Subunit Composition of the High Conductance Calcium-activated Potassium Channel from Smooth Muscle, a Representative of the mSlo and slowpoke Family of Potassium Channels

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High conductance Ca\(^{2+}\)-activated K\(^+\) ( maxi-K ) channels from bovine tracheal and aortic smooth muscle membranes have been purified employing monooiodo-tyrosine charybdotoxin binding as a marker for the channel and conventional chromatographic techniques. This K\(^+\) channel is composed of two subunits, \(\alpha\) and \(\beta\), of 62 and 31 kDa, respectively. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the electro-eluted tracheal smooth muscle \(\alpha\)-subunit was subjected to tryptic cleavage and a number of fragments were isolated by microbore C\(_{18}\) high performance liquid chromatography. Several of these peptides were microsequenced using Edman degradation techniques. Amino acid sequence information obtained from these fragments reveals the existence of very high sequence homology with the recently cloned mSlo maxi-K channel (Butler, A., Tsunoda, S., McCobb, D. P., Wei, A., and Salkoff, L. (1993) Science 261, 221-224). A specific antipeptide antibody directed against the amino acid sequence of one of the fragments of the \(\alpha\)-subunit is capable of specifically immunoprecipitating not only the denatured \(^{125}\)I-Bolton-Hunter-labeled \(\alpha\)-subunit, but also, under nondenaturing conditions, the complex of \(\alpha\) and \(\beta\) subunits, demonstrating specific noncovalent association of both subunits. Thus, our results indicate that the \(\alpha\)-subunit of the purified tracheal smooth muscle maxi-K channel is a member of the mSlo family of K\(^+\) channels and forms a noncovalent complex with a \(\beta\)-subunit. It is concluded that the extensive biochemical information acquired to date on smooth muscle charybdotoxin receptors is pertinent to the structure of native maxi-K channels.

Potassium channels are ubiquitous integral membrane proteins that serve numerous functions in excitable and nonexcitable cells (1). Major types include voltage-dependent, Ca\(^{2+}\)-activated, and ATP-sensitive channels, although subtypes usually exist within these classifications, and some channels exhibit overlapping characteristics. Even though representatives of several different classes of K\(^+\) channels have been cloned (2-5), few have been investigated at the biochemical level due to both the paucity of selective, high affinity probes for K\(^+\) channels, and the low channel density present in most tissues (6, 7).

High conductance Ca\(^{2+}\)-activated K\(^+\) ( maxi-K ) channels are a group of proteins that have recently been characterized by biochemical means. These channels are blocked with high affinity by a number of peptide toxins (e.g., charybdotoxin (ChTX), iberiotoxin (IbTX), and limbatustoxin), which were isolated from scorpion venoms and which bind in the external vestibule of the channel to physically occlude the pore and prevent ion conduction. With the use of antibodies specific for charybdotoxin (\(^{125}\)I-ChTX), high affinity receptor sites have been identified in bovine aortic and tracheal smooth muscle sarcolemma and shown to be associated with maxi-K channels. Recently, the ChTX receptor has been purified to homogeneity from bovine tracheal smooth muscle membranes (8) and shown to consist of two noncovalently associated subunits with peak mobilities on SDS-PAGE corresponding to \(M_\text{r}\) values of 62,000 and 31,000. The smaller subunit, termed \(\beta\), is heavily glycosylated by N-linked sugars; after deglycosylation, the \(M_\text{r}\) of this subunit is altered to 21,400 (8). An \(\alpha\)-identical subunit composition has been observed for the purified ChTX receptor from bovine aortic smooth muscle.\(^*\) Recently, a member of the maxi-K channel family has been cloned from mouse brain and skeletal muscle (3) using molecular probes derived from the Drosophila slowpoke Ca\(^{2+}\)-activated K\(^+\) channel (9). Expression of the mSlo cDNA in Xenopus oocytes yielded a voltage-dependent and Ca\(^{2+}\)-activated K\(^+\) current that was sensitive to external application of ChTX and IbTX, and which had other characteristics expected of a maxi-K channel.

