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A highly charged voltage sensor helix translocates spontaneously across membranes**

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The molecular mechanism of gating in voltage-gated potassium channels lies at the intersection of biochemistry, structural biology and neurobiology, and for that reason it has been studied intensively. The first complete structure of a voltage-gated potassium channel, the K_vAP protein from the archeon *Aeropyrum pernix*, was solved in 2003^[1]. Based on that structure and on biochemical data, a controversial model was proposed for voltage gating that, surprisingly, placed the four arginines of the voltage sensing “S4 helix” in direct contact with the lipid environment^[2]. Furthermore, it was hypothesized that the S4 segment moved a distance of 20 Å *across the membrane* during channel gating, in direct disagreement with the idea that the hydrocarbon core of the bilayer will impart a strict barrier to charged moieties in general, and to the guanidinyll moiety of arginine in particular^[3–5]. Here, we use synthetic peptides to test the critical idea that the physical properties of the S4 sequence alone are sufficient to allow it to move easily across lipid bilayer membranes.

Support for the idea that the S4 segment interacts directly with the membrane hydrocarbon has increased in recent years. For example, the translocon-mediated integration of the S4 segment into the endoplasmic reticulum membrane was shown to have an apparent free energy close to zero^[6], suggesting that its insertion is not energetically prohibitive, at least in the context of a chimeric protein in the translocon machinery. Neutron diffraction measurements also showed that the S4 segment is, in fact, embedded in the bilayer in the context of the voltage sensor domain reconstituted into synthetic bilayers^[7]. Yet, some molecular dynamics simulations and other calculations still yield very high cost of Arg insertion into membranes, prompting questions about the interpretation of the experimental data and of the gating model^[3;8]. It is likely that arginines in membrane-bound peptides will be associated with counterions; either from solution or contributed by lipids^[9–12]. Likewise, it has been suggested that the S4 segment must be chaperoned by counterions from other parts of the voltage sensor domain (e.g. ^[13]). Despite intense interest in the subject, the idea that the S4 segment can make a large movement across the membrane while its Arg residues are in direct contact with lipids remains controversial.

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The arginines in the K_vAP S4 helix are found in a consensus sequence motif, $\Phi R \Phi R \Phi R \Phi R \Phi R$, composed of very hydrophobic residues (Φ) and arginine (Fig 1). Recently, we reported the discovery of a family of small spontaneous membrane translocating peptides (SMTPs) which also contain a single S4-like $\Phi R \Phi R$ motif (Fig 1)^[14]. These translocating peptides were selected in a high-throughput screen based on their membrane translocation efficiency in a lipid vesicle-based system. The ~10,000 member library from which they were selected contained hydrophobic and cationic residues in every position, yet the spontaneous translocating sequences that were selected frequently contained an S4-like $\Phi R \Phi R$ motif. Thousands of other cationic/hydrophobic peptides from the library did not translocate as efficiently. An engineered SMTP homolog with the arginines replaced by glutamate also did not translocate^[14]. Thus we hypothesize that the physical properties of the $\Phi R \Phi R$ sequence motifs could be responsible for the spontaneous movement of the SMTPs, as well as the S4 sequence, across membranes. If true, this would strongly support the channel gating model described above. Here we test the idea by directly measuring the ability of the isolated S4 sequence peptide to spontaneously translocate across synthetic lipid bilayers without the involvement of any other protein component.

To examine spontaneous membrane translocation, the S4 helix from K_vAP was synthesized along with three control peptides: an SMTP positive control^[14], an observed translocation negative peptide (ONEG) from the same library^[14], and an Arg-rich cell penetrating peptide (Arg9) which triggers endocytosis in cells, probably through the formation of multivalent anionic lipid domains^[15], but does not translocate spontaneously across synthetic membranes^[14]. A carboxyl-terminal cysteine residue (Fig. 1) on each peptide was labeled with either a large, zwitterionic dye, 6-carboxytetramethylrhodamine (TAMRA) or a small, neutral dye N-(7-nitro-2,1,3-benzoxadiazol-4-yl) (NBD).

