Medical Research Archives. Volume 4 Issue 6

WATER AND H^+ TRANSFER IN VOLTAGE GATED K^+ CHANNEL FUNCTION, WITH EVIDENCE FROM QUANTUM CALCULATIONS.

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ABSTRACT: Voltage gated K^+ channels have been the subject of intensive study for over a half century. They are found in all cells; together with Na^+ channels, they are responsible for the nerve impulse, and play a key role in other excitable tissue, particularly the heart. Malfunctions due to mutation lead to a range of diseases, referred to as channelopathies. The mechanism by which the channels open and close, called gating, has been studied extensively; there is a range of standard models. All have in common a transmembrane segment of the channel protein moving in response to depolarization of the membrane, thereby pulling open a section of the channel at the intracellular end of the membrane; this allows K^+ ions into the channel pore, producing a current of ions out from the cell. The motion of the ions is preceded by a capacitative current, the gating current, which is attributed to positive charges on the putatively mobile transmembrane segment. The evidence supporting this class of models is examined and reinterpreted to show that the evidence does not require the motion of a segment of protein. This and other evidence is instead considered in terms of a model in which protons provide the gating current; when these are at the intracellular terminus of the protein, they close the channel, while the channel is open when they are at the extracellular end. This model is supported by quantum calculations that are larger than have been previously reported for a protein system. Some of the calculations include most of the voltage sensing domain, and others the

pore of the channel, with the hydration and cosolvation of the ion specified.

Keywords: Ion channels, gating, solvation, voltage sensing domain, quantum calculations

Ion channels are protein pores in membranes of cells, and in some cases of organelles. They are found quite literally in all cells, including those of bacteria and archea, and one set has recently been discovered even in viruses (Moroni, A. and G. Thiel 2006). Everything alive must control the ion concentration within its membrane. In some cases, the channels control signaling, as in neurons. Malfunctioning channels lead to a large variety of diseases, or channelopathies. Thousands of mutants with a variety of symptoms are known. Let us begin with these, to see the medical relevance of the topic first.

1. **CHANNELOPATHIES**: There are a variety of channelopathies, diseases that result from malfunctions of ion channels. In some cases these may be gain of function mutations, in others loss of function. The diseases cover a very wide range, but for the channels with which we will be most concerned here, forms of familial epilepsies, as well as long QT syndrome of heart muscle, may be the most important, or at least the most well-known. These have been discussed in multiple articles; a sampling of examples, in which the defects are in potassium channels, and including excitable tissue, may be found in these articles, most of them concerning neurological defects (Akhtar, S., O. Shamotienko, M. Papakosta, F. Ali and J. O. Dolly 2002, Kosolapov, A., L. Tu, J. Wang and C. Deutsch 2004, Robbins, C. A. and B. L. Tempel 2012, Robbins, C. A. and B. L. Tempel 2012, Serratrice, G., J.-P. Azulay, J. Serratrice and S. Attarian 2004, Tigerholm, J. and E. Fransen 2011, Zhao, W. and Y. Chen 2016). Abbott has considered cardiac diseases related to potassium channels (Abbott, G. W. 2006). Kim has given a general review of channelopathies of multiple types, related to several types of channels, and in multiple organs(Kim, J.-B. 2014). A very recent paper finds that sleep in a fruit fly brain, and probably in mammalian brains as well, is controlled by addition and deletion of a K^+ channel (albeit not a gated channel) from the neuron membrane of a particular section of the brain (Pimentel, D., J. M. Donlea, C. B. Talbot, S. M. Song, A. J. F. Thurston and G. Miesenbock 2016). As this article is concerned with the molecular mechanisms by which channels function, we will not discuss channelopathies in detail. However, the fact that malfunctions of channels lead to disease phenotypes is no surprise, as the channels are required for cell function, and whichever channel has loss of function or gain of function mutations will cause the tissue in which it is expressed to be unable to perform normally. In this review, we are primarily concerned with voltage gated potassium channels. Consequently, the diseases that are most directly relevant are diseases of excitable tissue, nerve and heart in particular. These are not the only K_v^+ channelopathies, and Na_v^+ channelopathies of excitable tissue also exist. However, these examples are sufficient to point out that the subject is of considerable medical relevance.
2. **BACKGROUND**: It is possible to begin the story of ion channels in a number of places. This is not a historical review, something which is available from many sources, probably the best being Hille's classic work(Hille, B. 2001). However, we can note the major step forward that led to the postulated existence of ion channels, in connection with the work of Alan Hodgkin

and Andrew Huxley on the nerve impulse (Hodgkin, A. L. and A. F. Huxley 1952, Hodgkin, A. L. and A. F. Huxley 1952, Hodgkin, A. L. and A. F. Huxley 1952). In fitting the electrochemical observations on nerve impulse to a set of equations, they postulated that there were sodium channels that opened first, allowing Na^+ to enter the cell, followed by the opening of K^+ channels that produced a flow of K^+ out of the cell. The Na^+ channels were postulated to have three gating particles plus an additional parameter while the K^+ channels had four identical particles. With no structural information whatever, this was an amazing insight, and they were awarded the 1963 Nobel Prize in Physiology and Medicine (shared with John Eccles). The early work on channels had to be carried out on preparations of nerve that contained multiple channels. It became possible to study single channels after the development of patch clamping by Ernst Neher and Bert Sakmann (E, N., Sakmann B and S. JH 1978) for which they also received the Nobel Prize (1991). Once it became possible to study single channels the field advanced rapidly. One earlier discovery is worthy of note: Hodgkin and Huxley had postulated that the opening of channels would be preceded by a capacitative current in response to the depolarization of the membrane, as the gating charges moved. This “gating current” was observed in 1974 by two groups: Armstrong and Bezanilla (Armstrong, C. M. and F. Bezanilla 1974) and Keynes and Rojas (Keynes, R. D. and E. Rojas 1974). The first channel gene was cloned in 1984 by Noda et al (Noda, M., T. Shimizu, T. Tanabe, T. Takai, T. Kayano, T. Ikeda, H. Takahashi, H. Nakayama, N. Kanaoka, N. Minamino, K. Kangawa, H. Matsuo, M. A. Raftery, T. Hirose, S. Inayama, H. Hayashida, T. Miyata and S. Numa 1984).

By 1990 the field was advancing rapidly. It was known that the K^+ channel inactivated (stopped conducting) not long after opening, without returning to the closed state, entering an “inactivated state” that did not conduct, until such time as it could return to its closed, or resting, conformation. There were two mechanisms involved, one fast, one slow, and in 1990 the fast mechanism was found to depend on a “ball and chain” that was attached to the N-terminal of the voltage sensing domains (VSD) of the channels, and that plugged the pore in a relatively short time after opening.(Zagotta, W.N,, H. Toshi, and R. W. Aldrich, 1990, Zagotta, W. N., T. Hoshi and R. W. Aldrich 1994). Earlier, Armstrong and Bezanilla showed that there must be a section of the channel protein that was responsible for fast inactivation, because pronase removed inactivation (Armsttrong, C.M., and F. Bezanilla 1977). However, it was difficult to propose a molecular mechanism, as no structure of a channel was known. In 1998, MacKinnon and coworkers(Doyle, D. A., J. M. Cabral, R. A. Pfuetzner, A. Kuo, J. M. Gulbis, S. L. Cohen, B. T. Chait and R. MacKinnon 1998) provided the first X-ray crystal structure of an ion channel, the prokaryotic, pH gated, KcsA channel from Streptomyces Lividans. MacKinnon received the 2003 Nobel Prize in Chemistry (shared with Peter Agre, who discovered the aquaporin water channels). The KcsA channel lacked a VSD, but showed the pore, with the selectivity filter; this was essentially identical to that in eukaryotic K^+ channels; X-ray structures of the eukaryotic channels followed within several years. It was this, along with these subsequent studies that added complete channels with VSDs, which made it possible to carry out

the modern studies, and modern modeling, which are the subject of this review. Obviously this "history" is barely a skeleton outline of well over a half century of effort by thousands of workers in the field, a little of which will be referred to in the main sections of this review. However, these milestones marked what are probably the major transition points in the development of the field, bringing us to the point where it makes sense to consider molecular models of gating. Absent structures for the channels, it was only possible to guess at the major parts of the mechanisms. The entire field of ion channels has been recently reviewed in detail, in the "Handbook of Ion Channels" (Zheng, J. and M. Trudeau 2015). Individual reviews on 43 topics are included. The reader may refer to this work for the standard view of all major topics in ion channels. Our review is specifically concerned with voltage gating of K^+ channels, almost certainly with relevance for Na^+ channels as well. We consider a view of ion channel gating, and response to membrane depolarization, that reinterprets certain well known properties of ion channels. In the course of the review, we will cover certain aspects of channel properties that have been previously reviewed by others, and will present the standard model of gating, with the interpretation of the evidence for the standard set of models. Not all these "standard" models are identical, and the evidence is not interpreted in the same way by all workers in the field. They all have in common a similar mechanism of response to voltage change, with somewhat similar conformational changes. The channel structure, as discussed in the next section, includes four voltage sensing domains (VSD), with 4 transmembrane (TM) segments each. In addition, each domain contributes 2 TM segments to form a pore, which therefore has a total of 8 TM segments, with a section linking the two segments of each domain forming a selectivity filter. However, we will make particular reference to a form of gating model we argue should be considered as a possible alternative to these standard models. We will first consider the evidence for the standard view of gating, then the reasons this evidence can be reinterpreted. The experiments themselves are not in question. We also observe, however, that certain experiments appear to be overlooked in interpreting the standard models. We are considering a model that differs in its central mechanism from the standard models. This is supported by not only reinterpretation of earlier evidence, but also by quantum calculations that are as extensive as are possible at the present time. We will argue that quantum calculations are more likely to be correct than the more common molecular dynamics (MD) calculations that have been used by many workers. Of course, most evidence in the field is experimental, and the interpretation of this evidence is crucial to any modeling.

