Heterotypic gap junction channel formation between heteromeric and homomeric Cx40 and Cx43 connexons

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Received 8 May 2001; accepted in final form 10 July 2001

Cottrell, G. Trevor, and Janis M. Burt. Heterotypic gap junction channel formation between heteromeric and homomeric Cx40 and Cx43 connexons. Am J Physiol Cell Physiol 281: C1559–C1567, 2001.—Recent evidence indicating formation of functional homomeric/heterotypic gap junction channels by connexin40 (Cx40) and connexin43 (Cx43) raises the question of whether data previously interpreted as support for heteromeric channel formation by these connexins might not instead reflect the activity of homomeric/heterotypic channels. To address this question and to further characterize the behavior of these channels, we used dual whole cell voltage-clamp techniques to examine the junctions formed between cells that express only Cx40 (Rin40) or Cx43 (Rin43) and compared the results with those obtained when either of these cell types was paired with cells that naturally express both connexins (A7r5 cells). Rin40/Rin43 cell pairs formed functional gap junctions that displayed a strongly asymmetric voltage-dependent gating response. Single-channel event amplitudes ranged between 34 and 150 pS, with 90- to 130-pS events predominating. A7r5/Rin43 and A7r5/Rin40 cell pairs had voltage-dependent gating responses that varied greatly, with most pairs demonstrating strong asymmetry. These cell pairs exhibited a variety of single-channel events that were not consistent with homomeric/homotypic Cx40 or Cx43 channels or homomeric/heterotypic Cx40/Cx43 channels. These data indicate that Cx40 and Cx43 form homomeric/heterotypic as well as heteromeric/heterotypic channels that display unique gating and conductance properties.

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mation of many homomeric/heterotypic channels. If such were the case, then the unique pH-dependent gating behavior would be that exhibited by homomeric/heterotypic, not heteromeric/heterotypic, channels.

The possibility of functional interactions between Cx40 and Cx43 has also been explored in mammalian cells. With the use of dye-transfer techniques, it was shown that HeLa cells expressing Cx40 were not capable of forming functional junctions with HeLa cells expressing Cx43 (8). Using electrophysiological techniques, Haubrich et al. (10) verified this observation and explored the structural basis for incompatibility. In light of these results, significant variability in voltage-dependent gating and single-channel conductance and the presence of heteromeric connexons in cells that coexpress Cx40 and Cx43 were interpreted by He et al. (11) as evidence for formation of functional heteromeric channels. However, recent evidence of homomeric/heterotypic channel formation by Cx40 and Cx43 in HeLa and Rin cells (22) raises the question of whether the heteromeric connexons detected biochemically by He et al. were nonfunctional and whether the reported variability in gating and single-channel behavior is a property of functional homomeric/heterotypic channels.

If heteromeric/heterotypic Cx40/Cx43 channels do form, then cell pairs formed between coexpressing cells and cells expressing only Cx40 or Cx43 would have unique voltage-dependent gating and single-channel conductance properties that could not be attributed to homomeric/homotypic, homomeric/heterotypic, or a combination of these channel types. In the present study, we verify that Cx43 and Cx40 form homomeric/heterotypic channels and further characterize the voltage-dependent gating behavior and transitional amplitudes of these channels. In addition, by demonstrating channel behaviors and voltage-dependent gating properties distinct from homomeric/heterotypic and homomeric/homotypic channels, we provide strong evidence that Cx40 and Cx43 form functional heteromeric/heterotypic channels.

