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Effective charge of melittin upon interaction with POPC vesicles

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The binding of bee venom melittin to small unilamellar vesicles and large nonsonicated multilamellar bilayer membranes composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was studied by means of circular dichroism, ^{31}P-NMR and electrophoretic mobility. The melittin binding isotherm for small unilamellar vesicles (SUV) could be described by a partition equilibrium with $K_p = (6 \pm 1) \cdot 10^4$ M^{-1}. Electrostatic effects were taken into account by means of the Gouy-Chapman theory. Combining the partition equilibrium with the Gouy-Chapman analysis suggested an effective charge for melittin of $z_p = 1.9$, which is lower than the true electric charge of 5–6. The variation of the ^{31}P-NMR signal of SUV showed the change in potential at the phosphodiester moiety of the lipid upon addition of melittin. This potential change was lower than that for an ion with an electrical charge of 5–6 and corresponded to a charge of 1.5. Electrophoretic mobility measurements with multilamellar vesicles confirmed the charge reduction effect. These experimental results show that the use of the simple Gouy-Chapman theory requires an effective electrical charge of the melittin which is lower than the formal charge.

Introduction

Melittin, a small peptide of 26 amino acids, is a main component of bee venom. The amino acid sequence of melittin is:

(+)Gly^1-Ile-Gly-Ala-Val-Leu-Lys(+)-Val-Leu-Thr-Thr-Gly-

Leu-Pro-Ala-Leu-Ile-Ser-Trp^{19}-Ile-Lys(+)-Arg(+)-Lys(+)-

Arg(+)-Gln-Gln^{26}-CONH$_2$

Hydrophobic and polar residues are unevenly distributed: the 20 residues on the N-terminal side are mainly hydrophobic, whereas the other 6 at the C-terminal are polar. Because of its amphiphilicity (high solubility in water and strong interaction with lipids and detergents), melittin is a suitable model for lipid–protein interaction.

Many binding studies of melittin with lipids have been carried out [1–3]. A binding constant, and number of lipids per bound melittin between 2 and 60, were evaluated using linear Scatchard plots, but because of statistical effects such an evaluation of data is not applicable for the binding of ligands covering more than one receptor [4–6]. Moreover, although at neutral pH melittin has 5–6 positive charges, the electrostatical effects of the attraction of melittin to negatively charged lipids or repulsion from positively charged lipids were not taken into consideration. A more realistic model of the interaction of melittin with neutral lipids [7,8] or with negatively charged lipids [9] has been proposed. A simple partition equilibrium between aqueous and lipid phases was assumed. The concentration of melittin near the lipid surface was corrected according to the Gouy-Chapman theory. An effective charge of melittin of about 2 was found, which is much lower than the electrical charge of 5–6 which is expected at pH = 7.4.

Evidence that the lipid surface 'senses' a much lower charge than 5–6 has been obtained from ^{2}H-NMR of lipid headgroups [8,9]. It is important to confirm experimentally this as yet inexplicable charge reduction effect.

In this work we have studied the interaction of melittin with vesicles of pure 1-palmitoyl-2-oleoyl-*sn*-

Abbreviation: POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; NMR, nuclear magnetic resonance; CD, circular dichroism; SUV, small unilamellar vesicles.

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glycero-3-phosphocholine (POPC): the CD signal of melittin was utilized to determine the binding parameters of melittin to small unilamellar vesicles (SUV) of POPC. We have also measured the change of surface potential of SUV after the binding of melittin, using the ^{31}P-NMR signal of lipid vesicles in the presence of paramagnetic ions [10]. The absolute values of surface potential were obtained from electrophoretic measurements of multilamellar vesicles of POPC upon addition of melittin.

Materials and Methods

Materials. Melittin was purchased from Sigma (St. Louis, MO) (grade 2, phospholipase free) and purified according to Refs. 2 and 8. The melittin used in the microelectrophoresis measurements was purchased from Mack Chemical (Illertissen, F.R.G.) and was purified according to Ref. 7. Control experiments showed no differences between the two samples. The peptide concentration was determined by UV spectroscopy using an absorption coefficient of 5570 M^{-1} cm^{-1} at 280 nm [11].

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids (Birmingham, AL) and used without further purification.

