Interaction of Charybdotoxin S10A with Single Maxi-K Channels: Kinetics of Blockade Depend on the Presence of the β1 Subunit†

Kathleen M. Giangiacomo,*,‡ Valerie Fremont,‡ Theodore J. Mullmann,‡ Markus Hanner,§ Robert H. Cox,|| and Maria L. Garcia§

Department of Biochemistry, Temple University School of Medicine, 3420 North Broad Street, Philadelphia, Pennsylvania 19140, Department of Membrane Biochemistry and Biophysics, Merck Research Laboratories, Rahway, New Jersey, and Department of Physiology, The University of Pennsylvania, Philadelphia, Pennsylvania

Received December 14, 1999; Revised Manuscript Received March 9, 2000

ABSTRACT: The maxi-K channel from bovine aortic smooth muscle consists of a pore-forming α subunit and a regulatory β1 subunit that modifies the biophysical and pharmacological properties of the α subunit. In the present study, we examine ChTX-S10A blocking kinetics of single maxi-K channels in planar lipid bilayers from smooth muscle or from tsA-201 cells transiently transfected with either α or α+β1 subunits. Under low external ionic strength conditions, maxi-K channels from smooth muscle showed ChTX-S10A block times, 48 ± 12 s, that were similar to those expressing α+β1 subunits, 51 ± 16 s. In contrast, with the α subunit alone, ChTX-S10A block times were much shorter, 5 ± 0.6 s, and were qualitatively similar to previously reported values for the skeletal muscle maxi-K channel. Increasing the external ionic strength caused a decrease in ChTX-S10A block times for maxi-K channel complexes of α+β1 subunits but not of α subunits alone. These findings indicate that it may be possible to predict the association of β1 subunits with native maxi-K channels by monitoring the kinetics of ChTX blockade of single channels, and they suggest that maxi-K channels in skeletal muscle do not contain a β1 subunit like the one present in smooth muscle. To further test this hypothesis, we examined the binding and cross-linking properties of [^{125}I]-IbTX-D19Y/Y36F to both bovine smooth muscle and rabbit skeletal muscle membranes. [^{125}I]-IbTX-D19Y/Y36F binds to rabbit skeletal muscle membranes with the same affinity as it does to smooth muscle membranes. However, specific cross-linking of [^{125}I]-IbTX-D19Y/Y36F was observed into the β1 subunit of smooth muscle but not in skeletal muscle. Taken together, these data suggest that studies of ChTX block of single maxi-K channels provide an approach for characterizing structural and functional features of the α/β1 interaction.

The large-conductance, calcium-activated potassium (maxi-K)[1] channel is an integral membrane protein that couples transmembrane ionic flux to physiological stimuli. Both depolarizing membrane potentials and increasing levels of intracellular calcium promote transitions to the open, potassium-permeant channel conformation. Because of this synergistic relationship between calcium and voltage, the maxi-K channel provides an important link between calcium- and voltage- dependent cellular processes.

Recent cloning and biochemical approaches have revealed tremendous insight into the molecular underpinnings of maxi-K channel function. The maxi-K channel purified from either tracheal or aortic smooth muscle consists of a complex of α and β1 subunits in a one to one stoichiometry (*1, 2*). The α subunit is the pore-forming subunit that, as a homotetramer, contains all of the gating and permeation properties associated with functional maxi-K channels (*3*−*5*). It belongs to the *Slo* family of potassium channels (*4*) which contain three functionally important domains: the permeation pore (*6*), the voltage-sensor (*4*−*6*), and the "calcium-bowl" (*7*). The β1 subunit is a regulatory subunit, consisting of two transmembrane segments (*8*), that has been shown to increase the calcium-sensitivity of the α subunit and change its pharmacological properties (*9*). Recently, a new β subunit, β2, has been identified from a BLAST search of expressed sequence tag databases for homology with β1 (*10, 11*). β2 diplays the same topology as β1, enhances the calcium-sensitivity of the α subunit, and confers inactivation properties to the α subunit due to the existence of a specific domain at the N-terminal part of the protein. Maxi-K channels with inactivating properties, such as those expressed in chromaffin cells, appear to consist of α/β2 subunits (*10*). Because of its effects on calcium-dependent gating, the tissue-specific expression of the β subunit likely plays a critical role in defining maxi-K channel function. Indeed, a broad range of calcium-sensitivity has been reported for maxi-K channels (*12*) that may be related to β subunit coexpression.

† This work was supported in part by NIH Grant GM52179.

* To whom correspondence should be addressed (phone: 215-707-8170; fax: 215-707-7536; e-mail: giang@unix.temple.edu).

‡ Temple University School of Medicine.

§ Merck Research Laboratories.

|| The University of Pennsylvania.

[1] Abbreviations: α-KTx, α-K toxin peptides; α-KTx 1.1, charybdotoxin; α-KTx 1.3, iberiotoxin; ChTX, charybdotoxin; [^{125}I]-ChTX, monoiodocharybdotoxin; maxi-K channel, large-conductance calcium-activated potassium channel; IbTX, iberiotoxin; [^{125}I]-IbTX-D19/Y36F, monoiodoiberiotoxin-D19Y/Y36F; K_d, equilibrium dissociation constant; k_{on}, second-order association rate constant; k_{off}, dissociation rate constant; SM, smooth muscle; SK, skeletal muscle; SDS−PAGE, sodium dodecyl sulphate−polyacrylamide gel electrophoresis; T_{block}, time constant for block; TsA-201 cells, a subclone of the human embryonic kidney cell line HEK293 that expresses the SV40 T antigen.

