FEBS Letters 382 (1996) 84–88 FEBS 16765

A calcium switch for the functional coupling between α (*hslo*) and β subunits ($K_{V,Ca}\beta$) of maxi K channels

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Received 11 January 1996

Abstract $K_{V,Ca}\beta$ subunit dramatically increases the apparent calcium sensitivity of the α subunit of MaxiK channels when probed in the micromolar $[Ca^{2+}]_i$ range. Analysis in a wide range of $[Ca^{2+}]_i$ revealed that this functional coupling is exquisitely modulated by $[Ca^{2+}]_i$. Ca^{2+} ions switch MaxiK α+β complex into a functionally coupled state at concentrations beyond resting $[Ca^{2+}]_i$. At $[Ca^{2+}] \leq 100$ nM, MaxiK activity becomes independent of Ca^{2+}, is purely voltage-activated, and its functional coupling with its β subunit is released. The functional switch develops at $[Ca^{2+}]_i$ that occur during cellular excitation, providing the molecular basis of how MaxiK channels regulate smooth muscle excitability and neurotransmitter release.

Key words: MaxiK channel; Calcium-independent activation; Calcium-dependent activation; Human myometrium; Subunit interaction α–β

1. Introduction

Cellular excitability is determined by the precise activation, deactivation and inactivation of voltage-sensitive ion channels. MaxiK channels are unique because they are modulated not only by voltage, but also by calcium in the micromolar range. For this reason it has been proposed that they play a role in the repolarization phase of the action potential and in setting the pace of cell firing in neurons. This type of K channels is conformed by at least two subunits, the pore-forming α subunit [1] and a regulatory β subunit [2–4]. Unlike other voltage-dependent K channel β subunits ($K_v\beta$), which are cytoplasmic [5], the β subunit of the MaxiK channel ($K_{v,Ca}\beta$) has two putative transmembrane regions [2] similar to the TipE *para* sodium channel subunit [6]. We now demonstrate that intracellular calcium controls the functional coupling between β and α subunits of the MaxiK channel complex, in a concentration range relevant to cellular excitation. Further, at low $[Ca^{2+}]_i$ (≤ 100 nM) MaxiK channels become Ca-independent and purely voltage gated.

2. Materials and methods

The human β subunit was cloned by homology using a cDNA library from human myometrium and the bovine β subunit [2] as probe. Human myometrial α and β subunits (GenBank accession numbers: U11058 and U25138) cRNAs were injected into collagenase treated *Xenopus laevis* oocytes. α and β subunits were usually injected at 1:2 molar ratio to ensure that all α subunits were interacting with β subunits. Recordings were made using the patch clamp technique 2–4 days after injection. The pipettes had 1–3 MΩ resistance and were filled with (mM): 105 potassium methanesulfonate (KMES), 5 KCl, 10 HEPES, pH 7.0. The composition of the bath solution (intracellular membrane face in inside-out patches) was (mM): 105 KMES, 5 KCl, 10 HEPES pH 7.0. $CaCl_2$ and $MgCl_2$ were added to the desired pCa and pMg 3. Solutions from 10 nM to 3 μM free Ca^{2+} were buffered with 5 mM HEDTA, calculated according to Fabiato and checked with either a Ca^{2+} electrode (World Precision Instruments, Sarasota, FL) or with Fura-2. Solutions beyond 3 μM were made without a Ca^{2+} buffer; free Ca^{2+} was measured with a Ca^{2+} electrode. Solutions in the picomolar $[Ca^{2+}]_i$ range were calculated according to Fabiato [7] and were obtained using isotonic EDTA (70 mM, 3 pM free Ca^{2+}), isotonic EGTA (70 mM, 20 pM free Ca^{2+}) or 10 mM EGTA (141 pM free Ca^{2+}), 10 mM HEDTA (580 pM free Ca^{2+}).

