Not what you thought: How H^+ ions combine with taurine or other aminosulfonates to close Cx26 channels

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Gap junctions are aggregates of microscopic channels that connect the cytoplasmic compartments of the coupled cells. A gap junction channel is formed of two hemichannels, one contributed by each cell. Hemichannels can be formed in a post-Golgi compartment and transported to the cell surface in vesicles that fuse with the external membrane (Musil and Goodenough, 1993). Hemichannels then find a hemichannel in an apposed membrane and dock to form a cell–cell channel. For a long time, hemichannels were thought not to open to the exterior and to open only when joined to a hemichannel from an apposed membrane. Electrophysiological measurements showed that the hemichannels forming the first cell–cell channel had not been open to the medium before the channel formed (Bukauskas and Weingart, 1993). A teleological argument was that if hemichannels had the same large diameter pore as in a cell channel, they would have provided a deleterious link with the external medium, allowing loss of K^+, ATP, and other metabolites and influx of Ca^{2+} and Na^+. Now it is clear that isolated, unapposed hemichannels can open under specific physiological as well as pathological conditions, and metabolites coming out through hemichannels can mediate paracrine as well as autocrine signaling (Sáez et al., 2010). The open probability is generally low, and increased opening can contribute to cell death, as presumed in arguing that the hemichannels should not open.

Gap junctions are regulated at the levels of gene transcription, RNA translation, membrane insertion, cell–cell channel formation, channel gating, and degradation. For the electrophysiologist, channel gating is perhaps the most attractive of the regulatory processes, and it represents a relatively fast and reversible means of control of intercellular communication. Gap junction channels are usually maximally open at zero transjunctional voltage, V_j, and gating to a closed state can be induced by V_j, low cytoplasmic pH, high cytoplasmic pCa, and various exogenous blocking agents such as long chain alcohols, carbenoxolone, oleamide, and mefloquine. Gating agents that have been examined in this respect decrease conductance by reducing open probability; unitary conductance of open channels is not affected. Admittedly, the pharmacology is not very clean, and the available blocking agents are somewhat nonspecific. In most cases, junctional conductance, g_j, is dependent on V_j and independent of the voltage between the inside of a cell and the external medium, $V_{i\text{-}o}$ (or V_m). To complicate the picture more, most gap junctions exhibit two forms of voltage gating: fast gating to a subconductance state and slow or loop gating to complete closure (Bukauskas and Verselis, 2004). Moreover, voltage and pharmacological gating are modulated by pH in several connexins (Palacios-Prado et al., 2010; Skeberdis et al., 2011).

In addition to many connexins (21 in humans), mammals have three proteins, pannexins 1–3, that are homologues of innexins, the gap junction–forming proteins that are found in protostomes and less derived deuterostomes (Alexopoulos et al., 2004). These proteins are not homologous to the connexins, although there are many convergent features. The name, pannexin, has also been applied to the entire innexin/pannexin family, as the prefix, pan-, refers to the wide (but not universal) distribution (Shestopalov and Panchin, 2008). Connexins first appear in a common ancestor of ascidians and chordates after divergence of amphioxus (Putnam et al., 2008). In mammals, the pannexins appear to operate primarily as (hemi)channels. Some authors maintain that pannexin channels crossing a single membrane should be not be called hemichannels because they do not (or are not known to) form gap junctions when endogenously expressed. They do form gap junctions when overexpressed in mammalian cell lines (Lai et al., 2007) and *Xenopus laevis* oocytes (Bruzzone et al., 2005), and it is early to say that endogenous pannexins don't ever form gap junctions in mammals. The reason for including pannexins here is that the basic tetraspan structure is similar in connexins and pannexins, and several blocking agents work on both classes of channel. Differentiation where both connexins and pannexins occur can be somewhat problematic. The nomenclature will sort itself out, as more data accumulate. I favor the term hemichannels applied to pannexins based on

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their similarity to connexins and homology to innexins, which of course do form gap junctions as well as functional hemichannels, i.e., hemichannels that open to the exterior under specific conditions (e.g., Luo and Turnbull, 2011). Yet, one must agree that an unapposed hemichannel providing a pore between cell interior and exterior is indeed a channel.

In Cx43, the most common connexin in mammals, the cytoplasmic C terminal (CT), is implicated in pH gating, i.e., reduction of conductance at low pH (Morley et al., 1997; Hirst-Jensen et al., 2007). Truncation of the CT greatly reduces pH-mediated closure, and coexpression of the truncated form and the CT as separate peptides restores pH sensitivity. The implication is that the CT behaves like a ball and chain analogous to the mechanism of inactivation of several voltage-sensitive ion channels. The CT of several other, but not all, connexins tested has been implicated in pH gating (Stergiopoulos et al., 1999). Another way of closing gap junction channels is to raise intracellular calcium. The effect of calmodulin inhibitors indicates that Ca^{2+} acts through calmodulin, but the linkage to channel closure is not established (Lurtz and Louis, 2007; see Dodd et al., 2008, for calmodulin binding to Cx32 domains).

