Conductance and Permeability of the Residual State of Connexin43 Gap Junction Channels

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ABSTRACT We used cell lines expressing wild-type connexin43 and connexin43 fused with the enhanced green fluorescent protein (Cx43-EGFP) to examine conductance and perm-selectivity of the residual state of Cx43 homotypic and Cx43/Cx43-EGFP heterotypic gap junction channels. Each hemichannel in Cx43 cell–cell channel possesses two gates: a fast gate that closes channels to the residual state and a slow gate that fully closes channels; the transjunctional voltage (Vj) closes the fast gate in the hemichannel that is on the relatively negative side. Here, we demonstrate macroscopically and at the single-channel level that the IV relationship of the residual state rectifies, exhibiting higher conductance at higher Vj that are negative on the side of gated hemichannel. The degree of rectification increases when Cl− is replaced by Asp− and decreases when K+ is replaced by TEA+. These data are consistent with an increased anionic selectivity of the residual state. The Vj-gated channel is not permeable to monovalent positively and negatively charged dyes, which are readily permeable through the fully open channel. These data indicate that a narrowing of the channel pore accompanies gating to the residual state. We suggest that the fast gate operates through a conformational change that introduces positive charge at the cytoplasmic vestibule of the gated hemichannel, thereby producing current rectification, increased anionic selectivity, and a narrowing of channel pore that is largely responsible for reducing channel conductance and restricting dye transfer. Consequently, the fast Vj-sensitive gating mechanism can serve as a selectivity filter, which allows electrical coupling but limits metabolic communication.

KEY WORDS: intercellular communication • dye transfer • EGFP • voltage gating • permeability

INTRODUCTION

Connexins (Cx),* a large family of homologous membrane proteins, form gap junction (GJ) channels that provide a direct pathway for electrical and metabolic signaling between cells (Bennett et al., 1991; Elfgang et al., 1995). Each GJ channel is composed of two hemichannels that, in turn, are composed of six connexin subunits. Connexins are predicted to have four α helical transmembrane domains (TM1–TM4), intracellular NH2 and COOH termini (NT and CT, respectively), two extracellular loops (E1 and E2), and a cytoplasmic loop (Unwin and Zampighi, 1980; Yeager and Nicholson, 1996; Yeager, 1998). Freeze fracture electron microscopy has shown that GJ channels cluster tightly into 2-D arrays or plaques (Revel and Karnovsky, 1967; Peracchia, 1977). In cells transfected with connexins that are fused with enhanced green fluorescent protein (EGFP), GJ plaques can be visualized in living cells by fluorescence microscopy (Jordan et al., 1999; Falk, 2000), and it was shown that only channels assembled into junctional plaques are functional (Bukauskas et al., 2000). In invertebrates, GJ channels are formed of innexins, another large family of integral membrane proteins, and it is predicted that they also have four transmembrane domains and oligomerize into hemichannels composed of six subunits. Despite a lack of primary sequence homology with connexins, innexins form GJ channels with surprisingly similar functional properties (Verselis et al., 1991; Bukauskas et al., 1992; Bukauskas and Weingart, 1994; Phelan and Starich, 2001).

Junctional conductance (gj) of GJ channels formed from all connexins is sensitive to transjunctional voltage (Vj) the voltage difference between the cells. In homotypic junctions, formed by the docking of identical hemichannels, reductions in gj with Vj are typically symmetric about a maximum at Vj = 0. In heterotypic junctions, where asymmetry is formed by the docking of hemichannels differing in connexin composition, asymmetry in gating about Vj = 0 usually results. It was proposed that each hemichannel in a formed GJ channel has its own Vj gate and, for each polarity of Vj, closure can be ascribed to one hemichannel (Harris et al., 1981). A distinct property of Vj gating is that gj does not decline to zero with increasing Vj, leaving a residual conductance that varies from ~5 to 30% of its maximum depending on the connexin type (Werner et al., 1989; Willcke et al., 1991; Rook et al., 1992; Moreno et al., 1995;
White et al., 1995; Steiner and Ebihara, 1996; Revilla et al., 1999). Single-channel studies have shown that the residual $g_j$ is explained by gating of GJ channels to a long-lived substate (Bukauskas and Weingart, 1994; Moreno et al., 1994). Weingart and Bukauskas (1993) termed this substate the “residual” state, and demonstrated it to be a property common to vertebrate and invertebrate GJs (Bukauskas et al., 1995). GJ channels can close completely as well (i.e., to a nonconducting state in response to $V_j$), but has been shown to occur by a different mechanism (Banach and Weingart, 2000; Bukauskas et al., 2001). Gating to the residual and closed states is not only distinguished by the degree of channel closure, but also by kinetics and we have termed these two mechanisms “fast” and “slow” $V_j$ gating, respectively.

Bukauskas and Weingart (1994) proposed that the residual state represents the most closed conformation for the fast $V_j$ gate. Depending on the connexin composition, this gate has been shown to close for either polarity of $V_j$, so that some hemichannels will close on relative negativity and others on relative positivity on their cytoplasmic sides. Molecular studies have shown that charged residues in the NT domain form the voltage sensor for fast $V_j$ gating and that the sign of the charge on the sensor confers gating polarity (Verselis et al., 1994; Oh et al., 2000). Closure of the gate has been proposed to occur by movement of the NT domain into the cytoplasmic vestibule of the hemichannel pore transduced through a straightening of a proline-kink in TM2 (Ri et al., 1999). The result is a local narrowing of the pore at the cytoplasmic vestibule that should also increase the effective charge density in this region. Oh et al. (1999) demonstrated in Cx32 that although open channel current is linear with $V_j$, the residual state rectifies such that current increases when the closed hemichannel is made relatively more negative. This observation is consistent with an increase in the electrostatic effect of a positive charge at the cytoplasmic vestibule of the hemichannel closed by fast $V_j$ gate.

Previously, using Cx43 with EGFP fused to its CT domain, we determined that the hemichannel in response to applied $V_j$ closes to the residual state on the relatively negative side. Accordingly, the sensor should be positively charged. Here, we examined whether the residual state exhibited rectification that is consistent with the introduction of a positive charge at the closed end of the Cx43 channel. Furthermore, we extended these studies to examine whether the Cx43 channel in the residual state indeed represents a narrowed pore and whether there is a change in the charge selectivity compared with the open state, as might be expected by the translocation of the NT domain. These were accomplished at the level of the single channel as well as macroscopically using protocols designed to close the fast, but not the slow, $V_j$ gate (Bukauskas et al., 2001).

