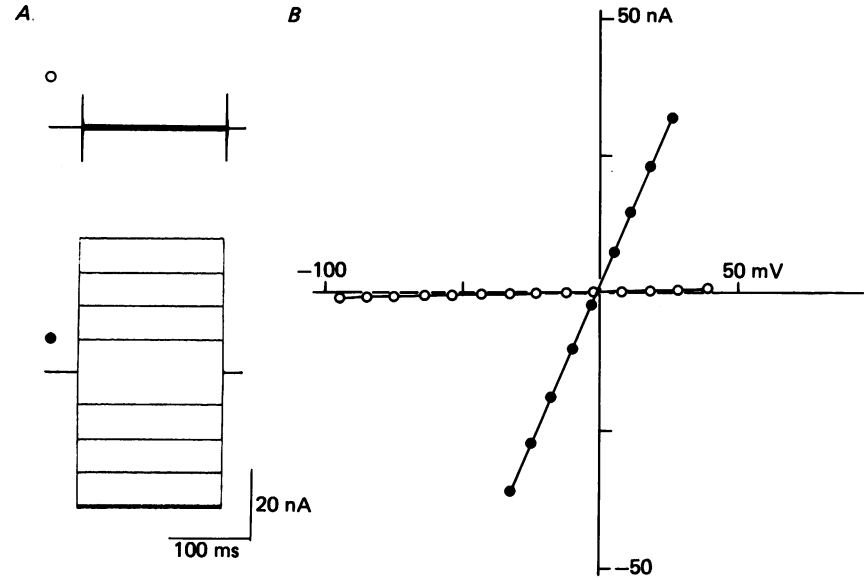

Fig. 3. *B*, *I–V* relationships measured before (open circles) and after (filled circles) the disruption of the membrane of the partner cell. *A*, original current traces in response to eight clamp pulses of 10 mV steps were superimposed before (upper panel) and after (lower panel) rupture. The holding potential was −43 and 0 mV in the upper and lower records, respectively. The paired cells were superfused with an internal solution containing 1 mM-EGTA.

1110 nS on crushing the cell. The current during the stepped changes in potential showed no obvious relaxation. The *I–V* relationship was linear over the potential range examined in every experiment. When the junctional conductance was small, a linear *I–V* relationship was obtained over the range of ±100 mV without saturation of the recording system (40 nA). Although the capacitive current interfered with the resolution of transient events within several milliseconds of the potential jumps, it may be concluded that the gap junction channels in the cardiac muscle have no time- or voltage-dependence. This is different from the voltage dependence of the gap junction channel observed in the electrotonic synapse (Furshpan & Potter, 1959) and in frog and fish blastomeres (Spray, White, Carvalho, Harris & Bennett, 1984).

That the measured conductance is due to that of the gap junction is indicated by the 'healing-over phenomenon' observed when the membrane was disrupted in a solution of pCa 6·4 (Fig. 4). The upper panel of Fig. 4 shows the *I–V* relationships, measured before (indicated by *a*) and at 18, 27 and 54 s (indicated by *b*, *c* and *d*, respectively) after disruption of the partner cell using ramp command potentials. As shown in the lower graph, after an initial rapid increase resulting from the crushing

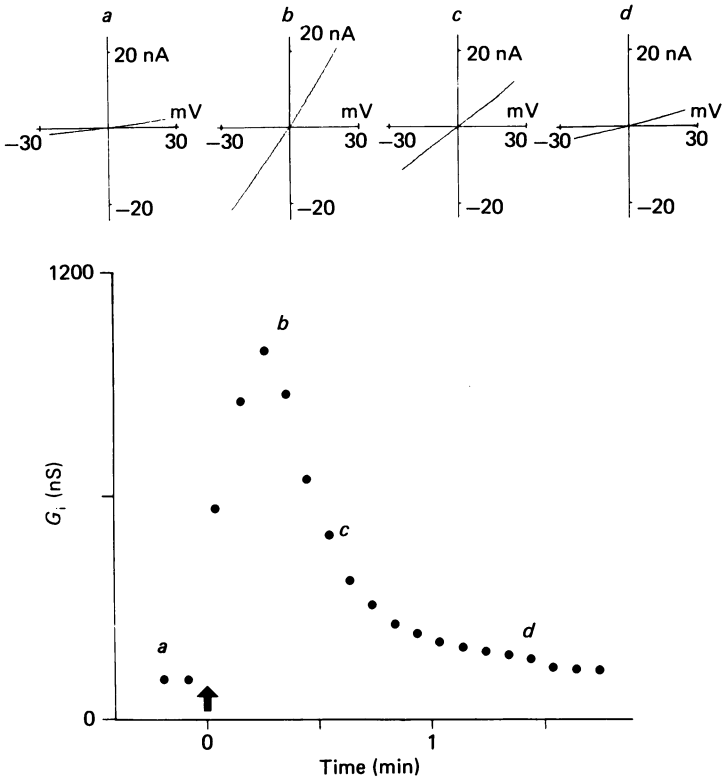

Fig. 4. Time course of the change in the input conductance (G_i) after disruption of the membrane of the partner cell at pCa 6·4. The pH of the solution was 7·4. The input conductance was measured by giving ramp command potentials of 2·5 s duration every 6 s and the original traces obtained at the times indicated by *a*, *b*, *c* and *d* in the lower graph are illustrated in the upper panel. The arrow at time zero indicates the crushing of the partner cell.

