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High-Conductance Calcium-Activated Potassium Channels; Structure, Pharmacology, and Function

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High-conductance calcium-activated potassium (maxi-K) channels comprise a specialized family of K^+ channels. They are unique in their dual requirement for depolarization and Ca^{2+} binding for transition to the open, or conducting, state. Ion conduction through maxi-K channels is blocked by a family of venom-derived peptides, such as charybdotoxin and iberiotoxin. These peptides have been used to study function and structure of maxi-K channels, to identify novel channel modulators, and to follow the purification of functional maxi-K channels from smooth muscle. The channel consists of two dissimilar subunits, α and β. The α subunit is a member of the *slo* Ca^{2+}-activated K^+ channel gene family and forms the ion conduction pore. The β subunit is a structurally unique, membrane-spanning protein that contributes to channel gating and pharmacology. Potent, selective maxi-K channel effectors (both agonists and blockers) of low molecular weight have been identified from natural product sources. These agents, together with peptidyl inhibitors and site-directed antibodies raised against α and β subunit sequences, can be used to anatomically map maxi-K channel expression, and to study the physiologic role of maxi-K channels in various tissues. One goal of such investigations is to determine whether maxi-K channels represent novel therapeutic targets.

KEY WORDS: maxi-K channels; charybdotoxin; iberiotoxin; smooth muscle; ion channel purification; *slo* channels; β-subunit; K channel agonists; K channel blockers; ion channel pharmacology.

INTRODUCTION

Calcium-activated potassium channels are a diverse class of K^+ channels that share the common feature of being gated by intracellular Ca^{2+}. Many types of Ca^{2+}-activated K^+ channels have been described. They can be divided loosely into three subclasses based on differences in single-channel conductance, pharmacological properties, and voltage dependence of channel opening. Small-conductance channels (10–20 pS) are typically blocked by the bee venom toxin, apamin, and their open probability is unaffected by membrane potential. Intermediate-conductance channels (25–100 pS) are often blocked by charybdotoxin and their gating is not voltage-dependent. High-conductance (maxi-K) channels (100–300 pS) are usually blocked by charybdotoxin and their gating is voltage-dependent. The maxi-K (or BK) channel is perhaps the best studied of the different types of Ca^{2+}-activated K^+ channels in terms of its biochemical, biophysical, and pharmacological properties, because of its prevalence and existence of high-affinity blockers of this channel.

Maxi-K channels are found in a variety of both electrically excitable and nonexcitable cells (for a review see Latorre *et al.*, 1989; McManus, 1991). These channels are opened by membrane depolarization and by μM concentrations of intracellular Ca^{2+}. A number of maxi-K channel subtypes can be distinguished based on differences in single-channel conductance, Ca^{2+} sensitivity, and sensitivities to inhibitors

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(Latorre *et al.*, 1989; McManus, 1991). Cloning of the gene responsible for the *slopoke* phenotype in *Drosophila* (Atkinson *et al.*, 1991) led to cloning and expression of cDNAs encoding insect (Adelman *et al.*, 1992) and vertebrate maxi-K channels (Butler *et al.*, 1993; Dworetzky *et al.*, 1994; Pallanck and Ganetzky, 1994; Tseng-Crank *et al.*, 1994; Wallner *et al.*, 1995). Many alternatively spliced cDNAs encoding vertebrate maxi-K channels were identified, providing a molecular basis for the different maxi-K channel phenotypes observed in electrophysiological experiments. Regulated expression of maxi-K channel variants in different tissues may allow these channels to participate in an array of cellular processes.

In order to identify the molecular components of the maxi-K channel and to determine the physiologic function of maxi-K channels in target tissues of interest, specific high-affinity probes of this class of channels are needed. During the last few years, we and others have identified a family of maxi-K channel inhibitors from scorpion venoms. In this review, we focus on use of these ligands to determine the structure and function of maxi-K channels in vertebrate smooth muscle. Through application of these tools, the maxi-K channel has been purified to homogeneity and its subunit composition determined. In addition, the first selective nonpeptide agonists and antagonists of this channel were identified by screening with a radiolabeled peptide channel blocker. These ligands have been and continue to be of use in investigating the role of maxi-K channels in the physiology of various tissues.

PROBES FOR MAXI-K CHANNELS

Charybdotoxin

It is fortunate that a number of peptide inhibitors of maxi-K channels have been discovered in scorpion venoms. The first report of such an agent was made by Miller and colleagues who demonstrated that a component of venom from the scorpion *Leiurus quinquestriatus hebraeus* blocked maxi-K channels according to a simple bimolecular binding reaction (Miller *et al.*, 1985). This inhibitor, termed charybdotoxin (ChTX), is a minor venom component. Subsequently, ChTX was purified to homogeneity from *Leiurus* venom by a combination of ion exchange and reversed phase HPLC chromatographic techniques, and its primary sequence was determined by Edman degradation (Gimenez-Gallego *et al.*, 1988). ChTX is a 37 amino acid peptide with a blocked N-terminus (pyroglutamic acid), three disulfide linkages (giving it a highly compact structure), and multiple positively charged residues (net charge of +5 at physiological pH; Fig. 1). The basic amino acids are critical for an electrostatic interaction which occurs between ChTX and negatively charged residues in the pore of the channel (Anderson *et al.*, 1988; MacKinnon and Miller, 1989; Park and Miller, 1992b). Several laboratories independently confirmed the primary structure of ChTX (Lucchesi *et al.*, 1989; Schweitz *et al.*, 1989; Strong *et al.*, 1989).

To develop the potential of ChTX, it was necessary to produce the peptide, rather than relying on purification from venom, since the peptide is present in small quantities in venom (ca. 0.2%). This was first accomplished by synthetic methods using solid-phase techniques (Lambert *et al.*, 1990; Sugg *et al.*, 1990). This approach yields large quantities of ChTX whose properties are indistinguishable from homogeneous

