Consequences of phosphate-arginine complexes in voltage-gated ion channels

Michael E. Green
Department of Chemistry, City College of New York; New York, New York USA

Key words: ion channel gating, potassium channel, phosphate-arginine complexes, gating current, proton cascade

There are two reasons for suspecting that phosphate complexes of arginine make it very difficult to derive gating charge in voltage gated potassium (and presumably sodium) channels from the motion of charged arginines. For one thing, the arginines should be complexed with phosphate, thereby neutralizing the charge, at least partially. Second, Li et al. have shown that there is a large energy penalty for putting a charged arginine into a membrane. Ion channel gating current is generally attributed to S4 motion, in that the S4 segment of the voltage sensing domain (VSD) of these channels contains arginines, some of which are not (or at least not obviously) salt bridged, or otherwise charge compensated. There is, however, good reason to expect that there should be a complex of these arginines with phosphate, very probably from lipid headgroups. This has consequences for gating current; the complexed arginines, if they moved, would carry too much of the membrane along. This leads to the suggestion that an alternative to S4 physical motion, H+ transport, should be considered as a possible resolution of the apparent paradox. The consequences for a gating model that was proposed in our earlier work are discussed; there is one major difference in the model in the present form (a conformational change), but the proton cascade as gating current and the role of water in the closed state are reinforced.

Introduction and Statement of the Problem

The mechanism by which voltage gated ion channels actually use the transmembrane electric field, and its changes, to open, or gate, is still controversial. The channel is tetrameric, and can be thought of in two parts: a pore, with two transmembrane (TM) segments in each of the monomers, and a voltage-sensing domain (VSD) with four TM segments. The linker between these segments is also important. It is known that there is a set of arginines on one of the TM segments in the VSD, S4. It has been argued that the arginines are held “in” (toward the intracellular side) by the transmembrane potential in the closed state, and then released to move outward, generating a capacitative current (gating current) that precedes the actual opening of the channel pore. However, there is considerable evidence that phosphate complexes the arginines, which changes the charge drastically. The linkage between VSD and pore has also had a rather extensive discussion. Here, an alternative mechanism for the generation of gating current, and for the linkage to pore opening, is suggested, and discussed in some detail. We start with the observation that the standard models will have difficulty with the phosphate that is present in the neighborhood of the channel, especially in the phospholipid of the membrane itself.

Also, Li et al. have calculated the energy costs of placing charged arginine in a lipid membrane, and this has significant consequences for ion channel gating. They find a barrier of approximately 17 kcal/mole for motion of a single arginine through a membrane. If the arginine would enter or partially cross the membrane, as in most standard gating models for voltage gated ion channels, this would become effectively an activation energy for the motion of an S4 segment of a VSD. For a process with 17 kcal as activation energy, the rate constant would have a factor of $\approx 10^{-12} = \exp(-17/0.6)$ at room temperature, leading to a rate of membrane crossing several orders of magnitude slower than the rate of ion channel gating, which corresponds to a time constant $\approx 10^{-4}$ seconds. Even if the calculation leads to a higher than realistic energy (it is necessary to remember that the MD simulation may not be exact), and the error is as large as 25% (this should be an adequate upper limit on the magnitude of the error) one gets, for a single arginine, a kinetic factor of $\approx 10^{-9}$, which is still too small. In addition, since more than one charged arginine is required to produce the observed gating current, the barrier is still larger; even one arginine provides a barrier effectively too large to surmount.

No easy fix for the standard models seems to be available. There is no evidence, and no suggestion, in any of the “classical” theories in which the arginine charges provide the gating current, that an arginine leaves the membrane. Balancing an entering arginine with a leaving arginine does not help. It is hard to see how a distant arginine leaving would reduce the activation energy for an entering arginine, although it would alter the thermodynamic equilibrium. Here we are concerned with kinetics. Thermodynamics would become relevant if the question concerned the formation of the channel and its insertion into the membrane, but then all the charges would be required, and the problem would be as difficult.

