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Voltage gating and anions, especially phosphate: A model system

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Abstract

The voltage sensor of voltage gated sodium and potassium channels consists of four sets of transmembrane segments, of which one, called S4, contains at least four arginines; these are presumed to each carry positive charges. The channel opening is usually attributed to the outward (i.e., toward the extracellular side of the membrane) motion of S4. The evidence for this motion is based on certain experiments that appear to show differential access to parts of S4 from the intracellular and extracellular sides of the membrane in the open and closed states. A newly available structure [S.B. Long, E.B. Campbell and R. MacKinnon, Crystal structure of a mammalian voltage-dependent *Shaker* family K^+ channel. Science 309 (2005) 897–903; S.B. Long, E.B. Campbell, R. MacKinnon, Voltage sensor of Kv1.2: structural basis of electromechanical coupling. Science 309 (2005) 903–908][1,2] has now been used to argue for a large scale motion, although, as a static structure, it is not conclusive. In this paper, we consider the effect of anions in the surrounding medium. Phosphate is present in the intracellular as well as the extracellular fluid, apparently at hundreds of micromolar concentration, or more. There is evidence in the literature suggesting that phosphate–arginine complexes are rather strong. In a recent calculation one of us [M.E. Green, A possible role for phosphate in complexing the arginines of S4 in voltage gated channels. J. Theor. Biol. 233 (2005) 337–341][3] has shown that a model peptide with a 2:1 arg:phosphate complex should have a favorable geometry. Here, we present NMR evidence of the existence of phosphate complexes of a model peptide with two arginines separated by two hydrophobic residues, the same spacing as in S4 segments. The complexes (there are different complexes for HPO_4^{2-} and for $H_2PO_4^-$ [3]) form with concentrations of peptide in the range of hundreds of micromolar, making it significant in the biological context. NMR spectra provide changes in chemical shift as functions of both phosphate concentration and pH. The resulting curves show titration of the phosphate, with its standard pK. Possible implications for other anion–S4 interactions, including ion pairs rather than complexes, as with Cl^-, are also discussed.

Keywords: Voltage gating; Ion channel; S4; Anion; Phosphate; Arginine complex

1. Introduction

The gating mechanism of voltage-gated channels is a matter of immense interest and has been the subject of considerable experimental and theoretical effort. The standard mechanisms that have been proposed start from the existence of positively charged residues, generally arginines (the occasional lysine is apparently not involved in gating), every third position in the S4 transmembrane (TM) segments of each of the four transmembrane domains of the channels. These mechanisms then assume that some form of outward motion of the S4 domains physically carries the gating charge across the membrane, or at least its electric field [4–12]. There is one set of experiments that are often considered the strongest evidence for the motion of S4 from the internal to the external side of the membrane. The arginines can be mutated to cysteines, which can, in turn, react with methanethiosulfonate (MTS) reagents; there is a difference in the accessibility of the first two, and last two, positions, in the open and closed states; there is more accessibility externally in the open state, internally in the closed state. This was interpreted most simply by assuming physical motion of the S4 across the membrane, although this is not the only possibility.

MacKinnon and coworkers have proposed a "paddle" model in which the arginines carry the gating current in an outward motion, based on the structure of an archebacterial potassium channel, KvaP; this model of S4 motion is quite different than in other, "classical", models [13]. A more recent structure of a eukaryotic K^+ channel (Shaker-type) [1,2] has been published, and its gating interpreted similarly.

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The gating current in a model proposed by one of us involves the motion of protons along the S4 segment [14–16]; it does not require physical motion of the S4 segment to produce the gating current. The MacKinnon models differ from the conventional models in that the large scale motion they postulate does not require as much contact of the S4 segment with the external solution in the open state. This is not entirely obvious in the channel in the new Shaker-type structure [1,2], and the empty space is characterized as being in contact with lipid molecules. The contact could be with water (albeit water that is not tightly bound in the open state, i.e., non-crystallographic water). However, EPR data tend to disfavor water, but with some ambiguity, as an environment for some of the critical residues [17].

