Multiple Facets of Maxi-K^+ Channels: The Heme Connection

José López-Barneo and Antonio Castellano

Laboratorio de Investigaciones Biomédicas, Hospital Universitario Virgen del Rocío, Universidad de Sevilla, E-41013, Seville, Spain

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In the classic era of electrophysiology, two distinct classes of ion channels were thought to exist in cell membranes: one class accounted for the generation of action potentials and their propagation along nerve fibers (voltage-gated channels); the other class accounted for the electrical signals at the chemical synapses (transmitter-gated channels). The advances made for the last decades in terms of elucidating the structure and function of ion channels show how simplistic this view was. Several hundreds of ion channel types, encoded by dozens of gene families expressed in all tissues, are known to be gated by elaborate processes related not only to membrane voltage changes or transmitter release but also to membrane deformation, direct coupling to G proteins, or the presence of intracellular ligands such as Ca^{2+}, H^+, nucleotides, and lipids, among others. In addition, in most channels, gating and/or ion permeation are modulated by phosphorylation, redox modification, nitrosylation, and even by gaseous oxygen and carbon monoxide. Ion channels do not work on isolation; they are intimately involved in most signaling pathways and their function is finely tuned by the metabolic state of the cells.

The maxi-K^+, or BK, channel offers a most enticing example of the multifaceted nature of how ion channel function is regulated. These channels are highly sophisticated molecular machines, gated synergistically by voltage and Ca^{2+}, that exhibit both a high K^+ selectivity and a large single-channel conductance. Each maxi-K^+ channel is formed by four α-subunits and up to four auxiliary β-subunits (Atkinson et al., 1991; Vergara et al., 1998). The maxi-K^+ channel α-subunit is encoded by a single gene (Slo1) with several spliced isoforms, which are expressed rather ubiquitously. Each α-subunit has seven transmembrane segments (S0–S6), which, like other voltage-gated K^+ channel α-subunits, provide the voltage sensor and pore domains, a small extracellular amino terminus, and an expanded cytosolic carboxyl terminus containing two regulators of K^+ conductance (RCK) domains separated by a large nonconserved linker (Meera et al., 1997; Jiang et al., 2002). Several high- and low-affinity Ca^{2+} binding sites on the COOH terminus confer upon maxi-K^+ channels Ca^{2+} (and to a less extent Mg^{2+}) sensitivity (Zeng et al., 2005).

Depolarization and Ca^{2+} serve as allosteric regulators of channel activation by independently altering the energetics of channel opening (see below). This dual regulation by two physiologically relevant variables allows maxi-K^+ channels to display a remarkable diversity in their properties among different cells and tissues and to participate in multiple cellular processes. Maxi-K^+ channels play a fundamental role in the control of membrane potential and cellular excitability. In some cases, as with smooth muscle contraction and exocytosis, maxi-K^+ channel–mediated hyperpolarization acts as a negative feedback mechanism, which decreases further Ca^{2+} entry through voltage-gated Ca^{2+} channels.

Functional diversity among maxi-K^+ channels also results from the selective tissue distribution of several types of auxiliary β-subunits, which modulate important aspects of channel function. For example, the β1 subunit appears to mediate the regulation of maxi-K^+ channels by estrogens (Valverde et al., 1999) and increases the sensitivity of the α-subunit to Ca^{2+}. Other β-subunits contain a large intracellular domain that can interact with the internal mouth of the pore to produce N-type inactivation (Wallner et al., 1999).

Among the numerous intracellular signals modulating maxi-K^+ channel function, H_2O_2, CO, NO, and O_2 have received special attention due to their possible participation in specialized homeostatic processes or in the pathophysiology of disease (Bolotina et al., 1994; Wang and Wu, 1997; López-Barneo et al., 2001; Tang et al., 2004; Williams et al., 2004). Yet, although the many facets of maxi-K^+ channels have already been explored to some degree, there is room for surprises. Recently, it has been reported that the Slo1 channel possesses a conserved heme binding sequence motif (Wood and Vogeli, 1997; Tang et al., 2003) in the linker between the two RCK domains and that free intracellular heme markedly decreases the frequency of channel opening (Tang et al., 2003). In this issue of *The Journal of General Physiology*, Horrigan and colleagues (Horrigan et al.,

Correspondence to José López-Barneo: jose.l.barneo.sspa@juntadeandalucia.es

Abbreviations used in this paper: HO-2, heme oxygenase-2; RCK, regulator of K^+ conductance.

