Heme Regulates Allosteric Activation of the Slo1 BK Channel

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Large conductance calcium-dependent (Slo1 BK) channels are allosterically activated by membrane depolarization and divalent cations, and possess a rich modulatory repertoire. Recently, intracellular heme has been identified as a potent regulator of Slo1 BK channels (Tang, X.D., R. Xu, M.F. Reynolds, M.L. Garcia, S.H. Heinemann, and T. Hoshi. 2003. Nature. 425:531–535). Here we investigated the mechanism of the regulatory action of heme on heterologously expressed Slo1 BK channels by separating the influences of voltage and divalent cations. In the absence of divalent cations, heme generally decreased ionic currents by shifting the channel’s G–V curve toward more depolarized voltages and by rendering the curve less steep. In contrast, gating currents remained largely unaffected by heme. Simulations suggest that a decrease in the strength of allosteric coupling between the voltage sensor and the activation gate and a concomitant stabilization of the open state account for the essential features of the heme action in the absence of divalent ions. At saturating levels of divalent cations, heme remained similarly effective with its influence on the G–V simulated by weakening the coupling of both Ca2+ binding and voltage sensor activation to channel opening. The results thus show that heme dampens the influence of allosteric activators on the activation gate of the Slo1 BK channel. To account for these effects, we consider the possibility that heme binding alters the structure of the RCK gating ring and thereby disrupts both Ca2+- and voltage-dependent gating as well as intrinsic stability of the open state.

INTRODUCTION

Heme, composed of an iron center and a surrounding protoporphyrin IX, plays critical roles in protein function. Many proteins, including hemoglobin, cytochromes and soluble guanylate cyclases, contain heme as a functionally indispensable prosthetic group. The presence of heme often confers novel properties to the proteins, such as high sensitivity to biologically important gases, including oxygen, nitric oxide, and carbon monoxide (Rodgers, 1999; Jain and Chan, 2003). While the majority of cellular heme may be bound to proteins, some “uncommitted” or “free” heme likely exists (Ponka, 1999). Precise estimates of free intracellular heme concentration are not widely available because of technical problems, but an estimate of >1 μM has been reported for reticulocytes (Garrick et al., 1999). In neuronal cells, intracellular heme concentration may dramatically increase following hemorrhagic strokes. These vascular accidents lead to a breakdown of hemoglobin and release of heme in the extracellular medium (Wagner and Dwyer, 2004). Extracellular heme is transported across the cell membrane by a protenacious mechanism, thereby increasing the intracellular concentration (Worthington et al., 2001).

Increasing evidence suggests that intracellular heme acts as a signaling molecule (Padmanaban et al., 1989). For example, heme reversibly binds to selected transcription factors and initiates cellular signal transduction events involving diverse classes of proteins (Zhang and Hach, 1999). Aside from the selected heme-binding transcription factors, how other proteins involved in signal transduction, such as ion channels, are acutely regulated by heme is not well understood. As a first step toward identification of the effectors of intracellular heme, we have recently shown that heme binds to a cytoplasmic domain of a large conductance Ca2+-dependent potassium (Slo1 BK) channels, a key inhibitory component in neuronal and muscle excitability, and drastically reduces the channel activity (Tang et al., 2003). While the detailed mechanism of the heme-mediated inhibition of heterologously expressed Slo1 BK channels is not yet known, the effect is exquisitely potent, with a typical IC50 value of <80 nM. This high sensitivity suggests that heme or a heme-like endogenous substance may be a potent modulator of Slo1 BK channels, especially during “heme stress,” such as that following hemorrhagic strokes (Wagner and Dwyer, 2004). Such injuries are often followed by cerebral vasospasm, in which an inhibition of the Slo1 BK channel function may play a critical role (Aihara et al., 2004; Williams et al., 2004).

Gating of the Slo1 BK channel is allosterically controlled by voltage and divalent cations (e.g., Cox et al., 1997; Rothberg and Magleby, 2000; summarized in Magleby, 2003; Rothberg, 2004). The activation gate of the Slo1 BK channel may be opened by depolarization alone without Ca2+, or Ca2+ alone without depolarization, but under physiological conditions, both Ca2+ and
depolarization work synergistically to activate the channel (Cui et al., 1997). This multidimensional allosteric characteristic allows Slo1 BK channels to participate in multitudes of physiological phenomena, generally exerting a finely tuned negative influence on cellular excitability (Sah, 1996; Vergara et al., 1998).

The physiological versatility of the Slo1 BK channel based on its multidimensional allosteric property complicates studies of its modulation. A given modulator, such as heme, may alter the channel function by affecting any one of the allosteric interactions, and its overall effect may be excitatory or inhibitory, depending on voltage and the divalent ion concentration. Here we investigated how heme inhibits the Slo1 BK channel by separating the influences of voltage and divalent cations. Measurements and simulations of ionic and gating currents show that heme decreases the allosteric coupling of voltage sensor activation and Ca\(^{2+}\) binding to channel opening.

**MATERIALS AND METHODS**

**Channel Expression**

For ionic current measurements, human Slo1 (hSlo1) BK channels (U11058) were expressed transiently in HEK-tsA cells using the FuGene method (Roche) as previously described (Avdonin et al., 2003). The cells were used for electrophysiological experiments typically within 18–36 h of transfection. For gating current measurements, mouse Slo1 (mSlo1) (Horrigan and Aldrich, 2002) was heterologously expressed in HEK-tsA cells.

**Ionic Current Measurements**

Macroscopic and single-channel ionic currents were recorded in the inside-out configuration using an AxoPatch 200B amplifier (Axon Instruments) essentially as previously described (Avdonin et al., 2003). When filled with the solutions described below, the typical input resistance of electrodes for macroscopic tail currents started to decay exponentially with time constants greater than 5 s.

