Membrane Proteins

DOI: 10.1002/anie.201202741

A Highly Charged Voltage-Sensor Helix Spontaneously Translocates 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 arginine residues 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; this hypothesis is 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 guanidinyl moiety of arginine in particular.[3–5] Herein, 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 core 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] thus 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, thereby prompting questions about the interpretation of the experimental data and of the gating model.[3;8] It is likely that arginine residues 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. Ref. [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.

The arginine residues in the K_VAP S4 helix are found in a consensus sequence motif, ΦRΦΦRΦΦRΦΦR, composed of very hydrophobic residues (Φ) and arginine (Figure 1). Recently, we reported the discovery of a family of small spontaneous membrane-translocating peptides (SMTPs) that also contain a single S4-like ΦRΦΦR motif (Figure 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 approximately 10.000 member library from which the peptides were selected contained hydrophobic and cationic residues in every position, yet the spontaneous translocating sequences that were selected frequently contained an S4-like ΦRΦΦR motif. Thousands of other cationic/hydrophobic peptides from the library did not translocate as efficiently. An engineered SMTP homologue with the arginine residues replaced by glutamate also did not translocate.[14] Thus we hypothesize that the physical properties of the ΦRΦΦR sequence motifs could be responsible for the spontaneous movement of the SMTPs, as well as the S4 sequence, across membranes. If this hypothesis was true, it would strongly support the channel gating model described above. Herein 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 (SMTP 1),[14] an observed translocation-negative peptide (ONEG) from the same

S4:	LGLFRLVRLLRFLRILLIIC(TAMRA)
SMTP 1:	PLIYLRLLRGQFC(TAMRA)
ONEG:	PLGRPLQRRC(TAMRA)
Arg9:	RRRRRRRRRC(TAMRA)

Figure 1. Sequences of the peptides studied herein. S4: The S4 sequence from the K_VAP potassium channel; SMTP 1: A spontaneous membrane-translocating peptide identified in a high-throughput screen;[14] ONEG: A negative, non translocating peptide from the library that yielded the SMTP 1; Arg9: a widely studied, non translocating, cell-penetrating peptide.[14] The ΦRΦΦR motifs in S4 and SMTP 1 are underlined. Arginine residues are shown in blue. TAMRA = 6-carboxytetramethylrhodamine.

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[**] This work was funded by NIH grants GM060000 (W.C.W.) and GM095930 (K.H.) and NSF grants DMR-1003411 (W.C.W.) and DMR-1003441 (K.H.). We thank Stephen H. White (UC Irvine) and Chris Miller (Brandeis) for critically reading the manuscript.

library,[14] and an Arg-rich cell-penetrating peptide (Arg9) that triggers endocytosis in cells, probably through the formation of multivalent anionic lipid domains,[15] but does not translocate spontaneously across synthetic membranes.[14] A carboxy-terminal cysteine residue (Figure 1) on each peptide was labeled with either a large zwitterionic dye, TAMRA or a small neutral dye *N*-(7-*n*itro-2,1,3-benzoxadiazol-4-yl) (NBD).

We conducted two types of translocation experiments. In the first experiment, we prepared multilamellar vesicles (MLV) that are up to 40 μm in diameter and have at least 10–15 partly concentric bilayers with closed interior vesicular structures (Figure 2a). Peptide and dye translocation into MLVs was assessed using laser scanning confocal fluorescence microscopy.[14] When dye-labeled S4 (2 μM) was added to MLVs (6 mM) composed of 100% zwitterionic phosphatidylcholine (PC; Figure 2b) or PC with 10% anionic phosphatidylglycerol (PG; Figure 2c) the peptide equilibrated across all of the bilayers; equal accumulation of the peptide was observed on all of the interior bilayers, and to a lesser extent in interior aqueous spaces (Figure 3a). The halftime of translocation was three to five minutes. Both S4-TAMRA and S4-NBD behaved similarly, thus the dye properties do not contribute significantly to the 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

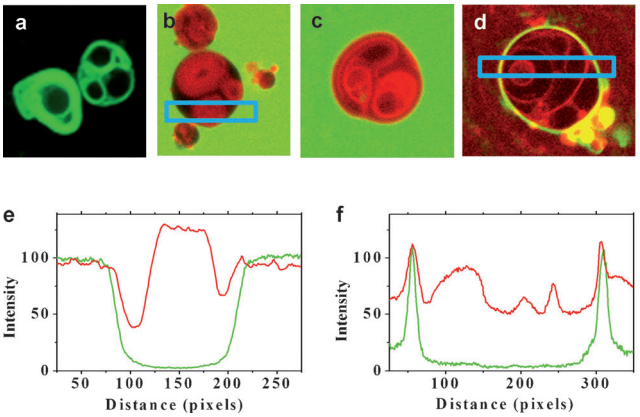

Figure 2. Translocation into multilamellar vesicles. a) For initial characterization, MLVs were made with a trace of lipid dye (green) and imaged with laser scanning confocal fluorescence microscopy to show typical internal structures. b, c) Two examples of MLVs (without lipid dye) incubated simultaneously with S4-TAMRA (red) and fluorescein dextran (FD3, green) for approximately 30 min. FD3 is a 3000 Da fluorescein dextran that is used to track the external solution. Translocation experiments were done at 2 μM peptide, 10 μg mL^{-1} FD3, and 6 mM lipid. The vesicles in (b) are 100% zwitterionic phosphatidylcholine (PC). The vesicle in (c) is 90% PC with 10% anionic phosphatidylglycerol (PG). d) A preformed MLV with 10% PG after simultaneous incubation with a trace of dye-labeled NBD-lysolipid (green) and S4-TAMRA (red) for 30 min. The vesicles shown in these images are 10–40 μm in diameter. e) Intensity scan across the vesicle shown in (b; blue box). 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 (d; blue box). Red is TAMRA-peptide intensity and green is NBD-lysolipid intensity.

