Phospholemman Transmembrane Structure Reveals Potential Interactions with Na\(^+\)/K\(^+\)-ATPase

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Phospholemman (PLM) is a 72-residue bitopic cardiac transmembrane protein, which acts as a modulator of the Na\(^+\)/K\(^+\)-ATPase and the Na\(^+\)/Ca\(^2+\) exchanger and possibly forms taurine channels in nonheart tissue. This work presents a high resolution structural model obtained from a combination of site-specific infrared spectroscopy and experimentally constrained high throughput molecular dynamics (MD) simulations. Altogether, 37 experimental constraints, including nine long range orientational constraints, have been used during MD simulations in an explicit lipid bilayer/water system. The resulting tetrameric \(\alpha\)-helical bundle has an average helix tilt of 7.3\(^\circ\) and a crossing angle close to 0\(^\circ\). It does not reveal a hydrophilic pore, but instead strong interactions between various residues occlude any pore. The helix-helix packing is unusual, with Gly\(^{19}\) and Gly\(^{20}\) pointing to the outside of the helical bundle, facilitating potential interaction with other transmembrane proteins, thus providing a structural basis for the modulatory effect of PLM on the Na\(^+\)/K\(^+\)-ATPase. A two-stage model of interaction between PLM and the Na\(^+\)/K\(^+\)-ATPase is discussed involving PLM-ATPase interaction and subsequent formation of an unstable PLM trimer, which readily interacts with surrounding ATPase molecules. Further unconstrained MD simulations identified other packing models of PLM, one of which could potentially undergo a conformational transition to an open pore.

Human phospholemman (PLM)\(^2\) is a member of a family of single-span transmembrane proteins characterized by the invariant extracellular motif FXYD (1) and is also known as FXYD1. It is found in liver, skeletal muscle, and most abundantly in the cardiac sarcolemma (2). Over recent years, conclusive experimental evidence has been presented that PLM acts as a tissue-specific modulator of the Na\(^+\)/K\(^+\)-ATPase similar to the other members of the FXYD family, each of which is prevalent in a different tissue, summarized in several recent reviews (3, 4). The Na\(^+\)/K\(^+\)-ATPase consists of a catalytic \(\alpha\)-subunit with 10 transmembrane segments and a \(\beta\)-subunit involved in membrane insertion and as a modulator of transport properties (5). In particular, cross-linking and modeling studies revealed the direct interaction between the Na\(^+\)/K\(^+\)-ATPase \(\alpha\)-subunit and members of the FXYD family (\(\gamma\) and CHIF), which involves the binding of a single transmembrane domain into a groove formed by M2, M6, and M9 transmembrane helices of the Na\(^+\)/K\(^+\)-ATPase (6, 7). PLM appears to be a major control point in the function of heart cells, although its specific physiological role is unclear, complicated by the possibility that its regulatory effect on the Na\(^+\)/K\(^+\)-ATPase may depend on the phosphorylation state of PLM (8) as well as other less well established functional roles (e.g. interaction with the Na/Ca\(^2+\) exchanger (9, 10) or independent channel formation). PLM knock-out mice expressed a complex response, including increased cardiac mass, larger cardiomyocytes, and ejection fractions in the absence of hypertension, whereas the overall Na\(^+\)/K\(^+\)-ATPase activity was reduced by 50% (11). There is growing evidence that PLM regulates the activity of Na/Ca\(^2+\) exchanger 1 (NCX1) from heterologous expression studies of PLM and NCX1 in HEK239 cells (10) and overexpression of PLM in rat cardiomyocytes (12, 13) using different approaches to measure NCX1 activity. It appears that phosphorylation of PLM abolishes its inhibitory effect on the Na\(^+\)/K\(^+\)-ATPase (14, 15), whereas the phosphorylated form of PLM inhibits NCX1 (9, 12).

Electrical measurements of PLM in artificial lipid bilayers and oocytes showed that PLM facilitates the membrane flux of ions (16) and taurine transport (17), which leads to the possibility that PLM has a function in the regulation of cell volume either as a modulator of a swelling-activated signal transduction pathway or directly facilitating osmolyte influx in noncardiac tissues (18).

