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Membrane-perturbing properties of two Arg-rich paddle domains from voltage-gated sensors in the KvAP and HsapBK K⁺ channels

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ABBREVIATIONS

Kv-channel, voltage-gated K⁺-channel; VSD, voltage-sensing domain; CPP, cell-penetrating peptide; P/L ratio, peptide-lipid ratio; LUV, large unilamellar vesicle; KvAP, A. pernix K⁺ channel; HsapBK, H. sapiens BK K⁺ channel; CD, circular dichroism; NMR, nuclear magnetic resonance; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-sn-glycero-3-phospho-(1’-rac-glycerol); DPC, dodecylphosphocholine; DPG, dodecylphospho-rac-glycerol sodium salt; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1’-rac-glycerol); DHPC, 1,2-dihexanoyl-d₂₂-sn-glycero-3-phosphocholine; Δν_q, quadrupolar splitting; S_CD, order parameter; T₁, longitudinal relaxation time; T₂, transverse relaxation time; NOE, nuclear Overhauser effect; S², generalized order parameter; MD, molecular dynamics
ABSTRACT: Voltage-gated K⁺ channels are gated by displacement of basic residues located in the S4 helix that together with a part of the S3 helix, S3b, forms a “paddle” domain, whose position is altered by changes in the membrane potential modulating the open probability of the channel. Here, interactions between two paddle domains, KvAPp from the Kᵥ channel from Aeropyrum pernix and HsapBKp from the BK channel from Homo sapiens, and membrane models have been studied by spectroscopy. We show that both paddle domains induce calcein leakage in large unilamellar vesicles and we suggest that this leakage represent a general thinning of the bilayer making movement of the whole paddle domain plausible. That HsapBKp induces more leakage than KvAPp, may be explained by the presence of a Trp residue in HsapBKp. Trp residues generally promote localization to the hydrophilic/hydrophobic interface and disturb tight packing. In magnetically aligned bicelles KvAPp increases the order along the whole acyl chain, while HsapBKp affects the morphology, also indicating that KvAPp adapts more to the lipid environment. Relaxation NMR measurements for HsapBKp show that overall the sequence has anisotropic motions. The S4 helix is well-structured with restricted local motion, while the turn between S4 and S3b is more flexible and undergoes slow local motion. Our results indicate that the calcein leakage is related to the flexibility in this turn region. A possibility for HsapBKp to undergo structural transitions is also shown by relaxation NMR, which may be of importance for the gating mechanism.
Voltage-gated $K^+$-channels, $K_v$-channels, are gated by charged amino acid residues (Arg or Lys), whose positions are altered by changes in the membrane potential.\textsuperscript{1,2} This modulates the probability of the channel being in the open state. The gating charges are found in the S4 transmembrane segment of the voltage-sensing domain (VSD). The S4 helix forms, together with a part of the S3 helix, S3b, a domain known as the “paddle” domain or the “arginine-rich paddle”\textsuperscript{2-4}. The first structure of a full-length $K_v$-channel revealed that the arginine-rich paddle forms a helix-turn-helix structure situated within the hydrocarbon core of the membrane.\textsuperscript{2,3} This positioning is controversial, due to the great hydrophilicity of arginine. Many experimental\textsuperscript{5-7} and theoretical studies\textsuperscript{8-10} have aimed at understanding the molecular basis for the positioning of the Arg-rich paddle. It has been argued that the S4 helix in the paddle motif is fairly independent of the rest of the VSD, and that the structure of this helix is governed by protein-lipid interaction.\textsuperscript{11} It has also been demonstrated that the voltage sensor motif can function on its own as a voltage-activated proton channel without a separate pore domain\textsuperscript{12,13} and that the paddle domain in $K_v$ and Na\textsubscript{v} channels are modular and can be swapped between different ion channels.\textsuperscript{14-16} Several studies of isolated paddle domains, or of the S4 helix alone have been conducted to shed light on the interaction between this Arg-rich sequence and the lipid bilayer.\textsuperscript{11,17} Other studies have shown that the S4 helix alone from the the $K_v$ channel KvAP (from the thermophilic archaeabacteria \textit{Aeropyrum pernix})\textsuperscript{2,3,6} have properties that resemble those of the intact VSD.\textsuperscript{11,17} Despite several studies of VSDs during the last ten years, it is still debated whether a change in the membrane potential induces a small charge movement, which rotates and tilts the S4 transmembrane segment,\textsuperscript{1} or if a larger charge movement, that alters the position of the whole paddle domain (S3b-S4),\textsuperscript{2} is induced. A third possibility is that the thickness of the lipid bilayer is significantly reduced by the paddle domain due to hydrophobic mismatch, which
means that the movement needs not to be that large.\textsuperscript{8,9,18}

The aim of present study was to investigate how paddle domains from two different channels, the \( K_v \) channel KvAP (from \textit{Aeropyrum pernix}, GI:14601099)\textsuperscript{2,3,6} and the BK channel HsapBK (from \textit{Homo sapiens}, GI:2570854)\textsuperscript{19} interact with membranes, to be able to draw general conclusions about the mechanism of gating and to be able to compare paddle domains from two different subtypes. We have previously determined the high-resolution NMR structure of the paddle-domain from HsapBK (HsapBKp, corresponding to HsapBK(233-260)) in DPC micelles.\textsuperscript{19} The solution structure revealed a helix-turn-helix structure similar to the structure of the corresponding sequence in KvAP as determined by X-ray crystallography.\textsuperscript{2,3,19} In the present study the interactions between the paddle domains from KvAP (KvAPp, corresponding to KvAP(112-146)) and from HsapBK (HsapBKp) and lipids were studied by fluorescence, circular dichroism (CD) and NMR spectroscopy. To investigate bilayer perturbation induced by the paddle domains, leakage of the fluorophore calcein from large unilamellar vesicles (LUVs) was monitored by fluorescence spectroscopy\textsuperscript{20-23}. Circular dichroism was used to examine the lipid-dependent structure induction, while deuterium NMR spectra of acyl-chain deuterated DMPC and DMPG in magnetically aligned bicelles were acquired to investigate the effects of the paddle domains on bilayer order and integrity. The dynamics of selectively \(^{15}\text{N}\)-labeled HsapBKp in micelles composed of either DPC or DPC/DPG 9:1 was also investigated by \(^{15}\text{N}\) spin relaxation under the same conditions as used in the solution structure.