Due to its stable conformation, as evidenced from its long dwell time, the residual state can play a significant functional role in regulating the transmission of electrical signals and the cell–cell transfer of metabolites.

### M A T E R I A L S  A N D  M E T H O D S

#### Cell Lines and Culture Conditions

Experiments were performed on HeLa cells (a human cervix carcinoma cell line; ATCC No. CCL-2) transfected with Cx43 or Cx43-EGFP and on Novikoff cells, a rat hepatoma cell line, that endogenously express Cx43 (Meyer et al., 1991). HeLa cells were stably transfected with cDNAs encoding rat Cx43 or rat Cx43-EGFP. The cDNA encoding Cx43-EGFP was constructed as described by Jordan et al. (1999). HeLa cells were grown in DMEM supplemented with 10% FBS. All media, sera, and culture reagents were obtained from Life Technologies (GIBCO BRL). The transfection procedure has been described previously (Jordan et al., 1999; Bukauskas et al., 2001). Novikoff cells were grown in Swim’s S-77 medium with 4 mM glutamine, 20% horse serum, and 5% FBS. To study homotypic junctions, cells of one type were seeded at a density of $\sim 10^4$ cells/cm$^2$ onto sterile coverslips placed in culture dishes. To study Cx43/Cx43-EGFP heterotypic junctions, Novikoff cells were mixed with HeLa/Cx43-EGFP cells in equal quantities and seeded on coverslips at $\sim 10^4$ cells/cm$^2$. Novikoff cells were transfected to Dulbecco’s medium 3 wk before coculturing with HeLa cells.

#### Electrophysiological Measurements

For simultaneous electrophysiological and fluorescence recording, cells were grown on 22 × 22-mm number 0 coverslips and transferred to an experimental chamber (Bukauskas, 2001) mounted on the stage of an inverted microscope (model Olympus IX70; Olympus America) equipped with phase-contrast optics and a fluorescence imaging system. The chamber was perfused with a modified Krebs-Ringer’s solution containing the following (in mM): 140 NaCl, 4 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 5 HEPES, 5 glucose, and 2 pyruvate, pH 7.4. In most of the experiments, patch pipettes were filled with our standard pipette solution containing the following (in mM): 10 NaCl, 130 KCl, 0.26 CaCl$_2$, 1 MgCl$_2$, 3 MgATP, 5 HEPES, pH 7.2, and 2 EGTA ([Ca$^{2+}$] = 5 × 10$^{-8}$ M). Table I shows the compositions of the pipette solutions used in ion-substitution studies. Solutions were adjusted to pH 7.2 by using KOH for solutions 1 and 2, and TEAOH for solution 3. In addition, we measured conductivity of all pipette solutions using conductometer (Accumet model-30, Fisher Scientific).

$g_j$ was measured using the dual whole-cell patch clamp (Neyton and Trautmann, 1985). Briefly, each cell of a pair was voltage-clamped independently with a separate patch clamp. By stepping the voltage in one cell and keeping the other constant, junctional current ($I_j$) is measured directly as a change in a current in the unstepped cell. Thus, $g_j$ is obtained by dividing the change in

#### Table I: Composition of the Pipette Solutions Used in Ion Substitution Studies

| Pipette solution | KC1 (mM) | KAsp (mM) | TEACl (mM) | CaCl$_2$ (mM) | EGTA (mM) | MgCl$_2$ (mM) | HEPES (mM) | Conductivity (S/cm) |
|----------------|---------|---------|-------------|-------------|-----------|-------------|-----------|------------------|
| #1, KC1 | 140 | 0.26 | 2 | 1 | 5 | 17.2 |
| #2, KAsp | 140 | 0.26 | 2 | 1 | 5 | 12.1 |
| #3, TEACl | 140 | 0.26 | 2 | 1 | 5 | 11.3 |
I_j by the change in V_j. With low levels of coupling, unitary junctional currents can be recorded as discrete quantal changes in the unstepped cell that are accompanied by equal and opposite quantal changes in the stepped cell. Voltages and currents were recorded on videotape using a daq recorder (model V10-10K; Instruch), and digitized in parallel using a converter (model MIO-16X A/D; National Instruments) and our own acquisition software. Records were digitized at 5 kHz and filtered at 1 kHz. Junctional conductance is plotted in two ways: as the chord conductance (g_j) calculated as I_j/V_j and slope conductance (g_slope) calculated as ΔI_j/ΔV_j, where ΔI_j = I_{j+1} - I_j, and ΔV_j = V_{j+1} - V_j; n is the nth point of a digitized current or voltage recording. To increase the signal to noise ratio, the values of ΔI_j and ΔV_j were calculated by using a running average procedure in which ~10–20 sequential digitized data points were averaged. We used g_slope preferentially to study a rectification of macroscopic residual conductance by superposing repeated V_j ramps of small amplitude onto the V_j steps (see Fig. 1 C). g_slope has some advantages over g_j because of the following: (1) slow variations of non-junctional membrane as well as seal leak currents do not have an impact on calculated g_slope value, whereas g_j depends on an absolute value of I_j; (2) g_slope can be measured at V_j that are close to zero, whereas g_j deviates to infinity; and (3) g_slope is better applicable to study conductance dependence on V_j for the processes with nonlinear I-V relationships.

Cx43-EGFP Fluorescence and Dye Transfer Measurements

Fluorescence signals were acquired and analyzed using an Olympus IX70 microscope, a Spectramaster high speed monochromator, and UltraVIEW software for image acquisition and analysis (Perkin Elmer Life Sciences). The wavelengths used for excitation of EGFP, Alexa Fluor, and ethidium bromide were delivered by a monochromator and were 480, 530, and 518 nm, respectively. Appropriate filter cubes were used for monitoring emission (Chroma Technology Corp.).

