REVIEW

Large conductance Ca\(^{2+}\)-activated K\(^+\) (BK) channel: Activation by Ca\(^{2+}\) and voltage

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ABSTRACT

Large conductance Ca\(^{2+}\)-activated K\(^+\) (BK) channels belong to the S4 superfamily of K\(^+\) channels that include voltage-dependent K\(^+\) (Kv) channels characterized by having six (S1-S6) transmembrane domains and a positively charged S4 domain. As Kv channels, BK channels contain a S4 domain, but they have an extra (S0) transmembrane domain that leads to an external NH\(_2\)-terminus. The BK channel is activated by internal Ca\(^{2+}\), and using chimeric channels and mutagenesis, three distinct Ca\(^{2+}\)-dependent regulatory mechanisms with different divalent cation selectivity have been identified in its large COOH-terminus. Two of these putative Ca\(^{2+}\)-binding domains activate the BK channel when cytoplasmic Ca\(^{2+}\) reaches micromolar concentrations, and a low Ca\(^{2+}\) affinity mechanism may be involved in the physiological regulation by Mg\(^{2+}\). The presence in the BK channel of multiple Ca\(^{2+}\)-binding sites explains the huge Ca\(^{2+}\) concentration range (0.1 \(\mu\)M-100 \(\mu\)M) in which the divalent cation influences channel gating. BK channels are also voltage-dependent, and all the experimental evidence points toward the S4 domain as the domain in charge of sensing the voltage. Calcium can open BK channels when all the voltage sensors are in their resting configuration, and voltage is able to activate channels in the complete absence of Ca\(^{2+}\). Therefore, Ca\(^{2+}\) and voltage act independently to enhance channel opening, and this behavior can be explained using a two-tiered allosteric gating mechanism.

Key terms: BK channel, Ca\(^{2+}\)-binding sites, voltage dependence, allosteric models.

PROLOGUE: ON SQUIDS AND THE AMAZING MONTEMAR LABORATORY

If you ask me (RL) why the laboratory in Montemar was so important in the development of biophysics in Chile, I would immediately say: because the seniors were doing good science, and they let the juniors do whatever they wanted. In this regard, our professors made it clear from the onset of our research work that we were supposed to take one idea from the many floating around in the lab in those years and develop the experimental and theoretical framework in an absolutely independent manner. Freedom is a terrible thing when one is young and insecure, and learning to be on my own was the first thing I was forced to master in Montemar. Our teachers were wise enough and brave enough to give us sufficient intellectual ammo to make us scientifically independent. In other words, all the scientists at Montemar followed the Peter Medawar’s dictum to the young scientist apprentice: choose an important problem and become apprenticed to a senior scientist. I fully agree with Perutz when he states that “creativity in science, as in the arts, cannot be organized. It arises spontaneously from individual talent”. Well-run laboratories, as was the case of Montemar, can foster talent, but hierarchical organizations, inflexible bureaucratic rules that plague our universities, and mountains of futile paperwork can kill it, as is usually the case in Chile.

The next thing I learned during my Ph.D. years at Montemar was that biology is above all an experimental field and that no theory is as valuable as a well-done experiment. Actually, many experiments were needed, and the squid season was short, so you were
forced to work hard and efficiently and even built your own equipment when necessary (voltage clamps were not commercially available at the time!). The whole lab environment forced you to realize that “experimentation is a form of thinking and that a wrong interpretation of an experiment is forgivable, but an unrepeatable experimental result is not” (Medawar, 1979).

Paul Ehrlich, the father of immunology, used to say that scientists need the four Gs: Geschick, Geduld, Geld, und Glück (skill, patience, money, and luck). Science at Montemar was undertaken with considerable skill, patience and luck but very little money; our advantage over the rich laboratories of the United States was the squid (Dosidicus gigas). Biophysics first became a Chilean specialty because “that’s where the squids were” as Chris Miller stated in an interview that appeared in Science in 1995. Using the squid giant axon, Mario Luxoro and his pupil Eduardo Rojas (“Guayo” to his colleagues and students) were the first to claim that proteins were involved in the electrical excitability of nerve (Rojas and Luxoro, 1963). The axons of this monster, about one millimeter in diameter, were, during the 1960s, an attraction difficult to resist for scientists interested in nerve excitability. And this is another important reason why the Montemar laboratory was so successful. Every summer, we were visited by scientists of the stature of Clay Armstrong, Ichigi Tasaki, Bob Taylor, and Gerry Ehrenstein. Clay, Gerry, and Guayo were my first scientific idols, Clay because of his amazing wit, Gerry because of the economy and precision of his thinking, and Guayo because he was able to play Beethoven with a voltage-clamp set-up. Clay, Gerry and Guayo were the first to introduce me to the amazing field of ion channels. Thanks to the squid, Montemar was a window to the world, and Cecilia Hidalgo, Pancho Bezanilla, and I had the opportunity (Glück) of doing postdocs in very good laboratories in the U.S. Thus, the “little ones” also learned at Montemar that if you have a unique biological preparation that enables you to understand a fundamental biological process, you can use this knowledge to your advantage in order to overcome poverty.

