The β Subunit of the High Conductance Calcium-activated Potassium Channel

IDENTIFICATION OF RESIDUES INVOLVED IN CHARYBDOTOXIN BINDING*

Markus Hanner‡§, Rosane Vianna-Jorge‡, Augustus Kamassa§, William A. Schmalhofer‡, Hans-Günter Knaus‡¶, Gregory J. Kaczorowski‡, and Maria L. Garcia‡**

From the ‡Department of Membrane Biochemistry and Biophysics, Merck Research Laboratories, Rahway, New Jersey 07065 and the ¶Institute for Biochemical Pharmacology, University of Innsbruck, Peter-Mayr Strasse 1, A-6020 Innsbruck, Austria

Coexpression of α and β subunits of the high conductance Ca\(^{2+}\)-activated K\(^{+}\) (maxi-K) channel leads to a 50-fold increase in the affinity for 125I-charybdotoxin (125I-ChTX) as compared with when the α subunit is expressed alone (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). To identify those residues in the β subunit that are responsible for this change in binding affinity, Ala scanning mutagenesis was carried out along the extracellular loop of β, and the resulting effects on 125I-ChTX binding were determined after coexpression with the α subunit. Mutagenesis of each of the four Cys residues present in the loop causes a large reduction in toxin binding affinity, suggesting that these residues could be forming disulfide bridges. The existence of two disulfide bridges in the extracellular loop of β was demonstrated after comparison of reactivities of native β and single-Cys-mutated subunits to N-biotin-maleimide. Negatively charged residues in the loop of β, when mutated individually or in combinations, had no effect on toxin binding with the exception of Glu94, whose alteration modifies kinetics of ligand association and dissociation. Further mutagenesis studies targeting individual residues between Cys76 and Cys103 indicate that four positions, Leu90, Tyr91, Thr93, and Glu94 are critical in conferring high affinity 125I-ChTX binding to the α-β subunit complex. Mutations at these positions cause large effects on the kinetics of ligand association and dissociation, but they do not alter the physical interaction of β with the α subunit. All these data, taken together, suggest that the large extracellular loop of the maxi-K channel β subunit has a restricted conformation. Moreover, they are consistent with the view that four residues appear to be important for inducing an appropriate conformation within the α subunit that allows high affinity ChTX binding.

---

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of Erwin Schrödinger Fellowships J-01108-MED and J-01460-MED from the Austrian Research Foundation.

¶ Supported by grants from the Austrian Research Foundation (P12863-MED, P-11187-MED), the Austrian National Bank Foundation (number 6269), and the European Union BIOMED 2 program (BMH4-CT96-2118).

** To whom correspondence should be addressed. Tel.: 732-594-7564; Fax: 732-594-3925; E-mail: maria_garcia@merck.com.

---

High conductance Ca\(^{2+}\)-activated K\(^{+}\) (maxi-K) channels purified from bovine aortic and tracheal smooth muscle are composed of two noncovalently linked subunits, α and β (1, 2). The α subunit is a member of the Slo family of K\(^{+}\) channels (3, 4), whereas the β subunit is a novel protein that modifies the biophysical and pharmacological properties of α. In coexpression experiments, the β subunit causes a hyperpolarizing shift of ~80–100 mV in the midpoint of channel activation (4–7). This shift is equivalent to that produced by a 10-fold rise in Ca\(^{2+}\) concentration. Thus, coexpression of α and β subunits in vivo could be a way of regulating maxi-K channel activity. Whereas maxi-K channels present in different smooth muscle tissues (e.g. aorta (2), trachea (1), coronary artery (8), colon (9), and presumably uterus (10)) appear to be composed of both α and β subunits, the composition of these channels in other tissues remains to be determined. For example, there is evidence in brain that α subunits are expressed in the absence of the β subunit that is present in smooth muscle (11, 12), and a similar situation appears to occur in skeletal muscle (12). However, brain maxi-K channels may possess a unique β subunit as indicated from cross-linking experiments employing rat brain membranes and the selective maxi-K channel blocker, moniodotyrosine-iberiotoxin-D19Y/Y36F (125I-IbTX-D19Y/Y36F; Ref. 13). The identity and functional characteristics of this putative novel β subunit must still be determined.

---

1 The abbreviations used are: maxi-K channel, high conductance Ca\(^{2+}\)-activated K\(^{+}\) channel; ChTX, charybdotoxin; 125I-ChTX, moniodotyrosine-charybdotoxin; 125I-IbTX-D19Y/Y36F, moniodotyrosine-iberiotoxin-D19Y/Y36F.
linking experiments, which place Lys on of β at a maximal distance of 11 Å from Lys of ChTX when toxin is bound in the vestibule of α, indicate that some residue(s) of the large extracellular loop of β are in close proximity to the vestibule of the channel’s pore (16, 17). These residues could either participate in the formation of the ChTX receptor or alter the conformation of α allosterically so as to cause an increase in the affinity for ChTX.

