Interaction Sites between the Slo1 Pore and the NH$_2$ Terminus of the $\beta$2 Subunit, Probed with a Three-residue Sensor$^{*\text{S}}$

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Calcium- and voltage-gated (BK) K$^+$ channels encoded by Slo1 play an essential role in nervous systems. Although it shares many common features with voltage-dependent Kv channels, the BK channel exhibits differences in gating and inactivation. Using a mutant in which FWI replaces three residues (FIW) in the NH$_2$ terminus of wild-type $\beta$2-subunits, in conjunction with alanine-scanning mutagenesis of the Slo1 S6 segment, we identify that the NH$_2$ terminus of $\beta$2-subunits interacts with the residues near the cytosolic superficial mouth of BK channels during inactivation. The cytosolic blockers did not share the sites with NH$_2$ terminus of $\beta$2-subunits. A novel blocking-inactivation scheme was proposed to account for the observed non-competition inactivation. Our results also suggest that the residue Ile-323 plays a dual role in interacting with the NH$_2$ terminus of $\beta$2-subunits and modulating the gating of BK channels.

Ca$^{2+}$ and voltage-gated K$^+$ channels (BK channels)$^4$ are encoded by mammalian Slo1 genes related to the Drosophila Slow-poke (Slo) gene (1, 2). These channels are abundantly distributed in the nervous system to regulate excitability in response to intracellular Ca$^{2+}$ and membrane potential. Rapid inactivation of BK channels results from mSlo1 pore-forming $\alpha$-subunits being coexpressed with $\beta$2-subunits (3–6).

BK channels probably share similar pore structural determinants and many kinetic characteristics with voltage-dependent K$^+$ channels (K$_V$ channels). For K$_V$ channels, N-type inactivation arises from the cytosolic NH$_2$ terminus of the pore-forming $\alpha$-subunits (7, 8) or the auxiliary $\beta$-subunits (9) inserting into the ion permeation pathway thereby blocking conduction ion permeation. Previous studies show that the NH$_2$ terminus of either the $\alpha$- or $\beta$-subunit requires an inactivation domain (ID) composed of a hydrophobic head group to inactivate channels, followed by several positively charged amino acids (10, 11). Xia et al. (12) reported that the uncharged hydrophobic head group (FIW) of the h$\beta$2 NH$_2$ terminus results in the inactivation of BK channels and proves that it is the only structural determinant required for inactivation.

Evidence supporting the idea that the ID must insert into the pore is derived from the blocking experiments of K$_V$ channels by cytosolic blockers, which compete with the ID for channel occupancy (13, 14), thus resulting in the slowing of inactivation kinetics. Another report from McKinnon’s laboratory (15) demonstrated that the first four residues of the NH$_2$ terminus of an inactivating K$_V$$\beta$ auxiliary subunit indeed interact with pore-lining residues of the K$_V$1.4 $\alpha$-subunit. By contrast with K$_V$ channels, cytosolic blockers of BK channels do not slow the inactivation kinetics, indicating that there is no competition between these blockers and the ID of h$\beta$2-subunits (3, 16, 17). For BK channels, there are two questions to be left, that is, how the ID interacts with the channel pore and why the cytosolic blocker of BK channels does not compete with the ID.

With a mutant FWI, which is obtained from substituting the initial three amino acids (FIW) of the h$\beta$2-subunit NH$_2$ terminus with FWI, and a systematic alanine-scanning mutagenesis of the mSlo1 S6 segment, we demonstrate that the residue Ile-323 of mSlo1 $\alpha$-subunits plays a dual role in interacting with the ID of h$\beta$2-subunits and modulating the gating of BK channels. We examined the binding sites of both the cytosolic blockers and the ID of h$\beta$2-subunits and developed a new model for explaining the non-competition mechanism of inactivating BK channels. The present findings may underlie the gating and inactivating mechanisms of BK channels.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—All mutations of mSlo1 and h$\beta$2 were generated through PCR reactions, and all constructs were verified by sequencing. The FWI mutation of the wild-type h$\beta$2
was generated by the traditional PCR reaction with the primers 5′-GGGCGGAGCTTACCAACCTAGTTTTTGATGAAACACT-3′ and 5′-GGGCGGAGCTTACCAACCTAGTTTTTGATGAAACACT-3′. 