For CD and zeta potential measurements the following buffer was used: 100 mM NaCl, 10 mM Tris-HCl (pH 7.4), 0.1 mM Na_2EDTA. For ^{31}P-NMR measurements 100 mM NaCl, 10 mM Tris-HCl (pH 7.4) buffer was taken.

Preparation of lipid samples for CD, NMR and microelectrophoresis experiments. Small unilamellar vesicles (SUV) of POPC were required for the CD and NMR measurements and were prepared as follows: a lipid dispersion (approx. 3 mg/ml for CD and 16 mg/ml for ^{31}P-NMR measurements) was sonicated under a nitrogen atmosphere in buffer for 40–50 min at 10 °C. Metal debris from the titanium tip was removed by centrifugation for 10 min in an Eppendorf centrifuge. Multilamellar vesicles for zeta potential measurements were prepared by the method of Bangham [12]. Usually 4 mg of POPC were dissolved in chloroform/methanol/water (20:9:1, v/v) (see also Ref. 13) and evaporated in a round-bottom flask by rotary evaporation for at least 30 min. After rotary evaporation the flask was put under high vacuum for at least 1 h. 5 ml buffer was added together with a few glass beads to the dried thin lipid layer and the suspension was shaken gently. The multilamellar vesicles formed in this way had a size of 1–10 μm. The desired quantity of melittin was added to the solution after the preparation of the vesicles.

CD and NMR measurements. CD measurements were made with a Cary 61 spectrometer calibrated with d(+)-10-camphorsulfonic acid. The optical length of the cuvette was 2 mm. $[\theta]$ represents the mean ellipticity per residue in deg cm^2/dmol. ^{31}P-NMR measurements were carried out on a Bruker MSL-400 spectrometer operating at 162 MHz. 50 mg of lipid was taken in buffer containing 20% D_2O for a field-frequency 'lock'. Cobaltcloride ($CoCl_2 \cdot 6H_2O$) was added to freshly prepared vesicles in a final concentration of 6 mM and were present only in the external medium. Low amounts of bound paramagnetic Co^{2+} ions broaden significantly the phosphorus line of the lipid in the outer monolayer. The broadening is proportional to the free concentration of cobalt in the aqueous phase adjacent to the phosphate group. The change of the linewidth of ^{31}P upon melittin binding was used to calculate the potential on the phosphodiester group. The phosphorus relaxation time T_1 in the outer monolayer was much reduced upon addition of Co^{2+} ions, while T_1 from the inner monolayer remained approximately the same. This difference in relaxation times T_1 was used to suppress the signal from the inner monolayer with a $(\pi\text{-}\tau\text{-}\pi/2)$ radio frequency pulse [10,14]. The 90° pulse width was 18 μs, the interpulse delay τ was 0.54 s and the recycle time was 4.5 s. The spectral width was 20000 Hz. The linewidth $\Delta\nu_p$ is determined as the difference between the ^{31}P-NMR linewidths of POPC vesicles in the presence and absence of cobalt.

Microelectrophoresis measurements. Electrokinetic mobilities were measured with a Rank Brothers Mark 2 microelectrophoresis apparatus, using a cylindrical cell and platinum electrodes. The zeta potential, ζ was calculated from the electrophoretic mobility, u, using the Helmholtz-Smoluchowski equation

$$\zeta = (u\eta)/(\epsilon_R\epsilon_0) \quad (1)$$

where η is the viscosity of the aqueous phase, ϵ_0 is the permitivity of free space and ϵ_R the dielectric constant of water. Care was taken to focus at the stationary layer [18]. The concentration of melittin in the aqueous phase, c_{eq}, was determined after the zeta potential measurements. The suspension of multilamellar vesicles was centrifuged at 300000 × g for 140 min using polyallomer vials, resulting in a clear and lipid-free supernatant. The concentration of melittin in the supernatant was determined with UV-spectroscopy at 280 nm.

All experiments were carried out at 25 °C.