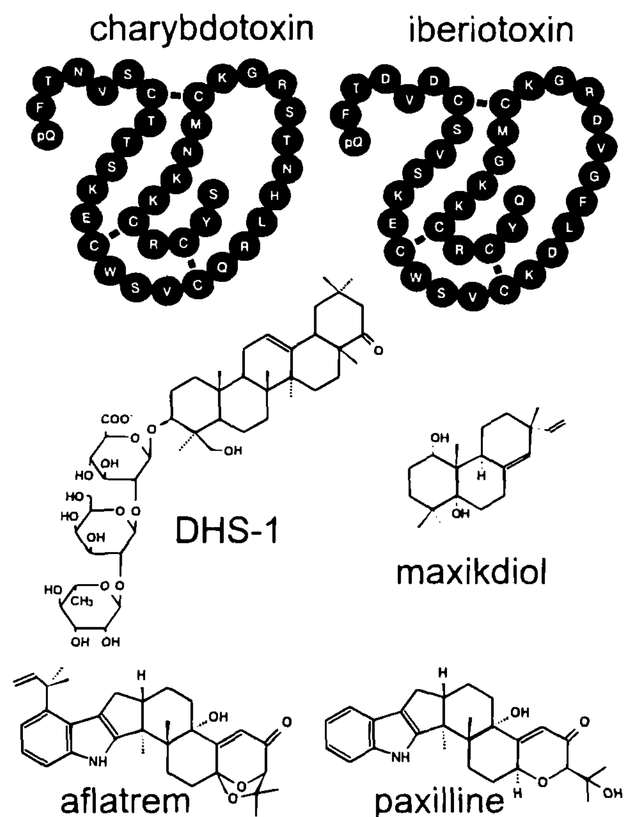

Fig. 1. Structures of maxi-K channel modulators. The structures of potent peptide (charybdotoxin, iberiotoxin) and small molecule (aflatrem, paxilline) blockers of the maxi-K channel are shown. Also depicted are two small-molecule maxi-K channel agonists (DHS-1, maxikdiol).

native peptide. Production of synthetic ChTX allowed verification of the peptide's primary structure. Synthetic material was further used to determine ChTX's disulfide bonding pattern and secondary structure. This biochemical data predicted that the peptide should be tightly folded, and this prediction was confirmed when the solution structure of ChTX was determined by 2D-NMR spectroscopy (Bontems *et al.*, 1991; Lambert *et al.*, 1990). The peptide displays a surface formed by three antiparallel β sheets which are attached through three disulfide bridges to a helix composed of residues 10–18 lying behind the plane of the β sheet structure. Similar structural features and peptide folding patterns have now been observed with a number of scorpion toxins (Bontems *et al.*, 1992).

ChTX has also been produced biosynthetically in *E. coli* as a fusion protein (Park *et al.*, 1991). After expression and purification of the fusion protein, ChTX is cleaved and its N-terminus cyclized to yield material identical to native toxin. This biosynthetic approach is important because it allows synthesis of numerous mutants that have proved useful for mapping residues that make contact with the external mouth of ChTX-sensitive K^+ channels (Park and Miller, 1992b; Stampe *et al.*, 1992, 1994). In this manner, residues Arg_{25}, Lys_{27} and Arg_{34} have been shown to be important for binding of ChTX to the maxi-K channel. Conversion of any of these amino acids to Gln dramatically reduces inhibitory activity of mutant peptides by increasing toxin dissociation rates. Mutation of Lys_{27} is particularly interesting because this residue is in close proximity to a K^+ binding site in the pore of the maxi-K channel; K^+ entering the ion conduction pathway from the inner surface binds to this site and destabilizes ChTX binding (MacKinnon and Miller, 1988; Park and Miller, 1992a). Further work revealed that modification of hydrophobic residues Phe_2, Trp_{14}, Met_{29}, and Tyr_{36} also destabilized ChTX-maxi-K channel interactions. Using this method, 8 of 37 amino acids of ChTX have been shown to be important for toxin block of the channel. These residues account for approximately 24% of ChTX's surface, and such data, combined with the NMR solution structure of the peptide, can be used to define a putative interaction surface for toxin with the channel; a triangular area formed by three antiparallel β strands of ChTX makes close contact with a complementary surface on the channel, perhaps spanning several subunits of what is expected to be a tetrameric complex (Stampe *et al.*, 1994). Binding of ChTX occludes the pore formed by the four-channel subunits and thereby prevents ion conduction.

ChTX was used to advance the biochemistry and molecular pharmacology of the maxi-K channel by labeling this probe to high specific activity with $[^{125}I]$, and using it to develop a radioligand binding assay in target tissues of interest (Vazquez *et al.*, 1989). Binding of $[^{125}I]$ChTX to maxi-K channels has been well described in purified sarcolemmal membranes from both bovine trachea and aorta. Toxin binding is time- and concentration-dependent, and, under low ionic strength conditions, $[^{125}I]$ChTX interacts with a single class of sites displaying a K_d of 100 pM and a B_{max} of approximately 1 pmol/mg protein. ChTX binding is a reversible bimolecular reaction, as predicted from electrophysiological studies, and K_d calculated from dissociation and association rate constants is equivalent to the K_d measured under equilibrium conditions. $[^{125}I]$ChTX binding in smooth muscle displays properties expected for toxin interacting with maxi-K channels: binding is inhibited by monovalent and divalent cations known to bind with sites located along the channel's ion conduction pathway (Cecchi *et al.*, 1987; Neyton and Miller, 1988; Vergara and Latorre, 1983); tetraethylammonium ion (TEA), which binds with moderate affinity (K_d = 100 μM) in the external pore of the channel (Miller, 1988; Villaroel *et al.*, 1988), inhibits $[^{125}I]$ChTX binding competitively with a K_i equivalent to its K_d in functional experiments; ionic strength affects $[^{125}I]$ChTX binding the same way that it affects toxin block in electrophysiological measurements (Anderson *et al.*, 1988); other peptidyl inhibitors of maxi-K channels (see below) also block binding. Taken together, these results strongly suggest that receptors characterized for $[^{125}I]$ChTX in bovine smooth muscle sarcolemma are directly associated with maxi-K channels.

Iberiotoxin

Unfortunately, ChTX is not a selective inhibitor of maxi-K channels; the peptide is known to block other types of K^+ channels, including small-conductance Ca^{2+}-activated K^+ channels in blood cells (Leonard *et al.*, 1992; Lucchesi *et al.*, 1989; Wolff *et al.*, 1988), and voltage-gated K^+ channels of the $K_v1.3$ type in lymphocytes (Sands *et al.*, 1989) and also presumably in brain (Vazquez *et al.*, 1990). Although the number of K^+ channels blocked by ChTX is limited, it was necessary to identify more selective probes for studying maxi-K channels. To accomplish this task, a screening strategy was employed in which various

