The Long Pore Gets Molecular

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In the dawning years of modern electrophysiology, closely following Hodgkin and Huxley's (1952) triumphant sightings of Na^+ and K^+ currents in the squid axon, the first indicators began to emerge regarding the pore-like character of the channels through which these ions move across the membrane. (Today's students and postdocs will gape with disbelief at the notion that molecular pictures were being drawn in those benighted days when DNA was still in its crib, myoglobin diffraction patterns had not yet been phased, and ion channel sequences were not even the stuff of dreams.) The most compelling of these indicators was the discovery of "long-pore" effects in unidirectional ionic fluxes through K^+ channels. The experiments were originally motivated by the simple wish to gain direct, chemical confirmation that the outward repolarizing currents of squid axon are carried by K^+, as the electrical measurements had concluded. To achieve this, Hodgkin and Keynes (1955) determined the inward and outward fluxes of radioactive $^{42}K^+$ in axons under voltage-clamp. While corroborating that K^+ did indeed carry the outward current, the results revealed an unexpected feature of these fluxes: a surprisingly high value for an arcane parameter called the flux-ratio exponent.

To get an idea of the meaning of this parameter, imagine the unidirectional fluxes of K^+ ions through an open channel. The net current observed electrically is the difference between the inward and outward fluxes. As voltage varies in the positive direction, say, the outward flux increases and the inward flux decreases in a manner that is impossible to predict quantitatively, as these individual fluxes reflect the specific, structure-dependent energy landscape along which the ions diffuse on their journey through the channel. However, as Ussing (1949) showed long ago, the ratio of these fluxes is expected to vary with voltage in a way that is much less dependent on the specific mechanism of ion transport. In particular, if ions diffuse independently of one another through the channel, this flux ratio is given by

$$J_{\text{inward}}/J_{\text{outward}} = [\exp(-FV/RT)]^n,$$

where n, the "flux-ratio exponent," represents the valence of the diffusing ion.

The big surprise of the $^{42}K^+$ flux experiments was that $n \approx 3$! Hodgkin and Keynes took this to mean that K^+ ions do not permeate the channel independently but, rather, interact strongly and march through in lockstep. They naturally explained the result by envisioning a pore that was so narrow that K^+ ions could not pass one another and long enough for at least three ions to be constrained to diffuse in single file. According to this picture, $^{42}K^+$ ions diffusing from the outside to the inside solution require that two other ions (and presumably some water molecules trapped between them) move through as well, thus producing an ionic "valence" of 3.

Since those early times, numerous investigators have made extensive, precise measurements of flux-ratio exponents in K^+ and Na^+ channels of squid axon and muscle (Begenisich and De Weer, 1980; Busath and Begenisich, 1981; Spalding et al., 1981), in Ca^{2+}-activated K^+ channels in red blood cells (Vestegaard-Bogind et al., 1985), and in gramicidin A (Finkelstein and Andersen, 1981). This latter "model" peptide channel of known structure provides a solid standard for the behavior expected for a narrow pore in which two substantially dehydrated ions diffuse in single file. These are exceedingly difficult measurements to make, but the fundamental nature of the flux-ratio exponent has motivated the required expenditure of experimental effort and has additionally provoked theoretical studies of multi-ion, single-file ionic diffusion through pores (Heckmann, 1972; Hille and Schwarz, 1978; Schumaker and MacKinnon, 1990). As a result, it became clear early on that K^+ channels in biological membranes are simultaneously occupied by three or four ions; in contrast, Na^+ channels are rarely occupied by more than a single ion. These results fit nicely with other indications that K^+ channels bind three to four K^+ ions strongly as a necessary condition for their remarkable combination of high selectivity and high transport rate (Hille and Schwarz, 1978; Neyton and Miller, 1988; Lu and MacKinnon, 1994).

