Commentary

Shedding Light on Voltage-dependent Gating

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Voltage-dependent channels respond to changes in the transmembrane electric field by rearranging their voltage sensors, triggering increases in selective permeability. The channels reside in three types of conformationally distinct populations: closed states, open states, and a variety of inactivated states. These populations are closely interrelated and, while significant advances have been made in understanding these relationships, their quantitative and molecular description has occupied most of the waking hours of channel biophysicists for the last five decades.

Initial studies of ion channel gating depended on the ability of channels to conduct ions at rates of 10^6–10^7 ions/s, allowing for exquisitely sensitive ionic current measurements both at the ensemble and single molecule levels (see Hille, 1992, for references). But, by definition, ionic currents give little or no information on the nonconductive steps of the activation pathway, thus limiting these studies to the transitions to and from the open state. The first direct evidence of voltage-gated conformational changes, gating current measurements (Armstrong and Bezanilla, 1973; Keynes and Rojas, 1974), opened a rich source of information on the multiple nonconductive states that channels populate before the final opening transition. The catch is, however, that gating currents can only monitor channel conformational changes involving charge movements or dipole rearrangements; no information is obtained from electrically silent protein rearrangements, no matter how massive they might be.

Spectroscopic approaches, either global or local, through reporter group techniques enable investigators (in principle) to sample most of the conformational space of a macromolecule. Application of this idea to the study of voltage-dependent processes is not new. Hubbell and MacConnell (1968) first applied the reporter group approach to the study of excitable membranes by looking at the partition behavior of spin labels incorporated into vagus nerves placed inside a custom-made EPR resonator. The first global spectroscopic approach to the study of membrane excitation was the measurement of changes in birefringence from single axons (squid giant axons) or bundles of axons (crab leg nerves) by Cohen and co-workers (Cohen et al., 1968, 1971). These experiments were later superseded by measurements of membrane birefringence under voltage clamp by Landowne (1985), who observed voltage-dependent rearrangement of peptide bonds correlating with Na^+ channel gating current kinetics. Although exciting, these pioneering studies were intrinsically limited by their inability to (*a*) study and target a specific molecular entity, and (*b*) to probe a defined site within a given molecule.

Fast-forward 20–30 yr and introduce molecular cloning, heterologous expression systems, and site-directed mutagenesis. By taking advantage of sulfhydryl chemistry to specifically attach fluorophores, and cleverly timing channel membrane incorporation to minimize background fluorescence levels, Manuzzu et al. (1996) made a seminal contribution to the measurement of voltage-dependent conformational changes by providing direct physical evidence for voltage-dependent S4 movements in *Shaker* K^+ channels. These movements were detected as changes in fluorescence intensity of probes attached at positions 356 and 359 in *Shaker* channels (at the extracellular surface and at the membrane–water interface of the S4 transmembrane segment), and were found to track the kinetics and voltage dependence of gating current traces. This initial report was followed and extended by the work of Cha and Bezanilla (1997), who looked at similar residues in S4 and detected additional fluorescence changes from positions at the extracellular end of S2 (residue T276) and the pore (residues F425 and T449). Cha and Bezanilla (1997) also probed systematically the origins of the fluorescence changes, suggesting that these, in most cases, derive from collisional quenching from other parts of the channel. Isacoff and co-workers later coined the term voltage-clamp fluorimetry (VCF) to define the simultaneous measurement of fluorescence from specifically attached probes and ionic or gating currents in voltage-clamped cells (*Xenopus* oocytes, so far).

The key challenge of VCF, however, is not the detection of fluorescence changes after switching the transmembrane voltage, but the identification or correlation of these changes with specific steps along the activation/inactivation path of a channel. Two articles appearing in this issue of *The Journal* (Cha and Beza-

J. Gen. Physiol. © The Rockefeller University Press • 0022-1295/98/10/373/4 $2.00
Volume 112 October 1998 373–376
http://www.jgp.org

nilla, 1998; Loots and Isacoff, 1998) rise to the challenge and give additional insight into the physical and functional relations between areas of the extracellular face of *Shaker* K^+ channels involved in activation and slow inactivation. Although the driving force behind each of the articles is different, they both show compelling evidence for a physical interaction between the pore of the channel and its voltage sensor (S4 segment) during gating-related events. To this end, both groups cleverly use the nonconducting mutant W434F (Perozo et al., 1993), which very handily eliminates ion conduction but leaves gating currents intact, allowing a direct comparison between fluorescence changes and charge movements.

