Characterization of Tissue-expressed \( \alpha \) Subunits of the High Conductance \( \text{Ca}^{2+} \)-activated \( \text{K}^{+} \) Channel*

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Purified high conductance calcium-activated potassium (maxi-K) channels from tracheal smooth muscle have been shown to consist of a 60–70-kDa \( \alpha \) subunit, encoded by the slo gene, and a 31-kDa \( \beta \) subunit. Although the size of the \( \beta \) subunit is that expected for the product of the gene encoding this protein, the size of the \( \alpha \) subunit is smaller than that predicted from the slo coding region. To determine the basis for this discrepancy, sequence-directed antibodies have been raised against slo. These antibodies specifically precipitate the in vitro translation product of mslo, which yields an \( \alpha \) subunit of the expected molecular mass (135 kDa). Immunostaining experiments employing smooth muscle sarcolemma, skeletal muscle T-tubules, as well as membranes derived from GH3 cells, reveal the presence of an \( \alpha \) subunit with an apparent molecular mass of 125 kDa. The difference in size of the \( \alpha \) subunit as expressed in these membranes and the purified preparations is due to a highly reproducible proteolytic decay that occurs mostly at an advanced stage of the maxi-K channel purification. In the purified maxi-K channel preparations investigated, the full-length \( \alpha \) subunit, an intermediate size product of 90 kDa, and the 65-kDa polypeptide, as well as other smaller fragments can be detected using appropriate antibodies. Proteolysis occurs exclusively at two distinct positions within the long C-terminal tail of slo. In addition, evidence for the tissue expression of distinct splice variants in membrane-bound as well as purified maxi-K channels is presented.

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‡ The abbreviations used are: maxi-K channel, high conductance, \( \text{Ca}^{2+} \)-activated \( \text{K}^{+} \) channel; \text{ChTX}, charybotoxin; [\( ^{125} \text{I} \)]\text{ChTX}, [\( ^{125} \text{I} \)]monooiodotyrosine charybotoxin; \text{ELISA}, enzyme-linked immunosorbent assay; TBS, Tris-buffered saline; PVDF, polyvinylidene difluoride; PAGE, polyacrylamide gel electrophoresis.

Among the diverse family of \( \text{K}^{+} \) channels, high conductance \( \text{Ca}^{2+} \)-activated \( \text{K}^{+} \) (maxi-K) channels exhibit certain features that distinguish this class, such as high potassium conductance (200–300 picosiemens) and selectivity for this monovalent cation. These channels are activated by both membrane depolarization and intracellular \( \text{Ca}^{2+} \) (1) and are sensitive to inhibition by a number of peptidyl toxins (e.g. charybotoxin (\text{ChTX}) (2) and iberiotoxin (3)) and a series of alkaloid indole diterpene small molecules (4). Maxi-K channels from bovine tracheal and aortic smooth muscle sarcolemmal membranes have been purified to homogeneity by monitoring binding of [\( ^{125} \text{I} \)]\text{ChTX} (5). The purified preparation has been shown to consist of two individual subunits, \( \alpha \) and \( \beta \), with apparent molecular masses of 60–70 and 31 kDa, respectively (5). Reconstitution of this preparation into artificial lipid bilayers yielded channels whose properties are virtually identical to those of native maxi-K channels (5).

