Commentary

Voltage-sensing and Substate Rectification: Moving Parts of Connexin Channels

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The article by Bukauskas et al. (2002) in this issue of *The Journal* addresses several key issues in the structure–function of connexin channels, and suggests a structural link between voltage-sensitive gating and the charge in the pore. Previous work on connexin32 (Cx32) noted a correlation between the sign of voltage-sensing charges and the rectification of the subconductance state induced by movement of those charges. The present work investigates in detail the permeability properties of the subconductance state induced by voltage in channels composed of connexin43 (Cx43). The results impart generality to an intriguing structural relationship between voltage-dependent gating and modulation of pore charge selectivity. They also show how the single-channel gating physiology may relate to the molecular signaling function of connexin channels.

The importance of electrical and molecular signaling through connexin channels is widely recognized. An increasingly detailed functional and structural picture of connexin channels has been emerging recently (Verselis and Veenstra, 2000; Yeager and Nicholson, 2000; Harris, 2001), but the relation between this picture and the signaling properties of connexin channels has remained elusive.

There are ∼20 connexin isoforms, each producing channels with distinct unitary conductances and gating sensitivities. The maximal unitary conductances range from 15 to over 300 pS, and the fully open states of channels formed by the different isoforms have widely diverse ionic and molecular selectivities (Elfgang et al., 1995; Veenstra et al., 1995; for review see Harris, 2001). Connexin channels typically exhibit complex voltage-dependent gating with substantial occupancy of states with less than maximal conductance (substates).

The gating of connexin channels can be very sensitive to voltage, as much as e-fold for 2–3 mV, yet the sequences contain no S4-like domain. Therefore, the voltage sensitivity likely arises from a molecular mechanism distinct from that of most voltage-sensitive channels. Each hemichannel contains at least two distinct voltage-sensitive gating mechanisms. One, called "Vj gating" (or "fast Vj gating" in Bukauskas et al., 2002), governs rapid transitions between conducting states, typically between the fully open state and the smallest substate, or "residual" state. The conductance of this substate can be from 5 to 40% of the fully open state, depending on the connexin. A junctional channel can be in a substate due to Vj gating of either hemichannel.

For Cx32 and connexin26 (Cx26), the Vj-gating voltage sensor includes charges in the first several positions of the cytoplasmic NH_2-terminal domain and possibly two positions in the NH_2-terminal part of the first extracellular loop (Brink, 2000; Oh et al., 2000). This was established by studies in which the magnitude and polarity of voltage sensitivity were altered by single amino acid substitutions (Verselis et al., 1994; Oh et al., 1999, 2000; Purnick et al., 2000b). Due to sequence differences in these regions, hemichannels formed by some connexins close to substates only when depolarized, and some only when hyperpolarized (Verselis et al., 1994; Verselis and Veenstra, 2000). Including the positive charge of the NH_2-terminal methionine, the rule is that connexins with net neutral or negative charge in these regions close to substates with depolarization, and connexins with net positive charge close to substates with hyperpolarization. Cx32 and Cx43 are in the latter category, with net positive charge and closing to substates with hyperpolarization.

The NH_2-terminal residues involved in Vj gating are accessible from the cytoplasm, yet appear to sense the membrane voltage field, leading to the proposal that they reside within the pore when the channel is open. An NMR structure and mutagenesis suggest that the NH_2-terminal domain could adopt a hinged conformation that would allow such a configuration (Purnick et al., 2000a). In this model of Vj gating, the NH_2-terminal region inside the pore moves toward the cytoplasm in response to voltage. For example, hyperpolarization of Cx32 would cause movement of its positively charged NH_2-terminal domain toward the cytoplasmic end of the pore. Exactly how this movement induces the substate is not clear, but work on Cx32 suggests it is accompanied by the straightening of a proline kink in one of the transmembrane domains (Ri et al., 1999).

The fully open state of Cx32 channels does not rectify. Though not examined in detail, the earlier work

noted that the Cx32 substate rectified in a way that indicated enhanced influence of positive charge within the pore of the hemichannel whose Vj gate was closed. Putting this together with the net positive gating charge of Cx32 and its expected movement toward the cytoplasm with hyperpolarization, it was suggested that the NH_2-terminal residue itself provided the positive charge by occupying a position at the pore vestibule when the hemichannel was in the substate. Superficially, this mechanism resembles the voltage-dependent induction of rectifying substates in other channels by some peptide toxins (Tripathy et al., 1998), except that in this case the "peptide" is part of the protein.