In the present study, we performed Ala scanning mutagenesis of residues in the extracellular loop of β to identify those that contribute to high affinity binding of 125I-ChTX, after coexpression with the α subunit. Our results indicate that the four Cys residues in the loop form disulfide bridges, which should lead to a more restricted conformation of β, and that four residues, Leu90, Tyr91, Thr93, and Glu94, are crucial for high affinity binding of 125I-ChTX. Based on the effects of these mutations on kinetics of ligand association and dissociation, it is suggested that these residues are important for conferring an appropriate conformational change in α that provides the large increase in ChTX binding affinity. A preliminary report of these findings has appeared in abstract form (18).

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes were from Promega. Pfu DNA polymerase was purchased from Stratagene. pCI-neo vector was obtained from Promega. COS-1 cells (culture CRL 1650) were obtained from 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 are from Life Technologies, Inc. FuGENE™6 transfection reagent and streptavidin-conjugated alkaline phosphatase were from Boehringer Mannheim. Fetal bovine serum was purchased from Hyclone. 3-(N-Maleimidylpropionyl)biocytin was obtained from Molecular Probes Inc. DNA polymerase (Pfu DNA polymerase) was from Promega. 3-Maleimidylpropionyl)biocytin was obtained from Biosearch Technologies (Skokie, IL). GFC glass fiber filters were purchased from Whatman. ChTX was bought from Peninsula Laboratories and radioiodinated as described (19). ChTX mutants were a generous gift from Dr. Chris Miller, Brandeis University. All other reagents were obtained from commercial sources and were of the highest purity commercially available.

**Mutant Channel Constructs—**A HindIII restriction site was generated in the β subunit by altering amino acid Arg to Lys. A 9E10 c-Myc tag (20) was introduced at the C terminus using an oligonucleotide cassette containing HindIII and NotI restriction sites. Alanine scanning was performed by site-directed mutagenesis using the “overlap extension” technique (21). Polymerase chain reaction amplification was carried out using Pfu DNA polymerase. The integrity of all mutant constructs was verified by nucleotide sequencing (automated DNA sequencer, ABI 373A). The human α clone huR2 ( ) and the bovine β clones were subcloned into pCI-neo vector (15).

**Transfection of COS-1 Cells and Membrane Preparation—**COS-1 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with defined fetal bovine serum, penicillin/streptomycin, and l-glutamine under 10% CO2, 100% humidity at 37 °C, as described previously (15). For transfections, either 10 µg of α DNA, with or without 3 µg of β DNA, per T-252 cm2 flask was employed, together with 100 µl of LipofectAMINE™ (Life Technologies, Inc.). All conditions for transfection have been described previously (15). After transfection, cells were grown for 72 h. Cells were then scraped from the flasks and collected by centrifugation. Membranes were prepared as described (22). The final membrane pellet was resuspended in 100 mM NaCl, 20 mM Hepes-NaOH, pH 7.4. Aliquots were quickly frozen in liquid N2 and stored at −70 °C.

**Binding Assays**—The interaction of 125I-ChTX with COS-1 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 mM paxilline. For saturation experiments, membranes were incubated with increasing concentrations of 125I-ChTX at room temperature until equilibrium was achieved (e.g., 3–4 h). The kinetics of ligand dissociation were determined by addition of 10 mM ChTX to receptor-bound 125I-ChTX and incubating at room temperature for different periods of time. Separation of bound from free ligand was achieved using filtration protocols as described (19).

**Transfection of TsA-201 Cells—**TsA-201 cells, a subclone of the human embryonic kidney cell line HEK293 that expresses the SV40 T antigen, were maintained in Dulbecco’s modified Eagle medium/Ham’s F-12 medium, enriched with 10% fetal bovine serum, minimum Eagle’s medium nonessential amino acids, minimum Eagle’s medium sodium pyruvate, solution, and grown at 37 °C in 5% CO2. FuGENE™6 transfection reagent was used for transfection of TsA-201 cells. The transfection procedure was performed according to the manufacturer’s instructions with a FuGENE™6:DNA ratio of 3:1. After transfection, cells were grown for 44–52 h, scraped, collected by centrifugation, and frozen in liquid N2. After thawing at room temperature, cells were homogenized by using a glass tissue grinder in 250 mM sucrose, 5 mM MgCl2, 20 mM Tris-HCl, pH 7.4. The material was centrifuged at 500 × g for 10 min. The supernatant was saved and the pellet was homogenized again. Combined supernatants were centrifuged at 100,000 × g for 45 min. The final membrane pellet was resuspended in 100 mM NaCl, 20 mM Hepes-NaOH, pH 7.4. Aliquots were frozen in liquid N2 and stored at −70 °C.