In Montemar, I realized that science is not a quiet life. If you put together two Italian descendants (Mario and Mitzy) in the same space, you get an explosive mixture. Discussions about science, lab space, or politics were frequently heated and always won by Fernando Vargas because during the course of a debate he never lost his temper, and his logic was unbeatable. So, there was another lesson for us: scientists are generally passionate people who defend their territories and pet ideas as ferociously as bears. Above all, however, our teachers taught us that in Montemar the authority principle did not exist. If you were wrong, somebody was there to show you that you were mistaken, whether you were a graduate student, Luxoro, or the Pope himself. One of his characters in Fred Hoyle’s novel the Black Cloud remarks that scientists are always wrong, yet they always go on. What makes them to continue? I think it is something that was always latent in Montemar: the passion for solving problems aesthetically.

Eduardo Rojas deserves a special space in these remembrances. Guayo was fundamental in my life as a scientist. Due to the political situation in the country during the late 1960s, most of our professors left Montemar to take different posts at the Universidad de Chile, and Cecilia and I were left orphaned of advisers. During the summer of 1969, Guayo adopted us, and thanks to his support (emotional and scientific), we were able to finish our Ph.D. theses. His generosity and kindness made possible for me to be telling you my part of the Montemar adventure.

INTRODUCTION

When Mario Luxoro asked me (RL) to write a chapter in this issue of *Biological Research* dedicated to my teacher, colleague, and friend, Eduardo Rojas, I immediately thought that the best present for him would be to say something about the ion channel that is closest to my heart.
existence in a membrane preparation from muscle T-tubule (Latorre et al., 1982), we were confronted with a molecular Pandora’s box: once opened, its electrical language left all of us bewitched. Others (e.g., Marty, 1981; Pallota et al., 1981) were as fascinated as we were with this “monster” of a single-channel conductance closed to the ceiling imposed by simple diffusion combined with an exquisite K+ selectivity. BK channels essentially are impermeant to Na+ and conduct K+ 10- and 200-fold more effectively than Rb+ and Cs+, respectively (large conductance channels were not supposed to be so selective!) (Blatz and Magleby, 1984; Eisenman et al., 1986; Stefani et al., 1997). At the same time, the channel was activated by voltage and cytoplasmic Ca2+. This latter property led Meech (1978) to hypothesize that this conductance system was perfect link between cell metabolism and electrical activity, and he was right on the mark. Because of its large conductance, this voltage and calcium-activated K+ channel was christened “maxi K” (Latorre and Miller, 1983) or “BK” (for big K+; Blatz and Magleby, 1987).

Muscle contraction, neurosecretion, chromaffin cell electrical activity, and hair cell tuning are some of the key physiological processes that require an increase in cytoplasmic Ca2+ to develop. Most often, this Ca2+ increase is mediated by the Ca2+ entry into the cells through voltage-dependent Ca2+ channels (VDCCs). The increase in internal Ca2+, however, also puts into action a negative feedback that will serve to stop or to dampen excitatory phenomena induced by the opening of VDCCs. This negative feedback appears as a consequence of the activation of BK (about 250 pS in 100 mM symmetrical KCl; Marty, 1981; Pallota et al., 1981; Latorre et al., 1982; for reviews see Latorre et al., 1989; McManus, 1991). Thus, BK channels have the largest single-channel conductance of all K+ selective channels. To ensure maximum efficiency of the negative feedback, BK channels functionally co-localize with VDCCs (Marrion and Tavalin, 1998; Prakriya and Lingle, 1999). Despite being coded by a single gene (Slowpoke), the diversity of BK channels is great. Regulatory β-subunits, splicing, and metabolic regulation create this diversity fundamental to the adequate function of many tissues (Vergara et al., 1998; Latorre et al., 2000; Orio et al., 2002) In a bird’s-eye view of this fantastic molecular machine, we will discuss its molecular properties and how these properties determine the BK channel opening and closing.

GROSS BK CHANNEL STRUCTURE AND THE Ca2+-SENSING ELEMENTS

The cloning of the BK channel from Drosophila (Atkinson et al., 1991; Adelman et al., 1992) showed that the BK channel was a member of the S4 superfamily encompassing voltage-dependent K+ (Kv), Na+ and Ca2+ channels. The gene coding for BK was called Slowpoke or Slo and, after the cloning and expression of Slo2 (Yuan et al., 2000; Yuan et al., 2003) and Slo3 (Schreiber et al., 1998), was renamed Slo1. In the case of Kv channels, the channel-forming protein possesses six transmembrane domains (S1-S6) containing the pore-forming domain S5-P-S6 and an S4 voltage-sensing element. Like Kv channels, the BK channel is a tetramer (Shen et al., 1994); unlike Kv channels, however, the BK channel protein consists of seven transmembrane domains (S0-S6) that lead to an exoplasmic N-terminus (Fig. 1; Meera et al., 1997; Wallner et al., 1996; Toro et al., 1998). The large C-terminus (containing about twice as many amino acids as S0-S6) has four hydrophobic domains (S7-S10), and Salkoff’s group identified two molecular domains (S0-S8, “core” and S9-S10, “tail”) that, when expressed together, were able to produce functional channels (Wei et al., 1994).

