Maxi-K$_{Ca}$, a Unique Member of the Voltage-Gated K Channel Superfamily

L. Toro, M. Wallner, P. Meera, and Y. Tanaka

Large-conductance, voltage-, and Ca^{2+}-sensitive K$^+$ (maxi-K$_{Ca}$) channels regulate neuronal and smooth muscle excitability. Their pore-forming α-subunit shows similarities with voltage-gated channels and indeed can open in the practical absence of Ca^{2+}. The NH$_2$ terminus is unique, with a seventh transmembrane segment involved in β-subunit modulation. The long COOH terminus is implied in Ca^{2+} modulation.

Large-conductance, voltage-, and Ca^{2+}-sensitive K$^+$ (maxi-K$_{Ca}$) channels are ubiquitously distributed among tissues, except in heart myocytes. Their activity is triggered by depolarization and enhanced by an increase in cytosolic Ca^{2+}, providing a link between the metabolic and electrical state of cells. The physiological role of maxi-K$_{Ca}$ channels has been examined in smooth muscle, where they are particularly abundant and play a key role in setting contractile tone (7), and in neurons, where they are involved in spike shaping and neurotransmitter release (4). Their role in cellular processes has been determined in many cases using scorpion toxins like charybdotoxin and the selective blocker iberiotoxin (Ref. 4, see Fig. 4). Maxi-K$_{Ca}$ channels were first cloned from *Drosophila*; their primary sequence showed that they belong to the voltage-gated K$^+$ channel (K$_v$) superfamily (3, 4). Biochemical purification from smooth muscle tissues showed that maxi-K$_{Ca}$ channels are a protein complex formed by two integral membrane subunits, the pore-forming α-subunit and a regulatory β-subunit (4). Current molecular studies have established several novel structural and functional features of maxi-K$_{Ca}$ channels. We have recently shown that, in contrast to other members of the K$_v$ superfamily, the maxi-K$_{Ca}$ channel α-subunit has a seventh transmembrane segment, S0, that leads to an exoplasmic NH$_2$ terminus (6,13). This region is a critical determinant of β-subunit modulation (13). It is now established that, in accordance with their sequence homology to other voltage-gated ion channels, the activation machinery of maxi-K$_{Ca}$ channels is triggered by voltage but not by Ca^{2+} (9). Instead, Ca^{2+} acts as a modulator: micromolar Ca^{2+} switches the channel from a Ca^{2+}-independent state to a Ca^{2+}-modulated state. Only when the channel is in its Ca^{2+}-modulated state (Ca^{2+} above 1μM) can the β-subunit upregulate its activity (5). The requirement for micromolar Ca^{2+} suggests that colocalization or close approximation of maxi-K$_{Ca}$ channel α-subunits with or without β-subunits with Ca^{2+}-conducting proteins in the plasma membrane or internal stores may be necessary for the channel to regulate cell excitability.

General molecular properties

After the *Drosophila* maxi-K$_{Ca}$ channel α-subunit was cloned, many groups engaged in cloning maxi-K$_{Ca}$ (*slo*) channels from tissues of their interest, in the hope of finding the molecular basis of this channel function and diversity as related to specific cell types. The β-subunit was cloned after biochemical purification and increases the α-subunit apparent Ca^{2+} sensitivity (4).

Figure 1*A* shows a scheme of the α-subunit primary characteristics. The primary sequence of S1–S6 is homologous to the corresponding regions in K$_v$ channels; however, S0 is unique to maxi-K$_{Ca}$ channels and adds a seventh transmembrane segment that, together with the NH$_2$ terminus, allows β-subunit modulation (6, 13) (see Figs. 2 and 3). The long COOH terminus encompasses two-thirds of the protein and contains four hydrophobic segments (S7–S10) and several splicing sites (triangles in Fig. 1*A*). Only two mammalian splice variants seem to confer different Ca^{2+} sensitivities (8,11). However, the functional role of other splice insertions, including those containing possible phosphorylation sites (~P), is still unclear. One noteworthy characteristic of maxi-K$_{Ca}$ channels is their capability of being expressed as two separable domains: the "core" and the "tail." The tail is the highest

"...the maxi-K$_{Ca}$ channel α-subunit has a seventh transmembrane segment...."