The three-dimensional structure of PLM is not known, but on the basis of hydrophathy analysis of the amino acid sequence, a transmembrane domain from residue 18 to 37 has been predicted (2) and confirmed later by attenuated total reflection (ATR) FTIR spectroscopy (19). The C terminus is located intracellularly, resistant to protease digestion, whereas the N terminus is located extracellularly, as shown by antibody labeling (20). A PLM peptide of residues 12–39 comprising the transmembrane domain forms \(\alpha\)-helical tetramers in lipid bilayers, which are tilted by less than 17°
against the membrane normal (19). Here we present a detailed three-dimensional structure of the tetrameric transmembrane domain based on site-specific infrared dichroism (SSID) (21, 22) combined with a recently developed conformational search based on experimentally constrained high throughput molecular dynamics (MD) simulations in an explicit lipid bilayer (23, 24).

**EXPERIMENTAL PROCEDURES**

*Preparation of Labeled Peptides—*1-13C-Labeled amino acids were obtained from Cambridge Isotope Laboratories (Cambridge, MA) and further labeled by reaction with H318O catalyzed by gaseous hydrochloric acid (25, 26). The labeled amino acids were then Fmoc-protected by a standard procedure using Fmoc-N-succinimidyl-carbonate (27).

Ten peptides of the transmembrane domain sequence of PLM (residues 12–39 DYQSLQIGLVIAGILFLGILVLSRR) were obtained from Keck Laboratories (Yale University). Each peptide contains one 1-13C=18O-labeled residue in the positions shown in boldface type.

*Preparation of Proteoliposomes—*Peptides were mixed with dimyristoylphosphocholine lipids in hexafluoroisopropyl alcohol at a ratio of ~1:30 (w/w). Liposomes were then made by complete evaporation of the hexafluoroisopropyl alcohol and the addition of water to a lipid concentration of 10 mg/ml. The lipid suspension was then sonicated and subjected to a freeze-thaw cycle three times.

*Infrared Spectroscopy—*The liposome suspensions were deposited onto a trapezoidal germanium refraction element (50 × 2 × 20 mm), and bulk water was removed under a stream of nitrogen, forming a thick film compared with the penetration depth of the IR light on the element.

ATR FTIR spectra were obtained using a Bruker Tensor FTIR spectrometer (Bruker Optics, Coventry, UK) with a high sensitivity liquid nitrogen-cooled MCT/A detector. One spectrum was calculated from the average of 1000 interferograms at a resolution of 2 cm⁻¹ per data point in the 4000 to 1000 cm⁻¹ range of the electromagnetic spectrum. In each case, the spectrum was recorded at parallel and perpendicular polarization to the plane of incidence, and the dichroic ratio was calculated as the ratio between the integrated absorption of parallel and perpendicular polarized light for the amide I absorption band centered at a wave number of 1658 cm⁻¹ and for the 13C=18O absorption band centered at 1594 cm⁻¹. For each labeled peptide, at least three independent measurements were taken.

*Data Analysis—*The spectra were analyzed according to the theory of SSID presented elsewhere (22, 28, 29). Briefly, the dichroic ratios of the amide I absorption band and the absorption band of the label for two peptides with labels in positions 1 and 2 were analyzed together in order to obtain the local helix tilt angle β and the rotational pitch angle ω for the label in position 1. Another peptide with a label in position 3 was analyzed in order to give the orientational parameters for position 2, and so on. In that way, the following pairs of labels were analyzed: Gly20/Leu21, Leu21/Val22, Val22/Ile23, Ile23/Gly25, Gly25/Ile26, Ile26/Leu27, Leu27/Gly31, Gly31/Ile32, Ile32/Leu33.

The angle between the C=O bond and the z axis θ used as orientational constraint was calculated as \( \cos \theta = \cos \alpha \cos \beta - \sin \alpha \sin \beta \cos(\omega + 17°) \), with \( \alpha = 180° - 38° = 142° \) denoting the angle between the transition dipole moment and the molecular director given by Marsh et al. (30). All calculations were carried out using the Mathematica 5 software (Wolfram Research, Champaign, IL).