One possible way around this would be to have the arginines uncharged because of a pK shift in the membrane, but Li et al.
effectively rule this out; it would not help the gating current problem either, as it would not provide for the motion of charge. The major classical gating models, including the “paddle” of MacKinnon, and the Bezanilla and Roux model in which S4 rotates, or the variation proposed by Tombola and Isacoff, require large scale movement of charged arginines to provide gating current.

It is known that there are salt bridges with aspartate and with glutamate in neighboring TM segments that partially neutralize the charge of the arginines. In standard gating models, the arginines switch partners, with uncomplexed charges from “below” (intracellular side) moving up on channel opening to create new salt bridges, while the closed state salt bridged arginines move up to create gating charge. However, if the charges “below” are complexed to phosphate, this is not possible.

There is, in fact, a high probability that at least two of the key arginines are complexed to phosphate. Schmidt et al. have shown that negatively charged lipids are needed to have a functional channel. Xu et al. have found that removing the phospho-head of the phospholipid head group results in preventing the channel from functioning, which would be consistent with a requirement for a complex involving this group and, presumably, arginines at the boundary of the TM segment. While these authors offer a more complex interpretation of the nature of the interactions with phospholipids, they make it clear that there are such interactions, and these affect early and late steps in gating differently. Freites et al. have shown by MD simulation that the phosphate of a negatively charged lipid follows the arginines into the membrane, being complexed to it; their result looks very much like what one would expect from the Schmidt et al. study. Green has shown, by ab initio calculation, what the structure of such a 2-arginine/1 phosphate (+water) complex should be. Pradhan et al. have confirmed by NMR that the 2-arginine peptide used in Green’s calculation does form a complex with phosphate. Most important, Woods and Ferre, and Jackson et al. have shown that an arginine-phosphate complex will be far too strong to decompose with the energy involved in gating. They find the binding energy is much larger than 100 kcal/mole, indeed comparable to covalent bonds, while the 70 mV x 3 charges available for gating allows more like 6 kcal/mole; the complex could not decompose simply upon depolarization of the membrane. It seems very probable that a phosphate-arginine complex exists in S4, presumably at the intracellular end. Thus the salt bridges for the “upper” (more extracellular) arginines, to acidic residues in S2 and S3, cannot exchange as postulated in classical gating theories. The arginines that are supposed to replace the arginines in the salt bridge cannot do so; they are already linked to the negative charge of the phosphate. This means that insufficient, or possibly no, net charge would be provided by the motion of S4, leaving the source of the gating current undetermined. The connection to the lipid through the phosphate would also mean that motion of S4 is severely hindered, at least in the vertical (transmembrane) direction, by the necessity of pulling the lipid with it. This makes the argument against physical motion of S4 as the source of gating current stronger. Horizontal motion would be less hindered, as the lipid headgroup would not be pulled still further into the membrane, so that the lipid chain would be less distorted.

This seems to leave a paradox: if there is a complex, it will not decompose; even if it allows the S4 arginines to go to the positions they are assigned in standard gating models, it will not provide a gating current, or at least an adequate gating current (that is, a current large enough to match the measured value). If the complex somehow does not form, S4 remains charged. However, the motion of a charged S4 would require too much activation energy. Even if the activation energy is not exactly as calculated in the MD simulation of Li et al. it will be large; we have already noted that even if the error were as large as 25%, the resulting movement of the charges would be too slow for gating current. Aside from activation energy, the necessity for negatively charged phospholipids would remain to be explained. Either way, the gating current is not accounted for.