MATERIALS AND METHODS

Cells and cell culture. Experiments were performed with A7r5 cells (American Type Culture Collection, Rockville, MD), Cx43-transfected Rin (Rin43) cells (generously provided by Dr. Paolo Meda; see Ref. 1), and Cx40-transfected Rin (Rin40) cells. Rin40 cells were obtained after transfection of Rin 1046 cells (generously provided by Dr. Ron Lynch) with pcDNA3 (Invitrogen, Carlsbad, CA) containing a 1.1-kb fragment of rCx40 that included the entire coding domain. Using PCR and primers that added BamHI and EcoRI restriction sites at the 5’ and 3’ ends, respectively, we amplified the coding sequence of rCx40 from SP64T-Cx40 (kindly provided by Dr. David Paul; see Ref. 5). The amplified fragment was digested and subcloned into the BamHI and EcoRI sites of pcDNA3, and the sequence was confirmed. Transfection was accomplished using Lipofectamine PLUS reagent (Life Technologies, Rockville, MD). Stable transfectants were selected with 125 μg/ml of genetin (Life Technologies). Parental Rin cells displayed no background connexin expression when evaluated using electrophysiological techniques.

A7r5 and Rin cells were cultured in DMEM (catalog no. 1152, Sigma) and RPMI 1640 medium (catalog no. 1383, Sigma), respectively. All media were supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics (5% streptomycin and 3% penicillin). Cells were kept in a 5% CO2 humidified incubator at 37°C and were passaged weekly, or as required, using 0.25% trypsin in Ca2+- and Mg2+-free phosphate-buffered saline.

Electrophysiology. A fluorescent tag and cell morphology were used, in combination, to distinguish between cell types in coculture during electrophysiology experiments. Cells were first grown to confluence in 100-mm plates. Old medium was aspirated off and replaced with medium containing 5 μM calcein-AM (Molecular Probes, Eugene, OR). Cells were incubated in calcein-AM-containing medium for 30 min at 37°C and then washed, lifted, and replated onto coverslips as described below. Detectable calcein transfer did not occur at the level of junctional coupling required for our experiments (<8 nS); therefore, calcein labeling and morphological differences inherent in Rin and A7r5 cells were effective for distinguishing new cell pairs in coculture.

Dual whole cell voltage-clamp experiments were performed on Rin43/Rin43, Rin40/Rin40, A7r5/Rin43, A7r5/Rin40, and Rin40/Rin43 cell pairs. All cells were grown to confluence before trypsinization (0.25% trypsin in Ca2+- and Mg2+-free phosphate-buffered saline) and were replated at low density on glass coverslips. Cells were then incubated for 1–12 h at 37°C before electrophysiological analysis. The coculturing of different cell types required various incubation periods for development of junctional communication. Rin40 cells were plated at low density 24 h before electrophysiological study.

Dual whole cell voltage-clamp experiments were carried out as reported previously (11, 12). Electrodes were fabricated from 1.2-mm filament glass (AM Systems, Everett, WA) on a Sutter Instruments (Novato, CA) puller and filled with (in mM) 67.8 CsCl, 67.8 potassium glutamate, 10 tetraethylammonium chloride, 0.5 CaCl2, 3 MgCl2, 5 glucose, 10 HEPES, 10 EGTA, and 5 Na2ATP (320 mosM, 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 (gj). Voltage-dependent gating was evaluated as previously described (11) in cell pairs in which the gj 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 (gj < 0.5 nS) using a 40-mV transjunctional voltage (Vj) applied for >20 s. The uncoupling agent halothane was used only for Rin43/Rin43 experiments; no uncoupling agents were used for any other experiments. No differences in single-channel conductances are observed for Rin43 cell pairs in the presence vs. the absence of halothane.

Data analysis. Nonlinear regression of voltage-dependent gating was performed using the Boltzmann equation in two different ways. If all experiments within a given treatment group could be fit with the Boltzmann equation, the voltagesensitive component of conductance (Gmin) and half-maximal Vj (V0) were determined for each experiment, averaged across treatment groups, and compared using Student’s t-test. In treatment groups where data were irregular and could not be fit using a Boltzmann equation, all experiments were pooled and simultaneously fit. The pooling of all the data for a given treatment group did not permit statistical comparisons between groups but did provide Gmin and V0 values that could be subjectively compared and an R2 value that served as an overall indicator of variability within the treatment group. Boltzmann fits were considered adequate if
all outcome values were unaltered with changing initial parameters.

