Cross-linking of Charybdotoxin to High-Conductance Calcium-Activated Potassium Channels: Identification of the Covalently Modified Toxin Residue†

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ABSTRACT: High-conductance calcium-activated potassium (maxi-K) channels are composed of two subunits, α and β. The pore-forming α subunit is a member of the *mSlo* family of K^+ channels, whereas the β subunit is a novel protein that modulates the biophysical and pharmacological properties of the channel complex. In the presence of a bifunctional cross-linking reagent, monoiodotyrosine charybdotoxin ([^{125}I]ChTX) is covalently incorporated specifically into Lys_{69} of the β subunit, which is located in a large extracellular loop of this protein. Using variants of ChTX which retain their channel-blocking activity and in which individual Lys residues have been mutated, we have identified the corresponding amino acid in ChTX that is involved in the cross-linking reaction. All of the ChTX mutants investigated bind to the maxi-K channel and display the same pharmacological profile as native ChTX in competition binding experiments. Whereas substitution of amino acids at positions 11 and 31 of ChTX yields wild-type cross-linking patterns, the peptide without a Lys at position 32 fails to incorporate into the β subunit of the maxi-K channel. Given the model for the interaction between ChTX and the outer vestibule of the maxi-K channel that has been proposed (Stampe et al., 1994), our data constrain the maximum distance between the pore of this channel and the region in the extracellular loop of the β subunit where the cross-linking reaction takes place to 11 Å. This topological limit helps define structural features of the maxi-K channel that may aide in probing the functional interaction between α and β subunits of the channel complex.

Large-conductance Ca^{2+}-activated K^+ (maxi-K)[1] channels are a family of channel proteins that display both high selectivity for and high conductance of potassium ion (Latorre et al., 1989). These channels are gated by increasing intracellular Ca^{2+} levels and by membrane depolarization. Maxi-K channels are potently blocked by a number of peptidyl toxins [e.g., charybdotoxin (ChTX), iberiotoxin (IbTX), and limbatustoxin] that have been isolated from scorpion venoms [for a review, see Garcia et al. (1993)]. A large body of evidence suggests that these toxins bind in the outer vestibule of the channel to physically occlude the pore and prevent ion conduction (Giangiacomo et al., 1992; MacKinnon & Miller, 1988). The ability to produce large quantities of ChTX by recombinant techniques (Park et al., 1991), together with site-directed mutagenesis studies (Park & Miller, 1992a; Stampe et al., 1992), has led to the identification of those toxin residues that are critical for the peptide's interaction with the maxi-K channel. These studies, combined with knowledge of the three-dimensional structure of ChTX derived from NMR studies of the peptide in solution (Bontems et al., 1991a,b, 1992), have allowed predictions of the nature of the interaction surface between toxin and the channel (Stampe et al., 1994).

ChTX has also been very useful in identifying the molecular components of the maxi-K channel. With the use of monoiodotyrosine charybdotoxin ([^{125}I]ChTX), the maxi-K channel has been purified to homogeneity from bovine tracheal and aortic smooth muscle membranes and shown to consist of two noncovalently linked subunits, α and β (Garcia-Calvo et al., 1994). Amino acid sequence information obtained from proteolytic fragments of the α subunit (Knaus et al., 1994c) reveals that this is the pore-forming subunit of the channel because it displays a high degree of homology with predicted amino acid sequences from the previously cloned *slowpoke* and *mSlo* maxi-K channels (Adelman et al., 1992; Butler et al., 1993). The β subunit has recently been cloned from bovine aortic and tracheal smooth muscle (Knaus et al., 1994b). The cDNA encodes a novel 22 kDa protein predicted to consist of two transmembrane domains spanned by a large extracellular loop that contains two sites for N-linked glycosylation. Recent studies have shown that coexpression of this protein with the *mSlo* α subunit has profound effects on the gating and pharmacological properties of the resulting maxi-K channels (McManus et al., 1995; Meera et al., 1995). The β subunit represents the protein to which [^{125}I]ChTX is specifically and covalently incorporated in the presence of the bifunctional cross-linking reagent disuccinimidyl suberate (Garcia-Calvo et al., 1991). Proteolytic digestion of the [^{125}I]ChTX-cross-linked subunit, together with immunoprecipitation and deglycosylation studies, have identified Lys_{69} as the residue

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[1] Abbreviations: maxi-K channel, high-conductance Ca^{2+}-activated K^+ channel; ChTX, charybdotoxin; [^{125}I]ChTX, monoiodotyrosine charybdotoxin; IbTX, iberiotoxin; DSG, disuccinimidyl glutarate; DSS, disuccinimidyl suberate; EGS, ethylene glycobis(succinimidyl succinate); Taps, *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TEA, tetraethylammonium ion.