Of course, for the conventional ("classical") models, or the paddle model, to work, the arginines must remain positively charged. With the p*K* of arginine above 12, this does not appear to be much of a problem. However, if the arginines are complexed to a negative ion, this changes. Our earlier proposal [3] was that there is sufficient phosphate in the cell (0.1 to 0.3 mM), and in the extracellular fluid, that the arginines should be complexed if two of them are simultaneously exposed to this fluid. The channels have to function under all physiological concentrations, so the high concentration end of the range applies. Polyphosphate is present in appreciable concentration as well, at least in prokaryotes [18]. At lower concentrations, there may be polyphosphate present in eukaryotes also; there is very possibly enough that it could complex as well. Its access should be comparable to that of the reagents that are known to react. Even if polyphosphate is essentially absent in eukaryotes, ADP (adenosine diphosphate) and ATP (adenosine triphosphate) are present and could be expected to behave similarly.

There is a considerable literature on phosphate–arginine, or phosphate–guanidinium, complexes, some with quite tight binding constants, and some that have been crystallized [19,20]. Additional literature on such complexes includes work in biological contexts [21–25].

It is therefore reasonable to ask if similar complexes could form with the arginines arranged as in the S4 segment, where there are two hydrophobic residues separating alternate arginines. This was the arrangement that was studied in the ab initio calculation described in our earlier work [3], with exposure to several water molecules (not enough molecules to constitute bulk water). We have now obtained experimental NMR evidence for the existence of two complexes of phosphate with a model peptide that incorporates the arg–X–X–arg motif (X–X in the peptide we used in the experiment is ile-val, but the main point is that these are two hydrophobic residues) and find that the phosphates form a complex with the model peptide with phosphate concentrations in the 400 to 800 μM range; this range was studied for convenience with the NMR measurements, but the apparent binding strength is such that the complexes clearly exist at lower concentrations. It is not ruled out that the complex may be labile, but there is no question as to the existence of a significant interaction. This is in accord with our expectations, and suggests that the S4, if it moves while complexed to phosphate, would be likely to carry much less charge, possibly none, due to motion during gating. It would at the least make it more difficult to move S4 at all for steric reasons. This does not rule out S4 motion in a lipid or protein medium, provided there is no access for phosphate. However, as we will discuss below, this would present additional difficulties.

Other anions are also present in the intracellular and extracellular fluid. There are other phosphates, such as adenosine phosphates (ADP, the diphosphate, and ATP, the triphosphate, of the nucleotide adenosine). There is also several hundred millimolar salt, which has mostly Cl^- as anion; the Cl^- can interact with arginine as well, even if it is only an electrostatic interaction, without actual complex formation. This interaction also would hinder the motion of S4. All of these anions would have to be shielded from the S4 segment, if the S4 is to be mobile during gating. If S4 is not mobile, then there may be no shielding requirement, as the charge motion would consist of protons that would be able to move in the presence of anions.

2. Methods

2.1. Materials

Peptide: The model peptide ser–ser–ala–arg–ile–val–arg–ala–ser–ser (SSARIVRASS) was obtained from Anaspec at >98% purity (manufacturer's specification, not further tested). The termini were not capped; the amines had trifluoroacetic acid counterions, and the carboxy group was supplied as the acid. The NMR spectra were run in water containing 10% D_2O. The serines insured solubility for the peptide.

Control peptide: We are looking for a complex with a pair of arginines spaced as in the S4 segment. In the control peptide, one of the arginines was replaced by a serine. As an additional control, spectra as a function of pH were taken for phosphate, alone and in the presence of serine or arginine, as well.

Phosphate: Phosphate was analytical reagent grade, and without the peptide gave a single peak in the ^{31}P spectrum. Also, ^{31}P spectra of phosphate plus arginine (principally toward the upper end of the pH range) and phosphate plus serine (principally toward the lower end of the pH range) were also determined. The chemical shift was pH dependent. For the control peptide, at pH 4.4, the chemical shift was 1.1 ppm, at pH 9, it was 3.5 ppm. Other than phosphate, no salt was used in these experiments.

