A heme-binding domain controls regulation of ATP-dependent potassium channels

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Heme iron has many and varied roles in biology. Most commonly it binds as a prosthetic group to proteins, and it has been widely supposed and amply demonstrated that subtle variations in the protein structure around the heme, including the heme ligands, are used to control the reactivity of the metal ion. However, the role of heme in biology now appears to also include a regulatory responsibility in the cell; this includes regulation of ion channel function. In this work, we show that cardiac K_{ATP} channels are regulated by heme. We identify a cytoplasmic heme-binding $CXXHX_{16}H$ motif on the sulphonylurea receptor subunit of the channel, and mutagenesis together with quantitative and spectroscopic analyses of heme-binding and single channel experiments identified Cys628 and His648 as important for heme binding. We discuss the wider implications of these findings and we use the information to present hypotheses for mechanisms of heme-dependent regulation across other ion channels.

heme | heme regulation | K_{ATP} channel | SUR2A | potassium channel

Heme is a small organic molecule with iron in the center, and it plays a vital role in a wide range of biological systems. Heme-containing proteins form a large and biologically important group of enzymes: they are found in all living species and carry out a wide variety of functions, for example, in oxygen transport (the globins), electron transfer (the cytochromes), and in various heme-dependent catalytic processes (e.g., in the cytochrome P450s, nitric oxide synthases, peroxidases, dioxygenases). For many of these proteins, much structural and mechanistic information is available, and this has led to an established view that the role of heme in biology is as a prosthetic group, which means that it binds tightly to a particular protein (such as hemoglobin), thus conferring specific properties that vary according to the biological requirements. However, it is now becoming clear that this represents only one part of a more complex biological picture and that heme interacts with proteins in a variety of ways and has a much wider regulatory role in the cell (1–5). The mechanisms of heme regulation in biology are, at present, largely unknown.

One new regulatory role for heme is in control of ion channel function. Ion channels are central to the control of a vast range of biological processes ranging from neuronal signaling to regulation of blood pressure, and, as a consequence, defects in ion channel function lead to a variety of disease states. Ion channel gating (opening and closing of the pore) can be regulated by membrane potential and/or by ligand binding; furthermore, coassembly with regulatory subunits and modulation by signaling pathways yields an additional means of control.

The molecular basis for the heme-dependent regulatory control in ion channels has yet to be precisely defined. The complexity of the problem is in part because the target proteins and their sites of interaction with heme are poorly characterized and are probably different in different ion channels, and because the interactions with heme are often weak and give spectroscopic signatures that are much different to those in the more well-characterized heme proteins that are often used as a benchmark. Consequently, the development of ideas about precisely how heme regulates such complex biological events is at a preliminary stage, and the mechanisms of regulation in structurally diverse channels have yet to be unraveled.

In this paper, we examined heme-dependent regulation in cardiac ATP-sensitive K^+ channels (K_{ATP}). K_{ATP} channels regulate the excitability of cardiac ventricular myocytes, which is especially apparent during metabolic stress, such as ischemic heart disease and myocardial infarction (6). We find clear evidence for heme-dependent modulation of cardiac K_{ATP} channels, and our analyses indicate that heme interacts with a cytoplasmic regulatory domain to modulate channel activity. We use this information to present a mechanistic framework for heme-dependent regulation across other ion channels.

Results

The K_{ATP} family of ion channels responds to intracellular ATP and plays a pivotal role in linking cellular metabolism to excitability (7). The most abundant ventricular K_{ATP} channel is a heterooctameric complex consisting of four pore-forming K^+ channel subunits of the inward rectifier family (Kir6.2) and four regulatory sulphonylurea receptor subunits (SUR2A), which are members of the ATP-binding cassette (ABC) transporter superfamily (8, 9), Fig. S1*A*. The modulation of K_{ATP} channel activity is an important process that enhances the cardiac muscle response to oxidative stress (10).

Heme Increases Whole-Cell K_{ATP} Currents. The effect of heme on the K_{ATP} current from isolated cardiac myocytes was investigated

Significance

Heme-containing proteins are found in all living species and carry out a wide variety of biological functions. It is becoming clear that heme has a wider regulatory role in the cell; one such regulatory role is in control of ion channel function. In this paper, we demonstrated heme-dependent regulation of K_{ATP} channels and identified the heme-binding location as being on a sulphonylurea receptor subunit of the channel. We use this information to present a hypothesis for how heme regulation across numerous ion channels may occur.