SEARCHING FOR THE HIGH AFFINITY Ca2+-BINDING SITES

Taking advantage of the fact that the Drosophila Slo (dSlo1) channel has a different Ca2+ sensitivity than the mouse
Figure 1. Schematic diagram of the α (black) and β (gray) subunit of BK channels. A. D362/D367, M513 and the calcium bowl define sites that when mutated decrease the high-affinity Ca\(^{2+}\) sensitivity of the BK channel. E374/E377 is a site that when mutated decreases the low affinity Ca\(^{2+}\) and Mg\(^{2+}\) sensitivity. The first two sites are located in the RCK domain. The RCK domain is defined as the amino acid stretch comprised between the C-terminus of S6 and the C-terminus of S7. B. BK channels are tetramers. C. Primary sequence of the Ca\(^{2+}\) bowl aligned with the corresponding partial sequence of the C-terminus in Slo3 and the mutant D5N5.
Slo (mSlo1) channel, Wei et al. (1994) showed that coexpression of mSlo core together with dSlo tail produced channels with a Ca$^{2+}$ sensitivity similar to that of the dSlo channel. These experiments provided the first indication that the tail was playing the role of the calcium-sensing element. Closer inspection of the tail showed the presence of a domain consisting of 28 amino acids containing nine acidic residues including a string of five conserved aspartates residues, the “calcium bowl” (Fig. 1A, C). Partial deletion or point mutations of the aspartates contained in the calcium bowl produced BK channel that were less sensitive to Ca$^{2+}$ at the same Ca$^{2+}$ concentration, the calcium bowl mutant conductance-voltage (G-V) curves were right-shifted compared to the wild-type BK channel G-V curve (Schreiber and Salkoff, 1997; Bian et al., 2001; Braun and Sy, 2001). If the Ca$^{2+}$ bowl is disrupted by deleting crucial aspartates, this high Ca$^{2+}$ affinity regulatory site is lost, but the mutant and the wild-type BK showed the same sensitivity to Cd$^{2+}$ ions. This finding provided evidence of the presence of two different Ca$^{2+}$-binding sites; a site sensitive to Ca$^{2+}$ but not to Cd$^{2+}$ and another site able to bind with similar affinity Ca$^{2+}$ and Cd$^{2+}$ (Schreiber and Salkoff, 1997). Clearly, it would be difficult to explain the observation that BK activation is sensitive to a range of Ca$^{2+}$ concentrations that spans over four orders of magnitude on the basis of a single Ca$^{2+}$-binding site (see Fig. 6). The Slo3 channel lacks the calcium bowl (Fig. 1C) and is not sensitive to Ca$^{2+}$. Coexpression of mSlo1 core and mSlo3 tail gave origin to Ca$^{2+}$-insensitive channels in the range comprised between 0-10 μM internal Ca$^{2+}$, but the data presented indicated that the chimeric Slo channels were activated by Ca$^{2+}$ at concentrations ≥300 μM (Schreiber et al., 1999). Interestingly, mSlo1 core-mSlo3 tail channels opened at much lower voltages than mSlo1 at zero internal Ca$^{2+}$ (conductance-voltage curves for the chimeric channel were shifted by about 60 mV to the left along the voltage axis). These results suggest the presence of a Ca$^{2+}$-binding site different from the Ca$^{2+}$ bowl and that, in the absence of Ca$^{2+}$, the Slo1 tail inhibits voltage-dependent BK channel gating, inhibition that is relieved by Ca$^{2+}$. This inhibition may help to keep the channel closed in the resting cell (low internal Ca$^{2+}$ and negative membrane potential). A detailed single-channel analysis confirmed the results of Schreiber et al. (1999) and suggested that in addition to playing a role as a Ca$^{2+}$ sensing domain, the tail domain also modulates the gating and conductance properties of BK channels (Moss and Magleby, 2001).

In all the experiments described above, Ca$^{2+}$-binding sites were inferred from the effects of Ca$^{2+}$ on channel activation (i.e., changes in the BK open probability). Bian et al. (2001) measured direct binding of radioactive calcium to a COOH-terminus fragment of the Drosophila BK containing the calcium bowl and showed that mutating the aspartates to asparagines (D5N5 mutant; Fig. 1C) was able to reduce the Ca$^{2+}$-binding affinity by about 60%. This result demonstrates a direct correlation between Ca$^{2+}$ binding and Ca$^{2+}$ sensitivity for channel activation (see also Braun and Sy, 2001). The D5N5 mutant expressed in cells exhibits a lower Ca$^{2+}$ sensitivity for activation and reduces the dSlo channel’s Hill coefficient 2-fold, as if one binding site per monomer is lost in the D5N5 mutant. The most economic way to account for these results is to assume the existence of two distinct Ca$^{2+}$-binding sites per channel monomer (Bian et al., 2001; cf. Schreiber and Salkoff, 1997). Bao et al. (2004) explored the relative importance of the different acidic amino acids contained in the calcium bowl using alanine scanning mutagenesis of 20 residues contained in this domain. These experiments were done in the background mutant, M513I; a mutant that eliminates one of the high Ca$^{2+}$sensitivity regulatory sites (see below) allowing determining the isolated Ca$^{2+}$-binding properties of the calcium bowl. They found that mutation to alanines of two critical aspartates (D898 and D900; Fig. 1C) has the greatest effect on the change in the G(V) curve half voltage (ΔV$\text{0.5}$) upon addition of Ca$^{2+}$. The point mutations also were able to decrease binding of $^{45}$Ca by
about 50% to a peptide composed of a fusion protein consisting of glutathione-S-transferase and a 207-amino acid part of the mSlo1 tail that includes the calcium bowl. These results indicate that there are “hot” residues in the calcium bowl and gave further support to the hypothesis that this domain is the one of the high affinity sites that couples Ca$^{2+}$ binding to channel opening. (1)

The molecular origin of the remaining BK Ca$^{2+}$ sensitivity after neutralization of most of the acidic residues present in the Ca$^{2+}$ bowl was elucidated by mutating negatively charged residues outside the Ca$^{2+}$ bowl and, in particular, in the regulator of conductance for K$^+$ (RCK; Jiang et al., 2001; Fig. 1-3). The RCK domain in the BK channel was unveiled by MacKinnon’s group (Jiang et al., 2001) by multiple sequence alignment of the BK channel with prokaryotic K$^+$ channels and other proteins known to possess the RCK domain structure. The structure of the RCK domain of a six transmembrane domain K$^+$ channel from E. coli (Fig. 2A) solved at 2.4 Å resolution has a Rossmann-fold topology, a very common structural motif found in enzymes and ligand-binding proteins. Rossmann-fold secondary structures are organized into two linked $\beta-\alpha-\beta-\alpha-\beta$ units (see Figs. 2B and 3A) and were first identified in a number of NAD$^+$-dependent dehydrogenases (Darby and Creighton, 1993).