L. Toro, M. Wallner, and P. Meera are in the Dept. of Anesthesiology at the University of California at Los Angeles School of Medicine, Los Angeles, CA 90095–1778, USA. Y. Tanaka is in the Dept. of Pharmacology, School of Pharmaceutical Sciences, Toho University, 2-2-1 Miyama, Funabashi-City, Chiba 274, Japan.
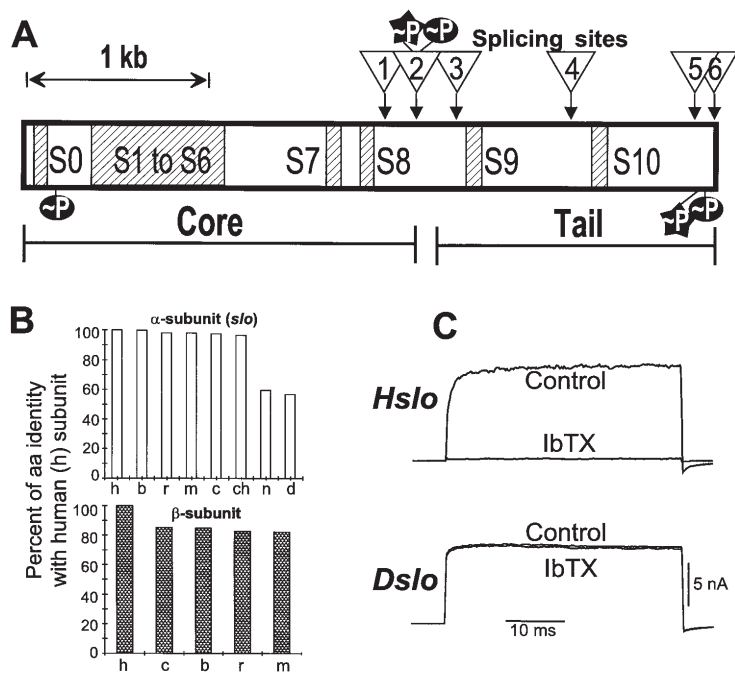
FIGURE 1. Basic properties of large-conductance, voltage-, and Ca^{2+}-sensitive (maxi-K_{Ca}) channels. *A*: diagram of *slo* channels. S0–S6 mark NH_2-terminal transmembrane segments. S0 is unique for maxi-K_{Ca} channels, and S1–S6 are homologous to voltage-dependent ion channels. S7–S10 are hydrophobic regions at the COOH terminus (two-thirds of the protein). Two separable domains, the "core" and "tail," are marked. Triangles *1–6* mark alternative splice sites that lead to the insertion of 3–59 amino acids into the primary sequence. Putative sites for phosphorylation (~P) are in stars (protein kinase G) and in circles (protein kinase C). *B*: bar graph comparing the amino acid (aa) identity of human α- (*slo*) and β-subunits, respectively, are as follows: h (human), U11058 and U25138; b (bovine), U60105 and L26101; r (rat), U55995 and AF020712; m (mouse), L16912 and AF020711; c (canine), U41001 and U41002; ch (chicken), U73189; n (nematode, *C. elegans*), see Ref. 14; d (*D. melanogaster*), JH0697. *C*: blockade of *Hslo*, but not of *Dslo*, by 100 nM iberiotoxin (IbTX). Dissociation constant was ~1 nM (6).

conserved region among *slo* clones and seems to be involved in Ca^{2+} modulation (14,15).

Because native K_{Ca} channels have different properties within and among tissues, the failure to clone more than one maxi-K_{Ca} channel gene has been surprising. In contrast to other types of voltage-gated ion channels (Ca^{2+}, Na^+, and K^+ channels), which show a variety of isoforms, the maxi-K_{Ca} channel α-subunit seems to be encoded by a single gene. Mammalian maxi-K_{Ca} channel α-subunits have almost identical amino acid sequences among different species (>97%, Fig. 1*B*), whereas mammalian β-subunits have only 82–85% sequence identity. The striking sequence conservation of the α-subunit may reflect a high evolutionary pressure to maintain an optimized function in mammals.