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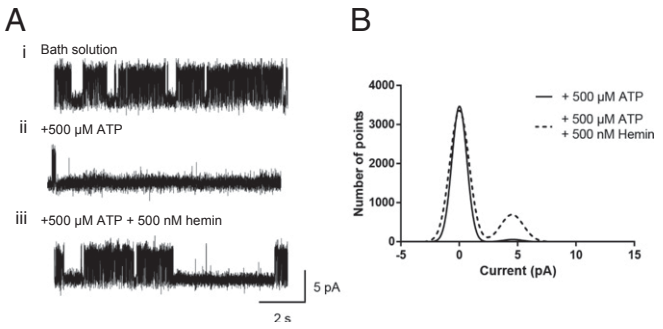

Fig. 1. Heme increases K_{ATP} single channel open probabilities. (*A, i*) K_{ATP} channel currents recorded from an inside-out patch of a ventricular myocyte at +70 mV exposed to normal bath solution containing 0 μM ATP, illustrating high channel activity. (*A, ii*) Local perfusion of the same patch with 500 μM ATP reduced channel activity. (*A, iii*) Local perfusion of the same patch with 500 μM ATP and 500 nM hemin increased channel activity. (*B*) Overlaid amplitude histograms from the above patch, (fitted with Gaussian distributions) illustrating an increase in single channel activity with application of 500 nM hemin (dashed line).

using whole-cell patch-clamp recordings. Cardiac myocytes were held at 0 mV and bath application of the K_{ATP} channel opener, P1075 (10 μM), resulted in a whole-cell current that was sensitive to bath application of the selective K_{ATP} channel blocker, glibenclamide (10 μM), that completely blocked the current (Fig. S2*A*). The application of heme (500 nM) resulted in an increase in the K_{ATP} current, producing a 1.6-fold increase in K_{ATP} current (Fig. S2 *B* and *C*). Heme increased the K_{ATP} current in a dose-dependent manner with a maximal response achieved with 500 nM heme; the half maximal increase in K_{ATP} channel open probability in response to heme is ~100 nM (Fig. S2*C*), which is in the physiological range for heme concentration within the cell (11). Bath application of protoporphyrin IX (Fig. S2*F*), Zn-protoporphyrin IX (Fig. S2 *D* and *F*), or Sn-protoporphyrin IX (Fig. S2*F*) did not result in any change in the K_{ATP} currents. In further experiments, $FeSO_4$ (500 nM) was superfused onto cardiac myocytes during the recording (Fig. S2*E*). However, in contrast to the heme-induced increases in K_{ATP} current, application of $FeSO_4$ resulted in a significant decrease in K_{ATP} current (Fig. S2 *E* and *F*). We conclude that the increases in current are specific to heme and are not a consequence of the porphyrin ring or iron alone.

Depletion of Intracellular Free Heme Reduces K_{ATP} Current. The effect of depleting intracellular heme was also tested by incubating myocytes with succinylacetone (SA), which inhibits heme biosynthesis by inhibiting the ALA dehydratase enzyme (the second enzyme in the heme biosynthesis pathway). When intracellular heme levels are decreased a feedback loop causes an increase in expression of aminolevulinate synthase-1 (ALAS-1, the first enzyme in the pathway). After incubation with SA, an increase in the expression of ALAS-1 was observed by quantitative PCR (qPCR; Fig. S3*A*). The K_{ATP} channels were opened by the channel opener P1075 (10 μM), and the resulting currents were recorded from myocytes in the whole-cell configuration. The currents recorded from the myocytes treated with SA (1 mM) for 4 h were significantly smaller in amplitude compared with the amplitude of control untreated myocytes (Fig. S3 *B* and *C*).

Heme Increases Cardiac K_{ATP} Single Channel Activity. K_{ATP} channels from ventricular myocytes were recorded in inside-out patches at a holding potential of 70 mV. Immediately after patch excision, the K_{ATP} channel open probability (P_{open}) was high due to lack of ATP in the bath solution (Fig. 1 *A*, *i*). The mean amplitude of single channel currents was ~4.5 pA with P_{open} of 0.454 ± 0.063, $n = 4$, for endogenous K_{ATP} channels in cardiac myocytes. A localized perfusion pipette, enabling rapid solution changes to the intracellular side of the patch, delivered a solution containing ATP (500 μM) directly to the excised patch. The addition of ATP to the patch reduced P_{open} to 0.014 ± 0.009, $n = 4$ (Fig. 1 *A*, *ii* and *B*). Following this, application of hemin (500 nM) with ATP (500 μM) revealed a significant increase in channel activity, P_{open}, to 0.104 ± 0.030, $n = 4$ (Fig. 1 *A*, *iii* and *B*).

