New Roles for Connexons

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Connexons or gap junction hemichannels are large, nonselective ion channels that reside in the nonjunctional plasma membrane before their assembly into gap junction channels. Increasing evidence suggests that these channels can open under certain conditions and may participate in a number of cellular processes, including the release of small metabolites such as ATP and NAD$, which are involved in paracrine signaling.

Gap junction channels are intercellular channels that allow the passage of ions and other small molecules between neighboring cells. They are formed from two multimeric subunits called hemichannels or connexons that reside in the plasma membranes of two closely opposed cells. Connexons are composed of six transmembrane protein subunits called connexins. The connexins belong to a multigene family composed of at least 19 human members. All of the connexins have four membrane-spanning domains, and both the NH$_2$ and COOH terminals reside on the cytoplasmic side of the membrane. The regions of the proteins corresponding to the transmembrane-spanning and extracellular domains are highly conserved among connexins. In contrast, the regions corresponding to the central cytoplasmic loop and carboxy tail show much less homology.

The first step in gap junction formation involves the assembly of newly synthesized connexons into connexons in either the endoplasmic reticulum or the trans-Golgi network, as shown in Fig. 1 (20). The connexons then travel in vesicular structures along microtubules to the plasma membrane, where they insert. Surprisingly, this insertion process appears to occur randomly all over the plasma membrane instead of being specifically targeted to the gap junctions (12). The final step in gap junction formation involves the lateral diffusion of connexons in plasma membrane to the outer margins of the gap junction plaques, where they can dock to form complete gap junction channels.

Experimental evidence for functional hemichannels

The existence of single connexons in the nonjunctional plasma membrane raises the intriguing possibility that connexons may function as transmembrane ion channels in addition to serving as precursors in the formation of gap junction channels. The first evidence for functional hemichannels came from a study by DeVries and Schwartz (4), who showed that solitary horizontal cells from the catfish retina express a novel dopamine-sensitive current that could be observed only when extracellular calcium was removed. This current showed many of the properties that would be expected of a current flowing through gap junction hemichannels, including permeability to the large, anionic dye Lucifer yellow and regulation by agents such as voltage, pH, cAMP, cGMP, and dopamine that are known to modulate gap junction channels.

A current with very similar properties has been observed in horizontal cells from the skate retina (14). At the same time as DeVries and Schwartz were studying hemichannels in catfish horizontal cells, Paul et al. cloned a gap junction protein, rat connexin 46 (Cx46), that was expressed primarily in the lens, where it forms gap junctions between fiber cells. Unexpectedly, expression of Cx46 in the Xenopus oocyte pair system resulted in cellular depolarization and osmotic lysis. Voltage-clamp experiments revealed that Cx46 cRNA-injected oocytes developed a large, nonselective cation current that was activated on depolarization and blocked by external divalent cations. In addition, Cx46 was able to induce the formation of gap junction channels if calcium was taken to block the nonjunctional current by elevating extracellular calcium concentration (7). The ability to form functional connexons in oocytes is not unique to Cx46. Several other connexins, including skate Cx35, perch Cx35, and perch Cx34.7, have been reported to form functional hemichannels when expressed in oocytes (5, 16). Functional connexons have also been identified in freshly isolated and cultured mammalian cells (13, 19).

Functional properties of hemichannels

A common feature of all of these channels is that they are blocked by external divalent cations and in most cases by hyperpolarizing transmembrane potentials. These two effects appear to act synergistically to prevent the opening of hemichannels under physiological conditions, as shown in Fig. 2. The effect of external calcium on connexon structure has been recently studied at molecular resolution (<2 Å vertical resolution and <10 Å lateral resolution) by using atomic force microscopy in aqueous solution in the presence and absence of calcium (15). Images of single connexon layers composed of Cx26 showed a reversible reduction in the diameter of the external pore during incubation with millimolar concentrations of external calcium. The time course of this conformational change was extremely slow, which is consistent with the slow time course of the calcium response observed in studies on Cx46 hemichannels expressed in oocytes (6). Interestingly, this phenomenon appears to be distinct from the effect of elevated cytoplasmic calcium on intact gap junction channels.

