Intramolecular Interactions Mediate pH Regulation of Connexin43 Channels

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ABSTRACT We have previously proposed that acidification-induced regulation of the cardiac gap junction protein connexin43 (Cx43) may be modeled as a particle-receptor interaction between two separate domains of Cx43: the carboxyl terminal (acting as a particle), and a region including histidine 95 (acting as a receptor). Accordingly, intracellular acidification would lead to particle-receptor binding, thus closing the channel. A premise of the model is that the particle can bind its receptor, even if the particle is not covalently bound to the rest of the protein. The latter hypothesis was tested in antisense-injected *Xenopus* oocyte pairs coexpressing mRNA for a pH-insensitive Cx43 mutant truncated at amino acid 257 (i.e., M257) and mRNA coding for the carboxyl terminal region (residues 259–382). Intracellular pH (pH_i) was recorded using the dextran form of the proton-sensitive dye seminaphthorhodafluor (SNARF). Junctional conductance (G_j) was measured with the dual voltage clamp technique. Wild-type Cx43 channels showed their characteristic pH sensitivity. M257 channels were not pH sensitive (pH_i tested: 7.2 to 6.4). However, pH sensitivity was restored when the pH-insensitive channel (M257) was coexpressed with mRNA coding for the carboxyl terminal. Furthermore, coexpression of the carboxyl terminal of Cx43 enhanced the pH sensitivity of an otherwise less pH-sensitive connexin (Cx32). These data are consistent with a model of intramolecular interactions in which the carboxyl terminal acts as an independent domain that, under the appropriate conditions, binds to a separate region of the protein and closes the channel. These interactions may be direct (as in the ball-and-chain mechanism of voltage-dependent gating of potassium channels) or mediated through an intermediary molecule. The data further suggest that the region of Cx43 that acts as a receptor for the particle is conserved among connexins. A similar molecular mechanism may mediate chemical regulation of other channel proteins.

INTRODUCTION

Gap junction channels allow for the passage of ions and small molecules between adjacent cells. This traffic of molecular information from cell to cell is thought to mediate many vital functions, such as the synchronous contraction of the cardiac muscle (Delmar et al., 1986, 1987), the regulation of cell division in the ovary (Granot and Dekel, 1994), the proper development of the embryonic heart (Reaume et al., 1995; Britz-Cunningham et al., 1995), the control of tumoral cell growth (Esinduy et al., 1995), the harmonic contraction of the uterus during labor (Tabb et al., 1992), and the synchronization of exocytosis in gland cells (Meda et al., 1995).

Gap junction channels are formed by multimers of an integral membrane protein called connexin. Data from antibody, protease, and mutagenesis studies (see Stauffer and Unwin, 1992; Bennett et al., 1991, for review) show that connexins share the same membrane topology: four transmembrane domains, two extracellular loops, one intracellular loop, and both the amino and carboxyl termini located in the intracellular space. The primary sequences of the cytoplasmic loop and carboxyl terminal regions differ significantly among connexins (Bennett et al., 1995). In the adult mammalian cardiac ventricles, gap junction channels mostly consist of a 43-kDa, 382-amino acid protein named connexin43 (Cx43; see Beyer et al., 1987). Cx43 is also found in tissues such as neuroglia, uterus, and ovary (Lang et al., 1991; Risek et al., 1990; Yamamoto et al., 1990).

Acidification-induced closure of cardiac gap junction channels has been extensively documented (see Spray and Bennett, 1985; Bennett and Verselis, 1992; Delmar et al., 1994, for review). However, the molecular mechanisms mediating this process are not yet understood. The importance of studying pH regulation of cardiac intercellular communication is warranted by the fact that pathophysiological processes associated with lack of oxygen supply (i.e., myocardial ischemia and infarction) lead to acidification of the intracellular environment. Acidification may in turn cause gap junction channel closure and loss of functional synchronization between cells (Delmar et al., 1994; Gettes et al., 1985; Saffitz et al., 1995).

Previous results from this and other laboratories have shown that truncation of the carboxyl terminal of Cx43 up to amino acid 245 permits gap junction channel formation, indicating that the carboxyl terminal is not an essential component of the channel pore (Fishman et al., 1991; Dunham et al., 1992). However, truncation at amino acid 257 prevented the normal pH-dependent closure (Liu et al., 1993). We therefore proposed that the carboxyl terminal acts as an independent domain (a "binding particle") that upon intracellular acidification recognizes and nonco-

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valently binds to a specific peptide sequence at or near the pore (acting as a receptor for the particle), thus closing the channel. This particle-receptor hypothesis was further strengthened by the finding that mutations in another region of Cx43 (histidine 95) could alter pH gating despite the presence of an intact carboxyl terminal (Ek et al., 1994). This would suggest that at least two regions of the molecule act in concert to bring about acidification-induced uncoupling. This intramolecular particle-receptor interaction would be similar to the ball-and-chain mechanism of voltage-dependent gating of membrane channels (Armstrong and Bezanilla, 1977; Hoshi et al., 1990; Zagotta et al., 1990).

