CORRECTIONS

COMMENTARY

Due to a printer’s error, the title appeared incorrectly. The title should instead appear as “Complex nature of malaria parasite hemoglobin degradation.” The online version has been corrected.

www.pnas.org/cgi/doi/10.1073/pnas.1305990110

EVOLUTION

The authors note that Fig. 2 should have the following credit line: “Photo © NSW Department of Trade & Investment, Primary Industries Division artist Pat Tully.”

www.pnas.org/cgi/doi/10.1073/pnas.1304910110

MEDICAL SCIENCES
Correction for “Primary aldosteronism and impaired natriuresis in mice underexpressing TGFβ1,” by Masao Kakoki, Oleh M. Pochynyuk, Catherine M. Hathaway, Hirofumi Tomita, John R. Hagaman, Hyung-Suk Kim, Oleg L. Zaika, Mykola Mamenko, Yukako Kayashima, Kota Matsuki, Sylvia Hiller, Feng Li, Longquan Xu, Ruriko Grant, Alejandro M. Bertorello, and Oliver Smithies, which appeared in issue 14, April 2, 2013, of Proc Natl Acad Sci USA (110:5600–5605; first published March 15, 2013; 10.1073/pnas.1302641110).

The authors note that the affiliation “bDepartment of Integrative Biology and Pharmacology, University of Texas Health Science Center, San Antonio, TX 78229” should instead appear as “bDepartment of Integrative Biology and Pharmacology, University of Texas Health Science Center, Houston, TX 77030.” The corrected author and affiliation lines appear below. The online version has been corrected.

Masao Kakokia, Oleh M. Pochynyukb, Catherine M. Hathawayb, Hirofumi Tominita, John R. Hagamana, Hyung-Suk Kimb, Oleg L. Zaikab, Mykola Mamenkob, Yukako Kayashimab, Kota Matsuki, Sylvia Hillerb, Feng Lib, Longquan Xub, Ruriko Grantb, Alejandro M. Bertorellob, and Oliver Smithiesb

aDepartment of Pathology and Laboratory Medicine, University of North Carolina, Chapel Hill, NC 27599; bDepartment of Integrative Biology and Pharmacology, University of Texas Health Science Center, Houston, TX 77030; and cMembrane Signaling Networks, Atherosclerosis Research Unit, Department of Medicine, Karolinska Institutet, Karolinska University Hospital-Solna, 171 76 Stockholm, Sweden

www.pnas.org/cgi/doi/10.1073/pnas.1305878110

RETRACTION

BIOPHYSICS AND COMPUTATIONAL BIOLOGY

The authors wish to note the following: “The contrast of our final projection map was inverted, so that we interpreted the background density rather than the actual protein density in terms of structural features of the potassium channel-Fv complex. In addition, we indexed the 2D crystals with unit cell parameters of a = b = 175 Å, while the correct indexing would be a = b = 124 Å. Given these analysis errors, the resulting density map and our interpretation of the structural features are not correct. Accordingly, we would like to retract this paper. We acknowledge Yoshinori Fujiyoshi, Rod MacKinnon, Kazutoshi Tani, and Tom Walz for identifying the errors and pointing them out to us.”

Liang Shi
Hongjin Zheng
Hui Zheng
Brian A. Borkowski
Dan Shi
Tamir Gonen
Qiu-Xing Jiang

www.pnas.org/cgi/doi/10.1073/pnas.1304582110
Voltage sensor ring in a native structure of a membrane-embedded potassium channel

Liang Shi, Hongjin Zheng, Hui Zheng, Brian A. Borkowski, Dan Shi, Tamir Gonen, and Qiu-Xing Jiang

*Department of Cell Biology, University of Texas Southwestern Medical Center, Dallas, TX 75390; and Janelia Farm Research Campus, Howard Hughes Medical Institute, Ashburn, VA 20147