2005) provide a compelling analysis of this finding and conclude that heme is a potent, but subtle, regulator of allosteric coupling in maxi-K^+ channels.

The interaction of heme with maxi-K^+ channels was studied by recording single-channel and macroscopic ionic currents as well as gating currents generated by channels formed by human (hSlo1) or mouse (mSlo1) maxi-K^+ α-subunits transiently expressed in HEK cells. The study was designed based on the conceptual framework provided by Horrigan, Cui, and Aldrich (denoted here as HCA model; Horrigan and Aldrich, 1999, 2002; Horrigan et al., 1999; see also Rothberg and Magleby, 2000 and the enlightening review by Magleby, 2003). The essence of this model is that voltage- and Ca^{2+}-sensors act relatively independently of one another and that they move through successive states that are separate from the open–closed channel transition. In the absence of Ca^{2+}, the maxi-K^+ functions as a voltage-dependent channel. The steeply voltage-dependent transitions resulting from the movement of each voltage sensor allostericaly regulate the intrinsic opening/closing process of the channel, which in itself is weakly voltage dependent (see Fig. 1 in Horrigan et al., 2005). In this gating scheme, the most likely activation pathway for moderate to large depolarizations is through the closed states (C_0–C_4). Brief depolarizing pulses (lasting a few hundred microseconds) generate gating currents associated with voltage sensor movements before the channels open. After prolonged depolarizing pulses, 5 ms or longer, a slow component in the off gating current appears reflecting the voltage-dependent backward transitions between open states (O_4 to O_0 in the bottom row of horizontal transitions in Fig. 1 of Horrigan et al., 2005). Cytosolic Ca^{2+}, which binds at several sites located in (or near) the RCK domains, has almost no effect on the gating currents; yet Ca^{2+} can increase channel open probability more than 1,000-fold at extreme negative potentials when the voltage sensors are not activated. Maxi-K^+ channels thus seem to be activated either by membrane depolarization or Ca^{2+} acting through independent allosteric mechanisms that converge on a common step, the opening of the channel gate. Under normal conditions for mammalian cells, the two variables (membrane potential and cytosolic [Ca^{2+}]) act synergistically to induce conformational changes that convert the energy stored in the membrane electric field and the free energy of Ca^{2+} binding into the mechanical work that actually opens the channel (Fig. 1, top).

Horrigan and colleagues found that intracellular heme (or the oxidized form hemin used in most experiments) produces a marked, dose-dependent (50% inhibition at <100 nM), and reversible decrease in the amplitude of the macroscopic maxi-K^+ currents. Importantly, heme elicits changes in specific gating parameters, thus providing clues for understanding how it modulates channel function. In the complete absence of Ca^{2+} (or any other divalent cations), the most salient effects of heme are as follows. First, a decrease in the steepness and depolarizing shift of the conductance–voltage (G–V) curve, without appreciable change in the gating currents generated by brief depolarizing pulses. The slow component of the off gating current recorded at the end of prolonged depolarizations is, however, diminished by heme, consistent with the decrease in the fraction of channels open during depolarization. Second, a decrease of the channel open probability during moderate to large depolarizations without changing the single-channel conductance. This is accompanied by a notable increase in the number of blank records that, together with the shift of the G–V curve, cause the decrease of K^+ current amplitude. Third, a deceleration of the activation kinetics at extreme positive voltages and deactivation kinetics at extreme negative voltages. Fourth, an increase of the channel open probability at negative voltages (by 10–20-fold) with ~50% increase in mean channel open duration. This effect at the single channel level is consistent with the slower deactivation kinetics of macroscopic currents seen at negative voltages.

