Mechanisms of Two Modulatory Actions of the Channel-binding Protein Slob on the *Drosophila* Slowpoke Calcium-dependent Potassium Channel

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Slob57 is an ion channel auxiliary protein that binds to and modulates the *Drosophila* Slowpoke calcium-dependent potassium channel (dSlo). We reported recently that residues 1–39 of Slob57 comprise the key domain that both causes dSlo inactivation and shifts its voltage dependence of activation to more depolarized voltages. In the present study we show that removal of residues 2–6 from Slob57 abolishes the inactivation, but the ability of Slob57 to rightward shift the voltage dependence of activation of dSlo remains. A synthetic peptide corresponding in sequence to residues 1–6 of Slob57 blocks dSlo in a voltage- and dose-dependent manner. Two Phe residues and at least one Lys residue in this peptide are required for the blocking action. These data indicate that the amino terminus of Slob57 directly blocks dSlo, thereby leading to channel inactivation. Further truncation to residue Arg[16] eliminates the modulation of voltage dependence of activation. Thus these two modulatory actions of Slob57 are independent. Mutation within the calcium bowl of dSlo greatly reduces its calcium sensitivity (Bian, S., I. Favre, and E. Moczydlowski. 2001. *Proc. Natl. Acad. Sci. USA.* 98:4776–4781). We found that Slob57 still causes inactivation of this mutant channel, but does not shift its voltage dependence of activation. This result confirms further the independence of the inactivation and the voltage shift produced by Slob57. It also suggests that the voltage shift requires high affinity Ca^{2+} binding to an intact calcium bowl. Furthermore, Slob57 inhibits the shift in the voltage dependence of activation of dSlo evoked by Ca^{2+}, and this inhibition by Slob57 is greater at higher free Ca^{2+} concentrations. These results implicate distinct calcium-dependent and -independent mechanisms in the modulation of dSlo by Slob.

INTRODUCTION

The modulation of neuronal membrane excitability is critical for the regulation of neural circuits, and ultimately of behavior. Neuromodulation can result from changes in the properties of membrane ion channels, often as a result of their interactions with auxiliary subunits and signaling proteins that can influence channel function. Among the ion channels that are subject to such regulation is the large conductance calcium-dependent potassium (BK) channel $K_{Ca}1.1$, whose activity is controlled by both the membrane potential and intracellular free calcium. Although calcium binding is not necessary to activate the BK channel, it facilitates the response of the channel to membrane depolarization (Meera et al., 1996; Cui et al., 1997; Cox et al., 1997; Braun and Sy, 2001).

The *Drosophila* slowpoke BK channel (dSlo) is involved directly in the excitability of neurons and muscle cells, and plays important roles in many physiological phenomena, including neurotransmitter release and

muscle contraction (Warbington et al., 1996; Atkinson et al., 2000). Like other members of the BK channel family, dSlo contains a large carboxyl-terminal tail region (Atkinson et al., 1991; Adelman et al., 1992) that is critical for regulating channel activity. This tail domain contains binding sites for calcium (Bian et al., 2001) and modulatory proteins (Schopperle et al., 1998; Xia et al., 1998), and can be phosphorylated by several protein kinases (Wang et al., 1999). Among the modulatory proteins that bind to the dSlo channel is Slob, a protein discovered by a yeast two-hybrid screen using the entire carboxyl-terminal tail region of dSlo as bait (Schopperle et al., 1998). There are several Slob proteins, resulting from alternative splicing and multiple translational start sites (Jaramillo et al., 2006), that modulate dSlo channel activity in different ways (Zeng et al., 2005a).

Interestingly, Slob mRNA (McDonald and Rosbash, 2001; Claridge-Chang et al., 2001; Ceriani et al., 2002; Lin et al., 2002; Ueda et al., 2002) and protein (Jaramillo et al., 2004) cycle in a circadian manner in vivo, and different Slob variants are expressed in different subsets of neurons (Jaramillo et al., 2006) that are hypothesized

Abbreviations used in this paper: BK channel, large conductance, voltage-gated, calcium-dependent potassium channel ($K_{Ca}1.1$); dSlo, *Drosophila* Slowpoke calcium-dependent potassium channel.
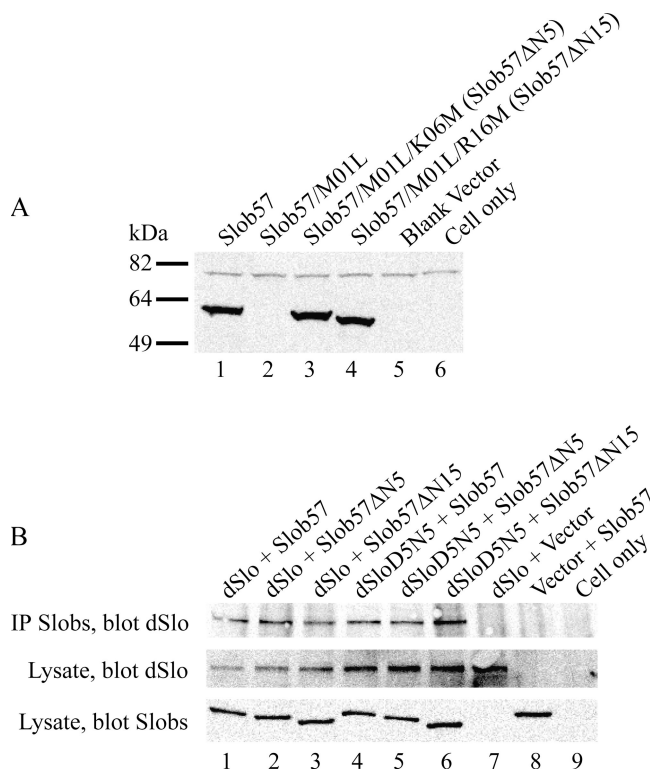
Figure 1. Expression of Slob57ΔN5 and Slob57ΔN15 and coimmunoprecipitation with dSlo or dSloD5N5. (A) Slobs were transfected into CHO cells. Slob expression was analyzed in cell lysates with anti-Slob antibody. Lane 1, wild-type Slob57; lane 2, Slob57 with Met[1] to Leu mutation; lane 3, Slob57 with Met[1] to Leu and Lys[6] to Met mutations (Slob57ΔN5); lane 4, Slob57 with Met[1] to Leu and Arg[16] to Met mutations (Slob57ΔN15); lane 5, cells transfected with blank vector; lane 6, nontransfected cells. (B) Different Slobs were cotransfected into CHO cells together with dSlo or dSloD5N5. Cell lysates were then incubated with anti-Slob antibody and immunoprecipitated by protein A/G agarose beads. Coimmunoprecipitated proteins were assayed by Western blot with anti-dSlo antibody (top). Slobs and dSlo in cell lysates were analyzed for expression level by Western blot with anti-Slob or anti-dSlo antibody (bottom two panels). dSlo cotransfected with Slob57 (lane 1), with Slob57ΔN5 (lane 2), or with Slob57ΔN15 (lane 3). dSloD5N5 cotransfected with Slob57 (lane 4), with Slob57ΔN5 (lane 5), or with Slob57ΔN15 (lane 6). Lane 7, dSlo cotransfected with blank vector used for Slobs; lane 8, Slob57 cotransfected with blank vector used for dSlo channels; lane 9, nontransfected CHO cells.