The product was digested with HindIII and XhoI and then ligated into HindIII_XhoI vector pBF. Other point mutations of mSlo1 and hß2 were generated by QuikChange protocol (Stratagene). For example, PCR reactions of mSlo1 were performed with mSlo1 as a template and a pair of complementary mutagenesis primers. The PCR reaction mixture was then cut with the enzyme DpnI to digest the template mSlo1. After DpnI digestion, the PCR mixture was used to transform competent bacterial cells to amplify the mutant plasmid of mSlo1.

Expression in Xenopus Oocytes—After DNA was linearized with MluI, SP6 RNA polymerase (Roche Applied Science) was used to synthesize cRNA for oocyte injection. The stage V–VI Xenopus oocytes were injected with 5–10 ng of cRNAs and then incubated in ND-96 solution at 18 °C for 2–7 days. To keep ß2 (or ß2 mutants)–subunits at a saturating concentration, we co-injected mSlo1 α (or α mutants) and ß2 (or ß2 mutants) mRNAs into oocytes in a ratio of at least 1:2 by weight.

Electrophysiology—Macroscopic currents were recorded in inside-out patches at room temperature (22–25 °C). Pipettes were filled with a solution containing the following (in millimolar): 160 MeSO₃K, 10 H⁺–HEPES, and 2 MgCl₂, adjusted to pH 7.0 with MeSO₃H. Intracellular solutions with different free Ca²⁺ concentrations (for 0.5, 5, 10, 15, 20, 50, or 100 μM) were prepared by adding CaMeSO₃ and EGTA or HEDTA. Inside-out patches were initially charged with the intracellular solution. Experiments were performed and recorded using an EPC-9 patch clamp amplifier and PULSE software (HEKA Elektronik, Lambrecht/Pfalz, Germany). Currents were typically digitized at 20 kHz. All macropatch records were filtered at 2.9-kHz during digitization. For displays, currents were filtered digitally at 2 kHz (Bessel 8-pole). All single-channel currents were recorded at 10 kHz. During recording, solutions with or without the drugs QX-314 (2 mM) or tetraethylammonium (TEA, 50 mM) were bubbled locally onto the inside-out patches via a pipette containing seven solution tubes. The tip (∼300 μm diameter) of the pipette was located about 120 μm from the patches. As determined by the conductance tests, the solution around a patch under study was fully controlled by the application of a solution with a flow rate of 100 μl/min or greater. All pharmacological experiments met this criterion. Chemicals were obtained from Sigma-Aldrich.

Data Analysis—Data were analyzed with IGOR (Wavemetrics, Lake Oswego, OR), Clampfit (Axon Instruments, Foster City, CA), and SigmaPlot (SPSS Science, Chicago, IL) software. Unless stated otherwise, the data are presented as mean ± S.E., significance was tested by Student’s t test, and differences between the mean values were considered significant at a probability of ≤0.05. G–V curves for activation were generated from steady-state currents, converted to conductance and then fitted by the single Boltzmann function with the form,

\[
\frac{G}{G_{\text{max}}} = \left(1 + \exp((-V - V_{\text{50}})/\kappa)\right)^{-1}
\]

where \(V_{\text{50}}\) is the voltage at which the conductance \(G\) is half the maximum conductance \(G_{\text{max}}\), and \(\kappa\) is a factor affecting the steepness of the activations. Recovery curves were fitted with the bi-exponential function (Equation 2),

\[
\text{Fractional recovery} = a_{\text{fast}} \times (1 - \exp(-t/\tau_{\text{fast}})) + a_{\text{slow}} \times (1 - \exp(-t/\tau_{\text{slow}}))
\]

where \(a_{\text{fast}}\), \(\tau_{\text{fast}}\), \(a_{\text{slow}}\), and \(\tau_{\text{slow}}\) are the percentages and time constants of fast and slow recovery components, respectively. The first term on the right of Equation 2 was used for the mono-exponential recovery.