Identification of the Heme-Binding Location. Identification of heme-binding domains within heme-dependent ion channels presents a considerable experimental challenge. The K_{ATP} subunits Kir6.2 and SUR2A do not contain any CXXCH cytochrome *c*-like heme-binding motifs, as identified in the large conductance Ca^{2+}-activated K^+ channel (BK channels) (12). However, SUR2A contains a $CXXHX_{16}H$ motif (nomenclature follows that in ref. 13) (Fig. S1*B*). SUR2A (UniProt ID: sp|O60706|ABCC9_HUMAN) is a 1,549-residue protein that belongs to the *c*-subfamily of the ABC family, an ABC subfamily also referred to as multidrug resistance-associated proteins (MRPs). Based on sequence alignments, SUR2A contains two ATP-binding domains of ABC transporters (Pfam domain ID: PF00005) in the regions 684–835 and 1325–1473, and two transmembrane domains of ABC transporters (Pfam domain ID: PF00664) in the regions 303–582 and 990–1260 (Fig. 2 and Fig. S1*B*). The $CXXHX_{16}H$ motif of the SUR2A subunit (residues 628–648) is located between the first transmembrane domain and the first nucleotide-binding domain (Fig. S1). The hypothesis that the $CXXHX_{16}H$ motif is involved in heme binding was therefore tested by mutagenesis (residues Cys628, His631, and His648).

Inside-out patches from HEK293 cells heterologously expressing WT Kir6.2 and SUR2A show K_{ATP} channel currents with a robust response to heme. In the presence of ATP (500 μM), the K_{ATP} channels had a low open probability (0.023 ± 0.009, $n = 9$; Fig. 3 *A* and *B*) compared with the addition of heme (500 nM) when an increase in open probability is observed (0.109 ± 0.020, $n = 9$; Fig. 3*B*). The increase in open probability was observed over all voltages tested in macropatches (Fig. S4*D*). The channels expressed in HEK293 cells were 4.5 pA at 70 mV and were sensitive to glibenclamide (50 μM), which is indicative of the K_{ATP} channel currents (Fig. S4 *A*, *iv*). The effect of mutations in the $CXXHX_{16}H$ motif (at C628S, H631A, and H648A) was also tested (Fig. 3 *C*–*G*). Single channel analysis revealed that both single mutations C628S and H648A had a substantial effect on the K_{ATP} channel response to heme (Fig. 3 *A* and *B* and Fig. S4 *E*

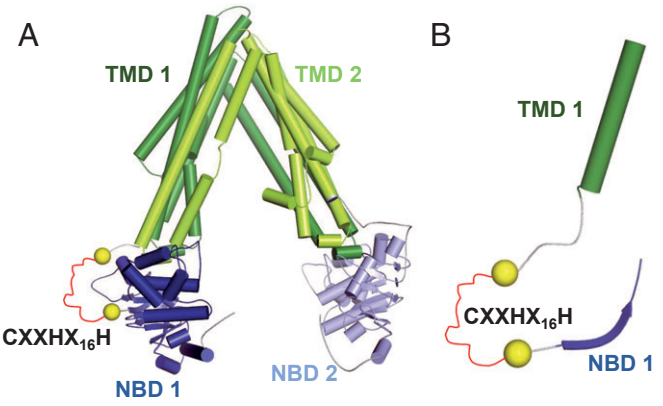

Fig. 2. (*A*) SUR2A homology model based on *C. elegans* MRP PGP-1. α-Helices are displayed as barrels, and β-strands as arrows using the color scheme introduced in Fig. S1*A*. The $CXXHX_{16}H$ motif (red), which could not be modeled, is schematically indicated as a stretch of residues between the two yellow spheres. (*B*) Zoom into the SUR2A homology model. Visualization as in *A*, but only residues that are also present in the multiple sequence alignment in Fig. S1*B* are shown.