to participate in the generation of circadian rhythms (Helfrich-Forster, 2003). In view of the emerging evidence that membrane excitability is a critical determinant of circadian rhythmicity (Nitabach et al., 2002; Pennartz et al., 2002; Cloues and Sather, 2003), these findings raise the intriguing possibility that Slob plays an important role in the generation and/or regulation of circadian behavior.

We demonstrated previously that Slob can have several distinct modulatory actions on dSlo, and that the amino terminus of Slob57, the predominant slob variant, is critical for the modulation (Zeng et al., 2005a).

In the present study, we used serial truncations from the amino terminus of Slob57, as well as a peptide corresponding in sequence to the first six amino acids of Slob57, to dissect further the molecular determinants of Slob modulation of the dSlo channel.

MATERIALS AND METHODS

Constructs

Slob57 cDNA constructs used in this study were described previously (Zeng et al., 2005a). Site-directed mutagenesis was performed with the QuikChange site-directed mutagenesis kit, according to the manufacturer's specifications (Stratagene). All Slob constructs and site-directed mutations were confirmed by DNA sequencing. The wild-type dSlo was as described previously (Schopperle et al., 1998), and the mutated channel dSloD5N5 (Bian et al., 2001) was provided by E. Moczydlowski (Clarkson University, Potsdam, NY).

Cell Culture and Western Blot

CHO cells were used to heterologously express dSlo and Slob. In brief, CHO cells were maintained in Ham's F-12 nutrient mixture (Invitrogen) containing 10% FBS (Invitrogen) and 100 U/ml penicillin and streptomycin (Invitrogen). Plasmids were transfected into CHO cells using Lipofectamine 2000 reagent (Invitrogen). 1 d after transfection, cells were harvested, washed, and lysed in lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM NaF, 50 mM KCl, 1% CHAPS, 1 mM phenylmethylsulfonyl fluoride, 2 mM DTT, 1 mg/ml each aprotinin, leupeptin, and pepstatin A). Supernatants of the cell lysates, separated by centrifugation at 16,000 g for 10 min, were used for analysis of protein expression, or were incubated for 2 h with anti-Slob antibody with rotation at 4°C for immunoprecipitation. After this incubation, protein A/G agarose beads were added, and the incubation was allowed to proceed with rotation at 4°C overnight to precipitate the immunocomplex. Beads were then isolated by centrifugation at 5,000 g for 10 min. After four washes with PBS, the beads were used for Western blot analysis.

Proteins in the cell lysates or on the beads were solubilized in SDS loading buffer as described previously (Zeng et al., 2004) and loaded onto 4–15% gradient or 7.5% SDS polyacrylamide gels (Bio-Rad Laboratories), separated by gel electrophoresis, and transferred to nitrocellulose membranes for Western blotting. After blocking in TBST (Tris-HCl–buffered saline with 0.1% Tween 20) containing 5% nonfat milk, membranes were incubated with primary antibody (Schopperle et al., 1998) for 1 h at room temperature. After washes with TBST, membranes were incubated with horseradish peroxidase–conjugated donkey anti-rabbit antibody at room temperature for 1 h. Labeled proteins were visualized using an enhanced chemiluminescence reagent according to the manufacturer's specifications (GE Healthcare), and the images were acquired with a Kodak Image Station 4000R and Kodak Imaging Software (version 4.0).

Peptide Synthesis

The Slob57 amino-terminal peptide MFKFNK and all other synthetic peptides described in the text were synthesized at the Tufts University Core Facility.