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in the large extracellular loop of the β subunit to which [^{125}I]-ChTX is covalently attached (Knaus et al., 1994a). These studies have also confirmed the proposed topology of the β subunit as well as the presence of N-linked carbohydrate residues at the two putative extracellular glycosylation sites.

In the present investigation, ChTX variants with normal channel-blocking properties were used in which individual Lys residues have been neutralized in order to determine the amino acid(s) that participate in the cross-linking reaction. The results presented herein demonstrate that Lys_{32} of ChTX is the residue responsible for the cross-linking profile. These results place constraints on the distance between the pore of the maxi-K channel and a specific region of the β subunit, and such data may be of use in future studies probing the interaction of the maxi-K channel's two subunits. A preliminary report of some of these findings has been made in abstract form (Munujos et al., 1995).

EXPERIMENTAL PROCEDURES

Materials. ChTX was purchased from Peninsula Laboratories. The ChTX mutants Lys_{11}Asn, Lys_{31}Gln, and Lys_{32}-Gln/Arg_{19}Gln were a generous gift from Dr. Chris Miller, Brandeis University. ^{125}INa was obtained from DuPont NEN, whereas digitonin, special grade (water-soluble), was from Biosynth AG, Switzerland. The cross-linking reagent disuccinimidyl suberate (DSS) was from Pierce, while paxilline was purchased from Sigma. GF/C glass fiber filters were purchased from Whatman and prestained molecular weight standards were obtained from Bio-Rad. All other reagents used were of the highest purity commercially available.

Iodination of Toxins. All of the ChTX analogs were iodinated by employing a glucose oxidase/lactoperoxidase methodology as previously outlined (Garcia et al., 1992). Separation of the monoiodotyrosine derivatives was achieved as described for ChTX. Toxins were resuspended at a concentration of 500 μCi/mL in 100 mM NaCl, 20 mM Tris-HCl, pH 7.4, and 0.1% bovine serum albumin. Aliquots were rapidly frozen in liquid N_2 and stored at −70 °C until their use.

Maxi-K Channel/ChTX Receptor Preparations. Purified sarcolemmal membrane vesicles derived from either bovine aortic or tracheal smooth muscle were prepared as outlined (Slaughter et al., 1989). The details of the ChTX receptor purification scheme have been previously described (Garcia-Calvo et al., 1994). Briefly, maxi-K channels from tracheal smooth muscle sarcolemmal membranes were solubilized after six consecutive exposures to 1% digitonin at 4 °C, in the presence of protease inhibitors. After centrifugation at 180 000g, the resulting supernatants were loaded onto a DEAE Sepharose CL-6B column which was eluted batchwise with 200 mM NaCl. The ChTX receptor was incubated with wheat germ agglutinin-Sepharose and the bound material biospecifically eluted with 200 mM *N*-acetyl-D-glucosamine. This material was applied to a MonoQ HR 10/10 (Pharmacia) ion exchange column and eluted with a linear NaCl gradient. The fractions active for ChTX binding were loaded onto a Bio-Gel HPHT (Bio-Rad) hydroxylapatite column and eluted with a sodium phosphate gradient. Fractions containing the ChTX receptor were dialyzed, concentrated, and separated on a 7−25% continuous sucrose density gradient. Fractions active in binding were pooled, rapidly frozen in liquid N_2, and stored at −70 °C.

Binding Assays. Binding of [^{125}I]ChTX to membrane-bound or solubilized maxi-K channels was carried out using well described procedures (Garcia-Calvo et al., 1994). Binding studies with radiolabeled mutant variants of ChTX were performed as with [^{125}I]ChTX, except that the incubation volume was reduced in all cases to 100 μL.

