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Voltage-dependent pore activity of the peptide alamethicin correlated with incorporation in the membrane: salt and cholesterol effects

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Strong aggregation of incorporated alamethicin in the bilayer of lipid vesicles has been observed spectroscopically at aqueous peptide concentrations above a critical value c^*. On the other hand, in conventional gating studies with planar lipid films, the onset of conducting pore formation can be characterized by a threshold voltage V^*. We present experimental evidence of a direct correspondence between the effects on c^* and V^* when these parameters are modulated by adding NaCl (to the aqueous medium) or cholesterol (to the lipid moiety). A quantitative analysis supports the idea that the measured aggregation actually results in pore formation, the voltage-dependence being due to an electric field effect on the partition equilibrium of the peptide between the aqueous and the lipid phases.

Introduction

Despite enormous efforts, it has not yet been possible to unravel the molecular details of voltage gating of conducting membrane channels. In order to circumvent the complexities of large cellular membrane proteins, smaller peptides, just long enough to span a lipid bilayer, were introduced as model systems. The most intensively studied among such peptides is the antibiotic alamethicin (see reviews on conductance properties [1] and molecular structure [2] and further references given in previous publications [3,4]). However, even for this comparatively simple compound, the voltage gating (which can be impressively observed on planar lipid films) remains open to debate and speculation as far as its molecular machinery is concerned (cf. Refs. 5–8).

Recently we have measured the incorporation of alamethicin into lipid vesicles with the help of spectroscopic techniques (circular dichroism, fluorescence) and found a markedly anomalous behavior. Incorporation isotherms start relatively flat at low aqueous peptide concentrations, c, and bend sharply upwards once a certain critical concentration, c^*, is reached (cf. Fig. 1). This phenomenon could be rationalized by assuming that alamethicin molecules tend to aggregate in the membrane. At c above c^* the concentration in the membrane becomes sufficient for massive aggregate formation [3,4]. This interpretation of our data was also suggested considering the fact that the conducting pores of alamethicin are actually thought to be aggregates of variable size, built in a 'barrel stave' manner [5,9].

From our results readily evolved a fresh view of the voltage gating process for alamethicin: interaction of the dipole moment of the helical peptide (of the order of 70–80 Debye [10,11]) with the electric field would increase the partitioning into the membrane upon application of an electric membrane potential, V, of appropriate polarity. Thus, the critical aqueous concentration for strong aggregation, c^*, would be lowered. Accordingly, the probability of some aggregation and possibly

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pore formation would increase already at very low aqueous concentrations (in the nanomolar range) as they are typical for experiments on planar lipid films. Such an interpretation is consistent with the observation that the voltage needed to induce pore formation decreases with increasing aqueous peptide concentration, until pores form spontaneously even in the absence of a transmembrane potential as micromolar peptide concentrations are reached [12]. In addition, the correct orientation of the dipoles is consistent with the well-known gating asymmetry of alamethicin [13].

Nevertheless, despite the plausibility of such a hypothesis, it relies on spectroscopic data obtained with a vesicle system at micromolar concentrations of alamethicin, whereas pore activity has been measured on planar bilayers in a range of much smaller c. Experimentally, it is not easy to bridge that gap, because the spectroscopic signal is too weak at very low concentrations and the planar bilayers break at high peptide concentrations, where large pores form spontaneously.

As a way out of this dilemma, we have tried to find, at least, quantitative correlations between the pore activity observed on lipid films and the critical concentration, c^*, measured spectroscopically with vesicle systems. In this context we note that c^* varies as a function of the NaCl concentration in the aqueous medium or of the membrane composition, respectively [4]. For instance, increasing the salt concentration causes c^* to be reduced; parallel to it the threshold for pore formation in lipid films is reached at lower voltages. On the other hand, an increase of the threshold voltage has been reported if cholesterol is incorporated in glycerylmonooleate bilayers [14]. We therefore decided to make a quantitative comparison between the variations of c^* and a suitable threshold voltage $V^\bullet$ as a function of (i) the aqueous NaCl concentration and (ii) the cholesterol content in the membrane.