(2) pH was adjusted by adding dilute HCl or dilute NaOH solutions and measured with an Accumet AB15 pH meter from Fisher Scientific instruments.

2.2. Methods

(3) ^1H decoupled ^{31}P-NMR spectra were recorded at frequency 202.31 MHz, on a Varian Inova AS500 spectrometer with a 500 SWPFG (switchable pulse field gradient) probe, and are reported in ppm using 85% H_3PO_4 solution (0 ppm) as an external standard. All data were recorded at 25 °C using a relaxation delay of 6 s between subsequent transients. The other parameters are: spectral width, 44518.6 Hz; acquisition time, 1 s; 64 scans per transient.

(4) Linewidths were computed by fitting the NMR results to a Lorentzian. The software was written here by one author (RG) specifically for this purpose. The peaks could not be resolved into separate Lorentzians in a statistically significant manner.

(5) Each point on the graph is a single chemical shift measurement at a particular pH.

3. Results

(1) pH measurement: A measurement of the pH of a 0.4-mM peptide solution gives a pH of 3.9. This corresponds to a weak acid with a pK_a of approximately 4.4, which is

presumably the C terminus. The positive charge on the arginines could be responsible for a shift of several tenths of a pK_a unit toward more acid values for the peptide C-terminus.

(2) NMR results:

(a) Chemical Shifts: The peptide concentration for all results reported here was 0.4 mM with phosphate at 0.4 or 0.8 mM, or 0.8 mM, with phosphate at 0.8 or 2.0 mM; we did not use spectra at other concentrations, although a few were measured; these did not indicate anything unusual, nor any reason to extend the measurement range. Fig. 1 shows the chemical shift vs. pH for several concentrations, plus the corresponding controls: Fig. 1A shows the chemical shifts for the phosphate with the peptide as a function of concentration over the pH range tested, thus showing the individual complexes separately, with comparison to the one-arginine peptide. The chemical shift differs from that of the controls at the concentrations given in the figure captions. Furthermore, each titration curve shows that there are two species, one with $H_2PO_4^-$, the other with HPO_4^{2-}. The results given in Fig. 1 still look like a titration curve, as the single peak is actually a mixture of the two complexes in the middle of the pH range; Fig. 1B shows results for two concentrations, with controls. (b) The chemical shift at the low end of the pH range was 0.75±0.03 (experimental peptide), 1.10±0.02 (control peptide), 1.11 for the other controls (serine control: serine, arginine, and phosphate together gave a titration curve, although only serine was taken to low pH, and the 1.11 corresponds to the serine value); these results all fell on the same curve, and are shown in Fig. 4.

(b) At the high end of the pH range, the corresponding shifts were 3.31±0.06 (experimental peptide), 3.53±0.03 (control peptide), 3.54±0.01 (arginine control; this had several additional points at the high pH end, hence a relatively low error). There is no question that the experimental and control results differ throughout the pH range with the pure amino acids behaving similarly to the control peptide. Although there is some variation in the exact pH taken to be the end of the pH range, in every case, the plateau region was reached, indicating no further chemical shift change with pH. Sample spectra are shown in Fig. 2.

(c) Linewidth: Fig. 3 shows the linewidth as a function of pH for 0.8 mM peptide, with two phosphate concentrations. The linewidth is clearly greater in the middle of the range, where two complexes, with different chemical shifts, are mixed. At the ends of the range, only one complex is present, so the linewidth is essentially the intrinsic linewidth of a single complex. There is insufficient data to justify fitting two Lorentzians to the broader peaks, although the existence of two peaks is almost certainly the reason for the broadening.