Single-channel recordings were digitized for analysis using the software developed by Ramanan and Brink (19), as reported by He et al. (11). Single-channel event transitions were manually counted, normalized, and arranged into 10-pS bins for histogram comparisons. To limit the effect of voltage polarity-dependent rectification of single-channel conductances on variability within groups, single-channel event frequencies were compared between groups under a similar gating charge influence.

RESULTS

Voltage-dependent gating. The ability of a gap junction channel to gate closed in response to a change in the voltage potential between cells is variable, with each type of gap junction channel being associated with a unique voltage response “signature.” Examination of the macroscopic transjunctional current-voltage response in cell pairs permits determination of the types of gap junctions that may be present between a given cell pair.

Rin43 cell pairs \( (n = 6, g_j = 1.9 \pm 0.5 \text{ nS}) \) had a consistent voltage response across experiments (Fig. 1A). The data were Boltzmann fit using individual and pooled fitting approaches with comparable outcomes. There was no polarity-dependent asymmetry in the gating response, and \( G_{\text{min}} \) (negative \( = 0.33 \pm 0.03 \), positive \( = 0.37 \pm 0.03 \)) and \( V_0 \) (negative \( = 64 \pm 3 \text{ mV} \),

![Fig. 1. Voltage-dependent gating of gap junction channels with normalized junctional conductance (steady state relative to maximal \( \frac{G_{ss}}{G_m} \)) plotted as a function of transjunctional voltage \( (V_j) \) for Rin43 \( (A, n = 6) \), Rin40 \( (B, n = 5) \), A7r5/Rin43 \( (C, n = 8) \), A7r5/Rin40 \( (D, n = 7) \), and Rin40/Rin43 \( (E, n = 10) \) cell pairs. Solid line, mean normalized conductance across experiments; symbols, individual experiments; error bars, SE. \( V_j \) is plotted relative to the Rin43 cell in \( C \) and \( E \) and relative to the Rin40 cell in \( D \). Rin43 and Rin40 cells have uniform, symmetrical voltage responses. A7r5/Rin43 and A7r5/Rin40 cell pairs showed highly variable and asymmetric voltage responses. Rin40/Rin43 cell pairs displayed a strongly asymmetric voltage-dependent gating response opposite to that observed for A7r5/Rin43 experiments.](image-url)
The voltage-dependent gating of A7r5/Rin43 cell pairs was strongly asymmetric, with a polarity of asymmetry opposite to that predicted by the Cx40/Cx43 homomeric/heterotypic junction (compare Fig. 1C with 1E). When the Rin43 cell was held at a negative potential, \( G_{\text{min}} \) and \( V_0 \) values were \( 0.45 \pm 0.09 \) and \( 79 \pm 3 \) mV, respectively (\( R^2 = 0.65 \)) compared with \( G_{\text{min}} \) and \( V_0 \) values of \( 0.06 \pm 0.29 \) and \( 63 \pm 19 \) mV when the Rin43 cell was commanded with a positive potential (\( R^2 = 0.64 \), pooled fits). Boltzmann fits for all individual A7r5/Rin43 experiments could not be accomplished (3 of 8 A7r5+/Rin43− experiments and 4 of 8 A7r5−/Rin43+ experiments), thus precluding further statistical comparisons. The variability between experiments for A7r5/Rin43 pairs (\( R^2 = 0.65 \)) was greater than that for the Rin40/Rin43 and the Rin43/Rin43 pairs when the Rin43 cell was held at a negative potential, further differentiating these cell pairs from the homomeric/heterotypic and homomeric/homotypic phenotypes.

The voltage-dependent gating behavior of A7r5/ Rin40 cell pairs (\( n = 7, g_j = 5.9 \pm 2.3 \) nS) was more variable than that observed in homomeric/homotypic and homomeric/heterotypic pairs in the presence of a gating charge (Rin40+) and demonstrated only slight asymmetry (compare Fig. 1D with 1B and 1E). When the Rin40 cell was held at a positive potential, \( G_{\text{min}}, V_0, \) and \( R^2 \) values were \( 0.3 \pm 0.07, 44 \pm 6 \) mV, and 0.6, respectively, compared with \( G_{\text{min}}, V_0, \) and \( R^2 \) values of \( 0.23 \pm 0.2, 64 \pm 13 \) mV, and 0.57 when the Rin40 cell was commanded with a negative potential (pooled fits).

