Cx40 and Cx43 expression ratio influences heteromeric/heterotypic gap junction channel properties

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Cottrell, G. Trevor, Yan Wu, and Janis M. Burt. Cx40 and Cx43 expression ratio influences heteromeric/heterotypic gap junction channel properties. Am J Physiol Cell Physiol 282: C1469–C1482, 2002; 10.1152/ajpcell.00484.2001.—In cells that coexpress connexin (Cx)40 and Cx43, the ratio of expression can vary depending on the cellular environment. We examined the effect of changing Cx40:Cx43 expression ratio on functional gap junction properties. Rin cells transfected with Cx40 or Cx43 (Rin40, Rin43) were cocultured with 6B5n, A7r5, A7r540C1, or A7r540C3 cells for electrophysiological and dye coupling analysis. Cx40:Cx43 expression ratio in 6B5n, A7r5, A7r540C1, and A7r540C3 cells was ~1:1, 3:1, 5:1, and 10:1, respectively. When Rin43 cells were paired with coexpressing cells, there was an increasing asymmetry of voltage-dependent gating and a shift toward smaller conductance events as Cx40:Cx43 ratio increased in the coexpressing cell. These observations could not be predicted by linear combinations of Cx40 and Cx43 properties in proportion to the expressed ratios of the two Cxs. When Rin41 cells were paired with coexpressing cells, the net voltage gating and single-channel conductance behavior were similar to those of Rin40/Rin40 cell pairs. Dye permeability properties of cell monolayers demonstrated that as Cx40:Cx43 expression ratio increased in coexpressing cells the charge and size selectivity of dye transfer reflected that of Rin40 cells, as would be predicted. These data indicate that the electrophysiological properties of heteromeric/heterotypic channels are not directly related to the proportions of Cx constituents expressed in the cell; however, the dye permeability of these same channels can be predicted by the relative Cx contributions.

Though communal sharing of ions, second messengers, and metabolites between cells, gap junctions influence organism development, growth, death, and homeostatic control. Unfortunately, the specific mechanisms by which gap junctions regulate such cellular and tissue responses are unclear and how intercellular flux of molecules is regulated and differentiated in different cell systems has not been determined.

Twenty mammalian gap junction genes (connexins; Cxs) have been identified to date (K. Willecke, J. Eiserger, J. Deyen, D. Eckhardt, A. Romualdi, M. Gueldenagel, U. Deutsch, and G. Soehl, unpublished observations), with Cxs being expressed in almost all mammalian organ systems. Different patterns of Cx expression in differing tissue systems suggest that there may be a specificity of molecular movement through gap junctions. Such specificity of signaling control is complicated by the coexpression of multiple Cxs in a given cell. Coexpression of more than one Cx in a cell creates the potential for the interaction of multiple Cx types in the formation of a heteromeric connexon. In addition, dissimilar connexons, contributed by neighboring cells, can dock to form heterotypic channels, thus adding to channel complexity. The degree to which Cx heterooligomerization can occur between differing Cxs is currently unclear; however, many heterologous interactions of this nature have been identified to date (2, 6, 7, 13, 31). The physiological consequences of heterologous gap junction channel formation on intercellular communication remain unclear.

Cx40 and Cx43 are gap junction proteins that are frequently colocalized in cells of the cardiovascular system (26, 29, 32) and have been demonstrated to interact in the formation of heteromeric/heterotypic gap junction channels (12, 16, 24, 25). A convenient model for the study of these interactions is the neonatal rat aortic smooth muscle cell line A7r5, which naturally coexpresses both Cx40 and Cx43 (20). Recently, it was observed that growth-arrested A7r5 cells have an increased ability to transfer Lucifer yellow dye compared with cells cultured in the presence of growth serum (18). These differences occurred despite comparable electrical coupling under the two conditions. In essence, the growth signal limited the ability of A7r5 cells to transfer large molecules while at the same time maintaining electrical excitability between cells, thus suggesting that there was an alteration in permselectivity of the channels. These observations correlated with more recent data showing that as the Cx40:Cx43 expression ratio increased in genetically modified A7r5 cells, dye permeability decreased (9). Together these observations suggest that the chronic presence of a growth stimulus results in alterations in expression/degradation rates of Cx40 and Cx43 in A7r5 cells such that the Cx40:Cx43 ratio increases, and this in turn decreases large molecule transfer between cells.
Little work has been done to characterize the effects of changing Cx expression ratios on channel function. Bevans et al. (5) examined reconstituted Cx26/Cx32 heteromeric connexons in liposomes and concluded that there was a relationship between Cx26 fraction in the liposome and cGMP permeability but not cAMP permeability. Gu et al. (15) studied pH sensitivity of Cx40/Cx43-coexpressing oocytes and found that the Cx expression ratio could predict pH gating sensitivity; however, the extent of heteromerization in this model is uncertain. Oh et al. (21) used a heteromeric gap junction model to successfully determine the stoichiometry of gap junction voltage gating. These studies commonly suggest a predictability of gap junction assembly in coexpressing cells, such that changing expression ratios result in a shift in channel behavior to that of the dominantly expressed Cx.

In theory, if a cell type expresses both Cx40 and Cx43 isoforms, and if they are free to associate in a random fashion to form connexons, the cell could contain at least 14 different connexons, 12 heteromeric and 2 homomeric. If each of these 14 connexons can dock with all of the others, then pairwise two such coexpressing cells could result in as many as 196 different channel types, more if the connexons are allowed to rotate relative to one another. The predominance of a given channel type in this setting would depend on the isoform expression ratio. If random assembly occurs in coexpressing cells, and if channel behavior reflects the majority composition of its constituents, then as the Cx40:Cx43 ratio is increased in a coexpressing cell, overall channel behavior should shift to reflect a Cx40 channel phenotype. Alternatively, channel assembly may not be random and channel properties may not reflect relative expression ratios.

We explored the effect that altering the Cx40:Cx43 expression ratio has on electrophysiological and dye permeability properties of cells coexpressing these Cxs. It was determined that channel electrophysiological properties do not reflect behaviors of the predominant Cx; however, the dye permeability characteristics in these same cell types are predicted by the relative expression ratio of the Cx constituents.

