Modes of Operation of the BK\textsubscript{Ca} Channel β\textsubscript{2} Subunit

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The β\textsubscript{2} subunit of the large conductance Ca\textsuperscript{2+}- and voltage-activated K\textsuperscript{+} channel (BK\textsubscript{Ca}) modulates a number of channel functions, such as the apparent Ca\textsuperscript{2+}/voltage sensitivity, pharmacological and kinetic properties of the channel. In addition, the N terminus of the β\textsubscript{2} subunit acts as an inactivating particle that produces a relatively fast inactivation of the ionic conductance. Applying voltage clamp fluorometry to fluorescently labeled human BK\textsubscript{Ca} channels (hSlo), we have investigated the mechanisms of operation of the β\textsubscript{2} subunit. We found that the leftward shift on the voltage axis of channel activation curves (G(V)) produced by coexpression with β\textsubscript{2} subunits is associated with a shift in the same direction of the fluorescence vs. voltage curves (F(V)), which are reporting the voltage dependence of the main voltage-sensing region of hSlo (S4-transmembrane domain). In addition, we investigated the inactivating mechanism of the β\textsubscript{2} subunits by comparing its properties with the ones of the typical N-type inactivation process of Shaker channel. While fluorescence recordings from the inactivated Shaker channels revealed the immobilization of the S4 segments in the active conformation, we did not observe a similar feature in BK\textsubscript{Ca} channels coexpressed with the β\textsubscript{2} subunit. The experimental observations are consistent with the view that the β\textsubscript{2} subunit of BK\textsubscript{Ca} channels facilitates channel activation by changing the voltage sensor equilibrium and that the β\textsubscript{2}-induced inactivation process does not follow a typical N-type mechanism.

INTRODUCTION

The large conductance voltage- and Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (BK\textsubscript{Ca}) are widely distributed in cells and tissues (Salkoff et al., 2006), particularly in smooth muscles and in the central nervous system where their level of expression is significantly higher than in other tissues. In central neurons, BK\textsubscript{Ca} channels control cell excitability and neurotransmitter release, coupling the membrane potential with intracellular Ca\textsuperscript{2+} levels (Gribkoff et al., 2001; Latorre and Brauchi, 2006). BK\textsubscript{Ca} channels share many structural features with the family of voltage-gated K\textsuperscript{+} channels with six membrane-spanning domains, such as the presence of the positively charged S4 segment that encodes for a functional voltage sensor (Stefani et al., 1997; Diaz et al., 1998), and the tetrameric association of four identical subunits (α) to form a functional channel (Shen et al., 1994). However, BK\textsubscript{Ca} channels are unique in that they possess a seventh transmembrane domain (S0) that brings the N terminus extracellularly (Wallner et al., 1996) (Fig. 1A) and a long intracellular C terminus domain that encodes for the Ca\textsuperscript{2+} sensitivity of the channel (Wei et al., 1994; Schreiber and Salkoff, 1997; Moss and Magleby, 2001; Xia et al., 2002; Zeng et al., 2005; Qian et al., 2006). Although only one gene (hSlo) encodes for the human BK\textsubscript{Ca} channel (Wallner et al., 1995), the phenotypic variability of BK\textsubscript{Ca} currents observed in different tissues is derived from alternative splice variants of the pore-forming α subunit (Zarei et al., 2001) and from the interaction with a variety of modulatory partners, such as the β subunits (Lu et al., 2006). In humans, four genes have been identified that encode for β subunits, reported as β\textsubscript{1}, β\textsubscript{2}, β\textsubscript{3}, and β\textsubscript{4} (Tseng-Crank et al., 1996; Jiang et al., 1999; Wallner et al., 1999; Behrens et al., 2000; Brenner et al., 2000; Weiger et al., 2000). They share structural similarities: two transmembrane segments are connected by an extracellular loop and the N and C termini are intracellular (Fig. 1A) (for review see Orio et al., 2002). Functional BK\textsubscript{Ca} channels are thought to be formed by the association of four pore-forming α subunits and one to four accessory β subunits (Wang et al., 2002). The coexpression of α with β\textsubscript{1}, β\textsubscript{2}, and β\textsubscript{4} subunits modifies the apparent Ca\textsuperscript{2+}/voltage sensitivity, while all the β subunits alter the ionic current kinetics and the pharmacological properties of BK\textsubscript{Ca} channels (Wallner et al., 1995, 1999; Hanner et al., 1997; Nimigean and Magleby, 1999; Behrens et al., 2000; Brenner et al., 2000; Meera et al., 2000; Xia et al., 2000; Lippiat et al., 2003; Ha et al., 2004; Wang et al., 2006). In addition, it has been recently shown that both the β\textsubscript{1} and the β\textsubscript{2} subunits can also modulate hSlo expression level through an endocytic mechanism.

Abbreviations used in this paper: ANCOVA, analysis of covariance; COVG, cut-open oocyte technique; HP, holding potential; MES, methanesulfonate; TMRM, tetramethylrhodamine-5′-maleimide.
A recent work by Bao and Cox (2005) has demonstrated that the $\beta_1$ co-expression stabilizes the active state of the voltage sensor, consequently increasing the apparent $\text{Ca}^{2+}$/voltage sensitivity of the channel. A striking feature of the $\beta_2$ and isoforms of $\beta_3$ subunits is their ability to confer fast inactivating properties to the channel (Wallner et al., 1999; Xia et al., 1999, 2000; Uebele et al., 2000; Hu et al., 2003; Zeng et al., 2007). The NMR structure of the $\beta_2$ N terminus domain shows that this region consists of a helical core (residues 20–45, chain domain) and a flexible disordered motif (residues 1–18, ball domain) (Bentrop et al., 2001). The mechanism proposed is an N-type–like inactivation (i.e., “ball and chain” mechanism) in which 18 amino acids from the $\beta_2$ N terminus rapidly occludes the cytoplasmic mouth of the channels when they are in the open state (Walhner et al., 1999).