Electrophysiology

Whole-cell recording used the same procedures as described previously (Zeng et al., 2005a). In brief, CHO cells were transiently transfected with different Slob and/or different dSlo cDNAs in a 1:1 molar ratio using Lipofectamine 2000 following the manufacturer's instructions (Invitrogen). 1 d after transfection, whole cell
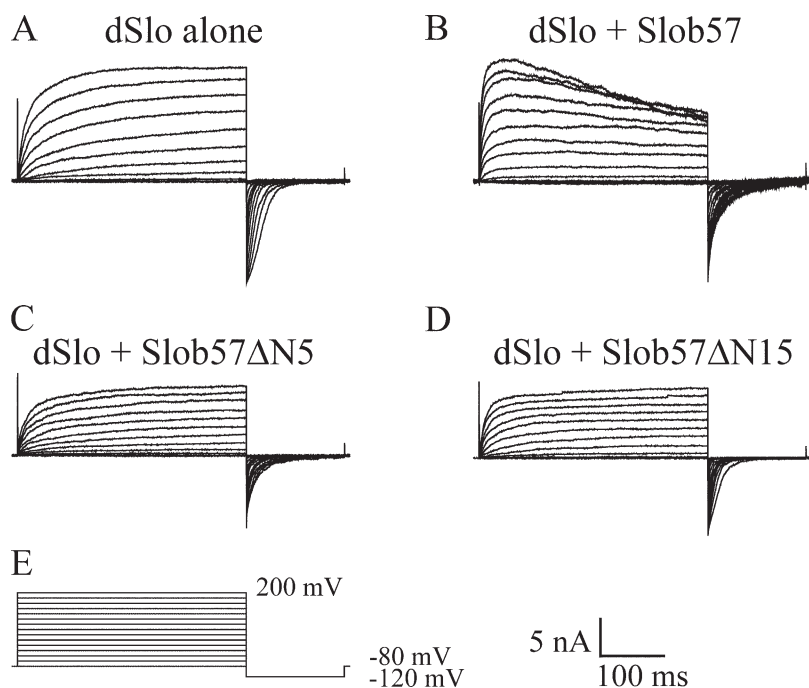
A   dSlo alone

B   dSlo + Slob57

C   dSlo + Slob57ΔN5

D   dSlo + Slob57ΔN15

E

200 mV

-80 mV
-120 mV

5 nA

100 ms

Figure 2. The first six amino-terminal residues of Slob57 are essential for the inactivation of dSlo. Whole cell currents in cells transfected with dSlo and Slob57 or its mutants were evoked, in the presence of 110 μM free Ca^{2+}, by a 350-ms test pulse to different voltages from a holding potential of −80 mV, followed by hyperpolarization to −120 mV. (A) dSlo alone, (B) coexpression of dSlo and Slob57, (C) coexpression of dSlo and Slob57ΔN5, (D) coexpression of dSlo and Slob57ΔN15, (E) pulse protocol.

currents were recorded at room temperature with an Axopatch 200A amplifier (Axon) and an Axiovert 25 inverted fluorescence microscope (Carl Zeiss MicroImaging, Inc.). Glass pipettes with resistances of 2–4 MΩ were pulled from thinwall glass (WPI) using a two-stage vertical puller (Narishige). Both the bath and pipette solutions contained (in mM) 150 KCl, 0.5 HEDTA, 2 $MgCl_2$, and 10 HEPES, pH 7.2 (symmetrical conditions). The pipette solution also contained different amounts of free Ca^{2+} (20, 40, 80, 110, or 300 μM). The final concentration of free Ca^{2+} was measured as described previously (Zeng et al., 2005a).

Inside-out patch recording used the following conditions unless noted otherwise. Patches were held at −80 mV, and then stepped to +140 mV. Current was measured by taking the mean amplitude of the current during the last 10 ms of the pulse. Each patch served as its own control before peptide application. The bath and pipette solutions both contained (in mM): 150 KCl, 0.5 HEDTA, 1 Mg^{2+}, 10 HEPES, pH 7.2, and 7 μM free Ca^{2+}. The free calcium concentration was measured as described above. The MFKFNK peptide and its variants were dissolved in the bath solution. All experiments were performed at room temperature. Data are expressed as mean ± SEM, and were compared with a One Way Anova followed by Bonferroni's multiple comparison test.

To generate the dose–response curve for the Slob57 amino-terminal peptide MFKFNK, inside-out patches were depolarized to +140 mV from a holding potential of −80 mV. Current amplitude was normalized to that in the same patch before peptide application. Data points at various concentrations of MFKFNK were fitted with the Hill equation: $Y = 1 - ([x^H]/[x^H + IC_{50}^H])$, where H is the Hill coefficient and IC_{50} is the half maximal concentration.

Woodhull Model

Inside-out patches were held at −80 mV and depolarized in steps of 20 mV from +40 to +120 mV. Peak current amplitude was measured before and after application of 100 μM MFKFNK. Data were fitted to the Woodhull model: $Y = 1/((1 + 100)/K_d0*exp(z*\delta*X*(F/RT)))$, where $K_d0 = K_d$ at 0 mV, z = number of charges, δ = effective electrical distance, and F, R, and T have their standard meanings (Woodhull, 1973).

Amino-terminal Truncations of Slob57 Alter its Modulatory Actions

To determine the roles of different parts of the Slob57 amino-terminal domain in the modulation of dSlo, we first mutated Met[1] of Slob57 to Leu, which expresses no Slobs (Fig. 1 A, lane 2). We then mutated Lys[6] or Arg[16] to Met in this background, to express the amino-terminal five residue–truncated (deletion of residues 2–6) Slob57ΔN5 (Fig. 1 A, lane 3) or the amino-terminal 15 residue–truncated (deletion of residues 2–16) Slob57ΔN15 (Fig. 1 A, lane 4). Both Slob57ΔN5 and Slob57ΔN15 express well, with no expression of full-length Slob57, and these two truncated Slob57 proteins still bind to dSlo (Fig. 1 B, lanes 2 and 3). As we demonstrated previously (Zeng et al., 2005a), Slob57 causes inactivation of dSlo (Fig. 2, compare A with B). When Slob57 is replaced by Slob57ΔN5, no inactivation is observed (Fig. 2 C). Similarly, dSlo coexpressed with Slob57ΔN15 does not inactivate (Fig. 2 D). These results clearly implicate amino acid residues 2–6 of Slob57 in the inactivation of dSlo.

