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The hemolytic activity of six arachnid cationic peptides is affected by the phosphatidylcholine-to-sphingomyelin ratio in lipid bilayers

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Abstract

The hemolytic activity of six cationic amphipathic peptides (Oxki1, Oxki2, Pin1, Pin2, IsCT1 and IsCT2) from arachnids strongly depends on the source of red blood cells. The hemolytic activity of the amphipathic peptides was correlated to the phosphocholine-to-sphingomyelin ratio (PC/SM) content, the potency order of which on mammal erythrocytes ranked as follows Guinea pig>pig>sheep. The spider peptides, Oxki1 and Oxki2, prefer small unilamellar vesicles (SUV) composed of PC, but they could not disrupt SUVs made of SM only. Moreover, the membrane-disrupting activity of the scorpion peptide Pin1 was affected by increasing concentrations of SM. Only the scorpion hemolytic peptide Pin2 was able to disrupt SUVs composed merely of SM at high concentrations. Finally, the short scorpion peptides IsCT1 and IsCT2 seem to tolerate high concentrations of SM in the presence of PC for disruption of SUVs; however, the disrupting activities of IsCT1 and IsCT2 are much lower than that of the other four hemolytic peptides. The hemolytic activity caused by all six cationic peptides in mammalian erythrocytes was positively correlated to increases in temperature and increases in the concentration of benzyl alcohol, a membrane fluidizing agent. It was concluded that the hemolytic activity of the cationic peptides strongly depends on the PC/SM content of mammalian erythrocytes, in which cell membranes with a low PC/SM ratio (i.e., of low fluidity) were less disturbed than membranes with a high PC/SM ratio (i.e., of high fluidity).

Keywords: Cationic amphipathic peptide; Phosphatidylcholine to sphingomyelin ratio; Hemolysis; Membrane fluidity

1. Introduction

Amphipathic and cationic α-helical peptides are broadly found in animals as a part of their biological defense system [1]. Since the discovery of melittins [2] and cecropins [3], a large number of amphiphilic and cationic peptides have been characterized in invertebrates, especially from the phyla Arthropoda [4] and in vertebrates from the class Amphibia [5]. Linear cationic peptides with α-helical conformation share some common characteristics such as ''pore-forming'' activities at low micromolar concentrations and α-helix formation in hydrophobic environments. Moreover, other features distinguish them such as their hydrophilic/hydrophobic amino acid distribution along the structure rendering its amphipathic characteristics, and their disruptive activity towards eukaryotic cells, particularly red blood cells. The erythrocyte outer leaflet membrane bilayer is mainly composed of phosphatidylcholine (PC) and sphingomyelin (SM) together with cholesterol. Zwitterionic choline-containing phopholipids such as PC and SM ranged between 50% and 70% of the total phospholipids in most red blood cell membranes [6,7]. Other phospholipids such as phosphatidylserine, phosphatidylethanolamine, phosphatidyl inositol and phosphatidic acid are present in an average of 15.8%, 26%, 3.0% and 0.89%, respectively [8]. However, the total lipid composition of the erythrocyte cell membranes differs in mammalian species, and the PC-to-SM (PC/SM) ratio varies widely in membranes of the same cell from different species. The PC/SM balance plays a significant role in regulating the dynamic properties of biological membranes and, in particular, membrane fluidity [9]. Cholesterol is also a fundamental component of the erythrocyte cell membranes at approximately 26% of the total lipid content [8]. It has been reported that the differences in the amounts of phospholipids in the cell membranes play a

Abbreviations: PC, phosphatidylcholine; SM, sphingomyelin; SUV, small unilamellar vesicle; BA, benzylalcohol

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Table 1
Structural properties of antimicrobial peptides

Peptide	Peptide sequence[a]	Molecular mass (Da)	Length (aa)	Mean hydrophobicity[b]	Net charge[c]
IsCT1	ILGKIWEGIKSLF	1503.9	13	2.150	+1
IsCT2	IFGAIWNGIKSLF	1465.8	13	2.930	+1
Pin2	FWGALAKGALKLIPSLFSSFSKKD	2612.1	24	0.560	+3
Oxki2	GKFSGFAKILKSIAKFFKGVGKVRKQFKEASDLDKNQ	4146.8	37	−2.000	+7
Pin1	GKVWDWIKSAAKKIWSSEPVSQLKGQVLNAAKNYVAEKIGATPT	4799.6	44	−1.490	+4
Oxki1	FRGLAKLLKIGLKSFARVLKKVLPKAAKAGKALAKSMADENAIRQQNQ	5221.3	48	−1.580	+10

[a] IsCT1 and IsCT2 are from the ischnurid scorpion venom *Opisthacanthus madagascariensis* [37,38]. Pin1 and Pin are from the scorpion venom *Pandinus imperator* [13]. Oxki1 and Oxki2 are from the oxyopid spider venom *Oxyopes kitabensis* [39]. All six peptides markedly differ from each other in their amino acid sequences, molecular mass, net charge, and hydrophobicity. These peptides strongly inhibited the growth of both Gram-positive and Gram-negative bacteria, and they are lytic to erythrocytes membranes.

[b] Hydrophobicity was calculated using HydroMcalc by Sandri L. (http//www.bbcm.univ.trieste.it/~tossi/HydroCalc/HydroMCalc.html) on the basis of CCS, a corrected hydrophobicity scale derived from two consensus scales; the GCS scale, based on 160 scales found in the literature; and the XCS scale, based only on 33 strictly experimental hydrophobicity scales.

