Fluorescence-based clamp approaches provide powerful techniques to directly report structural dynamics underlying gating processes in Shaker Kv channels. Here, following on from work carried out in Shaker channels, we have used voltage clamp fluorimetry for the first time to study voltage sensor motions in mammalian Kv1.5 channels, by attaching TMRM fluorescent probes to substituted cysteine residues in the S3–S4 linker of Kv1.5 (A397C). Compared with the Shaker channel, there are significant differences in the fluorescence signals that occur on activation of the channel. In addition to a well-understood fluorescence quenching signal associated with S4 movement, we have recorded a unique partial recovery of fluorescence after the quenching that is attributable to gating events at the outer pore mouth,1 that is not seen in Shaker despite significant homology between it and Kv1.5 channels in the S5-P loop-S6 region. Extracellular potassium is known to modulate C-type inactivation in Shaker and Kv channels at sites in the outer pore mouth, and so here we have measured the concentration-dependence of potassium effects on the fluorescence recovery signals from A397C. Elevation of extracellular K+ inhibits the rapid fluorescence recovery, with complete abolition at 99 mM K+, and an IC50 of 29 mM K+o. These experiments suggest that the rapid fluorescence recovery reflects early gating movements associated with inactivation, modulated by extracellular K+, and further support the idea that outer pore motions occur rapidly after Kv1.5 channel opening and can be observed by fluorophores attached to the S3–S4 linker.

Voltage clamp fluorimetry (VCF) is a relatively new technique, applied mainly to potassium channels, that has been used to examine channel gating in a manner that is not possible with traditional electrophysiological techniques. VCF utilizes a class of molecules with unique properties of light absorption and emission, collectively referred to as fluorophores or dyes. When attached to sulfhydryl-linker moieties, these dyes can be covalently attached to cysteine residues introduced at specific positions within the channel as a fluorescent probe. Emitted light is a function of energy retained by the fluorophore following photon absorption, and can thus be influenced by any interactions with the probe resulting in a loss of energy; in other words, measured emission is highly sensitive to changes in fluorophore environment. Measurements of changes in emitted light (fluorescence) during changes in membrane potential can therefore provide direct readings of protein movements near the fluorophore in response to the potential change, and the kinetics with which they occur, due to voltage-dependent modifications in the environment.

The sensitivity of the fluorophore to its environment is quite high, as even adjacent mutated and labelled amino acids can exhibit significantly different emission profiles during depolarization.

Data collected from labelled residues in Shaker and other ion channels has shed light on the fundamental gating processes associated with channel opening and inactivation. In particular, fluorophores located in or near the pore and external end of S4 have been used to further understand how these regions move in sensed voltage and modulating activation and inactivation. In Shaker channels, simultaneous recording of ionic current along with changes in fluorescence emission from tetramethylrhodamine-5-maleimide (TMRM) attached to sites in the S3–S4 linker have shown that the fluorescence changes provide a faithful report of the time and voltage dependence of voltage sensor movements.2,3 Similar fluorescence reports of voltage sensor dynamics have also been described in hSKM1 Na+ channels, hERG, eag, BKCa K+ channels and spHCN channels.4,7 Specific fluorescence reports on inactivation have also been reported, with much slower time scales than activation-related changes. Inactivation can be reported with fluorophores attached in S4 as well, but have been most prominently shown with fluorophores attached in the external pore domain of Shaker using labelled S424C mutant Shaker channels.8
Here, we present a further description of the first fluorescence reports from mammalian Kv1 channels. TMRM attached to the S3–S4 linker of Kv1.5 revealed a unique and complex time course of fluorescence change upon depolarization that was unexpected due to the high sequence homology between Kv1.5 and Shaker channels. In addition to a rapid fluorescence quenching that became faster with larger depolarizations, similar to that seen in Shaker channels, and attributable to voltage sensor movement, the TMRM fluorophore attached to sites in the Kv1.5 S3–S4 linker reported a secondary, rapid fluorescence increase upon channel activation (Fig. 1B). The peak fluorescence-voltage (F-V) relationship did not fit that expected for gating charge movement (and thus a record of S4 conformational change), as it overlay the conductance (G-V) relation rather than being left-shifted, and suggested that the overall signal was a composite of at least two events occurring around the same time. The F-V relationship for the rapid fluorescence increase (F peak-plateau) is shifted to more positive potentials than channel opening itself (Fig. 1C). We have shown that a number of experimental interventions, such as exposure to 4-aminopyridine, the ILT triple mutation that separates gating from pore opening, and other point mutations that stabilize the outer pore of the channel, but not the inner activation gate, are all capable of preventing the secondary fluorescence increase upon channel activation.1 The fluorescence signal upon activation was transformed into the monotonic decrease reported for Shaker channels, and the F-V hyperpolarized to the left of the G-V relationship to a position that clearly reflects voltage sensor movement. As a result, we concluded that the rapid de-quenching of the fluorescence after channel activation reflected rapid structural events at the selectivity filter gate or the outer pore that changed the fluorophore environment at A397 in the nearby S3–S4 linker.

