Gap junction channels formed by coexpressed connexin 40 and connexin 43

Valiunas, Virginijus, Joanna Gemel, Peter R. Brink, and Eric C. Beyer. Gap junction channels formed by coexpressed connexin 40 and connexin 43. Am J Physiol Heart Circ Physiol 281: H1675–H1689, 2001.—Many cardiovascular cells coexpress multiple connexins (Cx), leading to the potential formation of mixed (heteromeric) gap junction hemichannels whose biophysical properties may differ from homomeric channels containing only one connexin type. We examined the potential interaction of connexin Cx43 and Cx40 in HeLa cells sequentially stably transfected with these two connexins. ImmunobLOTS verified the production of comparable amounts of both connexins, cross-linking showed that both connexins formed oligomers, and immunofluorescence showed extensive colocalization. Moreover, Cx40 copurified with (His) 6-tagged Cx43 by affinity chromatography of detergent-solubilized connexons, demonstrating the presence of both connexins in some hemichannels. The dual whole cell patch-clamp method was used to compare the gating properties of gap junctions in HeLa Cx43/Cx40 cells with homotypic (Cx40-Cx40 and Cx43-Cx43) and heterotypic (Cx40-Cx43) gap junctions. Many of the observed single channel conductances resembled those of homotypic or heterotypic channels. The steady-state junctional conductance (gj,ss) in coexpressing cell pairs showed a reduced sensitivity to the voltage between cells (Vj) compared with homotypic gap junctions and/or an asymmetrical Vj dependence reminiscent of heterotypic gap junctions. These gating properties could be fit using a combination of homotypic and heterotypic channel properties. Thus, whereas our biochemical evidence suggests that Cx40 and Cx43 form heteromeric connexons, we conclude that they are functionally insignificant with regard to voltage-dependent gating.

Heteromeric channel; intercellular communication; ion channel; electrophysiology

GAP JUNCTION CHANNELS are critical for the passage of current between cells in the working myocardium and specialized conducting tissues of the heart. Gap junctions are also present and implicated in the function of other cardiovascular cell types including endothelial cells and vascular smooth muscle cells. A gap junction channel is formed by the meeting of two hemichannels (connexons) located in the plasma membranes of opposed cells. A hemichannel is composed of six subunit proteins [connexins (Cx)]. Several different connexins are expressed in cardiovascular cells, including connexin Cx37, Cx40, Cx43, and Cx45. Each of these connexins can form functional gap junctions by themselves (homomeric/homotypic channels). However, many cells coexpress more than one connexin, suggesting that they might participate in the formation of mixed channels. One specialized mixed channel (a heterotypic channel) could be formed by the pairing of two hemichannels that contain different connexins. A more complicated type of mixing would occur if multiple connexins mixed within the same hemichannel (a heteromeric channel).

Biochemical studies have demonstrated the formation of heteromeric gap junction channels in expression systems and in various mature tissues. Stauffer (45) expressed recombinant Cx32 and Cx26 in insect cells and used gel filtration to detect mixing in detergent-solubilized connexons, whereas Jiang and Goddenough (21) used coimmunoprecipitation to show mixing of Cx46 and Cx50 in the lens. Bevans et al. (4) found heteromeric mixing of Cx26 and Cx32 in the rodent liver when Cx26 was coisolated with Cx32 by affinity chromatography on an anti-Cx32 column. He et al. (18) showed coimmunoprecipitation of Cx40 with Cx43 in A7r5 cells. The observation of heteromeric forms has lead to speculation that some or all of the biophysical properties of the resultant gap junction channels might be different from homomeric gap junction hemichannels containing only one connexin type. We (5) previously utilized electrophysiological studies of Cx37 and Cx43 cotransfected into N2A cells to show the existence of a population of heteromeric Cx37-Cx43 channels. Dual whole cell patch-clamp analysis of coexpressing cells demonstrated that the voltage dependence was weaker and the range of single channel conductances was broader than could be explained as arising from conventional homotypic or heterotypic gap junction channels. Other studies have suggested that Cx43 and Cx40 can also make heteromeric channels. In A7r5 rat vascular smooth muscle cells, which normally coexpress Cx40 and Cx43, the macroscopic voltage dependence...
was weaker than that of either homotypic form, and some of the single channel conductances could not be explained easily as homotypic Cx43 or Cx40 forms (18). These authors concluded that the single channel data provided evidence for putative heteromeric Cx40-Cx43 gap junction channels. However, they did not consider heterotypic Cx40-Cx43 channels, whose existence has been subsequently established (37).

A tempting hypothesis is that the disparate properties of heteromeric channels relative to homotypic and/or heterotypic channels could affect multicellular function. For example, in excitable cells (such as the myocytes found in cardiac conducting and pacemaker regions), the type of gap junction channel (homotypic, heterotypic, or heteromeric) could potentially have a profound influence on action potential propagation (22, 34, 37).

The purpose of the current study was to examine biochemically the possible heteromeric mixing of Cx40 with Cx43 and to analyze the electrophysiological properties of gap junction channels formed between cells coexpressing these two connexins. We studied these two connexins because they are abundantly coexpressed in various cells (including atrial myocytes and some endothelial and smooth muscle cells). Double whole cell patch-clamp methods were used to compare the gating properties of channels formed between cells coexpressing Cx40 and Cx43 with those of homotypic Cx40 (Cx40-Cx40) and Cx43 (Cx43-Cx43) and heterotypic Cx43-Cx43 forms.

MATERIALS AND METHODS

Cells and culture conditions. Experiments were carried out using HeLa cells transfected with DNA coding for rat Cx40, rat Cx43, or mouse Cx45. Cx40 or Cx43 DNA was subcloned into the eukaryotic expression plasmid pcDNA3.1/mygro (Invitrogen) or pSFFV-neo (15). Constructs containing a His_{6} tag appended to the Cx43 COOH-terminal were obtained using PCR methods. Cells were stably transfected with linearized DNA using lipofectin (Life Technologies) as described earlier (39). Cells that stably expressed both Cx43 and Cx40 were generated by sequential transfection with the two different expression plasmids. HeLa cells were grown in mini-

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were filled with saline containing (mmol/l) 110 CsCl, 0.1 MgCl₂, 0.1 CaCl₂, 3 EGTA, and 10 HEPES (pH 7.2).