[c] The peptide net charge was calculated under the assumption that at physiological conditions Lys, Arg and the N-termini are positively charged, and Glu, Asp and C-terminals are negatively charged.

major role in the specificity of cationic peptides towards cell membranes [10]. The positive charge of the amphipathic α-helical peptides facilitates their hemolytic behavior towards cell membranes [11]. However, the mode of action of amphipathic cationic α-helical peptides is not fully understood, and the basis for their selectivity towards cell membranes is still a matter of some controversy [12]. We report in this work the hemolytic activities of six amphipathic and cationic α-helical peptides from arachnids (Table 1) towards mammalian erythrocytes and their membrane-disrupting activities towards artificial lipid membranes rich in PC, and we associated these results with direct effects on membrane fluidity.

2. Materials and methods

2.1. Lipids and peptides

Egg L-α-phosphatidylcholine, benzyl alcohol (BA) and calcein (fluorexone) were purchased from Nacalai Tesque (Kyoto, Japan). Egg SM was obtained from Sigma (St. Louis, MO). Sheep, pig and guinea pig erythrocytes were purchased from Nihon Seibutsu Zairyo Center (Osaka, Japan). Sheep and rat plasma was purchased from Nihon Biotest Research (Tokyo, Japan). Peptides were chemically synthesized by a solid phase method using the Fast-Fmoc methodology on an Applied Biosystems 433A peptide synthesizer according to previously published procedures [13].

2.2. Hemolytic assays

Hemolytic activity was determined by incubating a 10% (v/v) suspension of sheep, pig, or guinea pig red blood cells with serial dilutions of each selected peptides. Red blood cells (10% v/v) were rinsed several times in PBS by centrifugation for 3 min at $3000 \times g$ until the OD of the supernatant reached the OD of the control (PBS only). Red blood cells were counted by a hemacytometer and adjusted to approximately $7.7 \times 10^6 \pm 0.37 \times 10^6$ cell/ml. Erythrocytes were then incubated at room temperature for 1 h in 10% Triton X-100 (positive control), in PBS (blank), or with the appropriate concentrations of peptide (0–30 or 60 μM). The samples were then centrifuged at $10{,}000 \times g$ for 5 min; the supernatant was separated from the pellet and its absorbance measured at 570 nm. The blank supernatant absorbance was almost negligible, indicating the lack of spontaneous hemolysis during centrifugation. The relative OD compared to that of the suspension treated with 10% Triton X-100 defined the percentage of hemolysis.

2.3. Incubation of sheep erythrocytes with plasma

Sheep erythrocytes were incubated with plasma by the method of Borochov [9]. Briefly, cell suspensions (approximately 10^9 cell/ml) were mixed with equal volumes of rat or sheep plasma (as control) previously inactivated by incubation at 56 °C for 30 min in the presence of penicillin (500 μg/ml) and EGTA (2 mM) to avoid microbial contamination and lecithinase activity, respectively. Erythrocytes suspended in rat or sheep plasma were further incubated at 37 °C with constant shaking for 42 h. The modified erythrocytes were then collected and the hemolytic assays were performed as mentioned; the modified erythrocyte membranes were isolated for lipid analysis.

2.4. Benzyl alcohol and temperature experiments

For the benzyl alcohol experiments, sheep red blood cells were incubated in the presence of different concentrations (10, 20, and 50 μM) of benzyl alcohol (BA), and the appropriate concentrations of each of the six arachnid peptides. For the temperature experiments, pig red blood

cells were incubated at 5, 12, 18, and 25 °C, in the presence of each of the six arachnid peptides, and the hemolytic activity was measured as previously indicated.

2.5. Artificial vesicles

Small unilamellar vesicles (SUV) containing calcein were prepared according to Wieprecht et al. [14]. Calcein leakage from vesicles was fluorometrically monitored using a Hitachi Fluorescence Spectrophotometer F-4500 (Tokyo, Japan) by measuring the time-dependent increase in the fluorescence of calcein release. As calcein leaks from the vesicles due to the disrupting activity of the cationic peptides, it becomes diluted and therefore dequenched; the increase in fluorescence is recorded (excitation = 490 nm; emission = 520 nm). A final volume of 0.95 ml of the SUVs was placed in a stirred cuvette at room temperature. An aliquot of peptide solution (50 μl) was added to the cuvette. The percentage of calcein released by the addition of each peptide was evaluated by the equation: $100(F-F_o)/(F_t-F_o)$, where F is the fluorescence intensity achieved by the peptides, F_o is the fluorescence intensity observed without the peptides, and F_t is the fluorescence intensity corresponding to 100% calcein release determined by the addition of 50 μl of 10% Triton X-100. The phosphorus content in the phospholipid vesicles was estimated by spectrophotometric analysis [15].