As with the A7r5/Rin43 pairs, the data from some A7r5/Rin40 pairs were not fit by the Boltzmann equation (1 of 7 data sets from A7r5+/Rin40−, 2 of 7 data sets from A7r5−/Rin40+), thus precluding statistical comparisons.

**Single-channel conductance.** Each type of gap junction channel has a unique ability to conduct current. This single-channel unitary conductance can be used to characterize which gap junction channels are present in a given cell pair. Amplitudes of Cx43 and Cx40 homomeric/homotypic channel events were evaluated. In the Rin43 cell pairs, 100% of single-channel event transitions were measured in the 60- to 90-pS range, with 75-pS events predominating (Fig. 2, A and C, Table 1). In contrast to previous studies of Cx43 channel conductance (11, 21), the Rin43 cells did not have repeatedly observable subconductance (or residual) states in the 20- to 30-pS range. The reason for this is unclear but may be related to an absence of phosphorylation-mediated reductions in channel conductance (17).

Rin40 cell pairs typically displayed main and residual states of \( \sim 170 \) and 25–30 pS, respectively, with frequent transitions of \( \sim 140 \) pS (Fig. 2, B and D). The long-lived, low-conductance state (residual state) of \( \sim 25 \) pS was observed in almost all records and could be measured only as the \( V_j \) was returned to zero; therefore, these events were not reflected in the event frequency histogram. There was significant variability in event amplitudes between and within experiments; however, 88% of the transitions occurred between 120 and 180 pS (Fig. 2B, Table 1), which could reflect the difference between full open and transition states.

A7r5/Rin43 cell pairs had channel events that were similar in amplitude to homomeric/homotypic Cx43 channel events (66, 74, 88, and 91 pS) as well as many atypical event amplitudes (98, 105, 116, and 130 pS; Fig. 3, A and C). With 49% of the events in the 60- to 120-pS range and 45% of event transitions <60 pS, it was clear that these channel events were separate from those of typical homomeric/homotypic Cx43 channels (Table 1). A7r5/Rin40 cell pairs had channel events that were similar to Cx40/Cx40 channels (129, 140, 164, and 170 pS), as well as many dissimilar events (62, 79, 86, 103, 115, 119, and 190 pS; Fig. 3, B and D). Events of low amplitude (<120 pS) occurred at a much higher frequency than in the Cx40/Cx40 settings (32% vs. 5%), which suggests that these events were unlikely to represent the activity of homomeric/homotypic channels (Table 1).
for A7r5/Rin43 cell pairs (Table 1). Similar to previous reports, the homomeric/heterotypic channels displayed \( V_j \) polarity-dependent rectification. Such rectification is evident in Fig. 4, where the Rin40 cell of a Rin40/Rin43 cell pair was held alternately at \(-40\) mV (Fig. 4B), where 122- and 80-pS events were observed, and at \(+40\) mV (Fig. 4C), where 150- and 108-pS events were observed. This observation was consistent between experiments; however, the large variability of channel transitions, both within and between experiments, did not permit a clear distinction of polarity-specific channel conductances.

**DISCUSSION**

To convincingly demonstrate that Cx40 and Cx43 form heteromeric/heterotypic channels, the properties of such channels must be distinct from the homomeric/homotypic channels (Cx40/Cx40 and Cx43/Cx43) and the homomeric/heterotypic channels (Cx40/Cx43). Distinct voltage-dependent gating and single-channel behaviors were observed in settings where formation of heteromeric/heterotypic channels was favored, which leads us to conclude that Cx40 and Cx43 form heteromeric/heterotypic channels.