Electrical measurements. Glass coverslips with adherent cells were transferred to an experimental chamber perfused with bath solution at room temperature (21–23°C). The chamber was mounted on the stage of an inverted microscope (Olympus IMT2). Patch pipettes were pulled from glass capillaries (code 7052, A-M Systems) with a horizontal puller (Sutter Instruments). The resistance of the filled pipettes measured 1–1.5 MΩ. Experiments were carried out on mixed cell pairs. A dual voltage-clamp method and whole cell recording were used to control the membrane potential of both cells and to measure currents (6, 36). Each cell was attached to a patch pipette connected to a separate micromanipulator (WR-88, Narishige Scientific Instruments) and amplifier (Axopatch 200). Initially, the membrane potentials of cell 1 and cell 2 were clamped to the same value: the voltage of cell 1 (V₁) = the voltage of cell 2 (V₂). V₂ was then changed to establish a transjunctional voltage (Vⱼ) = V₂ - V₁. Currents recorded from cell 2 represent the sum of two components, the junctional current (Iⱼ) and the membrane current of cell 2; the current obtained from cell 1 corresponds to Iⱼ. To measure Iⱼ, both cells were held at the same holding potential (Vₕ), i.e., Vₕ = 0 mV. The voltage of one of the cells was then stepped to different levels (37). A bipolar pulse protocol was used as described previously (5, 33). The amplitudes of Iⱼ were determined at the beginning (instantaneous Iⱼ (Iⱼ,inst)) and at the end of each pulse (steady-state Iⱼ (Iⱼ,ss)) to estimate the instantaneous and steady-state conductances (gⱼ,inst and gⱼ,ss, respectively). Because the "apparent instantaneous" current rapidly decays with larger Vⱼ, gⱼ,inst and gⱼ,ss were normalized with respect to a 10-mV prepulse conductance (constant with time). We used this as our standard for gⱼ,inst and gⱼ,ss normalization. We used Iⱼ,ss or gⱼ,ss to indicate junctional current or conductance at the 400-ms or 10-s time points, where gⱼ,ss represents an approximation of the steady state.

Signal recording and analysis. Voltage and current signals were recorded on chart paper (Gould RS 2400, Gould Instruments) and videotape (Neurocorder DR-384, Neuro Data Instruments). For off-line analysis, the current signals were filtered at 1 kHz (low-pass filter), digitized with a 12-bit analog-to-digital converter (DT21EZ, Data Translation), and stored with a personal computer. Data acquisition and analysis were performed with custom-made software (6, 25). Curve fitting and statistical analysis were performed using SigmaPlot and SigmaStat (Jandel Scientific), respectively. Macroscopic conductances were compared using the Mann-Whitney rank sum test. Unless otherwise noted, data are presented as means ± SE. Single channel currents were studied in low-conductance cell pairs as previously described (5, 6, 12, 36, 37). The single channel data were fit using the analytical approach of Kullman (26).
RESULTS

Double expression of Cx40 and Cx43 in HeLa transfec-tants. The properties of channels formed by cloned connexins can be examined by their exogenous expression in transfected HeLa cells, because these cells are virtually communication deficient. To examine the properties of Cx40 and Cx43 channels alone and after potential mixing with each other, we generated HeLa cell lines that were stably transfected with Cx40, Cx43, or both Cx43 and Cx40 (HeLa Cx43/Cx40) with the use of two different selectable markers. To facilitate isolation of expressed connexins, we also prepared a Cx43 construct in which a His₆ tag was appended to the carboxyl terminus. We isolated HeLa clones expressing the His₆-tagged Cx43 alone [HeLa Cx43(His)] or with Cx40 [HeLa Cx43(Cx40)/Cx43(His)].

The production of the transfected connexins was confirmed immunologically. As expected from previous studies (17, 20, 37), immunofluorescent staining of the parental HeLa cells showed no reaction with anti-Cx40 or anti-Cx43 antibodies (data not shown). In contrast, anti-Cx40 antibodies localized to appositional membranes (as expected for gap junctions) and perinuclear regions (likely within the protein synthesis/export pathway) in both HeLa Cx40 cells (Fig. 1A) and HeLa Cx43/Cx40 cells (Fig. 1C and E). Anti-Cx43 antibodies showed a very similar staining pattern in HeLa Cx43 cells (Fig. 1B), HeLa Cx43(His) cells (data not shown), and HeLa Cx43/Cx40 cells (Fig. 1D and F). Cx40 and Cx43 showed virtually identical intercellular and gap junctional distributions with double-label immunofluorescence in the coexpressing cells (Fig. 1, E and F).

Production of Cx40 and Cx43 proteins in HeLa transfectants was also confirmed by immunoblotting (Fig. 2). Neither anti-Cx40 nor anti-Cx43 antibodies showed reactivity with the parental HeLa cells. Anti-Cx40 antibodies blotted immunoreactive bands in HeLa Cx40 cells but showed no reactivity with HeLa Cx43 cells (Fig. 2A), and, conversely, anti-Cx43 antibodies blotted immunoreactive bands in HeLa Cx43 cells but showed no reactivity with HeLa Cx40 cells (Fig. 2B), confirming the specificity of these antibodies. The levels and immunoblot patterns of Cx40 and of Cx43 showed only modest variations between the different transfectants.

The abundance of Cx43 or Cx40 in the transfectants was assessed by quantification of immunoblots using a phosphorimager and comparison to the immunoblot intensities produced by bacterial fusion proteins containing carboxy terminal portions of rat Cx40 or Cx43. As shown in Fig. 3, there was less than a fourfold variation in the absolute abundance of Cx40 or Cx43 proteins in the different clones studied. Moreover, in the cotransfected cells, the abundance of Cx40 and Cx43 were indistinguishable.

The presence of Cx40 and of Cx43 within oligomeric connexons was confirmed by chemical cross-linking. HeLa Cx43/Cx40 cells were harvested, and a Triton X-100 supernatant containing solubilized connexons was prepared according to Musil and Goodenough (32). Approximately 50% of the total cellular immunoreactive connexin was solubilized by this Triton X-100

Fig. 2. Immunoblot analysis of Cx40 (A) and Cx43 (B) in HeLa cells transfected with connexins. Whole cell lysates from parental HeLa cells or HeLa cells transfected with Cx40, Cx43, or Cx43 tagged with His₆ [Cx43(His)] were resolved by SDS-PAGE, transferred to membranes, and blotted with anti-Cx40 (A) or anti-Cx43 (B) antibodies. Migration of molecular weight markers is indicated to the left of the blot.

