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ARTICLE

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Effect of membrane-partitioned n-alcohols and fatty acids on pore-forming activity of a sea anemone toxin

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Abstract Equinatoxin II, a 19.8 kDa pore-forming toxin from the sea anemone *Actinia equina*, was examined for hemolytic activity and permeabilization of small unilamellar lipid vesicles (SUV) in the presence of increasing amounts of n-alcohols (methanol to n-octanol) and fatty acids (palmitic and palmitoleic acid). We observed an enhancement of toxin activity which was dependent on the concentration of the membrane partitioning additive. An exception was palmitic acid which exerted a bimodal role. While at low bulk concentrations it increased toxin-induced hemolysis, above 3 μM bulk concentration it was inhibitory; in neither case was it efficient in promoting release of the fluorescent marker calcein from SUV. The increased permeabilization activity was correlated with an increase in the amount of toxin bound as indicated by changes in the intrinsic toxin fluorescence. In the case of n-alcohols, at least, these effects appeared to depend on the actual amount of alcohol present inside the membrane rather than on its specific chemical nature. This suggests that the observed effects could be due to changes of the biophysical properties of the lipid bilayer, such as thickness, lipid acyl-chain ordering, and dielectric constant induced by the partitioned additives.

Key words Cytolysin · Hemolysis · Alcohol · Fatty acid · Membrane permeabilization · Lipid vesicles · Anesthetics

Abbreviations C_1–C_8, alcohols from methanol to n-octanol · C_{16} and $C_{16:1}$, palmitic and palmitoleic acid · EDTA, ethylenediaminotetraacetic acid · EqTx II, equinatoxin II · DPPC, 1,2-dipalmitoyl-sn-glycero-phosphocholine · POPC, 1-palmitoyl-2-oleyl-sn-glycero-phosphocholine · SM, sphingomyelin · SUV, small unilamellar vesicles

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1. Introduction

Pore-forming toxins (Bernheimer and Rudy 1986; Harvey 1990; Menestrina et al. 1994) are water-soluble polypeptides which insert into cells or model lipid membranes and permeabilize them. Transmembrane fluxes of normally impermeant solutes through the newly created discrete toxin openings may cause osmotic imbalance, loss of intracellular metabolites, and finally, cell deterioration. The formation of a toxin pore in a lipid bilayer generally involves several steps: adsorption to the membrane surface, insertion into the hydrophobic core, and finally, aggregation of several toxin molecules to create a water filled oligomeric pore spanning the lipid bilayer. During this process one or more segments of the protein should dip into and more across the lipid bilayer. Although, on average, such segments must be hydrophobic (for example see Singer and Yaffe 1990; De Kroon et al. 1990; McLean et al. 1991) they may also contain polar amino acid residues, either neutral or charged, as also reported for transmembrane stretches of integral proteins (Landolt-Marticorena et al. 1993; Sipos and Von Heijne 1993; Zhang et al. 1995). Such residues are expected to oppose the partitioning into the lipid bilayer. Therefore, properties of the host lipid membrane such as its polarizability, fatty acid chain ordering, and thickness may modulate insertion and translocation of polypeptide segments across the bilayer.

In the present work we addressed this question experimentally by studying whether solutes partitioned into a lipid membrane could alter the insertion and formation of pores by a cytolytic toxin. We used n-alcohols and fatty acids in different concentrations to modify erythrocyte and model lipid membranes. The toxin chosen was equinatoxin II (EqTx II), a 19.8 kDa cytolysin from the sea anemone *Actinia equina* L. (Maček and Lebez 1981;

Maček and Lebez 1988; Belmonte et al. 1994), that damages cell and model membranes by a pore-forming mechanism (Zorec et al. 1990; Belmonte et al. 1993; Maček et al. 1994). We demonstrate here that modification of either natural or model membranes by partitioned additives can significantly influence the rate of hemolysis and permeabilization induced by this and other toxins.

2. Materials and methods

2.1. Materials

The series of n-alcohols, from methanol to n-octanol, were obtained from Kemika (Croatia) or Merck (Germany). Palmitic or palmitoleic acid (Sigma, USA) were dissolved in n-propanol to give a 50 mM stock solution. When appropriate, alcohols and fatty acid stock solutions were diluted in an incubation buffer. Palmitoyl-oleoyl-phosphatidylcholine and spingomyelin were purchased from Avanti Polar Lipids, USA. The fluorescent dye calcein was from Sigma, USA. All chemicals were at least analytical grade.