Results

CD experiments

In aqueous solution melittin exists in monomeric and tetrameric forms [11,15]. The equilibrium between the two forms depends upon the salt concentration, the pH of the aqueous medium and the melittin concentration. In our CD experiments melittin was always pre-

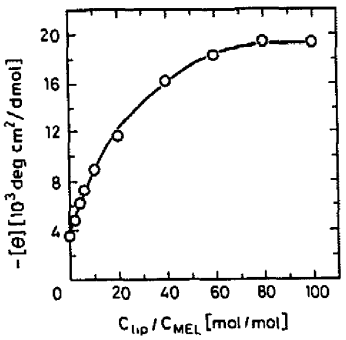

Fig. 1. Variation of the negative ellipticity per residue of melittin at 222 nm versus the total lipid to peptide ratio. The melittin concentration was 10 μM. The data have been obtained at pH 7.4 (10 mM Tris-HCl, 100 NaCl, 0.1 mM Na_2EDTA).

sent as monomer in buffer. The monomeric form of melittin has a low degree of secondary structure and shows no significant signal at 222 nm ($\theta_f = -3800$ deg cm^2/dmol). Upon titration of melittin with SUV it becomes more helical and the signal at 222 nm reaches a plateau ($\theta_b = -19500$ deg cm^2/dmol) (see Fig. 1).

Melittin binds to the outer monolayer of vesicles [16,17]. The change of the CD signal of melittin upon addition of SUV was utilized to determine the binding isotherm, i.e. the amount of bound melittin per lipid as a function of free aqueous peptide concentration. Fig. 2 shows the association isotherm of melittin to SUV of POPC, where x_b^* is the extent of binding corrected for the asymetrical binding of melittin to vesicles, i.e. $x_b^* = x_b/0.6$ [9], where x_b = concentration of bound melittin/concentration of lipid. The binding isotherm was fitted with the theoretical curve (see Discussion and also Refs. 7, 9 and 38).

^{31}P-NMR experiments

The addition of cobalt ions to SUV causes broadening and a small shift of the ^{31}P signal from the outer monolayer [10]. The broadening is proportional to the

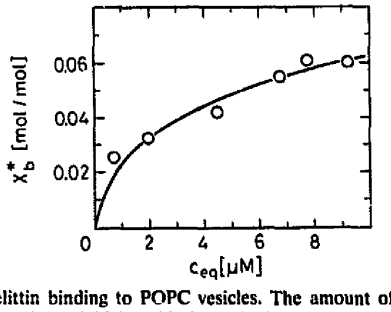

Fig. 2. Melittin binding to POPC vesicles. The amount of melittin bound per mole of lipid (considering only the outer vesicle layer) is plotted versus the equilibrium concentration of free melittin in solution. The solid line is the theoretical binding isotherm with $K = 60000\ M^{-1}$ and $z_p = 1.9$ (see Discussion).

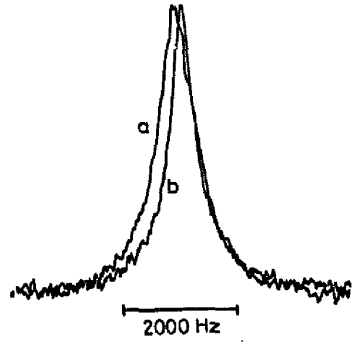

Fig. 3. ^{31}P-NMR spectra of the outer monolayer of sonicated POPC vesicles with (spectrum b; $x_b^* = 0.019$) and without (spectrum a) melittin. The spectra have been obtained at pH 7.4 (10 mM Tris-HCl, 100 mM NaCl). The total lipid concentration was 21.3 mM. Cobalt ions were present only in the external aqueous medium ($c_0 = 6$ mM).

amount of bound cobalt ions, which in turn is proportional to the concentration of free cobalt in the aqueous solution close to the lipid surface. After addition of cobalt we added melittin to the SUV. In the ^{31}P-NMR experiments all added melittin was bound to the SUV because of the high lipid to melittin ratio. The concentration of melittin was kept low enough to prevent penetration of Co^{2+} to the inner volume of SUV (the maximum value of x_b^* was 0.02). Such a low melittin/lipid ratio also ensures the stability of the SUV during the experiments, and excludes the likehood of the other effects influencing the spectrum. Control experiments showed that for the duration of the experiments (approx. 3 h) the binding of cobalt remained asymmetrical. The binding of melittin leads to an increase in positive surface potential whose magnitude depends upon the effective charge of melittin. This positive potential will repel cobalt ions from the lipid surface and the ^{31}NMR linewidth becomes narrower. We have indeed observed the narrowing of the ^{31}P-NMR linewidth upon binding of melittin as shown by the ^{31}P-NMR spectra of the outer monolayer of SUV with and without bound melittin (see Fig. 3). This narrowing of the ^{31}P-NMR spectrum of the outer monolayer was accompanied by a shift of the resonance position of approx. 055 ppm (Fig. 3a) to 0.25 ppm (Fig. 3b) to the ^{31}P peak of SUV without melittin and cobalt ions. The shift is not so suitable for evaluation as the narrowing because it can be determined much less precisely.