RESULTS

The Mutation FWI Is a More Sensitive Probe for Screening the Interaction Sites—The pore-forming segment (S6) of the α-subunits of BK channels shares homology with the K⁺ channels, several of whose crystal structures have been well resolved (18–20). After aligning the sequences of mSlo1 with the potassium channels KcsA, Kv₁.2, and MthK (Fig. 1A), we inferred by structural homology that the pore-lining residues in BK channels (12), are shown in gray. The sequence is: hß2, human KCNMB2, acc PIR NM_005832.

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To determine interaction between the ID and pore, the wild-type hß2 (FWI) usually serves for detecting the interacting residues in the pore by means of alanine mutagenesis of mSlo1 α-subunits. The time constant of recovery from inactivation is a good character for this purpose. On the basis of our preliminary results, however, it is difficult to use the FIW to determine the interacting sites due to its doubtful time constants in recovery from inactivation (data not shown). Therefore, it is necessary to find a more sensitive probe for the goal mentioned previously.

After examining wild-type hß2 (FIW) and three rearranged constructs FWI, IWF, and WIF, we find that they largely share

\[
\frac{G}{G_{\text{max}}} = \left(1 + \exp((-V - V_{\text{50}})/\kappa)\right)^{-1}
\]

where \(V_{\text{50}}\) is the voltage at which the conductance \(G\) is half the maximum conductance \(G_{\text{max}}\), and \(\kappa\) is a factor affecting the steepness of the activations. Recovery curves were fitted with the bi-exponential function (Equation 2),

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where \(a_{\text{fast}}\), \(\tau_{\text{fast}}\), \(a_{\text{slow}}\), and \(\tau_{\text{slow}}\) are the percentages and time constants of fast and slow recovery components, respectively. The first term on the right of Equation 2 was used for the mono-exponential recovery.
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Most of kinetic characteristics in activation, inactivation, and recovery, except that the construct FWI exhibits a bi-exponential recovery different from the other mutations (12) (supplemental Fig. S1). For recovery from inactivation, the wild-type hβ2 and IFW and WIF exhibited similar time constants in a mono-exponential recovery (average τr = 185 ± 4.4 ms at −140 mV), whereas the mutation FWI exhibited bi-exponential recovery components with a fast component (τr,fast = 6.5 ± 0.6 ms at −140 mV) and a slow component (τr,slow = 323 ± 46 ms at −140 mV), ~50-fold slower (Fig. 2, A and C, and supplemental Table S1). We infer that the bi-exponential kinetics of FWI in recovery from inactivation mostly results from the residue Trp-3, because tryptophan is bulky and may have significant impact on the pore-lining residues in the S6 region during the recovery process. Despite the differences in the recovery kinetics, we believe that FWI interacts with a similar region of the channel as FIW, because their kinetic characteristics are very similar except for recovery.

Considering that the FWI as an infrequent mutation of the native hβ2 (FIW) exhibits a bi-exponential recovery, we infer that there should be a specific residue in the pore interacting with the FWI more strongly to induce the slow recovery. Furthermore, two components of FWI, especially the slow one, can be used to identify the interaction sites, and these are much better than one component of hβ2 (FIW). Comparing with the results from native hβ2 (FIW), we found in this study that the FWI was a better sensor for exploring interaction sites.