**Solutions**

For ionic current measurements, the extracellular solution contained (in mM) 140 KCl, 2 MgCl\(_2\), 10 HEPES, pH 7.2 with NMDG. The “zero” divalent internal solution contained (in mM) 140 KCl, 11 EGTA, 10 HEPES, pH 7.2 with NMDG. The internal solution with 1 mM Ca\(^{2+}\) contained (in mM) 140 KCl, 10 HEDTA, 2.4 CaCl\(_2\), 10 HEPES, pH 7.2 with NMG. The “saturating” Ca\(^{2+}\)/Mg\(^{2+}\) solution contained (in mM) 140 KCl, 10 MgCl\(_2\), 0.12 CaCl\(_2\), 10 HEPES, pH 7.2 with NMG. Free [Ca\(^{2+}\)] levels were calculated by Patcher’s Power Tools v1.0 (F. Mendez; http://www.mpibpc.gwdg.de/abteilungen/140/software/).

For gating current measurements, the extracellular solution contained (in mM) 130 TEA, 20 HEPES, 2 MgCl\(_2\), 6 HCl, pH 7.2 with methanesulfonic acid (MES). The internal solution contained (in mM) 140 NMDG, 5 EGTA, 20 HEPES, 25 HCl, pH 7.2 with MES.

Heme was applied in the form of iron protoporphyrin IX chloride (hemin; Sigma-Aldrich). Heme was dissolved, diluted with the desired internal solution to 10 μM, and stored at −80°C. For each experiment, a fresh tube was thawed immediately before use and diluted to the final concentrations. Electrophysiology experiments with heme were performed with a minimum of illumination. To change the bath heme concentration, the recording chamber (~150 μl) was washed with 1 ml of a new solution and 4 min was allowed to elapse before measurements. The effects of heme were partially reversible with wash but the recovery time course was variable. The time course observed was generally faster than that reported by Tang et al. (2003). It is not clear what accounts for the difference. To obtain concentration dependence data, each patch was treated with increasing concentrations of heme.

**Data Analysis**

Data were analyzed using PatchMachine (Tang et al., 2001, 2004; Avdonin et al., 2003) and IgorPro (Wavemetrics) running on Mac OS 10. Tail currents at −40 mV following pulses to different voltages were fit with a single exponential to extrapolate instantaneous current amplitudes. The voltage dependence of the instantaneous current size was then fit with a Boltzmann equation to estimate the maximum macroscopic conductance (G\(_{\text{max}}\)) values and the normalized G–V curve. Macroscopic kinetics of Slo1 currents was characterized using a single exponential, and the voltage dependence of the time constant was in turn fitted with an exponential function. To measure open probability and dwell times, the openings were idealized using the hidden Markov method as implemented in PatchMachine (Avdonin et al., 2000). First latency distributions were corrected for the number of channels present (Aldrich et al., 1983), and the fractional number of open channels was obtained from the corrected distribution. The free energy contribution of divalent ions to channel activation was determined using the formulation of Cui and Aldrich (2000) as implemented previously (Tang et al., 2004). Gating current data were analyzed as described in Horrigan and Aldrich (2002).

**Simulation**

Simulated currents were generated and analyzed using PatchMachine and IgorPro as performed with experimental data. The effects of leak and capacitance subtraction, series resistance, and the built-in filter of the amplifier were not considered. The single-channel conductance was set at 250 pS. Simulated single-channel data contained Gaussian noise whose RMS noise level corresponded to that of typical experimental data (1 pA).
The values of the parameters in the HCA model (Horrigan et al., 1999) were estimated in the following manner. The macroscopic tail kinetics and the open probability values estimated from the single-channel data at extreme negative voltages determined the initial values of $\gamma_0$ and $L_0$. The voltage dependence of the deactivation kinetics at negative voltages determined the partial charge associated with $\gamma_{-1}$. The macroscopic activation kinetics at extreme voltages estimated the value of $\delta_i$. Then the values of $D$, $\delta_2$, and $\delta_3$ (see Fig. 1) were adjusted so that the simulated macroscopic G–V, kinetics of the ionic currents, and the single-channel open probability matched the respective average experimental data as judged by eye. The $\gamma_i$ values in this study are smaller than that reported previously for mSlo1 (Horrigan et al., 1999), but it is unclear what accounts for the difference. As in Horrigan et al. (1999), the values of $\gamma_0$ and $\gamma_1$ were assumed to be the same. The values of $\delta_2$ and $\delta_3$ were adjusted to fit the voltage dependence of the kinetics of macroscopic current relaxation with the constraint that the values of $\delta$'s are a monotonic function of voltage. The simulation process did not consider the heme-mediated decrease in the maximum macroscopic conductance.

Simulations of G–V curves based on the model of Horrigan and Aldrich (Horrigan and Aldrich, 2002) involving the full allosteric interactions among the channel gate, voltage sensor, and Ca$^{2+}$ binding were performed using IgorPro. The values of the Ca$^{2+}$-dependent parameters in the model were taken from Horrigan and Aldrich (2002). The G–V curves predicted by the model of Horrigan and Aldrich were not corrected for the data processing procedure.

**Statistics**

Statistical comparisons were made using $t$ test or paired $t$ test as appropriate with DataDesk (Data Description). Statistical significance was assumed at $P \leq 0.05$. Where appropriate, data are presented as mean ± SEM.

**RESULTS**

The seemingly complex gating of the Slo1 channel may be interpreted using the concept that the channel’s gate is allosterically controlled by voltage, and cytoplasmic Ca$^{2+}$ and Mg$^{2+}$ (summarized in Magleby, 2003). In the absence of divalent cations, the Slo1 channel functions essentially as a voltage-dependent channel, and the gating properties are well described by the model of Horrigan et al. (1999) (HCA model) (Fig. 1). The HCA model postulates that the weakly voltage-dependent intrinsic opening/closing processes of the channel gate (vertical transitions) are allosterically regulated by the steeply voltage-dependent movement of the voltage sensor (horizontal transitions). In this study, we used the framework of the HCA model to elucidate the mechanism of the heme action on channel function and performed the initial set of experiments in the virtual absence of divalent cations.