2.1. Data analysis

To estimate $V_{1/2}$ values (from FP_o vs. voltage curves) at $[Ca^{2+}]_i$ in the pM to 300 nM range, maximum conductances were obtained at high $[Ca^{2+}]_i$ (≥ 10 μM). The latter were then used to fit the experimental data to Boltzmann distributions and estimate the $V_{1/2}$ value. Since the maximum conductance is dependent on the number of channels (N) active in the patch, we collected I-V curves at low $[Ca^{2+}]_i$, followed by high $[Ca^{2+}]_i$ and returning to low $[Ca^{2+}]_i$ ('bracketing') to avoid errors caused by rundown. Data are expressed as mean values ± SD.

3. Results and discussion

To determine whether $[Ca^{2+}]_i$ is involved in the coupling of α and β subunits, we have coexpressed both subunits in *X. laevis* oocytes, and studied their function from the pM to the mM $[Ca^{2+}]_i$ range. We have previously shown that *hslo* is modulated by a bovine $K_{v,Ca}\beta$ subunit in experiments using micromolar $[Ca^{2+}]_i$ [4]. Fig. 1A shows the sequence alignment of the human (used in this work) and bovine β subunits indicating a high degree of homology (84% identity). As expected from this homology, the human β subunit was able to upregulate the activity of the human MaxiK channel α pore-forming subunit, *hslo*. Fig. 1B–E illustrates a typical experiment comparing currents induced by expression of human α vs. α+β subunits at 10 μM Ca^{2+}. Consecutive pulses from −199 mV, every 6 or 8 mV, were delivered from a holding potential of 0 mV. A large difference between steady-state currents in α vs. α + β injected oocytes is evident at negative potentials (Fig. 1B,D). The current vs. voltage (I-V) and fractional open probability vs. voltage (FP_o-V) curves (Fig. 1C,E) quantitate this finding and demonstrate that co-expression of the β subunit makes *hslo* respond much more efficiently to voltage. This change in efficacy is manifested as a diminution in the voltage necessary to half activate the channel ($V_{1/2}$). In this experiment, $V_{1/2}$ changed from 18 mV (α) to −77 mV (α+β), a 95 mV negative shift in the voltage-activation curve. The shift in $V_{1/2}$ is also expressed by a shift in the apparent $K_{1/2}$ for calcium. For example, at −60 mV near the resting potential of many cells, the apparent $K_{1/2}$ for calcium is in-

