Biophysical Chemistry 318 (2025) 107365

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: www.elsevier.com/locate/biophyschem

Trp residues near peptide termini enhance the membranolytic activity of cationic amphipathic α-helices

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ARTICLE INFO

Keywords:
Cationic antimicrobial peptide
Amphipathic α-helix
Anchoring Trp residues
Length-dependent peptide activity
Solid-state ^{15}N NMR
Transmembrane pore

ABSTRACT

KIA peptides were designed as a series of cationic antimicrobial agents of different lengths, based on the repetitive motif [KIAGKIA]. As amphiphilic helices, they tend to bind initially to the surface of lipid membranes. Depending on the conditions, they are proposed to flip, insert and form toroidal pores, such that the peptides are aligned in a transmembrane orientation. Tryptophan residues are often found near the ends of transmembrane helices, anchoring them to the amphiphilic bilayer interfaces. Hence, we introduced Trp residues near one or both termini of KIA peptides with lengths of 14–24 amino acids. Our hypothesis was that if Trp residues can stabilize the transmembrane orientation, then these KIA peptides will exhibit an increased propensity to form pores, with increased membranolytic activity. Using solid-state ^{15}N NMR, we found that peptides with Trp near the ends are indeed more likely to be flipped into a transmembrane orientation, especially short peptides. Short KIA peptides also exhibited higher antimicrobial activity when modified with Trp, while longer peptides showed similar activities with and without Trp. The hemolytic activity of KIA peptides of all lengths was higher with Trp near the ends. Vesicle leakage was also increased (sometimes more than 10-fold) for the Trp-mutants, especially in thicker membranes. Higher functionality of amphiphilic helices may thus be achieved in general by exploiting the anchoring effect of Trp. These results demonstrate that the incorporation of Trp increases membranolytic activities (vesicle leakage, hemolysis and antimicrobial activity), in a way compatible with a transmembrane pore model of peptide activity.

1. Introduction

Antimicrobial peptides (AMPs), also called host defense peptides, are found in most organisms and form a defense against pathogens [1–4]. They are of interest as possible new types of antibiotics against multi-resistent microbes [5]. In particular, peptides able to form amphipathic structures bind strongly to membranes and have often been shown to have strong membrane-destabilizing effects. Many such AMPs are proposed to be membrane-active and kill bacteria by permeabilizing their membranes [1,4,6–9]. Several studies have investigated properties of amphipathic α-helical peptides important for their activity [10–12].

We have recently studied the length-dependent activity of cationic, amphipathic AMPs by using a series of designed helices with a repetitive sequence [KIAGKIA] of varying length from 14 to 28 amino acids, called KIA peptides [13–17]. This series was based on an earlier model peptide MSI-103 [18], with the sequence $[KIAGKIA]_3$-NH_2, also called KIA21. These peptides have been proposed to form pores in the membrane, and as described below, we have found several indications that they indeed form toroidal wormhole pores with peptides lining the water-filled pore in a transmembrane orientation [13–17,19–21]. These may be short-lived, transient pores, and it is not clear if monomeric or oligomeric peptides are involved. It is conceivable that even a single inserted peptide could stabilize an otherwise fully lipidic toroidal wormhole, like a finger pushing into the membrane to trigger a more stable state.

Early studies showed a strong length-dependent effect of KIA peptides, namely that only those peptides that are long enough to span the membrane are able to induce vesicle leakage [13,15]. In vesicles of POPC/POPG (with a hydrophobic thickness of approximately 28 Å [22]), KIA15 (with 15 amino acid residues, and a length of 22.5 Å assuming an ideal α-helix) showed no leakage at P/L = 1/12.5, but

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https://doi.org/10.1016/j.bpc.2024.107365
Received 29 February 2024; Received in revised form 24 November 2024; Accepted 28 November 2024
Available online 2 December 2024

KIA17 (17 residues, length 25.5 Å) gave 100 % leakage. In thicker DErPC/DErPG membranes (hydrophobic thickness 34.4 Å [22]), KIA22 (33 Å) gave no leakage, but KIA24 (36 Å) induced almost 100 % leakage. On the other hand, in thinner DMoPC/DMoPG membranes (hydrophobic thickness 19 Å [23]), even the shortest KIA14 peptide (21 Å) induced full leakage. The fact that only peptides which are long enough to span the membrane are able to induce leakage, is a strong indication that the active peptide conformation is a membrane-spanning orientation, which is compatible with standard pore models. There is also a threshold length needed to kill bacteria or erythrocytes [15]. If the activity would be due to a carpet model with peptides lying on the membrane surface, then we could expect that a higher concentration of shorter peptides would be needed to get the same activity, i.e., that a certain coverage of the membrane surface would be needed. But this was not observed, as peptides which are too short to span the membrane had no activity even at very high concentrations.

In a later study, variants of the KIA peptides were constructed, called KIXA, which have a similar sequence to KIA peptides but where a constant charge was maintained for all peptide lengths. For the KIXA variants, the same length dependence was found, showing that any additional charges on the longer KIA peptides were not responsible for the length-dependent biological activity [13]. Solid-state ^{15}N NMR analysis in membranes containing lysolipids showed that the KIA peptides can insert into the lipid bilayers in a transmembrane orientation, and the helix tilt angle changes in exactly the way that would be expected from hydrophobic mismatch between the helix length and the bilayer thickness [14] – an effect previously seen only for transmembrane helices [19,20].

In a more recent continuation of our systematic analysis, so-called CKIA peptides were constructed, using the same repetitive sequence as KIA peptides. However, whereas the repetitive sequences of KIA peptides are extended towards the C-terminus and have a fixed N-terminus with a varying C-terminus, the CKIA peptides have a fixed C-terminus and are extended towards the N-terminus. It was found that charges on the termini have a strong effect on the membrane-permeabilizing ability of CKIA and KIA peptides [17]. When both termini of KIA/CKIA peptides are charged, the membranolytic activity is lower than when one terminus is charged and the other one hydrophobic. If the mechanism of action would be a carpet model, with peptides lying flat on the membrane being active, then it is hard to explain why such a change in the terminal charge would have an effect on activity. But this observation can be readily explained if the peptides have to flip from the lipid bilayer surface into a transmembrane orientation to become active, in accordance with the commonly accepted mechanism for a pore-forming peptide [17]. Indeed, it would only be favorable for a helical peptide to insert one end into and across the hydrophobic interior of the membrane, as long as there is no charge on that terminus.

Given the fact that the transmembrane insertion of KIA peptides is generally favored by positive lipid curvature as exerted by the presence of lysolipids, we recently investigated the three-dimensional architecture of pores formed by KIA21 in DMPC/lyso-MPC (2/1 mol/mol). It was found that a minimum amount of lysolipids and a minimum peptide concentration were needed for the formation of stable pores, and these pores were enriched in lysolipids and negatively charged lipids (which are attracted by the cationic peptides) [21]. Pore structures were proposed to be assembled from several monomeric helices or from several antiparallel dimers, with lipid head groups in between the peptides lining the pore [21].