The N-type inactivation mechanism described for fast inactivation in Na$^+$ and K$^+$ (Shaker) channels has been shown to be associated to the voltage sensor long-lasting permanence in its activated state, retarding its return to the resting position upon repolarization (“charge immobilization”) (Armstrong and Bezanilla, 1977; Bezanilla and Armstrong, 1977; Bezanilla et al., 1991; Perozo et al., 1992; Roux et al., 1998).

Using the voltage clamp fluorometry technique (Mannuzzu et al., 1996) we have recently characterized the voltage-dependent conformational changes of the voltage-sensing region of hSlo channels during activation (Savalli et al., 2006). In this work, in order to gain insights on the mechanisms by which $\beta_2$ subunit facilitates channel opening and produces inactivation, we have studied the conformational changes occurring in BKCa voltage-sensing region in the presence of its $\beta_2$ modulatory subunits. We found that the $\beta_2$ subunit affects the movements and the equilibrium of the S3–S4 region, suggesting that the $\beta_2$ subunit promotes channel opening by favoring the activated conformation of the voltage-sensing region of BKCa channels. In addition, we have investigated whether the coexpression of the $\beta_2$ subunit affects the voltage sensor return to the resting position upon repolarization, i.e., induces “charge immobilization.” We found no evidence of voltage sensor immobilization due to the docking of the $\beta_2$ inactivating particle into BKCa channel pore, suggesting an inactivating mechanism not homologous to the classically described N-type.

**Materials and Methods**

**Molecular Biology**

We have used the $\alpha$ subunit of human BKCa (hSlo) (GenBank/EMBL/DDBJ accession no. U11058), previously modified in a way that all native extracellular cysteines (C14S, C141S, and C277S) are substituted with serines and containing the R207Q mutation to increase open probability (Diaz et al., 1998). In hSlo cysteine-less R207Q background, a single cysteine was introduced at three positions in the S3-S4 linker (200, 201, and 202, see Fig. 1 B) by overlap mutagenesis PCR (Ho et al., 1989). The $\alpha$ subunit is cloned in the pBSTA vector, whereas the human $\beta_2$ subunit (GeneBank/EMBL/DDBJ accession no. AF099137) is in the pcDNA3 vector. The nonconducting Shaker $\text{K}^+$ channel mutant (Sh W434F) (Perozo et al., 1993) (GeneBank/EMBL/DDBJ accession no. M17211) and its inactivation-removed version (Sh-IR W434F) were also used. For site-directed fluorescent labeling of the S4 region with thiol-reactive fluorophores, we introduced a unique cysteine in the S3–S4 linker using the QuickChange site-directed mutagenesis kit (Stratagene), generating the Sh M356C W434F and Sh-IR M356C W434F mutants. All of the mutations were confirmed by sequence analysis. cRNAs were prepared in vitro (mMESSAGE mMACHINE; Ambion) and stored at −80°C.

**Expression and Labeling**

*Xenopus laevis* (NASC0) oocytes (stage V-VI) were prepared as previously described (Haug et al., 2004), injected with 50 nl of total cRNA either of the mutant $\alpha$ subunits (0.01–0.1 $\mu$g/µl) alone or $\alpha$+$\beta_2$ subunits (0.5–1 $\mu$g/µl) using a Drummond nanoinjector. Injected oocytes were maintained at 18°C in an amphibian saline solution supplemented with 50 $\mu$g/ml gentamycin (Invitrogen), 290 $\mu$M DTT, and 10 $\mu$M EDTA, 3–9 d after injection, oocytes were stained for 30–45 min with 10 $\mu$M membrane-impermeable thiol-reactive fluorescent dyes, tetramethylrhodamine-5’s-maleimide (TMRM) or PyMPO-maleimide (Molecular Probes) in depolarizing $K^+$ solution (in mM: 120 K-methanesulfonate [MES], 2 Ca$^{2+}$-maleimide (M DTT, and 10 $\mu$M EDTA, 3–9 d after injection, oocytes were stained for 30–45 min with 10 $\mu$M membrane-impermeable thiol-reactive fluorescent dyes, tetramethylrhodamine-5’s-maleimide (TMRM) or PyMPO-maleimide (Molecular Probes) in depolarizing $K^+$ solution (in mM: 120 K-methanesulfonate [MES], 2 Ca$^{2+}$, and 10 HEPES, pH 7). These fluorophores were dissolved in DMSO (100 mM stock concentration) and stored at −20°C. Changes in fluorescence emission were due to environmental differences sensed by the fluorophores.

**Electrophysiology**

**Patch Clamp.** Membrane patches of *Xenopus* oocytes in the inside-out configuration were perfused with bath solutions containing (in mM) 115 K-MES, 5 KCl, 5 HEDTA, 10 HEPES. The free [Ca$^{2+}$] was varied by adding CaCl$_2$. The free [Ca$^{2+}$] was first theoretically
with a glass pipette filled with solution containing (in mM) 3,000 K-glutamate and 10 K-HEPES (pH 7). The oocyte was impaled whereas the intracellular solution contained (in mM) 110 Na-MES, 2 Ca(MES)\(_2\), 10–50 K-MES, and 10 Na-HEPES (pH 7); for epifluorescence measurement (Cha and Bezanilla, 1998; Savalli technique (COVG) (Stefani and Bezanilla, 1998) implemented in voltage clamp condition using the cut-open oocyte. Fluorescence, ionic, and gating currents were re-
corded in voltage clamp condition using the cut-open oocyte.

−240 mV (25 ms) was delivered before the test pulse, as indicated in the figures.

The holding potential (HP) was −90 mV. All experiments were performed at 22–24°C.