3. STRUCTURE:

3.1) OVERALL STRUCTURE OF THE CHANNEL: In order to understand the models of gating it is necessary to begin the discussion with the structure of the channels. Fig. 1 shows the channel structure that is probably the best defined at present, pdb code 3Lut. This structure is derived from the X-ray structure of the channel (Long, S. B., E. B. Campbell and R. MacKinnon 2005) with addition of the hydrogen atoms by normal mode analysis (Chen, X., Q. Wang, F. Ni and J. Ma 2010). It

shows a tetrameric structure, with 4 VSDs in the membrane region of the channel, with substantial intracellular sections that belong to the N and C terminals of the protein. We will discuss part of this later, as it has been shown to be relevant to gating. It is important to note that ***all X-ray structures of the type of channels we are considering are in the open conformation***, or at least they are believed to be. It is not possible to impose a potential on a channel in a crystal, and it is necessary to use a crystal to get an X-ray structure, of course. It would be necessary to find a channel closed at 0 volts to get a closed state structure. Cryoelectron microscopy is just now becoming possible with adequate resolution, and the structure of one channel, of a somewhat different structure has just been reported using this technique; however, it too must be in the open form, for the same reason (Whicher, J. R. and R. MacKinnon 2016). A somewhat unusual family of two-pore channels (K2P), (Kintzer, A. F. and R. M. Stroud 2016) may be closed at zero volts, but it is not so clear how to compare structures to the standard K_v channels, so we do not consider the two pore channels further.

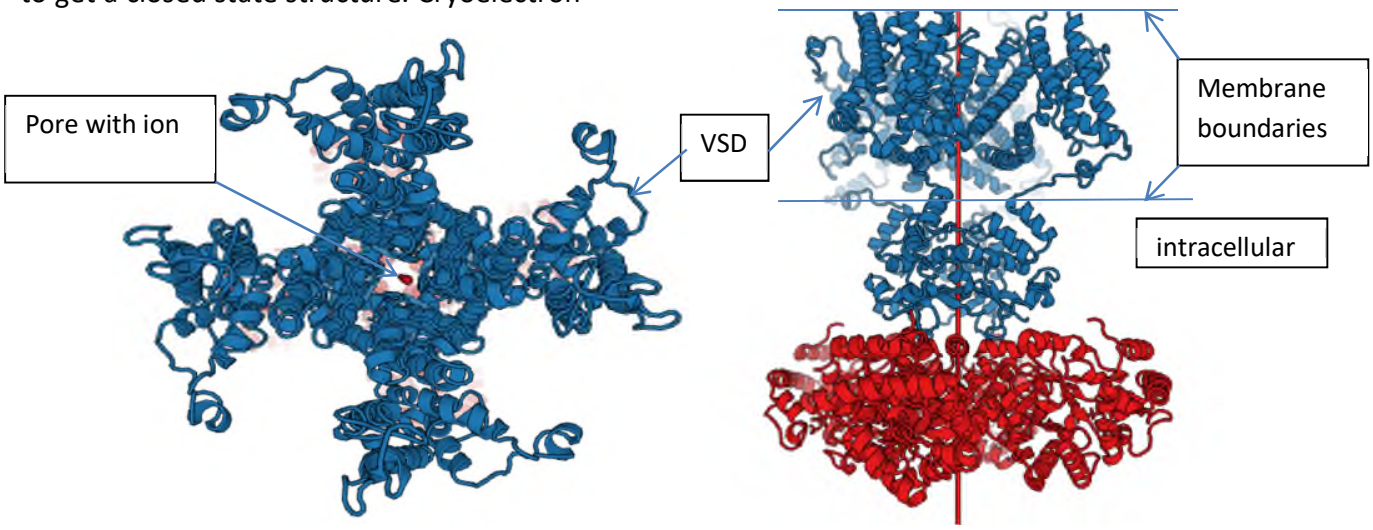

Fig. 1: K_v1.2 channel ribbon diagram from the protein data bank (www.rcsb.org/pdb) code 3Lut (Chen, X., Q. Wang, F. Ni and J. Ma 2010). A: view from the extracellular side, showing the four VSD, and a view down the pore in the center. B: Side view, with red line through the pore. The VSD is within the membrane. The approximate boundaries of the membrane are marked by the two horizontal lines, with the large section below on the intracellular side of the membrane. Each domain has 6 TM segments, 4 in the VSD, 2 contributing to the pore.

3.2) STRUCTURE OF A VSD: Each K_v channel has four identical VSDs. The VSD has four TM segments labeled S1 (leading to the C-terminal end of the protein) to S4; the latter is connected to a linker to the pore segment, S4-S5, which is at the intracellular side of the membrane.

S5 and S6 of each domain constitute the pore. The structure of S4 is critical. It contains a positively charged amino acid in every third position, almost always arginine, the first at the extracellular side of the segment (R1), and continuing to the intracellular end. A lysine, again three residues from the last arginine, is often found at the intracellular side. The side chains of the intermediate arginines, R2, R3, and R4, which are all considered to be involved in gating, point fairly consistently toward the interior of the VSD. Since an α-helix requires 3.6 turns, meaning that side chains in every third position would point in different directions, it has been suggested that the helix is closer to a 3_{10} helix, in which every third position points in the same direction. Some models have a 3_{10} to α-helix transition as a part of the conformational change that is supposed to accompany (or produce) gating. The two amino acids between the arginines are hydrophobic, and not as well conserved. Arginines are found in every third position in at least most, if not all, voltage gated potassium channels, as well as in voltage gated sodium channels (although the sodium channels are not exact tetramers—there are differences especially in Domain IV, from the other three domains).

The other TM segments, S1 to S3, in a VSD, have negatively charged amino acids, glutamate or aspartate, which form salt bridges to the arginines (Papazian, D. M., X. M. Shao, S.-A. Seoh, A. F. Mock, Y. Huang and D. H. Wainstock 1995). These salt bridges appear to be fairly strong, and in our calculations described below they play a central role. In a salt bridge, the base (here, arg) is positively charged, and the acid (glu, asp) is negatively charged. It is possible that the proton that is normally on the arginine shifts to the acid, making both neutral. This may happen under the influence of the electric field; in the closed state of the channel, the membrane potential is -70 mV (the minus means the intracellular side is negative), while in the open state the membrane is depolarized. The extent of depolarization required to open the channel depends on the channel, and different mutants may shift the required potential depolarization. In the channel shown in Fig. 1, the salt bridges are assumed in the standard gating models to always be ionized, and it is usually assumed that the charges do not shift in other channels as well. However, salt bridges may not always ionize (Liao, S. and M. E. Green 2011), especially in a hydrophobic environment. The extent to which the interior of the VSD is hydrophobic is not entirely clear. However, it appears that water can reach from either end of the VSD to a point interior to the membrane; there is a phenylalanine located fairly centrally within the membrane, where water cannot pass through, so that there is likely to be at least one salt bridge in the VSD that is not ionized. The electric field may also be localized to some extent. 70 mV across a 70 Å membrane means a field of 10^7 V m^{-1}, a high field but not

overwhelming. However, there is evidence that the field may be localized, reaching at least 10^8 V m^{-1} near R2 (second arginine)(Asamoah, O. K., J. P. Wuskell, L. M. Loew and F. Bezanilla 2003). This implies that much of the transmembrane region is at low field, since 10^8 V m^{-1} x 7 Å is already 70 mV, and 7 Å is only a fraction of the membrane thickness (the hydrophobic part is perhaps 40 Å, but the potential is measured at a point some distance away, thus including the head group region, so that the full thickness is about 70 Å). The moral of the story is that a proton can transfer, the state of ionization of the salt bridges is not certain, and we must look for evidence from other sources, including appropriate calculation. The amount of charge transferred across the membrane in gating (gating charge) is also significant. In *Shaker* the measured value is 12 – 13 charges, or about 3 per VSD. However, in the closely related $K_v1.2$ channel, it is close to 10 (Ishida, I. G., G. E. Rangel-Yescas, J. Carrasco-Zanini and L. D. Islas 2015). Since both have the same number of arginines that are supposed to cross the membrane field, the reason for this discrepancy is not immediately obvious in any of the standard models.

4. THE STANDARD MODELS, IN MORE DETAIL: At this point we include a brief discussion of the standard models, so that it will be possible to see the structure with the models that are usually used to describe gating in mind, and with the evidence that produced these models understood. The standard models of gating focus on the S4 arginine residues, considered to be always positively charged; arginine has a pK = 12.5 in solution, so this appears to be a reasonable assumption. The standard models then attribute the position of the S4 TM segment with the membrane polarized to the intracellular negative potential pulling the positive charges down. When the membrane is depolarized, the arginines are released and move in the extracellular direction, pulling the S4-S5 linker up, thus opening the gate by separating the S5 intracellular ends. The details change somewhat from one version of standard model to another, with some variation in the degree to which the gate opens.