In the present investigation, protein microsequencing was employed to obtain information regarding the composition of the \(\alpha\)-subunit of the purified smooth muscle ChTX receptor. The results presented herein demonstrate that this subunit is homologous to the recently cloned mSlo maxi-K channel. Moreover, it is directly shown by immunoprecipitation studies using site-directed antibodies against the \(\alpha\)-subunit that the purified maxi-K channel from smooth muscle is composed of two individual subunits, which are noncovalently associated.

EXPERIMENTAL PROCEDURES

Materials—Sequencing-grade modified trypsin (specific activity 15,500 units/mg) was purchased from Promega. Iodoacetic acid was obtained from Calbiochem. Wheat germ agglutinin-Sepharose 4B was from Pharmacia LKB Biotechnologies Inc. Protein-A Sepharose 4B, Dowex AG1X2, CHAPS, Triton X-100, iodoacetamide, phenylmethylsulfonyl acid, ChTX, charybdotoxin; IbTX, iberiotoxin; RP-HPLC, reversed-phase high performance liquid chromatography; RIA, radioimmunoassay.

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\(^\dagger\) The abbreviations used are: maxi-K channel, high conductance Ca\(^{2+}\)-activated K\(^+\) channel; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; ChTX, charybdotoxin; IbTX, iberiotoxin; RP-HPLC, reversed-phase high performance liquid chromatography; RIA, radioimmunoassay.

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forny fluorid, benzamidine, and N-acetyl-o-glucosamine were obtained from Sigma. 125I-Bolton-Hunter protein labeling reagent (2200 Ci/ mmol) was from DuPont NEN. All electrophoresis equipment was from Novex National Molecular weight standards were from Bio-Rad. All other reagents used were of the highest purity commercially available.

**Purification of the Maxi-K Channel**—A detailed description of the purification procedure has been published previously (8). In brief, maxi-K channels from either bovine tracheal or aortic smooth muscle membranes were solubilized after six consecutive exposures at 4°C to 1% digitonin in the presence of 1 mM iodoacetamide, 0.1 mM phenylmethylsulfonyl fluoride, benzamidine, and 0.1 mM EDTA. After centrifugation at 180,000 × g, the resulting supernatants were loaded onto a DEAE Sephacel CL-6B column and eluted batchwise with 170 mM NaCl. The eluted ChTX receptor was incubated overnight with wheat germ agglutinin-Sepharose and the bound material biospecifically eluted with 5% N-acetyl-o-glucosamine. The sample was subsequently applied to a Mono Q HR 10/10 (Pharmacia) ion exchange column and eluted with a linear NaCl gradient. The resulting active fractions were separated on a Bio-Gel HPHT (Bio-Rad) hydroxyapatite column and eluted with a sodium phosphate gradient. The fractions containing 125I-ChTX binding activity were dialyzed, concentrated, and separated on a 7–25% continuous sucrose density gradient. The active fractions were further purified by ion exchange chromatography on a Mono S HR5/5 column, dialyzed, and applied to a second continuous sucrose density gradient as described above.

**Bolton-Hunter Labeling**—Fractions (5 μl) from the final sucrose density gradient centrifugation step were dialyzed against 10 mM sodium borate, pH 8.8, 0.05% Triton X-100 and reacted with 5 μCi of 125I-Bolton-Hunter labeling reagent (2200 Ci/mmol) for 15 min on ice. The reaction was quenched by addition of Tris-HCl, pH 7.4, to a final concentration of 100 mM. Samples were subjected to immunoprecipitation studies as described below.

**Electroelution and Tryptic Fragmentation of the Bovine Tracheal Maxi-K Channel a-Subunit**—Fractions from the final sucrose density gradient centrifugation step containing ~70 pmol of 125I-ChTX binding activity were dialyzed overnight against 5 mM Tris-HCl, pH 7.4, 0.05% SDS, concentrated, and then subjected to SDS-PAGE. The gel was stained with Coomassie Blue, destained for 12 h, and slices corresponding to the 62-kDa a-subunit or a blank were cut out from the gel, minced, and electroeluted as described (12). After electroelution, samples were reduced (1% β-mercaptoethanol) in the presence of 4% SDS, alkylated with iodoacetic acid and dialyzed for 18 h against 6 M urea, 10 mM sodium phosphate, pH 7.2, containing 20 μl/g Dower AG1X2 resin. Dialysis was continued for 24 h against 5 mM Tris-HCl, pH 8.5, 0.05% CHAPS. Samples were concentrated about 20-fold and incubated with 4 μg of trypsin (80 μg/ml) for 15 h at 37°C.