procedure, the conductance decreased exponentially and recovered almost completely within a few minutes. This finding is in contrast to that obtained in single cells (not illustrated). When the membrane of a clamped single cell was ruptured, the increased input conductance remained almost constant, indicating that the ruptured membrane did not reseal. It was concluded that the almost complete recovery observed in the paired cells was caused by the closure of the gap junction channel under the influence of elevated Ca^{2+}.

The conductance of the gap junction was measured according to eqn. (2). The values ranged from 90 to 3900 nS and their distribution is given in Fig. 5. A gap junctional conductance of about 1000 nS was most frequently observed.

Closure of the gap junction at lower pH

Since the molecular mechanism for gating of the gap junction had been attributed to the binding of H^+ or divalent cations to receptor sites, it was of interest whether H^+ and Ca^{2+} would compete for binding sites on the receptor molecule. To see the possible interactions, the effects of H^+ or Ca^{2+} on the junctional conductance were examined at various concentrations of the other ion.

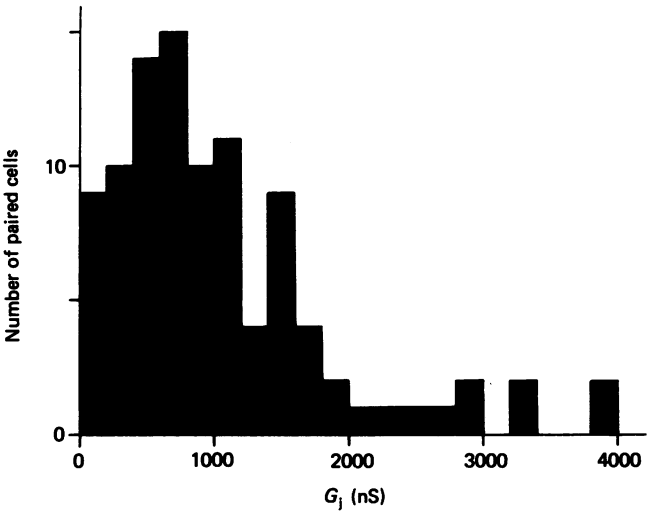

Fig. 5. Histogram of the junctional conductance (G_j) for ninety-eight paired cells.

The effects of H^+ were studied at pCa 6·8, 7·0 or 'zero Ca^{2+}'. The 'zero Ca^{2+}' solution contained 2 mM-EGTA, and no Ca^{2+} was added. To keep the free-Ca^{2+} concentration at pCa 6·8 and 7·0, 10 mM-EGTA was used as a buffer. In these solutions, the concentration of free Mg^{2+} varied between 0·08 and 0·8 mM but its effect on the junctional conductance was neglected, because the threshold of Mg^{2+} for affecting the gap junction was higher than this variation (see later).

Four experiments of varying pH were carried out at pCa 6·8 or 7·0, and another four with the 'zero Ca^{2+}' solution. There was no obvious difference between the two groups of results. Fig. 6 shows the results of an experiment with the solution of pCa 6·8. The pH of the bath solution was changed in the sequence indicated at the top of the Figure. On switching the bath solution from pH 7·4 to various lower pH values, the gap junctional conductance decreased to respective new levels. The amount of the conductance drop depended upon each pH value and almost complete closure of the gap junction was obtained at pH 5·6. These closing effects of acid were almost fully reversible.

The conductance measured at each pH was normalized with reference to the average of two values obtained at pH 7·4 before and after the application of the test solution. Fig. 7 shows data with pCa 6·8 or 7·0 (filled triangles) and without Ca (open circles). In both cases, the junctional conductance changed little from pH 7·4 to 6·5 and decreased with further acidification up to pH 5·4.

The least-squares fitting of the Hill equation to the pH–conductance relationship gave a half-maximum concentration (pK'_H) of about 6·1 and a Hill coefficient of 2·4.