Fig. 4 shows the variation of ^{31}P-NMR linewidth $\Delta\nu_p$ with increasing amounts of bound melittin, x_b^*. The two theoretical curves show the variation of linewidth for effective charge of melittin equal to 1.5 or 5 (see Discussion).

Microelectrophoresis experiments

The zeta potential is the average electrostatic potential at the hydrodynamic plane of shear, which lies in

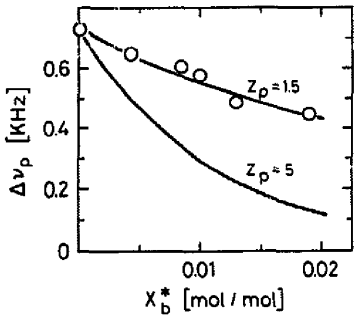

Fig. 4. Variation of ^{31}P-NMR linewidth ($\Delta\nu_p$) of POPC molecules in the outer monolayer of SUV with the amount of bound melittin (x_b^*). The two solid lines are the theoretical ^{31}P-NMR linewidth variations for two different effective charges of melittin (see Discussion).

the aqueous phase 2 Å from the surface in 0.1 M monovalent salt solution [18]. At small values of the surface potential, $\psi_0 \leq 25$ mV, the variation of electrostatic potential in the aqueous phase, $\psi(x)$, can be described by the following equation [19]:

$$\psi(x) = \psi_0 e^{-\kappa x} \tag{2}$$

where κ is the reciprocal Debye length.

Fig. 5 shows the increase of the surface potential ψ_0 with the increasing of equilibrium concentration of melittin in the aqueous phase, c_{eq}. The surface potential was calculated from the zeta potential using Eqn. 2. The bound melittin can extend some amino acids into the water phase. These amino acids act as a hydrodynamic drag and can decrease the electrophoretic mobility [20]. A contrary effect increases the mobility. Charges located at some distance from the surface have a larger influence on the mobility than charges located at the surface [40]. We neglect both these effects. The zeta potential measurements carried

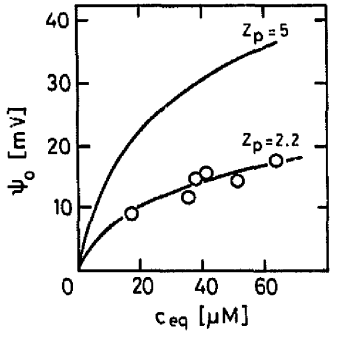

Fig. 5. Zeta potential measurements. Variation of the surface potential with concentration of melittin in the aqueous phase. The solid lines are theoretical curves for two different effective charges of melittin (see Discussion). The data have been obtained at pH 7.4 (10 mM Tris-HCl, 100 mM NaCl, 0.1 mM Na_2EDTA).

out with gentamicin and spermine showed no such effects [21]. The two theoretical curves in Fig. 5 show the variation of surface potential in the case of the effective charge of melittin equal to 2.2 or 5 (see Discussion).

Discussion

The analysis of binding of melittin to neutral lipids by means of the Gouy-Chapman theory has been given previously [8]. Briefly, the binding of melittin and cobalt ions create a positive charge at the outer monolayer of SUV. The positive charge density is:

$$\sigma = \left(\frac{e_0}{A_L}\right)\left(\frac{Z_p x_b^* + 2x_{Co}^*}{1 + x_b^*\left(\frac{A_p}{A_L}\right)}\right) \tag{3}$$

z_p is the effective charge of melittin, e_0 is the elementary electrical charge, A_L is the surface area of POPC molecule and $A_L = 68$ Å^2, A_p/A_L is a factor to correct for the expansion of the lipid surface upon melittin binding, where $A_p = 150$ Å^2, [9,22] and x_{Co}^* is the extent of Co^{2+} ion binding at the outer lipid monolayer.