Many natural and synthetic membrane interacting peptides, such as cell-penetrating peptides (CPPs), i.e. peptides that can transport biological therapeutics into cells, antimicrobial peptides and toxins contain a high number of basic residues but are
still able to interact with bilayers, causing membrane perturbation and even translocate through a bilayer. CPPs are characterized by this high Arg content together with a great average hydrophobicity, which are properties shared by the paddle domains (Table 1). A general understanding of the influence of hydrophobicity and basic amino acid residues on membrane properties is therefore of great interest. Hence, we have compared our results for the paddle domains with results for other Arg-containing peptides: the cell-penetrating peptides M918, and the N-terminal fragments of mouse (mPrPp) and bovine prion protein (bPrPp). The results are thus important for understanding the role of the basic amino acid residues in membrane interactions in general, as well as for understanding how these paddle domains in the intact potassium channels may influence the lipid bilayer.

EXPERIMENTAL PROCEDURES

Materials. Synthetic peptides corresponding to either HsapBK(233-260) or KvAP(112-146), including fluorescein-labeled βAla-KvAp and ¹⁵N-Leu-labeled HsapBKp, were obtained from PolyPeptide Group (Strasbourg, France) and used without further purification. Calcein, a fluorescein derivative (C30H26N2O13, 622.5 Da) was obtained from Molecular Probes (the Netherlands).

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (POPG) were used to produce large unilamellar vesicles (LUVs). 1,2-dihexanoyl-d₂₂-sn-glycero-3-phosphocholine (DHPC) was used as the short-chained phospholipid in the phospholipid bicelles. Both deuterated (d₅₄) and undeuterated 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (DMPG) were used in different samples to produce the bilayer in phospholipid bicelles. All lipids were
obtained from Avanti Polar Lipids (Alabaster, AL, USA), while the detergent $d_{38}$-dodecylphosphocholine (DPC) was obtained from Cambridge Isotope Laboratories, dodecylphospho-rac-glycerol sodium salt (DPG) was obtained from Alexis (Switzerland) and Triton X-100 was from Sigma–Aldrich (Stockholm, Sweden).

**Preparation of large unilamellar vesicles.** LUVs with a diameter of 100 nm were used for calcein leakage experiments and CD measurements. In order to produce zwitterionic and 30% negatively charged LUVs, POPC or a 7:3 mixture of POPC/POPG were initially dissolved in chloroform to obtain homogenous solutions. The solvent was then removed by evaporating under high vacuum for 3 h. The resulting dried lipid films were subsequently resuspended in phosphate buffer at pH 7.2 and vortexed for 10 minutes to obtain a more defined size distribution. These solutions were then subjected to five freeze-thaw cycles to decrease lamellarity. Finally, to obtain uniform LUVs, the samples were extruded using an Avanti manual extruder approximately 20 times through a polycarbonate microfilter with a pore size of 100 nm.\(^{36}\)

**Calcein release from large unilamellar vesicles.** For the calcein leakage experiments LUVs were prepared as described above. The buffer used was a 50 mM potassium phosphate buffer (pH 7.2) with 55 mM calcein dissolved. Calcein not entrapped inside the LUVs was removed by filtering through Sephadex-G25 columns two times. The fluorescence intensity of calcein should be low due to self-quenching, and increase upon dilution. After measuring the background intensity, KvAPp or HsapBKp was titrated to either 0.1 mM POPC or 0.1 mM POPC/POPG 7:3 at 25 °C, from 0.67 mM stock solution in ddH$_2$O/methanol 2:1, yielding concentrations of 5 and 10 µM. The methanol was added due to the low solubility of the paddle domains in buffer, and as a
control, methanol was added to LUVs to make sure that this did not influence the leakage. The release of calcein from the vesicles, as a consequence of adding the paddle domains were monitored over 10 min as an increase in fluorescence intensity using a Horiba Jobin Yvon Fluorolog-3 spectro-fluorometer with wavelengths set at 490<sub>ex</sub> and 516<sub>em</sub> nm. The background fluorescence, <i>F</i><sub>0</sub>, was used as a measure of 0% leakage. In the final step 100% leakage, i.e. destruction of the vesicles was induced by addition of 10% (w/v) Triton X-100, giving an upper reference level of the fluorescence, <i>F</i><sub>r</sub>. The degree of leakage induced by the paddle domains was then calculated using the following equation:

\[
\text{%leakage} = \left[ \frac{(F - F_0)}{(F_r - F_0)} \right] \times 100
\]  

(1)

where <i>F</i> is the fluorescence intensity in the presence of KvAPp or HsapBKp. The experiments were performed three times and the same trend was seen in all repeats.

**Circular dichroism spectroscopy.** CD samples with a concentration of 0.1 mM of POPC or POPC/POPG 7:3 and a potassium phosphate buffer concentration of 50 mM (pH 7.2) were prepared as described above. The low concentration of LUVs was chosen to diminish disturbances due to light scattering. The paddle domains were titrated to these solutions from 0.67 mM stock solutions in ddH<sub>2</sub>O/methanol 2:1, yielding concentrations between 5µM and 55 µM and spectra were acquired. In this way the methanol content in the final sample was kept to a minimum. In addition, CD spectra of 48 µM KvAPp and HsapBKp, respectively, in 48 mM DPC-micelles in 50 mM (pH 7.2) sodium phosphate buffer were acquired, and compared to spectra where methanol was added, yielding a solution of buffer/methanol 2:1, to make sure that methanol had no
The far-UV CD spectra were acquired on a Chirascan CD spectrometer using a 2 mm quartz cell. The temperature was maintained at 25 °C with a TC 125 temperature control. Wavelengths ranging from 190 to 260 nm were measured with a 0.5-nm step resolution. Spectra were collected and averaged over ten measurements. Background spectra of vesicles or micelles without the paddle domains were subtracted. To estimate the percentage of different secondary structures in the CD spectra the CDSSTR method\textsuperscript{37-39} in DICHROWEB\textsuperscript{40} was utilized using reference set 7 optimized for 190-240 nm.