Concentration-dependent action of Ca^{2+} and its pH dependence

The effects of Ca^{2+} on the junctional conductance were examined at pH 7·4, 7·0 and 6·5 with free-Mg^{2+} concentration at around 0·1 mM. At pH 7·4, measurement of the gap junction was frequently disturbed by contracture of the partner cell, which was induced by application of a higher concentration of Ca^{2+}. The Ca^{2+}-induced contracture was, however, markedly suppressed when the test solution was more

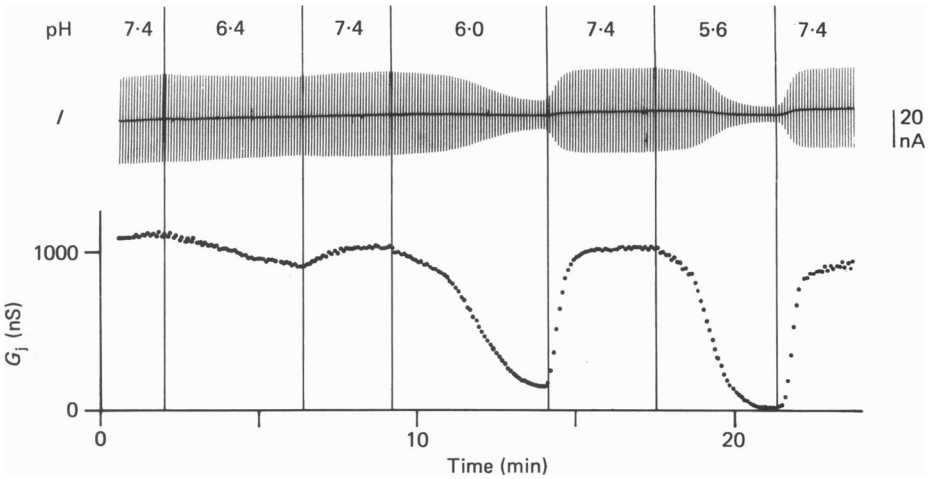

Fig. 6. The effects of lowering pH on the junctional conductance (G_j). The pH of the bath solution was changed to the values indicated at the top. The pCa of all the solutions was 6·8. The upper panel shows the chart recording of the current induced by the ramp command potentials, which were ± 20 mV in amplitude and applied every 6 s from the holding potential of 0 mV. The lower panel shows the time course of the change in the junctional conductance of the clamped cell, which was measured as the slope conductance during each ramp command potential. The conductance of the gap junction was measured using eqn. (2).

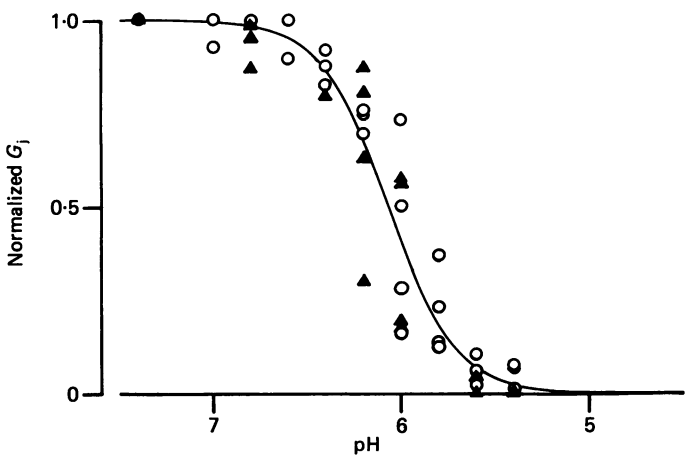

Fig. 7. The relationship between pH and the junctional conductance, G_j. The conductances at various pH values were measured using the protocol illustrated in Fig. 6 in eight experiments. In four experiments, data were obtained with test solutions containing no Ca^{2+} (open circles). In the other four (filled triangles), measurements were done in the presence of Ca^{2+} (pCa 6·8 or 7·0). Note that there are no obvious differences between these two groups. The smooth curve is a least-squares fit of equation $y = 1-1/(1+(K'_H/[H])^n)$ to all of the data points, with $K'_H = 7{\cdot}94 \times 10^{-7}$ M and $n = 2{\cdot}4$.

acidic (Fabiato & Fabiato, 1978). Thus, the typical effect of Ca^{2+} was obtained at pH 6·5 as illustrated in Fig. 8, where the solutions were changed sequentially to the indicated pCa values. The control solution was buffered with 10 mM-EGTA and no Ca was added (pCa ~ 9·4). When the bath solution was changed from the control