Equinatoxin II was isolated from the sea anemone *Actinia equina* L. and the hemolytic activity was assayed as reported previously (Maček and Lebez 1988). Alpha-toxin (M. Wt. 34 kDa) from *Staphylococcus aureus* was a generous gift of Dr. Hungerer (Behring, Germany), melittin (M. Wt. 2847 Da) from bee venom was purchased from Sigma.

2.2. Kinetics of hemolysis

Bovine erythrocytes were prepared from citrated blood obtained from a local slaughter-house. The cells were routinely isolated as described previously (Belmonte et al. 1993) and used within a day or kept at 5 °C for not more than three days.

The rate of hemolysis was determined with a turbidimetric method (Maček and Lebez 1981) using an approximately 0.05% (v/v) erythrocyte suspension in 130 mM NaCl, 20 mM Tris.HCl, pH 7.4, with an apparent absorbance of 0.5 at a wavelength of 700 nm and 1-cm path length. Hemolysis was followed at 25 °C as a decrease of the apparent absorbance in a Pye Unicam SP30 (UK) spectrophotometer equipped with a thermostated cell holder. The rate of hemolysis was evaluated as $1/t_{0.5}$ (min^{-1}), where $t_{0.5}$ is the time required for 50% lysis (Belmonte et al. 1993; Maček et al. 1994). In a typical experiment, 3 ml of the erythrocyte suspension were first preincubated with an appropriate volume of alcohol or fatty acid. We ensured that even at the highest concentrations used neither alcohols nor fatty acids caused hemolysis or membrane permeabilitation by themselves. Two minutes later, hemolysis was started by mixing 10 µl of EqTx II in water into the preincubated suspension; the final toxin concentration was 60 ng/ml (3 nM). We performed controls in which the additives were immediately followed by the toxin in order to determine the effect of the preincubation period on the rate of hemolysis. No significant differences were observed. Furthermore, preincubation of the toxin itself with the additives at 25 °C for 10 min did not significantly change its activity.

2.3. SUVs preparation

We studied vesicle permeabilization using calcein loaded SUV composed of POPC/SM 9:1 (molar ratio) while toxin binding was studied on POPC/SM 1:1 SUV devoid of the fluorescent marker, which otherwise strongly quenched toxin tryptophan fluorescence. Both kinds of SUV were prepared by sonication as described in detail elsewhere (Menestrina 1988; Forti and Menestrina 1989). The lipid concentration of SUV, either calcein loaded or calcein-free, was 2 mg/ml in 140 mM NaCl, 20 mM Tris.HCl, 1 mM EDTA, pH 8.0.

2.4. Calcein release assays

The time course of EqTx II permeabilization of the calcein loaded SUV was recorded as an increase in fluorescence due to the dequenching of calcein released from the vesicles. The assay was performed at room temperature with a fluorescence microplate reader (Fluostar from Labinstruments GmbH, Austria, supported by FLUOsoft program). Excitation and emission filters were chosen at 485 and 538 nm, respectively. The assay was run on flat-bottom 96-well microplates as follows. To 200 µl of 2-fold serially diluted additives (alcohols or fatty acids) in 140 mM NaCl, 20 mM, 1 mM EDTA, pH 8.0, 150 µl of SUVs were added and incubated for 2 min. The vesicles were then permeabilized by adding 50 µl of 40 µg/ml EqTxII. The final lipid concentration in the permeabilization mixture was 2 µg/ml. Controls without toxin were also run. Maximal release of calcein was achieved by adding 4 µl of 100 mM Triton X-100 to each well, and the percentage of permeabilization, P(%), was calculated as

$$P(\%) = [(F_{fin} - F_{in})/(F_{100} - F_{in})] \times 100 \,, \qquad (1)$$

where F_{fin} is the final fluorescence intensity after toxin treatment, F_{in} the initial fluorescence of vesicles, and F_{100} the fluorescence after detergent permeabilization.

