The molecular basis for the actions of $K_{\beta 1.2}$ on the opening and closing of the $K_{v1.2}$ delayed rectifier channel

Christian J. Peters, Moninder Vaid, Andrew J. Horne, David Fedida and Eric A. Accili

Department of Cellular and Physiological Sciences; and Department of Anesthesiology, Pharmacology and Therapeutics; University of British Columbia; Vancouver, BC Canada

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Cytosolic $K_{\beta}$ subunits co-assemble with transmembrane $K_{v1}$ channel $\alpha$-subunits and have complex effects on channel function. Fast inactivation, the most obvious effect conferred, is due to fast open channel block resulting from the binding of the N-terminus within the inner mouth of the pore. $K_{\beta}$ subunits also slow current deactivation, enhance slow inactivation and shift channel activation to more negative voltages, but the mechanisms underlying these actions are not known. Here we use voltage clamp fluorimetry at sites near the extracellular end of the S4 helix, the channel’s primary voltage sensor, in combination with voltage clamp electrophysiology, to independently track the movement of the S4 helix along with ionic current, and thus identify the structural and mechanistic means by which the $K_{\beta}$1.2 subunit confers its actions on the $K_{v1.2}$ channel. We show that the negative shift in current activation is not due to direct actions of $K_{\beta}$1.2 on the S4 segment. Instead, this shift results from an apparent saturation of channel activation at depolarized potentials as the extent of open channel block by the $K_{\beta}$1.2 N-terminus progressively increases. The return of fluorescence to baseline is slowed along with current deactivation. According to our data, this is due to an inability of the activation gate to close while the $K_{\beta}$1.2 N-terminus occupies the pore and strong coupling of the gate with the S4 segment. Together with data from previous studies, our findings provide a complete and coherent picture of the functional and structural interactions between $K_{\beta}$1.2 and $K_{v1.2}$.

Introduction

In native tissue, the $Shaker$-related ($K_{v}1$) family of channels exists as pore-forming $\alpha$-subunits in heteromeric complexes with auxiliary cytosolic proteins termed $K_{\beta}$-subunits. According to recent crystallographic studies, these are positioned below the associated channels in 1:1 stoichiometry with four-fold symmetry and form a so-called ‘hanging gondola’. To date, three mammalian $K_{\beta}$ genes ($K_{\beta}1-3$) have been cloned, as well as a $K_{\beta}$ homolog from $Drosophila$ melanogaster called Hyperkinetic, and these can be considered as two distinct regions based on primary sequence and function. The C-terminus is highly conserved among the three $K_{\beta}$ genes and co-translationally forms the primary contacts with the T1 domain of the N-terminus of $K_{v1}$ subunits. In contrast, the N-terminus is highly variable in primary structure among the three $K_{v}$ genes, including three splice variants of $K_{v}1$, and does not form any long-lasting interactions with the $K_{v1}$ T1 domain. Elegant structural studies have shown that the N-termini of $K_{v}$1 subunits reach the central cavity and inner pore of an N-terminally truncated $K_{v1.4}$ channel as extended peptides to confer rapid channel block. Rapid block of $K_{v1.2}$ by the $K_{\beta}$1.2 N-terminus reduces whole-cell currents by up to ~90%, which can be explained completely by a corresponding reduction in open probability.

Functional interactions between $K_{v}$1 and $K_{\beta}$1 subunits are complex. All three $K_{\beta}$1 subunits convert $K_{v1}$ channels from the delayed rectifier phenotype observed with the $\alpha$-subunits alone to a rapidly decaying transient outward current, and greatly slow channel closing upon repolarization. Fast inactivation and slowed channel closing can be abolished by the removal of the N-terminus, and the remaining $K_{v}$1 C-termini differentially regulate the surface trafficking of $K_{v1}$ channels, with a sizable upregulation of current and cell surface expression conferred upon $K_{v1.2}$ by $K_{\beta1}$ subunits. A negative shift in the activation curve is also observed when $K_{\beta}$1 subunits are co-expressed with $K_{v1}$ channels, but the structural mechanism underlying this effect is equivocal. Ablation of the unique N-terminus eliminates the negative shift produced by $K_{\beta}$1.2 on $K_{v1.2}$, and mutations in $K_{\beta}$1.3 that remove fast inactivation make the shift in $K_{v1.5}$ activation less pronounced. Modeling of ionic current data suggests that open channel block by the N-terminus, in addition to causing fast inactivation, can produce saturation of activation at depolarized potentials and thus an apparent negative shift in activation. Slowed channel deactivation is consistent with the inability of the channel gate to close while the $K_{v}$1 N-terminus...
resides in the pore, as has been demonstrated for fast inactivation produced by the N-terminus of Kv1.4 and Shaker channels, except that the extent of slowing induced by the Kvβ1 N-terminus is far greater. Allosteric interactions between Kvβ subunits and Kv1 channels may contribute to the shift in channel activation\(^{12,30}\) or to the slowing of channel deactivation, for example, by acting directly on the S4 segment or activation gate. Conformational changes in the outer pore that are associated with slow inactivation and augmented by the Kvβ1 N-terminus\(^{27,30}\) could also inhibit the closure of the activation gate as well as the return of the movement of the S4 helix to its resting position. However, there is no structural or functional evidence, as of yet, identifying any direct or indirect interaction of Kvβ with the voltage sensing elements.

