Purification and Reconstitution of the High-conductance, Calcium-activated Potassium Channel from Tracheal Smooth Muscle*

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The high-conductance Ca**-activated K* ( maxi-K ) channel from bovine tracheal smooth muscle was purified to apparent homogeneity by a combination of conventional chromatographic techniques and sucrose density gradient centrifugation. Fractions with the highest specific activity for binding of monoiodotyrosine charybdotoxin, [*'I]ChTX, were enriched ~2000-fold over the initial digitonin-solubilized material up to a specific activity of 1 nmol/mg protein. Silver staining after SDS-polyacrylamide gel electrophoresis of the fractions from the last step of the purification indicates that binding activity is correlated with a major component of the preparation that displays an apparent molecular weight of 62,000. Labeling the same preparation with [*'I]Bolton-Hunter reagent reveals the existence of both 62 (α) and 31 (β)-kDa subunits, in an apparent stoichiometry of 1:1, comigrating with binding activity. The β subunit is heavily glycosylated. Deglycosylation studies indicate that the β subunit represents the protein to which [*'I]ChTX is covalently incorporated in the presence of the bifunctional cross-linking reagent disuccinimidyl carbonate. Binding of [*'I]ChTX to the purified ChTX receptor displayed the same pharmacological profile that has been found previously for toxin binding to native membranes, including inhibition by iberiotoxin, limbatustoxin, tetraethylammonium, potassium, cesium, and barium. The purified preparation was reconstituted into liposomes which were then fused with artificial lipid bilayers. Single channels were readily observed with liposomes that were blocked by ChTX. The open probability of these channels was increased by depolarizing membrane potentials and by raising the internal calcium concentration. These data suggest that the maxi-K channel purified from tracheal smooth muscle is composed of two subunits.

Potassium channels comprise a family of proteins that can be subdivided into different categories based on their biochemical and pharmacological properties. Two broad classes of potassium channels can be distinguished dependent on the physiological stimuli that cause the channels to open: voltage-gated and ligand-gated channels. Structural information is available regarding the family of voltage-gated channels due to the cloning and functional expression of several channel types encoded by the genes responsible for mutant phenotypes in Drosophila (1). Mammalian homologues of these Drosophila channels have been identified using low stringency hybridization and expression cloning techniques (2). Regions in these proteins that form the ion-conducting pore (3, 4), that confer sensitivity to channel inhibitors (3, 5–10), and that are involved in channel activation (11–15) and inactivation (16, 17) have been identified. Functional voltage-gated potassium channels are formed by association of four identical or dissimilar subunits (18–21), and this may contribute to the diversity of potassium channels found in different tissues. The subunit composition of native K* channels, however, is still unclear, since the only type of voltage-dependent K* channel that has been purified to date is the dendrotokyn receptor from rat and bovine brain (22, 23). This protein is composed of two units with molecular weights of 76,000–80,000 and 38,000. NH2-terminal protein sequence of the larger subunit is identical to one of the previously cloned mammalian K* channels (24), although there is evidence from reconstitution experiments of heterogeneity in the biophysical properties of the preparation (25). No structural information is yet available regarding the smaller subunit, and its role in channel function is unknown. Antibodies directed against a different type of subunit are precipitated not only the subunit encoded by drk1, but an additional 38-kDa protein, suggesting that this channel is formed by a complex of two or more subunits (26).