Edited* by Christopher Miller, Howard Hughes Medical Institute, Brandeis University, Waltham, MA, and approved January 17, 2013 (received for review October 18, 2012)

Voltage-gated ion channels support electrochemical activity in cells and are largely responsible for information flow throughout the nervous systems. The voltage sensor domains in these channels sense changes in transmembrane potential and control ion flux across membranes. The X-ray structures of a few voltage-gated ion channels in detergents have been determined and have revealed clear structural variations among their respective voltage sensor domains. More recent studies demonstrated that lipids around a voltage-gated channel could directly alter its conformational state in membrane. Because of these disparities, the structural basis for voltage sensing in native membranes remains elusive. Here, through electron cryo-EM crystallographic analysis of membrane-embedded proteins, we present the detailed view of a voltage-gated potassium channel in its inactivated state. Contrary to all known structures of voltage-gated ion channels in detergents, our data revealed a unique conformation in which the four voltage sensor domains of a voltage-gated potassium channel from *Aeropyrum pernix* (KvAP) form a ring structure that completely surrounds the pore domain of the channel. Such a structure is named the voltage sensor ring. Our biochemical and electrophysiologic studies support that the voltage sensor ring represents a physiological conformation. These data together suggest that lipids exert strong effects on the channel structure and that these effects may be changed upon membrane disruption. Our results have wide implications for lipid–protein interactions in general and for the mechanism of voltage sensing in particular.

The key elements in all excitable membranes are voltage-gated ion channels (1–3). Voltage sensor domains (VSDs) are nanomachines in the voltage-gated ion channels. They use electrostatic potential energy to drive the structural rearrangements in the channel pore, thus resulting in permeability changes. Their response can happen within a narrow range of transmembrane voltage, as observed by Hodgkin and Huxley decades ago (4, 5), or through a broader range as seen in voltage-sensitive proton channels and phosphatases, both of which are weakly controlled by one voltage sensor (6–8). The amino acid sequences of all known VSDs are fairly conserved. In the superfamily of voltage-gated calcium (Cav), sodium (Nav), and potassium (Kv) channels, a total of four VSDs work together to control one highly selective ion-conducting pore (Fig. S1). These four VSDs can come from four subunits each having one VSD (in the case of Kv), or from four tandem domains in a single molecule (eukaryotic Nav and Cav) (9). In both cases, the four VSDs are thought to exist as discrete units, but act together on the same ion channel pore (10, 11). Topologically, each VSD is composed of four transmembrane helices (S1 to S4). The channel pore is formed by transmembrane helices 5 and 6 (S5–S6) with the connecting loop between S5 and S6 forming the selectivity filter (12, 13). The ion conduction pathway is always found at the axis of symmetry.

A number of X-ray crystallographic studies were successful in determining the structures of various Kv and Nav channels in detergents (14–19). A common feature of these channel structures is that the channel pore has four VSDs around it, where each VSD forms a four-helix bundle. However, despite these structures, it remains unclear how the VSDs sense voltage and signal the channel pore to open or close (20). Understanding the voltage-driven allosteric movement of the VSDs in relation to the pore domain is therefore key to elucidating the voltage-sensing mechanism. The current biophysical models suggest that the VSDs switch between at least two distinct gating conformations, which are named based on whether the charged S4 residues (mainly arginines in four highly conserved positions) face the intracellular (“down”) or extracellular side (“up”) of the transmembrane voltage profile (3, 10, 11, 21–23). The down state is also called “resting” or “hyperpolarized” and the up state, “activated” or “depolarized.”