**Labeling with 3-(N-Maleimidylpropionyl)biocytin—**TsA-201 membranes were biotinylated by incubation with 0.2 mM 3-(N-maleimidylpropionyl)biocytin, at room temperature for 5 min. 3-(N-Maleimidylpropionyl)biocytin was added from a fresh 50 mM stock solution prepared in dimethyl sulfoxide. The reaction was quenched by addition of 2% (v/v) 2-mercaptoethanol. Membranes were pelleted by centrifugation at 150,000 × g for 15 min and solubilized with 1% Triton X-100 in a medium consisting of 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, for 1 h on ice. Insoluble material was removed by centrifugation at 150,000 × g for 20 min. Soluble material was diluted 4-fold with 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, and applied to an immobilized 9E10 c-Myc antibody column (Immunopure® protein G IgG orientation kit, Pierce; immobilization of the antibody was performed according to the manufacturer’s instructions) equilibrated with 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.1% Triton X-100. Under these conditions, membranes were washed with equilibration buffer, and bound material was eluted with 0.1 M glycine/HCl, pH 2.8, 0.1% Triton X-100. The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and biotinylated proteins were detected by using streptavidin-conjugated alkaline phosphatase.

**Antibody Production and Synthesis of Anti-β118–132 Antibody Column—**Polyclonal serum raised against residues 913–926 of the α subunit (Anti-α913–926), and residues 118–132 of the β subunit (Anti-β118–132) of the maxi-K channel were obtained as described (17, 23). 2 ml of anti-β118–132 was affinity-purified on the corresponding peptide column, as described previously (23). Antibodies were eluted in the presence of 0.1 M borax, pH 2.5, and the pH was neutralized immediately by addition of NaOH. Affinity-purified antibodies were coupled to 0.5 g of CNBr-activated Sepharose (Amersham Pharmacia Biotech) in 0.25 M NaHCO3, 0.5 M NaCl, according to the manufacturer’s protocol. After 120 min of coupling at 22 °C, the remaining reactive groups were blocked with 0.1 M glycine, pH 8.5. After incubating for 120 min, the resin was washed with 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.1% Triton X-100.

**Construction of COS-1 Membranes and Immunoaffinity Chromatography of αβ Subunit Complexes—**COS-1 membranes were solubilized with 2% Triton X-100 in a medium consisting of 0.5 M NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, 0.1 mM benzamidine, 1 mM iodoacetamide for 30 min on ice. Insoluble material was removed by centrifugation at 100,000 × g for 60 min. Soluble αβ complexes (600–1000 ml) were diluted 3-fold by addition of chilled water and incubated with an anti-β118–132, antibody column that had been equilibrated in 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.1% Triton X-100, for 12 h at 4 °C. Afterward, the column was extensively washed with equilibration buffer, and bound material was eluted stepwise by addition of 0.2 M glycine/HCl, pH 2.5, 150 mM NaCl, 0.1% Triton X-100, neutralized by addition of NaOH and subjected to Western blot analysis using either affinity-purified anti-α (913–926) or anti-β118–132, as described previously (15).

**Analysis of Data—**Data from saturation experiments were analyzed according to the equation \( B = B_{\text{max}}/(1 + (K_d/L)^n) \), where \( B_{\text{max}} \) is the maximum number of receptors, \( K_d \) is the dissociation constant, \( n \) is the pseudo-Hill coefficient, and \( L \) is the free ligand concentration. The dissociation rate constant (\( k_\text{off} \)) was determined directly from the first order plot of ligand dissociation versus time.