The family of ligand-gated potassium channels contains members whose open probability is regulated by intracellular messengers such as ATP and calcium, neurotransmitters, GTP-binding proteins, and other mediators. One member of the ligand-gated family of potassium channels that has been characterized biochemically is the apamin receptor that forms a small conductance Ca**-activated K* channel. Cross-linking and photoaffinity labeling experiments carried out with [*'I]apamin derivatives and rat brain membranes suggest that the receptor is composed of two subunits of 86 and 30 kDa (27–30). The high-conductance calcium-activated potassium ( maxi-K ) channel is a special member of the family of ligand-
gated potassium channels because its gating is both ligand- and voltage-dependent (31). Channel opening requires calcium binding to sites on the cytoplasmic face of the channel, and in the presence of calcium, channel opening is increased by membrane depolarization. Recently, the gene responsible for the slowpoke phenotype in Drosophila has been cloned (32) and shown to express Ca\(^{2+}\)-activated K\(^{+}\) channels (33). This gene can potentially encode a large number of alternatively spliced variants giving rise to a variety of possible channel proteins. However, the only way to determine directly the physical properties and subunit composition of the channels as they are expressed in different tissues is via biochemical isolation and analysis. Biochemical analysis of maxi-K channels is made possible by discovery of peptide toxins (e.g., charybdotoxin (ChTX),iberitoxin (IbTX), and limbus-toxin (LbTX)) isolated from scorpion venoms that are potent blockers of these channels (34, 35). High affinity binding sites for moniodirotysine ChTX ([\(\text{[^{125}I]}\)ChTX]) with moderate density have been identified in smooth muscle membrane preparations, and the pharmacological properties of these receptors suggest that they are associated with maxi-K channels (36, 37). The bovine aortic smooth muscle ChTX receptor has been solubilized in the presence of digitonin and characterized as a glycoprotein that displays a high sedimentation coefficient (23 S) upon sucrose density gradient centrifugation (38). In addition, a protein of 51 kDa has been identified as the ChTX receptor after covalent incorporation of \([\text{[^{125}I]}\)ChTX into either membranes or solubilized receptor in the presence of the bifunctional cross-linking reagent diisuccinimidyl suberate.

In the present study we describe the purification of the tracheal smooth muscle ChTX receptor to apparent homogeneity by a combination of conventional chromatographic techniques and sucrose density gradient centrifugation. Analysis of the purified preparation indicates the presence of two subunits, \(\alpha\) and \(\beta\), of 62 and 31 kDa, respectively, that copurify with binding activity. The purified receptor was reconstituted into liposomes which were fused with planar lipid bilayers. This preparation formed large conductance potassium-selective channels whose open probability was increased by calcium and membrane potential and which were blocked by ChTX. The properties of the purified reconstituted channels resemble those of native maxi-K channels, suggesting that the two subunits of 62 and 31 kDa represent the molecular components of the maxi-K channel from smooth muscle. A preliminary report of some of these findings has been made in abstract form (39).

**Experimental Procedures**

**Materials**—ChTX and IbTX were purchased from Peninsula Laboratories. LbTX was purified from venom of the scorpion Centruroides limatus as described previously (35). \([\text{[^{125}I]}\)ChTX (2200 Ci/mmole) and \([\text{[^{125}I]}\) Bolton-Hunter reagent (2200 Ci/mmole) were obtained from DuPont NEN. Recombinant N-glycans was bought from Genscience. WGA-Sepharose was supplied by Pharmacia LKB Bio-technologies Inc. Digitonin special grade (water soluble) was obtained from Amersham. Palmitoyl-2-oleoylphosphatidylethanolamine (POPE) and l-palmitoyl-2-oleoylphosphatidylcholine (POPC) were obtained from Avanti Polar Lipids, Inc. Decane, from Fisher, was 99.9% mol purity. The protein-gold reagent was from Integrated Separation Systems. All other reagents were obtained from commercial sources and were of the highest purity commercially available.