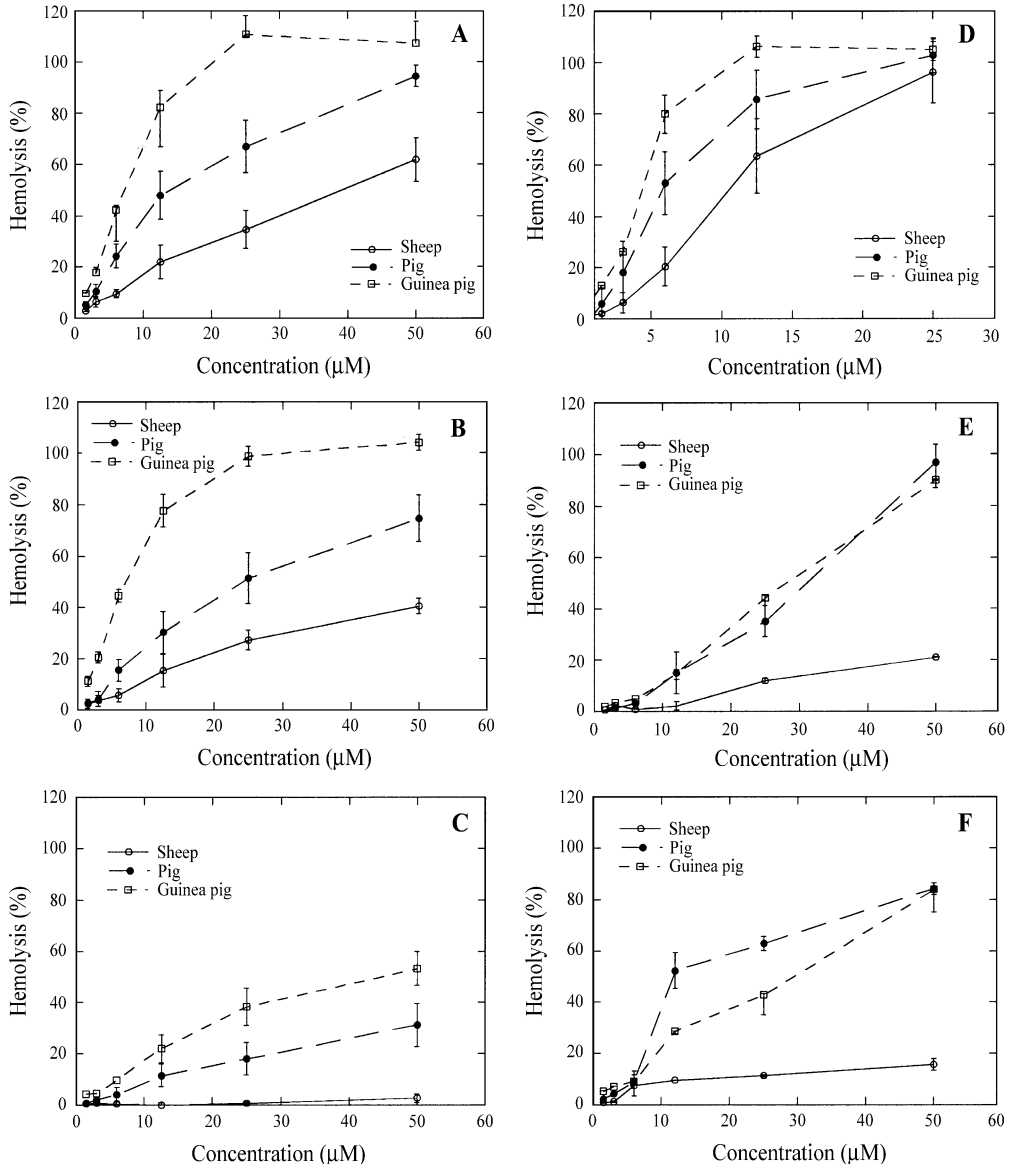

Fig. 1. Hemolytic activity of arachnid peptides towards different mammalian red blood cells. (A) Oxki1, (B) Oxki2, (C) Pin1, (D) Pin2, (E) IsCT1 and (F) IsCT2. The values represent the average mean ± S.D. ($n = 2$). A positive control was determined using a 10% solution of Triton X-100.

2.6. Lipid analysis

Lipids were extracted with chloroform and methanol as described by Bligh and Dyer [16] and separated by two-dimensional thin-layer chromatography, following the technique of Broekhuyse [17]. The molar content of phospholipids in big spots was determined by phosphorus analysis using the Fiske–Subba Row procedure [15].

2.7. Statistical analysis

The last significant difference method was used to determine whether statistically significant differences occurred among the mean values obtained.

3. Results

3.1. Hemolytic activities towards different mammalian erythrocytes

The hemolytic effects of the six different cationic peptides towards three different mammalian erythrocytes species was observed (Fig. 1). All six peptides induced hemolysis of the three types of erythrocyte membranes. Overall, the peptides induced the hemolysis of the erythrocyte membranes in the following order; guinea pig> pig>sheep. Sheep erythrocyte membranes were more resistant to the hemolytic activity of all six peptides than guinea pig erythrocyte membranes. However, the hemolysis caused by IsCT1 against pig and guinea pig erythrocytes did not differ significantly ($P>0.05$). Moreover, the hemolytic activity of IsCT2 on guinea pig erythrocytes was significantly lower ($P<0.05$) than that of pig erythrocytes (Fig. 1F). Pin1 exhibited the lowest hemolytic activity of all peptides, whereas Pin2 was found to be the most potent hemolytic peptide (Fig. 1C,D). The order of peptide potency towards the erythrocyte membranes was Pin2>Oxki1>Oxk2>IsCT2>IsCT1>Pin1. The hemolytic activities of the arachnid peptide results were not correlated to their chemical characteristics such as amino acid residue length, mean hydrophobicity and net charge of the peptides (Table 1). However, they correlated well to the PC/SM ratio found in the three different erythrocyte membranes where the PC/SM ratio in the cell membranes of sheep, pig and guinea pig erythrocytes is 0.020, 0.88, and 3.72, respectively [8].