An Amino-terminal Peptide Derived from Slob57 Inhibits dSlo Current

To further study the inactivation of dSlo by Slob57, we used a synthetic peptide corresponding in sequence to the first six residues of Slob57 (M[1]FKFNK[6]) in inside-out patches. In the presence of 100 μM MFKFNK on the cytoplasmic side of the channel, current amplitude is reduced substantially and a marked increase in noise is observed (Fig. 3 A). The inhibition by the peptide is
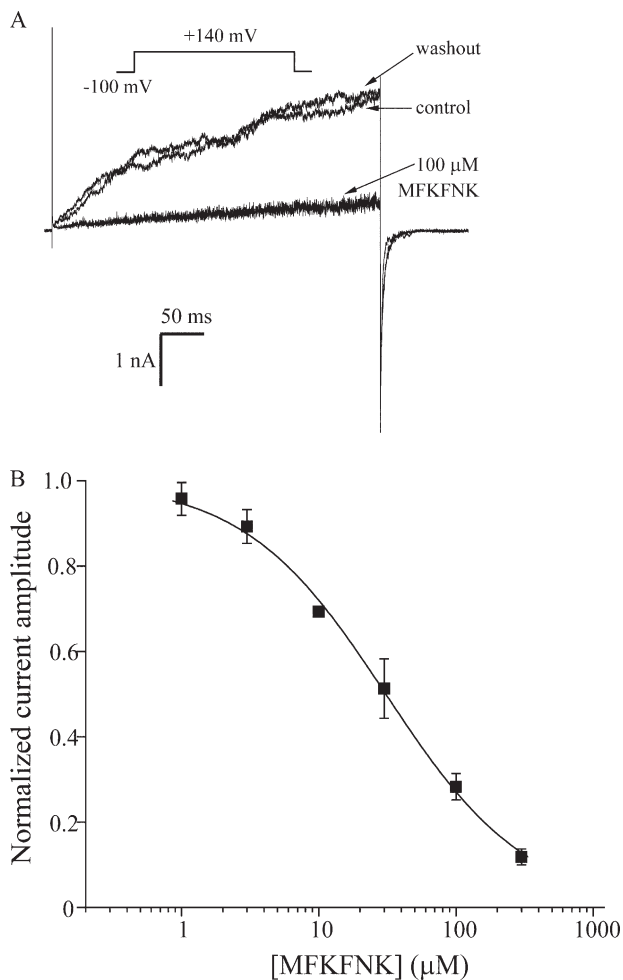
Figure 3. The peptide MFKFNK blocks dSlo. (A) dSlo current traces from an inside-out patch before and during application of 100 μM MFKFNK (note the increase in noise in the current trace), and after wash as indicated in the figure. (B) MFKFNK blocks dSlo in a dose-dependent manner. Current amplitudes in the presence of different concentrations of MFKFNK were normalized to that in its absence. The data were fit with the Hill equation as described in Materials and methods. IC_{50} is 31 μM, and the Hill coefficient is 0.84 ($n = 3$). Error bars illustrate SEM.

Figure 4. MFKFNK inhibits dSlo in a voltage-dependent manner, and independent of internal Mg^{2+} or external K^+ concentrations. (A) The dSlo current amplitude in the presence of 100 μM MFK-FNK was normalized to that in the same patch before addition of the peptide. The normalized current was plotted against voltage and analyzed using the Woodhull model as described in Materials and methods. The x axis represents the voltage to which the cell was stepped from the holding potential of −80 mV. The K_d at 0 mV is 162 μM and the effective electrical distance δ is 0.17 ($n = 7$). (B) Normalized current amplitude of dSlo at +140 mV in the presence of 100 μM MFKFNK in control solutions, 0 Mg^{2+} internal solution, or 5 mM K^+ external solution are shown ($n = 3$).

immediate and is not use dependent (not depicted); it is fully reversible upon washing (Fig. 3 A). The dose–response relation for the inhibition by MFKFNK is shown in Fig. 3 B. An IC_{50} value of 31 μM and a Hill coefficient of 0.84 were obtained from a fit of the data to the Hill equation (Fig. 3 B). In addition, the inhibition of dSlo current by MFKFNK is enhanced at higher voltages (Fig. 4 A). These data were fit with the Woodhull model (Woodhull, 1973), and a K_d of 162 μM at 0 mV and an effective electrical distance δ of 0.17 were obtained (Fig. 4 A). Elimination of magnesium ions in the bath solution or reducing the extracellular potassium concentration from 150 to 5 mM have no effect on the inhibition of current by MFKFNK (Fig. 4 B). When either of the two Phe residues in the peptide is substituted by Ala, the blocking
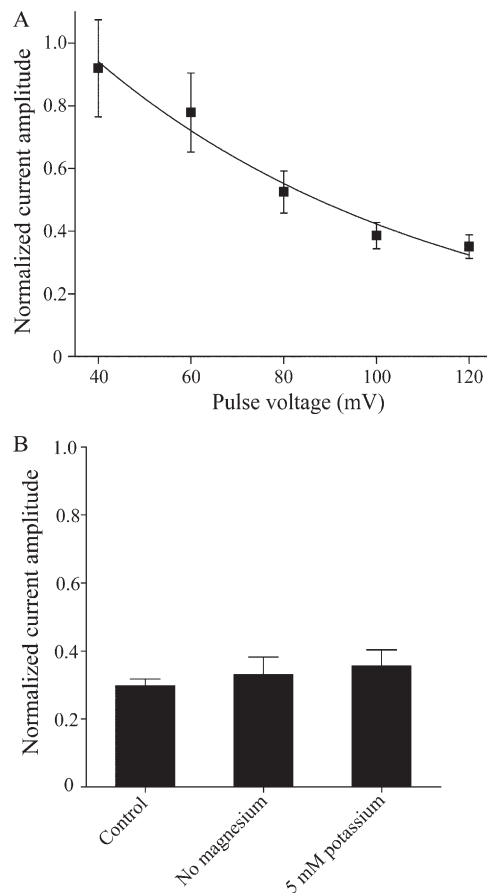
effect is abolished completely (Fig. 5). Substitution of Phe^2 with Tyr also eliminates the inhibition by the peptide. Furthermore, at least one of the two Lys residues is required to obtain some inhibition of dSlo current, and the inhibition is greater when both are present. Finally, the exchange of the positions of Phe^2 and Lys^3 significantly reduces the inhibition by the peptide (Fig. 5).