The fly *slo* channel (*Dslo*) differs from mammalian *slo* channels in its primary sequence, which is reflected in functional aspects such as single-channel kinetics, lack of β-subunit modulation (see Fig. 3), and insensitivity to nanomolar charybdotoxin and iberiotoxin (Fig. 1*C*).

Given the fact that the native maxi-K_{Ca} channels' functional differences in kinetics, Ca^{2+} sensitivities, and responses to phosphorylation cannot be explained by the existence of various genes, they may be explained by the expression of splice variants of the same gene and/or differential association with a modulatory subunit(s). Functional properties related to alternative splicing or the distribution of splice variants in tissues is starting to emerge. Changes in kinetics and/or apparent Ca^{2+} sensitivities have been reported for splice variants in brain (related to a 4-amino acid insert in splice *site 1*, Fig. 1*A*) (11) and in chromaffin cells (related to a 59-amino acid exon at splice *site 2*, Fig. 1*A*) (8). Also, their relative distribution in tissues has been examined using splice-site-specific polymerase chain reaction (8, 11). For example, the splice variant from chromaffin cells that has increased apparent Ca^{2+} sensitivity is present in brain and other tissues but not in spinal cord or smooth or skeletal muscles (8). However, the functional significance and the physiological relevance of most of the splice variants are still an open question.

In contrast to the maxi-K_{Ca} channel α-subunit that has several splicing sites, analysis of the human $β_{K,Ca}$ gene shows no evidence for alternative RNA splicing. Up to now, cloning of different genes for β-subunits has been elusive. However, related sequences for both subunits can be found in public data bases (14).

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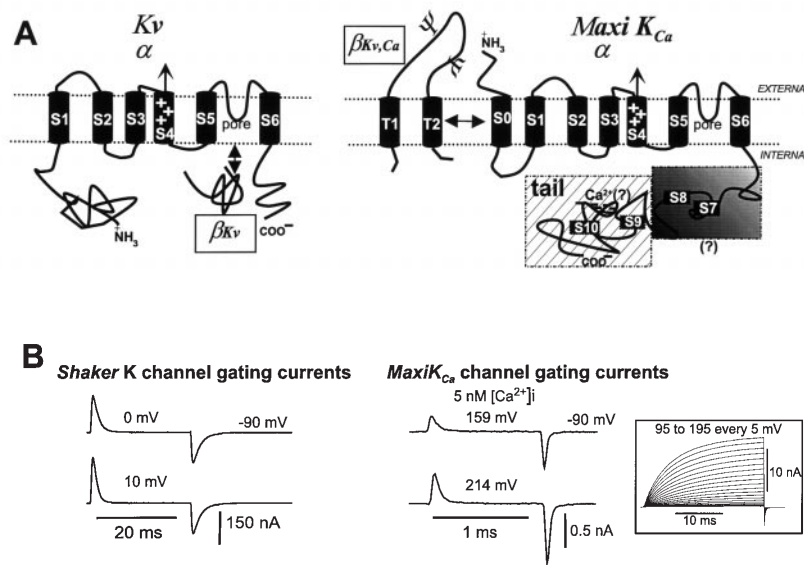
FIGURE 2. Two voltage-gated K$^+$ channels of the S4 superfamily: K$_v$ and maxi-K$_{Ca}$ channels. *A, left*: K$_v$ channels have intracellular NH$_2$ and COOH termini. K$_v$ modulatory β-subunit(s) is cytosolic. Some of them carry sequences resembling the inactivation "ball" and are thought to interact with the pore region. *Right*: maxi-K$_{Ca}$ channels α- and β-subunits. β$_{Kv,Ca}$, Transmembranal β-subunit. Ψ, glycosylation sites. S0–S6, α-subunit 7 transmembrane segments. S0 and part of the exoplasmic NH$_2$ terminus determine functional coupling between α- and β-subunits. ?, Regions with uncertain topology (S8-S7, shaded box) and uncertain Ca^{2+} binding site. Hatched box, cytosolic "tail" region with hydrophobic S9–S10 regions. *B*: voltage-dependent gating of *Shaker* K (typical K$_v$) and maxi-K$_{Ca}$ channels. K$_v$ and maxi-K$_{Ca}$ channels have an intrinsic voltage sensor whose movement generates gating currents. Increasing evidence suggests that depolarization induces the outward movement of S4 (arrows in *A*), leading to pore opening. *Inset*: maxi-K$_{Ca}$ currents in the practical absence of Ca^{2+} (5 nM).

