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Channel-Forming Activity of Cecropins in Lipid Bilayers: Effect of Agents Modifying the Membrane Dipole Potential

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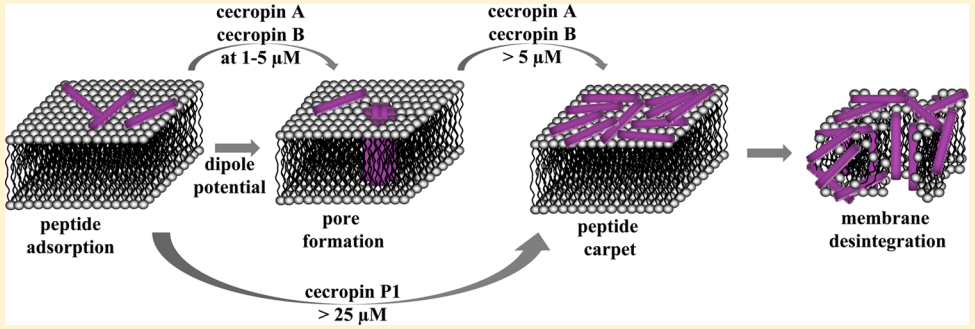
Supporting Information



ABSTRACT: Cecropin A (CecA) and cecropin B (CecB) added to one side of a bilayer formed from equimolar mixtures of DOPS and DOPE, DPhPS and DPhPE, or DOPS, DOPE, and Chol leads to the formation of well-defined and well-reproducible ion channels of different conductance levels while cecropin P1 (CecP1) does not induce pore formation at micromolar concentrations. We found three populations of CecA channels: pores with weak cationic selectivity, pores with weak anionic selectivity, and pores that were nonselective. The dipole modifiers, flavonoids and styryl dyes, were used to modulate the channel-forming activity of CecA and CecB. The mean conductance of single CecA channels is affected by the influence of dipole modifiers on the lipid packing in the membrane. A decrease in the membrane dipole potential is accompanied by a decrease in the steady-state transmembrane current induced by CecA and CecB in cholesterol-free and cholesterol-containing bilayers. The observed changes in the channel-forming activity might be caused by an increase in the energy barrier for the interfacial accumulation of cecropin monomers. This finding indicates that the negative pole of the cecropin dipole is inserted into the membrane.

INTRODUCTION

Cecropins are antibacterial peptides containing 31−39 L-amino acid residues isolated from silkworm species (*Hyalophora cecropia*, *Antheraea pernyi*, and *Bombyx mori*).[1,2] They consist of an amphipathic N-terminal domain connected to a hydrophobic C-terminal domain by a flexible hinge region formed by residues Gly 23 and Pro 24, which are conserved in all cecropin sequences. The charges of N-terminal and C-terminal domains in cecropin A (CecA) are equal to +6e and +1e, respectively. In cecropin B (CecB), the C-terminal domain is neutral.[3−5] Cecropins are unstructured in aqueous solutions, but they are α-helical in lipid environments. The helix−bend−helix structure is strongly required for antimicrobial activity.[5]

Although the secondary structure and the functional properties of cecropins have been well characterized, the detailed mechanism of antibiotic action is still a subject of intensive research because of several conflicting models. Studies with all-D-, retro-, and retroenantio-cecropins provided evidence against antibiotic binding to a specific receptors.[6] Two general models have been proposed to explain the antimicrobial properties of cecropins: the formation of transmembrane pores and the so-called carpet model.

Pore formation has been proposed to proceed via two distinct mechanisms: a barrel-stave structure formed by cecropin oligomers and a toroidal pore composed of both cecropin and lipid molecules.[7] Christensen et al. demonstrated that in planar lipid membranes cecropins predominantly form anion-selective channels.[8] The observed voltage dependence of the membrane conductance forced the authors to conclude that the process of pore formation by cecropins involve the association of N-terminal helices. Silvestro et al. found the concentration dependence of CecA action on liposomes. They suggested that a peptide/lipid ratio determined whether the ion channels or large pores (that were able to pass probe molecules) were formed.[9,10] Durell et al. developed computer models of cecropin channels.[11] A cecropin-melittin hybrid peptide was found to form pores via a toroidal mechanism.[12] It is believed that cationic cecropin analogs are also able to form pores while hydrophobic ones act via a carpetlike mechanism.[13−15]

The carpet model postulates the contact of litic peptides with only the phospholipid headgroups.[16] A membrane breaks when the peptide molecules occupy the entire surface. The measured inhibitory concentration of cecropin does not contradict the carpetlike model.[17] The data of different spectroscopic studies

