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Cx30.2 can form heteromeric gap junction channels with other cardiac connexins

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Abstract

Since most cells in the heart co-express multiple connexins, we studied the possible heteromeric interactions between connexin30.2 and connexin40, connexin43 or connexin45 in transfected cells. Double label immunofluorescence microscopy showed that connexin30.2 extensively co-localized with each co-expressed connexin at appositional membranes. When Triton X-100 solubilized connexons were affinity purified from co-expressing cells, connexin30.2 was isolated together with connexin40, connexin43, or connexin45. Co-expression of connexin30.2 with connexin40, connexin43, or connexin45 did not significantly reduce total junctional conductance. Gap junction channels in cells co-expressing connexin30.2 with connexin43 or connexin45 exhibited voltage-dependent gating intermediate between that of either connexin alone. In contrast, connexin30.2 dominated the voltage dependence when co-expressed with connexin40. Our data suggest that connexin30.2 can form heteromers with the other cardiac connexins and that mixed channel formation will influence the gating properties of gap junctions in cardiac regions that co-express these connexins.

Keywords

Gap Junction; Intercellular Communication; Connexin

Current passage between cells in the heart is facilitated by intercellular channels clustered in gap junctions. These channels are formed by docking of two hemichannels (connexons) provided by the opposing cells. Each connexon is a hexamer of subunit proteins called connexins (Cx). As demonstrated in cells expressing individual connexins, each connexin can form channels by itself (homomeric/homotypic channels), and different connexins form channels with different conductance, permeability and gating properties [1]. In cells co-expressing different connexins, heteromeric channels (containing different connexins in the same connexon) can potentially be formed [2].

Many studies have shown the presence of three well characterized connexin proteins in the heart, Cx40, Cx43, and Cx45. Cx43 is most abundant in atrial and ventricular myocytes and in Purkinje fibers [3,4]. Cx40 is expressed in atrial myocardium, atrioventricular bundle and

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its branches [5,6]. Cx45 is detected in sinoatrial and atrioventricular nodes, atrioventricular bundle and branches [7,8].

Recently, another cardiac connexin, mouse Cx30.2 (human Cx31.9) was discovered [9–11]. Cx30.2 expression was detected within the sinoatrial and atrioventricular nodes [11,12]. Expression of Cx30.2 in *Xenopus* oocytes or transfected cells has shown that this connexin forms functional channels with rather unique properties including a low sensitivity of gating to transjunctional voltage and a smaller unitary conductance than other connexins (~9 pS) [10,11]. Since we have been interested in the formation of heteromeric channels between connexins expressed in heart and its consequences mixing upon cardiac intercellular communication [13,14], we designed the current study to examine the possible heteromeric mixing of Cx30.2 with Cx40, Cx43, or Cx45.

MATERIALS AND METHODS

Connexin-expressing cells

Different cell lines were utilized because of their relative advantages for different analyses (N2a cells for electrophysiology; HeLa and HEK293 cells for biochemical and immunohistochemical analysis). Generation of HeLa cells stably expressing Cx43 or Cx45 [13,14] and culturing of N2a, HeLa, and HEK293 cells have been described previously [15, 16].

Cx30.2 was amplified from mouse genomic DNA by PCR (using sense primer, ATGGGGGAGTGGGCGTTCCTAGGCTCCCTG and anti-sense primer, CTAGATGGCCAGGTCCTGGCGCACGGTAGC) yielding a sequence identical to GeneBank accession number AJ414561. Cx30.2 DNA was subcloned into pTracer-CMV2 and pcDNA3.1/+ (Invitrogen, Carlsbad, CA). Rat Cx40 DNA was subcloned into pcDNA3.1/hygro (Invitrogen). Constructs containing an HA tag (YPYDVPDYA) appended to the C-termini of Cx30.2 or Cx40 were obtained using PCR methods.

