A structural basis for the unequal sensitivity of the major cardiac and liver gap junctions to intracellular acidification: The carboxyl tail length

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ABSTRACT The regulation of junctional conductance (G_j) of the major cardiac (connexin43; Cx43) and liver (connexin32; Cx32) gap junction proteins by intracellular hydrogen ion concentration (pH; pH_i), as well as that of a truncation mutant of Cx43 (M257) with 125 amino acids deleted from the COOH terminus, was characterized in pairs of *Xenopus laevis* oocytes expressing homologous channels. Oocytes were injected with 40 nl mRNAs (2 $\mu g/\mu l$) encoding the respective proteins; subsequently, cells were stripped, paired, and incubated for 20–24 h. G_j was measured in oocyte pairs using the dual electrode voltage-clamp technique, while pH_i was recorded simultaneously in the unstimulated cell by means of a proton-selective microelectrode. Because initial experiments showed that the pH-sensitive microelectrode responded more appropriately to acetate than to CO_2 acidification, oocytes expressing Cx32 and wild type and mutant Cx43 were exposed to a sodium acetate saline, which was balanced to various levels of pH using NaOH and HCl. pH was changed in a stepwise manner, and quasi-steady-state G_j–pH_i relationships were constructed from data collected at each step after both G_j and pH_i had reached their respective asymptotic values. A moderate but significant increase of G_j was observed in Cx43 pairs as pH_i decreased from 7.2 to 6.8. In both Cx32 and M257 pairs, G_j increased significantly over a wider pH range (i.e., between 7.2 and 6.3). Further acidification reversibly reduced G_j to zero in all oocyte pairs. Pooled data for the individual connexins obtained during uncoupling were fitted by the Hill equation; apparent 50%-maximum (pK; pK_a) values were 6.6 and 6.1 for Cx43 and Cx32, respectively, and Hill coefficients were 4.2 for Cx43 and 6.2 for Cx32. Like Cx32, M257 had a more acidic pK_a (6.1) and steeper Hill coefficient (6.0) than wild type Cx43. The pK_a and Hill coefficient of M257 were very similar to those of Cx32. These experiments provide the first direct comparison of the effects of acidification on G_j in oocyte pairs expressing Cx43 or Cx32. The results indicate that structural differences in the connexins are the basis for their unequal sensitivity to intracellular acidification in vivo. The data further suggest that a common pH gating mechanism may exist between amino acid residues 1 and 256 in both Cx32 and Cx43. However, the longer carboxyl tail of Cx43 relative to Cx32 or M257 provides additional means to facilitate acidification-induced gating; its presence shifts the pK_a from 6.1 (Cx32 and M257) to 6.6 (Cx43) in the conductance of these channels.

INTRODUCTION

Gap junctional channels provide an intercellular low resistance pathway for cells to communicate and share their individual pools of low molecular weight molecules, including ions, amino acids, and metabolic products. Since Loewenstein's (1) first demonstration of regulation of gap junctional channels by calcium in salivary gland cells, intercellular communication in many cells has been shown to be modulated by a wide variety of agents, including intracellular hydrogen ion concentration (pH_i)[1] (2), transjunctional voltage (3), phosphorylation (4–6), alkanols (7), low ATP (8), and arachidonic acid (9). Moreover, synergistic actions of various such regulators have also been suggested (10).

The molecular basis of cellular regulation of cell-to-cell communication has been promoted by analysis of primary amino acid sequences of junctional proteins. Recently, many cDNA clones, encoding a variety of gap junction proteins in different cell types, have been characterized. Comparative studies have demonstrated 32–71% homology among various gap junction proteins (11), which comprise a family referred to as "connexins" (Cx's) (12). Among them, Cx43 and Cx32 have been identified as the major cardiac and liver gap junction proteins (12, 13), respectively. Members of the gap junction family share certain structural motifs: the peptide chain of a connexin spans the membrane four times to give rise to two well-conserved extracellular loops and one intracellular loop; both amino and carboxyl ends are identified on the cytoplasmic surface (14–17). The length of the carboxyl tail is variable; for example, Cx26 has a carboxyl tail of 11 amino acid residues (18), whereas the tail of Cx43 is 149 residues long (12); the amino acid sequence also shows great diversity of the carboxyl tail in various tissues. Whether the tail defines tissue-specific functions is not known as yet. However, morphologically, the long carboxyl tail seems to contribute to the fuzzy appearance observed on the cytoplasmic surface of the junctional membranes isolated from cardiac tissues (19, 20), since the fuzzy surface disappears under proteolytic conditions with the loss of a 17-kD carboxyl segment. The absence of the fuzzy structure in liver preparations (19) has led to the hypothesis that the long carboxyl tail of the cardiac gap junction protein

This work represents partial fulfillment by S. Liu of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology, State University of New York Health Science Center at Syracuse, Syracuse, New York.

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[1] *Abbreviations used in this paper:* Cx, connexin; G_j, junctional conductance; i, intracellular; I_j, junctional current; o, extracellular.

0006-3495/93/05/1422/12 $2.00

might be responsible for certain tissue-specific properties of myocardium (20). To assess the relationship between tail length and function of gap junctional channels, genes encoding wild type Cx43 and its mutants (with various lengths of carboxyl tail) have been functionally expressed in either *Xenopus* oocytes (23) or in stably transfected cell lines (21, 22). Various levels of macroscopic and microscopic conductance have been observed for functionally expressed mutant Cx43 channels having various carboxyl tail lengths (22, 23), which indicates that the carboxyl tail of Cx43 plays a role in determining microscopic conductances. Whether the carboxyl tail is involved in other functional properties of gap junctional channels, such as pH or calcium sensitivity of channel conductance, remains to be determined.