There are acid, presumably negatively charged, amino acids in the neighboring TM segments S2 and S3. Then the S4 positive charges are expected to exchange partners with alternate acids, so that they can move with respect to the stationary S2 and S3 (we ignore the “paddle” model of MacKinnon, which also has part of S3 moving; this model has even more of the protein unfolding and refolding, making it even less plausible, in our view; however, it appears that this model has not been abandoned). The possibility that the salt bridge components may have exchanged protons so that they are not charged is not considered.

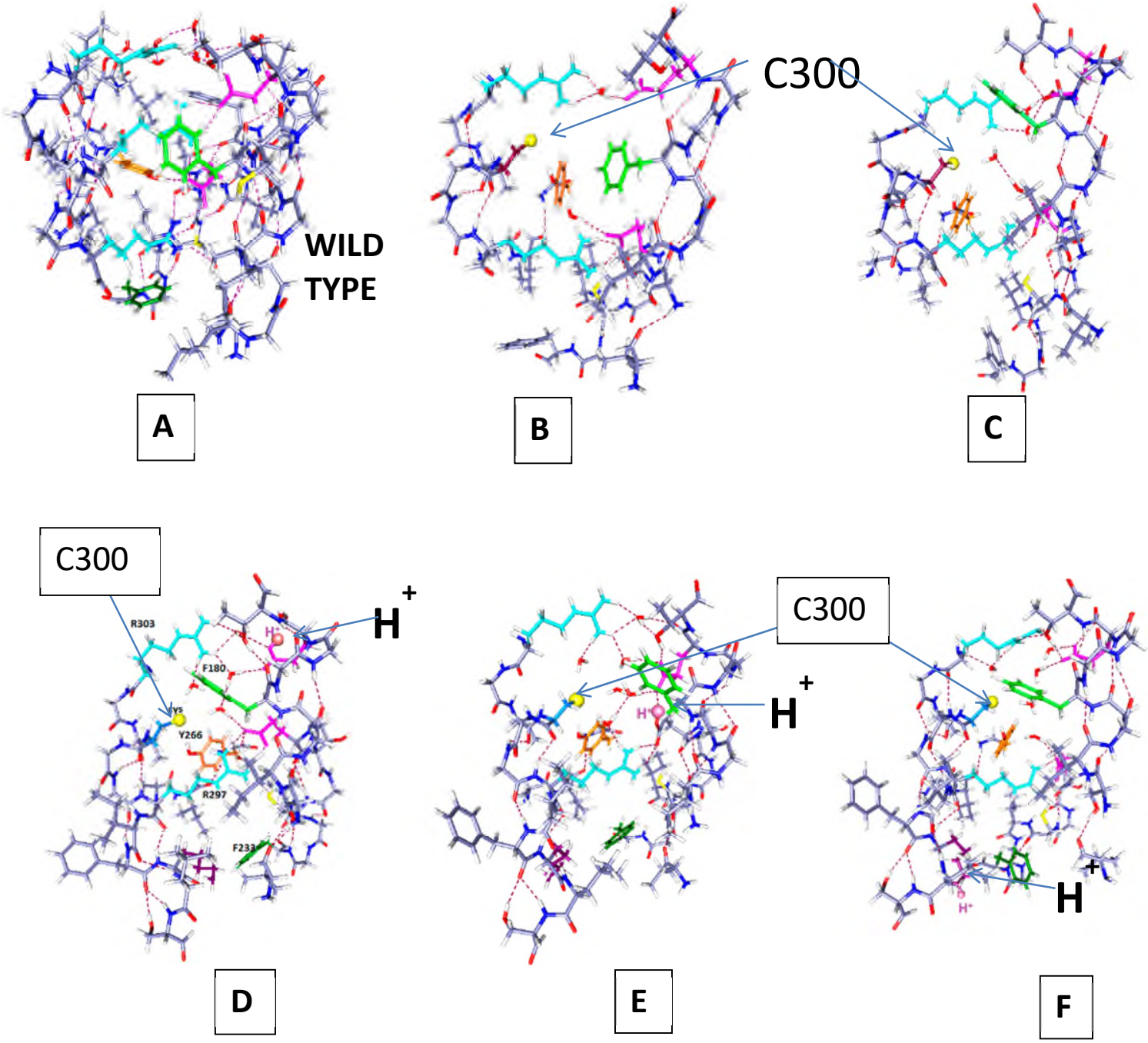

Fig. 2: A, B, C: *Quantum calculations of the central part of the voltage sensing domain, side view from R297 (**top**) to R303 (**bottom**) on the S4 TM segment (3Lut numbering), and nearby residues on S1, S2 and S3. Most of the protein backbone is shown as dark blue (N) and gray (C). Arginines 297 and 303 are light blue, tyrosine (Y266) is orange, phenylalanine F233 green; in **B**,**C**, which are R300C mutants, C300 has the sulfur as a large yellow sphere. Glutamate side chains are magenta. Water molecules are red (O) and white (H). H-bonds are dashed lines. The calculations optimize parts (**B**,**C**) at HF/6-31G** level from a*

*starting position that allowed salt bridges to be maintained where possible; part (**A**) was similarly optimized directly from the 3Lut structure, and the changes are small. The extra water molecules in **C** cause the side chains, especially of F180, to bend out of the way (dihedral angle at carbon changes 270.4°), and the structure contracts slightly.* ***D, E, F:*** *Calculations (same level) on three R300C mutants with added 6 H_2O plus one H^+: The three positions of H^+ are labeled on the figure (D, on E183 at the extracellular end, E, on E266 at mid-cavity, F, intracellularly, on K306. Blockage of the cys changes with the position of the proton; cys appears to be, and probably is, more accessible extracellularly in D and E (in E possibly accessible from both sides); in F, access appears likely from the intracellular side only. Further computations would be needed to confirm the access directions. Water molecules have also rearranged. Shifting a proton can change access to a cysteine attached to S4. The net result is that state dependent access of MTS reagents, consistent with experimental results, can be a consequence of H^+ shifts. All of Fig. 2 is reproduced from (Kariev, A. M. and M. E. Green 2015).*

The evidence for this comes from a few sources.

4.1) SUMMARY OF EVIDENCE SUPPORTING THE STANDARD MODEL: Probably the most important line of evidence, and the first, used a mutation of the arginines to cysteines, one at a time. The cysteine could react with methanethiosulfonate (MTS) reagents which kill the channel. This was based on the SCAM method developed by Karlin and Akabas (Karlin, A. and M. H. Akabas 1998). The cysteine must ionize to react, something that the method assumes requires the cysteine to move to the membrane surface, with its higher dielectric constant. The results of the R→C mutation experiments showed that the reaction was state dependent: the reaction seemed to be more probable at the external surface when the channel was open, at the intracellular surface when it was closed. This is interpreted to mean that the cysteine residues had moved to the extracellular surface when the channel was open, the intracellular surface when closed, in accord with the expectation from the positions proposed for S4 in the standard models. However, as we shall discuss below, the cysteine side chain, essentially a single atom after ionization, is tiny compared to the arginine side chain it replaces. As a result the mutation leaves behind a cavity that can be filled by water, or by the reactive head group of the MTS reagent, making the interpretation of the access experiments much less clear (see Fig. 2).

We observe that the cavity that remains behind is large in Fig. 2, which shows the difference between the wild type and the mutant. In earlier proposals for gating we have suggested that protons could move, thus constituting the gating current. We observe in the calculations that the response of the channel to the mutation can depend on the position of the hydrogen (Fig, 2b); therefore the observed state dependence is still consistent with protons acting as the gating current.

4.1.1) Note an apparent inconsistency in the standard model assumptions. *i)* These models require that the arg-asp or arg-glu salt bridges are ionized; this in turn requires that there be at least some water present (Liao, S. and M. E. Green 2011) (or at least that the dielectric constant not be too low). *ii)* The SCAM assumption that requires the cysteine to react at the membrane surface assumes that the dielectric constant in the VSD is too low to

allow the cysteine residue incorporated as a consequence of R→C mutation to ionize in the VSD interior. In order for the two assumptions to be consistent, the cysteine ionization would require a dielectric constant higher than that for the salt bridges to ionize, and this would have to be true even with the large cavity left by the R→C mutation, a cavity that almost certainly has to be filled by water; at least 3 molecules would fit, enough to allow cysteine to ionize, but more than 3 molecules could fit if suitably configured. If the ionization probability of the cysteine is less than that of the salt bridges, it is possible that both assumptions could be simultaneously true. We have not seen any discussion of this assumption, and it is not so clear how to test it, by any of the standard experiments. It seems odd that the apparent contradiction has not been discussed in the literature. We have made an attempt to test this; we did a small quantum calculation in which the salt bridge was tested with one and with two waters. We have already done a number of calculations with salt bridges(Kariev, A. M. and M. E. Green 2015, Liao, S. and M. E. Green 2011). These are discussed below (see Fig. 7) in the context of discussing the source of gating current; the result we find is that some of the salt bridges may be ionized, but not all. The cysteine is harder to calculate. Cysteine itself has a pK of 8.3. It will be only slightly ionized at physiological pH even in pure water. Not surprisingly, with only a couple of water molecules, the ionization probability is not high. However, as water molecules fill the cavity left by the mutation, the probability of ionization should approach that of cys in water, adequate for reaction with MTS reagents. A quantum calculation will be too sensitive to the absence of surrounding parts of the system to give a pK accurate for cysteine with the protein severely truncated, and with the field shifting so that the side chains rearrange. Therefore we do not present results from such a calculation here. However, we observe that with the water present it is to be expected that the reaction with MTS reagents should be able to occur in situ with a probability not very different from that which it has in solution, or at a membrane surface. Furthermore, because in larger calculations we observe that side chains rearrange with the field, the accessibility of the cysteine would be state dependent. What we have not yet done is to demonstrate that the state dependence really is that which is observed experimentally; so far, our results make this plausible, but are not conclusive.