3.2. Incorporation of PC into the membrane of sheep erythrocytes

To observe the effect of the PC/SM ratio in erythrocyte membranes, the hemolytic activity of all six cationic peptides was examined in PC-enriched sheep erythrocytes. Rat serum, which contains an appreciable amount of phospholipids [18], was used as the source for incorporating PC into sheep erythrocyte membranes. The treatment of sheep red blood cells with lecithinase-inactivated rat plasma resulted in up to a 0.18 increase in the PC/SM ratio, which is threefold higher than the control (Fig. 2). On the other hand, incubation of sheep erythrocytes with lecithinase-inactivated sheep plasma did not significantly increase ($P>0.05$) the PC/SM ratio (Fig. 2). During the incubation of sheep red blood cells with sheep and rat plasma, the SM content did not change significantly (data not shown), suggesting that SM degradation during incubation was negligible. Therefore, the observed increase in PC/SM ratio was caused by the enrichment of sheep red blood cell membranes with PC. The hemolytic effects of the six different cationic peptides on the PC-enriched and on the unmodified sheep erythrocytes were observed (Fig. 3). Overall, all cationic peptides were more hemolytic toward the PC-enriched sheep erythrocytes than toward the unmodified sheep erythrocytes. These results indicate a hemolytic preference for cellular membranes with a rich content of PC.

3.3. Leakage from PC/SM vesicles

SUVs composed of PC and SM were used to observe the ‘‘pore-forming’’ or disrupting activities of all six amphipathic peptides. The extent of calcein leakage induced by different amounts of cationic peptides was observed at a constant phospholipid concentration (84 μM). The arachnid peptides strongly permeate SUVs containing different PC/SM ratios in the order Pin2> Oxki1>Oxki2>Pin1>IsCT1>IsCT2 (Fig. 4). For all peptides, calcein leakage from the SUVs was dependent on the peptide concentration. In vesicles composed of only PC, 50% calcein leakage for Oxki1 and Pin2 was observed at a peptide-to-lipid (P/L) ratio of 0.0004, whereas the 50% calcein leakage for IsCT1 and IsCT2 was 0.04 (100-fold) (Fig. 4A). SUVs composed only of SM (84 μM) did not

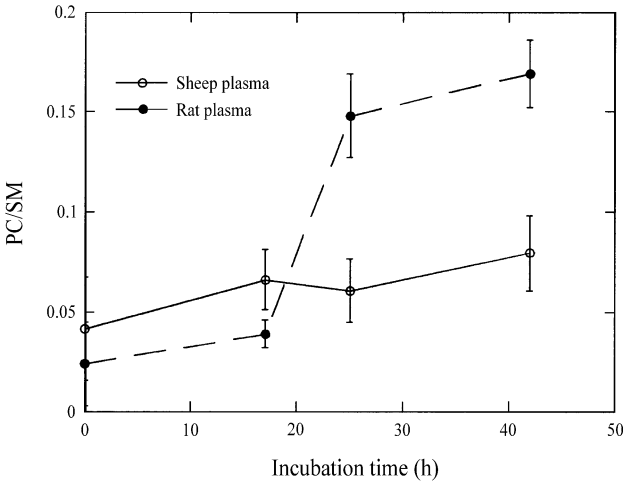

Fig. 2. Increase of the PC/SM mol ratio of intact sheep erythrocytes. Cells were incubated at 37 °C in inactivated sheep or rat serum plasma.