Cross-Linking Experiments. A partially purified ChTX receptor preparation was incubated with [^{125}I]ChTX in medium consisting of 10 mM NaCl, 10 mM Taps-NaOH, pH 9.0, and 0.1% digitonin, in the absence or presence of other agents for 2 h at room temperature. Then NaCl was added to a final concentration of 300 mM, followed by addition of cross-linking reagent to a final concentration of 0.18 mM. After incubation at room temperature for 1 min, the reaction was quenched by addition of Tris-HCl, pH 7.4, at a final concentration of 200 mM. Samples were dialyzed against 10 mM Tris-HCl, pH 7.4, and 0.05% digitonin, concentrated 10-fold, and subjected to SDS−PAGE using 12% gels. Gels were dried and exposed to Kodak XAR-film at −70 °C for 24 h. Under the experimental conditions described above, the amount of toxin remaining bound to the receptor after 1 min incubation in 300 mM NaCl is 69% for control ChTX, 30% for Lys_{11}Gln, 23% for Lys_{31}Gln, and 63% for Arg_{19}Gln/Lys_{32}Gln.

Analysis of Data. Data from saturation experiments were subjected to a Scatchard analysis, and linear regression was performed to obtain the equilibrium dissociation constant (K_d) and maximum receptor concentration (B_{max}). The kinetic data from ligand association and dissociation experiments were subjected to the analysis of Weiland and Molinoff (1981). The rate of ligand association (k_1) was determined from the equation $k_1 = k_{obs}([LR]_e/([L][LR]_{max}))$, where [L] is the concentration of ligand, $[LR]_e$ is the concentration of the complex at equilibrium, $[LR]_{max}$ is the maximum number of receptors present, and k_{obs} is the slope of the pseudo-first-order plot obtained by graphing ln $([LR]_e/([LR]_e - [LR]_t))$ versus time. The dissociation rate constant (k_{-1}) was determined directly from a first-order plot of ligand dissociation versus time. Data from competition experiments were analyzed by the method of Cheng and Prusoff (1973) to determine K_i values.

Protein Determination. Protein concentration was determined using the Bradford method with bovine serum albumin as the standard (Bradford, 1976).

RESULTS AND DISCUSSION

Characterization of ChTX Mutants. It has previously been demonstrated that [^{125}I]ChTX is specifically incorporated into the β subunit of the maxi-K channel after reaction with the bifunctional cross-linking reagent disuccinimidyl suberate (DSS) which contains a fully extended spacer arm of 11 Å (Garcia-Calvo et al., 1991; Knaus et al., 1994a). Given the chemical nature of the cross-linking reaction which is specific for primary amines, covalent modification can only occur through the ϵ-amino group of Lys residues located on both the β subunit and the toxin itself. Analysis of proteolytic fragments of the [^{125}I]ChTX-cross-linked β subunit has identified Lys_{69} as the sole residue to which the toxin is covalently attached in the presence of DSS (Knaus et al., 1994a). This residue is part of a large extracellular loop that spans two hydrophobic transmembrane domains. Covalent incorporation of [^{125}I]ChTX into the β subunit of the

maxi-K channel has also been obtained with two other cross-linking reagents, disuccinimidyl glutarate (DSG) and ethylene glycobis(succinimidyl succinate) (EGS) which possess fully extended methylene bridge spacers of 8 and 16 Å, respectively (Knaus et al., 1994a). However, the corresponding Lys residue(s) on the β subunit has not been identified for these reactions. Given that a model of the ChTX−α subunit interaction has been proposed for toxin bound in the outer vestibule of the maxi-K channel (Stampe et al., 1994), it is important to determine the corresponding residue on ChTX that is involved in the cross-linking reaction with DSS. Although the β subunit does not appear to contribute to the interaction surface for ChTX, knowledge of the distance between these residues would allow positioning of the β subunit's Lys_{69} residue at some predicted distance from the pore of the channel.