**MATERIALS AND METHODS**

**Cells and cell culture.** Experiments used the following stable cell lines, which were previously characterized: Rin43, rat insulinoma cell line transfected with Cx43 (1); Rin40, rat insulinoma cell line transfected with Cx40 (12); A7r5, neonatal rat aortic smooth muscle cell naturally coexpressing Cx40 and Cx43 (American Type Culture Collection, Manassas, VA); and 6B5n, A7r5 cell line transfected with Cx43 antisense oligomer (9). To develop cell lines with varied Cx40: Cx43 expression ratios, A7r5 cells were transfected with pcDNA3 (Invitrogen, Carlsbad, CA) containing a 1.1-kb fragment of rCx40 that included the entire coding domain. Transfection was accomplished with Lipofectamine Plus reagent (Life Technologies, Rockville, MD). Stable transfectants were selected with 300 μg/ml Geneticin (Life Technologies). Four subclones were harvested and labeled as A7r540C1, A7r540C2, A7r540C3, and A7r540C4. A7r5-derived cell lines and Rin-derived cell lines were cultured as previously described (12).

**Western blot analysis and protein quantification.** Polyclonal Cx40 antibody was raised in rabbits against amino acids 231–331 of Cx40 fused to glutathione S-transferase protein. Antibody purification was performed as described previously (14). The antibody failed to react with Cx43 or Cx43 COOH terminus standard but did react well with Cx40 and Cx40 COOH terminus peptide. A7r5 transfected subclones were assayed for Cx40 and Cx43 content as previously described (9). Briefly, total protein isolate was electrophoretically separated on 10% SDS gels and transferred to nitrocellulose (Hybond ECL, Amersham Pharmacia Biotech). After a blocking step, nitrocellulose membranes were exposed to polyclonal, affinity-purified Cx43 (Sigma, St. Louis, MO) or Cx40 antibodies. Blots were then incubated with 35S-labeled anti-rabbit IgG (0.4 μCi/ml; Amersham Pharmacia Biotech). Blots were visualized for position-specific radioactivity with an Instant Imager (Packard Instruments). Each gel was loaded with several samples, protein ladder, and either Cx40 or Cx43 standards. The standards were loaded in a staggered fashion, in the same lane, in 0.5-, 1-, 2-, and 4-pmol amounts. The counts from these standards were used to construct a standard curve against which the Cx content of the samples could be compared.

**Immunofluorescence.** For immunofluorescence labeling, cells were subcultured at high density on coverslips in six-well plates. After a 24- to 48-h incubation period, cells were washed three times in divalent cation-free phosphate-buffered saline (PBS) and fixed with methanol at −20°C for 5 min. Coverslips were washed, permeabilized with 0.2% Triton X-100 in PBS for 30 min, washed again, and then incubated for 15 min in 0.5 M NH4Cl in PBS. Cells were then washed twice with PBS and incubated for 10 min with a blocking reagent featuring 4% fish skin gelatin (Sigma) and 1% goat serum (Sigma) in PBS. Primary colabeling was performed with mouse monoclonal anti-Cx43 antibody (Chemicon) and rabbit polyclonal anti-Cx40 antibody suspended in blocking reagent at dilutions of 1:200 and 1:300, respectively. Two to three hours of antibody treatment was sufficient for effective labeling, although some slides were incubated overnight with equal effect. After a washing step, secondary antibody colabeling was performed with either goat anti-rabbit Cy2 antibody and goat anti-mouse Cy5 antibody or goat anti-mouse Cy2 and goat anti-rabbit Cy5, all at a 1:200 dilution in blocking buffer (all secondary antibodies from Jackson Immunoresearch Laboratories). Cells were then washed twice in blocking buffer and once in PBS for 10 min. Coverslips were mounted on microscope slides in Fluoromount G (Southern Biotechnology Associates) and examined with an Olympus BX50WI microscope with appropriate filters. Images were captured with a SenSys KAF1401 charge-coupled device (CCD) camera (Photometrics, Tucson, AZ) and analyzed with V++ imaging software (Digital Optics).

**Electrophysiology.** A fluorescent tag was used to distinguish between cell types in coculture during electrophysiology experiments (12). Dual whole cell voltage clamp experiments were performed on Rin43/Rin43, Rin40/Rin40, Rin40/Rin43, Rin43/6B5n, Rin43/A7r540C1, Rin43/A7r540C3, Rin40/6B5n, Rin40/A7r540C1, and Rin40/A7r540C3 cell pairs. All cells were grown to confluence before trypsinization (0.25% trypsin in Ca2+/-Mg2+/-free PBS) and were replated at low density on glass coverslips. Cells were then incubated for 1–12 h at 37°C before electrophysiological analysis.
Dual whole cell voltage clamp was carried out as reported previously (16, 18). Electrodes were fabricated from 1.2-mm filament glass (A-M Systems, Everett, WA) on a Sutter Instruments puller (Novato, CA) and were filled with (in mM) 124 KCl, 14 CsCl, 9 HEPES, 9 EGTA, 0.5 CaCl₂, 5 glucose, 9 tetraethylammonium chloride, 3 MgCl₂, 5 Na₂ATP (320 mosmol/kg H₂O, pH = 7.2). After the dual whole cell voltage-clamp configuration was achieved, both cells were held at 0 mV and then alternately stepped to −10 mV to determine macroscopic junctional conductance (g_j). Voltage-dependent gating was evaluated as previously described (16) in cell pairs in which the g_j and series resistance values ensured accurate space clamp with an error rate of ≤10% in the normalized data. Single-channel events were studied in cell pairs with one or a few functional channels (g_j < 0.5 nS) with a 40-mV transjunctional voltage (V_j) applied for durations >20s. The uncoupling agent halothane was used only for Rin43/Rin43 and Rin40/Rin40 experiments; no uncoupling agents were used for any other experiments. No differences in single-channel conductances were observed for Rin43 or Rin40 in the presence or absence of halothane.

Dye transfer. Dye transfer was evaluated in both homogeneous and heterogeneous cell monolayers. For homogeneous cell monolayers, cells were plated at confluent density (15,000–20,000 cells/cm²) onto glass coverslips in combination with the same cell type, prelabeled with the cell marker DiD (Molecular Probes), and plated at a lower density (~1,000 cells/cm²). Prelabeling of cells with DiD was accomplished with a 10 μM DiD solution in PBS for 30 min at 37°C before lifting and replating.

For heterogeneous cell monolayers, Rin40 or Rin43 cells were plated on coverslips at confluent density (~20,000 cells/cm²) in combination with 6B5n, A7r5, or A7r540C3 cells, prelabeled with DiD, at a low density (~1,000 cells/cm²). The goal of both homogeneous and heterogeneous cell coculturing was to have 1 DiD-labeled cell surrounded by 20–50 unlabelled cells. After a 24-h incubation period, coverslips were mounted in a perfusion chamber and visualized on a microscope equipped with differential interference contrast and fluorescence optics.