2.5. Kinetics of toxin binding to SUV

EqTx II is fluorescent mainly due to its five tryptophan residues (Belmonte et al. 1994). Recently, we found that binding of EqTx II to SUVs resulted in a significant increase in this fluorescence (Maček et al. 1995). Therefore, here we employed fluorimetry to monitor toxin association with POPC/SM (1:1) SUV in the presence of the additives. Toxin intrinsic fluorescence was measured at 25 °C with a Fluoromax photon counting spectrofluorimeter (Spex, USA) operated by a PC and equipped with a thermostated

cell and a magnetic stirrer. Excitation and emission wavelengths were set at 295 and 335 nm, respectively, with 2 nm slits on both beams. The apparent rate constant of EqTx II binding to SUVs was derived by fitting experimental fluorescence data to the mono-exponential function:

$$F/F_0 = 1 + [(F_{max} - F_0)/F_0][1 - \exp(-kt)] , \quad (2)$$

where F_0 is the fluorescence intensity before mixing, F the fluorescence at time *t* (see Fig. 5), F_{max} the asymptotic value at infinite *t* and *k* the rate constant.

In the binding experiments, an accurate determination of F_0 required a change of the mixing protocol. To 1 ml of 140 mM NaCl, 20 mM Tris.HCl, 1 mM EDTA, pH 8.0, in a semimicro quartz cuvette, appropriate amounts of alcohol or fatty acid were first added, followed by 5 µl of 1 mg/ml EqTx II (final concentration 250 nM) and later by 2.5 µl of concentrated SUV. This experimentally faster and simpler procedure was used only after we had ensured that a 2 min preincubation of SUV with the additives before toxin addition gave the same final results, at least within experimental error. The same was true also for hemolysis (not shown). Both observations suggest a very fast partitioning and diffusion of n-alcohols and fatty acids across cell and vesicle membranes. This is consistent with data reported in the literature which have indicated that partitioning occurs within tens of ms for alcohols (Brahm 1983), and fatty acid diffusion has $t_{1/2} < 1$ s (Kamp and Hamilton 1992, 1993; Anel et al. 1995; Langner et al. 1995). Since the insertion of the toxin into the lipid phase occurs in the range of seconds (Fig. 5), the fact that exchanging the order of additive and SUV, doesn't change the results, is not surprising. Among all the n-alcohols and fatty acids assayed, only methanol and ethanol had an effect per se on the intrinsic fluorescence of EqTX II in solution, which was increased. This implies that solutes larger than ethanal have only poor accessibility to the toxin emitting centers.

Membrane concentration of alcohols, C_m, were calculated from the bulk concentration, C, according to:

$$C_m = K C , \quad (3)$$

where K is a partition coefficient that has been tabulated (Seeman 1972).

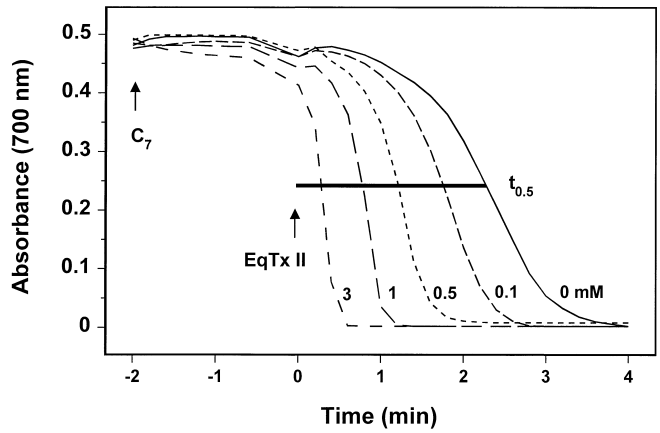

Fig. 1 Time course of hemolysis induced by equinatoxin II in the presence of an n-alcohol. Bovine erythrocytes in 130 mM NaCl, 20 mM Tris.HCl, pH 7.4, were preincubated for 2 min with n-heptanol, C_7, at the indicated final concentration and then lysed with 60 ng/ml EqTx II as described in Materials and methods. Arrows depict the additions of alcohol, and toxin. *Horizontal bar* indicates the time required to reach to 50% hemolysis, $t_{0.5}$. The rate of hemolysis was then calculatd as the reciprocal of this time, $1/t_{0.5}$