Maxi-K channels (Slo) have been cloned from smooth muscle, skeletal muscle, and brain of different mammalian species (6–11). Comparison of the predicted primary sequence of these channels with that of voltage-gated \( \text{K}^{+} \) channels revealed a core structure that has six putative transmembrane domains, termed S1 to S6, with a conserved region, between S5 and S6, defining a presumed pore structure. However, the slo maxi-K channel genes encode a subunit of >1100 amino acid residues in length, twice the typical length of voltage-gated \( \text{K}^{+} \) channels. This is due to the presence of a long tail sequence located at the C-terminal side of the core, which does not exist in other voltage-gated \( \text{K}^{+} \) channels. One remarkable feature of this C-terminal tail domain is the occurrence of four additional hydrophobic regions, termed S7 through S10, of unknown topology and the existence of several splice sites. Recently, it has been shown that injection of mRNA for either C-terminal or N-terminal truncated forms of mslo (the channel cloned from mouse brain and skeletal muscle) into Xenopus oocytes does not lead to the appearance of functional channels (12). However, coinjection of mRNA encoding both truncated forms produces functional maxi-K channels indistinguishable from those obtained from the gene encoding full-length mslo. These studies have led to the suggestion that open time, conductance, and voltage dependence of maxi-K channels are determined by the N-terminal core, whereas \( \text{Ca}^{2+} \) sensitivity of the channel appears to involve a region of several negatively charged residues at the C-terminal core. In addition, co-expression experiments with mslo and the \( \beta \) subunit of the maxi-K channel indicate that the later subunit affects the \( \text{Ca}^{2+} \) sensitivity of mslo by shifting the mid-point of activation about 80 mV to more hyperpolarized potentials (13).

An alignment of internal amino acid sequences obtained from the purified tracheal maxi-K channel \( \alpha \) subunit (14) demonstrates that this protein belongs to the slo family of \( \text{K}^{+} \) channels (6–11). However, the smooth muscle maxi-K channel \( \alpha \) subunit appears upon SDS-PAGE as a band of 60–70 kDa, which corresponds to a protein of only 600–700 residues (5). In the present study, we have employed a battery of sequence-directed antibodies to resolve the discrepancy in apparent size between slo and the purified \( \alpha \) subunit of the smooth muscle.
maxi-K channel. Our results indicate that the membrane-bound α subunit, as expressed in native smooth muscle tissue, has a size of 125 kDa and that the purified α subunit is due to a highly reproducible proteolytic decay that occurs at an advanced stage of maxi-K channel purification. Despite this proteolytic event, all of the resulting fragments remain associated throughout the purification procedure and can only be resolved after denaturation of the preparation with SDS under reducing conditions. Moreover, our results demonstrate, for the first time, the level of channel expression in different tissue preparations and provide evidence for the occurrence of distinct splice variants at the protein level in native tissue. A preliminary report of these findings has been made in abstract form (15).
splice site A. Anti-$\alpha_{(913-926)}$ is directed against a sequence within a putative extracellular loop between transmembrane segments S9 and S10, while anti-$\alpha_{(978-992)}$ recognizes a sequence that is thought to be located on an intracellular site of the $\alpha$ subunit shortly beyond the putative S10 segment. ELISA analyses indicate that each of the antisera displayed a significant titer and that each reacted specifically with its corresponding peptide antigen.

To determine the utility and specificities of these affinity-purified, sequence-directed antibodies, they were further analyzed by immunoblotting 1–10 fmol of maxi-K channels present in bovine tracheal smooth muscle membranes. All of the antibodies listed above reacted in Western blots with the $\alpha$ subunit of the maxi-K channel (Figs. 2, 4, and 5). As shown in Fig. 2, a single major immunoreactive protein of 125 kDa is detected in this smooth muscle tissue (Fig. 2A). To investigate the tissue distribution and the level of maxi-K channel expression in different tissues, membranes derived from different smooth, cardiac, and striated muscle, as well as GH3 cells and kidney, were screened for the presence of maxi-K channel $\alpha$ subunits with anti-$\alpha_{(913-926)}$. As already suggested by recent cloning (8, 11) and biochemical (5) studies, maxi-K channel $\alpha$ subunits are expressed in bovine aortic smooth muscle (Fig. 2B), rat uterus smooth muscle (Fig. 2C) and rat skeletal muscle T-tubule membranes (Fig. 2E). In all cases, this polypeptide has an apparent molecular mass of 125 kDa. Furthermore, this polypeptide in these tissues was also immunostained by all other antibodies employed in this study, although in some cases additional polypeptides were nonspecifically labeled to certain extents (data not shown). The specificity of these antibodies was confirmed by inclusion of the corresponding antigenic peptide (1 mM), which virtually abolished the immunostaining reaction (Figs. 2, 4, and 5).