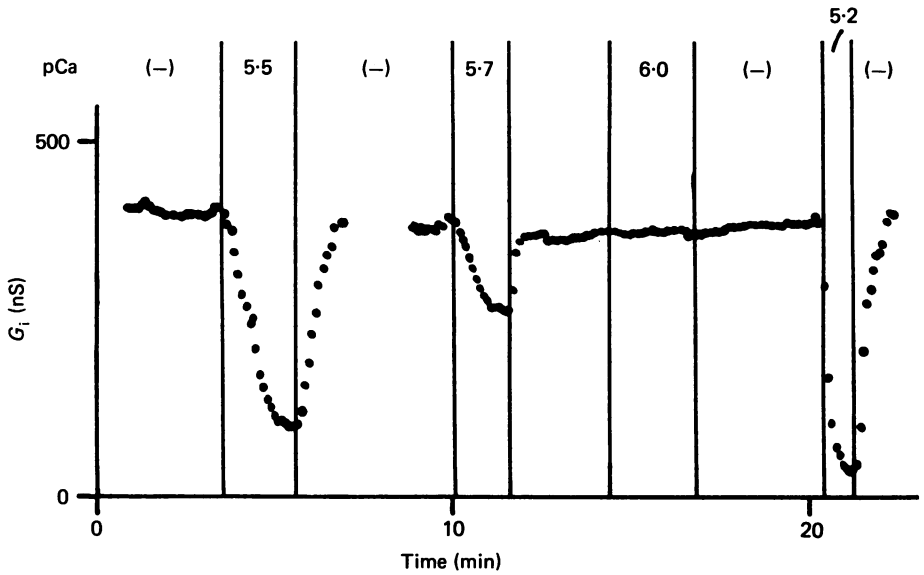

Fig. 8. The effects of Ca^{2+} on the junctional conductance (G_j). The bath solutions were changed to the indicated pCa values with pH held at 6·5. The junctional conductance, measured in the same way as in Fig. 6, is plotted against time. The control test solution (indicated as (−) in the Figure) was buffered with 10 mM-EGTA and contained only the contaminated Ca^{2+} so that the pCa of the solution was estimated to be 9·4. During the second control test, the measurement was interrupted by artifactual noise in the command signal.

solution to test solutions of pCa 5·5, 5·7 or 5·2, the junctional conductance decreased exponentially to a new steady level, which was dependent on the pCa of each test solution. On returning the bath solution to the control, the conductance recovered. It should be noted that perfusion of pCa 6·0 solution did not affect the junctional conductance.

Similar experiments were performed at pH 7·0 and 7·4. In the case of pH 7·4, however, Ca^{2+}-induced contracture prevented us from testing various Ca^{2+} concentrations in one experiment. Thus, one concentration of Ca^{2+} was applied to each pair of cells in the following manner. Before rupturing the membrane, the paired cells were superfused with the test solutions of a given pCa. On rupturing the membrane, the control junctional conductance was initially obtained as the peak value of the input conductance as shown in Fig. 4. Thereafter, the diffusion of the test solution into the partner cell decreased the junctional conductance to a new quasi-stable level. This level was dependent on the pCa of the test solution as shown in Fig. 9.

When the bath solution had a pCa of less than 6·7, following the initial exponential decay as stated above, the conductance again started to decrease accompanying the mechanical detachment caused by the contracture of the partner cell. When the cells

were separated, the clamped cell remained relaxed and its input conductance was smaller than the original value obtained before the rupture of the cell membrane. The input conductance of the separated clamped cell remained small even when the cell was superfused with an internal solution containing EGTA, suggesting a completely

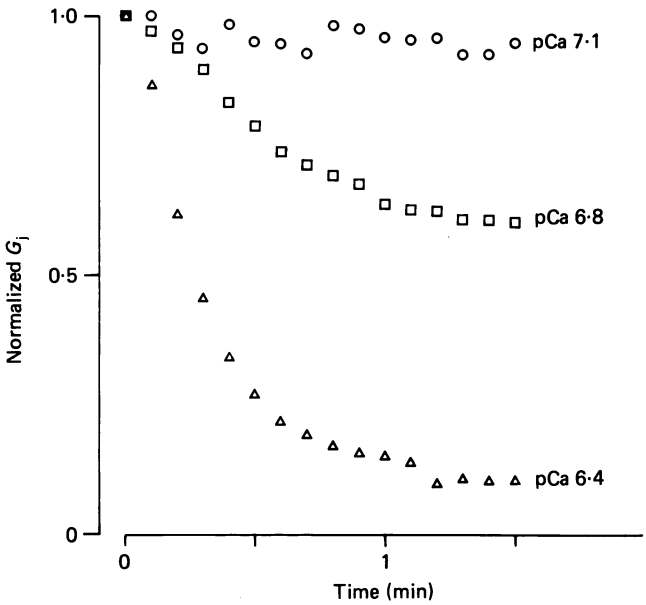

Fig. 9. Concentration-dependent decrease of the junctional conductance (G_j) by Ca^{2+} at pH 7·4. The gap junctional conductances obtained at different times using eqn. (2) were normalized with reference to the initial value of G_j. Different symbols indicate different experiments. The recovery of the gap junctional conductance on washing out Ca^{2+} was incomplete (not illustrated), because contracture of the cell caused stretching which partially damaged the gap junction. The data in this Figure were obtained before the gap junction was obviously deformed.

different mechanism for the closure of the mechanically separated gap junction than the regulation by Ca^{2+} (for example, see Mazet, Wittenberg & Spray, 1985). In experiments in which obvious mechanical detachment started before any stable level of the conductance was reached, the data were discarded.

In Fig. 10, pCa–conductance relationships from forty-four experiments are summarized. At pH 7·4 (filled triangles) the junctional conductance decreased over the pCa range 7–6 with a half-maximum concentration (pK'_{Ca}) of 6·6. Lowering the pH from 7·4 to 7·0 shifted the pCa–conductance curve slightly to the right with a pK'_{Ca} of 6·4 (open squares). The pCa–conductance curve at pH 6·5 (open circles) shifted markedly toward a higher Ca^{2+} concentration range with a pK'_{Ca} of 5·6, so that a higher free Ca^{2+} was required to obtain the same closing effect when the pH was lowered. The slopes of these curves at pH 7·4, 7·0 and 6·5 were fitted with Hill coefficients of 3·4, 3·6 and 3·3, respectively.

