Interfering amino-terminal peptides and functional implications for heteromeric gap junction formation

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INTRODUCTION

Gap junctions facilitate the metabolic, biochemical, and electrical integration of component cells into functional tissues, because they contain intercellular channels that link them while excluding access to the extracellular milieu. The task of coupling the cells of the various tissues in the body is accomplished by 20 different connexin (Cx) proteins. Although the requirement for so many different connexins is not well understood and there are significant differences in functional properties among the channels formed of different connexins (including conductance, permeability, and gating).

Essentially all mammalian tissues (and most of the cells within them) contain more than one connexin. The expression of multiple connexins provides the opportunity for interactions with each other to form heteromeric and heterotypic channels. The properties of the resulting hetero-oligomeric channels can have a diversity of functional properties determined by their different subunits, the interactions of those subunits, and the stoichiometries of the interactions.

In previous studies, we have particularly focused on Cx43 and its interactions with other co-expressed connexins. Cx43 is one of the most widely expressed connexins. It has been found in some cells in most organs of the body, and it has been implicated in significant functions in smooth muscle, myocardium, astrocytes, lens epithelium, endothelium, etc. Many of these cells also contain other connexins. Cx43 is most commonly found with the other connexins that have the most similar sequences including Cx37, Cx40, Cx46, and Cx50 (that are members of the α-subfamily of connexins encoded by the gap junction α (GJA) gene family; Kumar and Gilula, 1992; Beyer and Berthoud, 2009).

The functional interactions of Cx43 with other connexins have been extensively studied by expression of the connexins in Xenopus oocytes or in transfected communication-deficient cells. Such studies have consistently shown that Cx43 will form functional heteromeric and/or heterotypic gap junction channels with three α-connexins, Cx37, Cx46, Cx50, and with Cx45 (now classified as a γ-connexin), but not with the β-sub-family connexins, Cx26 and Cx32 (White et al., 1994, 1995; Elfgang et al., 1995; Brink et al., 1997; Berthoud et al., 2001; Martinez et al., 2002; Gemel et al., 2004). These functional interactions between Cx43 and other connexins may have significant functional consequences, like generation of a large variety of different channel sizes (Brink et al.,

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Valiunas et al. (2001) observed a rather low total conductance of Cx43–Cx40 heterotypic interactions (Lin et al., 2011; and unpublished results), arguing for functional Cx43–Cx40 heterotypic interactions in pairs of neuro2a (N2a) cells (Valiunas et al., 2000) and rat insulinoma (RIN) cells (Cottrell and Burt, 2001). When Vennstra and colleagues paired Cx43 with a Cx40 mutant containing substitutions of two charged residues (Musa et al., 2001), they observed symmetrically convergent alterations of voltage-dependent gating, arguing for Cx43–Cx40 heterotypic interactions (Lin et al., 2011; and unpublished results). But, in contrast, a study of connexins tagged with fluorescent proteins at their C-terminus concluded that Cx40 and Cx43 only appeared to make heterotypic gap junctions when Cx45 (which could interact with either connexin) was co-expressed (Rackauskas et al., 2006).

However, the issue of heterotypic interactions between these connexins should only have importance in the rare case of a cell producing only Cx40 contacting another expressing only Cx43. In contrast, the possible heteromeric interaction of these connexins may occur frequently in cells (such as atrial myocytes and some endothelial cells) that co-express both connexins.

Several studies have supported the abilities of Cx40 and Cx43 to form functional heteromers. Mixed heteromers of these two connexins were identified by affinity purification or co-immunoprecipitation studies performed using co-expressing cells (He et al., 1999; Vaniunas et al., 2001). In transfected N2a cells, Vaniunas et al. (2001) observed a rather low total conductance in pairs of cells expressing both Cx40 and Cx43, with only a rather small variation in single channel conductances and alterations of voltage-dependent gating; they suggested that Cx40–Cx43 heteromers might form inefficiently and many might be non-functional. Burt and colleagues have extensively studied the consequences of Cx40 and Cx43 co-expression in A7r5 and RIN cells, including pairs of cells with different relative expression ratios (Cottrell and Burt, 2001; Cottrell et al., 2002; Burt and Peel, 2003; Heyman et al., 2009). Their data suggest that these two connexins readily form heteromeric channels and that the composition of these channels influences many properties including gating, conductance, permeability, charge selectivity, and response to drug antagonists (PDGF).