All these studies indicate that KIA peptides are able to adopt a transmembrane orientation and form probably transient pores in membranes. Based on the mechanistic concept that these pores are responsible for vesicle leakage, antimicrobial activity and hemolysis, we hypothesized that if the peptides could be modified such that their transmembrane insertion is favored, then they should exhibit higher membrane activity.

Integral membrane proteins usually contain one or more transmembrane α-helices (TMHs). It is well-known that these hydrophobic helices often carry aromatic residues at their ends, i.e., close to the amphiphilic membrane interface between lipid head groups and acyl chains [24,25]. It has been proposed that these aromatic residues, particularly Trp, serve as anchors for transmembrane helices due to their strong affinity for the bilayer interface region [26–28]. There seems to be a particular affinity of Trp for the membrane interface, where it prefers a special position and orientation, which has been attributed to cation-π or anion-π interactions, hydrogen bonds to lipid headgroups, hydrophobic effects, or interactions with the helix dipole [29]. It has been found that a Trp residue at the end of a singular TMH influences not only the tilt angle but also the azimuthal rotation angle of the helix, in a way that the helix tends to tilt towards that face where the Trp residue is attached [30]. The anchoring effect of Trp has been studied in detail using model peptides that form regular transmembrane helices, called WALPs or GWALPs, consisting of a transmembrane stretch of Ala-Leu residues, with one or two Trp residues near each end [31–33]. From these studies, it also seems that Trp has a very specific favored position in the membrane.

Our hypothesis here is that Trp, which is known to stabilize a transmembrane orientation of hydrophobic helices in membrane proteins and model peptides, may also promote a transmembrane orientation of amphipathic helices, such as our KIA peptides. As a consequence, it is expected that this would then stabilize the transmembrane pores evidenced previously for KIA peptides, and increase their membranolytic activity as measured, for example, by vesicle leakage, hemolysis and antimicrobial assays.

The influence of Trp on AMPs has been examined in numerous studies, and there are also reviews on the subject [34,35]. The studied peptides were usually very short and rich in Trp and did not form α-helices [36–38]. Some helical AMPs have also been studied, but usually these had mostly Trp in the middle of the sequence, or had many Trps spread in the sequence [39–41]. To the best of our knowledge, Trp has not been added to the ends of helical amphipathic AMPs to stabilize and modulate their pore-forming properties.

To examine our hypothesis, we investigated KIA peptides with Trp residues at the ends and compared them with normal KIA peptides without Trp. As a first test, we used a single Trp at either end or in the middle of the peptide, and in further experiments, we used two Trp residues, one near each end of the peptide. To minimize any change in hydrophobicity of these Trp-mutants, we always replaced Ile residues with Trp. KIA peptides of different lengths were used, in order to obtain additional information about the effect of Trp on length-dependent activity.

In this study, we used Trp fluorescence to determine the binding affinity of peptides to membranes, and circular dichroism spectroscopy to confirm folding to a helical secondary structure of the peptides in membranes. To test the hypothesis that Trp near the end of the helices stabilizes a transmembrane orientation of peptides in membranes, solid-state ^{15}N NMR was used in model membranes. The propensities of peptides to insert in NMR experiments were correlated to membranolytic activity by a fluorescence-based vesicle leakage assay. Antimicrobial and hemolysis assays were used to observe the biological activity as a function of peptide length and presence of Trp.

2. Experimental section

2.1. Materials

The lipids 1,2-dierucoyl-*sn*-glycero-3-phosphatidylcholine (DErPC), 1,2-dierucoyl-*sn*-glycero-3-phosphatidylglycerol (DErPG), 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylglycerol (DMPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol (POPG) were purchased from NOF Corporation (Grobbendonk, Belgium). 1-Myristoyl-2-hydroxy-*sn*-glycero-3-

phosphatidylcholine (lyso-MPC) was purchased from Avanti Polar Lipids (Alabaster, AL, US).

2.2. Methods

This study is a continuation of our previous studies of KIA and CKIA peptides. Peptide synthesis, circular dichroism spectroscopy (CD) measurements, minimum inhibitory concentration (MIC) assays, hemolysis assays, fluorescence-based vesicle leakage assays and solid-state NMR experiments were performed as described in previous publications about KIA, KIXA and CKIA peptides [13–15,17]. The NMR chemical shifts were referenced using the ^{1}H NMR water signal of the sample, which was set to 4.65 ppm at 35 °C; for other nuclei, the chemical shift was calculated using the known gyromagnetic ratios of the nuclei. Binding measurements were performed using the chromatosolvic shift of the Trp fluorescence signal as described in detail previously [42]. Details of the methods are given in the Supplementary Information.

3. Results and discussion

3.1. Peptide synthesis

KIA peptides of different lengths were synthesized with a single Trp at the end or in the middle of the sequence, or with Trp residues at each end. Since we had previously shown that charged or hydrophobic residues at the N- and C-termini critically affect the functional activity [17], we used only KIA peptides starting and ending with the sequence KIA. To investigate the length effect, we thus used KIA peptides with 14, 17, 21 or 24 amino acids. The sequences of all peptides used are given in Table 1. As a class, KIA peptides with Trp substitutions will be called WKIA peptides. Peptide names are given as KIAxW$^{i(,j)}$, where x is the number of residues of the peptide, and i (and j if present) indicate the positions of a Trp residue, which always replaces an Ile residue. This substitution does not significantly change the peptide hydrophobicity, as seen from the HPLC retention times (Table 1) which depend on peptide length, but are only slightly larger for peptides containing Trp, and in some cases even a bit lower than for peptides without Trp.

3.2. Circular dichroism spectroscopy

CD spectra (Fig. 1) showed that all the peptides are unstructured in phosphate buffer (PB), as seen from the minimum near 198 nm, which is typical of random coil spectra. The peptides predominantly form α-helices in the presence of DMPC/DMPG vesicles at a peptide-to-lipid molar ratio (P/L) of 1/50, indicated by a maximum close to 192 nm and minima at 208 nm and 222 nm. A deconvolution of the CD spectra using several algorithms [43–50] showed that the peptides are 79–93 % helical in the presence of lipid vesicles, indicating that only 1–3 residues are not part of the helix (Table S1 in the Supplementary Information).

3.3. Antimicrobial activity

The antimicrobial activity of the WKIA peptides was tested by a standard MIC assay, where the MIC was determined by growing bacteria in a twofold dilution series of peptide concentrations. The assay was carried out with Gram-negative *Escherichia coli* (DSM 1116) and *Enterobacter helveticus* (DSM 18390) and Gram-positive *Bacillus subtilis* (DSM 347) and *Staphylococcus xylosus* (DSM 20287), as previously reported [17,51,52]. The results are shown in Fig. 2 and Table S2. The helix length has a clear effect on MIC values, with longer peptides being more active, as reported previously for KIA peptides [13,15]. There was also a length-dependent effect when Trp was added to the termini, with an improvement in the activity of short peptides, though not of long peptides. For KIA14, CKIA17 and KIA17, the MIC was reduced by a factor of 4 or more when Trp was added, in the case of *E. coli*, *E. helveticus*, and *B. subtilis* (in *B. subtilis,* the effect is not observed for KIA17). For *S. xylosus,* there was a reduction in MIC for these peptides by a factor of 2, which is within the error margin of the method and might therefore not be significant. For the longer KIA21 and KIA24, adding Trp showed no effect (within a factor of 2); presumably because these peptides were already more active than the shorter peptides. Thus, it seems that adding Trp at the helix termini can improve the activity of less active peptides, while it will not improve any peptides further that already have good activity (the limiting MIC seems to be approximately 16 μg/mL for *S. xylosus*, and 4 μg/mL for the other tested bacteria). The reason may be that peptides with 21 or 24 amino acids are already long enough to be preferentially inserted into the membrane without Trp, hence the added Trp has little effect.