Novel topology

Comparison with K$_v$ channels. K$^+$ channels are formed by the association of four α-subunits; maxi-K$_{Ca}$ channels seem to share this property. K$_v$ as well as maxi-K$_{Ca}$ channels are regulated by β-subunits (3). In contrast to K$_v$ channels whose β-subunits are intracellular proteins and modulate inactivation (3), the β-subunit of maxi-K$_{Ca}$ channels is a transmembrane protein that modulates Ca^{2+} sensitivity (see Fig. 3*B*), kinetics, and toxin binding (2, 4, 5) (Fig. 2*A*). The maxi-K$_{Ca}$ channel α-subunit shares sequence homology with K$_v$ channels and other members of the S4 superfamily of voltage-gated ion channels, from transmembrane regions S1 to S6 (Fig. 2). The most striking similarities are *1*) an S4 region with three critical charges that largely account for the voltage sensor gating current, *2*) conserved charged residues in S1, S2, and S3, which may form ionic interactions with charges in S4, and *3*) a typical pore loop between S5 and S6 that forms the channel conduction pathway (3, 13).

These similarities point to the view that maxi-K$_{Ca}$ channels possess an intrinsic voltage sensor and thus are voltage-gated channels. In fact, as is discussed below, depolarization of maxi-K$_{Ca}$ channels leads to the outward movement of charged residues in the voltage sensor (Fig. 2*A*, arrow in S4), as in K$_v$ channels, generating gating currents before pore opening (Fig. 2*B*) (9).

Novel features of maxi-K$_{Ca}$ channel α-subunit topology. We have demonstrated that maxi-K$_{Ca}$ channels possess an additional transmembrane region (S0) that leads to an exoplasmic NH$_2$ terminus (6, 13). We have introduced a c-*myc* epitope (epitope tag) at the NH$_2$ terminus and detected antibody binding of nonpermeabilized transfected cells using beads coated with secondary antibody. Because antibodies cannot cross cell membranes, labeling of living (nonpermeabilized) cells by antibodies is an indication that the epitope is extracellular. Figure 3*A* outlines the strategy and shows an example of bead-labeled cells.

Because S0 is highly conserved in all maxi-K$_{Ca}$ (*slo*) channels, including the *Drosophila* and *Caenorhabditis elegans* homologues, the presence of a seventh transmembrane domain S0 leading to an exoplasmic NH$_2$ terminus seems to be a key feature of this class of channels. As is discussed below, this additional domain S0 and the exoplasmic NH$_2$ terminus are crucial for β-subunit modulation of the mammalian *slo* (Fig. 3*B*).

At the COOH terminus, maxi-K$_{Ca}$ channels possess four hydrophobic regions (S7–S10). Our recent experiments show that at least S9 and S10 are cytoplasmic, since the in vitro translated protein containing these two regions (tail) (Fig. 1*A*) is soluble. Furthermore, in vivo reconstitution of channel activity can be obtained if a membrane