Cells were transiently or stably transfected with connexin DNA using lipofectamine (Invitrogen/GIBCO) [15]. HEK293 cells stably expressing both Cx30.2 and Cx40 were generated by sequential transfection with two different expression plasmids [15,16]. For many of the electrophysiological experiments, N2a or HeLa cells were transiently transfected with connexin DNA in pTracer-CMV2 (in which GFP is driven by the EM-7 promoter and the subcloned sequence is driven by the CMV promoter); transfected cells were identified by GFP fluorescence.

Antibodies used for connexin detection

Cx30.2 was detected using rabbit polyclonal antibodies directed against a peptide derived from the C-terminal region of mouse Cx30.2 (40–7400; Invitrogen/Zymed). Anti-Cx30.2 antibodies showed no reactivity with untransfected N2a, HeLa or HEK293 cells (not shown). Cx43 was detected using a mouse monoclonal antibody directed against amino acids 252–270 (MAB 3068, Millipore/Chemicon, Billerica, MA) for immunofluorescence or using rabbit polyclonal antibodies directed against amino acids 363–382 of human/rat Cx43 (C6219, SIGMA Chemical Company, St. Louis, MO) for immunoblotting. Cx40 was detected using a mouse monoclonal antibody (39-1700, Invitrogen/Zymed) for immunofluorescence or rabbit antibodies directed against a bacterially expressed fusion protein containing the Cx40 carboxyl tail [17] for immunoblotting. Cx45 was detected using a mouse monoclonal antibody directed against amino acids 354–367 of human Cx45 (MAB 3101, Millipore/Chemicon).

Immunofluorescence analysis of connexin expression

For microscopy, cells were cultured on multiwell slides. For double-labeling experiments, cells were incubated simultaneously with both rabbit anti-Cx30.2 antibodies and mouse anti-Cx43, Cx45 or Cx40 monoclonal antibodies followed by anti rabbit Cy2- and anti mouse Cy3-conjugated secondary reagents. Confocal microscopy was performed as described [16].

Affinity purification of HA-tagged proteins

For studies of potential connexin co-purification, one HA-tagged connexin and a second untagged connexin were co-expressed. One protein was expressed stably, another one was introduced by transient transfection or both were expressed transiently. 72 hours following transfection, connexins and connexons were solubilized in 1% Triton X-100 [18]. Samples were centrifuged at 100,000g_{ave} for 30 min, and the supernatant (containing solubilized connexons) was affinity purified using the μ MACS HA isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) [19]. Samples were analyzed by immunoblotting. We have previously utilized this procedure to identify heteromeric interactions between connexins and demonstrated the specificity of this procedure [13,14,16,19] including a variety of “negative controls”. When the supernatant from cells expressing only an untagged connexin was applied to the MicroBeads, no connexin protein was found in the eluate; all connexin protein was recovered in the “flow-through”. Moreover, for combinations of connexins that do not form heteromers (eg. Cx43 and Cx26) the untagged connexin does not co-purify with the tagged connexin [16].

Electrophysiological recordings

Double whole cell patch clamp recordings of junctional currents (I_j) were performed as described previously [20,21]. All transjunctional voltage (V_j) errors resulting from the patch electrode series resistance (R_{el}) were corrected during the junctional conductance (g_j) calculations according to the expression:

$$g_j = \frac{-\Delta I_2}{V_1 - (I_1 \bullet R_{el1}) - V_2 + (I_2 \bullet R_{el2})} \quad [21]$$

The maximum g_j ($g_{j,max}$) was determined from the linear regression fit of the steady state I_j - V_j curve from each experiment between ± 5 and ± 20 mV. Normalized g_j (G_j) was calculated from the ratio of $g_j/g_{j,max}$ for each V_j polarity and normalized $I_j = G_j \times V_j$ for each experiment. All currents were digitized at 1–4 kHz after low pass filtering at 100–500 Hz (LPF 202A, Warner Inst., Hamden, CT). Curve fitting procedures were performed using Clampfit software (pClamp version 8.2, Axon Instruments, Inc.) using the sum of squared errors minimization procedure and the standard errors for each estimated parameter are provided.