**Deuterium NMR of magnetically partially oriented lipid bilayers.** Magnetically aligned phospholipid bicelles with a total lipid concentration of 300 mM and q=3.5 were prepared. The bilayer forming part was made of DMPC-d\textsubscript{54}, DMPC-d\textsubscript{54}/DMPG 7:3 or DMPC/DMPG-d\textsubscript{54} 7:3. These lipids were vortexed in 50 mM sodium phosphate buffer at pH 7.2 to yield uniform slurries. 66 mM DHPC was then added to all solutions, which were vortexed again and stored at 4°C over-night. To yield clear solutions the samples were subjected to cooling and heating cycles between 4°C and 50°C. Background spectra of all three bicelle samples were measured. 1 mM KvAPp or HsapBKp in ddH\textsubscript{2}O/methanol 2:1 were then added to the samples, which were subsequently lyophilized to remove the methanol. The lipid films created in this way were dissolved in ddH\textsubscript{2}O, incubated at 4°C degrees, subjected to new heating and cooling cycles (between 4°C and 40°C) and vortexed until all lipids were dissolved.

Deuterium experiments were performed at 61.4 MHz $^2$H frequency (9.39 T) using a Bruker Avance spectrometer equipped with a tunable broad-band probe. All
NMR samples were incubated in the spectrometer, first at 15°C for 15 minutes yielding an isotropic solution and then at 37°C for 30 minutes yielding magnetically aligned bicelles, before spectra were recorded. 37°C is well above the gel to liquid crystal phase transition temperature, which is 24°C for DMPC and 20.5°C for DMPC-d<sub>54</sub>, assuring that the bicelles are in the lamellar liquid crystalline state, L<sub>α</sub>. Standard quadrupolar delay spectra (π/2-τ<sub>1</sub>-π/2-τ<sub>2</sub>-acq) were acquired for both bicelle solutions with and without peptides, using a π/2 pulse width of 14 µs, and with the delays set to τ<sub>1</sub>=50 µs and τ<sub>2</sub>=48 µs. 1024 scans were measured and averaged and a 2s delay was used in between scans. The FID was multiplied with an exponential line-broadening function prior to Fourier transformation.

Quadrupolar splittings (∆ν<sub>q</sub>) were measured in the spectra. In concentrated mixtures of DMPC/DHPC well above the gel to L<sub>α</sub> transition temperature the dominating bilayer species is aligned with its normal perpendicular to the static magnetic field, yielding the following relation between the ∆ν<sub>q</sub> and the order parameter, S<sub>CD</sub>:

\[
\Delta \nu_q = \frac{3}{4}\left(\frac{e^2qQ}{\hbar}\right)S_{CD}
\]

(2)

where (e<sup>2</sup>qQ/\hbar) is the quadrupolar coupling constant for a C-D bond (167 kHz). Since the quadrupolar splitting (∆ν<sub>q</sub>) is related to the segmental order parameter, S<sub>CD</sub>, an increase in the ∆ν<sub>q</sub> is generally associated with an increase in the order of the system, while a decrease in ∆ν<sub>q</sub> is associated with a decrease in the order.

**Dynamics studied by <sup>15</sup>N spin relaxation.** <sup>15</sup>N-<sup>1</sup>H longitudinal (T<sub>1</sub>) and transverse
(T2) relaxation times and heteronuclear NOE were measured for 15N Leu-labeled HsapBKp (Leu8, Leu13, Leu15, Leu18, Leu21 and Leu23) in DPC and DPC/DPG 9:1 micelles in 10% 2H2O/90% 50 mM sodium phosphate buffer, pH 7.2. In DPC the paddle domain concentrations were 500 µM or 1 mM and the DPC concentrations were 50 mM and 100 mM, respectively. In the DPC/DPG sample, the peptide concentration was 200 µM and the detergent concentration was 20 mM, keeping the P/L ratio constant in all measurements. The experiments were acquired using Bruker Avance spectrometers at 16.4, 14.1 and 11.7 T. R1 NOE and R2 15N-relaxation rates were determined using standard sensitivity enhanced pulse sequences.44,45 Further, a 3D edited 15N-H NOESY-HSQC was acquired at 11.7 T for 15N shift assignment purposes. All experiments were carried out at 37 °C. The T1 relaxation was determined from the following series of relaxation delays: 10, 30, 60, 100, 150, 300, 500, 700, 1000, 1400, 2000 and 2500 ms. The T2 relaxation, was measured using CPMG delays of 16, 32, 80, 95, 143, 190 and 238 ms. Two of the T1 and T2 experiments were repeated to estimate the error which was set to 0.1 for R1 and 1 for R2. All spectra were evaluated using Topspin (version 3.0). Single exponential decays were fitted using GraFit version 3.09b. Heteronuclear NOEs were calculated by dividing the intensity of the 15N-H peaks in a NOE enhanced spectrum with the corresponding intensities in the unsaturated spectrum. Errors of 10% were used for the NOE values. The relaxation data was subsequently analyzed with Modelfree 4.1546,47 to yield the motional parameters using the Lipari-Szabo model-free approach.48,49

RESULTS

Calcein leakage induced by the paddle domains. The effect of the concentration of the two paddle domains, KvAPp and HsapBKp, on the induced leakage from LUVs
composed of either zwitterionic lipids (POPC) or a mixture of POPC and negatively charged lipids (POPG) 7:3 was investigated. Figure 1 shows the percent of calcein leakage, calculated according to Eq (1), as a function of paddle domain concentration and lipid composition. For POPC LUVs, we see that HsapBKp induces more leakage of calcein than KvAPp at both concentrations (Figure 1A). Further, the leakage increases with an increased HsapBKp concentration. For POPC/POPG LUVs, HsapBKp still induces more leakage than KvAPp (Figure 1B). Here, the leakage increases with an increased paddle domain concentration for both KvAPp and HsapBKp. When comparing the results in POPC with the results in POPC/POPG 7:3 it is seen that KvAPp show a similar leakage in both types of vesicles while HsapBKp induces a greater leakage in POPC (Figure 1 and Table 2). From these results we conclude that both paddle domains perturb the membrane integrity. However, HsapBKp causes a larger membrane disrupting effect, which is concentration-dependent and appears to be larger in POPC bilayers than in POPC/POPG bilayers.