crude scorpion venom extracts were tested for inhibition of [^{125}I]ChTX binding to smooth muscle sarcolemmal membranes. Using this approach, fractionation of venom from the old world scorpion, *Buthus tamulus*, yielded a peptide with appropriate properties; iberiotoxin (IbTX) was purified to homogeneity, and subsequently characterized both structurally and functionally (Galvez *et al.*, 1990). IbTX is a 37 amino acid peptide 68% homologous with ChTX, but because it possesses several acidic residues, the peptide has an overall lower net positive charge than ChTX (Fig. 1). In spite of the high degree of homology between the two peptides, IbTX is apparently selective for the maxi-K channel; it does not block other ChTX-sensitive K^+ channels tested to date. IbTX inhibits maxi-K channels by the same mechanism as does ChTX, but the dissociation rate of IbTX is much slower than for ChTX (Candia *et al.*, 1992; Giangiacomo *et al.*, 1992). IbTX has been made in biologically active form, both synthetically and biosynthetically. The NMR structure of synthetic IbTX indicates that the backbone configuration of the peptide is nearly identical to that of ChTX (Johnson and Sugg, 1992). A radiolabeled derivative of IbTX has recently been constructed by alkylating an IbTX mutant having Cys at position 19 with [^{3}H]N-ethylmaleimide (Knaus *et al.*, 1996).

Other maxi-K selective peptides have also been identified using the screening strategy outlined above (Novick *et al.*, 1991). The structure of limbatustoxin (LbTX), purified from the new world scorpion, *Centruroides limbatus*, was determined and shown to be 57% and 70% homologous with ChTX and IbTX, respectively. LbTX blocks maxi-K channels with nM affinity. Through identification of new maxi-K channel peptidyl inhibitors, synthesis of chimeric peptides incorporating sequences from different toxins (Giangiacomo *et al.*, 1993), and site-directed mutagenesis studies with existing well-characterized peptides, it may be possible to better define those residues which are critical for the peptide–maxi-K channel interaction. Moreover, complementary mutagenesis studies with the channel can be used to identify amino acids comprising the pore and external mouth of the maxi-K channel. Such studies are ongoing in many laboratories.

PURIFICATION AND STRUCTURE OF THE MAXI-K CHANNEL

Development of [^{125}I]ChTX as a ligand, along with identification of other peptidyl probes, has made it possible to obtain biochemical information about the molecular structure of the smooth muscle maxi-K channel. As a first approach to identifying components of the channel, [^{125}I]ChTX was crosslinked to its receptor in bovine smooth muscle sarcolemmal membranes using a bifunctional crosslinking reagent (Garcia-Calvo *et al.*, 1991). This strategy was used because it had not been possible to incorporate a reactive functionality directly into ChTX without destroying biological activity. To prevent significant levels of nonspecific labeling due to interaction between positively charged ChTX and anionic membrane sites, ChTX was prebound to sarcolemmal vesicles, before treatment with disuccimidyl suberate. This bifunctional reagent forms a covalent bridge between [^{125}I]ChTX and a single membrane protein which migrates upon SDS-PAGE with apparent molecular weight of 31 kDa (when the mass of ChTX is subtracted). Crosslinking ChTX to this protein is specific because it is inhibited by all agents which block the binding reaction such as IbTX, TEA, and K^+. The same size species is identified using either aortic or tracheal sarcolemmal membranes.

Purification Scheme

The maxi-K channel was purified to homogeneity from bovine tracheal smooth muscle by a combination of conventional chromatographic techniques and sucrose density gradient centrifugation while using [^{125}I]ChTX binding as a marker for the channel protein (Garcia-Calvo *et al.*, 1994). This approach was taken in order to identify and ultimately clone the molecular components of the maxi-K channel. The channel was solubilized from membranes using sequential digitonin extraction procedures; binding properties of the ChTX receptor, in terms of ligand affinity and effector pharmacology, are maintained in solubilized preparations. This is an initial indication that the maxi-K channel is not altered by removal from its native environment. Solubilized receptor was subjected to a seven-step purification scheme employing ion exchange, lectin affinity, and hydroxylapatite chromatographies, followed by two sucrose density gradient centrifugation steps. Starting from 10 grams of purified sarcolemmal membrane protein, representing vesicles prepared from 250 bovine trachea, the ChTX binding site was purified approximately 2000-fold to a specific activity of ca. 1 nmol [^{125}I]ChTX bound/mg protein. Peak fractions of binding activity from the final sucrose density gradient

yield 1% of initial [^{125}I]ChTX binding activity, and approximately 200 μg of apparently homogeneous protein. After SDS-PAGE and silver staining of the resulting gel, ChTX binding activity correlates with a single major component of 62 kDa. However, alkylation of this preparation with [^{125}I] Bolton–Hunter reagent followed by SDS-PAGE reveals the presence of two protein species in a 1:1 stoichiometry co-migrating with [^{125}I]ChTX binding activity: 62-kDa (α subunit) and 31-kDa (β subunit) proteins. The 31-kDa protein is heavily glycosylated, which may account for its poor staining properties upon silver imaging of SDS gels. Deglycosylation with endoglycosidase H converts the β subunit, in two steps, to a protein which migrates upon SDS-PAGE with apparent molecular weight of 22 kDa. This pattern suggests that the protein possesses two N-linked carbohydrate residues. In addition, the β subunit is the protein into which [^{125}I]ChTX is incorporated in the presence of a bifunctional cross-linking reagent. Identical results have been obtained after purification of the ChTX receptor from bovine aortic sarcolemma (Giangiacomo *et al.*, 1995).

Reconstitution of the Purified ChTX Receptor

The purified ChTX binding site from bovine trachea and aorta displays binding properties that are identical to receptors present in native smooth muscle sarcolemma in terms of ligand affinity and pharmacological profile. [^{125}I]ChTX binding is blocked by ChTX, IbTX, LbTX, TEA, K^+, Ba^{2+}, and Cs^+. Furthermore, after reconstitution into proteoliposomes, followed by fusion with planar lipid bilayers, robust maxi-K channel activity is observed (Garcia-Calvo *et al.*, 1994; Giangiacomo *et al.*, 1995). Single channels are observed with a conductance of approximately 240 pS in symmetrical 150 mM KCl. Open probability of reconstituted channels is increased by both membrane depolarization and increasing internal Ca^{2+}. When open, the channels are highly selective for K^+ over Na^+ and Cl^-. Channel activity is blocked by ChTX, IbTX, and TEA. Together, these results strongly suggest that the purified ChTX receptor consists of two subunits. These two subunits are sufficient to form channels with properties identical to native maxi-K channels. The robustness of the maxi-K channel, compared with other ion channels that have been purified, is illustrated by the fact that channel activity is not altered during a purification scheme which requires seven steps performed over a two-week period.