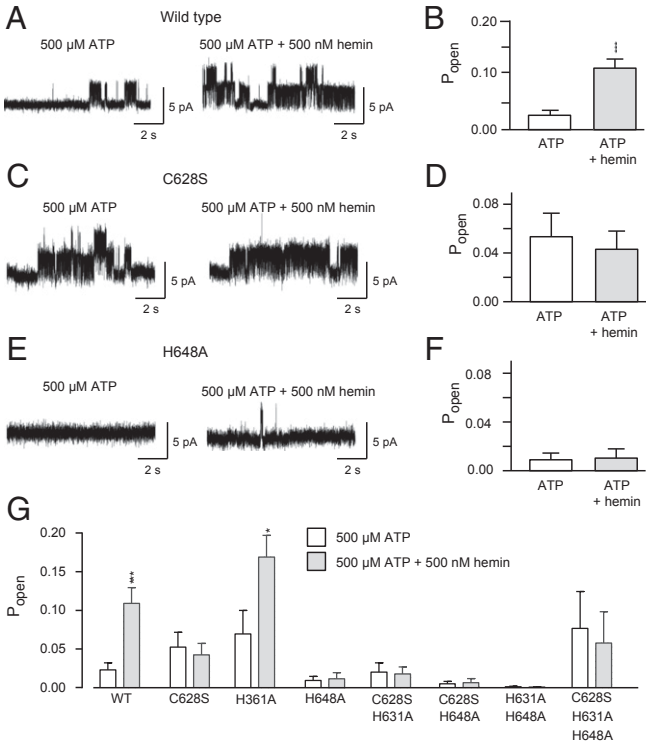

Fig. 3. Effect of mutagenesis of the $CXXHX_{16}H$ region of SUR2A on K_{ATP} channel activity. (*A*) Inside-out patch of WT perfused with 500 μM ATP, and 500 μM ATP with 500 nM hemin, and (*B*) plot mean P_{open} with empty bars indicating 500 μM ATP and gray bars indicating 500 μM ATP with 500 nM hemin, $n = 9$ (***$P \leq 0.001$). (*C*) Representative inside-out patch of C628S with 500 μM ATP, and 500 μM ATP with 500 nM hemin, and (*D*) mean P_{open}, $n = 7$. (*E*) Representative inside-out patch of H648A with 500 μM ATP, and 500 μM ATP with 500 nM hemin, and (*F*) mean P_{open} for 500 μM ATP and 500 μM ATP with 500 nM hemin, $n = 5$. (*G*) Mean P_{open} for all mutants: empty bars for 500 μM ATP and gray bars indicating 500 μM ATP with 500 nM hemin.

and *F*), whereas the H631A mutation had a minor effect on the heme response (Fig. 3*G*). Double and triple mutations showed that any combination of mutations that contained C628S and H648A affected the heme-dependent increases in K_{ATP} channel open probability (Fig. 3*G*). These data are consistent with the SUR2A subunit of K_{ATP} channel being the site of heme binding and suggest a role for C628 and H648.

Heme Binding to SUR2A. To further quantify the interaction of heme with the SUR2A subunit, we examined heme binding to two model peptides and to a fragment of the SUR2A subunit (residues S615-L933) containing the entire $CXXHX_{16}H$ region. In assessing the spectroscopic properties of these heme-bound species, it is important to note that the spectra for regulatory heme proteins often differ from those of well-known heme proteins such as the globins and cytochrome *c*, most likely because the heme binds more weakly.

Peptide A (LPFES**C**KKHTGVQSKPINRKQPGRY**H**LDNYE) contains the residues corresponding to C628/H648 (in bold in the sequences). At a 1:1 hemin:peptide ratio, peptide A binds heme to form a complex that is clearly red-shifted ($\lambda_{max} = 417$ nm; Fig. 4*A*) compared with free hemin ($\lambda_{max} = 385$ nm). Similar absorption patterns ($\lambda_{max} = 412$–417 nm) have been reported for heme binding to peptides with a single cysteine residue (14) and to proteins with Cys/His coordination (Table S1). Heme binding to a control peptide B (LPFES**S**KK**A**TGVQSKPINRKQPGRF**A**LDNFE) in which Cys and His residues have been replaced did not show any evidence of heme binding under the same conditions.