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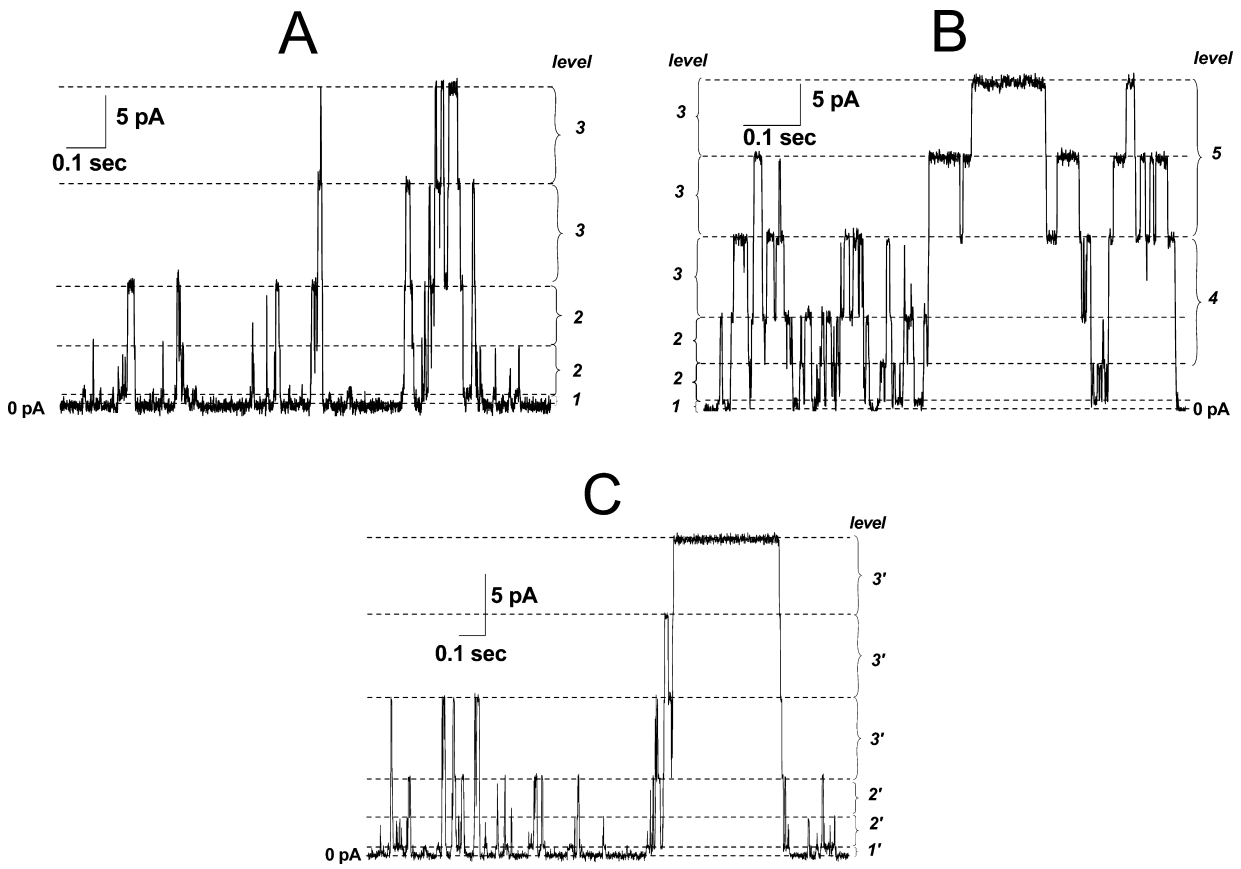

Figure 1. Current fluctuations of the CecA channels of different conductance levels (A and B). The membranes were made from DOPS/DOPE (1:1) (A, B) and DPhPS/DPhPE (1:1) (C) and bathed in 0.1 M KCl, 5 mM HEPES, pH 7.4. The transmembrane voltage was 25 mV.

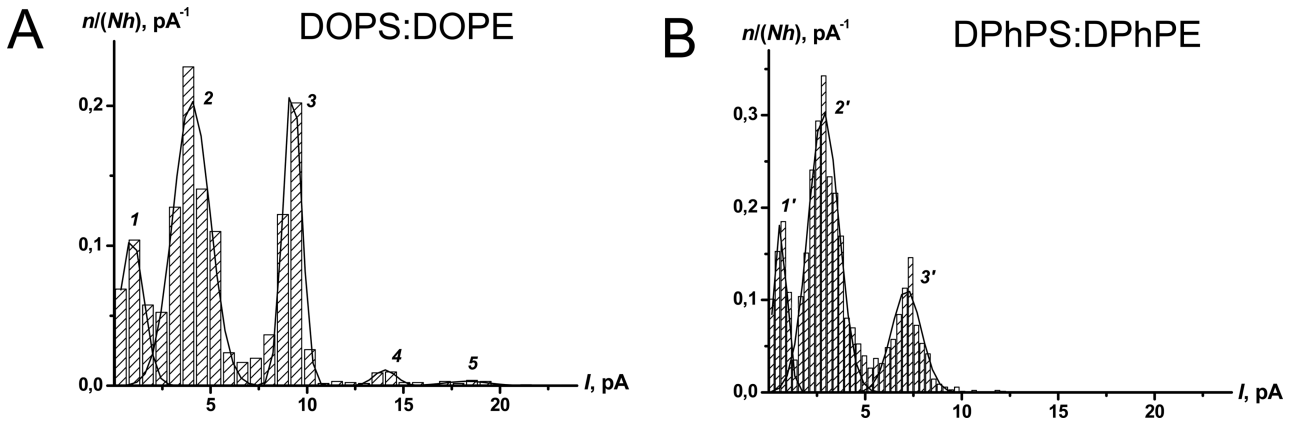

Figure 2. Current-transition histograms of CecA channels. The membranes were made from DOPS/DOPE (1:1) (A) and DPhPS/DPhPE (1:1) (B) and bathed in 0.1 M KCl, 5 mM HEPES, pH 7.4. The transmembrane voltage was 25 mV.

clearly indicate that cecropins are not embedded into the hydrophobic core of the membrane.[18−20] Using a spectroscopic technique and molecular dynamic simulation, it was also shown that a cecropin-like 31 residue peptide isolated from the small intestine of a pig, cecropin P1 (CecP1), does not penetrate the hydrocarbon core of the membrane.[21]

To make the choice between these two conflicting models, one should take into account that the membrane disintegration caused by peptides that initially interact with membranes in a carpetlike manner may involve the formation of toroidal pores.[7,22] The findings by Marassi et al. are in agreement with such an assumption.[23]

Alternative therapeutic strategies may include studies on membrane active agents that would aim to enhance the activity of cecropins. The present study is an attempt to make the first steps in this direction. For these purposes, dipole-modifying agents, which are able to change the potential drop at the membrane−solution interface,[24] were used to modulate the membrane activity of cecropins. A similar approach has been successfully applied to the studies of antimicrobial lipopeptides

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and toxins and has provided substantial information on the mechanisms of their membrane activity.[25−36]

MATERIALS AND METHODS

All chemicals were of reagent grade. Synthetic 1,2-dioleyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleyl-*sn*-glycero-3-phosphoserine (DOPS), 1,2-diphytanoyl-*sn*-glycero-3-phosphoethanolamine (DPhPE), 1,2-diphytanoyl-*sn*-glycero-3-phosphoeserine (DPhPE), and cholesterol (Chol) were obtained from Avanti Polar Lipids, Inc. (Pelham, AL). Cecropin A (CecA), cecropin B (CecB), cecropin P1 (CecP1), phloretin (3-(4-hydroxyphenyl)-1-(2,4,6-trihydroxyphenyl)-1-propanone), myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone), ionophores valinomycin (Val), and nonactin (NonA) were purchased from Sigma Chemicals (St. Louis, MO). RH 421 (*N*-(4-sulfobutyl)-4-(4-(4-(dipentylamino)phenyl)butadienyl) pyridinium, inner salt) and RH 237 (*N*-(4-sulfobutyl)-4-(6-(4-(dibutylamino)phenyl)-hexatrienyl)pyridinium, inner salt) were obtained from Molecular Probes (Eugene, OR). Water was distilled twice and deionized. A solution of 0.1 M KCl was buffered with 5 mM Hepes, pH 7.4.