**Internal Amino Acid Sequence of the Bovine Tracheal Maxi-K Channel a-Subunit**—The digested a-subunit was loaded onto an Applied Biosystems Protein Sequencing Column (300 μm) using an Applied Biosystems model 130A separation system. The reversed-phase column was equilibrated with 2% acetonitrile, 7 mM trifluoroacetic acid at a flow rate of 30 μl/min. Elution was achieved in the presence of a linear gradient from 2 to 98% acetonitrile (0.66% /min). The collected peptides were loaded onto Porton peptide filter supports and subjected to automated Edman degradation employing an integrated microsequencing system (Porton Instruments PI 2000) with an on-line detection system. Typical repetitive yields during sequencing of the various peptide fragments was 92%.

**Sequence Comparison**—The amino acid sequences obtained were aligned to the deduced mSlo and slowpoke sequences using the GAP program of the University of Wisconsin Genetic Computer Group software package (13).

**Sequence-directed Antibodies**—A polyclonal antiserum, anti-[421–435], was raised in rabbits against a synthetic peptide corresponding to the sequenced tryptic fragment HFTQVEFYQGSVLNP of the bovine tracheal maxi-K channel α-subunit with a cysteine residue added to the NH2 terminus to facilitate coupling. The peptide was synthesized by the solid-phase method, and its structure was confirmed by sequence analysis. Antibody was affinity purified. For immunofluorescent experiments, the peptide was coupled to keyhole limpet hemocyanin through its amino-terminal cysteine residue using the bifunctional reagent m-maleimidobenzo-2-nitrophenylhydroxysuccinimidyl ester. Peptide-keyhole limpet hemocyanin conjugate emulsified in Freund's adjuvant was injected subcutaneously into each of two rabbits. This procedure was repeated 1 month later, and serum collection was begun 2 weeks thereafter. Antibody production was monitored by enzyme-linked immunosorbent assay.

**Immunoprecipitation of Maxi-K Channel Subunits**—For all immunoprecipitation studies, 125I-Bolton-Hunter labeled α-subunit or the 125I-labeled maxi-K channel subunit complex. In all the experiments where the preparation had been denatured by boiling in SDS in the presence of β-mercaptoethanol, the final SDS concentration was never allowed to exceed 0.05% (w/v). The labeled α-subunit or native 125I-Bolton-Hunter-labeled purified maxi-K channels were incubated with the protein A-Sepharose-bound antibodies for 12 h at 4°C under gentle rotation. The immunoprecipitated samples were collected by sedimentation in a microcentrifuge and then washed three times with 1 ml of ice-cold RIA buffer. The immunoprecipitated radiolabeled subunits were first quantified by γ-counting and subsequently analyzed by SDS-PAGE after denaturation of the protein for 15 min at 56°C in SDS sample buffer containing 1% (v/v) β-mercaptoethanol. The radiolabeled subunits were separated on 12% SDS-PAGE gel and stained with Coomasie Blue R-250. To visualize the immunoprecipitated radiolabeled subunits, the gels were dried and exposed for 7 days at ~80°C to Kodak XAR film.

**Protein Determination**—Protein concentration was determined using either the Bradford (14) or the Lowry method (15) with bovine serum albumin as standard.

**RESULTS AND DISCUSSION**

**Maxi-K Channel α-Subunit Is a Representative of mSlo Family of K+ Channels**—To obtain internal amino acid sequence information from the α-subunit of the bovine tracheal smooth muscle maxi-K channel, substantial quantities of this protein were purified by a combination of classical chromatographic steps and sucrose density gradient centrifugation. The individual chromatographic steps included ion exchange, lectin affinity, and hydroxyapatite chromography in the presence of protease inhibitors. A detailed description of this purification scheme has appeared previously (8). A typical purification yields approximately 2000-fold enrichment in 125I-ChTX binding as compared to solubilized receptor. This preparation achieves a final specific activity of 1200 pmol of 125I-ChTX binding sites/mg of protein, with a recovery of about 3% of the binding sites relative to the crude solubilized extract. Binding of 125I-ChTX to the purified receptor preparation demonstrates the presence of a single class of sites with a pharmacological profile identical to that of membrane-bound receptors. The subunit composition of the purified preparation was ascertained by SDS-PAGE in the presence of reducing agents, followed by silver staining of the proteins or by autoradiography of the 125I-Bolton-Hunter-labeled samples. Whereas a polypeptide of approximately 62 kDa was clearly visible with either staining procedure, only the latter visualization method revealed the presence of two major bands (the 62-kDa α-subunit and a 31-kDa β-subunit), which copurify with 125I-ChTX binding activity (8). Deglycosylation of the radiolabeled purified ChTX receptor preparation with recombinant N-glycosidase F led to a size reduction of the β-subunit to 21.4 kDa as determined after SDS-PAGE. This high degree of N-linked glycosylation may account for the lack of staining of this protein with conventional methods. In contrast, the lack of change in electrophoretic mobility of the M, 62,000 α-subunit after identical treatment with N-glycosidase F suggests that it does not contain N-linked sugars (8).