**Residue Ile-323 in the Cavity of the mSlo1 Channel Interacts with the ID of the FWI**—Because the ID produced inactivation through hydrophobic interaction, we systematically mutated each hydrophobic residue in S6 of BK channel to alanine. Each mutant of the mSlo1 coexpressed with the mutation FWI was termed “mutant mSlo1-FWI,” for example, I323A-FWI in this study. For each coexpression such as mutant mSlo1-FWI, all the recovery experiments were performed from inside-out patches by the paired-pulse recovery protocol shown at the bottom of Fig. 2A (Also see supplemental Fig. S2). Among the sixteen mutants of mSlo1, the coexpressed mutation I323A-FWI showed pronounced alterations in the slow dissociated constant Kd,slow, and the mutations V319A-FWI and M314A-FWI in the fast dissociated constant Kd,fast = 43.6 ms is the closest to the monoexponential recovery time constant of the wild-type mSlo1-hβ2 (τr = 21.9 ms). Considering the fact that the residue Ile-323 is a pore-lining residue, thus, we infer that Ile-323 possibly plays a significant role as one of the interaction sites in prolonging recovery from the inactivation. The fast recovery time constants of M314A-FWI and V319A-FWI (τr,fastM314A = 1.9 ± 0.4 ms; τr,fastV319A = 1.8 ± 0.5 ms) were significantly faster than those of any other mutations (supplemental Table S2). Both the amino acids Val-319 and Ile-323 probably contribute to the lining toward the inner wall of the pore, whereas the residues Met-314 may not (18). One explanation may be that the mutation M314A allosterically translocates the pore-lining residue Phe-315, which then may alter its contact with the ID of hβ2. Unfortunately, we failed to express mutant F315A (21), which may indicate an unusual structure between the Met-314 and Phe-315. An NMR structure of the hβ2 NH2-terminal peptide (22) indicates that the first ten residues of hβ2 are flexible and extendable into a linear peptide structure. Making a rough estimate on the basis

![FIGURE 2. Mutational analysis of the ID of hβ2 subunits and hydrophobic residues in the S6 pore region of mSlo1 channels](image-url)
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Because the FWQ has the lesser hydrophobicity, the time constants of over 80 ms, which are 4-fold that of mSlo1-FWQ and mSlo1-hβ2 demonstrate a specific steric requirement for Ile-4 in the ID to directly interact with the pore-lining residue Ile-323.

We also compared the G-V curves of each pore mutation with the wild-type mSlo1 and found that the V_{50} of I323A shifted only about +20 mV with 10 μM Ca^{2+} (Fig. 3B) relative to the mSlo1. Furthermore, we noticed that the relative V_{50} of the pore mutations compared with mSlo1 shifted variously at 10 μM Ca^{2+} (Fig. 3C). Comparing Fig. 3C with Fig. 2B, we found that the slow recovery component was not tightly related to the changes of V_{50} but mainly depended on the interaction between the pore and the ID. Taking L312A as an example, even though its V_{50} shifted ~−130 mV, the recovery of L312A still contained a slow recovery component.

No Mutations in the S6 Pore Significantly Affect the Binding Affinities of Cytosolic Blockers—Xia et al. (3) and Solaro et al. (17) reported that the cytotoxic blockers of BK channels did not slow the inactivation process and further inferred that they did not share the binding sites with the ID based on the competition model of K_{V} channels. However, it is still unknown whether the blockers of BK channels have specific binding sites in pore and why they do not compete with the ID.

To determine whether blockers slow the inactivation process and where their binding sites are at the same time, we examined the inactivating currents of coexpression of the mSlo1 mutants and FWI in most experiments. Cytosolic application of 2 mM QX-314 reduced the averaged peak currents to 20–50% but caused insignificant differences in inactivation time constants τ_{i} (Fig. 4, A and B). The inactivation time constants τ_{i} varied from 20 to 36 ms with an average time constant τ_{i} = 25.3 ± 4.3 ms. According to competition model, the 20–50% unblocking currents are predicted to produce a 2- to 4-fold slowing of τ_{i} (3, 13, 14). In Fig. 4 (A and B), however, 2 mM QX-314 resulted in no significant changes in τ_{i} for all the mSlo1 mutants. Similar results were obtained by cytotoxic application of 50 mM TEA (Fig. 4, C and D).