Fig. 3. Absolute abundances of Cx40 and Cx43 in HeLa transfec-tants. Immunoblots of whole cell lysates were performed as in Fig. 2. Blots also contained serial dilutions of bacterially expressed fusion proteins containing COOH-terminal portions of Cx40 or Cx43. Reaction products were quantitated using a phosphorimager, and abundances of Cx40 and Cx43 were determined by comparison to these standard curves. Each bar represents the mean value ± SE based on 2–4 determinations.
extraction as indicated by comparison of immunoblots of whole cell homogenates and Triton X-100 supernatants and pellets (data not shown). The Triton X-100 supernatant was reacted with the cross-linker DSS and then analyzed by SDS-PAGE and immunoblotting (Fig. 4). Immunoblots reacted with anti-Cx40 or anti-Cx43 antibodies exhibited rather similar patterns in the DSS-treated samples: both blots showed a ladder of major bands that migrated to the positions expected for connexin monomers, dimers, and higher oligomers. Reaction with solvent alone yielded none of the higher forms.

The association of Cx40 with Cx43 within oligomeric connexons was analyzed using HeLa Cx43(His)/Cx40 cells. Connexons were solubilized with Triton X-100. This material, which contained both immunoreactive Cx43 and Cx40 (Fig. 5, “before column”), was applied to a Ni-NTA affinity column. The column was extensively washed and then eluted with imidazole. The eluted material contained both Cx43 and Cx40 (Fig. 5, “eluate”). In contrast, when Triton X-100-solubilized material derived from HeLa-Cx40 cells was applied to the Ni-NTA column, only traces of immunoreactive Cx40 bound to the column (data not shown). Moreover, incubation of HeLa Cx43(His)/Cx40 cell extracts with SDS (0.6% for 1 h at room temperature) before column purification dramatically reduced the amount of Cx40 in the eluate, confirming that Cx40 binding to the column occurred through association with Cx43(His).

Voltage dependence of Cx43/Cx40 gap junction currents. Homotypic Cx40 or Cx43 channels have been characterized in primary cell cultures (36) and in transfected cells (1, 6, 8, 37). The formation of heterotypic channels containing Cx40 and Cx43 has been more recently demonstrated and analyzed (37).

Macroscopic intercellular currents (Ij) were examined in 32 pairs of HeLa cells double transfected with Cx43 and Cx40 (HeLa Cx43/Cx40). In 27 preparations, the cells were coupled by gap junctions (see Table 1). Analysis of these cell pairs yielded a gap junction conductance (gj) of 14.3 ± 1.1 nS. The remaining five cell pairs contained cytoplasmic bridges. To distinguish between gap junctions and cytoplasmic bridges, the preparations were uncoupled by exposure to CO2 (10). The average junctional conductance of coexpressing HeLa Cx43/Cx40-HeLa Cx43/Cx40 cell pairs was less than that of either homotypic form (14.3 vs. 24.8 or 20.5 nS for HeLa Cx43/HeLa Cx43 and HeLa Cx40/HeLa Cx40, respectively) despite the similar abundances of Cx40 and Cx43 in the cotransfected cells relative to the singly transfected cells (Fig. 3). The

Table 1. Total gj in different pairs of transfected cells

<table>
<thead>
<tr>
<th>Cell 1</th>
<th>Cell 2</th>
<th>gj, nS</th>
<th>Investigated Cell Pairs</th>
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<tr>
<td>Cx43</td>
<td>Cx43</td>
<td>20.5 ± 2.3 †</td>
<td>16</td>
</tr>
<tr>
<td>Cx40</td>
<td>Cx40</td>
<td>24.8 ± 3.9 †</td>
<td>16</td>
</tr>
<tr>
<td>Cx40*</td>
<td>Cx43</td>
<td>3.8 ± 1.6 †</td>
<td>12</td>
</tr>
<tr>
<td>Cx40*</td>
<td>Cx43</td>
<td>6.8 ± 2.9 †</td>
<td>8</td>
</tr>
<tr>
<td>Cx43/Cx40</td>
<td>Cx43/Cx40</td>
<td>14.3 ± 1.1</td>
<td>27</td>
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<td>Cx43/Cx40</td>
<td>Cx40</td>
<td>7.6 ± 3.1 †</td>
<td>10</td>
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<td>Cx43/Cx40</td>
<td>Cx43</td>
<td>8.0 ± 1.5 †</td>
<td>17</td>
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<td>Cx43/Cx40</td>
<td>Cx45</td>
<td>11.0 ± 1.5 †</td>
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Values are means ± SE. Pairs of stably transfected HeLa cells (or RIN cells as indicated) were examined for total gap junction conductance (gj). *Data from Valiunas et al. (37). †Significantly different (P < 0.05) compared with cotransfected HeLa Connexin (Cx43/Cx40 and HeLa Cx43/Cx40 cell pairs (Mann-Whitney rank sum test).

Fig. 4. Cross-linking of connexins in HeLa Cx43/Cx40 cells. Triton X-100-soluble extracts (containing connexons) from HeLa Cx43/Cx40 cells were reacted with disuccinimyl suberate (DSS) cross-linker dissolved in DMSO (+) or were incubated with DMSO solvent alone (−). These samples were resolved by SDS-PAGE, and Cx43 and Cx40 were detected by immunoblot analysis using rabbit anti-connexin antibodies. Migration of molecular weight markers is indicated to the left of the blot.

Fig. 5. Affinity purification of His-tagged Cx43 and associated Cx40. A Triton X-100-soluble extract from HeLa Cx43(His)/Cx40 cells (containing connexons) was affinity purified using a Ni-NTA column (as described in MATERIALS AND METHODS). Cx43 and Cx40 in the extract applied to column and in the eluted fractions were detected by immunoblotting using anti-Cx43 and anti-Cx40 antibodies. Migration of molecular weight markers is indicated to the left of the blot.
heterotypic Cx40-Cx43 junctional conductance determined by Valiunas et al. (37) was 6.8 nS, which is a similar value to our new data obtained for HeLa Cx43/Cx40 and HeLa Cx43/Cx40-HeLa Cx43 pairs (Table 1).

The relationship between \( V_j \) and \( g_j \) was studied systematically in 12 cotransfected HeLa Cx43/Cx40 cell pairs. The protocol adopted was as follows. Both cells were clamped to the same \( V_h \), so that \( V_1 = V_2 = 0 \) mV. Starting from a \( V_{j,0} \) of 0 mV, bipolar pulses of 800-ms duration were administered to establish \( V_j \) gradients of identical amplitude with either polarity. \( V_j \) was then altered from \( \pm 10 \) to \( \pm 150 \) mV using increments of 20 mV (Fig. 6). In some preparations, the duration of the pulse was prolonged up to 10 s, and \( V_j \) was altered from \( \pm 10 \) to \( \pm 110 \) mV.