The present study was aimed at testing one critical premise of the particle-receptor hypothesis: that pH gating should occur even if the particle (presumably the carboxyl terminal region) is expressed separately from the rest of the Cx43 molecule. We have also tested whether addition of the particle could modify the pH gating of the less pH-sensitive connexin Cx32 (Liu et al., 1993; Werner et al., 1991). Our results are consistent with the particle-receptor hypothesis and further suggest that the region acting as a receptor is conserved among connexin molecules. We propose that a similar particle-receptor mechanism may mediate connexin gating caused by other factors, as well as the chemical regulation of other ion channels.

MATERIALS AND METHODS

cDNA and mRNA preparation

Rat cardiac Cx43 cDNA (originally provided by Dr. Eric Beyer) was subcloned into pBluescript IISK+ (Stratagene). Cx32 cDNA was kindly provided by Dr. David Paul. Amino acids 259–382 of rat Cx43 were amplified by polymerase chain reaction, followed by subcloning into pBluescript SK- (Stratagene). The forward primer was designed to incorporate an ATG codon 5′ to amino acid 259. Unless otherwise indicated, in vitro transcription of Cx43 and of cDNA coding for amino acids 259–382 of Cx43 was carried out using T3 RNA polymerase and the mCAP mRNA capping kit (Stratagene). Transcription of Cx32 was performed using T7 RNA polymerase and the mCAP mRNA capping kit.

Oocyte preparation

Experiments were performed on stage V or VI *Xenopus laevis* oocytes (Dumont, 1972). The procedure used for oocyte preparation has been described previously (Liu et al., 1993; Ek et al., 1994). Briefly, oocytes were surgically removed by an abdominal incision and placed in L-15 (0.5×) culture medium. The follicular layer was manually stripped from selected oocytes. Oocytes were then digested with type II collagenase (10 mg/ml) for 30 min and allowed to recover from the enzymatic treatment for at least 1 h. After the recovery period oocytes were injected with 50 ng of a *Xenopus* connexin38 (Cx38) antisense oligonucleotide (Barrio et al., 1991) and maintained in L-15 medium for 3–6 days. Oocytes were injected with 30 ng of the proper RNA 48 h before recording. Twenty-four hours before recording, the oocytes were placed in a hyperosmotic solution and the vitelline layer was stripped. Immediately after removal of the vitelline membrane, oocytes were returned to the L-15 medium, where they were paired for electrophysiological measurement of junctional conductance.

Intracellular acidification

Intracellular acidification was induced by superfusion with a bicarbonate-buffered solution, gassed with a predetermined mixture of 95%/5% O_2/CO_2 and 100% CO_2. The composition of the superfusate was (in mM): NaCl, 88; KCl, 1; $MgSO_4$, 0.82; $CaCl_2$, 0.74; $NaHCO_3$, 18. The outlets of the 95:5 gas source and of the 100% CO_2 source were connected to a programmable valve (Automatic Scientific). The valve was programmed to change extracellular pH from 7.4 to 6.2 in five similar steps of 10 min each, causing a progressive acidification of the intracellular space from control values of around 7.2 to 6.3–6.4 after 60 min. By controlling the rate and extent of extracellular acidification, similar acidification ramps were applied to all oocyte pairs tested. We chose to use a slow acidification rate to avoid recording artifacts due to time delays in the response of the junctional conductance to a given pH (Delmar et al., 1994; Liu et al., 1993).

Electrophysiology

Electrophysiological measurements of junctional conductance (G_j) were made at room temperature using the dual voltage clamp technique, as previously described (Spray et al., 1981). The voltage recording electrode was filled with 3 M KCl and had resistances of 10 to 20 MΩ. Current-passing electrodes had a resistance of 0.4 to 0.6 MΩ and were filled with a solution containing (in mM): KCl, 150; $MgCl_2$, 1; ATP (disodium salt), 5; phosphocreatine (disodium salt), 5. The voltage clamp protocol and data analysis hardware and software were the same as previously described (Liu et al., 1993).

Averaged data are reported as mean ± standard error of the mean.

Fluorescent Imaging

A total of 50 nl of a 357 μM solution of dextran-seminaphthorhodafluor (SNARF) (molecular weight 70,000; Molecular Probes) was injected into oocytes 2 days before recording. Intracellular SNARF concentration was 17 μM. Excitation of the pH-sensitive fluorophore and recording of its light emission were carried out using a customized SPEX Fluorolog system, coupled to a Nikon Diaphot inverted microscope equipped for epifluorescence. Excitation light was maintained at a wavelength of 534 nm. Emission spectra were obtained before and after all experiments for calibration purposes using a monochromator placed before a single photomultiplier tube, and mounted on the camera port of the microscope. Emission ratios were obtained in real time from two separate photomultipliers, placed after emission filters with selected cut-offs (585/30 and 640/40) mounted on the side arm of the microscope. Switching between emission spectra and emission ratios was achieved by directing the emitted light either to the camera port or to the side arm. Oocytes were placed on the stage of the inverted microscope; light excitation and optical recordings were carried out through the objective, and microelectrodes were placed from above using conventional microelectrode techniques. Oocytes were visualized for impalement using a separate stereomicroscope.