**Purification of the ChTX Receptor**—Purified sarcolemmal membrane vesicles derived from bovine tracheal smooth muscle were prepared as described previously (40). All buffers employed for solubilization and during purification contained 1 mM iodoacetamide, 0.1 mM phenylmethanesulfonyl fluoride, 0.1 mM benzamidine. Membranes derived from 250 cow tracheas were solubilized with 0.5% digitonin for 10 min at 4 °C, followed by centrifugation at 180,000 \(\times\) g for 50 min. The supernatant was removed and discarded. The remaining pellet was homogenized in 20 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1% digitonin and the mixture was incubated at 4 °C for 10 min. Solubilized membranes were resuspended in 20 mM Tris-HCl, pH 7.4, 0.1% digitonin. This process was repeated a total of five times. The resulting supernatants \((S_a)\) were pooled, adjusted to 50 mM NaCl, and loaded onto a DEAE-Sepharose CL6B column (500 ml of packed gel) equilibrated with 50 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.1% digitonin. Bound receptor was eluted batchwise with 70 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.1% digitonin. The eluted ChTX receptor was incubated overnight at 4 °C with 200 ml of WGA-Sepharose in 200 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.1% digitonin. The suspension was then placed in an empty column, and the fluid phase was collected until the WGA-Sepharose resin was packed. Unbound material was removed by washing with 10 bed volumes of equilibration buffer. Glycoproteins were biospecifically eluted with 200 mM N-acetyl-d-glucosamine in equilibration buffer. The eluate was dialyzed against 20 mM Tris-HCl, pH 7.4, 0.05% digitonin, concentrated 20-fold, and adjusted with NaCl to a final concentration of 200 mM. Subsequently, the sample was applied in eight consecutive runs to a Mono Q HR10/10 (Pharmacia) ion exchange column equilibrated with 100 mM NaCl, 30 mM Tris-HCl, pH 7.4, 0.05% digitonin. A linear gradient was applied from 0.1 to 0.5 M NaCl over 70 min at a flow rate of 2 ml/min. Fractions displaying ChTX binding activity, between 0.21 and 0.31 M NaCl, were adjusted to 80 mM sodium phosphate, pH 7.5, and loaded onto a Bio-Cell HPHT (Bio-Rad) 100 \(\times\) 7.5-mm hydroxylapatite column, equilibrated with 80 mM sodium phosphate, pH 7.0, 10 mM NaCl, 0.05% digitonin. Bound material was eluted at a flow rate of 0.5 ml/min with a linear gradient from 80 to 160 mM sodium phosphate in 10 mM NaCl within 12 min, followed by a gradient from 160 mM sodium phosphate in 10 mM NaCl to 560 mM sodium phosphate, 70 mM NaCl within 10 min. Fractions containing ChTX binding activity eluted between 200 and 440 mM sodium phosphate. These fractions were dialyzed against 20 mM Tris-HCl, pH 7.4, 0.05% digitonin, concentrated, and separated on a continuous 7–25% (w/v) sucrose gradient. Active fractions were dialyzed against 20 mM Tris-HCl, pH 7.4, 0.05% digitonin and loaded onto a Mono S HR5/5 (Pharmacia) ion exchange column preequilibrated with the same buffer. Bound material was eluted with a linear gradient from 0 to 700 mM NaCl within 20 min at a flow rate of 0.5 ml/min. Fractions containing ChTX binding activity, which eluted between 120 and 280 mM NaCl, were dialyzed against 20 mM Tris-HCl, pH 7.4, 0.01% digitonin, concentrated to 0.3 ml, and applied to another continuous sucrose gradient as described above.

**Binding Assays**—The interaction of \([\text{[^{125}I]}\)ChTX with bovine tracheal sarcolemmal membrane vesicles was monitored as described previously (36). Binding of radiolabeled toxin to solubilized receptor was measured by incubating aliquots of solubilized material in 0.05% digitonin with \([\text{[^{125}I]}\)ChTX as outlined previously (38). At the end of the incubation period, protein was precipitated by addition of 10% (w/v) poly(ethylene glycol) \((M, \approx 8000)\) in the presence of \(\gamma\)-globulin, and the precipitate was immediately collected onto GF/C glass fiber filters that had been presoaked in 0.5% polyvinylalcohol. Nonspecific binding was determined in the presence of 10 nM ChTX. For each experiment, triplicate assays were routinely performed, and the data were averaged. The standard error of the mean of these replicates was typically less than 3%.

**Cross-linking Experiments**—Covalent incorporation of \([\text{[^{125}I]}\)ChTX into either membranes or solubilized material in the presence of the bifunctional reagent diisuccinimidyl suberate was carried out as described previously (38).

**Bolton-Hunter Labeling**—Fractions from the last sucrose density gradient centrifugation containing 31–158 ng of protein were subjected to SDS-PAGE and dried gels were exposed to Kodak XAR-5 film.