For cell–cell dye transfer studies, three patch pipettes were used: two for dual whole-cell voltage-clamp recording, and a third for loading one cell of a pair with fluorescent dye. Dye was added to the third pipette at a concentration of ~0.1 mM. A dual whole-cell recording was established first, and a V_j was imposed before introducing the dye-filled pipette. This experimental design allowed us to load dye in one cell and monitor cell–cell transfer with G_j channels predominantly in the residual state. Off-line analysis of fluorescence in both cells of a pair was accomplished using UltraVIEW software. Fluorescence intensity in each cell of a pair was measured as the average within a region of interest that included most of the area of the cell (see Fig. 7). Background fluorescence was measured in region of interest that is located outside the cell pair. All data are plotted as background-subtracted intensities.

**RESULTS**

Rectifying Properties of Macroscopic Residual Conductance of Cx43/Cx43-EGFP Channels

Recently, we showed that the fast gate, which closes channels to the residual state, and the slow gate, which closes channels completely, operate in series such that activation of the slow gate depends on whether the fast gate is open or closed (Bukauskas et al., 2001). At small V_A (up to ~60 mV), the fast gate remains open and most gating events reflect the operation of the slow gate. At V_S between ~60 and 100 mV, the faster and more steeply V_S-sensitive fast gate closes channels to the residual state, preventing closure of the slow gate due to a reduction in V_j across the slow gate. At sufficiently large V_S (i.e., >100 mV), the slow gate can close even with the fast gate closed. Thus, we used heterotypic Cx43/Cx43-EGFP junctions and the V_j “window” between ~60 and 100 mV at which most Cx43 channels reside in the residual state to examine and compare I-V characteristics of open and residual states. In this heterotypic pairing configuration, only the Cx43 hemichannels express fast V_j gating (i.e., close to the residual state; Bukauskas et al., 2001). Cx43/Cx43-EGFP cell pairs were obtained from cocultures of Novikoff cells, which endogenously express only Cx43, and HeLa cells transfected with Cx43-EGFP. Heterotypic cell pairs were identified by having only one cell of a pair displaying EGFP fluorescence (Fig. 1 A). The arrow in the example of the cell pair shown indicates a junctional plaque in the region of cell–cell contact. Fig. 1 B shows the asymmetric G_j-V_j dependence characteristic of Cx43/Cx43-EGFP junctions. Data are pooled from 19 cell pairs. Fast V_j gating of Cx43 hemichannels is manifest macroscopically as a reduction in g_j to a residual plateau value at large negative V_S on the Cx43 side. Conversely, g_j decreases to zero at large positive V_S, which is indicative of a lack of fast V_j gating to the residual state in Cx43-EGFP hemichannels (Bukauskas et al., 2000). The solid lines are fits of the data for each polarity of V_j to a Boltzmann relation of the form: G_j = (1 - G_{min})/ (1 + exp[(A · (V_j - V_0)]) + G_{min}, where G_{min} is the normalized residual g_j. A is a measure of voltage sensitivity in mV^{-1}, and V_0 is the V_j at which G_j is halfway between the maximum and minimum values. The Boltzmann parameters are as follows: V_0 = 49 ± 2 mV, A = 0.07 ± 0.01 mV^{-1}, and G_{min} = 0.2 ± 0.02 for negative V_S and V_0 = 66 ± 2 mV, A = 0.07 ± 0.02 mV^{-1} and G_{min} = 0.12 ± 0.03 for positive V_S. The dashed line shows the Boltzmann G_j-V_j relation obtained for homotypic Cx43 junctions (Bukauskas et al., 2001).

Examples of changes in I_j upon application of long duration V_j steps show that I_j declines rapidly to a residual steady-state level with negative V_j on the Cx43 (Novikoff) side. For the opposite polarity (i.e., positive on the Cx43 side), I_j declines considerably more slowly and fails to reach steady state within the 60-s duration of the V_j step (Fig. 1 C). These properties at negative and positive V_S relative to the Cx43 side are characteristic of gating ascribed to Cx43 and Cx43-EGFP hemichannels, respectively (Bukauskas et al., 2001). Correlative single-channel studies by Bukauskas et al. (2001) have shown that the rapid decline in g_j to the residual steady-state level reflects closure of Cx43 hemichannels to their residual state and the continued decline in g_j to near zero shows full closures of Cx43-EGFP hemichannels.
To examine the I-V characteristics of junctions in open and residual states, repeated small amplitude \( V_j \) ramps (Fig. 1 C, inset) were applied at times in between and superimposed onto the \( V_j \) steps (Fig. 1 C). Corresponding \( I_j-V_j \) and \( g_{\text{slope}}-V_j \) scatter plots were obtained from ramps applied during the indicated intervals, I–IV, (Fig. 1, D and E; for \( g_{\text{slope}} \) calculations see MATERIALS AND METHODS). The I-V relations obtained from ramps applied with cells held at \( V_j = 0 \) (interval I) were stable and linear, giving a \( g_{\text{slope}} \) of \( \sim 6.8 \) nS. The I-V re-
lations obtained from $V_j$ ramps superimposed onto the long duration $V_j$ steps of $-80$ and $-100$ mV relative to the Cx43 side (intervals II and III, red and green, respectively) were also stable, but reduced in slope due to gating to the residual state and give $g_{\text{slope}}V_j$ relations that show dependence on $V_j$ (Fig. 1 E). The mean values of all data points calculated separately for $g_{\text{slope}}$ measured at $V_j$ steps of $-80$ and $-100$ mV were $1.86 \pm 0.01$ nS ($n = 18,426$), and $2.03 \pm 0.01$ nS ($27,384$), respectively. The $I-V$ relations obtained from $V_j$ ramps superimposed onto the long duration $V_j$ step of $+100$ mV relative to the Cx43 side (interval IV, blue) showed a reduced slope and, in addition, high variability among the individual ramps due to the fact that $I_j$ was not at steady state and continued to decline. However, the $I-V$ relations are linear, giving constant $g_{\text{slope}}V_j$ relations much like that at $V_j = 0$. The mean value for $g_{\text{slope}}$ in this interval was $1.20 \pm 0.01$ nS ($n = 7,762$). Similar results of $I-V$ characteristics were observed in nine other cell pairs. Given that these channels are predominantly open near $V_j = 0$ and gate only between open and closed states at $V_j$s relatively positive on the Cx43 side, the linear $I-V$ characteristics obtained at intervals I and IV are the result of current flow through open channels. Conversely, at $V_j$s relatively negative on the Cx43 side (intervals II and III), channels are predominantly in the residual. These data are consistent with linear and rectifying properties of single channels in open and residual states, respectively.