To accomplish this task, ChTX analogs have been employed in which individual Lys residues of the toxin were made nonreactive by conversion to either Gln or Asn. If one of these amino acids is the acceptor for the cross-linking reagent, neutralization of this residue should prevent incorporation of radioactivity into the β subunit. The ChTX mutants that were used for these studies are Lys_{11}Asn, Lys_{31}Gln, and Arg_{19}Gln/Lys_{32}Gln. These toxins have been shown to be functional as blockers of the skeletal muscle maxi-K channel reconstituted into planar lipid membranes (Park & Miller, 1992b), and only minor differences in activity were observed with respect to wild-type ChTX. These mutated peptides display substitutions at three of the four Lys residues present in ChTX. The fourth Lys residue, at position 27 of ChTX, has been shown in functional studies to be critical for interaction of toxin with the maxi-K channel (Park & Miller, 1992a). Since binding of peptide modified at Lys_{27} is of very low affinity, ca. 500-fold reduced when compared with the native toxin due to a very enhanced dissociation rate which causes a destabilization of the bound state, this molecule was not considered for use in the present investigation. Although a double mutant, Arg_{19}Gln/Lys_{32}Gln, was used to determine the contribution of Lys_{32} in the cross-linking reaction, the validity of this approach is supported by several observations: (a) data have been presented which indicate that this peptide is folded in the same fashion as native toxin because it blocks maxi-K channels with high affinity (Park & Miller, 1992b); (b) Arg is not a substrate for the cross-linking reagent; (c) position 19 is located on the side of the molecule which is not important for an interaction with the receptor since substitutions at this position do not modify channel blocking activity when compared with native toxin (Shimony et al., 1994).

Interaction of the ChTX-Lys variants with the smooth muscle maxi-K channel was studied after radiolabeling each toxin molecule with ^{125}INa. The resulting monoiodotyrosine derivatives were purified and characterized in binding studies using either bovine aortic or tracheal smooth muscle sarcolemmal membrane vesicles or the partially purified tracheal ChTX receptor. Although the size of the α subunit in membrane vesicles is 125 kDa, which is in excellent agreement with the predicted size of this protein from translation of the open reading frame of its cDNA, the partially purified preparation employed in these studies has undergone proteolytic degradation (Knaus et al., 1995). However, the maxi-K channel complex in this preparation appears to remain intact due to the fact that all fragments are associated through disulfide linkages (Knaus et al., 1995), and consequently, the preparation mantains full biological activity (Garcia-Calvo et al., 1994). Binding of each toxin was found to be saturable and reversible, displaying the same pharmacology as previously determined for interaction of [^{125}I]ChTX with maxi-K channels. Thus, binding is inhibited by increasing concentrations of native ChTX, IbTX, or TEA, with identical K_i values (Table 1) as those found for interaction of [^{125}I]ChTX with maxi-K channels (Garcia-Calvo et al., 1991). It has previously been demonstrated that certain indole alkaloid members of the tremorgenic mycotoxin family are the most potent and selective nonpeptidyl inhibitors of maxi-K channels identified to date (Knaus et al., 1994d). However, the way in which these compounds modulate binding of ChTX to its receptor is unique in that some agents (e.g., paxilline and verruculogen) stimulate [^{125}I]-ChTX binding, whereas other members of the family (e.g., aflatrem, paspalitrem C) are inhibitors of the binding reaction; all of these agents function through an allosteric mechanism. As expected for a similar mechanism of interaction with maxi-K channels, binding of the ChTX-Lys analogs is affected by these indole alkaloids in the same manner as they affect the interaction of [^{125}I]ChTX with its receptor (Table 1). The maximal stimulation of binding produced by either paxilline or verruculogen was found to be similar for all the toxin variants. These values range between 5.8- and 6.6-fold for each of the toxins with either compound.

Table 1: Modulation of Binding of ChTX-Lys Variants to the Maxi-K Channel[a]

effector	ChTX	Lys_{11}Asp	Lys_{31}Gln	Arg_{19}Gln/ Lys_{32}Gln
ChTX (pM)	30	32	30	25
IbTX (pM)	80	72	85	80
TEA (μM)	100	102	115	120
paxilline (nM)[b]	100	100	100	100
verruculogen (nM)[b]	100	100	110	100
aflatrem (nM)	150	130	120	130

[a] A partially purified tracheal maxi-K channel preparation was incubated with either [^{125}I]ChTX or the indicated [^{125}I]ChTX variant in the absence or presence of increasing concentrations of known effectors of the binding reaction. Inhibition of toxin binding was assessed relative to an untreated control. The K_i values reported in the table were determined as indicated under Experimental Procedures. [b] Concentration of the drug that produces half-maximum stimulation of toxin binding.