*High Throughput Molecular Dynamics Conformational Searching—*The conformational search of PLM tetrameric helical bundles was carried out as described elsewhere for a dimeric system (23, 24). Briefly, α-helical bundles were generated by symmetrically rotating the helices in 10° increments in order to produce an array of starting structures for MD simulation. Both left- and right-handed crossing bundles (crossing angle of ±25°) were generated, giving 72 different orientations, with each being simulated at four different initial random velocities, resulting in a total of 288 starting structures. Each structure was subjected to a 200-ps MD simulation using the GROMACS software (31) in a dimyristoylphosphocholine bilayer/water system containing 128 dimyristoylphosphocholine molecules and 3655 water molecules. The initial coordinates of the dimyristoylphosphocholine bilayer were obtained from Gurtovenko et al. (32). Constraints were placed on each α-helical bundle, and the angle between the z axis and the C=O bond of the label was constrained to the derived value from SSID. The local tilt angle was also constrained by defining the angle between the z axis and a vector between the Ca atoms of the residue in question and a residue seven positions along in the helix. Also, since the peptide is known to be α-helical (as detected from IR), the distances between hydrogen-bonding pairs of amine hydrogens and carbonyl oxygens were constrained between 1.6 and 2.3 nm, and a harmonic potential was applied outside these boundaries. All constraints were added to the GROMACS force field with a force constant of 5000 kJ mol⁻¹. For the unconstrained search, the same procedure was followed without applying any constraints. All MD simulations were performed in parallel on an eight-node dual 2.66-GHz Xeon processor cluster (Streamline Computing, Warwick, UK).

*Clustering of Structures—*Ca root mean square deviation comparisons were made between all resulting structures from the search. Structures were then clustered according to their root mean square deviation values. The adopted criteria for structure clustering were that of a maximum root mean square deviation value of 2 Å between two structures. For a cluster to be created, a minimum of five structures possessing root mean square deviation values within this limit was required.

The structures contributing to this cluster were then used to create an average structure, which was subjected to experimentally constrained MD simulation for 2 ns in a lipid bilayer followed by energy minimization.

**RESULTS**

*ATR Infrared Spectroscopy—*Altogether, 10 PLM transmembrane peptides containing residues 12–39 with 13C=18O-labeled residues in the positions indicated in Fig. 1...
have been reconstituted in dimyrystoylphosphocholine vesicles and analyzed by polarized attenuated total reflection infrared spectroscopy. The symmetric amide I absorption band centered at ~1658 cm\(^{-1}\) revealed that in all cases, the peptide adopted a predominantly \(\alpha\)-helical conformation (33), confirming earlier observations (19). The absorption band of the labeled sites centered at 1594 cm\(^{-1}\) showed that the label is also in an \(\alpha\)-helical environment (26). In some cases, a shoulder existed at 1618 cm\(^{-1}\) (Fig. 1) representing the absorption of a residue with a \(^{13}\)C-\(^{16}\)O carbonyl bond, which is caused by incomplete labeling of the amino acid with \(\mathrm{H}_2\mathrm{^{18}}\)O prior to peptide synthesis. However, in all cases, the \(^{13}\)C-\(^{18}\)O bond was of sufficient intensity for analysis.

The dichroic ratio of the amide I peak varied from 2.5 to 4.2, depending on sample order, whereas the dichroic ratio of the \(^{13}\)C-\(^{18}\)O peak varied from 2.1 to 5.8, depending on position and sample order. The dichroic ratio of the label is in all cases different from the dichroic ratio of the unlabeled amide I peak, which is indicative of oligomerization. A label in a monomeric helix would adopt an average dichroic ratio similar to the unlabeled residues due to rotational symmetry with respect to the membrane normal. The local helix tilt shows variation between (3 ± 4\(^\circ\)) and (10 ± 4\(^\circ\)), whereas the change in rotational pitch angle \(\omega\) is in the region of 100° for successive residues in accordance with \(\alpha\)-helical geometry (Table 1). The angle between the C=O bond and the z axis, calculated from \(\omega\) and \(\beta\), is used as an experimental constraint for the MD simulation.