The pH-induced uncoupling of junctional conductance may be physiologically meaningful, especially in the cardiac tissue. In ischemia-affected cells, the pH_i may reach values as low as 6.2 (24). This ischemia-induced low pH may be one of the factors involved in blocking communication between injured and normal cells to protect the functional state of the latter. Sensitivity of cell-to-cell communication to pH was first reported by Turin and Warner in 1977 (2). Subsequent studies have demonstrated that intracellular acidification causes electrical uncoupling in a variety of preparations (25). The demonstration of an S-shaped curve describing the pH–junctional conductance relation led to the hypothesis that, in general, the proton-mediated response is a cooperative process that should be described by the Hill equation (26). However, the specific degree of pH sensitivity of the gap junctions varies significantly among individual tissues (25, 27). Indeed, the intracellular acidity needed to reduce macroscopic junctional conductance (G_j) to 50% of the maximum value (hereafter referred to as apparent pK or pK_a) in liver gap junctions (pK_a 6.3; see references 25 and 28) is different from that of cardiac gap junctions (pK_a 6.7; see reference 25); the Hill coefficient describing the cooperativity in the G_j response to changes of pH_i obtained for hepatocytes (7; see reference 28) is also different from that in cardiac myocytes (2; see reference 27). However, whether such a difference results from different cellular environments or resides in the intrinsic amino acid sequence of the respective gap junction proteins has not been established. Indeed, although functionally expressed gap junctional channels have been shown to be sensitive to intracellular acidification (29–31), no detailed quantitative analysis has been done, except in an abstract by Ebihara (31), who reported that Cx38 and Cx43 channels expressed in *Xenopus* oocytes showed unequal sensitivity to pH. Similar observation was obtained by Werner et al. (32) using different CO_2 concentrations. Furthermore, previous experiments (23) have demonstrated that the macroscopic G_j measured in oocyte pairs expressing M257, a mutant of Cx43 with 125 amino acids deleted from the carboxyl tail, is similar to that in oocyte pairs expressing Cx43, while mutants with shorter carboxyl tails express lower macroscopic G_js (23). Whether or not the response of M257 channels to pH_i is similar to that of Cx43 channels has not been determined as yet.

In this study, we established a method of simultaneously recording pH_i and G_j to assess the steady-state pH_i–G_j relation in oocyte pairs expressing rat Cx32, rat Cx43, or M257. Our results show that the pH sensitivity of Cx43 and Cx32 channels is indeed different, and that the difference resides within their amino acid sequences; the carboxyl tail of Cx43 plays an essential role in determining higher pK_a of G_j decrease of cardiac gap junctional channels.

MATERIALS AND METHODS

cDNA and mRNA preparation

Cx43 and Cx32 cDNAs (courtesy of Drs. Eric Beyer and David Paul, respectively; see references 12 and 13) were subcloned into the phagemid pBluescript IISK+ (Stratagene, La Jolla, CA). Mutant M257 cDNA was produced as described previously (23). pBluescript containing a given cDNA insert was transcribed in vitro using T3 RNA polymerase and the mCAP mRNA capping kit (Stratagene). The prepared mRNAs were routinely tested for their activity by in vitro translation, as has been described previously (23, 35).

Oocyte preparation

Adult female *Xenopus laevis* frogs (Nasco Biologicals, Fort Atkinson, WI) were used. After anesthesia with 0.2% MS-222 (3-aminobenzoic acid ethyl ester), frogs were subjected to an abdominal incision for collection of unfertilized ovarian oocytes and the oocytes were placed in Leibovitz L-15 medium (with L-glutamine and without Na_2CO_3; GIBCO BRL Life Technologies, Inc., Grand Island, NY). The follicular layer of stage VI oocytes was stripped manually with fine forceps under a dissecting microscope. Oocytes were then digested with 10 mg/ml type II collagenase (Sigma Chemical Co., St. Louis, MO) for 20 min, injected with 40 nl of mRNA (2 $\mu g/\mu l$), and then incubated overnight. The following day, oocytes were immersed in a hypertonic stripping solution (33) where they were stripped of the vitelline layer. Subsequently, pairs of oocytes were gently placed in apposition (with vegetal poles facing each other) and incubated in L-15 medium at 19°C for 20–24 h before electrophysiological recording.

Leibovitz L-15 medium was prepared as instructed by the supplier. Hepes (20 mM) was added to the medium, which was then adjusted to pH 7.4, filtered, and stored at 4°C. Before use, the medium was diluted to 50% of its original composition; 10 mg/ml gentamicin and 10 mg/ml penicillin were added and the medium was filtered again. The diluted medium could be stored at 4°C for 5 d.

Preparation of pH-sensitive electrodes

The method used to prepare proton-selective microelectrodes was similar to that reported previously by Peracchia (34). Briefly, clean glass pipettes were used to make microelectrodes that, if filled with 3 M KCl, would have resistances ranging between 10 and 15 MΩ. Electrodes were baked at 200°C in a metal container (~547 ml) for ≥2 h and subsequently silanated by adding 80 ml of 5% dimethyl-dichloro-silane (dissolved in xylene) into the metal container. Thereafter the electrodes were left at 200°C for another 5 min, after which they were baked at 100°C for 2 h. Silanized electrodes were stored in the presence of desiccant, activated silica gel. Electrodes were filled with a solution of 2.5 M KCl, 20 mM Hepes, and 103 mM sodium acetate adjusted to pH 7.0. Positive pressure was applied from the shank until the solution was forced to the very tip of the electrode. The tip was then immersed in a

drop of the proton-selective resin (catalog number IE010; World Precision Instruments, Sarasota, FL) and negative pressure was applied through the shank of the electrode to fill 200–500 μm of the tip with the proton-selective resin. Electrode tips were subsequently immersed in a sodium acetate saline solution (NaAcS; see composition below). The pH of NaAcS was adjusted to 7.0, and the solution was stored overnight at 4°C.

All pH-sensitive electrodes were calibrated at pHs 5.0, 6.0, and 7.0, and the calibrated voltage slope (V_{slope}) of the function describing the potential change sensed by the pH electrode per pH unit was calculated for each. Electrodes with $V_{slope} < 50$ mV/pH unit were discarded. All pH-sensitive electrodes used in the experiments responded linearly to pH changes between 5.0 and 8.0.

Electrophysiological recording

Unless otherwise stated, voltage clamp recordings were obtained in a NaAcS solution of the following composition (mM): 103 sodium acetate, 20 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.82 $MgSO_4$, 0.74 $CaCl_2$, and 20 Hepes. Benzyl penicillin (10 mg/ml) and streptomycin sulfate (10 mg/ml) were added. The pH of the solution was adjusted using either HCl or NaOH. The rate of superfusion was constant at 2 ml/min. G_j was measured using the dual electrode voltage clamp technique (36) as previously described by our laboratory (23). Briefly, each oocyte of a pair was impaled with two microelectrodes, one for voltage recording and the other for current injection. Microelectrodes were filled with a solution containing 150 mM KCl, 1.0 mM $MgCl_2$, 5 mM Na_2ATP, and 5 mM Na_2-phosphocreatine; tip resistances ranged between 10 and 20 MΩ for voltage electrodes and between 0.5 and 3 MΩ for current-passing electrodes. Cell pairs were held at a common holding potential of −20 mV. Voltage-step pairs (positive step followed by negative step) of 10 mV in amplitude and 5 s in duration, with an interstep interval of 5 s, were delivered to one oocyte (cell 1) at the rate of one/min. Junctional current (I_j) was measured as the current recorded in the unstimulated cell 2 (36). Resting membrane potential was routinely checked at the outset of each experiment. Only oocyte pairs having G_js > 5 μS and resting membrane potential more negative than −30 mV were used.

pH_i measurements

In all experiments, the pH-sensitive microelectrode was introduced into the unstimulated cell (cell 2). Voltage signals were input to an electrometer (World Precision Instruments), filtered at 1 Hz, and amplified 10 times. The pH_i was calculated from the following equation:

$$pH_i = 7.0 - (V_{pH} - V_m)/V_{slope}, \quad (1)$$

where V_{pH} is the potential sensed by the pH electrode and V_m is the membrane potential of the same cell containing the pH electrode.