**I-V Characteristics of the Residual State of Cx43 at the Single-Channel Level**

$I-V$ curves at a single-channel level were examined in poorly coupled Novikoff/HeLaCx43EGFP cell pairs. In the example shown in Fig. 2 A, a $V_j$ protocol consisting of repeated 600-ms ramps from $-120$ to $+120$ mV initiated and terminated by 50-ms epochs at $-120$ and $+120$ mV. The $I_j$ record shows gating transitions between open and residual states only at negative $V_j$. The channel typically reopens when $V_j$ approaches 0 mV and stays open at positive $V_j$. The $I-V$ scatter plot of all data points from A. (C) Representative example of a $V_j$ polarity reversal protocol used to examine the residual state current at positive $V_j$s. Stepping to $+85$ mV shows the open channel current giving $\gamma_{\text{open}} = 115$ pS; no gating to the residual substate is observed. Reversal to $V_j = -85$ mV results in equal, but opposite, current giving the same $\gamma_{\text{open}}$ before eventually gating to the residual state ($\gamma_{\text{res}} = 28$ pS, dashed line). Upon reversal back to $V_j = +85$ mV, the channel briefly remains in the residual state before opening, and shows a reduced $\gamma_{\text{res}}$ of 15 pS (dotted line). (D) $I-V_j$ scatter plot obtained from the record presented in C. The solid line is a fit of the data by using single exponential function, $I_{\text{res}} = I_{\text{open}} - \left( \exp(b \cdot V_j) - 1 \right)$. The fitting parameters were as follows: $I_{\text{open}} = 3.3 \pm 0.4$ pA, $b = -0.097 \pm 0.001$ mV$^{-1}$ ($n = 2,489$); $\gamma_{\text{res}}$ of Cx43/Cx43-EGFP channel rectifies decreasing nearly twofold when $V_j$ changes from $-100$ to $100$ mV.
mained in the residual state until \( V_j \) decreased to about -50 mV whereupon it reopened and remained open throughout the remainder of the ramp. A plot of all the \( I_j \) data points versus \( V_j \) obtained from the three consecutive ramps in Fig. 2 A illustrates this behavior (Fig. 2 B) and is consistent with fast \( V_j \) gating being operational only in Cx43 hemichannels at relatively negative \( V_j \) on the Cx43 side. The lack of full closures characteristic of slow \( V_j \) gating at either \( V_j \) polarity is due to the slow kinetics of this gating mechanism; closures would be rare within the duration of ramps imposed.

Because the fast \( V_j \) gate in Cx43 hemichannels invariably reopens when the voltage ramps approach 0 mV, it was not possible using a ramp protocol to observe the I-V relation of the residual state at positive \( V_j \)s. Alternatively, we applied a pulse protocol in which a single channel was driven into the residual state by applying a large conditioning \( V_j \) step negative to the Cx43 side and then reversing the \( V_j \) polarity. Typically, a channel remained in the residual state sufficiently long after polarity reversal to measure conductance at \( V_j \)s positive on the Cx43 side (Fig. 2 C). An example is shown for an -85-mV conditioning step followed by reversal to +85 mV applied to the Novikoff (Cx43) cell of a Novikoff/Cx43-EGFP cell pair. Junctional conductance is plotted above the \( I \) record. For both positive and negative 85-mV \( V_j \) steps, open channel conductance (\( \gamma_{open} \)) was found to be the same (\( \sim 115 \) pS), which is consistent with \( V_j \) independence of the open state conductance, whereas the residual conductance (\( \gamma_{res} \)) was \( \sim 28 \) pS for -85 mV (Fig. 2 C, dotted line) and \( \sim 15 \) pS for +85 mV (Fig. 2 C, dotted line), which is consistent with the rectification previously indicated in macroscopic experiments. Fig. 2 D shows a \( \gamma_{res}/V_j \) scatter plot of the data presented in Fig. 2 C. The solid line is an exponential fit to the data constrained to go through the origin.

Fig. 3 (A and B) combines scatter plots of all data points that were collected by using voltage ramp and step protocols for open and residual states, respectively. The \( I_j-V_j \) scatter plot for the open state is well fit by a linear relation (Fig. 3 A, solid line) giving \( \gamma_{open} = \sim 115 \) pS (35 ramps from four different cell pairs). The \( I_j-V_j \) plot for the residual state shown in Fig. 3 B was collected from 25 records using voltage ramp protocol (four experiments) and 12 records (three experiments) using voltage step protocol. The \( I_j-V_j \) data are fit well by an exponential function with \( \gamma_{res} \) decreasing from \( \sim 28 \) pS at \( V_j = -100 \) mV to \( \sim 15 \) pS at \( V_j = 100 \) mV. Correspondingly, the ratio, \( \gamma_{res}/\gamma_{open} \), decreases from 0.23 to 0.13 (Fig. 3 C).

We sought to confirm our findings in homotypic Cx43 channels because of the possibility that the residual state of Cx43 hemichannels has different characteristics when paired heterotypically with Cx43-EGFP hemichannels. Such measurements were accomplished by \( V_j \) polarity reversal experiments designed to capture the residual state of one hemichannel over a wide range of \( V_j \). Fig. 4 A shows an example of a single-channel current record in which a \( V_j \) of -70 mV was applied by hyperpolarizing cell 1 to place the hemichannel in this cell into the residual state (Fig. 4 A, dashed line). Holding potentials of -75 and -5 mV were applied to cell 1 and cell 2, respectively. Intermittent, short duration (100 ms) steps to a positive \( V_j \) were applied to measure \( I_j \) of the residual state upon polarity reversal. The insert shows an expanded view of one of the \( V_j \) reversals. The residual state displays a conductance of \( \gamma_{res} \) at \( V_j = -70 \) mV (Fig. 4 A, dashed line) and decreases to \( \sim 18 \) pS at +35 mV. Fig. 4 B shows an I-V scatter plot of data obtained from four cell pairs (two Novikoff cell pairs and two Cx43-transfected HeLa cell
pairs), and is indistinguishable from that obtained in heterotypic Cx43/Cx43EGFP channels, with the Cx43 side made relatively negative. Unlike the heterotypic channel, the same degree of rectification, but opposite in direction, could be obtained in the homotypic channel for either polarity of $V_j$. These results indicate that the conductance and I-V characteristics of the residual