**RESULTS**

The β Subunit of the Maxi-K Channel: Role of Cys Residues—The bovine maxi-K channel β subunit contains five Cys residues, all of which are conserved among various species such as...
Interaction between α and β Subunits of the Maxi-K Channel

Fig. 1. Mutation of Cys residues in the maxi-K channel β subunit. A, saturation kinetics. Membranes derived from COS-1 cells transiently transfected with α + β subunits of the maxi-K channel were incubated with increasing concentrations of 125I-ChTX at room temperature, until equilibrium was achieved. Non-specific binding was determined in the presence of 10 nM ChTX. Specific binding data were analyzed as described under "Experimental Procedures." Data are presented as the ratio of Kd values for wild type and mutant β versus the corresponding Cys residue mutated and represent the mean ± S.E. of at least two different transfection experiments. The Kd of the α/β control was 3.0 pm. B, dissociation kinetics. Membranes derived from COS-1 cells transiently transfected with α and β subunits of the maxi-K channel were incubated with 3 pm 125I-ChTX at room temperature. Dissociation kinetics were initiated by addition of 10 nM ChTX, and samples were incubated at room temperature for different lengths of time. The k−1 values were determined directly from first order plots of ligand dissociation versus time. Data are presented as the ratio of k−1 values for wild type and mutant β versus the corresponding Cys residue mutated. The k−1 of the α/β control was 4 × 10−4 s−1.

rat, dog, and human. Four of these Cys residues are located in the large extracellular loop of the protein that connects the two transmembrane domains, whereas the fifth residue resides near the intracellular surface at the beginning of the first transmembrane domain. To determine whether any of these residues plays a role in the process by which the β subunit influences 125I-ChTX binding activity, individual Cys residues were substituted with Ala, and the effects of these mutations on 125I-ChTX binding were analyzed after coexpression with the α subunit. These studies were performed under two experimental conditions: (a) using saturation binding protocols where measurements were made at equilibrium and (b) by monitoring the time course of 125I-ChTX dissociation. Fig. 1A shows the results of saturation experiments in which the ratio of the equilibrium dissociation constant (Kd) of mutant versus Kd of wild type is plotted against the corresponding Cys residue changed for each mutation. In Fig. 1B, a plot of the ratio of the dissociation rate constant (k−1) of mutant versus wild type is also presented as a function of the Cys residue mutated. In these plots, a ratio of 1 indicates that the behavior of the mutant is identical to wild type, whereas a ratio >1 reflects decreased binding at equilibrium or an increase in the rate of dissociation as a result of the amino acid substitution. When the behavior of the Cys18→Ala substitution is considered, it is clear that this mutation has no effect on the ability of the β subunit to modulate 125I-ChTX binding. However, the results are markedly different when mutations at the other Cys residues are investigated. Thus, substitution of individual Cys residues, Cys53, Cys76, Cys103, or Cys135, leads to dramatic changes in the properties of the 125I-ChTX binding reaction; both Kd and k−1 are altered in these mutants. Since the effect of these mutations on the Kd value is larger than on k−1, it is predicted that the rate constant of association (k+1) is also decreased in these mutations. Multiple substitution of Cys residues produce similar effects on 125I-ChTX binding as those observed with single amino acid changes (Fig. 1, A and B).

The large effects observed with the four Cys mutations in this β subunit could be explained if these residues are forming pairs of disulfide bridges that are important for maintaining the protein’s tertiary structure. In this case, the conformation of the loop would be largely restricted, and altering such conformation might diminish the contribution of the β subunit to stimulating 125I-ChTX binding. To determine whether or not extracellular Cys residues of the maxi-K channel β subunit are forming disulfide bridges, we subjected membranes derived from TsA-201 cells that had been transiently transfected with both α and β subunits to reaction with the sulfhydryl reagent, N-biotin-maleimide. Subsequently, the β subunit was immunoprecipitated, separated by SDS-polyacrylamide gel electrophoresis, and visualized using a streptavidin-conjugated alkaline phosphatase. To facilitate these studies, we employed in the control the substitution Cys18→Ala, since this mutation has no effect on 125I-ChTX binding and eliminates a potential sulfhydryl reactive center in the protein. Fig. 2 shows the results of these experiments. The β subunit Cys18→Ala mutant did not display any significant reaction under these detection conditions (lane 2), although the presence of the protein could be detected in parallel Western blot experiments employing an antibody against the β subunit (data not shown). Likewise, the β subunit in which all Cys residues have been mutated did not give a reaction (lane 3). In marked contrast, all β subunit constructs in which one of the extracellular Cys residues had been changed to Ala gave a strong signal upon reaction with streptavidin-conjugated alkaline phosphatase (lanes 4–7). These results strongly suggest that extracellular Cys residues in the β subunit form disulfide linkages, because there is no reaction of the native protein with the sulfhydryl reagent.

