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, cytogenes 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 cyto- plasmic 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; Rothenberg and Magleby, 2000; summarized in Magleby, 2003; Rothenberg, 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
scopic capacitative and leak currents were subtracted using a
were excluded from analysis. Unless otherwise stated, macro-
within 60–70
cially, macroscopic tail currents started to decay exponentially
electronically compensated in the macroscopic recordings. Typi-
was 1.5 M
al., 2001; Avdonin et al., 2003). When filled with the solutions de-
(Axon Instruments) essentially as previously described (Tang et
(2002) was heterologously expressed in HEK-tsA cells.
channels were excised into nominally K
channels were expressed transiently in HEK-tsA cells using the FuGene method (Roche) as previously described (Avdonin
nels (U11058) were expressed transiently in HEK-tsA cells using
for ionic current measurements, human Slo1 (hSlo1) BK chan-
that heme decreases the allosteric coupling of voltage
characteristic allows Slo1 BK channels to participate in
depolarization work synergistically to activate the chan-
channel (Cui et al., 1997). This multidimensional allosteric characteris-
ting isotonic TEA in the extracellular solution to block any resid-
ul ionic currents. Voltage commands were filtered at 20 kHz to
limit capacitive transients. Currents were filtered at 20 kHz, sam-
presented 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
subsequent measurements, mouse Slo1 (mSlo1) (Horrigan and Aldrich,
characterizes studies of its modulation. A given modulator, such as
heme decreases the allosteric coupling of voltage sensor activation and Ca2+
channel opening.

MATERIALS AND METHODS

Channel Expression

For ionic current measurements, human Slo1 (hSlo1) BK chan-
previously described (Avdonin et al., 2003). The cells were used for electrophysiological exper-
typically within 18–36 h of transfection. For gating current
measurements, mouse Slo1 (mSlo1) (Horrigan and Aldrich,
mSlo1) (Horrigan and Aldrich,
Gating currents were measured as previously described (Horrigan
Gating 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 (Tang et
was heterologously expressed in HEK-tsA cells. The physiological versatility of the Slo1 BK channel
based on its multidimensional allosteric property compli-
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results. The voltage dependence of the instantaneous current was then fit with a Boltzmann equation to esti-
to channel activation (G
max
). First latency distributions were corrected for the number of chan-
channels present (Aldrich et al., 1985), and the fractional number of blank sweeps is obtained from the corrected distribution. The free energy contribution of divalent ions to channel activation
was determined using the formulation of Cai 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 PatchMa-
and IgorPro as performed with experimental data. The ef-
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).

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 instanta-
neous current amplitudes. The voltage dependence of the instant-
anous current size was then fit with a Boltzmann equation to es-
timate the maximum macroscopic conductance (G
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) values and the normalized G–V curve. Macroscopic kinetics of Slo1 cur-
rents was characterized using a single exponential, and the volt-
age 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
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channel data contained Gaussian noise whose RMS noise level corresponded to that of typical experimental data (1 pA).
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 (∼21 ± 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 ± 0.08 ms (n = 24) in the control condition (Fig. 2 C), and application of heme increased the time constant value by ∼81 ± 13% (P ≤ 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 heme, the fractional inhibition of the current was unaltered (∼21 ± 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 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 γ0 and L0. The voltage dependence of the deactivation kinetics at negative voltages determined the partial charge associated with γ0. The macroscopic activation kinetics at extreme voltages estimated the value of Δγi. Then the values of D, Δδ, and δi (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 γ0 value in this study is 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 γ0 and γ1 were assumed to be the same. The values of δ0 and δ1 were adjusted to fit the voltage dependence of the kinetics of macroscopic current relaxation with the constraint that the values of δ’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 Ca2+ binding were performed using IgorPro. The values of the Ca2+-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 ≤ 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 Ca2+ and Mg2+ (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 ∼36 ± 19% (n = 17) of the control amplitude with a pseudo second-order rate constant of 2.2 × 105 ± 1.2 × 104 s−1 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

![Figure 1](image-url)