**Figure 4.** Residual conductance dependence on $V_j$ in Cx43 homotypic channels. (A) $I_j$ record obtained in the late stage of CO$_2$-induced uncoupling in a HelaCx43 cell pair when only a single channel was operating. The holding potential in cell 1 was $-75$ mV, and in cell 2 was $-5$ mV; repeated pulses of $+105$ mV were applied to cell 1. $V_j$ of $-70$ mV was maintained throughout except for intermittent pulses. The channel predominantly resides in the residual state at $V_j = -70$ (dashed line). Upon $V_j$ reversal to $+35$ mV, the channel briefly remains in $\gamma_{res}$ before opening fully (lower solid line). At the end of this record, the channel closed fully (i.e., to a nonconducting state), as evidenced by a lack of a change in $I_j$ in response to the last $V_j$ step. Inset shows $I_j$ during $V_j$ reversal at an extended time scale. $I_j$ at $V_j = +35$ mV gave a residual conductance of $\sim 18$ pS (dotted line) compared with $\gamma_{res}$ of $\sim 30$ pS at $V_j = -70$ mV (dashed line). (B) $I_j$-$V_j$ scatter plot of the residual state obtained from the data shown in A and three other similar experiments. The solid line shows the fit of the data to a single exponential function. The fitting parameters were as follows: $I_o = 1.5 \pm 0.5$ pA, and $b = -0.011 \pm 0.003$ mV$^{-1}$ ($n = 894$ data points).

**Figure 5.** Rectification of the residual state in symmetric KAsp. (A) Single-channel currents in a Novikoff cell pair in response to repeated $V_j$ ramps (from $-85$ to $85$ mV) demonstrates gating transitions between open and residual states. (B) Summarized $I_j$-$V_j$ scatter plot of data points from the open state. A linear regression (solid line) gives an open channel conductance of $46$ pS ($n = 9,100$ data points; $r^2 = 0.99$). (C) Summarized $I_j$-$V_j$ scatter plot of data points from the residual state (collected from five cell pairs). A fit to a single exponential function gave parameters as follows: $I_o = 0.22 \pm 0.03$ pA, and $b = 0.016 \pm 0.002$ mV$^{-1}$ ($n = 2,920$ data points; solid line). (E) $\gamma_{res}/\gamma_{open}$ scatter plot calculated from the fitted curves shown in B and C. $\gamma_{res}/\gamma_{open}$ declines from $\sim 0.18$ to $\sim 0.07$ when $V_j$ changes from $-100$ to $+6$ mV.
state of a Cx43 hemichannel are the same whether incorporated in a homotypic Cx43 channel or heterotypic Cx43/Cx43-EGFP channel.

**The Open and Residual States Differ in Selectivity**

To assess selectivity of the residual state, we examined changes in conductance of open ($\gamma_{\text{open}}$) and residual ($\gamma_{\text{res}}$) states upon ion substitution. Compositions of the pipette solutions used in these experiments are shown in Table I. Removal of MgATP and NaAsp from the standard KCl solution (solution #1), had no appreciable affect on conductance of either the open or residual states, as well as on sensitivity of $V_j$ gating of homotypic Cx43 channels. Upon replacement of Cl\(^-\) with Asp\(^-\), the I-V relation of the open channel, obtained from repeated voltage ramps, remained linear, but conductance decreased from 115 pS to ~45 pS, which is suggestive of a substantial anionic component to the current flowing through the open state (Fig. 5 A). Data showing $I_j$-$V_j$ plots of open and residual state obtained from five experiments are shown in Fig. 5 (B and C, respectively). The slope of the regression line from (Fig. 5 B, solid line) yields a conductance of 46 pS ($n = 9,100$ data points; $r^2 = 0.99$) for the open state. Conductance of the residual state decreased from ~10 pS at $V_j = -120$ mV to ~3 pS at $V_j = 0$ mV; at positive $V_j$s, current in the residual state was too small to measure reliably (Fig. 5 C). A plot of the ratio, $\gamma_{\text{res}}/\gamma_{\text{open}}$, illustrates the steeper rectification in KA\(_2\) compared with KCl and is consistent with an increase in anion selectivity of the residual state (Fig. 5 D).

We performed the same set of experiments upon replacement of K\(^+\) with TEA\(^+\) (Fig. 6). Like in KA\(_2\), conductance of the open channel in TEACL also decreased substantially, from ~115 to ~40 pS, and the open state I-V curve remained linear (Fig. 6 B). These data suggest that K\(^+\) contributes to the conductance of the open state comparably to Cl\(^-\). The conductance of the residual state also decreased, but the I-V relation in TEACL showed substantially weakened rectification, giving a nearly constant value for $\gamma_{\text{res}}/\gamma_{\text{open}}$ between -100 and +100 mV (Fig. 6 D). Thus, at positive $V_j$s relative to the side of a closed hemichannel, $\gamma_{\text{res}}$ is reduced substantially more upon replacement of Cl\(^-\) than K\(^+\), which again is consistent with an increased anion selectivity of the residual state.

Confirmation of the selectivity properties of the residual state using measurement of reversal potential was not successful because large $V_j$s were needed to keep channels in the residual state and they tended to open in the $V_j$ range where current reversal would occur. At the single-channel level, reversal potential was difficult to assess with confidence as the substate currents in the substituted salts were small and often within the noise of the whole-cell recording.