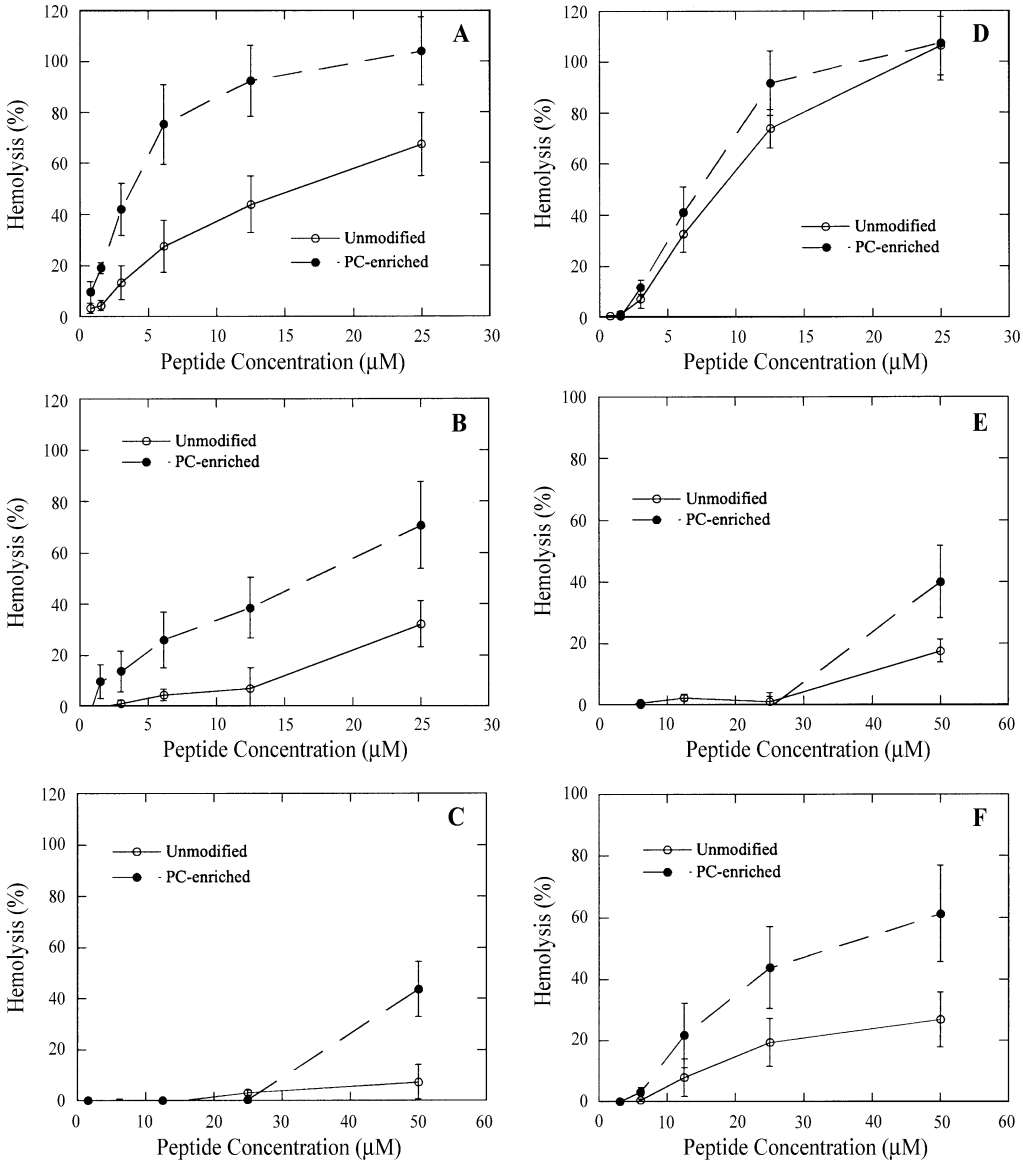

Fig. 3. Hemolytic activity of arachnid peptides towards PC-enriched and unmodified sheep erythrocytes. (A) Oxki1, (B) Oxki2, (C) Pin1, (D) Pin2, (E) IsCT1, and (F) IsCT2. The values represent the average mean ± S.D. ($n=2$); a positive control was determined using a 10% solution of Triton X-100.

leak calcein on the addition of Oxki1, Oxki2, Pin1, IsCT1 and IsCT2 even at a high P/L ratio (P/L = 0.05); moreover, only the addition of Pin2 at a P/L of 0.08 led to the 50% leakage of calcein from SM vesicles (data not shown). The decrease in PC up to 50% did not significantly affect ($P>0.05$) the lytic activities of Oxki1, Oxki2, Pin1 and Pin2 (Fig. 4B). For IsCT1, an increase of 10% in calcein release was registered, but this was not observed for IsCT2 (Fig 4B). Furthermore, a decrease in the PC composition of SUVs up to 20% did not significantly influence the shape and parameters of the calcein leakage curves for Oxki1, Pin2 and Oxki2 (Fig. 4C). A drastic fall in the lytic activity of Pin1 attributed to the lower concentration of PC in the vesicles was observed. Surprisingly, IsCT1 and IsCT2 showed considerably enhanced calcein leakage in the SUVs at a PC/SM of 0.25. Nevertheless, the disrupting activities of IsCT1 and IsCT2 were much lower than those of the other four peptides (Fig. 4C).

3.4. Membrane fluidity experiments using mammalian erythrocytes

To determine the role of membrane fluidity on the hemolytic susceptibility of red blood cells, sheep erythrocytes were incubated in the presence of both benzyl alcohol (BA) and each hemolytic peptide (Fig. 5A). BA, a fluidizing agent, increases membrane fluidity due to a general increase in the freedom of lipid movement caused by the partitioning of BA into the cell membranes [19]. The hemolytic activity of all six arachnid peptides increases in the presence of BA. Hemolysis of sheep erythrocytes in the presence of Oxki1, Oxki2, Pin1 and