It seems unnecessary to use the FWI for determining the binding affinity of blockers. However, we will find something interesting after comparing how they differ. In Fig. 4B, 2 mM QX-314 showed less inhibition on the currents of the mSlo1-hβ2, M314A-FWI, and V319A-FWI than that of the mSlo1, M314A, and V319A, respectively, which suggests that the ID prevents QX-314 from entering the cavity. In Fig. 4D, however, 50 mM TEA did not show the statistic difference on inhibition probably due to a very high concentration used in this experiment. No matter whether we have used FWI or not, there is no significant difference in their binding affinities. It indicates that no mutations along the ion conduction pathway significantly affect the binding affinities of QX-314 or TEA.

A Kinetic Model for the Cytosolic Blocker QX-314 Blocking the Inactivating BK Channels—On the basis of our experiments, a schematic, which is presented with only two opposite subunits and one inactivation domain, illustrates a kinetic process that includes both the inactivation and the blocking and inactivation of BK channels (Fig. 5). For a competition model of K_{V} channels, the channel can only alter from the open state to the inactivated state or to the blocked state, but not to the...
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FIGURE 4. The currents of mutations in the cavity of BK channels exhibit similar sensitivity to both QX-314 and TEA. A and C, the representative traces of currents as indicated were elicited from inside-out patches in 10 \( \mu M \) Ca\(^{2+} \) by the voltage protocol shown at the top. Each trace is labeled with control/wash, 2 mM QX-314 (in A), 50 mM TEA (in C) and 0 \( \mu M \) Ca\(^{2+} \), respectively. B and D, summary data showing the inhibitory effects of 2 mM QX-314 (in B) and 50 mM TEA (in D) on channel activity from mSlo1-h\( \beta \)2, mSlo1-FWI and some of S6 mutations coexpressed with FWI. The left panel shows the fractional unblocking currents of mSlo1 and other combinations as indicated. In B and D, the fractional unblocking currents of M314A and V319A were obtained at 300 \( \mu M \) Ca\(^{2+} \) and others at 10 \( \mu M \) Ca\(^{2+} \). The right panel shows the ratio of 100* \( \frac{I_{\text{C}}}{I_{\text{W}}} \) to allow a comparison between the inactivation time constants with and without QX-314 and TEA. The number on the top of the bars is the number of patches.

FIGURE 5. Diagram of the one-step non-competition model accounting for the non-competitive blocking of BK channels by QX-314.

In our non-competitive scheme for BK channels, the channel cannot only go from the open state to either the blocked state or the inactivated state, but also from the blocked state to the blocked and inactivated state. Therefore, the one-step non-competitive scheme for BK channels ("one-step" means one inactivation step) can be modeled as in Scheme 1, where states are represented by: O, open; I, inactivated; B, blocked; and BI, blocked-inactivated. Rate constants are: \( k_i \), inactivation; \( k_{-i} \), returning from inactivation; \( k_{ib} \), binding; and \( k_{-ib} \), dissociation.