α

β

Figure 2. Lack of voltage-dependent fluorescence changes in the hSlo C-less background channel. Representative K\(^+\) current traces from oocytes expressing α (A) and α+β\(_2\) (C), elicited by 100-ms depolarization from −160 mV to the indicated potential. The corresponding TMRM fluorescence traces are shown in B and D. Holding potential (HP) was −90 mV. In C, the time-dependent decay of the ionic current is caused by the inactivating properties conferred to the BK\(_{Ca}\) channel by β\(_2\) subunits. Note that no fluorescence changes (ΔF) detected in hSlo C-less. (E) Averaged G(V) curves for α (○) and α+β\(_2\) (□) and the best fits to one Boltzmann distribution are shown superimposed (see Materials and Methods). Coexpression of the β\(_2\) subunit produced ∼20 mV shift of the G(V) curve toward more negative potentials (fitting parameters: α, \(V_{half} = 1.44\) mV and \(z = 0.84\); α+β\(_2\), \(V_{half} = −26.13\) mV and \(z = 0.83\)). Error bars represent SEM.

calculated with WEBMAXC v2.10 (http://www.stanford.edu/~cpatton/maxc.html) and then measured using a Ca\(^{2+}\) electrode (World Precision Instruments). Solutions were titrated to pH 7.0. The borosilicate pipettes (World Precision Instruments) were filled with the bath solution at lowest free [Ca\(^{2+}\)] (0.067 μM).

The holding potential (HP) was −90 mV. All experiments were performed at 22–24°C. A prepulse to −160 mV (100 ms) or to −240 mV (25 ms) was delivered before the test pulse, as indicated in the figures.

**RESULTS**

The Cysteine-less hSlo Coexpressed with the β\(_2\) Subunit Does Not Elicit Voltage-dependent Fluorescence Changes

We have first evaluated the possibility that the cysteine-less hSlo clone (C-less) (used as a background construct in this study) expressed alone or together with the β\(_2\) subunit could elicit voltage-dependent fluorescence signal after incubation with thiol-reactive fluorophores (TMRM and PyMPO). As shown in Fig. 2 A, under voltage clamp conditions, the expression of the C-less channel gave rise to a robust K\(^+\) current elicited by membrane depolarizations. C-less channels constituted only by α subunits lacking all of the extracellularly exposed cysteines (C14S, C141S, and C277S) did not display any voltage-dependent change in V\(_{half}\) (for a range of 1.5 mV) induced by the coexpression of the β\(_2\) subunit.

\[
G(V) = \frac{G_{max}}{1 + e^{[(V_{half} - V_m)/z]}}
\]

\[
F(V) = \frac{F_{max} - F_{min}}{1 + e^{[(V_{half} - V_m)/z]}} - F_{min};
\]

\[
Q(V) = \frac{Q_{max} - Q_{min}}{1 + e^{[(V_{half} - V_m)/z]}} - Q_{min};
\]

where \(G_{max}\), \(F_{max}\), and \(Q_{max}\) are the maxima G, F, and Q; \(F_{min}\) and \(Q_{min}\) are the minima F and Q; \(z\) is the effective valence of the distribution; \(V_{half}\) is the half-activating potential; \(V_m\) is the membrane potential; and F, R, and T are the usual thermodynamic constants.

The analysis of covariance (ANCOVA) was performed to assess statistical significance of the change in V\(_{half}\) (for a range of 1.5 mV) induced by the coexpression of the β\(_2\) subunit.
The β2 Subunit Affects the Kinetics and Voltage Dependence of both Ionic Current and Voltage Sensor Movements

Position 202. Based on the alignment with Shaker channel, Díaz et al. (1998) proposed that the outmost residue in BKCa S4 segment is W203. We first investigated the effect of β2 on the conformational changes reported by PyMPO labeling S202C mutant. As shown in Fig. 3, the expression of S202C mutant gave rise to large ionic currents (A) associated to a rather slow voltage-dependent rearrangements during depolarization and/or channel gating (Fig. 2 D). The normalized activation curves (G(V)) constructed from channels composed by only α (○) or α+β2 (□) subunits labeled with TMRM are shown in Fig. 2 E. The coexpression of β2 with C-less R207Q facilitated channel opening as shown by the parallel leftward shift of the G(V) curve on the voltage axis by ~20 mV (α, \(V_{\text{half}} = -3.97 \pm 10.15\) mV, \(z = 0.91 \pm 0.07\), \(n = 4\); and α+β2, \(V_{\text{half}} = -25.91 \pm 6.31\) mV, \(z = 0.88 \pm 0.04\), \(n = 3\)). This effect of β2 subunits is in general agreement with previous studies in WT hSlo and mSlo channels (Wallner et al., 1999; Xia et al., 1999). Similar results were obtained after incubation with a different fluorophore, PyMPO (Savalli et al., 2006; unpublished data).

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dependence of fluorescence signal was negatively shifted by β₂ by ~20 mV in the same direction (Fig. 3 C; Table I).

(c) The fluorescence signal was significantly attenuated by ~55% after coexpression with β₂ subunits: the ratio (for α alone) (∆F \(_{\text{max}}\)/F)/G\(_{\text{max}}\) = 61.1 ± 13.1 μS\(^{-1}\) (n = 5) decreased to 27.6 ± 12.3 μS\(^{-1}\) (n = 5) in the presence of β₂ (Fig. 3 E), where ∆F \(_{\text{max}}\)/F is the maximum fluorescence change and G\(_{\text{max}}\) the limiting membrane conductance.

(d) The kinetics of both ionic current activation (Meera et al., 1996; Brenner et al., 2000; Orio and Latorre, 2005) and fluorescence rising phase were significantly slowed down by the presence of the auxiliary subunit (Fig. 3 F). For example, at 40 mV, the time constants (τ) of ionic current activation (approximated to a single exponential function) were τ\(_{\alpha}\) = 1.91 ± 0.18 ms (n = 6) and τ\(_{\alpha+\beta_2}\) = 3.89 ± 0.62 ms (n = 4) for α and α+β₂, respectively. Similarly the time constants for the fluorescence onset of α and α+β₂ (well fitted to a single exponential function) were τ\(_{\alpha}\) = 200.0 ± 13.24 ms (n = 6) and τ\(_{\alpha+\beta_2}\) = 392.67 ± 19.83 ms (n = 4). In contrast, the effective gating charge (z) was not affected by β₂ (Table I), in agreement with recent findings (Orio and Latorre, 2005).