4.1.2) Molecular Dynamics (MD) simulations are used to determine the path followed by the S4 segment. Various simulations, sometimes with an exaggerated field strength, showed S4 moving (Delemotte, L., M. L. Klein and M. Tarek 2012, Jensen, M. O., V. Jogini, D. W. Borhani, A. E. Leffler, R. O. Dror and D. E. Shaw 2012, Tronin, A., C. Nordgren, J. Strzalka, I. Kuzmenko, V. Lauter, J. Freites, D. Tobias and J. Blasie 2016). Not all simulations showed equal motion; in other words, the partners of the arginines in salt bridges were not always the same. Some showed S4 more nearly associated with the intracellular membrane when closed, and less accessible when open, others had the S4 nearer the extracellular membrane when open, less accessible when closed. The extent of pore opening was not the

same in all cases. MD simulations solve Newton's equations of motion (F=ma) for the protein-water-lipid system stepwise, with each step approximately 10^{-15} s. This is repeated to get trajectories that in the most recent simulations can reach into the microsecond range, with of the order of 10^5 atoms. Some of the largest simulations may do as much as hundreds of microseconds. This should be adequate to describe the S4 trajectory and gating, and most other properties of interest, accurately. Unfortunately, there is a fundamental problem with the method: the forces have to be assigned as partial charges to atoms to calculate the Coulombic forces, which are typically large. The state of ionization during a run is fixed; proton transfer cannot be considered. Polarization of the atoms by local electric fields may in principle be considered, but in practical terms is too time consuming, so it is not included in large simulations. Partial covalent character of short hydrogen bonds cannot be included; in fact, hydrogen bonds are central to the behavior of the VSD, and they cannot be adjusted as they change during the simulation. These are some of the main reasons why MD simulations do not agree with each other, even though they are all done correctly, and why there is reason to doubt that they actually correspond to the behavior of the physical system. There is another problem with extended MD trajectories: it appears that there have been no simulations that reverse the process and show that the protein can refold properly. Some neurons fire at 20 Hz or faster, more or less continuously. If a channel opens at that rate, in a channel with a lifetime of 10 minutes (which seems minimal—it is expensive to produce protein continuously), the channel, with 4 domains, would have to have domains that refolded correctly 48,000 times per channel lifetime. Even if this is somehow an overestimate, getting of the order of 10^4 correct refoldings, with the VSD never trapped in a local energy minimum, seems extremely improbable; trapping in a local minimum would kill the channel. It would be interesting to get an estimate from MD of the probability of the correct refolding by running trajectories backward, but that appears not to have been done.

4.1.3) Fluorescence quenching (FRET, for fluorescence resonance energy transfer) is a third line of evidence. Two fluorescent moieties are conjugated to the channel, normally attaching them by something that reacts with a cysteine. The extent to which the fluorescence of one of these is quenched by the other shows how close they are; if there is a conformational change so that they become further apart, there is less quenching; the quenching falls sharply beyond what amounts to a cutoff distance, quenching falling as R^{-6}. From this, the distances are deduced. As with every other method this too has its defects. For one thing, the fluorescent moieties often have dimensions over 10 Å, so that uncertainties in orientation of a fluorescent group can be comparable to the distances measured. One of the two moieties can be a single lanthanide atom (leading to LRET as the acronym, for lanthanide resonance energy transfer), which helps. The fact that changes in fluorescence quenching occur is clear; whether these correspond to large movements of the S4 backbone may be less certain. Side chain rotation, and

orientation of the fluorescent dye used, may produce results showing motion, but not corresponding to motion of the backbone.

4.1.4) Cross linking of double cysteine mutants of channels has also been suggested as a method for determining which arginine residues of S4 might relate to which negatively charged residues in the rest of the VSD. DeCaen et al offer an example(DeCaen, P. G., V. Yarov-Yarovoy, Y. Zhao, T. Scheuer and W. A. Catterall 2008). There are at least two questions that may be raised with respect to such experiments. First, the timing of the experiment is uncertain; a protein, given enough time, can move such that it allows segments that are not related functionally, or geometrically, to eventually come into contact. Second, substituting cys for arg and for an acid leaves a great deal of space as we discussed above, into which parts of the protein may move, so that even if the experiments are done quickly (<1 ms), the protein has time to collapse such that non-natural contacts become possible. The probability of the motion may be affected by an external field, such that there is an apparent state dependence that accords with expectations, even though there would be no such shift if the full channel, with extended side chains, were still present.

While these are some of the main experiments that have given rise to the standard models, there are a number of other experiments that are much harder to interpret on any form of the standard model. We discuss these in Section 4.5 below.

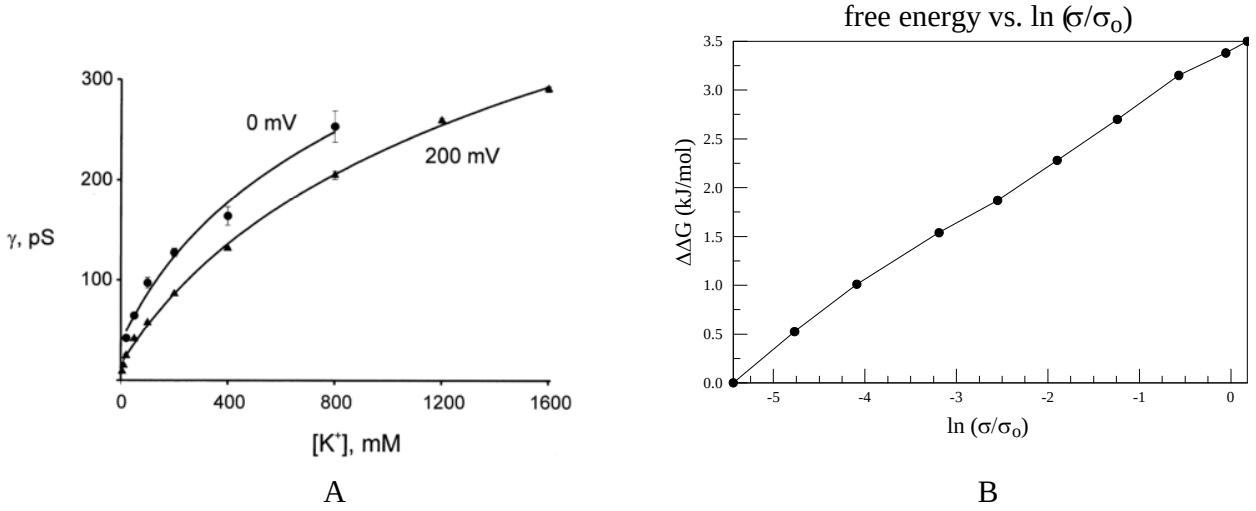

Fig. 3*: **Conductance-concentration curve for KcsA in K^+.** A) From Le Masurier et al. showing the pore conductivity of a typical K^+ channel(LeMasurier, M., L. Heginbotham and C. Miller 2001) B) The 200 mV data replotted, using –RT log $[K^+]$ = ΔG. This shows the conductivity is proportional to the free energy of the $[K^+]$ ion, which is consistent with the existence of a single barrier for conduction of the ion (the plot is corrected for activity coefficients, but this makes insufficient difference to actually see on the plot).Fig 3B from (Kariev, A. M. and M. E. Green 2012).*

4.2) THE PORE: We noted that the VSD had 4 TM segments. The pore has 8, 2 from each of the four domains. These are designated S5 and S6. In addition, the linker between S5 and S6, which is at the extracellular side of the pore, forms the pore lining section that functions as the selectivity filter. This section is characterized by a sequence of amino acids, TVGYG, that is almost perfectly conserved through evolution for K channels, back to bacteria. This sequence presents its backbone carbonyls so as to complex a K^+ ion, and reject a Na^+ ion. The ion, as it proceeds from the intracellular gate up the pore, is hydrated, until it reaches the selectivity filter, described below. Conductivity in the channel is illustrated in Fig. 3.

We can discuss the pore in three sections: 1) at the intracellular end, the ion enters when the gate is open. In our calculations, the gate does not open wide to allow a free flow of water and ion into the gate, but instead opens fairly narrowly to keep the ion complexed. This is required because an ion typically resides in the middle section, above the gate, where the channel widens to provide a cavity that hydrates the ion fully. There is a conserved PVPV sequence at the gate, and the nitrogen on the proline ring appears to be absolutely required to properly solvate the ion. In another calculation we considered the effect of an *in silico* mutation to PVVV, and found that the solvation of the ion was partially lost, and the water column through the gate was seriously disrupted. 2) The cavity: Above the pore and below the selectivity filter there is space for the fully hydrated K^+ ion discussed above. The ion is found in some X-ray structures at least. Some time ago, it was suggested by Armstrong that there should be a "knock-on" mechanism such that the incoming ion would push the ion in the cavity up into the selectivity filter, then take the place of the previous ion in the cavity, continuing the ion conduction. However, the ion in the cavity would knock an incoming ion back if the incoming ion were not held at the gate. This requires that the ion be essentially complexed there, so that the gate can open only about 4 Å in diameter beyond its closed position, which seems to already be about 8 Å diameter. Fig. 4 shows a calculation of the lower and middle part of the pore. As can be seen in the figure, the opening at the gate is just large enough for a hydrated K^+ ion. Fig. 5 shows the consequences for the sequence of positions occupied by the ion as it passes through the gate and the central part of the pore. Having the ion complexed at the gate has as a consequence the ability to push an ion in the cavity onwards into the filter; a fully open filter would almost certainly cause an entering ion to be "knocked-back" instead of the knock-on (*i.e.* forward) effect required for conduction. Even with the ion trapped at the gate, it is not clear that "knock-on" is a good description, as the interaction is not entirely a Coulombic push on the central ion; the water molecules turn out to provide effective screening. The trapping of the ion at the gate prevents a "knock-back" effect, however, and a stochastic transfer of the ion from cavity to selectivity filter, followed by transfer of an ion from the gate to the cavity, would then suffice to allow conduction (Fig. 5). 3) As the ion reaches the selectivity filter, the first backbone carbonyls begin to cosolvate it. As it enters the selectivity filter to the first binding site, solvation has been completely

taken over by the carbonyls of the selectivity filter.