For the purpose of obtaining amino acid sequence information from the α-subunit, approximately 70 pmol of purified tracheal smooth muscle ChTX receptor were separated under...
denaturing conditions by SDS-PAGE. The region corresponding to the position of the α-subunit was electroeluted, and the recovered material was subjected to proteolytic cleavage with trypsin. The resulting fragments were separated by microbore C18 RP-HPLC and then subjected to microsequencing using automated Edman degradation techniques. Fig. 1 shows the chromatographic profile obtained from the C18 RP-HPLC microbore column. All the individual peaks were subjected to microsequencing. Most of the peaks did not yield any sequence, and only in those labeled we were able to determine unambiguously amino acid sequences. The numbering of these peaks correspond to the sequences of the peptides shown in Fig. 2. The material corresponding to a large absorbance peak with a retention time between 20 and 30 min contains CHAPS and retention time between 20 and 30 min contains CHAPS and, as a consequence, two other peaks that yielded sequence were separated (data not shown). All of the identified tryptophan cleavage products can be aligned unequivocally with position 421–435 of mSlo. The only discrepancies that occur between the amino acid sequences determined in this study and those of mSlo are found in positions that involve Arg, Ser, or Cys residues. This could represent real differences between the two proteins or could be the result of difficulties in distinguishing these residues at the low levels of material employed for sequencing. When the molecular mass of the purified α-subunit from bovine tracheal smooth muscle (i.e. 62 kDa, as determined by SDS-PAGE) is compared with the deduced mass of mSlo (i.e. 135 kDa), there is a remarkable discrepancy. Possible explanations for these differences include post-translational modification of the extensive carboxyl-terminal region of mSlo or proteolytic degradation of the α-subunit of the ChTX receptor during purification. The latter possibility cannot be ruled out. However, the purified ChTX receptor preparation forms functional maxi-K channels as demonstrated from reconstitution experiments with artificial planar lipid bilayers (8). Another possibility that cannot be discarded is that cleavage takes place within the α-subunit, but the complex remains intact until exposed to denaturing conditions. Further studies are required to establish the size of the maxi-K channel α-subunit expressed in smooth muscle.

**Composition of ChTX-sensitive Aortic Smooth Muscle Maxi-K Channel**—To provide further evidence that the α- and β-subunits of the maxi-K channel are specifically associated with each other in a complex, a sequence-directed antibody, anti-α(421–435), was raised against a synthetic peptide corresponding to one of the identified fragments of the α-subunit of the bovine tracheal channel. This peptide can be aligned unequivocally with position 421–435 of the deduced amino acid sequence of mSlo, a region about 130 residues into the long carboxy-terminal tail of the protein. Fig. 3 shows that when the radio-labeled maxi-K channel complex is dissociated into its individual polypeptides after boiling the preparation in SDS with β-mercaptoethanol present, anti-α(421–435) precipitated exclusively and specifically the α-subunit (lanes A–C). This result clearly demonstrates that anti-α(421–435) recognizes the α-subunit but not the SDS-denatured β-subunit.