Negative Charged Residues in the Extracellular Loop of the β Subunit—To determine which residue(s) of the β subunit participate in the large increase in 125I-ChTX binding affinity that is observed after coexpression with the pore forming subunit, we first focused on anionic residues that are present in the extracellular loop of the protein. We reasoned that since ChTX is a highly positively charged molecule, some or all of the effects of the β subunit on toxin binding might be due to interaction between positively charged residues in the toxin with negatively charged residues in the β subunit. There are 16 anionic residues in the extracellular loop of the bovine β sub-
unit. Of these 16 residues, only five are not conserved in the rat and human homologues. All 16 residues of the bovine β subunit were mutated individually, or in combination, to Ala and the consequences of these mutations were analyzed with respect to $^{125}$I-ChTX binding after their coexpression with the α subunit. Results of these experiments are presented in Fig. 3. Neutralization of the charge on most residues, whether done individually or as combinations, has no effect on $^{125}$I-ChTX dissociation kinetics. Similar results were obtained by monitoring $^{125}$I-ChTX binding under equilibrium conditions (data not shown). However, Glu$^{94}$ → Ala produces marked changes in toxin binding. This alteration is observed regardless of whether Glu$^{94}$ is neutralized by itself or if it is neutralized along with other negatively charged residues. In the later case, the effects noted on toxin binding were no different from those observed with the single Glu$^{94}$ → Ala mutation. It is worth mentioning that Glu$^{94}$ is a conserved residue among different species. Interestingly, Asp$^{95}$, which is also a conserved residue and which lies next to Glu$^{94}$, does not appear to significantly influence toxin binding.

Role of Residues Located between Cys$^{76}$ and Cys$^{103}$ in the β Subunit—Ala scanning mutagenesis of negatively charged residues in the extracellular loop of the β subunit demonstrated that only Glu$^{94}$ appears to be important for modulating $^{125}$I-ChTX binding. Since these mutagenesis studies cover residues spanning almost the entire extracellular region of β, we next concentrated our attention on those residues surrounding Glu$^{94}$ that are located between Cys$^{76}$ and Cys$^{103}$. As before, the effects of mutations of these residues were analyzed on $^{125}$I-ChTX binding after coexpression of each modified β subunit with the pore forming subunit of the channel; the results of these experiments are presented in Fig. 4. Most of these Ala substitutions produced no effect on either equilibrium binding (Fig. 4A) or toxin dissociation kinetics (Fig. 4B). There are, however, three additional residues that when mutated caused large changes in $^{125}$I-ChTX binding affinity. In addition to Glu$^{94}$, which has been reported above, mutations at Leu$^{90}$, Tyr$^{91}$, and Thr$^{93}$ caused pronounced effects on the ability of the β subunit to increase $^{125}$I-ChTX binding. The major effect is observed for Leu$^{90}$, whose alteration results in a destabilization of toxin binding by about 1.75 Kcal/mol when equilibrium binding is assessed. All of these mutations are also expected to cause changes in association kinetics, since the effects observed on $K_d$ are larger than those found on $k_+ \cdot k_-$ values. It is interesting to note that mutation of His$^{92}$, located between the four critical residues, is itself without effect. Moreover, the four critical residues are all conserved between different species.

An important question arises as to whether the effects produced by these mutations are caused by lack of association of α and β subunits into a functional complex or are the consequence of changing the binding properties conferred by a particular residue. Preliminary evidence, which favors the idea that α and mutant β subunits are indeed associated together, and that the observed effects are due to specific changes in binding affinity caused by these amino acid substitutions, comes from the analysis of $^{125}$I-ChTX dissociation kinetics. Fig. 4C shows results from such experiments in which wild type α, or α and β, are compared with mutations containing Leu$^{90}$, Tyr$^{91}$, and Thr$^{93}$ substitutions. It is apparent that in all cases the data can be fit to first order reactions; this is consistent with the existence of a single class of toxin binding sites for all α/mutant β pairs examined. All relevant mutations significantly enhance toxin dissociation kinetics, but the rates of dissociation never reach the level found when the α subunit is expressed in the absence of β (see also Fig. 4B). These data then are consistent with the idea that the mutations in β under investigation do not prevent a physical interaction between α and β subunits.