The four-helix bundle VSD models in X-ray structures are all supposed to represent the physiological up conformation and thus should all adopt similar structures. However, the positions and orientations of the S4 arginine residues in these structures vary substantially (15–19). Electrophysiologic studies have shown that membrane-embedded voltage-gated potassium channel from *Aeropyrum pernix* (KvAP), Kv2.1, and voltage-gated sodium channel from *Aerobacter butzleri* (NavAb) held at 0 mV become inactivated over time (17, 22, 24). Therefore, the X-ray structures of these three channels, if completely physiological, should all reflect the inactivated states (state I), not necessarily the states to which they were assigned. Furthermore, these structures exhibit striking differences compared with the recently reported structures of bacterial Nav channels that were assigned to rather disparate inactivated states (17, 18). These discrepancies between structure and function have contributed to the long-standing uncertainty regarding the conformational assignment of the X-ray structures as well as the possible uncoupling between the VSDs and the pore domain in detergent-saturated channels.

Others and we have reported a strong influence of lipids on the structure and function of voltage-gated channels (25–27). Our data showed that lipids may directly change the conformation of the VSDs in Kv channels (25), suggesting that the surrounding membranes may have profound effects on the structure (and therefore function) of VSDs. Logically, it has become clear that a structure of a voltage-gated channel in membrane is necessary to sort out the differences among the published studies, and will likely reveal novel and significant insights into the mechanism of voltage sensing. Next, we will present a structural view of a Kv channel in a lipid bilayer, and evaluate its physiological relevance to voltage-dependent gating.


The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

Freely available online through the PNAS open access option.

To whom correspondence may be addressed. E-mail: qiu-xing.jiang@utsouthwestern.edu or gonen@janelia.hhmi.org.

This article contains supporting information online at www.pnas.org/cgi/suppl/doi/10.1073/pnas.1218203110/-/DCSupplemental. 

www.pnas.org/cgi/doi/10.1073/pnas.1218203110

PNAS | February 26, 2013 | vol. 110 | no. 9 | 3369–3374
Results and Discussions

Electron Crystallography of the KvAP Channel in Membranes. In electron crystallography, membrane proteins of interest are crystallized within lipid bilayers that provide a more native environment than detergent micelles (20). Extensive lipid–protein interactions exist in such 2D crystals, and these interactions often affect both the structure and function of the proteins in membrane. Thus, we performed an electron crystallographic study of the KvAP channel (28). For the successful crystallization in membranes we sought to stabilize the channels in a defined, homogeneous conformation. Previous functional data suggest that at 0 mV multiple states exist for the KvAP (C, O, and I) in which the VSDs remain up while the pore domain undergoes significant conformational changes (Fig. S1A). We recently characterized a single-domain Ig protein (Fv) that recognizes the extracellular side of the S3/S4 voltage sensor paddle, and whose presence stabilizes the KvAP channel in the inactivated state (I) with the VSDs up and the channel pore closed (25) (Fig. S1D). We took advantage of this conformational homogeneity and pursued the 2D crystallization of the KvAP/Fv complex in membrane.

We successfully grew large and very well-ordered 2D crystals of the KvAP/Fv complex. Purified protein complex was mixed with detergent-solubilized lipids and allowed to reach equilibrium. The detergents were removed by slow dialysis against crystallization buffer over 7 d. When the samples were examined by negative-stain electron microscopy (EM), we observed large single-layered membrane sheets, many of which were ∼5 μm in diameter and showed a rather coherent square lattice (Fig. L4 and details in SI Methods).

To investigate whether the channel proteins in our crystals were really embedded in lipid bilayers, small quantities of crystals were treated with 1.0 M sodium carbonate (pH 11) followed by sucrose-gradient fractionation. Some of the Fv molecules were stripped off, but the KvAP protein remained in vesicles (Fig. S2). These results indicate that the channels in the 2D crystals are indeed membrane-embedded, whereas the Fv molecules are not. Instead, the Fv molecules adhere to membranes through their attachment to the membrane-embedded channels.