There are four main binding sites, and it is usually considered that alternate sites, either 2 and 4 or 1 and 3, are occupied by ions, with the other sites occupied by water molecules. Whether this is exactly correct or not (there is some question), it appears to be at least a somewhat accurate description of the selectivity filter. Water and ions move through the selectivity filter together.

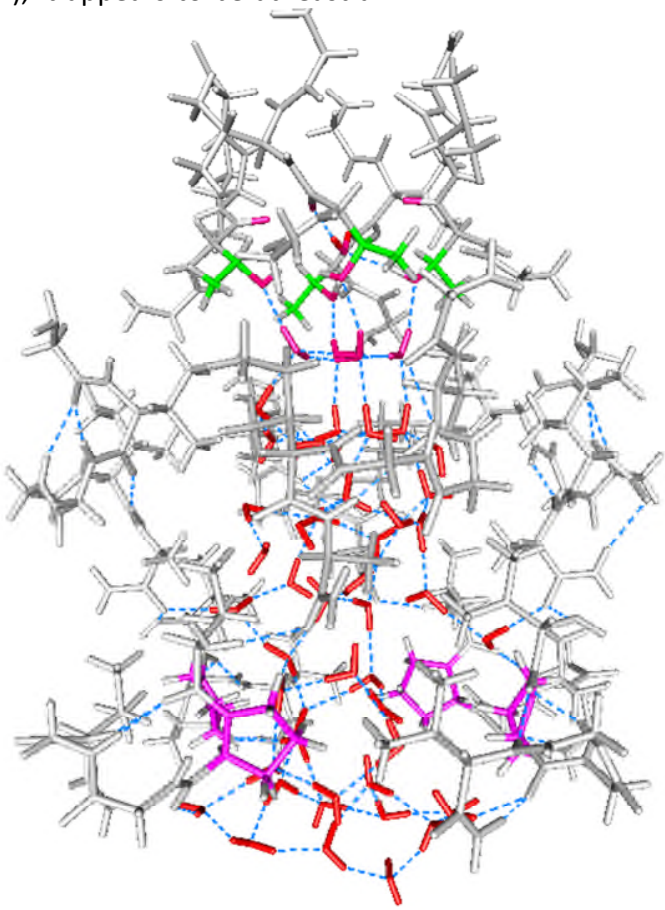

Fig. 4: A single quantum calculation of the gate plus cavity region of the pore: The structure is optimized (energy minimized) using HF/6-31G level quantum calculation, adequate to give a good structure. Water molecules are shown in red, the prolines of the highly conserved PVPV gate sequence in magenta, at the intracellular end of the pore (bottom). The threonine of the selectivity filter, in green, is where the ion would be first cosolvated, and enter the selectivity filter. Additional parts of the protein that were included in the calculation are in gray. In all, 872 atoms are included, and the calculation ran for about 6 months.*

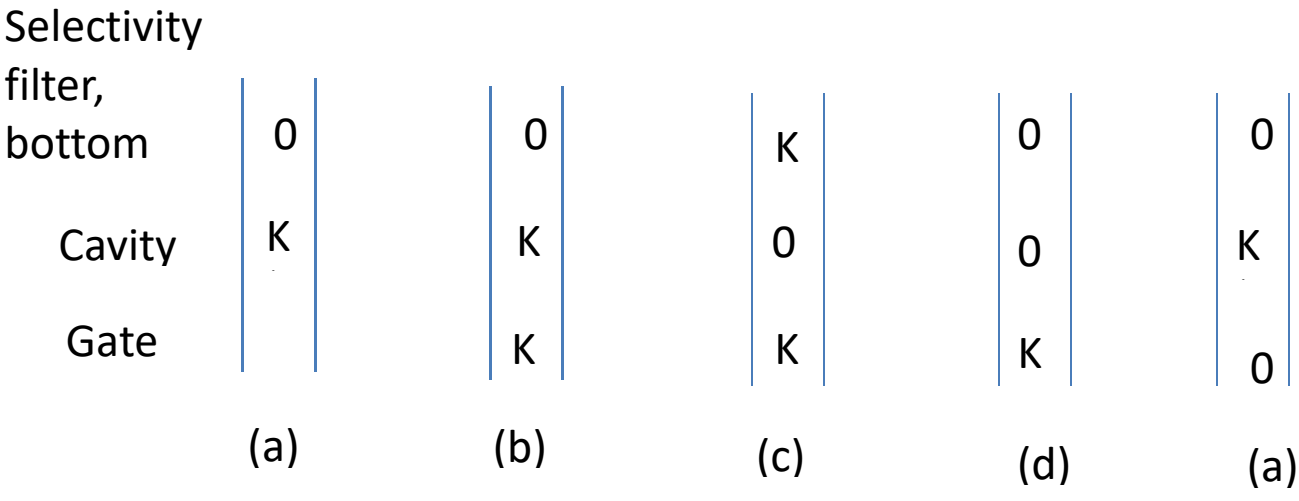

Fig. 5: The succession of positions for ions in the channel pore: (a) the cavity is occupied, but the gate and the lowest position of the selectivity filter are not. (b) the gate is also occupied. This is the crucial configuration. The ion at the gate is supposed to push the ion in the cavity up; however, unless the ion at the gate is held tightly enough, it will be pushed back out instead. This means that a wide open gate is impossible if the "knock on" mechanism is at all valid, and it appears that it should be (c) The cavity ion has moved up to the lowest position of the selectivity filter (d) and then further into the selectivity filter, allowing (a) the ion from the gate to enter the cavity, restoring the first configuration, so that the cycle can repeat. This figure is reproduced from (Kariev, A. M. and M. E. Green 2012)

4.3) THE GATE: The gate itself has, as its closest residues, the highly conserved PVPV sequence mentioned above. We have just noted that the nitrogen from one of the prolines, (there are 4 such, one from each domain) appears to complex the ion at the gate, to make possible the "knock-on" mechanism. There are other residues near the gate that also appear to exist in several K^+ channels, and a water column is crucial. The PVVV mutant discussed earlier showed a broken water column as the most obvious difference from the native structure. Other residues of particular interest consist of glutamines or asparagines near the gate. The KcsA channel is gated by a drop in pH, *i.e.,* by protons, rather than by membrane depolarization, and contains glutamines (Q119) just inside the gate. Another newly characterized channel (Whicher, J. R. and R. MacKinnon 2016), which is voltage gated but shut by Ca^{2+} interacting through calmodulin, the Eag1 channel belonging to the K_v10 family, also has glutamines (Q462) that appear to play a role in gating. Even the $K_v1.2$ channel that we have been discussing, (pdb 3Lut) has asparagines (N414) near the gate, with the side chains pointing in to the pore. This is very near the H418 residue needed to have a functioning channel, discussed in the next section. We have done calculations on KcsA, and found a "basket" of four water molecules hydrogen bonded to the Q119 residues (Kariev, A. M. and M. E. Green 2009). This appears to be a stable motif; it also appeared in calculations on $K_v1.2$, attached to the glutamines there. It is not clear whether there is a functional relation between this basket and the channel conduction, but it is a matter worth noting, pending further

examination. KcsA, like $K_v1.2$, has a histidine (H124) near the 4 glutamines, in approximately the same relation to them as H418 in $K_v1.2$ has to the asparagines there. It is known experimentally that a distant histidine, H25, is crucial for gating in KcsA (Thompson, A. N., D. J. Posson, P. V. Parsa and C. M. Nimigean 2008), but it is not so clear what the relation of these glutamines and histidine is to gating. However, it is not unreasonable to suggest that there is a path for protons to reach the glutamines from H25; there are glutamates above and below the glutamines, which could be part of this path on the way to the histidine, which is close to the lower (intracellular) glutamate. If the glutamines become partially charged (not impossible if the activity of H^+ is high enough (*i.e*., pH low enough), they can separate by the approximately 2 Å needed to open the gate. At this time, the evidence for this is still uncertain.

4.4) THE INTRACELLULAR REGION, AND THE S4-S5 LINKER: In all standard models of gating, the gate opens when the S4 segment pulls the S4-S5 linker aside, increasing the diameter of the gate so that the ion may enter. As we have just seen, too large an opening would prevent conduction. Although not all models take this into account, in several the opening is small enough that the gate could form the necessary complex. A variety of connections between S4 and the linker have been proposed. The careful work of Blunck and coworkers suggests that a change in radius of 3 - 4 Å at the gate is sufficient to allow gating to occur (Batulan, Z., G. A. Haddad and R. Blunck 2010, Blunck, R. and Z. Batulan 2012, Faure, E., G. Starek, H. McGuire, S. Berneche and R. Blunck 2012). This is only slightly larger than the 2 Å which appears adequate based on our calculations, and which would allow the complex suggested by Fig 4 to form. Given the limits of error on LRET (Lanthanide Resonance Energy Transfer) experiments, 2 Å is possible. In addition, the histidine of the S4-S5 linker (H418 in the 3Lut numbering) is required to have a functioning channel (Zhao, L.-L., Z. Qi, X.-E. Zhang, L.-J. Bi and G. Jin 2010). The S4-S5 linker in the $K_v1.2$-$K_v2.1$ chimera, and in *Shaker*, is long enough to stretch between domains, and it is supposed to act as a lever in some standard models.