RESULTS

Characterization of Cx30.2 expressed alone

Cx30.2 was expressed by transient transfection of N2a or HeLa cells (which are connexin- and communication-deficient). Cx30.2 protein production was verified by immunoblotting (Supplemental figure). Immunoblots showed a major immunoreactive Cx30.2 band of approximately 30 kDa in homogenates of these transfected cells (that migrated similarly to the band detected in samples prepared from mouse heart. In untransfected cells, no immunoreactive Cx30.2 was detected. Immunofluorescence demonstrated immunoreactive Cx30.2 at appositional membranes in transfected cells (data not shown).

Double whole cell patch clamp studies showed that expression of Cx30.2 produced robust currents in transfected N2a cells. The junctional current (I_j) increased at transjunctional

voltages ($V_j \geq 40\text{mV}$), whether the g_j was high or low, causing an increase in normalized junctional conductance (G_j). This increasing I_j and V_j was observed whether a continuous V_j ramp or pulse protocol was used (Fig. 1). The steady-state junctional conductance-voltage ($G_j - V_j$) relationship was distinct from those of other cardiac connexins (e.g. Cx43, Cx40 and Cx45), but was well described by a modified Boltzmann equation:

$$G_{j,ss} = 2 - [G_{j,max} \cdot [\exp(zF/RT \cdot (V_j - V_{1/2}))] + G_{j,min}]/[1 + [\exp(zF/RT \cdot (V_j - V_{1/2}))]]$$

where $G_{j,max} = 1$, $G_j = g_j/g_{j,max}$, $G_{j,min}$ is the minimum value of $g_j/g_{j,max}$, z is the valence in elementary charge units (q), F is Faraday's constant (in coulombs/mol), R is the molar gas constant [in J/(mol · K)], T is temperature (in K), $V_{1/2}$ is the half-inactivation voltage.

Immunochemical studies of the heteromeric compatibility of Cx30.2 with other cardiac connexins

We used double label confocal immunofluorescence microscopy to examine the localization of Cx30.2 and Cx43, Cx45 and Cx40 in co-expressing cells. Co-expression of Cx30.2 with the other cardiovascular connexins was studied by stable transfection of HEK293 cells (which endogenously express Cx43 and Cx45) or by stable expression of both Cx30.2 and Cx40 into these cells. While no immunoreactive Cx30.2 was detected in untransfected HEK293 cells, homogenates of stably transfected cells contained a Cx30.2 band of approximately 30 kDa (Supplemental figure).

There was substantial overlap (co-localization) of Cx30.2 immunoreactivity with each of other connexins at appositional membranes (Fig. 2).

The potential heteromeric association of Cx30.2 with other connexins was also examined using an affinity purification strategy analogous to that previously used to study formation of heteromeric connexons between Cx43 and other connexins [13,14,16]. In each experiment, one of the two co-expressed connexins contained an HA epitope tag. The 100,000g supernatant of a 1% Triton X-100 cell extract was affinity purified using an HA column which allowed binding and elution of the tagged connexin and any tightly associated proteins. Co-elution of a co-expressed connexin suggests heteromeric association between them [19]. Cx30.2 co-purified with co-expressed Cx43, Cx45 or Cx40 (Fig. 3).