Closure of the gap junction by Mg^{2+}

The effects of free Mg^{2+} were examined over the pMg range 4·0–1·5 at pH 7·4 in the absence of Ca^{2+}. In Fig. 11, the solutions were changed sequentially to the

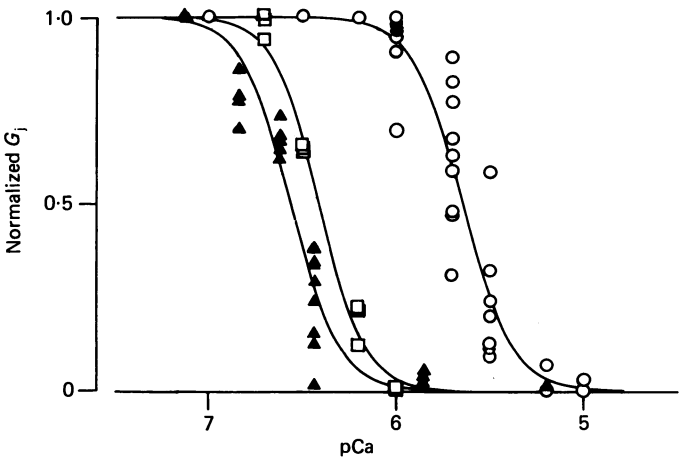

Fig. 10. Relationships between pCa and the junctional conductance (G_j) at different pH. In experiments at pH 7·4 (filled triangles), each point was obtained from different experiments ($n = 27$). At pH 7·0 (open squares) and 6·5 (open circles), data were obtained from four and thirteen experiments, respectively. The smooth curves are least-squares fits of equation $y = 1-1/(1+(K'_{Ca}/[Ca])^n)$, with $K'_{Ca} = 2{\cdot}51 \times 10^{-7}$, $3{\cdot}98 \times 10^{-7}$ and $2{\cdot}00 \times 10^{-6}$ M and $n = 3{\cdot}4$, 3·6 and 3·3 at pH 7·4, 7·0 and 6·5, respectively.

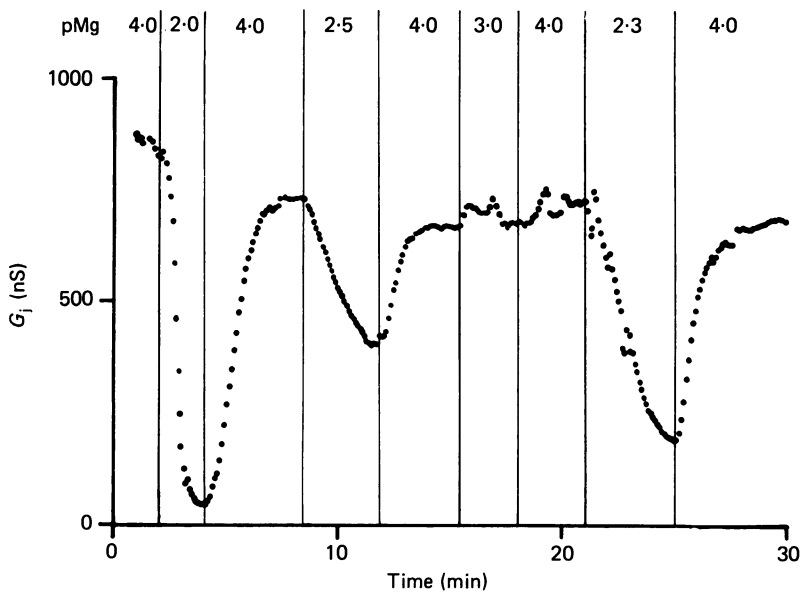

Fig. 11. The effects of increasing Mg^{2+} on the junctional conductance (G_j). The junctional conductance was measured with the ramp-clamp method. The pMg of the test solution was changed to the indicated values while keeping the pH at 7·4 and pCa at about 9·4.

indicated pMg values. The junctional conductance was little changed at pMg 3·0 and decreased in response to increases of free-Mg^{2+} concentration from pMg 4·0 to 2·0, 2·5 and 2·3 in a dose-dependent manner. The effects of Mg^{2+} were reversible. The junctional conductance measured at a given pMg was normalized with respect to the value at a pMg of 4·0.

In Fig. 12 the normalized conductances at each pMg were plotted. The junctional conductance decreased over the pMg range 3·0–2·0 with a pK'_{Mg} of 2·5 (3·2 mM). By fitting Hill plots to the data, a Hill coefficient of 3·0 was obtained.