These experimental evidences suggest that the β₂ subunit facilitates voltage sensor activation by altering its equilibrium such that BK\(_{Ca}\) channel can reach higher open probability for the same membrane depolarization.

Position 201. In hSlo channel, the extracellular linker between S3 and S4 transmembrane segments is probably formed by no more than three residues (N200-R201-S202) (Diaz et al., 1998; Wallner et al., 1999; Ma and Horrigan, 2005). All these residues can report voltage-dependent conformational changes related to the movement of the main BK\(_{Ca}\) voltage sensor (Savalli et al., 2006). We speculate that the β₂ effects observed on S202C construct should be similar to the ones reported by the other two positions in the S3–S4 linker. To test this hypothesis, we labeled R201C with PyMPO and simultaneously recorded the ionic current and the fluorescence signals from oocytes expressing the pore-forming α subunit alone (Fig. 4, A and B) or together with β₂ subunits (Fig. 4, C and D). The onset of the fluorescence signal during depolarization was faster in R201C than in S202C, thus 100-ms pulses were adequate to reach steady state in this clone (Fig. 4, B and D, vs. Fig. 3, B and E). Similar to that observed for the adjacent position 202, PyMPO fluorescence reported by position 201 was reduced by ~30% when β₂ was coexpressed (Table I). Also in this mutant, the presence of β₂ subunit produced a parallel leftward shift of both ionic current activation and fluorescence curves, as shown in Fig. 4 E. The midpoint of the F(V) was F\(_{\text{V_half}}\) = −22.55 ± 3.58 mV (n = 10) for α alone (●) and F\(_{\text{V_half}}\) = −57.19 ± 4.45 mV (n = 8) for α+β₂ (■) (Fig. 4 E; Table I). As for S202C construct, the slowing of the ionic current activation kinetics induced by the auxiliary subunit was accompanied by a similar (but more pronounced) slowing of the fluorescence onset (Fig. 4 F). For depolarizations to 40 mV, the time constants of ionic current activation approximated to a single exponential function were τ\(_{\alpha}\) = 2.75 ± 0.18 ms (n = 8) and τ\(_{\alpha+\beta_2}\) = 5.22 ± 1.05 ms (n = 6) for α and α+β₂, respectively. The time constants for the fluorescence onset of α and α+β₂ were τ\(_{\alpha}\) = 3.47 ± 0.09 ms (n = 8) and τ\(_{\alpha+\beta_2}\) = 17.35 ± 3.69 ms (n = 6).

Position 200. As reported previously (Savalli et al., 2006), position 200 did not elicit significant voltage-dependent ∆F when labeled with PyMPO. Therefore, we investigated the conformational changes involving N200C construct using TMRM. Ionic current and fluorescence recordings from oocytes expressing α alone (Fig. 5, A and B) or α+β₂ (Fig. 5, C and D) show that, in this mutant, the coexpression of β₂ subunit produced a relatively smaller shift of the channel activation curve toward hyperpolarized potentials (~5 mV). Nevertheless, this change in voltage dependence was associated to a well-resolved shift of the F(V) curve by ~20 mV in the same direction (Fig. 5 E; Table I). As for the other positions,
ionic current activation kinetics was slowed down by the presence of \( \beta_2 \) subunit (at 40 mV, \( \tau_\alpha = 1.99 \pm 0.30 \) ms, \( n = 5 \), and \( \tau_{\alpha+\beta_2} = 4.19 \pm 0.45 \) ms, \( n = 3 \)). Similarly, the time constant of the fluorescence onset slightly increased in the presence of \( \beta_2 \) subunit (at 40 mV, \( \tau_\alpha = 20.00 \pm 3.80 \) ms, \( n = 5 \), and \( \tau_{\alpha+\beta_2} = 25.06 \pm 3.06 \) ms, \( n = 3 \)) (Fig. 5 F).

In summary, for all the positions tested, we have consistently observed a left shift in the activation curve, associated with a shift of the fluorescence vs. voltage curve in the same direction, when the modulatory \( \beta_2 \) subunit was coexpressed with the pore-forming \( \alpha \) subunit. The G(V) shift observed is rather small, raising the question whether the oocyte internal \([Ca^{2+}]_i\) is low (in the submicromolar range) or the effect of \( \beta_2 \) subunit on the R207Q mutant is somewhat quantitatively different from the WT. We have addressed this point in the next section.

**Ca\(^{2+}\) Dependence of the hSlo C-less R207Q S202C Mutant: Effect of the \( \beta_2 \) Subunit**

The \( \beta_2 \) subunit facilitates WT BK\(_{\alpha} \) channels opening in a wide range of Ca\(^{2+}\) concentrations. However, in the submicromolar range, the \( \beta_2 \) effect on the half activation potential is limited (Wallner et al., 1999; Orio and Latorre, 2005). Thus, a very low oocyte internal Ca\(^{2+}\) concentration could be the reason for the relatively small shift of the G(V) curves in the presence of the \( \beta_2 \) subunit observed in the COVG experiments.