Association of α and β Subunits—The fact that mutations in
the β subunit that cause large effects on 125I-ChTX binding do not disrupt an interaction between α and β has been investigated in a more direct fashion. For these experiments, membranes solubilized in Triton X-100 were incubated with an anti-β(118–132) affinity column. Material retained by the column was then eluted at acidic pH, separated by SDS-polyacrylamide gel electrophoresis, and tested for the presence of α and β subunits by Western blotting employing affinity-purified antibodies directed against each protein. The presence of the β subunit is expected in all cases, since an anti-β(118–132) affinity column was used to retain solubilized material. However, the presence of α subunits in the eluted material can only be explained if this subunit is bound to β. Fig. 5 shows the results of experiments in which solubilized control membranes containing α and β subunits of the maxi-K channel were incubated with increasing concentrations of 125I-ChTX at room temperature until equilibrium was achieved. Other experimental conditions are given under “Experimental Procedures.” Data are presented as the ratio of Kd values for wild type and mutant β versus the corresponding residue mutated. The Kd of toxin binding to the α/β control was 3.0 pm. B, dissociation kinetics. Membranes were incubated with 3 pm 125I-ChTX at room temperature, and dissociation kinetics were determined as indicated under “Experimental Procedures.” Data are presented as the ratio of k−1 values for wild type and mutant β versus the corresponding residue mutated. The k−1 values measured for toxin dissociation from α/β or α alone were 4 × 10−7 s−1 and 3 × 10−3 s−2, respectively. C, Time course of 125I-ChTX dissociation. Membranes were incubated with 3 pm 125I-ChTX, and dissociation kinetics were initiated by addition of 10 nM ChTX. Samples were incubated at room temperature for the indicated periods of time. ●, α; □, α + β; ○, α + βLeu90Ala; ◆, α + βTyr91Ala; ●, α + βThr93Ala. Experimental data have been fitted to a first order reaction, where B0 and Bt represent 125I-ChTX bound at time 0 and t, respectively.

Although there are differences in the amount of protein in the starting material due to variations in the efficiency of the transient expression system, there do not appear to be significant differences for a given construct with respect to the amount of either α or β subunit present in starting solubilized material versus that eluted from the antibody affinity column. Similar results have been obtained with the Thr93 mutation (data not shown). These data are consistent with physical association of α and β subunits in a complex for all mutants investigated.

An alternative way of detecting the presence of the β subunit in the maxi-K channel complex is to carry out cross-linking experiments with either 125I-ChTX or 125I-IbTX-D19Y/Y36F in the presence of a bifunctional cross-linking reagent such as disuccimidyl suberate. It has previously been demonstrated that either 125I-radiolabeled ligand is covalently incorporated solely into the β subunit of the maxi-K channel complex (1, 24), despite the fact that binding requires the obligatory presence of the α subunit. The results of such experiments conducted with
membranes derived from COS-1 cells transiently transfected with α and β subunits are consistent with the close physical association of these subunits in a complex for all β subunit mutants examined, since successful toxin cross-linking was achieved in each case (data not shown).

**DISCUSSION**

The results presented in this study provide new information concerning the means by which the β subunit of the maxi-K channel produces a large increase in the binding affinity for 125I-ChTX, when it is coexpressed with the pore forming α subunit. Detailed Ala scanning mutagenesis studies have provided structural information regarding the existence of two disulfide bridges in the large extracellular loop of the maxi-K channel β subunit and have identified four residues, Leu 90, Tyr 91, Thr 93, and Glu 94 that are critical in conferring high affinity 125I-ChTX binding to the αβ subunit complex of this channel (Fig. 6).

The existence of two disulfide bridges in the extracellular loop of the β subunit has been elucidated by mutagenesis of single Cys residues and, subsequently, determining the reactivity of the modified protein to sulfhydryl reagents. These experiments were facilitated by the use of Cys18 mutant that is not altered in its functional coupling to α, but which eliminates a potential sulfhydryl reactive group in the molecule. The modified Cys18 mutant does not show any reactivity toward sulfhydryl reagents. However, further single amino acid mutations in any of the four Cys residues present in the extracellular loop of β yield proteins that are able to react with N-biotin-maleimide. These results are consistent with a model where the four Cys loop residues form two disulfide bridges in the native β subunit structure. The presence of these disulfide bonds should restrict the conformation of the loop and produce a defined interaction surface that could affect the conformation of the ChTX receptor. Consistent with this idea, mutagenesis of these Cys residues causes large changes in the properties of 125I-ChTX binding. These four residues are all conserved among various species, and they may be critical in the formation of a maxi-K channel complex where the β subunit influences other functional aspects of channel activity (4, 6, 7, 9, 12).