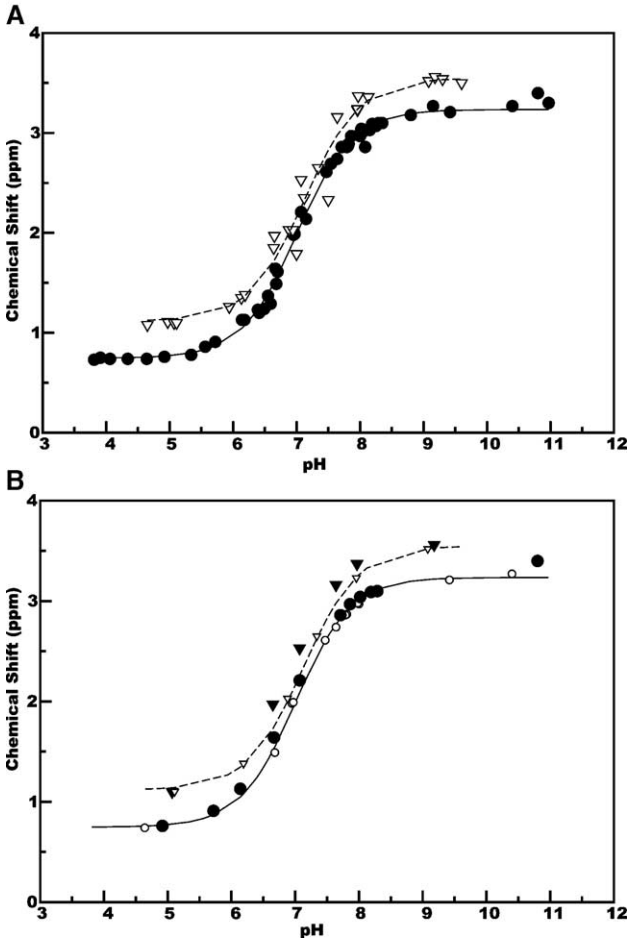

Fig. 1. Chemical shift (ppm) for the two-arginine peptide, and the one-arginine peptide (control). (A) The two arginine peptide (test peptide), four concentrations combined: 0.8 mM peptide/2.0 mM PO_4; 0.8 mM peptide/0.8 mM PO_4; 0.4 mM peptide/0.8 PO_4; and 0.4 mM peptide/0.4 PO_4. All are plotted on the same scale, and produce chemical shifts identical within experimental error at each pH, and are shown as filled circles. The corresponding controls, in which the peptide is changed to one with a serine in place of one of the arginines are shown as inverted triangles. Note that the low pH plateau for the control case is approximately 0.35 ppm greater than that of the test peptide (1.10 compared to 0.75), while at the high pH end the difference is approximately 0.2 (3.50 vs. 3.30). Individual points have errors of approximately 0.05, making the difference significant. (B) The titration curve for 0.8 mM peptide/0.8 mM PO_4 (filled circle), compared to the corresponding control (filled triangle). The titration curves for 0.8 mM peptide/2.0 mM PO_4 (open circle) plus control (open triangle) also show that difference between experimental peptide and control is maintained throughout the pH range. The lines shown are obtained by fitting the Henderson–Hasselbalch equation to the data, with residual error as shown in Table 1.

(d) The pH-chemical shift curve can be fit to the Henderson–Hasselbalch equation for a titration; the pK_a values for the fits were 7.01±0.02 for the two-arginine peptide, 7.12±0.06 for the control peptide. For the free serine, arginine, and phosphate plotted together, the NMR data give pK_a=6.97±0.05, from the same treatment (Fig. 4). Thus, the serine, arginine, and pure phosphate show no specific interaction. The literature value for the $H_2PO_4^- \rightarrow HPO_4^{2-} + H^+$ equilibrium pK_a is 7.20 [27], but this is in pure water; the presence of 10% D_2O leads to a marginal shift. Taken