Optical recordings were obtained using a Nikon Fluor 20 20× (0.75 160/0.17) objective. An effort was made to consistently place the center of the field of view at the site of cell-cell apposition. Our emission recording system only allows for recordings of localized regions of the membrane. For the experiments reported in this paper, the field of view was approximately 600 μm. The emitted light was passed through a pinhole diaphragm, which reduces the area from which light is connected to a circular spot that is 1/50th of the original field. Thus, consistent with observations from other laboratories (Takahashi et al., 1987), emitted light was collected from a circular area that was approximately 30 μm in diameter. The focal plane was moved from below the chamber upward, until maximum intensity was recorded, and then moved back down to record a signal intensity of about 80% from maximum. Following this procedure, Sakmann and his colleagues (Takahashi et al., 1987) localized the focal plane to coincide within ~10 μm with the bottom of the chamber.

SNARF calibration

Calibration curves for the emission spectra of SNARF as a function of proton concentration were obtained in a solution containing (in mM) KPO_4, 50; HEPES, 10, as well as in oocytes. Fig. 1 shows the results. The continuous line depicts the best fit of data (*closed symbols*) obtained from the emission spectra of SNARF dissolved in a solution buffered to various pH values. The results agree closely with data previously reported (Blank et al., 1992), thus confirming the reliability of our recording hardware. The calibration curve of dextran-SNARF emission ratios recorded from oocytes is shown with the broken line. Intracellular pH (pH_i) was measured using pH-sensitive microelectrodes, as previously described (Liu et al., 1993). The open symbols show calibration measurements made from oocytes that were placed in a solution containing (in mM) sodium acetate, 103; KCl, 1; $NaHCO_3$, 2.4; $MgSO_4$, 0.82; $CaCl_2$, 0.74; HEPES, 15; NaCl, 20; clearly, the pH dependence of SNARF emission depends on whether the fluorophore is inside a cell or just dissolved in solution. A similar shift has been reported for SNARF in cardiac cells (Blank et al., 1992) and emphasizes the importance of calibrating optical signals within the cell system under study. The value of pH_i measured with the pH-sensitive microelectrode was also correlated with emission ratios (data not shown). The accuracy of the emission ratios measured on every experiment was confirmed by obtaining emission spectra at the beginning and the end of our recording, showing that the pH_i values estimated by both methods concurred. pH_i on each experiment was calibrated according to the sigmoidal function depicted by the broken line. Given the pH dependence of emission ratios, measurements of pH_i below 6.35 were not taken into account.

RESULTS

Acidification-induced uncoupling: junctional conductance as a function of pH_i

Fig. 2 *A* shows simultaneous measurements of G_j (*top trace*) and pH_i (*bottom trace*), recorded from an oocyte pair expressing Cx43. Cells were superfused with a bicarbonate-containing solution that was progressively acidified with increasing concentrations of CO_2 (see Materials and Methods). The rate of extracellular acidification was slow enough to prevent changes in pH_i that were faster than the changes in junctional conductance. The traces show that acidification led to a progressive decrease in G_j, with maximum uncoupling occurring at a pH_i of 6.55. Fig. 2 *B* shows the data obtained by correlating G_j and pH_i at every sample point. Ordinates show the value of G_j relative to $G_{j\,max}$, i.e., the maximum G_j value recorded. The data clustering and the smooth slope of the sigmoidal relation confirm that junctional conductance responded to pH_i at the same point in time.

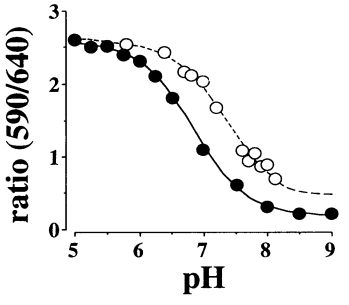

FIGURE 1 Calibration of Dextran-SNARF. The spectral ratio (intensity @ 590 nm/intensity @ 640 nm) is plotted against pH. Two calibration curves were generated: one for SNARF dissolved in solutions buffered at various pHs (*solid line; closed circles*), and another one for SNARF injected into *Xenopus* oocytes (*open circles; broken line*). For the latter, intracellular pH was independently measured using a proton-sensitive microelectrode, as in Liu et al. (1993). The lines represent best fits from a Hill equation. Notice that the measurement of intracellular pH loses resolution below pH 6.35. Given the pH dependence of the emission ratios, measurements of pH_i below 6.35 were not taken into account.