In addition to the distribution of this protein in muscle tissue, the $\alpha$ subunit of the maxi-K channel has been detected in GH3 cell membranes, a hypophysis adenoma cell line. In marked contrast, membranes from kidney, liver, testis, and cardiac sarcoplasmic membranes (Fig. 2, D and F, and data not shown) did not show any specific immunostaining signal, regardless of which sequence-directed antibody was employed. To determine the relative level of maxi-K channels that are expressed in these tissues, identical amounts of membrane protein were subjected to SDS-PAGE, transferred to PVDF membranes, and probed in immunostaining experiments employing anti-$\alpha_{(913-926)}$. The highest expression levels were observed in bovine trachea, bovine aorta, and rat uterus smooth muscle membranes, whereas only intermediate levels were seen in membranes from GH3 cells (Fig. 2G). A significantly lower tissue expression level was found in skeletal muscle T-tubule membranes (Fig. 2E). Taken together, these data quantitate the relative levels of maxi-K channels expressed in these tissues and support data from earlier studies using electrophysiological or molecular biological methods (8, 21).

Characterization of the Translation Product of mslo—The data presented above indicate that the molecular mass of membrane-bound $\alpha$ subunits, independent of the tissue under investigation, is in the 125-kDa range. This value is in good agreement with the deduced Mr predicted for the channel from translation of the open reading frame of bslo (11), the bovine homologue of mslo, mslo (8), and the human hslo (10). Thus, when mslo is translated in vitro in the presence of $[^35]S$ methionine and separated by SDS-PAGE, the $[^35]S$ methionine-labeled translation product of mslo migrates with the 133-kDa protein standard (Fig. 3). To verify that the labeled product is indeed encoded by mslo, immunoprecipitation studies using anti-$\alpha_{(421-435)}$, anti-$\alpha_{(993-710)}$, and anti-$\alpha_{(913-926)}$ were performed. All three antibodies specifically immunoprecipitated mslo (some data not shown), whereas a sequence-directed antibody against the $\beta$ subunit of the maxi-K channel (17), was not capable of recognizing this polypeptide (Fig. 3B).

Recognition of the $\alpha$ Subunit and Its Proteolytic Fragments in a Partially Purified Maxi-K Channel Preparation from Smooth Muscle—The purified $\alpha$ subunit of the bovine tracheal maxi-K channel has been shown to display a molecular mass of 60–70 kDa (5). As shown in Fig. 2, all smooth muscle membrane preparations possess an $\alpha$ subunit with a molecular mass of about 125 kDa. Since the membrane-bound $\alpha$ subunit displays a higher apparent molecular mass, experiments were performed to address this discrepancy. For these studies, membrane-bound or partially purified maxi-K channels were separated by SDS-PAGE, transferred to a PVDF membrane, and immunostained with anti-$\alpha_{(421-435)}$. After subjecting the bovine tracheal smooth muscle $\alpha$ subunit to solubilization and purification as previously outlined (5), the staining pattern remains unchanged up to and including the third purification step, which corresponds to ion exchange chromatography on a Mono Q ion exchange column (Fig. 4, lanes 1 and 2). However, after the fourth purification step, which employs a hydroxylapatite
Fig. 4. Proteolysis of the α subunit of the bovine tracheal smooth muscle maxi-K channel. Approximately 18 fmol of [125I]ChTX binding sites were subjected to 12% SDS-PAGE and transferred to a PVDF membrane. Thereafter, the α subunit was visualized by immunoblotting employing anti-α(421–443) after each individual purification step, 1, after chromatography on a wheat germ agglutinin-Sepharose column (second purification step); 2, after chromatography on a Mono-Q ion exchange column (third purification step); 3 and 4, after chromatography on a hydroxyapatite column (fourth purification step) in the absence (3) or in the presence (4) of 1 μM of the corresponding competing peptide. Fractions after the hydroxyapatite purification step were also immunostained with anti-α(459-475) (5 and 6) in the absence (5) or presence (6) of 1 μM of corresponding peptide. The relative mobility of molecular mass standards (in kDa) are indicated by arrows.