The 2D crystals were then prepared for analysis under cryogenic conditions (details in SI Methods). Both image data and electron diffraction data were collected from untitled crystals (Fig. 1 B and C, respectively). We then performed crystallographic analysis as described by Henderson and coworkers (29–31). Some of the large 2D crystals yielded data to ∼5 Å resolution (Fig. S3). When data from multiple 2D crystals were merged, reliable phase information to 6.5- to 7-Å resolution was obtained (Table S1). Crystallographic analysis revealed the unit cell parameters of \( a = b = 175 \, \text{Å} \) and \( \gamma = 90^\circ \). Our 2D crystals adopted the planar group symmetry \( P4_2 \) (Table S2), requiring the channel proteins to alternate their orientations regularly in a bilayer membrane (Fig. 1 D and E). A projection map of one channel is presented in Fig. 1D with its extracellular side facing the reader. Details on the handedness determination, helix assignment, and the exclusion of possible crystallographic artifacts are elaborated in the SI Methods.

We built a tentative structural model of the KvAP in membranes that best fits the experimental projection map. The structural model was based on the KvAP X-ray structures and its single-particle reconstruction by cryo-EM, as well as the functional studies of the KvAP and Shaker-like channels in membranes (14, 22, 32–34) (leftmost panels in Fig. 2 A and B). The position of the voltage sensor helices was modified manually in Coot (35) to overlay the experimental density map. After rigid-body refinement, we obtained a structural model that closely resembles the experimental map (Fig. 1E and Fig. S4). A complete unit cell with the structural models embedded in the density is presented in Fig. 1E. The channel molecule at the center of the unit cell has its extracellular side facing the reader while the surrounding four channels are oriented with their intracellular side facing the reader (Fig. 1E, gold versus red). The Fv molecules are found between channels where they mediate crystal contacts (gray density in a schematic rendition in Fig. 1E). Because our data show that the channels are membrane-embedded, but the Fv molecules are not (Fig. S2), the neighboring KvAP channels must have no direct crystal contact with each other inside the membrane and are instead completely surrounded by lipids. The density for one channel viewed from the extracellular side is extracted for further analysis (the leftmost panel in Fig. 24).

Voltage Sensor Ring Formed by Four Voltage Sensor Domains in Membrane. The membrane-embedded KvAP appears structurally different from all X-ray structures of Kv and Nav channels determined to date (14–19, 36). In the projection structure of the KvAP channel in membrane, each channel is composed of two concentric rings of density (Fig. 24). The inner density belongs to the pore domain with the ion-conducting pathway at the very center (Fig. 2 A and B). This structure is reminiscent of the pore structure observed previously in a projection map calculated for the 2D crystals of the KcsA channel in membrane (37). An outer ring of density, likely belonging to the four VSDs, surrounds the
pore domain of the KvAP molecule (Fig. 2A). From the distribution of electron density, it is clear that the voltage-sensor helices are evenly arranged around the channel pore. X-ray structures of three voltage-gated channels are presented for comparison in Fig. 2: Kv1.2/2.1 chimera, MlotiK (a cyclic nucleotide-regulated potassium channel from Mesorhizobium loti), and NavAb (15, 16, 19). We calculated 7.0-Å projection maps from these structures and presented them in Fig. 2A. The corresponding structural models are presented in Fig. 2B. Strong peaks in the projection map were assigned to the helices because the KvAP voltage sensor has very short loops between the transmembrane segments (details in SI Methods). The striking difference between the structures of membrane- and detergent-embedded channels is schematized in Fig. 2C, which illustrates the helical arrangements of the four VSDs around the channel pore.