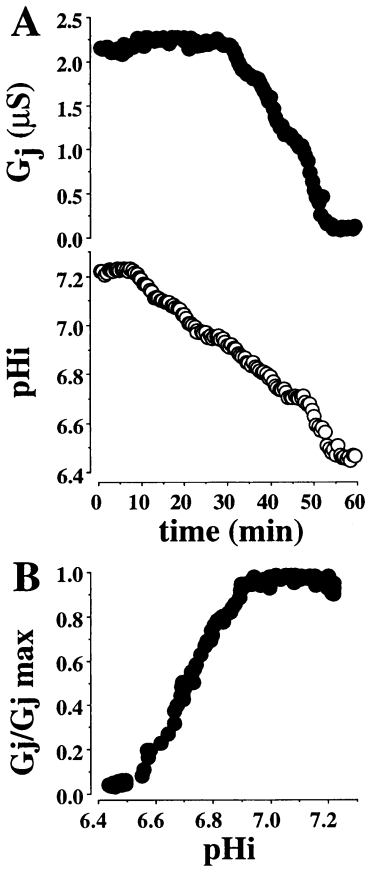

FIGURE 2 Changes of junctional conductance and pH_i recorded simultaneously from a pair of oocytes expressing Cx43. (*A*) Time course of junctional conductance (G_j; *top trace*) and intracellular pH (pH_i; *bottom trace*) during slow acidification with CO_2. The initial 4 min were recorded during superfusion with a bicarbonate-buffered solution gassed with a mixture of 95% O_2, 5% CO_2 (pH of the solution was 7.4). As of minute 4, the concentration of CO_2 was progressively increased in a stepwise fashion, until at minute 45, continuous gassing of the solution with 100% CO_2 was begun. (*B*) Point-by-point correlation of the values of pH_i and G_j recorded during the experiment illustrated in *A*. The smooth slope and data clustering show that the junctional conductance is following changes in pH_i.

In Fig. 3 we have plotted the average pH_i sensitivity curve obtained from five oocyte pairs expressing Cx43 (average G_j control: 1.84 ± 0.3 μS). Symbols represent the average value of relative G_j (±SEM) obtained after grouping all measurements of $G_j/G_{j\,max}$ recorded within a window of 0.1 pH units (e.g., the data point presented at a pH of 6.7 represents the average of all G_j data collected between pH_i values of 6.650 and 6.749, in all five Cx43 experiments).

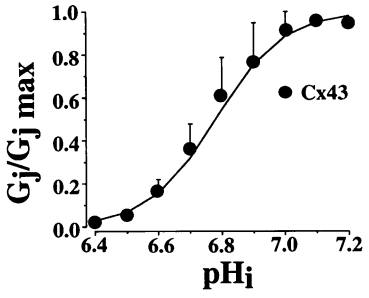

FIGURE 3 pH sensitivity of wild-type Cx43. Symbols represent the average data obtained from oocytes expressing wild-type Cx43. Vertical lines on each data point correspond to the standard error of the mean. The abscissa corresponds to pH_i and the ordinate indicates the relative magnitude of junctional conductance measured for a given pH_i. All data were plotted relative to the maximum G_j recorded and were fitted by a Hill equation. The average pK_a (±SEM) measured from 5 Cx43 experiments was 6.78 ± 0.07. The average Hill coefficient was 5.68 ± 0.84.

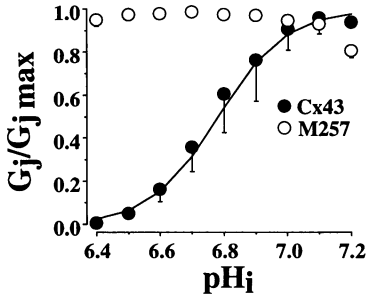

FIGURE 4 Open circles depict the pH sensitivity of a mutant of Cx43 where the carboxyl terminal was truncated at amino acid 257 (i.e., M257; n = 6). Data for wild-type Cx43 are shown by the closed circles and continuous line (same data as in Fig. 3). Vertical lines on each data point correspond to the standard error of the mean. The figure shows that truncation of the carboxyl terminal domain prevented acidification-induced uncoupling within the range of pH_i tested.

The sigmoidal solid line depicts the best fit of a Hill equation describing the average data obtained from Cx43 pairs. In some instances, acidification led to an initial increase in G_j before progressive uncoupling ensued. In those cases, the maximum G_j value ($G_{j\ max}$) was recorded at pH_i values slightly more acidic than the control. Thus, for the purposes of fitting the data to a sigmoidal function, only those data obtained during the uncoupling phase of G_j were included (Bennett et al., 1988; Liu et al., 1993). The average pK_a (calculated from the individual pK_a values recorded in all five experiments) was 6.78 ± 0.07. These results are consistent with those previously published from this laboratory using different methodology (Liu et al., 1993), although quantitative differences are indeed apparent (see Discussion).

Impaired pH sensitivity after truncation of the carboxyl terminal region

In Fig. 4 we compare the pH sensitivity of Cx43 (*solid circles; continuous line*) with that of a mutant of Cx43 where the carboxyl terminal was truncated at amino acid 257 (mutant M257; *open circles*). Average G_j control for the mutant channel was 4.36 ± 1.07 μS (n = 6). Consistent with previous observations from our laboratory (Liu et al., 1993), the data show that deletion of the carboxyl terminal caused the loss of pH sensitivity within the pH range tested, thus demonstrating that the carboxyl terminal is an essential structure for the normal pH gating of Cx43.