3.4. Hemolysis

A hemolysis assay was performed to study the unwanted side effects of the peptides on red blood cells. Results are presented in Fig. 3 and Table S3. For all the original KIA and the Trp-modified WKIA peptides, hemolysis increased with peptide length. We can note that KIA17 induced a bit more hemolysis than CKIA17, which correlates with a somewhat higher antimicrobial activity, and a more inserted orientation into membranes according to ^{15}N NMR in DMPC, of KIA17 compared to CKIA17. At a low peptide concentration of 8 μg/mL, only a small extent of hemolysis was observed (<10 %) for the KIA peptides (Fig. 3). When Trp was added to both ends of KIA17, KIA21 and KIA24, hemolysis increased. A single Trp in KIA21 had no effect. At a high peptide concentration of 256 μg/mL, KIA14, CKIA17 and KIA17 still exhibited only low hemolysis. When Trp was added to each end, CKIA17 and KIA17 showed a large increase in hemolysis, but KIA14 did not. KIA21 exhibited 3–5 times higher hemolysis when Trp was added at either end

Table 1
Sequences of all peptides used. Trp residues are shown underlined and in bold. In all peptides, Ala-10 (marked in bold) was labeled with ^{15}N at the backbone.

Peptide	Sequence	Length [a] [Å]	Charge	Retention time (min)
KIA14	KIAGKIA KI**A**GKIA-NH_2	21	+5	8.5
KIA14W^{2}	K**W**AGKIA KI**A**GKIA-NH_2	21	+5	8.7
KIA14W^{2,13}	K**W**AGKIA KI**A**GK**W**A-NH_2	21	+5	8.7
CKIA17	KIA KIAGKI**A** KIAGKIA-NH_2	25.5	+6	9.5
CKIA17W^{2,16}	K**W**A KIAGKI**A** KIAGK**W**A-NH_2	25.5	+6	9.7
KIA17	KIAGKIA KI**A**GKIA KIA-NH_2	25.5	+6	9.8
KIA17W^{2,16}	K**W**AGKIA KI**A**GKIA K**W**A-NH_2	25.5	+6	10.1
KIA21	KIAGKIA KI**A**GKIA KIAGKIA-NH_2	31.5	+7	11.2
KIA21W^{2}	K**W**AGKIA KI**A**GKIA KIAGKIA-NH_2	31.5	+7	11.3
KIA21W^{13}	KIAGKIA KI**A**GK**W**A KIAGKIA-NH_2	31.5	+7	10.4
KIA21W^{20}	KIAGKIA KI**A**GKIA KIAGK**W**A-NH_2	31.5	+7	11.0
KIA21W^{2,20}	K**W**AGKIA KI**A**GKIA KIAGK**W**A-NH_2	31.5	+7	11.1
KIA24	KIAGKIA KI**A**GKIA KIAGKIA KIA-NH_2	36	+8	12.4
KIA24W^{2,23}	K**W**AGKIA KI**A**GKIA KIAGKIA K**W**A-NH_2	36	+8	12.5

[a] The peptide length is estimated from an ideal α-helix with a length of 1.5 Å per residue.

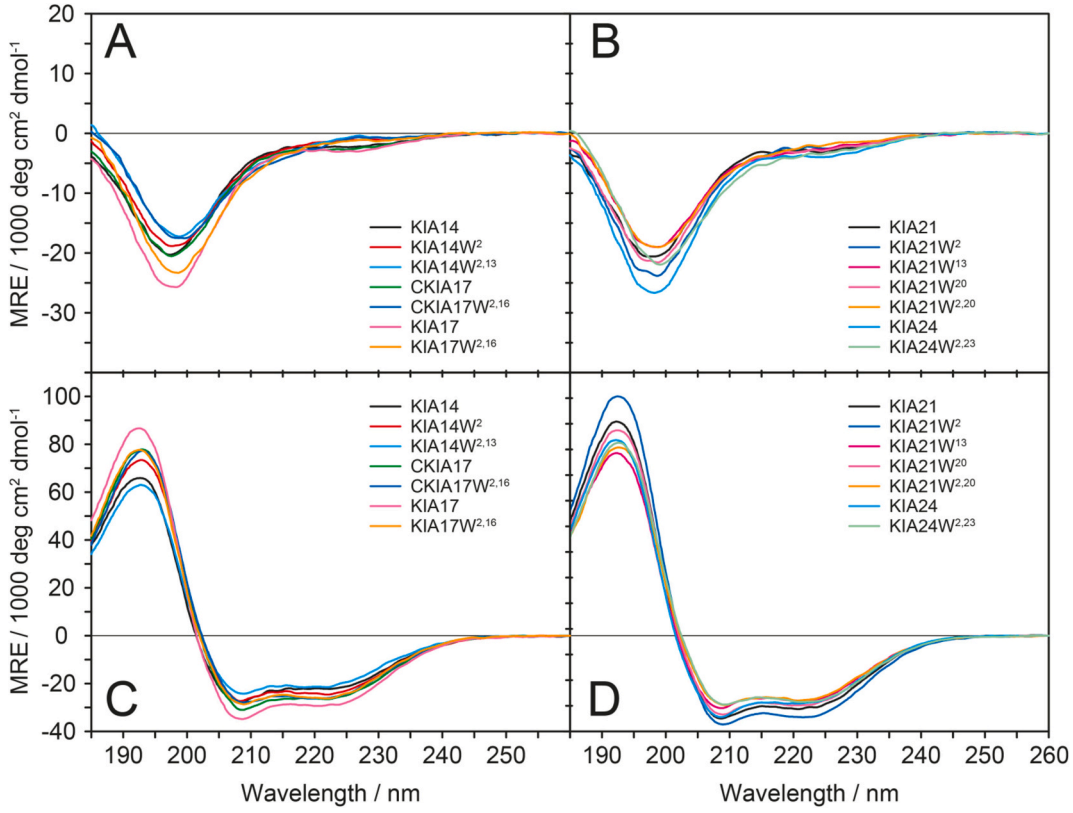

Fig. 1. CD spectra of KIA and WKIA peptides. (A, B) In 10 mM PB at 25 °C. The peptide concentration was 0.1 mg/mL. (C, D) In DMPC/DMPG (3/1) vesicles at 30 °C. The lipid concentration was 1 mg/mL, and the peptide-to-lipid molar ratio was 1/50.