Macroscopic junctional conductance and V_j -dependent gating in cells co-expressing Cx30.2 with Cx43, Cx45 and Cx40

The effects of Cx30.2 upon the total junctional conductance (g_j) when co-expressed with other cardiac connexins were examined by transient transfection into cells expressing no connexin (N2a) or stably expressing each of the connexins individually (N2a-Cx43, HeLa-Cx45, or N2a-Cx40). Expression of Cx30.2 alone in N2a cells produced a macroscopic junctional conductance of $1.1 \pm 1.9 \text{ nS}$ ($n=6$) (Fig. 4A). A wide range of conductances was observed in cells expressing Cx43, Cx45, or Cx40 alone or in combination with Cx30.2. On average, there was a decrease in g_j when Cx30.2 was transfected into cells stably expressing one of the other connexins, however the differences did not achieve statistical significance (p -values > 0.07 , one-way ANOVA) (Fig. 4A).

The ability of Cx30.2 to influence the voltage gating of other cardiac connexins was tested by co-transfecting Cx30.2 into cells expressing Cx43, Cx45, or Cx40. In some cell pairs co-expressing Cx30.2 and Cx43, voltage-dependent gating was intermediate ($0.45 < G_{min} < 1.0$, $g_j = 3.94 \pm 2.09 \text{ nS}$, $n=4$) between that of cells expressing either connexin alone (Fig. 4B); some other cell pairs showed $G_j - V_j$ curves similar to those seen in cells expressing only Cx30.2 ($G_{min} > 1.0$, $g_j = 2.67 \pm 2.44 \text{ nS}$, $n=3$, Fig. 4B). In most pairs of cells co-expressing Cx30.2 and Cx45, V_j -dependent gating was intermediate between that in cells expressing either

connexin alone ($0.12 < G_{\min} < 0.55$, $g_j = 7.25 \pm 8.53$ nS, $n=7$, Fig. 4C); a single cell pair ($G_{\min} > 1.0$, $g_j = 12.9$ nS) exhibited gating rather similar to that of Cx30.2 alone (Fig. 4C). In contrast, all pairs of cells co-expressing Cx40 and Cx30.2 showed V_j -dependent gating similar to that of Cx30.2 alone (Fig. 4D). Because of the variability, Boltzmann fits of the steady state G_j - V_j curves could not be determined for cells co-expressing Cx43 and Cx30.2 or Cx45 and Cx30.2. The parameters of the Boltzmann fits for Cx30.2-Cx40 co-expressing cell pairs were very similar to these of cells expressing only Cx30.2 (Supplemental Table)

Single channels in co-expressing cells

Single channel events in cells co-expressing Cx30.2 with Cx43, Cx40, or Cx45 were difficult to resolve from junctional whole cell recordings. However, brief examples of unitary current fluctuations were observed in one case each for low g_j N2a-Cx43 and N2a-Cx40 cell pairs co-transfected with Cx30.2 (Fig. 4 E, G). For comparison, recordings from homotypic N2a-Cx43 and N2a-Cx40 cell pairs are shown in Fig. F, H to indicate that, in the absence of Cx30.2, unitary gap junction currents were readily observable under similar circumstances.

DISCUSSION

This project was undertaken to study the properties of Cx30.2 after expression in transfected cells and the consequences (potential heteromeric interactions) of co-expression with Cx40, Cx43, and Cx45. In cells expressing Cx30.2 alone, functional gap junction currents were detected. The Cx30.2 currents activated at transjunctional potentials ≥ 40 mV (Fig. 1). Previously, investigators had shown that Cx30.2 gap junction channels exhibit little gating sensitivity to transjunctional voltage [9–11]. The insensitivity or activation of Cx30.2 G_j at high V_j may be important for the preservation of slow impulse propagation through the atrioventricular node. During fast impulse propagation in the atrium or ventricle, which expresses mainly Cx40 and Cx43, large inactivating V_j gradients are not supposed to develop under normal physiological conditions. Inactivation may serve as a protective mechanism by which healthy cells uncouple from their compromised neighbors; partial uncoupling can also promote conduction slowing, unidirectional block, and arrhythmias [22,23]. In the atrioventricular node where conduction is normally slow, inactivation of G_j could promote partial AV block whereas activation above > 60 mV V_j would preserve slow conduction.