Data acquisition, analysis, and display

Signals were acquired by a PC/386 Zenith data system computer through an A/D converter (Axon Instruments, Burlingame, CA). Voltage and current signals recorded from each cell of an oocyte pair were amplified by Axoclamp amplifiers (Axon Instruments). The output signals of the amplifiers together with the recorded pH signal were fed into the computer through a TL-1 DMA interface (Axon Instruments) at a rate of 20 Hz. Electrical stimulations were delivered from the computer through a D/A circuit of the TL-1 DMA interface. All the parameters of stimulation, data acquisition, and subsequent data analysis were set up using PCLAMP software (Axon Instruments).

A first-order form of the Hill equation (26) was used to fit the data obtained in individual groups of experiments during uncoupling of G_j to determine the sensitivity of G_j to acidification, as follows:

$$G_j = K^n/(K^n + [H^+]^n), \quad (2)$$

where G_j is the junctional conductance normalized with respect to its maximum value, K is the apparent dissociation constant, and n is the Hill coefficient, which may be considered to represent the number of cooperative sites with respect to proton binding (26). Comparisons among groups were made using analysis of variance (ANOVA). Unless otherwise indicated, data are reported as mean ± SEM percent change from control.

RESULTS

Validation of the acidification technique

Extracellular pH measurements

Sodium acetate and saturation with 100% carbon dioxide have been used previously as alternative methods to achieve intracellular acidification (34). While both can reliably reduce the pH in the bathing solution, it has been suggested that CO_2 may induce artifacts in the pH measurement when proton-selective microelectrodes are used (34). Hence, to avoid such artifacts it was important to carry out control experiments to compare the response of pH-sensitive electrodes to two different solutions (CO_2-saturated and NaAcS) in a cell-free system (Fig. 1 *A*). In this experiment, a pH-sensitive electrode was immersed in a Petri dish that was continuously perfused with a saline solution containing 88 mM NaCl and no sodium acetate. After 5 min control recording, the solution was saturated with 100% CO_2 (pH 5.8). After a 2-min lag, the pH_i recorded by the microelectrode quickly declined, but did not reach the expected value of 5.8. Moreover, in spite of the continuous presence of CO_2 in the perfusate, pH gradually increased by ~0.5 U after it had achieved its lowest value, from ~6.2 to 6.7. During washout with normal saline solution (pH 7.4), there was an overshoot whereby pH rapidly increased above baseline, and then more gradually returned to the initial baseline. Subsequent superfusion with NaAcS balanced to pH 6.0 induced a stable and predictable pH-selective electrode response. These results are similar to those of Peracchia (34), who suggested that diffusion of CO_2 through the pH-sensitive resin into the pipette would change the intrapipette pH, and would thus change the baseline in the pH measurement. Consequently, since superfusion with saline solution saturated with CO_2 would introduce a significant error in the pH_i measurements (≥0.5 units), all subsequent intracellular acidification experiments were always performed by superfusion of cell pairs with NaAcS balanced at various pH values.

Intracellular versus extracellular pH

A steady-state pH_i versus extracellular pH (pH_o) plot was constructed from 19 experiments (Fig. 1 *B*). The normal pH_i in our oocytes was 7.12 ± 0.03 (mean ± SEM), which is similar to previously reported values of 7.11–7.2 in oocytes at various stages of maturity (31, 37). Linear fitting of the data produced a slope of 0.78, with a regression coefficient of 0.97. Similar linear relations between pH_i and pH_o have been observed in other tissues, although with slightly different slopes (0.52, see

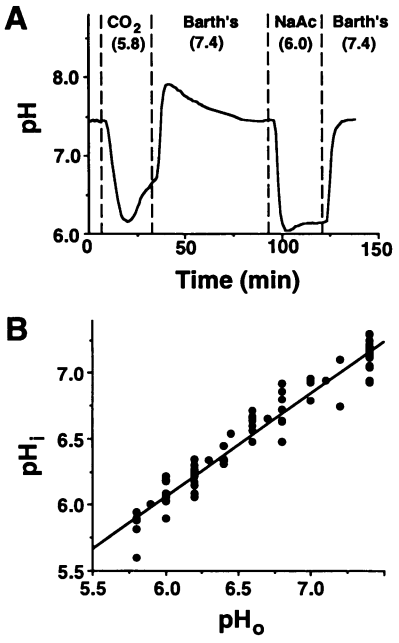

FIGURE 1 Validation of the technique of pH_i measurement. (*A*) The response of a pH-sensitive electrode to acidification induced by CO_2-saturated Barth's solution (pH 5.8) and to a NaAcS solution balanced at pH 6.0. The response of the pH-sensitive electrode to CO_2 is less than expected and changes continuously with time. On the other hand, the response of the pH-sensitive electrode to NaAcS (pH 6.0) is more accurate and predictable. Barth's solution (see reference 35) was modified to achieve the following composition: 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.74 mM $CaCl_2$, 10 mg/ml benzyl penicillin, 10 mg/ml streptomycin sulfate, and 20 mM Hepes. Acidification was obtained by adding appropriate amounts of sodium acetate or bubbling the solution with 100% CO_2. (*B*) relationship between pH_o and pH_i measured at the asymptotic state of each superfusion step of known extracellular pH_o. Data are pooled from the experiments of intracellular measurement in all 19 Cx43, Cx32, and M257 pairs. A linear relation between pH_i and pH_o is apparent.

reference 38; 0.63, see reference 39). The linear relationship between pH_i and pH_o and consistency of the data with those in the literature validate the use of the pH-sensitive microelectrode and demonstrate the accuracy of the pH_i measurement.

G_j expressed in oocyte pairs

Initially, the G_js of different connexins expressed in oocyte pairs were measured to ensure equivalent functional expression of channels. After overnight pairing (20–24 h), G_j was measured at the beginning of each experiment using dual electrode voltage-clamp technique under control conditions. The average G_j (mean ± SEM) was 18.4 ± 6.8 μS for Cx43 pairs (n = 6), 21.6 ± 3.4 μS for Cx32 pairs (n = 7), and 21.2 ± 4.5 μS for M257 pairs (n = 6).