The goal of the present study was to examine the substate selectivity of Cx43, which, like Cx32, closes to a substate with hyperpolarization, to see if Vj gating to the substate induced a similar change in pore selectivity. Comparing these particular connexins makes sense; other than the common polarity of Vj gating sensitivity, the pores of channels formed by Cx32 and Cx43 are quite different. Cx43 junctional channels have a maximal conductance of ~120 pS and are significantly cation-selective, whereas Cx32 channels have a smaller conductance (50 pS) and are slightly anion-selective. Cx43 channels have a larger limiting diameter (Goldberg et al., 1999; Nicholson et al., 2000). In addition, Cx32 and Cx43 are the prototypical exemplars of the two major groups of connexins as defined by sequence homology, specific sequence motifs, and gene structure (Kumar and Gilula, 1992; Bennett et al., 1994; Beyer and Willecke, 2000).

A second gating mechanism mediates slow transitions (~10–30 ms) into and out of nonconducting state(s). This mechanism is not at issue in these studies, except that it complicates the experiments. These transitions can occur in response to voltage (when they are called "loop-gating" or "slow Vj gating" [Trexler et al., 1996]), chemical factors such as lipophiles and lowered pH (when they are called "chemical gating" [Bukauskas and Peracchia, 1997]), and the docking of two hemichannels (Bukauskas and Weingart, 1994). The slow transitions may reflect a common structural change induced by these several effectors (electrical, chemical, and structural). Alternatively, they could reflect distinct structural changes that are indistinguishable at the single-channel level and have yet to be resolved mechanistically. The loop-gating process is typically much less voltage-sensitive than is the Vj-gating process, and is always in response to hyperpolarization. The presence of both mechanisms means that a single hemichannel in which Vj gating and loop gating respond to different voltage polarities can exhibit bipolar gating: closing to a substate with one polarity, and closing fully with the other (Oh et al., 2000).

Because of these voltage dependencies, examination of substate permeation properties of a junctional channel can be difficult. A voltage that closes one of the hemichannels to a substate will also close the loop gate of the same, or the other, hemichannel. Furthermore, the opposite voltage polarity will have the same effect, but on the hemichannel gates unaffected by the first polarity. One would like to determine the single-channel I-V relation for the substate of one of the two hemichannels without having the apposing hemichannel enter its substate (or fully closed state).

Bukauskas et al. worked around this problem by using junctional channels in which one hemichannel is wild-type and the other has EGFP attached to its (cytoplasmic) COOH terminus, a modification previously shown to inhibit the function of the Vj gate of the Cx43 hemichannel (Bukauskas et al., 2001). With this channel construct, it was possible to investigate the single-channel I-V relations over a broader voltage range than would be otherwise possible.

The data confirm previous work showing that the I-V relation of the fully open state is linear. More significant, they show that the I-V relation of the substate rectifies, decreasing at voltages positive on the side of the hemichannel that is in the substate. The rectification was enhanced by replacement of Cl^- by aspartate, and reduced by replacement of Na^+ by TEA^+. Neither substitution affected the I-V relation of the fully open state. These data are most readily explained by the substate having greater selectivity for anions than the fully open state. The change in selectivity argues against the possibility that the substate is only an apparent one caused by rapid fluctuations between closed and fully open states (Dani and Fox, 1991).

A simple mechanism for the change in charge selectivity is an increased relative positive charge within the pore of the hemichannel that is in the substate. This idea is given qualitative support from an application of 1-D PNP theory (Chen and Eisenberg, 1993). This theory could not account for the magnitude of the reduced conductance of the substate purely on the basis of a charge barrier, suggesting that the pore is physically narrowed as well.

Moving on to molecular permeability, further experiments showed that larger molecules of either charge (ethidium bromide, 314 D, charge of +1; Alexa Fluor® 350 carboxylic acid, succinimidyl ester, 410 D, charge of -1) that could permeate the fully open state were essentially impermeable through junctional channels in the substate, confirming that the substate has a narrowed pore.

Thus far, these data make a nice story: voltage induces a conformational change that both narrows the pore of the affected hemichannel and increases the influence of positive charge within the pore of that

hemichannel. Is the latter is a consequence of the former? The authors think not, drawing on the studies of the Vj-gating sensor of Cx32. As mentioned above, that work showed that the first few positions of the cytoplasmic NH_2-terminal domain contain charges that determine the magnitude and polarity of sensitivity to voltage. This suggests that they lie inside the pore in the fully open state, and that a negative voltage caused them to move toward the cytoplasm and induce the substate. This mechanism dovetails nicely with the current experimental results on Cx43, leading to the proposal that the "gating charges" of the NH_2-terminal domain are the ones that cause the change in charge-selectivity by moving to the cytoplasmic vestibule. One could imagine that inside the open channel, the charge forms a charge-pair with a pore-lining residue, and that this is disrupted by the effects of membrane voltage.