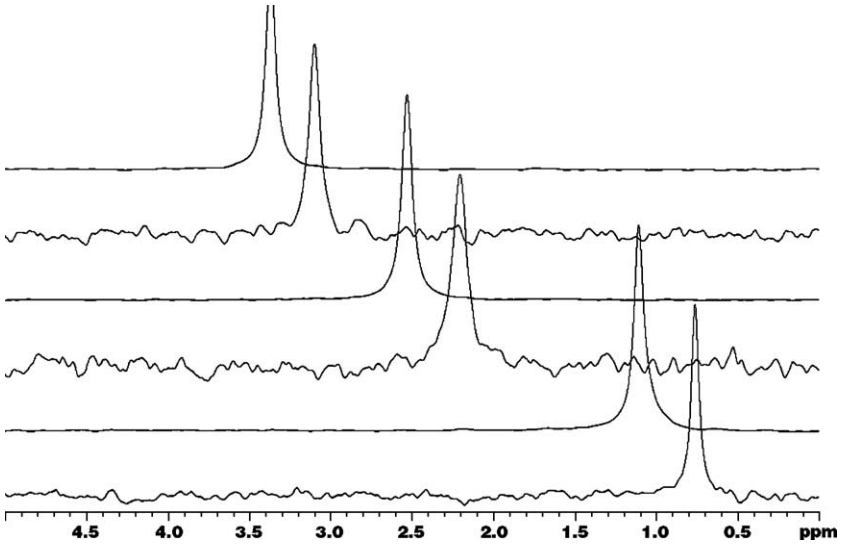

Fig. 2. Sample spectra of the test peptide plus phosphate, and the corresponding concentrations of the 1-arg (control) peptide: From bottom to top: (a) test peptide, pH 4.92; (b) control peptide, pH 5.07; (c) test peptide, pH 7.07; (d) control, pH 7.07; (e) test peptide pH 8.29; (f) control peptide pH 7.97. The baselines have been displaced to allow the spectra to be distinguished. The difference in signal-to-noise (S/N) ratio is apparent, but the S/N ratio is in all cases $\gg 1$.

together, it is hard to use the apparent pK values to draw significant conclusions, other than that good titration curves are obtained, suggesting that there are two species, and only two species, of complexes. The pK results are summarized in Table 1. Because the chemical shift values corresponding to the low and high pH complexes are clearly different with the test (two arginine) peptide from the value with the control, a pair of complexes must form when that peptide was allowed to react with phosphate, while there is no indication that any complex has formed with serine or arginine plus phosphate, pure phosphate, or the control peptide, which give chemical shifts indistinguishable from each other.

4. Discussion

The complexes of phosphate with the experimental peptide exist well within the physiological range of phosphate concentration. They evidently have a lifetime in the fast to intermediate NMR time scale, as the spectra could not be resolved into separate peaks.

Results from a number of experiments on ion channel gating have been interpreted to mean the large scale motion of the S4 segments in the extracellular direction as constituting the gating current [7,8,12,27–32]. We agree that the experiments suggest the differential exposure of the S4 residues to the intracellular and extracellular solutions in the closed and open states. We do not agree that this requires physical motion of the S4 segments with respect to the membrane, but only that the water and hydrogen bonds may be sufficiently differently configured to affect accessibility of large reagents to the S4 arginines, as discussed below. Even the ESR results [17] do not rule out additional access to the S4 segment, as the accessibility probe they used, Ni-EDDA, required 5–6 Å solution, and the phosphate ion is about 2 Å smaller than that. (Cl^- is a non-complexing ion that could nevertheless form ion pairs, and would go even further into a crevice, as discussed below). However, the larger phosphates, like ADP, can possibly be ruled out. The arginines of S4, when two residues are exposed either intracellularly or extracellularly, should be complexed to phosphate in some form, at least most of the time, or at least ion-paired. A large fraction of the time, their physical movement could not make their charge dependably part of the gating current, unless they are very thoroughly sequestered from the aqueous solution, much more so than is suggested by the ESR experiments.

The newly reported Shaker-like structure (Kv1.2) would be free of this effect only if no form of the motion of this segment allowed ions in. This seems doubtful, as it would require rather tight packing of the lipid around S4, with the arginines charged; in spite of this, the S4 would have to be unable to attract negatively charged ions from a relatively short distance. This seems to require the following: the lipid has to be packed tightly enough to deny access to oppositely charged ions and water, but loosely enough to allow relatively rapid concerted motion. Second, the motion must come near the lipid headgroups, but there are negatively charged headgroups. It is difficult to understand why these would not trap the arginines in the open position; all phospholipids, including those that are neutral, like phosphatidylcholine (PC), have negative charges on their phosphates; PC is a zwitterion, the negative charge close to the membrane hydrophobic layer, with the positive choline several angstroms distant. Even if the closed position of the S4 in this model does not bring the arginines near headgroups, the open position seems to. This is not a question that arises in the “classical” models, as the lipids are not involved; instead, the phosphate complexes become an issue.