Cha and Bezanilla (1998) made the surprising discovery that manipulations affecting ion flow also have remote effects on S4 fluorescence changes. By comparing voltage-evoked fluorescence changes in the presence and absence of specific K^+ channel blockers (TEA or Agitoxin II), or by directly mutating the pore maintaining the W434F mutation, they detected a component of the fluorescence signal that disappears in the absence of ionic flow. A systematic analysis of this component revealed that, although its voltage dependence was essentially identical to that of the conductance vs. voltage curve, its kinetics were much slower than those of equivalent ionic currents, as if presaging an entry into the slow inactivated state. The work by Cha and Bezanilla (1998) is also significant from a purely technical standpoint. Changes in their previous experimental setup, including vastly different optics, produced a large increase in the signal-to-noise ratio (10×), allowing for routine spectral analysis of the fluorescence signals and, for the first time, in situ membrane protein fluorescence anisotropy measurements.

Loots and Isacoff (1998) also detected this pore–S4 interplay, taking it a step further by thoroughly dissecting the slow inactivation process through the study of fluorescence signals during extended depolarizations. They find that changes in fluorescence at either the NH_2-terminal end of the S4 segments (residue A359) or in the pore region (residue A424) closely follow the development and recovery of slow inactivation. These long depolarizations produce no further changes in fluorescence at position 424, but affect the recovery from the inactivated state. At position 359, a small but measurable decrease in fluorescence intensity was observed. Based on these and other findings, Loots and Isacoff (1998) suggest that the development of the slow inactivated state involve two sequential rearrangements affecting a single inactivation gate located in the external mouth of the pore. In the first rearrangement, the inactivation gate closes within a few seconds, abolishing ion conduction. This state is reminiscent of the "P-inactivation" described by De Biasi et al. (1993). In the second step, the inactivation gate is stabilized by its interaction with the S4 segment, thus generating a shift in the voltage dependence of the gating charge movement, which defines the "true" C-inactivated state. The suggestion of a sequential relationship between entry into the P- and C-inactivated states correlates well with earlier findings by Olcese et al. (1997), who showed that gating charge immobilization during slow inactivation is likely to develop through a sequential mechanism. The results of Loots and Isacoff (1998) also support the notion put forward by Yang et al. (1997) that the W434F mutation locks the channel into an inactive state equivalent to the P inactivation.

A structural perspective of the VCF studies in *Shaker* K^+ channels based on the results of both articles is shown in Fig. 1. The positions of residue α carbons showing large voltage-dependent fluorescence changes (*spheres*) are shown against the α carbon tracing (*black trace*) from a model of the last two transmembrane segments of *Shaker*, constructed based on the crystal structure of the *Streptomyces lividans* K^+ channel (Doyle et al., 1998). (Segments S2 and S4 added arbitrarily at the periphery of the channel core, *gray traces*.) Positions with signals that correlate with channel activation are shown as light spheres: T276 in S2 (Cha and Bezanilla, 1997), M356 in S4 (Cha and Bezanilla, 1997, 1998; Loots and Isacoff, 1998; Mannuzzu et al., 1996), and F425 in the pore region (Cha and Bezanilla, 1997). A position that