Application of heme (100 nM) to the cytoplasmic side of the channel progressively decreased the Slo1 current size at 160 mV to $\sim 36 \pm 19\%$ ($n = 17$) of the control amplitude with a pseudo second-order rate constant of $2.2 \times 10^5 \pm 1.2 \times 10^4 \text{s}^{-1} \text{M}^{-1}$ ($n = 17$; Fig. 2 A). As reported earlier (Tang et al., 2003), the fractional inhibition of the current and the time course of the development of the current inhibition were notably variable among the patches examined. When heme was applied without repeated depolarization (Fig. 2 B), the fractional inhibition of the current was unaltered ($\sim 21 \pm 14\%$, $n = 3$, $P = 0.17$). The effectiveness of heme in the absence of divalent ions suggested that the underlying mechanism likely involved the intrinsic and/or voltage-dependent gating of the Slo1 channel. Furthermore, the inhibitory efficacy of heme did not require depolarization-mediated channel opening.

The reduction of the current size caused by heme was accompanied by a consistent slowing of the deactivation process at $-100$ mV (Fig. 2 C). The tail currents at $-100$ mV were well described by a single exponential with a time constant of $0.19 \pm 0.08 \text{ms}$ ($n = 24$) in the control condition (Fig. 2 C), and application of heme increased the time constant value by $\sim 81 \pm 13\%$ ($P \leq 0.001$). This slowing of the deactivation kinetics will be described in detail later (see Fig. 8).

**Open-channel i–V**

Despite the dramatic reduction in the macroscopic current described above, the open-channel i–V properties of Slo1 remained largely unaltered. The composite i–V curves for the main conductance state in the control condition and the experimental condition with heme (100 nM; Fig. 3 A, left and right sweeps 1 and 2) were indistinguishable (Fig. 3 B). However, in the presence of hor...
of heme, smaller conductance levels, corresponding to \(\sim 60 \) and \(40\%\) of the full level (Fig. 3 A, sweeps 3 and 4), were more frequently observed. The increased occurrence of these substates was unequivocal, but the extent of the increase was, however, variable from one patch to the next and difficult to quantify. The rectification properties of these substates were not markedly different from those of the main state. While the occurrence of these substates probably contributed to the macroscopic current inhibition by heme, most of the inhibitory effect of heme was likely mediated by changes in the channel gating as described below.

**Slo1 Channels with Heme Open with Depolarization**

Heme (100 nM) inhibited Slo1 currents recorded at a variety of voltages (Fig. 4 A), but even in the presence of heme, greater depolarization elicited larger and more rapidly activating currents (Fig. 4 A and B). Comparison of the normalized macroscopic G–V curves inferred from tail current measurements before and after heme application showed that the channels in the presence of heme retained depolarization-activated gating (Fig. 4 C). The normalized G–V curve obtained in the presence of heme was well described by a simple Boltzmann function but it was noticeably shallower and shifted to the right along the voltage axis.

**A Single Population of Channels**

The kinetics of the tail currents was invariant with the prepulse voltage both without and with heme (Fig. 4...
D). For example, in the presence of heme, the time course of the tail current following a prepulse to 120 mV, where the normalized conductance value was 0.1, was virtually identical to that following 220 mV, where the normalized conductance was 0.75. Similar results were obtained using different prepulse voltages, tail voltages, and heme concentrations (100 nM). The invariance of the tail kinetics from the prepulse voltage suggests that a kinetically single population of channels contributes to the shallower G–V curve observed with heme at 100 nM (Swartz and MacKinnon, 1997); the shallower G–V does not result from activation of unmodified channels at the foot of the G–V and heme-bound channels at more positive voltages.

**Single-channel Gating**

Consistent with the idea that heme alters the gating of Slo1, application of heme (100 nM) drastically decreased the peak open probability (Fig. 5). At 170 mV, heme decreased the peak probability value from 0.62 ± 0.05 to 0.08 ± 0.02 (P ≤ 0.0001). The decrease in open probability was accompanied by a significant decrease in the mean open duration from 2.3 ± 0.3 to 0.70 ± 0.17 ms (P = 0.004).

With large depolarization, the maximum open probability of Slo1 in the control condition saturated at ~0.8–0.9 (200 mV in Fig. 5 C). Qualitatively similar openings were also observed in the presence of heme (100 nM) but greater depolarization (240 mV in Fig. 5 C) was required. The absolute maximum open probability was slightly lower because of the greater occurrence of short flicker closures. Nonetheless, the Slo1 channel in the presence of heme retained the ability to open.

More notably, heme significantly increased the number of blank sweeps (depolarization epochs during which a channel failed to open) (Fig. 5 D).
control condition, ~10% of the pulses to 170 mV (≥50 ms in duration) failed to elicit at least one opening. In the presence of heme (100 nM), up to 60% of the pulses to 170 mV produced no opening. Greater depolarization to 240 mV (≥40 ms), where the normalized macroscopic conductance is 0.85–0.9 (Fig. 4 C), did not appreciably decrease the number of blank sweeps (Fig. 5 D). As shown later, these pulse durations (≥40 ms) were at least 10 times greater than the time constant of the macroscopic current activation (see Fig. 8). The presence of these blank sweeps suggested that heme remained functionally associated with the channels even at extreme positive voltages and contributed to the macroscopic current inhibition.

The blank sweeps are likely the result of a very slow gating component and might be eliminated if the depolarization duration was increased. However, the patch instability associated with repeated extreme depolarization prevented us from testing this idea. In some macroscopic currents, we observed but did not quantify a minor and variable slow component.