Fig. 5. Characterization of the α subunit fragments. Fractions after the fourth purification step of the bovine tracheal smooth muscle maxi-K channel purification were subjected to SDS-PAGE employing a 15% gel and transferred to a PVDF membrane. The α subunit core fragments (α65, α60, and α90), as well as all α subunit tail fragments (α’28, α’29, and α’33), are immunodetected employing the indicated sequence-directed antibodies. Please note that the tail fragments, α’28 and α’29, are clearly resolved into two major fragments of distinct molecular masses. The relative mobility of molecular mass standards (in kDa) are indicated by arrows.

α(939-710) does not recognize α65, even though this antibody is clearly capable of staining the full-length α subunit as well as α90 (Fig. 5). Therefore, fragment α’48 must account for the difference in molecular mass between α65 and α125, and represents the remaining C terminus derived from the full-length α subunit. In addition to α’48, a doublet of proteolytic tail fragments with molecular masses of ~22 and ~25 kDa (α’23) are also detected by anti-α(939-710) (Fig. 5). Among all the antibodies employed in this study, only anti-α(939-710) is capable of detecting this 23-kDa doublet fragment derived from the tail domain of Slo. Therefore, these fragments must possess exclusively the recognition sequence for this antibody, and their origin can only be explained by the fact that they account for the difference in molecular mass between α65 and α90 (Fig. 7). Inspection of the mslo coding sequence for the position of putative splice sites reveals that splice site A should be included within fragment α’23.

To characterize the second site of proteolysis (α125 → α90), experiments were performed with anti-α(913-926) and anti-α(978-992). These antibodies detect the full-length α subunit, α125, but neither of the core fragments, α60 or α90 (Figs. 5 and 6), implying that the smaller fragments lack recognition sites for these antibodies. In addition to the full-length α subunit, two proteolytic tail fragments with molecular masses of ~45–50 kDa (α’48) and a doublet fragment of ~27 and ~29 kDa (α’23) are also detected by these antibodies (Figs. 5 and 6). α’48 very likely is identical to the fragment previously stained by anti-α(939-710) (see above). Given the characteristic staining pattern and the molecular mass of this fragment, it could account for the difference in molecular mass between α125 and α60, and should possess as its N terminus residues between 640–690. Furthermore, this fragment ought to contain the recognition sites for the antibodies anti-α(939-710), anti-α(913-926), and anti-α(978-992) and extend to the C terminus of the tissue-expressed Slo, which should be located close to residue 1100. The newly detected doublet fragment, α’29, can clearly be resolved into two individual peptide species. They exhibit a sequence recognized only by anti-α(939-926) and anti-α(978-992), and, therefore, originate with high likelihood from the very C-terminal end of the α subunit. This would account for the difference in molecular mass between the full-length α subunit, α125, and the larger core fragment, α90. The position of splice site B should also be contained within this fragment. Keeping in mind that the N terminus is intact and that the overall

column, the α subunit undergoes significant proteolytic decay (Fig. 4, lane 3). Identical results were obtained with anti-α(459-475) (Fig. 4, lanes 5 and 6). At this stage, maxi-K channels have been purified almost 100-fold when compared to the digitonin-solubilized starting material. A significant part of the 125-kDa full-length α subunit is proteolyzed into two distinct α subunit core fragments of ~65 kDa (α65) and ~90 kDa (α90), which are clearly recognized by anti-α(421–443). The specificity of this antibody is indicated by the fact that 1 μM of the corresponding antigenic peptide virtually abolished the immunostaining reaction (Fig. 4, lane 4). Depending on the scale of the purification, up to 30% of the full-length maxi-K channel α subunit (α125) remains unproteolyzed. The reason for this reproduce but incomplete proteolysis is not well understood at the present time.

To investigate whether proteolysis occurs at either the N- or the C-terminal domain of the α subunit, a sequence-directed antibody (anti-α(28–40)) raised against the predicted first 13 N-terminal residues of the protein was employed in immunoblots. The same proteolysis pattern detected with anti-α(421–443) can also be demonstrated with anti-α(28–40) (Fig. 5, lane 1), indicating that all α subunit core fragments (α125, α90, and α65) possess a common N terminus. This suggests that proteolysis occurs within the long C-terminal tail domain of Slo.