Four separate dyes were used to assess gap junction function: 2-[4-(nitro-2,1,3-benzoxadiol-7-yl)aminoethyl]trimethylammonium [NBD-TMA; mol wt 280, net charge 1⁺, 5 mM; kindly provided by Dr. Stephen Wright, University of Arizona; see Bednarczyk et al. (4)], Alexa 350 (mol wt 326, net charge 1⁻, 10 mM), Alexa 488 (mol wt 548, net charge 2⁻, 10 mM), and Alexa 594 (mol wt 736, net charge 2⁻, 10 mM) (all Alexa dyes from Molecular Probes). Microelectrode tips were filled by capillary action with one of the four dyes, backfilled with 200 mM KCl, and lowered onto the surface of the cell. Cells were impaled by the overcompensation of the capacitance feature of the amplifier (A-M Systems), withdrawn after 10 s, and the cells receiving dye were counted after 3 min. The percentage of cells in contact with the injected cell that received dye was compared between groups.

Data analysis. Nonlinear regression of voltage-dependent gating was performed with the Boltzmann equation of the form

\[ \frac{G_{\text{norm}}}{G_{\text{norm}}} = \frac{G_{\text{max}} - G_{\text{min}}}{1 + \exp[(V_j - V_{\text{fit}})/z]} + G_{\text{min}}, \]

where \(G_{\text{norm}}\) is the normalized steady-state conductance \((G_{\text{norm}}/G_{\text{max}})\) and \(G_{\text{max}}\) and \(G_{\text{min}}\) are normalized maximum and minimum conductance, respectively. \(z = q/kT\) reflects equivalent charge movement, with \(q\) being the number of charges moving through the applied voltage field, \(k\) representing the valence electron charge, \(K\) is the Boltzmann constant, and \(T\) is temperature in degrees Kelvin. \(V_j\) represents transjunctional voltage, and \(V_{\text{fit}}\) is the half-maximum inactivation potential. \(G_{\text{norm}}\) values for each voltage polarity and each treatment group were pooled and simultaneously fit with SigmaPlot 2001 software (SPSS; iterations = 100, step size = 100, tolerance = 0.0001). \(G_{\text{norm}}\), \(z\), and \(V_{\text{fit}}\) parameters were initially estimated to test the accuracy of fit, with fits being considered adequate if all outcome values were unaltered with changing initial parameters. The pooling of all the data for a given treatment group did not permit statistical comparisons between groups but did provide \(G_{\text{min}}\) and \(V_{\text{fit}}\) values that could be subjectively compared and an \(R^2\) value that served as an overall indicator of variability within the treatment group.

Single-channel recordings were digitized for analysis with the software developed by Ramanan and Brink (23) as previously reported by He et al. (16). Single-channel event transitions were manually counted, normalized, and arranged into 10-pS bins for histogram comparisons.

Dye transfer was compared for each dye across treatment groups with a single factorial ANOVA. \(t\)-Tests were used to compare NBD-TMA vs. Alexa 350 (similar size, opposite charge), and Alexa 350 vs. Alexa 594 (different size, same charge) transfer within treatment groups.

**RESULTS**

**Protein quantification.** Quantification of protein expression ratios with a protein standard and 35S-labeled secondary antibody was previously shown to be both highly accurate and reliable (9). Four Cx40-transfected A7r5 subclones were evaluated on two separate occasions, with representative Western blots presented in Fig. 1, A and B. A7r540C1, A7r540C2, A7r540C3, and A7r540C4 subclones had average Cx40:Cx43 ratios of 5.3:1, 10.7:1, 10.6:1, and 14.4:1, respectively (Fig. 1C).

Nontransfected parental A7r5 cells have an average Cx40:Cx43 expression ratio of 2.7:1 (9), indicating that Cx40 transfection was sufficient to increase the expression ratio in the subclones examined. 6B5n cell Cx40:Cx43 expression ratio was previously reported as 1.5:1 (9); therefore, to study a range of expression ratios, 6B5n, A7r5, A7r540C1, and A7r540C3 cell lines were used in further experiments.

**Immunofluorescence.** There is evidence that A7r5 cells display significant colocalization of Cx40 and Cx43 protein (11). To confirm that the transfected cells had both Cx40 and Cx43 localized to the membrane and that these Cxs were colocalized, immunofluorescence was performed. Coimmunolabeling of 6B5n cells revealed prominent staining of gap junction plaques for both Cx40 and Cx43 (Fig. 2A). Overlaying of the Cx40- and Cx43-immunolabeled images showed many areas of colocalization (yellow regions) as well as regions where only Cx40 or Cx43 was observed independently, although the resolution inherent in the staining procedure may limit the levels at which proteins can be visualized. A7r5 cells had strong Cx40 staining and relatively weaker Cx43 staining (Fig. 2B), whereas A7r540C1 and A7r540C3 cells had dominant Cx40 immunostaining with less observable Cx43 in plaque regions (Fig. 2, C and D). In all cases, the immunofluorescence data strongly correlated with protein expression levels in the same cell types. On rare occasions, some cells had strong Cx43 plaques colocalized to regions of weak Cx40 staining (not shown). These differ-
ences may be indicative of changing Cx expression with progress through the cell cycle. In either case, all cell types appeared to form gap junction plaques in which Cx40 and Cx43 proteins were colocalized.

**Voltage-dependent gating.** Rin43/Rin43, Rin40/Rin40, and Rin40/Rin43 cell pairs had voltage-dependent gating responses similar to those previously described for these channel types under similar conditions (Fig. 3, Table 1; Refs. 1, 8, 12, 16). When Rin43 cells were paired with 6B5n cells, there was increased variability between experiments, as denoted by a shift to lower $R^2$ values, and a slight asymmetry in gating response, with the greatest mean voltage gating effect occurring when the Rin43 cell was relatively positive (6B5n negative, with $V_o$ and $G_{min}$ values progressively decreasing. When the Rin43 cell was relatively negative, there was an overall decrease in $V_j$ sensitivity, as indicated by higher $G_{min}$ and $V_j$ values (Table 1), and a loss of ability to reduce conductance to the voltage-insensitive state with the $V_j$ ranges used in these experiments. Despite the progressively increasing Cx40: Cx43 ratio in 6B5n, A7r540C1, and A7r540C3 cells, respectively, the resultant asymmetry in $V_j$ gating is opposite to that of the homomorphic/heterotypic Cx40/Cx43 channels.