Figure 6, A–D, shows records from pairs of cotransfected HeLa Cx43/Cx40 cells. The behavior of \( I_j \) was varied. Two types of current-voltage relationships were seen using both short (400 ms; Fig. 6, A and B) and long (10 s; Fig. 6, C and D) pulse protocols; some preparations showed symmetrical currents, whereas others were asymmetric. Figure 6, A and C, shows an example with symmetrical currents (short pulse, Fig. 6A; long pulse, Fig. 6C). The associated currents \( (I_j) \) increased proportionally with \( V_j \) and showed a voltage- and time-dependent inactivation for positive and negative \( V_j \). Figure 6, B and D, illustrates asymmetrical gap junction currents (short pulse, Fig. 6B; long pulse, Fig. 6D). Negative \( V_j \) gave rise to an outward current with pronounced exponential decay at high voltages. Positive \( V_j \) of the same amplitudes produced an inward current with only marginal inactivation. The amplitudes of \( I_j \) were determined at the beginning \( (I_{j,\text{inst}}) \) and end of each pulse \( (I_{j,\text{ss}}) \) to estimate the conductances \( g_{j,\text{inst}} \) and \( g_{j,\text{ss}} \), respectively. The values of \( g_{j,\text{inst}} \) and \( g_{j,\text{ss}} \) were normalized and plotted versus \( V_j \).

Figure 6, E and F, summarizes the data gathered from HeLa Cx43/Cx43 preparations with symmetrical and asymmetrical \( I_j \), respectively. They show the normalized relationships \( g_{j,\text{inst}} \) versus \( f(V_j) \) and \( g_{j,\text{ss}} \) versus \( f(V_j) \), obtained using the short pulse protocol, and \( g_{j,\text{ss}} \) versus \( f(V_j) \), obtained using the long (10 s) pulse protocol. Figure 6E shows a quasisymmetrical relationship from nine preparations. The analysis revealed the following Boltzmann (continuous curve in Fig. 6E) pa-
parameters for the 400-ms data sets: initial $V_j$ ($V_{j,0}$) = −83/72 mV, voltage-insensitive component of $g_j$ ($g_{j,min}$) = 0.33/0.28, and gating charge ($z$) =1.5/1.6, for negative/positive $V_j$, respectively. For the longer duration data (5 preparations), Boltzmann fits (continuous dashed line in Fig. 6E) yielded the following values: $V_{j,0} = −72/66$ mV, $g_{j,min} = 0.28/0.23$, and $z = 1.7/1.8$, for negative/positive $V_j$, respectively.

When compared with the respective homotypic Cx40-Cx40 and Cx43-Cx43 channels under the same conditions (400-ms pulse protocol), the gap junctions from pairs of doubly transected (HeLa Cx43/Cx40) cells exhibited a broader voltage sensitivity ($V_j = 56$ and 66 mV for Cx40-Cx40 and Cx43-Cx43, respectively) (37).

Plots from three asymmetrical cases obtained using the short pulse protocol are shown in Fig. 6F. The $g_{j,ss}$ declined in a sigmoidal fashion to a quasistable level at a $V_j$ of −150 mV when $V_j$ was made negative, and, in contrast, it decreased gradually without reaching a plateau at positive $V_j$. The continuous curves in Fig. 6F at negative values of $V_j$ represent the best fit of the data to the Boltzmann equation using the following parameters: $V_{j,0} = −104$ mV, $g_{j,min} = 0.29$, maximum $g_j$ ($g_{j,max}) = 1.02$, and $z = 1.7$. In the case of the long pulse protocol, a Boltzmann fit (continuous dashed line in Fig. 6F) yielded the following parameters: $V_{j,0} = −76$ mV, $g_{j,min} = 0.27$, $g_{j,max} = 1.0$, and $z = 2.5$. The pronounced asymmetry of the $g_{j,ss} = f(V_j)$ relationship implies the existence of more than one population of channels distributed between cell pairs. The currents and the relationship between $V_j$ and $g_j$ from asymmetrical cases resembled heterotypic Cx40-Cx43 channel currents and their $g_j$-$V_j$ relationship (37).

From the data shown here, we conclude that the 400-ms step duration and 10-s step duration give qualitatively similar results: symmetric or asymmetric current-voltage relationships. We refrained from assuming the 400-ms data are representative of the steady state, but rather we used the same pulse duration to compare the behavior of the different channel types.

Voltage dependence of Cx43/Cx40-Cx40 and Cx43/Cx40-Cx43 gap junction currents. We occluded cotransfected cells (HeLa Cx43/Cx40) with singly transfected cells (HeLa Cx40 or HeLa Cx43). This strategy allowed us to observe channels in which one hemichannel potentially contained two connexin types but the other contained only a single connexin (was homomeric) to determine whether this configuration would distinguish heteromeric forms from homotypic or heterotypic channels.

Like the pairs of double-transfected HeLa Cx43/Cx40 cells, recordings from these mixed cell pairs consisting of one HeLa Cx43/Cx40 cell and one HeLa Cx40 or HeLa Cx43 cell revealed quasisymmetrical and asymmetrical gap junction currents. Figure 7, A and B, shows data obtained from mixed pairs of HeLa Cx43/Cx40 and HeLa Cx43 cells. Figure 7A, top, illustrates an example with quasisymmetrical $I_j$, and Fig. 7B, top, shows asymmetrical $I_j$. In the former case, the $g_{j,ss} = f(V_j)$ relationship was nearly bell shaped and tended to symmetry. In the latter case, it was strongly asymmetrical.

Figure 7A, bottom, summarizes the results of six complete experiments, including the most symmetrical cases. The smooth curve corresponds to the best fit of the data to the Boltzmann equation using the following values: $V_{j,0} = −71/92$ mV; $g_{j,min} = 0.39/0.28$, $g_{j,max} = 1.01/1.06$, and $z = 2.2/0.7$, for negative/positive $V_j$, respectively. In this case, negative $V_j$ indicates positive potential inside the HeLa Cx40 cell.