10.1021/bi992865z CCC: $19.00
Published on Web 04/26/2000

The α-K toxin, α-KTx, peptides are invaluable tools for the identification and purification of maxi-K channel complexes of α and α+β subunits. Charybdotoxin (ChTX or α-KTx 1.1) and iberiotoxin (IbTX or α-KTx 1.3) are highly basic peptides that block K^+ current through the maxi-K channel by occluding the extracellular pore of the channel (*13, 14*). Monoiodinated ChTX, [^{125}I]-ChTX, and monoiodinated IbTX-D19Y/Y36F, [^{125}I]-IbTX-D19Y/Y36F, can be covalently incorporated into the β1 subunit using a bifunctional cross-linking reagent (*15, 16*). This covalent linkage occurs through Lys32 in the toxin and Lys 69 in the β1 subunit (*17, 18*). Thus, these radiolabeled toxins can be used to identify and quantitate maxi-K channel complexes of α and α+β subunits.

Another way to determine the presence of the β1 subunit is to monitor the properties of ChTX binding. Under low ionic strength conditions, the equilibrium binding interaction of [^{125}I]-ChTX to membranes expressing maxi-K channel complexes is enhanced ~50-fold by coexpression of α and β1 subunits (*19*). Several residues in the extracellular loop of β1 have been identified as being critical for conferring this high-affinity interaction (*20*).

In the present study, we use the α-KTx peptides ChTX-S10A and [^{125}I]-IbTX-D19Y/Y36F to evaluate the functional expression of maxi-K channel complexes in native membranes or in membranes derived from cells transiently transfected with either α or α+β1 subunits. Our results indicate that the kinetics of ChTX block of single maxi-K channels provide a powerful diagnostic indicator of β1 subunit expression, and they suggest that maxi-K channel complexes expressed in rabbit skeletal muscle do not contain a β1 subunit.

MATERIALS AND METHODS

Materials. Sarcolemmal membranes from bovine aortic smooth muscle (*21*) and from rabbit skeletal muscle (*22*) were prepared as described previously. IbTX-D19Y/Y36F was prepared and iodinated as described (*15*). ChTX-S10A was a generous gift from Dr. Christopher Miller (Brandeis University).

For RT-PCR, RNasin was from Promega and AMV reverse transcriptase from Pharmacia. For PCR of the rabbit β1 clone, Taq Plus Long polymerase was from Stratagene. For mutant constructs of bovine β1, *Pfu* DNA polymerase was from Stratagene. Restriction enzymes were from Promega. pCI vector was obtained from Promega. COS-1 cells (culture CRL 1650) were obtained from the American Type Culture Collection. TsA-201 cells, a subclone of the human embryonic kidney cell line HEK293 that expresses the SV40 T antigen, were a gift of Dr. Robert DuBridge. All tissue culture media and the LipofectAMINE reagent were from Gibco. FuGENE6 transfection was from Boehringer Mannheim. Digitonin special grade (water soluble) was bought from Biosynth Technologies (Skokie, IL). GF/C glass fiber filters were purchased from Whatman.

Delrin cuvettes for bilayer experiments contained either a 100 or a 150 μm aperture and were purchased from Warner Instruments, Inc. (Hamden, CT). 1-Palmitoyl-2-oleoylphosphatidylethanolamine (POPE) and 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) were from Avanti Polar Lipids, Inc. (Birmingham, AL). Decane from Fisher Scientific, Inc. (Springfield, NJ), was 99.9% mole purity. All other reagents were of the highest purity commercially available.

Mutant Channel Constructs. A *Hind*III restriction site was generated in the β1 subunit by altering amino acids Arg191 to Lys. A 9E10 c-myc tag (*23*) was introduced at the C-terminus using an oligonucleotide cassette containing *Hind*III and *Not*I restriction sites. Site-directed mutagenesis was performed using the "Overlap extension: technique" (*24*). Polymerase chain reaction amplification was carried out using P*fu* DNA polymerase. The integrity of all mutant constructs was verified by nucleotide sequencing (automated DNA sequencer, ABI 373). The human α clone huR2(+) and the bovine β clones were subcloned into pCI-neo vector (*19*).

Transfection of TsA-201 and COS-1 Cells and Membrane Preparation. The procedures for handling cells, their transfection with FuGENE6 transfection reagent, and preparation of membrane vesicles have been previously described (*20*). The final membrane pellet was resuspended in 100 mM NaCl, 20 mM Hepes−NaOH, pH 7.4. Aliquots were frozen in liquid N_2 and stored at −70 °C.

Binding Assays. The interaction of [^{125}I]-IbTX-D19Y/Y36F with membranes was measured in a medium consisting of 10 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.1% bovine serum albumin, 0.1% digitonin, 5 μM paxilline. For saturation experiments, membranes were incubated with increasing concentrations of [^{125}I]-IbTX-D19Y/Y36F at room temperature until equilibrium was achieved (e.g., 20 h). Nonspecific binding was determined in the presence of 10 nM ChTX. Separation of bound from free ligand was achieved using filtration protocols as described (*25*).

Cross-Linking Experiments. Rabbit skeletal and bovine aortic smooth muscle membranes were incubated with 9 pM [^{125}I]-IbTX-D19Y/Y36F in a medium consisting of 10 mM NaCl, 20 mM Hepes−NaOH, pH 7.4, 0.04% bovine serum albumin, 0.1% digitonin, 5 μM paxilline, in a total volume of 10.3 mL, for 18 h at room temperature. The amount of specific radioactivity bound in 0.5 mL of reaction media at equilibrium was 10 492 and 4035 cpm for smooth muscle and skeletal muscle membranes, respectively. Membranes were collected by centrifugation, resuspended into 0.5 mL of 200 mM NaCl, 10 mM Taps−NaOH, pH 9.0, and reacted with disuccinimidyl suberate (DSS). Samples were treated as described previously (*15*), and final pellets were resuspended into 300 μL of SDS−PAGE sample buffer. Samples were subjected to SDS−PAGE, and gels were dried and exposed to XAR-film at −70 °C.