**Enzymatic Deglycoylsation of the \(\beta\) Subunit**—Samples were dialyzed for 12 h at 4 °C against 5 mM Tris-HCl, pH 7.4, 0.05% digitonin and denatured by heating for 5 min at 65 °C in the presence of 0.5% Nonidet P-40 in 100 mM NaCl, 20 mM \(\beta\)-mercaptoethanol. Demembranated samples were subjected to 8% polyacrylamide gel electrophoresis (SDS-PAGE) and deglycoylation was started by addition of 1 IU recombinant \(\beta\)-galactosidase. F. After incubation at 37 °C for different periods of time, the reaction was stopped by addition of boiling SDS-
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sample buffer. Samples were subjected to SDS-PAGE and dried gels were exposed to Kodak XAR-5 film.

Reconstitution of the ChTX Receptor into Liposomes—Aliquots (300 μl, containing 121–185 μg of protein/ml) of purified ChTX receptor in 0.05% digitonin were incubated on ice with 0.3% bovine serum albumin, 0.34% L-α-phosphatidylcholine, 0.85% CHAPS for 50 min. The mixture was loaded onto a 7 ml of 2.5 ml of a Linear Gradient of G-25 Sephacryl S-2000 (Pharmacia Chemical Co.) equilibrated with 100 mM NaCl, 20 mM Hepes-NaOH, pH 7.4, 0.2% bovine serum albumin and eluted with 1.5 ml of 100 mM NaCl, 10 mM MgCl₂, 20 mM Hepes-NaOH, pH 7.4. Proteoliposomes were precipitated by the addition of polyethylene glycol (Mw = 8000) to give a final concentration of 25% and collected by centrifugation at 100,000 rpm (Beckman TL-A 100-3 rotor) for 20 min. Proteoliposomes were washed once in 100 mM NaCl, 20 mM Hepes-NaOH, pH 7.4, frozen in liquid N₂ and stored at −80 °C.

Recordings of Maxi-K Channels in Planar Lipid Bilayers—Planar lipid bilayers (200–300 μF) were formed by applying a mixture of POPG and POPC in a 7/3 molar ratio diluted in decane at 50 mg/ml to a 250-μm hole separating two aqueous compartments. Currents flowing across the bilayer were measured with standard voltage clamp methods. Proteoliposomes containing the purified ChTX receptor were thawed at room temperature, sonicated for 10 s, and fused with the bilayer in the presence of an osmotic gradient consisting of 150 mM NaCl, 10 mM MgCl₂, 50 mM DTT, and incubated at 37 °C for 120 min. Samples were subjected to SDS-PAGE using either continuous or discontinuous 5% gels (41).

Analysis of Data—Data from saturation binding experiments were subjected to Scatchard analysis, and linear regression was performed to obtain the equilibrium dissociation constant (Kd), and the maximum receptor concentration (Bmax). The correlation coefficients were greater than 0.97. Data from competition experiments were analyzed by the method of Cheng and Prusoff (42), to determine Ki values.

Protein Determination—Protein concentration was determined using either the Bradford (43) or the Gold method (44) with bovine serum albumin as standard.

RESULTS

Purification of the Bovine Tracheal ChTX Receptor—[125I]ChTX binds to a single class of receptor sites in aortic and tracheal smooth muscle sarcolemma that are known to be associated with maxi-K channels (36, 37). These receptors from bovine tracheal sarcolemma membranes can be solubilized in functional form in the presence of digitonin. As found previously when aortic sarc olemmal membranes were used (38), optimal solubilization of membrane-bound ChTX receptors is achieved by six consecutive extractions with digitonin. The solubilized material was fractionated using a combination of different chromatographic techniques and sucrose density gradient centrifugation. A typical purification scheme is outlined in Table I. A large amount of starting material (~250 bovine tracheas that yield ~10 g of purified sarcolemmal membrane vesicles) was used for each purification, because the density of ChTX receptors in the membrane preparation is modest (0.5–0.8 pmol/mg of protein). The first step in purification, DEAE-Sepharose CL6B is intended to remove large quantities of other proteins that would decrease the binding capacity of the WGA-Sepharose. Using this purification scheme, we have purified the ChTX receptor almost 2000-fold with recovery of 3.3% of the initial binding activity. It is worth noting that total recoveries at each individual step during purification are close to 100%, indicating that no significant loss of activity occurs during the time involved in the purification procedures. This purification scheme has been repeated three more times yielding identical results.