The three most pronounced asymmetrical cases are summarized Fig. 7B, bottom. It shows the normalized relationships $g_{j,inst}$ versus $f(V_j)$ and $g_{j,ss}$ versus $f(V_j)$. Both plots were strongly asymmetrical. The $g_{j,inst}$ increased when the cell expressing Cx40 was made positive inside and decreased when it was made negative. The increase in $g_{j,inst}$ peaked at −1.29 at $V_j = −150$ mV. The decrease in $g_{j,inst}$ reached a value of −0.94 at $V_j = 150$ mV. In contrast, the $g_{j,ss}$ declined when the Cx40 cell was made positive. It decreased in a sigmoidal fashion to a quasistable level at a $V_j$ of −150 mV. When the HeLa Cx40 cell was made negative, $g_{j,ss}$ decreased without reaching a plateau, i.e., it closely followed $g_{j,inst}$. The continuous curves in Fig. 7B at negative values of $V_j$ represent the best fit of the data to the Boltzmann equation using the following parameters: $V_{j,0} = −103$ mV, $g_{j,min} = 0.33$, $g_{j,max} = 1.03$, and $z = 2.2$.

Both the relationships $g_{j,inst} = f(V_j)$ and $g_{j,ss} = f(V_j)$ for the asymmetric cases were similar to those obtained from heterotypic Cx40-Cx43 gap junctional channels (37). The kinetics of current inactivation and $V_j$ polarity dependence were also reminiscent of heterotypic Cx40-Cx43 channels.

Figure 7D summarizes the results of 12 experiments where $I_j$ was symmetric for the HeLa Cx43/Cx40-HeLa Cx43 cell pair configuration. The continuous curve in Fig. 7D corresponds to the best fit of the data to the Boltzmann equation using the following values: $V_{j,0} = −82/87$ mV; $g_{j,min} = 0.26/0.42$, $g_{j,max} = 1.04/1.05$, and $z = 1.3/1.0$, for negative/positive $V_j$, respectively. When the HeLa Cx43 cell was made negative inside (negative $V_j$), $g_j$ was 0.26; in contrast, stepping the inside of the HeLa Cx43 cell to positive yielded a $g_j$ of 0.42.

Time- and conductance-dependent transition from asymmetric to symmetric voltage dependence. The total gap junction conductance between cell pairs often declined with time. As already indicated, the degree of asymmetry of $I_j$ was variable and dependent on total gap junction conductance. In some cases, the changes of total gap junction conductance between cell pairs led to transformation from asymmetrical currents to symmetrical ones. This was observed for all cell types investigated (i.e., HeLa Cx43/Cx40-HeLa Cx43/Cx40, HeLa Cx43/Cx40-HeLa Cx40, and HeLa Cx43/Cx43-HeLa Cx43). Figure 8, A and B, illustrates an example of a mixed HeLa Cx43/Cx40-HeLa Cx40 cell pair. At the beginning of the measurements, the total conductance of the cell pair was −3 nS (Fig. 8A, top). Stepping $V_j$ to make the inside of the HeLa Cx40 cell positive resulted in a large $I_j$ with pronounced inactivation.
Conversely, stepping \( V_j \) to make the inside of the HeLa-Cx40 cell negative led to a smaller \( I_{j,\text{inst}} \) with marginal or no inactivation. When spontaneous total conductance dropped to \( \approx 2 \text{nS} \), \( I_j \) became more symmetrical (Fig. 8A, bottom). Figure 8B shows the analysis of the current records depicted in Fig. 8A. The curves represent the normalized \( g_j \) versus \( V_j \). A: quasisymmetrical relationship with Boltzmann fit: \( V_{j,0} = -71.92 \text{mV}, g_{j,\text{min}} = 0.39/0.28, \) and \( z = 2.2/0.7, \) for negative/positive \( V_j \), respectively. B: asymmetrical relationship with Boltzmann fit for negative \( V_j \): \( V_{j,0} = -103 \text{mV}, g_{j,\text{min}} = 0.33, g_{j,\text{max}} = 1.03, \) and \( z = 2.2. \) C: \( I_j \) elicited in response to a series of voltage steps (\( V_j \)) in HeLa Cx40/Cx43-HeLa Cx43 cell pairs. Top, symmetrical current inactivation; bottom, asymmetrical currents voltage dependence. Currents were measured from the HeLa Cx43/Cx40 cell. D: instantaneous (\( \circ \)) and steady-state plots (\( \bullet \)) of normalized \( g_j \) versus \( V_j \). Boltzmann fit: \( V_{j,0} = -82/87 \text{mV}, g_{j,\text{min}} = 0.26/0.42, g_{j,\text{max}} = 1.04/1.05, \) and \( z = 1.3/1.0, \) for negative/positive \( V_j \), respectively.

Properties of Cx43/Cx40-Cx45 gap junction currents. The gating properties of heteromeric Cx43/Cx40 channels were difficult to distinguish from homotypic forms of Cx40 or Cx43 and heterotypic Cx40-Cx43. This prompted us to try to unmask heteromeric gating by pairing Cx45-expressing HeLa cells with HeLa Cx43/Cx40 cells. The bipolar pulse protocol was used to study multichannel currents from a HeLa Cx43/Cx40-HeLa Cx45 cell pair (Fig. 8C). Stepping \( V_j \) to make the inside of the HeLa Cx45 cell negative or the inside of the HeLa Cx43/Cx40 cell positive resulted in an \( I_{j,\text{inst}} \) with pronounced inactivation. Conversely, stepping \( V_j \) to make the inside of the HeLa Cx45 cell positive and the inside of the HeLa Cx43/Cx40 cell negative led to an \( I_{j,\text{inst}} \) with distinct activation.

Figure 8D shows the results from five cell pairs. The plots represent the normalized relationships \( g_{j,\text{inst}} \) versus \( V_j \) and \( g_{j,\text{ss}} \) versus \( V_j \). The \( g_{j,\text{inst}} \) decreased slightly when the cell expressing Cx45 was made negative. The \( g_{j,\text{ss}} \) increased when the HeLaCx45 cell was made positive inside and decreased when it was made negative. Note that \( g_j \) was far from steady state at the end of the...
400-ms pulse applied (see Fig. 8C). The continuous curves in Fig. 8C at positive values of $V_j$ represent the best fit of data to the Boltzmann equation using the following parameters: $V_{j,0} = -59$ mV, $g_{j,\text{min}} = 0.05$, $g_{j,\text{max}} = 1.01$, and $z = 2.9$.