To estimate the free intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in the COVG experiments, we have characterized the Ca\(^{2+}\) dependence of the half activation potential (V\(_{\text{half}}\)) of the hSlo C-less R207Q S202C coexpressed with and without the \( \beta_2 \) subunit in *Xenopus* oocytes. Excised membrane patches in the inside-out configuration were perfused with solutions containing different free [Ca\(^{2+}\)]\(_i\), ranging from 0.067 to 138 \( \mu \)M. K\(^+\) currents were recorded during depolarizations ranging from -240 to 240 mV (Fig. 6). In average, the coexpression of the \( \beta_2 \) subunit facilitated channel opening as shown by the plot in Fig. 6 E reporting the V\(_{\text{half}}\) vs. free [Ca\(^{2+}\)]\(_i\), for all the experiments. The lowering of the V\(_{\text{half}}\) induced by the \( \beta_2 \) subunit is significant (P < 0.0001) as revealed by the analysis of covariance (ANCOVA) adjusted for the log([Ca\(^{2+}\)]\(_i\)). The difference in V\(_{\text{half}}\) for [Ca\(^{2+}\)]\(_i\) = 1 \( \mu \)M was -22.3 mV. These results are qualitatively in agreement with that previously reported for the WT channel coexpressed with the non-inactivating \( \beta_2 \) mutant (\( \beta_2\)IR, inactivation removed), although the overall effect of the \( \beta_2 \) subunit on the activation of this mutant appears significantly smaller.
From the $V_{\text{half}}$ vs. $[\text{Ca}^{2+}]_i$ plot, we estimated the free $[\text{Ca}^{2+}]_i$ of the COVG experiments. By rough interpolation, we calculated that the $[\text{Ca}^{2+}]_i$ is 3–4 μM. This value is in excellent agreement with the contaminant $[\text{Ca}^{2+}]_i$ of the internal solution (120 mM K-glutamate) measured with a Ca$^{2+}$-sensitive electrode (4.9 μM contaminant $[\text{Ca}^{2+}]_i$). Note that the K-glutamate solution faces the saponine-permeabilized oocyte membrane. Thus, the effect of the $\beta_2$ subunit observed in the fluorescence experiments using the COVG technique (5–15 mV $G(V)$ shift toward hyperpolarized potentials) is consistent with the results from the excised patch.

A Typical N-type Mechanism for $\beta_2$?
The N-type mechanism of channel inactivation has been well characterized and shown to directly affect the charge movement in Na$^+$ (Armstrong and Bezanilla, 1977) and in K$^+$ (Shaker) channels (Bezanilla et al., 1991; Perozo et al., 1992; Roux et al., 1998). Upon depolarization and channel opening, the inactivation particle binds to its docking site in the inner mouth of the pore, slowing the return of the voltage sensor to the resting conformation, thus preventing the channel deactivation. The electrical manifestation of this process is the so-called “OFF charge immobilization,” since gating charge cannot return readily at the end of the depolarizing pulse, until the inactivating particle has been released from the inner pore. Since large part of the charge movement is produced by the translocation of the charged residues of the S4 segment, the effect of charge immobilization is indeed a partial immobilization of the S4 segment itself.

To learn about the mechanism of inactivation in BK$_{\text{Ca}}$ channel, we have investigated whether the $\beta_2$ subunit produces immobilization of the BK$_{\text{Ca}}$ voltage sensor by directly assessing the movement of the S4 segment using an optical approach. As a reference, we have first characterized the immobilization of the voltage-sensing region in Shaker K$^+$ channel, a classical model for N-type inactivation (Hoshi et al., 1990; Bezanilla et al., 1991; Hoshi et al., 1991; Roux et al., 1998).

Optical Detection of Shaker S4 Segment Immobilization
After N-type Inactivation
In the nonconducting (W434F) Shaker mutant (Perozo et al., 1993) we have fluorescently labeled position 356 in the S3–S4 linker using TMRM to track the movement of the voltage sensor. After N-type inactivation, we have observed a reduction in the rate of current activation at all potentials tested (at 40 mV, $\tau_a = 2.00 \pm 0.28$ ms, $n=5$, and $\tau_{a+\beta_2} = 4.19 \pm 0.45$ ms, $n=3$). Error bars represent SEM.
Affects Voltage Sensor Equilibrium

of the main voltage-sensing region (Mannuzzu et al., 1996; Cha and Bezanilla, 1997). In Fig. 7 (A and B), we show families of gating currents recorded from Shaker channel having the N terminus deletion ∆6–46 that lacks N-type inactivation, Sh-IR M356C W434F (A), and from channels with intact N terminus, Sh M356C W434F (B). While, for all potentials tested, the ON gating currents are practically identical in the two clones, the OFF gating currents following voltage steps leading to channel opening (e.g., >−40 mV) display an extremely slow component reflecting a “charge immobilization” due to the inactivating N terminus (Fig. 7 B). On the other hand, in the mutant lacking the N-terminal inactivating domain, the OFF charge returns with a faster kinetics (i.e., it does not display charge immobilization) (Fig. 7 A). The corresponding fluorescence recordings that report the conformational changes of the voltage-sensing region for the two Shaker mutants are shown in Fig. 7 (C and D).

The steady-state properties of both charge and fluorescence changes are summarized in Fig. 8 A for the two Shaker mutants. The presence of the inactivating N terminus does not seem to affect the voltage dependence of the charge movement (Q(V)) or of the F(V) curves (Fig. 8 A).

The onset of the fluorescence change is practically identical in the two mutants in the range of potentials explored (from −60 to 20 mV) (Fig. 8 B). For example, at 10 mV the ON fluorescence time constants for inactivating (Sh) and noninactivating (Sh-IR) Shaker channels were τ_sh = 6.18 ± 0.42 ms and τ_sh,ir = 6.90 ± 0.53 ms (n = 5). On the contrary, the OFF fluorescence reveals dramatic differences in the kinetic behavior of the two channels (Fig. 8 C). In the Sh-IR channels, during repolarization to −90 mV after pulsing to 10 mV, the fluorescence follows a monoexponential decay, with a rather fast time course (τ = 3.44 ± 0.38 ms at 10 mV, n = 5) (Fig. 7 C and Fig. 8 C). On the other hand, in the channel with the intact N terminus, TMRM fluorescence reports the progressive S4 immobilization that becomes more prominent with larger depolarizations (Fig. 7 D). This is revealed by the appearance of a second slow component in the fluorescence relaxation that is absent in Sh-IR (during repolarization to −90 mV after pulsing to 10 mV, τ_fast = 2.74 ± 0.25 ms and τ_slow = 32.71 ± 0.20 ms, Amp_fast = 22.9% ± 2.9% and Amp_slow = 77.1% ± 2.9% at 10 mV, n = 5) (Fig. 8, C and D).