Materials and methods

Circular dichroism measurements on sonicated vesicles and conductance experiments on planar lipid films were performed using in both cases the same lipid, dioleoylphosphatidylcholine (DOPC) from Avanti, Birmingham, AL, and the same alamethicin, the electrically neutral component, purified and characterised as described previously [4]. The same reference gives a detailed account of the experimental and evaluation procedures to obtain incorporation isotherms from circular dichroic data. Recently a more general evaluation method has been described [15] which avoids the original two-state assumption and extrapolation of the limiting signal at full peptide incorporation; instead, the difference between two titration curves obtained at different peptide concentrations is used. Applying this approach, we arrived at exactly the same results as with the simple two-state assumption. The present c^* values were obtained by fitting to the isodesmic incorporation/aggregation model of Rizzo et al. [4]. However, critical concentrations could also be determined in terms of other models or even estimated by simple inspection of the isotherms; although the absolute values turn out somewhat different [3], the ratios of c^* values relevant to Fig. 3 remain essentially unchanged.

Planar lipid bilayers were painted from a 10 mg/ml solution of DOPC in n-decane on holes in teflon septa, pretreated with a 5 mg/ml solution of DOPC in hexane. After adding alamethicin and stirring for 10–15 min, we waited for a further 30–45 min before measuring current-voltage curves (cf. Ref. 16). Only those curves were used which remained reproducible for several trials, even with intermediate stirring. In the case of cholesterol containing bilayers, the same proportion of cholesterol was added to the pretreatment and to the membrane forming solution. In analogy to results reported for other lipids [14], we assumed that the cholesterol/DOPC ratio in the planar film was half that in the membrane forming solution. This assumption was supported by the observation of large changes occurring between membranes formed from 1.3 : 1 and 2 : 1 cholesterol/DOPC mixtures, respectively (cf. Table I where 40% refers to a 4 : 3 and 50% to a 2 : 1 mixture in the membrane-forming solution). We consider it unlikely that the cholesterol content in the bilayer ever exceeds 50 mol%, and this maximum value appears to be attained only with the 2 : 1 mixture.

The characteristic threshold voltage $V^\bullet$ was defined as the voltage where the alamethicin con-

ductance reached a value of 8 $\mu S \cdot cm^{-2}$ (i.e. 10^{-8} S on a hole of 0.4 mm diameter). Similarly defined threshold voltages are known to decrease with increasing aqueous alamethicin concentration, shifting by -26 mV to -30 mV as the peptide concentration is doubled [16–18]. Using a 1 ml cell, we found increased shifts of $V^{\bullet}$ at low peptide concentrations (cf. also the data given by Menestrina et al. [18]). We attribute this effect to adsorption of peptide to the walls of the teflon cell which appears to be stronger in the case of uncharged alamethicin than with the charged form of the antibiotic. In principle, this should not influence our determination of salt or cholesterol dependence since they were evaluated at fixed concentrations of the peptide. Nevertheless we repeated the majority of the experiments with a larger, 10 ml cell, having a reduced surface to volume ratio. As expected, the salt and cholesterol dependence turned out to be the same as with the small cell. In addition, we did some control experiments with alamethicin kindly provided by the Upjohn Co., Kalamazoo, MI, which contains mainly the charged component. The adsorption problem was much less severe with that sample. The salt and cholesterol dependences were very similar to those with the uncharged species, at least when using NaCl concentrations above or equal to 0.1 M. The shift of $V^{\bullet}$ was -28 mV per factor of 2 in the alamethicin concentration under all conditions (not determined in the complete absence of NaCl).

Circular dichroism as well as conductance experiments were all performed at 20 °C in 10 mM Tris-HCl (pH 7.4) with NaCl added as indicated in each case.Thus zero NaCl refers to an ionic strength of 8 mM due to dissociated buffer, $Tris^{+}$ and Cl^{-}, at that pH.