Even a labile complex will require a considerable expenditure of energy to move; the motion would be unlikely unless the S4 sheds its phosphate (the Cl^- ion can form ion pairs, so it is an alternative in an aqueous crevice; we will consider this

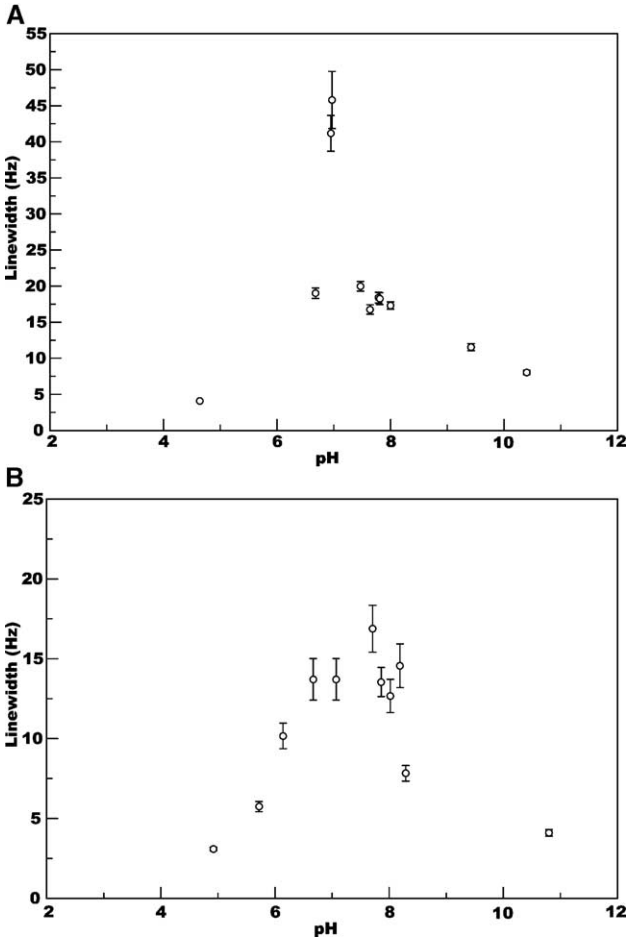

Fig. 3. The linewidth of the peaks for two cases: (A) 0.8 mM/0.8 PO_4; (B) 0.8 mM/2.0 PO_4. As expected for a transition from one species to another, the peaks for the pure species (the extremes of the pH range) are relatively narrow, while the mixture has wide peaks, stretching from the edge of one to the opposite edge of the other. The reason for the two unusually wide peaks for case B) in the center of the pH range is not clear (note the difference in ordinate scales), but the trends are as expected. Peak widths were determined by a Lorentzian fit to the data of each spectrum, using local software. Linewidths are in Hz; 270 Hz=1 ppm, so the maximum linewidth is <0.2 ppm.

possibility in more detail below, but note here that ion pair energetics may be significant also). If the S4 carries an anion, it lacks the charge that the standard gating models must assume, even if it were able to move; the motion would be unlikely if the anion had to be dragged along. The "paddle" has similar problems, with the details depending on exactly what the relative location to lipid or aqueous medium is.