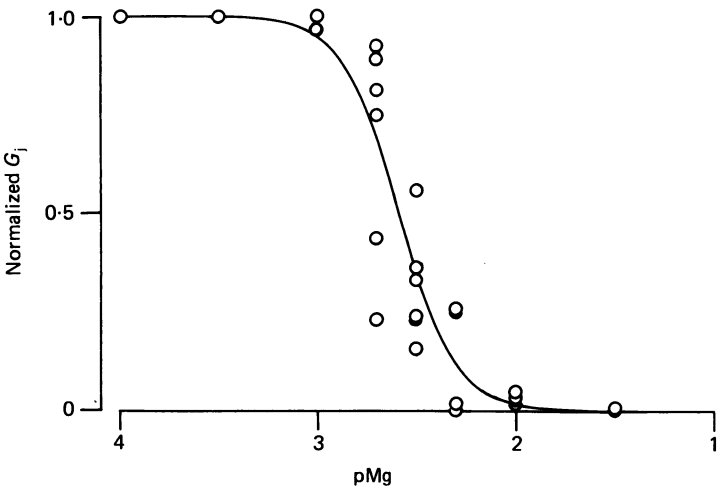

Fig. 12. Dependence of the junctional conductance (G_j) on pMg. Data obtained from ten experiments are shown. The smooth curve is a least-squares fit of equation $y = 1-1/(1+(K'_{Mg}/[Mg])^n)$, with $K'_{Mg} = 3{\cdot}16 \times 10^{-3}$ M and $n = 3{\cdot}0$.

DISCUSSION

The magnitude of the junctional conductance varied between different paired cells over a range between 90 and 3900 nS. This large variation might be caused by an error in the estimation of the non-junctional membrane conductance or by the involvement of the series resistance in the measurements. However, the non-junctional membrane conductance was made minimum (Fig. 3) and the rupture of the cell membrane in the partner cell was made as large as possible. In fact, the frequency histogram of the gap junctional conductance is quite similar to that obtained from essentially the same preparation but with a different technique (Kameyama, 1983). The result is also in good agreement with the value of the nexal membrane resistance (2·12 MΩ) measured most recently by Metzger & Weingart (1985) in dissociated paired cells from rat ventricle. We attribute the wide variation to loss of a fraction of the gap junctional channels during the dissociation procedure, as has been suggested by Kameyama (1983).

No voltage or time dependence of the junctional conductance was observed over the wide voltage range used in the present study. This finding is consistent with and extends the previous observations on guinea-pig (Kameyama, 1983) and rat hearts (White, Carvalho, Spray, Wittenberg & Bennett, 1983; Metzger & Weingart, 1985), but is in contrast to the clear voltage and time dependence of the gap junction channel of frog and fish blastomeres (Spray *et al.* 1984). In these embryonic cells, the junctional conductance declines sharply with the increase of the trans-junctional voltage in either direction. It may be concluded that gating of the cardiac gap junction is operated only by chemical ligands. The present study, therefore, was

focused on the relationship between the gap junctional conductance and ion concentration.

Different receptor sites for divalent cations and H^+

The present study showed that lowering the pH shifted the pCa–G_j curve toward the higher Ca^{2+} concentration range. This finding can be explained on the basis of a simple competition between Ca^{2+} and H^+ in a binding reaction to the Ca^{2+} receptor, which is involved in the control of the gap junction. When the negative charge of the receptor site is neutralized by H^+, the affinity of Ca^{2+} for the binding site would be reduced. Such a mechanism has been proposed for the high-affinity binding of Ca^{2+} to the plasma membrane in the uterine smooth muscle of the rat, which is highly pH dependent (Grover, Kwan & Daniel, 1983).

A simple co-operative model was proposed to explain the competitive interaction between Ca^{2+} and H^+:

$$H_{2n}R \underset{2n.H}{\rightleftharpoons} R \underset{n.Ca}{\rightleftharpoons} Ca_n R,$$

and the Ca–G_j relationship is:

$$G_j = 1 - \frac{1}{1 + (K_{Ca}/[Ca])^n . (1 + ([H]/K_H)^{2n})}, \quad (3)$$

where G_j is the normalized junctional conductance, K_{Ca} the Ca^{2+} concentration required to close 50 % of the maximum fraction of gap junction channels, K_H the H^+ concentration required to induce 50 % protonation of the Ca receptor (R), and n the number of Ca-binding sites per receptor. [Ca] and [H] represent respective ion concentrations. The apparent half-maximum Ca^{2+} concentration (K'_{Ca}) obtained from the experiment is given as,

$$(K'_{Ca})^n = (K^n_{Ca} . (1 + ([H]/K_H)^{2n}). \quad (4)$$

From the relationship between $(K'_{Ca})^n$ and H^{2n} (the value of n was assumed to be 3), K_{Ca} and K_H were estimated to be $3{\cdot}16 \times 10^{-7}$ and $1{\cdot}12 \times 10^{-7}$ M, respectively.

On the other hand, the pH–G_j relationship was not influenced by the presence of Ca^{2+}. The result suggests that there are at least two kinds of binding sites for the modulation of junctional conductance, one binding divalent cations and the other binding H^+. The following results also support this view. (1) The Hill coefficient for the divalent Ca^{2+} (3·4) was not half that for the monovalent H^+ (2·4). (2) The K_H value on closing the gap junction ($7{\cdot}94 \times 10^{-7}$ M) was different from the value obtained from the effect of H^+ on the pCa–G_j relationship ($1{\cdot}12 \times 10^{-7}$ M).