**Permeability of the Residual State of Cx43 Channels to Dyes**

A different assessment of perm-selectivity also can be obtained from dye flux studies. To examine dye permeability of the residual state, we needed to impose a large $V_j$ between Novikoff cells during dye transfer and...
modified the protocol to include the use of three patch pipettes: two for dual whole-cell voltage-clamp recording, and a third pipette to load one of the cells with dye. Fig. 7 A shows a phase-contrast image of a cell pair with positions of the three pipettes outlined (Fig. 7 A, dashed lines). After gigaohm seals were formed with all three pipettes, whole-cell recordings were established with pipettes 1 and 2. Both pipettes were held at a common voltage of −55 mV to maintain \( V_j = 0 \) mV. A \( g_o \) of 43 nS, corresponding to ~390 open channels on average, was measured by applying repeated small, brief \( V_j \) steps to cell 2. A large, long duration 90-mV \( V_j \) step was then applied by depolarizing cell 2 to close channels to their residual state (Fig. 7 E). This \( V_j \) step would tend to close the Cx43 hemichannels in cell 1 to their residual state. Upon reaching a steady-state current, a whole-cell recording in current clamp mode was established in cell 1 with the third patch electrode filled with Alexa Fluor to initiate dye loading (Fig. 7 E, arrow). Fluorescence images taken 2 and 15 s after the start of dye loading are shown in Fig. 7 (B and C). The time course of fluorescence changes in both cells is plotted in Fig. 7 D. Fluorescence intensity rapidly rose in cell 1 after opening the patch in pipette 3 (arrow) and reaches a plateau after ~70 s. NFI in cell 2 shows little change during the time \( V_j \) is imposed when channels mainly reside in the residual state. Upon reopening channels by removal of \( V_j \), NFI begins to rise immediately and reaches ~14% of the maximum within 60 s (inset shows an expanded scale of NFI). Concomitant with an increase in NFI in cell 2, there is a decrease in cell 1, presumably due to rapid dye transfer to cell 2. Reimposition of \( V_j \) caused an immediate decline in NFI in cell 2 due to loss of transfer from cell 1 and dialysis with patch pipette 2. (E) Records of \( I_j \) and \( V_2 \) over time corresponding to fluorescence plot in D. \( V_j \) of +90 mV was applied to cell 2 which caused \( g_j \) to decline to a steady-state value of ~10 nS. Between 90-mV \( V_j \) steps, small repeated ±10-mV \( V_j \) steps were applied to cell 2 to assess \( g_j \), which remained constant at 43 nS. Conductance recovered rapidly upon removal of the +90-mV \( V_j \) step. Arrow indicates opening of the patch in pipette 3.
DISCUSSION

In this paper, we sought to examine the conductance and permeability properties of the residual state of Cx43 GJ channels. The finding that GJ channels are sensitive to Vj demonstrated the distinctive property of residual conductance in which gj declines to a nonzero conductance plateau (Harris et al., 1981). The residual gj resembles a voltage-insensitive conductance whose magnitude has been shown to be connexin-specific, ranging from a small fraction to as large as ~50% of the maximum gj (Werner et al., 1989; Willecke et al., 1991; Rook et al., 1992; Moreno et al., 1995; White et al., 1995; Steiner and Ebihara, 1996; Revilla et al., 1999). Single-channel studies have shown that the residual gj is explained by gating of GJ channels to a long-lived substate (Weingart and Bukauskas, 1993; Moreno et al., 1994). Weingart and Bukauskas (1993) termed this substate as the “residual state” and demonstrated it to be a property of different connexins (Bukauskas et al., 1995; Bukauskas and Peracchia, 1997; Valiunas et al., 1997), and proposed that the residual state represents the maximally closed state for this gate. GJ channels also can close completely (i.e., to a nonconducting state in response to Vj or Vm; Bukauskas and Weingart, 1994; Banach and Weingart, 2000), but we have shown that full closures are the result of a separate gate (Bukauskas et al., 2000, 2001).

Although in most homotypic channels large Vjs are often necessary to invoke gating to the residual state, heterotypic channels can have gj-Vj relations that are shifted so strongly that most of the time the channels reside in the residual state at Vjs close to zero. Our unpublished data as well as data of Elenes et al. (2001) show that a substantial fraction of heterotypic Cx45/Cx43 or Cx45/Cx47 channels are closed at Vj = 0 mV by reason of high Vj sensitivity of the slow gating mechanism. In addition, Vj gating sensitivity of Cx45/Cx43 or Cx45/Cx47 channels at Vj is relatively negative on Cx45 hemichannel side is almost twice higher than Vj gating sensitivity of homotypic Cx45 channels. This effect may be due to the relatively small unitary conductance of Cx45 hemichannel in comparison with unitary conductance of Cx43 or Cx47 hemichannel. Thus, het-

than that expected by the decrease in conductance, and can be explained by a small number of channels opening occasionally (Fig. 7 D, inset). Upon removal of Vj, conductance rapidly recovered and fluorescence immediately started to rise in cell 2 concomitant with a decrease in fluorescence in cell 1. These results are indicative of rapid flux of dye from cell 1 into cell 2. A second depolarization of cell 2 by 90 mV again halted dye flux into cell 2; the decrease in fluorescence in cell 2 is likely due to dye loss into the patch pipette. Four additional experiments similarly showed no appreciable transfer of Alexa Fluor through Cx43 GJ channels residing in the residual state.

A similar result was obtained using ethidium bromide, a positively charged dye, is restricted when channels are in the residual state. (A) Schematic of a cell pair and the arrangement of pipettes used for recording and dye-loading. Fluorescence was measured in Novikoff cell pairs as described in Fig. 7. (B) Plot of NFI in cell 1 and cell 2 over time. NFI in the cell 1 increases upon opening the patch in pipette 3 (solid arrow) and approaches a plateau in ~100 s. NFI in cell 2 shows no change during the time a Vj step of +90 mV was imposed and channels mainly reside in the residual state. Upon reopening channels by removal of Vj, NFI begins to rise and reaches ~5% of the maximum within 150 s (inset shows an expanded scale of NFI; horizontal dotted line indicates zero fluorescence level). (C) Records of Ij and Vj over time corresponding to records of Ij and Vj over time corresponding to expanded scale of NFI; horizontal dotted line indicates zero fluorescence plot in B. Vj of +90 mV was applied to cell 2, which caused gj to decline to a steady-state value of ~9 nS. Small repeated ±30 mV Vj steps were used to assess gj, which recovered rapidly up to 35 nS level upon a removal of long +90 mV Vj step.

Figure 8. Intercellular transfer of ethidium bromide, a positivelycharged dye, is restricted when channels are in the residual state.