The voltage gating data for A7r5/Rin43 cell pairs provide the most striking evidence for heteromeric/heterotypic gap junctions formed by the oligomerization of Cx40 and Cx43. In this pairing, the dominance of the Cx43 connexon is lost in the gating response. When the Rin43 cell is held negative, the gating effect is much less than that observed in the Cx43/Cx43 setting (compare Fig. 1A with 1C). Comparatively, the greatest average gating response is observed when the Rin43 cell is positive, indicating that the Cx43 connexon has shifted to a positive gating sensitivity or the coexpressing cell is dominating the gating effect. In either case, this implies that the channels observed in the coexpressing cell are not just homomeric Cx40 or Cx43 but unique hemichannel types that are derived from heteromeric connexon formation. This is further supported by an opposite asymmetric voltage gating response in the A7r5/Rin43 cells from that predicted by a combination of homomeric/homotypic and homomeric/heterotypic channels. With the assumption of an equal combination of Cx43/Cx43 and Cx40/Cx43 channels, macroscopic voltage gating responses should result in a \( G_{\text{min}} \) of \(-0.2\) and \( V_0 \) of 39 mV when the Rin43 cell is held at a negative potential. This was not observed. In fact, there are no possible combinations of Cx43/Cx43 and Cx40/Cx43 channels that would account for the observed A7r5/Rin43 voltage gating response. As well, the variability between cell pairs did not reflect the voltage response of heterotypic channels or an average distribution between homomeric/homotypic and homomeric/heterotypic channels. The high variability in gating response between cell pairs, loss of the Cx43-dominated gating typical of the Cx43/Cx40

<table>
<thead>
<tr>
<th>Cell Pair</th>
<th>Conduction Bin</th>
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<tr>
<td></td>
<td>&lt;60 pS</td>
</tr>
<tr>
<td>Rin43/Rin43</td>
<td>0</td>
</tr>
<tr>
<td>Rin40/Rin40</td>
<td>0</td>
</tr>
<tr>
<td>Rin43/A7r5</td>
<td>45</td>
</tr>
<tr>
<td>Rin40/A7r5</td>
<td>6</td>
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<tr>
<td>Rin40/Rin43</td>
<td>12</td>
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Values are percentages. Events were counted for each cell pair, normalized to the total events for that cell pair, averaged across experiments, and grouped into conductance bins.
cell pairs, and opposite asymmetric voltage dependence provide direct evidence for the presence of heteromeric connexons in the coexpressing cells.

A7r5/Rin40 cell pairs do not demonstrate dramatic shifts in asymmetric voltage response; however, they do display high variability between experiments. As well, the upward shift in $G_{\text{min}}$ when the Rin40 cell is held with a positive potential is opposite to that which is predicted by a combination of Cx40/Cx40 and Cx40/Cx43 channels. The lack of significant asymmetry in A7r5/Rin40 cell pairs, despite the purported heteromeric channels in the coexpressing cells, implies that Cx40 maintains a dominant gating effect in the heteromeric and homomeric connexon populations. It could also be the result of a greater proportion of Cx40 connexons in the coexpressing cell. Cx40 and Cx43 protein content has been quantified for confluent A7r5 cells, with the Cx43-to-Cx40 expression ratio being...
calculated as 0.41 (7). This value falls in subconfluent cells to levels as low as 0.17. Little is known about heteromeric channel assembly, but at the 0.41 expression ratio, with the assumption that channel assembly can be described by probability theory, as is true for other channel types (14), 13% of connexons in the A7r5 cells would be homomeric Cx40, 0.06% homomeric Cx43, and 81% heteromeric connexons containing three, four, or five Cx40 subunits. Thus, when paired with cells that express only Cx40, >94% of the channels would contain nine or more subunits of Cx40, whereas when paired with cells that express only Cx43, none of the channels would contain this many Cx40 subunits. Instead, 13% of the channels would be homomeric/heterotypic, and 81% of the channels would contain three, four, or five subunits of Cx40. If channel behavior is increasingly Cx40-like as the number of Cx40 subunits in the channel increases, then it would be expected that the A7r5/Rin40 cells pairs would display far less channel diversity than the A7r5/Rin43 cell pairs. These assumptions on channel assembly and behavior require further testing, but certainly they are consistent with the available data.