**TABLE 1**

Experimentally determined orientations: rotational pitch angle \(\omega\), local tilt angle \(\beta\), and the derived angle of the carbonyl bond to the z axis \(\theta\) for all labels in the transmembrane domain, calculated from the SSID data.

| Label   | \(\beta\) (degrees) | \(\omega\) (degrees) | \(\theta\) (degrees) |
|---------|---------------------|---------------------|---------------------|
| Gly\(^{20}\) | 8.9                 | 64                  | 40.2                |
| Leu\(^{21}\) | 8.3                 | 119                 | 44.3                |
| Val\(^{22}\) | 4.9                 | 235                 | 36.8                |
| Ile\(^{23}\) | 8.8                 | 327                 | 46.6                |
| Gly\(^{25}\) | 8.7                 | 206                 | 32.0                |
| Ile\(^{26}\) | 10.1                | 302                 | 46.0                |
| Leu\(^{27}\) | 6.2                 | 15                  | 43.4                |
| Gly\(^{31}\) | 2.5                 | 213                 | 36.4                |
| Ile\(^{32}\) | 7.3                 | 298                 | 43.4                |

**FIGURE 1.** Infrared spectra for each peptide containing \(^{13}\)C-\(^{18}\)O label in the position indicated in the top right. The amide I area is displayed, whereas the inset shows the magnified amide I absorption band of the label.

**Constrained High Throughput MD**—At each stage of the MD simulation and energy minimization, a total of 37 experimental constraints have been applied, composed of nine orientational constraints obtained from SSID of 10 labeled peptides (Table 1), in order to set the local helix tilt and distance constraints between the carbonyl oxygen of residue \(n\) and the amide hydrogen of residue \(n + 4\) in order to maintain the experimentally determined \(\alpha\)-helical geometry, although unconstrained simulations of helices in lipid bilayers have shown that \(\alpha\)-helices are retained even after long term MD simulations (23, 24). Following the MD simulation...
of 72 different starting structures, each at four different random initial atom velocities (288 simulations), a cluster analysis provided six structures (Fig. 2). Of these, structure 6 showed the closest agreement with the rotational pitch angles obtained from SSID experiments (Table 2), although structure 6 was of slightly higher energy than other structures. It is generally accepted that similarity to experimental

Table 2
Comparison of rotational pitch angles from SSID with the structures produced from the MD search

| Label | \( \theta_{\exp} \) | \( \theta_{\text{model}1} \) | \( \theta_{\text{model}2} \) | \( \theta_{\text{model}3} \) | \( \theta_{\text{model}4} \) | \( \theta_{\text{model}5} \) | \( \theta_{\text{model}6} \) |
|-------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Gly\(^{20}\) | 64 | 57 | 259 | 322 | 10 | 203 | 313 |
| Leu\(^{21}\) | 119 | 243 | 341 | 52 | 71 | 340 | 27 |
| Val\(^{22}\) | 235 | 334 | 92 | 160 | 172 | 31 | 126 |
| Ile\(^{23}\) | 327 | 46 | 200 | 352 | 301 | 240 | 304 |
| Gly\(^{25}\) | 206 | 347 | 49 | 23 | 12 | 147 | 177 |
| Ile\(^{26}\) | 302 | 294 | 355 | 151 | 9 | 338 | 315 |
| Leu\(^{27}\) | 15 | 352 | 19 | 10 | 21 | 54 | 62 |
| Gly\(^{31}\) | 213 | 168 | 108 | 117 | 85 | 154 | 156 |
| Ile\(^{32}\) | 298 | 271 | 250 | 279 | 207 | 262 | 249 |
| Deviation | 1069 | 1054 | 879 | 794 | 880 | 669 |

In the transmembrane region, the residues in the helices adopt \( \alpha \)-helical geometry throughout, but close to the termini, some are locally distorted. This region does not possess any constraints, and therefore this effect is most likely due to the greater freedom of movement these residues will have. The center of structure 6 is occluded by interactions between residues Tyr\(^{27}\), Gln\(^{31}\), Leu\(^{35}\), Phe\(^{42}\), Ile\(^{46}\), Leu\(^{50}\), and Arg\(^{53}\).