The transition rates between the B and the BI states are same to that between the open (O) and the inactivated (I) states. There is no link between the BI and I states, because the charged blockers cannot penetrate through cell membrane. Therefore, blockers are trapped in the cavity of the pore when the inactivation site is occupied. Following Choi et al. (14), we also ignore the voltage-dependent activation steps, which are rapid and thus do not complicate measurements of inactivation at the positive voltages studied. For a complete inactivation, we make the assumption that \( k_{-i} \) is close to zero. The blockers association and dissociation is typically much more rapid than the inactivation process (i.e. \( k_{ib} \) and \( k_{-ib} \) \( \gg \) \( k_i \)). The channel goes to the inactivated state from the open state or to the blocked-inactivated state from the blocked state with the same inactivation time constant \( \tau_i \equiv 1/(k_i + k_{-i}) \) (for the case \( k_i \gg k_{-i} \), \( \tau_i \approx 1/k_i \)). The difference between the competitive and the non-competitive model is that the blocked channels are protected from rapid equilibrium with the pool of open channels and return to the blocking-inactivation state and "permanently" lost with the same rate as that between the open and inactivation state. Therefore, this scheme can predict that the inactivation time constant with and without blockade will be the same (the unblocked fraction of channels \( f = k_{-ib}/(k_{ib} + k_{-ib}) \) and the inactivation time constant in the presence of blockers \( \tau_{ib} \equiv 1/k_i \).

A similar consideration can be directly used to describe the two-step inactivation model. Two-step inactivation model (O \( \leftrightarrow \) O* \( \leftrightarrow \) I) is revised from the one-step inactivation model (O \( \leftrightarrow \) I) (13–16). O, O*, and I are the open, pre-inactivation, and inactivation states, respectively. However, the two-step inactivation model can be simplified into a one-step inactivation model when the transition rate between O and O* is much larger than between O* and I (15, 23).

Simulations in Fig. 6 illustrate two different cases, i.e. one-step non-competition and two-step non-competition. \( \tau_i \) is defined as the control inactivation time constant (without...
Residue Ile-323 Plays a Dual Role in BK Channels—By analogy to the shaker-type K⁺ channel Kᵥ1.4-IR, residue Ile-323 in BK channels corresponds to Tyr-569 in Kᵥ1.4-IR channels (15). Closer examination of I323A macroscopic and single-channel currents reveals additional critical differences from the wild-type BK channels. The I323A tail currents exhibit an outward rectified current, strikingly different from the wild-type channels (Fig. 7, A and B) (26). The results of the macroscopic currents are consistent with the outward rectification of unitary conductance measured from the maximum single-channel level shown in Fig. 7 (C and D). In contrast, the single-channel currents of the wild-type mSlo1 show little rectification in Fig. 7 (C (right) and D). Resembling a dSlo-like flickery behavior (27, 28), the single channel of I323A shows very noisy opening in contrast to the mSlo1 single-channel opening shown in Fig. 7C. We also observed that the open probability of the largest level reduced more rapidly at negative voltages. Consequently, the rectification property of the I323A mutant was possibly caused by the very short open time, the very low open probability, and the relatively low filtering frequency. More detailed work is clearly required to illuminate the nature of rectification. In addition, rectification will not affect the G–V curve due to very low open probability at negative voltages under conditions we currently used (Fig. 3B).

As the corresponding residue in the flickery dSlo (A2, C2, E2, G5, 10 splice variant) is Thr-337 rather than Ile (28, 29), we substituted Thr for Ile-323 in wide-type mSlo1 and found that I323T induced the flickery single-channel currents similar to the flickery dSlo.⁵ This mechanism may help us to understand the behavior of the flickery dSlo single channels.

Simulation of an Interaction between hβ2 (FIW and FWI) and mSlo1 Subunits of BK Channels—To further understand the molecular basis for interaction between the hβ2 subunit and BK-type channels, docking simulations were performed with the wild-type hβ2 (FIW) NH₂ terminus and its mutant FWI interacting with mSlo1 α-subunits, using the bimolecular complex program 3D-DOCK (see also “Methods” in the supplemental materials). The most favorable docking conformations were guided by our experimental results described above (Fig. 8) based on the Kᵥ1.2 channel data (PDB code 2A79) and the NMR data (PDB code 1JO6) of the hβ2 NH₂ terminus (22, 30). The computational models for complexes of FIW-mSlo1 and FWI-mSlo1 were further analyzed, using the LIGPLOT program (31, 32) (Fig. 9). As shown in Fig. 8, the FIW and FWI side chains of the hβ2 subunits are tightly packed together to form a hydrophobic core. Each component of the hydrophobic core makes close contact to a corresponding residue of the mSlo1 S6 segment and fully blocks the mouth of the BK channels. The interactions in the mSlo1-hβ2 (mSlo1-FIW) structural model mainly involved hydrophobic contacts, which include Phe-2 with Val-319 (B), Ile-323 (B) and Val-319 (D), Ile-3 with P320 (D), and Trp-4 with Ile-323 (C). The interactions in the mSlo1-FWI structural model predict similar hydrophobic contacts: Phe-2 with Val-319 (B), Trp-3 with Ile-323 (B) and Ile-323 (D), and Ile-4 with Pro-320 (B) and Ile-323 (C). In addition, the