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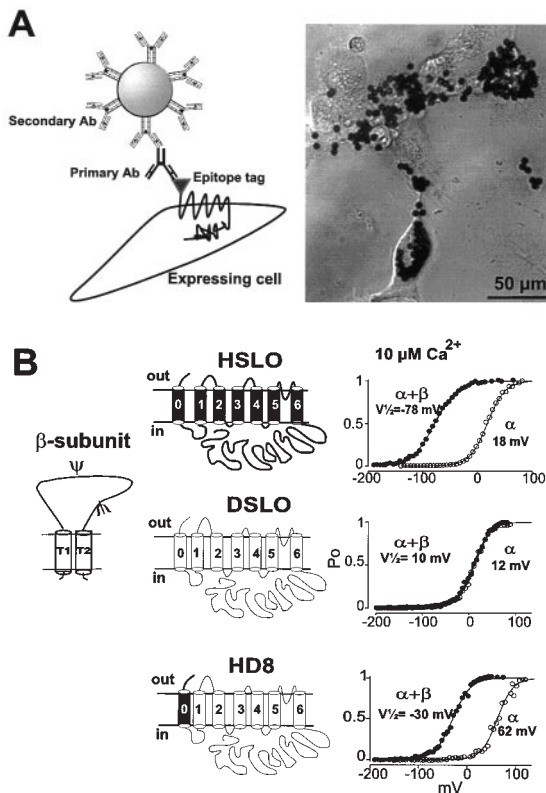
FIGURE 3. Maxi-K_{Ca} channel unique NH_2 terminus. *A*: extracellular NH_2 terminus. Diagram depicts a cell expressing maxi-K_{Ca} channels with a c-*myc* epitope tag at the NH_2 terminus. Bead coated with secondary antibody (Ab) binds to the cell through a bridge formed by the primary antibody (anti-c-*myc* antibody). Picture at *right* illustrates intact cells expressing c-*myc*-tagged (at the NH_2 terminus) maxi-K_{Ca} channel profusely labeled with beads, as in diagram. *B*: extracellular NH_2 terminus and S0 are structural determinants of β-subunit modulation. Coexpression of *Hslo* (α, bold lines, filled segments) and β-subunit shifts the voltage activation curves of maxi-K_{Ca} currents by ~100 mV ($V_{1/2}$, half-maximal voltage). *Dslo* (thin lines, open segments) currents are not affected by coexpression with β-subunit. *Hslo* NH_2 terminus and S0 in the backbone of *Dslo* (HD8) restore β-subunit modulation. Experiments were done in isotonic 140 mM K^+ and 10 μM Ca^{2+}.

patch of an oocyte expressing the "core" (Fig. 1*A*) is introduced into an oocyte expressing the tail (6).

Function-structure relationships

Expression of a large number of maxi-K_{Ca} channels in heterologous systems has allowed the dissection of functional mechanisms that have been elusive for years. For example, it has been possible to answer the long sought after question: Are maxi-K_{Ca} channels opened (gated) by voltage or by Ca^{2+}?

Voltage-dependent gating. The measurement of gating currents, which reflect the movement of the voltage sensor by depolarization, is the experimental proof of a channel being voltage dependent. As shown in Fig. 2*B*, we have recently demonstrated that maxi-K_{Ca} channels possess an intrinsic voltage sensor, since gating currents can be measured on cell depolarization (9). Gating currents and ionic currents (Fig. 2*B*, *inset*) are elicited independently of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) when this divalent cation is practically absent ($[Ca^{2+}]_i \leq 100$ nM) (5, 9); a large depolarization is required to maximally open the channel. If Ca^{2+} increases to the micromolar level, the channel switches to a Ca^{2+}-regulated state or "mode" in which less electrical energy is required to open the channel (5).

$[Ca^{2+}]_i$ modulation. As stated before, Ca^{2+} modulation of maxi-K_{Ca} channels occurs only at concentrations above 100 nM free $[Ca^{2+}]_i$ (5). It appears that the channel changes conformation between two states, from a Ca^{2+}-independent state to a Ca^{2+}-modulated state. The amino acid residues or structural determinants responsible for Ca^{2+} binding are still an open question. A stretch of aspartic acids (D) between the S9 and S10 regions could be involved in Ca^{2+} binding and sensitivity (14). However, natural splice variants, outside this aspartic acid reach region, have different apparent Ca^{2+} sensitivities (8, 11), suggesting that the Ca^{2+} binding site(s) is formed by a complex three-dimensional interaction of various protein sites.

β-Subunit modulation. The Ca^{2+} sensitivity of maxi-K_{Ca} channels is modulated by their β-subunit. This regulation takes place when the channel is in its Ca^{2+}-modulated mode and occurs at concentrations higher than 1 μM (5). In addition to the effects on the channel's apparent Ca^{2+} sensitivity, the β-subunit also alters kinetics (5) and toxin binding (2) and makes the channels susceptible to activation by nanomolar dehydrosoy-asaponin I (DHS-I; Ref. 4). Of these properties, the change in kinetics induced by the β-subunit is observed even at Ca^{2+} concentrations lower than 1 μM, whereas upregulation by DHS-I occurs only if $[Ca^{2+}]_i > 1$ μM (5, 10). Using chimeric constructs from the nonregulated *Dslo* channel and the responsive *Hslo* channel (Fig. 3*B*), we have recently demonstrated that S0 and part of the exoplasmic NH_2 terminus of the α-subunit determine β-subunit modulation (13).