To narrow the site of proteolysis within the α subunit, several other antibodies directed against synthetic maxi-K channel peptides were employed (see Fig. 1). The immunostaining pattern of anti-α(939-710) reveals that only the core fragments α125 and α90 contain the recognition sequence for this antibody, but α65 is not detected (Fig. 5). A staining pattern that is virtually identical is obtained by employing anti-α(978-992) (data not shown). However, fragments of molecular masses between 45 and 50 kDa (α’48) are detected by anti-α(939-710) (Fig. 5) and by anti-α(913-926) (Fig. 5; see below). Given that all protein sequences obtained from the purified 60–70-kDa α subunit are identical to those present in Slo (14) and that the most downstream fragment identified corresponds to position 635–640 of mslo, the α subunit core fragment α65 must extend, at least, to this sequence. These data narrow the site of the proteolytic cleavage that generates α65 to a stretch of residues starting at position 640 and ending at position 690, since anti-
molecular mass of the full-length subunit is 125 kDa, the second proteolytic fragmentation must occur approximately at residue 850 of mslo. This would explain the observed molecular masses of both α90 and α’28.

Although two incomplete proteolytic clips occur at position 640–690 and near position 850 of slo to partially degrade the full-length α subunit and to generate α90, α95, and three smaller size tail fragments (α’48, α’28, and α’23), the maxi-K channel subunit complex remains tightly associated for three additional purification steps after the hydroxylapatite column (two sucrose density gradients and an additional ion exchange chromatographic step). This proteolytic cleavage is not paralleled by any significant decay in receptor density or change in the resulting pharmacological profile of the purified receptor (5). In fact, all fragments remain associated as demonstrated by Western blot analysis (Fig. 6). The association of the fragments with the maxi-K channel core likely occurs through disulfide bonding (data not shown). All generated fragments possess several cysteine residues through which such bonding to the core fragment could occur. It will be interesting to investigate the disulfide bonding between different extra- or intracellular loops of the native channel protein in further detail, taking advantage of the well defined pattern of proteolysis that is produced during purification procedures.

**DISCUSSION**

Characterization of Tissue-expressed α Subunits of the Maxi-K Channel with Sequence-directed Antibodies—In the present study, we have characterized for the first time the maxi-K channel α subunit as expressed in several smooth muscle membrane preparations, as well as in skeletal muscle T-tubule and GH3 membranes, by employing a battery of site-directed antibodies. An overall molecular mass of 125 kDa was detected for all tissue-expressed α subunits, which is in agreement with the predicted molecular mass from translation of the open reading frame of the cDNA-encoding maxi-K channels. Immunoblotting data indicate that the N terminus of the α subunit is intact and that the C terminus of α125 must be located near position 1100 of the predicted mslo sequence.

Proteolysis of the α Subunit upon Maxi-K Channel Purification—In this study, we have also demonstrated that the α subunit of the maxi-K channel undergoes partial proteolytic decay upon purification. This occurs at the fourth purification step, which corresponds to chromatography upon a hydroxylapatite column. The reproducibility in size of the proteolytic breakdown products and their relative abundance appears to be highly consistent when preparations from individual purifications were monitored. The putative protease causing this proteolytic degradation may therefore co-chromatograph with smooth muscle maxi-K channels through DEAE-Sepharose, wheat germ agglutinin-Sepharose, and Mono-Q ion exchange chromatographies. However, no significant decay of the α subunit can be detected until the hydroxylapatite purification step. It is possible that the conformation of the maxi-K channel is altered during this step in such a way that previously masked sites become accessible to the protease. At this moment, we favor the hypothesis that the α subunit possesses distinct recognition sites for what may be a Ca2+-activated protease that copurifies with the maxi-K channel through three purification steps. Immobilization of the maxi-K channel on a hydroxylapatite column either exposes these cleavage sites to this protease and/or activates the protease in the presence of the high Ca2+ found on this chromatography matrix. Moreover, the proteolytic cleavage is never complete, which could be explained by either rapid separation of the protease from the partially purified maxi-K channel preparation on the hydroxylapatite column or inactivation of the enzyme due to a fast increase of the phosphate concentration employed for elution of protein from the column.