### Unconstrained High Throughput MD
In order to explore the range of all possible packing models of PLM in lipid bilayers, we performed a high throughput MD search without any constraints applied. In this simulation, we found nine clusters of structures and calculated an average structure for each cluster (Fig. 4A). The starting structure for the helix rotation \( \phi = 0^\circ \) is the same as shown in Fig. 2B. Each cluster average structure reveals a well packed \( \alpha \)-helical tetramer. In order to find a model that would support the experiments of PLM ion channel activity, we analyzed visually each structure for the possibility of the formation of a hydrophilic pathway through the center of the tetramer. For most structures, the center of the tetramer is completely occluded by interactions between several residues, but in structure number 4 only the side chains of Val\(^{34}\) and Tyr\(^{27}\) occlude the center of the structure as shown in Fig. 4B, whereas other side chains are either pointing to the outside or are involved in close packing interaction at the helix-helix interface. Tyr\(^{27}\) is located in the water phase at the N-terminal end of the peptide and shows a high conformational flexibility in MD simulations and thus would not contribute significantly to occlusion of the pore, whereas Val\(^{34}\) is located in the transmembrane domain. Structure number 4 has the lowest energy of all unconstrained packing models of \(-1299\) kJ, whereas the next lowest energy structure is structure 3 with \(-1253\) kJ.

Figure 2. A, polar plot of the energies of structures obtained from MD simulation of PLM in dependence of the helix rotation angle \( \phi \) changing clockwise from 0 to 360°. The distance from the center indicates negative energy (E) in kJ/mol. Each individual structure is indicated by a triangle, whereas the clustered averages are shown as numbered circles. The arcs represent the movement of structures from their starting positions with respect to the helix rotation angle \( \phi \) during the MD simulation. B, the rotation of the helix tetramer defined as \( \phi = 0 \), Phe\(^{48}\) residues shown in wire frame.
**DISCUSSION**

**Constrained High Throughput MD Simulation**—The conformational search combining MD simulations in a realistic environment with long range orientational constraints allowed us to obtain an experimentally defined structural model of the PLM tetrameric transmembrane domain with atomic detail.

However, a comparison of the rotational pitch angles between the experiment and the best model structure reveals some deviations (Table 2). This is caused on the one hand by the interplay between the experimental constraints and the tendency of the structure to adopt an energy minimum in the “imperfect” computational force field, which also takes into account side chain packing and protein-lipid interaction, for which we have no experimental data. On the other hand, the rotational pitch angle for a residue is a derived parameter assuming ideal α-helix geometry, which is not the case for PLM and other helices; thus, calculation of this angle leads to deviations, whereas the angles between the C=O bond and the z axis used as actual constraints in the simulation were matched precisely (data not shown). However, since the rotational pitch angle of a residue defines the interaction side of helices in a bundle, comparisons based on this angle have been proven most reliable, even allowing the determination of the preferred oligomerization state (34).

**PLM as Na+/K+-ATPase Regulator**—PLM has three regions of its transmembrane sequence conserved in human, canine, mouse, and rat versions of the protein (1, 35, 36), residues 18–21 (IGGL), residues 23–34 (IAGILFILGILI), and residues 36 and 37 (LS). Val22 is mutated to Thr in mice and rats and to Ile in canine species, whereas Val35 is maintained in canine and mutated to Ile in mice and rats. In our model, the residues Ile18, Gly19, and Gly20 all point outward toward the lipid environment and therefore do not form interhelical interactions; however, the Leu21 residues are in the interior of the α-helical complex and appear to pack uniformly. The Leu36 and Ser37 residues protrude outward into the surrounding environment; however, this region is in the water phase. In the residue 23–34 region, the Gly25, Phe28, and Ile32 residues form the interhelical binding motif. Perhaps surprisingly, only one glycine residue packs in the interior of the complex, whereas all others point outward into the lipid environment. One possible explanation is that this arrangement allows PLM to interact with other transmembrane proteins (e.g. the Na+/K+-ATPase). Of the nonconserved residues, Val22 and Val35 are in the interfacial region.