We conducted two types of translocation experiments. In the first, we prepared multilamellar vesicles (MLV), which are up to 40 μ m in diameter and have at least 10–15 partly concentric bilayers with closed interior vesicular structures (Fig. 2a). Peptide and dye translocation into MLVs was assessed using laser scanning confocal fluorescence microscopy^[14]. When 2 μ M dye-labeled S4 was added to 6 mM MLVs composed of 100% zwitterionic phosphatidylcholine (PC) (Fig. 2b) or PC with 10 % anionic phosphatidylglycerol (PG) (Fig. 2c) the peptide equilibrated across all of the bilayers, accumulating equally on all of the interior bilayers, and to a lesser extent in interior aqueous spaces (Fig. 3a). The halftime of translocation was 3–5 minutes. Both S4-TAMRA and S4-NBD behaved similarly, thus the dye properties do not contribute significantly to translocation rate. Similarly, we observed translocation into MLVs made from pure PG lipids as well as 1:1 PC:PG (not shown), thus the lipid headgroup net charge is not a critical parameter. These observations indicate rapid, spontaneous translocation of S4 across bilayers. Bilayer permeabilization or disruption is not expected at the very low peptide:lipid ratios (1:3000) used in these experiments^[16] and was never observed. Polar probes in the aqueous phase during peptide translocation always remained outside the vesicles (Fig. 3a), including free dye molecules with molecular weights less than 500 Da.

In the second type of translocation experiment we incubated dye-labeled peptides (Fig. 1) with large unilamellar vesicles that contained an entrapped protease, chymotrypsin, with an excess of external protease inhibitor^[14]. Translocation was measured by assessing peptide cleavage using reverse phase HPLC. As shown in Fig. 3b, S4 and the SMTP translocated rapidly into the unilamellar vesicles while the control peptide, ONEG, did not translocate measurably. Arg9 does not have a chymotrypsin cleavage site and was not studied in LUVs. Pre-incubation of S4 with a large excess of protease-free vesicles for several hours did not

significantly slow the cleavage by protease-containing vesicles added later, indicating that translocation is reversible.

These experiments show that the highly cationic S4 voltage sensor helix has the remarkable ability to spontaneously translocate across membranes without disrupting them. Translocation occurs at very low peptide concentration, and in the absence of any other protein. The membrane hydrocarbon core is not an effective barrier to the movement of the highly charged S4 sequence. This observation is consistent with the proposed role of the S4 helix movement in voltage gating, and in strong disagreement with the idea that the cost of inserting arginine into membranes is prohibitive. The results also show that the Arg residues in the S4 segment do not have to interact with, or be chaperoned by, other parts of the voltage sensor domain in order to pass through the hydrocarbon core of the membrane.

The guanidinyll group in the side chain of arginine will likely interact with counterions, perhaps including lipid headgroup moieties, when embedded in lipid bilayers^[9–11]. In fact, it has been shown that hydrophobic anions can chaperone arginine-like groups across membranes^[10;12]. Yet, translocation of S4 in our experiments does not require anionic lipids, and it occurs in phosphate buffer, in TrisHCl buffer and even in distilled water (not shown). These results support recent literature suggesting that the lipid phosphate group and interfacial water molecules may maintain interactions with arginine residues at all depths in the bilayer^[8;17] even if it requires severe local distortion of the lipids^[8;17]. The guanidinyll moiety of Arg is probably never directly exposed to lipid hydrocarbon. Thus Arg residues in bilayers are effectively less polar than expected^[18;19]. We propose that, in the overlapping $\Phi R\Phi\Phi R$ motifs of the S4 helix, the reduced effective polarity of the arginines in membranes due to counterion effects in combination with the abundance of the most hydrophobic Φ residues^[20;21] (Fig 1) allow for free movement of the S4 voltage sensor helix across the membrane whether as a free peptide or in the context of a potassium channel's voltage sensor domain.

Experimental Section

Multilamellar vesicle translocation

Multilamellar vesicles (MLV) were prepared as described elsewhere^[14]. Briefly, lipids in chloroform were dried under vacuum and then resuspended in phosphate buffered saline at 8 mM lipid followed by ten cycles of freezing and thawing. In a translocation experiment aliquots of MLV solution were added to a small Eppendorf tube, followed by fluorescein dextran (FD3) in PBS and concentrated peptide in DMSO to bring the concentrations to 6 mM lipid, 10 μ g/ml FD3 and 2 μ M TAMRA-peptide. DMSO content was less than 5%, which we showed has no effect on vesicle integrity or translocation (Fig. 3b). For time course experiments, 3 μ l of the lipid peptide mixture was spotted immediately after preparation between a glass slide and cover slip and the slide was mounted on a Nikon laser scanning confocal microscope using a 60X oil immersion lens. A large vesicle was located as quickly as possible and the same vesicle was imaged at 1–2-minute intervals for the next 20 minutes. For overall translocation measurements, lipid peptide-samples were incubated for 40–60 minutes before being placed on a slide. Multilamellar vesicles that were between 5 and 50 μ m diameter and spherical in shape were located and imaged. Imaging was done without washing free peptide. The focal plane was always adjusted to give the maximum vesicle diameter. Imaging was done using a 488 nm laser and 520 nm band pass filter (for fluorescein and NBD) and a 543 nm laser with a 580 nm band pass filter for TAMRA. Under these conditions, bleed-through between channels is negligible and background intensities in the absence of dye are negligible. Neutral density filters were used to attenuate laser intensities to reduce photo bleaching.