The surface charge at the lipid surface generates a surface potential (ψ_0), which can be calculated by means of the Gouy-Chapman theory [23,24]:

$$\sigma^2 = 2000\epsilon_0\epsilon_R RT\sum_i c_{i,eq}(e^{-z_i F_0\psi_0/RT} - 1) \tag{4}$$

where $\epsilon_R = 78$ is the dielectric constant of water, ϵ_0 the permittivity of free space, R the gas constant, F_0 the Faraday constant, $c_{i,eq}$ the concentration of the ith electrolyte in the bulk aqueous phase (in moles per liter), and z_i the signed charge of the ith species.

CD experiments

In these experiments no Co^{2+} ions were added, i.e. $x_{Co}^* = 0$. The positively charged membrane will repel melittin molecules from the lipid surface and the melittin concentration in the aqueous solution at the plane close to the lipid surface (c_M) will be lower in comparison with the concentration of melittin in the bulk solution (c_{eq}):

$$c_M = c_{eq}\exp(-z_p F_0\psi_0/RT) \tag{5}$$

We obtain a simple partition equilibrium between two phases

$$K = x_b^*/c_M \tag{6}$$

by setting $z_p = 1.9 \pm 0.1$. Combining Eqns. 3–6 we calculated the binding constant $K = (6 \pm 1)\cdot 10^4$ M^{-1} (see Fig. 2).

The binding between melittin and nonsonicated bilayer membranes of POPC has been investigated [8]. The binding constant of $K = (2.1 \pm 0.2) \cdot 10^3$ M^{-1} is lower compared with our present result. We suggest that this difference arises from the different lipid packing in SUV and in planar lipid bilayers. Similar effects were observed with other proteins [25,26]. The interaction of melittin with SUV of a POPC/POPG mixture has also been investigated [9]. After correction of electrostatic effects a similar binding constant of $K = 47000$ M^{-1} was found.

^{31}P-NMR experiments

Using a Langmuir binding law with the Gouy-Chapman theory after some simple algebra we obtain for the concentration of bound cobalt ($c_{b,Co}$):

$$c_{b,Co} = \frac{K_{Co} \cdot c_{lip} \cdot 0.6 \cdot c_0 \cdot \exp(-2F_0\psi_0/RT)}{1 + K_{Co} \cdot c_{lip} \cdot 0.6 \cdot \exp(-2F_0\psi_0/RT)} \quad (7)$$

K_{Co} is the binding constant of cobalt ions and was taken to be 1 M^{-1} [27], c_{lip} is total lipid concentration (21.3 mM), 0.6 is the factor correcting for asymmetrical binding of cobalt to SUV, and c_0 is the total concentration of cobalt ions (6 mM).

In calculations we assumed that the free lipid concentration was equal to the total lipid concentration in the outer lipid monolayer (the extent of cobalt binding was less than 1%) and we used an equation for cobalt ions similar to that for melittin:

$$C_{M,Co} = c_{eq,Co} \exp(-2F_0\psi_0/RT) \quad (8)$$

In the absence of melittin ($x_b^* = 0$) we obtain $x_{Co}^* \approx 0.005$ ($x_{Co}^* = c_{b,Co}/(0.6\ c_{lip})$) and $\psi_0 = +2.7$ mV according to Eqns. 3, 4, and 7. An addition of melittin leads to an increase in the positive potential at the lipid surface and this causes the narrowing of the ^{31}P-NMR linewidth. From Eqn. 7 we obtain the following expression for the ratio of two linewidths with and without melittin:

$$\frac{\Delta\nu_p^0}{\Delta\nu_p} = \exp[2F_0(\psi_1 - \psi_0)/RT] \left[\frac{1 + 0.6K_{Co}c_{lip}\exp(-2F_0\psi_1/RT)}{1 + 0.6K_{Co}c_{lip}\exp(-2F_0\psi_0/RT)}\right] \quad (9)$$

where $\Delta\nu_p^0$ and $\Delta\nu_p$ are the ^{31}P-NMR linewidths without and with melittin, respectively, and ψ_1 is the surface potential upon binding of melittin.

The term in the square brackets yields a small correction (about one percent). Solving Eqns. 3, 4 and 9 we can fit the experimental points on Fig. 4. The best fit was with the effective charge of melittin $z_p = 1.5 \pm 0.4$. This value agrees well with the value of 1.9 from CD experiments.