To rule out cell-specific interactions causing the behaviors observed between Rin43 and A7r540C3 cells, normal rat kidney (NRK) cells (17) and A7r540C3 cells were cocultured. Similar voltage-dependent gating was observed in these cell pairs, in which $G_{min}$ and $V_o$ were 0.49 and $-74$ mV, respectively, when the NRK cell was relatively negative and 0.28 and 50 mV, respectively, when the NRK cell was relatively positive ($n = 5$; data not shown). The similarity of voltage gating asymmetry between Rin43/A7r540C3 and NRK/A7r540C3 cell pairs suggests that the unique voltage gating events were not a cell-specific phenomenon.

Pairing Rin40 cells with A7r540C3, A7r540C1, and 6B5n cells resulted in little change in voltage-dependent gating response despite the progressive decrease in Cx40:Cx43 ratio associated with these cell types (Fig. 5). Although these cell pairs had slightly more variability between experiments than the Rin40/Rin40 cell pairs, there were no noticeable differences in mean voltage gating parameters compared with the homomorphic/homotypic Cx40 channels (Table 1).

**Single-channel analysis.** Trends in single-channel event amplitudes among Rin43/Rin43, Rin40/Rin40, and Rin40/Rin43 cell pairs were similar to those previously reported (12) and are summarized in Fig. 6. In Rin43 cell pairs, 96% of single-channel events had amplitudes between 90 and 130 pS, with no low-conductance residual state being recorded. Rin40 cell pairs had a concentration of channel events in the 150- to 180-pS range (85%). Small residual conductance events of $\sim-35$ pS were only consistently observed when $V_j$ was returned to 0 mV and therefore were not reflected in the frequency histograms. Rin40/Rin43 cell pairs had predominant conductance events in the 100- to 140-pS range; 32% of events in these cell pairs were between 130 and 160 pS, making them different from Rin43/Rin43 and Rin40/Rin40 cell pairs that had only 4–6% of events in this range. Close analysis of single-channel records revealed a 150-pS main state and a 25-pS residual state, with frequent 125-pS transitions between main and residual states being measured. Similar to previous observations, there was a voltage polarity-dependent rectification of channel conductance (20% difference in conductance), which resulted in increased variability within the event frequency histogram.

When Rin43 and 6B5n cells were paired, there was an increase in the variability of single-channel event amplitudes compared with Rin43/Rin43 pairs, although, similar to Rin43 cells, the predominant events
were within the 90- to 130-pS range (63%; Fig. 7, A and B, top). Rin43/A7r540C1 cell pairs also displayed a wide range of single-channel event amplitudes; however, the predominant events were shifted to a lower conductance (61% in 80- to 110-pS bins; Fig. 7, A and B, middle). Rin43/A7r540C3 cell pairs also displayed considerable variability of event amplitudes, and there was a further shift to a predominance of low-conductance events, with 60% of transitions occurring between 40 and 90 pS and only 37% of the events in the 90- to 130-pS bins (Fig. 7, A and B, bottom). We hypothesized that as the Cx40:Cx43 ratio increased there would be a shift in single-channel events to amplitudes larger than those observed with Rin43/Rin43 cell pairs, similar to what was observed in the heterotypic Rin40/Rin43 setting. The opposite occurred in this case. To rule out cell-specific interactions causing the behaviors observed between Rin43 and A7r540C3 cells, NRK cells were cocultured with A7r540C3 cells. These cell pairs displayed single-channel event distributions

Fig. 2. Representative expression patterns of Cx40 (green; left) and Cx43 (red; center) in 6B5n (A), A7r5 (B), A7r540C1 (C), and A7r540C3 (D) cells. Right, overlay of Cx40 and Cx43 staining, with strong areas of colocalization appearing yellow. Cx40 plaques are easily distinguished in all 4 cell types (green arrows), with Cx43 plaques only staining strongly in 6B5n cells (red arrow). There was strong colocalization of Cxs in all cell types. Scale bar, 10 μm.
similar to those observed in Rin43/A7r540C3 cell pairs; 86% of the events were between 30 and 100 pS, with peak event frequency being observed in the 50- to 60-pS bin (22%, n = 3; data not shown). The similar predominance of relatively small single-channel events between Rin43/A7r540C3 and NRK/A7r540C3 cell pairs suggests that the unique single-channel conductances observed were not a cell-specific phenomenon.

Rin40/A7r540C3 cell pairs had single-channel events that were similar to Rin40/Rin40 cell pairs, with 75% of channel events occurring within 150- to 220-pS bins; however, there was a slight increase in the proportion of smaller channel events occurring within 150- to 220-pS range; Fig. 8, A and B, middle). Rin40/6B5n cell pairs demonstrated a larger variability in single-channel events than Rin40/A7r540C3 or Rin40/A7r540C1 cells; however, the 150- to 220-pS events still predominated (Fig. 8, A and B, bottom). As the Cx40:Cx3 ratio progressively decreased in A7r540C3, A7r540C1, and 6B5n cell pairs, respectively, the channel populations were predicted to shift from those of Rin40/Rin40 cell pairs to events more similar to Rin40/Rin43 cell pairs. Although such a shift is evident, especially in the Rin40/6B5n cell pairs, the Cx40 phenotype still predominates. Perhaps ratios that favored Cx43 over Cx40 would have revealed greater variability, comparable to that observed when Rin43 cells were paired with coexpressing cells of 5:1 and 10:1 Cx40:Cx43 expression ratios.

**Dye transfer.** Alexa 350, Alexa 488, and NBD-TMA all transferred equally well between Rin43 cells, indicating absence of charge selectivity. Cx43 channels did display significant size selectivity, with Alexa 594 transferring to fewer neighboring cells than Alexa 350 (73.3 ± 5.6% vs. 91.1 ± 2.4%; Fig. 9A). When 6B5N, A7r5, or A7r540C3 cells were cocultured with Rin43

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### Table 1. Summary of macroscopic junctional conductance measures and Boltzmann fit data for each treatment group