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SSDI S0014-5793(96)00151-2

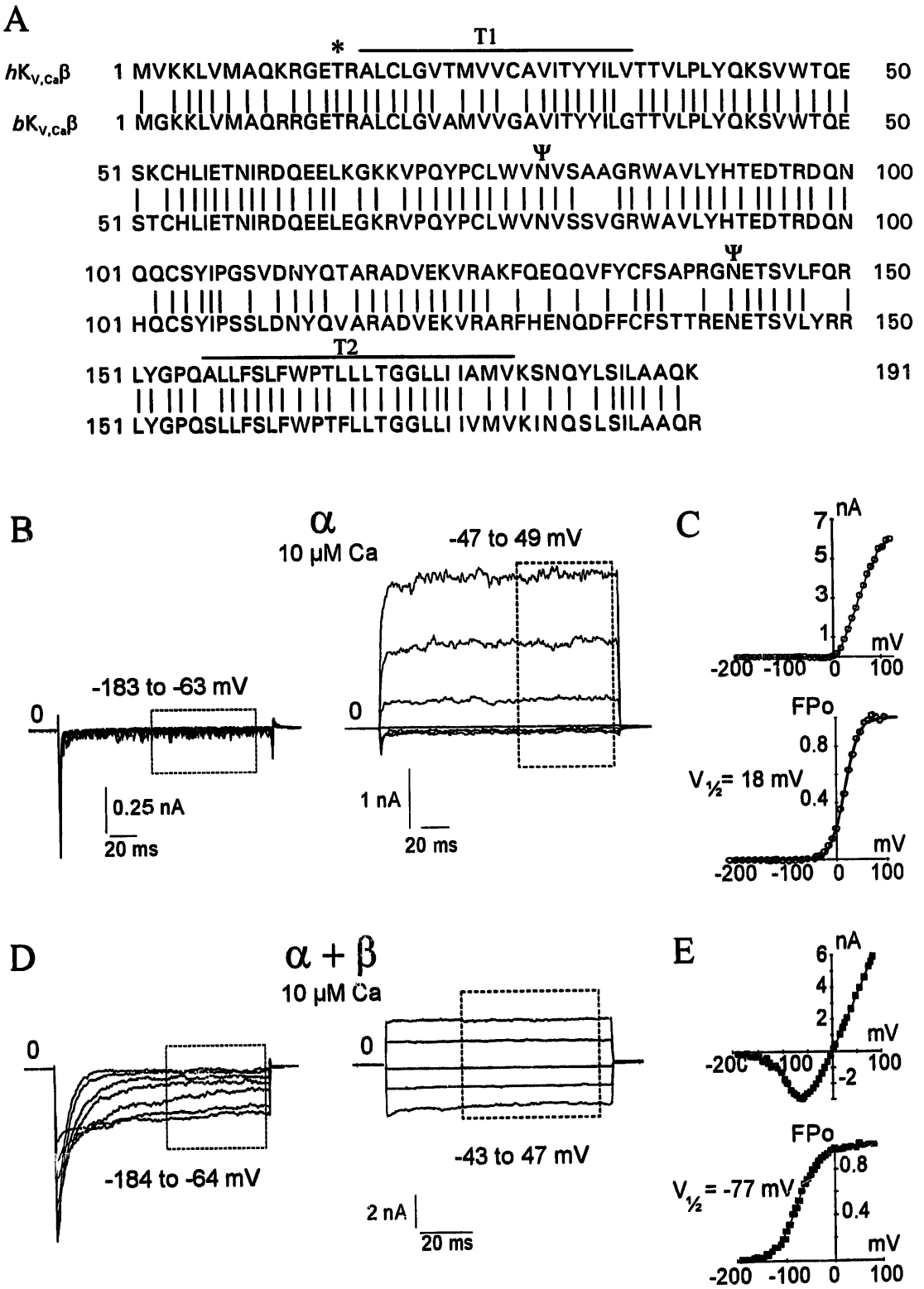

Fig. 1. *hSlo* activation by its β subunit in micromolar Ca^{2+}. (A) Sequence alignment of human and bovine $K_{V,Ca}β$ subunits. (ψ) Consensus sequence for N-linked glycosylation. (*) Consensus sequence for PKA-phosphorylation. (T1,T2) Putative transmembrane domains. (B,D) Ionic currents in inside-out patches of *Xenopus laevis* oocytes expressing α or α + β subunits. Holding potential (V_h) = 0 mV (reversal potential for K). $[Ca^{2+}]_i$ = 10 μM. Mean steady-state currents measured near to the end of pulses (dashed lines) were used to construct current to voltage (*I-V*) relationships. Inward transient currents recorded at the beginning of negative pulses reflect the time course of channel closure of the fraction of channels that were open at V_h. (C,E) *I-V* curves and corresponding FP_o-*V* plots. FP_o-*V* curves were obtained by normalizing *G-V* curves with respect to the maximum conductance and fitting to a Boltzmann distribution (continuous lines correspond to the best fit). At 10 μM $[Ca^{2+}]_i$, $V_{1/2}$ was 12.5 ± 18 mV (n = 64) for α subunit alone and −77 + 10 mV (n = 14, ± SD) when α + β subunits were co-expressed.