**Figure 1.** Allosteric gating model of Slo1 in the absence of divalent ions proposed by Horrigan et al. (1999). (A) HCA model with individual rate constants. (B) HCA model using equilibrium constants. The allosteric factor D is defined as D = k, the weakly voltage-dependent open–closed equilibrium constant is defined as L0 = k0 = k0 at 0 mV, and the strongly voltage-dependent equilibrium constant J is defined as J = α/β at 0 mV. For details, see Horrigan et al. (1999). The most likely activation pathway on moderate to large depolarization is C0-C1-C2-C3-C4-O4 and the most likely deactivation pathway on repolarization to a very negative voltage is O4-O3-O2-O1-C0.
of heme, smaller conductance levels, corresponding to \(\sim 60\) and \(\sim 40\%\) 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 \( \frac{1}{H_{11601}} 0.1 \), was virtually identical to that following 220 mV, where the normalized conductance was \( \frac{1}{H_{11601}} 0.75 \). Similar results were obtained using different prepulse voltages, tail voltages, and heme concentrations (\( \frac{1}{H_{11350}} 100 \text{nM} \)). 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 \( \frac{1}{H_{11350}} 100 \text{nM} \) (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 (\( P \leq 0.0001 \)). 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 \( \sim 0.8–0.9 \) (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). In the con-
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 they 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 Gmax estimated from the tail current size (Fig. 6 C). The decrease in Gmax 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, V0.5 and the apparent charge movement (Qapp), are summarized in Fig. 6 (E and F). The rightward shift of G–V became noticeable at ~30 nM heme, which produced a 20 mV shift in V0.5. The shift saturated with 300 nM heme, producing a V0.5 shift of 80 to 100 mV. Concomitantly with the rightward shift in V0.5, heme also decreased Qapp so that the G–V curves were markedly shallower. The effect of heme in

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 saturate in size following pulses to 200 to 400 mV in 10 mV increments in the presence of 1 μM heme. The pulse to 200 mV was 50 ms in duration, and the duration decreased by 2 ms for each 10 mV increment so that the rightmost tail current was recorded following depolarization to 200 mV and the leftmost current was recorded following depolarization to 400 mV. (C) Changes in Gmax caused by different concentrations of heme. To limit prolonged extreme depolarization, the pulse duration decreased by 2 ms for every 10 mV depolarization starting from a 75 ms pulse to 0 mV. (C) Normalized macroscopic G–V curves obtained from the currents shown in A. The symbols are as in A. (D) Changes in V0.5 by different concentrations of heme. The mean V0.5 value in the control condition was 155 ± 2 mV. (E) Fractional changes in Qapp by different concentrations of heme. The mean Qapp value in the control condition was 1.2 ± 0.04 e. Smooth curves in B, C, and D reflect Kd/60 nM assuming a single binding site. n = 3–20.

reducing Qapp 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 Qapp persisted even with the highest concentration of heme tested, supporting the idea that heme-bound channels are characterized by a shallow G–V.
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 ms 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 ($Q_cV_{0.5}$) shifted to more positive voltages by $\sim 20$ mV. On average, $Q_cV_{0.5}$ increased by only $+22 \pm 3$ 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 ($Q_{cmax}$) also decreased slightly (Fig. 7 B). However, this decrease of $7 \pm 2\%$ ($n = 4$) 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 $Q_cV_{0.5}$ and $Q_{cmax}$.

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 $\sim 40\%$ decrease in $Q_{\text{app}}$ 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 $Q_{\text{cmax}}$ (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 $Q_{\text{cmax}}$ and/or positive shifts in $Q_cV_{0.5}$ 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).