<table>
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<tr>
<th>Treatment</th>
<th>n</th>
<th>g_{0}, nS</th>
<th>G_{min}</th>
<th>z</th>
<th>V_{m}, mV</th>
<th>R^2</th>
<th>G_{min}</th>
<th>z</th>
<th>V_{m}, mV</th>
<th>R^2</th>
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<tbody>
<tr>
<td>Rin43/Rin43</td>
<td>6</td>
<td>1.9 ± 0.6</td>
<td>0.33 ± 0.02</td>
<td>-5.9 ± 3.3</td>
<td>-64 ± 3</td>
<td>0.94</td>
<td>0.36 ± 0.02</td>
<td>4.3 ± 1.8</td>
<td>61 ± 1</td>
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<td>8</td>
<td>3.2 ± 0.8</td>
<td>0.29 ± 0.03</td>
<td>-4.1 ± 1.4</td>
<td>-40 ± 2</td>
<td>0.82</td>
<td>0.3 ± 0.03</td>
<td>5.2 ± 2.4</td>
<td>40 ± 2</td>
<td>0.82</td>
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<tr>
<td>Rin40/Rin43</td>
<td>6</td>
<td>1.9 ± 0.7</td>
<td>0.15 ± 0.06</td>
<td>-2.2 ± 0.7</td>
<td>-42 ± 4</td>
<td>0.74</td>
<td>0.26 ± 0.11</td>
<td>1.9 ± 0.6</td>
<td>69 ± 6</td>
<td>0.82</td>
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<tr>
<td>Rin43/6B5n</td>
<td>15</td>
<td>3.7 ± 0.7</td>
<td>0.3 ± 0.05</td>
<td>-2.5 ± 0.5</td>
<td>-67 ± 3</td>
<td>0.8</td>
<td>0.23 ± 0.06</td>
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<td>55 ± 4</td>
<td>0.76</td>
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<td>15</td>
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<td>0.3 ± 0.05</td>
<td>-2.5 ± 0.5</td>
<td>-67 ± 3</td>
<td>0.8</td>
<td>0.23 ± 0.06</td>
<td>1.9 ± 0.4</td>
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<td>0.76</td>
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<td>11</td>
<td>3.3 ± 0.7</td>
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<td>0.73</td>
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<td>3.9 ± 1.3</td>
<td>0.43 ± 0.07</td>
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<td>-72 ± 3</td>
<td>0.84</td>
<td>0.22 ± 0.13</td>
<td>1.3 ± 0.7</td>
<td>31 ± 9</td>
<td>0.41</td>
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<td>31 ± 9</td>
<td>0.41</td>
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</tbody>
</table>

Values are means ± SE. Negative or positive transjunctional voltage (V_j) refers to the holding potential of the homomeric connexon-expressing cell (Rin40 or Rin43) when paired with coexpressing cells. In Rin40/Rin43 cell pairs it refers to the holding potential of the Rin43 cell and is arbitrarily assigned to the pulsed cell of Rin43/Rin43 and Rin40/Rin40 cell pairs. G_{min}, residual conductance of voltage-inactivated junction; z, gating charge; V_{m}, transjunctional membrane potential at which one-half of the channels are gated closed; R^2, variance of fit for the nonlinear regression.

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Fig. 3. Voltage-dependent gating of gap junction channels with normalized junctional conductance (G_j/G_{m,max}) plotted as a function of transjunctional voltage (V_j) for Rin43/Rin43 (A), Rin40/Rin40 (B), and Rin40/Rin43 (C) cell pairs. Solid line represents the mean normalized conductance (±SE) across experiments, with the symbols representing individual experiments. In C, V_j steps are represented in the Rin43 cell.
cells, an increasing Cx40:Cx43 ratio resulted in increased charge and size selectivity (Fig. 9A). Rin43/6B5N junctions did not differ significantly from Rin43/Rin43 junctions, whereas Rin43/A7r540C3 junctions displayed significant charge selectivity (NBD-TMA = 84.1 ± 6.1% vs. Alexa 350 = 59.8 ± 12.1%) and increasing size selectivity (Alexa 350 = 59.8 ± 12.1% vs. Alexa 594 = 11.2 ± 4.8%). Rin40 cells showed significant charge selectivity, permitting efficient NBD-TMA transfer (70.8 ± 6.9%) but little Alexa 350 transfer (11.2 ± 4.1%) and no Alexa 488 or Alexa 594 transfer (Fig. 9B). When 6B5N, A7r5, or A7r540C3 cells were cocultured with Rin40 cells, the homomeric Cx40-expressing cell dictated size and charge selectivity. Thus junctional permeability in these cocultures was comparable to that observed in Rin40 cell monolayers. 6B5N, A7r5, and A7r540C3 cell monolayers independently displayed charge and size selectivity that increased with increasing Cx40:Cx43 expression ratio (Fig. 9C). Nontransfected parental Rin1046 cells displayed background levels of dye coupling of 2–3% for all dyes (data not shown).

There were no differences in the extent of electrical coupling between Rin40/Rin40 and Rin43/Rin43 cell pairs (Fig. 10); therefore, the dye coupling differences between these cell types cannot be attributed to differ-

Fig. 4. Voltage-dependent gating of gap junction channels with normalized junctional conductance plotted as a function of $V_j$ for Rin43/6B5n (A), Rin43/A7r540C1 (B), and Rin43/A7r540C3 (C) cell pairs when the Rin43 cell was held at a relatively positive or negative voltage potential. The solid line represents the mean normalized conductance (±SE) across experiments, with the symbols representing individual experiments. Rin43/6B5n cell pairs are more variable but have mean gating characteristics similar to Rin43/Rin43 cell pairs (see Fig. 3A). Rin43/A7r540C1 and Rin43/A7r540C3 cell pairs show a progressive asymmetric shift in voltage sensitivity away from the symmetry displayed by Rin43/Rin43 and Rin43/6B5n cell pairs. This shift is opposite to that which would be predicted by Rin40/Rin43 cell pairs (compare with Fig. 3C).

Fig. 5. Voltage-dependent gating of gap junction channels with normalized junctional conductance plotted as a function of $V_j$ for Rin40/A7r540C3 (A), Rin40/A7r540C1 (B), and Rin40/6B5n (C) cell pairs when the Rin40 cell was held at a relatively positive or negative voltage potential. The solid line represents the mean normalized conductance (±SE) across experiments, with the symbols representing individual experiments. Although slightly more variable, all plots have voltage-dependent gating behavior similar to Rin40/Rin40 cell pairs.
ences in the product of channel number and channel open probability. However, the formation of homo-
meric/heterotypic Cx40/Cx43 channels does not appear to be favored (Fig. 10), which may explain why none of the dyes transferred between Rin40 and Rin43 cells in coculture (data not shown).