In our PLM model, the Phe28 residues reside in the interior of the complex. Interestingly, previous experimental cross-linking studies of the related γ-subunit identified a Phe residue that was in close proximity to neighboring helices of the Na+/K+-ATPase (6). Also, Leu42 and Ile43 were identified from modeling studies (in the γ-subunit) to be close to other helices in this complex. These residues are conserved in PLM (as Leu33 and Ile34). In the PLM model presented here, both of these residues protrude into the lipid environment. The biological consequences of these findings are that even in its oligomeric form, PLM would be able to interact with the Na+/K+-ATPase, either by stable interaction of the whole PLM tetramer or by forming an intermediate complex from which a PLM monomer is abstracted, leaving behind a trimer.

**Two-stage Model of PLM-Na+/K+-ATPase Interaction**—Based on the structure obtained in this report and earlier
investigations (19), we present a model of interaction between PLM and the Na\(^+/K^+\)-ATPase that involves a transient intermediate complex between the PLM tetramer and the Na\(^+/K^+\)-ATPase. This interaction provides the energy to strip out a subunit of the tetramer in a slow process followed by a fast step of association between the remaining PLM trimer and surrounding Na\(^+/K^+\)-ATPase molecules (Fig. 5). The molecular surface of the PLM tetramer presents a hole at positions Gly\(^{19}\) and Gly\(^{20}\) (Fig. 5A), which would enable PLM to pack closely against other transmembrane helices in this region. On the other hand, Leu\(^{33}\) and Ile\(^{34}\) are pointing to the outside of the PLM tetramer, and those residues are believed to be in close proximity to the Na\(^+/K^+\)-ATPase transmembrane helices (in the related γ-subunit (6)). Thus, we postulate that the PLM tetramer is able to interact with the Na\(^+/K^+\)-ATPase at a groove formed by M2, M6, and M9 transmembrane helices (Fig. 5B). Also, the Phe\(^{28}\) was identified by cross-linking experiments (in the related γ-subunit) to be in close proximity to the Na\(^+/K^+\)-ATPase, whereas our PLM tetramer model would not allow the formation of cross-links to other residues of interacting transmembrane helices. Phe\(^{28}\) in our model is buried in the interior of the helical bundle (Fig. 3C). Thus, we must postulate either a conformational change or the dissociation of a PLM trimer from the ATPase-PLM tetramer complex. The latter possibility is supported by the experimental evidence that a PLM monomer co-immunoprecipitates with the ATPase (10). Oligomerization studies did not show the existence of a PLM trimer (19); thus, it would be unstable and readily interact in a second fast stage with other Na\(^+/K^+\)-ATPase molecules in the lipid membrane (Fig. 5B). The biological advantage of our model is a very responsive mode of control of the Na\(^+/K^+\)-ATPase by PLM reminiscent of a positive cooperative effect. Once PLM-ATPase interaction has been initiated, by a mechanism that remain to be investigated, PLM interacts fast and efficiently with ATPase molecules in the surroundings, which is facilitated by clustering of the Na\(^+/K^+\)-ATPase molecules observed by fluorescence microscopy and immunogold electron microscopy (37).