In conclusion, the fluorescent labeling of Shaker channel directly demonstrates the immobilization of the S4 segment, as a consequence of N-type inactivation.

The Inactivating Process of BKCa β2 Subunit Does Not Involve the S4 Segment Immobilization in its Active State

We have applied to hSlo the same fluorescence-based strategy used for detecting S4 immobilization in Shaker. In BKCa channels labeled with PyMPO at position 202, we have compared the OFF fluorescence kinetics in the absence and in the presence of the inactivating β2 subunit. As shown in Fig. 9 (B and D), the OFF fluorescence kinetics of the α subunit alone, during repolarization to −160 mV, is slightly faster than the one in the presence of the β2 subunit, as expected, since the auxiliary
subunit slows down the kinetics of both activation and deactivation of the channel (Meera et al., 1996; Brenner et al., 2000; Orio and Latorre, 2005). For example, after 1-s depolarization to 80 mV the OFF fluorescence time constants measured at −160 mV were $\tau_\alpha = 40.22 \pm 3.69$ ms ($n = 3$) (●) and $\tau_{\alpha+\beta_2} = 51.31 \pm 2.51$ ms ($n = 3$) (■) (Fig. 9 I). However, the coexpression of the $\beta_2$ subunit does not cause the appearance of a second slower component in the fluorescence return, as found for Shaker channel, supporting the view that $\beta_2$-inactivating ball domain does not interfere with the voltage sensor deactivation. We obtained similar results for repolarization to $-90$ mV (Fig. 9, F, H, and I), where $\tau_\alpha = 78.93 \pm 2.29$ ms ($n = 3$) (●) and $\tau_{\alpha+\beta_2} = 92.46 \pm 2.30$ ms ($n = 3$) (■). Note that the OFF fluorescence during repolarization to $-90$ mV does not return to baseline ($-160$ mV), due to the significant $\Delta F$ present between $-160$ and $-90$ mV, which is larger in $\alpha+\beta_2$ than in $\alpha$ alone (see F(V) curves in Fig. 3 C). Since it is possible that the OFF rate of the inactivating $\beta_2$ N-terminal “ball peptide” during repolarization is faster than the return of the voltage-sensing region, we have used a double pulse protocol to estimate the time course of the recovery from inactivation at different membrane potentials: $-160, 120,$ and $-90$ mV (Fig. 10, A–E). We found that at $-160$ mV, 90% of the channels recovered from inactivation with a time constant of $\tau = 5.24 \pm 0.35$ ms ($n = 4$; Fig. 10 A). This time constant is $\sim 10$ times faster than the one describing the OFF fluorescence kinetics, suggesting that at $-160$ mV, when the S4 segments are returning to their resting position, all the channels have already recovered from inactivation.

On the other hand, the recovery of the channel conductance from inactivation at $-90$ mV is biexponential with fast and slow component equally represented: $\tau_{\text{fast}} = 43.47 \pm 3.99$ ms (55%), $\tau_{\text{slow}} = 248.91 \pm 0.23$ ms (45%) ($n = 4$) (Fig. 10, F and G). Nevertheless, at $-90$ mV the OFF fluorescence relaxes with a time constant of $\tau = 92.46 \pm 2.30$ ms (Fig. 9 H), significantly faster than the overall recovery from inactivation at this potential. Thus, at $-90$ mV, the S4 segments appear to return to their resting position, while most of the channels are still inactivated. These results strongly suggest that the docking of the $\beta_2$ inactivating “ball” into the inner pore does not immobilize the BKCa voltage sensor in the active position, opposite to that observed in Shaker channel. In this aspect, BKCa channels do not follow a classically described N-type inactivation mechanism.

The lack of influence of the $\beta_2$ inactivation on the S3–S4 region conformational changes is also supported
DISCUSSION

The intracellular regions of $\beta_1$ and $\beta_2$ subunits are responsible for most aspects of the modulation on BK$_{Ca}$ channels (Orio et al., 2006). However, the mechanism by which $\beta$ subunits exert their regulation on channel gating is still under scrutiny. To investigate the possibility that the $\beta_2$ subunit interacts directly or indirectly with BK$_{Ca}$ main voltage sensor (S4), we have taken advantage of an optical method to directly monitor its effect on the voltage-sensing region of the channel.

As previously described for the $\beta_2$ modulation on BK$_{Ca}$ channels (Xia et al., 1999; Wallner et al., 1999), also the cysteine mutants used in this study undergo fast inactivation when coexpressed with $\beta_2$ (Fig. 3 D). In addition, the voltage dependence of channel activation (G(V) curve) was shifted toward more negative potentials in the presence of the auxiliary subunit (Fig. 2 E, Fig. 3 C, Fig. 4 E, and Fig. 5 E). Thus, for the same membrane potential, the channel open probability is higher when the $\beta_2$ is present. Interestingly, the extent of the G(V) shift observed in this study seems smaller than the one reported for WT channels (Wallner et al., 1999; Orio and Latorre, 2005). One possible explanation is that the presence of an intact $\beta_2$ subunit (not with the N terminus deletion [IR, inactivation removed]) may be responsible for the reduced effect on the G(V) curves. Alternatively, the smaller G(V) shift could be a consequence of the R207Q mutation that by itself induces a shift of the activation curves toward more negative potentials. In agreement with this hypothesis, the mutation F315Y that increases channel open probability several times (similarly to the R207Q mutation) reduces the $\beta_1$ effect on the BK$_{Ca}$ channel voltage sensitivity (Wang et al., 2006). Nevertheless, we found that the voltage dependence of the fluorescence changes (F(V) curves) consistently shifted on the voltage axis in the