Do Kvβ1 subunits modify the movement of the S4 segment? If so, how is S4 movement modified and what parts of the Kvβ subunit are responsible? To answer these questions and establish the mechanisms underlying the effects conferred by the Kvβ1.2 subunit on Kv1.2 channel activation and deactivation, movement of the S4 segment and ion flow were independently tracked by combining voltage clamp fluorimetry and current recording. Voltage clamp fluorimetry, carried out here for the first time with Kv1.2, permits the visualization in real time of protein conformational changes in response to stimuli, while simultaneously recording ionic currents with two electrode oocyte voltage clamp.\(^{32,33}\) Our changes in response to stimuli, while simultaneously recording permits the visualization in real time of protein conformational shift of activation and slowed deactivation in Kvβ1.2.

The Kvβ1.2 N-terminus induces fast inactivation, a negative shift of activation and slowed deactivation in Kv1.2. In order to establish the effects of the Kvβ1.2 subunit on Kv1.2 in our system, oocytes were injected with cRNA encoding Kvβ1.2 alone, or mixed with either full length Kvβ1.2, or a mutant of Kvβ1.2 lacking the first 77 residues, called Kvβ1.2ΔN (Fig. 1A). The sequence of Kvβ1.2ΔN corresponds to a C-terminal region that is identical among the three Kvβ1 splice variants and thus is referred to as Kvβ1 C-terminus in previous studies from our group (e.g., Accili et al. 1997). The cysteine-substituted Kv1.2 mutants were utilized for these ionic studies as well as for the fluorescence-tracking experiments, which were performed in parallel.

Typical delayed rectifier currents were recorded from Kv1.2-expressing oocytes and showed little to no inactivation over the 100 ms pulse duration (Fig. 1B). In the presence of Kvβ1.2, but not the truncated Kvβ1.2ΔN, ionic currents from Kv1.2 measured at depolarized potentials were converted from delayed outward rectifying currents to transient outward currents followed by a rapid decay to a steady state value, reminiscent of N-type inactivation (Fig. 1B). Tail current amplitudes, measured immediately following the pulse to -40 mV, were normalized to the maximum amplitude, plotted against test voltage and fitted to a Boltzmann function (Eq. 1; Fig. 1C). We noted a leftward shift of the activation curve, along with markedly slower kinetics in the ionic tail currents, when Kvβ1.2 was co-injected with Kv1.2 (Fig. 1D). When Kvβ1.2ΔN was used in place of the full length Kvβ1.2, the recorded ionic currents were kinetically indistinguishable from those recorded from Kv1.2 injected alone, though they were significantly larger in amplitude (data not shown), consistent with previous observations of Kv1.2 current upregulation by Kvβ1 and Kvβ2 in Xenopus oocytes.\(^{21,27}\) Upregulation of current has been used as functional evidence for direct interactions between Kv1 subunits and Kvβ C-termini, which has been demonstrated in a number of studies utilizing biochemical assays (see Introduction).

These data show that the fast inactivation, activation shift and slowed deactivation observed when Kv1.2 was co-expressed with Kvβ1.2 were due to its N-terminus, as was shown previously.\(^{24}\) Moreover, the similarity of these data with those obtained from wild type Kv1.2 channels show that the cysteine substitutions do not alter the interaction between these subunits.