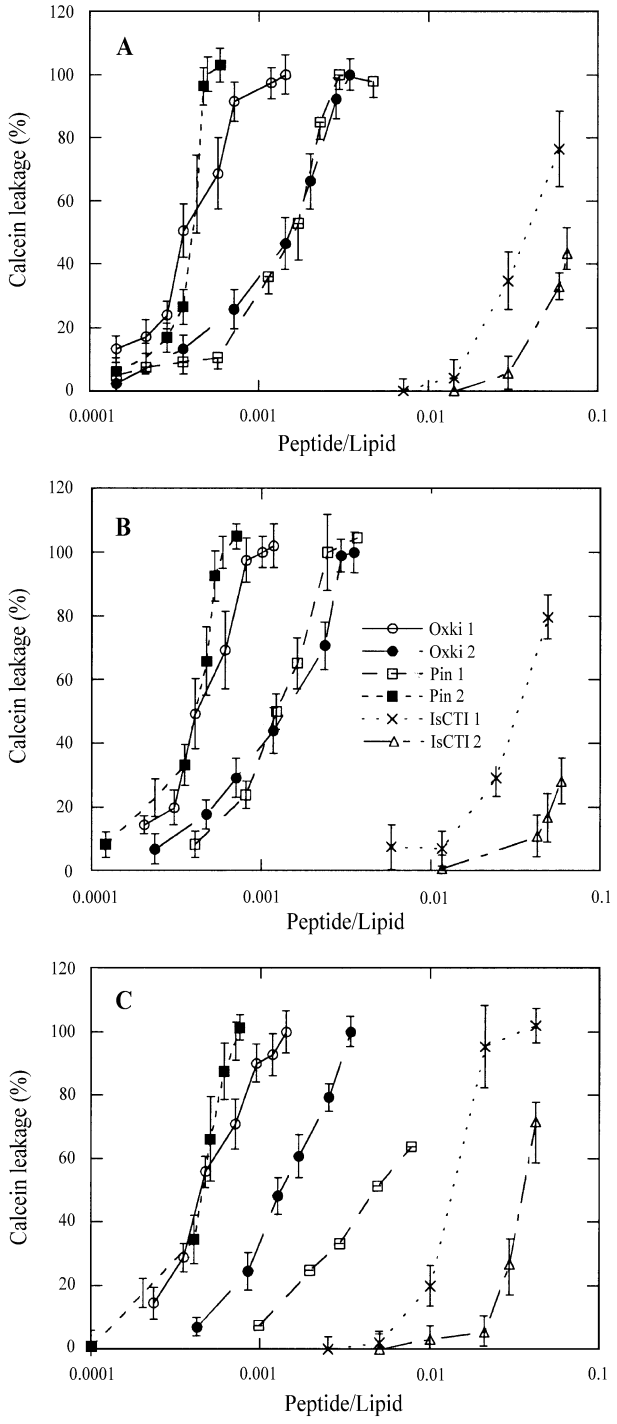

Fig. 4. Calcein leakage caused by cationic peptides towards artificial PC/SM vesicles. PC content; (A) 100%, (B) 50%, and (C) 20%. The calcein leakage was plotted as a function of the peptide to lipid (P/L) ratio. The lipid concentration employed was 84 μM. The values represent the average mean ± S.D. ($n = 2$).

Pin2 gradually increased with the increase in BA concentration. However, the hemolytic activity of IsCT1 and IsCT2 resulted in a slight decrease in the presence of 10 mM BA. Only at the highest concentration of BA (50 mM), IsCT1 and IsCT2 induced a pronounced increase in sheep erythrocyte hemolysis. These results demonstrate that the presence of BA in sheep erythrocytes induced an increase in membrane susceptibility to the hemolytic peptides up to 40% for Oxik1, Oxik2, Pin2 and Pin1, 30% for IsCT1, and 3.5% for IsCT2. The three different concentrations of BA in the absence of hemolytic peptides (BA positive control) did not cause hemolysis of the sheep erythrocyte membranes (data not shown).

The effect of membrane fluidity was also observed on incubating pig erythrocytes membranes at different temperatures in the presence of the hemolytic peptides (Fig. 5B). Hemolysis of pig erythrocytes gradually decreased with the decrease in temperature in the presence of each arachnid peptide. Under the assumption that the rate of peptide hemolysis was limited only by the rate of mem-

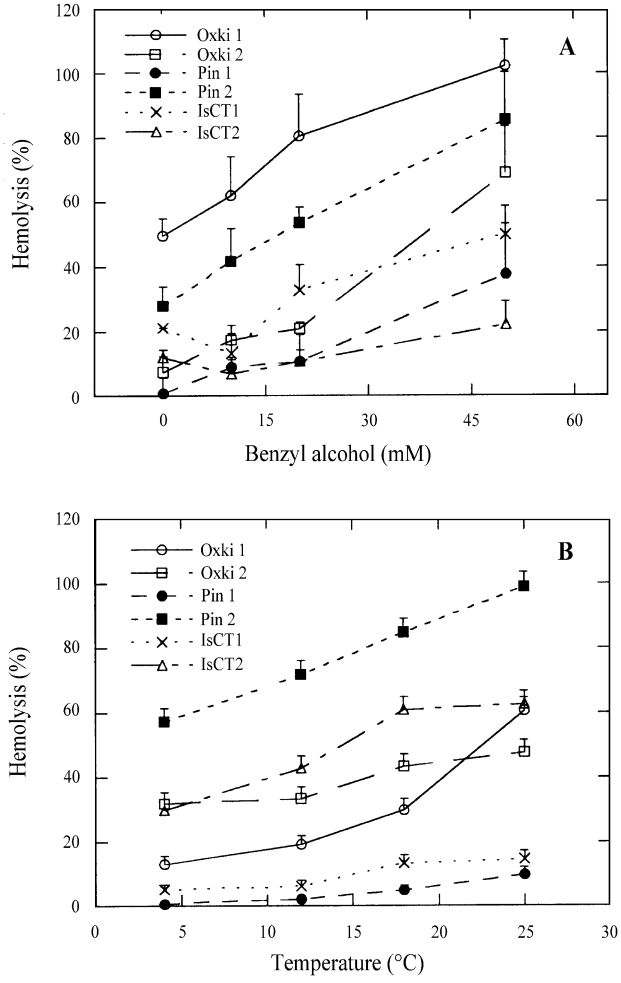

Fig. 5. Effects of membrane fluidity in the hemolysis of erythrocytes. (A) Hemolysis of sheep erythrocytes in the presence of benzyl alcohol. The peptide concentration for Oxki1, Oxki2, and Pin2 was 12.5 μM. The peptide concentration for Pin1, IsCT1, and IsCT2 was 50 μM. (B) Hemolysis of pig erythrocytes at 5, 12, 18, and 25 °C. The peptide concentration for all six peptides was 12.5 μM. The values represent the average mean ± S.D. ($n = 2$), and a positive control was determined using a 10% solution of Triton X-100.