Amino Acid Residues 7–16 Contribute to the Modulation of the Voltage Dependence of Activation of dSlo

Because the inactivation of dSlo complicates measurement of the conductance–voltage relationship from tail currents, we used a shorter, 100-ms depolarizing pulse to measure the voltage dependence of activation of dSlo, as described previously (Zeng et al., 2005a). Under these conditions, Slob57ΔN5 causes a rightward
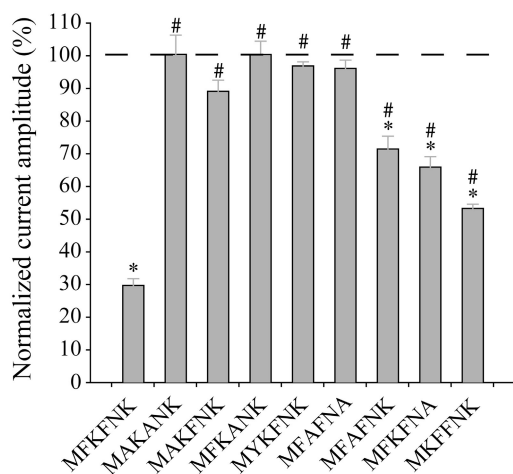
Figure 5. A specific sequence is required for peptide block of dSlo. Current amplitude of dSlo at +140 mV with 100 μM MFKFNK or its variants (as indicated in the figure) was normalized to control current in the same patch before peptide application ($n = 5$–6). *, significantly different from control (100%, dashed line), P < 0.05; #, significantly different from wild type MFKFNK, P < 0.05.
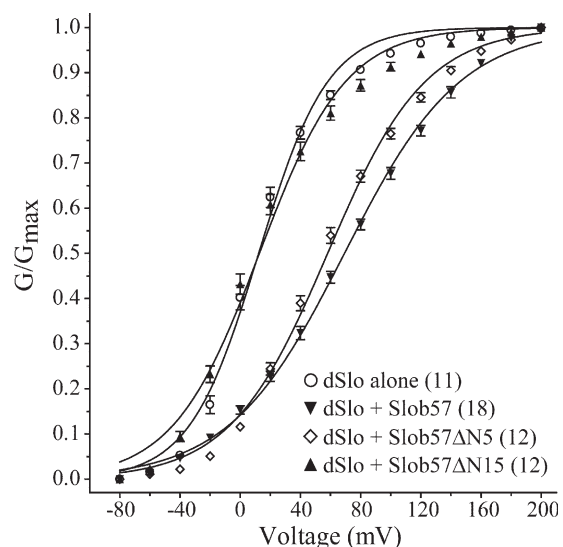
Figure 6. Conductance–voltage relationships for dSlo in the presence of Slob57 or its N-terminal truncation mutants. The conductance–voltage relationships for dSlo transfected alone (◯) or together with Slob57 (▼), Slob57ΔN5 (◇), or Slob57ΔN15 (▲) were measured from tail currents with 110 μM free Ca^{2+}. To prevent dSlo inactivation caused by Slob57 in longer test pulses, a 100-ms test pulse was used (see Results). The conductance–voltage relationship was generated from peak tail currents as described in Materials and methods. The number of experiments is shown in parentheses, and the error bars illustrate SEM. Some of the error bars are so small that they are masked by the symbols.

shift of the voltage dependence of activation of dSlo, as does wild-type Slob57 (Fig. 6). However, upon further truncation to residue Arg^{16} of Slob57, the resulting Slob57ΔN15 no longer rightward shifts the conductance–voltage relationship of dSlo (Fig. 6), even though it still binds to dSlo (Fig. 1 B).

Ca^{2+} Dependence of Slob57 Modulation of the Voltage Dependence of Activation of dSlo

Ca^{2+} plays a fundamental role in regulating the dSlo response to membrane depolarization. One of the three known Ca^{2+} binding sites (Xia et al., 2002; Bao et al., 2002; Zeng et al., 2005b), known as the calcium bowl (Schreiber and Salkoff, 1997), contributes to the high-affinity Ca^{2+} sensitivity of the channel (Schreiber and Salkoff, 1997; Schreiber et al., 1999; Braun and Sy, 2001; Bao et al., 2002; Niu and Magleby, 2002; Xia et al., 2002; Bao et al., 2004; Zeng et al., 2005b). We used a mutated dSlo channel with substitution of Asp^{966}-Asp^{970} by Asn^{966}-Asn^{970} within its calcium bowl, which greatly reduces the channel's calcium sensitivity (Bian et al., 2001), to determine whether Ca^{2+} participates in Slob57 modulation of dSlo. This mutated channel, dSloD5N5, still binds to full-length and truncated Slobs (Fig. 1 B, lanes 4–6). During a 350-ms test pulse (Fig. 7 E), Slob57 causes dSloD5N5 inactivation (Fig. 7 B), consistent with our previous finding that the inactivation caused by Slob57 is Ca^{2+} independent (Zeng et al., 2005a). After deletion of the amino-terminal residues 2–6 of Slob57, the inactivation of dSloD5N5 is eliminated (Fig. 7 C), and further truncation of Slob57 to Arg^{16} also eliminates the inactivation (Fig. 7 D), just as observed with wild-type dSlo (Fig. 2, C and D).