Dependence of Macroscopic G–V on Heme Concentration
Heme progressively inhibited the currents recorded at >50 mV in a concentration-dependent manner (Fig. 6 A). Even in the presence of heme, the macroscopic conductance saturated with very large depolarization. This is illustrated in Fig. 6 B where the tail currents recorded at ~40 mV following prepulses to different voltages pulses are shown superimposed. Depolarization to >350 mV did not further increase the tail current, indicating that the macroscopic conductance saturated.

As predicted from the single-channel observation that heme increased the number of blank sweeps, heme decreased macroscopic apparent G_max estimated from the tail current size (Fig. 6 C). The decrease in G_max was notably variable, but a 40–50% decrease was typically observed with heme (≥100 nM) (Fig. 6 C). This reduction is similar to the fractional number of blank sweeps observed in the single-channel data (Fig. 5).

When the G–V curves were normalized to infer the properties of the channels that opened in the presence of heme, we found that the inhibition of the Slo1 current was accompanied by shallower and right-shifted normalized G–Vs (Fig. 6 D). Each normalized G–V curve was characterized by a simple Boltzmann function as a data descriptor function, and the values of the two parameters, V_0.5 and the apparent charge movement (Q_app), are summarized in Fig. 6 (E and F). The rightward shift of G–V became noticeable starting at ~30 nM heme, which produced a 20 mV shift in V_0.5. The shift saturated with 300 nM heme, producing a V_0.5 shift of 80 to 100 mV. Concomitantly with the rightward shift in V_0.5, heme also decreased Q_app so that the G–V curves were markedly shallower. The effect of heme in reducing Q_app saturated around 100 nM, producing a ~40% decrease from 1.2 ± 0.04 e in the control condition to 0.78 ± 0.02 e (100 nM; Fig. 6 F). It is noteworthy that the decrease in Q_app persisted even with the highest concentration of heme tested, supporting the idea that heme-bound channels are characterized by a shallow G–V.

Figure 6. Concentration dependence of the inhibitory effect of heme. (A) Peak I–V curves obtained with different concentrations of heme in a representative patch. (B) Tail currents recorded at ~40 mV following prepulses to different voltages pulses are shown superimposed. Depolarization to >350 mV did not further increase the tail current, indicating that the macroscopic conductance saturated.

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The concentration dependence of the changes in $G_{\text{max}}$, $V_{0.5}$, and $Q_{\text{app}}$ was adequately described with an apparent $K_d$ value of 60 nM, assuming binding of one heme molecule was sufficient to induce the effects (Fig. 6, C, E, and F). However, this assumption is not specifically addressed by the results available.

Gating Currents
The changes in G–V caused by heme (Fig. 6) could be explained in several ways. One possibility is that heme directly interferes with the voltage-sensing charge movements involving S4, which are represented by the horizontal transitions in the HCA model (Fig. 1; Horrigan et al., 1999). If these voltage-dependent steps that normally precede channel opening are impeded by heme, ON gating currents ($I_{\text{gON}}$) may be reduced in size and slower at a given voltage. Representative gating currents elicited by brief 0.5-ms pulses from −80 to 200 mV before and after application of heme (300 nM) are compared in Fig. 7 A. This concentration of heme reduced the peak ionic currents by up to 75% (Fig. 6) but had a much smaller effect on the gating currents. The peak $I_{\text{gON}}$ and $I_{\text{gOFF}}$ were reduced by <15%, and their kinetics were not appreciably altered by heme.

Heme (300 nM) only slightly altered the voltage dependence of the Slo1 gating charge movement ($Q_{C-V}$) (Fig. 7 B). $Q_{C}$ was measured by fitting the first 60–100 μs of the $I_{\text{gON}}$ decay with an exponential function and determining the area under the fit (Horrigan and Aldrich, 1999). Slo1 gating currents are much faster than ionic currents, reflecting that voltage sensors are largely equilibrated before channels open. Therefore $Q_{C}$ indicates the steady-state properties of voltage sensor activation while channels are closed, the top row of horizontal transitions in the HCA model (Fig. 1), reflecting the voltage sensor charge ($z_{J}$) and equilibrium constant ($J$) associated with these transitions.

In the experiment shown, Boltzmann fits to the $Q_{C-V}$ curves indicated that the gating charge half-activation voltage ($Q_{C-V_{0.5}}$) was shifted to more positive voltages by ∼20 mV. On average, $Q_{C-V_{0.5}}$ increased by only +22 ± 3 mV ($n = 4$), which is markedly less than the 80–100 mV shift in $V_{0.5}$ (see Fig. 6). The total charge movement approximated by the amplitude of the $Q_{C-V}$ fit ($Q_{C_{\text{max}}}$) also decreased slightly (Fig. 7 B). However, this decrease of 7 ± 2% ($n = 4$) was small compared with the effect of heme on $G_{\text{max}}$ (Fig. 6 C). The $Q_{C-V}$ curves in the presence and absence of heme were well fit by Boltzmann functions with identical voltage sensor charge ($z_{J} = 0.58 e$) (Fig. 7, B and C). The value of $z_{J}$ was fixed to the mean value determined from many control experiments (Horrigan and Aldrich, 1999) because $z_{J}$ determined from individual experiments is very sensitive to scatter in the data and, if allowed to vary, would obscure small changes in $Q_{C-V_{0.5}}$ and $Q_{C_{\text{max}}}$. It is clear from Fig. 7 (B and C) that any change in $z_{J}$, if any, must be small and cannot account for the reduced steepness of the G–V. If the ∼40% decrease in $Q_{C-V}$ resulted from a 40% reduction in voltage sensor charge, then a decrease in the limiting logarithmic slope of $Q_{C-V}$, which is directly proportional to $z_{J}$, would be clearly evident (Fig. 7 C, dashed trace). Similarly, the failure to observe a large decrease in $Q_{C_{\text{max}}}$ (Fig. 7 B) is inconsistent with a large decrease in $z_{J}$.