![Figure 8. Evidences of S4 immobilization in Shaker channel from voltage clamp fluorometry. (A) Averaged Q(V) and F(V) curves for Sh (Q(V), ○, and F(V), ●) and Sh-IR (Q(V), □, and F(V), △). Data points are fitted to two Boltzmann distributions and normalized to the respective maxima and minima (see Materials and Methods). Note that the inactivation process is not affecting the voltage dependence of both charge and fluorescence. The averaged parameters are as follows: for Sh-IR, for the Q(V) $V_{\text{half}} = -56.40 \pm 1.58$ mV, $z_1 = 1.68 \pm 0.06$, $Q_1 = 22.50 \pm 1.59\%$, $V_{\text{half}} = -21.01 \pm 1.31$ mV, $z_2 = 1.86 \pm 0.05$, $Q_2 = 77.50 \pm 1.59\%$ and for the F(V) $V_{\text{half}} = -44.53 \pm 1.93$ mV, $z_1 = 1.17 \pm 0.08$, $F_1 = 30.37 \pm 2.62\%$, $V_{\text{half}} = -20.38 \pm 1.50$ mV, $z_2 = 2.79 \pm 0.19$, $F_2 = 69.63 \pm 2.62\% - \Delta F/F = 2.72 \pm 0.59\%$ ($n = 5$); in Sh, for the Q(V) $V_{\text{half}} = -61.72 \pm 2.04$ mV, $z_1 = 1.50 \pm 0.17$, $Q_1 = 18.44 \pm 2.26\%$, $V_{\text{half}} = -16.11 \pm 1.87$ mV, $z_2 = 1.78 \pm 0.15$, $Q_2 = 81.56 \pm 2.26\%$ and for the F(V) $V_{\text{half}} = -43.38 \pm 2.24$ mV, $z_1 = 1.66 \pm 0.18$, $F_1 = 34.90 \pm 3.44\%$, $V_{\text{half}} = -22.34 \pm 0.55$ mV, $z_2 = 3.24 \pm 0.17$, $F_2 = 65.70 \pm 3.44\% - \Delta F/F = 21.01 \pm 2.62\%$ ($n = 5$). (B) Averaged time constants ($n = 5$) of the ON fluorescence for Shaker (○) and Shaker-IR (●) at different membrane potentials ($HP = -90$mV) (fit to a monoexponential function). (C) Averaged time constants ($n = 5$) of the OFF fluorescence for Shaker (○, ●) and Shaker-IR (■) at different membrane potentials ($HP = -90$mV). The OFF fluorescence was fitted to single exponential function for Sh-IR and to the sum of two exponential functions for Sh. In the presence of the inactivating “ball” a second, slower component appears in the fluorescence traces during repolarization. Note that the time constant of the fast component of Sh (○) closely follows the one of Sh-IR (■), while the second component in Sh (●) (generated by inactivated channels) is ~10-fold slower. (D) Percentage of fast (○) and slow (●) components of the OFF fluorescence in Sh. Error bars represent SEM. When the bars are not visible, they are inside the symbols.

by the observation that, despite the prominent time-dependent decay of the ionic current, its time constant does not correlate with any components of the fluorescence kinetics. We have compared current inactivation kinetics with both ON and OFF fluorescence kinetics during 1-s depolarization in oocytes expressing the S202C mutant. These three processes were well fitted by a single exponential function and their time constants plotted against the membrane potential, as shown in Fig. 9 L. The time constants characteristic of the ionic current inactivation do not have corresponding or related time constants in the fluorescence signals but lay in between the time constants of ON and OFF fluorescence.

Different membrane potentials (HP = -90mV) (fit to a monoexponential function). (A) Averaged Q(V) and F(V) curves for Sh (Q(V), ○, and F(V), ●) and Sh-IR (Q(V), □, and F(V), △) at different membrane potentials (HP = -90mV). The OFF fluorescence was fitted to single exponential function for Sh-IR and to the sum of two exponential functions for Sh. In the presence of the inactivating “ball” a second, slower component appears in the fluorescence traces during repolarization. Note that the time constant of the fast component of Sh (○) closely follows the one of Sh-IR (■), while the second component in Sh (●) (generated by inactivated channels) is ~10-fold slower. (D) Percentage of fast (○) and slow (●) components of the OFF fluorescence in Sh. Error bars represent SEM. When the bars are not visible, they are inside the symbols.
same direction as the G(V) curves (Fig. 3 C, Fig. 4 E, and Fig. 5 E). Since the fluorescence measurements report the voltage sensor movement (Savalli et al., 2006), our results suggest that the \( \beta_2 \) subunit facilitates an activated conformation of the voltage sensor. These findings are in agreement with the conclusions drawn by Bao and Cox (2005) from gating current measurements in channels formed by coexpression of the mouse BK channel (mSlo) with \( \beta_1 \) subunits. In that study, the Q(V) curves were shifted toward more negative potentials when \( \beta_1 \) was coexpressed, leading to the hypothesis that a physical interaction between the \( \beta_1 \) extracellular loop and the S4 can account for the stabilization of the voltage sensor in the active state. Our study, using a completely different experimental approach, suggests a similar mechanism for the \( \beta_2 \) subunit. The significant reduction in \( \Delta F \) observed in S202C and R201C when the \( \alpha \) and \( \beta_2 \) subunits are coexpressed supports the hypothesis of a close interaction between \( \beta_2 \) extracellular loop and the upper S4 region (Table I). In general, the extracellular loops of BKCA \( \beta \) subunits seem long enough to interact with the pore region, e.g., to induce rectification of the ionic current (Zeng et al., 2003) or reduce toxin accessibility to the channel outer mouth (Meera et al., 2000). We speculate that the drastic reduction in \( \Delta F \) observed when \( \beta_2 \) is coexpressed is either due to a decreased upper S4 labeling efficiency (because of the steric hindrance of the long extracellular loop) or to a quenching effect on the fluorophores attached to residues S202C or R201C. Interestingly, the averaged \( \Delta F \) of 200C labeled with TMRM was not affected by \( \beta_2 \) coexpression (Table I), possibly because fluorophore accessibility to this position is not reduced by the \( \beta_2 \) subunits.