Results

(a) NaCl dependence

Typical isotherms obtained from circular dichroism titrations are shown in Fig. 1. The critical concentrations evaluated under five different salt conditions are given in Table I. Obviously, c^{*} decreases upon increasing the NaCl concentration. This means that c^{*} gets closer to any given low alamethicin concentration (in the nanomolar range, typical for conductance measurements on lipid films). Accordingly we expect gating to be promoted if it is connected with aggregation. In fact, the threshold voltage, $V^{\bullet}$, needed to induce pore activity on DOPC bilayers, decreased with increasing NaCl concentration as is evident from Fig. 2 and the compiled data in Table I. The form of the conductance-voltage curves was indepen-

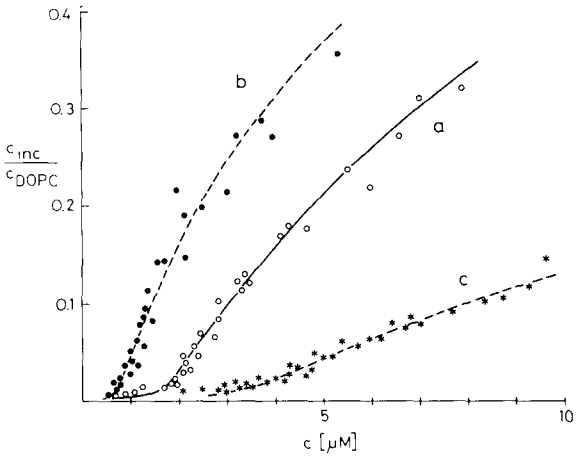

Fig. 1. Molar ratio of incorporated alamethicin per DOPC, plotted against the aqueous peptide concentration, as determined from (circular dichroism) titrations. Dashed and solid curves: fits to the model presented in Ref. 4, using the c^{*} values of Table I. Conditions: (a) 0.1 M NaCl, (b) 0.5 M NaCl, (c) 0.1 M NaCl, 25 mol% cholesterol in bilayers.

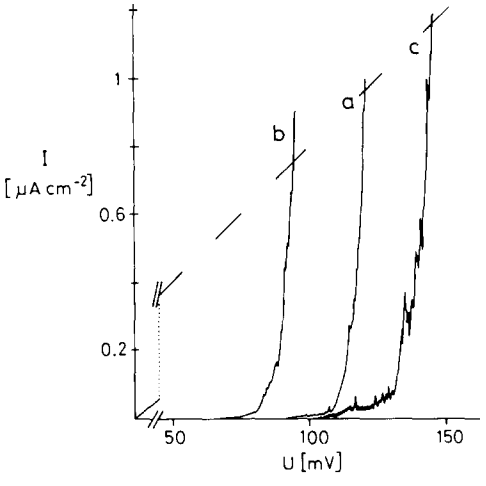

Fig. 2. Current-voltage curves obtained with planar DOPC bilayers in the presence of about 150 nM alamethicin. Conditions (a), (b), (c) as in Fig. 1. The dashed line corresponds to a conductance of 8 $\mu S \cdot cm^{-2}$ at which $V^{\bullet}$ was evaluated.

dent of the NaCl concentration, the conductance g being proportional to $\exp(V/V_e)$ with V_e between 4 and 4.5 mV (cf. Refs. 12, 16–18). Only in the absence of NaCl, in pure 10 mM buffer, was the slope of the conductance-voltage curve markedly decreased, with an exponential form as above and V_e being of the order of 7–10 mV (cf. Ref. 19). In addition, V_e was much more variable than in the presence of NaCl, from one bilayer to another and even sometimes on one single bilayer as a function of time (in agreement with other authors [19]). Nevertheless, $V^\bullet$ as defined under Materials and Methods remained essentially invariant (the range of inaccuracy being a few millivolts).