Single-step acidification and changes in G_j

To estimate the effects of pH_i on cell-to-cell communication, G_j and pH_i were measured simultaneously while superfusing cell pairs with low-pH bathing solution (Fig. 2; see also references 10, 26, 31, and 34). Oocyte pairs expressing Cx43 (Fig. 2 *A*) or Cx32 (Fig. 2 *B*) were initially superfused with NaAcS at pH 7.4 for a period of 5 min, and then exposed to a similar solution adjusted to a lower pH (6.0 for Cx43 pair and 5.8 for Cx32 pair). As illustrated in Fig. 2, G_j increased in all cell pairs, expressing Cx43 or Cx32 channels before any sign of electrical uncoupling became apparent. A similar early increase in G_j has been reported previously by other investigators (31, 32, 40) during mild intracellular acidification. Continued superfusion of cells with low pH solution produced a rapid decline in G_j, which became zero after 40 min. Subsequent washout with NaAcS of pH 7.4 led to a gradual increase of G_j in both Cx43 and Cx32 pairs, which, in the particular case depicted in Fig. 2 *A*, reached 70% from control. The recovery of G_j was variable. However, mean washout values were not different from control. Indeed, upon return to NaAcS of pH 7.4 in 5 Cx43 experiments, G_j recovered to 97.1 ± 11.5% (see also reference 41) of the control.

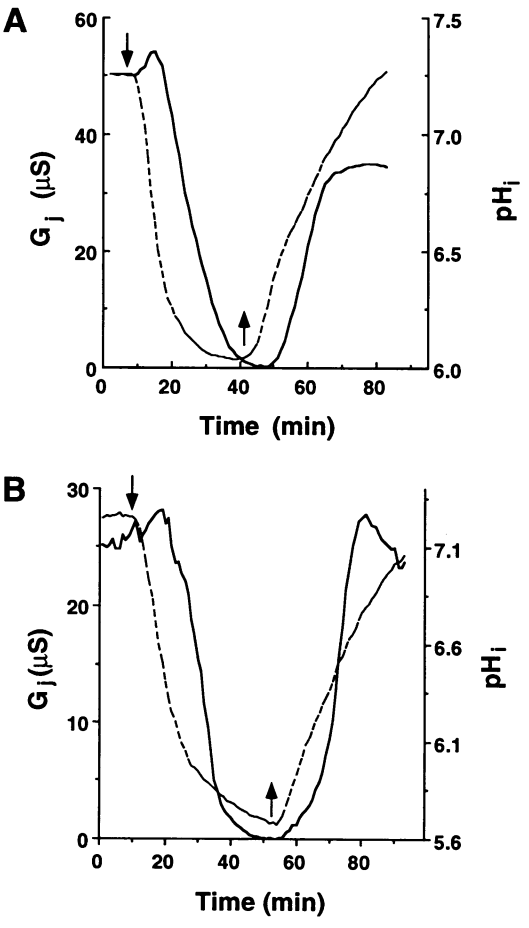

FIGURE 2 Uncoupling and recovery of G_j (*solid line*) of oocyte pairs expressing Cx43 (*A*) and Cx32 (*B*) gap junction proteins. pH_i (*broken line*) was simultaneously monitored. In both panels, *downward arrows* indicate the start of superfusion with low pH NaAcS and *upward arrows* indicate the onset of washout.

Quasi-steady-state G_j–pH_i relations

The results presented in Fig. 2 demonstrate that abrupt changes in the pH (e.g., 7.4–6.0 in Fig. 2 *A*) are accompanied by a small but immediate increase in G_j, which is interrupted by a protracted decrease. To explore more systematically the pH_i dependence of such complex changes in G_j, we carried out a new group of experiments in which pH_i was changed in relatively small steps in an attempt to approach a steady-state G_j response at each pH_i. In most instances, it was possible to reach asymptotic values of G_j before switching solutions; however, to reduce the total duration of the experiments, there were cases in which, during the acidification-induced increase in G_j (see below), the pH of the extracellular solution was changed before a steady state was reached. Yet, the asymptotic states were always reached during acidification-induced uncoupling. In the experiments illustrated in Fig. 3, stepwise changes in pH_i were induced in oocyte pairs expressing Cx43 (Fig. 3 *A*) or Cx32 (Fig. 3 *B*). Each set of experiments was carried out in oocytes obtained from the same group of frogs and

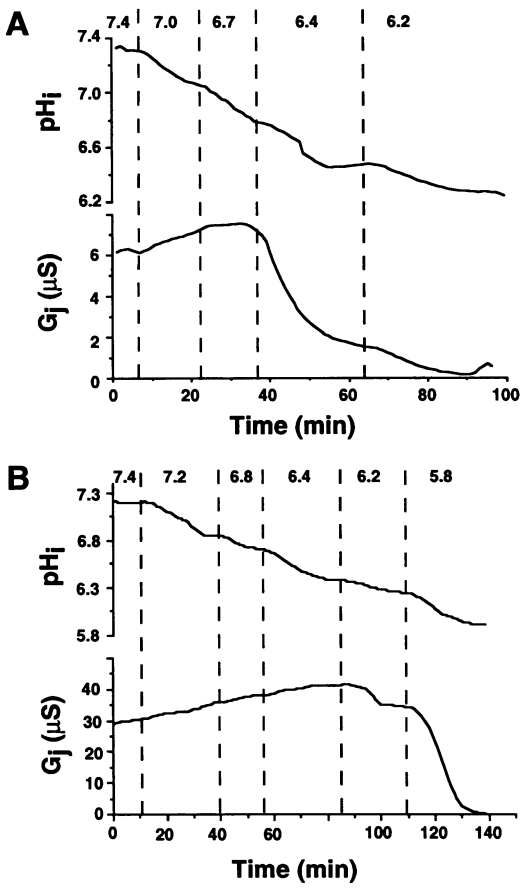

FIGURE 3 Time course of changes of G_j and pH_i in oocyte pairs expressing Cx32 and Cx43 channels. Numbers above each diagram indicate the pH of NaAcS at each superfusion step. G_j increased in the Cx43 pair as pH was reduced to 6.7; G_j increased in the Cx32 pair as pH was lowered to 6.4. Further decrease in pH led to uncoupling in both cases.