For the charge to be shed, the cost of complete charge separation would be large compared to k_BT. For a phosphate complex, while we cannot say with certainty what fraction of the time the complex exists if it is labile, it appears to be a large

Table 1
pK values for phosphate 2nd ionization

System	pK	Error (±)
Test peptide	7.01	0.02
Control peptide	7.12	0.06
Ser, arg, phosphate	6.97	0.05
Literature [26]	7.199	–

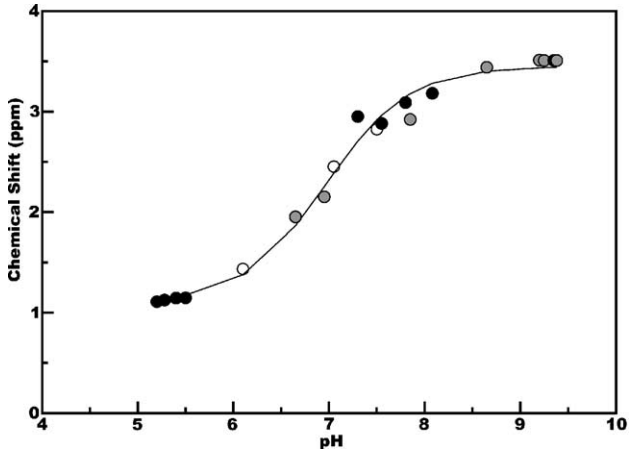

Fig. 4. Controls other than single arginine peptide: The NMR shifts for phosphate with either no additional component (open circles), or with serine (solid circles), or arginine (gray circles), alone. The line is the single Henderson–Hasselbalch fit to all points, giving the pK value cited in Table 1 (pK=6.97).

enough fraction to cause a substantial shift in the peak, therefore on the order of half the time or better. Nevertheless, suppose (extremely conservatively) that the complex exists only 1/4 of the time. For four S4 segments, the chance that all four are uncomplexed is then $(3/4)^4 \approx 0.32$. Presumably, therefore, under these conditions, only about 1/3 of the channels would be able to gate at any given time. This is probably a fairly generous estimate, given the independence of phosphate concentration in the range 0.4 to 0.8 mM, which actually suggests that the complex is not very labile, and has a K_D appreciably less than 0.4 mM. This would make the real probability that all four S4 are uncomplexed at any given time $\ll 0.32$. The main point is that even a complex weaker than the data provided indicates incompatibility with usual gating models. The MacKinnon paddle is not ruled out by this argument if the motion occurs in a fairly tight environment, well protected from the aqueous environment by lipid; it seems to be ruled out by an analogous argument pertaining to the phospholipid headgroups. We also do not understand how the MTS reagent experiments, showing access from solution, would work with accessible parts of S4 buried in lipid.

4.1. Other anions

Some gating experiments have been done with reconstituted systems not containing phosphate, and produced results similar to those in cells. These, however, almost always have sulfonate buffers (usually HEPES or PIPES) that might replace the phosphates. We have not done any experiments with sulfonates, but it would be fairly surprising if they could not replace phosphate. The size of the sulfonate group on the end of the molecule, and charge density, are comparable to phosphate; these sulfonates form buffers with pK comparable to those of the $H_2PO_4^-/HPO_4^{2-}$ buffer, so are comparable as acids as well.

This returns us to accessibility issues. One way in which these complexes may fail to exist for S4 in real channels would be for the accessibility of the arginines to the solutions to be extremely limited, as in the MacKinnon and coworkers proposal; we have commented on that extensively above. We

would like to propose an alternate limitation on access, however. There may be strong hydrogen bonding among water molecules in an aqueous crevice neighboring the S4 segments. If the full S4, corresponding to the two-arginine peptides we have examined here, is protected from contact with large reagents by tightly hydrogen-bonded water, then the complexes may be avoided. Mutating one arginine to cysteine may allow the MTS reagents to enter the crevice, which loses its hydrogen bonds, in part. Phosphates may also enter the crevice, but then they would be unable to complex the single-arginine S4 that remains. The gating current is affected by the mutation; the complexation would necessarily be affected also. The mutated channel may complex or not but it differs in significant respects from the wild-type channel, limiting the usefulness of the interpretation of the results of the MTS reagent experiments.