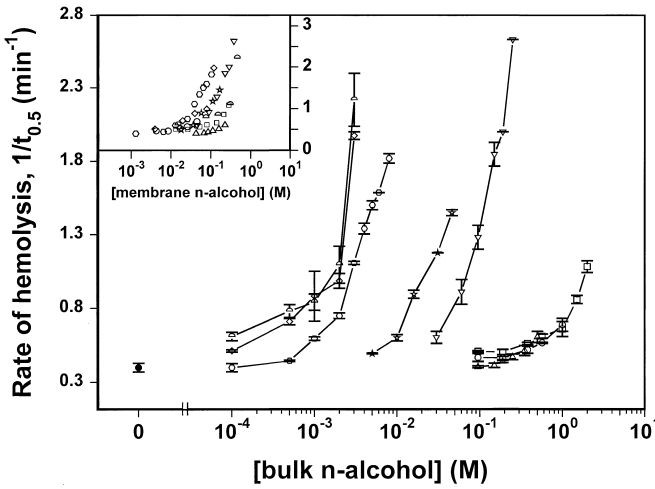

Fig. 2 Effect of n-alcohols on the rate of equinatoxin II-induced hemolysis. At various bulk concentrations of alcohols, the rate of hemolysis $1/t_{0.5}$ was obtained as shown in Fig. 1. Alcohols from methanol to n-octanol are numbered accordingly to C-atoms. *Open symbols* and *vertical bars* indicate mean ± s.d. of 3–4 measurements, *solid circle* is mean ± s.d. of 16 hemolytic experiments without alcohol. In the *inset*, the same data are reported as a function of the membrane alcohol concentration, instead of the bulk concentration, using Eq. (3) and published partition coefficients (Seeman 1972)

3. Results

Alcohols, used at concentrations not producing hemolysis by themselves (Seeman 1972), regularly enhanced EqTx II-induced hemolysis of bovine erythrocytes. Representative time courses of hemolysis are shown in Fig. 1 for n-heptanol. As compared to the control without alcohol, the half-time for 50% hemolysis, $t_{0.5}$, was shortened by the alcohol without changing the sigmoid shape of the time course. The effect of a series of n-alcohols on the rate of hemolysis is summarized in Fig. 2. It is apparent that the potency of alcohols increases both with their bulk concentration and with their chain-length. Thus, on the basis of its bulk concentration, n-octanol appeared to be more effective than short-chained methanol, ethanol, and n-propanol by about four orders of magnitude. However, it is important to note that these differences were largely eliminated if the membrane, rather than the bulk, concentration of the additive was considered (Fig. 2 inset). This was done by taking into account the membrane partition coefficients for alcohols (Seeman 1972). Such enhance-

ment of hemolytic activity is not limited to EqTx II. We observed the same effect with two other polypeptidic cytolysins, i.e. melittin and *S. aureus* α-toxin (Table 1). Notably, the lower the molecular weight of the toxin, the larger is the enhancement of hemolysis by alcohols.

The permeabilization of SUV by EqTx II was also promoted by alcohols. Kinetics of calcein release in the presence of different amounts of methanol are shown in Fig. 3. The dependence of the percentage of final release on the dose of different n-alcohols is reported in Fig. 4. In the present experimental conditions, less than 5% of calcein release was usually achieved with the toxin alone, while in the presence of alcohols the release could reach 100%. As for hemolysis, the potency of alcohols, based on their bulk concentration, uniformly increased in the order C_1 to C_8 but the differences largely disappeared when their membrane concentration was considered (Fig. 4 inset).

Table 1 Promotion by alcohols of the rate of hemolysis induced by pore-forming toxins

Alcohol	Melittin 3.5 µg/ml	Equinatoxin II 60 ng/ml	*S. a.* α-toxin 2.0 µg/ml
No alcohol	1 (0.50 min^{-1})	1 (0.41 min^{-1})	1 (0.20 min^{-1})
Methanol, 2 M	10	2.9	4.2
Ethanol, 1 M	3.3	2.7	2.0
n-propanol, 1 M	20	4.6	1.9
n-butanol, 100 mM	6.7	2.9	1.2
n-pentanol, 25 mM	4.0	2.2	1.4
n-hexanol, 2 mM	10	3.0	1.8
n-heptanol, 1 mM	4.5	2.7	1.5
n-octanol, 1 mM	4.5	1.6	1.5

Rate of hemolysis $1/t_{0.5}$ was determined with bovine erythrocytes as described in Materials and Methods using toxins at the indicated final concentration. For each toxin, the control rate shown in brackets was used to calculate the relative rates reported. Numbers are representative of 1–3 replicates. The variation was ±8% of the mean