A Possible Resolution

Green and coworkers have suggested that proton motion along S4 is the source of gating current, and this requires only the existence of a water wire anchored to S4. The binding of MTS reagents and the biotin binding experiments of Ruta et al. would then require further discussion. However, if charge is suitably neutralized some S4 motion is not incompatible with the energetics. The structure shown in Figure 1 is based on a projection of the Kv1.2 structure (pdb structure 2A79). S4 motion would, from the point of view of gating current, be likely to be an “epiphenomenon”, associated with gating but not the source of gating current. In the present form of the model, it appears that some horizontal motion is required to move certain residues (discussed below) close enough to the center of the pore to support the water structure blocking the pore. It is not necessary for such motions to be rigid body motions; they do not carry charge, and the details of the motion are not critical to this model. If, for example, there is a 310 to α-helix transformation of S4, it would be still consistent, although not a prediction of the model.

Since some of the pore segment S6 must move, at least at the intracellular end, to open the channel, it is possible that either S4 is displaced to some extent, or that neighboring transmembrane segments may be displaced, opening a cleft next to S4, allowing differential access to the S4 residues in open and closed states. If the primary motion of the S4 segment is horizontal, then the accessibility can be accounted for, while the gating current remains to be understood.

Proton transport as the source of gating current resolves the apparent paradox discussed here; it is easier to understand the evidence for S4 motion without requiring this motion to provide the gating current than to account for the phosphate binding and lipoprotein results while still attributing the gating current to S4 “vertical” motion. The data on phosphate complexes are more consistent with the proton transport hypothesis for gating current than with any of the standard models.

How the Model Functions

We need to know how the protons could move, and how this motion results in opening the channel. Secondarily, we must understand how they could undo the salt bridges that appear to hold the S4 TM segment and the rest of the VSD in place in the closed state, and where new salt bridges in the open state might form to hold S4 in a different location. Moving S4 would allow understanding the reaction data on differential access to cysteine substituted S4 in open and closed states, even if it would not account for the gating current. The vertical motion has been reported to be large, or, based
Protons move. There is substantial reason to understand that protons move. (A) Histidine scanning: Histidine scanning experiments by Starace and Bezanilla\textsuperscript{26-29} show that protons can move along the S4 segment; substituting one of the histidines (which one depends on whether the channel is open or closed) turns the VSD into a proton channel. The full complement of arginines in the unmutated channel is sufficient to prevent the escape of the proton from the VSD, with the end arginine evidently blocking the proton path. When any non-arginine residue (thus not in the proton path) is mutated to histidine, no proton path is created, consistent with motion of the protons along the arginine face of the S4 segment. (We do not consider mutations on other segments here). The complete traversal of the membrane with the mutated channel by protons shows that the remaining portion of the transmembrane region, in either open or closed states, is accessible to protons, even if not to larger reagents. A recent review by Chanda and Bezanilla\textsuperscript{30} discusses a number of instances of proton motion through what appear to be analogs of VSDs,\textsuperscript{30} giving several lines of evidence. Traversal of part of the membrane would produce a capacitative current. It is easy to see how to produce a barrier that would limit proton motion at a given point, and an arginine, if charged, could do this. One barrier is sufficient to prevent continuous current (if a proton could not get past a given point, the charge would prevent further current; continuous current requires that the proton leaves, and another arrives to take its place).

(B) The H\textsubscript{V} channel: Another mutation turns the VSD into a voltage gated proton channel that is found in a number of biological species.\textsuperscript{31,32} This also suggests that protons can move through the VSD, although it is less specific than the histidine scanning result.

(C) \textit{Ab initio} calculations show that transferring a proton between arginines does not encounter a major barrier, as long as water is present.\textsuperscript{20,33}

(D) Classical gating theories have difficulty in understanding the extremely fast initial step (the "piquito") in gating found by Stefani and Bezanilla.\textsuperscript{34-36} If this step were thermally activated, it would require too low a barrier to thermal activation of the channel, and one would expect more random openings of the channel. However, it is possible to understand this step as the initial traversal of a barrier to proton motion by quantum tunneling, something that would require only a small change in a large electric field at a single residue in S4; we showed that a proton could transfer between two methyamines given a small change in a large field.\textsuperscript{37} The large field exists, and a location at one of the arginines has been found for it in \textit{Shaker}.\textsuperscript{38} The proton cascade model would lead to the expectation that the high field should be in the proton pathway. Proton tunneling has been previously discussed in this context.\textsuperscript{37,39,40}