Cloning of the β1 Subunit from Rabbit. First-strand cDNA was synthesized from 1 μg of rabbit brain poly A+ RNA (Clontech) using random hexamer primers in a 20 μL reaction. The first round of PCR (PCR1) was performed using 2 μL of this template with the degenerate primers [sense (107−126): AA(A/G)AA(A/G)(T/C)TNGTNATGGCNCA; antisense (584−603): A(A/G)NA(A/G)(A/G)AANGTNGGCCA(A/G)AA] encoding regions of amino acid identity between the bovine, dog, and rat maxi-K β1 subunits (GenBank accession numbers AF26101, U41002, and U79661, respectively). Cycling paramaters were 25 cycles of 94 °C for 1 min, 37 °C for 2 min, and 72 °C for 3 min followed by a final extension of 7 min at 72 °C. Reamplification of the PCR1 product was performed using nested primers [sense (224−243): TA(C/T)CA(A/G)AA(A/G)(A/T)(G/C)NGT-

NTGGAC; antisense (512−531): GT(C/T)TC(A/G)TT(C/T)TCNC(G/T)NGTNGT]. Products of the two PCR reactions were analyzed by Southern blotting using a ^{32}P-labeled random hexamer primed probe derived from bovine trachea cDNA. The fragment that hybridized to the probe was isolated from an agarose gel, cloned into pBluescript II KS+ (Stratagene), and sequenced on both strands by standard techniques (*26*). These experiments yielded an unambiguous 309 bp sequence encoding amino acids 42−144 of the rabbit β1 subunit. This sequence was then used to design nested, gene-specific primers and probes for use in RACE reactions (*27*) to clone the remaining 5′ and 3′ regions of the cDNA. For 5′RACE, the following primers were used: RP1, AGCAGTAGAAAACCTGGC; RP2, GTGGAATTTGGCTCTGAC; and RP3, TGCAGCCGGTCCCGGTAG. For 3′-RACE, the following primers were used: FP1, AGGAATCCTTGTGTCGGC; and FP2, GAGACCAACATCAGGGAC. RACE reactions were carried out using a commercially available kit with conditions supplied by the manufacturer (GibcoBRL). PCR conditions were as described above except the melting temperature was increased to 56 °C. Amplification products were detected by Southern analysis, then cloned, and sequenced on both strands by standard techniques.

Using these approaches, a 1031 bp cDNA encoding the rabbit β1 subunit was cloned. The nucleotide sequence has been deposited in the GenBank database and assigned accession number AF107300. The open reading frame encodes a 191 amino acid protein that shares 87−90% sequence identity with other known species variants.

Recordings of Maxi-K Channels in Planar Lipid Bilayers. Planar lipid bilayers were formed, and maxi-K channels from membranes were fused with the artificial bilayer as described previously (*14*). The orientation of the maxi-K channel was determined by the voltage- and calcium-dependence of channel gating as described (*14*). The lipid composition of the planar lipid bilayer consisted of POPE/POPC in a 7:3 molar ratio. The exact compositions of the solutions on either side of the bilayer are as described in the figure legends.

Currents through single maxi-K channels were detected and amplified using a Dagan 3900A integrating patch clamp amplifier as described (*14*). Currents were recorded onto a video cassette tape and were digitally encoded on-line as described (*28*).

The single-channel currents were refiltered off-line as described (*28*), and opening and closing events were detected using a threshold detection algorithm in TAC (Bruxton Corp.). The toxin blocked times were discriminated as described previously (*28*), and toxin blocked and unblocked times were fitted as sums of exponential components using TACfit (Bruxton Corp.). The time constants for toxin block and unblock were each obtained from an average of 159 events.

The rate constants for toxin dissociation (k_{off}) and association (k_{on}) were calculated from the time constants for block and unblock, respectively, as described (*28*). The toxin equilibrium dissociation constant, K_d, was caclulated from toxin k_{off} and k_{on} as described (*28*).

RESULTS

β1 Subunit Expression Slows the Rate of ChTX-S10A Unbinding from the Channel Vestibule. Previously, it has been shown that coexpression of α and β1 subunits tightened the binding interaction between [^{125}I]-ChTX and maxi-K channels ~50-fold as compared to α subunits alone (*19*). However, the corresponding functional effects have not been determined at the single-channel level. For this purpose, we examined the blocking kinetics of single maxi-K channels either from native smooth muscle membranes or from membranes derived from cells transiently transfected with either α or α+β1 subunits. Under our transfection conditions (see Materials and Methods), maxi-K channels formed by cotransfection of α+β1 subunits resembled native smooth muscle channels in their functional stoichiometry as illustrated by the presence of both a single class of high-affinity sites for [^{125}I]-ChTX and monoexponential toxin dissociation kinetics (*19*). ChTX-S10A was used for these experiments because it displays a ~10-fold faster dissociation rate constant (k_{off}) and hence a ~10-fold weaker binding affinity relative to wild-type ChTX (*29*). This faster k_{off} value allows a larger number of toxin blocking events to be recorded per unit time and thus a robust measure of toxin blocking kinetics.

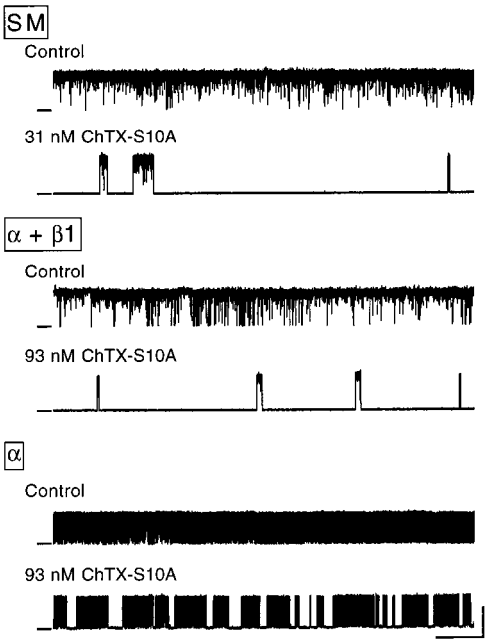

FIGURE 1: Effect of β1 subunit expression on ChTX-S10A block of single maxi-K channels. Currents through single maxi-K channels in the absence and presence of ChTX-S10A. Membranes were prepared from bovine aortic smooth muscle (SM) and from tsA-201 cells transiently transfected with either α or α+β1 subunits. The zero current level is indicated by the lines to the left. Horizontal and vertical scale bars represent 30 s and 10 pA, respectively. Currents were filtered at 60 Hz for display. Conditions were as follows: 10 mM KCl outside, 150 mM KCl inside, 10 mM Hepes, pH 7.2, inside and outside; the membrane potential was 0 mV. $CaCl_2$ inside was 305 μM for SM and α+β1 maxi-K channels and 605 μM for maxi-K channel complexes of α subunits alone.