The domains within the connexin protein that influence oligomerization between subunits to form a hexamer and between different connexins to form a heteromeric hexamer have not been clearly defined. Various biochemical and mutagenesis studies have implicated residues within the amino-terminal (NT) and within the first and third transmembrane domains (Lagger et al., 2003; Maia et al., 2005; Martinez et al., 2011). The NT has also been implicated in contributing to various channel properties including voltage-dependent gating, unitary conductance, and perm-selectivity (Oh et al., 2000; 2004; Vennstra, 2003; Musa et al., 2004; Dong et al., 2006; Gemel et al., 2006; Tong and Ebihara, 2006; Vennstra and Lin, 2006).

Some gap junction channels are very sensitive to block by polyamines like spermine and spermidine (Musa et al., 2001; Musa and Vennstra, 2003). However, this block is selective among connexins with Cx40 being very sensitive and Cx43 insensitive (Musa and Vennstra, 2003). Mutagenesis studies suggest that this connexin-specific difference is imparted by N-terminal amino acids (Musa et al., 2004; Gemel et al., 2006; Lin et al., 2006). Specifically, replacement of two negatively charged residues (E9 and E13) in Cx40 with the corresponding positively charged residues (K9 and K13) of Cx43 abolished spermine block (Musa et al., 2004). This also suggested that the block of Cx40 (but not Cx43) channels by spermine might involve interaction with these NT residues.

Polyamines (including putrescine, spermidine, and spermine) are ubiquitous polybasic molecules that interact with a wide variety of cellular molecules including nucleic acids, nucleotides, phospholipids, and acidic proteins. The NT domains of several connexins are shown in alignment in Figure 1. The structure of the NT domain was initially investigated by studying synthetic peptides using circular dichroism and nuclear magnetic resonance (Purlick et al., 2000; Arita et al., 2006; Kalmarosky et al., 2009; Kyle et al., 2009). Each of these studies showed that much of the beginning of the NT is a-helical, although they differ in the exact helical region. Purlick et al. (2000) concluded that the a-helix in Cx26 extended from position 1 to 10 while Kyle et al. (2009) suggested that it extended between amino acids 5 and 15 in Cx37. When Maeda et al. (2009) determined the structure of a Cx26 channel at 3.5 Å resolution, they observed that the NT regions of the six subunits lined the pore entrance and formed a funnel, which restricts the diameter at the entrance of the pore. The beginning of the NT is located deep within the pore. The NT helix extends beyond the cytoplasmic side of the membrane and then forms a loop (including the highly conserved amino acids corresponding to the serine and threonine at positions 18 and 19 in Figure 1) that bends back to the membrane where TM1 begins. Although all of the connexins may have homologies, their NT domains do not necessarily assume identical conformations. The β-connexins were proposed to have a glycine hinge including and following amino acids 12 and 13 (Purlick et al., 2000; that correspond to amino acids 13 and 14 in the α-connexins); however, insertion of the SG/GG (β-connexin) motif into Cx40 or Cx43 NT abolishes Vg gating, suggesting a structural/functional disparity between the sub-families (Gemel et al., 2006).
In the current study, we examined the possibility of differential regulation of channels made of different connexins through interactions of their NT domains. Based on their inhibition by spermine, we hypothesized that Cx40 channels might be blocked by short peptides that contained similarly spaced positively charged residues, like the Cx43 sequence from residues 9–13 (KLLDK as shown in Figure 1). We synthesized this potentially interfering peptide (designated Cx43iNT1) and tested its ability to inhibit gap junction channel formed of various α-connexins. Because Cx37, Cx45, Cx46, and Cx50 all contain multiple glutamate residues (like Cx40 as shown in Figure 1), we hypothesized that they might also be susceptible to block by spermine or “interfering” NT (iNT) peptides based on the sequences of other connexins (Figure 1).

The data presented suggest that corresponding sequences of acidic and basic amino acid residues within NT domains of Cx43, Cx37, Cx40, Cx45, Cx46, and Cx50 may influence connexin-specific interactions and their abilities to function as heteromeric channels.