Recovery of pH sensitivity of mutant M257 by coexpression of the carboxyl terminal of Cx43

It is our hypothesis that the carboxyl terminal specifically interacts with a separate region of Cx43 to close the channel. We further postulate that this protein-protein interaction should occur even if the particle is not covalently bound to the rest of the Cx molecule. The experiments presented in Fig. 5 were designed to test the latter hypothesis. The data show the pH sensitivity of M257 channels when coexpressed with mRNA coding for the carboxyl terminal (CT) region. As in the previous figures, ordinates represent the junctional conductance (relative to maximum), and the abscissae, pH_i as measured by optical methods. Open circles show data from oocytes that were injected with two separate transcripts: mRNA coding for the pH-insensitive M257 mutant channel, and mRNA coding exclusively for the carboxyl terminal region (amino acids 259 to 382). The broken line represents the best fit of the average data obtained from six coexpression experiments. The average control G_j of this group was 4.5 ± 1.59 μS. The pH sensitivity curve of wild-type Cx43 is provided for comparison (*closed circles; continuous line*). Clearly, the junctional conductance formed by M257 channels was significantly reduced by acidification when the channel protein was coexpressed with the carboxyl terminal region. The average pK_a from the

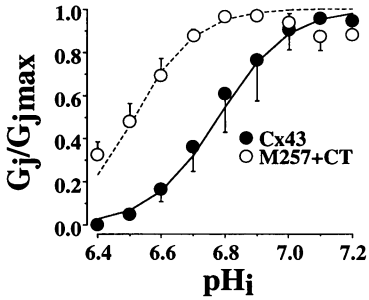

FIGURE 5 Rescue of pH sensitivity of Cx43 by coexpression of the carboxyl terminal region. Open circles depict average data (±SEM) obtained when mutant M257 was coexpressed with mRNA coding for the carboxyl terminal (CT) region (amino acids 259–382 of Cx43). For these experiments, 30 ng mRNA coding for M257 was coinjected with 0.6 ng mRNA coding for the CT region. mRNA was produced using the mCAP mRNA capping kit (Stratagene). The broken line corresponds to the best fit for the Hill equation describing data obtained from coexpression experiments. pK_a= 6.57 ± 0.04. Average Hill coefficient = 4.67 ± 0.69. Continuous line and closed circles correspond to data obtained from oocytes expressing wild-type Cx43 (same data as Figs. 3 and 4).

coexpression experiments was 6.57 ± 0.04 (n = 6). These data strongly support the hypothesis that the carboxyl terminal specifically interacts with a separate region of the Cx molecule to bring about acidification-induced closure of gap junctional channels.

The ratio of mRNAs injected in the coexpression experiments shown in Fig. 5 was 50/1 (30 ng mRNA coding for M257/0.6 ng mRNA coding for the 259–382 region). Injection of equal amounts of both mRNAs (30 ng), or of a higher amount of mRNA expressing the carboxyl tail, prevented the functional expression of gap junctional channels when tested 24–34 h after pairing (48–58 h after mRNA injection). On the other hand, injection of less than 0.6 ng mRNA coding for the carboxyl terminal, together with 30 ng M257 mRNA, failed to enhance the pH sensitivity of the M257 channels. However, this result varied widely, depending on the transcription product being used. Fig. 6 shows data from experiments similar to those presented in Fig. 5; however, mRNA was prepared using a different in vitro transcription kit (Ambion). Control G_j for this group was 4.23 ± 0.78 μS (n = 3). The pH sensitivity of M257 was rescued when using a 1/10 mRNA ratio (5 ng mRNA coding for M257/50 ng mRNA coding for the carboxyl terminal). Gap junction channels expressed in oocytes injected with mRNA ratios of 1/1 (10 ng of both transcripts) were insensitive to pH in the range tested. The results show that the phenomenon of recovery of pH sensitivity by coexpression of the carboxyl terminal is reproducible; however, the concentrations of mRNA that are needed to reproduce the same phenomenon may vary from one transcript to another. Consequently, the ratio of mRNA concentrations injected should not be interpreted as an indication of the actual stoichiometry of the reaction.

The carboxyl terminal of Cx43 enhances pH gating of Cx32 channels

Previous results have shown that Cx32 is less sensitive to acidification-induced uncoupling than Cx43 (Liu et al., 1993); site-directed mutagenesis studies suggest that the molecular mechanisms mediating pH gating of Cx32 (and, perhaps, of all β connexins) are intrinsically different from those of Cx43 (Werner et al., 1991; Delmar et al., 1994). Nevertheless, it is possible that the region of Cx43 acting as a receptor for the pH gating particle is conserved in the Cx32 sequence. We tested whether the pH sensitivity of Cx32 could be enhanced by coexpressing this channel protein with mRNA coding for the carboxyl terminal of Cx43. For these experiments, we injected 30 ng Cx32 and 0.06 ng mRNA coding for the CT domain (ratio 50:1). Transcripts were prepared with the Stratagene kit. The ratio chosen was based on our previous experience with the M257+CT coexpression experiments (see Fig. 5). The data are presented on Fig. 7. Fig. 7 *A* depicts the pH sensitivity of Cx32 (*closed triangles*); G_j control was 8.7 ± 2.4 μS (n = 8). Data for Cx43 are displayed for comparison (*closed circles*). As previously noted (Liu et al., 1993) Cx32 channels also close upon intracellular acidification, but a larger concentration of protons was required (average pK_a for Cx32 was 6.47 ± 0.03; n = 8). As shown in Fig. 7 *B*, coexpression of the carboxyl terminal of Cx43 enhanced the pH sensitivity of Cx32 channels (*open triangles*), causing a small displacement of the curve toward higher pH values (pK_a = 6.63 ± 0.046; n = 5). The difference between the two pK_a values was statistically significant ($p < 0.05$; two-tailed unpaired t-test). Data from Cx32-expressing pairs (*closed triangles*) are presented for comparison. Average control G_j for the coexpression group was 4.74 ± 0.68 μS (n = 5). These results support the hypothesis that differences in the primary structure of the carboxyl terminal regions are responsible, at least in part, for the differences in pH sensitivity between