The currents and conductances of the HeLa Cx43/Cx40-HeLa Cx45 cell pairs behaved similarly to HeLa Cx40-HeLa Cx45 and HeLa Cx43-HeLa Cx45 cell pairs [see Valiunas et al. (37)] with regard to $V_j$ dependence, gating polarity, current patterns, kinetics of inactivation, and the presence of activation of $I_j$. The data shown here support the conclusion of Valiunas et al. (37): that Cx45 gates negatively.

**Simulation of macroscopic gap junction conductance involving Cx40 and Cx43 channels.** We modeled our data to examine whether the $g_j = f(V_j)$ relationship for cells cotransfected with Cx40 and Cx43 could be explained under some conditions where only heterotypic and homotypic channels were present.

In Fig. 9A, we attempted to fit the averaged steady-state symmetric data (shown in Fig. 6C) assuming different proportions of homotypic Cx43 and Cx40 channels and heterotypic Cx40-Cx43 channels. In Fig. 9A, the solid line represents a smooth curve fit to the actual data obtained from symmetrically behaving HeLa Cx43/Cx40 cell pairs. The other curves in Fig. 9A represent the predicted results [using actual data from Valiunas et al. (37)] where the percentages of homotypic and heterotypic channels were varied. For the heterotypic population, we assumed that the two possible polarities (Cx43-Cx40 and Cx40-Cx43) always existed in equal amounts. The dashed dotted line in Fig. 9A (with the closest fit to our data) represents the results predicted by the case where 70% of the channels were heterotypic (35% of each polarity) and 15% were homotypic Cx43-Cx43 and 15% were homotypic Cx40-Cx40. The short dashed line in Fig. 9A represents the results predicted by the case where 30% of the channels were heterotypic (15% of each polarity) and 35% were homotypic Cx43-Cx43 and 35% were homotypic Cx40-Cx40. The two extremes shown represent the cases where 100% of the channels were heterotypic.
Conductances of single channels. To study single channel currents, we selected cell pairs with one or two to three operational channels. The pulse protocol adopted involved an inversion of the \( V_j \) polarity. Figure 10 illustrates experiments in weakly coupled pairs or in normally coupled pairs after advanced spontaneous uncoupling. Figure 10, A–C, shows data from HeLa Cx43/Cx40-HeLa Cx40 cell pairs, and Fig. 10, D–F, shows data from HeLa Cx43/Cx40-HeLa Cx43/Cx40 cell pairs.

Figure 10A shows a record from a weakly coupled HeLa Cx43/Cx40-HeLa Cx40 cell pair. Biphasic pulses (±50 mV, 400 ms) were applied to cell 1 (\( V_1 \), Cx40 cell) while gap junction currents were recorded from cell 2 (\( I_2 \), Cx43/Cx40 cell). The continuous line in Fig. 10A indicates zero junctional current. Initially, the cell pair had one channel (\( I_2 \), trace) and, after inversion of the of the \( V_j \) polarity, a second gap junction channel was observed. The analysis led to the following conductances: outward current, 64 pS; inward current, 102 pS; and second channel, 138 pS. The \( V_j \) polarity dependence was characteristic of heterotypic channels (5, 9, 37). This suggested the presence of two different types of channels: heterotypic Cx40-Cx43 with conductances 64/102 pS, for negative/positive \( V_j \), respectively, and homotypic Cx40 with a conductance of 138 pS.

Figure 10B illustrates another example that may be explained by the presence of homotypic Cx40 and heterotypic Cx40-Cx43 channels. Starting from a common \( V_h \) (\( V_1 = V_2 = 0 \) mV), a depolarizing pulse was administered to cell 1 (Cx40-expressing cell) followed by a hyperpolarizing pulse. The inward and outward current showed abrupt transitions giving rise to several discrete current levels attributable to current flow through the different types of the channels. The analysis yielded the following conductances: inward current, 128 and 92 pS; and outward current, 65, 31, and 131 pS.

Figure 10C shows a single channel record obtained from a mixed HeLa Cx43/Cx40-HeLa Cx40 pair where a \( V_j \) of 60 mV was maintained for many seconds. In this case, the HeLa Cx40 cell was made negative inside. The selected current segment \( I_j \) shows an episode that started and ended by slow transitions in the channel closed state (solid lines in Fig. 10C). The channel gated between the main state and the residual state (dashed lines in Fig. 10C). Analysis of the current amplitude histogram yielded a \( \gamma_{j,\text{main}} \) and \( \gamma_{j,\text{residual}} \) of 109 and 18 pS, respectively. These data are similar to what we observed for heterotypic Cx40-Cx43 channels, which exhibit a unitary channel conductance of 100 pS for the main state (\( \gamma_{j,\text{main}} \)) and 14–20 pS for the residual state (\( \gamma_{j,\text{residual}} \)) when the Cx40 side is negative (37).

Figure 10D shows single channel activity during short (400 ms) biphasic \( V_j \) of 110 mV. The current analysis revealed the following conductances (arrows in Fig. 10D): inward current, 143, 106, and 45 pS; and outward current, 60 and 62 pS. These conductances may be interpreted as follows: 143 pS, Cx40 homotypic; 106 pS, Cx40-Cx43 heterotypic; 45 pS, heteromeric,
Figure 10, E and F, shows single channel records obtained from Cx40/Cx43 cell pairs at a maintained $V_j$ of 50 and 60 mV, respectively. In the former case, current histograms revealed conductance states corresponding to 44 and 105 pS; these may correspond to a heterotypic Cx40-Cx43 channel (105 pS) and an unknown channel (endogenous/possibly heteromeric). In Fig. 10F, histograms revealed peaks yielding conductance levels of 32, 49, and 78 pS; these values could be interpreted as follows: 78 pS, homotypic Cx43-Cx43 channel; 49 pS, unknown homotypic channel (heteromeric or heteromeric, because there is no $V_j$ polarity dependence, characteristic of heterotypic channels); and 32 pS, substate, endogenous or heteromeric.