(E) Proton tunneling would be a first step in transferring protons along the water wire that appears to exist neighboring the S4 TM segment. Even prior to calculations, we should expect such a water wire, as there is clearly space for it, and given the results on access to S4, essential certainty that water must be able to reach this volume. The proton that has been transferred according to (D) above will start a cascade of protons to generate the proton current, with consequences that are considered in the next section. There would not be a continuous proton current if another proton could not enter the VSD; in the unmutated VSD the last arginine can act as a barrier to entrance of a proton. The charge that is left in the original position remains fixed, and only a capacitative current is observed, not a proton pore.

\textbf{Added H\textsuperscript{+} can open a channel.} (A) To understand this, it is possible to look at the KcsA channel, a bacterial K\textsuperscript{+} channel with an almost standard pore segment, and no VSD. As would be expected from a channel with no VSD, on the hypothesis that protons are responsible for gating, this channel gates with a drop in pH. While there have been several hypotheses presented in attempts to understand the gating mechanism, the one relevant here is our proposal for a water structure (containing 14 water molecules plus
one K\(^+\) ion, as a “nanocrystal”) at the bottom of the gate. The structure is anchored to four glutamines (one from each monomer in the channel), and appears to be stable, based on DFT calculations.\(^{41}\) Adding protons (one per monomer) destroys the structure, so that the glutamines move aside, and the channel opens.

(B) While KcsA lacks a VSD, adding a VSD with a suitable linker should make it a voltage gated channel, and this is the case.\(^{42}\) This is consistent both with the proton cascade model and classical models; we will consider below whether the restrictions on the linker are consistent with our model. The proton cascade in the voltage gated channel can add a proton to the structure closing the channel in the same manner as the drop in pH presumably does in KcsA, so the analogy is reasonably exact.

(C) This appears to imply that the voltage gated models should have a structure in the closed state in which there would be amino acids, preferably glutamine or asparagine, that can anchor a “nanocrystal” like that which we calculated for KcsA. Further calculations will be needed to confirm this. A candidate has been found (see section D below); it is too soon to propose it in detail, pending further calculations, but a sketch may be attempted. The open state at least has an X-ray structure (pdb 2A79) to work from; however, it is incomplete. With the remaining residues we use a program that will insert side chains, and create a structure; it is not as accurate as a quantum calculation. It will, however, allow us to make a plausible hypothesis. We have chosen DeepView\(^{43}\) to accomplish the positioning of the side chains, and a reasonable approximation to the backbone. The orientations of side chains are of limited reliability, but it is possible to tell whether a salt bridge could exist or not.

(D) For the closed state, absent an X-ray structure, the best we can do is postulate the residue, N414 in the K\(_{v}1.2\) structure, that would anchor the water “nanocrystal” we hypothesize by analogy with what we calculated for KcsA. The extent of horizontal motion required seems reasonable. Two key salt bridges appear to exist in the open state that should be relevant to gating; in the K\(_{v}1.2\) (pdb 2A79) structure, they are numbered as follows (it should be understood that calculations have not been completed on these, and what follows is plausible, but not confirmed):

1. K312 (S5)—E420 (S6). This salt bridge is in the pore region, but it should be reachable by protons that come down a path from S4 and diffuse across an aqueous cleft below S4.
   2. K134 (T1)—E422 (S6). This salt bridge is again accessible by protons across the same cleft, and helps anchor the pore segment S6. The basic residue in both cases is lysine, which is easier to deprotonate than arginine. It remains to be seen whether this is significant; possibly a K312R or K134R mutant would have more difficulty opening, which in turn suggests testing these mutants. It is early as yet to state which residues comprise the salt bridges that lock the closed state; for this either a structure will have to be found, or a very extensive calculation will be required.