Figure 7. Heme does not markedly alter Slo1 gating currents. (A) Representative gating currents elicited by depolarization from $-80$ mV to 200 mV before (thin) and after (thick sweep) heme (300 nM). Note that the stimulation duration used is too short to noticeably activate ionic currents. (B) $Q_c$–$V$ is only slightly affected by heme. Smooth traces are fits to Boltzmann functions with voltage sensor charge $z_J = 0.58 e$ (control, open symbol, $Q_cV_{0.5} = 207.5$ mV; $Q_{cmax} = 13.4$ nC; heme, filled symbol, $Q_cV_{0.5} = 228.0$ mV; $Q_{cmax} = 12.8$ nC). (C) $Q_cV$ from B is plotted on a semilog scale to show that the limiting slope is not appreciably altered and is inconsistent with a 40% decrease in $z_J$ to 0.35 (dashed trace) analogous to the 40% decrease in G–V slope produced by heme. (D) The slow component of $I_{\text{gOFF}}$ (dashed lines) following prolonged depolarization to +200 mV is reduced by heme, consistent with a marked decrease in open probability. Traces represent the average $I_{\text{gOFF}}$ following pulses of 5, 10, 15, and 20 ms duration. Individual records were indistinguishable for pulses of 5 ms or longer. A, B, C, and D were all from the same patch.
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 \( \geq 50\% \) by heme (300 nM), indicating a marked decrease in open probability. Thus, the small effects of heme on \( Q_c \) 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 (\( \tau \)) is illustrated in Fig. 8 B. Consistent with the previous finding of Horrigan et al. (1999), \( \tau \) in the control group had three exponential components. Each component was characterized by the equation \( \tau(V) = \tau_0 e^{qF/RT} \) 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 \( O_0 \) to \( C_0 \) and that at extreme positive voltages in part reflects the preferred opening transition \( C_4 \) to \( O_4 \) in 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 \( O_0 \) to \( C_0 \) transition. Much smaller changes were observed with the activation process at extreme positive voltages (Fig. 8 D).

Figure 8. Changes in macroscopic Slo1 kinetics caused by heme. (A) Representative Slo1 currents at \(-220, 100, \) and 250 mV before and after heme application. In each set, the current recorded with heme (100 nM) is denoted by \( \bullet \) (thick sweep). (B) Voltage dependence of the time constant of current relaxation at different concentrations of heme. Currents were fitted with single exponentials, and the time constant values are plotted as a function of voltage. (C) Concentration dependence of deactivation kinetics at \( \leq -100 \) mV. The estimated deactivation time constant values at \( \leq -100 \) mV were fitted with an exponential, and the extrapolated values at 0 mV (\( \tau_0 \)) normalized to the control values are plotted as a function of heme concentration (left). The fractional changes in the equivalent charge (\( q \)) movement associated with the deactivation process are also shown (right). The mean values of \( \tau_0 \) and \( q \) in the control condition were \( 0.35 \pm 0.01 \) ms and \( 0.14 \pm 0.01 \) e. (D) Concentration dependence of activation kinetics at \( \geq 210 \) mV. The estimated activation time constant values at \( \geq 210 \) mV were fitted with an exponential and the extrapolated values at 0 mV (\( \tau_0 \)) normalized to the respective control values are plotted as a function of the heme concentration (left). The fractional changes in the equivalent charge movement associated with the activation process are also shown (right). The mean values of \( \tau_0 \) and \( q \) in the control condition were \( 19.0 \pm 3.8 \) ms and \( 0.24 \pm 0.01 \) e. \( n = 3–21 \).
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 at more positive voltages, heme strikingly increased the open probability, typically by 10–20-fold without a significant change in the voltage dependence (Fig. 9 B). 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 Qapp−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 γ and/or smaller δ, 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 γ 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.
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 \(\mu\)M [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 \(\mu\)M 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.
when heme was present. Similar inhibitory effects of heme were observed at an intermediate concentration (1 μM) of Ca2+ (unpublished data).