Planar Lipid Bilayers. Virtually solvent-free planar lipid bilayers were prepared according to a monolayer-opposition technique[37] on a 50-μm-diameter aperture in a 10-μm-thick Teflon film separating two (cis and trans) compartments of a Teflon chamber. The aperture was pretreated with pure hexadecane. Lipid bilayers were made from equimolar mixtures of DOPS and DOPE; DPhPS and DPhPE; or DOPS, DOPE, and Chol. After the membrane was completely formed and stabilized, CecA, CecB, or CecP1 from stock solutions (1 mg/mL in water) was added to the aqueous phase on the cis side of the bilayer to obtain a final concentration ranging from 2 to 50 μM. Ag/AgCl electrodes with 1.5% agarose/2 M KCl bridges were used to apply the transmembrane voltage (V) and measure the transmembrane current (I). Positive voltage refers to the case in which the cis-side compartment is positive with respect to the trans-side compartment. All experiments were performed at room temperature.

Current measurements were carried out using an Axopatch 200B amplifier (Axon Instruments) in the voltage clamp mode. Data were digitized by Digidata 1440A and analyzed using pClamp 10 (Axon Instruments) and Origin 7.0 (Origin Lab). Data acquisition was performed with a 5 kHz sampling frequency and low-pass filtering at 200 Hz. The current tracks were processed through an eight-pole Bessel 100 kHz filter. The filtered tracks were reduced by a factor of 100 for figure drawing.

The dipole potential of the membrane was reduced by the two-side addition of 20 μM flavonoids (phloretin or myricetin) to the bilayer bathing solution. The two-side addition of 5 μM styryl dyes (RH 421 or RH 237) to the membrane bathing solution was used to increase the dipole membrane potential of the bilayers. The final concentration of ethanol in the chamber did not exceed 0.2%. An ethanol concentration below 1% does not affect the stability and conductance of the membranes.

Current transition histograms were constructed for tested voltages. Routinely, the amplitude of the current transition was calculated as the difference between average current values of 2 ms records collected just before and just after the current transition. A ratio $n/(Nh)$ was set as the histogram ordinate, where n is the number of current fluctuations corresponding to a particular current level, N is the total number of fluctuations, and h is the bin size. The total number of events used for the analysis was in the range of 10 000−90 000. Histogram peaks were fitted with the normal density function. The distribution hypothesis was verified by χ^2 minimization ($P < 0.05$). A mean normalized current, I_{SC}, defined as follows,[32]

$$I_{SC} = \sum_k I_k P_k \tag{1}$$

where k is the peak number and P_k is the area under the kth peak or, in other words, the probability of finding the kth channel state with a mean current amplitude equal to I_k. The corresponding mean normalized conductance was estimated as $G_{SC} = I_{SC}/V$.

Transfer numbers of K^+ (t^+) and Cl^- ($t^- = 1 - t^+$) were estimated using the general expression[38] $V^{rev} = (RT/F)(2t^+ - 1)\ \ln(\gamma_1 C_1/\gamma_2 C_2)$

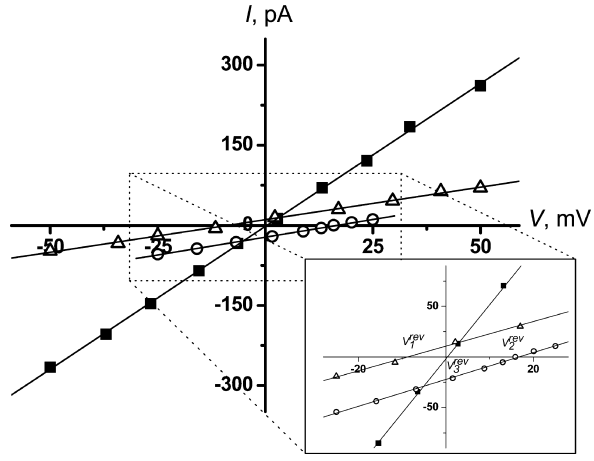

Figure 3. Anion/cation selectivity of the CecA channels. $I(V)$ curves of anion-selective (Δ), nonselective (■), and cation-selective (○) CecA channels are presented. The intercepts on the V axis (V_1^{rev}, V_2^{rev}, and V_3^{rev}) are at −11, 0, and 18 mV, respectively. The membranes were made from DOPS/DOPE (1:1). Bilayer bathing solutions in the cis and trans compartments contain 0.1 and 1.0 M KCl (5 mM HEPES, pH 7.4), respectively.

Table 1. Changes in the Membrane Dipole Potential after the Addition of Various Modifiers

		$\Delta\varphi_d$, mV	
concentration	modifiers	DOPS/DOPE (1:1)	DOPS/DOPE/Chol (1:1:1)
20 μM	phloretin	−90 ± 10	−75 ± 10
	myricetin	−20 ± 5	
5 μM	RH 237	100 ± 5	
	RH 421	60 ± 10	45 ± 5

where V^{rev} is the reversal potential; R, T, and F have their usual meanings; and γ_1 and γ_2, C_1 and C_2 indicate activity coefficients and KCl concentrations in the cis and trans compartments (C_1 = 0.1 M KCl, C_2 = 1 M KCl), respectively.