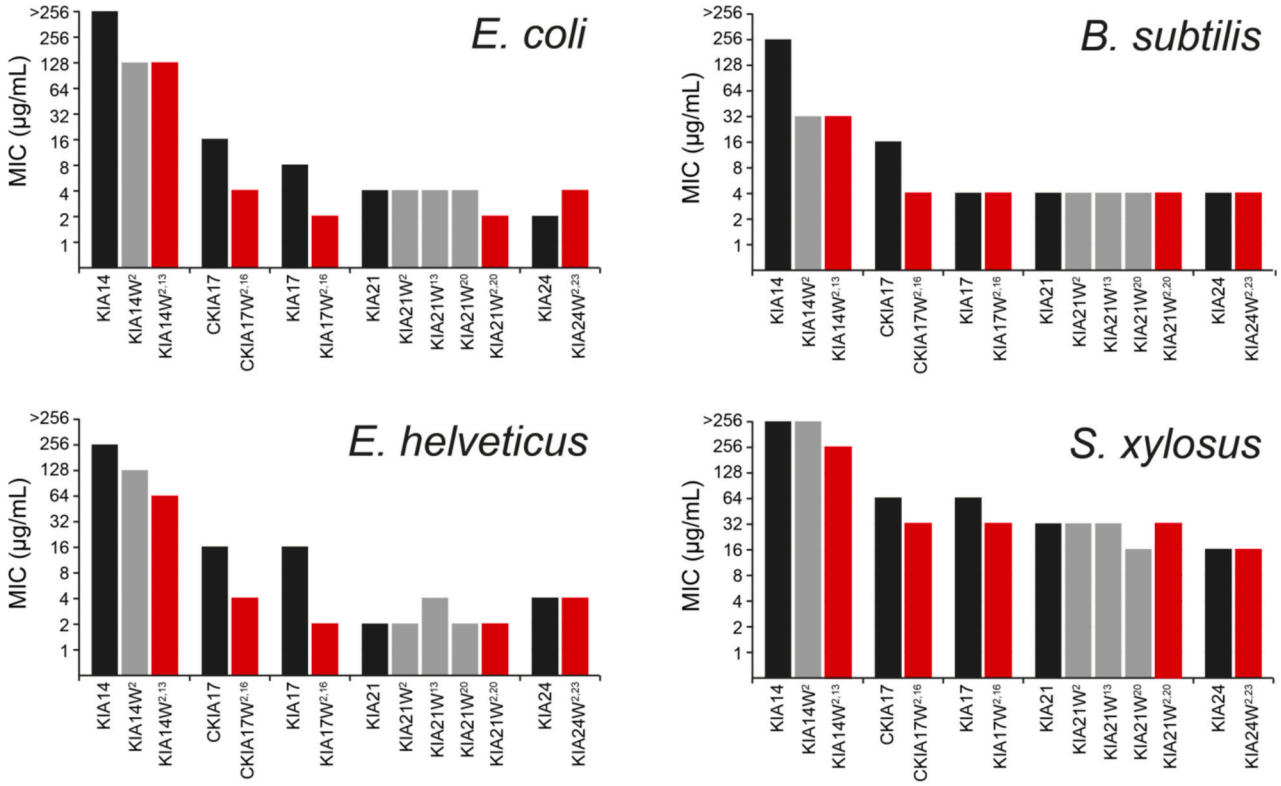

Fig. 2. MIC values of the different KIA peptides against four different bacterial strains. KIA peptides without Trp are shown with black bars, WKIA peptides with one Trp in gray, and WKIA peptides with two Trps in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

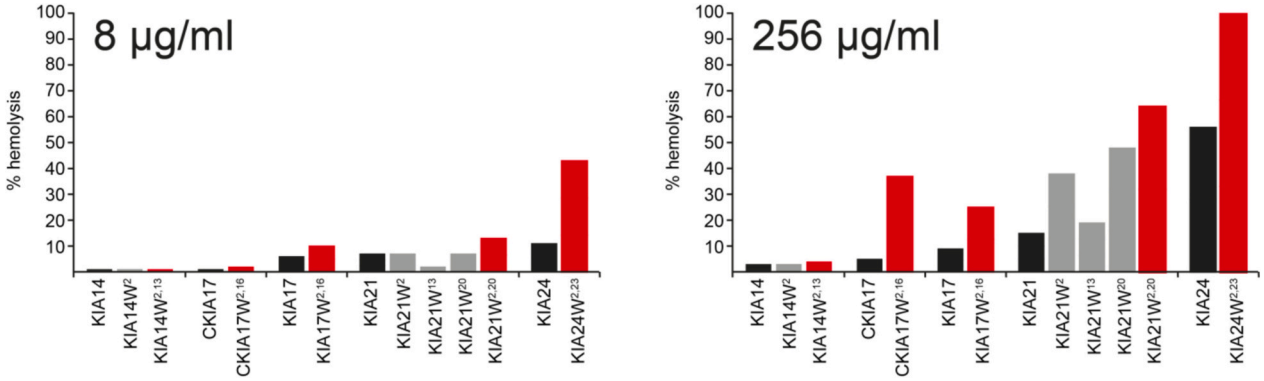

Fig. 3. Hemolysis of the different KIA peptides at high and low peptide concentrations. KIA peptides without Trp are shown with black bars, WKIA peptides with one Trp in gray, and WKIA peptides with two Trps in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

or at both ends, but Trp at position 13, in the middle of the sequence, had almost no effect. For KIA24, there was a large increase in hemolysis when Trp was added at both ends, reaching 100 % at the highest tested concentration. Clearly, adding Trp at the ends of KIA peptides leads to a strong increase in hemolysis, except for the very short KIA14.

3.5. Vesicle leakage

Vesicle leakage experiments were performed using a fluorescence-based assay, where the leakage of small dye molecules from vesicles was measured after the addition of peptides. We have previously shown that vesicle leakage is strongly dependent on KIA peptide length and can only take place when peptides are long enough to span the hydrophobic thickness of the membrane [13,15]. Thus, in POPC/POPG (1/1) vesicles, KIA peptides need at least 17 amino acids to be active, and in thicker DErPC/DErPG (1/1) membranes, at least 24 amino acids are needed [15]. Here, we observed an increase in leakage for peptides with Trp at the ends (see Fig. 4 and Table S4), except for KIA14, which was too short to induce leakage. In POPC/POPG, the longer peptides show an increase by a factor of 2–5 in leakage with Trp at both ends, compared to peptides without Trp. KIA21 with a single Trp showed a smaller increase. Without Trp, there was a clear length-dependent effect, as KIA24 causes much more leakage than KIA17, but with Trp at both ends, the difference was smaller, as peptides with 17, 21 or 24 amino acids all caused similarly strong leakage.

In very thick DErPC/DErPG membranes, without any Trp-modification only the original KIA24 was active, whereas the shorter peptides without Trp caused only 1–3 % leakage. With Trp at both ends, KIA14 was still completely inactive, while KIA17 and CKIA17 gave some weak 10 % leakage. For KIA21, leakage increased by almost a factor of 40 when Trp was included at both ends. Notably, with a single Trp at position 2, there was a large effect (a factor of 10), whereas Trp at position 13 or 20 had only a minor effect. For KIA24, leakage increased by a factor of 3 when Trp was added to both ends. It is clear that in all cases, adding Trp to both ends leads to an increase in leakage, sometimes a dramatic increase. Even peptides that are usually too short to cause leakage, are able to produce some significant leakage when Trp is included, except for the very short helix of KIA14.