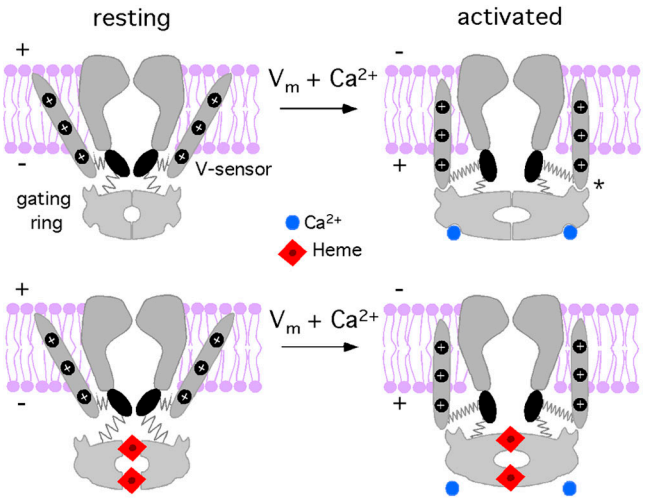

Figure 1. Schematic cartoon of the structural/molecular model proposed to explain the gating modifications induced by heme/hemin on maxi-K^+ channels. The drawings, representing two α-subunits, are inspired by those in Fig. 13 of Horrigan et al. (2005). See text for details.

Taken together, these results suggest that heme does not impede the movement of the voltage sensors when the channels are closed. During depolarizing pulses, however, heme binding seems to disfavor the open channel state, thereby causing a decrease in the single-channel open probability, a depolarizing shift, and a reduction in the slope of the G–V curve and a decrease in the off gating current after prolonged depolarizing

pulses. Surprisingly, therefore, heme favors the open state of the channels at negative voltages. Based on the HCA model, Horrigan et al. (2005) simulated these effects of heme by decreasing the strength of allosteric coupling (factor D in the HCA model) between the channel gate and the voltage sensor, and by shifting the resting closed–open equilibrium toward the open state (O_0 in Fig. 1 of Horrigan et al., 2005, see below). The higher occurrence of blank records in the presence of heme could not be satisfactorily fitted with the simulations and was assumed to result from slow gating modifications.

The modulatory effect of heme on maxi-K^+ channel gating persists at saturating Ca^{2+} concentrations, suggesting that the channels are less sensitive to Ca^{2+} when heme is bound. In terms of the HCA model, this could be simulated assuming that heme produces an additional decrease in the allosteric coupling strength between either the channel gate or the voltage sensor and Ca^{2+} binding (factors C and E, respectively, in the HCA model).

After the biophysical analysis, Horrigan et al. (2005) provided a possible molecular interpretation for their observations based on the high-resolution structure of prokaryotic RCK domains (Jiang et al., 2002) and the mechanical spring model of maxi-K^+ channel gating (Niu et al., 2004). As shown schematically in Fig. 1 (top), it is proposed that the four RCK dimers in the maxi-K^+ channel complex form a gating ring structure that can expand or constrict depending on cytosolic [Ca^{2+}]. The movement of the gating ring is envisioned to contribute to channel opening and closing by exerting force on the channel gates (possibly the cytoplasmic end of S6) through a molecular spring formed by the S6-RCK1 linker (Jiang et al., 2002; Niu et al., 2004). Another spring-like connector transfers energy from the voltage sensor to the activation gate. In accord to the HCA model, two separated linkages with the gate are required to account for the additive effect of voltage and Ca^{2+} on channel activation.

A central idea in Horrigan et al.'s article is that part of the coupling between the voltage sensor and the activation gate is mediated through the interaction of the voltage sensor with the gating ring. Specifically, it is proposed that channel opening causes the voltage sensor to interact with the cytosolic gating ring, which then stabilizes the open channel conformation (asterisk in Fig. 1, top right). Heme binding to the segment between RCK1 and RCK2 (Tang et al., 2003) is thought to alter the structure of the gating ring making it more expanded (Fig. 1, bottom).