The channel-forming activity of CecA or CecB in the absence and after the introduction of the modifier (flavonoids or styryl dyes) was characterized by a steady-state transmembrane current (I_∞) under the given experimental conditions (V = 50 mV). Mean ratios (I_∞/I_∞^0) of steady-state transmembrane currents induced by cecropins in the presence (I_∞) and in the absence of modifiers (I_∞^0) were averaged for three to nine bilayers (mean ± se).

Measurement of the Membrane Dipole Potential. The steady-state K^+-nonactin- or K^+-valinomycin-induced membrane conductance was modulated via the two-sided addition of flavonoids (phloretin or myricetin) or styryl dyes (RH 421 or RH 237) from millimolar stock solutions in ethanol to the membrane-bathing solution (0.1 M KCl, 5 mM Hepes, 7.4) to concentrations of 20 μM for different flavonoids and 5 μM for various styryl dyes. The final concentration of ethanol in the chamber did not exceed 0.8%. We used nonactin in the experiments with phloretin and myricetin because phloretin is less effective in the transfer of K^+-valinomycin than is K^+-nonactin in phospholipid- and sterol-containing membranes.[24] As we found that RH 421 is less effective in the transfer of K^+-nonactin than K^+-valinomycin by a factor of approximately 4, valinomycin was used in the experiments with RH 421 and RH 237. The conductance of the lipid bilayer (G) was determined by the transmembrane current (I) at a constant transmembrane voltage of V = 50 mV.

The corresponding calculations were performed by assuming that the membrane conductance is related to the membrane dipole potential (φ_d) by the Boltzmann distribution[24]

$$\frac{G_m}{G_m^0} = \exp\left(-\frac{q_e \Delta\varphi_d}{kT}\right) \tag{2}$$

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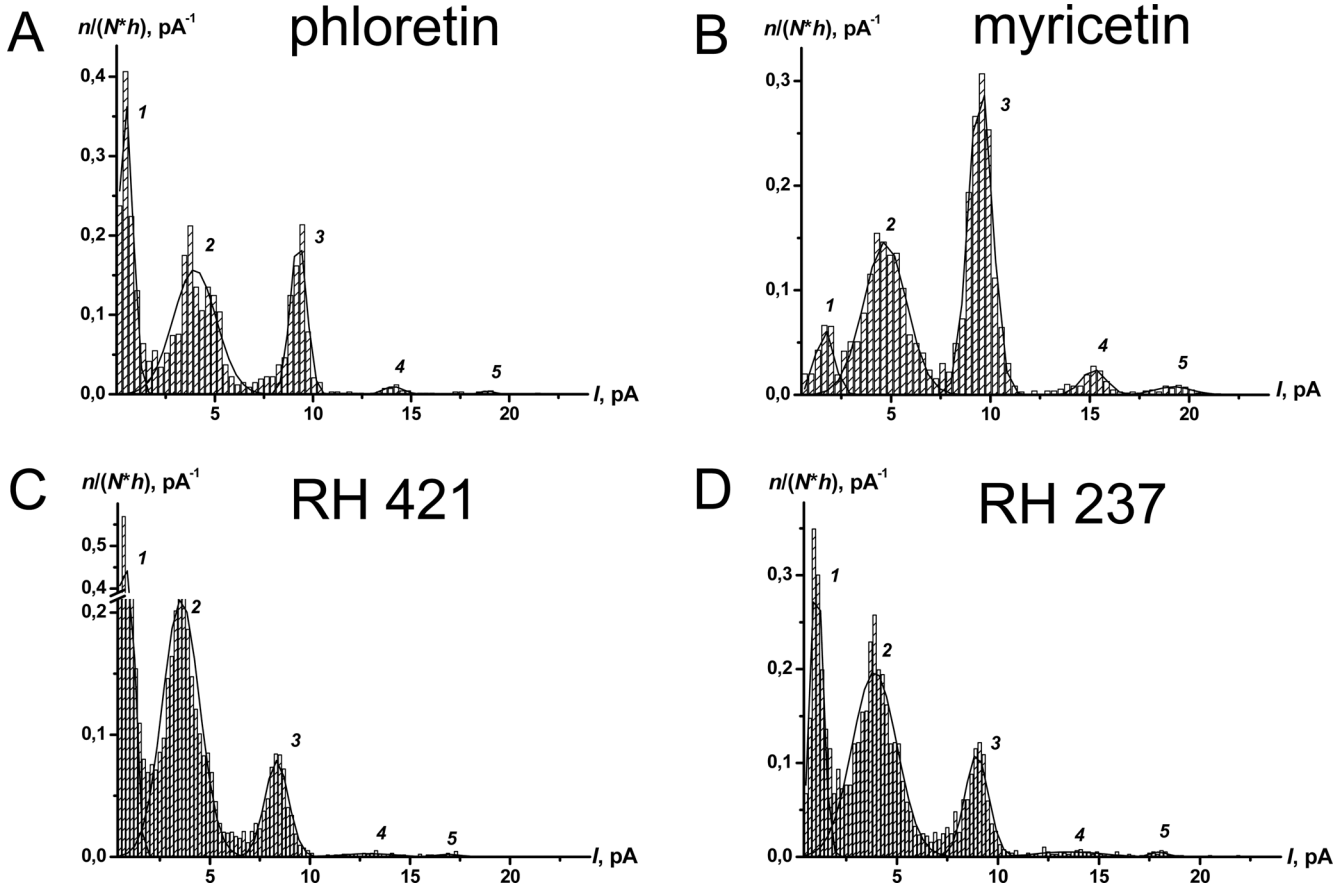

Figure 4. Current-transition histograms of CecA channels in the presence of 20 μM phloretin (A), 20 μM myricetin (B), 5 μM RH 237 (C), and 5 μM RH 421 (D) in the bilayer bathing solution. The membranes were made from DOPS/DOPE (1:1) and bathed in 0.1 M KCl, 5 mM HEPES, pH 7.4. The transmembrane voltage was 25 mV.

where G_m and G_m^0 are the steady-state membrane conductance induced by nonactin or valinomycin in the presence and absence of dipole modifiers, respectively; $\Delta\varphi_d$ is the change in φ_d after the addition of modifier to a bilayer bathing solution; and q_e, k, and T have their usual meanings. The values of $\Delta\varphi_d$ for given experimental conditions were averaged for three to five bilayers (mean ± sd). Control experiments were performed to demonstrate that cecropins do not affect the bilayer dipole potential ($\Delta\varphi_d$ = 0.6 ± 0.4 mV at 1.5 μM CecA or CecB).