The data shown in Fig. 10 represent the spectrum of conductive states observed from HeLa Cx43/Cx40-HeLa Cx40 cotransfectant pairs and partial heterotypic HeLa Cx43/Cx40-HeLa Cx40 pairs. Data were collected from many records, because in each individual single channel recording, the likelihood of observing multiple conductance states was low. The conductance values obtained from single channel records were sampled in 5-pS bins and plotted as frequency histograms. Figure 11 shows the histograms for HeLa Cx43/Cx40-HeLa Cx40 pairs (Fig. 11A, 6 cell pairs) and heterotypic HeLa Cx43/Cx40-HeLa Cx40 cell pairs (Fig. 11B, 5 cell pairs). The single channel data yielded a complex distribution in both cases. In the case of cotransfected HeLa Cx43/Cx40-HeLa Cx43/Cx40 pairs, the data were best fitted by five Gaussians using the...
analytical approach of Kullmann (26) with the following conductances: $37 \pm 6, 59 \pm 6, 75 \pm 4, 98 \pm 7$, and $128 \pm 8$ pS. Fitting of the data from the partial heterotypic HeLa Cx43/Cx40-HeLa Cx43/Cx40 cell pairs (A), the sum of 5 Gaussians was used; in the case of heterotypic HeLa Cx43/Cx40-HeLa Cx40 gap junctions (B), the sum of 4 Gaussians was used. For specific data, see text.

**Fig. 11.** Histograms of single channel conductances. The smooth curves represent the theoretical fits of data to Gaussian distributions. In the case of HeLa Cx43/Cx40-HeLa Cx43/Cx40 cell pairs (A), the sum of 5 Gaussians was used; in the case of heterotypic HeLa Cx43/Cx40-HeLa Cx40 gap junctions (B), the sum of 4 Gaussians was used. For specific data, see text.

**DISCUSSION**

The potential physiological consequences of formation of mixed channels by Cx40 and Cx43 may have importance for understanding cardiovascular function, because these two proteins are coexpressed in a number of cell types. Conflicting data have been published regarding the heterotypic and heteromeric interactions of these proteins. The initial electrophysiological observations suggested that Cx40 and Cx43 did not interact to form heterotypic channels in *Xenopus* oocytes (7, 41). Other studies performed in HeLa cell transfectants utilized dye injection (14) or the induction of channels in approximated cells (17). Unfortunately, these approaches may not have allowed detection of heterotypic channel formation, because later studies by Valiunas et al. (37) revealed that Cx40 and Cx43 do form functioning heterotypic gap junction channels in transfected HeLa and RIN cells.

Recently, He et al. (18) have argued for functional mixing of Cx40 and Cx43. They studied A7r5 rat vascular smooth muscle cells, which normally coexpress Cx40 and Cx43. The macroscopic voltage dependence that they detected in these cells was weaker than that produced by either homotypic form, and some of the observed single channel conductances could not be readily explained as homotypic Cx43 or Cx40 forms. These authors concluded that their data provided evidence for heteromeric Cx40/Cx43 gap junction channels. However, they did not consider the possible existence of heterotypic Cx40-Cx43 channels, which we (37) recently detected and studied.

In the present study, we extensively analyzed the biochemical properties of HeLa cells coexpressing Cx40 and Cx43. Immunoblotting experiments showed that cells produced both Cx40 and Cx43 proteins, and quantitation of immunoblots showed that the cotransfected cells produced very similar amounts of the two proteins. Immunofluorescence microscopy showed that both Cx40 and Cx43 localized to virtually identical cell surface locations, consistent with their participation in the same gap junctions. Cotransfected cells were disrupted with Triton X-100 under conditions that solubilize connexons (hexameric hemichannels); chemical cross-linking of this material showed that both Cx40 and Cx43 formed oligomers in these cells. Finally, when the Triton X-100 extracts were affinity purified using Ni-NTA-Sepharose, Cx40 copurified with Cx43(His); this result implied that Cx43(His)-containing connexons also contained Cx40. Thus our cotransfected cells abundantly expressed both Cx40 and Cx43 and contained biochemically detectable heteromeric connexons. Our data extend the data of He et al. (18), who showed coimmunoprecipitation of Cx40 with Cx43 from A7r5 cells, and support their conclusion of heteromeric mixing. One caveat of both studies is that they are based on Triton X-100 solubilization, which has become a standard procedure to examine connexon assembly (3, 24, 32). However, this is not a quantitative procedure; moreover, as noted in earlier studies by Musil and Goodenough (31), some cellular connexin (including some material in gap junction plaques) is not solubilized by this procedure. Much of the Triton X-100-solubilized material may derive from connexons within the assembly/export pathway and from hemichannels and channels that have not been incorporated into plaques. An assumption is that the mixing of connexins to form heteromers is similar in the synthetic pathway and in the plasma membrane.

We also extensively analyzed the electrophysiological properties of HeLa cells coexpressing Cx40 and Cx43. Our double whole cell patch-clamp data are less striking than might have been anticipated in suggesting unique electrophysiological properties conferred by heteromeric mixing.
One of our findings was that in HeLa cell pairs with one or more member coexpressing Cx40 and Cx43, the junctional conductance was substantially less than in pairs where each cell was transfected with only a single connexin (Table 1). For example, the junctional conductance of the cotransfected HeLa Cx43/Cx40 cell pairs (14.3 nS) was only 56–66% of the conductance obtained in homotypic Cx40 or Cx43 cell pairs. These results are somewhat surprising compared with our connexin protein data. The HeLa Cx43/Cx40 cell pairs were prepared by transfecting Cx40 into the same HeLa Cx43 cell clone used for the homotypic studies. Thus the HeLa Cx43/Cx40 cells contain indistinguishable amounts of Cx43 compared with the HeLaCx43 cells plus the introduction of a similar amount of Cx40 (Fig. 3). We might have expected an increased (approximately doubled) conductance in the HeLa Cx43/Cx40 pairs. The observed reduction cannot be due to impaired trafficking of Cx43 produced by introduction of Cx40, because both connexins were detected in similar plasma membrane locations (Fig. 1). Therefore, it must be that some of the heteromeric Cx43/Cx40 connexons are nonfunctional as hemichannels or are unable to pair with other connexons to make functionally complete gap junctional channels.

We observed even further reduced conductances in the partial heterotypic cell pairs formed when HeLa Cx43/Cx40 cells were paired with cells expressing only Cx40 or Cx43 (14.3 vs. 7.6–8.0 nS; Table 1). These data suggest a reduced efficiency of channel formation/interaction for the heterotypic channel.