Additionally, the nucleotide-binding domain 1 of SUR2A including the heme-binding region (residues S615-L933; Fig. S1*B*) was expressed in *Escherichia coli* and titrated with heme (Fig. 4*B*). The absorption spectrum shows a peak at 413 nm; a similar peak of lower intensity was observed in the absorption spectrum of the H648A mutant. As mentioned above, similar absorption patterns have been reported for heme binding to proteins with Cys/His coordination (Table S1), typical for low-spin type 2 heme thiolate Fe(III) Soret bands (15). Overall, these data are consistent with heme binding to the SUR2A subunit of the K_{ATP} channel and are in agreement with the electrophysiology data.

Resonance Raman experiments were used to provide further insight on the SUR2A-hemin interaction. In the high-frequency region, the spectrum of free hemin displays the distinctive features of a ferric five-coordinate high-spin complex (5c-HS) with characteristic bands at 1370 (ν_4), 1490 (ν_3), and 1571 cm^{-1} (ν_2) (Fig. 4 *C, i* and Table S2). The ν_{10} mode of 5c-HS heme is hidden by the vinyl stretching bands expected in the 1610- to 1640-cm^{-1} region (1618 cm^{-1} for the in-plane and 1628 cm^{-1} for the out-of-plane vinyl group) (16). When titrated with a 0.8 equivalent of hemin, ferric SUR2A(S615-L933) shows significant differences with free hemin (Fig. 4 *C, ii*), indicating that heme is bound specifically. Besides the bands at 1490 (ν_3) and 1573 cm^{-1} (ν_2) assigned to a 5c-HS hemin complex that probably originates from some unbound hemin, the ν_4 band shifts to a higher frequency (1373 cm^{-1}), and there are additional bands at 1507 (ν_3), 1589 (ν_2), and 1641 cm^{-1} (ν_{10}) that clearly indicate the presence of a six-coordinate low-spin complex (6c-LS) (Table S2). The proportion of 6c-LS heme is significantly increased when the concentration of SUR2A(S615-L933) is in excess, as indicated by increases in intensity of the ν_3 (1505 cm^{-1}), ν_2 (1586 cm^{-1}), and ν_{10} (1640 cm^{-1}) bands (Fig. 4 *C, iii*). Time-dependent resonance Raman spectra of the ferric SUR2A(S615-L933)-hemin complex reveal a build-up of the 6c-LS species (Fig. S5), as evidenced by an increase in the intensity of the ν_3 (1506 cm^{-1}) and ν_{10} (1641 cm^{-1}) bands (Fig. S5 *ii* and *iii* and Table S2). The spectroscopic data indicate that hemin binding to the channel occurs rapidly, but that complete formation of low-spin heme is slower and presumably involves a conformational rearrangement that "locks" the heme into place. The H648A mutation (Fig. 4 *C, iv*) leads to a significant decrease of the 6c-LS species compared with the WT protein (Fig. 4 *C, ii*), as indicated by the decrease of the intensity of the bands at 1506 (ν_3), 1590 (ν_2), and 1641 cm^{-1} (ν_{10}). These data are consistent with a decreased intensity of the Soret absorbance band at 413 nm for the H648A-hemin complex and with the electrophysiology data. Together, the data suggest that heme binds to SUR2A(S615-L933) at a specific location and that H648 plays a significant role.

Electron paramagnetic resonance (EPR) data support the conclusions above. The spectrum of the ferric SUR2A(S615-L933)-hemin complex confirms the presence of low-spin heme ($g = 2.47$, 2.28, and 1.86; Fig. S6*ii*). As observed in the resonance Raman data above (Fig. 4 *C, iii*), the proportion of 6c-LS heme increases in the presence of higher concentrations of SUR2A (Fig. S6*iii*). These g values are typically observed for His-Cys heme ligation, as demonstrated in Fig. S7 (15). Some high-spin heme is visible ($g = 6$; Fig. S6*ii*), which probably arises from a small amount of free hemin (compare with Fig. S6*i*). EPR data show that the 6c-LS signals essentially disappear in the H648A mutant (Fig. S6*iv*), in agreement with the resonance Raman spectra (Fig. 4 *C, iv*), and confirm the importance of this residue in heme binding. The mutation of the C628S residue had less of an impact on the EPR spectra (Fig. S6*v*) (resonance Raman data for C628S showed a similar pattern), but mutation of H631 decreases the amount of 6c-LS species (Fig. S6*vi*). The implications of these findings are discussed below.