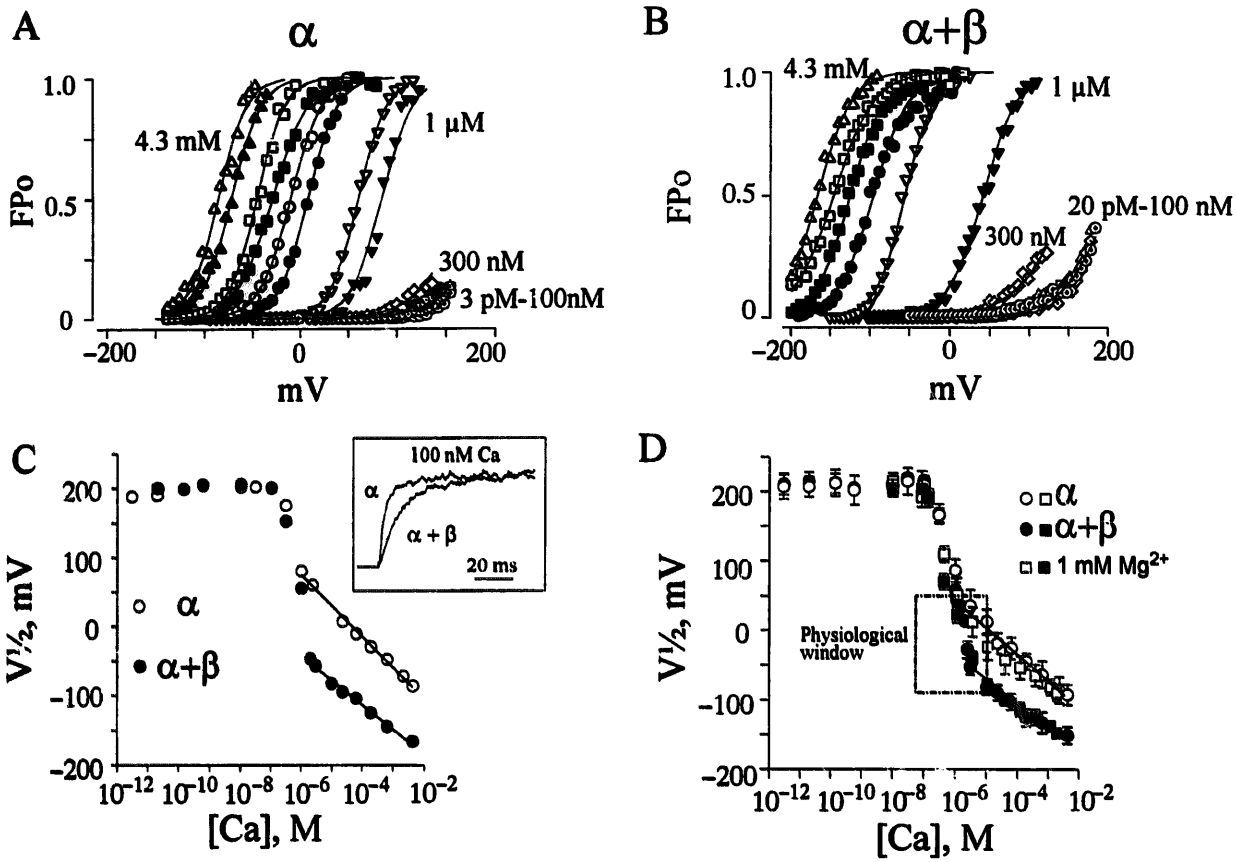

Fig. 2. Ca^{2+} switch in *hSlo*: functional coupling of α and β subunits, and from Ca-independent to Ca^{2+}-dependent mode. (A,B) FP_o as a function of voltage at different $[Ca^{2+}]_i$ in single inside-out patches. In A (α): 4.3 mM, △; 2.2 mM, ▲; 647 μM, □; 194 μM, ■; 64 μM, ○; 23 μM, ●; 2.3 μM, ▽; 1 μM, ▼; 300 nM, ◇; 100 nM, ◆; 30 nM, ▽ and · ; 10 nM, □ and · ; 20 pM, △ and · ; 3 pM, ⊕. In B (α+β), symbols are as in A, except 141 pM, ◇ and · ; and 580 pM, ⊕; symbols for 100 nM, 10 nM, and 20 pM are superimposed. Continuous lines are the best fit to Boltzmann distributions. (C) $V_{1/2}$ vs. $[Ca^{2+}]_i$ plot from A and B. (Inset) Normalized currents in inside-out patches. $[Ca^{2+}]_i = 100$ nM. $\tau_\alpha = 4$ ms, $\tau_{\alpha+\beta} = 12$ ms. $V_h = 0$ mV; pulse to 134 mV. (D) Mean $V_{1/2}$ values vs. $[Ca^{2+}]_i$. Bars, S.D.; number of experiments = 3–62. Two processes are switched ON when $[Ca^{2+}]_i$ is larger than 100 nM. One is the Ca^{2+} dependency of the MaxiK channel activity, and the other is the functional coupling with its β subunit. Box delimits predicted physiological ranges.

creased 300 fold from 1.2 mM to 4 μM, bringing the Ca sensitivity of MaxiK channels to the physiological range.