Subunit Composition of the ChTX Receptor—In order to identify the molecular components of the ChTX receptor, the distribution of [125I]ChTX receptors and protein concentration in fractions obtained from the final sucrose density gradient procedure were determined (Fig. 1). The purified ChTX receptor migrates in sucrose gradients as a large particle with an apparent sedimentation coefficient of 23S (Fig. 1B), and most of the binding activity was contained in fractions 14 and 15. When fractions from the sucrose gradient were subjected to SDS-PAGE, staining with silver revealed a single component with an apparent molecular weight of 62,000 that comigrated with [125I]ChTX binding activity (Fig. 1A).

A component of 31,000 was specifically labeled in tracheal sarcolemmal membranes with [125I]ChTX in the presence of the bifunctional cross-linking reagent disuccinimidyl carbonate (Fig. 2A), and in cross-linking experiments carried out with purified receptor, a component of 31,000 was also specifically labeled (not shown). Labeling of this protein is abolished by agents such as bolton-hunter, TEA, and potassium that are known to inhibit ChTX binding to maxi-K channels (Fig. 2A). Since this component is heavily glycosylated (see below), it may not stain well by conventional protein staining techniques. Therefore, fractions from the sucrose gradient were labeled with [125I]bolton-hunter reagent, subjected to SDS-PAGE, and analyzed by autoradiography. Results of these experiments are shown in the inset of Fig. 1B. From the distribution of [125I]Bolton-Hunter labeled polypeptides, it is evident that ChTX binding activity correlates with the presence of two subunits, α and β, of 62,000 and 31,000 apparent molecular weights. Although other minor components appear to be present in some of the fractions, their pattern across the gradient clearly does not correlate with binding activity. Given the staining properties of the smaller subunit, it was difficult to attempt to estimate the relative stoichiometry of both components using the classical approach of densitometric scanning of a stained gel. As an alternative approach, we determined the amount of [125I]Bolton-Hunter reagent incorporated into each subunit after their separation by SDS-PAGE. The amount of radioactivity incorporated into the α subunit was 1.8 times higher than that in the β subunit which suggests a stoichiometry of 1:1 for both components assuming that labeling is proportional to the size of the protein.

Characterization of the β Subunit—We examined the relationship between the β subunit identified after Bolton-Hunter labeling of the purified preparation and the component labeled with [125I]ChTX in cross-linking experiments. Deglycosylation experiments were carried out with both preparations. Recombinant N-glycanase caused a time-dependent conversion of the [125I]ChTX cross-linked protein (apparent molecular weight of 35,000; 31 kDa for the core protein plus 4.4 kDa contributed by the radiolabeled toxin) into an intermediate form of 28.9 kDa and a final product of 25.6 kDa (Fig. 2B). These experiments indicate that this protein is heavily glycosylated, most probably at two different glycosylation sites by N-linked sugars. The same experiment was repeated with purified 125I-Bolton-Hunter-labeled receptor (Fig. 2C). The β subunit displayed an identical time course of deglycosylation to that of the [125I]ChTX cross-linked protein. The apparent molecular weight of the deglycosylated 125I-Bolton-Hunter labeled core protein was determined inde-
The presence of detergent, suggesting that the receptor preferably potassium, barium, cesium, and TEA (Fig. 3A). Determined from reversible toxin binding to proteoliposomes reconstituted into liposomes and then fused with artificial planar lipid bilayers. The efficiency of reconstitution, as the receptor retains the properties described previously for ChTX Ki values with increasing concentrations of [125I]ChTX (Fig. 3B). The properties of [125I]ChTX binding to the purified receptor, and the Ki values for inhibition determined from saturation experiments were carried out with increasing concentrations of [125I]ChTX (Fig. 3A). Linear transformation of the specific binding data in a Scatchard representation indicates the presence of a single class of binding sites that display an affinity of 100 pmol/mg of protein (Fig. 3B). This pA2 constant is similar to the value (36 pm) determined for [125I]ChTX binding to tracheal smooth muscle membranes under identical conditions. IbTX (46) and LbTX (35) are potent blockers of maxi-K channels and do not block other known ChTX-sensitive K+ channels (34). For this reason, these toxins are useful tools in identifying maxi-K channels. IbTX and LbTX (Fig. 3C), as well as native ChTX, inhibit the binding of [125I]ChTX to the purified receptor, and the K+ values for inhibition (156, 107, and 8.4 pm) were virtually identical to those determined in intact membranes. In addition, [125I]ChTX binding to the purified receptor is inhibited by potassium, barium, cesium, and TEA (Fig. 3D) with similar K+ values (11.2, 5.4, 25, and 168 μM) to those determined for their interaction with the maxi-K channel in sarcosomal vesicles (36). These data suggest that the purified ChTX receptor retains the properties described previously for ChTX binding to maxi-K channels in intact membranes.