Extensive Ala scanning mutagenesis of other residues in the extracellular loop of β have identified four amino acids that appear to be critical for functional coupling to the α subunit as defined by their ability to enhance 125I-ChTX binding when coexpressed with the pore-forming subunit. These four residues are clustered in one region of the molecule between Cys76 and Cys103. Although we have not studied the consequences of eliciting more than one mutation at a time, individual changes have pronounced effects on toxin binding. For instance, Leu90 causes a 20-fold decrease in the affinity of 125I-ChTX for its receptor, as determined under equilibrium binding conditions; this is equivalent to a destabilization effect of ca. 1.75 Kcal/mol. Both the kinetics of ligand association and dissociation appear
to be affected by this substitution and the other three critical alterations in primary structure. What is the mechanism by which these residues of β provide the functional coupling with the α subunit to enhance toxin binding? Two possibilities should be considered. First, a region within β comprising these residues may directly participate in the formation of the ChTX receptor. In this scenario, such critical residues in β could contribute to an interaction with residue(s) in ChTX to enhance affinity of toxin for the αβ subunit complex. Another possibility is that the β subunit changes the conformation of the α subunit’s vestibule where ChTX binds, but it itself does not participate directly in the formation of the receptor. We have tested for the first possibility by doing complementary mutagenesis between the β subunit and ChTX. Lys₁₁, Lys₃¹, Arg₁⁹, and Lys₃² of ChTX are not critical for interaction with the maxi-K channel from rabbit t-tubule skeletal membranes (25), a channel that does not appear to contain a β subunit (15). The finding that Glu⁹⁴ of β has pronounced effects on ¹²⁵I-ChTX binding prompted us to investigate a putative interaction between this residue and the positively charged residues of ChTX noted above. Of all the ChTX mutants investigated (Lys₁¹ → Asn, Lys₃¹ → Asn, Arg₁⁹ → Gln/Lys₃² → Gln, Ser₁₀ → Gln, Asn₃₀ → Leu, Asp₃⁰ → Gly, Tyr₉⁶ → Gln, and Ser₉⁷ → Gln), no evidence has been found for a direct interaction between the critical residues of β Tyr₉¹ and Glu⁹⁴ and those of ChTX (data not shown). Although we cannot exclude the possibility that the limited scope of these studies and the nature of the mutations generated may have missed such an interaction, we favor the idea that the β subunit causes a conformational change in the α subunit, and that this change modifies not only the biophysical, but also the pharmacological properties of α. Supporting this hypothesis is the finding that both Cys mutations, as well as substituting a limited number of critical residues in the β subunit, have similar effects on ¹²⁵I-ChTX binding; all modifications alter the kinetics of ligand association and dissociation. This behavior is not unexpected for the Cys mutations, since they are predicted to produce large changes in the conformation of the loop of β. However, for the other critical residues, the changes in the kinetics of ligand association might be the result of alterations in the spatial localization of negatively charged residues that electrostatically drive ChTX into its receptor site (26). This is consistent with the idea of the β subunit changing the conformation of α to enhance ChTX binding. There is, however, still the formal possibility that, in addition to changing the conformation of α, these critical residues also participate in the formation of the ChTX receptor by interacting directly with residue(s) still unidentified in the toxin. Regardless of the mechanism, Leu₉⁰, Tyr₉¹, Thr₉³, and Glu⁹⁴ are all conserved between different species, which is consistent with their importance in the functional coupling of β to the α subunit. The results of this investigation should be compared with previous findings, which indicate that Lys⁹⁹ in the β subunit is the residue that cross-links to ¹²⁵I-ChTX bound in the vestibule of the α subunit, suggesting close proximity of regions of the extracellular loop of β to the site where ¹²⁵I-ChTX binds (16, 17).

A striking finding of these studies is the observation that most of the negatively charged residues in the extracellular loop of β, with the exception of Glu⁹⁴, have no effect on ¹²⁵I-ChTX binding. This is true whether these residues are mutated individually or in combination, exclusive of Glu⁹⁴. Such data suggest that the electrostatic mechanism which drives ¹²⁵I-ChTX binding most likely is derived from negatively charged residues present in the α subunit. It is also noteworthy to mention that Asp⁹⁵, which lies next to Glu⁹⁴, has no effect on ¹²⁵I-ChTX binding and that His⁹², which is located between two other pairs of critical residues, also appears to have no effect on the coupling of β to the α subunit.

Recently, it has been shown that the extracellular domain of the α subunit of the maxi-K channel, hSlo, together with the first transmembrane domain S₃, are important for the shift in the activation curve of the channel to hyperpolarized potentials caused by the presence of the β subunit (14). The predicted topology of hSlo differs from that of other voltage-gated K⁺ channels in that hSlo has an additional transmembrane domain, S₄, but it is not known how the overall α-helical packing of the protein occurs (27). It is possible that S₄ in hSlo is involved in an interaction with one of the putative α-helical transmembrane domains of the β subunit and that this interaction could account for the large overall conformation change in hSlo, which favors gating transitions to the open state, while also altering the pharmacological properties of the αβ subunit complex (4, 6, 7, 15). Further studies are necessary to detail the molecular determinants of the αβ subunit interaction that mediate such dramatic changes in maxi-K channel activity.