It came to our attention, however, that expressed currents recorded in the cell-attached mode, where $[Ca^{2+}]_i$ is in the nM range, showed no difference in $V_{1/2}$ when coinjected with the β subunit. The estimated $V_{1/2}$ value (see section 2) was 221 ± 19 mV ($n = 6$) when the α pore-forming subunit alone was expressed compared to 215 ± 13 mV ($n = 5$) when both α and β subunits were co-injected[1]. When the same patches were excised and exposed to 10 μM $[Ca^{2+}]_i$, a huge increase in current and a prominent shift in $V_{1/2}$ were observed as illustrated in Fig. 1. These experiments show that α and β subunits were functionally uncoupled at 'resting' calcium concentrations.

Although there is no change in open probability of the channel at resting calcium in the presence of the β subunit, the activation kinetics of the currents is altered (see inset in Fig. 2C). For example, in the cell-attached mode, the τ of activation for α-induced currents at 130 mV is 3 ± 0.3 ms ($n = 8$), compared to 14±4 ms ($n = 4$) when α+β subunits are co-expressed. Similar values were obtained at 100 nM Ca^{2+} ($n = 9$). The simplest explanation to this finding is that even though there is no effect of the β subunit at low $[Ca^{2+}]_i$ on the steady-state current, a physical interaction does exist between both α and β subunits, which is not Ca^{2+}-dependent. This interpretation is consistent with the tight association of both subunits demonstrated during the biochemical purification of the MaxiK channel [8].

Fig. 2A–D demonstrate unequivocally that the functional coupling of α and $K_{v,Ca}\beta$ subunits is $[Ca^{2+}]_i$-dependent. Fig. 2A and B are examples of voltage-activation curves of α and α+β induced currents, respectively, in single inside-out patches at different $[Ca^{2+}]_i$. Note that in both cases a parallel shift along the voltage axis is attained without a change in slope, suggesting that the β subunit does not modify the ef-

[1]McManus et al. [3] using the bovine β subunit and *mslo*19 observed an increase in activity in α+β injected oocytes with the two-electrode voltage clamp technique that should be equivalent to the cell-attached configuration of our experiments. This different behavior may be due to the differences in the clones used or due to a higher $[Ca^{2+}]_i$ in those oocytes. In our experiments using *hslo*, either with the bovine or the human β subunit, equivalent $V_{1/2}$ values were obtained. For example, at 100 nM $[Ca^{2+}]_i$ $V_{1/2}$ was 209 ± 12 mV ($n = 4$) when the bovine β subunit was used and 216 ± 6 mV ($n = 3$) when the human β subunit was expressed. Similarly, at 10 nM $[Ca^{2+}]_i$ $V_{1/2}$ was 211 ± 14 ($n = 4$) for the bovine β subunit and 210 ± 6 ($n = 5$) for the human β subunit.