Figure 1 shows the effects of ChTX-S10A on currents through single maxi-K channels derived from bovine aortic smooth muscle (SM) cells and from tsA-201 cells transiently transfected with either α+β1 subunits or α subunits alone. In control, the open probabilities for maxi-K channels from SM and from TsA-201 cells with α+β1 subunits are both high, >0.95. In contrast, the open probability for the maxi-K channel from tsA-201 cells with the α subunit alone is much lower, 0.7. These effects on single-channel open probability

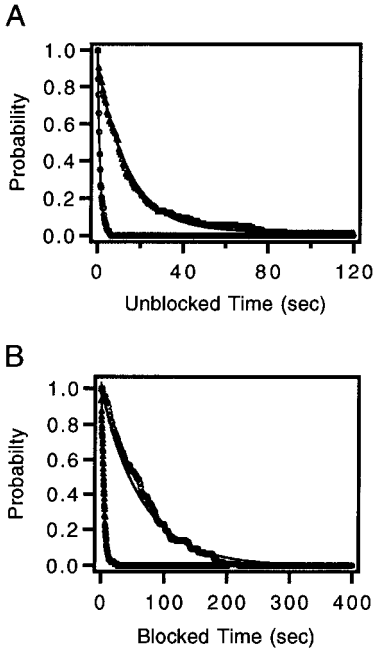

FIGURE 2: Cumulative dwell-time distributions for ChTX-S10A block of complexes of α alone and α+β1 subunits. The durations of unblocked (A) and blocked (B) times for 93 nM ChTX-S10A are plotted as cumulative dwell-time distributions. Maxi-K channels were obtained from TsA-201 membranes expressing either α alone (triangles) or α+β1 (circles) subunits. The solid lines represent the best fit of the data to a single-exponential component. For the α subunit alone, the time constants for ChTX-S10A block and unblock were 4.9 and 14 s, respectively. For α+β1 subunits, the time constants for ChTX-S10A block and unblock were 68 and 1.3 s, respectively. Distributions were constructed from a minimum of 107 events. Conditions were as described in Figure 1.

are consistent with the effects of the β1 subunit on maxi-K channel gating (*9*). Addition of ChTX-S10A to the outside of the channel causes the appearance of long, nonconducting silent periods that are interrupted by periods of normal channel activity. The average durations of these silent periods represent the time when toxin is bound to the channel and therefore are inversely related to the first-order, dissociation rate constant, k_{off} (*14*, *30*). For maxi-K channels from SM and from tsA-201 cells expressing α+β1 subunits, the average durations of these silent periods are similar, around 50 s. In contrast, the average duration of these silent periods for the α subunit alone was much shorter, ~5 s. Thus, under these conditions of low external potassium, expression of the β1 subunit increases the amount of time the toxin spends bound to the maxi-K channel outer vestibule.

The quantitative effects of β1 subunit expression on ChTX-S10A blocking kinetics of single maxi-K channels are shown by the cumulative dwell-time distribution in Figure 2B. The time constants for ChTX-S10A block obtained from these distributions were 64 s for maxi-K channels expressing α+β1 subunits versus 4.6 s for those expressing only the α subunit. Similarly, the time constants for block averaged from several experiments, shown in Table 1, were 48, 51, and 4.8 s for smooth muscle (SM), α+β1 subunit, and α subunit maxi-K channels, respectively. Thus, the β1 subunit stabilizes the toxin:channel complex approximately 10-fold in maxi-K channels from SM or from transiently transfected TsA-201 cells.

The quantitative effects of β1 subunit expression on the ChTX-S10A association rate are shown by the cumulative

Table 1: ChTX-S10A Blocking Kinetics and Open Probabilities from Recordings of Single Maxi-K Channels When the Membrane Potential Was 0 mV[a]

membranes	open probability	K_d (nM)	k_{on} ($\times 10^6$ $M^{-1}s^{-1}$)	T_{block} (s)
SM	0.971 ± 0.007	9.5 ± 3.8	3.3 ± 0.1	48 ± 12
α+β1	0.97 ± 0.01	10.6 ± 7.8	4.7 ± 2	51 ± 16
α+β1(L90A)	0.91 ± 0.04	61.1± 8.6	3.23 ± 0.15	5.2 ± 0.5
α	0.657 ± 0.078	200 ± 17	1.1 ± 0.09	4.8 ± 0.63

[a] Maxi-K channels fused with the bilayer were obtained either from bovine aortic smooth muscle (SM) membranes or from membranes obtained from TsA-201 cells transiently transfected with α alone or α+β1 subunits or from COS-1 cells transfected with wild-type α subunits and mutated β1 subunits, α+β1(L90A). Open probabilities were averaged from a control representing 10 min of single-channel recording before addition of ChTX-S10A. The first-order dissociation rate constants, k_{off}, and the second-order association rate constants, k_{on}, were obtained from the time constants for block (T_{block}) and unblock, respectively, as described (*28*). The equilibrium dissociation constant (K_d) values were calculated from the ratio of k_{off} and k_{on} values where $K_d = k_{off}/k_{on}$. Each value was the average of three to five separate measurements, with ± representing the standard error of the mean. Experimental conditions were as described in Figure 1.

dwell-time distributions for ChTX-S10A unblock (Figure 2A). At the same concentration of 93 nM ChTX-S10A, the time constants for unblock of maxi-K channels expressing either α or α+β1 subunits were 14.4 and 1.3 s, respectively. Table 1 shows that the average k_{on} values derived from these time constants for unblock are 3−4-fold faster for maxi-K channels expressing α+β1 subunits than for those expressing only the α subunit. This suggests that the β1 subunit also facilitates the ingress of ChTX-S10A into its site on the maxi-K channel vestibule. Together, the combined effects of β1 subunit expression on toxin binding and unbinding produced a 20-folder tighter K_d for toxin block of current through single maxi-K channels.