The hypothesis that BK channels contain a RCK domain was put to test by disturbing a well-conserved salt bridge, not commonly present among Rossmann-fold proteins (K448-D481 in hSlo1; Fig. 3B). If either position is mutated (e.g., K448D or D481K), the channel becomes less Ca$^{2+}$ sensitive. However, the double mutant K448D/D481K recovers the Ca-sensitivity shown by the wild-type BK channel. Therefore, these results strongly suggest the presence of a salt bridge as predicted from the E.coli K$^+$ channel RCK structure and support the hypothesis that the BK channel contains an RCK domain on its C-terminus.

The double mSlo1 RCK mutant D362A/D367A (Fig. 3C) produced a marked reduction in the Ca$^{2+}$ sensibility of the BK channel, and the mutant D362A/D367A-D5N is still gated by voltage, with a voltage dependence similar to that of the wild type, but Ca$^{2+}$ is unable to activate the channel at [Ca$^{2+}$] < 10$^{-3}$ M (Xia et al., 2002). Another mutation, M513I, a residue immediately following the S7 domain (Figs. 2B and 3B), together with a deletion mutant that eliminates most of the aspartates contained in the calcium bowl (D896-903), similar to the D362A/D367A-D5N mutant, was shown to completely remove high affinity responsiveness to Ca$^{2+}$ (Bao et al., 2002). The behavior of the M513I and D896-903 mutants was analyzed in terms of allosteric models (Cox and Aldrich, 2000; Rothberg and Magleby, 2000; Horrigan and Aldrich, 2002; see below) that allow for the quantitative determination of the contribution of the different mutations to the BK channel activation. The main finding of this study was that in the absence of an applied voltage, each regulatory site contributes almost equally to the free energy difference between open and closed states. The estimated dissociation constants were in the micromolar range. Thus, although no direct evidence supports the idea that D362/D367 or M513 form part of a structure able to coordinate Ca$^{2+}$ ions, the data of Xia et al. (2002) and Bao et al. (2002) suggest that the BK channel contains at least two high affinity Ca$^{2+}$-binding sites. The results of Schreiber and Salkoff (1997) pointed towards the existence of a second site able to bind Cd$^{2+}$ and gave a clear indication of the existence of a second Ca$^{2+}$-binding site with different divalent cation selectivity. Oberhauser et al. (1988), on the other hand, found that the BK channel can be activated by a series of divalent cations including Sr$^{2+}$, Cd$^{2+}$, Mn$^{2+}$, Fe$^{2+}$, and Co$^{2+}$. Taking advantage of these observations, Zeng et al. (2005) performed a series of elegant divalent cation selectivity experiments using the D362A/D367A mutant to investigate the selectivity of the Ca$^{2+}$ bowl or the D5N5 mutant to determine the divalent cations able to activate the channel by interacting with the D362/D367 regulatory mechanism. The results indicated that the calcium bowl was able to selectively bind Ca$^{2+}$ and Sr$^{2+}$, while
Figure 2. mSlo1 RCK secondary structure prediction.

A. Sequence alignment used for the homology modeling. mSlo1 C-terminus primary sequence used was directly taken from the protein data bank (sequence number NP_034740). Available KCH_ECOLI-RCK domain coordinates (P31069. 1ID1) obtained previously by X-ray crystallography (Jiang et al., 2001) were used as template. The alignment used for the homology modeling was performed using LIALIGN. There is ~20% identity between these proteins, but they are similar regarding their secondary structure. The homology modeling was performed using Modeler 7v7 (Marti-Renom et al., 2000). LOOPS routine was used for the unaligned sequences. The models that show the lowest energy profiles were selected. WATH IF web interface was used to check the obtained structures and remove bumps.