Is this the only possible explanation? It is possible that the movement of the NH_2-terminal voltage sensor causes the pore to narrow by allosteric means, and that this narrowing enhances the electrostatic influence of preexisting positive charges in the wall of the pore on permeating ions. Since the narrowing would be asymmetric (i.e., would not occur in the opposing hemichannel), rectification would result. No data argue against this idea, but it is more complicated structurally and does not explicitly account for the effects of movement of the voltage-sensing charges within the pore.

As appealing as the favored explanation is, some questions remain. The previous work (Purnick et al., 2000a) on Cx32 indicated that formation of the hinge that would allow the first eight to ten NH_2-terminal residues to fold into the pore required a glycine (in the wild type) or a proline at position 12 to facilitate the bend; other substitutions did not yield functional channels. In Cx43, this position is occupied by an aspartate and there are no glycines or prolines adjacent. If the Cx43 NH_2-terminal sequence does not permit the formation of a structure that allows folding into the pore, other structural models must be considered.

Looking more closely, one notes that the NH_2-terminal domain of Cx43 has a negative charge at position three (D3; whose charge presumably offset by the positive charge of K8, neither of which is present in Cx32). If substate rectification is due to movement of the NH_2-terminal domain toward the cytoplasm, one must conclude that, in the substate conformation, D3 is positioned so that its charge does not influence permeation. On this basis, one might posit that the only charge that matters in this regard is the NH_2-terminal methionine. If so, one would expect all substates induced by this structural mechanism to rectify in the same manner as those of Cx32 and Cx43, no matter what other charges contribute to the voltage-sensor. But, if positions other than the NH_2-terminal methio-

nine are involved in the rectification, one could expect that for some connexins, particularly those in which Vj gating responds to the opposite voltage polarity (i.e., closes to the substate with depolarization) the substate would be cation selective.

Caveats aside, any mechanism in which the gating charges also modulate the substate conductance pathway gains support from the finding that Cx32 and Cx43, whose Vj gates close with the same polarity and whose fully open pores are quite different, are moved by Vj gating into substates with the same kind of conductance rectification. There are no published data on the substate selectivity of connexin channels with the opposite Vj-gating sensitivity.

The dye studies suggest that the substates of connexin channels may function to eliminate intercellular molecular signaling while maintaining some degree of electrical coupling and ionic continuity. This particular point is established explicitly in recent work by Qu and Dahl (2002), demonstrating for Cx43 and Cx46 that cAMP is permeable through the fully open states but does not significantly permeate the substates. This finding is particularly significant because of the poor correlation between pore size estimated from dye studies and the permeability of endogenous cytoplasmic molecules (Goldberg et al., 1999; Nicholson et al., 2000). One should note that while the junctional conductance is reduced by Vj gating, electrical coupling can be substantially preserved, depending on the particular junctional and nonjunctional conductances (Bennett, 1966).

Except in excitable tissues, voltage-induced closure to a substate is not likely to be biologically important for homomeric channels (composed of a single connexin isoform) because the junctional voltages required are greater than those that are usually seen between coupled cells. For homomeric Cx43 channels, substate occupancy does not contribute significantly to junctional conductance at physiological junctional voltages (Christ and Brink, 1999). Even so, the voltages that induce substate occupancy can be modest (e.g., 15–25 mV) compared with those that modulate other voltage-dependent channels. Therefore, in energetic terms, these substates may be relatively accessible from the open state, and may be induced by specific biochemical interactions or cellular conditions that have yet to be identified. In fact, phosphorylation of Cx43 by PKC at a serine in the COOH-terminal cytoplasmic domain has been shown to enhance occupancy of a dye-excluding substate (Lampe and Lau, 2000). Whether this is the same substate as that induced by Vj gating is not clear.

Heterotypic junctional channels (in which each hemichannel is composed of a different connexin) can show dramatic shifts in voltage sensitivity, such that junctional conductance is substantially less than maximal at zero junctional voltage (Elenes et al., 2001). Cer-

tain amino acid substitutions cause similar effects (Oh et al., 1999). Even though brief sojourns into substates near zero voltage have been observed (Trexler et al., 1996; Pfahnl and Dahl, 1998), to date there has been no direct demonstration of substantial substate occupancy in the absence of a junctional potential.