The secondary structure of the paddle domains depends on the surrounding lipid environment. To monitor the induced secondary structure in different membrane mimetics and at different P/L ratios used in this study, CD spectra were acquired. Spectra of KvAPp or HsapBKp (between 5 and 55 µM) in 0.1 mM POPC and in 0.1 mM POPC/POPG 7:3 (Figure 2 and Table 2) were measured under the same conditions as the leakage studies. CD spectra of KvAPp and HsapBKp in DPC micelles were also acquired at the same conditions as used in the relaxation studies and in the previously determined solution structure.

For KvAPp in POPC, all spectra show the minimum around 222 nm characteristic for α-helical secondary structure (Figure 2A). However, the 208 nm
minimum, characteristic for α-helix is not so distinct, which indicates that β-structure with a minimum at 215 nm might also be present. Secondary structure estimation using DICHROWEB (Table 2), show that the helical content is highest and the β-sheet content is lowest at the lowest KvAPp concentration (P/L=1/20). When adding more paddle domain the helical content is decreased and the β-sheet content is increased until a P/L ratio of 1/4 is reached. The spectra for HsapBKp in 0.1 mM POPC are all very similar (Figure 2B) but differ from the spectra of KvAPp. They show a minimum around 215 nm, indicating mainly β-structure, which is confirmed by estimations using DICHROWEB.

The spectra for the paddle domains in POPC/POPG 7:3 are similar to those in POPC (Figs 2C, 2D). However, the CD signal is significantly decreased. The low helical content and the high β-sheet content seen at a P/L ratio of 1/4 in POPC, is reached already at a P/L ratio of 1/20. Hence, β-sheet content can be induced either by introducing charge in the membrane or by elevating the peptide concentration. Estimations of the secondary structure of HsapBKp in POPC/POPG 7:3 LUVs give the same results as in POPC: the dominating secondary structure component is β-strand. A large variation in the relative signal intensity was however observed, indicating that at higher P/L ratio, the peptide aggregates, which results in loss of CD signal.

CD measurements for the two paddle domains in DPC (with a peptide/detergent ratio of 1/100), used in the relaxation NMR measurements (Figure 3) were also acquired. CD spectra of both KvAPp and HsapBKp in DPC micelles showed clear α-helical features, which were very similar to earlier observations in bicelles. Addition of methanol did not affect the secondary structure significantly, suggesting that the methanol added to the LUV samples does not affect the secondary structure significantly either.
In summary, KvAPp has a significant α-helical content in POPC at low peptide concentrations. By adding peptide or by adding charge to the LUVs the amount of β-sheet is increased, while the α-helical contribution decreases. Both types of LUVs induce mainly β-sheet structure in HsapBKp. In micelles at P/L 1:100 the paddle domains form helix-turn-helix motifs and thus helical features are seen in the CD spectra. Hence, the structure of these two paddle domains depends critically on the P/L ratio and the features of the surrounding lipid environment.

**Deuterium NMR experiments show that both paddle domains affect the bilayer.** To study the influence of the two paddle domains on bilayer order, $^2$H quadrupolar spectra were recorded at 37°C for 300 mM magnetically aligned bicelles (q=3.5 (DMPC-d$_{54}$/DHPC, (DMPC-d$_{54}$/DMPG 7:3)/DHPC or (DMPC/DMPG-d$_{54}$ 7:3)/DHPC mixtures) (Figure 4) with and without the addition of 1 mM of either KvAPp or HsapBKp. In Figures 4A and B, deuterated DMPC (DMPC-d$_{54}$) is monitored and in Figure 4C deuterated DMPG (DMPG-d$_{54}$) is monitored. The magnetically aligned bicelles show typical $^2$H spectra for the deuterated lipid chains with quadrupolar splittings, $\Delta \nu_q$, for the outermost peaks of DMPC-d$_{54}$ (plateau) of 21 kHz, and splittings of the innermost peaks (the methyl deuterons) of 2.4 kHz in both bicelle types. For DMPG-d$_{54}$ in (DMPC/DMPG 7:3)/DHPC bicelles the corresponding splittings are 20 kHz and 2.4 kHz, respectively (Table 3).

The paddle domains both have small, which is not surprising considering the low P/L ratio (1/300), but significant effects on the $^2$H spectra. In DMPC bicelles (Figure 4A), KvAPp has a slight ordering effect for all carbons along the acyl chain with an average increase in the splitting, and thus in the order parameter, of +1.6% when adding 1 mM KvAPp (When adding 3 mM KvAPp this increases to 5.2%, data
not shown). This implies that KvAPp interacts along the whole acyl chain. The effect of HsapBKp on the splittings is more modest.

In DMPC/DMPG 7:3 bicelles, the effect on DMPC-d_{54} (Figure 4B) and DMPG-d_{54} (Figure 4C) was measured separately. KvAPp increased Δν_q for DMPC-d_{54} by +3.2% on average along the acyl chain, and is thus seen to have an ordering effect. This effect is greater in the middle of the acyl chain, with a maximum increase of +4.6% and a minimum when approaching the outermost peak. This indicates that KvAPp is situated in the mid-part of the acyl chain further away from the head-group. HsapBKp, on the other hand, has both modest ordering and disordering effects on DMPC-d_{54}. The main effect, however, is to decrease the peak resolution in the spectrum. This indicates a change in T_2 or a change in mosaic spread, i.e. the distribution of angles relative to B_0, when adding the paddle domain. When adding KvAPp to the negatively charged bicelles the order of DMPG-d_{54} increases only slightly. KvAPp does thus influence DMPC more than DMPG in the DMPC/DMPG bicelles. Addition of HsapBKp causes a decrease of the Δν_q of DMPG-d_{54} and has thus a disordering effect. The average difference in Δν_q is -1% with a maximum decrease being -3.4% for position 13 in the acyl chain. Further, the peak resolution is also decreased for DMPG-d_{54}.