A paper in the September 2011 issue of this journal by Locke et al. establishes a new twist on gating at low pH. The Harris group some years ago implicated aminosulfonates in acid-induced closing of homomeric channels formed of Cx26 and of heteromeric channels formed of Cx26 and Cx32 (Bevans and Harris, 1999). In the basic observation, hemichannels reconstituted into liposomes were found not to be closed at low pH unless there was a protonated aminosulfonate present. Aminosulfonates, exemplified by Good's pH buffers (e.g., HEPES), include the common cytoplasmic constituent, taurine. The data indicated that taurine, which is largely protonated at physiological pH, and other aminosulfonates when protonated, acted directly on the hemichannel, and that an intermediate molecule, such as calmodulin, was not required. In a nice series of measurements, changing taurine concentration at constant pH and changing pH at constant taurine both blocked hemichannels to the extent predicted from the concentration of protonated taurine. A direct hydrogen ion effect was excluded, as pH as low as 4 or 5 with other buffers such as MES and Tris did not cause channel closure. Furthermore, with different aminosulfonates, concentration–response curves depended on the concentration of the protonated form and not directly on pH. The earlier studies depended on a novel but "robust" assay, "transport-specific fractionation" (TSF). Liposomes with hemichannels were reconstituted in urea solution at a lipid to protein ratio such that few liposomes had more than one hemichannel. When laid on an isosmotic urea to sucrose gradient, the opening of a hemichannel permeable to the denser sucrose allowed the vesicles to sink (aided by centrifugation) as the denser molecule entered. The liposomes stop at a level determined by the buoyancy of the liposome membrane. Of course liposomes without channels do not sink. The fraction of open channels was assayed as the relative density of the lower to the upper band, and aliquots of the same liposome preparation were compared in treated and untreated (control) preparations. It was argued that "TSF is an all-or-none assay of per-liposome hemichannel permeability and reports only . . . complete or near complete pore closure. . . . Inhibition of activity because of AS [aminosulfonates] is therefore assessed as the decrease in the fraction of liposomes in the lower band compared with the corresponding control" (Locke et al., 2011). Channel closure must be essentially complete for the TSF assay, and the titration curves obtained are ascribed to different sensitivity among hemichannels rather than to different degrees of closure. Although the requirement for all-or-none closure pushes the concept of equilibria between closed and open hemichannels to an extreme, the suggestion of virtually complete closure at different concentrations of blocker because of differences among channels is reasonable. The threshold differences giving an apparent titration curve for heteromeric hemichannels can be a function of the number of Cx26 monomers in the hemichannel hexamers, if differences in this number result in intermediate sensitivities and a distribution of closed channels as a function of protonated aminosulfonate concentration.

The new results in Locke et al. (2011) provide further mechanistic insight into the gating process. In brief, the CT of Cx26 binds to the cytoplasmic loop (CL) at normal pH, and the binding affinity increases under acidic conditions, although the pH dependence of this effect appears unrelated to closure. In the absence of aminosulfonate, the channel is insensitive to low pH. Protonated aminosulfonates bind to the CL and displace the CT, which then blocks the channel, perhaps by direct binding or by inducing a conformational change. This action is rather independent of pH, as block is a function of protonated aminosulfonate concentration and not pH over a relatively wide range of pH. Another clue came from use of a 28–amino acid purification tag on the CT of Cx26. Adding this tag to the CT prevents protonated aminosulfonate closure, and a direct action of the CT seems likely. Cleaving the tag to leave a four–amino acid addition to the CT changes the action in Cx26 homomers to a narrowing of the channel. Somewhat surprisingly, coexpression of Cx26 with the shortened His tag and wild-type Cx32 rescues complete closure of the channel. One possibility is that the much longer CT of Cx32 can substitute for a normal length Cx26 CT when the Cx26 CT is made nonfunctional by the addition of four amino acids. Structural characterization of the various closed channels with a protonated

aminosulfonate present may be required to clarify the issue. Although a crystal structure of Cx26, presumably in the open configuration has been obtained, the CL and CT domains were not resolved, suggesting flexibility of the structure in this region (Maeda et al., 2009). In any case, a direct action by taurine on the CL to free the CT and allow it to block the channel is the most plausible mechanism at this time.