In order to gain further insight into the rearrangement that is occurring at the selectivity filter upon Kv1.5 gating, we attempted to modulate the structure of the outer pore mouth by altering the concentration of external potassium. Shaker and Kv1 channel currents are strongly modulated by extracellular K+, and elevation results in an inhibition of C-type inactivation in a “foot-in-the-door” manner,9,10 that is affected by substitutions at T449 in the outer pore.11 Data in Figure 2 show reports from TMRM attached to site A397C in the S3–S4 linker of Kv1.5. The same cell was pulsed to +60 mV for 100 ms in different concentrations of external K+, as shown, and the fluorescence signal was recorded. As was shown in Figure 1, on depolarization to +60 mV there is an immediate fluorescence quenching followed by a rapid phase of recovery of the fluorescence signal that is incomplete and fails to reach the baseline level until the oocyte membrane is repolarized. There is little apparent change in the instantaneous fluorescence quenching at different concentrations of external K+, but clearly the amplitude of the secondary fluorescence increase is dependent on the concentration of external K+, and as it is elevated, this secondary component is reduced in amplitude until it is completely abolished in 99 mM K+. In order to describe the K+ dependence of the fluorescence increase, the normalized amplitude of the fast fluorescence increase is plotted vs. the external K+ concentration (Fig. 2B). This curve was fit with a Hill equation, and the rapid fluorescence increase was found to be inhibited with an IC50 of 29 ± 6 mM K+.

Figure 1. Voltage-clamp fluorimetry report and analysis of TMRM emission at residue A397C in Kv1.5. (A and B) Representative ionic current (A) and fluorescence (B) traces obtained from Xenopus oocytes injected with Kv1.5 A397C cRNA, labelled with TMRM. Methodological details are similar to those described previously.1 Voltage-clamp depolarizations were from a holding potential of -80 mV, up to +60 mV in 10 mV increments, for 100 ms. Only selected currents are shown here for clarity. Fluorescence emission is reported as a percent change from the baseline emission magnitude prior to any change in cell membrane potential (dotted line). (C) Conductance- (G-V) and fluorescence-voltage (F-V) relationships for the cell pictured in (A and B), normalized to peak values. V1/2 and k values were -11.0 mV and 39.6 mV and 41.9 mV and 30.0 mV respectively, for the two relationships.
Our previous work suggested that the rapid fluorescence recovery reported on a structural rearrangement at the selectivity filter occurring soon after channel opening. The K+ concentration dependence of the transient fluorescence reported here provides further insight into this structural rearrangement. It has been described that the selectivity filter of KcsA can reside in two conformations and the occupancy of each state is dependent on the concentration of external K+. Interestingly, the midpoint for the transition between the two conformations is around 20 mM external K+, which is similar to the concentration effect described for KV1.5. Our data seem consistent with a similar phenomenon occurring in KV1.5, which is reported on by a novel transient phase of fluorescence. At present, it is unclear as to why this transition is reported upon by TMRM attached within the S3–S4 linker of KV1.5 channels and not by Shaker or other mammalian KV1 channels. However, it is interesting to note that the S3–S4 linker region in KV1.5 channels is unusually short and flexible. In KV1.5 channels this linker is 19 amino acids long (compared with 32 in Shaker, and 21 and 22 amino acids in KV1.2 and KV1.4, respectively), and almost one third (six amino acids) is formed by glycine residues. It is possible that restraints on the movement of the S3–S4 linker after channel opening allow it to sense conformational changes at the outer pore that are missed by the analogous linker in Shaker. Such unique reports of gating events in different channels further exemplify the potential power of fluorimetric techniques to resolve and scrutinize channel gating dynamics.

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