As further validation that all of the toxin molecules under present investigation interact with the same receptor entity, saturation experiments were carried out with a partially purified tracheal smooth muscle ChTX receptor preparation in the presence of increasing concentrations of each radiolabeled toxin, and these data were subjected to a Scatchard analysis. Since paxilline enhances toxin binding by increasing ligand affinity, this agent was included in the incubation medium to optimize the binding conditions. Under this protocol, [^{125}I]ChTX interacts with a single class of binding sites that display a K_d of 6.2 pM and a B_{max} of 51 pmol/mg of protein (Figure 1B, inset). Data from experiments with the double mutant Arg_{19}Gln/Lys_{32}Gln are illustrated in Figure 1A and indicate that toxin binding is a saturable process. A linear transformation of the specific binding data is shown in Figure 1B in the form of a Scatchard representation and illustrates that this toxin binds to a single class of receptors with a K_d of 220 pM and B_{max} of 51 pmol/mg of protein.

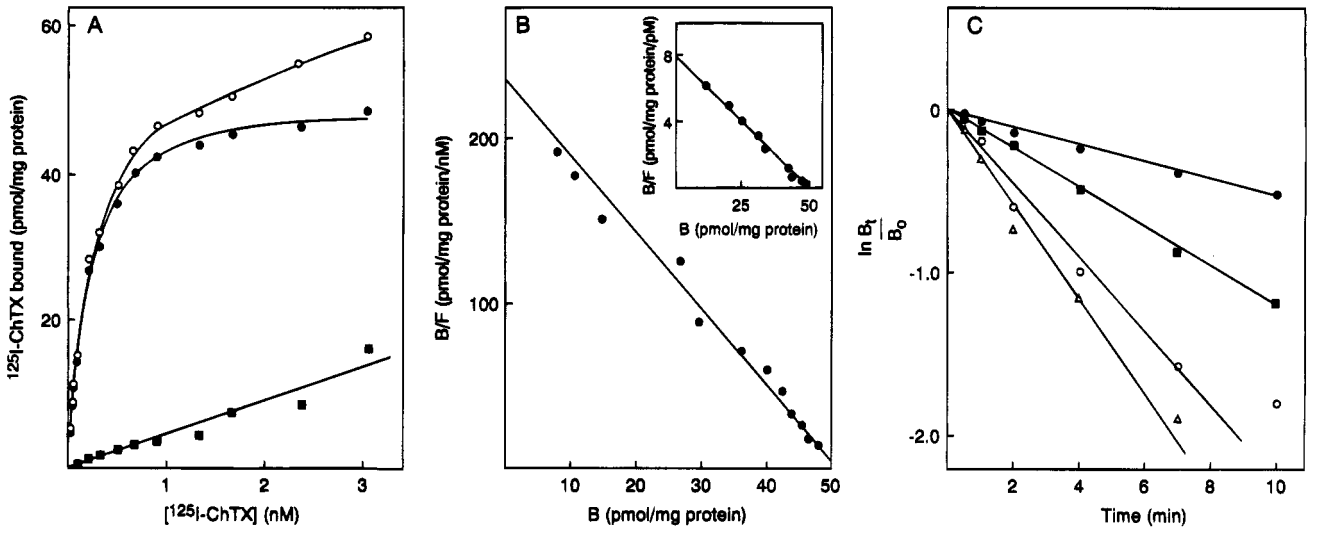

FIGURE 1: Binding of ChTX and ChTX-Lys mutants to a partially purified maxi-K channel preparation from bovine tracheal smooth muscle. A) Saturation experiments. A maxi-K channel preparation was incubated in a medium consisting of 10 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.1% bovine serum albumin, 0.1% digitonin, and 5 μM paxilline with increasing concentrations of [^{125}I]ChTX $Arg_{19}Gln/Lys_{32}Gln$, in the absence (○) or presence of 10 nM ChTX (■). Samples were incubated at room temperature, and separation of bound from free ligand was accomplished as described under Experimental Procedures. Specific binding data (●) are defined as the difference between total binding (○) and nonspecific binding (■). B) Scatchard analysis. Specific binding data from panel A are presented in the form of a Scatchard representation. (Inset) Linear representation of the specific binding data for [^{125}I]ChTX. (C) Dissociation kinetics. The maxi-K channel preparation was incubated in the same medium as described above with either 17 pM [^{125}I]ChTX (●), 135 pM [^{125}I]ChTX(Lys_{11}-Asn) (○), 162 pM [^{125}I]ChTX($Lys_{31}Gln$) (△), or 151pM [^{125}I]ChTX($Arg_{19}Gln/Lys_{32}Gln$) (■) for 2 h at room temperature. Ligand dissociation was initiated by the addition of 10 nM ChTX while incubating at room temperature for different periods of time.