Finally, as we have noted, the most common anion in the cell is chloride, and a high concentration may form an ion pair with an arginine, though not a complex. Concentrations of hundreds of mM Cl^- are expected intra- and extracellularly, roughly 10^3-fold greater than that of phosphate. The arginine charges, in the absence of a complexing counterion, should produce what is essentially a charged surface, possibly within a pore. Charged surfaces facing an electrolyte attract counterions, creating a charge double layer. Here, generally, only two charges are exposed, likely in a relatively narrow cleft in the protein. If the neighborhood of the S4 arginines is largely aqueous, it is hard to see how this could fail to attract a counterion. In the absence of a phosphate ion, a Cl^- ion should be present within a few angstroms. The most generous assumption (for the point of view that the S4 really moves physically and thus produces the gating current) is to use a dielectric constant of 80, as in bulk solution, with a Debye length of 5 Å, appropriate for 400 mM ionic strength. Separating one charge in bulk at a distance of 5 Å with $\varepsilon = 80$ costs only somewhat more than k_BT in energy for a single charge, not enough to be very important. A 3 Å ion pair would be held by >2 k_BT. However, a more realistic treatment, assuming a relatively narrow cleft, with a smaller dielectric constant, with ordered water molecules, if not lipid, and taking into account the effect of having two charges, would lead to a much higher energy. It is easy to see how binding energies of >20 k_BT could arise. It seems likely that even Cl^- would interact with a pair of arginines strongly enough, simply through electrostatics, to make charge separation difficult; a full analysis is left for future work, when a structure that clarifies both the closed state and the location of lipid becomes available.

What about the cysteine scan/MTS reagent results? It seems most likely that the S4 arginines may be exposed as suggested by the cysteine results, but also likely that they are complexed most of the time in the wildtype channel. When mutated to cys, the single arginine channel would not be complexed, so the results are in some danger of misinterpretation. Arginines may be (and, in our opinion, probably are) shielded, when not exposed, by networks of water, hydrogen bonded so as to limit access to the crevices containing the arginines; with a mutated cysteine the result might be less restricted. The hydrogen bonding would likely be weaker with cysteine, making access easier with the mutated channel. The differences in hydrogen bonding would have effects analogous to those found by Bezrukov and Kasianowicz in α-hemolysin channels [33], in which access of polyethyleneglycol (PEG) was limited by changing pH. The pH shift changed the charge on the channel wall, tying up a layer of water molecules, which in turn limited the PEG access—this was detected by the fact that it limited the size of the largest PEG that could go through the channel. Here, access is for a different type of molecule, but the effect of hydrogen bonding is likely to be similar, in a similarly constricted environment. Two other papers have very recently led to similar results, in which water partially crystallizes in a protein cleft [34], or, with the aid of an electric field roughly an order of magnitude smaller than that of the resting potential of a nerve cell membrane, between two electrodes with nanometer spacing [35]. The basic phenomenon seems well established.

We have postulated the motion of protons as the source of gating current. It is easy to see how removing or adding protons to the intracellular or extracellular end of the S4 segment by having the protons themselves move as the gating current could produce in a voltage-gated channel an analogous consequence to that in the α-hemolysin channel.

The suggestion that there is a static (or nearly so) S4, with the apparent motion produced by differences in accessibility caused by changes in hydrogen bonding, seems to us consistent with the rather limited S4 motion (<2 Å) found by LRET and FRET in some studies [36,37]. Taken together, these considerations would leave, as the most likely alternative, the motion of protons along the S4 segment as the source of gating current.

5. Conclusions

The results in our model experiment are consistent with S4 not moving physically to carry gating current, in spite of the "classical" and paddle models. The role of anions must be considered when examining the motion of a cationic protein segment. Natural channels would have phosphate present and would be likely to be complexed to phosphate. The most obvious alternative would not be the free cationic peptide, but a peptide ion-paired to Cl^-. Either would appear to make it impossible to produce adequate gating current by S4 motion. Additional work on the role of anions in affecting the gating of cation channels would be highly useful in resolving the question of the extent to which they interact with S4 arginines, and whether they prevent the physical motion of the S4 segments from becoming the gating current in voltage gated channels.

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