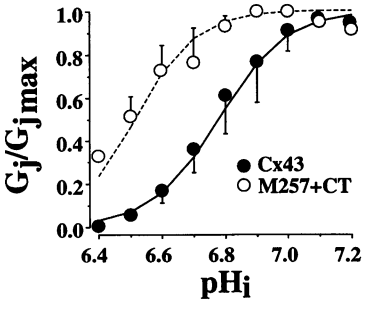

FIGURE 6 Coexpression of M257 and of mRNA coding for the carboxyl terminal (CT) region. For these experiments, 5 ng mRNA coding for the M257 channel was coinjected with 50 ng mRNA coding for the carboxyl terminal regions. Vertical lines on data points correspond to standard error of the mean. In contrast with the experiments presented in Fig. 5, mRNA for these experiments was produced using the mMessage mMachine capped RNA transcription kit (Ambion). The data show the reproducibility of the experiments shown in Fig. 5, although the RNA concentrations needed to rescue the pH sensitivity of M257 channels may vary from one transcript to another.

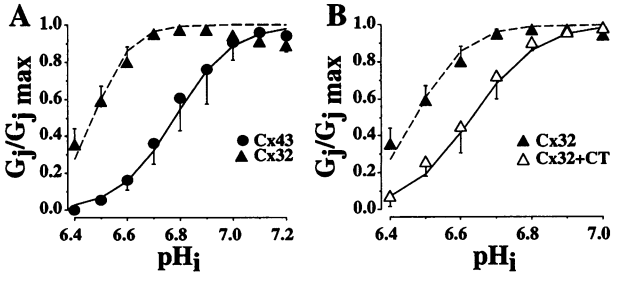

FIGURE 7 (*A*) pH sensitivity of Cx32 (*closed triangles; broken line*) measured from eight oocyte pairs. Average pK_a was 6.47 ± 0.03. Average Hill coefficient was 6.1 ± 0.52. Cx43 data are provided for comparison (*closed circles; solid line*). (*B*) Enhanced pH sensitivity of Cx32 channels by coexpression of the carboxyl terminal of Cx43 (data labeled Cx32+CT). The average pK_a for this group (n = 5) was 6.63 ± 0.05; the average Hill coefficient was 7 ± 0.61. Vertical lines on each data point correspond to the standard error of the mean. Data for pH_i values between 6.4 and 7.0 are shown, to accentuate the differences between the two curves.

these connexins. Differences in the affinity of the receptor, or additional pH gating mechanisms present only in Cx32, cannot be discarded. The data also suggest that the region of the connexin molecule that interacts with the carboxyl terminal of Cx43 may be conserved, at least in part, among connexins.

DISCUSSION

The results presented in this manuscript are consistent with the hypothesis that pH gating of Cx43 results from intramolecular interactions between the carboxyl terminal and a separate region of the connexin molecule. The data fit a particle-receptor model whereby the carboxyl terminal specifically recognizes and noncovalently binds to specific amino acids that are associated with the pore-forming structure. Some general aspects of this study, however, need to be considered further.

The pH sensitivity of Cx32: quantitative discrepancies with previous results

Our results confirm a previous observation that Cx43 channels are more sensitive to intracellular acidification than Cx32. However, there is a difference between the pK_a s previously measured (6.6 and 6.1 for Cx43 and Cx32, respectively; Liu et al., 1993) and those reported here (6.8 for Cx43 and 6.47 for Cx32). This discrepancy may result from the different experimental protocols and, most importantly, macroscopic conductances. First, the acidification protocol previously used allowed for the recording of only a few data points per individual experiment, possibly causing an error in the estimated value of pK_a. Currently, we use slow acidification ramps to acquire all values of pH_i and G_j so that complete pH sensitivity curves, including individual pK_a s, can be recorded from each experiment. A second experimental consideration relates to the possibility of transitory drifts in calibration when using the pH-sensitive electrode for prolonged experiments. Third, and perhaps most importantly, it is possible that some of the experiments previously reported were affected by the loss of isopotentiality and voltage control at the junction. Indeed, the experimental measurements presented by Liu et al. (1993) showed that oocyte pairs with estimated G_j values as high as 40 μS were used for some of the experiments. In addition, pipette resistances for the current electrodes were as high as 3 MΩ. A consequent loss of voltage control at the junctional site can result, leading to an underestimation of the actual value of $G_{j\ max}$ and pK_a. The present study was limited to oocyte pairs with G_j values of $\leq$17 μS; pipette resistances ranged between 0.4 and 0.6 MΩ. The average G_j values are similar to those used by other investigators to compare the extent of electrical coupling among oocytes expressing various connexins (White et al., 1995; Bruzzone et al., 1994; White et al., 1994) and to study acidification-induced uncoupling of Cx32 and its mutants (Werner et al., 1991). Recent data from our laboratory show that the susceptibility to acidification-induced uncoupling is independent of the recording conditions when G_j stays below 25 μS (Ek et al., unpublished observations).