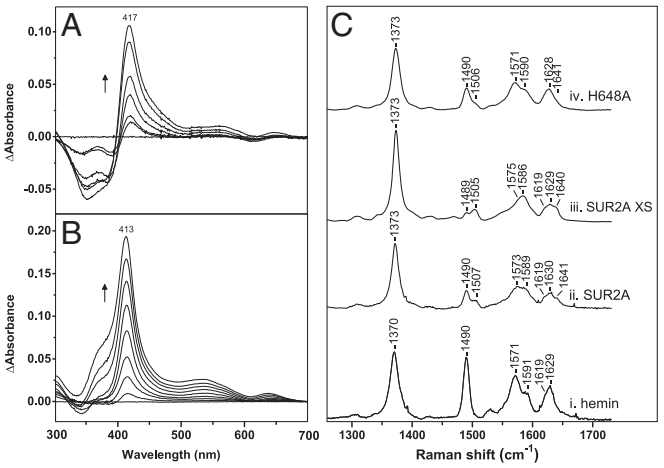

Fig. 4. Spectroscopic analysis of heme bound to SUR2A and a synthetic peptide. (*A*) Spectrophotometric titrations of the synthetic peptide LPFESCKKHTGVQSKPINRKQPGRYHLDNYE with heme. (*B*) Corresponding heme titration of the SUR2A truncated protein (residues S615-L933) containing the heme-binding $CXXHX_{16}H$ region; the arrows represent the directions of the absorbance changes with increasing heme concentration. (*C*) Room temperature high-frequency resonance Raman spectra of (*i*) hemin, (*ii*) ferric SUR2A (S615-L933)-hemin at substoichiometric hemin concentrations, (*iii*) ferric SUR2A (S615-L933)-hemin with the protein in four- to fivefold excess, and (*iv*) H648A-hemin. All spectra collected with 413.1-nm laser excitation.

Discussion

Most of what is known about the role of heme iron in biology has emerged over many decades from structure/function studies on well-known heme proteins, for example, the globins, the cytochromes, and the many and varied catalytic heme enzymes. However, there is evidence in the literature that heme acts as a signaling molecule and also regulates a variety of other complex biological events in the cell, which extends the role of heme far beyond the limits of current understanding. As well as roles in control of ion channel function, there is very good evidence for the involvement of heme in circadian control (day/night cycle), in control of transcriptional events and gene expression, and in regulation of phosphorylation and kinase activity (see refs. 1, 3, 17, and 18 for reviews).

The mechanisms of heme-dependent regulation are very unclear. It is not yet known how heme is stored and mobilized within the cell for regulatory control: heme is insoluble in aqueous solutions and is most likely bound (weakly) to as yet unknown chaperone/transporter proteins, but the mechanisms of heme transport across the membrane are not established in detail. Heme concentrations in the cell have not been measured precisely (estimates are in the range ∼100 nM or less (4, 11, 19) and probably increase during hypoxia and after thrombosis/stroke (20–22) so that local changes in heme concentration in the cell cannot, at present, be reliably quantified.

Heme Binding Interactions in Ion Channels. In the case of ion channel regulation, it has recently emerged that there is a role for heme (12, 23–26), but the observations are largely empirical, so that the molecular basis for the regulatory control within individual channel proteins has yet to be properly defined. A number of heme-responsive motifs (27) have been suggested to be involved: these include Cys/Pro (CP) motifs using thiolate ligation to the heme as in the P450s or Cys/His motifs (3, 14). In the case of the Slo1 channels (12), a cytochrome *c*-like CXXCH motif has been implicated (12, 28). However, this raises immediate questions that do not chime with established patterns of behavior in other heme proteins, because most proteins bind heme reversibly (i.e., noncovalently), whereas cytochrome *c* uses complex and specialized biosynthetic machinery to bind heme irreversibly (i.e., covalently) through thioether bonds from the Cys residues of the CXXCH motif to the heme vinyl groups (29, 30). There is as yet no evidence that ion channels proteins use similarly specialized biosynthetic machinery; thus, it seems unlikely that their heme is covalently attached. Neither the epithelial sodium channels (24) nor the A-type K^+ channels (23) contain a CXXCH motif, which we interpret to mean that the mode of heme binding is most likely not the same across all ion channels.