The single-channel event transitions for A7r5/Rin43 cell pairs provide further evidence for Cx40 and Cx43 heteromerization. As shown in Table 1, 100% of Rin43/Rin43 single-channel amplitudes were 60–90 pS, and 88% of Rin40/Rin43 amplitudes were 60–150 pS. If the channels formed between A7r5 and Rin43 cell pairs are a combination of homomeric/homotypic and heteromeric/heterotypic channels, then 88–100% of the A7r5/Rin43 transition events should be in the 60- to 150-pS range. Instead, only 55% of the channel events fall in this range for these cell pairs. Furthermore, 45% of the channel amplitudes were <60 pS in the A7r5/Rin43 pairs, compared with 0% in Rin43/Rin43 and 12% in Rin40/Rin43 cell pairs. No combination of Cx43/Cx43 and Cx40/Cx43 channels can account for these data, providing further support for heteromeric Cx40 and Cx43 connexon formation in the coexpressing cells.

The single-channel evidence was less convincing for A7r5/Rin40 cell pairs. As shown in Table 1, 88% of Rin40/Rin40 and 37% of Rin40/Rin43 channel events were in the 120- to 190-pS range. It was also shown that 64% of the A7r5/Rin40 events fell in this same range, which represents almost exactly what would be predicted for an equal contribution of Cx40/Cx40 and Cx40/Cx43 channels. There are several possible explanations for this: 1) similar to the voltage gating data, Cx40 dominates channel conductance even in heteromeric channels, 2) there are more Cx40 channels because of the low Cx43-to-Cx40 expression ratio, 3) heteromeric Cx40/Cx43 connexons, when paired with a homomeric Cx40 connexon, have a dominant conductance that is coincidentally in the midrange between Cx40/Cx40 and Cx40/Cx43 channels, and 4) the channels measured are not heteromeric and, instead, reflect a combination of homomeric/homotypic and homomeric/heterotypic channels, although this latter explanation is not supported by the A7r5/Rin43 findings.

It is interesting to note that if the voltage-dependent gating and single-channel event frequency data of Rin40/A7r5 and Rin43/A7r5 cell pairs are combined, the results are similar to those previously reported for A7r5 cell pairs (11, 13, 15). This observation provides evidence that the gap junction channels observed in A7r5 cell pairs are representative of a large variety of heterologous channel assemblies.

Our data permitted characterization of homomeric/heterotypic Cx40/Cx43 channels beyond that previously published by Valiunas et al. (22). The asymmetry of the voltage-dependent gating response of homomeric/heterotypic Cx43/Cx43 channels (Fig. 1E) was expected from the positive gating of Cx40 and negative gating of Cx43 (4, 5, 16); however, the presence of a slight, yet inconsistent, gating effect when the Rin43 cell was held positive (Rin40) was not. This latter observation suggests that either voltage sensing components of the individual hemichannels are not clearly defined or the formation of a heterotypic channel somehow changes the voltage-sensing component(s) of the individual connexons. The lower \( V_0 \) and \( G_{min} \) values of Cx40/Cx40 than Cx43/Cx43 channels might have predicted that the heterotypic channel’s voltage dependence would resemble that of Cx40/Cx40 channels (5). The similarity of \( V_0 \) values reported here supports this expectation; however, the disparity in \( G_{min} \) values suggests that the heterotypic channels display unique properties not readily predicted by the homomeric/homotypic channels. A shift to a lower \( G_{min} \) value in the heterotypic setting is provocative, in that it suggests that either the non-voltage-sensitive substate had a lower conductance than in the homotypic setting or the open channel probability was lower than typical of the homomeric/homotypic channel. Unfortunately, neither of these factors could be resolved in these experiments. Valiunas et al. also observed asymmetric gating behavior but reported that heterotypic channels were less voltage sensitive than homotypic channels. They used 800-ms bipolar pulses. Our observations with >10-s pulses suggest that an 800-ms pulse is insufficient for a true steady state to be achieved. Consequently, they only partially characterized the voltage-dependent gating, the portion termed “fast inactivation,” while changes in slow inactivation were not assessed.