**PLM as a Hydrophilic Channel**—The possibility of the formation of a hydrophilic channel by PLM for ions or taurine has been discussed in the literature (16–18). Visual analysis of the experimentally obtained PLM model shows that no noticeable cavity exists and certainly not one that would accommodate a taurine molecule that has a diameter of >2.2 Å (38, 39) (also shown in Fig. 3D for comparison). There are strong interactions between various residues occluding the central pore of the tetramer; thus, we believe that any conformational change to an open pore would require major structural rearrangements and disruption of interresidue interactions, which are incompatible with the typical delicate balance between open-close conformations in voltage- or ligand-gated transmembrane channels. However, it has been shown that hyperpolarization activates anion currents through PLM (40), and based on further experiments with embryonic kidney cells, it has been hypothesized that PLM may facilitate osmolyte influx in renal tissue (18). Indeed, the clustering of four negative charges at the extracellular entrance would provide a binding site for the zwitterionic osmolyte taurine. In order to explore other possible packing models of PLM not encountered in the ATR-FTIR experiments, we performed an unconstrained MD conformational search. Among nine packing models, the lowest energy structure 4 shows a narrow central pore, which is only occluded by the Val\(^{34}\) residue. Although structure 4 clearly shows a closed pore, the energy barrier for a conformational transition to an open pore might be overcome by voltage gating or binding of a ligand to the PLM molecule. We predict that mutation of Val\(^{34}\) (V34G) could abolish the gating behavior and lead to a permanently open channel. This should be explored with further electrophysiology experiments using kidney cells as well as PLM transmembrane peptides in artificial lipid bilayer systems.

In summary, the first experimental high resolution structural investigation of PLM gives credence to the hypothesis of a PLM tetramer as an inactive storage form that nevertheless allows...
very efficient regulation of the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase in a process reminiscent of a positive cooperative effect. An experimentally unconstrained modeling study identified another model of biological significance forming a potential closed conformation of a transmembrane channel.