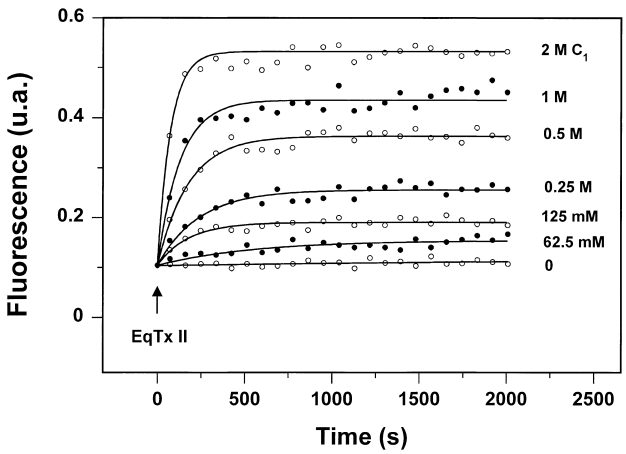

Fig. 3 Time course of equinatoxin II-induced permeabilization of SUV in the presence of methanol. In a 96-well microplate, calcein loaded POPC/SM 9:1 SUV (2 µg/ml in 130 mM NaCl, 20 mM Tris.HCl, 1 mM EDTA, pH 8.0) were preincubated with different amounts of methanol as indicated. After 2 min vesicles were treated with EqTx II (indicated by an *arrow*) to a final concentration of 5 µg/ml and the fluorescence change was recorded as described in Materials and methods. 100% permeabilization was achieved by adding 4 µl of 100 mM Triton X-100. Solid lines are best fit to a monoexponential function

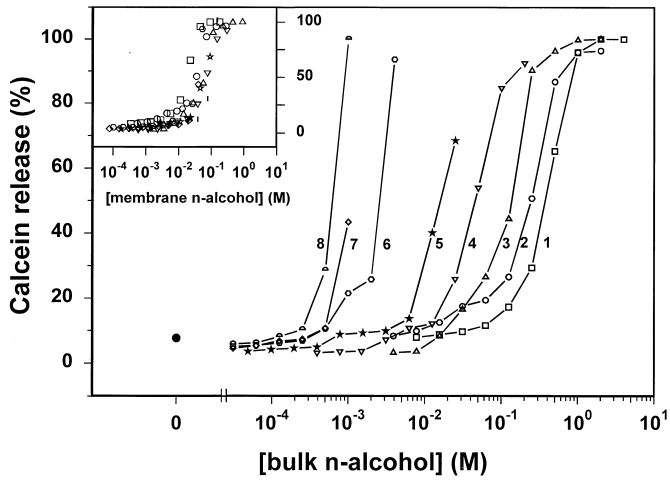

Fig. 4 Effect of n-alcohols on equinatoxin II-induced permeabilization of SUV. In a 96-well microplate assay, as described in Fig. 3, SUV were preincubated with a series of alcohols, from methanol to n-octanol, labeled as in Fig. 2. After 2 min, vesicles were treated with EqTx II (final concentration 5 µg/ml) and the percentage of release after a 45 min incubation was calculated using the 100% permeabilization value obtained with Triton X-100. Shown is one representative experiment of a series of three which gave similar results. Numerals refer to the number of alcohol C-atoms. In the *inset*, the data are reported as a function of the membrane alcohol concentration as in Fig. 2

As we have recently described (Maček et al. 1995), combining POPC/SM 1:1 SUV with EqTx II resulted in an increase in the intrinsic fluorescence of the toxin (here approximately 60–70%) as indicated by the relative fluorescence intensity ratio F/F_0 (curve 0 in Fig. 5). The increase of F/F_0 occurs within a few of seconds, and appears to represent toxin binding to the lipid layer. Alcohols and fatty acids additionally increased this ratio in a concentration dependent manner. The time courses of such increases are shown in Fig. 5 for the case of n-heptanol. Effect of n-alcohols on $(F/F_0)_{max}$ derived by fitting the time courses to Eq. (2) are presented in Fig. 6 A bimodal effect of alcohols, in particular those of C_1 to C_5, on $(F/F_0)_{max}$ could also be observed, which was not the case either in hemolysis (Fig. 2) or in vesicle permeabilization (Fig. 4).