The two other [^{125}I]ChTX-Lys variants also bind to a single class of binding sites with K_d's of 185 and 180 pM and B_{max}'s of 49 and 48 pmol/mg of protein for the analogs, $Lys_{11}Asn$, and $Lys_{31}Gln$, respectively (not shown). Thus, although the apparent affinity for each radiolabeled mutant toxin is reduced ca. 30-fold with respect to [^{125}I]ChTX, the maximum density of binding sites labeled in each case is the same.

To determine which kinetic parameter of the binding reaction is altered leading to the decreased binding affinity of the ChTX-Lys mutants, time courses of toxin association and dissociation were determined for each peptide. In all cases, toxin association is a pseudo-first-order reaction, whereas toxin dissociation can be fit to a single monoexponential relationship that is indicative of a first-order reaction. The results of the toxin dissociation experiments are shown in Figure 1C and illustrate that all of the mutant peptides display enhanced off-rates when compared to wild-type ChTX. However, the magnitude of the differences are larger for $Lys_{11}Asn$ and $Lys_{31}Gln$ than for $Arg_{19}Gln/Lys_{32}Gln$. These data correlate qualitatively with what has been observed for inhibition of maxi-K channels by these toxins in functional studies (Park & Miller, 1992b). The rate constants for toxin association were also calculated from the slope of the pseudo-first-order binding reaction as indicated under Experimental Procedures. These values are 1.6×10^8, 1.6×10^7, 1.5×10^7, and 1.1×10^7 M^{-1} s^{-1} for ChTX and the $Lys_{11}Asn$, $Lys_{31}Gln$, and $Arg_{19}Gln/Lys_{32}Gln$ variants, respectively. Thus, the on-rates for the mutants are decreased about 10-fold with respect to ChTX. When K_d values for these peptides were calculated from the ratio of dissociation and association rate constants, the K_d's agree well with values determined under equilibrium binding conditions. These data, taken together, indicate that all the toxins employed in the present investigation bind to maxi-K channels with characteristics similar to those found in functional studies.

Cross-Linking of [^{125}I]ChTX-Lys Mutants to the Maxi-K Channel. To determine which Lys residue in the ChTX peptide is involved in cross-linking of toxin to the β subunit of the maxi-K channel in the presence of DSS, each of the [^{125}I]ChTX variants characterized above as well as native [^{125}I]ChTX were incubated with the partially purified tracheal maxi-K channel and subjected to cross-linking conditions as described under Experimental Procedures. Importantly, since all of the ChTX variants bind with ca. 30-fold lower affinity than ChTX, incubation conditions were adjusted so that the amount of toxin bound at equilibrium before addition of DSS was equivalent in each case. The results of these experiments are shown in Figure 2. The pattern of incorporation of radioactivity into the β subunit is the same for $Lys_{11}Asn$ and $Lys_{31}Gln$ ChTX mutants as it is for native ChTX. The efficiency of the cross-linking reaction is ca. 5−10% for each of the three toxins as determined after excising the corresponding area from the gel and determining the amount of radioactivity incorporated. In all cases, labeling of the β subunit is prevented by including an excess amount of nonradiolabeled ChTX in the incubation media. However, in the presence of the variant $Arg_{19}Gln/Lys_{32}Gln$, labeling of the β subunit is marked reduced to 10−17% of native ChTX. The same pattern was observed for the four peptides after exposure of the film for longer periods of time (data not shown). Since the amount of modified ChTX bound at equilibrium is identical in all cases, these results cannot be attributed to the lower affinity of this variant peptide for the maxi-K channel. Moreover, out of all the ChTX derivatives tested, $Arg_{19}Gln/Lys_{32}Gln$ is the one peptide that displays the slowest dissociation kinetics, and, by this criterion, it should therefore be the most favorable of the modified toxins to participate in the cross-linking reaction. Although these experiments were carried out with a partially purified tracheal smooth muscle ChTX receptor