B. The secondary structure prediction of the hSlo1 RCK domain was performed using JPred (Professor Barton group, Dundee University, Scotland, UK) and PredictProtein Server (Columbia University Bioinformatics Center) (Karplus, 2003; Rost, 2003). Secondary structure prediction was used to evaluate the structural similarity between RCK-Domain from E.coli 6TM channel (KCH_ECOLI) and mSlo1 RCK. Previous alignment between these two sequences was carried out by Jiang et al. (2001). Secondary structure prediction is marked as E (Strand) and H (Helix). The order of Rossmann structures $\beta-\alpha-\beta$ is predicted for mSlo1 from $\alpha$D, after this point, the a helices from Jiang et al. (2001) original structure are conserved but there are no more predicted beta formations. Moreover, original $\beta$E in Jiang et al. (2001) structure was replaced by a $\alpha$ helix in our prediction, named $\alpha$X (highlighted). Buried (B) amino acids also are predicted and indicate that the hydrophobic segment S7 is buried almost completely. Important charges involved in calcium sensitivity are highlighted with red asterisks. Residues forming salt bridges are marked with dotted lines. Notice that E374 is predicted to form a salt bridge with H350. Solid line over the conserved sequence IMRVI, shows a sequence involved in the interaction between domains.
Figure 3. mSlo1 RCK-Domain. Different views of the obtained structure are shown.
A. General view for the obtained structure. The original $\beta-\alpha-\beta$ motif is conserved, and the protein is highly packed. Predictions for buried amino acids are in agreement with the structure in which $\alpha$-helices form a shelter for $\beta$ strands. It is possible to distinguish two domains in the structure, the first one is comprised by $\beta A$ through $\alpha D$ and the second one is defined by what we called $\alpha X$ through $\alpha G$. S7 hydrophobic segment corresponds to $\alpha G$ and is predicted to be buried. It is possible that this structure is in close contact with the next not-modeled portion of carboxyl terminal. B. Several important residues were highlighted. Yellow sticks are the amino acids involved in the interaction with the other subunit. The interaction between K448 and D481 is conserved, as there is a similar pair present in the E.coli RCK. Residue M513 is shown in a CPK representation (yellow). C. Details of the domain involved in calcium sensitivity. Amino acids D362, D367, E374, and E399 are showed. The amino acids indicated as responsible for $\text{Ca}^{2+}$ low affinity E374 and E399 are accompanied by histamines (H344/H350). (D) A complete view of all the salt bridges formed in the structure. These salt bridges may play a key role in maintaining the structure of the RCK domain. Molecular modeling protocols were performed using Hyper mem 7.5 professional (Hypercube, Inc.) with CHARMM 27 force field. The best model obtained previously was minimized using steepest descent protocol in vacuum in the presence of counter ions. Problematic loops were improved as follows; four simple 20 pHs annealing protocols were done (heating 300k, cooling 100k, cooling steps 1k/0.01ps). Each annealing was followed by 30 cycles of conjugate gradients minimization and 30 cycles of steepest descent minimization or until convergence was reached. Finally, salt bridges were calculated using WHAT IF web interface. Image handling was performed using DS Viewer 5.0 (Acers Inc.)
regulation defined by D362/D367 can be activated by Ca\(^{2+}\), Sr\(^{2+}\), and Cd\(^{2+}\). A third, low affinity mechanism (discussed below), can be put into action by Ca\(^{2+}\), Sr\(^{2+}\), Cd\(^{2+}\), Mn\(^{2+}\), Ni\(^{2+}\), and Co\(^{2+}\). In the absence of binding assays like those performed with BK carboxyl terminus fusion proteins (Bian et al., 2001; Braun and Sy, 2001), there exists the possibility, however, that all the results are due to coincidental allosteric effects on Ca\(^{2+}\) binding to a region that lies outside the RCK region. Independent of these considerations, the unique divalent cation selectivity of the different BK regulatory mechanisms supports the existence of at least three distinct divalent cation binding sites and that the sites act independently of each other.

Although rooted in solid experimental data, this picture of a BK channel containing all his Ca\(^{2+}\)-sensing machinery located in the carboxyl terminal, needs further confirmation both from binding and structural studies, in particular, in view of the report by Piskorowski and Aldrich (2002), who claimed that a channel lacking the calcium bowl and RCK domain can still be activated by Ca\(^{2+}\). However, by constructing chimeric channels in which the carboxyl termini between the Slo1 channel (Ca\(^{2+}\) activated) and the Slo3 (pH dependent) were switched, Lingle’s group showed that the C-terminus of Slo1 is a requisite for channel Ca\(^{2+}\) sensitivity (Xia et al., 2004). The chimeric channel Slo3-C-terminus Slo1 was activated by Ca\(^{2+}\), and the chimeric channel Slo1-C-terminus Slo3 induced pH-sensitive currents.

LOW AFFINITY Ca\(^{2+}\)-BINDING SITES AND THE MECHANISM OF Mg\(^{2+}\) ACTIVATION OF BK

Golowash et al. (1986) found that millimolar amounts of Mg\(^{2+}\) were able to potentiate BK channel activation by Ca\(^{2+}\) and to increase the Hill coefficient describing the BK Ca\(^{2+}\) activation curves. Other divalent cations, such as Cd\(^{2+}\), Mn\(^{2+}\), Fe\(^{2+}\), Co\(^{2+}\), and Ni\(^{2+}\), also were able to enhance activation and increase the Hill coefficient of channels already activated by Ca\(^{2+}\) (Oberhauser et al., 1988). Recently, it was discovered that most of these cations are able to activate the BK channel in the absence of Ca\(^{2+}\) by binding to a low affinity site (Shi and Cui, 2001; Zhang et al., 2001; Shi et al., 2002; Zeng et al., 2005).