We next used a 100-ms test pulse (during which no inactivation occurs) to measure the voltage dependence of activation of dSloD5N5 in the presence of 110 μM free Ca^{2+}, as only at high concentrations of free Ca^{2+} can tail current saturation be achieved with the mutant channel (Fig. 8). As shown in Fig. 8, Slob57 does not rightward shift the conductance–voltage relationship of dSloD5N5. Because dSloD5N5 can still respond to an increment of free Ca^{2+} at high concentration (Bian et al., 2001), we then increased the concentration of free Ca^{2+} to 300 μM. As shown in Fig. 8, the conductance–voltage relationship of dSloD5N5 shifts to less depolarized voltages when the concentration of free Ca^{2+} is increased from 110 to 300 μM, but again the $V_{1/2}$ is not modulated by Slob57.

We next measured the voltage dependence of activation of wild-type dSlo, in the presence or absence of Slob57, at free Ca^{2+} concentrations ranging from 20 to 300 μM. As shown in Fig. 9 A (and by many previous investigators), in the absence of Slob57, the voltage–conductance relationship of dSlo shifts to less depolarized voltages as the free Ca^{2+} concentration is increased in this range. The $V_{1/2}$ decreases from 85 to −22 mV, a change of 107 mV, when the concentration of free Ca^{2+} is increased from 20 to 300 μM. In contrast, when dSlo is coexpressed with Slob57, this increment of free Ca^{2+} concentration shifts the $V_{1/2}$ to

A dSloD5N5 alone

B dSloD5N5 + Slob57

C dSloD5N5 + Slob57ΔN5

D dSloD5N5 + Slob57ΔN15

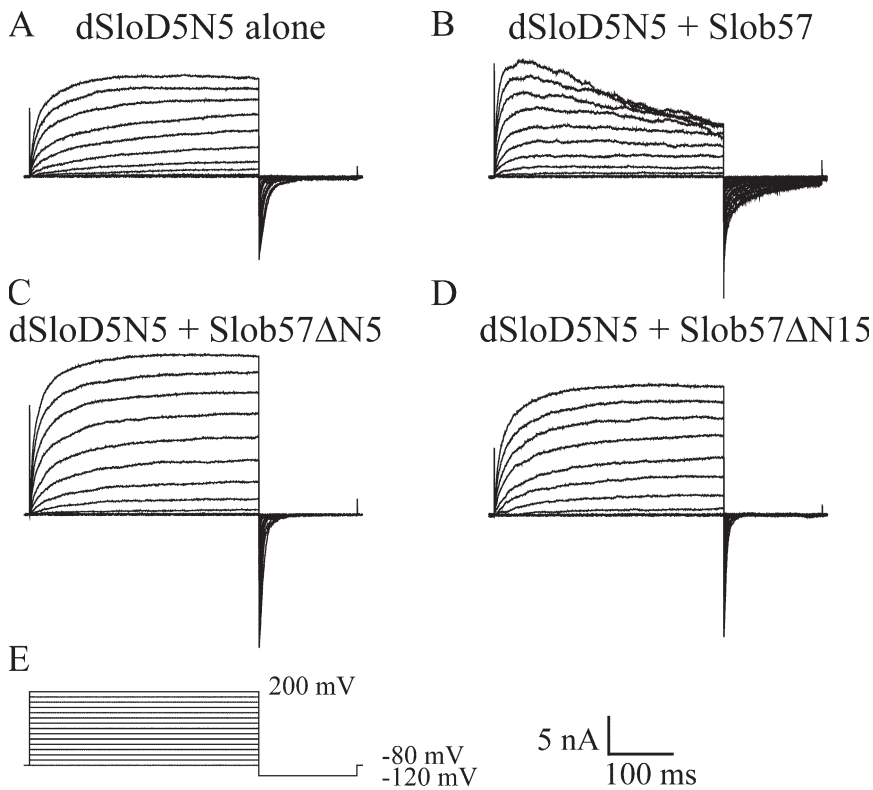
E

200 mV

-80 mV
-120 mV

5 nA

100 ms

Figure 7. Slob57 causes inactivation of dSloD5N5. Same as Fig. 2, except that the mutated channel dSloD5N5 was used instead of wild-type dSlo. (A) dSloD5N5 alone, (B) dSloD5N5 together with Slob57, (C) dSloD5N5 together with Slob57ΔN5, (D) dSloD5N5 together with Slob57ΔN15, (E) pulse protocol.

a much lesser extent (Fig. 9 B and Fig. 10 A). Note also that the effect of Slob57 on the $V_{1/2}$ is more apparent at higher free Ca^{2+} concentrations (Fig. 10 A). To illustrate this better, we plotted the $V_{1/2}$ change evoked by Slob57 ($\Delta V_{1/2}$) as a function of the free Ca^{2+} concentration (Fig. 10 B) to demonstrate that the magnitude of the modulatory shift in $V_{1/2}$ evoked by Slob57 itself is calcium dependent.