The junctional conductance decreased in response to an increase of free Mg^{2+} over the millimolar range in a reversible manner. Oliveira-Castro & Loewenstein (1971) have shown, in an open-cell system of insect salivary gland, that free Mg^{2+} higher than 0·12 mM could uncouple the cells. Using isolated membranes from calf lens fibres, Peracchia & Peracchia (1980) showed reversible changes in the gap junctional structure corresponding to the functional uncoupling when the preparation was incubated in medium containing 1 mM or higher Mg^{2+}. Thus, the closing effect of Mg^{2+} seems to be one of the general characteristics of the gap junctional channel. The

slope of the pMg–G_j curve was similar to that of the pCa–G_j curve, suggesting that Ca^{2+} and Mg^{2+} bind to the same receptor site.

The hypothesis involving two different receptor sites well explains the species difference in the relative sensitivities to Ca^{2+} and H^+. In fish blastomeres the sensitivity to H^+ (pK_H 7·3) is much higher than to Ca^{2+} (pK_{Ca} 3·3; Spray, Stern, Harris & Bennett, 1982). In contrast, in our preparation, the threshold pCa was 6·7 and threshold pH was 6·4.

Sensitivity to Ca^{2+}

It should be noted that Ca^{2+} was applied to one cytoplasmic aspect of the gap junctional channel in the open-cell system, while in the conventional multicellular preparation the intracellular concentration was varied along the entire length of the preparation. Nevertheless, the measurements of the threshold concentration (0·2 μM; Weingart, 1977; 0·5 μM; Dahl & Isenberg, 1980) are quite similar to that obtained in the present study. If the normal intracellular pH is between 7·0 and 7·2 (Roos & Boron, 1981), the present study gives a threshold concentration of about pCa 6·7. Nishiye, Mashima & Ishida (1980) measured the binding of Ca^{2+} to membranes isolated from cardiac ventricular cells, and obtained two components with small and large pK_{Ca} values. The component with the larger pK_{Ca} is rather similar to that obtained in the present study. These findings may be consistent with the hypothesis that the gap junctional channel is composed of two symmetrical units and that the binding of H^+ or divalent cations to one component blocks the channel (Loewenstein, 1981).

The present result is also consistent with the electron microscopic finding in calf lens fibres (Peracchia & Peracchia, 1980) that the threshold Ca^{2+} concentration for crystallization of the gap junction particles was 0·5 μM. In the insect salivary gland there was a high degree of uncoupling at 10^{-5} M-Ca^{2+} (Rose & Loewenstein, 1976).

Physiological implications

It has been considered that the threshold concentration of the intracellular Ca^{2+} for electrical uncoupling would be higher than that for tension development (Weingart, 1977; Nishiye *et al.* 1980; Kameyama, 1983). This hypothesis was based on the finding that the internal resistance of ventricular muscle did not change throughout the course of a single action potential (Weidmann, 1970). However, in the present study the conductance of the gap junction channels decreased with a threshold concentration of pCa 6·7, which is quite similar to the threshold for tension development (Fabiato & Fabiato, 1975).

Two possible mechanisms might be proposed in order to reconcile these two findings. One is that the rate of uncoupling would not be sufficiently fast to achieve sizeable uncoupling during each Ca^{2+} transient. The results that the Ca^{2+}-induced uncoupling proceeded with a time course of more than several seconds might support this view. However, we could not resolve the time course into the response time of the gap junction channel and the diffusion time of the test solution through the ruptured cell in the present method. Recently very slow, voltage-dependent kinetic properties of the gap junction channel have been demonstrated in single-channel recordings of the junction of rat lacrimal glands (Neyton & Trautmann, 1985).

An alternative possibility is that free Ca^{2+} in the vicinity of the gap junction is maintained at a low enough concentration to keep the gap junction patent, while the Ca^{2+} concentration within the cell transiently increases up to 2 μM during each contraction of the cardiac muscles (Allen & Blinks, 1978). This view may be supported by the fact that release and sequestration of Ca^{2+} are carried out by intracellular organelles such as mitochondria and sarcoplasmic reticulum (Endo, 1977; Carafoli & Crompton, 1978) and that an increase in Ca^{2+} in the cytosol may be effectively compartmentalized (Meech, 1974; Rose & Loewenstein, 1975). When the cell is overloaded with Ca^{2+} by the application of ouabain or by inhibition of metabolism, however, the sequestering capacity may become saturated leading to a rise in the free-Ca^{2+} concentration at the junction (Dahl & Isenberg, 1980). This would result in the decoupling of cardiac cells and depress the conduction of excitation (Weingart, 1977; Wojtczak, 1979; Dahl & Isenberg, 1980).