DISCUSSION

Cx40 and Cx43 expression ratios are dynamically regulated in numerous cells that coexpress these Cxs (19, 22). It is not known what effect this regulation has on gap junction channel behavior or whether changes in expression ratio yield channel behaviors that can be predicted based on probability theory of hemichannel assembly and the dominant Cx phenotype of each resulting gap junction channel. By examining electrophysiological and dye coupling properties of cells coexpressing varying levels of Cx40 and Cx43, we demonstrate here that 1) voltage-dependent gating and single-channel conductance behaviors of hetero-
meric/heterotypic Cx40/Cx43 channels cannot be predicted by the relative contribution of the constituent Cxs to total protein; 2) in Rin43/coexpressor pairs, voltage-dependent gating and single-channel conductances were determined by the heteromeric connexons of the coexpressing cell; 3) in Rin40/coexpressor pairs, voltage-dependent gating and single-channel conductances were determined by the homomeric Cx40 connexons; and 4) the permeability of heteromeric/hetero-
typic channels to dyes of different size and charge can be predicted by the relative proportion of Cx constituents in the total protein of the coexpressing cell.

Table 2 contains a schematic representation of the 14 possible hemichannel conformations that probability theory predicts exist in a cell coexpressing Cx40 and Cx43. With the assumption of random assembly and an equal likelihood of interaction between Cx subunits, the percent contribution of each of the connexon iso-
forms for Cx40:Cx43 expression ratios of 1:1, 3:1, 5:1, and 10:1 is displayed in Table 2. A shift in expression ratio from 1:1 to 5:1 is predicted to result in an almost complete loss of connexons composed of four or more Cx43 subunits, with 73% of the connexons being composed of five or more Cx40 subunits. We hypothesized that as the Cx40:Cx43 expression ratio increased, the channels formed by coexpressors paired with cells ex-

Fig. 6. A: multichannel records obtained from Rin43/Rin43 (top), Rin40/Rin40 (middle), and Rin40/Rin43 (bottom) cell pairs. Asterisks indicate $V_i = 0$ mV. B: single-channel event amplitudes (at $V_j = 10$ mV) for Rin43/Rin43 (top; $n = 10$, mean number of events $= 145 \pm 27$), Rin40/Rin40 (middle; $n = 6$, mean number of events $= 176 \pm 56$), and Rin40/Rin43 (bottom; $n = 6$, mean number of events $= 154 \pm 23$) cell pairs illustrating their unique single-channel conductance characteristics. Events were counted for each cell pair and normal-
ized to the total events for that cell pair. The mean event frequency across cell pairs for each 10-pS bin is plotted; 32% of events in Rin40/Rin40 cell pairs fell midway (130–160 pS) between those of Rin43/Rin43 (96% in 90– to 130-pS bins) and Rin40/Rin40 (85% in 150- to 220-pS bins) cell pairs. The gray shaded bar corresponds to the unique event amplitudes in the Rin40/Rin43 cell pairs (130–160 pS) and can be used for comparisons between Figs. 6, 7, and 8.
pressing Cx43 only would be similar to homomeric/heterotypic Cx40/Cx43 channels whereas the channels formed by coexpressors paired with Cx40-expressing cells would display behaviors similar to those of homomeric/homotypic Cx40 channels.

Voltage-dependent gating behavior of Rin43/coexpressor pairs did not approach the behavior of homomeric/heterotypic pairs as the Cx40:Cx43 expression ratio increased (Fig. 4). In Rin40/Rin43 cell pairs, voltage sensitivity was greatest when the Rin43 cell was relatively negative (Rin40 positive; Fig. 3). In contrast, in Rin43/A7r540C3 cell pairs, voltage sensitivity when the Rin43 cell was relatively negative was less than when the cell was relatively positive. These observations suggest that the heteromeric connexon dominated the $V_j$ gating effect and that interactions between the two connexons changed the Cx43 connexon’s voltage gating behavior.

These results are intriguing in that they demonstrate flexibility in channel behavior that is dependent on the interaction of two hemichannels. The loss of voltage gating sensitivity in Rin43/A7r540C3 cell pairs when the Rin43 cell is held relatively negative implies that the allosteric interactions of the two connexons either alter the ability of Cx43 to gate effectively in response to a negative transjunctional voltage potential or effect a loss in the ability of Cx40 subunits in the

Table 2. Predicted connexon frequency for different Cx40:Cx43 expression ratios

<table>
<thead>
<tr>
<th>Cx40:Cx43 Expression Ratio</th>
<th>6.0</th>
<th>5.1</th>
<th>4.2</th>
<th>3.3</th>
<th>2.4</th>
<th>1.5</th>
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<td>9.4</td>
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<td>31.3</td>
<td>23.4</td>
<td>9.4</td>
<td>1.6</td>
</tr>
<tr>
<td>3:1</td>
<td>0.0</td>
<td>0.4</td>
<td>3.3</td>
<td>13.2</td>
<td>30</td>
<td>35.6</td>
<td>17.8</td>
</tr>
<tr>
<td>5:1</td>
<td>0.0</td>
<td>0.1</td>
<td>0.9</td>
<td>5.6</td>
<td>20.6</td>
<td>40.2</td>
<td>32.7</td>
</tr>
<tr>
<td>10:1</td>
<td>0.0</td>
<td>0.0</td>
<td>0.1</td>
<td>1.1</td>
<td>8.3</td>
<td>33.7</td>
<td>56.8</td>
</tr>
</tbody>
</table>

Connexon frequency is represented as a percentage of total connexons. Cx43, solid circles; Cx40, shaded circles.

Fig. 7. A: multichannel records obtained from Rin43/6B5n (top), Rin43/A7r540C1 (middle), and Rin43/A7r540C3 (bottom) cell pairs. Asterisks indicate $V_j = 0$ mV. B: single-channel event amplitudes (at $V_j = 40$ mV) for Rin43/6B5n (top; $n = 8$, mean number of events = 121 ± 24), Rin43/A7r540C1 (middle; $n = 5$, mean number of events = 195 ± 66), and Rin43/A7r540C3 (bottom; $n = 9$, mean number of events = 186 ± 24) cell pairs illustrating their unique single-channel conductance characteristics. Events were counted for each cell pair and normalized to the total events for that cell pair. As the Cx40: Cx43 expression ratio increases in the coexpressing cell, the frequency of smaller channel conductance events increases. The gray shaded bar corresponds to the unique event amplitudes in the Rin40/ Rin43 cell pairs (130–160 pS).
heteromeric channel to gate with a positive potential. Such an observation is not unprecedented. Our study as well as previous studies have demonstrated that homomeric/heterotypic Cx40/Cx43 channel voltage gating exhibits a lower $G_{\text{min}}$ than Cx43 homotypic channels when the Cx43 expressing cell is held relatively negative (Cx40+) (12). This implies that the interaction of Cx40 and Cx43 connexons altered the voltage sensing and gating properties of the constituent Cxs.