These effects of β1 subunit expression on ChTX-S10A blocking kinetics are similar to those observed for the kinetics of [^{125}I]-ChTX binding to membranes containing either α or α+β1 subunits of the maxi-K channel (*19*). Recently, alanine scanning mutagenesis of the β1 subunit revealed four residues (L90, Y91, T93, and E94) in the extracellular loop that weakened equilibrium [^{125}I]-ChTX binding to COS cell membranes expressing α and mutated β1 subunits (*20*). Of these four critical residues, the L90A mutation showed the largest effect on [^{125}I]-ChTX equilibrium binding, a ~20-fold increase in K_d. Given these data, we examined the effects of ChTX-S10A on maxi-K channels expressing the mutated L90A β1 subunit, α+β1(L90A). Table 1 shows that the time constants for ChTX-S10A block of maxi-K channels expressing α+β1(L90A) are very similar, 4.7 s, to those expressing the α subunit alone, 4.6 s. Interestingly, these α+β1(L90A) complexes showed high channel open probabilities in control that were similar to those observed for wild-type α+β1 complexes, 0.9 versus 0.97, respectively. These results suggest that the effects of β1 subunit expression on ChTX-S10A block of current through a single maxi-K channel result from specific interactions between α and β1 subunits. Further, these results show that the effects of β1 subunit expression on electrophysiological and equilibrium binding studies arise from similar molecular determinants.

[^{125}I]-IbTX-D19Y/Y36F Binding and Cross-Linking Studies Reveal Differential β1 Subunit Expression in Smooth and

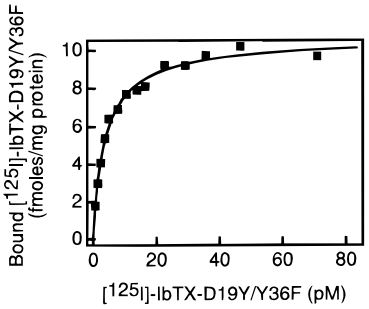

FIGURE 3: Membranes from skeletal muscle contain a single class of high-affinity [^{125}I]-IbTX-D19Y/Y36F binding sites. Specific binding of [^{125}I]-IbTX-D19Y/Y36F to rabbit skeletal muscle membranes is plotted as a function of increasing concentrations of [^{125}I]-IbTX-D19Y/Y36F. The solid line represents the best fit of the data to the equation: $B = B_{max}/[1 + (K_d/L)^{nH}]$, with a maximum receptor density, B_{max}, value of 10.7 fmol/mg of protein, an equilibrium dissociation constant, K_d, value of 3.9 pM, and a pseudo-Hill coefficient, nH, of 0.92. L is the free [^{125}I]-IbTX-D19Y/Y36F concentration. Membranes were incubated in a medium consisting of 10 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.1% bovine serum albumin, 0.1% digitonin, 5 μM paxilline, and different concentrations of [^{125}I]-IbTX-D19Y/Y36F. Incubations were carried out at room temperature for ca. 20 h. Separation of bound from free ligand was achieved by filtration through GF/C glass fiber filters that have been presoaked into 0.5% polyethylenimine. Nonspecific binding was determined in the presence of 10 nM ChTX.

Skeletal Muscle. The subunit composition of maxi-K channels expressed in native tissues influences the biophysical and pharmacological properties of the channel and therefore the physiologic role of the channel in cell function. For instance, in rabbit skeletal and aortic smooth muscle, the calcium sensitivity of maxi-K channel gating differs by an order of magnitude (*12*). This difference in calcium sensitivity may reflect a tissue-specific difference in the expression of the β1 subunit.

We monitored expression of maxi-K channel complexes in rabbit skeletal muscle with the use of [^{125}I]-IbTX-D19Y/Y36F. Binding of [^{125}I]-IbTX-D19Y/Y36F occurs with similar affinities to membranes containing either α or $\alpha+\beta$1 subunits (not shown). Figure 3 shows the specific binding of [^{125}I]-IbTX-D19Y/Y36F to membranes from rabbit skeletal muscle (SK) as a function of ligand concentration. The specific binding was best fit to a Langmuir binding isotherm for [^{125}I]-IbTX-D19Y/Y36F with a pseudo-Hill coefficient of 1, an equilibrium dissociation constant, K_d, value of 3.9 pM, and a B_{max} of 10.7 fmol/mg of protein. These findings reveal that rabbit skeletal membranes contain a single class of [^{125}I]-IbTX-D19Y/Y36F binding sites with a K_d value similar to that observed for smooth muscle membranes (*15*).

Specific, covalent incorporation of [^{125}I]-IbTX-D19Y/Y36F into the β subunit provides a means for directly monitoring the expression of β1 subunits that are functionally associated with α subunits (*15*). For these experiments, skeletal and smooth muscle membranes were incubated in the presence of [^{125}I]-IbTX-D19Y/Y36F, reacted with the bifunctional cross-linking reagent DSS, and subjected to SDS−PAGE. Exposure of the dried gel to XAR-film for 11 days reveals covalent incorporation of radioactivity into the β1 subunit of smooth muscle membranes over a range of membrane concentrations, 3−30 μL, while no radioactivity can be detected with the skeletal muscle membranes over the same range (Figure 4). Identical results were obtained

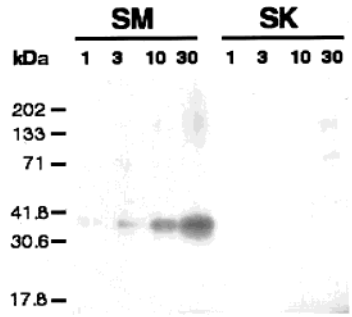

FIGURE 4: Cross-linking of [^{125}I]-IbTX-D19Y/Y36F to bovine smooth and rabbit skeletal muscle membranes. Bovine aortic smooth muscle (SM) or rabbit skeletal muscle (SK) membranes were incubated with 9 pM [^{125}I]-IbTX-D19Y/Y36F, reacted with DSS, and subjected to SDS−PAGE as described under Materials and Methods. Different amounts of final membrane suspension (1, 3, 10, and 30 μL) were loaded into 12% polyacrylamide gels. Gels were dried and exposed to XAR-film for 11 days at −70 °C. The migration of molecular weight markers is indicated by the lines to the left.