MATERIALS AND METHODS

N2a CELL CULTURES AND CONNEXIN EXPRESSION

Stable N2a cell clones expressing human Cx37, Cx40 (hCx40), or rat Cx40 (rCx40) were prepared and cultured as previously described (Veenstra et al., 1994; Lin and Veenstra, 2007; Xu et al., 2012). Mouse Cx45 (mCx45), Cx46, and Cx50 were

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**Table 1**

| Connexin | Amino-terminal Sequence | iNT Peptide | Peptide Name |
|----------|-------------------------|-------------|--------------|
| Cx37     | H2N-N6GWSFLQNLVEHVKQST...(19) | Ac-EFLEE-DH2 | iNT-Cx40a |
| hCx40    | H2N-N6GWSFLQNLVEHVKQST...(19) | Ac-EFLEE-DH2 | iNT-Cx40b |
| rCx40    | H2N-N6GWSFLQNLVEHVKQST...(19) | Ac-EFLEE-DH2 | iNT-Cx40c |
| Cx43     | H2N-N6GWSFLQNLVEHVKQST...(19) | Ac-KLLRK-DH2 | iNT-Cx43  |
| Cx45     | H2N-N6GWSFLQNLVEHVKQST...(19) | Ac-KLLRK-DH2 | iNT-Cx45  |
| Cx46     | H2N-N6GWSFLQNLVEHVKQST...(19) | Ac-KLLRK-DH2 | iNT-Cx46  |
| Cx50     | H2N-N6GWSFLQNLVEHVKQST...(19) | Ac-KLLRK-DH2 | iNT-Cx50  |

"interfering" NT (iNT) peptides based on the sequences of other connexins (Figure 1).
transiently expressed in N2a cells using the pTracer™ vector (Chen et al., 2011).

**CONNEXIN INT PEPTIDE PRODUCTION**

Connexin-specific N-terminal domain peptides were synthesized in 5 mg quantities, high-performance liquid chromatography (HPLC) purified to >95%, and stored (−20°C) as lyophilized powder until needed (Anaspexc, San Jose, CA, USA). The peptides were dissolved in diethylpyrocarbonate (DEPC)-treated sterile distilled water to create a stock concentration of 10 mM (except −20°C- and 40 μl aliquots were diluted with 140 mM KCl internal pipette solution (IPS) as needed for daily patch clamp experiments. The relevant connexin N-terminal domain and peptide sequences are provided in Figure 1A. The amino and carboxyl termini of two peptides (INT-Cx43 and INT-Cx40b) were acetylated (amino) and amidated (carboxyl) to protect them from hydrolysis in aqueous solution. Pentameric N-terminal domain peptides for Cx43, Cx40, and Cx50 were prepared and tested in dual whole cell patch clamp experiments on homotypic Cx37, Cx40, Cx43, Cx45, Cx46, and Cx50 gap junctions expressed in N2a cells.

**GAP JUNCTION CONDUCTANCE (gj) MEASUREMENTS**

Dual whole cell patch clamp experiments were performed on connexin-transfected N2a cells pairs using established procedures (Veenstra, 2001). The connexin INT peptides were added to the patch pipette receiving the ±V1 voltage clamp step for quantitative gj measurements and calculation of the fraction of unblocked junctional current (gj) using the previously developed spermine block equation: 

\[
g j = \frac{\Delta I_j}{(V_j - R_{g,j} I_j)} = \frac{V_j - R_{g,j} I_j}{V_j - R_{g,j} I_j}
\]

where \(\Delta I_j\) is the change in whole cell current (Ij) during the ΔVj step. Rg,j and Rg,j are the respective whole cell patch electrode resistances, and I1 and I2 correspond to the respective whole cell currents (Veenstra, 2001). To determine the fraction of Ij block induced by the INT-Cx43 peptide or spermine, \(\Delta V_j\) was alternately stepped negative, positive, and negative to the common holding potential (−40 mV) in 5 mV increments from 5 to 50 mV (Figure 1B). The duration of each ±ΔVj−ΔVj sequence was 90 s with a 500-ms step to −40 mV occurring every 20 s. The fraction of unblocked \(I_j = (\Delta I_j / (V_j + \Delta I_j)) / (\Delta I_j / (V_j - \Delta I_j))\). Only those experiments where the \(I_j = 0\) baseline remained stable throughout the total 20 min duration of the cation block protocol were used in the final analysis.