Primary Structure of the α Subunit

Structural information was obtained from 70–100 pmoles of the purified tracheal maxi-K channel α subunit by electroeluting the protein from a gel after SDS-PAGE, and subjecting it to proteolytic digestion with trypsin (Knaus *et al.*, 1994c). A number of fragments were isolated by reversed-phase HPLC employing microbore column techniques, and these were microsequenced using Edman degradation. Sequence information was obtained from seven fragments; these data reveal very high homology between sequences from the tracheal α subunit and those deduced from cDNA sequences of the Ca^{2+}-activated K^+ channel, *slowpoke*, cloned from *Drosophila* (Adelman *et al.*, 1992; Atkinson *et al.*, 1991), and vertebrate maxi-K channels, cloned by homology to *slowpoke* (Butler *et al.*, 1993; Dworetzky *et al.*, 1994; Pallanck and Ganetzky, 1994; Tseng-Crank *et al.*, 1994; Wallner *et al.*, 1995). Both *slowpoke* and vertebrate *slo* cDNAs produce TEA-sensitive Ca^{2+}-activated K^+ channels when expressed in *Xenopus* oocytes, indicating that the α subunit is the pore-forming unit of the channel complex. However, there is significant difference between the size of the purified subunit (62 kDa) and the predicted size of this protein based on the *slo* gene coding region (135 kDa).

This discrepancy has recently been resolved by employing a battery of sequence-directed antibodies raised against synthetic peptides encompassing the entire *slo* sequence (Knaus *et al.*, 1995). As expected, these antibodies specifically immunoprecipitate the *in vitro* translation product of *mslo*, a protein of 135 kDa. Furthermore, Western blotting experiments reveal the presence of an α subunit with apparent molecular weight of 125 kDa in smooth muscle sarcolemma, as well as in skeletal muscle t-tubules and plasma membranes from GH_3 anterior pituitary cells. The difference in size of these α subunits from that of the purified maxi-K channel preparation is due to a highly reproducible proteolytic cleavage which occurs at the hydroxylapatite chromatography stage of purification. Using appropriate antibodies, it is possible to detect a full-length α subunit, an intermediate size product of 90 kDa, and the 62-kDa polypeptide, as well as smaller fragments in some preparations. Proteolysis occurs exclusively in two regions within the long C-terminal tail of *slo*, near two alternative splice sites. Importantly, proteolytic fragments remain associated upon SDS-PAGE under nonreducing conditions, presumably a result of association of fragments through disulfide bridges within the α subunit core fragment. Biochemi-

cal characterization of these fragments provides direct evidence for the functional expression of distinct splice variants of the channel. Based on hydropathy analysis, the putative topology of the pore-forming subunit may resemble that of other voltage-gated K^+ channels; beginning with the N-terminus, six transmembrane-spanning regions, including a pore domain, are predicted. The extended C-terminal region may possess an additional four transmembrane domains. Cloning of the bovine aortic smooth muscle α subunit has recently been reported (Moss *et al.*, 1995). Inspection of the predicted amino acid sequence indicates the presence of a protein kinase A recognition site, multiple phosphorylation sites for protein kinase C, and a putative glycosylation site whose substitution has not been defined. The predicted structure of the maxi-K channel α subunit is illustrated in Fig. 2. With availability of site-directed antibodies, it should now be possible to confirm the topology of this protein.

Isolation and Cloning of the β Subunit

The structure of the maxi-K channel β subunit was determined by separating this protein from the α subunit through SDS-PAGE, electroeluting the protein from the gel, and subjecting it to cleavage with endoproteinase Glu-C (Knaus *et al.*, 1994b). After purifying the resulting peptide fragments by reversed-phase HPLC and performing microsequencing, a unique sequence of 28 amino acid residues was obtained. From this sequence, degenerate oligonucleotide probes were used to amplify a unique cDNA sequence encoding the peptide fragment. This probe was then used to screen a cDNA library prepared from bovine tracheal smooth muscle. A cDNA encoding the β subunit was thus isolated. This cDNA encodes a unique protein of 191 amino acids. Hydropathy analysis suggests the presence of two hydrophobic regions which presumably span the membrane, a large extracellular loop, and two short cytoplasmic domains at the N- and C-termini of the protein (Fig. 2). Two glycosylation sites for N-linked carbohydrate are present in the extracellular domain, consistent with biochemical studies (see above), as are four Lys residues which are potential sites for crosslinking to ChTX via a bifunctional reagent. There is also a protein kinase A phosphorylation site in the N-terminal cytoplasmic region that may be involved in maxi-K channel regulation (see below).

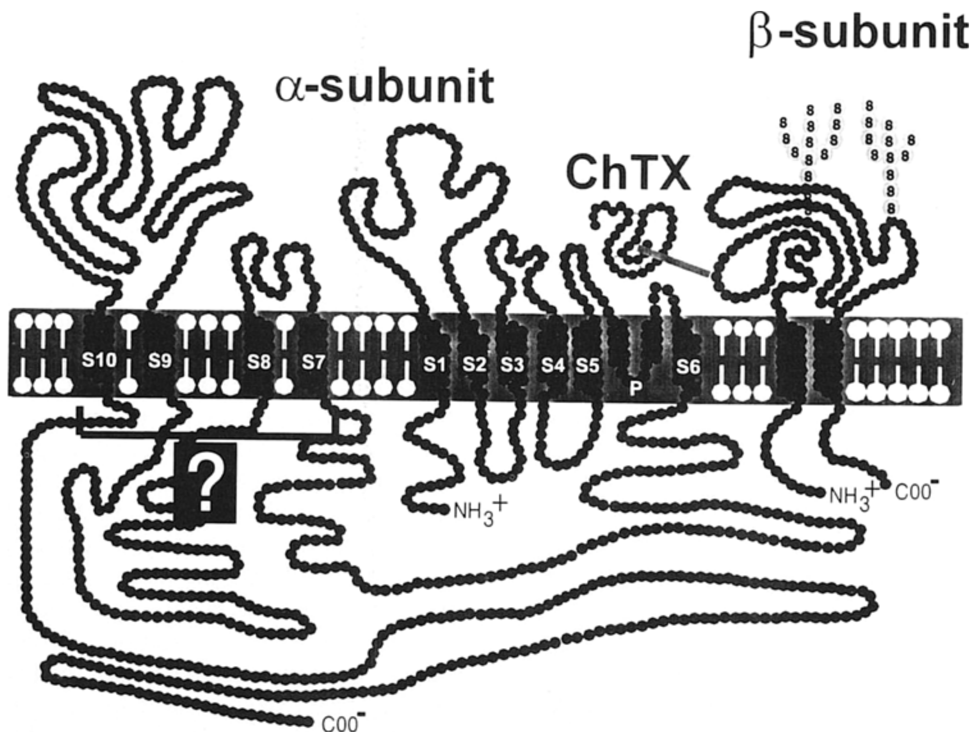

Fig. 2. Hypothetical structure of a maxi-K channel α-β heterodimer. The putative transmembrane topology of the α- and β-subunits of the maxi-K channel is illustrated. Also shown is charybdotoxin binding to the pore of the α-subunit and its crosslinking to the β-subunit with a bifunctional reagent. Four such complexes are expected to associate and form a functional maxi-K channel. See text for a full description of the data supporting this model.