The four VSDs of a KvAP channel in membranes form a continuous ring of density that completely surrounds the channel pore. Henceforth, we name this structural arrangement the “voltage sensor ring.” In X-ray crystallographic studies of Kv and Nav channels in detergent, VSDs always form individual four-helix bundles as shown in Fig. 2B. However, in membrane we observed an alternative structural arrangement in which the four helices of each voltage sensor are almost evenly distributed around the channel pore (Fig. 2 and Fig. S4). The voltage sensor ring structure predicts that in a tetrameric channel the S1 and the S4 from two neighboring voltage sensors are in close proximity to each other, whereas this would not be true in two neighboring discrete four-helix bundles (Fig. 3). Second, in the voltage sensor ring the S1 and S4 helices appear to be peripherally exposed, whereas in the X-ray structures they are buried between the channel pore and the S2/S3 helices (Fig. 4). To test these predictions, we introduced cysteine residues at specified sites of the VSD in a cys-less KvAP mutant (C247S) and tested their ability to cross-link. To avoid crystallization artifacts, we performed biochemical and functional analysis as well as structural modeling to test the hypothesis that it might have resulted from crystallization artifacts. The results of our analyses were inconsistent with the crystallization artifacts being responsible for the observed structure (Figs. S5–S7 and SI Methods). Nevertheless, we expect that once a 3D map is obtained from images of crystals tilted at different angles, our structural model will be further refined and the exact register and tilting of individual helices can be determined.

We next sought to test whether the voltage sensor ring structure from our modeling represents a true, physiological state for the functional KvAP channels in membranes. The helical arrangement in the voltage sensor ring suggests two testable predictions that would differentiate it from the four-helix bundles. First, the voltage sensor ring structure predicts that in a tetrameric channel the S1 and S4 from two neighboring voltage sensors are in close proximity to each other, whereas this would not be true in two neighboring discrete four-helix bundles (Fig. 3). Second, in the voltage sensor ring the S1 and S4 helices appear to be peripherally exposed, whereas in the X-ray structures they are buried between the channel pore and the S2/S3 helices (Fig. 4). To test these predictions, we introduced cysteine residues at specific sites of the VSD in a cys-less KvAP mutant (C247S) and tested their ability to cross-link. To avoid crystallization artifacts, we reconstituted the channels without the Fv into vesicles at a low protein–lipid ratio such that the channels were free to diffuse in...
Intrachannel Cross-Linking. Introducing cysteine pairs at proper positions between the S1 and the S4 should yield cross-linked KvAP dimers in the voltage sensor ring conformation, but only KvAP monomers in VSD four-helix bundles (Fig. 3A). This is because in a voltage sensor ring the S1 from one monomer is near the S4 of a neighboring monomer and thus they should be able to cross-link. Following oxidation, a disulfide bond could form within an individual tetrameric channel, and one would expect the appearance of cross-linked dimers or even high-order oligomers (Fig. S8). However, in the four-helix bundle conformation the S1 of one monomer is more than 3 Å away from the S4 of another monomer, preventing any cross-linking between two monomers in one channel. We analyzed multiple cysteine pairs (Table S3). When the KvAP T47C/L118C double mutant was assayed, the tetrameric channels isolated by size exclusion HPLC contained a significant portion of cross-linked dimers (Fig. 3B). We obtained similar results in T47C/G108C and T47C/A111C, both of which represent the cross-linking between the S1 and S3 from neighboring voltage sensors (Table S3), indicating that the S3/S4 paddle of one VSD is adjacent to the S1 of the neighboring VSD. As negative controls, multiple pairs of cysteine residues were introduced around the T47/L118, T47/G108, or T47/A111 pairs. These did not permit good intrachannel cross-linking (Table S3), nor did the single-cysteine mutants (e.g., Fig. 3C). Under strong oxidizing conditions, cross-linked trimers and even tetramers were observed together with substantial protein aggregation (e.g., Fig. S8). In all cases, mutant channels were fully functional under reducing conditions, suggesting that the cross-linking results are physiologically relevant (Fig. 3D). These data suggest that the voltage sensor ring likely exists in membrane in which the S1 and the S3/S4 (paddle) from two neighboring subunits face each other in close proximity.