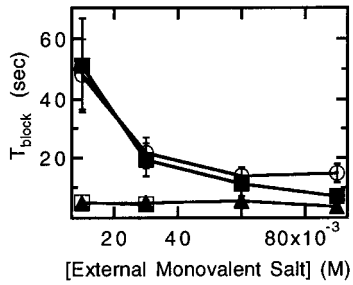

FIGURE 5: Effects of external monovalent salt concentration on ChTX-S10A blocked times. The time constants for ChTX-S10A block are plotted as a function of external monovalent salt concentration for maxi-K channels from SM (opened circles) or from tsA-201 cells expressing subunits of α alone (filled triangles), $\alpha+\beta$1 (filled squares), and $\alpha+\beta$1(L90A) (opened squares). Time constants for toxin block represent values averaged from two to five separate measurements, and error bars represent standard errors of the mean. Conditions were as described in Figure 1 except the external monovalent salt concentration was increased from 10 mM KCl by addition of NaCl.

after a much longer exposure of the gel to film except that, in this case, the lowest amount of smooth muscle membrane concentration, 1 μL, also produced a detectable signal (data not shown). Since the difference in the amount of ^{125}I-labeled receptor sites between smooth and skeletal muscle membranes was only 2.5-fold (see Materials and Methods), these findings suggest that β1 subunits do not functionally associate with maxi-K channels in rabbit skeletal muscle.

Electrostatic Interactions Modulate the β1:Toxin Binding Interaction. We have shown that the β1 subunit is expressed in SM but not in SK muscle. In addition, we have demonstrated that this differential expression of β1 has dramatic functional consequences on ChTX-S10A block of current through single maxi-K channels, causing an ~10-fold increase in toxin blocked times. However, the reported ChTX toxin blocked times for SM (*31*) and SK (*29*) maxi-K channels differ by only ~4-fold under conditions of 150 mM external KCl. This apparent discrepancy may arise from effects of external ionic strength on the β1 toxin binding site. Figure 5 shows that as the external monovalent salt concentration is raised from 10 to 90 mM, the ChTX-S10A block times decreased ~4-fold for maxi-K channels from either SM or from tsA-201 cells expressing $\alpha+\beta$1 subunits.

In addition, we have observed similar ionic strength effects with increasing external magnesium chloride concentration (not shown). In contrast, for maxi-K channel complexes of either α subunits alone or α+β1(L90A) subunits, toxin blocked times show no diminution as the external monovalent salt concentration is raised. Similar to previously published effects on ChTX association rates with the skeletal muscle channel (*30*), increasing external ionic strength causes a large dimunition in the ChTX-S10A association rate. And, k_{on} values for maxi-K channel complexes of α and α+β1 subunits behave similarly with increasing external monovalent salt concentration (not shown). Thus, the profound effects of the β1 subunit on the ChTX-S10A dissociation rate are modulated by electrostatic interactions.

DISCUSSION

The β1 Subunit Promotes a High-Affinity ChTX:Maxi-K Channel Interaction. In this study, we showed that β1 subunit expression has profound effects on ChTX-S10A blocking kinetics for single maxi-K channels incorporated into planar lipid bilayers. Under conditions of low external ionic strength, i.e., 10 mM KCl, the average ChTX-S10A block time was ∼10-fold slower for α+β1 complexes of the maxi-K channel than for the α subunit complex. This effect on toxin block times provides a striking diagnostic tool for β1 subunit expression. Indeed, under these same conditions, wild-type ChTX displays block times ∼600 s in duration for bovine aortic smooth muscle channels (not shown). These longer block times seen with ChTX-S10A and ChTX show that the β1 subunit decreases k_{off} and stabilizes the toxin:channel complex.

Expression of the β1 subunit also increases the rates of ChTX-S10A binding to the maxi-K channel. The k_{on} values for ChTX-S10A, calculated from unblocked times, were ∼3.5-fold faster for complexes of α+β1 subunits than for α subunits alone. However, much of this effect is likely to arise from the effect of the β1 subunit on channel open probability since the ChTX association rate constant is known to increase with single-channel open probability (*30*). As expected, we observed higher single-channel open probabilities for maxi-K channel complexes consisiting of α+β1 subunits than for α subunits alone, 0.97 and 0.66, respectively. Taken together, these results suggest that the effects of the β1 subunit on the ChTX-S10A association rate result from effects of β1 subunit expression on maxi-K channel gating.

The combined effect of β1 subunit expression on toxin blocking and unblocking rates is manifested in an ∼20-fold tigher K_d value for maxi-K channel complexes of α+β1 subunits than α subunits alone. Moreover, the ChTX-S10A blocked time distributions for maxi-K channel complexes of α+β1 subunits were well-described by single-exponential functions. Under low ionic strength conditions, where stabilization of k_{off} is most pronounced, 14 of 15 blocked time distributions were best fit with a single exponential. These effects of β1 subunit expression on ChTX-S10A blocking kinetics are similar to effects reported on the kinetics of [^{125}I]-ChTX binding to COS cells transiently transfected with either α or α+β1 subunits (*19*).