Even in the absence of internal Ca^{2+} or any voltage sensor movement, deformation of the gating ring by heme would impose tension on the activation gate, thus favoring the open channel state at negative membrane potentials. Expansion of the gating ring might also decrease the affinity for Ca^{2+} while preventing any further expansion required for the normal interaction between the ring and the voltage sensor (Fig. 1, bottom right). Hence, by acting on the gating ring, heme would diminish the strength of voltage- and Ca^{2+}-dependent allosteric coupling. The molecular scheme proposed by Horrigan et al. (2005) is attractive because it provides an intuitive explanation to most of the biophysical observations simply as a result of the interaction of heme with the gating ring. Nevertheless, it must be noted that (as recognized by the authors) the structural underpinning for this model remains speculative and lacks direct experimental support. Expansion of the gating ring and decrease of Ca^{2+} affinity induced by heme are reasonably expected structural changes because the heme-binding segment is located between the two RCK domains and in the proximity of the Ca^{2+} binding sites. In accord with this notion, even discrete chemical modification (i.e., cysteine oxidation) of residues near the Ca^{2+} bowl should interfere with the Ca^{2+}-dependent activation of the channels (Tang et al., 2004). However, it is difficult to visualize how the voltage sensor can contact directly the intracellular gating ring, as structural models suggest that the S4 segment moves outward during activation. The voltage sensor/gating ring interaction therefore could be indirect because, as discussed by Horrigan et al. (2005), mutations in the S4 or S4–S5 loop disrupt Mg^{2+}-dependent activation of the channels involving the cytoplasmic S6-RCK1 linker.

The exquisite sensitivity of maxi-K^+ channels to heme, raises far-reaching questions regarding its physiological impact and significance. Do heme's effects on channel function simply reflect an interesting toxic/pharmacological action or is heme a physiological modulator of maxi-K^+ channel function? In addition, could heme be "permanently" bound to the Slo1 protein as a prosthetic group and confer to maxi-K^+ channels sensitivity to the physiologically important molecules O_2, CO, and NO (Fig. 2)? In red blood cells, heme is bound to hemoglobin and in skeletal muscle cells to myoglobin, but heme also is present in nonerythroid/muscle cells as a cofactor of numerous proteins, such as cytochrome-containing enzymes, catalase, glutathione reductase, soluble guanylate cyclase, or nitric oxide synthase (for cell biology of heme see reviews by Padmanaban et al., 1989, Ponka, 1999). Heme (Fe^{2+}) and its oxidized form hemin (protophorphyrin IX with a bound Fe^{3+}) also exist as free cell-signaling molecules that can bind to "heme-responsive motifs" in transcription factors that regulate the expression of cytochrome-P_{450}–containing enzymes. Heme/hemin also are believed to play an important role in the posttranscriptional regulation of heme and nonheme proteins

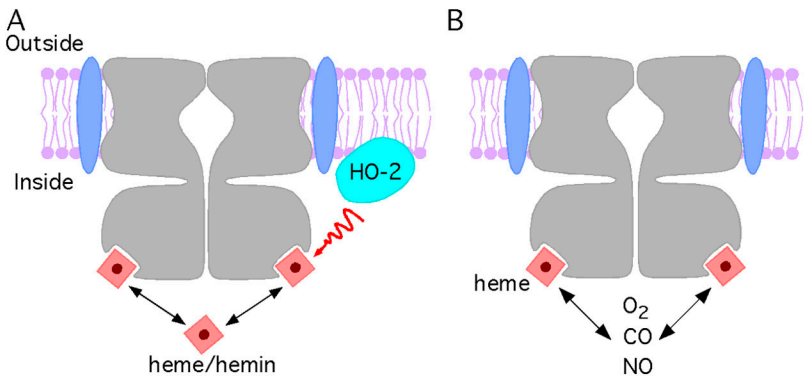

Figure 2. Possible interactions of heme with maxi-K^+ channels. (A) Heme and its oxidized form hemin act as reversible modulators of the channels by interacting with the conserved heme-binding cytoplasmic site. The cartoon illustrates two α and two β-subunits. The heme-catabolizing enzyme HO-2 is depicted as being attached to the maxi-K^+ channel macromolecular complex. (B) Maxi-K^+ channel as a heme protein. Ferrous heme is permanently bound to each α-subunit and confers to maxi-K^+ channel sensitivity to gaseous (oxygen, carbon monoxide, and nitric oxide) heme ligands.