We analyzed the cell biological and biochemical properties of cells co-expressing Cx30.2 with Cx40, Cx43, or Cx45. Double-label confocal immunofluorescence microscopy showed near perfect co-localization of all studied connexin combinations in HEK-293 cells (Fig. 2); this implies that Cx30.2 can mix (nearly homogeneously) within the same gap junction plaques with the other cardiac connexins. Affinity purification experiments using Triton X-100 solubilized material (containing connexons) consistently demonstrated co-purification of Cx30.2 with Cx40, Cx43, or Cx45 (Fig. 3); these results provide biochemical evidence for the ability of Cx30.2 to hetero-oligomerize with the other cardiac connexins. These data add to our previous observations (using similar strategies) showing evidence for heteromer formation between other pairs of co-expressed cardiac connexins including Cx40 and Cx43 [13] or Cx43 and Cx45 [14].

Previously, the potential participation of Cx30.2 in mixed channels was characterized physiologically by expressing it in heterotypic combinations with other connexins. HeLa cells transfected with mCx30.2 formed functional heterotypic channels when paired with cells expressing Cx40, Cx43 or Cx45. Voltage-dependent gating was asymmetric, consistent with the contributions of the two different hemi-channels [11]. Single channel conductances were small (17–18 pS) for all three combinations [11] and the channels exhibited limited permeability to Alexa Fluor-350 [24] reflecting the limited permeability of Cx30.2 to this dye.

We also characterized the physiological properties of the gap junctions in co-expressing cells. Our double whole cell patch clamp studies showed that the most poorly coupled cells were these that expressed Cx30.2 alone. Co-expression of Cx30.2 with Cx43, Cx45 or Cx40 led to reductions in total junctional conductance in all cases; however the differences were not significant because of the large variation of the data for all types of cell pairs (Fig. 4A). It has previously been hypothesized that co-expression of Cx30.2 with other connexins might reduce junctional conductance in co-expressing cardiac cells such as those within the sinoatrial and atrioventricular nodes [25]. Our data suggest that these reductions may be relatively modest. In cells expressing Cx43 and Cx45, introduction of Cx30.2 did alter the properties of the gap junction currents (Fig. 6). In most cell pairs, voltage-dependent gating was intermediate between that of either connexin alone. These intermediate properties might reflect formation of heteromeric channels, similar to the situation of some connexins that make heteromeric channels (eg, Cx37/Cx43 and Cx43/Cx45) [2,14]; alternatively, they might correspond to the aggregate of a mixture of channels formed exclusively of the two different connexins. In some cell pairs, gating was similar to that of Cx30.2 alone; this might best be explained by an imbalance in expression levels of the two connexins with Cx30.2 being much greater. Thus, for the combinations of Cx30.2 with Cx43 or Cx45, while biochemical evidence suggests that they can form heteromeric channels, it is not necessary to invoke the presence of heteromers to explain the alterations of total gap junction channel activity in co-expressing cells. This situation resembles that observed for the co-expression of Cx40 and Cx43 [13].

In contrast, the physiological properties of gap junction channels in cells co-expressing Cx30.2 and Cx40 can only be explained easily by the formation of heteromeric channels. When Cx40 and Cx30.2 were co-expressed, the voltage-dependence closely resembled that in cells expressing Cx30.2 alone (Fig. 4D); thus, although these cells contained both proteins, Cx30.2 dominated this property. This is reminiscent of previous cases in which one connexin dominates the dye permeability or kinase sensitivity properties of mixed channels [14]. Similar to other connexin pairs that form heteromeric channels (e.g., Cx37/Cx43 and Cx43/Cx45; [2,14]) Cx30.2 and Cx40 co-expression may result in new single channel conductances, since these cells did not contain distinct large channels as expected for Cx40 alone.