The voltage-dependences of fluorescence deflections upon depolarization from TMRM-labeled Kvβ1.2 S4 mutants are unaffected by Kvβ1.2. To track the movement of the S4 segment independently from ionic current, two sites at the extracellular end of the S4 helix were labeled with TMRM dye, and voltage clamp fluorimetry recording was performed alongside two electrode voltage clamp. As mentioned in the Experimental Procedures, cysteine residues were introduced at sites M288 and A291, as the equivalent residues (M356 and A359; Fig. 2A) were found to faithfully track S4 movement in Shaker.\(^{33}\) The addition of TMRM to wild type channels produced little fluorescence quenching in response to depolarizing test pulses (Fig. 2B), despite the presence of an external cysteine residue (C181) in wild-type Kv1.2. We generated a mutant Kv1.2 C181V A291C, and found that fluorescence signals therefrom were kinetically equivalent to Kv1.2 A291 with C181 intact (see overlapping traces in Fig. 2C). However, channel surface expression was reduced for the C181V A291C mutant. Therefore, C181 was left intact for these studies.

TMRM-labeled Kv1.2 M288C and Kv1.2 A291C yielded voltage-dependent fluorescence deflections in a voltage-clamp fluorimetry configuration (Fig. 3A). Here, oocytes were subjected to a protocol similar to the one used to generate G(V) curves in Figure 1, except that after depolarization, oocytes were returned to -100 mV to allow complete channel deactivation and return of the voltage sensor. As seen in traces from both M288C and A291C, robust fluorescence that varied as a function of applied voltage could be recorded under these conditions. In order to examine effects of the Kvβ1.2 N-terminus on voltage sensor movement and on channel activation simultaneously and separately, we co-injected cRNA encoding Kvβ1.2 (or Kvβ1.2ΔN) with Kv1.2 M288C or A291C, and recorded fluorescence deflections.

To compare the voltage dependencies of the fluorescence deflections associated with voltage sensor movement, signal amplitudes from Kv1.2 A291C and M288C were normalized to those at 0 mV, plotted against membrane potential and fit with a Boltzmann function (Equation 1) to generate “F(V)” curves, from which values of mid-activation voltage and slope factor were determined. For Kv1.2 A291C and Kv1.2 M288C, no differences in the
The primary sequence of the deleted amino acids of Kβ1.2 is shown in the right panel. (A) Current traces recorded from oocytes expressing Kvβ1.2 A291C alone, with Kvβ1.2 or with Kvβ1.2ΔN. Traces were recorded in response to depolarizing test pulses from a holding potential of -80 mV to voltages ranging from -120 mV to +60 mV in 10 mV steps, then to -40 mV to elicit outward tail currents, before being returned to the holding potential. (C) A plot of normalized tail current amplitudes versus test voltage, fitted with a single order Boltzmann function (Equation 1). This relation (the G(V) curve) is steeper and shifted to more negative potentials significantly (p < 0.05, Student’s t-test) when Kvβ1.2 A291C is co-injected with Kvβ1.2 (circles, dashed line; V1/2 = -17.50 ± 1.69 mV, k = 13.30 ± 0.61 mV, n = 16) as compared with Kvβ1.2 (squares, solid line; V1/2 = -5.78 ± 2.78 mV, k = 15.15 ± 0.64 mV, n = 23), or with Kvβ1.2ΔN (triangles, dotted line; V1/2 = -8.30 ± 3.26 mV, k = 14.92 ± 0.44 mV, n = 9). (D) A plot of deactivation time constants determined from single exponential fits of tail currents recorded at -40 mV following test pulses from -20 mV to +40 mV, in 10 mV increments. The values for Kvβ1.2 A291C + Kvβ1.2 (circles) are considerably larger at all potentials (p < 0.0001 at all potentials tested, Student’s t-test, n values as in C) compared to for Kvβ1.2 + Kvβ1.2ΔN (triangles) or Kvβ1.2 A291C alone (squares).