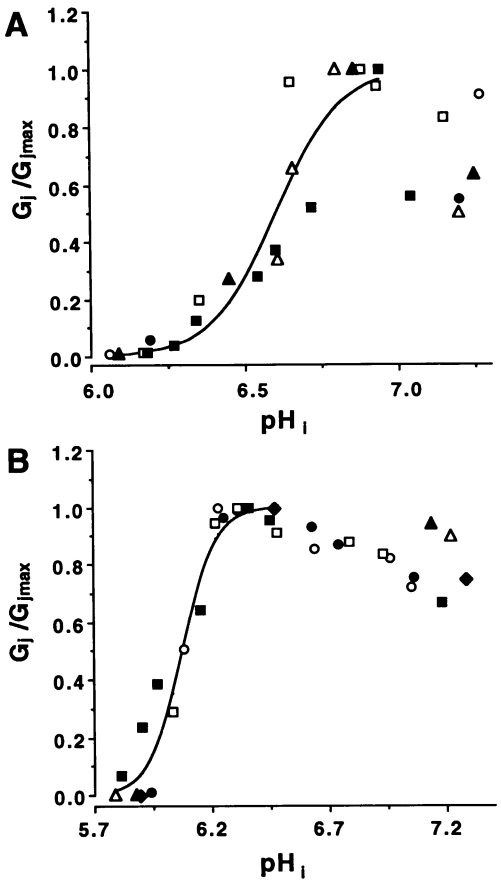

FIGURE 4 G_j–pH_i relation of Cx43 and Cx32 channels expressed in oocyte pairs. (*A*) G_j–pH_i relation of the Cx43 channel ($n = 6$). A Hill's coefficient of 4.2 and a pK_a of 6.6 were obtained. (*B*) G_j–pH_i relationship of the Cx32 channel ($n = 7$). $pK_a = 6.1$ and Hill's coefficient = 6.2. Values of G_j and pH_i were collected at the end of each perfusion step demonstrated in Fig. 3. $G_j/G_{j\,max}$ are G_j values normalized to the highest G_j measured during each experiment. Each symbol corresponds to a different experiment. (*Solid lines*) Curves produced by fitting Hill's equation to the G_j–pH_i data obtained during the acidification-induced uncoupling of G_j.

with mRNA prepared the same day. The superfusion solution was switched to one with a lower pH only after G_j and pH_i had reached their respective asymptotic value at each pH_i step. In the experiment shown in Fig. 3 *A*, G_j of the Cx43 pair increased at each step between pH_i values of 7.2 and 6.7, whereas in the Cx32 pair (Fig. 3 *B*) it did so between 7.2 and 6.4. Further acidification uncoupled both connexin channels. Similar results were obtained in other Cx43 ($n = 7$) and Cx32 ($n = 6$) experiments. In all Cx43 experiments, conductance increased (61.4 ± 14.9%, range 14–96%) upon acidification to pH_i 6.8. In all Cx32 experiments, conductance increased by 24.7 ± 5.9% (range 6–50%) when pH_i was decreased to 6.3.

Data points representing G_j and pH_i values were collected at the end of each superfusion step and were used for the analysis of G_j–pH_i relations. Fig. 4 depicts the relations obtained with both Cx43 (Fig. 4 *A*) and Cx32

(Fig. 4 *B*) during stepwise acidification. Individual symbols correspond to separate experiments; only those data obtained during the uncoupling phase of G_j were fitted with Hill's equation (*solid line*) (26). G_j values were normalized to the maximal G_j achieved during each experiment. The pK_a was 6.6 for Cx43 and 6.1 for Cx32 pairs, and the Hill coefficient was 4.2 for Cx43 and 6.2 for Cx32. The pK_a values obtained here for Cx32 and Cx43 are similar to those obtained previously in hepatocytes (6.3; see references 25, 28) and cardiac myocytes (6.7; see reference 25), respectively. Moreover, the difference in the pK_as of Cx43 and Cx32 (~0.5 pH units) demonstrated in our oocyte experiments is similar to the difference in the pK_as observed in hepatocyte and cardiac myocyte cell pairs (~0.4 pH units) (25). Since in these experiments the electrical properties of Cx43 and Cx32 were compared by expressing their homologous channels in the same cellular environment, the results suggest that the unequal pH_i sensitivity of Cx43 and Cx32 channel conductances must represent a difference in their intrinsic properties, particularly in relation to their individual amino acid sequences. However, at this point it is difficult to discard the possibility that the natural environment provided by the cardiac myocyte and hepatocyte for Cx43 and Cx32, respectively, also influences pH_i-dependent gating. The slightly different Hill coefficients obtained here in relation to those of hepatocytes and cardiac myocytes (8 and 2, respectively; see reference 27) will be considered in the Discussion.

Role of the carboxyl tail

Recent experiments from this laboratory (23) have demonstrated that deletion of 135 amino acids from the carboxyl tail of Cx43 reduces functional expression in paired oocytes, whereas deletion of 125 amino acids (M257 mutant) does not alter functional expression of this channel in terms of the measured G_j values, relative to wild type Cx43. However, the effect of this mutation on responsiveness to known regulation of channel function was not examined. Since the length of the mutated protein approaches that of Cx32, it is of great interest to determine whether the response to known regulators of gating properties of the Cx43 channel is altered as well. This aspect is particularly important in relation to Cx43 since, to our knowledge, no specific regulatory function has been demonstrated previously that may be attributed to its very long cytoplasmic tail, except for the tyrosine phosphorylation–induced downregulation of Cx43 channels, which is absent in Cx32 channels (4). Thus, we undertook an additional study of the pH_i gating properties of M257 expressed in oocytes in an effort to determine whether truncation of the carboxyl tail affects responsiveness to pH_i change.

The effects of stepwise changes in pH_i on macroscopic conductance of M257 channels in a single oocyte pair are illustrated in Fig. 5 *A*. As with the two wild-type proteins, there was an initial increase of G_j with intracellular

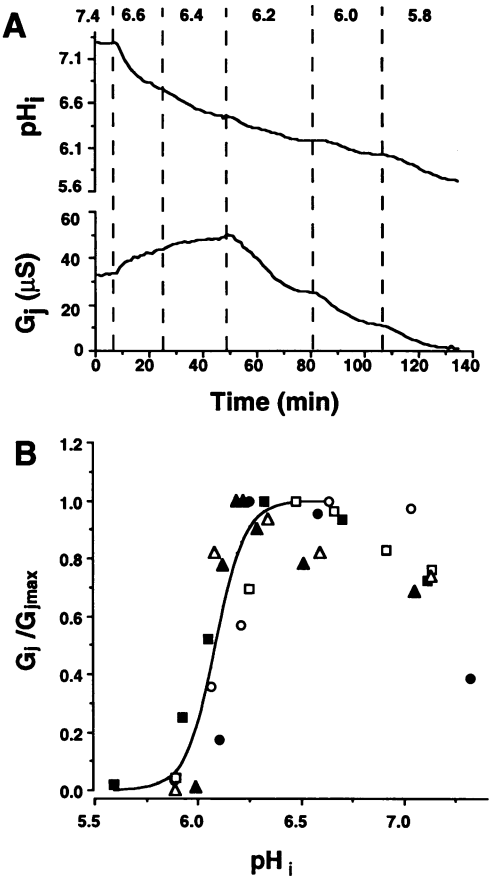

FIGURE 5 pH sensitivity of M257 channels. (*A*) Time course of changes of G_j and pH_i. Numbers above each diagram indicate the pH values of NaAcS at each perfusion step. G_j increased with mild acidification. Further reduction in pH led to uncoupling. (*B*) G_j–pH_i relation of the M257 channel ($n = 6$). $G_j/G_{j\,max}$: G_j values were normalized to the highest G_j value measured during each experiment. Different symbols represent specific experiments. Hill's equation fitting of the data gave rise to a Hill's coefficient of 6.0 and a pK_a of 6.1. Only those data corresponding to the uncoupling phase were used in the fitting procedure.