However, should this occur, electrical coupling would be modified in a way that also could have biological significance. The I-V relation of the substate defines the character of the intercellular currents that remain when molecular signaling is shut off. Due to the rectifying substate conductance, the instantaneous junctional conductance would be greater in one direction than another (Oh et al., 1999).

What are the implications for regulation of intercellular molecular signaling? The fully open state of junctional channels is typically wide enough to allow intercellular diffusion of second messengers and other small cytoplasmic molecules. Indeed, it has been demonstrated that for specific connexins, hemichannels or junctional channels can be permeable to cyclic nucleotides, nucleotide triphosphates, calcium ion, inositol trisphosphate, glucose and its metabolites, glutamate and glutathione (for review see Harris, 2001). Perhaps more important, different connexins can have different selectivities among these molecules (Bevans et al., 1998; Goldberg et al., 1999; Nicholson et al., 2000). With this information in mind, one can extrapolate from the work of Bukauskas et al. (2002) and Qu and Dahl (2002) that the substates induced by Vj gating provide connexin channels with the machinery to dynamically regulate differential permeability to cytoplasmic signaling molecules.

Connexin channel structures are approaching a resolution that will enable the known transmembrane amino acid sequences to be assigned to the imaged transmembrane α-helices. Electron cryomicroscopy of junctional channels formed by a COOH-terminal truncated connexin has achieved resolution of better than 7.5 Å in the plane of the membrane (Unger et al., 1999), and there are rumors of a forthcoming higher resolution map. The published map shows that each monomer exposes two transmembrane α-helices to the pore lumen. Identification of these helices is being actively pursued by several groups using mutagenesis and SCAM. One looks forward to the identification of the pore-lining helices and the particular residues exposed to the lumen. Such information will permit more detailed exploration of the structural relation between movement of the voltage-sensor and modulation of channel selectivity among ions and second messengers.

REFERENCES

Bennett, M.V.L. 1966. Physiology of electrotonic junctions. *Ann. NY Acad. Sci.* 137:509–539.

Bennett, M.V.L., X. Zheng, and M.L. Sogin. 1994. The connexins and their family tree. *Soc. Gen. Physiol. Ser.* 49:223–233.

Bevans, C.G., M. Kordel, S.K. Rhee, and A.L. Harris. 1998. Isoform composition of connexin channels determines selectivity among second messengers and uncharged molecules. *J. Biol. Chem.* 273: 2808–2816.

Beyer, E.C., and K. Willecke. 2000. Gap Junction Genes and Their Regulation. *In* Gap Junctions. E.L. Hertzberg, editor. Jai Press, Inc., Stamford. 1–30.

Brink, P.R. 2000. Gap junction voltage dependence: A clear picture emerges. *J. Gen. Physiol.* 116:11–12.

Bukauskas, F.F., and R. Weingart. 1994. Voltage-dependent gating of single gap junction channels in an insect cell line. *Biophys. J.* 67:613–625.

Bukauskas, F.F., and C. Peracchia. 1997. Two distinct gating mechanisms in gap junction channels: CO_2-sensitive and voltage-sensitive. *Biophys. J.* 72:2137–2142.

Bukauskas, F.F., Q. Shao, A. Bukauskiene, M.V.L. Bennett, D.W. Laird, and V.K. Verselis. 2001. Gating properties of gap junction channels assembled from connexin43 and connexin43 fused with green fluorescent protein. *Biophys. J.* 81:137–152.

Bukauskas, F.F., A. Bukauskiene, and V.K. Verselis. 2002. Conductance and permeability of the residual state of connexin43 gap junction channels. *J. Gen. Physiol.* 119:171–185.

Chen, D.P., and R.S. Eisenberg. 1993. Flux, coupling, and selectivity in ionic channels of one conformation. *Biophys. J.* 65:727–746.

Christ, G.J., and P.R. Brink. 1999. Analysis of the presence and physiological relevance of subconducting states of connexin43-derived gap junction channels in cultured human corporal vascular smooth muscle cells. *Circ. Res.* 84:797–803.

Dani, J.A., and J.A. Fox. 1991. Examination of subconductance levels arising from a single ion channel. *J. Theor. Biol.* 153:401–423.

Elenes, S., A.D. Martinez, M. Delmar, E.C. Beyer, and A.P. Moreno. 2001. Heterotypic docking of Cx43 and Cx45 connexons blocks fast voltage gating of Cx43. *Biophys. J.* 81:1406–1418.