None of the proteolytic tail fragments that are observed seem to be formed by a single peptide species (Figs. 5 and 6). In essentially all experiments, the bands in a Western analysis appear to be a well resolved doublet with a shadow on the trailing edge. This phenomena could be explained by multiple proteolytic cleavages within close proximity to each other but could more likely be explained by the presence of tissue-expressed splice variants of slo. This observation is consistent with the fact that both putative splice sites are known to be located within the tail fragments α’48, α’28, and α’23 that result from proteolytic processing (Fig. 7). These splice variants would differ by just 2–3 kDa in molecular mass (8, 10), and the reason for not resolving them as part of the full-length α subunit is explained by the inferior resolution of SDS-PAGE in the

**Fig. 6. Immunoblot analysis of purified maxi-K channels.** Fractions from the final purification step of the maxi-K channel were subjected to SDS-PAGE under reducing conditions and electrophoretically transferred to PVDF membranes. Immunoblotting data employing the N-terminal antibody anti-α28–40 (A) and a C-terminal antibody (anti-α913–926) (B) are presented. The amount of maxi-K channels in each fraction from the sucrose gradient that were applied to the gel, as determined by reversible [3H]ChTX binding, are as follows: 11, 0.51 fmol; 12, 1.67 fmol; 13, 7.75 fmol; 14, 10.4 fmol; 15, 5.01 fmol; 16, 2.21 fmol; 17, 1.7 fmol; and 18, 1.41 fmol. The tail fragment α28 is clearly resolved into two major polypeptide fragments. Positions of molecular mass standards (in kDa) are shown.

**Fig. 7. Schematic representation of the α subunit of smooth muscle maxi-K channels.** The three α subunit core fragments (α125, α90, and α65) are shown as filled bars. The recognition sites of the sequence-directed antibodies employed in this study are depicted above the bar symbolizing the full-length α subunit (α125), whereas all putative transmembrane domains (S1–S10) and the pore region (P) are indicated below the α125 bar. Internal peptide sequences obtained from the α subunit core fragment α90 (6) are shown below the bar, representing the core fragment α90. Splice site A (residues 661–663) and B (residue 902) are indicated in gray (A) and white (B) boxes within the corresponding α subunit tail fragments, respectively. All detected tail fragments (α’48, α’28, and α’23), their respective positions within the maxi-K channel α subunit, as well as the occurrence of putative splice sites within these fragments are indicated.
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high molecular mass range. However, the separation of distinct polypeptide species improves as lower molecular mass fragments are generated (Figs. 5 and 6).

All the resulting proteolytic fragments appear to remain associated with the maxi-K channel subunit complex, indicating that a tight interaction must be present. This interaction is probably the result of bonding of the fragments via disulfide bridges to the α subunit core fragments, since only a full-length α subunit is observed when the samples are separated on SDS-PAGE under non-reducing conditions (data not shown). Additional membrane-spanning segments (S7–S10) have been suggested by hydropathic analysis of Slo. All α subunit tail fragments harbor at least one of the additional putative transmembrane segments S7–S10. To conclusively prove the proposed transmembrane topology of Slo, further in vitro labeling studies employing these sequence-directed antibodies are required.

The maxi-K channel α subunit of bovine aortic smooth muscle tissue has recently been cloned (11). It displays an extremely high degree of homology with mSlo (8) and hSlo (10), but appears to possess 56 less residues than mSlo at its C terminus. Thus, species-specific splice variants could account for the slight difference in the observed molecular mass between the tissue-expressed α subunit and the in vitro translation product of mslo. Moreover, splice variants of the maxi-K channel may have different biophysical and biochemical properties, and this could have functional consequences for the regulation or physiological function of the channel in various target tissues. These alternatively spliced constructs differ significantly in their single channel properties, such as kinetics of activation or single channel conductance, and Ca2+ sensitivity (8, 10, 12), but all show very slow inactivation over a millisecond time range. Thus, alternative RNA splicing and, perhaps, association with other accessory subunits could contribute to the diversity of Slo channels in general.

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