Interchannel Cross-Linking. In the voltage sensor ring, the peripheral surface of the S1 and the S4 is exposed to the surrounding lipids. Thus, these two helices from two independent channel tetramers in the same membrane could come together and become cross-linked if cysteine residues are introduced in the right positions. However, in the four-helix bundle model, both the S1 and the S4 are buried, and should not allow interchannel cross-linking due to steric hindrance from the S2/S3 (Fig. 4A). We systematically introduced single cysteine residues into different positions of the S1 and S4 and tested their peripheral accessibility by examining interchannel cross-linking (Table S4). For membranes. Using the C247S mutant, which functions the same as the native wild-type channel (Fig. S1D), allowed us to analyze both disulfide bond formation and channel function. We analyzed disulfide bond formation in reconstituted channels using mild air oxidation (Fig. S8 and SI Methods).

Fig. 3. Intrachannel cross-linking: close proximity of the S1 and S4 in a voltage sensor ring. (A) Schematic presentation of T47 in S1 and L118 in S4 in voltage sensor ring and the four-helix bundle model, respectively. Intrachannel S-S bonds can only form in voltage sensor ring. (B) Upper) Size-exclusion HPLC purified tetrameric KvAP. (Lower) Nonreducing SDS-PAGE of KvAP tetramers that were air-oxidized in vesicles, blocked, and extracted with detergents before gel filtration. Lane 1, molecular weight (MW) markers; lane 2, sample before gel filtration; lanes 3–10, HPLC fractions of the tetramer peak. Intrachannel cross-linked dimers seen in lanes 6–10. More sites analyzed and listed in Table S3. (C) No intrachannel cross-linking for single cysteine mutants T47C or L118C. (D) T47C/L118C in bilayers showed voltage-dependent activities (Left) under reducing conditions. Holding potential, −80 mV; pulses, −20 to +60 mV at 20-mV steps. Oxidized channels had very little activity. Recordings from T47C and L118C are shown as control.

Fig. 4. Interchannel cross-linking: peripheral accessibility of the S1 and S4. (A) Schematic representation of F124C with the central pore complex surrounding the S3 paddle. (B) Upper) Size-exclusion HPLC separating KvAP octamers from tetramers. (Lower) Nonreducing SDS-PAGE of the octamers. Lane 1, molecular weight (MW) markers; lane 2, sample before gel filtration; lanes 3–10, HPLC fractions. Interchannel cross-linked dimers seen in the octamer peaks. More sites analyzed and listed in Table S4. Control sites on S2, S3, and S5 are also there. (C) Three sites near F124C (L122C and L125C on S4; G108C on S3) failed to cross-link two channels into an octamer. (Upper) Oxidized G108C assayed after gel filtration (silver-stained). (Lower) L122C and L125C mutants in vesicles before and after oxidation were assayed in Coomassie blue-stained nonreducing SDS-PAGE gels. (D) F124C currents in lipid bilayers under reducing conditions. Holding potential, −80 mV; pulses: −20 to +60 mV at 20-mV steps. Currents from G108C and L125C are shown as controls.
example, F124 is a residue located within the extracellular half of the S4 helix. In the four-helix bundle structural model of the KvAP channel (Fig. S1C), F124 is buried and physically segregated from its equivalent sites in other channels such that it could not form interchannel disulfide bonds, either in the same bilayer or between two membranes. However, following weak air oxidation, F124C mutants reconstituted in membranes were found to form interchannel disulfides as a significant fraction of KvAP octamers were observed (Fig. 4B). The same did not happen in several positions next to the F124 residue (Fig. 4C), suggesting strong site specificity. The F124C channel was fully functional in the reduced state, indicating that the results are physiologically significant (Fig. 4D). After cross-linking, the mutant channel became nonfunctional, probably due to the impaired voltage sensor movement. Likewise, the V39C mutation, which is located in the extracellular half of the S1, yielded similar results to those of the F124C (Table S4). When multiple sites in the extracellular halves of the S1 and S4 were compared with each other, only a few sites were able to support disulfide bond formation between two tetrameric channels. The apparent helical periodicity in the peripheral accessibility of these sites is consistent with the two helices adopting α-helical structures in membranes. More importantly, our results demonstrate that, in membranes, the S1 and S4 helices are not buried as in the four-helix bundle model of the VSD; instead they are peripherally exposed as indicated by the voltage sensor ring (Fig. 2C).