Connexons composed of different connexins show differ-
ences in their sensitivity to external calcium. For example, connexons composed of chicken Cx45.6 were blocked by much lower concentrations of external calcium than connexons composed of rat Cx46 or chicken Cx56 (8). The Cx45.6 hemichannel currents could only be detected when the external calcium concentration was reduced to nominally zero, whereas the Cx46 and Cx56 hemichannel currents were present at normal external calcium concentrations (0.7 mM Ca2+). Furthermore, Cx56, Cx46, and Cx45.6 hemichannel currents exhibit differences in activation and deactivation kinetics and steady-state voltage dependence in calcium-free bath solutions.

The structural features that determine whether a gap junction protein can form functional hemichannels are not well defined. To further complicate matters, certain connexins, such as Cx43, which has been reported to form functional hemichannels in a wide variety of mammalian cell lines, fail to induce detectable macroscopic ionic currents when expressed in oocytes (17).

FIGURE 1. Schematic representation of the steps involved in the assembly of connexin (Cx) 43 gap junctions based on current literature. Step 1: connexins are synthesized in the endoplasmic reticulum. Step 2: oligomerization of Cx43 into connexons occurs in the trans-Golgi network. Step 3: connexon-containing transport vesicles travel along microtubules to the nonjunctional plasma membrane, where they fuse. Step 4: the connexons that are delivered to the plasma membrane can reach the outer margins of the gap junction plaques by lateral diffusion. Modified from Ref. 20, with permission.

FIGURE 2. Effect of extracellular calcium ([Ca2+]o) and voltage on human Cx46 hemichannel currents. Representative hemichannel currents recorded from a wild-type human Cx46 cRNA-injected oocyte in solutions containing zero added magnesium and 0.01 mM (A) or 0.1 mM (B) [Ca2+]o are shown. The cell was held at the hemichannel equilibrium potential (~10 mV) between pulse sequences to minimize holding currents. A 20-s conditioning voltage clamp pulse was applied to voltages between 20 and ~100 mV, followed by a test pulse to ~80 mV. C: normalized conductance-voltage curves at different calcium concentrations. △, 0.01 mM; ○, 0.1 mM; ■, 1.0 mM. Normalized conductance (G/Gmax) was determined at each potential by measuring the initial amplitude of the tail current during the test pulse to ~80 mV and dividing it by the maximum value of the tail current obtained after a depolarization to +20 mV in the presence of 0.01 mM [Ca2+]o. Values are means ± SE; n = 3–6 experiments. The solid lines in C are fits to the Boltzmann equation: \[ y = \frac{G(V_{rev})}{G_{max}} = \frac{1}{1 + \exp[-k(V - V_{1/2})]} \]. For 0.01 mM [Ca2+]o, G(V_{rev})/G_{max} = 1.02, V_{1/2} = -50 mV, k = 0.102; for 0.1 mM [Ca2+]o, G(V_{rev})/G_{max} = 0.385, V_{1/2} = -27.7, k = 0.112; for 1.0 mM [Ca2+]o, G(V_{rev})/G_{max} = 0.61, V_{1/2} = -0.9, k = 0.096. Reproduced with permission from Ref. 6.
Proposed physiological roles of hemichannels

Although it widely acknowledged that functional hemichannels exist, their biological significance is still not well understood. Studies on intercellular calcium wave propagation, mainly in astrocytes and C6 glioma cells, have shown that connexin expression is necessary for propagation of calcium waves (3, 18). It was originally thought that interastrocyte signaling was mediated by the diffusion of calcium or inositol 1,4,5-trisphosphate through gap junction channels. However, recent studies indicate that calcium wave propagation can occur between cells that are poorly coupled or are not physically in contact with each other, suggesting an extracellular paracrine pathway involving a diffusible second messenger such as ATP. According to the hypothetical scheme shown in Fig. 3, ATP is released from astrocytes through open connexons into the extracellular fluid. The ATP then acts on purinergic receptors that are expressed on surrounding astrocytes to trigger an increase in intracellular calcium.

Another tissue in which functional connexons have been postulated to play an important role is the retina. Ganglion cells have a receptive field that consists of a circular zone at the center and an antagonistic surround. Light applied to the surround inhibits the effect produced by the illumination of the center. Previous studies have shown that the antagonistic surround response involves the direct interaction of horizontal cells with cone photoreceptors in the center of the receptive field. However, the cellular mechanisms underlying this response remain controversial. It has been proposed that the negative feedback mechanism involves modulation of presynaptic voltage-dependent calcium channels of the cones by changes in extrasynaptic potential (10). In this model, hemichannels located at the tips of the horizontal cell dendrites act as a current sink by which the extrasynaptic potential can be modulated.