Taken together with data regarding expression patterns of cardiac connexins, our new results suggest that co-expression of Cx30.2 with some of the other connexins may influence cardiac intercellular communication. Cx30.2 has been identified in the sinoatrial node, atrioventricular node, and atrioventricular bundle [11,12]. Cx43 is probably absent from these regions; thus interactions between Cx30.2 and Cx43 may have limited relevance to cardiac physiology in vivo. Cx45 shows the most extensive overlap with the Cx30.2 expression pattern; thus Cx30.2-Cx45 interactions may influence conduction in all those areas. However, the greatest consequences of heteromerization of Cx30.2 with other connexins may occur in those cells of the atrioventricular node and atrioventricular bundle where it has an overlapping distribution with Cx40 [11] because Cx30.2 co-expression so substantially alters the behavior of Cx40 channels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Double whole cell patch clamp characterization of gap junction currents in N2a cells transfected with Cx30.2

A. Representative junctional whole-cell current ($I_j = -\Delta I_2$) responses to ± 100 mV V_j pulses applied to cell 1. Dashed line indicates the $I_j = 0$ baseline.

B. Average I_j - V_j curve from five low g_j N2a-Cx30.2 cell pairs ($g_j = 0.27 \pm 0.07$ nS) obtained in response to a 200 ms/mV V_j ramp applied to cell 1. I_j increased above $V_j > \pm 40$ mV.

C. Average steady-state G_j - V_j relationship for five N2a-Cx30.2 cell pairs demonstrating the increase in G_j as the V_j ramp exceeded ± 40 mV.

Figure 2.

Double label immunolocalization of Cx30.2 and other cardiac connexins in stably transfected HEK cells. Individual immunoreactive connexins are detected in first two columns and appear green (Cx30.2) or red (Cx43, Cx45 or Cx40). Merged images are shown in the right panels where overlap appears yellow. Cx30.2 and each of the co-expressed cardiac connexins localize to gap junction plaques and show substantial overlap. Bar represents 6 μ m for A–F, 5 μ m for G–I.

Figure 3.

Affinity purification of HA tagged and associated connexins from HeLa-Cx43 cells transfected with Cx30.2HA (A), HeLa-Cx45 cells transfected with Cx30.2HA (B), or HeLa cells co-transfected with Cx30.2 and Cx40HA (C). Connexins were detected by immunoblotting. Gels were loaded with 25 μ g of protein (corresponding to 1/10 of the amount incubated with MicroBeads) in the lane labeled “before column”; 1/10 of the fraction collected as a “flow through”, 1/5 of each of the “washes”; and 1/10 of the “eluate” (corresponding to 25 μ g of protein applied to the purification column).

Figure 4. Gap junction conductances and currents in cells co-expressing Cx30.2 with other connexins. In all co-expression experiments, the first connexin was stably transfected, and Cx30.2-pTracer DNA was introduced transiently

A. Macroscopic junctional conductance (g_j) in cells expressing individual cardiac connexins or co-expressing Cx30.2 with Cx43, Cx45 or Cx40. Open circles, g_j from individual cell pairs; closed squares, means of open circles; brackets, $\pm$ S.D; n, number of cell pairs.

B–D. Relationships between normalized steady state junction conductance (G_j) and transjunctional voltage (V_j) in cells co-expressing Cx30.2 with Cx43 (B), Cx45 (C) or Cx40 (D) compared with those connexins alone.

E–H. Single gap junction channel events in N2a cells expressing Cx43+Cx30.2 (E), Cx43 (F), Cx40+Cx30.2 (G), or Cx40 (H). While unitary channel events were rarely resolved in recordings from the co-expressing cells (E, G), the N2a-Cx43 cell pair (F) contains identifiable unitary current fluctuations with conductances of 60, 85, or 115 pS and the N2a-Cx40 cell pair (H) contains two identifiable unitary channel currents with a conductance of 170 pS. The dotted lines indicate the zero I_j current baselines. Mirror image I_1 traces (not shown) confirmed the junctional nature of these current fluctuations.