The effects of heme on $Q_{C}$ were small and may even be overestimated by our experiments. Small slow decreases in $Q_{C_{\text{max}}}$ and/or positive shifts in $Q_{C-V_{0.5}}$ were sometimes observed in the absence of heme. Because heme acts slowly and is poorly reversible, we could not rule out that such spontaneous effects contribute to the observed changes in $Q_{C-V}$ (e.g., Fig. 7 B). However, heme did account for at least some of the $Q_{C-V}$ changes because they were partially reversed by heme washout (unpublished data).
Heme had little effect on gating currents evoked by brief voltage pulses (Fig. 7 A) but it had noticeable effects on IgOFF following prolonged (5–20 ms) depolarization (Fig. 7 D), consistent with the ability of heme to inhibit channel opening. IgOFF was slowed following pulses that open Slo1 channels, reflecting transitions among the bottom row of horizontal transitions in the HCA model (Fig. 1). Consequently, IgOFF decays exponentially following a brief pulse, but an additional slow component, whose amplitude reflects the fraction of open channels, appears following prolonged depolarization (Horrigan and Aldrich, 1999). The slow component of IgOFF (Fig. 7 D, dashed lines) was reduced by >50% by heme (300 nM), indicating a marked decrease in open probability. Thus, the small effects of heme on Q_0 do not represent a failure of heme to effectively inhibit channel gating under the conditions used to study gating currents. These results taken together suggest that heme did not markedly impede the voltage sensor function when channels are closed.

Heme Slows Macroscopic Activation and Deactivation

Heme altered the kinetics of Slo1 ionic currents in a voltage-dependent manner (Fig. 8 A). The current relaxation time courses were slower after heme application at extreme negative and positive voltages, but at intermediate voltages they were faster (Fig. 8 A).

The time course of Slo1 current relaxation at each voltage was adequately described by a single exponential and the voltage dependence of the current relaxation time constant (τ-V) is illustrated in Fig. 8 B. Consistent with the previous finding of Horrigan et al. (1999), τ-V in the control group had three exponential components. Each component was characterized by the equation $\tau = \tau_0 e^{(qF/RT)}$ where $\tau_0$ represents the time constant value at 0 mV and q represents the partial charge movement. The component at extreme negative voltages likely reflects the preferred closing transition O\textsubscript{0} to C\textsubscript{0} and that at extreme positive voltages is part of the HCA model (Fig. 1 B). The three exponential components were also clearly observed in the presence of heme (Fig. 8 B). Heme markedly slowed the deactivation process at extreme negative voltages by increasing $\tau_0$ and decreasing q (Fig. 8 C), suggesting that heme slows the O\textsubscript{0} to C\textsubscript{0} transition. Much smaller changes were observed with the activation process at extreme positive voltages (Fig. 8 D).
Heme Increases the Open Probability at Negative Voltages

Heme reduced the steepness of the Slo1 G–V curve (see Fig. 6). If the reduced steepness is maintained at more negative voltages, the G–V curves in the control and experimental conditions may cross over so that the channel open probability may be in fact greater after heme treatment at the negative voltages. A similar prediction about the enhanced open probability with heme can be made from the observation that heme slowed the deactivation kinetics at very negative voltages (Fig. 8). Representative openings recorded before and after application of heme (100 nM) at −50, −100, and −150 mV, where the voltage sensor–mediated activation of the channel should be negligible, are shown in Fig. 9. In contrast with the results obtained with heme, the values of D and L0 shift the G–V to more positive voltages (Fig. 10). The increase in the open probability was associated with a significant increase in the mean open duration by ~50% (P < 0.01; Fig. 9 C) at each voltage. The mean open durations observed at these voltages were smaller than the time constants of the macroscopic tail currents (Fig. 8).

Simulations with the HCA Model

The gating properties of Slo1 in the absence of divalent cations are well simulated by the HCA model (Horrigan et al., 1999). To account for the heme action on Slo1 using the HCA model, we considered the following three lines of observations critical. First, heme decreases the steepness of the G–V but does not markedly affect IgON or Q–V. Second, heme increases the open probability at very negative voltages. Third, heme slows the kinetics of activation at extreme positive voltages and deactivation at extreme negative voltages. We did not consider the decreased Gmax (Fig. 6 C) caused by blank sweeps (Fig. 5) or the small shift in Q–V0.5 (Fig. 7) in our analysis.

A shallower and right-shifted G–V could be simulated in multiple ways using the HCA model. Changes in the steeply voltage-dependent rate constants α and β (the equilibrium constant J) in the HCA model could induce changes in G–V similar to those experimentally observed. However, because the kinetic and steady-state properties of gating currents were not greatly affected by heme (Fig. 7), we deemed that the changes in α and β were unlikely to underlie the heme effect. Changes in the equilibrium constant between the closed state C0 and the open state O0, L0 in the HCA formulation, could also alter the steepness of G–V. A decrease in L0 caused by greater γh and/or smaller δ0 shifts the G–V position and decreases the steepness as found with heme. However, this G–V modification also decreased the open probability at negative voltages, exactly the opposite of what was observed. A decrease in the allosteric factor D in the HCA model decreases the G–V steepness, but this change does not appreciably increase open probability at negative voltages as experimentally observed. Thus, alterations in D or L0 alone fail to account for the experimental findings.

However, a decrease in D and a concomitant increase in L0 together do explain the three key observations listed above. A 73% decrease in D leads to a 45% decrease in Qapp and shifts the G–V to more positive voltages (Fig. 10). A 10-fold increase in L0 accounts for the enhanced open probability, the slower deactivation kinetics, and the longer mean open time at negative voltages (Fig. 10). In addition, the partial charge movement associated with γh is reduced by 50% to account for the reduced voltage dependence of the deactivation time course at very negative voltages. The essential features of the heme action were well simulated using the HCA model by these changes in the following parameters: the allosteric factor D becomes smaller and the equilibrium constant L0 becomes greater (Fig. 10).