RESULTS AND DISCUSSION

CecA and CecB produce ion channels in bilayer lipid membranes made from equimolar mixture of DOPS and DOPE in the concentration range of 2 to 5 μM. A further increase in the peptide concentration causes the destabilization of the bilayer and its subsequent breakdown at 10 μM concentration. These data are in agreement with the assumption that the formation of pores may be the first step in membrane disintegration by CecA and CecB, while the dense peptide carpet precedes complete bilayer destruction.[7,22] CecP1 does not induce ion channel formation; it leads to membrane destabilization at 25−50 μM and bilayer disruption at concentrations above 50 μM. These findings are in agreement with the data showing that the peptide does not penetrate the hydrocarbon core of the membranes.[21] Thus, CecP1 is believed to function by the carpetlike mechanism.

Figure 1 represents the records of current fluctuations induced by CecA in the DOPS/DOPE membrane bathed by 0.1 M KCl (pH 7.4) at V = 25 mV. One can see the well-defined cecropin channels of different conductance sublevels and that they are similar to the pores formed by alamethicin. (Compare these pores to those depicted in Figure 2, bottom, from ref 39.) Figure 1A presents the smallest conductance substates of CecA channels, i.e., approximately 1 pA (level 1), 4.5 pA (level 2), and 9 pA (level 3). Figure 1B also shows the largest sublevels of CecA channels, i.e., approximately 14 pA (level 4) and 18 pA (level 5). From Figure 2, it is observed that the histogram of current fluctuations is characterized by the existence of five channel substates at a transmembrane potential of V = 25 mV.

Christensen et al. showed that the cecropin channels possess weak anionic selectivity that most likely results from the existence of six positively charged groups in the N-terminal helix, which is meant to be pore-forming.[8] We performed a detailed study of the selectivity of CecA channels and found three populations of channels: pores with weak cationic selectivity, pores with weak anionic selectivity, and pores that were nonselective (Figure 3). Typically, nonselective pores have a higher conductance than do selective pores.

Our data are in agreement with the results of Durell et al.[11] The authors predicted two types of CecA channels. The first type (I) is formed by at least four membrane-bound dimers arranged in a circular pattern. The pore is formed by the association of C-terminal helices, with the polar faces oriented toward the center (Figure 1sA, Supporting Information). The narrowest part of the pore is formed by several Gln 31 and Gln 34 residues, which form two stacked, hydrogen-bonded rings. A similar single-ring structure occurs in models of the alamethicin

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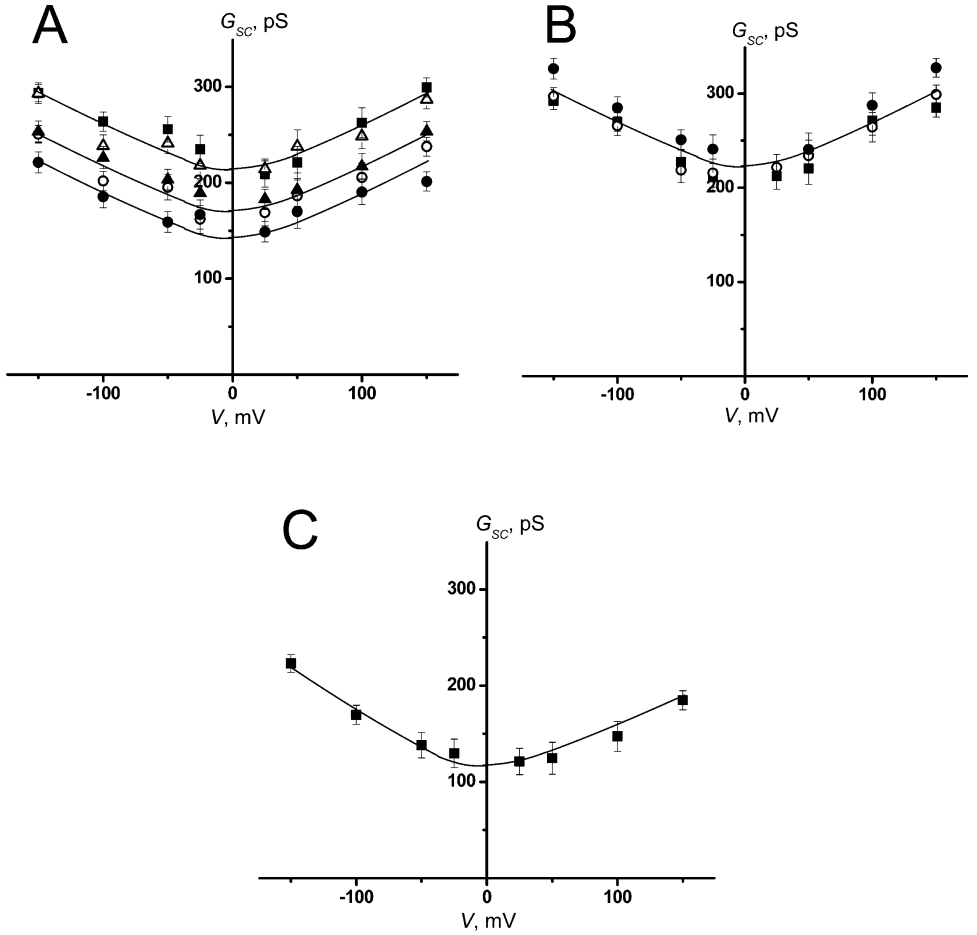

Figure 5. Mean normalized conductance−voltage curves of the CecA channels. The membranes were made from DOPS/DOPE (1:1) (A), DOPS/DOPE/Chol (1:1:1) (B), and DPhPS/DPhPE (1:1) (C) and were bathed in 0.1 M KCl, 5 mM HEPES, pH 7.4. Bilayer bathing solutions contain (■) no dipole modifiers, (○) 20 μM phloretin, (△) 20 μM myricetin, (●) 5 μM RH 421, and (▲) 5 μM RH 237.

channel, in which the Gln 7 residues are postulated to regulate the pore size and conductance.[40] Furthermore, alamethicin displayed weak cationic selectivity. Thus, the cation selectivity of type I CecA channels might be predicted. The second class of channel models (type II) predicted by Durell et al. is formed by the N-terminal helices (Figure 1sB, Supporting Information).[11] The net positive charge of the N-terminal helices would likely cause the anionic selectivity observed. One would expect that increasing the number of monomers in the conductive oligomers of both types would result in a loss of their selectivity and that nonselective pores are thus observed. Apparently, for this reason the steady-state multichannel current induced by CecA is nonselective (transfer number of cations is equal to 0.52 ± 0.02). This observation makes it impossible to distinguish which mechanism of channel formation is more probable.