**RESULTS**

Cx43 INT peptide selectively inhibits Cx40 gap junction channels

The INT-Cx43 peptide had some structural similarity to spermine: namely terminal amino groups separated by at least 10–C– or C–N bonds. Therefore, we initially tested whether INT-Cx43 peptide possessed any inhibitory activity toward rCx40 gap junctions. The Cx43 INT peptide was a potent \(V_j\)-dependent inhibitor of junctional conductance (gj) in cells expressing rCx40; indeed, equivalent block was observed at peptide concentrations of 10 μM, 100 μM, and 1 mM (Figures 2A,C). Although Glu-9 contributes to the spermine block of rat Cx40 channels (Musa et al., 2004), this residue is replaced with an asparagine (N9) in human Cx40 (hCx40). Therefore, we hypothesized that hCx40 might show a different effect of INT-Cx43 peptide. Surprisingly, we found that 100 μM INT-Cx43 peptide showed a similar inhibition of both human and rat Cx40 gap junctions (Figures 2B,D).

This unexpected result led us to test the ability of the Cx43 INT peptide to interfere with the function of other connexins that also contain an ExxxE or ExxxD motif in their N-termini. Cx43 iNT peptide selectivity inhibits Cx40 gap junctions (Musa et al., 2004), this residue is replaced with an asparagine (N9) in human Cx40 (hCx40). Therefore, we hypothesized that hCx40 might show a different effect of INT-Cx43 peptide. Surprisingly, we found that 100 μM INT-Cx43 peptide showed a similar inhibition of both human and rat Cx40 gap junctions (Figures 2B,D).

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Cx43 INT peptide effects do not correlate with spermine block

Since Cx40 (but not Cx43) exhibits \(V_j\)-dependent spermine block (Musa and Veenstra, 2003; Musa et al., 2004; Lin et al., 2006; Lin and Veenstra, 2007), we tested the possible relationship of INT-Cx43 block to spermine block by examining the effects of unilateral application of 2 mM spermine on the other five connexins. Not surprisingly, hCx40 gj was reduced in a \(V_j\)-dependent manner by 2 mM spermine (Figure 4A, black squares).

Interestingly, spermine also caused at least some inhibition of each of the other connexins. The spermine inhibition curve for
FIGURE 2  (A) The inhibition of rCx40 gap junction currents ($I_j$) by unilateral addition of 10 μM (■), 100 μM (●), or 1.0 mM (▲) INT-Cx43 peptide increased in a transjunctional voltage ($V_j$)-dependent manner. There was no statistical difference between the three curves based on comparison of the means at each $V_j$ value. (B) The $V_j$-dependent blockade of hCx40 gap junctions (●) by 100 μM INT-Cx43 peptide was not significantly different from rCx40 (continuous gray line). (C) Whole cell current traces recorded from cell 2 of a rat Cx40 cell pair with 100 μM INT-Cx43 peptide added to cell 1 during the Δ$V_1$ cation block voltage clamp protocol diagrammed in Figure 1B. The Δ$I_2$ current ($=−I_j$) decrease during the +$V_1$ voltage steps (only 10 mV incremental steps shown) illustrates the block induced by the INT-Cx43 peptide. (D) Actual Δ$I_2$ current recordings from a hCx40 cell pair during an INT-Cx43 peptide experiment illustrating a similar $V_j$-dependent block of hCx40 gap junction currents. Gap junction channel currents are visible at ±50 mV with reduced open probability during the positive (blocking) $V_j$ step.

Cx50 appeared similar to that of hCx40, and the maximum inhibition of Cx50 $g_j$ was ~80% (Figure 4A, gray circles). Cx46 $g_j$ achieved 60% inhibition (Figure 4A, open circles). Cx37 $g_j$ was reduced by ~70% at low $V_j$ values and then plateaued at ~50% inhibition (Figure 4A, gray open diamonds). Spermine inhibited Cx40 $g_j$ by <50% (data not shown).

Thus, the selective ability of INT-Cx43 peptide to inhibit Cx40, but not other connexin channels, does not correlate with spermine inhibition.