DISCUSSION

We reported recently that the amino-terminal region of Slob57 plays critical roles in rightward shifting the voltage dependence of activation of dSlo, and in causing dSlo inactivation (Zeng et al., 2005a). This is likely to be of physiological significance, because multiple Slobs that differ in their amino termini are present in flies as a result of both alternative splicing and the use of different transcriptional start sites (Jaramillo et al., 2006). Here, we report that the two modulatory actions of Slob57 are independent, as residues 2–6 of Slob57 contribute directly to the inactivation of dSlo, while residues 7–16 participate in shifting the channel's voltage dependence of activation. Studies with synthetic peptides are consistent with the hypothesis that residues 2–6 of Slob57 cause the voltage-dependent inactivation of dSlo. Furthermore, Slob57 greatly inhibits the leftward shift of the voltage dependence of activation of dSlo caused by an increase of free Ca^{2+}, and mutation of the high affinity calcium-binding site in dSlo's calcium bowl

reveals that Slob57 does not shift the voltage dependence of activation of this mutated channel. These results imply that Slob57 modulates the coupling between the dSlo voltage sensor and Ca^{2+} binding to the calcium bowl, and indicate that Slob57 has both calcium-dependent and -independent modulatory actions on dSlo.
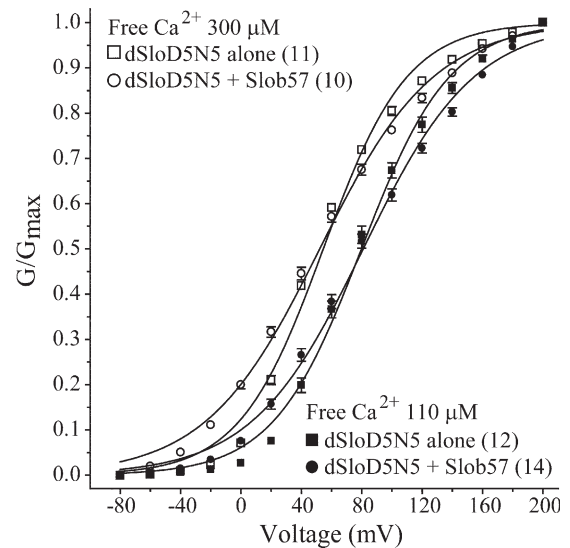

Figure 8. Conductance–voltage relationships for dSloD5N5. Same protocol as Fig. 6, except that the mutated channel dSloD5N5 was used in the presence (circles) or absence (squares) of Slob57, and the concentration of free Ca^{2+} was either 110 μM (filled symbols) or 300 μM (open symbols).
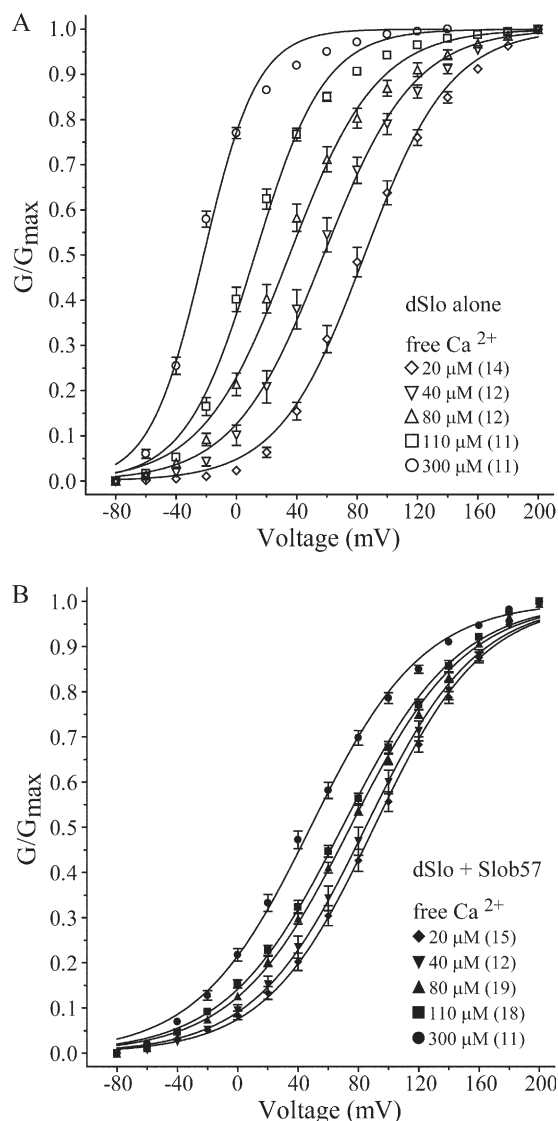
Figure 9. Effect of calcium on dSlo voltage dependence of activation in the absence or presence of Slob57. Same as Fig. 6, except that the measurements of dSlo expressed alone (A) or together with Slob57 (B) were performed in the presence of different concentrations of free Ca^{2+}: 20 μM (diamonds), 40 μM (inverted triangles), 80 μM (triangles), 110 μM (squares), and 300 μM (circles).