(b) Cholesterol dependence

The cholesterol dependence was studied at 0.1 M NaCl. Typical isotherms are again shown in Fig. 1, whereas Table I presents the complete set of c^* values obtained at three different cholesterol concentrations. Apparently, c^* shifts to higher values as the cholesterol content of the bilayer is increased. With the same argument as above, one should expect gating to become less pronounced. Indeed, the characteristic voltage, $V^\bullet$, increases (cf. Fig. 2 and Table I). The slope of the conductance-voltage curves was independent of the cholesterol content of the bilayers.

The complete set of $\Delta V^\bullet$ (i.e., differences with respect to the cholesterol-free bilayer at 0.1 M NaCl) was measured at two different alamethicin concentrations. No systematic deviations could be found between the two sets, demonstrating that the alamethicin concentration dependence of $V^\bullet$ is independent of the cholesterol content. In Table I, the average of the two sets is given.

(c) Comparison between spectroscopic and conductance experiments

Let us propose that the conducting pores are formed through the aggregation of incorporated peptide, a process characterized by the critical concentration c^*. The membrane conductance, g, can then be expressed as

$$g = A \cdot g_P[(c/c^*)\exp(V/V_1)]^m \qquad (1)$$

(see Appendix). Here, A and V_1 are constants, g_P is the single-pore conductance and m stands for the aggregation number of a pore aggregate. This result is consistent with the well-known concentration and voltage dependence of g. In particular we note a shift of the threshold voltage $V^\bullet$ upon changes of c according to the relation

$$\Delta V^\bullet = -V_1 \cdot \Delta \ln c \qquad (2)$$

In our system, $V_1 = 40$ mV, in agreement with literature data ranging between 37 and 42 mV [12,16,17].

In the present experiments the alamethicin concentration was held fixed, but its critical value c^* was varied by adding salt or cholesterol. Under these circumstances, Eqn. 1 predicts, in analogy to Eqn. 2, that

$$\Delta V^\bullet = V_1 \cdot \Delta \ln c^* \qquad (3)$$

involving the same parameter V_1. Eqn. 3, as it stands, has been derived under the assumption of

TABLE I

PARAMETERS OF BILAYER INCORPORATION AND PORE ACTIVITY OF ALAMETHICIN UNDER VARIOUS CONDITIONS OF SALT CONCENTRATION AND CHOLESTEROL CONTENT IN THE BILAYER

All experiments were done in 10 mM Tris-HCl (pH 7.4) buffer. All cholesterol experiments were done with 0.1 M NaCl added. The $\Delta V^\bullet$ values are averages of values obtained at different alamethicin concentrations. $\Delta V^\bullet$ and c^*/c^*_{ref} refer to 0.1 M NaCl without cholesterol as a reference point. Experimental errors are of the order of 7 mV for $\Delta V^\bullet$ and of 10% for c^* but about twice as large where indicated by [a].

Conditions	$\Delta V^\bullet$ (mV)	$\Delta V^\bullet$ (mV)	c^* (μM)	c^*/c^*_{ref}
0.1 M NaCl, no Chol. (reference point)	0	0	2.1	1
0 M NaCl	30	12	2.7	1.3
0.05 M NaCl	7	5	2.3	1.1
0.2 M NaCl	−10.5	−9	1.65	0.8
0.4 M NaCl	−23	−20		
0.5 M NaCl	−31	−27	0.95	0.4
10% Chol.	9 [b]	9 [b]	3.0	1.4
25% Chol.	21	22	4.4	2.1
40% Chol.	52	53.5	8 [a]	3.8 [a]
50% Chol.	80 [a]	82 [a]		

[a] Approximate values.
[b] At 12.5% cholesterol content.