Other experiments investigated the interactions of taurine and the CL and CT of Cx26. ELISA was used to assay the binding of peptides corresponding to the CT and to segments of the CL; the data indicated that the CL region close to the third transmembrane region binds to the CT and that the binding was tighter at low pH, the latter point not being obviously relevant to the aminosulfonate block as measured in this paper. In nuclear magnetic resonance studies, taurine binds to the CL but not the CT. Taurine displaces the CT from the CL in a primarily competitive interaction, and the freed CT somehow blocks the hemichannel as noted above. An earlier study by the Harris group (Tao and Harris, 2004) showed that protonated amino groups without a sulfonate competitively inhibit aminosulfonate action; i.e., they appear to inhibit the binding of protonated aminosulfonate and thereby reduce channel blocking. Sulfonates without a protonated amino group are less effective in this inhibition.

For those feeling a little uncomfortable with TSF as a means of studying channel function, new experiments on intact cells implicate protonated taurine in pH block of hemichannel opening and gap-junctional communication in living cells (Locke et al., 2011). Extracellular protonated taurine reduces hemichannel-mediated dye uptake in low Ca^{2+} medium by cells expressing wild-type Cx26 but not by cells expressing Cx26 with the purification tag. Uptake is much less in control cells not expressing Cx26. HEPES, which blocks the taurine transporter, prevents the taurine effect. Similar results are obtained by Locke et al. with gap junction–mediated dye transfer between cells expressing Cx26 with and without the purification tag, and again, the taurine effect is prevented by HEPES.

What does all this mean? Well, it provides further indications that our 21 connexins are not entirely redundant and that there are meaningful differences in a variety of gating properties, rather than solely in promoters and control of expression and trafficking. An intriguing aspect is the potential interplay between cytoplasmic constituents that act in the same way as taurine or, conversely, competitively inhibit its action. This complexity allows for a wide range of tissue-specific effects, and physiological levels of taurine are known to vary widely in different cell types (e.g., Huxtable, 1992).

For gap junction aficionados, there is the surprise that the short CT of Cx26 really is important in gating. Cx26 is expressed in many tissues, including liver, pancreas, skin, and cochlear supporting cells. It is often expressed with other connexins, although cooligomerization in hemichannels may only occur with β (Group I) connexins.

Surprisingly, most channels in functional gap junctions appear to be closed (Bukauskas et al., 2000). This inference is based on number of open channels determined from single-channel conductance and macroscopic junctional conductance, and number of channels present at the junction. In HeLa cells, the number of channels formed by fluorescently tagged channels has been determined from total fluorescence and the fluorescence per channel based on estimated channel density. From these data, it is clear that only a small fraction (<10%) of channels are open under the experimental conditions. Similar calculations have been made for club endings on the Mauthner cell, based on junctional and single-channel conductances and freeze-fracture estimates of number of channels at a single ending (Pereda et al., 2004). In contrast, the application of atomic force microscopy to isolated and split-open Cx26 junctions indicates that a large fraction of channels show changes consistent with gating by high Ca^{2+} or protonated aminosulfonate (HEPES) (Sosinsky and Nicholson, 2005; Thimm et al., 2005; Yu et al., 2007).

The fraction of channels that open in gap junctions between living cells is quite remarkably low compared with the fraction that appears to gate in preparations subjected to atomic force microscopy. The TSF liposome protein/lipid ratio may provide an additional datum, although many hemichannels may be damaged in the isolation procedure and closed in the unphysiological conditions. The ratio of sinking to unaffected liposomes compared with the number of liposomes with hemichannels should give an estimate of the fraction of hemichannels that are open. However, this information is not available in the Locke et al. (2011) paper, where responses are normalized to control values.

What else would we like to know? Single-channel measurements should provide much additional information for both hemichannels and cell–cell channels. In a perfused preparation with whole cell patch pipettes, taurine concentration would be "clamped" at the concentration in the pipette, and pH could be measured and modified by widely used methods. This approach would likely yield an improved taurine titration curve, along with time courses of block and recovery. It would then become clear whether block was a gradual reduction in open-channel probability or a decrease in single-channel conductance, the latter being observed in other connexins examined. Interactions between cytoplasmic constituents could also be evaluated. The modulation of V_j gating by cytoplasmic acidification (Young and Peracchia, 2004) should be reinvestigated in light of the new findings. Then, there is site-directed mutagenesis to evaluate intra- and intermolecular interactions. This work should be guided by the many known mutations of Cx26 that

cause human disease. The bottom line, however, is that we have a new mechanism of connexin gating and many more connexins to be investigated in this respect.

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