**Differential access to residues mutated to cysteine in S4.** There are two classes of experiment that start by mutating arginines in S4 to cysteine. In one, methanethiosulfonate (MTS) reagents are used; if they can reach the cysteine, the channel is destroyed. The other, used in the MacKinnon laboratory,\(^{5}\) provides access measurement using biotin, which can complex avidin. These experiments show more apparent access to S4 from the intracellular side in the closed state, and from the extracellular side in the open state. Some residues are simply not accessible. Water structures could in principle block access alternately from the intracellular and extracellular sides. However, in this case they are a little difficult to imagine; for one thing, there is no obvious place to anchor these structures. Also, blocking access to the internal volume of the VSD on the time scale of these experiments (minutes) would be difficult. However, there is another path to differential access, without requiring that the arginines carry charge across the membrane. If the closed state has a cleft between the pore and the VSD domains, with access to the S4 largely possible as the pore segment (from the S4-5 linker through S5 and S6) is pulled in, supporting the internal structure formed in that state, the residues on the intracellular side of S4 should be available to a reagent coming from the intracellular medium.

**The T1 segment.** It is known that the intracellular section of the channel at the C-terminus of the S6 segment, T1, is involved in gating, although the details remain somewhat unsettled.\(^{44-47}\) The proton cascade model, with salt bridge switching, for the first time suggests how T1 may be involved; one proposed salt bridge includes K134, which is in T1. We can postulate a path for the opening of the channel based on the known open structure of K\(_{v}1.2\) that includes a salt bridge with K134. However, the published work that shows the importance of T1 to gating does not definitively pinpoint a particular residue as critical.

**The analogy to KcsA.** We can surmise reasonable distances for an arrangement of asparagines similar to the glutamines of KcsA; although more extended calculations are beginning, at present it appears reasonable that asparagines N414 play a role similar to that of the KcsA glutamines to which the “nanocrystal” that we calculated is anchored. There are also asparagines two residues away (both N412 and N416), and residues with hydroxyls presumably on the same side of an alpha helix (S411 and Y417). Lu et al.\(^{42}\) found that in the Shaker sequence they used as linker in their Shaker-KcsA chimera, the corresponding group of amino acids was necessary for the channels to express current.

**Protein motion in the proton cascade model.** Nothing said so far suggests that a proton cascade is incompatible with protein conformational changes, including motion in the VSD. S4 may move. If N414 of K\(_{v}1.2\) is the anchor of the water nanocrystal blocking the channel in the closed state, this suggests substantial horizontal motion. The distance between diagonally opposite oxygens in the N414 amide is 22 Å in the open state X-ray structure, compared to 6 Å for the anchor glutamates (Q119) in the KcsA closed structure. This leads to an inward horizontal motion of 8 Å for the N414. The inward motion would expel S4, making it possible for MTS reagents to reach cysteines substituted for arginines there. Therefore, some S4 motion is now expected, mostly horizontally, and the space that exists between the VSD and the pore segments can be partially filled by water. There is very likely to also be a phosphate group in this region.

There are too many possibilities for salt bridges in the closed state to be certain which particular ones exist, based on the data now available, but those suggested above in section IID1 seem most probable, given what is known. Some form of reservoir for about 3 H\(^+\) is needed; it must help hold the channel together, in a manner analogous to that shown by the salt bridges in which, in KcsA, the E120 and E122 residues participate. In voltage gated channels like K\(_{v}1.2\), removing protons, not adding them, would destroy the salt bridges,
allow the water blocking the channel to become free, and thus open the channel. There exist also HCN channels that open in response in increasing rather than decreasing membrane polarization. In HCN channels, the gating current moves in the opposite direction, so that protons would be intracellular when the channel is open, assuming a model consistent with this one. Salt bridges involved in gating these channels have been pointed out by Craven and Zagotta.48 This is as we should expect. There is no reason the salt bridges must operate such that only protons moving up open the channel; either direction is possible. No new principle of motion is required for the proton cascade model. It is also noteworthy that another type of channel entirely, the bacterial outer membrane channel OmpA, has been reported to gate by switching salt bridges;49 salt bridge switching may be a more general mechanism than has been considered so far.