The deuterium spectrum of residual HDO was also investigated (Figure 4, right panel). Incomplete averaging of the large ²H quadrupolar splitting (=16 Hz) caused by rapid exchange between water molecules in the partially aligned hydration shell and in the bulk water creates a doublet spectrum, indicating an ordered bilayer phase.⁵⁰,⁵¹ KvAPp increases the Δν_q of the HDO peak in (DMPC/DMPG 7:3)/DHPC bicelles suggesting an ordering effect. This requires a well-defined peptide-lipid interaction between KvAPp and the bilayer. HsapBKp decreases Δν_q of the HDO peak in (DMPC/DMPG 7:3)/DHPC bicelles (Figures 4B and C, right panel), implying that this
paddle domain reduces the order of the bilayer. In DMPC/DHPC bicelles the HDO signal is not as affected (Figure 4A, right panel). However, a previous study in DMPC/DHPC bicelles using a P/L ratio of 1:100 shows that at this higher paddle domain concentration the quadrupolar splitting of the HDO peak is decreased. These findings together suggest that HsapBKp interacts stronger with 30% negatively charged bicelles than with zwitterionic bicelles, and the interaction leads to a decrease in bilayer order.

Together, the results indicate that the two paddle domains have opposite effect on the order of the bilayer. KvAPp induces a slight ordering for all carbons along the acyl chain of DMPC in both types of bicelles, while less effect on DMPG is observed. This implies a well-ordered interaction between this paddle domain and the bilayer. HsapBKp, on the other hand, is observed to have very modest effects on the size of the quadrupolar splittings for DMPC, while a decrease in quadrupolar splittings is seen for DMPG. A decrease in peak resolution is seen for both DMPC-d_{54} and for DMPG-d_{54} in negatively charged bicelles indicating that HsapBKp changes the morphology of the phospholipid bicelles.

**The dynamics in HsapBKp depend on lipid environment.** The interaction between HsapBKp and membrane mimetics was further studied by relaxation measurements in DPC as well as in DPC/DPG 9:1 micelles. Studies in bicelles were not feasible due to the excessive line broadening, probably due to strong interaction and/or unfavorable exchange. The six Leu residues (Leu8, Leu13, Leu15, Leu18, Leu21 and Leu23) in HsapBKp were specifically labeled with $^{15}$N (Figure 5) and in a $^{15}$N-filtered HSQC spectrum these six residues gave well-resolved cross-peaks. Assignments for the protons were available from previous investigations and assignments for the backbone
amides were obtained by the aid of a 3D $^{15}$N-H NOESY-HSQC, which was sufficient since all six Leu residues are preceded by different amino acids. $^{15}$N-$^1$H longitudinal ($T_1$) and transverse ($T_2$) relaxation times as well as heteronuclear NOE were measured for each Leu residue.

The $R_1$ ($1/T_1$) rates, the $R_2$ ($1/T_2$) rates and the NOE for HsapBKp in DPC are shown in the left panel of Figure 6. The relaxation parameters are uniform, with one exception, Leu13, which is characteristic for a structured globular protein, indicating that HsapBKp indeed is a self-folding domain of its own. Notably, Leu13 has a different $R_2$ rate than the other residues, suggesting a different dynamic behavior for this residue. Leu13 differs from the others as it has no Arg residue in the close vicinity, and is situated close to Trp12 and Gly14 in the turn region of the helix-turn-helix motif observed in the solution structure.

The relaxation data was modeled by the model-free approach. First we estimated the overall correlation time for the molecule ($\tau_M$) to 9.4 ns. Leu13 and Leu18 were excluded from this calculation, since the relaxation data for these residues differed significantly from the other four. To see if these differences were a consequence of anisotropic motion, the overall correlation time for each residue was calculated separately by using a spectral density function including an overall correlation time ($\tau_Mi$), a generalized order parameter ($S^2$) and a local correlation time for the local motion of each $^{15}$N-$^1$H bond vector ($\tau_{loc}$). The overall correlation times for each residue are shown together with the $S^2$ parameters in Table 4. The large spread in the $\tau_{Mi}$ times does indeed indicate that the motion is anisotropic, or the presence slow local motions that are difficult to separate from the overall motion, making the model-free spectral density function in principle invalid. The correlation time for in particular Leu13 deviates from the others, suggesting that either the $^{15}$N-$^1$H bond vector in Leu13 is
modulated by a different motion due to anisotropic overall molecular motion, and/or the presence of slow internal motion, or exchange. The latter is indeed indicated by the significantly faster $R_2$ relaxation (Figure 6). The average value for the correlation time, 10.7 ns, corresponds to a particle with a hydrodynamic radius of 23 Å, in agreement with estimates based on translational diffusion measurements of the peptide in DPC. This corresponds to a volume of 50 nm$^3$, which can be accounted for by considering the paddle domain volume (estimated to 4 nm$^3$) together with a somewhat larger DPC micelle. Hence, the micelle aggregation number is slightly increased as compared to without the peptide, which has also been seen for DPC micelles interacting with other peptides. The fact that the overall correlation times agrees well with the size of a peptide in a DPC micelle, indicates that the analysis appears to be valid.

All six Leu residues have heteronuclear NOEs close to 1, i.e. very limited NOEs, indicating a well-defined rigid structure in the presence of DPC, which translates into relatively high order parameters (ranging between 0.65 and 0.85). Figure 5 shows the structure of HsapBKp in DPC with the Leu residues color-coded according to their order parameters in DPC. Leu18, Leu21 and Leu23, situated in the S4 helix, have order parameters that are relatively high (0.80-0.85). Leu8, situated in the S3b part of the sequence, has a significantly lower order parameter (0.71) and Leu13 and Leu15, in the hinge between the two helices, both have even lower $S^2$ (0.65 and 0.66 respectively). The greater flexibility allowed for Leu13 and Leu15 suggests that this part of the domain may be responsible for the membrane perturbation by moving and hence breaking the tight seal between the surrounding lipids and the paddle domain. The $\tau_{loc}$ were all long, between 1.1 ns and 3.1 ns, indicating, as indicated above, difficulties in correctly separating local and overall motion.