The β subunit cloned from a bovine aortic cDNA library is identical to the tracheal protein. Although transmembrane topology of the β subunit is similar to that of auxiliary subunits of other ion channels, there is no significant sequence homology with these proteins.

Co-assembly of α and β Subunits

To demonstrate that α and β subunits of the tracheal maxi-K channel are tightly associated in a complex, antibodies prepared against the α subunit were employed in immunoprecipitation studies (Knaus *et al.*, 1994c). Using this approach, it was possible not only to immunoprecipitate specifically the denatured α subunit that was prelabeled with [^{125}I] Bolton–Hunter reagent, but also under nondenaturing conditions, the labeled α/β subunit complex. These results suggest that tracheal maxi-K α and β subunits are complexed through strong noncovalent interactions, as might be expected since both subunits remain together during a seven-step purification procedure.

Site-directed antibodies have been produced against sequences in the putative extracellular domain of the β subunit (Knaus *et al.*, 1994b). Under denaturing conditions, these antibodies specifically immunoprecipitate β subunit labeled with either [^{125}I] Bolton–Hunter reagent or crosslinked with [^{125}I]ChTX. In addition, under nondenaturing conditions, these antibodies immunoprecipitate both β and α subunits, consistent with results obtained using α subunit antibodies, suggesting tight association between the two proteins. Taken together, these data indicate that the maxi-K channel most likely consists of a multimer of β and α subunits, and that despite binding in the pore of the α subunit, ChTX is specifically and covalently incorporated only into the β subunit during crosslinkng procedures. Therefore, part of the β subunit must be in close proximity to the pore-forming structure.

Mapping the ChTX-Crosslinking Site

To determine the site of incorporation of ChTX into the β subunit, the purified maxi-K channel was crosslinked with [^{125}I]ChTX, and labeled protein was isolated by size-exclusion reversed-phase HPLC. Using a combination of proteolytic digestion with various endoproteinases, site-directed antibodies for immunoprecipitation studies, and deglycosylation procedures, the site of crosslinking was identified as Lys_{69} (Knaus *et al.*, 1994a). These results confirm the predicted topology of this subunit and demonstrate that both putative extracellular N-glycosylation sites are occupied with complex carbohydrate chains of 5–6 kDa each. Further, these data imply that a region of the β subunit's extracellular loop is in close proximity to the ChTX receptor site on the α subunit of the channel.

In the corollary of these studies, variants of ChTX which retain their channel blocking properties and in which individual Lys residues had been mutated to Gln, were used to identify the corresponding amino acid of ChTX involved in crosslinking (Munujos *et al.*, 1995). All ChTX mutants used bind to the maxi-K channel with physical and pharmacological properties similar to that of native ChTX. Upon reaction with bifunctional reagents, ChTX substituted at Lys_{11} and Lys_{31} still yield wild-type crosslinking patterns, but peptide without a Lys at position 32 fails to incorporate with high efficiency into the β subunit. In examining the model proposed for interaction of ChTX with the external pore of the maxi-K channel (Stampe *et al.*, 1994), Lys_{32} lies just above the channel's outer vestibule wall. Data obtained employing disuccimidyl suberate constrain the distance between the pore and Lys_{69} in the extracellular loop of the β subunit to a maximum of 11Å (Fig. 2).

Heterologous Expression of α and β Subunits

Expression of the α subunit (*slo*) of the maxi-K channel alone in *Xenopus* oocytes yields Ca^{2+}-activated K^+ channels (Butler *et al.*, 1993; Dworetzky *et al.*, 1994; Tseng-Crank *et al.*, 1994; Wallner *et al.*, 1995). However, coexpression of β and α subunits in this system has profound effects on the biophysical properties of the channel (McManus *et al.*, 1995). Channels from oocytes injected with cRNAs encoding both subunits are more sensitive to activation by voltage or intracellular Ca^{2+} than channels encoded by the α subunit alone; the midpoint of the current–voltage curve is shifted by nearly 100 mV in the hyperpolarizing direction by coexpression of the β subunit. Expressed another way, the change in the voltage dependence of channel activation in the presence of the β subunit is equivalent to that produced by a 10-fold increase in intracellular Ca^{2+}. Similar effects have been noted after coexpression of β subunit with α subunit cloned from human myometrium (Wallner *et al.*, 1995). In contrast, neither expression levels, single-channel conductance, nor ionic selectivity are affected by β subunit coexpression. The β subunit also has

profound effects on pharmacological properties of the maxi-K channel (see below). Thus, both α and β subunits contribute to the functional properties of maxi-K channels, and are required to reconstitute channel behavior that is observed in smooth muscle. Perhaps regulated coassembly of α and β subunits, or tissue-specific expression of variants of these subunits, gives rise to the functional diversity characteristic of Ca^{2+}-activated K^+ channels.

Heteromeric Assembly

We suggest that the oligomeric composition of the maxi-K channel most likely reflects coassembly of four α and four β subunits to form a heteromultimeric complex, by analogy with putative structures of voltage-gated K^+ channels. There are several lines of evidence which support this notion: α and β subunits exist in a 1:1 stoichiometry in a purified channel preparation; the size of the purified ChTX receptor, as measured by sucrose density gradient centrifugation, is consistent with a molecular mass of approximately 500–600 kDa. In addition, the Hill coefficient for Ca^{2+} in activating maxi-K channels is approximately 4, and the Hill coefficient for certain maxi-K channel agonists is also approximately 4 (see below).

MOLECULAR PHARMACOLOGY OF THE MAXI-K CHANNEL

Even though several peptidyl inhibitors of maxi-K channels have been discovered, relatively few small-molecule channel modulators, besides TEA, are known. In an attempt to further expand the molecular pharmacology of this channel, [^{125}I]ChTX binding to smooth muscle sarcolemmal membranes was used to screen synthetic molecules and natural product extracts for potential channel effectors. In this way, either competitive or allosteric modulators of [^{125}I]ChTX binding may be identified. Subsequently, these agents must be tested in functional assays to ascertain their effects on channel activity. Using this strategy, both potent maxi-K channel agonists and antagonists have been discovered.