3.6. Peptide binding to vesicles

Can the difference in leakage and other membrane activities for peptides of different length be explained by a difference in binding? Previously, this question could not be addressed experimentally for the KIA peptides, but with Trp residues in the sequences, binding could now be investigated. We used Trp fluorescence to study the binding of peptides to POPC/POPG (1/1) vesicles under conditions as similar as possible to those in the leakage experiments. The Trp fluorescence signal depends on the polarity of the environment, and the emission maximum changes from approximately 360 nm in aqueous solution to approximately 343 nm when the peptide is bound to the membrane. The signal intensity also increases in the bound state. The signal at 343 nm was measured at different lipid concentrations, and the bound fraction of peptide was determined. The partitioning constant K_p was then

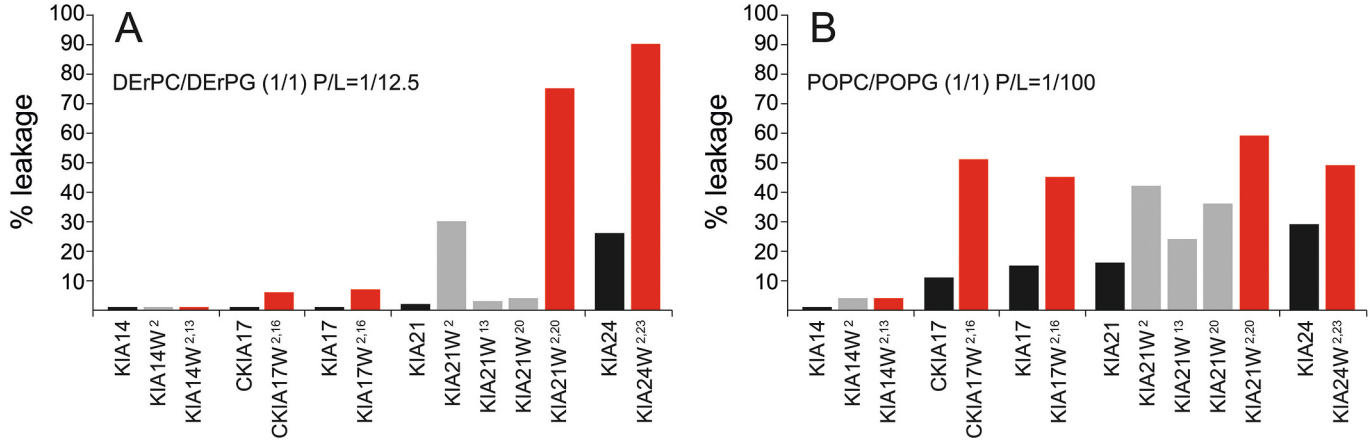

Fig. 4. Vesicle leakage induced by the different KIA peptides in (A) DErPC/DErPG (1/1) and (B) POPC/POPG (1/1) vesicles. KIA peptides without Trp are shown with black bars, WKIA peptides with one Trp in gray, and WKIA peptides with two Trps in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

determined from the fit to the data of the binding equation:

$$X_L = C_L K_p \gamma_L / (1 + C_L K_p \gamma_L) \quad (1)$$

where X_L is the molar fraction of peptides bound to the lipids, C_L is the concentration of the lipids, and γ_L is the molar volume of the lipids [42,53]. The association constant K_a can be calculated from $K_a = K_p \gamma_L$. For POPC/POPG (1/1), γ_L can be estimated to be 0.765 L/mol [54,55]. From K_p, the free energy of binding can be calculated according to:

$$\Delta G_p = -RT\, ln(K_p) \quad (2)$$

where R is the gas constant and T is the temperature (in K) [56].

Binding was determined for the doubly modified KIA peptides with Trp at both ends. Experiments were repeated three times, and the average value and the standard deviation are given in Table 2. The K_p of KIA14W^{2,13} was clearly the lowest at 71,000 ± 16,000. KIA17W^{2,16} had a K_p of 630,000 ± 100,000, which was slightly less than that of KIA21W^{2,20}, which had a K_p of 730,000 ± 130,000, though the difference was within the error margin. KIA24W^{2,23} had the strongest binding with a K_p of 2,250,000 ± 80,000.

In the leakage experiments, 100 μM lipids were used. As seen in the binding curves (Figs. S5 and S6), approximately 85 % of KIA14 was bound at this lipid concentration, and close to 100 % of the longer peptides. This small difference in binding, leading to somewhat fewer peptides in the membrane, is not enough to explain why KIA14 causes less than 10 % of the leakage of KIA17 in POPC/POPG (1/1).

3.7. Solid-state ^{15}N NMR

1D ^{15}N NMR on macroscopically oriented samples can be used to estimate the orientation of a ^{15}N-labeled α-helix in a membrane [57–60]. A peak at approximately 80–90 ppm indicates that the helix lies flat on the membrane surface, and a peak at approximately 180–200 ppm indicates that peptides are in a fully upright transmembrane orientation. Depending on the peptide length and membrane thickness, the helix may span the membrane with a certain tilt angle without being completely upright in the membrane, giving a peak at less than 180 ppm for a transmembrane peptide [14]. For example, the peak at 130 ppm found for KIA24 in DMPC/lyso-MPC still corresponds to a transmembrane orientation of this long peptide, as discussed more in detail previously [14]. In this study, the orientation determined by ^{15}N NMR was used to test the hypothesis that peptides with Trp at the ends can more easily insert into the membranes. If peptides which are more inserted are also more active, this would support the hypothesis that activity is due to the formation of pore-like structures with peptides in a transmembrane orientation.

^{15}N NMR was performed on all WKIA peptides at P/L = 1/50 to determine their orientation in several representative lipid systems. The spectra are shown in Figs. S1-S4 in the Supplementary Information, and the chemical shifts of the peptide peaks are illustrated in Fig. 5 for the different peptides in selected lipid systems. All the WKIA peptides, independent of the length and number of Trp, lie flat on the surface of POPC/POPG (1/1) membranes, as indicated by the chemical shift of 80–90 ppm in all cases (Fig. S1). Thus, no effect of Trp was observed.

Table 2
Binding results for WKIA peptides with Trps near both termini. The association constant K_a, the partitioning constant K_p, and the free energy of binding ΔG_p are determined. The values are averages of three measurements (± standard deviation).