In this paper, we quantified the effect of heme on ATP-dependent potassium channels. These channels couple electrical activity at the membrane to energy metabolism in numerous cells, such as pancreatic β cells and cardiac myocytes, and in this way regulate a number of key physiological processes including secretion and muscle contraction. We showed that heme at physiological concentrations is a robust activator of K_{ATP} channels (Fig. 1 and Fig. S2). We identified a $CXXHX_{16}H$ heme-binding motif on the SUR2A domain as a potential heme-binding location. Quantitative analyses of heme binding to model peptides as well as to a fragment of the SUR2A subunit expressed in *E. coli* demonstrate that heme binds to SUR2A(S615-L933), specifically, at this location. At least as far as the isolated SUR2A domain is concerned (i.e., not within the channel), formation of a low-spin heme species is not instantaneous and probably occurs after initial formation of a more loosely associated, nonspecific heme-protein complex, by a slow conformational transition that locks the low-spin SUR2A-hemin complex in place. Mutagenesis, together with single channel data, implicates Cys628 and His648 as involved in the heme regulatory response, Fig. 3. The spectroscopic data are clearly consistent with His648 as a potential ligand to a low-spin heme species, which, on the basis of EPR *g* values (Fig. S7), we tentatively assigned as His/Cys ligated species. Our spectroscopic data for the Cys628 variant do not unambiguously confirm this residue as a ligand to the low-spin heme species, even though a role for Cys628 is clearly evident from the single channel electrophysiology data. Other heme-binding modes are most likely possible (as also indicated by our findings on the H631 mutant), so that alternative residues may bind to the heme in the absence of Cys628. Such flexibility of heme binding—particularly flexibility of Cys ligation and particularly redox-linked ligation changes (15)—seems to be a feature of these regulatory heme proteins; it probably reflects an intrinsic mobility of the protein structure (as evidenced by the conformational changes leading to low-spin heme formation) that can, potentially, absorb binding of heme in more than one orientation. A mixture of binding modes have been suggested for the Kv1.4 ball peptide-hemin complex (23).

The $CXXHX_{16}H$ motif (residues 628–648) is located between the first transmembrane domain and the first nucleotide-binding domain (Fig. S1). Sequence alignment of the 12 human *c*-subfamily ABC proteins (Fig. S1*B*) illustrates that the $CXXHX_{16}H$ motif is part of an insertion that is present only in SUR2A and SUR1. SUR1 is the closest homolog to SUR2A (67% identity) within the ABC subfamily *c*. However, although SUR1 also has the insertion, the $CXXHX_{16}H$ motif is not conserved between SUR2A and SUR1. Hence, this particular mode of heme binding is specific to SUR2A and does not occur in other members of this family of ABC transporters. There is evidence for heme binding to ABC-transporter proteins (31–35) in other (unrelated) heme transport systems, but the heme-binding sites are not established.

Looking at all of the heme-dependent ion channels identified thus far, we note that cysteine is implicated as important for heme binding in all cases (Table S1). There is, as yet, no structural information on how heme binds to any ion channel protein. Modeling of SUR2A based on the crystal structure (36) of the homolog MRP1 from *Caenorhabditis elegans* illustrates the orientation of the two transmembrane and two nucleotide binding domains (Fig. 2). The region containing the $CXXHX_{16}H$ motif cannot be modeled meaningfully (and might be conformationally