Pore blockers used to identify the pore domain. The region assigned as the pore domain of maxi-K_{Ca} channels has the signature sequence (VGYGD) for the pore of K^+ channels (14). This assignment can be tested experimentally by modifying the response to known pore blockers. Ionic currents flowing through the pore of maxi-K_{Ca} channels are blocked by tetraethylammonium, iberiotoxin (Fig. 1*C*), and/or charybdotoxin in all *slo* channels tested. One exception is *Dslo*, which is insensitive to nanomolar concen-

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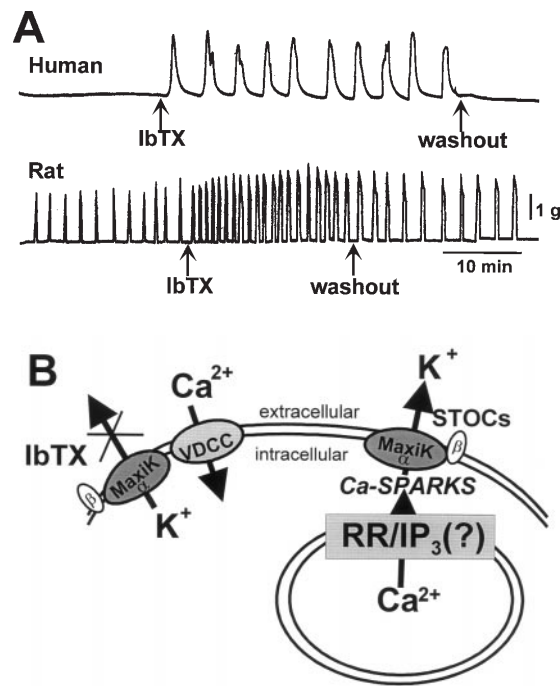
FIGURE 4. Physiological role of maxi-K_{Ca} channels. *A*: iberiotoxin (IbTX) that blocks maxi-K_{Ca} channels induces contractions of human uterine strips and increases the frequency of contractions of rat uterine strips. *B*: possible models of how maxi-K_{Ca} channels open in response to localized increases in Ca^{2+}. Colocalization with voltage-dependent Ca^{2+} channels (VDCC) and/or close approximation with subsarcolemmal Ca^{2+} stores allows a local rise in Ca^{2+} that induces maxi-K_{Ca} channel opening and hyperpolarization. In smooth muscle, spontaneous Ca^{2+} release from ryanodine receptors (RR), "Ca^{2+} sparks," open maxi-K_{Ca} channels, generating spontaneous transient outward currents (STOCs) (7). This causes hyperpolarization and relaxation, which can be reversed by maxi-K_{Ca} channels blockers (e.g., iberiotoxin). Inositol trisphosphate (IP_3) receptors as an alternative Ca^{2+} store with similar function need to be explored. [Modified from K. Anwer et al. *Am. J. Physiol.* 265 (*Cell Physiol.* 34): C976–C985, 1993.]

"…maxi-K_{Ca} channels are modulated by various protein kinases."

trations of both iberiotoxin (Fig. 1*C*) and charybdotoxin. Experimental evidence showing that the assigned pore region in *slo* channels indeed forms the pore includes a chimeric construct where *Hslo* loses its sensitivity to iberiotoxin by exchanging its pore with the pore of *Dslo* (6).

Other mechanisms of regulation. Many studies in native cells have shown that maxi-K_{Ca} channels are modulated by various protein kinases. However, in cloned channels, this property is not yet clear. The majority of possible phosphorylation sites in the α-subunit of maxi-K_{Ca} channels are present in the COOH terminus (~P, Fig. 1*A*). With the use of a low-stringency sequence for protein kinase A (PKA; R-$X_{1–2}$-S/T), *Hslo* was found to have 11 PKA possible phosphorylation sites but none using a strong consensus sequence (R-R/K-X-S*/T*). There is one strong protein kinase G (PKG) phosphorylation site (R/K-R/K-

$X_{1–2}$-S*/T*) near the COOH terminus and two strong protein kinase C (PKC) sites (R/K-$X_{2–0}$-S*/T*-$X_{2–0}$-R/K), one in the S0–S1 linker and the other at the COOH terminus. Sequences introduced by alternative splicing at splicing *site 2* add strong potential sites for PKG (8, 11) and PKC phosphorylation (11). None of these sites has been proven to be directly phosphorylated. It is tempting to suggest that splice variations produce different maxi-K_{Ca} channel phenotypes not only by changing Ca^{2+} sensitivities but also by introducing sites for phosphorylation.