Peptide	K_a (M^{-1})	K_p	ΔG_p (kcal/mol)
KIA14W^{2,13}	54,000 ± 12,000	71,000 ± 16,000	−6.7 ± 0.1
KIA17W^{2,16}	480,000 ± 80,000	630,000 ± 100,000	−8.0 ± 0.1
KIA21W^{2,20}	560,000 ± 100,000	730,000 ± 130,000	−8.1 ± 0.1
KIA24W^{2,23}	1,720,000 ± 60,000	2,250,000 ± 80,000	−8.8 ± 0.03

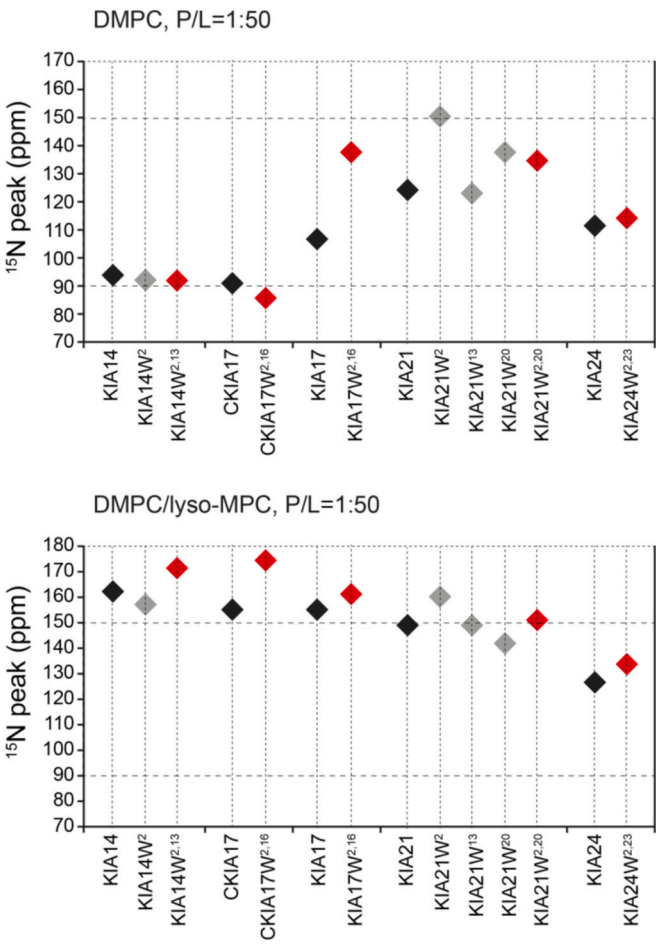

Fig. 5. ^{15}N NMR chemical shifts of ^{15}N-labeled peptides in two different lipid systems (pure DMPC, and DMPC/lyso-MPC 2/1), at a total peptide-to-lipid molar ratio of 1/50. KIA peptides without Trp are represented by black symbols, WKIA peptides with one Trp by gray symbols, and WKIA peptides with two Trps by red symbols. The ^{15}N NMR chemical shifts were referenced using the ^{1}H NMR signal of the water peak of the sample. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In DMPC/lyso-MPC (2/1) (Figure 5, lower panel), chemical shifts of 150–180 ppm were observed for peptides with 14–21 amino acids, indicating a transmembrane orientation of the helices. Peptides modified with Trp had a chemical shift similar to or higher than that of peptides of the same length without Trp, indicating a more upright orientation in the membrane. Peaks from the longest set of KIA peptides, with 24 amino acids, had a lower chemical shift of 130–140 ppm. Since these peptides are significantly longer than the hydrophobic lipid bilayer thickness, this most likely still corresponds to a transmembrane orientation but with a considerable helix tilt angle [14].

The most interesting results were found in DMPC bilayers, where the original KIA peptides did not insert, but when modified with Trp, the chemical shifts increased in many cases (Figure 5, upper panel). For the short KIA14 and CKIA17, a chemical shift close to 90 ppm indicated that the helices remained on the bilayer surface, and there was no difference observed with or without Trp. For KIA17, the chemical shift was 110 ppm, but with Trp at both ends, it shifted to 140 ppm. This large change indicates that peptides are at least partly inserted into the membrane. Interestingly, KIA17 inserts more easily than CKIA17, showing that even a small change in the amino acid sequence can influence the insertion. KIA21 without Trp has a peak at 125 ppm, but with Trp at position 2, 20 or both 2 and 20, it gets more inserted, whereas a Trp at position 13 has no effect. For the long KIA24, the chemical shift was 115 ppm with or

without Trp. To determine whether WKIA peptides were inserted into DMPC even at a lower peptide concentration, further experiments were performed at P/L = 1/100, but in this case, all peptides were found to remain on the membrane surface, giving a ^{15}N NMR peak close to 90 ppm (Fig. S4).

The expectation that KIA peptides are more likely to be inserted in membranes when Trp residues are positioned close to the ends could largely be validated by ^{15}N NMR in DMPC and DMPC/lyso-MPC membranes at P/L = 1/50. For KIA14 and CKIA17, the increase in tilt with Trp was larger in DMPC/lyso-MPC, whereas for KIA17 and KIA21 a more inserted orientation for peptides with Trp was more clearly seen in DMPC. Comparing with the activity assays above, we can note that the more inserted Trp containing peptides are also more active.

It could be expected that the peptide orientation determined from ^{15}N NMR data in POPC/POPG would give the best correlation with leakage in POPC/POPG vesicles. However, since in POPC/POPG all peptides have the same orientation, flat on the surface of the membrane, this data has no correlation to the varying leakage values found for the different peptides. There is a much higher correlation between the orientation in DMPC or DMPC/lyso-MPC and leakage, with more inserted peptides according to NMR giving more leakage. We propose two explanations for this puzzle.

According to studies from Wimley et al., a stable pore made of 10 peptides with a diameter of 10 Å would be enough to give 100 % leakage of a typical LUV with 100 nm diameter in less than a second [6]. In our POPC/POPG leakage assay, we use P/L = 1/100, the LUVs have around 100.000 lipids, and almost 100 % of the peptides are bound to the vesicles (see Table 2 and Fig. S6), meaning that around 1000 peptides are bound to each vesicle [6]. In our case, there is at most 60 % leakage after 10 min, meaning that in case pores are responsible for leakage, leakage can be explained by a very small part of the peptides forming short-lived transient pores, on average, possibly only one out of 1000 peptides. The rest of the peptides would presumably be bound to the surface. In the ^{15}N NMR experiment, we observe all peptides, and if a small part of them would be inserted and the majority are flat on the surface, we would see a main peak at around 80 ppm and a small peak for the more inserted peptides, but this peak would be hard to see as the signal to noise is not very high in ^{15}N NMR (see Fig. S1).

In DMPC and especially DMPC/lyso-MPC bilayers, it is easier for peptides to insert compared to POPC/POPG bilayers, as seen in many previous studies (for a review see [61]). Therefore, a larger proportion of peptides are in the inserted state and give the main ^{15}N NMR signal. In effect, we can trap the inserted state by using suitable lipids with a positive spontaneous curvature [61]. However, not all tested peptides get inserted even in these lipid systems, and we can observe differences in orientation between the different peptides. For example, KIA17W^{2,16} is more inserted than KIA17, and we can note that KIA17W^{2,16} also leads to more hemolysis and leakage, and a higher activity against most of the tested bacteria.

If the active state of the peptides is some kind of carpet model with peptides on the membrane surface, then we would not expect that the inserted states found in NMR would correlate with activity. But since there is a correlation, and the more inserted peptides are also more active, this is an indication that the inserted state is involved in the mechanism of action, which in turn indicates that pores may be responsible for activity.