Acidification-induced increase in G_j and data standardization to $G_{j\ max}$

An increase in junctional conductance during the initial stages of acidification has been reported by several authors (e.g., Bennett et al., 1988; Liu et al., 1993; Ek et al., 1994; White et al., 1994). It has been proposed that the observed increase in G_j is due to enhanced incorporation of functional channels in the membrane, and that the overall changes in G_j observed during acidification reflect the balance between increased channel incorporation and channel closure (see Delmar et al., 1994 for review). An increase in G_j during the initial stages of acidification was also observed in our experiments, although it is our impression (not evaluated quantitatively) that the use of slow acidification ramps seems to reduce the extent of the initial increase in G_j. Further studies would be necessary to confirm this observation and to determine whether the acidification protocol may influence the overall shape of the G_j-pH_i relation.

We have used the value of $G_{j\ max}$ as the relative standard for each experiment. A similar procedure was used by Bennett et al. (1988) when fitting the pH sensitivity of the gap junction channels in amphibian blastomeres, which also show an initial increase on acidification. Although the use of $G_{j\ max}$ as a standard is empirically established, we believe that it offers the closest approximation to the total number of functional channels that need to be closed by the pH gating reaction and, consequently, a more accurate estimation of the actual pK_a. The other alternative, to use the control G_j as the standard, would underestimate the extent of acidification-induced uncoupling, because the initial increase in junctional current would not be taken into account.

On the validity of the Hill equation to determine cooperativity in the pH gating reaction

Our data have been fit using the Hill equation, which characterizes the degree of cooperativity in a ligand-protein reaction (Creighton, 1993). We have chosen this fitting procedure so that comparisons could be made with pH sensitivity curves obtained by different laboratories using various systems (e.g., Bennett et al., 1988; Spray et al., 1986; Noma and Tsuboi, 1987). However, caution should be used when interpreting the slope parameter (or "Hill coefficient") yielded by our calculations. Indeed, at this point, we do not know whether pH gating involves cooperativity between particle(s) and receptor(s). Moreover, it is uncertain to what extent the Hill plot could be affected by the experimental protocol or by additional processes (e.g., increased G_j probably due to enhanced channel incorpora-

tion) that may overlap with the protein-protein binding reaction.

Cross-reactivity of the carboxyl terminal of Cx43 with Cx32

Previous results have shown that the susceptibility to acidification-induced uncoupling varies among connexins (Liu et al., 1993; White et al., 1994). In particular, Cx32 is less pH sensitive than Cx43 (Liu et al., 1993). Our results show that coexpression of the carboxyl terminal of Cx43 enhances the pH sensitivity of Cx32. This suggests that the region of Cx43 that acts as a receptor for the gating particle is conserved, at least in part, in Cx32. Similar cross-reactivity of particle and receptor has been demonstrated for voltage-dependent inactivation of potassium channels (Toro et al., 1994; Kramer et al., 1994). As in the latter case, the possibility that the carboxyl terminal affects the stability of the Cx32 molecule, although unlikely, cannot be completely discarded.

These results further open the possibility that gating of various connexins could be modified by coexpression of the active regions of the carboxyl terminal of Cx43. These interactions could even be present in cells showing heteromeric channels (Stauffer, 1995; Zhang and Nicholson, 1994; Koval et al., 1995). Heterologous interactions between different connexin domains could be another way of precisely regulating the extent of intercellular communication between cells according to conditions in the intra- or extracellular environment.

Intramolecular interactions mediate pH gating of Cx43: the particle-receptor hypothesis

Our results show that separate expression of the carboxyl terminal region rescues the pH sensitivity of the wild-type connexin. These data indicate that the carboxyl terminal constitutes a distinct domain, whose function and structure may be preserved even if independently expressed from the rest of the connexin protein. The data further show that pH gating results from a reversible interaction between two regions of the same molecule: the carboxyl terminal domain, and another area of the connexin protein that is associated with the pore-forming structure. Given the limited amounts of both channel protein and carboxyl terminal domain presumed to be available in the oocyte, this intramolecular interaction is likely to involve specific recognition and noncovalent binding between the two regions. Our results could be explained by a "particle-receptor" mechanism, similar to that in the "ball-and-chain" model of voltage-dependent channel gating (Armstrong and Bezanilla, 1977; Hoshi et al., 1990; Zagotta et al., 1990). We propose that the carboxyl terminal domain acts like the particle. Upon intracellular acidification, the particle moves toward the mouth of the channel, where it interacts with specific residues acting like a receptor site. Coupling of the particle to its receptor leads to channel closure and to the loss of a functional pathway between adjacent cells. Alternatively, the particle-receptor interaction may not be direct; rather, it may be mediated by an intermediary protein that couples the particle to the receptor. The latter model further implies an additional recognition step in the binding of the intermediary molecule to both elements of the connexin protein. Although the possibility that pH regulation of gap junctional channels is mediated by other proteins and/or cations has been postulated (Arellano et al., 1988; Lazrak and Peracchia, 1993), no study has demonstrated that pH gating of Cx43 requires the presence of an intermediary molecule. Further experiments are needed to determine whether the protein-protein association between the particle and the receptor is exclusively mediated through intramolecular, noncovalent forces, or whether an intermediary element is required.