To investigate the effect of introducing negatively charged DPG into the
micelles, relaxation for HsapBKp in DPC/DPG 9:1 micelles was measured (Figure 6, right panel). The sample aggregated over time, indicating a stronger interaction between the micelle and HsapBKp, which was also evident from increased line-widths in the NMR spectra, and only $R_1$ and NOE at 700 MHz and $R_1$ at 600 MHz were measured successfully. In addition, the relaxation parameters were more difficult to fit with the model free approach. Nevertheless, by employing the same model as for the measurements in DPC, rotational correlation times on the same order (with an average of 9.85 ns) were observed. The smaller overall correlation time compared to in DPC is likely to be consequence of not having $R_2$ rates in the fit. Here, an even more striking difference between Leu13 and the other residues was observed (Table 4). Further, Leu8, Leu13 and Leu15 needed addition of an exchange term to produce a reasonable fit, but due to the lack of transverse relaxation parameters, this model was not used to estimate the overall rotational correlation time. Therefore, a fixed correlation time of 8 ns for the entire peptide was used, optimized based on data for Leu8, Leu18, Leu21 and Leu23. Using this value we obtained very high order parameters (0.91 – 0.97) for four residues (Leu8, Leu18, Leu21 and Leu23) using the same model, including $S^2$ and $\tau_{loc}$. These high order parameters are at least partly likely to be a consequence of an unreasonably short overall correlation time, related to not having $R_2$ relaxation data in the fit. For Leu13 and Leu15, however, this procedure did not result in a usable fit. Together, this suggests that the interaction of the peptide with partly negatively charged micelles is different from the interaction with DPC micelles, leading to a dynamic behavior that is difficult to model from standard relaxation parameters using the model-free approach. Most likely, it involves slow motions with correlation times comparable to that of the overall rotation of the micelle, which makes the analysis fail.

In summary, the Leu residues located in the S4 segment, Leu18, Leu21 and
Leu23 have overall correlation times close to the average $\tau_M$, suggesting a uniform motion of this part of the paddle domain in DPC. The S4 segment is rigid with $S^2$ parameters between 0.8 and 0.85 indicating that it is well structured. However, significant internal motions on the nanosecond time-scale are also present, possibly due to small conformational changes. The $^{15}$N-$^1$H bond vectors in Leu13 and Leu15, situated in the turn of the helix-turn-helix motif close to the Trp residue, experience different overall motion, as indicated by differences in the estimated overall correlation times. This can be explained by anisotropic motion leading to different relaxation parameters in this part of the paddle domain, and/or slow local motion that is impossible to separate from the overall motion. Also, the order parameters are lower for this part of the sequence, indicating more flexibility. When negative charge is introduced in the micelles the dynamics of the turn is harder to fit, which further indicates the presence of slow local motions that are difficult to separate from overall motion. This is also evidenced by the markedly increased line-widths in the spectra.

**DISCUSSION**

The combined results from observing membrane perturbation effects (from induced leakage from vesicles and effects on bilayer order), dynamics and structure induction provide evidence for two main observations: the two paddles do not have the same effects on a bilayer, and the effects on the membrane depend on structural rearrangements and dynamics. The Arg-rich paddles from the KvAP and the HsapBK potassium channels differ only slightly from each other with respect to hydrophobicity and number of Arg residues. Yet, they are in this study observed to affect the membrane in different ways.

KvAPp is significantly $\alpha$-helical in zwitterionic vesicles and is seen to cause a
modest membrane perturbation at P/L=1/20. Further, KvAPp increases the order of magnetically aligned bicelles, which suggests a well-defined peptide-lipid interaction, along the whole perdeuterated chain. Together, these results suggest that KvAPp is positioned as a transmembrane helix spanning the bilayer, which does not disrupt the membrane significantly. By increasing the KvAPp concentration or by adding charged lipids to the vesicles, the amount of β-sheet is increased and also the effect on the membrane. When KvAPp has a high β-sheet content together with a low α-helical content, the membrane perturbation increases with increasing paddle domain concentration.

HsapBKp, on the contrary, is mainly β-structured at the concentrations used in the leakage studies and disrupts the membrane in a concentration-dependent manner. Due to the location of a Trp residue in the turn of the helix-turn-helix motif, HsapBKp is likely to be located in the water-lipid interface and might in this way be able to affect the membrane more than KvAPp is, which most likely is transmembrane under these conditions. HsapBKp induces a greater calcein leakage in zwitterionic vesicles than in 30% negatively charged vesicles and, contrary to KvAPp, affects the morphology of magnetically aligned bicelles. When adding DMPG to the magnetically aligned bilayers or increasing the paddle domain concentration the change in morphology is more severe, hence indicating a different mode of interaction with zwitterionic and negatively charged bilayers. The binding to negatively charged bilayers is thus stronger, while the leakage is stronger in zwitterionic bilayers. Hence, a tighter binding of the peptide to the bilayer is correlated with less membrane perturbation.

Next, we turn to our second observation, that bilayer perturbations are correlated with dynamics. The analysis of relaxation data for HsapBKp showed that the turn in the helix-turn-helix motif is more dynamic than the rest of the motif. Further, the calcein
leakage is seen to increase with increased flexibility in the turn region of the helix-turn-helix motif, which indicates that this part may be responsible for the bilayer perturbations. The turn is the part of the paddle domain that is most variable between channels, and this may in part explain the difference in the degree of perturbation between the two paddle domains studied here. The well-structured S4 helix, on the contrary, is a strong candidate for being a spontaneous membrane-translocating peptide that does not cause perturbation. The presence of a Trp residue in the turn region seems to increase the leakage significantly. Furthermore, we see that the S4 segment of HsapBKp is well-structured, but that the internal motions for all residues occur on a nanosecond time-scale, which are likely to be due to structural transitions. Transitions between α-helical and 3_10 helical structures have previously been demonstrated for the S4 helix and are suggested to be part of the gating mechanism. This small conformational change, where the α-helix becomes more tightly wound, is prolonged and thus becomes thinner has been reported to occur at an order of nanoseconds. Our previous structure of HsapBKp in DPC indicated both α-helical and 3_10 helical structure in the S4 segment supporting such a structural transition. We believe that our present observations suggest that paddle domains appear to be able to undergo structural transitions, have enhanced flexibility in the turn, and that there is a correlation between these properties and membrane perturbing effects. Together this provides a possible mechanism for how the paddle moves in a bilayer. A model for how this bilayer perturbation occurs as a consequence of increasing paddle domain concentration and negatively charged lipids is shown in Figure 7.