Elfgang, C., R. Eckert, H. Lichtenbergfrate, A. Butterweck, O. Traub, R.A. Klein, D.F. Hülser, and K. Willecke. 1995. Specific permeability and selective formation of gap junction channels in connexin-transfected HeLa cells. *J. Cell Biol.* 129:805–817.

Goldberg, G.S., P.D. Lampe, and B.J. Nicholson. 1999. Selective transfer of endogenous metabolites through gap junctions composed of different connexins. *Nat. Cell Biol.* 1:457–459.

Harris, A.L. 2001. Emerging issues of connexin channels: Biophysics fills the gap. *Quart. Rev. Biophys.* 34:325–472.

Kumar, N.M., and N.B. Gilula. 1992. Molecular biology and genetics of gap junction channels. *Semin. Cell Biol.* 3:3–16.

Lampe, P.D., and A.F. Lau. 2000. Regulation of gap junctions by phosphorylation of connexins. *Arch. Biochem. Biophys.* 384:205–215.

Nicholson, B.J., P.A. Weber, F. Cao, H.C. Chang, P. Lampe, and G. Goldberg. 2000. The molecular basis of selective permeability of connexins is complex and includes both size and charge. *Braz. J. Med. Biol. Res.* 33:369–378.

Oh, S., J.B. Rubin, M.V.L. Bennett, V.K. Verselis, and T.A. Bargiello. 1999. Molecular determinants of electrical rectification of single channel conductance in gap junctions formed by connexins 26 and 32. *J. Gen. Physiol.* 114:339–364.

Oh, S., C.K. Abrams, V.K. Verselis, and T.A. Bargiello. 2000. Stoichiometry of transjunctional voltage-gating polarity reversal by a negative charge substitution in the amino terminus of a connexin32 chimera. *J. Gen. Physiol.* 116:13–31.

Pfahnl, A., and G. Dahl. 1998. Localization of a voltage gate in connexin46 gap junction hemichannels. *Biophys. J.* 75:2323–2331.

Purnick, P.E.M., D.C. Benjamin, V.K. Verselis, T.A. Bargiello, and T.L. Dowd. 2000a. Structure of the amino terminus of a gap junction protein. *Arch. Biochem. Biophys.* 381:181–190.

Purnick, P.E.M., S.H. Oh, C.K. Abrams, V.K. Verselis, and T.A. Bargiello. 2000b. Reversal of the gating polarity of gap junctions by

negative charge substitutions in the N-terminus of connexin 32. *Biophys. J.* 79:2403–2415.

Qu, Y., and G. Dahl. 2002. Function of the voltage gate of gap junction channels: selective exclusion of molecules. *Proc. Natl. Acad. Sci. USA*. In press.

Ri, Y., J.A. Ballesteros, C.K. Abrams, S. Oh, V.K. Verselis, H. Weinstein, and T.A. Bargiello. 1999. The role of a conserved proline residue in mediating conformational changes associated with voltage gating of Cx32 gap junctions. *Biophys. J.* 76:2887–2898.

Trexler, E.B., M.V.L. Bennett, T.A. Bargiello, and V.K. Verselis. 1996. Voltage gating and permeation in a gap junction hemichannel. *Proc. Natl. Acad. Sci. USA*. 93:5836–5841.

Tripathy, A., W. Resch, L. Xu, H.H. Valdivia, and G. Meissner. 1998. Imperatoxin A induces subconductance states in Ca^{2+} release channels (ryanodine receptors) and cardiac skeletal muscle. *J. Gen. Physiol.* 111:679–690.

Unger, V.M., N.M. Kumar, N.B. Gilula, and M. Yeager. 1999. Three-dimensional structure of a recombinant gap junction membrane channel. *Science*. 283:1176–1180.

Veenstra, R.D., H.-Z. Wang, D.A. Beblo, M.G. Chilton, A.L. Harris, E.C. Beyer, and P.R. Brink. 1995. Selectivity of connexin-specific gap junctions does not correlate with channel conductance. *Circ. Res.* 77:1156–1165.

Verselis, V.K., C.S. Ginter, and T.A. Bargiello. 1994. Opposite voltage gating polarities of two closely related connexins. *Nature*. 368:348–351.

Verselis, V.K., and R.D. Veenstra. 2000. Gap Junction Channels: Permeability and Voltage Gating. *In* Gap Junctions. E.L. Hertzberg, editor. Jai Press, Inc., Stamford. 129–192.

Yeager, M., and B.J. Nicholson. 2000. Structure and Biochemistry of Gap Junctions. *In* Gap Junctions. E.L. Hertzberg, editors. Jai Press, Inc., Stamford. 31–98.