Membrane dipole potential modifying agents, i.e., flavonoids (phloretin and myricetin) and styryl dyes (RH 237 and RH 421), were employed for the further investigation of the channel-forming activity of CecA. The quantitative effects of these dipole modifiers on the dipole potential of DOPS/DOPE and DOPS/DOPE/Chol bilayers are presented in Table 1. Phloretin substantially decreases the dipole potential of DOPS/DOPE membranes, myricetin weakly influences it, and the introduction of RH 237 and RH 421 increases the dipole potential of bilayers. The presence of Chol in the membrane insignificantly affects the ability of phloretin and RH 421 to change the bilayer dipole potential.

Figure 4 presents the current transition histograms in the presence of dipole modifiers, flavonoids, phloretin, and myricetin, and styryl dyes, RH 237 and RH 421, at V = 25 mV. One can see that the addition of phloretin, RH 237, and RH 421 leads to a reduction in the probabilities of the higher conductance sublevels (4 and 5) and a redistribution of the probabilities of sublevels 1−3 in favor of the smallest one, especially in the case of styryl dyes (compare Figure 2 to Figure 4A,C,D). Myricetin does not influence the substate probabilities (compare Figure 2 to Figure 4B). To characterize the changes in probabilities of all of the populations of the channels observed in the absence and in the presence of dipole modifiers, we introduced a mean normalized current, I_{SC}, and corresponding mean conductance of CecA channels, G_{SC} (Materials and Methods). Figure 5A shows the dependence of G_{SC} on transmembrane voltage for all of the above-mentioned systems. The addition of 20 μM phloretin, 5 μM RH 237, or 5 μM RH 421 leads to a decrease in G_{SC}. Myricetin does not practically affect the mean CecA channel conductance. These data may indicate that the oligomerization process might be sensitive to the mechanical properties of the bilayer. It is known that phloretin decreases while myricetin does not affect the melting temperature of pure DMPC.[41] Moreover, using confocal fluorescence microscopy of phase separation in giant unilamellar vesicles, Ostroumova et al. have recently shown that phloretin fluidizes the membrane whereas myricetin does not significantly alter bilayer fluidity.[42] Apetrei et al. hypothesized that electrostatic repulsion between

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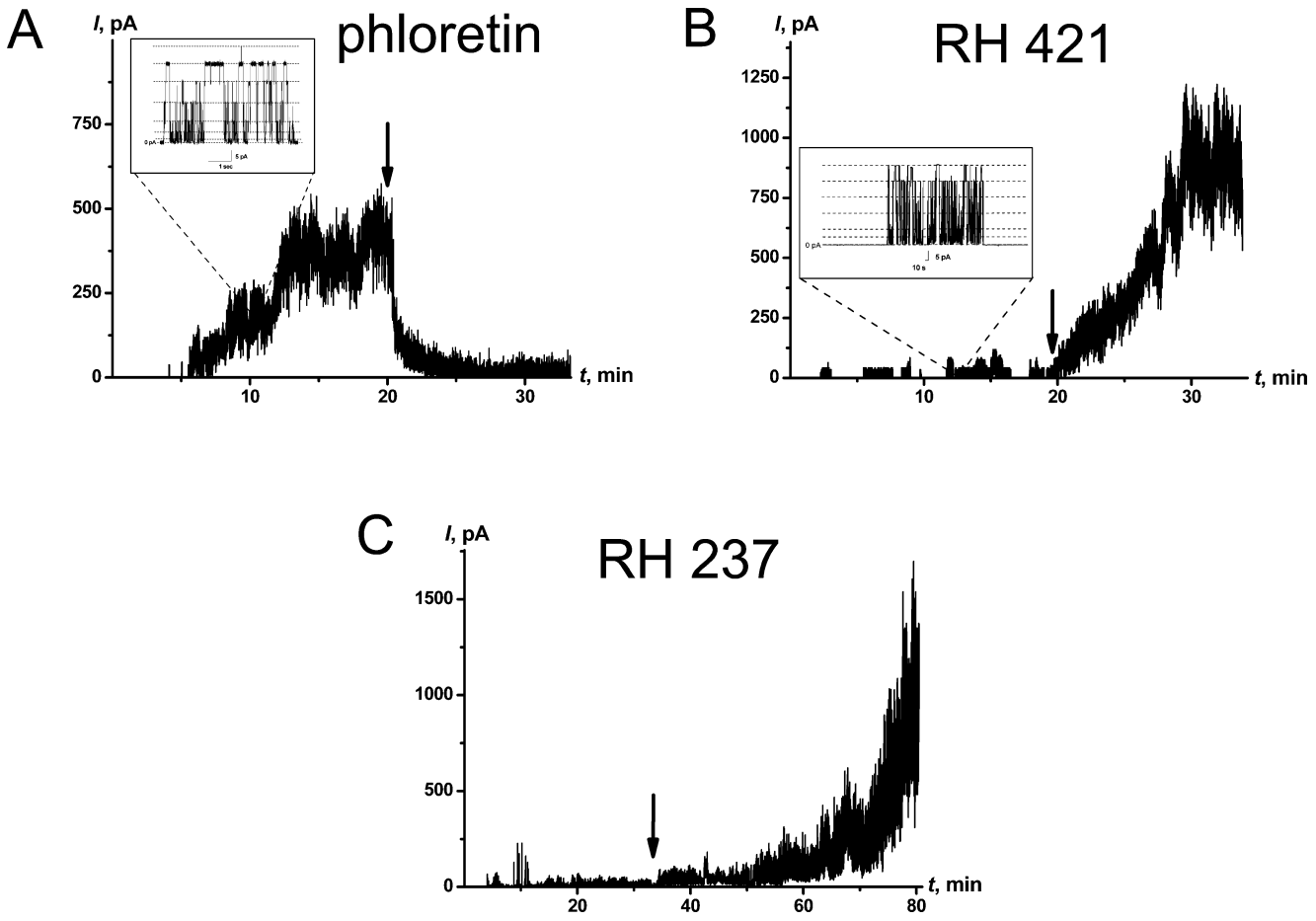