Glycosylated Triterpenes

Screening of medicinal herbs used in traditional medicine has yielded interesting ion-channel modulators in the past (e.g., the L-type Ca^{2+} channel blocker, tetrandrine; King *et al.*, 1988). Recently, this approach identified the first potent natural product maxi-K channel modulator. Alcoholic extracts of the plant *Desmodium adscendens* are used in Ghana for treatment of asthma and dysmenorrhea (Ampofo, 1977). Extracts of this plant inhibit contractility of intestinal smooth muscle elicited by electrical field stimulation, or of sensitized airways smooth muscle exposed to prostanoids or antigens (Addy, 1989; Addy and Burka, 1988, 1989). Even though *Desmodium adscendens* contains a number of identified agents which can contribute to smooth muscle relaxation (e.g., inhibitors of arachidonic acid metabolism), its extracts also block [^{125}I]ChTX binding in tracheal sarcolemmal membranes (McManus *et al.*, 1993b). Using ChTX binding to follow activity, three putative maxi-K channel modulators were purified to homogeneity from this plant and their structures were determined by NMR and mass spectroscopy techniques. The three compounds are known glycosylated triterpenes; dehydrosoyasaponin I (DHS-1; Fig. 1), soyasaponin I, and soyasaponin III. The most potent of these agents in blocking binding is DHS-1; K_i = 120 nM, 60% maximal inhibition of [^{125}I]ChTX binding. The other two soyasaponins inhibit binding with K_i's of 6 and 1 μM respectively, while the aglycone is completely inactive (>100 μM). Such data indicate a defined SAR for inhibition of toxin binding and illustrate the importance of both carbohydrate and triterpene moieties for supporting an interaction with the maxi-K channel. These compounds inhibit [^{125}I]ChTX binding by an allosteric mechanism; DHS-1 is a partial inhibitor of binding, it displays noncompetitive inhibition in a Scatchard analysis, and it increases rate of toxin dissociation from the ChTX receptor.

When functional effects of DHS-1 are evaluated on maxi-K channels reconstituted in planar lipid bilayers (McManus *et al.*, 1993b), addition of test compound at the normally extracellular face of the channel (1 μM) has no effect on channel activity. However, when added at the channel's normally cytoplasmic face, very low concentrations of DHS-1 (10 nM) reversibly increase maxi-K channel open probability. Since DHS-1 acts intracellularly and ChTX binds to the external side, this is an example of allosteric coupling between two drug binding sites. Consistent with this idea, single-channel experiments indicate that addition of DHS-1 at the cytoplasmic face of the channel can elicit a 3- to 8-fold increase in the rate of ChTX dissociation from the channel's pore, similar to results

observed in [^{125}I]ChTX dissociation experiments. DHS-1 cannot substitute for Ca^{2+} in activating channels; in the absence of Ca^{2+}, DHS-1 does not cause maxi-K channel opening. Starting with a low Ca^{2+} concentration and low initial channel open probability, DHS-1 can cause maximal channel activation. The slope of the relation between channel open probability and DHS-1 concentration in log-log plots varies from 2 to 4 (McManus *et al.*, 1993a), suggesting that at least four DHS-1 molecules can bind to a channel to cause maximal activation.

The effects of DHS-1 were examined on maxi-K channel α and β subunits heterologously expressed in *Xenopus* oocytes. In excised patch recordings, DHS-1 has no effect on activity of a neuronal maxi-K channel α subunit expressed alone, but markedly potentiates channel activity in oocytes coexpressing both α and β subunits (McManus *et al.*, 1995). These data demonstrate the requirement for the β subunit to confer sensitivity to DHS-1. A possible explanation is that the binding site for the glycotriterpene is located on the β subunit, or alternatively, presence of the β subunit modifies the α subunit so that DHS-1 can bind. The apparent Hill coefficient of 4 for DHS-1 activation of native channels is consistent with four β subunits in each channel complex (McManus *et al.*, 1993a).

In specificity tests, DHS-1 does not affect various voltage-gated K^+ channels, ATP-dependent K^+ channels, small-conductance Ca^{2+}-activated K^+ channels found in human T lymphocytes, L-type Ca^{2+} channels, or various membrane transporters (McManus *et al.*, 1993b). Thus, this glycotriterpene is the first example of a potent and selective maxi-K channel agonist, and, when compared with other types of K^+ channel agonists (e.g., the benzopyran series of agonists of ATP-dependent K^+ channels), this natural product is the most potent K^+ channel opener discovered to date. The ability of DHS-1 to open maxi-K channels may be related to the therapeutic efficacy of *Desmodium adscendens* in treating asthma and dysmenorrhea. Activation of maxi-K channels should suppress electrical excitability and promote relaxation of smooth muscle by hyperpolarization of the sarcolemmal membrane (Quast, 1993). In addition, activation of maxi-K channels should inhibit release of tachykinins (see below). However, the intracellular site of action of DHS-1, together with its relatively poor membrane permeability, predict that *in vivo* effects of glycotriterpenes should have a slow onset and display less potency than are observed in *in vitro* measurements. Interestingly, this medicinal herb must be given chronically to elicit therapeutic benefit. Perhaps these agents are metabolized to other species which can more easily penetrate cells. Nevertheless, finding that DHS-1 and its congeners are active as maxi-K channel agonists helps define a site on this protein complex where agents can bind to activate the channel. It is conceivable that structurally dissimilar agonists may be identified which also bind at this site.

Maxikdiol

A second, structurally unique agonist of the maxi-K channel was detected in a fermentation broth of an unidentified coelomycite, based on its ability to inhibit [^{125}I]ChTX binding to smooth muscle membranes (Singh *et al.*, 1994). Using this bioassay to guide purification, a novel dihydroxylisoprimane diterpene, termed "maxikdiol," was isolated and its structure determined (Fig. 1). Like DHS-1, maxikdiol is a partial inhibitor of [^{125}I]ChTX binding (K_i = 1 μM) and appears to act as an allosteric modulator of toxin binding; in a Scatchard analysis maxikdiol inhibits binding through a K_d effect, and it also markedly increases the dissociation rate of ChTX from the channel. When examined in functional experiments, maxikdiol increases maxi-K channel activity in excised inside-out patches from bovine aortic smooth muscle cells (1 μM threshold effect; clear increases in channel activity at 3–10 μM). Activation of maxi-K channels is reversible and occurs when this agent is added at the cytoplasmic face of the channel. This compound does not affect other types of K^+ channels tested, but does display significant Ca^{2+} entry blocker activity at 10 μM. In contrast to the effects of DHS-1, action of maxikdiol is observed when the maxi-K channel α subunit is expressed by itself in *Xenopus* oocytes (O.B. McManus and M. Sanchez, unpublished observations).