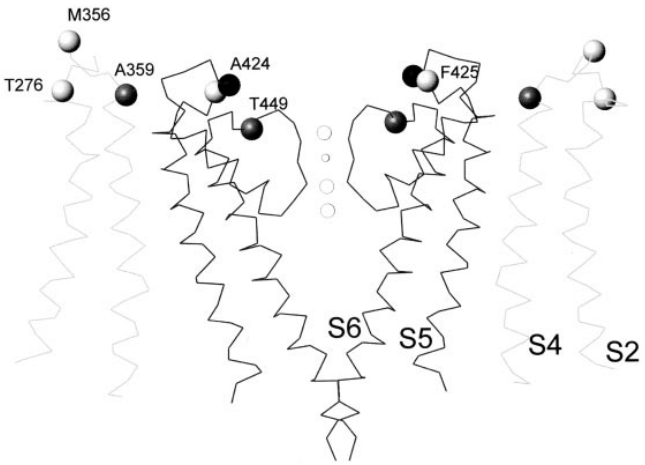

FIGURE 1. Structural correlates of conformationally active positions during activation and slow inactivation mechanisms. The putative locations of *Shaker* K^+ residues showing voltage-dependent fluorescence changes were mapped on a structural model of the *Shaker* K^+ based on the crystal structure of the *Streptomyces* K^+ channel. Shown are the α traces for the core of the channel (*black lines*) and the putative locations of the S2 and S4 transmembrane segments (*gray traces*). The labeled spheres correspond to the position of the α carbons of residues able to follow activation conformational changes (*light gray spheres*), slow inactivation conformational changes (*black spheres*), or both (*dark gray spheres*).

mostly tracks the slow inactivation process is shown as black spheres: A424 in the pore region (Loots and Isacoff, 1998). Finally, positions that respond to both activation and slow inactivation events are shown as dark gray spheres: A359 in S4 (Cha and Bezanilla, 1997, 1998; Loots and Isacoff, 1998; Mannuzzu et al., 1996), and T449 in the pore (Cha and Bezanilla, 1997). In the absence of direct structural information on the conformation of the S4 segment and the extracellularly located S3–S4 loop, it is impossible to establish the nature and extent of the physical connection between the S4 segment and the pore responsible for the cross talk evidenced by the fluorescence measurements. However, the residues in the pore (A424, F425, and T449) are spatially clustered but nevertheless report differentially on activation, slow inactivation, or both, which implies the existence of very specific interactions between the voltage sensor and the extracellular inactivation gate.

Although the results and conclusions in the two articles tend to agree on the broad strokes, there are a number of discrepancies that need to be addressed in future work. Key among them is the accessibility of the residues along the S3–S4 loop that show strong changes in fluorescence upon depolarization. The Isacoff group favors the notion that the extracellular portions of S4 and the adjacent S3–S4 loop are somewhat buried at rest but exposed during activation (Larsson et al., 1996; Mannuzzu et al., 1996; Baker et al., 1998). In contrast, the anisotropy measurements of Cha and Bezanilla (1998) indicate larger motional constraints at the extracellular end of S4 (residues M356 and A359) than in its putatively membrane embedded regions (residue 363), with M356 becoming more constrained and A359 less constrained upon depolarization. This led Cha and Bezanilla (1998) to invoke the presence of a protein vestibule at the extracellular end of the S4 segment, which would interact differentially with S4 as the voltage sensor reorients with changes in the transmembrane electric field. D_2O accessibility studies indicate that this vestibule is always water filled, regardless of the state of the channel, a suggestion that would challenge earlier data by Manuzzu et al. (1996), who hypothesized that the S4 rearrangements were accompanied by changes in the hydrophobicity of S4's microenvironment.

Currently, there are few drawbacks in the use of VCF to dissect voltage-dependent events on ion channels. The main limitation of VCF lies in its inability to study fluorescence changes from the intracellular portions of channels. This is because, so far, it has been impossible to label intracellularly exposed cysteine-containing channels without also labeling the large number of intrinsic cysteines in an oocyte. A plausible alternative could be the selective incorporation of unnatural amino acids, as introduced by Noren et al. (1989) and Mendel et al. (1992), and recently adapted to the oocyte expression system by Nowak et al. (1995). Also, there are concerns regarding the size of the maleimide tether (7–8 Å): that the fluorophores are relatively large and the fluorescence signal from these reporter molecules is intrinsically delocalized. Consequently, the position of the fluorophore may not correlate spatially with the putative position of the residue, imposing clear restrictions on carefree interpretations of the fluorescence changes. Still, the intrinsic capability of VCF to monitor protein conformational changes in real time, in vivo, and under voltage control clearly outweighs these and other possible drawbacks. As the interest in VCF increases and other groups join in the excitement, we should see improved understanding of the voltage-dependent activation and inactivation mechanisms, as novel approaches match the number of technical challenges that lie ahead. There is a bright future ahead for the application of fluorescence techniques to voltage-dependent phenomena.

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