in eukaryotic cells. In this context, it is quite possible to think of cytosolic heme (or hemin) as physiologically relevant modulators of maxi-K^+ channels (Fig. 2 A). It is in this respect tantalizing that the biophysical analysis of Horrigan et al. (2005) suggests that heme/hemin could have a dual effect on maxi-K^+ channels: being potent activator(s) at negative membrane potentials, but inhibitor(s) when the channels are activated by Ca^{2+} and/or cell depolarization. Such effects of heme/hemin could be manifested differentially in nonexcitable and excitable cells. It is possible, for example, that in nonexcitable cells, which normally have a negative membrane potential and relatively low cytosolic [Ca^{2+}], heme/hemin preferentially act as maxi-K^+ channel activators facilitating cell hyperpolarization. This could have an adaptive role by providing electromotive force for transmembrane Ca^{2+} influx required for Ca^{2+}-dependent cellular functions. In excitable cells, maxi-K^+ channel activation normally has a protective role by preventing excessive depolarization and Na^+/Ca^{2+} overload. In these cells, therefore, the effects of heme/hemin could be either homeostatic or toxic, depending on the particular functional or pathological scenario.

Specifically, maxi-K^+ channel openers have important pharmacological applications to prevent excitotoxicity in stroke (Gribkoff et al., 2001). During brain ischemic injury, hypoxia can trigger the intracellular release of micromolar levels of heme (Doré, 2002). In this situation (with cell depolarization and high cytosolic Ca^{2+}), inhibition of maxi-K^+ channels by heme/hemin might produce more serious deleterious effects. Delayed cerebral vasospasm, a frequent cause of morbidity and mortality after subarachnoid hemorrhage, is another condition in which the release of heme/hemin from blood clots into the subarachnoid space has been postulated to have an important pathophysiological role (Wagner and Dwyer, 2004). Inhibition of maxi-K^+ channels by heme/hemin transported into the cells may explain the drastic reduction of potassium permeability and the subsequent depolarization seen in cerebral arterial smooth muscle after subarachnoid hemorrhage (Harder et al., 1987). It may in this respect be important that heme/hemin catabolism mainly depends on hemeoxygenase-2 (HO-2) (Doré, 2002; Wagner and Dwyer, 2004), a ubiquitous enzyme that converts heme/hemin into free iron, biliverdin, and CO, which is in itself a maxi-K^+ channel activator. A recent proteomic study, reporting that HO-2 coimmunoprecipitates with heterologously expressed Slo1 (Williams et al., 2004), is quite provocative because it may indicate that HO-2 has become part of the maxi-K^+ macromolecular complex to mitigate heme/hemin inhibition of channel activation.

The heme-binding amino acid sequence motif typical of Slo1 channels (CXXCH, where X is any amino acid) overlaps another signature sequence that is characteristic for the immunoglobulin super gene family, and which has been implicated in maxi-K^+ α-subunit assembly (Wood and Vogeli, 1997). Within this sequence, histidine in position 616, one of the axial ligands of heme iron, appears to have an important role in maxi-K^+ channel function because its replacement with another residue not only abolishes the heme/hemin effect on maxi-K^+ channel (Tang et al., 2003) but also markedly decreases channel activity (Wood and Vogeli, 1997; Tang et al., 2003). Therefore, the heme-binding site in the maxi-K^+ α-subunit could have evolved independently of adaptive pressures related to heme homeostasis, in which case it might have only limited regulatory significance. There are, however, several reasons to believe this is not the case. The heme-binding motif characteristic of c-type cytochromes is conserved among Slo1 channels from several species and their splice variants. Furthermore, heme/hemin binds to Slo1 channels with high affinity (comparable to that of other heme-binding proteins for heme) and with high selectivity relative to free iron, protoporphyrins devoid of iron, and other chemicals (Tang et al., 2003). So, a fascinating hypothesis is that in normal circumstances, heme (with Fe^{2+} iron) is bound to the maxi-K^+ channel, which then would behave as a true heme protein, capable of reversibly binding gaseous ligands (Fig. 2 B). In fact, maxi-K^+ channels are activated by the heme-binding gases NO, CO, and O_2 independently of any

soluble cytosolic component (Bolotina et al., 1994; Wang and Wu, 1997; López-Barneo et al., 2001; Williams et al., 2004). Several mechanisms, including direct S-nitrosylation and interaction of CO with imidazole-containing residues, have been proposed but definitive demonstration of such interactions has yet to materialize. The connection between maxi-K^+ channels and heme has just begun. The inspiring analytical approach of Horrigan and colleagues provides an optimal conceptual framework for elucidating these interactions, which bridge biophysics with physiology and the mechanisms of disease.

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