Until now, all flux-ratio exponents have been mea-

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sured on channels in their natural membranes. This is problematic because "real" cells (even the specialized squid axon!) express a dog's breakfast of K^+ channels, molecular heterogeneity being the worst nightmare of the mechanistic channel biophysicist. Flux–ratio measurements had not taken advantage of heterologous high-level expression of molecularly defined channels, a now-standard approach in ion channel structure–function work. In this issue, however, Stampe and Begenisich, two masters of the flux–ratio method, describe such measurements on *Shaker* K^+ channels expressed in *Xenopus* oocytes, a system that is for ion channel workers what *Escherichia coli* is to molecular biologists. As with K^+ channels in squid axons, *Shaker* shows flux-ratio exponents of ~3, so the results are not surprising. However, they do relieve a low-level, chronic anxiety in the field that perhaps this number, so often cited in mechanistic arguments about K^+ channel conduction mechanisms, would not hold up once the system "got clean." The measurements thus conclude the symphony of classical ion channel biophysics with a satisfying coda.

Stampe and Begenisich's single-oocyte $^{42}K^+$ flux experiments are elegant, and the raw flux data are more beautiful than ever seen before, since now the K^+ channels are present at roughly 100-fold higher density in the oocyte membrane than in the squid axon. The authors push their measurements towards a structural conclusion that I think warrants some contemplation, since it touches on an important and timely issue: What does the selective pore actually look like? What is its length? For many years, channel researchers have exploited the advantages of electrophysiological analysis to draw conclusions about the lengths of K^+-selective pores. For instance, blockers were used on a cation-selective channel from sarcoplasmic reticulum to show (Miller, 1982) that the narrow part of the pore is very short (~12 Å) and that wide "vestibules" connect the narrow selectivity region to the bulk solutions; this picture provided a satisfying explanation for that single-ion channel's high conductance. A similar approach (Villarroel et al., 1989) on a Ca^{2+}-activated K^+ channel argued that this conventional three-ion K^+ channel is ~35 Å long in its narrow region. This makes intuitive sense because it would seem energetically prohibitive to cram three or four K^+ ions into a short length. The *Shaker* flux ratio measurements lead Stampe and Begenisich to propose as well that the single-filing region of *Shaker* is ~35 Å long, so as to accommodate four K^+ ions, each with two water molecules associated.

But there are two lines of modern evidence that restrain me from wholly embracing the idea of a classical long pore for K^+ channels, as intuitively appealing as that idea is. First is the realization (Kuo and Hess, 1993) that the narrow selectivity zone of Ca^{2+} channels is highly localized in space, even though this region of the pore accommodates two Ca^{2+} ions, i.e., four positive charges, as with a four-ion K^+ channel. The point-mutagenesis studies on L-type Ca^{2+} channels (Yang et al., 1993; Ellinor et al., 1995) show that both Ca^{2+} ions are bound physically close together by four glutamate residues located at about the same depth in the pore. Such a picture would have been considered electrostatically outrageous a decade ago, but today it gives us the most detailed and plausible view yet of a channel selectivity mechanism.

Second, several groups have been furiously probing the selectivity-determining deep-pore sequence of K^+ channels, and it has been found that rather large polar molecules can gain access to residues here, in this putatively narrow place. Specifically, from the external side of the membrane, peptide blockers make direct contact with known pore-associated residues on the *Shaker* channel (Naranjo and Miller, 1996; Ranganathan et al., 1996), as do polar thiols as large as glutathione (Lü, 1995). Surprisingly, large polar thiosulfonates (Pascual et al., 1995) can gain access from the cytoplasmic side to cysteine-substituted residues located in the K^+ channel's "selectivity signature sequence." Some of these externally accessible and internally accessible residues are separated by only one or two positions in the protein's linear sequence! So it may turn out that K^+ channels have the same sort of surprise in store for us as Ca^{2+} channels did: that they are much shorter than any sensible biophysicist would have thought (Goldstein, 1996). In the context of such a view, it will be a challenge to explain the long-pore single-filing behaviors shown by K^+ channels, such as the high flux-ratio exponent that Stampe and Begenisich have now nailed down.

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