To assess how well other values of D and L0 simulate the results obtained with heme, the values of D and L0

Figure 9.

Heme increases the open probability at negative voltages. (A) Representative channel openings at −50, −100, and −150 mV in the control condition and after application of heme (100 nM). Downward deflections represent opening transitions. Heme decreased the current from the same patch at 160 mV (right). The current after heme application is indicated by • (thick sweep). This patch contained ~150 channels assuming the open probability value of 0.5 at 160 mV and the unitary amplitude of 31 pA (see Fig. 3). (B) Voltage dependence of open probability. Open circles, control; filled circles, after heme (100 nM). Typically 60 s data were analyzed. n = 4–6 at each voltage. (C) Voltage dependence of mean open duration. The symbols are as in B. n = 4–6.
The values of $V_{0.5}$ and $Q_{app}$ for the simulated control and heme groups were 154 mV/1.3 and 16 Heme Action on Slo1 BK Channels

Heme Is Also Effective at Saturating Levels of Ca$^{2+}$ and Mg$^{2+}$

The virtual absence of Ca$^{2+}$ and Mg$^{2+}$ allowed us to study the effect of heme on the voltage-dependent activation pathway of the Slo1 BK channel in relative isolation. Alternatively, the voltage-dependent gating can be functionally isolated and studied at saturating levels of Ca$^{2+}$ and Mg$^{2+}$. In the presence of 120 μM [Ca$^{2+}$] and 10 mM [Mg$^{2+}$], which are expected to saturate the high- and low-affinity binding sites for divalent ions (Cox et al., 1997; Zhang et al., 2001; Zeng et al., 2005), heme effectively inhibited the currents (Fig. 11 A). As observed in the absence of divalent ions, the tail currents observed with heme saturated following large depolarization (Fig. 11 B). Heme (300 nM) noticeably altered the G–V curve (Fig. 11 C); the $V_{0.5}$ value shifted by 129 ± 9 mV and the $Q_{app}$ value decreased by 40 ± 5.6% to 0.64 e$^-$. The normalized conductance values at 0 mV (Fig. 11 D) indicated that the channels in the presence of heme (300 nM) were less sensitive to high concentrations of divalent cations; increasing [Ca$^{2+}$] and [Mg$^{2+}$] to 120 μM and 10 mM, respectively, produced a much smaller increase in the macroscopic conductance currents before and after (thick sweeps, denoted by •) heme application. Simulated assuming 20,000 channels. The scale bars represent 700 nA for the ionic currents and 100 pA for the gating currents. (F) Estimated $V_{0.5}$ values as a function of D. The ionic currents were simulated and analyzed using different values of D and $L_0$. The remaining parameter values are as in A. The thick curve describes how $V_{0.5}$ changes with D using $L_0 = 5 \times 10^{-4}$. The top thin curve describes $V_{0.5}$ changes with $L_0 = 7 \times 10^{-4}$ and the bottom thin curve describes those with $L_0 = 3 \times 10^{-4}$. The area between the two thin curves is shaded gray. The dotted lines indicate the values of D and $V_{0.5}$ as presented in A. The gray horizontal rectangle area represents the standard error associated with the experimentally estimated value of $V_{0.5}$ with 300 nM heme. (G) Estimated $Q_{app}$ values as a function of D. The thick curve describes how $Q_{app}$ changes with D using $L_0 = 5 \times 10^{-4}$. The top thin curve describes $Q_{app}$ changes with $L_0 = 7 \times 10^{-4}$, and the bottom thin curve describes those with $L_0 = 3 \times 10^{-4}$. The dotted lines indicate the values of D and $Q_{app}$ as presented in A. The gray horizontal rectangle area represents the standard error associated with the experimentally estimated value of $Q_{app}$ with 300 nM heme.

Figure 10. Simulation of the heme action in the absence of divalent ions using the HCA model. (A) The model parameters changed by heme application. The parameter values were adjusted to simulate the average results without heme and with heme (300 nM). As suggested by the results of the gating current measurements, the following parameters were kept constant: $\alpha = 1500$ s$^{-1}$, $\beta = 35370$ s$^{-1}$, $z_o = 0.275$ e$^-$ and $z_h = -0.275$ e$^-$. (B) G–V curves simulated by the model with the parameters shown in A. The values of $V_{0.5}$ and $Q_{app}$ for the simulated control and heme groups were 154 mV/1.3 and 231 mV/0.71 e$^-$. (C) Simulated single-channel currents at −100 mV. The open probability values for the simulated control and heme groups at 0.24 and 0.45 ms, respectively. (D) Voltage dependence of the simulated macroscopic current relaxation. In A, B, and C, the currents were simulated assuming 250 channels. (E) Simulated ionic and gating currents elicited by depolarization to 200 mV from −100 mV. The top sweep shows ionic currents and the bottom sweep shows gating.
when heme was present. Similar inhibitory effects of heme were observed at an intermediate concentration (1 μM) of Ca^{2+} (unpublished data).

With certain assumptions, comparison of the G–V curves recorded in 0 [Ca^{2+}]/0 [Mg^{2+}] and 120 μM [Ca^{2+}]/10 mM [Mg^{2+}] allows an estimation of the free energy contribution of the divalent ions to the channel activation (Cui and Aldrich, 2000). Such comparison indicates that the presence of heme (300 nM) significantly altered the free energy contribution of the saturating levels of Ca^{2+} and Mg^{2+} (ΔG_{Ca/Mg}) to the channel opening (P ≤ 0.0001); the mean ΔG_{Ca/Mg} value decreased by ~14 ± 1.0 kJ/mol or 51 ± 2.5%, confirming that the Slo1 channel was less sensitive to divalent ions when heme is bound.