Large Unilamellar vesicle translocation

Large unilamellar vesicles with entrapped chymotrypsin were prepared as described elsewhere^[14]. Briefly, lipids in chloroform were dried under vacuum and then resuspended in phosphate buffered saline (PBS) containing 10 mg/ml chymotrypsin followed by ten cycles of freezing and thawing. Extrusion through two stacked 0.1 μm polycarbonate filters was used to make 0.1 μm unilamellar vesicles. Elution of the vesicles over a gel filtration column^[14] was used to remove external chymotrypsin which we verified with the Enzchek assay. Titration of α -1 antitrypsin into detergentlysed vesicles was used to determine the amount needed to inhibit all of the chymotrypsin entrapped. In a translocation experiment aliquots of chymotrypsin LUVs, antitrypsin inhibitor, and plain LUVs (1 mM total lipid) were mixed with 1 μM dyelabelled peptide. The degradation of the peptide due to translocation was monitored by reverse phase HPLC. The normalized translocation rate is the cleavage rate in intact chymotrypsin vesicles with inhibitor divided by the cleavage rate in the presence of detergent without inhibitor. Control experiments showed that no cleavage occurred in the presence of detergent and inhibitor.

Data analysis

The program ImageJ was used to perform intensity scans across all large MLVs imaged. The translocation value for each vesicle is the average dye intensity inside the vesicle over the average intensity outside the vesicle. For SMTP translocation the intensity inside the vesicles is uniform; there is no strong peptide binding to membranes. Because S4 binds detectibly to membranes, especially PG-containing vesicles, MLVs incubated with S4 have peaks and troughs in the internal dye intensity (corresponding to lipid rich and lipid-poor areas of the vesicle interior, see Fig. 2) which we quantitated separately. For each probe molecule, translocation values were determined for at least 5–10 large vesicles from at least two independently prepared samples before averaging. For LUV translocation, the rate of proteolysis (i.e. translocation) was measured in HPLC chromatograms by monitoring the loss of peak area for full-length peptide.

References

1. Jiang Y, Lee A, Chen J, Ruta V, Cadene M, Chait BT, MacKinnon R. Nature (London). 2003; 423:33–41. [PubMed: 12721618]
2. Jiang Y, Ruta V, Chen J, Lee A, MacKinnon R. Nature (London). 2003; 423:42–48. [PubMed: 12721619]
3. Li L, Vorobyov I, Allen TW. J Phys Chem B. 2008; 112:9574–9587. [PubMed: 18636765]
4. Miller G. Science. 2003; 300:2020–2022. [PubMed: 12829759]
5. Johansson AC, Lindahl E. J Phys Chem B. 2009; 113:245–253. [PubMed: 19118487]
6. Hessa T, White SH, von HG. Science. 2005; 307:1427. [PubMed: 15681341]
7. Krepiy D, Mihailescu M, Freitas JA, Schow EV, Worcester DL, Gawrisch K, Tobias DJ, White SH, Swartz KJ. Nature (London). 2009; 462:473–479. [PubMed: 19940918]
8. Schow EV, Freitas JA, Cheng P, Bernsel A, von HG, White SH, Tobias DJ. J Membr Biol. 2010
9. Nishihara M, Perret F, Takeuchi T, Futaki S, Lazar AN, Coleman AW, Sakai N, Matile S. Org Biomol Chem. 2005; 3:1659–1669. [PubMed: 15858647]
10. Sakai N, Takeuchi T, Futaki S, Matile S. Chembiochem. 2005; 6:114–122. [PubMed: 15549725]
11. Sakai N, Matile S. J Am Chem Soc. 2003; 125:14348–14356. [PubMed: 14624583]
12. Som A, Xu Y, Scott RW, Tew GN. Org Biomol Chem. 2012; 10:40–42. [PubMed: 22025013]
13. Pless SA, Galpin JD, Niciforovic AP, Ahern CA. Nat Chem Biol. 2011; 7:617–623. [PubMed: 21785425]
14. Marks JR, Placone J, Hristova K, Wimley WC. J Am Chem Soc. 2011; 133:8995–9004. [PubMed: 21545169]