4.5) THERE IS ADDITIONAL INFORMATION FROM OTHER EXPERIMENTS: We have suggested a possible necessity for reinterpretation of the experiments described as used to support the standard model, but, if they are taken at face value, with the SCAM assumptions all accepted, and the other problems listed above ignored, then S4 would have move up and down as in the standard models. However, there are a number of other experiments that are harder to interpret in terms of the standard model. In each case, they are considered within the standard paradigm, with some geometric twist that makes this possible.

4.5.1) Below the membrane there are two substantial sections of the channel, which at least modulates its activity. The section immediately below the membrane is labeled T1, and has been shown to affect gating; it is not clear, on the basis of standard models, how this is possible, but experimentally it does matter (Minor, D. L., Jr., Y.-F. Lin, B. C. Mobley, A. Avelar, Y. N. Jan, L. Y. Jan and J. M. Berger 2000)

4.5.2) Substitutions of either sign of charge, for arginine, produce a very similar decrease in gating current (Gonzalez-Perez, V., K. Stack, K. Boric and D. Naranjo 2010). This seems to rule out a linear motion for S4, but it was suggested that this experiment could fit the standard models by a sort of twist that allows any change in charge to lower the gating current.

4.5.3) If S3, which contains negative charges, is given cysteine substitutions and reacted with MTS reagents, it produces the same differential access as S4 (Nguyen, T. P. and R. Horn 2002), in spite of the fact that the standard models suggest, on their face, that one should expect S3 moving in the reverse direction with the field. This too has been interpreted in terms of versions of the standard model. It is not so clear whether the S4 motions required for this experiment to fit the standard model and those for the experiment described in section 4.5.2 are compatible.

4.5.4) The T1 moiety affects gating(Minor, D. L., Jr., Y.-F. Lin, B. C. Mobley, A. Avelar, Y. N. Jan, L. Y. Jan and J. M. Berger 2000), and the histidine near the pore (H418 in the 3Lut numbering) is required to have a functional channel. Both the role of the terminal histidine, and the role of T1, are difficult to understand on the basis of the standard models. The histidine does extend close to the T1 section, so the two effects may well be related; it is easier to understand how both modulate, or abolish, gating, if they work together.

4.5.5) Proton transport in the VSD is possible: The VSD is capable of conducting protons at least part way across the membrane. If the end arginine is mutated to histidine the VSD becomes a proton conductor, and this is not a consequence of the histidine physically carrying the proton across a barrier (Starace, D. M. and F. Bezanilla 2001, Starace, D. M., E. Stefani and F. Bezanilla 1997). Furthermore there is a proton channel, H_v1, that has an upper section that is almost the same as in a VSD. In spite of the fact that the lower part of the channel is different (H_v1 must carry the H^+ completely across the membrane, while under normal conditions, the VSD must not do this), it is possible to see how the H^+ initial step could occur in the VSD. It is also possible to see a path for H^+ in the VSD. At least one other channel known to transmit protons, bacteriorhodopsin, has a very similar configuration of three ionizable amino acids at the origin of the proton path.

4.5.6) One other experiment concerns the gating current: the “piquito”, a very short pulse at the beginning of gating current that happens too quickly to measure its rise time (<2μs, the RC time constant of the membrane) (Sigg, D., F. Bezanilla and E. Stefani 2003, Stefani, E., D. Sigg and F. Bezanilla 2000). Efforts have been made to explain this in terms of conformational changes, especially of side chains, and their effect on the energy landscape in the VSD, but the details remain to be determined in these explanations (Sigg, D., F. Bezanilla and E. Stefani 2003). The pulse is more directly consistent with H^+ tunneling. In earlier work, we showed that if the field matched energy levels, such tunneling could occur between two methylamines, standing in for a channel (Yin, J. and M. E. Green 1998); we proposed that the “piquito” is generated by a proton transfer which in turn initiates gating. Tunneling should also imply a barrier, or threshold, something that had been thought to be ruled out by the “Boltzmann” curve that fit the measured gating current vs. voltage curve. However, if there is an approximately Gaussian distribution of threshold energies with

standard deviation roughly kT, as should be the case for an ensemble of channels (the Boltzmann curve measured for fairly large number of channels), then the cumulative distribution function of this threshold distribution, which is the relevant distribution for gating probability, gives a gating current curve that is very difficult to distinguish from the "Boltzmann" curve. A very slight perturbation of the Gaussian distribution could make it identical (Fatade, A., J. Snowhite and M. E. Green 2000). This also leads to a prediction of stochastic resonance if noise is added to the voltage, but this appears never to have been tested.

4.5.7) D_2O slows the last step in gating (Alicata, D. A., M. A. Rayner and J. A. Starkus 1990, Schauf, C. L. and J. O. Bullock 1980, Schauf, C. L. and J. O. Bullock 1982). This makes clear that water has a role in gating, something that is not obvious if gating is completely determined by S4 motion. As it is the last step in opening the gate, it may be related to the arrangement of the gate around the ion, with rearrangement of the hydration, but so far there is no direct evidence of this. There are a few other individual experiments, and they have been discussed in more detail in previous reviews (Kariev, A. M. and M. E. Green 2012, Kariev, A. M. and M. E. Green 2015). Here we note their existence, but do not repeat more detailed discussion.

One other question must be considered. If the S4 TM segment extends into the region outside the membrane, or even into the headgroup region, it is likely to be complexed by phosphate. It is known that channels require negatively charged lipids(Schmidt, D., Q.-X. Jiang and R. MacKinnon 2006). It is also known that arginines complex phosphates (Freites, J. A., D. J. Tobias, H. G. von and S. H. White 2005, Green, M. E. 2005, Green, M. E. 2008). If an arginine reached a negatively charged phosphate, it is hard to see how it would return, especially if it has to return in a reasonably short time—it may be complexed for a time long compared with the lifetime of the channel. If the phosphate is at least 0.05 M in the intra- or extracellular solution, this would allow a complex for an arginine that emerged from the headgroup region. However, most of even the standard models do not need the arginine to travel this far; it is the headgroups themselves that are the principal concern in this regard.

5) EVIDENCE FROM QUANTUM CALCULATIONS FOR AN ALTERNATIVE TO THE STANDARD MODELS: In this review we consider some results from quantum calculations, principally our own. We begin by considering the advantages, and the limitations, of these calculations. The advantages include: avoiding the problems with MD, since the polarization, the variation in hydrogen bonds, including covalent character, charge transfer (ions frequently turn out to allow some back transfer, for example K^+ turns out to have charge in the range $0.8 < q < 0.9$, depending on local environment, with electron density transferred from surroundings into the 4s, 4p, and 5s orbitals of K^+.) The amount of transfer may depend on the energy levels of the electrons in the surrounding atoms. This cannot be taken into account in MD, where charge cannot transfer. Salt bridges, including their state of ionization, are likely to be more accurate, and proton transfer can be studied. In short, in quantum calculations, results are likely to be

accurate. By use of natural bond orbital (NBO) calculations (Glendening, E. D., A. E. Reed, J. E. Carpenter and W. F. , Weinhold, F. 2012), one can obtain the charges on the atoms, and determine whether hydrogen bonds have acquired some covalent character. It is also possible to see what happens when a proton transfers within a salt bridge, such that the charges on arginine and glutamate (or aspartate) are zero (again, approximately, because of charge transfer). This could in principle also be done in MD by running separate trajectories with the proton in each position. This does not appear to have been done up to the present time.

However, quantum calculations have disadvantages as well. These include: 1) an energy minimum is obtained, not a trajectory or a mix of states. In other words, the result corresponds to 0 K temperature. One could in principle move a proton or side chain, calculate the energy at a series of positions, and try to map an energy landscape with all attendant possibilities, but the cost, so far, is prohibitive. There is an advantage to using the 0 K form; the X-ray structure is derived from a crystal at perhaps 100 K; the low temperature allows the structure to be determined with only limited vibrations smearing the electron density. Therefore the X-ray structure corresponds to a temperature well below that of the lowest water (ice) structural transition, which occurs around 220 K, and is therefore presumably quite close to the 0 K form, allowing the quantum calculation to be compared to the X-ray structure without error caused by vibrations. We expect the differences in entropy between different proton locations to be relatively small, so that the energy differences found in quantum calculations are probably not greatly different from the differences in free energy. This said, an error of a few kT is possibly introduced by use of the 0 K energy values, and the interpretation of the calculations must take into account the possibility of entropy differences that may be slightly too large to be neglected. 2) A second limitation of the quantum calculation is the finite number of atoms that it is possible to include in the calculation. While an MD calculation might have of the order of 100,000 atoms, and include lipid as well as water, the quantum calculations can only now begin to handle of the order of 1000 atoms, and this takes patience and a large computer. With new software, in which the time for HF calculations scales only as the square of the number of atoms, instead of the fourth power that has been limiting, the number that can be included may rise. The other major advance for some computers is the incorporation of graphics cards for straight computational purposes. Together these advances may make it possible to somewhat exceed 1000 atoms, but there is no immediate prospect of advancing as much as an order of magnitude. 3) MD allows some time dependence, with the calculation of ever increasing trajectory lengths reaching the microsecond range. Quantum calculations that we use are stationary. It is possible to do *ab initio* MD, but these are so computationally expensive that the number of atoms, and the length of the trajectories, are both too small to be of much use with ion channel computations.