3.8. Pore model

Based on previous results on KIA peptides, it has been proposed that the mechanism of action of these peptides is the formation of transmembrane pores, as will be described below. The structural and functional results on this novel set of WKIA peptides can be readily understood in terms of the tryptophan residues acting as anchors that stabilize the transmembrane alignment in the pore state. Upon binding to the membrane, amphipathic helices will initially lie flat on the surface, with the polar face pointing up towards the water and the hydrophobic face pointing down into the membrane core. Under suitable conditions, they can proceed to assemble into oligomeric transmembrane pores, where the polar face of each helix points into the water-filled center, while the hydrophobic faces point towards the surrounding lipid acyl chains. There is usually an equilibrium between these two states, which can be dramatically shifted by different features of the peptide and properties of the membrane. In Fig. 6 we give an overview of the different identified factors. This cartoon is a simplification of the complex situation in real membranes, and for simplicity pores are shown without lipids between the inserted peptides. Each part of the figure is based on publications where peptide orientations were determined with solid-state NMR.

One important factor we had identified earlier is the spontaneous curvature of the lipids constituting the bilayer [14,61–63]. Lipids with a negative spontaneous curvature (such as POPC/POPG or PE lipids) favor the surface state (Fig. 6A), whereas lipids with a positive spontaneous curvature (such as lysolipids) favor the pore state (Fig. 6B) [14,61–63]. Another factor is the hydrophobic matching between peptides and lipids, as we found that KIA peptides that are too short to span the membrane will stay on the surface, whereas sufficiently long peptides can form active pores (Fig. 6C) [13–15]. The length-dependence as well as the curvature-dependence mentioned above are clearly dominated by thermodynamics, i.e., by the relative stabilities (free energies) of the surface-bound state and the transmembrane pore state.

More recently, we also noticed that the distribution of charges at the peptide termini plays an important role in pore formation. When there is a single charge at each end of the helix, the surface state is favored and peptides are less active (Fig. 6D) [17]. A double charge at the N-terminus together with an uncharged C-terminus, on the other hand, readily enables insertion and pore formation. We found that this effect is strongest in leakage experiments (where peptides are added to vesicles, and leakage is monitored for 10 min), while it is less pronounced in the NMR data (where peptides and lipids are mixed from organic solvents, and samples are allowed to equilibrate 16–24 h before measurement). Hence, thermodynamic stability may not be enough to explain the observed influence of charge distribution, as there also seems to be a kinetic contribution, namely that the activation energy barrier for helix insertion and flipping represents a rate-limiting step of pore formation.

Here, we show that Trp residues near both termini are yet another important factor that contributes towards a favorable pore state (Fig. 6E). Using solid state ^{15}N NMR (which reflects the thermodynamically favored states in an equilibrated membrane sample), we found that KIA17 and KIA21 with Trp at both ends were inserted into a DMPC membrane to a higher degree than the original peptides without Trp (Fig. 5). In DMPC/lyso-MPC, both KIA14 and CKIA17 had a much more upright orientation when modified with Trp at both ends. In some cases, where the system was already close to the threshold of flipping, the presence of Trp resulted in a drastic orientational change of a majority of the peptides. For other peptides and lipid systems, the differences were smaller, but the anchoring Trp residues were generally seen to shift the equilibrium (as observed by NMR) towards a more inserted state. In the functional experiments, i.e., in antimicrobial and hemolysis assays as well as in vesicle leakage, kinetic parameters may play an additional role on top of the free energy that drives the insertion reaction. Yet, we also find in the functional experiments that the thermodynamic stability argument of a transmembrane pore can account for essentially all of our observations.

Interestingly, it seems that the Trp anchoring effect can override the length effect to some extent; i.e., peptides that are usually too short to form a pore without Trp are enabled to do so when Trp residues are present near the termini. Thus, in the leakage assay with POPC/POPG vesicles, the relatively short KIA17W^{2,16} is as active as KIA24W^{2,23}, while KIA17 is considerably less active than KIA24. Likewise, in DErPC/DErPG vesicles, KIA21W^{2,20} is almost as active as KIA24W^{2,23}, whereas KIA21 without Trp is essentially inactive. In hemolysis, the most