**Single Channel Recordings of Purified Maxi-K Channels**

In order to determine whether the purified ChTX receptor is sufficient to form functional maxi-K channels, fractions of the purified receptor with the highest specific binding activity were reconstituted into liposomes and then fused with artificial planar lipid bilayers. The efficiency of reconstitution, as determined from reversible toxin binding to proteoliposomes in the presence of digitonin to allow toxin access to both sides of the membranes, varied from 25 to 60%. In the absence of digitonin, toxin binding is ~30% of the amount observed in the presence of detergent, suggesting that the receptor preferentially reconstitutes in the inside-out orientation. Maxi-K channels were readily observed after applying the proteoliposomes to the cis side of the bilayer and incorporated into the bilayer with either polarity.

Fig. 4A shows a recording from a bilayer containing a single purified channel. The current fluctuates between two discrete levels, and the fraction of time spent in the open state increases as the internal calcium is raised from 10 to 20 μM or as the membrane is depolarized. A plot of open probability of the channel shown in Fig. 4A against membrane potential at different Ca²⁺ levels yields a family of curves (Fig. 4B) with similar slopes (e-fold change per 10–12 mV) in which Ca²⁺ shifts the midpoint of activation to more hyperpolarized potentials. Thus, the gating of the purified channel is regulated by calcium and membrane potential as expected for the maxi-K channel. The ionic selectivity and conductance of the channel shown in Fig. 4A is shown in Fig. 4C. In symmetrical 150 mM KCl and neutral lipids, the slope of the current-voltage relationship indicates a single channel conductance of 235 picosiemens. Decreasing the external potassium concentration to 25 mM caused a 42-mV shift in the reversal potential as predicted by the Nernst potential for a potassium selective channel and a decrease in single channel conductance to 184 picosiemens. External ChTX blocked the purified channel by causing long silent periods (not shown), which was similar to the pattern of ChTX block of maxi-K channels from other tissues (47). Thus the purified ChTX receptor is sufficient to reconstitute functional maxi-K channel activity.

**DISCUSSION**

The Purified ChTX Receptor Possesses the Same Properties as the Native Receptor—The ChTX receptor was purified to homogeneity from bovine tracheal smooth muscle plasma membranes using conventional chromatography and sucrose density gradient centrifugation. Presence of the ChTX receptor was assayed by measuring the number of binding sites for [125I]ChTX. After purification, the receptor concentration was increased approximately 2000-fold as compared with the solubilized membranes. The purification of the ChTX receptor from bovine tracheal smooth muscle described in this manuscript is a reproducible procedure hampered only by the large amounts of starting material required due to the moderate density of [125I]ChTX receptor sites in the native membranes. Since ligand affinity chromatography methods failed to purify this channel, a large number of steps was required to achieve a homogeneous preparation. The pharmacological properties of the ChTX receptor in the purified preparation are indistinct-

**TABLE I**

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<td>*Highest specific activity fraction from the gradient.</td>
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**Fig. 1. Subunit composition of the ChTX receptor.** A, silver staining after SDS-PAGE of the fractions from the final step of purification. Fractions were resuspended in SDS-PAGE sample buffer containing 50 mM DTT, incubated at 37 °C for 2 h, and subjected to SDS-PAGE using 10% acrylamide gels. The migration of molecular weight standards is shown. B, fractions from the last step of purification were analyzed for \[^{[125]}I\]ChTX binding activity (○) and protein concentration (●). The specific activities of various fractions in (cpm/μg protein) × 10⁷ as follows: 836 (lane 14); 1185 (lane 15); 1465 (lane 16); 1095 (lane 17); 902 (lane 18). Inset, aliquots of each fraction from the sucrose gradient were iodinated with \[^{[125]}I\]-Bolton-Hunter reagent and subjected to SDS-PAGE on a 12% acrylamide gel. The gel was dried and exposed to Kodak XAR-5 film for 150 min. The migration of molecular weight standards is shown.