The sequence properties of paddle motifs are shared with several biologically active peptides, such as antimicrobial peptides, and cell-penetrating peptides. The calcein leakage induced by KvAPp and HsapBKp was compared with previous leakage
studies performed for peptides found to function as CPPs: M918, mPrPp and bPrPp. M918 is less hydrophobic than both paddle domains but has more Arg residues. In previous studies M918 has been shown to induce a modest amount of leakage from both types of vesicles. The prion protein derived peptides, mPrPp and bPrPp, induce more leakage, comparable to the leakage induced by HsapBKp. These sequences are less hydrophobic than the paddle domains, but have a similar net charge (in this case Lys). Further, mPrPp and bPrPp have significant β-sheet content and the induced leakage increases with elevated β-sheet content. The β-sheet content is as for KvAPp increased when adding charge to the vesicle. The prion protein derived peptides contain a Trp residue and it has previously been seen that Trp content might be related to increase in leakage. In summary, a certain amount of β-sheet content together with Trp content, locating the peptide to the water-lipid interface, may be important factors related to membrane perturbation. Our data, together with previous work, show that the amount of leakage can generally not be predicted from the Arg (or Lys) content and hydrophobicity alone, even though basic residues and hydrophobicity seem to be necessary for leakage to occur in LUV model systems.

Finally, one can also draw conclusion about the two paddle domains as parts of the VSD in potassium channels. It has been shown experimentally and in MD simulations that the VSD induces a thinning of a bilayer, which may be a significant part of the gating mechanism. Studies have shown that the S4 helix alone also induces a thinning of the membrane, similar to what is observed for the VSD. A thinning mechanism is likely to be related to a more general membrane perturbation, as observed here. Some time ago we suggested a model in which leakage was induced by a transient rearrangement of a peptide in a bilayer, which gives rise to a graded leakage as in our present study. The induction of leakage by the isolated Arg-rich
paddle domains observed in our study may thus in fact be correlated with a more
general thinning effect produced by the whole VSD. Our results demonstrate that the
perturbation is caused by the dynamic region in the paddle. They also indicate that the
dynamics are likely to be related to structural transitions, which may also be of
importance for the gating mechanism. The thinning effect would then be compatible
with a gating mechanism where a movement of the whole paddle domain needs not to
be that large.
REFERENCES


TABLES

Table 1. Primary structures of the paddle domains and of selected CPPs

<table>
<thead>
<tr>
<th>Peptide</th>
<th>% Arg</th>
<th>% Trp</th>
<th>Tot.</th>
<th>Avg. hydrophobicity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>KvAPp</td>
<td>11</td>
<td>0</td>
<td>+5</td>
<td>0.40</td>
<td>PAGLLALIEGHLAGLGLFRLVRLRILLIIISR</td>
</tr>
<tr>
<td>HsapBKp</td>
<td>14</td>
<td>1</td>
<td>+4</td>
<td>0.65</td>
<td>PVFVSYNRSWLGLRLRALRILQFSE</td>
</tr>
<tr>
<td>M918</td>
<td>32</td>
<td>0</td>
<td>+7</td>
<td>0.93</td>
<td>MVTVFRLRLRIRASGPRVRV</td>
</tr>
<tr>
<td>mPrPp</td>
<td>4</td>
<td>2</td>
<td>+3</td>
<td>0.74</td>
<td>MANLGYWLLALFVTMWTMVGLCKKRKP</td>
</tr>
<tr>
<td>bPrPp</td>
<td>3</td>
<td>2</td>
<td>+5</td>
<td>0.80</td>
<td>MVKSKIGSWLVLFVAMWSDVGLCKKRKP</td>
</tr>
</tbody>
</table>

<sup>a</sup> Average hydrophobicity was calculated utilizing the von Heijne scale for biological hydrophobicity.<sup>65</sup>
Table 2. Summary of calcein release and secondary structures of the paddle domains and selected CPPs.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Lipids</th>
<th>P/L = 0.05 (1:20)</th>
<th>P/L = 0.1 (1:10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Secondary structure</td>
<td>% calcein leakage</td>
<td>Secondary structure</td>
</tr>
<tr>
<td></td>
<td>α</td>
<td>β</td>
<td>α</td>
</tr>
<tr>
<td>KvAPp</td>
<td>POPC</td>
<td>35</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>POPC/POPG 7:3</td>
<td>6</td>
<td>26</td>
</tr>
<tr>
<td>HsapBKp</td>
<td>POPC</td>
<td>4</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>POPC/POPG 7:3</td>
<td>4</td>
<td>36</td>
</tr>
</tbody>
</table>

*Estimation of the extent of helix and strand from CD spectra (Fig 2) as calculated by the CDSSTR method\textsuperscript{37-39} in DICHROWEB\textsuperscript{40}.