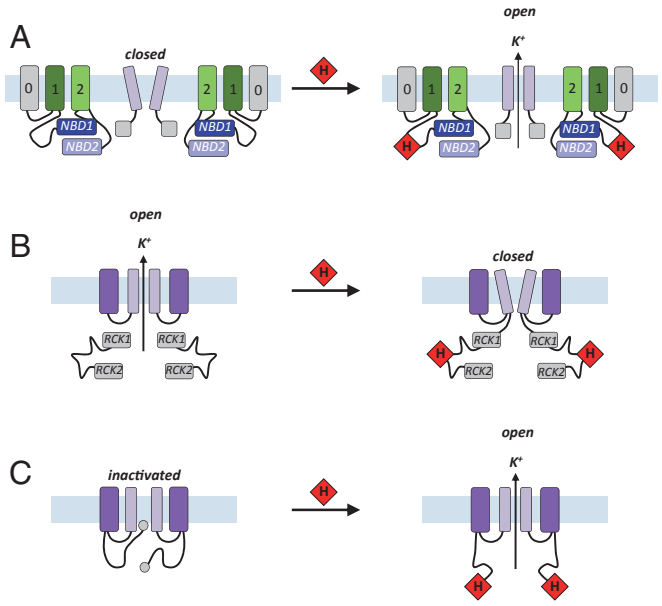

Fig. 5. Cartoon representation of heme interaction in various ion channels. (*A*) In the K_{ATP} channel (this work), heme binds to the $CXXHX_{16}H$ motif in the unique insertion (G622-P665 in the human protein) on the cytoplasmic domain of SUR2A and increases the open channel probability. (*B*) In Slo1 (BK) channel (5), heme binds to the cytochrome *c*-like motif CXXCH in the disordered region between the RCK1 and RCK2 domains, and increased concentrations of heme inhibit K^+ currents by decreasing the frequency of channel opening. Inhibition of channel activity as heme concentration increases is consistent with other observations (41) in which knockdown of heme oxygenase (which would increase heme concentration) also reduces channel activity. (*C*) In the Kv1.4 channel (23), heme binds to the "ball-and-chain" N terminus of the A-type potassium channel and impairs the inactivation process; a heme-responsive $CXXHX_{18}H$ motif is suggested as being responsible for heme binding, which introduces a stable configuration in the otherwise disordered region. The membrane is depicted in pale blue and the intracellular side is on the bottom. The light purple rectangles depict the conduction pore of the inward rectifier K^+ channel Kir6.2 subunit in the K_{ATP} channel and the Slo1 and Kv1.4 channels. The gray (TMD0), dark green (TMD1), and light green (TMD2) rectangles represent the transmembrane domains of the sulphonylurea receptor SUR2A in the K_{ATP} channel (color scheme as in (Fig. S1*A*), and the dark purple rectangles are the voltage-sensor domains in the Slo1 and Kv1.4 channels. Other transmembrane domains have been omitted for simplicity. NBD, nucleotide binding domains 1 and 2; RCK1/RCK2, regulator of conductance K domains 1 and 2. Heme is depicted as a red diamond.

mobile) because this insertion is missing in MRP1 *C. elegans*, but the model indicates that the heme-binding region is close to the first nucleotide binding domain (NBD1; Fig. 2 *A* and *B*). In this regard, there are similarities to the structure of human Slo1, because the proposed CXXCH heme-binding motif in Slo1 is either invisible or only partly visible in crystal structures (37–39) and is thus assumed to be located in an unstructured (conformationally mobile) region of the molecule.

Consideration of Heme-Dependent Regulatory Mechanisms Across Other Ion Channels. To begin to create a framework for the development of ideas on the mechanisms of heme-dependent ion channel regulation, we summarized schematically the information that has emerged thus far for K_{ATP} channels (this work), as well as for Slo1 (BK) (12) and Kv1.4 (23) channels (Fig. 5). In all three channels, we note that the heme interacts with a cytoplasmic domain to modulate channel activity, and, in each case, the heme is suggested to bind to a flexible region of protein structure. Thus, for the K_{ATP} channels, heme binds to the $CXXHX_{16}H$ motif in the cytoplasmic domain of SUR2A; for the Slo1 (BK) channels, heme binds to the cytochrome *c*-like CXXCH motif in the conformationally mobile region between RCK1 and RCK2 domains; and for the Kv1.4 channel, heme is suggested to bind to a similar $CXXHX_{18}H$ motif close to the N-terminal ball-and-chain inactivation domain, which introduces a stable configuration in an otherwise flexible region. Our results indicate that heme binding increases the open channel probability in K_{ATP} channels, but for BK channels the opposite effect is observed and for the Kv 1.4 channels heme binding impairs channel inactivation. Hence, although there may be similarities in the modes of heme binding across different channels, the functional consequences are not the same in each case and are a clear indication of the potential versatility of heme-binding processes in ion channel control. Additional layers of biological control can easily be envisaged when one considers that heme concentrations are linked to O_2 concentration by O_2-dependent heme degradation pathways (via heme oxygenase) (40), which itself produces CO. A three-way mechanism of control, linking heme concentrations to O_2 and/or CO binding to heme, could provide considerable versatility around the simple process of heme binding and would be a neat solution to a conceptually difficult biological problem.

Materials and Methods

Detailed methods on the following subjects are available in *SI Materials and Methods*: isolation of cardiac myocytes, transfection and cell culture, electrophysiology, RNA extraction and quantitative real-time PCR analysis, expression and purification of SUR2A NBD1, peptide synthesis, spectroscopy of heme-bound species, bioinformatics analyses, chemicals, and reagents.

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