Figure 1. Kβ1.2 confers a spike-and-decay “fast inactivation” to Kv1.2 currents, a hyperpolarizing shift of the activation curve, and a slowing of current deactivation. (A) The Kβ1.2 constructs used in this study. Kβ1.2ΔN lacks the first 77 amino acids that contain the region responsible for fast inactivation. Voltage dependence of fluorescence were observed when Kβ1.2, or Kβ1.2ΔN, were co-expressed with the α-subunit as compared to the α-subunit injected alone (Fig. 3B and C). In all cases, the F(V) curves span a range of voltages more negative than those of the G(V) curves (the fitted G(V) curve for Kv1.2 from Fig. 1B is shown in gray in Fig. 4), as would be expected for a signal correlated to voltage dependent S4 movement that precedes channel opening. Negatively shifted S4 fluorescence and gating charge as
were normalized, and plotted against voltages of the preceding pulses (Fig. 4B), they overlaid well with the $G(V)$ curve of $K_v1.2$ (from Fig. 1C). This suggests that slowing depends on the fraction of open channels that are available for fast block by the $K_v\beta_1.2\text{N-terminus}$ or, alternatively, for a slower inactivation process. If the slower component were due to the pore-localized $K_v\beta_1.2\text{N-terminus}$ and fast block, then the channels entering the fast inactivated state should leave this state slowly and the proportion of channels inactivated would depend upon how quickly they entered this state. Therefore, depolarizing pulses of varying duration were delivered and the amplitudes of slow components of the tails as a function of time upon relaxation to negative potentials were determined. Samples of fluorescence tails at -80 mV from $K_v1.2$ alone, or with $K_v\beta_1.2$ or $K_v\beta_1.2\Delta\text{N}$ following depolarizing pulses to +60 mV for 5 ms–40 ms are shown in Figure 5A. As before, fluorescence tails recorded with $K_v\beta_1.2$ were fitted with a double exponential function from which amplitudes for each component were determined. We reasoned that the slower component was due to the pore-localized $K_v\beta_1.2\text{N-terminus}$ and that the amplitude of this component reflects the proportion of available channels that had entered an inactivated state. When the ratios of the slower \(\tau\) amplitudes to the total fluorescence tail amplitudes were normalized, and plotted against voltages of the preceding pulses (Fig. 4B), they overlaid well with the $G(V)$ curve of $K_v1.2$ (from Fig. 1C). This suggests that slowing depends on the fraction of open channels that are available for fast block by the $K_v\beta_1.2\text{N-terminus}$ or, alternatively, for a slower inactivation process.

The fluorescence tails were considerably slowed by full length $K_v\beta_1.2$, resulting in the imposition of a second, slower phase of fluorescence at some potentials (Figs. 3A and 4A). These observations are consistent with a slowed second phase of fluorescence in the full length Shaker channel. To examine the voltage dependence of the onset of slowed fluorescence tails, the fluorescence tails were fitted with a double exponential function, and amplitudes for each component were determined. We reasoned that the slower component was due to the pore-localized $K_v\beta_1.2\text{N-terminus}$ and that the amplitude of this component reflects the proportion of available channels that had entered an inactivated state. When the ratios of the slower \(\tau\) amplitudes to the total fluorescence tail amplitudes were normalized, and plotted against voltages of the preceding pulses (Fig. 4B), they overlaid well with the $G(V)$ curve of $K_v1.2$ (from Fig. 1C). This suggests that slowing depends on the fraction of open channels that are available for fast block by the $K_v\beta_1.2\text{N-terminus}$ or, alternatively, for a slower inactivation process.

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These amplitudes, which likely represent the proportion of channels in the inactivated state, were plotted against prepulse duration (Fig. 5C). Although the maximum value was larger for
at depolarized membrane potentials; this is consistent with a model of open channel block to describe interactions of the KVβ1 N-terminus with KV1 channels, and does not support a direct effect of the KVβ1 C-terminus on the channel voltage sensor. Along with a corresponding slowing of current deactivation, the fluorescence deflections recorded during deactivation of KV1.2 exhibited a dramatic slowing by KVβ1.2. This slowing correlated with the proportion of open channels entering the fast inactivated state, on a time scale too quick for a slower inactivation process. These findings compare closely with those obtained using Shaker channels, which show that gating charge is "immobilized" during the intrinsic fast inactivation invoked by its own N-terminus; this occludes the pore and prevents gate closure. Immobilization likely results from physical associations between the activation gate and the S4 segments. Although it is possible that KVβ1.2 N-terminus may have inhibited fluorescence return ionic current, single exponential fits of these data yielded almost identical values of $\tau$ of 8.5 ± 1.3 ms (n = 6) for fluorescence and 8.8 ± 1.2 ms (n = 8) for ionic currents. These values likely reflect the progressive entry of channels into the fast inactivated state because they are similar to the order of onset of fast current inactivation at +60 mV (6.0 ± 0.8 ms, n = 11).