1. A.
2. B.
3. C.

| Vj (mV) | gj (nS) |
|--------|--------|
| -100   | 10     |
| 0      | 5      |
| 100    | 0      |

and cell 2 was less than that of Alexa Fluor (Fig. 8). The cell pair in the example shown had a gj = 35 nS. No transfer of ethidium bromide was detectable while the channels were in the residual state. Dye transfer immediately followed removal of the imposed Vj (Fig. 8 B, inset). As previously shown, recovery from closure to the residual state in Cx43 channels is rapid, within a few seconds (Bukauskas et al., 2001). In this experiment, Vj was such that cell 1 was relatively positive, which would assist transfer of the positively charged ethidium bromide. Similar results were obtained in four other cell pairs with conductance ranging from 20 to 55 nS.
Hemichannels that close at relatively negative Vj in Cx43 homotypic channels are largely gated to closed or to the residual states at Vj close to zero. Studies of the residual state must keep in mind that GJ channels are composed of two hemichannels that can be the same or different. Vj gating, being a property intrinsic to the hemichannel (Trexler et al., 1996; Oh et al., 2000), means that GJ channels possess two sets of gates that are oppositely oriented with respect to the field generated by Vj. Thus, imposition of a Vj of a given polarity tends to close one hemichannel and keep the other open or even promote its opening if it was closed. The opposite polarity of Vj reverses this configuration. I-V curves for the residual state derived from Ij transitions induced by positive and negative voltages applied to one cell of a pair are not likely the result of closure of the same hemichannel and, thus, do not provide a measure of the I-V characteristics of the residual state. Using a combination of Vj ramp and step protocols, we were able to show at macroscopic (Fig. 1) and single-channel (Figs. 2–4) levels that the residual state of Cx43, when generated by Vj gating of one of the hemichannels, displays Ij rectification. In symmetric KCl solutions, the Ij rectification is larger when the gated hemichannel side is relatively negative. Gating by Vj in Cx43 homotypic channels is such that hemichannel on the relatively negative side closes to the residual state. In Cx43 homotypic channels, rectification of the residual state is moderate, changing approximately twofold over a range of ±100 mV.

Oh et al. (2000) similarly demonstrated Ij rectification of the residual state in homotypic Cx32 GJ channels. Like in Cx43 GJ channels, gating by Vj in Cx32 is such that the hemichannel on the relatively negative side of the channel closes to the residual state. Interestingly, the direction of rectification is the same as in Cx43, with larger current flowing through the residual state when the closed hemichannel side is made relatively negative. These data are consistent with a mechanism proposed for Vj gating to the residual state that involves the formation of a gating barrier by charges in the NH2 terminus. Mutational studies have shown that the Vj sensor is composed of a charge complex located in the NH2 terminus whose net charge can be of either sign, and whose movement toward the channel pore is associated with hemichannel closure to the residual state (Verselis et al., 1994; Oh et al., 2000). Hemichannels that close at relative negativity on their side, such as Cx32 and Cx43, should contain a positively charged NH2-terminal sensor. This form of voltage dependence is only sensitive to Vj and is insensitive to the cells’ plasma membrane voltage (Verselis et al., 1991). For this reason, the voltage sensor has been proposed to be positioned in the pore where the transjunctional field would be constant for the same Vj regardless of the cells’ transmembrane voltage (Harris et al., 1981). From modeling studies with Poisson-Nernst-Plank (PNP) theory (Chen and Eisenberg, 1993), Oh et al. (1999) proposed that the conformational change associated with Vj gating involves a narrowing of the cytoplasmic entry of the channel that increases the electrostatic influence of the NH2-terminal sensor on ionic flux through the pore. This electrostatic influence creates an asymmetric charge profile within the pore that produces Ij rectification. For both Cx32 and Cx43, the gating barrier should be positively charged and would tend to produce the same direction of rectification in both types of channel. Our data are consistent with this mechanism for Vj gating.

According to this mechanism for Vj gating, closure of one hemichannel to the residual state should not only produce channel rectification, but also should change the charge selectivity characteristics of the channel. In addition, the concomitant narrowing of the pore should reduce the cutoff size for permeant molecules. Neither of these possibilities had been tested previously, and we used a combination of ion substitution and dye flux approaches. Replacement of either K+ with TEA+ or Cl− with Asp− results in a substantial reduction in the open state conductance. This result can be explained if both K+ and Cl− contribute substantially to the current flowing through the open channel and is consistent with our previous studies using measurements of Erev in KCl gradients that showed no appreciable selectivity between monovalent inorganic ions on the basis of charge (Trexler et al., 2000). However, the same substitutions had substantially different effects on the residual state. Fig. 9 A summarizes the data for three different pipette solutions, plotted as the ratio of residual to open channel conductance, γres/γopen as a function of Vj. Over most of the Vj range between ±125 mV, γres/γopen is substantially smaller in KAsp than in KCl and rectification is considerably steeper. Conversely, this ratio is larger in TEACL and rectification is nearly absent. A γρ plot shown in Fig. 9 B demonstrates the dependence of single-channel conductance on the conductivity of the pipette solutions. Closed circles correspond to the conductance of the open state, and open circles to γres evaluated at Vj = 0 mV. Closed and open triangles show γopen and γres, respectively, when pipette solutions contained mainly TEA+Asp− (Valiunas et al., 1997). Solid and dashed lines are regression curves of the second order calculated for open and residual states, respectively; dotted lines show confidential intervals separately for each regression line.

To qualitatively evaluate our results, we used 1-D PNP model (Chen and Eisenberg, 1993) to test whether a charge profile that introduces positive charge at one end of the channel can qualitatively explain the data. Fig. 10 A shows a schematic of the channel pore as a cylinder 100 Å in length and 10 Å in diameter. A charge locus is shown as a square, and z indicates the number of elementary charges. Fig. 10 B shows a family of Ij-Vj...
curves for the channel bathed in symmetric 140 mM KCl with $z = +6$ or $z = -6$ and located at different locations along the pore (symbol on the curve corresponds to the symbol shown in Fig. 10 A). These $I_{ij}-V_j$ plots show that a positive charge induces rectification of the $I-V$ plot, resulting in a conductance decrease when $V_j$ changes from negative to positive (i.e., $I_j$ rectification is in the same direction as we found experimentally). When the charge is negative (Fig. 10 A, closed circle), rectification of the $I_{ij}-V_j$ plot is opposite in direction. The degree of rectification is lower when the charge is located closer to the center of the channel. The solid line shows the $I_{ij}-V_j$ plot for the channel without charge (i.e., $z = 0$). These rudimentary evaluations simply show that the $\gamma_{res}$ dependence on $V_j$ observed with the fast gate closed can be induced by introducing a positive charge located closer to the cytoplasmic end of the hemichannel. This makes the gated channel preferentially selective for anions; Fig. 10 B (inset) shows the ratio of ionic fluxes for $Cl^-$ and $K^+$.