The single channel data are equivocal in terms of distinguishing between homotypic, heterotypic, and heteromeric forms. Many of the single channel events that we observed in pairs of HeLa Cx43/Cx40 cells were similar to ones that we detected in homotypic or heterotypic pairs of HeLa Cx40 and/or HeLa Cx43 cells; a relatively small number of events were unique. The conductances that are dissimilar from homotypic or heterotypic forms provide the strongest argument for heteromeric channel types, but interpretation of these data is unclear, because these events may also represent substates of any of the gap junction channels present or involvement of an endogenous connexin (such as Cx45). [In studies of cardiac myocytes, Elenes et al. (13) suggested that Cx45 might facilitate interactions of Cx40 and Cx43.] Thus the data do not unequivocally demonstrate unique conductances that can be defined as heteromeric Cx43/Cx40. Moreover, it is quite possible that heteromeric Cx43/Cx40 channels have similar or identical conductances to the homomorphic/homotypic or homomeric/heterotypic forms, as speculated by Hopperstad et al. (19). These data differ from our previous observations of many different single channel events when Cx43 and Cx37 were coexpressed; this difference suggests the possibility that less functional heteromeric channels are formed between Cx40 and Cx43 than between Cx37 and Cx43.

Brink et al. (5) calculated that 196 different gap junction channel forms are potentially formed when two connexins are equally coexpressed and have similar affinities for each other. In contrast, the maximum number of conductive forms would be only 14 for a pair between a cell coexpressing two connexins and a partner cell expressing only a single connexin (and therefore containing only homomeric connexons). Therefore, one expectation might be that the event histogram of channel conductances for the cotransfected cell pairs (HeLa Cx43/Cx40 paired with HeLa Cx43/Cx40) would show more conductive states than those detected between HeLa Cx43/Cx40 and HeLa Cx40 cells. The data shown in Fig. 11 qualitatively meet this criterion; however, in fact, there is only one additional conductive state demonstrable for the cotransfected pairs relative to the HeLa Cx43/Cx40-HeLa Cx40 cell pairs. The conductive state missing in the HeLa Cx43/Cx40-HeLa Cx40 cell pairs is in the range of 75 pS, which is similar to homotypic Cx43 observed under our conditions (37).

There are two alternative possible explanations for these data. First, it is possible that virtually all of the channels in the cotransfectants are heteromeric, but they function similarly to homotypic and heterotypic Cx43 and Cx40 channels. The conductances of the heteromers might not be distinguishable. Any of the peaks in the channel event histograms could represent multiple channel populations with similar or identical conductances but with very different connexin composition. Second, alternatively, it is possible that there is only a small population of heteromeric forms, which are not easily detected with our analysis; functional heteromer formation might be rare.

Interestingly, most, if not all, of the conductances in both histograms corresponded to conductances that are similar to the homotypic and heterotypic forms of Cx43 and Cx40. The least conductive peak in both histograms (33 and 37 pS) might represent channel activity of heteromeric forms. However, we cannot exclude involvement of subconductive states of either Cx40 or Cx43 channels or endogenous Cx45 channels.

Our new data regarding \( V_j \) dependence are consistent with previous reports (5, 18) utilizing cotransfected cell pairs in which the voltage dependence of coexpressing cells was shown to be broadened or less sensitive than the equivalent homotypic forms. We found that the junctional conductances observed in cells cotransfected with Cx40 and Cx43 showed weaker voltage dependence than their homotypic counterparts. A subset of cell pairs showed asymmetric voltage dependence similar to heterotypic Cx40-Cx43 channels. Similar observations were made in cell pairs where one hemichannel of the gap junction was homomeric (Cx40 or Cx43) and the other hemichannel was a cotransfected cell (HeLa Cx43/Cx40). Such cases with asymmetrical \( V_j \) dependence (as in Fig. 7, B and C) exhibited gating polarities corresponding to heterotypic Cx40-Cx43 channel gating polarities (37).

We tried to explain our data by fitting them with a combination of homotypic and heterotypic forms. The summarized data and fits shown in Fig. 9 do not distinguish between two possible situations where 1) the majority of the channels are heteromeric and behave like homotypic and/or heterotypic channels, or 2)
there is only a small heteromeric channel population and the total junctional conductance is dominated by homotypic and heterotypic channel types. Such conclusions are also supported by the data from Cx45-Cx43/Cx40 gap junctions (Fig. 8, C and D). Interestingly, the macroscopic properties observed were indistinguishable from heterotypic/homomeric gap junction channels, i.e., the recorded currents, their $V_j$ dependence, and gating polarities (Fig. 8, C and D) corresponded closely with those of heterotypic Cx40-Cx45 or Cx43-Cx45 gap junctions (37).

In terms of voltage gating, either the heteromeric hemichannel forms follow the behavior of their homomeric hemichannel counterparts or the heteromeric forms are functionally nonexistent.

It is possible that heteromeric mixing has a more profound influence on other properties of gap junction channels. For example, Gu et al. (16) presented data suggesting that the carboxyl tail of Cx43 can modulate the pH-dependent gating of Cx43 when the two connexins are coexpressed in *Xenopus* oocytes. Thus chemical gating is a potentially important physiological parameter with regard to heteromers. It is also possible that heteromeric Cx43/Cx40 channels might have altered permeability properties, because the two connexins individually form channels with some differences in permeability and selectivity (1, 2, 11, 38, 40), but a detailed characterization of these permeability/selectivity properties requires a full study of its own.

In summary, our data from expression of two major cardiac connexins (Cx40 and Cx43) in HeLa cells show biochemically that the two connexins can mix to form heteromeric connexons. However, reduced total conductances between pairs of coexpressing cells suggest that some heteromeric channels may be nonfunctional. Other electrophysiological analyses suggest that most properties of these cells can be understood based on the properties of homomeric and heterotypic channels. Single channel data revealed conductances that were consistent with the dominant channel forms being homomorphic/homotypic or heterotypic-like. Moreover, studies of the cotransfected HeLa Cx43/Cx40 cells yielded $g_{j,ss} = f(V_j)$ relationships that could be explained on the basis of homotypic and heterotypic channels alone, although the qualifier is that a high percentage of heterotypic channel types (between 30% and 70%) would be necessary to approximate the data. A related point is our observation that junctional conductances were initially asymmetrically voltage dependent but became symmetrically dependent as junctional conductance was reduced (Fig. 8, A and B). This is consistent with the presence of at least two distinct populations of voltage-dependent channels, one symmetric and other asymmetric. Thus either 1) heteromeric forms are the most prevalent channel type and have gating properties similar to heterotypic and homotypic channels, or 2) the population of functional heteromeric channels is so low that their gating behavior cannot be detected apart from the combination of homotypic and heterotypic channels. In either case, the heteromeric chan-

dels do not make a distinguishable contribution to voltage-dependent gating.

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