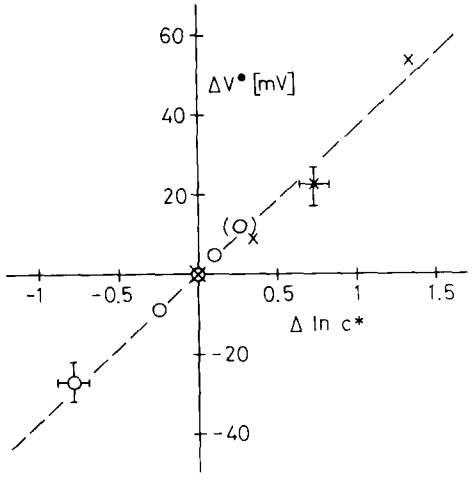

Fig. 3. Correlation between shifts of $V^\bullet$ (as obtained from conductance measurements with planar bilayers) and ln c^* (as determined from spectroscopic titrations with vesicles). The origin is given by the reference point (0.1 M NaCl, no cholesterol).The dashed line has been drawn by linear regression. Circles from upper right to lower left: 0, 0.05, 0.1, 0.2, 0.5 M NaCl; crosses from origin to upper right: 0, 12.5%, 25%, 40% molar cholesterol content of bilayers.

constant single pore conductance g_P. This is not true in reality, since the average pore conductance varies with applied potential (in PC bilayers [9]) and ionic strength, though the corrections are small due to the steep rise of the conductance-voltage curves. We corrected the raw data (column 1 of Table I) for changes in g_P by using the known potential dependence of average pores [9] and salt dependence of pore states [12]. The corrected values of $\Delta V^\bullet$ are compiled in Table I and plotted in Fig. 3 against the corresponding changes of ln c^*.

A linear relation is obtained (correlation coefficient 0.991) with a slope of 36.5 mV, in very good agreement with the prediction of Eqn. 3.

The entries corresponding to data obtained with pure buffer are put in parentheses in Table I and Fig. 3 to make aware of some pecularities: Corrections for changes in g_P were comparatively large. They were done using the Cl^-/Na^+ permeability ratio from the literature [12] and neglecting $Tris^+$ conductance [20]. The voltage was corrected for double-layer polarization according to Hainsworth and Hladky [21]. Even then, the results at zero NaCl concentration are difficult to compare to the other conditions because of the change in slope of the conductance-voltage curves. As long as a change in the external conditions only shifts this curve along the voltage axis without changing its form, the $\Delta V^\bullet$ values are independent of the particular conductance value at which $V^\bullet$ is read. However, this is no longer true if one wants to compare two curves with different slopes.We have nevertheless put the points corresponding to the pure buffer experiments in Table I and Fig. 3, using the same definition of $V^\bullet$ as before, with the following reasoning: The slope of conductance-voltage curves recorded at very low ionic strength tends to be larger at the beginning of the experiments and to decrease with time and number of trials, in agreement with results reported by Roy [19]. In such a way, we could record conductance-voltage curves of different slope from one single bilayer. As the slope changes, these curves crossed each other in the conductance range around $8 \cdot 10^{-8}$ S $\cdot$ cm^{-2}, i.e. the value chosen for our definition of the threshold voltage.

Discussion

Significant correlations have been found between the variation of pore forming activity (as measured by the characteristic threshold voltage $V^\bullet$ on planar bilayers) and the variation of incorporation and aggregation of alamethicin in bilayers (as measured by the critical concentration, c^*, determined from spectroscopic titrations). The variations of these parameters were induced by adding NaCl to the aqueous medium or cholesterol to the bilayers, in different amounts. Plotting $\Delta V^\bullet$ against Δ ln c^* (Fig. 3) yields a linear relation, in accordance with Eqn. 3. In addition the slope of this line, as determined by linear regression, is $V_1 = 36.5$ mV, in very good agreement with the value of $V_1 = 40$ mV obtained from the peptide concentration dependence of the alamethicin conductance.