Calcein release recorded 10 min after addition of KvAPp or HsapBKp to calcein-entrapping POPC and 30% negatively charged POPC/POPG LUVs, respectively.
Table 3. Quadrupolar splittings of DMPC-\textsubscript{d54} and DMPG-\textsubscript{d54} in magnetically aligned bicelles at 37 °C

<table>
<thead>
<tr>
<th>Bilayer forming part of the bicelle</th>
<th>Plateau (\Delta \nu_p) (kHz)</th>
<th>Difference (%)</th>
<th>Methyl (\Delta \nu_m) (kHz)</th>
<th>Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMPC-\textsubscript{d54}</td>
<td>20.73</td>
<td>2.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ KvAPp</td>
<td>21.05</td>
<td>1.5</td>
<td>2.37</td>
<td>-1.8</td>
</tr>
<tr>
<td>+ HsapBKp</td>
<td>21.07</td>
<td>1.7</td>
<td>2.31</td>
<td>-4.1</td>
</tr>
<tr>
<td>DMPC-\textsubscript{d54}/DMPG</td>
<td>21.00</td>
<td>2.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ KvAPp</td>
<td>21.30</td>
<td>1.4</td>
<td>2.42</td>
<td>2.7</td>
</tr>
<tr>
<td>+ HsapBKp</td>
<td>20.94</td>
<td>-0.3</td>
<td>2.32</td>
<td>-1.7</td>
</tr>
<tr>
<td>DMPC/DMPG-\textsubscript{d54}</td>
<td>19.76</td>
<td>2.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ KvAPp</td>
<td>19.35</td>
<td>-2.1</td>
<td>2.38</td>
<td>0.75</td>
</tr>
<tr>
<td>+ HsapBKp</td>
<td>19.59</td>
<td>-0.9</td>
<td>2.31</td>
<td>-2.1</td>
</tr>
</tbody>
</table>
Table 4. Overall correlation times ($\tau_{Mi}$) and squared generalized order parameters ($S^2$) for the six $^{15}$N labeled Leu residues in DPC and DPC/DPG 9:1 micelles.

<table>
<thead>
<tr>
<th>residue</th>
<th>DPC micelles</th>
<th>DPC/DPG 9:1 micelles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\tau_{Mi}$ (ns)</td>
<td>$S^2$</td>
</tr>
<tr>
<td>8</td>
<td>10.8 ± 1.8</td>
<td>0.71 ± 0.12</td>
</tr>
<tr>
<td>13</td>
<td>14.5 ± 1.8</td>
<td>0.65 ± 0.10</td>
</tr>
<tr>
<td>15</td>
<td>11.1 ± 1.9</td>
<td>0.66 ± 0.12</td>
</tr>
<tr>
<td>18</td>
<td>9.3 ± 1.3</td>
<td>0.85 ± 0.13</td>
</tr>
<tr>
<td>21</td>
<td>9.1 ± 1.3</td>
<td>0.83 ± 0.13</td>
</tr>
<tr>
<td>23</td>
<td>9.4 ± 1.4</td>
<td>0.80 ± 0.13</td>
</tr>
</tbody>
</table>

$^a$ Overall correlation times derived ($\tau_{Mi}$) from fitting $\tau_{Mi}$, $S^2$ and $\tau_{loc}$.

$^b$ Generalized order parameter squared derived using $\tau_{Mi} = 8.0$ ns, and a model including $S^2$ and $\tau_{loc}$. 
Figure legends

Figure 1.
Time-dependent fluorescence signals representing calcein leakage from LUVs containing 55 mM entrapped calcein at 25°C and pH 7.2. The effect of KvAPp (circles) and HsapBKp (squares) on LUVS composed of (A) 0.1 mM POPC. and (B) 0.1 mM POPC/POPG 7:3 are shown. Filled symbols indicated 5 µM peptide and open symbols indicate 10 µM peptide. The zero time point is ca 5 s after addition of the paddle domain to the LUVs. The apparent negative leakage shown in Fig 1B due to dilution of the sample when adding the paddle domain is visible due to the slower kinetics in POPC/POPG LUVs.

Figure 2.
CD spectra recorded for KvAPp (A, C) and HsapBKp (B, D) in either 0.1 mM POPC (A and B) or in 0.1 mM POPC/POPG 7:3 (C and D) at 25°C, pH 7.2. For the spectra in POPC, paddle concentrations of 5 µM (purple), 10 µM (blue), 15 µM (light blue), 25 µM (green), 35 µM (yellow), 45 µM (orange) and 55 µM (red) were used, yielding P/L ratios of 1/20, 1/10, 3/20, 1/4, 7/20, 9/20 and 11/20, respectively. For the spectra in POPC/POPG 7:3, paddle concentrations of 5 µM (purple), 10 µM (blue), 25 µM (green) and 55 µM (red) were used.

Figure 3. CD spectra of 48 µM KvAPp (red) and HsapBKp (blue) in 48 mM DPC, pH 7.2 (solid line) or in sodium phosphate buffer/methanol 2:1, pH 7.2 (dashed line)
recorded at 25°C.

**Figure 4.** Deuterium NMR spectra of 300 mM q=3.5 DMPC-d$_{54}$/DHPC bicelles (A), (DMPC-d$_{54}$/DMPG 7:3)/DHPC bicelles (B) and of (DMPC/DMPG-d$_{54}$ 7:3)/DHPC bicelles (C) at pH 7.2 and 37°C. The top spectrum is of bicelles only, the middle spectrum after the addition of 1 mM KvAPp and the bottom spectrum after the addition of 1 mM HsapBKp. The left-hand panel shows the spectrum of chain-perdeuturated DMPC or DMPG respectively, while the right-hand panel shows the residual HDO signal.

**Figure 5.** Solution NMR structure of HsapBKp in DPC (PDB: 24kk) with the six Leu (Leu8, Leu13, Leu15, Leu18, Leu21 and Leu23) that were specifically labeled with $^{15}$N color coded according to their order parameters derived from $^{15}$N-Leu-HsapBKp in DPC.

**Figure 6.** $^{15}$N Relaxation data for $^{15}$N-Leu-HsapBKp in DPC (left panel) and DPC/DPG 9:1 (right panel). Open triangles are relaxation parameters from measurements at 500 MHz, while closed triangles are from 700 MHz. In the right panel, closed diamonds are parameters measured at 700 MHz, while open diamonds are R$_1$-values measured at 600 MHz.

**Figure 7.** A model for how the HsapBKp paddle domain can undergo structural transitions, which affect the membrane perturbation. At low concentrations, the flexible turn in the largely $\alpha$-helical peptide does not cause severe leakage from LUVs. Increasing the concentration causes the peptide to undergo a structural transition, which
induces leakage. By adding PG lipids to the bilayer, the structure is stabilized, and the dynamics are more restricted which leads to a decrease in leakage.