Notice in Fig. 4A, that Ca\(^{2+}\) shift the probability of opening (\(P_o\)) vs voltage curves towards the left along the voltage axis and that the data is well fitted by using a Boltzmann function of the type:

\[
P_o = \frac{1}{1 + e^{-zF(V - V_{0.5})/RT}}
\]  

(1)

where \(z\) describes the channel voltage dependence, \(F\) is Faraday’s constant, \(V\) is the applied voltage, and \(V_{0.5}\), the voltage at which \(P_o = 0.5\). A convenient way of determining the ability of Ca\(^{2+}\) to activate the BK channel is by plotting the \(V_{0.5}\) as a function of Ca\(^{2+}\) concentration (Fig. 4B) because is directly related with the free energy, \(\Delta G\) to open the channel since

\[
\Delta G = zFV_{0.5}
\]  

(2)

where the values of \(z\) obtained from the fit of the data shown in Figure 4A to a Boltzmann function are given in Fig. 4C. This type of plot was extensively used in the characterization of BK channels and gave the first hint of the existence of a low affinity Ca\(^{2+}\) site (Wei et al., 1994; Cui et al., 1997). Fig. 4B shows that \(V_{0.5}\) shifts to more negative voltages as the Ca\(^{2+}\) concentration is increased, but above 10 \(\mu\)M, there is a tendency towards saturation, suggesting that a new binding site has began to play a role in BK channel activation. This low affinity site is unselective (see above) and Shi and Cui (2001) and Zhang et al. (2001) showed that Mg\(^{2+}\) was able to produce similar \(\Delta V_{0.5}\) in the presence or in the absence of Ca\(^{2+}\). In other words, Mg\(^{2+}\) activates BK channels independently of Ca\(^{2+}\). Mg\(^{2+}\) does not affect the activation but slows down the deactivation kinetics, suggesting that it binds preferentially to the open state. The possible location of the Mg\(^{2+}\) regulatory domain was revealed by co-expressing Slo1 core and Slo3 tail. These chimeric channels, which do not contain the Ca\(^{2+}\) bowl, are still sensitive...
to Mg$^{2+}$, indicating that the low affinity site resides in the RCK domain (Figs. 2 and 3; Shi and Cui, 2001). Sequence alignment of part of the RCK domain in Slo1 and Slo3 revealed several acidic amino acids present in the Slo1 RCK but not in the Slo3 domain. These residues that are conserved in mSlo1 and dSlo1 but not in Slo3 were mutated to the corresponding amino acids present in Slo3 with the result that two hot spots were found, E374A and E399N (Fig. 3C; Shi et al., 2002). Mutation of either of these two residues completely obliterated Mg$^{2+}$-dependent activation. Based on the structure proposed by Jiang et al. (2001) for the core of the RCK domain of the BK channel, Shi et al. (2002) concluded that the Mg$^{2+}$ ion is coordinated by the side chains of E374, E399, and Q397. Thus, the first RCK domain of the BK channel appears to contain high and low affinity Ca$^{2+}$-binding sites. Our own modeling of the RCK domain suggests that both E374 and E399 are very close to H350 and H344, respectively and that the pair E374/H350 forms a salt bridge (Fig. 3C and D). It would be interesting to test for the possibility that the low Ca$^{2+}$ affinity of these putative Ca$^{2+}$ and Mg$^{2+}$ sites is determined by the close proximity of the histidine residues.

Figure 4. Voltage and Ca$^{2+}$ dependence of the BK channel.
A. Averaged $P_o(V)$ curves at the indicated Ca$^{2+}$ concentrations. Lines are the best fit to a Boltzmann distribution (Eq. 1). Fitted parameters, $V_{0.5}$ and $z$, are shown in B and C. Solid line is the best fit to the allosteric model shown in Fig. 6 E.
B. $V_{0.5}$ plotted against Ca$^{2+}$ concentration.
C. $z$ values plotted against Ca$^{2+}$ concentration. Solid line is the best fit to the allosteric model shown in Fig. 6 E.
ACTIVATION BY VOLTAGE AND THE VOLTAGE SENSOR

Despite the fact that it contains an S4 domain, the molecular nature of the BK channel voltage-dependence remained unclear until it was unequivocally demonstrated that depolarizing voltages are able to activate the BK channel in the absence of Ca\(^{2+}\) (Pallota, 1985; Meera et al., 1996; Cui et al., 1997; Cox et al., 1997; Stefani et al., 1997; Horrigan and Aldrich, 1999; Rothberg and Magleby, 2000; Nimigean and Magleby, 2000; Taludker and Aldrich, 2000). One of the first observations in support of the presence of an integral voltage sensor in the BK channel-forming protein is depicted in Figure 4B. Figure 4B clearly shows a region comprised between 5 and about 100 nM Ca\(^{2+}\), where the \(V_{0.5}\) is independent of the Ca\(^{2+}\) concentration (Meera et al., 1996). Moreover, in this Ca\(^{2+}\) concentration interval, the channel can be maximally activated by voltage, indicating that in the absence of Ca\(^{2+}\), voltages high enough can increase the \(P_o\) to its maximum value (Cui et al., 1997).

In voltage-dependent channels, membrane depolarization promotes the displacement of the charged residues contained in the S4-inducing gating currents (Armstrong and Bezanilla, 1973; Schneider and Chandler, 1973; Keynes and Rojas, 1974; Yang and Horn, 1995; Aggarwal and MacKinnon, 1996; Seoh et al., 1996; Larsson et al., 1996; Yang et al., 1996; Yusaf et al., 1996; Ahern and Horn, 2004). Gating currents in BK channels were detected by Stefani et al., (1997) in the absence of Ca\(^{2+}\) and showed that like the G(V) curves, the gating charge-voltage (Q(V)) curves were left-shifted in the presence of Ca\(^{2+}\). The maximum number of gating charges per channel, obtained by dividing the limiting charge \(Q_{max}\) by the number of channels in the patch, was about 4, much less than those obtained in the case of the voltage-dependent Shaker K\(^+\) channel (12-14 electronic charges; Schoppa et al., 1992; Seoh et al., 1996; Aggarwal and MacKinnon, 1996; Noceti et al., 1996). The reason for this large difference in charge per channel between BK and Shaker is still unclear, since a primary alignment of hSlo and Shaker charged domains S2, S3 and S4 shows that most of the negative charges in S2 and S3 and positive charges in S4 are conserved (Fig. 5).