The cytosolic free-Mg^{2+} concentration in the cardiac tissue has recently been measured with an ion-selective micro-electrode or with nuclear magnetic resonance (Hess, Metzger & Weingart, 1982; Gupta, Gupta & Moore, 1984; Blatter & McGuigan, 1986). The reported values vary between 0·5 and 3·5 mM. Thus, it is possible that Mg^{2+} is also involved in regulation of the junctional conductance in cardiac tissues. The present results suggest that the free Mg^{2+} concentration in the normal cardiac cells would be lower than 1–2 mM. Otherwise the cells would be uncoupled.

Reber & Weingart (1982) showed that the internal longitudinal resistance of intact sheep Purkinje fibres changed significantly with pH changes between 6·7 and 7·6 induced by the external application of NH_4Cl or CO_2. On the other hand, De Mello (1983) reported that healing-over of cardiac cells was not observed at pH 6·5 in Ca^{2+}-free bathing solution and even at pH 5·5 healing-over was still incomplete. The present result is rather in accordance with the latter findings suggesting that H^+ does not play a major role in the regulation of the gap junctional conductance under physiological conditions. In internally perfused fish blastomeres, Spray *et al.* (1982) demonstrated that H^+ directly modulates the junctional conductance within the physiological pH range of the tissue.

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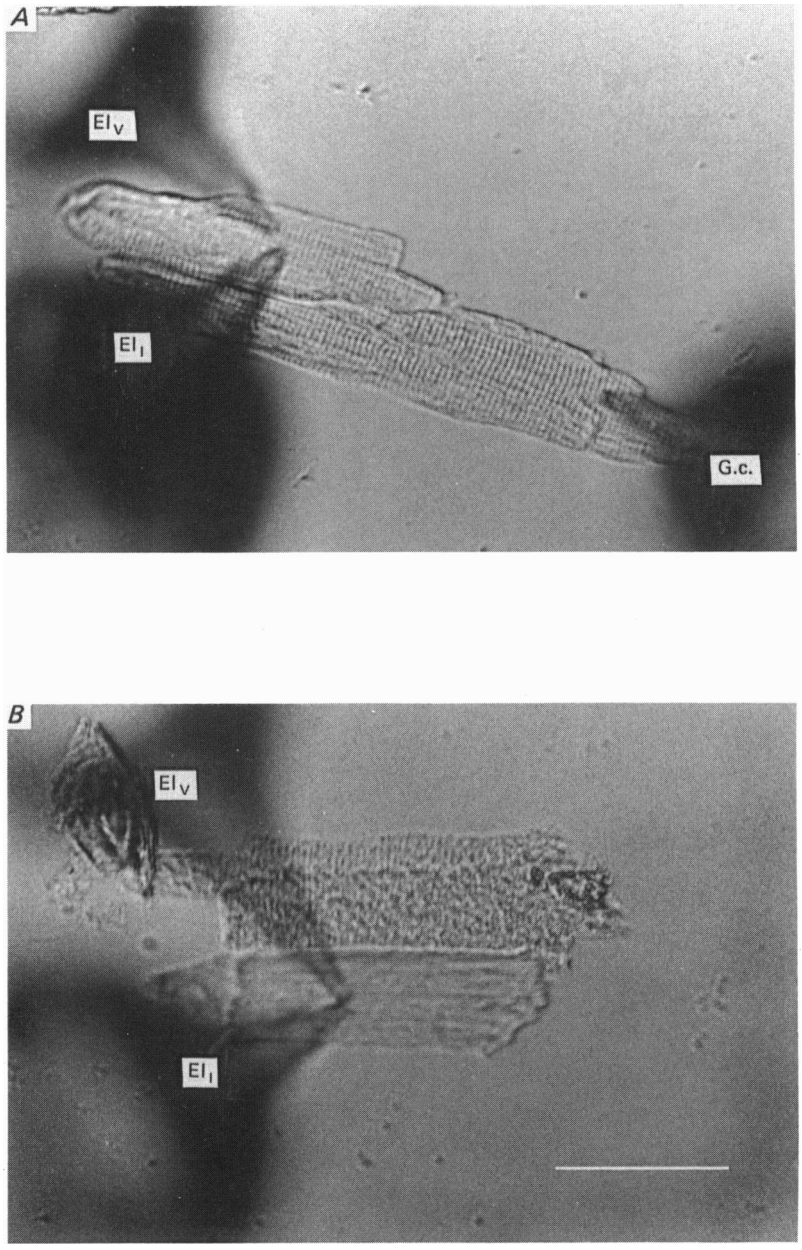
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EXPLANATION OF PLATE

Photomicrographs of paired ventricular cells. In *A*, two patch electrodes are attached to one of the paired cells for whole-cell voltage clamping and another capillary prepared for crushing the other cell is seen. *B* shows the granular appearance of the disrupted partner cell 10 min after the perforation of the cell membrane. In contrast, the clamped cell seems clear. Calibration, 50 μm. El_V: voltage-sensing electrode. El_I: current-feeding electrode. G.c.: glass capillary for crushing the membrane of the partner cell.