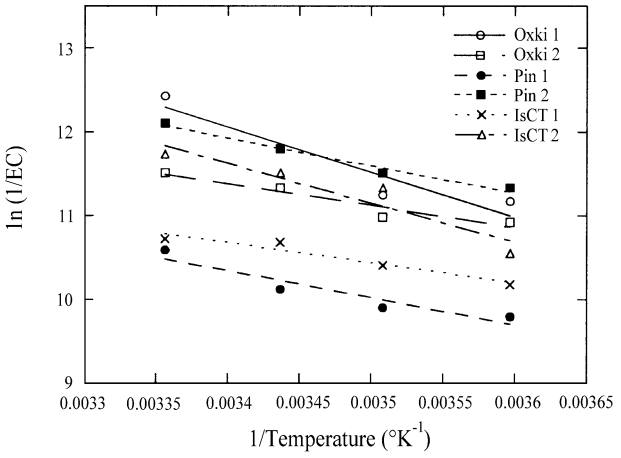

Fig. 6. Arrhenius plot of the hemolysis of pig erythrocytes induced by the six cationic peptides. The reciprocal of $EC_{10\%}$ (Oxki1, Pin1, IsCT2) and $EC_{20\%}$ (Oxki2, Pin2, IsCT1) values of each peptide at different temperatures were plotted as $\ln(1/EC_{10/20})$ versus $1/T$. The values represent the average mean ± S.D. ($n=2$).

brane disruption, the peptide concentrations causing 10% and 20% hemolysis ($EC_{10/20}$) were estimated from dose–response curves at different temperatures (5, 12, 18, and 25 °C). Considering that the peptide concentration causing hemolysis (an irreversible process) was proportional to the equilibrium constant of hemolysis, the activation energy of the hemolysis was estimated according to $k=Ae^{-\Delta E/RT}$, where k is the hemolytic rate proportional to 1/EC at temperature T (the greater the hemolytic rate the lower is the concentration required to achieve the same hemolysis), A is a preexponential factor, ΔE is the activation energy, and R is the gas constant (1.987 cal/mol). The reciprocal of $EC_{10\%}$ (Oxki1, Pin1, IsCT2) and $EC_{20\%}$ (Oxki2, Pin2, IsCT1) values of each peptide at different temperatures were plotted as $\ln(1/EC_{10/20})$ versus $1/T$ (Fig. 6). The activation energies (ΔE) of the hemolysis, as an irreversible process, were determined as the slopes of the linear fits. The calculated activation energies (ΔE), which are independent of the concentration of the cationic peptides were 10.6, 5.2, 6.4, 6.5, 4.7 and 9.4 kcal/mol for Oxki1, Oxki2, Pin1, Pin2, IsCT1 and IsCT2, respectively. The mean of ΔE for all six peptides was found to be 7.2 ± 2.3 kcal/mol. This result could be considered to be the reaction energy of pore formation on pig erythrocytes.

4. Discussion

The potency of the hemolytic activity of all six amphipathic peptides towards red blood cells was in the following order; guinea pig>pig>sheep erythrocytes. The PC/SM ratio of these red blood cells varies significantly; however, cholesterol concentration shows little species variation, the average content of which is 26% [8]. Other phospholipids such as phosphatidylethanolamine (24.6–29.7%), phosphatidylserine (14.1–17.8%) and phosphatidylinositol (1.8–2.9%) are present in a small range variation of the total phospholipid composition in those three red blood cells species [8]. Phosphatidic acid in these mammalian species is less than 0.3% except for guinea pig erythrocytes, which is 4.2% [8]. However, a comparison between the hemolytic effect of the six cationic peptides and the PC/SM ratio of the three types of mammalian erythrocytes indicated that the PC/SM ratio was associated with a flattening of their hemolytic activity. Similarly, melittin, an amphipathic peptide from honeybee venom, has strong hemolytic activity towards human blood cells (rich in PC), but lower hemolytic activity towards sheep blood cells (rich in SM) [10]. Given that cholesterol and other phospholipids in the outer leaflet erythrocyte membranes had low variation in their content, the PC/SM ratio was linked to its susceptibility to peptide hemolysis. According to Lowe and Coleman [20], the membranes of erythrocytes with a high mole fraction of SM showed an increase in the degree of fluorescent polarization (i.e., less fluid) compared to those erythrocyte membranes with lower relative concentrations of SM. Therefore, the different hemolytic susceptibility of red blood cells from various mammalian species to amphipathic peptides might be directly related to the differences in the PC and SM content and then to the membrane fluidity. To prove this hypothesis, the hemolytic susceptibility of erythrocytes was investigated by modifying their PC/SM ratio. The hemolysis of sheep erythrocyte membranes by the cationic peptides was considerably enhanced after incubation with inactivated rat plasma. It has been shown that an increase in the PC/SM molar ratio has been associated with a reduction in membrane microviscosity after the fluorescence polarization experiments [21]. Therefore, it was reasonable to attribute the observed increase in hemolysis to the enrichment of PC in sheep red blood cells and to the binding ability of these peptides towards PC. However, among all six peptides, Pin1 and IsCT1 required higher concentrations to induce hemolysis (Fig. 3C,E). Pin1 and IsCT1 may have a low preference for PC containing liposomes as seen in other amphipathic peptides. For example, Magainin, a cationic peptide from the skin of *Xenopus laevis*, has weak leaking activity towards PC vesicles [10]. Cecropins and dermaseptins do not interact with zwitterionic phospholipids, and they do not disrupt them [22,23].