Previous studies also showed that four residues of the extracellular loop of β1 (L90, Y91, T93, and E94) were found to be critical for conferring these functional effects (*20*). In this work, we showed that mutating just one of these four residues in the extracellular loop of the β1 subunit, L90A, caused a ∼10-fold decrease in the ChTX-S10A block time relative to the wild-type β1 subunit. Thus, the L90A mutation completely removed the stabilizing effect of the β1 subunit on the ChTX-S10A dissociation rate constant. Interestingly, single α+β1(L90A) channels show open probabilities in control that are similar to those seen for maxi-K channels expressing the wild-type β1 subunit, 0.9 vs 0.97, and these channels display k_{on} values similar to the native SM channel. This further supports that some of the effects of β1 on ChTX-S10A association rates result from its effects on channel gating. Thus, β1(L90A) appears to be critical for stabilizing the ChTX:channel interaction but not for modifying the gating behavior of the α subunit complexes.

These effects of L90 on ChTX binding, however, are not independent from those of the three other critical residues in the extracellular loop of β1 (*20*). Applying a simple formalism for additivity of binding free energy (*32*), we calculated a large interaction energy, ΔG_I, of −3.6 kcal/mol, for the four residues comprising the β1 toxin binding site (*20*). While we do not know the physical basis for this large interaction energy, it clearly indicates that these four critical residues in the extracellular loop of β1 interact to form a high-affinity toxin binding site.

The β1 Subunit and the ChTX Binding Site. ChTX blocks the flow of K^+ ions by binding to and occluding the external pore of the maxi-K channel (*13*). Thus, ChTX is in intimate contact with a complex of four α subunits that form a functional channel. Since the β subunit is not required for the formation of functional maxi-K channels, the question naturally arises: how does the β1 subunit stabilize this complex of one toxin molecule and four α subunits?

We have shown that the effects of the β1 subunit on k_{off} are modulated by electrostatic interactions. That is, when the external monovalent salt concentration is raised from 10 to 90 mM, the ChTX-S10A blocked time decreased from ∼50 to ∼10 s for α+β1 complexes, and at 150 mM salt, the blocked times for α+β1 complexes are similar to those for α complexes alone, ∼5 s (not shown). In contrast, the time constants for ChTX-S10A block of either α complexes or α+β1(L90A) complexes show no diminution over a similar range of external monovalent salt concentration. Thus, the stabilizing effects of the β1 subunit on the toxin/channel complex are maximal when external ionic strength is low and minimal when external ionic strength is high.

However, only one of the four residues in the putative β1 subunit site is ionized at physiological pH, E94, while the other three residues (L90, Y91, and T93) are neutral and hydrophobic. How can electrostatic forces influence a site that is primarily hydrophobic? One possible explanation is that ionic strength alters the conformation of the β1 toxin binding site either through changes in the extracellular loop of the β1 subunit alone or through changes in the α/β1 subunit quarternary structure.

β1 Subunit Expression Levels in Skeletal and Smooth Muscle. In this work, we have employed equilibrium [^{125}I]-IbTX-D19Y/Y36F binding to quantitate the maxi-K channel complexes present in rabbit skeletal muscle (SK) membranes.

These membranes contained a single class of high-affinity binding sites with a K_d value of 3.9 pM. This K_d value is similar to a previously reported value of 3.6 pM for [^{125}I]-IbTX-D19Y/Y36F binding to SM membranes (*15*). The [^{125}I]-IbTX-D19Y/Y36F receptor density in SK membranes, 10.7 fmol/mg of protein, is ~40-fold lower than the observed value for tracheal smooth muscle, 420 fmol/mg of protein (*15*). And, it is ~50-fold lower than the [^{125}I]-ChTX receptor density measured in aortic smooth muscle, 540 fmol/mg of protein (*16*). This suggests that maxi-K channel complexes are much less abundant in skeletal muscle than in either tracheal or aortic smooth muscle.

To test for coassembly of $\alpha+\beta$1 subunits, a specific cross-linking reaction was carried out between [^{125}I]-IbTX-D19Y/Y36 and its receptor in rabbit skeletal muscle. We showed that specifically bound [^{125}I]-IbTX-D19Y/Y36 could be covalently incorporated into the β1 subunit from aortic smooth muscle but not from skeletal muscle. The covalent cross-linking reaction between [^{125}I]-IbTX-D19Y/Y36 and the β1 subunit requires the presence of an acceptor in the β1 subunit. For ChTX, this acceptor is Lys69 (*18*) which is present in the bovine (*8*) and human (*33*) β1 subunit forms. The covalent linkage between β1 and IbTX-D19Y/Y36 has not been studied. However, given the similar pore-blocking mechanism for ChTX and IbTX (*13*, *14*, *28*, *29*), covalent linkage of [^{125}I]-IbTX-D19Y/Y36 to the β1 subunit also likely occurs between Lys32 in the toxin and Lys69 in the β1 subunit. To confirm the presence of Lys69 in rabbit, we cloned the rabbit cDNA for the β1 subunit and performed dideoxy sequencing as described under Materials and Methods. The deduced amino acid sequence for the rabbit β1 subunit reveals that Lys69 is present. Thus, the lack of covalent incorporation of [^{125}I]-IbTX-D19Y/Y36 into rabbit skelectal muscle membranes most likely derives from the absence of β1 in the maxi-K channel complex. Thus, these data and those describing the gating (*34*) and pharmacological (*35*) properties of the maxi-K channel suggest that the β1 subunit is expressed in and associated with α subunits in bovine aortic smooth muscle but not in rabbit skeletal muscle. Our findings extend previous northern blot analysis which showed that β1 subunit mRNA levels are much higher in aortic smooth muscle than in skeletal muscle (*33*). Further, in contrast to ChTX, the similar IbTX-D19Y/Y36F K_d values obtained for SM and SK membranes suggest that β1 subunit expression does not dramatically affect IbTX binding to maxi-K channel complexes.

In summary, we have shown that ChTX-S10A block of current through single maxi-K channels, on its own, can be a valuable diagnostic tool for β1 subunit expression. Further, we have used ChTX-S10A block of single channels and cross-linking reactions with IbTX-D19Y/Y36F to show that β1 subunits are functionally associated with maxi-K channels in smooth muscle but not skeletal muscle. Finally, we have shown that studies of ChTX block of single maxi-K channel complexes of α and mutated β subunits may provide new insight into the structural and functional relationship of α and β subunits.