Saturated $C_{16:0}$ and 9-cis-unsaturated $C_{16:1}$ modified the toxin induced permeabilization of the cells and SUV at bulk concentrations which were about two orders of magnitude lower than those of the most potent alcohol, i.e. n-octanol. Furthermore, $C_{16:0}$ exhibited a unique effect on EqTx II-induced hemolysis. Up to a micromolar concentration it accelerated hemolysis, whereas at higher concentrations inhibition occurred, bringing the activity to a level even lower than the control (Fig. 7A). SUV permeabilization was enhanced by the unsaturated $C_{16:1}$ but apparently not by $C_{16:0}$ (Fig. 7B). In some replicates (not shown) an increase of permeabilization was indeed detected with $C_{16:0}$; however, the unsaturated fatty acid was always consider-

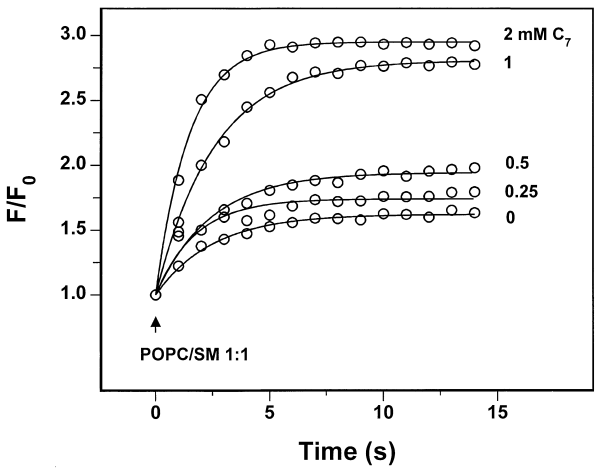

Fig. 5 Time course of equinatoxin II binding to SUV in the presence of an n-alcohol. The intrinsic fluorescence of EqTx II (5 μg/ml in 130 mM NaCl, 20 mM Tris.HCl, 1 mM EDTA, pH 8.0) was measured after the addition of 5 μg/m POPC/SM 1:1 SUV (indicated by the *arrow*) in the presence of the indicated concentration of n-heptanol, C_7. Excitation and emission were at 295 and 335 nm, respectively. *Curves* represent best fit of experimental points to Eq. (2), from which asymptotic values of F/F_0, i.e. $(F/F_0)_{max}$ were extracted (see Materials and methods for details)

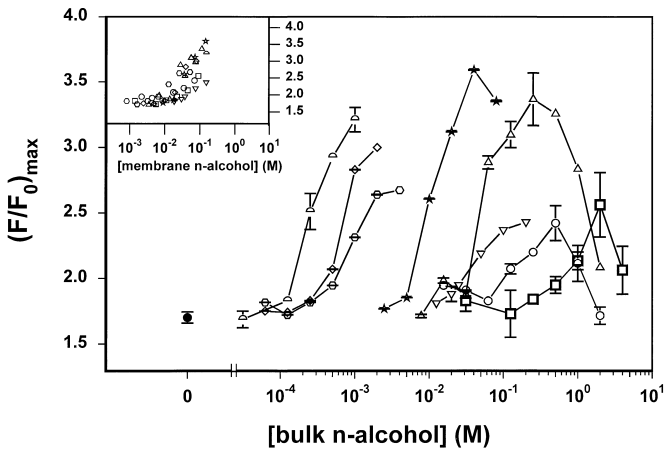

Fig. 6 Effect of n-alcohols on equinatoxin II binding to SUV. The increase of equinatoxin II intrinsic fluorescence upon addition of SUV (determined as described in Fig. 5) is given in the presence of n-alcohols which are designated by symbols as in Fig. 4. Maximal values of F/F_0 were obtained by fitting experimental data to Eq. (2) (see Materials and methods). Single measurements (*open symbols*) or means ± s.d. of 3–4 experiments (*open symbols* with *vertical bars*) are presented. Solid circle is control without additives and represents the mean ± 2 s.d. of 16 separate experiments. In the Inset, the same data are reported as a function of the membrane alcohol concentrations as in Fig. 2