Microelectrophoresis measurements

The binding of melittin to a coarse lipid dispersion of POPC was investigated earlier [8]. A binding constant of 2100 M^{-1} and an effective charge of $z_p = 2.2$ were found. We must keep in mind that from thermodynamic principles x_b^* is a definite function of c_{eq}. In both cases, i.e. when melittin penetrates through lipid bilayers in large multilamellar vesicles because of freeze-thaw and interacts with all the lipid [8], or when it interacts only with outer lipid layer as in our zeta potential measurements, this function stays the same. Taking $z_p = 2.2$ and surface potentials, which were obtained from our zeta potential measurements, we can calculate from Eqns. 3–6 the following binding constant: $K = 1900 \pm 300$ M^{-1} (see Fig. 5). It agrees excellently with the previously found value of 2100 M^{-1}. In other words, with $z_p = 2.2$ we obtain the same binding isotherm in both cases. If the effective charge of melittin was 5–6, then the surface potential calculated from the given binding curve, using the Gouy-Chapman theory, would be significantly higher (see Fig. 5). We can see with all the different experimental methods that the effective electrical charge of melittin is reduced.

A reduced electrical charge has also been observed with the other peptides [28,29,30,39]. There are several possible reasons for such a charge reduction. One is that charged amino acids of the bound peptide can lie rather far from the lipid surface and their contribution to the surface potential will be small. If we assume that the surface charge is placed in the water phase in the plane parallel to the lipid interface, then a distance of 6–7 Å would be enough to explain such a potential reduction on the lipid surface [31]. But, it has been pointed out that because of the dual nature of Lys^+ and Arg^+ (mixed hydrophobic/hydrophilic character) the transfer of these amino acids from water to the lipid interface is accompanied by a large reduction of free energy [32]. On the other hand, it was shown that di-, tri-, tetra- and penta-lysine bind weakly to PC membranes [41]. It has also been pointed out that arginine residues of melittin contribute little to the interfacial charge [38]. It is clear that the present state of knowledge does not allow this matter to be resolved.

Another reason could be the large size of melittin compared with the Debye length (the Gouy-Chapman theory assumes that ions in the diffuse double layer are point charges). A theory which takes into consideration effects of charge separation has been introduced [33,34], with which it was shown that ions with a large charge separation exert a smaller screening effect than point charges. The concentration of such molecules in the aqueous solution near the lipid surface is then lower than predicted by the Gouy-Chapman theory. It was also pointed out that by decreasing the ionic strength the discrepancy between the experimentally

observed effect of the large ions and the theoretical prediction of the Gouy-Chapman theory should decrease. But when the Debye length is varied from 7 Å to 31 Å the effective charge of melittin remains almost the same [7]. The effective charge of melittin about 2 (at pH ≈ 7) was found for the interaction of melittin with saturated lipids [38]. This effective charges stayes the same over similar variations of Debye length and decreases to as little as 0.3 at higher salt concentration (1 M NaCl). This shows that the finite size of melittin alone cannot account for the whole charge reduction.

One of the assumptions in the Gouy-Chapman theory is that charges at the surface are smeared out. Charged lipids and proteins introduce an essential discreteness of charge. An approach which takes into account the effects of the discreteness of charge has been presented by Nelson and McQuarrie [35]. None of the discreteness of charge effects predicted by this theory were, however, found in mixtures of neutral and charged lipids [36,37]. According to Nelson and McQuarrie, discreteness of charge leads to an overestimation of counterions near the lipid surface and an underestimation for coions compared with the Gouy-Chapman theory. This difference is greater for divalent than for monovalent ions. If discreteness of charge plays an essential role in the interaction of melittin with lipids then we would expect a significantly greater intrinsic binding constant of melittin with negatively charged lipids, than with neutral lipids. But no differences between intrinsic binding constants were found in the case of POPC/POPG (80:20, mol/mol; 90:10, mol/mol) [9] and pure POPC vesicles.

In conclusion, our binding model, constructed with the simple Gouy-Chapman theory, describes the interaction of melittin with neutral and charged lipids unexpectedly well, provided that an effective charge about a third of the formal value is assigned to the melittin. The reason for the low effective charge has yet to be found.

Acknowledgments

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