ACKNOWLEDGMENT

We extend our gratitude to Dr. Gregory Kaczorowski for his valued advice and support on this project. We thank Mr. William Schamlhofer for his help in performing the tissue culture. We thank Dr. Richard Swanson for his technical guidance on cloning the β1 subunit from rabbit.

REFERENCES

1. Garcia-Calvo, M., Knaus, H.-G., McManus, O. B., Giangiacomo, K. M., Kaczorowski, G. J., and Garcia, M. L. (1994) *J. Biol. Chem. 269*, 676−682.
2. Giangiacomo, K. M., Garcia-Calvo, M., Knaus, H.-G., Mullmann, T. J., Garcia, M. L., and McManus, O. B. (1995) *Biochemistry 34*, 15849−15862.
3. Adelman, J. P., Shen, M. P., Kavanaugh, R. A., Warren, Y. N., Wu, L. A., Bond, C. T., and North, R. A. (1992) *Neuron 9*, 209−216.
4. Atkinson, N. S., Robertson, G. A., and Ganetzky, B. (1991) *Science 253*, 551−555.
5. Butler, A., Tsunoda, S., McCobb, D. P., Wei, A., and Salkoff, L. (1993) *Science 261*, 221−224.
6. Jan, L. Y., and Jan, Y. N. (1997) *Annu. Rev. Neurosci. 20*, 91−123.
7. Schreiber, M., and Salkoff, L. (1997) *Biophys. J. 73*, 1355−1363.
8. Knaus, H.-G., Folander, K., Garcia-Calvo, M., Garcia, M. L., Kaczorowski, G. J., Smith, M., and Swanson, R. (1994) *J. Biol. Chem. 269*, 17274−17278.
9. McManus, O. B., Helms, L. M. H., Pallanck, L., Ganetzky, B., Swanson, R., and Leonard, R. J. (1995) *Neuron 14*, 645−650.
10. Xia, X.-M., Ding, J. P., and Lingle, C. J. (1999) *J. Neurosci. 19*, 5255−5264.
11. Wallner, M., Meera, P., and Toro, L. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 4137−4142.
12. McManus, O. B. (1991) *J. Bioenerg. Biomembr. 23*, 537−560.
13. MacKinnon, R., and Miller, C. (1988) *J. Gen. Physiol. 91*, 335−349.
14. Giangiacomo, K. M., Garcia, M. L., and McManus, O. B. (1992) *Biochemistry 31*, 6719−6727.
15. Koschak, A., Koch, R. O., Liu, J., Kaczorowski, G. J., Reinhart, P. H., Garcia, M. L., and Knaus, H.-G. (1997) *Biochemistry 36*, 1943−1952.
16. Garcia-Calvo, M., Vazquez, J., Smith, M., Kaczorowski, G. J., and Garcia, M. L. (1991) *Biochemistry 30*, 11157−11164.
17. Munujos, P., Knaus, H., Kaczorowski, G. J., and Garcia, M. L. (1995) *Biochemistry 34*, 10771−10776.
18. Knaus, H.-G., Eberhart, A., Kaczorowski, G. J., and Garcia, M. L. (1994) *J. Biol. Chem. 269*, 23336−23341.
19. Hanner, M., Schmalhofer, W. A., Munujos, P., Knaus, H.-G., Kaczorowski, G. J., and Garcia, M. L. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 2853−2858.
20. Hanner, M., Vianna-Jorge, R., Kamassah, A., Schmalhofer, W. A., Knaus, H.-G., Kaczorowski, G. J., and Garcia, M. L. (1998) *J. Biol. Chem. 273*, 16289−16296.
21. Slaughter, R. S., Shevell, J. L., Felix, J. P., Garcia, M. L., and Kaczorowski, G. J. (1989) *Biochemistry 28*, 3995−4002.
22. Moczydlowski, E., and LaTorre, R. (1983) *Biochim. Biophys. Acta 732*, 412−420.
23. Kolodziej, P. A., and Young, R. A. (1991) *Methods Enzymol. 194*, 508−519.
24. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene (Amsterdam) 77*, 51−59.
25. Vasquez, J., Katz, G., King, V. F., Reuben, J. P., Roy-Contancin, L., Feigenbaum, P., Slaughter, R. S., Kaczorowski, G., and Garcia, M. L. (1989) *J. Biol. Chem. 264*, 20902−20909.
26. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
27. Frohman, M., Dush, M., and Martin, G. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8998−9002.

28. Mullmann, T. J., Munojos, P., Garcia, M. L., and Giangiacomo, K. M. (1999) *Biochemistry 38*, 2395−2402.
29. Stampe, P., Kolmakova-Partensky, L., and Miller, C. (1994) *Biochemistry 33*, 443−450.
30. Anderson, C. S., MacKinnon, R., Smith, C., and Miller, C. (1988) *J. Gen. Physiol. 91*, 317−333.
31. Giangiacomo, K. M., Sugg, E. E., Garcia-Calvo, M., Leonard, R. J., McManus, O. B., Kaczorowski, G. J., and Garcia, M. L. (1993) *Biochemistry 32*, 2363−2370.
32. Giangiacomo, K. M., Gabriel, J., Fremont, V., and Mullmann, T. J. (1999) *Perspect. Drug Discov. Des. 15*/*16*, 167−186.
33. Tseng-Crank, J., Foster, C. D., Krause, J. D., Mertz, R., Godinot, N., DiChiara, T. J., and Reinhart, P. H. (1994) *Neuron 13*, 1315−1330.
34. Nimigean, C. M., and Magleby, K. L. (1999) *J. Gen. Physiol. 113*, 425−439.
35. Valverde, M. A., Rojas, P., Amigo, J., Cosmelli, D., Orio, P., Bahamonde, M. I., Mann, G. E., Vergara, C., and Latorre, R. (1999) *Science 285*, 1929−1931.

BI992865Z