⁵ H. Li, J. Yao, X. Tong, Z. Guo, Y. Wu, L. Sun, N. Pan, H. Wu, T. Xu, and J. Ding, unpublished data.
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A I323A

B I323A

mSlo

C FIW

D FWI

FIGURE 7. Representative currents expressed with cRNA of I323A exhibit a flickery outward-rectified feather. A, tail currents of I323A (left) and mSlo1 (right) were activated by a voltage protocol, which is shown at the bottom, with intracellular 10 μM Ca^{2+}. The dashed line represents zero current. B, I–V curves are plotted based on the instantaneous values of tail currents (C; I323A; mSlo1; the number of patches is n = 6). The standard errors often lie within the symbol. C, representative single-channel currents of I323A (left) and mSlo1 (right) show the maximum single-channel currents at voltages as indicated. Dotted lines labeled with a letter “c” represent the zero level, and those labeled with a letter “o” represent the maximum single-channel open level. All single-channel currents were recorded at 10 kHz. D, the I–V curves are plotted with the values of the maximum single-channel currents measured by eye (C; I323A (n = 2); dotted line, mSlo1).

FIGURE 8. The complex models of a channel composed of α subunit mSlo1 and its auxiliary NH₂ terminus (hβ2) generated by 3D_DOCK program. For clarity, two of the four subunits, B and D, for mSlo1 and the NH₂-terminal segment of hβ2 are presented. N and C indicate NH₂ terminus and C terminus of one subunit of mSlo1, respectively. A, the structure of mSlo1 with hβ2 (FIW). The interactions are mediated by hydrophobic contacts involving Phe-2 and Val-319 (B), Phe-2 and Ile-323 (B), Phe-2 and Val-319 (D), Ile-3 and Pro-320 (D), and Trp-4 and Ile-323 (C). B, the model of mSlo1 with hβ2 (FWI). The closest contacts between the proteins mainly include Phe-2 and Val-319 (B), Trp-3 and Ile-323 (B), Trp-3 and Ile-323 (D), Ile-4 and Pro-320 (B), Ile-4 and Ile-323 (C), and Thr-5 and Ile-323 (C). The results suggest the interactions between mSlo1 channel and hβ2 are mainly composed of hydrophobic contacts.

DISCUSSION

In this study, we show that the ID of the hβ2-subunit does not occupy the same site with channel blockers, which directly leads to a non-competitive inactivation. However, two critical questions need to be answered: why the channels in this work did not show any reopening during the deactivation process and by what pathway the ID can access the conducting entrance of the pore.

In KV channels, once the ID occupies its blocking position, it impedes closure of the channels to lead channels to reopen during the deactivation process (8, 13). In BK channels, during the process of deactivation, the fully inactivated channels with a hydrophobic inactivation domain such as hβ2 do not show reopening but do show reopening with some lesser hydrophobic inactivation domains such as hβ3b. It seems to depend on whether the recovery time constant is comparable to the closing time constant. Because the recovery process of hβ2 from complete inactivation (e.g., 20 ms) is usually much slower than its closing process (e.g., 0.1 ms), thus it can fully conceal the reopen process of BK channels. By contrast, we can anticipate that the reopening appears from the incomplete inactivated BK channels by hβ3b with a recovery time constant of τ_r = 0.38 ms. Moreover, recent work from a few laboratories has shown that the ID reaches the ion-conducting pore through lateral “side portals” in the cytoplasmic portion of the channel (20, 33, 34). In our modeling, we cannot exclude any pathway accessing to or withdrawing from the ion-conducting pore.