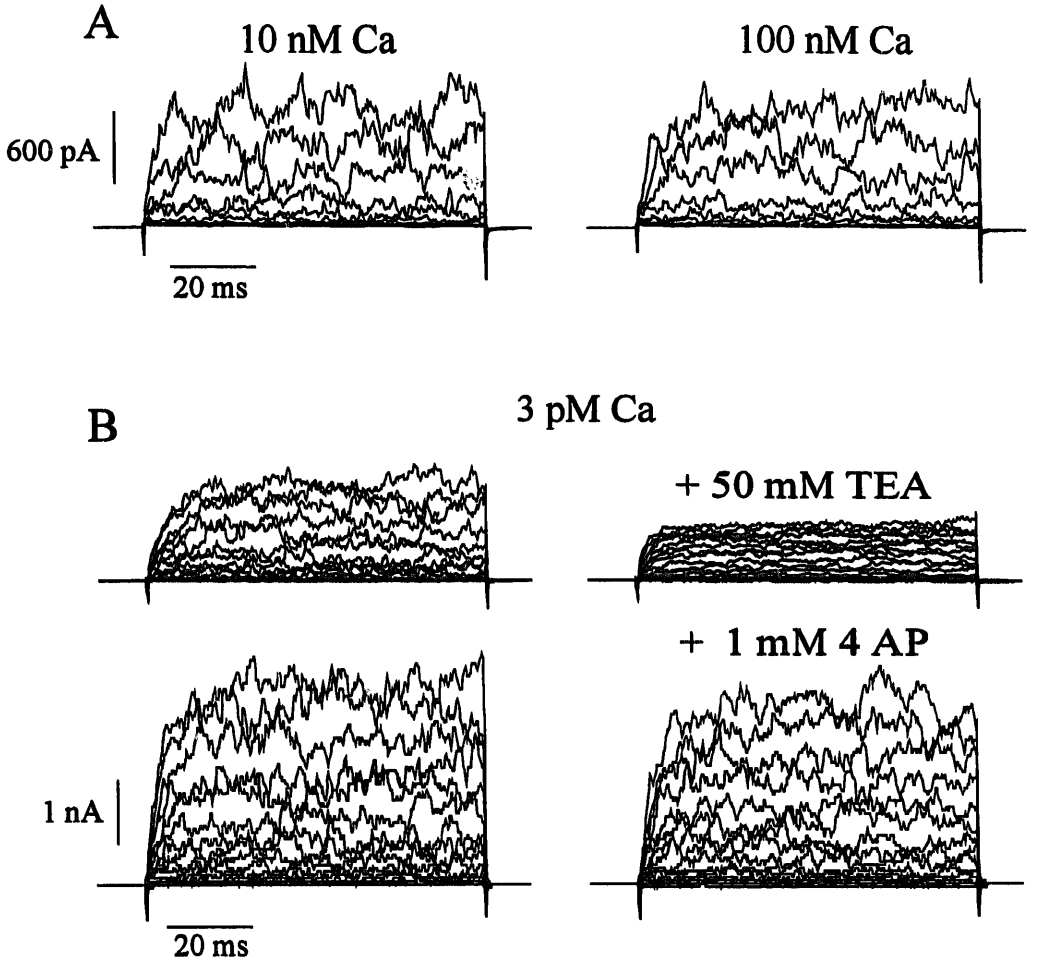

Fig. 3. Ca^{2+}-independent mode of gating of MaxiK α subunit. (A) Currents from the same patch are equivalent at 10 and 100 nM free $[Ca^{2+}]_i$. $V_h = 0$ mV. Pulses from 34 to 142 mV every 12 mV. (B) Pharmacology of *hslo* currents at low $[Ca^{2+}]$ (3 pM). Inside-out patches from different oocytes. $V_h = 0$ mV. Pulses, −50 to 154 mV (top); −100 to 164 mV (bottom). Internal TEA (50 mM) reduced the current by half; 1 mM 4-AP did not have a significant effect. Similar results were obtained in α+β induced currents.