These results give first experimental evidence of a direct correlation between the two phenomena, namely membrane incorporation and pore formation by alamethicin. Quantitative agreement is found with an elementary scheme (Eqns. 1 and 3) based on the assumption that the number of pores is proportional to the number of aggregates of a

certain size, in equilibrium with a pool of monomeric incorporated peptide; the latter is thought to be in a field-dependent partition equilibrium with the aqueous phase. Actually, the essential element leading to Eqn. 3 is that the membrane conductance g can be written as a function of the ratio c/c^*. Eqn. 2 (which can be regarded as an empirical relation between $V^\bullet$ and c) can then be stated in the form:

$$\Delta V^\bullet = -V_1 \cdot \Delta \ln(c/c^*) \quad (4)$$

which is the same as Eqn. 2 for changes in c at constant c^*, but yields Eqn. 3 when c^* is changed at fixed c. Thus c^* as determined from titrations with vesicle systems turns out to be a fundamental parameter which defines an intrinsic scale of the peptide concentration.

In view of the large technical differences between the planar bilayer (especially if containing solvent) and vesicle systems, such a result could not be anticipated a priori. In this respect, it is clearly important that relative changes, induced by variation of the system conditions, have been compared and not absolute quantities. In any event, the result validates our idea that a direct study of alamethicin incorporation in membranes by physical chemical techniques can yield information relevant to the electrical activity of this peptide.

Whereas this spectroscopic approach has been developed only recently [3,4], there exists an extensive literature on the conductance of alamethicin in planar lipid films. Our present conductance experiments are therefore mainly a repetition of standard procedures, the main objective being to use exactly the same lipid and peptide material as for the spectroscopic studies. The salt dependence of uncharged alamethicin in DOPC bilayers in the range 0.1 to 0.5 M NaCl is fully comparable to previous results obtained with the charged peptide [16]. Below 0.1 M NaCl, the salt dependence diminishes in our system, in contrast to data obtained on phosphatidylethanolamine bilayers [12], but in perfect agreement with data on phosphatidylcholine [19], both obtained with the charged peptide. Adsorption to lipid monolayers at the water/decane interface also shows the same trend of salt dependence [22].

A strong cholesterol dependence has been found with glyceryl monooleate bilayers [14], but no systematic study has been published on the cholesterol dependence in phosphatidylcholine (though the properties of single pores have been studied by Boheim et al. [23] in a phosphatidylcholine bilayer system). We found less pronounced effects of cholesterol in DOPC as compared with glyceryl monooleate [14], but the trend was the same. Since stable DOPC bilayers could only be formed with solvent (or with extensive pretreatment of the cell which induced strong adsorption artifacts), the cholesterol content of our membranes could only be adjusted approximately. The corresponding data may thus be considered as semiquantitative. Nevertheless, the agreement between the trends in the incorporation isotherms on one hand and the pore forming activity on the other hand is striking and clearly significant (cf. Fig. 3; the entries in this figure and in Table I correspond to the estimated cholesterol content in the bilayer, cf. Materials and Methods).

The slope of the straight line in Fig. 3 is directly related to the dipole moment per monomer in the pore complex , μ_1 (see Appendix):

$$V_1 = kT\,d/\mu_1 \quad (5)$$

(kT: Boltzmann term; d: membrane thickness at the location of the pore). The thickness of decane containing bilayers is quite large, about 4.8 nm [24]. A 20 amino acid α-helical peptide could hardly span such a distance. Therefore we assume that the width of the membrane is restricted at the location of the pores and set $d = 2.7$ nm for a solvent free bilayer [3]. With our value of V_1 we then obtain $\mu_1 = 88$ Debye close to the 70–80 Debye obtained from dielectric measurements in organic solvents [10,11].

The aggregation number, m, of an 'average pore' can be obtained by dividing V_1 by the potential dependence parameter V_e ($V_e = V_1/m$ according to Eqn. 1). A value of about 9 is obtained, independent of salt or cholesterol conditions (apart from the measurements in pure buffer). The constancy of the aggregation number m indicates that the aggregation in the membrane appears not to be strongly affected by varying salt or cholesterol concentrations. Instead, the main effects must come from the change of the partition

coefficient (cf. Eqn. A-5). In the case of NaCl, this may be understood as a simple salting out. Explanation of the cholesterol effect is less obvious and must await further study.