acidification. However, in contrast to Cx43 (see Fig. 3*B*), M257 conductance continued to increase even when pH_i was decreased to 6.3. The mean increase of G_j due to mild acidification was 52.5 ± 22.4% (range, 3–160%). Comparison of the increment by ANOVA did not yield a significant difference among oocyte pairs injected with Cx43, Cx32, and M257 mRNAs. Further acidification induced an abrupt decline of G_j. In Fig. 5 *B*, data from six individual experiments, which were obtained during the period when intracellular acidification induced a reduction in G_j, were graphed together and fitted by Hill's equation. Analysis of the M257 data resulted in a pK_a of 6.1 and a Hill coefficient of 6.0.

Finally, G_j–pH_i relations measured in oocyte pairs expressing homologous Cx43 (*closed circles*), Cx32 (*closed triangles*), and M257 (*open circles*) channels are replotted in Fig. 6 on the same scale. The data clearly demonstrate that deletion of the Cx43 carboxyl tail re-

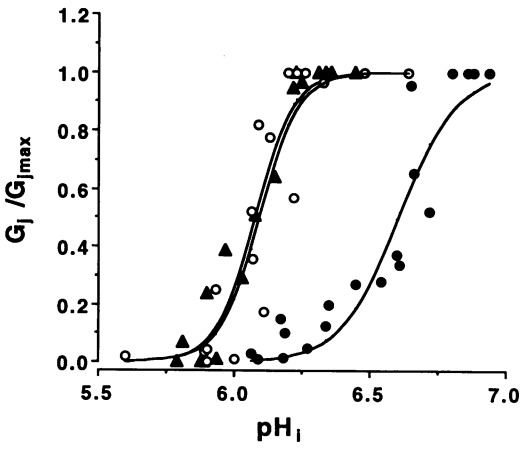

FIGURE 6 Comparison of G_j–pH_i relations of Cx43, Cx32, and M257 channels. Only the data obtained during the acidification-induced uncoupling of G_j are included. *Filled circles,* Cx43; *filled triangles,* Cx32; and *open circles,* M257. Note that deletion of carboxyl tail of Cx43 (M257) shifts the G_j–pH_i relation to more acidic levels and makes it indistinguishable from that of Cx32.

sults in a G_j–pH_i relation that is steeper and shifted to lower pH values. In fact, the M257 data points could be fitted by a curve that is indistinguishable from that used to fit the Cx32 results. The overall results suggest that the carboxyl tail plays a significant role in determining the gating response of Cx43 to acidification. However, since the deletion mutant M257 still retains pH sensitivity, it is likely that a common pH sensor and gating mechanism is located within the first 256 amino acid residues of the protein.

DISCUSSION

The uncoupling effect of pH_i on intercellular communication was first noticed by Turin and Warner in 1977 (2), when they observed that CO_2 (membrane-permeable) saturation of the superfusion medium led to a reduction in the electrical coupling between *Xenopus* embryonic cells, whereas low pH_o induced by membrane-impermeable acids did not alter electrical coupling over the pH range of 5.8–7.5. Subsequent studies confirmed the uncoupling effect of pH_i on intercellular communication in various tissues (42, 43), including heart (10, 44). Results from pH_i measurement predicted an S-shaped pH_i-coupling relationship (45), which was well fitted by a Hill equation (26). In addition, quantitative analysis has indicated that liver gap junctional channel conductance has a lower pK_a (6.3) than that of myocardial cells (6.7), and thus it is less sensitive to intracellular acidification than cardiac gap junctional channel conductance (25). On the other hand, it has been demonstrated that in the hepatocyte, the G_j–pH_i relationship shows a higher Hill coefficient than in the cardiac myocyte (27). However, previous work did not establish whether the observed unequal pH sensitivity of the two gap junction proteins represents a difference in their molecular structure or whether it is the result of any other unknown factors related to the dissimilar natural environments of these proteins. This study provides a characterization and comparison of individual Cx43 and Cx32 properties with respect to pH gating. By functionally expressing homologous channels of the major liver and cardiac gap junction proteins in an identical cellular expression system, we have conclusively demonstrated that a difference in their molecular structure, established by their unequal length, determines, at least in part, their dissimilar pH sensitivity. These data favor the idea that the difference in the pH-gating properties in liver versus cardiac gap junctions is the result of difference in the carboxyl tail of their respective major connexin proteins.

Acetate versus CO_2

Superfusion of cell pairs with solutions saturated with CO_2 has been commonly used to determine the effects of intracellular acidification on intercellular coupling (2, 26, 42, 43). Such a procedure, however, is not appropriate in experiments in which proton-sensitive electrodes are used for constant monitoring of pH_i and/or pH_o (see Fig. 1). Peracchia (34) has proposed that CO_2 may diffuse into the electrode, thus changing the pH of the filling solution and producing a baseline shift toward higher pH values. Our results further show that, as a result of such baseline shift, there is also an artificial overestimation of pH upon washout. The recorded value slowly returns to the original baseline, presumably as CO_2 diffuses away from the filling solution and into the superfusate. Moreover, the data show that the baseline error introduced by CO_2 superfusion is time dependent, and that such an error increases as a function of time of exposure to CO_2. For small cells, the uncoupling of G_j may require only a few minutes, in which case the baseline error would probably be small. However, for large cells such as *Xenopus* oocytes, steady-state G_j levels induced by intracellular acidification (from 7.2 to 6.0) may not be reached until after 30–40 min of CO_2 superfusion, which may lead to an unacceptable error of $\geq$0.5 pH units. Thus, as previously suggested by Peracchia (34), we used sodium acetate in all our experiments to lower the pH in the intracellular space.

The quasi-steady-state G_j–pH_i relationship

We directed our efforts toward determining the G_j–pH_i relationship in experiments in which stepwise changes in pH_i allowed measurements of G_j after it had reached an asymptotic value at each step. With this procedure it was possible to construct reliable and reproducible quasi-steady-state G_j–pH_i relations and to carry out the fitting procedure that enabled us to demonstrate appreciable differences in channel conductance sensitivity to acidification between liver and cardiac gap junction proteins

expressed in oocytes. This approach is quite similar to that used previously by Noma and Tsuboi (44) in their ventricular open-cell experiments.