**Discussion**

Our data demonstrate that distinct effects on KV1.2 channel opening and closing, and on S4 movement, result from transient interactions with the N-terminus of KVβ1.2. The voltage dependence of S4 movement was unaffected by that subunit despite the apparent shift of the G(V) curve to more negative voltages. This apparent shift is thus likely due to the progressive saturation of ionic tail currents from which channel activation is determined at depolarized membrane potentials; this is consistent with a model of open channel block to describe interactions of the KVβ1 N-terminus with KV1 channels, and does not support a direct effect of the KVβ1 C-terminus on the channel voltage sensor.

Along with a corresponding slowing of current deactivation, the fluorescence deflections recorded during deactivation of KV1.2 exhibited a dramatic slowing by KVβ1.2. This slowing correlated with the proportion of open channels entering the fast inactivated state, on a time scale too quick for a slower inactivation process. These findings compare closely with those obtained using Shaker channels, which show that gating charge is "immobilized" during the intrinsic fast inactivation invoked by its own N-terminus; this occludes the pore and prevents gate closure. Immobilization likely results from physical associations between the activation gate and the S4 segments. Although it is possible that KVβ1.2 N-terminus may have inhibited fluorescence return
by acting directly on the S4 segment, there are three reasons to suggest that this does not occur. First, the voltage-dependence of fluorescence movement is unaffected by KVβ1.2. Second, the observation that the slowing of ionic and fluorescence tails during deactivation are similar to the rate of onset of inactivation can be explained most easily by open channel block by the KVβ1.2 N-terminus, which makes comparative studies among the KVβ1.1, KVβ1.3 N-terminus, unlike KVβ1.1, may enter the pore as a hairpin and the inactivation conferred by this subunit and may be modified by the cellular metabolite PIP2. So while some similarity in mechanism seems evident from our data and those of others, there are undoubtedly significant differences, which makes comparative studies among the KVβ1 splice variants important in the future.

One ongoing challenge in constructing a cohesive picture of KVβ subunit behavior, and understanding the overall consequences of Kα and β associations, is the difficulty in performing unambiguous functional studies in native tissue. Indeed, the three Kβ proteins and their splice variants, which lack any measurable electrical behavior of their own, are known to interact promiscuously with known Kα subunit isoforms to alter trafficking and gating, as well as to enhance their sensitivity to the redox following a depolarizing pulse of increasing length made up a smaller proportion of the total amplitude compared to that for the ionic traces: even after the longer prepulses, a significant fraction of the fluorescence recovery remained fast. This implies that the fluorescence signal is reporting on an additional or separate component of movement of the S4 segment when the channel closes that is not slowed by the KVβ1 N-terminus. This is consistent with studies showing that the return of the S4 segment may be partitioned into several steps, some of which may not necessarily correspond temporally with activation gate closure. Alternatively, the KVβ1 N-terminus may not inhibit the returning movement of all four S4 segments. Functional inequivalence in voltage sensor movement within one channel has been reported in the voltage-gated sodium channel Nav1.4, in which fast inactivation preferentially slows the return of only two of four S4 segments to their resting position during channel closure.

Although the influence on KVβ1.2 gating behavior can be explained by open channel block by the KVβ1.2 N-terminus, the blocking process itself may be more complex and consist of at least two separate stages. The binding of the N-terminus to the cytoplasmic surface may occur initially, placing the channel in a pre-inactivated state. This initial interaction would be followed by the movement of the N-terminus into the pore as an extended peptide, such that the channel becomes blocked and fully-inactivated. The slowed return of fluorescence and current deactivation in our studies of KVβ1.2 could result from an interaction of the KVβ1.2 N-terminus with either the cytoplasmic surface or the inner pore but, because slowing is voltage independent, the binding of the extended peptide to the cytoplasmic face, an interaction on the outside of the membrane electric field may make the most sense intuitively. A two-step unblocking process also fits with our data showing that a fast fluorescence component remains, which may correspond to a fast exit of the KVβ1.2 N-terminus from the inner pore. While our results are in strong accordance with the findings of Zhou et al. using KVα1.4 and KVβ1.1, the variability in primary structure within the N-termini among different KVβ subunits and the involvement of other cellular elements may contribute to the complexity of the block by altering KVβ interaction at both the outer and inner pore mouth, with potentially different consequent effects on voltage sensor movement or its coupling to channel opening. For example, the KVβ1.3 N-terminus, unlike KVβ1.1, may enter the pore as a hairpin and the inactivation conferred by this subunit and may be modified by the cellular metabolite PIP2. So while some similarity in mechanism seems evident from our data and those of others, there are undoubtedly significant differences, which makes comparative studies among the KVβ1 splice variants important in the future.
Experimental Procedures