INT PEPTIDE ANTAGONISM OF SPERMINE BLOCK

Since Cx40 gap junctions were inhibited by both the Cx40 NT peptide and spermine, we examined whether INT peptides based on the NT sequences of Cx40 or Cx50 (containing the ExxEE motif) could antagonize the spermine block of Cx40 gap junctions. Spermine block experiments were performed with or without the addition of INT peptides.

The first Cx40 INT peptide (INT-Cx40a) was acetylated and amidated like the INT-Cx43 peptide (Figure 1). INT-Cx40a peptide was partially effective at reversing the 500 μM spermine block (Figure 4B, compare gray circles to spermine alone curve indicated with solid black squares). A second Cx40 INT peptide containing the EFLEE sequence, INT-Cx40b, 9–13 peptide was synthesized where the carboxy-terminus was hydroxylated instead of amidated to prevent the neutralization of the terminal glutamic acid group (Figure 1). The INT-Cx40b peptide totally prevented the
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FIGURE 3 | The ability of the iNT-Cx43 peptide to inhibit Cx37 (A), Cx45 (B), Cx46 (C), and Cx50 (D) gap junctions was tested using the same \( V_j \)-dependent block protocol as in Figure 2. As a reference, the blockade of rCx40 (continuous gray line) is illustrated in each panel. Unlike rCx40, none of these four connexin gap junctions were significantly inhibited by 100 \( \mu \text{M} \) iNT-Cx43 peptide. Actual \( \Delta I_2 \) current traces from Cx45 (E) and Cx50 (F) cell pair iNT-Cx43 peptide experiments illustrating the lack of \( I_j \) current block during the \( +\Delta V_1 \) step, in contrast to the Cx40 experiments shown in Figure 2. Cx45 and Cx50 gap junctions are more \( V_j \)-dependent than Cx40 (half inactivation \( V_j = V_j^{1/2} = \pm 30, \pm 40, \) or \( \pm 49 \) mV, respectively; Lin et al., 2006; Gonzalez et al., 2007). mCx45 gap junctions prominently display contingent hemichannel gating upon \( V_j \) polarity reversal, proposed by Harris et al. (1981), owing to the increased \( V_j \)-dependence and lack of fast \( V_j \)-gating kinetics of these gap junctions.

These findings suggested the hypothesis that block might result from the electrostatic interaction of the ExxEE sequence. To test this hypothesis, a hydroxylated peptide corresponding to residues 12–16 of Cx50 (EEVNE), iNT-Cx50b (Figure 1), was synthesized. INT-Cx50b had no detectable effect on the block of rCx40 gap junctions by 500 \( \mu \text{M} \) spermine (Figure 4E, gray circles). However, in contrast, a second hydroxylated Cx50 INT peptide, INT-Cx50a, corresponding to amino acids 9–13 (NILEE; Figure 1) effectively eliminated the \( V_j \)-dependent spermine block of rCx40 gap junctions (Figure 4F). This suggested a structural requirement for...
FIGURE 4 | (A) The sensitivity of four connexin-specific gap junctions was tested using the 2 mM spermine block assay. Human Cx40 (hCx40, ■) displayed similar Vj-dependent sensitivity to spermine as rCx40 despite the N9 substitution. Cx37 (∆), Cx46 (○), and Cx50 (•) gap junctions were all ≥60% inhibited by spermine. The maximum inhibition of Cx37 gj occurred at +20 mV, half the Vj required for maximal block of any other known connexin-specific gap junction. (B) The ability of iNT-Cx40 peptides to interfere with spermine block was tested by adding 500 μM spermine and iNT-Cx40a or iNT-Cx40b peptides to one patch pipette. The carboxyl-terminal hydroxylated (-OH, z = −4) form of the Cx40 peptide (Cx40b) effectively abolished the Vj-dependent spermine block, while the amidated form (Cx40a, −NH2, z = −3) was only partially effective (ANOVA, f-value < 0.05). (C) ΔI2 current traces from an rCx40 cell pair with 500 μM spermine added to cell 1. Ij decreased during the positive 30, 40, and 50 mV Vj pulses and returned to prepulse levels during subsequent negative Vj pulses. This illustrates the time- and Vj-dependent spermine block and unblock of rCx40 gap junctions. (D) ΔI2 current traces from an rCx40 cell pair experiment with 500 μM spermine and the iNT-Cx40b peptide added to cell 1. Accounting for the occurrence of Vj-dependent gating at Vj ≥±40 mV, instantaneous and steady state Ij increased in a stepwise (ohmic) fashion with increasing Vj amplitude, indicative of a lack of spermine block. (E) A negatively charged (z = −4) iNT-Cx50b peptide failed to significantly prevent the 500 μM spermine block of rCx40 gap junctions, suggesting that the bimolecular interactions between the rCx40 NT domain, spermine, and NT peptides are not purely based on electrostatic forces. (F) An iNT-Cx50a peptide (based on amino acids 9–13 and possessing a carboxyl-terminal valence (z = −3) significantly reduced the 500 μM spermine block of rCx40 gap junctions, suggesting a structural requirement for the interactions of iNT-Cx peptides with NT domains or spermine molecules.