The human β-subunit of maxi-K_{Ca} channels is also a putative substrate for phosphorylation. It has a strong PKG phosphorylation site at its intracellular NH_2 terminus. This site is also putative for PKA phosphorylation if a low-stringency motif is used. The possible role of β-subunit phosphorylation in the modulation of channel activity needs to be explored.

Another mechanism of metabolic regulation is the redox state of a molecule. *Hslo*, but not *Dslo*, channels seem to be activated by a reducing agent, dithiothreitol (1 mM), and inhibited by oxidation with hydrogen peroxide (1). It would be interesting to determine if natural reducing agents such as NADH or glutathione exert any effect on *Hslo* channels.

Tissue distribution and physiological role

Functional maxi-K_{Ca} channels are ubiquitously distributed among tissues, except in the heart myocytes. This has been common knowledge for electrophysiologists and has been recently confirmed at the mRNA level. Electrophysiological experiments have shown that maxi-K_{Ca} channels are particularly abundant in smooth muscles, where they are thought to set the pace of contractile activity. Although they are expressed to a lesser extent in neurons, it is thought that they play important roles in the regulation of transmitter release and spike shaping.

At the mRNA level, both α- and β-subunits coexist in most tissues, although in brain the level of β-subunit mRNA is much lower than the level of α-subunit. In smooth muscles, both α- and β-subunit signals are strong (12). However, studies at the protein level are few and need to be performed for each tissue of interest. Important physiological questions to be addressed are, In which tissues are α- and β-subunits colocalized? Are both proteins evenly distributed in cells or spotted to certain regions? Are most of the maxi-K_{Ca} channels formed by α- and β-subunits? In this regard, we have recently evaluated the molecular constituents of maxi-K_{Ca} channels in human coronary arteries (10). To investigate this

point, we took advantage of two results derived from molecular studies in the cloned α- and β-subunits: *1)* that DHS-I (nM range) only activates maxi-K_{Ca} channels composed of α- and β-subunits (4), and *2)* that the presence of the β-subunit makes the channel more sensitive to Ca^{2+}, requiring less voltage to open (5). Because ionic currents were activated by DHS-I and their Ca^{2+} sensitivities increased dramatically when $[Ca^{2+}]_i$ was in the micromolar range, mimicking the behavior of expressed α- and β-subunits, we came to the conclusion that in human coronary smooth muscle most of the channels are composed of α- and β-subunits.

Maxi-K_{Ca} channel blockers (e.g., iberiotoxin) effectively induce contractile activity of coronary and uterine smooth muscle (Fig. 4*A*). However, because channel activity in isolated cells is extremely low at resting Ca^{2+} (nM range), these experiments were difficult to explain in terms of maxi-K_{Ca} channels being "open at rest" and thus their blockade causing depolarization and contraction. The recent discovery of ryanodine-sensitive Ca^{2+} spikes that relax smooth muscle through maxi-K_{Ca} channels (7) and the fact that coexpression with the β-subunit dramatically increases the maxi-K_{Ca} channel α-subunit Ca^{2+} sensitivity, when tested in high external K^+ (5), seem to give an answer to this puzzle. A local increase in $[Ca^{2+}]_i$ to micromolar levels in the vicinity of maxi-K_{Ca} channels formed by α- and β-subunits may allow a significant K^+ flux through these channels, providing a very effective regulatory mechanism for the maintenance of smooth muscle tone. Studies using physiological ionic gradients and molecular anatomy studies should provide further evidence for this concept. It would be expected that maxi-K_{Ca} channel α- and β-subunits are colocalized with Ca^{2+} channels in the plasma membrane or in close approximation to Ca^{2+} channels of internal membranes (ryanodine or inositol trisphosphate receptors) (Fig. 4*B*).

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