Here we also want to emphasize that the models for both the competitive and non-competitive case may give a bi-exponential inactivation if the blocking rates are relatively smaller, that is, one of the inactivation time constants is faster.
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FIGURE 9. A depiction (generated by LIGPLOT) of the main interactions involved in hJ32 (FIW)-mSlo1 (A) and hJ32 (FW1)-mSlo1 (B).

and the another slower than the control. Slow blockers such as peptides used to be an example of the competition model. They clearly share the same sites with the NH2 terminus and typically induce a bi-exponential inactivation due to their low blocking rates.

It is interesting to know why the mutated construct FWI gives different dynamics in the period recovering from inactivation compared with the wild-type hJ2 (FIW). For FIW, the residue Trp-4, as the last residue involved in the inter-molecular interaction, is the most hindered component of the hydrophobic core maybe due to its large volume. It has less contact with the mouth of the channel (only one Ile-323) and is exposed to solvent. For construct FWI, the residue Trp-3 before Ile-4 as the second residue is also the most hindered component of the hydrophobic core. In comparison with FIW, the residue Trp-3 in FWI makes more contacts with the channels (interacting with two Ile-323 residues), is buried deeply and is less exposed to solvent. This kind of arrangement of the hydrophobic side chains in FWI might account for its distinct recovery behavior after inactivation. We expect that the last residue Ile-4 will firstly leave the pore at the beginning of the recovery, and then the buried residue Trp-3 will dissociate slowly with the Ile-323, which results in a slow recovery process. For construct FIW, the most hindered residue Trp-4 dissociates quickly due to the exposure to solvent and less interaction with the channel, and the residues Phe-2 and Ile-3 leave simultaneously to show monoexponential recovery process. This analysis may also explain why the complex mSlo1-FWQ shows identical recovery dynamics to that of the mSlo1-hJ2 complex. Because the glutamine (Gln) in the construct FWQ is a hydrophilic residue, the residue Trp-3 becomes solvent-exposed and so leads to a single exponential recovery. In complex I323A-FWI, the residue Ile-323 is replaced with a relatively weaker hydrophobic residue alanine, which leads to a reduced interaction between Trp-3 and Ile-323. Compared with the complex mSlo1-hJ2, the recovery of I323A-FWI shows a slight difference, which may be attributed to the shield effect of a following hydrophobic residue Ile-4.

It is interesting to consider why FWI shows a bi-exponential recovery with a 50:50 ratio. This feature may implicate that FWI has two interacting styles in the pore. At the recovery voltage of $-140$ mV with $10 \muM Ca^{2+}$, the $\sim 50\%$ of FWI may be trapped in a fast dissociating sub-state, which was not indicated in the present simulations of interaction between mSlo and hJ2 subunits (Fig. 9), and the remaining half in a slow dissociating sub-state to lead to a bi-exponential recovery with a ratio of about 50:50. However, FIW has only one stable interaction style. The reason for that is probably that the rhombus-like FW1 acts in an “unstable” style with two sub-states, but the cone-like FIW acts in a “stable” style with one state. Actually, the wide-type rJ2 (FIW) in rat chromaffin cells showed a much weaker bi-exponential recovery with a ratio of, on average, 25 (fast):75 (slow) at voltages more positive than $-100$ mV, although the bi-exponential recovery of hJ2 (FWI) is extraordinarily rare. Obviously, more experiments are needed to verify whether the bi-exponential phenomena are ubiquitous in inactivating BK channels.

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$^6$ J. Ding, unpublished data.
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