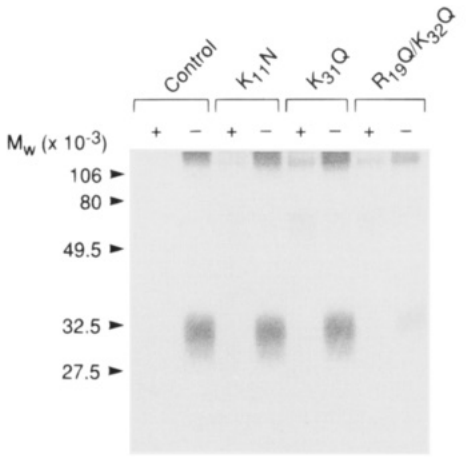

FIGURE 2: Cross-linking of ChTX and ChTX-Lys mutants to a partially purified bovine tracheal maxi-K channel preparation. A maxi-K channel preparation was incubated in a medium consisting of 10 mM NaCl, 10 mM Taps-NaOH, pH 9.0, 0.1% digitonin, and 5 μM paxilline with either 63 pM [^{125}I]ChTX, 320 pM [^{125}I]-ChTX(Lys11Asn), 432 pM [^{125}I]ChTX(Lys_{31}Gln), or 527 pM [^{125}I]ChTX(Arg_{19}Gln/Lys_{32}Gln), in the absence or presence of 10 nM ChTX, for 1 h at room temperature. At that point samples were adjusted to 300 mM NaCl, followed by the addition of DSS to a final concentration of 0.18 mM. After 1 min of incubation at room temperature, the reaction was quenched by addition of Tris-HCl, pH 7.4, at a final concentration of 200 mM. Samples were subjected to SDS−PAGE, and the dried gels were exposed to Kodak XAR-film at −70 °C for 24 h. Shown are data representative of four experiments carried out under identical conditions.

preparation, and by using an optimal concentration of paxilline identical results have been obtained in the absence of paxilline, or by employing either bovine aortic or tracheal sarcolemmal membrane vesicles as substrate (data not shown). Therefore, the proteolytic cleavage that occurs of the α subunit during purification (Knaus et al., 1995) does not appear to affect the pattern of the cross-linking reaction, since the same results are obtained with a preparation that displays the full size 125 kDa protein. The same pattern of toxin incorporation is observed when the four peptides are cross-linked with two other bifunctional reagents that have different fully extended spacer arms, DSG (8 Å) and EGS (16 Å) (data not shown), although, in these cases, the corresponding Lys acceptor residue(s) in the β subunit has not been identified. The cross-linking efficiencies for both DSG and EGS have been previously shown to be similar to that of DSS (Knaus et al., 1994a). Thus, these results suggest that Lys_{32} of ChTX is the residue that participates in covalent attachment of toxin to the β subunit of the maxi-K channel in the presence of a bifunctional cross-linking reagent. Although neutralization of Lys_{32} does not completely abolish the cross-linking reaction (i.e., 10−17% residual labeling is observed with this mutant), the Lys residue responsible for the minor reaction has not yet been characterized. However, previous work in which Lys_{69} was identified as the acceptor residue on the β subunit suggested that Lys_{11} in ChTX is not involved in the reaction (Knaus et al., 1994a). Moreover, all the digestion patterns of the ChTX-labeled β subunit are consistent with labeling occurring solely at Lys_{69} of β. Therefore, it is tempting to speculate that Lys_{31} of ChTX could be the residue responsible for 10−17% of the labeling noted above and that the acceptor residue in β is still Lys_{69}. Double Lys mutants of ChTX will be necessary to test this hypothesis.

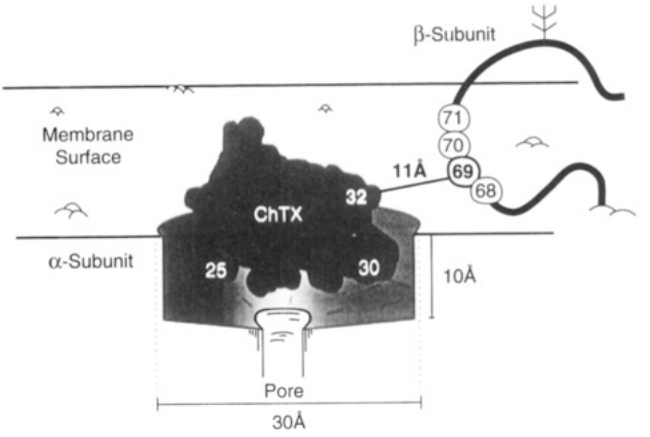

FIGURE 3: Schematic representation of the pore region of the maxi-K channel. ChTX is viewed sitting in the outer vestibule of the maxi-K channel. The dimensions of the vestibule are taken from those described by Stampe et al. (1994). A region of the extracellular loop of the β subunit is depicted highlighting residue 69. This residue is placed at a maximal distance of 11 Å from Lys_{32} of ChTX without contributing to the wall of the vestibule. The positions of two other critical residues of ChTX, Arg_{25} and Asn_{30}, are also indicated.