Simulations of G-V Curves at High Concentrations of Divalent Cations

The HCA model can be extended to account for the channel behavior in the presence of Ca^{2+} (HA model) (Horrigan and Aldrich, 2002). To simulate the effect of heme on the Slo1 G–V curve at the saturating levels of Ca^{2+} and Mg^{2+} (Fig. 11), the values of the HCA parameters obtained in the absence of divalent ions were applied to the HA model. Because Mg^{2+} was not included in the HA model and for the sake of simplicity, the effect of Mg^{2+} was not considered here. The remaining Ca^{2+}-dependent parameters in the HA model, the Ca^{2+} binding affinity, the allosteric coupling strength between the channel gate and Ca^{2+} binding (C), and the allosteric coupling strength between the voltage sensor and Ca^{2+} binding (E), were initially assumed to be the same in the control and heme conditions, and the values were taken from Horrigan and Aldrich (2002).

With this assumption, the HA model predicts that heme increases the channel open probability at [Ca^{2+}] = 120 μM (Fig. 12 A), exactly the opposite of what was experimentally observed (Fig. 11). The discrepancy between the experimental and simulation results suggests that heme may alter other aspects of channel gating in addition to D and I_{eq}. Consistent with this possibility, we found that simple decreases in the allosteric coupling strength between the channel gate and Ca^{2+} binding C and that between the voltage sensor and Ca^{2+} binding E by the same fraction as used for D (73%) described the effect of heme on the Slo1 G–V curve at high [Ca^{2+}], at least qualitatively (Fig. 12 B). The simulated steady-state G–V curve in the presence of heme is shifted markedly toward more positive voltages with low as well as high [Ca^{2+}] (Fig. 12), and this is consistent with the experimental results (Fig. 11). It should be noted that the G–V shift in high [Ca^{2+}] is strongly influenced by the allosteric factor C but less so by E. Therefore, it is necessary that C be reduced to reproduce the results in Fig. 11. It is possible that E is changed to a lesser extent or even unchanged. However, the experiments necessary to measure E are outside the scope of this study. Similarly, we did not determine whether heme altered the apparent affinity of Ca^{2+} binding. Such effects, if they occur, are unlikely to account for the reduced response of heme-bound channels to high concentrations of divalent cations in Fig. 11 because the [Ca^{2+}] used (120 μM) was in excess of the normal saturating concentration and channels are still sensitive to low (1 μM) Ca^{2+} in the presence of heme (not depicted). Thus the effects of heme on the Slo1 channel in the absence of divalent ions is consistent with an increase in the closed–open equilibrium.

**Figure 11.** Heme inhibits Slo1 currents in the presence of saturating levels of Ca^{2+} (120 μM) and Mg^{2+} (10 mM). (A) Representative currents at three different voltages before and after heme (100 nM) application with high Ca^{2+}/Mg^{2+}. In each set, the current recorded with heme (300 nM) is denoted by * (thick sweep). No leak or capacitative current subtraction. (B) Tail currents recorded at −160 mV saturate in size following pulses to −150 to 130 mV in 10 mV increments in the presence of 300 nM heme. The pulse to −150 mV was 75 ms in duration, and the duration decreased by 2 ms for each 10 mV increment. (C) Average G–V curves before (open symbols) and after heme (100 nM) application with high Ca^{2+}/Mg^{2+}. The estimated values of V_{0.5} and Q_{app} in the control and heme groups with high Ca^{2+}/Mg^{2+} were 80 ± 1.8 mV/1.1 ± 0.08 e and 49 ± 9.1 mV/0.64 ± 0.02 e, respectively. Without Ca^{2+}/Mg^{2+}, the parameter values were 160 ± 4.3 mV/1.2 ± 0.06 e and 229 ± 6.0 mV/0.72 ± 0.01 e, respectively. (D) Changes in normalized conductance caused by saturating levels of Ca^{2+} and Mg^{2+}. Average normalized conductance values at 0 mV estimated with no Ca^{2+} and Mg^{2+} and with high [Ca^{2+}] and [Mg^{2+}] before and after heme application (300 nM) are compared. Error bars are smaller than the symbols. n = 5 to 8.
constant $L_0$ and a decrease in the allosteric coupling factor between the channel gate and the voltage sensor $D$, and the effects at saturating levels of divalent ions require additional decreases in the $Ca^{2+}/Mg^{2+}$-dependent allosteric coupling factors $C$ and possibly $E$.

**DISCUSSION**

Heme is now emerging as an important intracellular signaling molecule; genomic effects of heme have been reported in several systems and nongenomic effects of heme are beginning to be elucidated. One such example is the acute modulatory effect of heme on the Slo1 BK channel (Tang et al., 2003). Heme reduces ionic currents at depolarized voltages through Slo1 channels in two ways. First, heme decreases $G_{\text{max}}$ so that effectively 40–50% less channels open. Second, the channels that open in the presence of heme do so with different characteristics. The $G$–$V$ curve of the Slo1 channel in the presence of heme is drastically shifted to more positive voltages and less steep. In contrast, Slo1 gating currents remain largely unaffected. At saturating concentrations of divalent cations, the potent efficacy of heme persists.

**Interpretations Using the HCA and HA Models**

While the overall effect of heme is largely inhibitory at the voltages where the open probability is appreciable, heme exerts an excitatory effect at more negative voltages; the channel open probability is greater when heme is present. Understanding the complex modulatory effects of heme may be facilitated by using well-developed allosteric models of Slo1 channel gating that describe the channel’s response to voltage and divalent cations. Simulations of the ionic currents obtained in the absence of divalent ions using the HCA model (Horrigan et al., 1999) suggest that heme markedly decreases the strength of allosteric coupling between the channel gate and the voltage sensor ($D$) and shifts the equilibrium between the closed and open states in the absence of voltage sensor–mediated activation ($L_0$) to the open state. The changes in these parameters in conjunction with a decrease in the partial charge movement associated with $\gamma_{\text{on}}$ reproduce the salient characteristics of the properties of the Slo1 channels that open in the presence of heme.