As mentioned in a previous publication [4], we could not find any significant temperature dependence of our spectroscopic isotherms between 20 °C and 60 °C, with DOPC. Moderate temperature effects are reported in the literature of the pore forming activity. From Fig. 3b of Boheim and Kolb [16] we estimate a change of 4–5 mV in $V^{\bullet}$ when going from 18 °C to 4 °C. Of these, about 2–3 mV are accounted for by changes in the single pore conductance, g_P, due to increased water viscosity and change in electric potential. The remaining 2 mV are below the experimental error. These experiments were done on solvent containing lipid films, and the results may be partly influenced by the strong temperature dependence of alkane solubility in bilayers [25]. Within these limits of comparability, there is thus good evidence that the analogy in the behaviour of $V^{\bullet}$ and c^* holds also for variations of the temperature.

In summary, we have found a definite correlation between membrane incorporation and pore activity of alamethicin under various conditions. We consider this result to support a molecular gating mechanism where the primary effect of the electric field is an increase in membrane partitioning of the peptide with concomitant aggregate formation. At least some of the higher aggregates appear to be conducting pores.

Clearly the present report is concerned only with the equilibrium properties of the alamethicin-lipid system. Some kinetic aspects have been investigated [15] which further support the proposed gating mechanism.

Appendix

The alamethicin conductance is known to be produced by pores of different sizes. However, for the purpose of our discussion it suffices to consider a homogeneous sample of pores of average size (cf. Ref. 12). The total observed conductance, g, is then the product of the average single-pore conductance, g_P, and the number of (open) pores, N_P:

$$g = g_P \cdot N_P \tag{A-1}$$

In order to conform with previous notation [3,4], we introduce the variable $r_P = N_P/N_L$ where N_L is the number of DOPC molecules in the bilayer. Strictly speaking, bilayer conductances are compared at constant area and not at constant N_L so that a correction should be applied for the dilution of DOPC in the presence of cholesterol. This will be omitted here, since the final results are found to be affected negligibly (less than 2% in the worst case). The simplest way to account for the aggregation mechanism in the membrane is to consider an equilibrium between alamethicin monomers (already incorporated in the membrane) and the pore complex, P, made up of m monomers:

$$m\ A_1 \rightleftharpoons P$$

with an equilibrium constant $K_P = K^m$. For the monomers, a partition equilibrium between bilayer and water phase is assumed; non-idealities can be neglected at the low peptide concentration relevant to conductance measurements (but are important for the spectroscopic isotherms in the micromolar range, as discussed previously [3,4]). Then, with a partition coefficient Γ:

$$r_P = K^m \cdot r_1^m \tag{A-2}$$

$$r_1 = \Gamma \cdot c \tag{A-3}$$

where r_1 is the number of incorporated peptide monomers per DOPC molecule. Alternatively, Eqn. A-2 may be written

$$r_P = (c/c^*)^m \tag{A-4}$$

with

$$c^* = (\Gamma \cdot K)^{-1} \tag{A-5}$$

Formally analogous results would be obtained from more sophisticated aggregation models, allowing for different aggregate sizes. The parameter K is chosen to facilitate comparison with such models (cf. Refs. 3,4).

Alamethicin is thought to enter the membrane with its more hydrophobic amino terminus. In a bilayer spanning position, its molecular dipole is then properly aligned to increase its partition

coefficient upon application of a transmembrane potential, positive at the side of peptide addition. Including possible electric field effects on the aggregation constant, we then have to take into account a Boltzmann factor so that

$$r_p = [c\ \Gamma K \exp(\mu_1 E/kT)]^m \qquad \text{(A-6)}$$

μ_1 is the dipole moment per monomer in the pore complex, parallel to the field direction, and ΓK denotes the product of the partition coefficient and aggregation constant in the absence of a potential. The electric field strength E equals the applied potential, V, divided by the membrane thickness, d. (At very low salt concentration, a correction has to be applied to account for double layer polarization, cf. Results). Eqn. 1 is then readily derived, with a parameter V_1 according to Eqn. 5.

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