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REFERENCES

1. Sweadner, K. J., and Rael, E. (2000) Genomics 68, 41–56
2. Palmer, C. J., Scott, B. T., and Jones, L. R. (1991) J. Biol. Chem. 266, 11126–11130
3. Geering, K. (2001) J. Bioenerg. Biomembr. 23, 387–392
4. Garty, H., and Karlish, S. I. D. (2006) Annu. Rev. Physiol. 68, 431–459
5. Geering, K. (2001) J. Bioenerg. Biomembr. 33, 425–438
6. Lindzen, M., Gottschalk, K. E., Fuzesi, M., Garty, H., and Karlish, S. J. D. (2006) J. Biol. Chem. 281, 5947–5955
7. Fuzesi, M., Gottschalk, K. E., Lindzen, M., Shainskaya, A., and Kuster, B. (2005) J. Biol. Chem. 280, 18291–19301
8. Mounsey, J. P., Lu, K. P., Patel, M. K., Chen, Z. H., Horne, L. T., John, J. E., III, Means, A. R., Jones, L. R., and Moorman, J. R. (1999) Biochim. Biophys. Acta 1451, 305–318
9. Zhang, X. Q., Ahlers, B. A., Tucker, A. L., Song, J., Wang, J., Moorman, J. R., Mounsey, I. P., Carl, L. L., Rothblum, L. L., and Cheung, J. Y. (2006) J. Biol. Chem. 281, 7784–7792
10. Ahlers, B. A., Zhang, X. Q., Moorman, J. R., Rothblum, L. I., Carl, L. L., Song, J., Wang, J., Geddis, L. M., Tucker, A. L., Mounsey, J. P., and Cheung, J. Y. (2005) J. Biol. Chem. 280, 19875–19882
11. Jia, L. G., Donnet, C., Bogaev, R. C., Blatt, R. J., McKinney, C. E., Day, K. H., Berr, S. S., Jones, L. R., Moorman, J. R., Sweadner, K. J., and Tucker, A. L. (2005) Annu. J. Physiol. 288, H1982–H1988
12. Song, J., Zhang, X. Q., Ahlers, B. A., Carl, L. L., Wang, J., Rothblum, L. I., Stahl, R. C., Mounsey, J. P., Tucker, A. L., Moorman, J. R., and Cheung, J. Y. (2005) Annu. J. Physiol. 288, H2342–H2354
13. Zhang, X. Q., Qureshi, A., Song, J., Carl, L. L., Tian, Q., Stahl, R. C., Carey, D. J., Rothblum, L. L., and Cheung, J. Y. (2003) Am. J. Physiol. 284, H225–H233
14. Despa, S., Bossuyt, J., Han, F., Ginsburg, K. S., Jia, L. G., Kutchai, H., Tucker, A. L., and Bers, D. M. (2005) Circ. Res. 97, 252–259
15. Silverman, B. Z., Fuller, W., Eaton, P., Deng, J., Moorman, J. R., Cheung, J. Y., James, A. F., and Shattock, M. J. (2005) Cardiovasc. Res. 65, 93–103
16. Chen, Z.-H., Jones, L. R., and Moorman, J. R. (1999) Receptors Channels 6, 435–447
17. Moorman, J. R., and Jones, L. R. (1998) Adv. Exp. Med. Biol. 442, 219–228
18. Davis, C. E., Patel, M. K., Miller, J. R., John, J. E., III, Jones, L. R., Tucker, A. L., Mounsey, J. P., and Moorman, J. R. (2004) Neurochem. Res. 29, 177–187
19. Beevers, A. J., and Kukol, A. (2006) Protein Sci. 15, 1127–1132
20. Chen, Z., Jones, L. R., O’Brien, J. J., Moorman, J. R., and Cala, S. E. (1998) Circ. Res. 82, 367–374
21. Kukol, A., Adams, P. D., Rice, L. M., Brunger, A. T., and Arkin, I. T. (1999) J. Mol. Biol. 286, 951–962
22. Kukol, A. (2005) Spectroscopy 19, 1–16
23. Beevers, A. J., and Kukol, A. (2006) J. Mol. Graph. Model. 25, 226–233
24. Beevers, A. J., and Kukol, A. (2006) J. Mol. Biol. 361, 945–953
25. Torres, J., Adams, P. D., and Arkin, I. T. (2000) J. Mol. Biol. 300, 677–685
26. Torres, J., Kukol, A., Goodman, J. M., and Arkin, I. T. (2001) Biopolymers 59, 396–401
27. Kortenaar, P. B. W., Van Dijik, B. G., Peeters, J. M., Raaben, B. J., Adams, P. J. H. M., and Tesser, G. I. (1986) Int. J. Protein Sci. 27, 398–400
28. Kass, I., Arbel, E., and Arkin, I. T. (2004) Biophys. J. 86, 2502–2507
29. Arkin, I. T., MacKenzie, K. R., and Brünger, A. T. (1997) J. Am. Chem. Soc. 119, 8973–8980
30. Marsh, D., Muller, M., and Schmitt, F. (2000) Biophys. J. 78, 2499–2510
31. Lindahl, E., Hess, B., and van der Spoel, D. (2001) J. Mol. Mod. 7, 306–317
32. Gurtovenko, A. A., Patra, M., Karrttunen, M., and Vattulainen, I. (2004) Biophys. J. 86, 3461–3472
33. Byler, D. M., and Susi, H. (1986) Biopolymers 25, 469–487
34. Kukol, A., and Arkin, I. T. (1999) Biophys. J. 77, 1594–1601
35. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Nucleic Acids Res. 25, 3389–3402
36. Bogaev, R. C., Jia, L., Kobayashi, Y. M., Palmer, C. J., Mounsey, J. P., Moorman, J. R., Jones, L. R., and Tucker, A. L. (2001) Gene (Amst.) 271, 69–79
37. Dalskov, S. M., Imenderl, L., Nielsen-Christiansen, L. L., Hansen, G. H., Schousboe, A., and Danielsen, E. M. (2005) Neurochem. Int. 46, 489–499
38. Allen, F. H. (2002) Acta Crystallogr. Sect. B 58, 380–388
39. Fletcher, D. A., McMeeking, R. F., and Parkin, D. J. (1996) J. Chem. Inf. Comput. Sci. 36, 746–749
40. Moorman, J. R., Ackerman, S. J., Kowdley, G. C., Griffin, M. P., Mounsey, J. P., Chen, Z., Cala, S. E., O’Brien, J. J., Szabo, G., and Jones, L. R. (1995) Nature 377, 737–740
41. Humphrey, W., Dalke, A., and Schulten, K. (1996) J. Mol. Graphics 14, 33–38