The observation that intermediate levels of acidity, which may be more physiologically relevant, can cause an immediate increase in G_j has been reported previously by other authors (32, 40). The molecular mechanism responsible for this phenomenon remains unknown. However, an interesting possibility may be that mild acidification enhances the incorporation of functional channels into the oolemma. Indeed, acidification-induced enhancement of channel assembly (46) and membrane localization (47) has recently been reported for other channel proteins, and similar changes in the structure of the channel could conceivably occur for the connexin protein. On the other hand, Dahl et al. (48) have reported that channel formation may be accelerated at high pH in the oocyte system. Moreover, our data do not discard possible direct effects of a given proton concentration on unitary conductance or open probability of the connexin channels.

Overall, our results suggest that protons exert two separate effects on the connexin channels: an immediate effect leading to an increase in conductance at intermediate levels of acidification, and a protracted effect that ultimately causes complete closure of the channel at critical pH levels. Further experiments, using cell systems in which the intracellular environment can be thoroughly controlled, would be necessary to further characterize the direct proton–connexin interactions that may be responsible for these actions, as well as the possible role of intermediary agents (e.g., calcium or calmodulin) on the pH_i-induced gating of the gap junctional channels.

The pK_a and the Hill coefficient

The Hill equation has been used frequently in the study of acidification-induced uncoupling of gap junctional channels (26, 40, 44). Yet, to our knowledge, no study has ever been performed to determine the validity of the model described by the Hill equation. Nevertheless, this equation does establish a standard so that data obtained in various laboratories can be easily compared. Hence, although a Hill equation of the second order has been suggested (26, 40), we have used the first-order form in our analysis with the sole purpose of facilitating comparison of our data with those in the literature.

In general, the pK_a values obtained in our experiments (6.1 for Cx32 and 6.6 for Cx43) are very similar to those measured by Spray et al. (25, 28), who reported values of 6.3 for hepatocytes and 6.7 for cardiac myocytes. However, the pK_a of Cx43 in our oocyte expression system is much higher than the 6.1 value reported by Noma and Tsuboi (44) for ventricular myocytes. It is possible that the open-cell technique used in the experiments of Noma and Tsuboi, in which one cell of a pair was patch clamped while the partner cell was torn apart to expose the intracellular space to the bathing solution, might have affected the results. Indeed, unilateral acidification has been reported to be less efficient than bilateral acidification (40), and it is possible that the proton concentration in the acidified cell at the location of the pH sensor near the channel pore may be diluted by the cytoplasmic fluid of the unacidified cell (40). Thus, it is reasonable to expect that the pK_a obtained from unilaterally acidified cell pairs, such as in the open cell system used by Noma and Tsuboi (44), should be more acidic than that obtained from oocyte pairs expressing Cx43 channels.

The Hill coefficient calculated here for Cx32 ($n = 6.2$) is similar to that reported previously for hepatocytes (6–8; see references 28). On the other hand, the Hill coefficient found for Cx43 ($n = 4.2$) is larger than that reported by Noma and Tsuboi ($n = 2.4$) for ventricular myocytes (44). As in the case of pK_a measurement, the different values reported in the literature may result from differences in the techniques used. Moreover, both hepatocytes and cardiac myocytes are known to express a variety of connexin proteins (49, 50); the combined pH sensitivity of all junctional channels between two liver or cardiac cells may be significantly different from the pH sensitivity of only one individual connexin.

Uncoupling and the carboxyl tail

It has been previously shown that *Xenopus* oocytes express an endogenous 38-kD protein (Cx38; references 23, 29, 51) that seems to be highly voltage (23, 29, 52) and pH sensitive (31, 32). The endogenous Cx38 may form either homotypic channels (with another Cx38) or heterotypic channels (with a different connexin) (23, 29, 53). Moreover, it has been postulated (53) that exogenous and endogenous connexins may form heteromeric connexons, although there is no experimental evidence that could substantiate such a hypothesis. The question then arises as to whether the pH-sensitive curves recorded in our experiments were somehow affected by the presence of heterotypic or heteromeric channels partly formed by endogenous Cx38. The latter possibility cannot be completely discarded. However, the probability of Cx38 significantly altering the pH-sensitive curves presented in this paper seems rather low since: (*a*) all the junctional currents recorded in our experiments were voltage independent in the ±50-mV range; the presence of Cx38 would be expected to confer voltage sensitivity to the channels (23, 29, 52). (*b*) Cx38 channels seem to be highly sensitive to pH. Recent results of Werner et al. (32) show that superfusion with 30% CO_2-saturated solution leads to complete uncoupling in oocytes expressing endogenous connexin channels. Moreover, preliminary data by Ebihara (31) indicate that electrical uncoupling of Cx38-expressing cell pairs can be nearly abolished at pH_i 6.8, whereas much lower pH_i values are necessary to induce uncoupling in our Cx43 experiments. (*c*) It is improbable that a mutation of the cytoplasmic tail of the protein would affect the ability of the connexin protein to form heterotypic channels. (*d*) In

our experiments, a large amount of mRNA (80 ng/oocyte) encoding Cx43, Cx32, or M257 was used, which probably led to the overproduction of exogenous protein. Thus, it is reasonable to expect that channels expressed in the injected oocytes correspond to those translated from the exogenous mRNA.

The carboxyl tail represents the region of greatest amino acid sequence diversity in the connexin family (11). The cytoplasmic location of the tail exposes it to a variety of cellular modulators, such as various second messenger systems, and this exposure makes the tail a good candidate for involvement in tissue-specific functions. Since sensitivity to pH is one of the gating properties of gap junctions that has been most frequently reported to vary in different tissue types, we sought to determine whether, other conditions being equal, truncation of the carboxyl tail of Cx43 would modify pH sensitivity. Deletion of 125 amino acid residues from the carboxyl end to yield M257 does not change macroscopic G_j of the expressed channels (reference 23; see also Results). However, the present results clearly show that the deletion mutant has a channel conductance that differs in its pH sensitivity from that of Cx43, which demonstrates that the carboxyl tail does play a significant role in pH-dependent gating. Furthermore, the results show that the G_j–pH_i curve of M257 is shifted in the acidic direction (see Fig. 6), and that its slope is steeper than that of Cx43. In fact, pH sensitivity of the mutant channels was indistinguishable from that of Cx32 channels, strongly arguing in favor of the idea that a major difference in pH-gating between the two wild-type proteins lies in the unequal length of their respective carboxyl tails. Yet, the conserved pH sensitivity in Cx32 and M257 suggests that a common pH_i sensor may be located somewhere between amino acid residues 1 and 256 of Cx43 and the counterpart region of Cx32. Such a sensor would be responsible for the G_j increase at moderate levels of acidification and for the uncoupling observed at pH_i 6 or lower. Alternatively, electrical uncoupling at pH 6.0 or lower may be an unspecific effect of acidification on the function and structural integrity of the connexin proteins. Our results show that segment 257–381 of Cx43 provides the protein with an enhanced sensitivity to increases in the intracellular proton concentration in that the pK_a value for conductance decay is shifted in the alkaline direction.