Figure 6. Effect of phloretin and RH 421 on the steady-state transmembrane current induced by CecA. The lipid bilayers were composed of DOPS/DOPE (1:1) and bathed in 0.1 M KCl, 5 mM HEPES, pH 7.4. The addition of 20 μM phloretin (A), 5 μM RH 421 (B,) or 5 μM RH 237 (C) to the bilayer bathing solution is indicated by arrows. V = 50 mV.

negatively charged moieties of the membrane-adsorbed RH molecules produces a disordering effect in the membrane.[31] Decreases in the membrane lipid condensation in the presence of phloretin and styryl dyes may influence cecropin aggregation/disaggregation. Cholesterol affects the fluidity and dipole potential of phospholipid membranes[43,44] and therefore might modulate the activity of membrane-inserted peptides. This knowledge forces us to study the effects of dipole modifiers on the channel-forming activity of cecropins in Chol-containing bilayers. Figure 5B presents the $G_{SC}(V)$ dependence of CecA channels in DOPS/DOPE/Chol membranes. It is observed that the effects of phloretin and RH 421 on the mean normalized conductance of CecA channels in Chol-enriched bilayers are negligible. Since these dipole modifiers almost identically alter the dipole potential of Chol-free and Chol-enriched membrane (Table 1) but have different effects on the G_{SC} of CecA channels in these bilayers (Figures 5A,B), it can be assumed that the latter is associated with a different ability of phloretin and styryl dyes to increase the fluidity of bilayers in the absence and in the presence of Chol. We have also tested the channel-forming activity of CecA in lipid bilayers made from an equimolar mixture of DPhPS and DPhPE. DPhPS and DPhPE differ from DOPS and DOPE by the branched structure of their acyl chains. Lipids with bulky tails take the shape of cone,[45] which leads to a less dense packing of lipids in the DPhPS/DPhPE membranes relative to that in DOPS/DOPE membranes. One can see that the substitution of DOPS and DOPE for DPhPS and DPhPE leads to an increase in the probabilities of the smaller conductance sublevels (1′, 2′, and 3′) (cf. Figure 2A,B), and the higher-conductance substates of the CecA channels (4′ and 5′) are not observed (Figures 1C and 2B). This results in a decrease in G_{SC} (cf. Figure 5A (the curve in the absence of dipole modifiers) to Figure 5C). Thus, one may conclude that a decrease in the membrane lipid condensation due to the presence of phloretin and RH 421[41,31] or the branched structure of lipid acyl chains may influence the oligomerization processes of cecropin molecules in the membrane during pore formation.

Table 2. Mean Ratios ($I_\infty/I_\infty{}^0$) of Steady-State Transmembrane Currents Induced by Different Cecropins in the Presence (I_∞) and Absence ($I_\infty{}^0$) of Dipole Modifiers (Flavonoids and Styryl Dyes) in DOPS/DOPE (1:1) Bilayers Bathed in 0.1 M KCl, 5 mM HEPES, pH 7.4

	modifiers			
cecropins	phloretin	myricetin	RH 237	RH 421
CecA	0.3 ± 0.2	1.1 ± 0.1	9.3 ± 3.9	7.2 ± 4.9
CecB	0.5 ± 0.3	0.9 ± 0.1	2.9 ± 0.8	3.7 ± 1.6

Figure 6A shows that the addition of 20 μM phloretin leads to more than a 10-fold decrease in the steady-state CecA-induced transmembrane current. Figure 6B shows that the introduction of 5 μM RH 421 increases I_∞ by a factor of 12. The addition of another styryl dye, RH 237, leads to a similar

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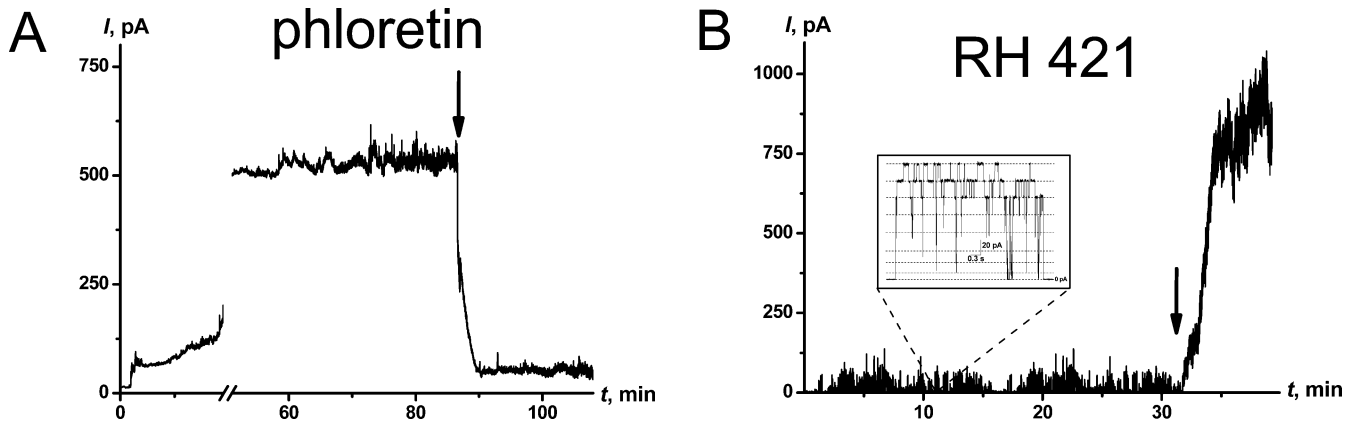