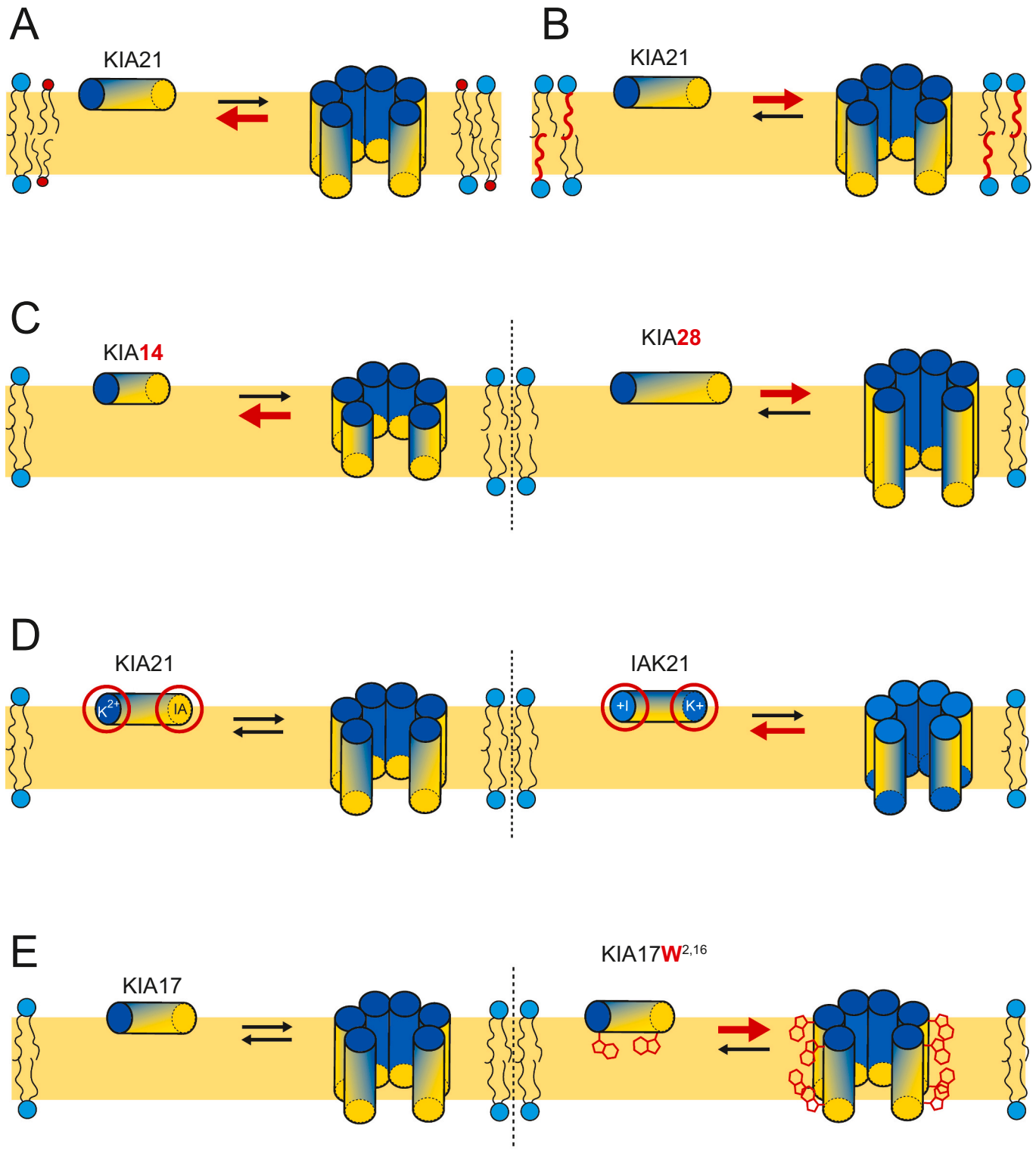

Fig. 6. Summary of factors influencing the equilibrium between the surface state and inserted state of KIA-type peptides. The arrows indicate the relative shift in the equilibrium between the two parts of each panel. (A) Lipids with a negative spontaneous curvature (such as PE lipids with a small head group) favor the surface state, but (B) lipids with a positive spontaneous curvature (such as lysolipids with a single acyl chain) favor the pore state [14,61–63]. (C) KIA peptides that are too short to span the membrane will stay on the surface (like KIA14), whereas peptides that are long enough (like KIA28) can form transmembrane pores [13–15]. (D) Peptides with one highly charged end (free N-terminus and Lys side chain) and one hydrophobic end (amidated C-terminal Ala or Ile, as in the original KIA21 can insert more easily than peptides with a symmetric charge distribution over both termini (like the permutated IAK21 peptide) [17]. (E) Peptides with Trp residues close to the N-terminus or both termini (like the WKIA peptides investigated here) tend to favor the transmembrane pore state more than peptides without Trp.

pronounced increase in activity upon Trp modification is observed for the longer peptides (17–24 residues), and in the antibacterial assay the strongest effects are seen for the shorter peptides (14–17 residues). In both cases, improved pore forming ability increases biological activity. The somewhat different length window of maximum response may be simply attributed to the fact that erythrocyte membranes are thicker than the bacterial membranes used here, as demonstrated in an earlier work [15].

Altogether, these observations support the hypothesis that transmembrane pores are responsible for biological activity. Only under some rare conditions when pores are readily formed per se by the parent KIA peptides, the incorporation of Trp does not improve the activity any further. For example, in the leakage of POPC/POPG vesicles, KIA17 and KIA24 show very different activities without Trp, but almost the same high activity with Trp at both ends. Longer peptides, which already have a good MIC, are not improved by the addition of Trp, but shorter peptides become more active against bacteria. The addition of Trp at the ends also increases hemolysis. Only the very short KIA14 peptide, which shows no leakage and no hemolysis, does not become active when Trp is added to the ends, which indicates that there is a limit to the effect of Trp. The anchoring effect can apparently compensate for insufficient length only up to a certain point, at least for the peptides that were studied here. It seems that Trp gives peptides a longer effective length, but if the peptide is much too short, even this additional effective length is not enough to confer activity. The longer effective length could be due to a stretching out of the Trp side chains, a thinning of the membrane, or a combination of both. This effect may well be attributed to the known propensity of Trp to be preferentially located in transmembrane helices of integral membrane proteins at a specific depth in the lipid bilayer, i.e., within the amphiphilic interface between headgroups and acyl chains.

3.9. Occurrence of Trp residues in natural AMPs

Are Trp residues near the peptide termini common in natural AMPs? There are some known AMPs with a Trp close to a terminus, for example the dermaseptins, a family of AMPs with a conserved Trp at position 3 [64]. To get a more general picture we made a database search. In the 3240 peptides within the APD3 database [65], Trp is the second least common amino acid with a frequency of 1.6 % [66]. This is slightly higher than the average for all proteins in the UniProt database, which have 1.1 % Trp [67], but much lower than the Trp frequency of approximately 4 % in transmembrane domains [68]. Of all peptides in the APD3 database, 1181 (36 %) contain at least one Trp. We thus selected from this database all helical, cationic peptides with 15–30 amino acids and obtained 223 hits, of which 71 (32 %) contained Trp. Thus, Trp is no more common in these helical, cationic peptides than in the database as a whole. Excluding synthetic peptides, 65 natural peptides were present in the selected set. Of those, 29 (45 %) contained a Trp as one of the three most N-terminal residues, 5 (8 %) contained a Trp as one of the three most C-terminal residues, one peptide had a Trp at both the N- and the C-termini, and 32 peptides (49 %) had Trp residues only in the central part of the peptide. Since the average lengths of these peptides were 23 amino acids, three residues correspond to 13 % of the length, hence it is clear that there is a distinct over-representation of Trp amongst the three most N-terminal residues in these peptides. On the other hand, there is no overrepresentation of Trp amongst the three most C-terminal residues.

Thus, in natural antimicrobial peptides, there seems to be a selection for Trp residues near the N-terminus, but not near the C-terminus. Interestingly, from our results (comparing KIA21W^{2} and KIA21W^{20}), it seems that Trp near the N-terminus has indeed a stronger effect on peptide orientation and leakage activity (especially in DErPC) than Trp near the C-terminus, while in the MIC and hemolysis assays, no clear trend was observed.

4. Conclusions

Trp residues near the ends of cationic helices of the KIA series tend to increase the membrane activity of these peptides to a larger or lesser degree. We found using ^{15}N NMR that the presence of Trp can in some cases (when the system is near the threshold of helix insertion) be the decisive trigger towards the insertion of peptides in membranes; antimicrobial MIC assays showed that this effect is particularly pronounced for the shorter range of peptides; hemolysis is seen to increase most effectively for longer peptides; and vesicle leakage is also found to increase, especially for those intermediate length peptides that induce only weak leakage without Trp.

Generally, we may conclude that Trp residues not only serve as membrane anchors in hydrophobic helices of typical integral membrane proteins, which is a well-known fact. Remarkably, Trp anchors can also contribute considerable to the stability of amphipathic helices that have assembled in a transmembrane orientation. Overall, our results confirm the hypothesis that KIA peptides can form pores in the membranes, and when Trp residues are present close to the ends, the probability of pore formation increases. We expect that this observation may be valid not only for KIA peptides, but also for many other similar cationic amphipathic helical peptides. The presence of Trp residues near the end of an amphiphilic helix, especially the N-terminus, should thus be considered as an important criterion when strategically designing de novo sequences or optimizing existing membrane-active peptides.

CRediT authorship contribution statement

Erik Strandberg: Conceptualization, Formal analysis, Investigation, Visualization, Supervision, Writing – original draft, Writing – review & editing. **Patrick Horten:** Investigation, Writing – review & editing. **David Bentz:** Investigation, Writing – review & editing. **Parvesh Wadhwani:** Investigation, Resources, Writing – review & editing. **Jochen Bürck:** Formal analysis, Investigation, Supervision, Writing – review & editing. **Anne S. Ulrich:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

We thank Andrea Eisele and Kerstin Scheubeck for assistance in the peptide lab, Kerstin Scheubeck for help in the microbiology lab, Dr. Stephan Grage and Markus Schmitt for maintaining the NMR infrastructure, and Siegmar Roth and Bianca Posselt for assisting in the CD lab. This work was supported by the Helmholtz Association Programs BIF-TM and NACIP, and by the German Research Foundation (DFG) grant INST 121384/58-1 FUGG. The funding sources were not involved in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bpc.2024.107365.

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