Some mammalian BK channels exhibit inactivation, characterized by trypsin sensitivity and Ca^{2+} dependence (Solaro and Lingle, 1992; Hicks and Marrion, 1998; Li et al., 1999; Ding and Lingle, 2002; Pyott et al., 2004), that is caused by auxiliary β subunits (Wallner et al., 1999; Uebele et al., 2000; Xia et al., 1999, 2000, 2003). The amino terminus of the mammalian β2 subunit may directly cause channel inactivation by blocking the channel (Xia et al., 2003). Similarly, dSlo expressed alone does not inactivate but shows inactivation in the presence of Slob57. Our truncation study indicates that the amino-terminal residues 2–6 (FKFNK) of Slob57 contribute directly to the inactivation of dSlo. Upon removal
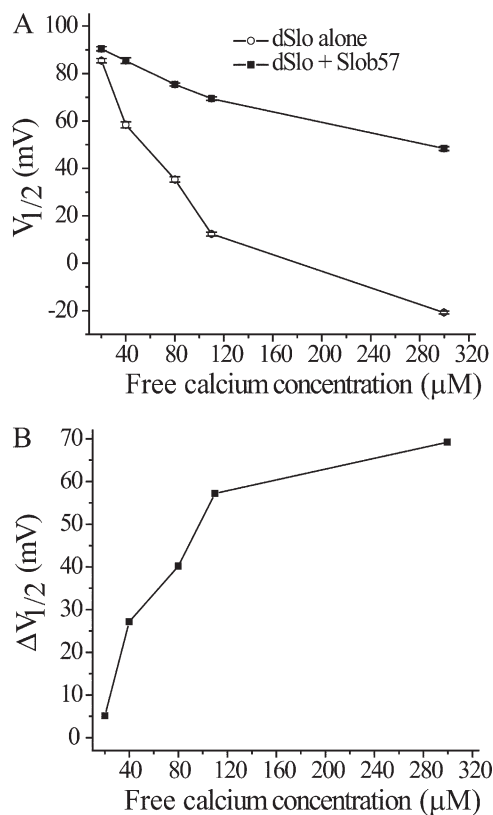

Figure 10. Interaction between Slob57 and calcium. The $V_{1/2}$ (A) of the conductance–voltage relationship for dSlo expressed alone (O) or together with Slob57 (■), taken from Fig. 9, and the difference in $V_{1/2}$ ($\Delta V_{1/2}$) evoked by Slob57 (B) are plotted as a function of the concentration of free Ca^{2+}.

of these five residues, the resulting Slob57ΔN5 still binds to dSlo but no longer causes channel inactivation.

The amino-terminal region of Slob57 contains both hydrophobic and positively charged residues, unlike the uniformly hydrophobic residues in this region of the mammalian β2 subunit (Xia et al., 2003). The peptide MFKFNK, corresponding in sequence to the amino-terminal 1–6 residues of Slob57, blocks dSlo from the cytoplasmic side in a voltage-dependent manner. The δ value of 0.17 obtained by Woodhull model fitting indicates that the peptide senses ~17% of the electrical field across the cell membrane. Neither removal of magnesium from the cytoplasmic side of the membrane nor lowering the potassium concentration on the extracellular side affects the block of dSlo by this peptide. This indicates that the blocking mechanism is neither C-type related nor magnesium dependent. Changes in the peptide sequence eliminate some or all of its blocking action. To obtain some block, at least one charged Lys residue and two hydrophobic Phe residues are required. Exchanging the positions of Phe[2] and Lys[3] attenuates the block by the peptide, and replacement of Phe[2] by Tyr completely abolishes the block. These results suggest that a particular conformation of the

amino terminus of Slob57 is required for it to cause inactivation of dSlo.

In the mutated channel dSloD5N5, the five Asp residues in its calcium bowl are substituted by five Asn residues (Bian et al., 2001), thereby eliminating one of the three Ca^{2+} binding sites in the carboxyl-terminal region of the channel (Zeng et al., 2005b). The calcium bowl contributes a large portion of the channel's high-affinity Ca^{2+} binding, and thus dSloD5N5 responds little to increases in the free Ca^{2+} concentration (Bian et al., 2001), similar to other BK channels with mutated calcium bowls (Schreiber and Salkoff, 1997; Braun and Sy, 2001; Bao et al., 2002; Niu and Magleby, 2002; Xia et al., 2002; Zeng et al., 2005b). We found that Slob57 still causes inactivation of dSloD5N5, in agreement with the idea that the inactivation caused by Slob57 is Ca^{2+} independent (Zeng et al., 2005a).

Several lines of evidence are consistent with the hypothesis that Slob57 shifts the voltage dependence of activation of dSlo by modulating the coupling between the channel's voltage sensor and high affinity Ca^{2+} binding to the calcium bowl. First, Slob57 does not influence the voltage dependence of activation of dSloD5N5, indicating the requirement for an intact calcium bowl for this modulatory effect. In contrast, the natural product mallotoxin modulates BK channels independent of Ca^{2+} binding, and its effects are similar on both wild type and calcium bowl mutant channels (Zakharov et al., 2005). Second, Slob57 clearly inhibits the shift in dSlo voltage dependence of activation normally evoked by increasing calcium concentrations, particularly in the concentration range at which Ca^{2+} binds to the calcium bowl (Schreiber et al., 1999). Finally, the extent of modulation of channel voltage dependence of activation by Slob57 is itself calcium dependent, most steeply so in the same high affinity binding concentration range, implying that this results from Ca^{2+} binding to the calcium bowl. It is notable that the voltage dependence of activation of dSloD5N5 can still be shifted by high concentrations of Ca^{2+}, via binding to lower affinity sites distinct from the calcium bowl (Schreiber et al., 1999; Bian et al., 2001; Bao et al., 2004; Zeng et al., 2005b). The fact that Slob57 does not modulate dSloD5N5, even at these very high Ca^{2+} concentrations, suggests that Slob57 discriminates among Ca^{2+} binding sites and selectively affects some step in the pathway that couples the channel voltage sensor with Ca^{2+} binding to the calcium bowl.

dSlo is dually regulated by the membrane potential and free intracellular Ca^{2+}. It is essential for dSlo to sense and correspondingly respond to changes of intracellular Ca^{2+} concentration. By modulating the coupling between Ca^{2+} binding to the calcium bowl of dSlo and the voltage sensor, Slob57 strongly inhibits the response of dSlo to membrane depolarization. Inactivation of the channel via voltage-dependent block by the amino terminus of Slob57 further dampens dSlo activity. Cycling of Slob57 protein in a circadian manner in vivo (Jaramillo et al., 2004) suggests that this complex regulation is a dynamic process that may play important physiological roles in neuronal function.

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