15. Palm-Apergi C, Lorents A, Padari K, Pooga M, Hallbrink M. FASEB J. 2009; 23:214–223. [PubMed: 18787109]
16. Wimley WC. ACS Chem Biol. 2010; 5:905–917. [PubMed: 20698568]
17. Freitas JA, Tobias DJ, White SH. Biophys J. 2006; 91:L90–L92. [PubMed: 17012321]
18. Hristova K, Wimley WC. J Membr Biol. 2010
19. Hessa T, Meindl-Beinker NM, Bernsel A, Kim H, Sato Y, Lerch-Bader M, Nilsson I, White SH, von HG. Nature (London). 2007; 450:1026–1030. [PubMed: 18075582]
20. Xu Y, Ramu Y, Lu Z. Cell. 2010; 142:580–589. [PubMed: 20691466]
21. Wee CL, Chetwynd A, Sansom MS. Biophys J. 2011; 100:410–419. [PubMed: 21244837]

S4: LGLFRLVRLLRFLRILLIIC(TAMRA)

SMTP: PLIYLRLLRGQFC(TAMRA)

ONEG: PLGRPLQRRC(TAMRA)

Arg9: RRRRRRRRRRC(TAMRA)

Figure 1.

Sequences of the peptides studied here. **S4:** The S4 sequence from the K_vAP potassium channel; **SMTP:** A spontaneous membrane translocating peptide identified in a high throughput screen^[14]; **ONEG:** A negative, non-translocating peptide from the library which yielded the SMTP; **Arg9:** a widely studied, non-translocating, cell penetrating peptide^[14]. The $\Phi R \Phi \Phi R$ motifs in S4 and the SMTP are underlined. Arginine residues are shown in blue.

Figure 2.

Multilamellar vesicle translocation. **a:** For initial characterization, multilamellar vesicles were made with a trace of lipid dye (green) and imaged with laser scanning confocal fluorescence microscopy to show typical internal structures. **b,c:** Two examples of multilamellar vesicles (without lipid dye) incubated simultaneously with S4-TAMRA (red) and fluorescein-dextran (FD3, green) for ~30 minutes. FD3 is a 3000 Da fluorescein-dextran which is used to track the external solution. Translocation experiments were done at 2 μ M peptide, 10 μ g/ml FD3 and 6 mM lipid. The vesicles in panel **b** are 100% zwitterionic phosphatidylcholine (PC). The vesicle in panel **c** is 90% PC with 10% anionic phosphatidylglycerol (PG). **d:** A preformed multilamellar vesicle with 10% PG after simultaneous incubation with a trace of dye labelled NBD-lysolipid (green) and S4-TAMRA (red) for 30 minutes. The vesicles shown in these images are 10–40 μ m in diameter. **e:** Intensity scan across the vesicle shown in panel **b**. External peptide has not been washed away. Red is TAMRA-peptide intensity and green is FD3 intensity. **f:** Intensity scan across the vesicle shown in panel **d**. Red is TAMRA-peptide intensity and green is NBD-lysolipid intensity.

Figure 3.

Translocation into vesicles. **a.** Translocation of dyes and dye-labeled peptides into multilamellar vesicles made with 100% zwitterionic phosphatidylcholine (PC), or 90% PC with 10% anionic phosphatidylglycerol (PG). The measured quantity is the ratio of the average fluorescence intensity inside the MLVs to the average intensity outside in solution after 30 minutes of incubation. Inside intensities for S4-TAMRA have been separated into obvious bilayer-rich areas and bilayer-poor spaces (see Fig. 2b–d for examples). Values are means $\pm$ SD from at least 5 vesicles and at least two independent experiments. **b.** Translocation into large unilamellar vesicles. The translocation rate is the rate of peptide cleavage by vesicle-entrapped protease divided by the rate of cleavage when the same amount of vesicles have been lysed with detergent. The maximum rate is around $1-3^{[14]}$.