Coexpression of α with the β subunit(s) in target tissues is a potential way of regulating maxi-K channel activity in vivo. Smooth muscle tissues such as colon, coronary artery, aorta, and trachea express both subunits of the maxi-K channel (1, 2, 8, 9), and in these cases it is expected that the channel should play a predominant role in controlling cellular excitability since it could respond to subtle changes in Ca²⁺ concentrations. In other tissues, such as in the CNS, in which α subunits are not always coexpressed with β subunits (11, 12), the channel would require much larger changes in Ca²⁺ concentration to become activated. It is possible that other β subunits exist which could mediate functional changes in channel activity as those observed with the smooth muscle β subunit, but these putative subunits remain to be identified.

The results of our study confirm and extend previous observations concerning the functional coupling between α and β subunits of the maxi-K channel. They also add new information concerning the structure of the extracellular loop of β and the molecular determinants involved in conferring high-affinity ¹²⁵I-ChTX binding to the αβ subunit complex.

Acknowledgment—We thank Maria Trieb for technical contributions.

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) Proc. Natl. Acad. Sci. U. S. A. 92, 5929–5933
3. Butler, A., Tsunoda, S., McCobb, D. P., Wei, A., and Salkoff, L. (1993) Science 261, 221–224
4. Wallner, M., Meera, P., Ottolia, M., Kaczorowski, G. J., Latorre, R., Garcia, M. L., Stefani, E., and Toro, L. (1995) Recept. Channels 3, 185–199
5. Meera, P., Wallner, M., Ottolia, M., Adelman, J. P., Kaczorowski, G., Garcia, M. L., and Toro, L. (1995) Biophys. J. 68, 3a
6. Dworetzky, S. I., Boissard, C. G., Lum-Ragan, J. T., McKay, M. C., Post-munson, D. J., Trojnacki, J. T., Chang, C.-P., and Griibkoff, V. K. (1996) J. Neurosci. 16, 4543–4550
7. McManus, O. B., Helms, L. H., Pallanic, L., Ganetzy, B., Swanson, R., and Leonard, R. J. (1995) Neuro 14, 1–20
8. Tanaka, Y., Meera, P., Song, M., Knaus, H.-G., and Toro, L. (1997) J. Physiol. (London) 502, 545–557
9. Vogalis, F., Vincent, T., Qureshi, I., Schmalz, F., Ward, M. W., Sanders, K. M., and Horowitz, B. (1996) Am. J. Physiol. 271, G629–G639
10. Meera, P., Wallner, M., Jiang, Z., and Toro, L. (1996) FEBS Lett. 382, 84–88
11. Chang, C.-P., Dworetzky, S. I., Wang, J., and Goldstein, M. E. (1997) Mol. Brain Res. 45, 33–40
12. Tseng-Crank, J., Godinot, N., Jefhansen, T. E., Ahring, P. K., strobaek, D., Mertz, R., Foster, C. D., Olesen, S. P., and Reinhardt, P. H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9200–9205
13. Koch, R. O. A., Koschak, A., Wanner, S. G., Kaczorowski, G. J., Wittka, R., Garcia, M. L., and Knaus, H. G. (1996) Soc. Neurosci. Abstr. 22, 1754
14. Wallner, M., Meera, P., and Toro, L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14922–14927
15. Hanner, M., Schmalhofer, W. A., Munujos, P., Knaus, H.-G., Kaczorowski, G. J., and Garcia, M. L. (1995) Proc. Natl. Acad. Sci. U. S. A. 94, 2853–2858
16. Munujos, P., Knaus, H.-G., Kaczorowski, G. J., and Garcia, M. L. (1994) Biochemistry 33, 10771–10776
17. Knaus, H.-G., Eberhart, A., Kaczorowski, G. J., and Garcia, M. L. (1994) Mol. Biol. Lond. 271, 545–557
Interaction between α and β Subunits of the Maxi-K Channel

23. Knaus, H.-G., Schwarzer, C., Koch, R. O. A., Eberhart, A., Kaczorowski, G. J., Glossmann, H., Wunder, F., Pongs, O., Garcia, M. L., and Sperk, G. (1996) J. Neurosci. 16, 955–963
24. 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
25. Park, C.-S., and Miller, C. (1992) Biochemistry 31, 7749–7755
26. Anderson, C. S., MacKinnon, R., Smith, C., and Miller, C. (1988) J. Gen. Physiol. 91, 317–333
27. Meera, P., Wallner, M., Song, M., and Toro, L. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14066–14071