**Fig. 2. Characterization of the \(\beta\) subunit.** A, cross-linking of \[^{[125]}I\]ChTX to bovine tracheal smooth muscle membranes. Covalent incorporation of \[^{[125]}I\]ChTX in the presence or absence of indicated agents was carried out as described under “Experimental Procedures.” Samples were subjected to SDS-PAGE using 10% acrylamide gels. The migration of molecular weight standards is shown. B, time course of deglycosylation of the \[^{[125]}I\]ChTX cross-linked subunit. \[^{[125]}I\]ChTX cross-linked material was incubated with 1 IU of recombinant N-glycanase for the indicated periods of time at 37 °C as indicated under “Experimental Procedures.” Samples were treated as outlined under “Experimental Procedures” and subjected to SDS-PAGE using 4-20% acrylamide gels. The migration of molecular weight standards is indicated by arrows. C, time course of deglycosylation of \[^{[125]}I\]-Bolton-Hunter-labeled purified ChTX receptor. Fraction 15 from the last sucrose density gradient was iodinated with \[^{[125]}I\]-Bolton-Hunter reagent and then incubated with recombinant N-glycanase for the indicated periods of time. Samples were resuspended in SDS-PAGE sample buffer and subjected to electrophoresis using 12% acrylamide gels. The migration of molecular weight standards is shown.

The purified ChTX receptor is composed of two subunits—separation of the purified ChTX receptor by SDS-PAGE revealed two subunits, a 62-kDa \(\alpha\) subunit and a 31-kDa \(\beta\) subunit. Both subunits comigrated with \[^{[125]}I\]ChTX binding activity in the final sucrose density gradient, suggesting that both proteins are part of the ChTX receptor. The \(\beta\) subunit did not stain using conventional methods, but could be visualized after \[^{[125]}I\]-Bolton-Hunter derivatization, where the protein is radiolabeled by covalent modification of its free amino groups. Treatment of this protein with recombinant N-glycanase resulted in a time-dependent two-step conversion to a core protein of 21 kDa, suggesting that the protein is glycosylated at least at two positions with N-linked carbohydrates. Extensive glycosylation of the \(\beta\) subunit may explain the inability to stain this protein using conventional methods and the broad appearance of this band on denaturing gels. The apparent mobility of the \(\alpha\) subunit during SDS-PAGE was not increased by treatment with N-glycanase. This behavior suggests that the \(\alpha\) subunit is not glycosylated by N-linked sugars. Since the \(\alpha\) subunit is retained on a WGA-Sepharose column which specifically retains glycoproteins, it appears that both subunits are tightly, but not covalently, associated and supports the finding that the ChTX receptor is composed of two different subunits.

Our finding that the ChTX receptor consists of two individual subunits is not surprising in light of previous results demonstrating that some voltage-dependent potassium channels may be composed of two or more different proteins. Antibodies specific for the drkl potassium channel gene product are able to precipitate both the 130-kDa protein likely encoded by the drkl gene and a 38-kDa polypeptide (26). The dendrotoxin receptor, that is associated to a subset of voltage-dependent potassium channels, has been purified from rat and bovine brain (22, 23) and is composed of two subunits with apparent molecular weights of 65,000 and 39,000 after...
The ChTX receptor in the purified preparation and in solubilized membranes (38) migrates as a 23 S particle upon sucrose density gradient centrifugation. Assuming that about half of the mass of this particle is contributed by detergent, the size of the receptor would be approximately 400 kDa. This estimate implies that the ChTX receptor is a heteromultimer likely composed of four or more α and β subunit pairs. Such a composition would be consistent with recent findings on the tetrameric structure of voltage-gated potassium channels (21).