**Influence of Lipids on the Structures of the Voltage-Gated Ion Channels.** Our data argue strongly that the voltage sensor ring seen in the 2D crystals represents a physiological, native conformation of the KvAP channel in membranes, where the channels are in steady-state inactivation at 0 mV. Because our 2D crystals were grown in the presence of Fv, the channel was stabilized in a defined conformation: the voltage sensors are in the up state and the channel pore in the “inactivated” state (Fig. S1). Thus, the KvAP in the inactivated state adopts a structure that is significantly different from those of all voltage-gated ion channels so far crystallized in detergents in the absence of a true bilayer membrane (14–19) (Fig. 2). In membrane, lipids surround the helices of the voltage sensor (S1–S4) such that the inner surface of the voltage sensor ring is in full contact with the channel pore, whereas the outer surface of the ring constitutes a robust interface with the surrounding lipids.

Our KvAP 2D crystals were grown in the presence of the Fv. In fact, the Fv molecules mediate all crystal contacts (Fig. 1E). A potential concern is that the voltage sensor ring structure could have been stabilized by the Fv, rather than representing a physiologically relevant conformation. Our data argue against this possibility. First, the Fv is not membrane embedded, whereas the KvAP is. The Fv molecules can be stripped from the membranes following incubation with sodium carbonate (Fig. S2). Second, a set of systematic cross-linking experiments were performed on freely diffusing KvAP channels in membrane, (noncrystalline with 15× higher lipid concentration than in the crystallization condition), and without any Fv (Figs. 3 and 4). If the voltage sensor ring structure were an artifact either of 2D crystallization or of Fv binding, we would expect no cross-linking under these conditions. Therefore, although a 3D dataset is still not available, our current data suggest that the tentative structure of the voltage sensor ring of the KvAP channel does exist in membranes, and is a structural arrangement that is probably stabilized by lipids, representing a physiological conformation (the inactivated state) in a native channel.

Previous studies based on electron paramagnetic resonance (EPR) measurements suggested strong lipid exposure of both V39 (S1) and F124 (S4) in the wild-type KvAP channels in membranes (39, 40). The results were interpreted by assuming a 180° rotation of the VSD helix bundles with respect to the channel pore, or by introducing a lipid-filled gap between the pore and the VSDs. Interpretation of the EPR data in light of the new voltage sensor ring does not require a 180° rotation. In fact, in the voltage sensor ring, the side chains of both V39 and F124 are exposed to peripheral lipids (and both could cross-link two independent channels; Table S4). Moreover, in both the KvAP and the Shaker channels the distances between each of the four VSD helices and the center of the channel pore in membrane-embedded channels, as determined by double electron electron resonance and fluorescence resonance energy transfer (as well as luminescence resonance energy transfer), indicate that the four helices (S1–S4) are roughly equidistant to the pore (41–43). Within the context of the four-helix bundle model, these results are quite perplexing (43). Indeed, in the four-helix bundle, both the S1 and the S4 are much closer to the channel pore than the S2 and S3, whereas in the voltage sensor ring, the S1–S4 helices are approximately equidistant from the channel pore.

Similarly, prior studies in Shaker-like Kv channels have identified sites on the S1 and S4 that move close to each other in a voltage-dependent manner or promote complete dimerization after strong oxidation (44–48). Again, these observations are puzzling within the context of the four-helix bundle structures, where both the S1 and the S4 are shielded from the periphery as well as from neighboring subunits. One possibility that was proposed was that the VSD four-helix bundles rotate by up to 180° to bring the S1 or the S4 in one VSD close to the equivalent sites in another VSD (either the same channel or another channel). This explanation inevitably introduces a great deal of flexibility to individual four-helix bundles, which would compromise the tight coupling between the voltage sensors and the pore (Fig. S1). In sharp contrast, the helical arrangement in the voltage sensor ring makes both S1 and S4 accessible to the periphery and permits them to form disulfide bonds at proper positions with another tetrameric channel. Thus, the voltage sensor ring structure offers a reasonable explanation of previous observations without requiring the detachment of the VSDs from the pore. Additionally, the large interface between the voltage sensor ring and the pore domain guarantees their tight coupling during gating transitions.