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the abilities of these pentameric peptides to oppose the spermene block of rCx40 gap junctions.

DISCUSSION

We began this series of experiments with a relatively simple ratio-

tale: Cx40 channels which contain the sequence Exxx4 in their N-termini are inhibited by the polyamine, spermene, and might also be inhibited by a pentamic peptide derived from the NT of Cx43 which has the motif ExxxK. Indeed, we observed potent block of rCx40 channels by INT-Cx43. The block occurred in a transjunctional voltage (Vj)-dependent manner that resembled spermene block. However, the simple model of electrostatic interaction between this peptide and the connexin NT is dispelled by human Cx40 channel was also blocked by INT-Cx43 despite the neutralization of the 9th residue in the human isoform (substitution of N for E). Moreover, INT- Cx43 peptide had no effect on Cx37, Cx46, or Cx50 gap junctions, despite the presence of a similar (Exxx4 or Exxx5) motif in these connexins. There also appears to be a structural requirement for the INT effects. While a Cx50 INT peptide containing similarly spaced negative charges had no effect, a Cx50 INT pep-
tide representing amino acids 9–13 antagonized spermene block of rCx40.

Our data support a selective inhibitory interaction between the NT domains of Cx40 and Cx43. INT-Cx43 only blocked Cx40 channels. This interaction appears to have a rather high affinity, since equivalent block was achieved with 10 μM peptide to that produced by higher concentrations. This putative selective NT interaction parallels the observed functional heteromeric interaction among this group of connexins. The Cx43 INT peptide exhibited no effect on Cx37, Cx45, Cx46, or Cx50 gap junctions, but blocked Cx40 channels. Similarly, functional heteromeric interactions between Cx43 and Cx37, Cx45, Cx46, and Cx50 have been extensively supported in the literature. We might have anticipated a reciprocal interaction of the Cx40 NT domain with Cx43 channels; however, we observed no effect when an INT- Cx40 peptide was prepared and applied to Cx43 gap junctions (data not shown). This negative result might have been anticip-
ted by the lack of effect of spermene on Cx43 gap junctions (Musa and Veenstra, 2003). The lack of direct reciprocity between Cx40 and Cx43 amino termini with alternately charged sequences implies that there is a structural difference between these two NT domains beyond their oppositely charged amino acids at positions 9 and 13.

Our INT peptide data support the conclusion that Cx40 and Cx43 can form heteromeric channels, but most (if not all) of them will be non-functional (closed) due to the interactions of their amino termini. It is likely that a single Cx43 subunit is sufficient to nullify the function of a heteromeric Cx40-Cx43 hemichannel (connexion), based on the stoichiometric study of N12D and N2E mutant Cx32/Cx43 hemichannels showing that a single NT domain is sufficient to induce Vj-dependent closure of a connexin hemichannel (Oh et al., 2000).

Finally, the connexin-specific effects of these INT peptides also suggest the possibility of designing drugs that serve as gap junction agonists or antagonists by altering modulatory Vj- or chemical gating interactions involving unique connexin domains. For instance, the Cx43 INT peptide may antagonize the abnormally high conductance of Cx40 hemi- or gap junction channels associated with atrial fibrillation (Grolib et al., 2000; Yang et al., 2010).

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