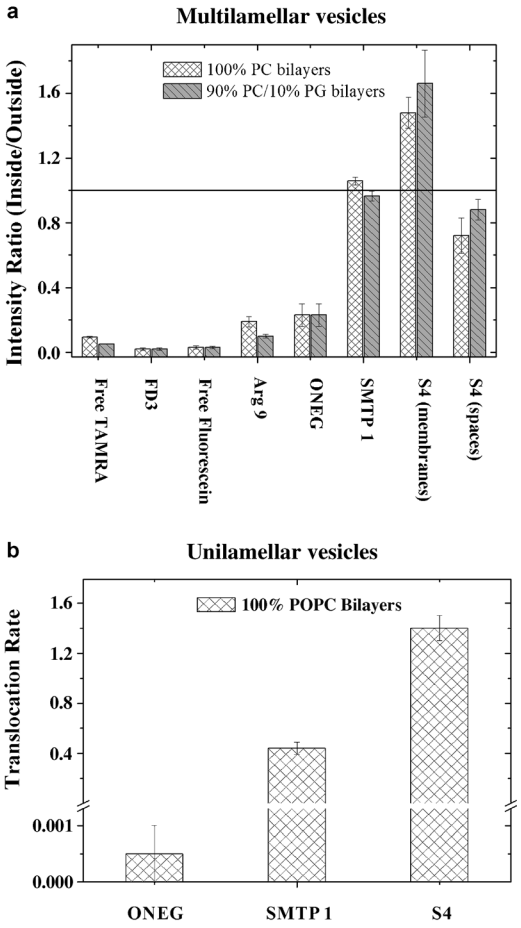

Figure 3. Translocation into vesicles. a) Translocation of dyes and dye-labeled peptides into multilamellar vesicles made with 100% PC, or 90% PC with 10% PG. The measured quantity is the ratio of the average fluorescence intensity inside the MLVs to the average intensity outside in solution after 30 min of incubation. Inside intensities for S4-TAMRA have been separated into obvious bilayer-rich areas (membranes) and bilayer-poor areas (aqueous spaces) (see Figure 2b–d for examples). Values are means ± standard deviation from at least five vesicles and at least two independent experiments. b) Translocation into large unilamellar vesicles made of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). 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]

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, including free dye molecules with molecular weights less than 500 Da, in the aqueous phase always remained outside the vesicles during peptide translocation (Figure 3a).

In the second type of translocation experiment we incubated dye-labeled peptides (Figure 1) with large unilamellar vesicles (LUVs) 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. S4 and SMTP 1 translocated rapidly into the unilamellar vesicles while the control peptide,

ONEG, did not translocate measurably (Figure 3b). Arg9 does not have a chymotrypsin cleavage site and was not studied in LUVs. Preincubation 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, thus indicating that translocation is reversible.

These experiments show that the highly cationic voltage-sensing S4 helix has the remarkable ability to spontaneously translocate across membranes without disrupting them. Translocation occurs at a 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 guanidinyl group in the side chain of arginine will likely interact with counterions, perhaps including lipid headgroup moieties, when embedded in lipid bilayers.[9–11] It has been shown that hydrophobic anions can chaperone arginine-like groups across membranes.[10;12] Yet, translocation of the S4 peptide in our experiments does not require anionic lipids, and it occurs in phosphate buffer, in tris(hydroxymethyl)aminomethane–HCl (Tris-HCl) 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 this interaction requires severe local distortion of the lipids.[8,17] The guanidinyl 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 ΦRΦΦR motifs of the S4 helix, the reduced effective polarity of the arginines in membranes, which is due to counterion effects in combination with the abundance of the most hydrophobic Φ residues[20,21] (Figure 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

Translocation into multilamellar vesicles. MLVs were prepared as described elsewhere.[14] Briefly, lipids in chloroform were dried in vacuum and then resuspended in phosphate-buffered saline (PBS) at a concentration of 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 dimethyl sulfoxide (DMSO) to bring the concentrations to 6 mM lipid, 10 $\mu g\,mL^{-1}$ FD3, and 2 μM TAMRA-peptide. DMSO content was less than 5%, which we showed has no effect on vesicle integrity or translocation (Figure 3b). For time course experiments, the lipid peptide mixture (3 μL) 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 min. For overall translocation measurements, lipid peptide mixtures were incubated for 40–60 min before being placed on a slide. MLVs that were between 5 and 50 μm in diameter and spherical in shape were located and imaged. Imaging was done without washing away the 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.

Translocation into large unilamellar vesicles. LUVs with entrapped chymotrypsin were prepared as described elsewhere.[14] Lipids in chloroform were dried in vacuum and then resuspended in PBS containing chymotrypsin ($10\,mg\,mL^{-1}$) 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 detergent-lysed 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 dye-labeled peptide (1 μM). The degradation of the peptide owing 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 1 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 Figure 2), which we quantitated separately. For each probe molecule, translocation values were determined for at least five to ten large vesicles from at least two independently prepared samples before averaging. For translocation into LUVs, the rate of proteolysis (i.e. translocation) was measured in HPLC chromatograms by monitoring the loss of peak area for full-length peptide.

Received: April 9, 2012
Published online: June 13, 2012

Keywords: ion channels · membrane proteins · peptides · translocation · voltage sensor

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