To test whether this simple model explains our experimental data demonstrating $I_j$ rectification dependence on ionic composition, we used a charge profile (Fig. 10 C) consisting of positive charge at either cytoplasmic end, representing NH$_2$-terminal charges, and negative charge closer to the center representing charges in E1 or near the TM1/E1 border (Oh et al., 1999; Trexler et al., 2000). In symmetric KCl, this channel exhibits a linear $I-V$ relation (Fig. 10 D, solid line) and nearly equal permeability to $K^+$ and $Cl^-$ (not shown). An increase in positive charge at one end, as a result of gating of one hemichannel to the substate, produces rectification such that current is larger when the side (hemichannel) that closes to the substate is made relatively negative (Fig. 10 D, dashed line). Narrowing of the pore was needed to substantially reduce the conductance. These data are plotted as the ratio $\gamma_{res}/\gamma_{open}$ in Fig. 10 E (solid line). By reducing the diffusion coefficient for the anion $\sim$2.5-fold, to account for substitution of $Asp^-$ for $Cl^-$, rectification is made slightly steeper (Fig. 10 E, dashed line). Conversely, reducing the diffusion coefficient for the cation the same amount reduces rectification (Fig. 10 E, dash-dot line). These changes in rectification are qualitatively similar to the experimental results, but considerably smaller in magnitude. Further reducing the diffusion coefficient of either anion or cation could not explain the results. Closer agreement with the data could be obtained by assuming that the organic ions were permeant and exhibited binding in the channel (Fig. 10 F). The binding was modeled as a distributed negative ($-0.2$) or positive charge ($+0.2$) for Asp and TEA, respectively. Such binding is also consistent with the observed changes in open channel conductance upon substitution with either TEA or Asp; the magnitude of the reduction in either case was $\sim$60%, greater than that expected by the reduced mobility of either the cationic or anionic component of the current, and suggestive of interactions within the channel that impede ionic fluxes. Although we did not attempt to replace both K and Cl, small but observable current flow through the residual state was reported in symmetric TEA-Asp salts, indicating these ions are permeable (Valiunas et al., 1997). Also, other studies have indicated that tetraalkylammonium ions and polyamines can display voltage-dependent block in homotypic Cx43 channels, which is consistent with their binding in the pore (Veenstra, 2001). Thus, our results suggest that the large changes in rectification upon substitution of larger organic ions can be explained by a combination of an electrostatic effect of the gating barrier and an electrostatic effect of the organic ions themselves permeating through the pore. We recognize that our model is rudimentary, and the assertion of a distributed charge contributed by binding of organic ions remains untested. More rigorous modeling will have to await more detail about the structure of the GJ channel pore.

Figure 9. (A) Summarized $\gamma_{res}/\gamma_{open}-V_j$ plots (from experimental data) for three different pipette solutions. Rectification is steeper in symmetric KAsp and reduced in symmetric TEACL. (B) Plot of single-channel conductance versus conductivity of the pipette solutions. Closed and open circles correspond to $\gamma_{open}$ and $\gamma_{res}$ respectively. Closed and open triangles are data for $\gamma_{open}$ and $\gamma_{res}$ respectively, in symmetric TEA-Asp taken from Valiunas et al. (1997). Solid and dashed lines are regression lines of the second order for $\gamma_{open}$ and $\gamma_{res}$ respectively; dotted lines show confidential interval.
Dye transfer between cells generally has been used to evaluate whether there is diffusional cell–cell communication mediated by GJs. It is well established that open Cx43 channels are permeable to both mono- and divalent negatively and positively charged dyes, which is consistent with its poor charge selectivity (Larson et al., 1992; Steinberg et al., 1994; Veenstra et al., 1995; Elfang et al., 1995, Bukauskas et al., 2000; Verselis et al., 2000). In addition, our unpublished data show that Cx43 channels, like channels formed by many other members of the connexin gene family, form quite large pores. Examining cell–cell transfer of Alexa Fluor, we show that the high permeability characteristic of the open state is nearly abolished in the residual state. We devised an experimental procedure where we could examine dye transfer in the same cell pair under conditions in which the channels reside primarily in the open state or in the residual state. $V_j$ steps were used to transfer the channel from the open to the residual state (Figs. 7 E and 8 C). Although we did not rigorously quantify permeability, it is clear from Fig. 7 D that intercellular flux of Alexa Fluor is reduced to a much greater extent in the residual state than predicted simply by the change in conductance. This occurs even with an increased preference for anions in the residual state and $V_j$ gradient that favored dye transfer. Thus, it is likely that Alexa Fluor is impermeant in the residual state. The very small amount of flux observed is likely due to a small fraction of channels that occasionally gate to the open state. The
same conclusion is likely true for ethidium bromide, but transfer rates were low even in the open state. Several factors may be responsible for the difference in dye transfer of Alexa Fluor and ethidium bromide including the following: (1) Alexa Fluor remains free in the cytoplasm and does not bind to intracellular compounds, whereas ethidium bromide binds strongly to DNA, and its free concentration remains low until binding saturates; (2) fluorescence intensity of Alexa Fluor remains constant, whereas fluorescence intensity of ethidium bromide increases when it is bound to DNA.

In summary, rectification of a Cx43 channel closed to the residual state can be explained by the introduction of positive charge in the pore. With the knowledge that the Cx43 channel is gated from the side with negative $V_j$ and the observation that $\gamma_{es}$ decreases when $V_j$ is made increasingly positive on the closed side, it would appear that the positive charge is most likely located close to the cytoplasmic vestibule of the gated hemichannel as proposed for Cx32 by Oh et al. (1999). In addition, gating to the residual state makes the Cx43 channel more anion-selective. Although the introduction of charge can explain the rectification, it cannot explain the reduced conductance of the gated channel, which is about five times smaller than that of the open state. Thus, the conformational change that results from gating to the residual state also leads to a significant narrowing of the channel pore that decreases channel conductance and reduces the cutoff size for permeant molecules. Our data indicate that molecules similar in size to fluorescent dyes (i.e., ~500 D) are excluded. Consequently, the $V_j$ gating mechanism can serve as a selectivity filter that preserves electrical cell-cell communication but can limit the communication of metabolic or biological signaling molecules.

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