The ChTX Receptor Is the Maxi-K Channel—Binding of [125I]ChTX to the purified ChTX receptor is inhibited by IbTX, LbTX, TEA, barium, cesium, and potassium. In electrophysiological experiments, these agents either block maxi-K channels by binding to sites in or near the entrance to the pore (47, 49–51), close to where ChTX binds to maxi-K channels (52, 53), or inhibit ChTX block of maxi-K channels (53). Thus, the pharmacological profile of inhibition of [125I] ChTX binding to the purified ChTX receptor is precisely the profile expected for ChTX binding to maxi-K channels.

Direct evidence that the purified ChTX receptor is the maxi-K channel comes from reconstitution experiments. When vesicles containing this preparation were fused with planar lipid bilayers, we readily observed large conductance (>200 picosiemens) potassium-selective channels that were blocked by ChTX. The open probability of these channels was regulated by calcium and membrane potential in a characteristic manner. Depolarization caused an e-fold increase in the presence of 10 nM ChTX (○) was calculated from the difference between total and nonspecific binding. B, specific binding data from A are presented in the form of a Scatchard transformation. C and D, effects of toxins and ions on [125I]ChTX binding to purified ChTX receptor. Purified receptor was incubated with 30 pM [125I]ChTX in a medium consisting of 10 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.05% digitonin, and 0.1% bovine serum albumin. Separation of bound from free ligand was performed as described under “Experimental Procedures.” Total binding (○) and nonspecific binding determined in the presence of 10 nM ChTX (△) are presented relative to an untreated control.

**FIG. 3. Pharmacological properties of the purified ChTX receptor.** A, saturation experiments. Aliquots of the fractions with the highest specific activity from the last step in purification were incubated with increasing concentrations of [125I]ChTX for 60 min at room temperature in a medium consisting of 10 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.05% digitonin, and 0.1% bovine serum albumin. Separation of bound from free ligand was performed as described under “Experimental Procedures.” Total binding (○) and nonspecific binding determined in the presence of 10 nM ChTX (△) are presented relative to an untreated control. 

B, specific binding data from A are presented in the form of a Scatchard transformation. C and D, effects of toxins and ions on [125I]ChTX binding to purified ChTX receptor. Purified receptor was incubated with 30 pM [125I]ChTX in a medium consisting of 10 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.05% digitonin, 0.1% bovine serum albumin for 60 min at room temperature in the absence or presence of increasing concentrations of ChTX (○, ●), IbTX (●, △), LbTX (●, ○), BaCl2 (●, ▲), KCl (●, △), CsCl (●, ▲), or TEA (○, △). Specific binding data in each case are presented relative to an untreated control.

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deglycosylation. The amino-terminal sequence of the 65 kDa subunit (24) is virtually identical to the deduced sequence of RCK5, a mammalian homologue of the Shaker family of voltage-dependent potassium channels. The 65-kDa subunit is likely to be the pore-forming subunit of the dendrotoxin receptor-channel complex, because RCK5 can encode the expression of functional voltage-dependent potassium channels in heterologous expression systems (48). By analogy, the 62-kDa α subunit of the ChTX receptor may be the pore-forming subunit of this channel complex.

We examined the stoichiometry of the subunits in the purified preparation by Bolton-Hunter labeling the preparation, excising the labeled α and β subunits from the gel after separation on SDS-PAGE, and determining the amounts of radioactivity incorporated into each protein. About 1.8-fold more radioactivity was incorporated into the α subunit compared with the β subunit. Assuming that the probability of covalent modification by Bolton-Hunter reagent is proportional to the molecular weight of the protein, we estimate that the α and β subunits are present in approximately a 1:1 stoichiometry.

The ChTX receptor in the purified preparation and in solubilized membranes (38) migrates as a 23 S particle upon sucrose density gradient centrifugation. Assuming that about half of the mass of this particle is contributed by detergent, the size of the receptor would be approximately 400 kDa. This estimate implies that the ChTX receptor is a heteromultimer likely composed of four or more α and β subunit pairs. Such a composition would be consistent with recent
that of the Ca²⁺-activated potassium channel responsible for the slowpoke phenotype in Drosophila, they have not yet provided structural information about other subunits that may be associated with these complexes. The purification of the maxi-K channel from bovine tracheal smooth muscle should allow cloning of both the α and β channel subunits after obtaining amino acid sequence information from these proteins. In this way, it will be possible through expression studies to define the role of each of the individual subunits in the maxi-K channel complex.

REFERENCES