The next goal was to confirm that a tight association exists between the 62-kDa α-subunit and 31-kDa β-subunit, under native conditions. For this purpose, a purified 125I-Bolton-Hunter-labeled maxi-K channel preparation was incubated with protein A-Sepharose-prebound anti-α(421–435), without boiling the preparation in SDS, and immunoprecipitation studies were performed as described under “Experimental Procedures.” Under these conditions, anti-α(421–435) precipitated the entire subunit complex (Fig. 3, lane E). Identical studies

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**Fig. 1.** RP-HPLC of the digested α-subunit of the maxi-K channel from bovine tracheal smooth muscle. The α-subunit from the bovine tracheal smooth muscle maxi-K channel was digested with trypsin and loaded onto a C18 microbore column as indicated under “Experimental Procedures.” Elution of bound material was achieved in the peak 2.

**Fig. 2.** Amino acid sequences derived from the α-subunit of bovine tracheal smooth muscle membranes. Amino acid sequences obtained from various peptides derived from the 62-kDa α-subunit are shown in comparison with homologous amino acid sequences deduced from the Drosophila slowpoke and the mouse mSlo K+ channels. The obtained sequences were aligned to the deduced sequences of these two K+ channels using the GAP program in the University of Wisconsin Genetic Computer Group software package (12). The numbering refers to the position of the residue within the deduced amino acid sequence of mouse mSlo. Identical residues are depicted in black, conservative substitutions in gray. The localization of the synthetic peptide for raising the sequence-directed antibody anti-α(421–435) is shaded and boxed.
mental Procedures." Part of this preparation was denatured by boiling for 5 min in 4% SDS in the presence of 2% β-mercaptoethanol, and the α-subunit was isolated by size exclusion chromatography on a Bio-Rad TSK-400 column. Lanes A, B, and C represent experiments performed with a denatured preparation, whereas in lanes D, E, and F, a non-denatured 35S-Bolton-Hunter-labeled preparation was used. Before performing the immunoprecipitation experiments, unound 35S-Bolton-Hunter reagent was quantitatively removed by size exclusion chromatography. The iodinated preparation was immunoprecipitated with preimmune serum did not yield any precipitate. SDS-PAGE as described under "Experimental Procedures" and autoradiographed for 7 days.

performed with preimmune serum did not yield any precipitation of the labeled subunit complex (Fig. 3, lane D). Moreover, the recognition of the labeled purified maxi-K subunit complex was blocked by inclusion of the synthetic peptide, against which the sequence-directed antibody was prepared (Fig. 3, lane F). Summarizing, the co-immunoprecipitation of the α- and β-subunits must result from a specific and tight interaction between the two polypeptides composing the purified smooth muscle maxi-K channel. All precipitation studies described above were performed in the presence of 1% Triton X-100. This detergent concentration can be considered partially denaturing any maxi-K channel subunit dissociation. These results demonstrate that mammalian maxi-K channels consist of two individual subunits, α and β, which form a functional complex.

The ChTX receptor migrates upon continuous sucrrose density gradient centrifugation with a sedimentation coefficient of approximately 23 S (11). Although a complete hydrodynamic analysis with purified smooth muscle maxi-K channels has not been performed so far, this high sedimentation coefficient suggests a molecular weight for the entire complex of ~400,000–500,000.

With these data and the present findings regarding the structure of the smooth muscle maxi-K channel, and in view of current ideas on the assembly of functional voltage-dependent K⁺ channels, we propose the following model for the maxi-K channel. The α-subunit is the central ion channel-forming element and contains the receptor for ChTX. In analogy with other potassium channels, four of these α-subunits are required to form a functional channel. This putative tetramer of α-subunits is non-covalently associated with four β-subunits in order to account for the high sedimentation coefficient of the entire complex. The β-subunits must be in close proximity (less than 12 Å) to the pore-forming and receptor-carrying subunit, since the bifunctional cross-linking reagent, disuccinimidyl carbonate, can bridge the distance between the α- and β-subunit and promote specific covalent incorporation of ChTX into the β-subunit (11). Unlike the α-subunit, the β-subunit contains approximately 30% carbohydrate. This maxi-K channel β-subunit appears to share certain characteristics with the β₁-subunit of rat brain Na⁺ channels (17) and the γ-subunit of skeletal muscle L-type Ca²⁺ channels (18), and may be analogous in structure and/or function. A polypeptide with similar features seems to be associated with the apamin-sensitive, calcium-activated K⁺ channel (19). It is interesting to speculate that this type of subunit may be a conserved constituent of many voltage- and Ca²⁺-dependent K⁺ channels.

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