The unique properties of Cx40/Cx43 heterotypic channels include not only gating differences but conductance differences (Fig. 4A). The high frequency of smaller events (<100 pS) might be explained by long-lived substrates, but the basis for larger events (>130 pS) is more difficult to discern. Because the conductance of a channel reflects its length, width (including access resistance), and selectivity, docking of the two dissimilar connexons by necessity results in an asymmetric channel. Such asymmetry is predicted to result in nonlinear conductance behavior (24), which may be further augmented by allosteric interactions that affect channel length, width, or selectivity. The resulting asymmetric channel displays a variety of event amplitudes, some larger than expected, and polarity-depen-
dent rectification. Valiunas et al. (22) also described rectification in their examination of heterotypic channels; however, the short time course of their holding potentials during single-channel analysis restricted their ability to observe the diversity of conductances described here for the heterotypic setting.

Cx40 and Cx43 are dynamically expressed in cells of the cardiovascular system in a species- and tissue-specific manner (23, 25). Expression levels in these tissues vary during development and in response to disease and injury (3, 18, 20). Because of the unique conductance and gating properties of heteromeric/heterotypic channels, these changes in connexin expression could result in dramatically altered intercellular communication and tissue function. Kurjiaka et al. (12) provided evidence of this, whereby proliferating A7r5 cells were less capable than growth-arrested A7r5 cells of communicating large molecules but maintained equivalent electrical coupling. Recent evidence (7) suggests that these shifts in communication are comparable to that observed with an increase in the Cx40-to-Cx43 ratio in proliferating cells and, therefore, may reflect an alteration in contribution of heteromeric/heterotypic channel populations. The heteromeric/heterotypic channel formation found here provides corroborating evidence supporting this hypothesis. The unique channel properties conveyed by heteromeric/heterotypic channels are likely to profoundly influence tissue function.

Unfortunately, the relationship between channel composition and channel conductance and gating has yet to be understood. It is not known whether a heteromeric channel with a single subunit of Cx43 and 11 subunits of Cx40 can be distinguished from another with a 50:50 mixture. If there are 196 different channel types, the possibility that each has unique conductance properties makes fitting the current data set with only a few Gaussian curves inappropriate. As a result, there are no appropriate statistical tools that would permit the differentiation of multiple channel types in a single-channel record. Establishing a relationship between channel conductance and channel composition will require examination of channel behavior in settings where the number of possible channel forms is restricted by altered expression ratios.

In previous research, five studies have concluded that Cx40 and Cx43 are incapable of forming functional homomeric/heterotypic channels (5, 8–10, 27), whereas one study has shown that they do form such channels (22). Here we confirm the ability of Cx40 and Cx43 to form homomeric/heterotypic channels. These channels have an altered voltage sensitivity, a multitude of single-channel conductance events, and a voltage polarity-dependent rectification of single-channel conductances that are partially obscured by the high variability in channel events. We also support the findings of He et al. (11) and demonstrate that, indeed, Cx40 and Cx43 form heteromeric/heterotypic channels and these channels display highly variable voltage sensitivities and single-channel events. It is not clear why the oocyte model fails to form detectable homomeric/heterotypic Cx40/Cx43 channels, nor is it clear why Elfgang et al. (8) and Haubrich et al. (10) did not see such channels in HeLa cells when Valiunas et al. (22) did. Nevertheless, heterologous assembly of connexins may be more robust than once thought, and the complexity of these interactions and their impact on cell function remain to be discovered.

This study was supported by National Heart, Lung, and Blood Institute Grant HL-58732 and American Heart Association Grant 0050020N.

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