So why the discrepancy between the four-helix bundle structure, seen in detergents, and the voltage sensor ring observed in a membrane-embedded channel? It is difficult to argue that the structure of a channel in a lipid bilayer is not physiologically relevant. Likewise, it is hard to argue that such a structure is less viable than crystal structures of membrane proteins in mixed detergent micelles. Our studies indicate that lipid–protein interactions are important for both the structure and function of the KvAP channel, and this scenario is likely true for many other membrane proteins. It is possible that detergent micelles, although critical in solubilization and purification of membrane proteins, do not mimic genuine lipid bilayers, nor can they fully satisfy the
intricate lipid–protein interactions that occur in nature and, at least in some cases, appear to be critical for membrane protein structure and function (49).

In the case of the KvAP channel, and perhaps other voltage-gated ion channels, we propose that lipid removal by detergent extraction may allow the strong hydrophobic interactions to drive the S1–S4 to collapse onto one another and form a four-helix bundle in detergent micelles. The lack of full lipid–protein interaction and the strong hydrophobic interactions between the S1–S4 helices may introduce a drastic lateral helical rearrangement from a voltage sensor ring into the four-helix bundles (Fig. 5). The four-helix bundle VSDs may represent the most stable conformation outside the membrane and hence prevail in all X-ray structures available to date. However, for channels fully embedded in membranes the voltage sensor ring likely represents the most stable structure of the inactivated channels. This scenario remains to be further refined experimentally with a 3D map, and expanded to other membrane proteins, especially those that are known to undergo significant allosteric conformational changes to perform their function.

Methods
Recombinant KvAP channel and its mutants were produced and reconstituted in lipid vesicles. The KvAPpv complexes were purified and mixed with lipids for 2D crystallization. The 2D crystals were embedded for cryoEM data collection. Electrical recordings were made from channels in bilayer lipid membranes. Details on experimental materials and methods are presented in SI Methods.

ACKNOWLEDGMENTS. We thank Dr. Roderick MacKinnon (The Rockefeller University) for his generous support in the early stage of the 2D crystallization of the KvAP in membranes, and Drs. Da-Neng Wang (New York University School of Medicine), David Stokes (New York University Structural Biology Center), Thomas Walz (Harvard Medical School), Yoshinori Fujiyoshi (Kyoto University), and Wah Chiu (Baylor College of Medicine) for offering support and advice in the project development and technical modifications, and providing access to their microscopes. We thank Dr. Puey Ounjai (currently at Berkeley National Laboratory) for performing some useful tests in improving the crystal quality and in cryoprotecting 2D crystals, and Dr. Anchi Cheng (The Scripps Research Institute) for her valuable advice in symmetrizing and testing during our crystallographic analysis. Dr. Richard Aldrich at University of Texas–Austin, Dr. Kenton Swartz at National Institutes of Health/National Institute of Neurological Disorders and Stroke, and Drs. Lily Huang, Lora Hooper, Youying Jiang, Sandra Schmid, and Michael Rosen at University of Texas Southwestern offered valuable comments on the manuscript. This research project has been supported by National Institutes of Health Grants R01GM088745 and R01GM093271 (to Q.-X.J.), American Heart Association Grant 12IRG900019 (to Q.-X.J.), and Welch Foundation Grant I-1684 (to Q.-X.J.). The T.G. laboratory is supported by the Howard Hughes Medical Institute.