Figure 5. Alignment of the S2, S3 and S4 transmembrane domains of the human (hSlo) and Shaker K\(^+\) channel. Horizontal bars denote the transmembrane segments in both channels. Numbers indicate the position of the far right residues in the primary sequence of their respective proteins. Amino acids in bold are the charged residues conserved in Kv channels and hSlo channels.
In *Shaker*, four arginines (R362, R365, R368, and R371) contribute to the gating charge (Seoh et al., 1996; Aggarwal and MacKinnon, 1996) and Bezanilla’s group presented elegant evidence that these four charges move the entire electric field (Bezanilla, 2000; Starace and Bezanilla, 2001; Starace and Bezanilla, 2004). In hSlo, three of these arginines are conserved (R207, R210, and R213) but only two of them (R210 and R213) were found to be able to alter the maximal slope of the $P_o(V)$ relationship (Diaz et al., 1998; cf., Cui and Aldrich, 2000). More recently, Ma and Horrigan (2005) reported that charge D153 in S2 and R213 in S4 reduce the maximum voltage dependence of $P_o$. (2) If R213 is the only gating charge, it should move all the way across the electric field (one charge per channel subunit). Considering that in Kv channels the voltage sensors structure (S1-S4) are essentially self-contained, independent domains inside the membrane (Long et al., 2005a, b) and that BK channels contain an extra transmembrane domain (S0), it is probable that the structure of the voltage sensor of the BK channel will prove to be slightly different from that of Kv channels. The clue of the large difference in gating changes between BK and Kv channels may lie in structural differences in the voltage sensor.

EXPLAINING BK CHANNEL ACTIVITY USING ALLOSTERIC MODELS

In this section, I will emphasize some of the highlights of the two-tiered allosteric model that is commonly used to explain the BK channel activity. For details, the reader should consult the excellent review by Magleby (2003). In the absence of Ca$^{2+}$, Nimigean and Magleby (2000) and Taludker and Aldrich (2000) found 4-5 exponential components in the dwell time distribution for the closed states and 2-3 components in the dwell time distribution for the open states for the BK channel. Linear models in which only one pathway led to the open states were excluded since McManus et al. (1985) found an inverse relationship between the duration of adjacent open and closed intervals: shorter closed intervals are preferentially adjacent to longer open intervals and longer closed intervals. Given the large number of closed and open states, the correlation between adjacent intervals and the fact that the channel is a tetramer, in the absence of Ca$^{2+}$, BK channel gating is consistent with the 10-state model indicated in Fig. 6A. One of the predictions of this model is that even in the absence of voltage sensor activation, described by the equilibrium constant $J$, the channel can open through the reaction described by the equilibrium constant $L$. (3) This prediction was confirmed by Horrigan et al. (1999), who found that even in the absence of Ca$^{2+}$ and at very negative voltages, the channel can open with a very low, but measurable, $P_o$ (about $10^{-6}$) and a low voltage dependence, $z_L$, not related to the voltage sensors. On the other hand, a detailed analysis of the gating currents in the absence of Ca$^{2+}$ suggests a two-state model, resting-activated (R-A) suffices to explain the voltage sensor movement (Horrigan and Aldrich, 1999). The simple behavior of the gating currents, $Q(V)$ curves which are well described by a Boltzmann function, the monoexponential kinetics of the fast component of the gating current, and the lack of a gating current rising phase also are consistent with the kinetic model proposed in Fig. 6A in which the voltage sensors act independently.

In the allosteric model described in Figure 6A, for each voltage sensor activated, the equilibrium constant for channel opening, $L$, is multiply by an allosteric factor $D$, so the opening process is facilitated as more voltage sensors are activated. The observation that even when all voltage sensor are resting, $P_o$ can be increased by augmenting intracellular Ca$^{2+}$ (Horrigan and Aldrich, 2002) is the basis for postulating the allosteric kinetic model depicted in Figure 6B under the assumption that there is only one Ca$^{2+}$-binding site per channel subunit. In this case, for each Ca$^{2+}$-binding site occupied the equilibrium constant $L$ is multiply by an allosteric factor $C$. Figures 5A and B define the key feature of BK channels:
neither Ca$^{2+}$ nor voltage are strictly necessary for channel activation and Ca$^{2+}$ binding and voltage sensor activation can act independently to enhance channel opening. Thus, we are in the presence of three processes, Ca$^{2+}$ binding, voltage sensor activation, and channel opening, which are independent equilibriums that interact \textit{allosterically} with each other. In support of the model shown in Fig. 6B, Niu and Magleby (2002), using channels with 1, 2, 3 or 4 Ca$^{2+}$ bowls (4), determined that the Hill coefficient increased in a stepwise fashion as the number of bowl increased from 1 to 4. This observation is consistent with models like the one shown in Fig. 6B in which the Ca$^{2+}$ binding to each of the sites is independent, and cooperativity arises as a consequence of the action of the allosteric factor C.