Therefore, while quantum calculations allow us to determine things we could not understand in any other way, these calculations too have limitations, and

cannot be regarded as the complete solution to understanding ion channels. Still, without quantum calculations, it would be far more difficult to interpret the effects of charges in these proteins.

Now that we have seen that there may be reason to look for an alternative to the standard models, we propose such an alternative. This has a fairly long history, going back about two decades. One of us first proposed that water had a key role in ion channel gating in 1989(Green, M. E. 1989), and this was a little later developed into a proposal that gating current consisted of a H^+ current rather than motion of S4 (Sapronova, A., V. S. Bystrov and M. E. Green 2003, Sapronova, A. V., V. S. Bystrov and M. E. Green 2003) The idea that the initial step in gating could be proton tunneling as a field matched energy levels was proposed by Yin and Green (Yin, J. and M. E. Green 1998), based on a quantum calculation in which the field was varied and proton transfer between methyl amines was calculated (now a small calculation, but it was not small at the time). We summarize the present state of this model:

5.1).There exists a path that can be seen for proton transfer between the extracellular (upper) and intracellular sides of the VSD. The upper section looks very much like the H_v1 proton channel (discussed in section 4a). The lower section in the VSD deviates, producing two alternate paths for protons. Since there are 2 to 3 charges per VSD, possibly both paths are used. Fig. 6 shows the proposed paths. This model is not a calculation, but a structure taken from the X-ray (therefore, open state) coordinates. However, if our model is correct the shift between open and closed backbone positions is ≤2 Å in the direction of the field (perpendicular to the membrane), so that there is not a major change in conformation. The side chains can rotate, so that atoms in the side chains can move more than 2 Å, especially if motion parallel to the membrane is included.

We postulate that the protons move from the upper to the lower section under the influence of polarization of the membrane, closing the channel by causing a side chain motion of about 2 Å near the PVPV conserved sequence, where this sequence is found in most K_v1, K_v2, K_v3 and K_v4 channels. In the 3Lut structure, there is a histidine, H418, near this sequence. Since the H418 deletion mutant channel does not function, and the T1 moiety affects gating, these facts are consistent with a proton path of the form proposed.

To test whether this proposal is plausible, we are carrying out quantum calculations on portions of the VSD.

One short test that can be performed on a fraction of the VSD is to determine whether the ionized or unionized form of the salt bridges found in the channel has lower energy. Based on previous work with salt bridges, we might expect that when there is no water present, the unionized form dominates(Liao, S. and M. E. Green 2011). This is the case, as shown in Fig. 7, and Table 1, which has the energies. In the neighborhood of R303, where the hydrophobic break appears, this should be the case.

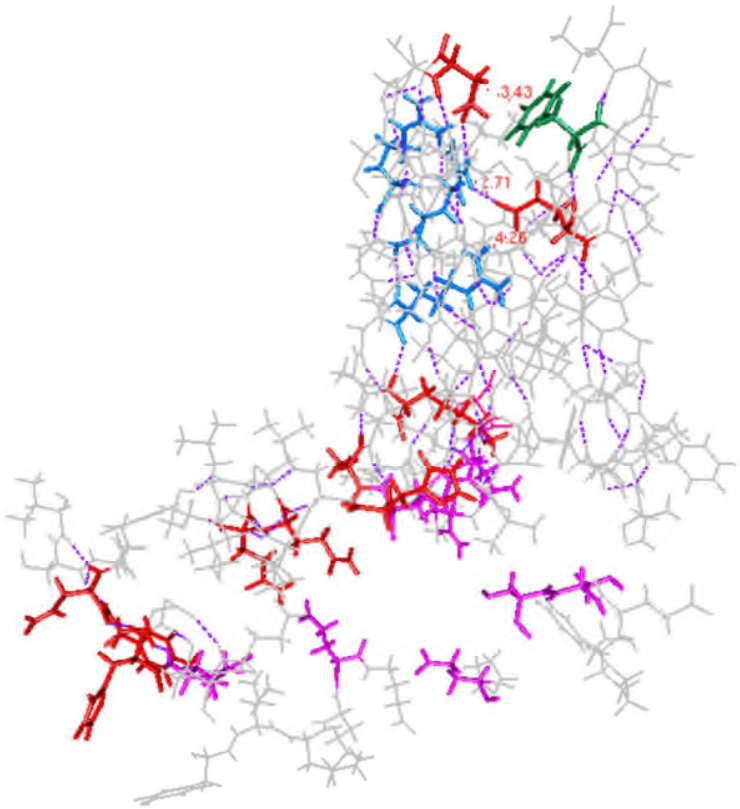

Fig. 6: Two proposed proton paths. The upper part of the VSD is almost identical to the upper part of the H_v1 channel, while the lower part deviates. The proton path in the H_v1 channel continues essentially straight down, while the paths here deviate down toward the gate. One path is shown in magenta, the other in dark orange. Water is needed to complete the lower part of the paths, but this is not a problem; water molecules are found in this region, which is at the intracellular edge of the VSD.

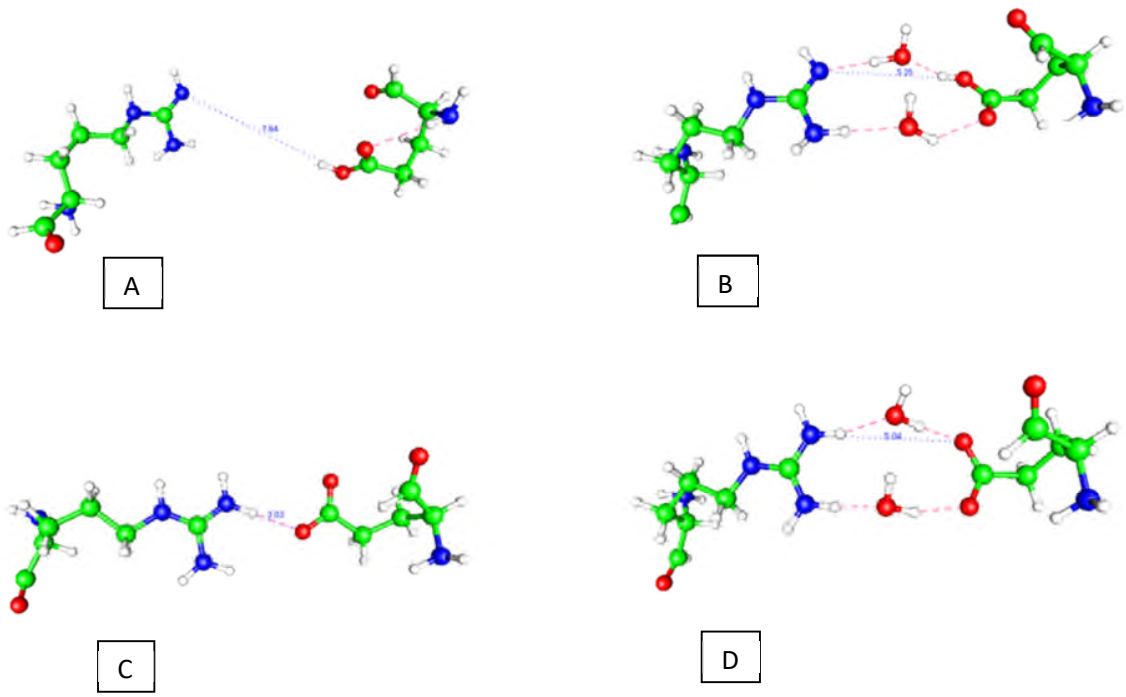

Fig. 7: The arginine-glutamate salt bridge in four forms: with no water (A, C) and with two water molecules (B,D). In A and B the salt bridge is not ionized, in C and D it is. The energies for each of these, ionized and not, are given in Table 1. Note that the unionized case A has the side chains much more separated than the ionized case C.

In the standard models, it is assumed that the arginines are ionized, generally with a full charge of 1, and these arginines exchange partners with the acid groups (aspartate, glutamate), moving up and down the VSD in response to voltage. Here we see that the assumption of ionization is questionable. There is not enough water for all the salt bridge partners to be charged; instead the proton remains on the acid for at least one pair, making both arginine and acid neutral. If the arginines are neutral, they cannot carry gating charge. If however, this is a transient state, as the proton moves on, then the gating current can be accounted for. If the arginines were to move, but the charge varied as they moved, it would be hard to estimate the gating current, but it would almost certainly not match the assumptions in the standard models. Finally, recall the discussion of the "piquito" (section 4.5.6), which is further evidence for the transfer of a proton, in this case to start the proton cascade.

TABLE 1

Energies of salt bridge cases shown in Fig. 7. (atomic units)

	Energy without water	Energy with 2 molecules of water
Ionized: Fig. 7 A and B	-1007.8945	-1160.8945
Not ionized: Fig. 7 C and D	-1007.9370	-1160.8858

*Notes on Table: Calculations: B3LYP/6-311G**. The two .8945 values in the ionized row are correct—they are equal by accident. The salt bridge has 43 atoms in the calculation; with two molecules of water, there are 49 atoms.*

In Table 1 the non-ionized cases are lower in energy; without water, by about 43 k_BT, with water, by about 9 k_BT (.001 atomic units ≈ 1 k_BT at room temperature). It is not the case that one can assume that salt bridges are always ionized; the presence of at least 3 water molecules is required to insure ionization(Liao, S. and M. E. Green 2011). This has implications for the VSD salt bridges as well. However, the translation to the protein case is not straightforward, as the local electric field depends on more than the number of water molecules, and the salt bridge may also transfer a proton to a neighboring residue that is not part of the salt bridge itself. The point of the present calculation is that caution is required in assigning charges in the VSD.