On the other hand, BKCA channel activation depends on the intrinsic closed to open equilibrium and is also allosterically coupled to voltage sensor activation and Ca\(^{2+}\) binding (Horrigan and Aldrich, 2002). Thus, it is possible that the \( \beta_2 \) subunit–induced facilitation of the voltage sensor activation arises from an indirect or allosteric effect.

**Figure 9.** The \( \beta_2 \)-induced inactivation process is not interfering with BKCA S4 segment return in the resting position. Representative K\(^+\) current traces from oocytes expressing the \( \alpha \) (A and E) and \( \alpha+\beta_2 \) subunit (S202C mutant) (C and G), elicited by 1-s depolarization from −160 to 80 mV, and repolarizations to −160 mV (A and C) and to −90 mV (E and G) (HP = −90 mV). The corresponding fluorescence traces and the best fits to a single exponential function of the OFF fluorescence are shown superimposed in B, D, F, and H. Note that the OFF fluorescence is well fitted to a single exponential function both in the presence and in the absence of \( \beta_2 \) subunits. (I) Time course of fluorescence return at different membrane potentials. (L) Time constants of current inactivation (○), best fitted to one exponential function from current peak to the end of the pulse (1-s pulses, as shown in Fig. 3 D), were compared with time constants of ON fluorescence (▲) and OFF fluorescence (■) (during repolarization to −160 mV) when \( \beta_2 \) is coexpressed, both best fitted to one exponential function. There is no correlation between the current inactivation kinetics and the fluorescence kinetics. Error bars represent SEM. When the bars are not visible, they are inside the symbols.
The coexpression with the modulatory BK<sub>Ca</sub> β subunits has been also shown to have kinetic effects on current activation and deactivation (Meera et al., 1996; Brenner et al., 2000; Cox and Aldrich, 2000; Cox and Aldrich, 2000; Orio and Latorre, 2005). We have observed a slowing of current activation kinetics of the cysteine mutants when the β<sub>2</sub> subunit was coexpressed (Fig. 3 F, Fig. 4 F, and Fig. 5 F). The analysis of fluorescence recordings has revealed a parallel slowdown of the fluorescence onset kinetics in the presence of the β<sub>2</sub> subunit (Fig. 3 F, Fig. 4 F, and Fig. 5 F), suggesting that the auxiliary subunit affects the overall kinetics of the voltage-sensing region, in turn affecting the rate of current activation.

In addition to the effects on the voltage dependence and kinetics of channel activation and S4 conformational changes, the β<sub>2</sub> subunit also confers a fast inactivation of the ionic current to BK Ca channels (Wallner et al., 1999), involving a mechanism similar to the one of N-type inactivation as in Na<sup>+</sup> (Armstrong and Bezanilla, 1977) and in K<sup>+</sup> (Shaker) channels (Bezanilla et al., 1991; Perozo et al., 1992; Roux et al., 1998). The immobilization of the voltage sensor is a signature of this process as the inactivating “ball,” once occupying the inner pore, impedes the return of the voltage sensor to its resting position (Bezanilla et al., 1991; Perozo et al., 1992; Roux et al., 1998). We have provided the first optical evidence that the inactivating particle in the N terminus of Shaker channels prevents the return of the S4 segments to their resting position in the inactivated channels. This conclusion could be derived from the appearance of a second, extremely slow, new component in the fluorescence return during repolarization when β<sub>2</sub> was coexpressed, suggesting an inactivating mechanism that is not the classically described N-type inactivation. The modest decrease in the time course of the OFF fluorescence during repolarization at −160, −120, and −90 mV observed in

![Figure 10](https://jgp.rupress.org)
α+β2 compared with α alone are compatible with the previously described effect of β2 subunits on the kinetics of BKCa activation and deactivation (Meera et al., 1996; Brenner et al., 2000; Oriol and Latorre, 2005). Supporting the view that β2-induced inactivation may not be the typical N type is also the finding that different intracellular blockers did not interfere with the inactivation process (Solaro et al., 1997), unlike that reported for Shaker channel (Choi et al., 1991). Although it would have been interesting to assess voltage sensor immobilization at positions other than 202, the OFF fluorescence kinetics for 200 and 201 mutants could not be reliably estimated due to the extremely small PyMPO fluorescence signal in the presence of the β2 subunit (R201C; Fig. 5 D) and the slow relaxation and fast bleaching rate of the TMRM fluorescence signals during long depolarizations (N200C) (Savalli et al., 2006).

There are both advantages and limitations in the fluorescence-based method we used to solve the mode of operation of the β2 subunit. Voltage clamp fluorometry has allowed us to directly track the S4 movements (for review see Tombola et al., 2006) and assess the properties of N inactivation without the use of pore blockers that could interfere with S4 movements and the inactivation mechanism. On the other hand, the hSlo BKCa channel needed to be engineered for fluorescence measurements (C-less+R207Q background). Therefore, although what we observed is consistent with previous studies, as for any mutagenesis-based study, these results should be ideally validated in the WT channel. Also, one of the assumptions of this approach is that the fluorescence reports the S4 movements, as the voltage dependence of the F(V) curve (always preceding the G(V) curve) suggests. Still, the possibility that other types of protein rearrangements can influence the fluorophore emission cannot be excluded. An alternative experimental strategy for this study could have been based on gating current measurements. However, limitations also apply to this approach; for example it requires the undesirable (as discussed above) use of K+ channel blockers to isolate the gating current. Additionally, it is uncertain that reliable information regarding S4 movement can be extracted from BKCa channel gating currents measurements since <50% of the total charge movement is contributed by the movement of the S4 segments (Ma and Horrigan, 2005).

In summary, the shift of the F(V) curves toward more negative potential upon β2 subunit coexpression supports the idea that the facilitation of BKCa channel activation by the β2 subunit is a result of a change in the equilibrium of the voltage sensor. We also provide evidence that the docking of the N terminus of the β2 subunit into the hSlo inner pore is not coupled to S4 segment immobilization. This is unlike the case of the Shaker channel and is consistent with the view that the BKCa channel inactivation process induced by the β2 subunit may not follow a typical N-type inactivation mechanism.

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