When Rin40 cells were paired with the coexpressing cells there were no dramatic shifts in mean voltage-dependent gating behavior with increasing Cx40:Cx43 expression ratio. We hypothesized that Rin40/A7r540C3 cell pairs would display $V_j$ gating similar to Rin40/Rin40 cell pairs whereas Rin40/6B5n cell pairs would be quite different, especially when the Rin40 cell was relatively negative. The symmetry in $V_j$ gating and consistency of gating when Rin40 was paired with the coexpressing cells suggests that either the Cx40 connexon or Cx40 within the heteromeric connexon dominated the gating effect. The interaction between a Cx40 connexon and a heteromeric Cx40-Cx43 connexon may reinforce the $V_j$ gating sensitivity of the Cx40 subunits in the heteromeric hemichannel such that the channel behaves much like a Cx40 homomeric/homotypic channel.

Single-channel events had trends between treatment groups that paralleled what was observed for the $V_j$ gating data, whereby channel behavior was determined by the heteromeric connexon when coexpressors were paired with Rin43 cells and by the Cx40 connexon when paired with Rin40 cells. In Rin43/coexpressor pairs, we hypothesized that an increased Cx40 contribution to channel populations would result in channel conductances approaching the homomeric/heterotypic channel (>120 pS); this was not the case. A resistors-in-series model of single-channel conductances states that when a Cx40 connexon is paired with a Cx43 connexon the resistance of the channel should be the sum of the resistances of each connexon. With a main-state conductance of 195 and 110 pS for Cx40 and Cx43, respectively, the predicted channel conductance for Rin40/Rin43 pairs would be 140 pS, which is essentially what was observed. The high incidence of 50- to 60-pS events in the Rin43/A7r540C3 cell pairs suggests that the predominant connexons in the coexpressing cells did not display resistances typical of Cx40 connexons. In fact, with the same resistors-in-series model and a single-channel event amplitude of 60 pS, the conductance of the dominant heteromeric connexon, if allowed to form a functional channel, is predicted to be

![Fig. 8. A: multichannel records obtained from Rin40/A7r540C3 (top), Rin40/A7r540C1 (middle), and Rin40/6B5n (bottom) cell pairs. Asterisks indicate $V_j = 0$ mV. B: single-channel event amplitudes (at $V_j = 40$ mV) for Rin40/A7r540C3 (top; n = 5, mean number of events = 268 ± 61), Rin40/A7r540C1 (middle; n = 8, mean number of events = 168 ± 39), and Rin40/6B5n (bottom; n = 7, mean number of events = 300 ± 45) cell pairs illustrating their unique single-channel conductance characteristics. Events were counted for each cell pair and normalized to the total events for that cell pair. There are only small shifts in conductance with changing Cx40:Cx43 ratios in the coexpressing cells, with overall channel amplitudes similar to those observed in Rin40/Rin40 cell pairs. The gray shaded bar corresponds to the unique event amplitudes in the Rin40/Rin43 cell pairs (130–160 pS).](http://ajpcell.physiology.org/)

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40 pS. These observations suggest that either the heteromeric connexons involved in gap junction formation between Rin43 and A7r540C3 cells are high-resistance hemichannels or the interaction of the homomeric and heteromeric connexons results in allosteric interactions that alter overall channel behavior in an unpredictable fashion.

The absence of large single-channel events (>130 pS) in Rin43/A7r540C3 cell pairs, in which 56% of the channels formed are predicted by probability theory to be homomeric/heterotypic Cx40/Cx43 channels, suggests that either the model of hemichannel distributions presented in Table 2 is not predictive of assembly/insertion processes or it is not predictive of the docking and channel formation process. Although Cx40/Cx43 homomeric/heterotypic channels clearly can form, the low levels of coupling observed between Rin40 and Rin43 cells shown in Fig. 10 suggest that this interaction is not favored. As a result, in Rin43/coexpressor pairs it is likely that Cx43 connexons preferentially dock with heteromeric connexons containing one or more subunits of Cx43. A similar model was suggested in the *Xenopus laevis* oocyte system, in which formation of heteromeric channels was induced by low-level expression of a docking-compatible Cx (15).

In contrast, when a Rin40 cell is paired with a coexpressing cell that favors Cx40 expression, despite shifts toward smaller conductance events, there are few differences in event frequencies with changing Cx40:Cx43 expression ratio. These observations suggest that Cx40 dominates the single-channel behavior, with little overt influence being contributed by any heteromeric connexons. It is not clear why single-chan-

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**Fig. 9.** Dye coupling in homogeneous and heterogeneous cell monolayers. A: Rin43 cells cocultured with DiD-labeled Rin43, A7r5, or A7r540C3 cells. B: Rin40 cells cocultured with DiD-labeled Rin40, A7r540C3, A7r5, or 6B5n cells. C: 6B5n, A7r5, and A7r540C3 homogeneous cell monolayers. DiD-labeled cells were injected with [2-(4-nitro-2,1,3-benzoxadiol-7-yl)aminoethyl]trimethylammonium (NBD-TMA; ○), Alexa 350 (○), or Alexa 594 (○). The percentage of cells in contact with the injected cell that received dye was compared between groups. As the Cx40:Cx43 expression ratio increased in the coexpressing cell, dye transfer approached that of the Rin40 cells, except when Rin40 cells were cocultured with the coexpressing cells, in which case dye transfer was similar to the Rin40 cells. *Significant difference from the homogeneous Rin43 cells (P < 0.05); †significant difference between NBD-TMA and Alexa 350 within each treatment group (P < 0.05); ‡significant difference between Alexa 350 and Alexa 594 within each treatment group (P < 0.05).

**Fig. 10.** Macroscopic conductance of Rin40/Rin40 (n = 14), Rin43/Rin43 (n = 11), and Rin40/Rin43 (n = 20) cell pairs. Cells were incubated for 24 h, and macroscopic conductance was measured. There are no differences in electrical coupling between Rin40/Rin40 and Rin43/Rin43 cells; however, the coupling between Rin40/Rin43 cells is significantly reduced (*P < 0.01), suggesting that the formation of these channels is not favored.
nel conductance (and voltage-dependent gating properties) does not change when cells of varying Cx40:Cx43 expression ratio are paired with Rin40 cells. Such a phenomenon could be due to one of the following factors: 1) the Cx40 connexon docks favorably only with Cx40 hemichannels in the coexpressing cells; 2) the expression ratios are not predictive of the distributions depicted in Table 2; or 3) the Cx40 hemichannel exerts a dominant effect when interacting with heteromeric Cx40-Cx43 connexons.