The "pore-forming" effect of all the six peptides using different PC/SM ratios in the SUVs was observed. For Oxki1, Oxki2, Pin2 and Pin1, the lytic activity was higher on SUVs containing only PC (Fig. 4A). The decrease in PC content to a PC/SM ratio of 1 did not significantly affect ($P>0.05$) the calcein leakage from artificial vesicles for Oxki1, Oxki2, Pin1 and Pin2 (Fig. 4B). However, the increase in the SM content to a PC/SM ratio of 0.25

decreased the slopes of the leakage curves significantly ($P < 0.05$) for Pin1 (Fig. 4C). Because increases in SM preferentially strengthen the phospholipid membrane rigidity, it might restrict the insertion of Pin1 into the lipid core, decreasing the "pore-formation" or calcein leakage. Pin1 leakage activity was higher in SUVs composed of only PC and SUVs with a PC/SM ratio of 1; therefore, it might be suggested that PC/SM ratios higher than 1 resulted optimal, but not PC/SM ratios lower than 1. This result agrees with the hemolytic activity of Pin1, which was higher towards guinea pig cells, the PC/SM ratio of which is 3.8. It has been reported that, for Sticholysin I, a potent cytolysin from sea anemone, an increase in SM in the PC/SM ratio favored membrane leakage, and this effect was optimal at equimolar concentrations, whereas the activity with pure SM or PC was extremely low [24]. Melittin also permeates vesicles at a PC/SM ratio of 1 better than pure PC or SM vesicles [25]. SUVs with a PC/SM ratio of 0.25 were better disrupted in the presence of IsCT1 and IsCT2 (Fig. 4B). Differences in the calcein leaking activity in SUVs might be related to structural characteristics of the amphipathic peptides. For example, IsCT1 and IsCT2 induce leakage in SUVs better at a PC/SM ratio of 0.25 than that of 1. IsCT1 and IsCT2 are shorter peptides (13 amino acid residues) than Oxki1, Oxki2, Pin1 and Pin2 (>20 amino acid residues). Amphipathic linear peptides larger than 20 amino acid residues can span the lipid bilayer [26,27]. Therefore, short hydrophobic peptides may differently act against SM-rich lipid bilayers. Oxki1, Oxki2, Pin1 and Pin2 might be pore-forming peptides whereas IsCT1 and IsCT2 might be detergent-like peptides, depending on the PC/SM membrane composition.

The incubation of sheep erythrocytes in the presence of BA increased the hemolytic susceptibility of cells to all cationic peptides (Fig. 5A). The presence of BA in sheep erythrocyte membranes resulted in a reduction in membrane rigidity [20]. Therefore, the observed increase in membrane hemolysis correlates with the increase in membrane fluidity. The calculated activation energy for all hemolytic peptides (7.2 ± 2.3 kcal/mol) is consistent with the reaction energy of several pore-forming peptides towards PC-enriched liposomes [28–30] and PC liposomes that preserve the liquid crystalline phase in the range of 0–50 °C with a ΔE value around 8 kcal/mol [31]. A reduction in the fluidity of erythrocyte membrane lipids due to a decrease in temperature had also been demonstrated [20]. Therefore, these data strongly suggest that the observed decrease in hemolytic activity with decreasing temperature is attributed to the reduction in the fluidity of erythrocyte membranes caused by the gel crystalline phase of PC [32]. Moreover, essentially parallel effects on the extent of hemolysis have been seen with the alterations in membrane fluidity resulting from the changes in membrane lipid composition, from the effects of temperature and from the presence of BA [9,19]. Therefore, the decrease in hemolytic activity of erythrocytes with an increase in membrane rigidity indicates that for all peptides studied, the effect on the restriction of peptide penetration into the lipid core generally would dominate in natural mammalian cell membranes rather than in artificial membranes. This could be attributed to other factors such as the content of cholesterol and other phospholipids in mammalian cell membranes. For example, the level of cholesterol is an important regulator of membrane fluidity and, therefore, the activity of various membrane peptides [33,34]. It has been shown that many eukaryotic membrane cells contain cholesterol-sphingolipid-rich microdomains, which act as concentration devices for some membrane-active peptides [35]. However, the cholesterol content could not be related to the differences in susceptibility of erythrocyte membranes because it shows little species variation, and it averages about 26% of the total lipids for several mammalian erythrocytes including the ones studied in this work [8]. Therefore, it seems that the variation in the PC/SM ratio is the most probable factor that causes the differences in susceptibility of erythrocytes to the amphipathic cationic peptides. The ratio of PC to SM may control the extent of gel phase lipid domain formation or the degree of acyl chain order as indicated by Lentz et al. [36]. They concluded that the SM and PC species with similar acyl chains show no analogous mixing behavior in either highly curved or flat bilayers except in the gel phase. Moreover, phase diagrams for analogous mixtures of pure phosphocholine species may substitute for each other in supporting the lamellar phase necessary to cell membrane structure. Finally, the ordering effect of SM in mammalian membranes is likely to be due to the saturated acyl chain composition of naturally occurring species rather than the SM backbone.

All peptides strongly permeated the zwitterionic PC SUVs. The shape of calcein leakage curves mimics well the shape of the corresponding curves of hemolysis. Thus the interaction of amphipathic peptides with PC is an important factor in membrane disruption. All six peptides permeate model membranes composed of PC in the rank order Pin2>Oxki1>Oxki2>Pin1>IsCT1>IsCT2, whereas they permeate erythrocyte membranes in the same order, but this slightly varies in pig red blood cells where IsCT1 was more active than IsCT2. The differences in the disruption of natural and artificial phospholipid membranes could be attributed to the physicochemical characteristics of each peptide, which also play an important role in the hemolysis of erythrocytes. Overall, it was demonstrated that the hemolytic activity of the six arachnid peptides strongly depends on the source of the erythrocytes. The perturbing activity of all six arachnid peptides increased with an increase in the PC/SM ratio, and this is directly linked to membrane fluidity. The low hemolytic activity of amphipathic peptides in SM-rich mammalian erythrocytes and in low-temperature erythrocytes could be attributed to the restriction of penetration of such peptides into the cell membranes.

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