An ancillary observation in this paper is that coinjection of M257 and carboxyl terminal mRNA at certain ratios prevented the formation of functional channels. At this point, we do not have a definite explanation for this finding. It is possible that translation of M257 was slowed down by the presence of an additional mRNA. Another possibility may be that the carboxyl terminal polypeptide alters M257 trafficking or its assembly into a channel. Alternatively, we may speculate that the carboxyl terminal can block M257 channels at a normal pH. Further experiments would be necessary to discern between these (and perhaps other) possibilities.

The results presented on Figs. 5 and 6 show that M257 regained pH sensitivity after coexpression of the carboxyl terminal domain. However, the pK_a values obtained in the coexpression experiments were still different from those recorded from Cx43-expressing oocytes. The latter may suggest that the intramolecular (i.e., full-length Cx43) particle-receptor reaction is more favorable than its intermolecular (i.e., M257+carboxyl terminal) counterpart (Creighton, 1993). The latter is a common observation when comparing intermolecular and intramolecular reactions, and it is mostly a consequence of the fact that in the intramolecular reaction, the standard entropy of approximation (i.e., the change in standard entropy due solely to bringing the separate reactants together) is negligible (Kyte, 1995).

The ball-and-chain hypothesis for voltage-dependent gating has been extensively studied. Yet, our data represent the first demonstration of a particle-receptor interaction as the mechanism mediating the chemical regulation of a membrane channel. This mechanism may not be unique to the pH gating of Cx43. Phosphorylation of residues within the carboxyl terminal domain of Cx43 by protein kinase C (Moreno et al., 1994) or by tyrosine kinase (Loo et al., 1995) is known to regulate conductance. It is possible that this mechanism of down-regulation, as well as the chemical regulation of other channel proteins, is mediated by a particle-receptor mechanism.

Our results support the hypothesis that pH regulation results from a particle-receptor binding reaction. However,

it is unknown whether the bound particle directly acts as an open channel blocker (Armstrong and Bezanilla, 1977; Hoshi et al., 1990; Zagotta et al., 1990) or whether it triggers an additional change in the molecular conformation that would bring about channel closure. Earlier studies (Unwin and Zampighi, 1980; Unwin and Ennis, 1984) suggested that closure of liver gap junction channels involved the sliding of entire connexins against each other, with the consequent closure of the pore in the cytoplasmic side. However, structural studies on pH gating of Cx43 are not available.

Ultrastructural modifications that result from chemical gating have recently been studied for the nicotinic acetylcholine receptor channel (NAChR). In that case, ligand binding leads to small rotations in the α-subunits, thus triggering changes in the orientation of the pore-lining α-helical (M2) regions that lead to the opening of the pore (Unwin, 1995). Similarly, it is possible that binding of the particle to the receptor could trigger changes in the alignment of the connexins, causing closure of the channel. Some functional and structural similarities exist between connexons and NAChR (Stauffer and Unwin, 1992; Suchyna et al., 1993). However, extending the gating mechanism of NAChR to connexons is limited by the lack of knowledge of the possible conformation of Cx43 and of gap junction channels in the open and closed states.

Possible location of the receptor in the primary sequence of Cx43

Previous results from our laboratory (Ek et al., 1994) showed that, as initially suggested by Spray and Burt (Spray and Burt, 1990), the presence of a histidine residue at position 95 is important in the pH gating process. We now propose that histidine 95 is part of the receptor and that electrostatic interactions, mediated in part by protonation of histidine 95, are involved in the particle-receptor binding reaction. Interestingly, the presence of a histidine residue at the N-terminal end of the cytoplasmic loop is highly conserved among connexins. Future experiments will determine whether mutations of such histidine in Cx32 could prevent the enhancement of pH sensitivity that is brought about by coexpression of the carboxyl terminal of Cx43.

CONCLUSIONS

In conclusion, we have demonstrated that pH gating of Cx43 results from an intramolecular interaction between the carboxyl terminal domain, acting as a gating particle, and a separate region of the Cx43 molecule, acting as a receptor. The data further suggest that the region acting as a receptor is conserved, at least in part, in Cx32. This is the first demonstration of an intramolecular particle-receptor interaction as the mechanism of chemical regulation of a membrane channel. A similar mechanism may mediate chemical regulation of other membrane channels. Finally, noncovalent binding between the carboxyl terminal domain and the channel protein may be a common mechanism for the regulation of intercellular communication in Cx43-expressing cells.

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