The consistency of single-channel events when Rin40 cells were paired with the coexpressing cells suggests that either the Cx40 connexon somehow stabilizes the pore when it pairs with a heteromeric connexon or the presence of Cx40 subunits in the heteromeric hemichannel maintains the pore resistance when paired with a Cx40 connexon. As stated above, the Rin43/A7r540C3 data present the possibility that the A7r540C3 pore is high resistance compared with Cx43 or Cx40 pores; however, this high-resistance behavior is not observed in the single-channel data from Rin40 and coexpressing cell pairs, therefore supporting the supposition that the Cx40 connexon stabilizes the overall pore behavior. In contrast, when the Cx43 connexons dock with heteromeric hemichannels the “flexibility” of the Cx43 connexon could lead to a loss of stabilization within the pore and result in a high-resistance channel.

Similar to previous studies examining gap junction charge selectivity (3, 27, 30), we have demonstrated that Cx43 gap junctions allow diffusion of positive and negative dyes equally well, whereas Cx40 gap junctions are cation selective. Figure 9C demonstrates that as the Cx40:Cx43 expression ratio increases in coexpressing cells, the permeability properties of the gap junctions shift from those of Cx43 gap junctions to those of Cx40 gap junctions. These data alone do not provide information regarding the permeability properties of heteromeric channels. However, the influence of the heteromeric connexon on channel permeability could be discerned in the Rin43/coexpressing cell cultures, whereby any deviation of permeability away from that of Rin43 cells is representative of homomeric/heterotypic or heteromeric/heterotypic gap junction permeability. The channels formed between coexpressing and Rin43 cells displayed a shift in dye permeability toward that characteristic of Rin40 cells as the Cx40:Cx43 expression ratio increased in the coexpressing cells (Fig. 9A). Thus, despite the unique and unpredicted electrophysiological properties of these cell types, dye permeabilities were predicted by the relative contribution of the most dye-restrictive Cx, in this case Cx40.

That Cx40:Cx43 expression ratio is predictive of dye permeability but not single-channel event amplitude suggests that single-channel conductances do not correlate with channel permeability characteristics. It was originally suggested that there was a relationship between dye permeability and single-channel conductance (28), with larger molecules traversing channels of larger unitary conductance. However, more recent evidence, and the data presented herein, show no relationship between the two (10, 27).

The comparable macroscopic conductance of Rin40 and Rin43 cell pairs (Fig. 10) suggests that differences in dye coupling between these cell types cannot be attributed to differences in the extent of coupling between these cells. In contrast, the low conductance of Rin40/Rin43 cell pairs indicates that these interactions are not favorable and may imply that low levels of dye coupling between Rin43 and A7r540C3 cells may be the result of decreased coupling between these cell types because of unfavorable docking of Cx43 with Cx40-dominant hemichannels in the coexpressing cell. Although favorable interactions may be playing a role in the types of channels formed, as the electrophysiological data suggest, the comparable levels of NBD-TMA transfer across all Rin43 coculture experiments indicate comparable levels of coupling and expression. If there were a loss of coupling between Rin43 and A7r540C3 cells in coculture, then it would be expected that the extent of NBD-TMA dye transfer in these experiments would be significantly diminished; this was not the case. Furthermore, as the Cx40:Cx43 ratio increased, size and charge selectivity increased. Thus the observed changes in dye coupling cannot be an artifact of reduced number or probability of open time of gap junction channels present between these cells but rather must reflect true changes in gap junction permeability.

The proportional protein composition of the functional population of gap junction channels formed by coexpressors and either Rin40 or Rin43 cells remains unknown. It is possible that the expression ratio in the functional channel population differs from that in total cell protein. Western blot data indicate that the Cx40 and Cx43 contents of 6B5n cells were both high, whereas in the A7r540C1 and A7r540C3 cells the Cx40 contents were equivalent to those in the 6B5n cells while Cx43 protein content was decreased. The immunofluorescent staining of these cells demonstrated strong Cx40 plaques in all three cell types, whereas Cx43 staining was noticeably diminished in the A7r540C1 and A7r540C3 cells compared with the 6B5n cells (Fig. 2). Therefore, the protein quantification and immunofluorescence results are compatible.

If the Cx40:Cx43 expression ratios of the functional channel populations were not different between cell types, then there should be few differences in voltage-dependent gating and single-channel conductances of Rin43 cells paired with the coexpressing cells. That these properties changed as Cx40:Cx43 ratio increased argues for a predictive relationship between Cx40:Cx43 ratio in total protein and Cx40:Cx43 ratio in functional channels. As well, predicted shifts in dye permeability when Rin43 cells were cocultured with coexpressing cells provide further evidence of increasing Cx40 predominance in functional channel composition. The combined immunofluorescence, electrophysiological, and dye coupling data confirm that expression ratios changed in a progressive fashion in the coexpressing cells; however, we cannot definitively
state the Cx40:Cx43 expression ratios of the functional channels in these cells.

The combined electrophysiological and dye coupling data demonstrate that heteromeric/heterotypic Cx40/Cx43 channels are not more restrictive to dyes than their homomeric/homotypic counterparts; instead, the permeation properties of these channels reflect the relative contribution of the most permeability-restrictive Cx, in this case Cx40. These findings support previous research demonstrating decreases in Lucifer yellow dye transfer with increasing Cx40:Cx43 ratio in coexpressing cells (9). They also coincide with dye permeability data from growth-arrested and proliferating A7r5 cells in which the cells grown in the presence of growth serum had a fivefold decrease in ability to transfer Lucifer yellow (18). These combined observations suggest that a growth stimulus that increases cellular Cx40:Cx43 expression ratio will cause shifts in gap junction channel populations that effect a progressive loss of permeability to anions in a precise and graded fashion. Such a change in permeability would result in the concentration of anionic signaling molecules, thus stimulating a continued growth response. Therefore, by controlling the expression of one of two expressed Cxs, a cell can control permeation of molecules between cells without compromising electrical conductivity, an essential property of excitable tissues.

In summary, altering Cx40:Cx43 expression ratio in coexpressing cells resulted in heteromeric channel populations with unique voltage-dependent gating and single-channel conductance properties that were not predicted by the relative contributions of each of the connexins to total cell protein. Instead, these properties of the channels were determined by the connexon with the highest Cx40 content, which suggests that allosteric interactions within and between connexons influence these properties of channels in unexpected ways. In contrast, dye permeability properties of the same channels were predicted by the expression ratio in total protein. Such changes in permeability properties brought about by alterations in Cx expression ratio are likely to play an important role in the control of intercellular growth signaling.

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