Figure 7. Effect of dipole modifiers on the steady-state transmembrane current induced by CecB. The lipid bilayers were composed of DOPS/DOPE/Chol (1:1:1) and bathed in 0.1 M KCl, 5 mM HEPES, pH 7.4. The addition of 20 μM phloretin (A) or 5 μM RH 421 (B) to the bilayer bathing solution is indicated by arrows. V = 50 mV.

increase in I_∞ (Figure 6C). Table 2 presents the mean ratios ($I_\infty/I_\infty{}^0$) of steady-state transmembrane currents induced by CecA and CecB in the presence and absence of modifiers (flavonoids and styryl dyes). The results obtained in Chol-enriched membranes were very similar to those obtained in Chol-free bilayers (Figure 7, Table 3). These results may demonstrate that the presence or absence of Chol does not determine the specificity of peptide action. The same conclusion was found by Silvestro et al., who showed that cholesterol delays but does not prevent channel formation by cecropins.[9]

Table 3. Mean Ratios ($I_\infty/I_\infty{}^0$) of Steady-State Transmembrane Currents Induced by Different Cecropins in the Presence (I_∞) and Absence ($I_\infty{}^0$) of Dipole Modifiers (Flavonoids and Styryl Dyes) in DOPS/DOPE/Chol (1:1:1) Bilayers Bathed in 0.1 M KCl, 5 mM HEPES, pH 7.4

	modifiers	
cecropins	phloretin	RH 421
CecA	0.4 ± 0.2	4.3 ± 2.4
CecB	0.3 ± 0.1	7.3 ± 6.9

A comparison of a decrease in G_{SC} (Figure 5) and I_∞ (Table 2) in the presence of phloretin at V = 50 mV (1.3 vs 3) allows one to conclude that phloretin insertion into the membrane is accompanied by both the reduction of open CecA channels (N_∞) and the decrease in their conductance (G_{SC}). Since the mean normalized conductance of CecA channels decreases while the steady-state multichannel cecropin-induced transmembrane current increases in the presence of styryl dyes, the latter effect is due to an increase in the number of open cecropin channels. It is obvious that N_∞ does not change after the addition of myricetin because this dipole modifier has no effect on the single channel and multichannel activity of CecA (Figure 5 and Table 2). The observed effects of dipole modifiers on the steady-state number of open cecropin channels are in agreement with the changes in the membrane dipole potential due to their adsorption (compare with Table 1).

Luchian and Mereuta also investigated the channel-forming activity of alamethicin in the presence of dipole modifiers, i.e., phloridzin and 6-ketocholestanol.[26] Phloridzin, similar to phloretin, decreases the membrane dipole potential, while 6-ketocholestanol increases it. The authors concluded that because alamethicin monomers have positively charged N-termini, a decrease in the membrane dipole potential is accompanied by a reduction in the energy barrier for the interfacial accumulation of alamethicin monomers. Following this logic, one can assume that in the case of cecropin the inverse relationship holds. The reduction in the membrane dipole potential due to phloretin addition is accompanied by a decrease in the steady-state cecropin-induced transmembrane current, i.e., by an increase in the energy barrier for the interfacial accumulation of cecropin monomers. An increase in the membrane dipole potential due to RH 421 introduction leads to an increase in the channel-forming activity of peptide, i.e., a decrease in the energy barrier for the interfacial accumulation of peptide molecules. Therefore, one can propose that the negative pole of the cecropin dipole is inserted into the membrane (Figure 2s, Supporting Information). This orientation is assumed for the type I CecA channels.[11] On the basis of this circumstance, one might infer that type I CecA channels are influenced by dipole modifiers. The preference for this type of pore might be a result of a more stable C-terminal helix rather than an N-terminal helix during the interaction with anionic lipid bilayers.[46]

CONCLUSIONS

The exact molecular mechanisms of membrane activity of cecropins have been under debate for more than 30 years of investigation. Two general models have been proposed to explain the antimicrobial action of cecropins: the formation of transmembrane pores and the carpet model. The present study clearly demonstrates that that CecA and CecB produce well-defined and well-reproduced ion channels of different conductance levels in bilayer lipid membranes made from equimolar mixtures of DOPS and DOPE; DOPS, DOPE, and cholesterol; or DPhPS and DPhPS at micromolar concentrations. A further increase in the peptide concentration causes the destabilization of the bilayer and its subsequent breakdown. Therefore, the formation of pores is the first step in membrane disintegration by CecA and CecB, while the dense peptide carpet precedes complete bilayer destruction. CecP1 does not induce ion channel formation but rather leads to membrane destabilization and disruption at concentrations above 50 μM. Thus, CecP1 is believed to function by the carpetlike mechanism.

Dipole-modifying agents, flavonoids and styryl dyes, which are able to decrease/increase the membrane dipole potential significantly modulate the channel-forming activity of CecA and

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CecB. The decrease in the membrane dipole potential leads to a reduction of the steady-state number of open CecA and CecB channels in cholesterol-free and cholesterol-enriched anionic lipid bilayers. The observed effects are related to changes in the energy barrier for the interfacial accumulation of cecropin monomers. This finding indicates that the negative pole of the cecropin dipole is inserted into the membrane. The preference for this type of pore might be a result of a more stable C-terminal helix rather than an N-terminal helix during the interaction with anionic lipid bilayers.

The observed increase in the channel-forming activity of CecA and CecB in the presence of styryl dyes may open new perspectives for the development of more effective cecropin-based formulations for medical applications.

■ ASSOCIATED CONTENT

Supporting Information

Cecropin channel models described by Durrel et al.[11] Simplified view of the possible physical mechanism through which a decrease in φ_d due to phloretin adsorption may lead to a reduction in cecropin activity. Current fluctuations corresponding to openings and closures of the single CecA channels in the planar lipid bilayers made from DOPC and DOPE. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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