Functional hemichannels may also play a role in cell damage under pathological conditions. It has been speculated that certain pathological insults such as ischemia or inflammatory injury might result in the opening of large numbers of hemichannels, leading to cell depolarization, collapse of ionic gradients, loss of small metabolites, and elevation of intracellular calcium. One example of a situation in which these events may occur is in the heart during myocardial ischemia. John et al. (9) described a nonselective conductance in cardiac ventricular myocytes that was permeable to calcein (\(M_r 660\)) but not to 1% dextran conjugated to fluorescein (\(M_r 1,500\)/G10,000). This conductance was activated by low extracellular calcium and blocked by lanthanum, properties similar to those of Cx43 hemichannels heterologously expressed in HEK-293 cells. Furthermore, both the endogenous conductance in cardiac myocytes and the exogenous conductance in Cx43-expressing HEK cells could be activated by metabolic inhibitors in the presence of normal extracellular calcium, raising the possibility that open hemichannels could contribute to myocardial injury and cardiac arrhythmias in ischemia. Opening of hemichannels has also been reported in primary cultures of rat and mouse astrocytes subjected to metabolic inhibition (1).

The mechanism by which metabolic inhibition triggers the opening of hemichannels remains controversial. Under normal conditions, Cx43 is phosphorylated on multiple serines, but the level of Cx43 phosphorylation can be markedly reduced by metabolic inhibition. This could lead to the opening of Cx43 hemichannels by either a direct effect of channel gating or by altering the rate of assembly or degradation of Cx43 gap junction channels. There have been a number of experimental observations that support the hypothesis that hemichannel gating can be modulated by phosphorylation. In reconstituted lipid vesicles, it has previously been shown that dephosphorylation of Cx43 leads to the opening of Cx43 hemichannels. Subsequent phosphorylation of Cx43 by MAP kinase resulted in channel closure (11). In Novikoff cells, TPA-activated PKC prevents dye uptake through Cx43 hemichannels (13). In solitary horizontal cells from the catfish retina,
application of dopamine, which activates a cAMP-dependent protein kinase, suppresses hemichannel currents (4). Nitric oxide acting through a guanylate cyclase/cGMP cascade also closes hemichannels. Alternatively, phosphorylation could act by modulating the number of hemichannels available for opening. For example, it has recently been reported that casein kinase I plays a role in gap junction channel assembly under basal conditions and that inhibition of casein kinase I activity results in an accumulation of nonjunctinal Cx43 in the plasma membrane (2).

The generation of free radicals secondary to oxidative metabolism of arachidonic acid via the cyclooxygenase or the lipoxigenase pathway might also play an important role in the opening of hemichannels during metabolic inhibition. It has been reported that nordihydroguaiaretic acid, a blocker of lipooxygenases, prevented ethidium bromide uptake by astrocytes subjected to metabolic inhibition, whereas indomethacin, a cyclooxygenase blocker, had no effect on dye uptake. These results suggest that arachidonic acid byproducts can activate hemichannels via the lipoxigenase pathway (1).

An important caveat in all of the studies discussed above is that interpretation of the data is complicated by the difficulty in distinguishing hemichannels from other ion channels in native cells. A property that has been widely used to identify hemichannels is permeability to small fluorescent dyes such as Lucifer yellow. However, this property is not unique to connexons. Other channels such as the P2Z/P2X purinergic receptor are also permeable to these molecules. Furthermore, there are no pharmacological interventions that specifically block hemichannels. All of the agents that are commonly used to inhibit gap junction channels (e.g., octanol, halothane, pH, calcium) affect other ion channels as well.

An alternative approach to this problem is to alter levels of connexin expression. For example, Contreras et al. (1) showed that metabolic inhibition induced dye uptake in primary cultures of astrocytes from wild-type mice but not from homozygous Cx43 knockout mice or mice with astrocyte-specific inactivation of the Cx43 gene. Although these findings strongly support the notion that the increase in dye permeability is due to the opening of Cx43 hemichannels, one cautionary note is that inactivating one gene sometimes leads to compensatory changes in the expression of other genes.

Future directions

In conclusion, these examples suggest that connexons may play other roles besides that of being precursors in the formation of gap junction channels. There is a high probability that some of the effects of targeted disruptions of specific connexin genes in mice that are currently being attributed to the disruption of gap junction communication are actually due to the disruption of signaling via connexons. Work in the next few years promises to uncover many more roles for connexons.

References