Indole Diterpene Alkaloids

The first structural series of potent nonpeptidyl maxi-K channel blockers was also discovered using [^{125}I]ChTX binding (Knaus *et al.*, 1994d). It is known that various tremorgenic indole alkaloids of fungal origin produce neurological disorders in animals (e.g., staggers syndrome) due to potentiation of release of certain neurotransmitters (Cole and Cox, 1981; Norris *et al.*, 1980; Selala *et al.*, 1991). Extracts containing such agents are positive in the [^{125}I]ChTX binding

assay, and the alkaloids aflatrem, paspalinine, paspalicine, paspalitrem A and C, paxilline, penitrem A, and verruculogen were identified as the active species in these extracts. These compounds affect ChTX binding in two distinct fashions: paspalitrem A and C, aflatrem, penitrem A, and paspalinine inhibit [^{125}I]ChTX binding with a defined rank order of potency, and inhibition can be either partial or complete; paxilline, verruculogen, and paspalicine enhance [^{125}I]ChTX binding in a concentration-dependent fashion. Consistent with their effects on [^{125}I]ChTX binding, these compounds either block or enhance crosslinking of [^{125}I]ChTX to the β subunit of the maxi-K channel, indicating a specific interaction for indole diterpenes with the channel.

The indole alkaloids modify [^{125}I]ChTX binding to maxi-K channels through an allosteric mechanism (Knaus *et al.*, 1994d). Compounds that enhance [^{125}I]ChTX binding do so by causing an increase in ligand affinity, while inhibitors function noncompetitively to decrease apparent receptor site density. Results of toxin off-rate experiments demonstrate that stimulators of binding decrease the rate of [^{125}I]ChTX dissociation from its receptor, while inhibitors can have mixed effects; some compounds markedly stimulate toxin dissociation, while others have no significant effect. These various alkaloids may interact at the same site because effects on [^{125}I]ChTX binding elicited by stimulators (e.g., paxilline or verruculogen) are prevented by inhibitors (e.g., aflatrem). These data suggest that indole diterpenes bind to a unique site(s) on maxi-K channels and can alter ChTX binding to the channel via either positive or negative allosteric interactions.

Although this group of indole diterpenes have different effects on [^{125}I]ChTX binding, all potently block maxi-K channel activity in functional experiments (Knaus *et al.*, 1994d). These compounds inhibit with IC_{50}'s of 0.1–10 nM in measurements of single channels present in either membrane patches excised from smooth muscle, or reconstituted in phospholipid bilayers. Block develops more readily when compounds are added at the inner surface of the channel, and appears to be state dependent; when $[Ca^{2+}]_i$ is elevated and channel open probability is high, indole alkaloids are weaker inhibitors. In addition, patterns of channel inhibition differ among the compounds. Paxilline produces long silent periods of block similar to peptide inhibitors, while paspalitrem C causes numerous brief (<100 msec) interruptions in channel gating.

In specificity testing (Knaus *et al.*, 1994d), paspalitrem C (100 nM) has no effect on small-conductance Ca^{2+}-activated K^+ channels or *n*-type K^+ current in human T lymphocytes. Paxilline weakly blocks delayed rectifier K^+ currents in mouse pancreatic β cells at 2 μM, but has no effect on L-type Ca^{2+} channels or neuronal voltage-gated Na^+ channels at 10 μM. Furthermore, small modifications in paxilline destroy maxi-K channel blocking activity, indicating a defined SAR for interaction with this channel. Some members of this structural class lack a C-19 hydroxyl group, which renders these compounds nontremorgenic *in vivo* (e.g., paspalicine), and they still potently block maxi-K channels. This suggests that tremorgenic effects of the indole diterpenes may not be due to block of maxi-K channels, and instead may be related to effects of indole alkaloids on GABA-gated Cl^- channels (Yao *et al.*, 1989).

Other Maxi-K Channel Modulators

Structurally novel maxi-K channel agonists have been identified by means other than effects on [^{125}I]ChTX binding activity. For the most part, members of the benzopyran series of ATP-dependent K^+ channel agonists do not affect maxi-K channels. One member of this class, BRL 55834, is now reported to open both ATP-dependent and Ca^{2+}-activated K^+ channels (Ward *et al.*, 1992). However, BRL 55834 is not particularly potent as a maxi-K channel agonist. A series of substituted benzimidazolones (NS004, NS1619) reversibly stimulate maxi-K channels (Olesen *et al.*, 1994; Sargent *et al.*, 1993). These agents can modulate the α subunit of the channel by itself in expression studies (Dworetzky *et al.*, 1994). Unfortunately, published compounds in this class are insufficiently potent or selective to serve as specific pharmacological probes (Edwards *et al.*, 1994). Niflumic acid (0.2–1 mM) also activates some maxi-K channels through an interaction with the α subunit (Wallner *et al.*, 1995). Recently, a unique imidazole pyrazine derivative, SCA 40, has been reported to function as a maxi-K channel agonist in guinea pig smooth muscle based on pharmacological profiles; both ChTX and high K^+ block the ability of SCA 40 to relax tracheal smooth muscle (Laurent *et al.*, 1993). However, no electrophysiological data have been presented to confirm direct activation of maxi-K channels. In fact, inspection of its structure suggests that SCA 40 might stimulate maxi-K channels indirectly through inhibition of phosphodiesterases and subsequent elevation in $[c\text{-}AMP]_i$ (see below). Currently, the number

of maxi-K channel modulators is small, but as work on this channel progresses, it is likely that more channel agonists and antagonists will be identified. Clearly, the maxi-K channel, like other ion channels, is a multidrug receptor possessing many allosterically coupled drug binding sites.