fective valence (zδ) of the voltage gating process of the α subunit. By plotting $V_{1/2}$ values obtained from curves A and B as a function of $[Ca^{2+}]_i$ (e.g. Fig. 2C), it is evident that at low $[Ca^{2+}]_i$ (≤ 100 nM) $V_{1/2}$ values are equal and independent of the presence of the $K_{v,Ca}\beta$ subunit. The functional coupling, reflected as a change in $V_{1/2}$, is sharply developed between 300 nM and 2 μM Ca^{2+} and is fully attained at concentrations ≥ 3 μM $[Ca^{2+}]_i$ (mean values are in Fig. 2D). These results demonstrate that a minimum $[Ca^{2+}]_i$ is required to switch α and β subunits to a functional activated mode. A rise in local $[Ca^{2+}]_i$ may induce a conformational change, either in the α and/or β subunits, that triggers the functional coupling between both of them, making the MaxiK channel α subunit respond much more efficiently to Ca^{2+} and voltage. During local Ca^{2+} elevations from a resting $[Ca^{2+}]_i$ of ≈ 50–100 nM to the micromolar range, which is likely to occur during Ca^{2+} spikes in arterial smooth muscle [9] or during transmitter release [10], MaxiK channels composed of α and β subunits would switch from a practically unresponsive mode to a highly responsive mode at physiological voltages. This would be consistent with the view that MaxiK channels are a powerful feedback mechanism that repolarizes the cell membrane.

Magnesium is known to increase the cooperativity of Ca^{2+} binding responsible for modulating the MaxiK channel [11]. However, this divalent ion was unable to substitute calcium in the functional coupling of the α and β subunits, suggesting that Ca^{2+} binds to a different site than Mg^{2+}in this coupling process. Fig. 2D illustrates that in the presence of 1 mM Mg^{2+} (squares), α and β subunits require about the same concentration of calcium to be functionally coupled.

MaxiK channels were thought to be calcium-and voltage-activated [12,13], in essence they would never open in the virtual absence of Ca^{2+}(lower than 100 nM). However, Figs. 2 and 3 show that the MaxiK channel α subunit indeed opens at low $[Ca^{2+}]_i$. Furthermore, they demonstrate that *hslo* becomes *independent* of $[Ca^{2+}]_i$ at concentrations lower than 100 nM, turning into a purely voltage-gated mode. In Fig. 2D, $V_{1/2}$ values remained the same from 3 pM to about 100 nM $[Ca]_i$. This behavior is illustrated in Fig. 3A where a 10 fold change in $[Ca^{2+}]_i$ from 10 to 100 nM did not induce any change in *hslo* currents. To demonstrate that currents measured at low Ca^{2+} (≤ 100 nM) correspond to *hslo* currents, we analyzed their sensitivity to tetraethylammonium (TEA) or 4-aminopyridine (4-AP) in inside-out patches. As expected for *hslo* activity [4], currents were half blocked by 50 mM internal TEA, and were unaffected by 1 mM 4-AP (Fig. 3B).

These results reveal that the MaxiK channel has two modes

of gating, one being Ca^{2+}-independent and purely voltage-gated and another mode that is Ca^{2+}-and voltage-dependent. Therefore, in a kinetic model (to be published elsewhere) the channel should have at least one closed (C) to open (O), C⇔O, transition that is Ca^{2+}-independent. The fact that the β subunit is unable to modulate the channel at low $[Ca^{2+}]_i$ where *hslo* is purely voltage-gated, indicates that the β subunit must favor an open state of a $C \overset{Ca^{2+},V}{\rightleftharpoons} O$ Ca^{2+}-and voltage-dependent transition. In comparison, in Ca^{2+} channels the α pore-forming subunit is modulated by its β subunit by favoring the final C⇔O transition and not affecting previous deeper C⇔C transitions [14]. It is possible that a general mechanism of modulation of ion channel's α subunits by their respective β subunits is to modulate the final C to O transitions without affecting voltage-gated conformational changes occurring prior to channel opening.

In conclusion, our results provide evidence for a Ca^{2+} mechanism that switches the MaxiK channel α subunit from a Ca^{2+}-independent to a Ca^{2+}-dependent mode and from a β subunit dull interaction to a β subunit activated mode. Since the functional switch develops at $[Ca^{2+}]_i$ that occur during cellular excitation (> 100 nM), it is likely that in neurons or other excitable tissues like vascular smooth muscle, K_{Ca} channels become highly active providing a very effective shutoff hyperpolarizing mechanism for repolarization.

Acknowledgements: We thank E. Stefani and R. Latorre for helpful discussions. Supported by NIH grant HL54970 to L.T.

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