The best compromise between simplicity and reproduction of the voltage and calcium dependence in a wide range of voltages and Ca$^{2+}$ concentrations, including very low $P_o$s, is probably the 50-state two-tiered gating mechanism shown in Fig. 6C (Rothberg and Magleby, 1999, 2000; Cox and Aldrich, 2000; Cui and Aldrich, 2000). If some allosteric coupling ($E$; Fig. 6D) between Ca$^{2+}$ binding and voltage sensor movement is included, the model increases to 70 states (Horrigan and Aldrich, 2002) and in several occasions, this has been the model of choice (e.g., Orio and Latorre, 2005). The beauty of the model is that it is possible to set experimental conditions to determine some of the different parameters unequivocally. For example, in the absence of Ca$^{2+}$ and at very negative voltages, channel gating kinetics is determined by the transition: 

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**Figure 6.** Allosteric activation model for BK channel.

A. The allosteric activation by voltage originates a 10-state Monod-Wyman-Changeaux (MWC) activation model. In this case, the allosteric factor is $D$ and the equilibrium constant $J$. B. The allosteric activation by Ca$^{2+}$ also originates a 10-state MWC model. For each Ca$^{2+}$-binding site occupied, the equilibrium constant for channel opening, $L$, is multiplied by the allosteric factor $C$. C. The combination of A and B produces a two-tiered, 50-state model. D. The complete 70-state model takes into account the interaction between the voltage sensor activation and Ca$^{2+}$ binding (allosteric factor $E$). When $E=1$, we recover the 50-state model (modified from Horrigan and Aldrich, 2002; Magleby, 2003).
\[
\frac{L}{C \leftrightarrow O}
\]

and

\[
P_\circ = \frac{O}{O + C} = \frac{1}{1 + L^{-1}}
\]

as \( L << 1 \), \( P_\circ \sim L = L_0 \exp(z_L F V/RT) \). Thus, under these experimental conditions, we are able to determine two parameters: \( L_0 \) and \( z_L \). This exercise allows us to arrive at another important conclusion: for the BK channel, the limiting slope is actually determined by the lesser voltage-dependent transition and does not reflect the voltage sensor charge effectively coupled to channel activation (see Almers, 1978; Sigg and Bezanilla, 1997). The existence of two or maybe three \( \text{Ca}^{2+} \)-binding sites with different affinities (see above) makes the picture more complicated, however, and raises almost exponentially the number of states in a model.

SOME INFERENCEs ABOUT THE MOLECULAR NATURE OF \( \text{Ca}^{2+} \) ACTIVATION

Jiang et al. (2002) determined the crystal structure of the \( \text{Ca}^{2+} \)-bound open *Methanobacterium thermoautotrophicum* K\(^+\) (MthK) channel. The structure shows that each of the four channel subunits contributes with two RCK domains forming a gating ring where two \( \text{Ca}^{2+} \) ions bind per subunit. The site behaves as the low \( \text{Ca}^{2+} \) affinity binding site in BK, since millimolar amounts of \( \text{Ca}^{2+} \) are necessary to activate the MthK channel. Jiang et al. (2002) put forward a model in which \( \text{Ca}^{2+} \) binding promotes displacement mediated by a flexible interface of the two RCK domains expanding the diameter of the gating ring; this expansion is used to mechanically open the pore to an open channel. Whether this mechanism applies to the BK channel is unclear, but the presence of RCK domains in BK channels is undeniable (Jiang et al., 2001; Figs. 2 and 3). Actually two RCK domains have been proposed to be present in BK channels, which can act as a gating ring similar to that of MthK channels (e.g., Zeng et al., 2005), but evidence for the second RCK domain is lacking.

Niu et al. (2004) studied the effect of changing the length of the linker joining the S6 with the RCK domain. The authors use a simple and elegant model in which the coupling could be explained by using a spring-like model. They found that shortening the linker increases channel activity and increasing linker length decreases channel activity. These observations suggest that, in the absence of \( \text{Ca}^{2+} \), the linker-RCK (gating ring?) complex behaves as a passive spring attached to the channel gates. This linker-gating complex in the absence of \( \text{Ca}^{2+} \) applies force to the gates, an observation consistent with the finding that compared to the Slo3 tail, the Slo1 tail inhibits channel opening. \( \text{Ca}^{2+} \) binding, as in the case of the MthK channel, transform the passive spring into a force generating that uses the free energy of \( \text{Ca}^{2+} \) binding to pull open the S6 helices to permit ion conduction.

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NOTES

1. The correlation between the DV0.5 induced by the mutations and the effect on 45Ca binding was lost when the mutation D899A was tested. This mutation has no effect on DV0.5 but a large effect on the 45Ca-binding signal. Bao et al. (2004) have
argued that the structure of the calcium bowl in the gel-overlay assay may take a conformation that is different to the one adopted in its native conformation.

2. In Shaker, the equivalent negative charge E283 does not contribute to the total gating charge but E293 does (Sheo et al., 1996); in the equivalent position of E293, hSlo contains a tyrosine (Y163).

3. Voltage sensor activation is described by the equilibrium constant $J = J(0)\exp(zjF(V - Vh(J)/RT))$, where $J(0)$ is the equilibrium constant at zero voltage; $zj$, the sensor charge; and $Vh(J)$, the half activation voltage for each sensor. $L = L(0)\exp(zL\nuF/RT)$, where $zL$ is the voltage dependence of $L$ and $L(0)$ is the equilibrium constant for channel opening with the voltage sensor in resting state and no Ca2+ bound at zero voltage.

4. The Ca2+ bowl of mSlo1 was disrupted by deleting aspartates 965 and 966, and the number of bowls per channel determined by mixing TEA-insensitive subunits with bowl-disrupted subunits. Stoichiometry is obtained from the single-channel current amplitudes (see also Shen et al., 1994).

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