Conclusions

Phosphate complexes of the arginines in S4 should exist, and make it possible to account for the gating current of the voltage gated K+ channel by conformational change of the S4 segment of the VSD. The gating current can be accounted for by the motion of protons within the VSD, although the arginines must play a role in establishing a water wire that is responsible for the conduction of the protons. A mechanism is proposed by which protons could be responsible for the transition from closed to open state. It involves a set of salt bridges that are formed or broken, depending on the position of the protons that move when the channel changes state. The protons constitute the gating current; the salt bridges are (partially?) responsible for the stability of the state. In the closed state, based on the KcsA structure, there should be a set of amino acids that anchors a fairly stable water structure that in turn blocks the entrance to the pore of the channel. The phosphate complexes of the S4 arginines are stable and should exist in both the open and closed states, neutralizing the arginine charge. Taken together with the salt bridges, these complexes provide a comprehensive, if still coarse-grained, picture of channel gating. Further details on the salt bridges await calculation.

Acknowledgements

The switch, which will be discussed in detail in a separate publication, was first suggested by Alisher Kariev, who contributed very useful scientific discussions to this work. I thank Dr. Vasily Znamenskiy for assistance with the DeepView program and with Figure 1. This work has been supported in part by a PSC-CUNY grant from the City University of New York. The calculations on phosphate in reference 14 were carried out at the Environmental Molecular Sciences Laboratory of the Pacific Northwest National Laboratory, a DOE facility.

References

1. Li L, Vorobyov I, MacKerril AD, Allen TW. Is arginine charged in a membrane? Biophys J 2008; 11-3.
2. Jiang Y, Ruta V, Chen J, Lee A, MacKinnon R. The principle of gating charge movement in a voltage-dependent K+ channel. Nature 2003; 423:42-8.
3. Ruta V, Chen J, MacKinnon R. Calibrated measurement of gating-charged arginine displacement in the KvAP voltage-dependent K+ channel. Cell 2005; 125:463-75.
4. Chanda B, Asanoma OK, Bluncr K, Roux B, Bezantia F. Gating charge displacement in voltage-gated ion channels involves limited transmembrane movement. Nature 2005; 436:852-6.
5. Campos-P pennon, Chanda B, Roux B, Bezantia F. Two atomic constraints unambiguously position the S4 segment relative to S1 and S2 segments in the closed state of Shaker K channel. Proc Natl Acad Sci USA 2007; 104:7904-9.
6. Tombola F, Patkh P, Miscon MM, Gorostiza P, Isacoff EY. The twisted ion-permeation pathway of a resting voltage-sensing domain. Nature 2007; 445:546-9.
44. Choe S, Cushman SJ, Baker D, Pfaffinger PJ. Excitability is mediated by the T1 domain of the voltage-gated potassium channel. Novartis Foundation Symposium 2002; 169-75.

45. Minor DL Jr, Lin Y-F, Mobley BC, Avelar A, Jan YN, Jan LY. The polar T1 interface is linked to conformational changes that open the voltage-gated potassium channel. Cell 2000; 102:657-70.

46. Strang C, Cushman SJ, DeRubeis D, Peterson D, Pfaffinger PJ. A central role for the T1 domain in voltage-gated potassium channel formation and function. J Biol Chem 2001; 276:28493-502.

47. GW, MC. Voltage-dependent gating rearrangements in the intracellular T1-T1 interface of a K+ channel. J Gen Physiol 2006; 127:391-400.

48. Craven KB, Zagotta WN. Salt bridges and gating in the COOH-terminal region of HCN2 and CNGA1 channels. J Gen Physiol 2004; 124:663-77.

49. Moroni A, Thiel G. Flip-flopping salt bridges gate an ion-channel. Nat Chem Biol 2006; 2:572-3.