Structure of the Maxi-K Channel. Site-directed mutagenesis studies with ChTX, combined with knowledge of the three-dimensional structure of the toxin in solution, have defined an interaction surface for the peptide with the maxi-K channel. Based upon a number of assumptions, a hypothetical view of the ChTX receptor has been formulated (Stampe et al., 1994). In this model, the vestibule is viewed as a recessed flat surface of about 30 Å x 20 Å in size, with a wall no more than 10 Å high. Since heterologous expression of *mSlo* cDNA yields ChTX-sensitive maxi-K channels (Butler et al., 1993), it is expected that the ChTX receptor would be formed solely from residues contributed by the α subunit. This idea is supported by data presented in the current study and by recent work in which toxin sensitivity could be transferred to the ChTX-insensitive channel, $K_v2.1$, by interchanging the residues between the S_5 and S_6 region of the channel (Gross et al., 1994). Lys_{32}, the ChTX residue cross-linked to the β subunit, is positioned 10 Å above the flat interaction surface and, therefore, could be within the outer limits of the channel's vestibule (Figure 3). This would place the cross-linked region of the β subunit, Lys_{69}, at a maximal distance of 11 Å from the vestibule's border. Although 10−17% of cross-linking could occur to Lys_{31} of ChTX, a residue which would be located within the channel's vestibule but above the toxin's interaction surface, the extracellular domain of the β subunit is likely to be facing the outside of the channel mouth. Even though the conformation of the toxin bound to the channel is not expected to change dramatically, the large extracellular loop of β could be more flexible and adopt certain conformations that favor cross-linking to Lys_{31} of the toxin. Furthermore, lack of reaction of the β subunit residue's Lys_{44} and Lys_{122} with the cross-linking reagent indicates that the regions of the extracellular domain containing these amino acids are more distant from the vestibule than the area containing Lys_{69}.

The stoichiometry of the maxi-K channel complex has not been fully established, but experimental evidence suggests that both subunits are present in a ratio of 1:1 (Garcia-Calvo et al., 1994). Therefore, a functional channel should be formed by association of four α and four β subunits. It is

interesting to note that, in a symmetric tetrameric channel, bound toxin imposes asymmetry. Therefore, there should be only one β subunit in the complex that is positioned at the appropriate distance for the cross-linking reaction to take place. Perhaps the residual reaction that is observed putatively with Lys_{31} of ChTX occurs via a different β subunit of the complex.

The topology of the maxi-K channel α subunit has not yet been defined. By analogy with other K^+ channels, it is expected to consist of six transmembrane domains (S_1-S_6) and a pore region located between S_5 and S_6 (Butler et al., 1993). In addition, there is a very large C-terminal domain, which is not found in other K^+ channels, that could form four additional transmembrane segments. Therefore, the number of extracellular residues that are contributed by the α subunit in the formation of the ChTX receptor is not known with certainty. Nevertheless, it may be safe to assume that only those residues within the first six transmembrane segments of the channel are important by analogy with results obtained from studies of other ChTX-sensitive K^+ channels. The topology of the β subunit is better defined. It contains a large extracellular domain consisting of residues 40−156 which connects two transmembrane segments. The two extracellular glycosylation sites are occupied by carbohydrate chains of ca. 5−6 kDa each. The number of extracellular amino acid residues contributed by each channel subunit could be similar. Although the β subunit may not be an intrinsic part of the ChTX receptor, it could, however, participate in toxin binding. For example, it is known that electrostatic forces contribute in the binding of ChTX to the maxi-K channel (Anderson et al., 1988). There are several negatively charged amino acid residues in the β subunit, some of which are located in proximity to Lys_{69}, that could influence the toxin−channel interaction under low ionic strength conditions. Clearly, definition of the role of the β subunit in maxi-K channel function will require further studies of the channel's properties after coexpression of the two subunits. The positioning of certain residues of the β subunit in relationship with the pore of the maxi-K channel as inferred from the present data should help in future studies directed at probing subunit interactions and channel function.

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