With certain assumptions, comparison of the G–V curves recorded in 0 [Ca2+] /0 [Mg2+] and 120 μM [Ca2+] /10 mM [Mg2+] 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 Ca2+ and Mg2+ (∆G_{Ca/Mg}) to the channel opening (P ≤ 0.0001); the mean ∆G_{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 Ca2+ (HA model) (Horrigan and Aldrich, 2002). To simulate the effect of heme on the Slo1 G–V curve at the saturating levels of Ca2+ and Mg2+ (Fig. 11), the values of the HCA parameters obtained in the absence of divalent ions were applied to the HA model. Because Mg2+ was not included in the HA model and for the sake of simplicity, the effect of Mg2+ was not considered here. The remaining Ca2+-dependent parameters in the HA model, the Ca2+ binding affinity, theallosteric coupling strength between the channel gate and Ca2+ binding (C), and the allosteric coupling strength between the voltage sensor and Ca2+ 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 [Ca2+] = 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 qw. Consistent with this possibility, we found that simple decreases in the allosteric coupling strength between the channel gate and Ca2+ binding C and that between the voltage sensor and Ca2+ binding E by the same fraction as used for D (73%) described the effect of heme on the Slo1 G–V curve at high [Ca2+], 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 [Ca2+] (Fig. 12), and this is consistent with the experimental results (Fig. 11). It should be noted that the G–V shift in high [Ca2+] 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 Ca2+ 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 [Ca2+] used (120 μM) was in excess of the normal saturating concentration and channels are still sensitive to low (1 μM) Ca2+ 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
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 $Ca^{2+}$-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 $\gamma_{\alpha4}$ 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 $\sim$70% at least qualitatively reproduces the heme effects at high concentrations of diva-
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-
vated (A) state. The voltage sensor interacts with both the activation gate and the gating ring. But the latter interaction is state dependent and occurs only when the gate is open and the voltage sensor is activated (OA state). This state-dependent interaction causes voltage sensor activation to stabilize the open state, functionally equivalent to the interaction represented by the allosteric factor D in the HCA model. Although not depicted, Ca$^{2+}$, as in the HA model, is assumed to bind with higher affinity to the open/expanded state than to the closed/constricted state, thereby favoring channel activation.

To account for the action of heme, we present the following speculative model that postulates that heme perturbs only the gating ring. We depict heme as binding to the segment between RCK1 and RCK2, altering the structures of the RCK1/RCK2 dimer and of the gating ring (Fig. 13 B). This positioning of heme is consistent with the suggestion by Tang et al. (2003) that heme binding depends on H616 in the RCK1–RCK2 linker segment. We suggest that heme binding makes the constricted conformation of the gating ring less constricted/more expanded, increasing tension on the closed gate to increase $L_0$. The expanded state of the gating ring may become less expanded, accounting for a decrease in the response to Ca$^{2+}$ (smaller C in the HA model) while also preventing the normal interaction between gating ring and voltage sensor (smaller D in the HA model). By acting on the gating ring, heme may thus act as a common regulator of allosteric coupling.

While speculative, the model in Fig. 13 accounts for the experimental findings presented in this study and is consistent with previous results about Slo1 gating. For example, one key observation is that heme has little impact on gating charge movement when the channels are closed (Fig. 7). This is implemented in the model by postulating that the interaction between voltage sensor and gating ring does not occur in the closed state. The model suggests that heme alters the gating ring structure by perturbing the RCK1/RCK2 dimer interface. This need not be the case but is consistent with the observation of Zhang and Horrigan (2005) that modification of C430 near the dimer interface in the RCK1 domain also alters allosteric coupling for both voltage- and Ca$^{2+}$-dependent activation.

Some details of the interaction between voltage sensor and gating ring in the model depicted in Fig. 13 are not well constrained by the experimental results. For instance, voltage sensor and gating ring may interact in all states so long as heme impacts these interactions in a state-dependent manner. In addition, a decrease in the allosteric factor D together with an increase in open probability at negative voltages (increasing $L_0$) could be produced by strengthening interaction between gating ring and voltage sensor in the OR state rather than weakening interaction in the OA state.

The model proposed in Fig. 13 accounts for heme’s action as a common regulator of allosteric coupling by postulating that binding of heme to the RCK1/RCK2 linker segment alters the conformational change of the gating ring. Alternatively, one may postulate that binding of heme acts merely to enhance the tension on the S6-RCK1 linker, perhaps modifying the passive mechanical properties of the gating ring/linker complex (Niu et al., 2004). This greater tautness is expected to increase open probability at negative voltages in the absence of Ca$^{2+}$, thereby increasing $L_0$ in the HCA model. However, the idea that heme simply increases the tension in the S6-RCK1 linker fails to account for the critical observation that heme modifies voltage-dependent gating, altering the steepness of the G–V (Fig. 6). The G–V steepness remains unaltered when the tension on the S6-RCK1 linker is changed by insertion/deletion mutations in the S6-RCK1 segment (Niu et al., 2004).

**Physiological Implications**

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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