USING MAXI-K MODULATORS TO UNDERSTAND CHANNEL FUNCTION

Smooth Muscle

The functional role of maxi-K channels has been studied primarily in smooth muscle, using peptide channel inhibitors (Suarez-Kurtz *et al.*, 1991; Winquist *et al.*, 1989). Myogenic activity of guinea pig bladder and ileum is enhanced by both ChTX and IbTX. In these tissues, maxi-K channels provide a pathway for cell repolarization following action potential-dependent elevation in intracellular Ca^{2+}. Other guinea pig smooth muscles (e.g., portal vein, uterus) are not affected by maxi-K channel blockers, even though these tissues express maxi-K channels. Thus, the role of maxi-K channels, relative to other conductances, in controlling smooth muscle excitation-contraction coupling is tissue-dependent (Suarez-Kurtz *et al.*, 1991). Maxi-K channels may contribute to cellular resting potential in some smooth muscles: ChTX and IbTX cause contracture of quiescent guinea pig aorta and trachea. The role of maxi-K channels in smooth muscle is not only tissue-dependent, but also species-dependent. Myogenic activity of rat portal vein is modulated by maxi-K channel blockers (Winquist *et al.*, 1989), while the same agents have no effect on guinea pig portal vein (Suarez-Kurtz *et al.*, 1991). Recently, effects of the indole diterpenes, paxilline and paspalitrem C, have been evaluated on smooth muscle contractility, and they have been found to elicit effects like the peptide channel inhibitors (F. de Farias and G. Suarez-Kurtz, personal communication).

Relaxation of carbachol-contracted guinea pig trachea is produced by a variety of agents such as β-agonists, phosphodiesterase inhibitors, membrane-permeant c-AMP analogs, and elevators of c-GMP. In the presence of peptide channel inhibitors (ChTX, IbTX), dose–response curves for these relaxants are shifted to the right, and, in some cases (e.g., with β agonists or c-GMP elevators) relaxation is completely abolished (Jones *et al.*, 1990, 1993). Similar data have been obtained from studies with human airways smooth muscle (Miura *et al.*, 1992). One interpretation of this pharmacological profile is that activation of maxi-K channels promotes tracheal relaxation. Consistent with this view are independent mechanistic studies demonstrating that open probability of maxi-K channels is enhanced by protein kinase A-dependent phosphorylation (Kume *et al.*, 1989), and by direct interaction with the GTP binding protein, α_s (Kume *et al.*, 1992). It may be relevant that GMP has been shown to directly stimulate maxi-K channel activity in patch clamp experiments (Williams *et al.*, 1988). All relaxants listed above elevate cellular c-AMP (or c-GMP) levels, and β agonists are also known to activate maxi-K channels through a G protein interaction. However, the antagonism of β agonist-induced relaxation by IbTX requires Ca^{2+} entry (i.e., it is prevented by nifedipine, Cd^{2+}, or low extracellular Ca^{2+}; Huang *et al.*, 1993). This implies a direct effect of IbTX on membrane potential. Apparently, IbTX depolarizes carbachol-contracted trachea. This opens voltage-gated Ca^{2+} channels, and subsequent entry of Ca^{2+} may prevent smooth muscle relaxation. Presently, it is not clear as to which of two β agonist effects, increased maxi-K channel activity vs. Ca^{2+} sequestration, contribute more to relaxation of airways smooth muscle. These results highlight some of the difficulties of using functional studies to characterize the role of maxi-K channels.

Synaptic Effects

In guinea pig airways, stimulation of sensory neurons causes release of neurokinins and substance P to promote smooth muscle contraction. In addition, these tachykinins produce mucus hypersecretion and plasma extravasation which are contributors to the pathophysiology of asthma. Several agents (e.g., α-agonists, μ-opioid agonists, neuropeptide Y) prevent release of tachykinins from sensory neurons by a mechanism which is blocked by ChTX and IbTX (Stretton *et al.*, 1992). These data suggest that release of tachykinins might be partially dependent on the activity of maxi-K channels at sensory nerve terminals. Thus, an activator of maxi-K channels could have therapeutic utility in asthma, not only as a smooth muscle relaxant, but also in preventing the neurogenic inflammatory component of the disease.

Limited studies have been carried out concerning the functional role of maxi-K channels in the central nervous system. Data obtained with the indole diter-

pene maxi-K channel antagonists (see above) suggest that such agents can modulate neurotransmitter release (Norris *et al.,* 1980; Selala *et al.,* 1991). This has already been demonstrated for peripheral neurons. Maxi-K and voltage-gated Ca^{2+} channels are co-localized at presynaptic motor nerve terminals, and block of maxi-K channels causes broadening of action potentials, enhanced Ca^{2+} entry, and increased neurotransmitter release (Robitalle *et al.,* 1993). Maxi-K channel expression has been mapped in rat brain using a combination of western blot analysis, *in situ* hybridization studies; immunohistochemistry, and binding of radiolabeled IbTX (Knaus *et al.*, 1996). The channel has a well-defined tissue distribution in brain, and immunocytochemical studies suggest that it is abundant at nerve terminals of certain fiber tracts. Maxi-K channel α subunits have been cloned from human brain (Dworetzky *et al.,* 1994; Tseng-Crank *et al.,* 1994). Nine different variants were identified, resulting from alternative RNA splicing of a single gene located on chromosome 10. Splice variants differ in their Ca^{2+} sensitivity, and may be targeted to different brain regions. Although a maxi-K β subunit has been identified in brain (Reinhart *et al.,* 1995), little is known about its role in this tissue. With the probes available, it may now be possible to define the physiologic function of maxi-K channels in the CNS.

CONCLUSIONS

Many significant advances have occurred during the last few years in the area of maxi-K channel research, and these form a solid foundation for promising future developments. The family of peptidyl inhibitors derived from venoms continue to be useful molecular probes for determining the architecture of the channel's pore, and for defining physiologic roles of maxi-K channels in various cells and tissues. Purification of the channel from smooth muscle not only independently identified the pore forming subunit as the mammalian homolog of the *slowpoke* gene product cloned from *Drosophila,* but also identified the first β subunit of Ca^{2+}-activated K^+ channels. Discovery of the β subunit highlights the importance of biochemical purification in order to identify all molecular components of native ion channel complexes. The β subunit has profound effects on biophysical and pharmacological properties of the maxi-K channel. Some of the diversity that exists within the Ca^{2+}-activated K^+ channel family may be due to formation of heteromultimers between various types of α and β subunits. Finally, production of reagents directed against maxi-K channels, both antibodies for topology and localization studies, and small molecules for exploring the channel's pharmacology, will be useful in future studies. It is likely that within the next few years, molecular components of small- and intermediate-conductance Ca^{2+}-activated K^+ channels will also be identified. As this field expands, it will be possible to more clearly define therapeutic opportunities available via modulation of Ca^{2+}-activated K^+ channels.

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