Water molecules: we also need to investigate whether water molecules are themselves a significant part of the channel from the point of view of affecting gating. To this end, several calculations with a cluster of water molecules at the extracellular end of the channel are shown in Fig. 8. This figure also shows the importance of the water molecules, in which the net dipole of the entire cluster of water reverses. The value of the dipole was not computed but the orientation of the water molecules was determined. Assuming roughly the normal water dipole of approximately 2 D (Debye) per molecule, with the orientations that were determined, there was a shift of approximately 5 D with the external field. This makes the local field at the extracellular end of the channel sufficiently different from that which is imposed externally to be significant for a charge that might move in response to a field. In other words, the water is functionally part of the channel, and must be considered in any model of channel gating. In these cases a very large field was applied. With about one-tenth the field applied in the central figure (one-fifth the left and right examples), as expected in a nerve, for example, the motion of S4, small in the center figure where it could move, would be expected to be even less.

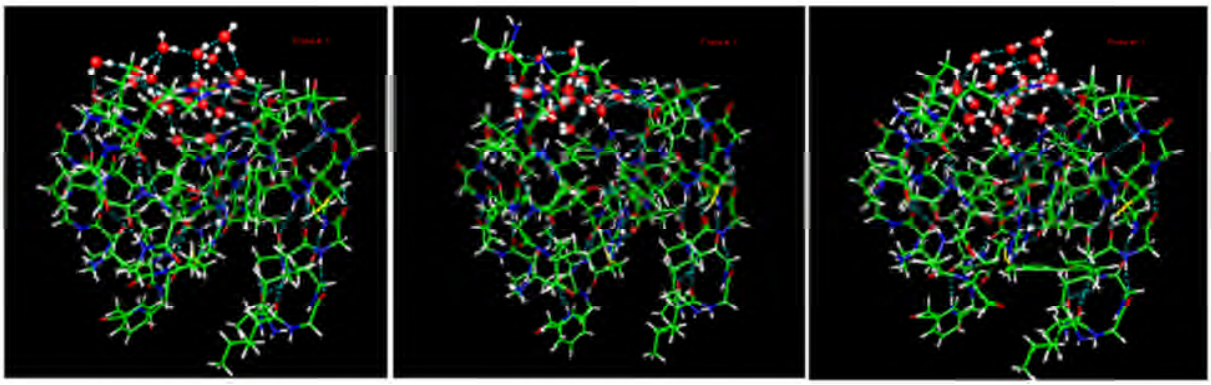

*Fig. 8: Three completed optimizations, two with electric fields that should close the channel. A single VSD is shown, with 609 protein atoms. Left: channel open (a field equivalent to 350 mV applied, intracellular positive, much larger than required to open the channel), with 21 water molecules at the extracellular end. Center: Closed, large field case (equivalent to 700 mV, intracellular negative), with 18 water molecules, and the S4 TM segment free; it shows minimal motion, especially in the vertical direction, which is contrary to the prediction of the standard models, although some sections move into space vacated by truncated side chains. Right: Closed, (350 mV intracellular negative), S4 fixed by several frozen atoms at each end, with 21 water molecules. All three have the same side chains truncated to keep the number of atoms to a level that could be computed. The most important result is the effect of the field on the water molecules. The energies for the two 21 water cases, calculated as single point values using B3LYP/6-31G** on the HF optimized structures, gave -17072.0905 (closed) and -17072.1007 (open) in atomic units. In other words, the open case has approximately 10k_BT lower energy, with no field, compared to the closed case with field.*

6) **QUANTUM CALCULATIONS**: Optimizations were generally done using Gaussian (Frisch, M. J. T., G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Mennucci, B.; Petersson, G. A.; Nakatsuji, H.; Caricato, M.; Li, X.; Hratchian, H. P.; Izmaylov, A. F.; Bloino, J.; Zheng, G.; Sonnenberg, J. L.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Montgomery, J. A., Jr.; Peralta, J. E.; Ogliaro, F.; Bearpark, M.; Heyd, J. J.; Brothers, E.; Kudin, K. N.; Staroverov, V. N.; Kobayashi, R.; Normand, J.; Raghavachari, K.; Rendell, A.; Burant, J. C.; Iyengar, S. S.; Tomasi, J.; Cossi, M.; Rega, N.; Millam, J. M.; Klene, M.; Knox, J. E.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Martin, R. L.; Morokuma, K.; Zakrzewski, V. G.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Dapprich, S.; Daniels, A. D.; Farkas, Ö.; Foresman, J. B.; Ortiz, J. V.; Cioslowski, J.; Fox, D. J. 2016)(all those reported here used Gaussian09, D or E versions). The level used for optimizations

was HF/6-31G* in esentially all cases. For determination of the charges and the energy of the optimized structure, NBO calculations (Glendening, E. D., A. E. Reed, J. E. Carpenter and W. F. , Weinhold, F. 2012) (again, as implemented in Gaussian09) were used at B3LYP/6-311G** level, as single point calculations.

7) **SUMMARY:**

7.1) The R→C mutation experiments that have seemed to establish the motion of all of S4 as the source of gating current can be understood in terms of the gap in the VSD that is left behind on replacing the very large R side chain with the single atom (when ionized) C side chain. This volume can contain at least 3 molecules of water, allowing the cys to ionize, followed by making space for an MTS headgroup, which can in turn displace the water. Access to the S^- of the cys is expected to be state dependent, based on the positions of H^+. The H^+ can transfer in response to the electric field. Calculations are in progress to determine the energetics of the proton transfer, to make sure that the transfer really would occur in response to the changes in field. We can postulate two pathways along which H^+ may transfer. Since the gating current may be >2 charges per VSD domain, there may be as many as three pathways. The terminus appears to be approximately at H418, which is near the PVPV conserved section that constitutes the gate. Other evidence that bears on the gating question can also be understood in terms of the motion of protons. We have discussed the most salient of this evidence, and found that at least some of it can be understood more easily in terms of the motion of protons than in terms of the motion of the entire S4. The H_v1 channel is generally taken to gate with an extended motion of the VSD, but it too can be understood as proton transitions producing a current. Some evidence is very difficult to interpret in terms of the motion of the VSD backbone, such as the importance of the H418 residue(Zhao, L.-L., Z. Qi, X.-E. Zhang, L.-J. Bi and G. Jin 2010), the fact that the T1 moiety makes a difference to gating(Minor, D. L., Jr., Y.-F. Lin, B. C. Mobley, A. Avelar, Y. N. Jan, L. Y. Jan and J. M. Berger 2000), that D_2O has an effect, that there is a an extremely brief pulse at the beginning of the gating current (the “piquito”), as well as several other experiments, some discussed above. We have also considered the reasons that MD simulations may not be accurate for a membrane protein in which protons may move, and charge transfer may occur (as well as other possible changes that are not possible with MD). Large scale motion of the S4 segment also requires that the segment refold properly, in some cases $>10^4$ times during the lifetime of the channel, something that has yet to be simulated; it appears overwhelmingly probable that the S4 would be trapped in some local minimum on the way back, at some point well short of the required lifetime of the channel. Putting all this together, the most probable gating current is the motion of protons, rather than the backbone motion of S4, pulling positively charged arginines along.

7.2) Quantum calculations have advantages over MD, in that they allow polarization, partial covalent H-bonds, charge transfer (e.g., back to K^+, making its charge <1), determination of the charge on salt bridges, and similar questions, such as charge, and partial covalency. They have some

difficulties, in comparison with MD: lack of any time dependence, the result is at 0 K, and the number of atoms is limited. This said, the quantum calculations are required to have a reasonable description of the behavior of the system.

7.3) The path of the ion through the pore also requires quantum calculations to determine the state of hydration of the ion, as well as the charge state. The ion must be held at the gate in order to push the ion above it, in the cavity of the pore, up into the selectivity filter ("knock-on" effect); a wide open gate would allow a "knock-back" effect, in which the incoming ion would be pushed back by the ion in the cavity, rather than the other way around. This sets limits on the extent of motion of the gate allowed to open the channel; gating and conduction are linked in this manner.

7.4) Finally, therefore, a gating mechanism in which the gating current is provided by H^+ motion is proposed. In this model, the gate opens only to a limited extent. In addition, the roles of what are usually ignored or at most given ancillary roles in gating, including hydrogen bond shifting and water, as well as the roles of some specific residues and sections (e.g., H418, the T1 moiety) are clarified, along with the meaning of several other experiments. We consider this ability to correlate more information to make the model more likely than the standard models. Further calculations to test this proposal are in progress.

Acknowledgements: This research was supported, in part, by a grant of computer time from the City University of New York High Performance Computing Center under NSF Grants CNS-0855217, CNS-0958379 and ACI-1126113, and this research used resources of the Center for Functional Nanomaterials, which is a U.S. DOE Office of Science Facility, at Brookhaven National Laboratory under Contract No. DE-SC0012704. Fig. 2, 3b, and 5 are reproduced from the International Journal of Molecular Science (Kariev and Green, 2012, 2015) under Creative Commons License.

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