Although the present data are too limited to enable presentation of a detailed explanation of the mechanism of uncoupling, the following issues may be considered. First, it has been previously suggested that pH sensitivity of the gap junctional channels is largely the result of the presence of titratable histidine residues in the intracellular loop of the connexin proteins (27). In fact, it has been hypothesized that there is a cooperative type of interaction between such residues, and that the larger Hill coefficient in liver hepatocytes may be the result of the larger number of histidines in the intracellular loop of Cx32 than in that of Cx43. The results presented in this paper suggest that the number of histidine residues in the intracellular loop is not the key factor regarding pH sensitivity of the connexins, since the intracellular loop region of M257 is identical to that of the wild-type Cx43 protein. Yet, the results presented in this paper demonstrate that pH sensitivity is drastically altered by the truncation of the cytoplasmic tail.

Second, it is possible that the deleted carboxyl tail in M257 mediates the purported synergistic action of hydrogen and calcium ions in inducing electrical uncoupling (10, 54). Indeed, although different authors emphasize the importance of one factor over the other (26, 34), it is well known that both low intracellular pCa (pCa_i) and low pH_i are capable of reducing G_j (44, 55). It is therefore possible that tail deletion abolishes the synergistic action of protons and calcium. Moreover, calmodulin may be involved in pH regulation to differing degrees in the two wild-type connexins. In fact, results by several investigators (56–58) have suggested that calmodulin is the actual mediator of acidification-induced uncoupling in some cell systems (however, see reference 40), and it has been postulated that a calmodulin-binding site may be present in the cytoplasmic tail of Cx32 (59). Thus, although no calmodulin consensus sequence has been identified in the cytoplasmic tail of Cx43, the possibility of a calmodulin-mediated effect cannot be entirely excluded.

Third, the acidification-induced uncoupling mechanism provided by the carboxyl tail might resemble the "ball-and-chain" gating model originally developed by Armstrong (60, 61) to explain the effect of quaternary ammonium ions on potassium channels. A similar model was subsequently invoked by the same group in their studies on the gating kinetics of sodium channels (62) and by Zagotta and coworkers (63, 64) to account for the time-dependent gating of the *Shaker* A–type potassium channels. According to the ball-and-chain model (62, 65), accumulation of charges on one side of the membrane may cause the physical displacement of a flexible terminal peptide ("the chain") toward the vestibule of the channel, whereby the end amino acid residues ("the ball") would occlude the hydrophilic pore. Shortening the terminal peptide would abolish this time-dependent gating process by eliminating the ball component. Such a process would also be abolished by replacing the flexible element acting as the chain. Although further experiments will be necessary to prove or disprove such a hypothesis, the metaphor seems appropriate for the cardiac connexin given that: (*a*) a long carboxyl tail is indeed present in the structure of Cx43; (*b*) the gating process is greatly displaced to lower pH values by significant shortening of the tail; and (*c*) although the length of the tail alters the channel kinetics, it does not seem to modify its macroscopic conductance (23), even though truncation of that tail seems to produce a significant change in the single-channel conduc-

tance (22). The gap junctional channel is normally comprised of six connexins (66), and, thus, pH_i-sensitive gating of Cx43 may be a special case of ball-and-chain kinetics, since there would be at least six balls ready to block each channel. However, in the case of the tetrameric *Shaker* K channel, entrance of one ball may be sufficient to totally block the channel (65). On the other hand, the case of Cx32 and of M257 would be different since closure of channels, upon further cytoplasmic acidification, would not depend on a ball-and-chain mechanism, but on a gating process regulated at a different region of the protein. Alternatively, the large acidification needed to demonstrate G_j closure in such proteins may have a nonspecific deleterious effect on the integrity of the connexin protein, although the complete recovery of conductance demonstrated for Cx32 (Fig. 2) and M257 (data not shown) upon return to control pH_i would argue against the latter.

Pathophysiological implications

Mild acidification leads to small but significant increases of junctional conductance in oocytes expressing either wild type Cx32, Cx43, or M257. The physiological implications of such an effect are not clear. However, it is possible that in the natural environment of cardiac and liver gap junction proteins, increased G_j may act as a normal response to physiologically induced reductions in pH_i, secondary to increased metabolic demand or mild ischemia. This would facilitate the interchange of small molecules, metabolic products, and ions between cells, which, in the case of the heart, would be manifest as an increase in conduction velocity of the electrical impulse (67, 68). In the past, small increases in conduction velocity associated with mild ischemia had been attributed solely to increases in the extracellular concentration of potassium (69), with consequent reduction of membrane potential and increased excitability secondary to reduced current requirements (69). It is possible that the increased G_j may contribute to the increased conduction velocity by enabling an increase in the electrotonic spread.

Severe acidification may have important pathophysiological consequences. Indeed, in acute cardiac ischemia, pH_i may be reduced to values as low as 6.2 (24), which is well below the pK_a of channel conductance of Cx43 (see Fig. 4). Moreover, since low pH_i normally parallels low pCa_i (10, 34, 42), the synergistic action of these two factors, together with the effects of other uncouplers released during the ischemic process (e.g., phospholipids from damaged cell membranes), would tend to stop communication between the injured cells and the surrounding normal tissue.

Conclusion

The present comparative study demonstrates that Cx43 channel conductance is more sensitive to acidification than Cx32 channel conductance and suggests that such a difference is related to the unequal structure of the two wild type proteins. Moreover, the experiments in which a deletion mutant was used further suggest that the carboxyl tail of Cx43 is indeed involved in the acidification-induced electrical uncoupling in cell pairs. However, the fact that the channels expressed by oocyte pairs injected with M257 remain pH sensitive indicates that a common pH gating mechanism, independent of the cytoplasmic tail, is present in the connexin proteins. The cytoplasmic tail of Cx43 may function as the most sensitive gating mechanism, responding to acidification more readily and thus helping the Cx43 channel to close at a relatively higher pH_i.

We thank Ms. L. Wang for her technical assistance in preparing cDNA derived mRNAs, and Ms. W. Coombs, Ms. J. Getchonis, and Miss C. Kapuscinski for their technical assistance both in experiments and in preparation of the manuscript.

This work is supported by grants HL-39707 and HL-29439 from the Heart, Lung, and Blood Institute of the National Institutes of Health.

Received for publication 28 August 1992 and in final form 18 November 1992.

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