The proposed mechanism of heme action based on the HCA model does not consider the decrease in $G_{\text{max}}$ (Fig. 6) caused by the blank sweeps in the single-channel data (Fig. 5). Even at very positive voltages with pulses much greater in duration than the macroscopic activation time constant, these blank sweeps are readily observed. They are likely caused by a very slow gating component operating independently of the voltage sensor movement because the total charge movement is only marginally decreased by heme (Fig. 7). The exact nature of this slow gating process and how it relates to the transitions described in the HCA model is not clear. What is certain is that these transitions are not created de novo by heme but their occurrence is drastically increased by heme because a small number of apparent blank sweeps are indeed observed without heme. The channels that do open in the presence of heme kinetically function as a single population, and the contributions from the blanks sweeps and the decrease in $G_{\text{max}}$ to our modeling and simulation were likely negligible.

The modulatory effects of heme on the Slo1 channel persist at saturating levels of $Ca^{2+}$ and $Mg^{2+}$. $Ca^{2+}$-dependent gating of the Slo1 channel is successfully described by the HA model (Horrigan and Aldrich, 2002), which builds on the HCA model and incorporates $Ca^{2+}$ as another allosteric dimension. The HA model includes allosteric interactions among the channel gate, voltage sensor, and $Ca^{2+}$-binding site, and the coupling strengths are described by the parameters $C$, $D$, and $E$. The changes in $L_0$ and $D$ described for the low-$Ca^{2+}$ condition when incorporated into the HA model do not adequately reproduce the results with high concentrations of divalent cations, suggesting that heme may modulate other functional characteristics of the channel. We find that an across-the-board reduction in $C$, $D$, and $E$ by $73\%$ and increases $L_0$ by 10-fold.
lent cations. The experiments necessary to rigorously estimate C and E are out of the scope of this study, but the possibility that heme may act as a common regulator of allosteric coupling in Slo1 gating suggests that heme may exert its action where the influences of the voltage sensor and divalent cation binding sites converge.

Molecular Mechanism of Heme Action

The biophysical interpretations of heme action on the Slo1 channel using the HCA and HA models may be given a molecular and structural connotation using the high-resolution structure of the prokaryotic MthK channel (Jiang et al., 2002) and the mechanical spring model of Slo1 gating (Niu et al., 2004). The Ca^{2+}-activated open structure of MthK suggests that the cytoplasmic RCK1 and RCK2 domains in each of the four Slo1 subunits in a channel complex dimerize and that the four RCK dimers in turn form a gating ring structure (Jiang et al., 2002). The expansion and constriction of the gating ring, caused by changes in the relative positions of the four RCK dimers, are envisioned to contribute to channel opening and closing by exerting force on the activation gate through the S6-RCK1 linker (Jiang et al., 2002). The idea that the S6-RCK1 linker exerts mechanical tension on the gate is supported by the observation that open probability, whether Ca^{2+} is absent or present, is drastically influenced by changes in the linker length (Niu et al., 2004). Based on this observation, Niu et al. proposed a mechanical spring model of Slo1 BK channel activation whereby each subunit component of the channel activation gate, likely the cytoplasmic end of S6, is coupled to the cytoplasmic gating ring and also to S4 via two separate spring-like connectors (Armstrong, 2003; Niu et al., 2004). The two separate linkages are required to account for the energetic additivity of voltage and Ca^{2+} in channel activation (Cui and Aldrich, 2000). The highly stylized diagrams in Fig. 13 A capture the essence of the mechanical spring model of Niu et al. (2004) in the absence of Ca^{2+} while incorporating an additional interaction between the voltage sensor and the gating ring composed of the RCK1/RCK2 domains. The presence of voltage sensor/gating ring interaction is consistent with the findings that mutations in S4 and the S4–S5 linker disrupt Mg^{2+}-dependent activation of the channel (Hu et al., 2005) involving the cytoplasmic RCK1 domain (Shi and Cui, 2001; Zhang et al., 2001) and that Mg^{2+} acts to enhance coupling between voltage sensor activation and channel opening (Horrigan, 2005). This model will be used to explain how heme binding to the cytosolic gating ring could influence voltage-dependent gating, and it draws on the idea that a portion of the coupling between voltage sensor and gate may be mediated by interaction between the voltage sensor and gating ring (Hu et al., 2003; Horrigan, 2005).

In the absence of heme (Fig. 13 A), the pore and gating ring are shown in a closed/constricted (C) or an open/expanded (O) conformation while the charged S4 voltage sensor is depicted in a resting (R) or an acti-
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The Slo1 channel is influenced by heme over a broad range of divalent cation concentrations. Therefore, heme is poised to act as a regulator of the channel function under a variety of physiological conditions. Information on dynamic changes in intracellular heme concentrations is not available but it is often speculated that the concentration may increase appreciably following hemorrhaging strokes (Wagner and Dwyer, 2004). Intracellular heme then may bind to Slo1 BK channels, modulating those physiological processes dependent on BK channels, such as vasorelaxation (Patterson et al., 2002) and oxygen sensing (Williams et al., 2004). Potential inhibition of BK channels by heme may account for the cerebral vasospasm frequently observed following hemorrhaging strokes (Aihara et al., 2004). However, the possibility that heme may play a regulatory or compensatory role during these vascular accidents cannot be excluded because heme actually enhances the channel activity at more hyperpolarized, and potentially more physiological, voltages. Future studies using native BK channels should provide further insights.

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