Molecular biology. Human Kβ1.2 was cloned from pcDNA3 into the vector pBluescript SK+ for expression in Xenopus laevis oocytes by digesting insert and vector with restriction enzymes XbaI and EcoRI (from New England Biolabs, Ipswich, MA). An N-terminal deletion mutant of Kβ1.2 missing residues 1–77 (KVβ1.2ΔN) was generated by simultaneous PCR amplification of the Kβ1.2 C-terminus and introduction of an EcoRI recognition sequence at its 5' end, after which the PCR product was digested with XbaI and EcoRI and re-inserted into pBluescript SK+. The modified pBluescript vector pEXO was used to express rat Kβ1.2 in oocytes. Cysteine residues were introduced at specific sites (M288 and A291) in the S3-S4 linker for fluorophore labeling. Point mutations were introduced using the Quikchange kit (Stratagene, Cedar Creek, TX) using primers synthesized by Integrated DNA Technologies (Coralville, IA), and were sequenced at the University of British Columbia core facility. cRNA was synthesized using the mMessage mMachine T7 Ultra transcription kit (Ambion, Streetsville, ON) from cDNA templates linearized with SacI (Kβ1.2) or NotI (Kβ1.2 and Kβ1.2ΔN). For co-injections of α and β subunit RNA, β-subunit RNA was pre-mixed with α-subunit RNA at a 50:1 environment.

Figure 5. Current deactivation and the return of fluorescence to baseline are coupled and slowed by the Kβ1.2 N-terminus. (A) Fluorescence traces from oocytes expressing Kβ1.2 M288C alone, or with Kβ1.2 or Kβ1.2ΔN, recorded at -80 mV following prepulses to +60 mV for variable durations between 5–40 ms (5, 20 and 40 ms traces are shown). Traces were fitted with a single (M288C alone or with Kβ1.2ΔN) or a double (with Kβ1.2) exponential function, which are overlaid in gray. (B) Current traces elicited from oocytes expressing Kβ1.2 M288C with Kβ1.2 as for fluorescence traces in A except at -40 mV to visualize them in the outward direction. (C) Plots of amplitudes of the slow τ from Kβ1.2 M288C + Kβ1.2 fluorescence (squares, n = 6) and current (circles, n = 8) deactivation tails (from A and B), as a fraction of the total amplitude (fast and slow) versus prepulse duration. Each set of values is fitted with a single exponential function (solid, fluorescence; dashed, ionic). To better compare these fitted curves, the fit for the fluorescence data was normalized to the maximum value for the ionic current fit and overlaid in gray.
Oocyte preparation. *Xenopus laevis* oocytes were prepared and isolated as has been described previously. Following removal of the follicular layer, oocytes were injected with 50 nl (10–200 ng) of cRNA and incubated in Barth’s solution, which contained (in mM) 88 NaCl, 1 KCl, 2.4 NaHCO3, 0.82 MgSO4, 0.33 Ca(NO3)2, 0.41 CaCl2, 20 HEPES (pH 7.5), for 1–3 days at 19°C. Prior to recording, injected oocytes were labeled with a reactive fluorescent dye, 5 μM tetramethylrhodamine-5-maleimide (TMRM), in a depolarizing solution containing (in mM) 99 KCl, 1 MgCl2, 2 CaCl2 and 5 HEPES (pH 7.5), for 30 min at 10°C.

Two electrode voltage clamp electrophysiology and fluorimetry. Ionic currents and fluorescence signals were recorded simultaneously using two electrode voltage clamp fluorimetry as described previously. The bath solution contained (in mM) 96 NaCl, 5 KCl, 1 MgCl2, 1 CaCl2 and 5 HEPES (pH 7.5). Voltage-dependent fluorescence changes were measured from TMRM bound via stable carbon-sulfur bond to cysteine residues introduced in the S3-S4 linker. Excitation and emission light were filtered with 525-nm bandpass and 560-nm longpass filters, respectively. Emitted light was detected using a 9124b Electron Tubes photomultiplier tube (ET Enterprises, Uxbridge, UK). Acquisition signals (ionic current and fluorescence) were sampled at 20 kHz and low-pass filtered off-line at 1–3 kHz. Fluorescence traces recorded from TMRM represent the average of at least five sweeps. Recording microelectrodes contained 3 M KCl and had resistances between 0.2–0.8 MΩ.

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