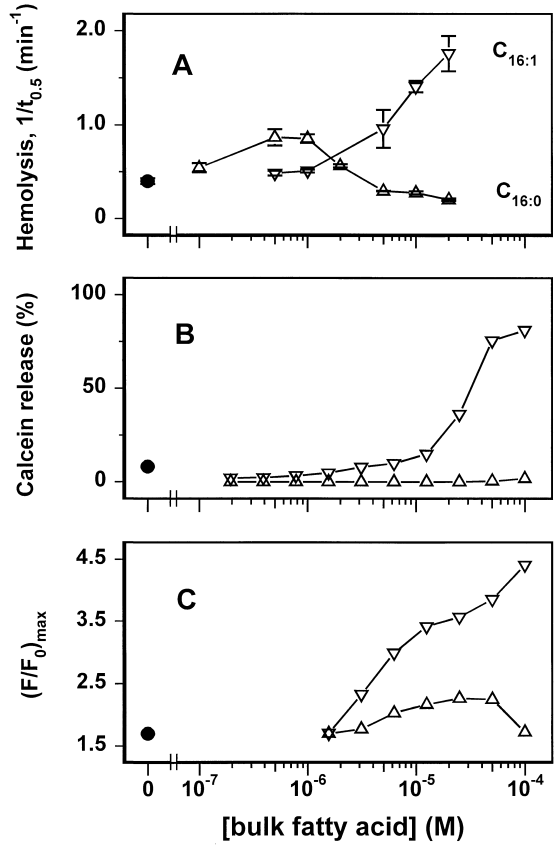

Fig. 7A–C Effect of palmitic ($C_{16:0}$) and palmitoleic ($C_{16:1}$) acid on the interaction of EqTx II with natural and model membranes. **A** Rate of toxin-induced hemolysis in the presence of various amounts of $C_{16:0}$ and $C_{16:1}$. n-Propanol was also present as a solvent for the fatty acids, always at a concentration well below that giving any effect by itself. Except for the use of fatty acids all other conditions and statistical evaluations are as in Fig. 2. **B** Effect of $C_{16:0}$ and $C_{16:1}$ on toxin-induced permeabilization of POPC/SM 9:1 SUV measured as a percentage of calcein release. Experimental conditions are the same as described in Fig. 4. A representative experiment of three similar ones is shown. **C** Effect of $C_{16:0}$ and $C_{16:1}$ on equinatoxin II binding to POPC/SM 1:1 SUV. Except for the additives, all other experimental conditions and statistical procedures are the same as in Fig. 6. *Solid circle* with *vertical bars* is mean ± 2 s.d. of 16 separate experiments

ably more active. A remarkable difference between the saturated and unsaturated fatty acid could also be noticed in the binding of EqTx II to SUV (Fig. 7C). At the bulk concentrations assayed, $C_{16:0}$ was much less potent than $C_{16:1}$ in increasing the intrinsic fluorescence of the toxin. It had a maximum effect around 50 μM, while there was no decline with palmitoleic acid.

4. Discussion

Cnidarian cytolysins use sphingomyelin as a membrane acceptor (Bernheimer and Avigad 1976; Linder et al. 1977); however, it was shown that membrane permeabilization is also dependent on the fatty acid composition of the membrane. In particular, the occurrence of unsaturated phospholipids facilitated the interaction (Michaels 1979). Recently, we noticed that calcein release from SUVs was markedly slower if normal fatty acid in phosphatidylcholine was replaced, even in small percentages, by the bulky pyrene-decanoic acid (Maček et al. 1995). These results suggest that the physical state of the bilayer can be important in modulating toxin-membrane interaction and pore formation.

We investigated these effects by modifying membrane properties with a series of lipophilic additives. We used n-alcohols and fatty acids, all known to be weak anesthetics, at concentrations not hemolytic by themselves (Seeman 1972; Brahm 1983; Chi et al. 1992) and not producing phospholipid flip-flop (Schwichtenhoevel et al. 1992). Interestingly, these concentrations overlapped those reported to protect erythrocytes from hypo-osmotic hemolysis (Seeman 1972). In contrast to this, n-alcohols and palmitoleic acid enhanced SUV permeabilization and hemolysis induced by EqTx II which has been shown to be of a colloid-osmotic type (Zorec et al. 1990; Belmonte et al. 1993).

Except for palmitic acid, the enhancement of permeabilization of both red blood cells and SUV correlates well with the increase of toxin intrinsic fluorescence upon combination with the SUV. We have recently shown that such an increase results from the partitioning of EqTx II into the lipid phase, close to the interphase (Maček et al. 1995). Accordingly, it appears that the presence of membrane additives increases the amount of toxin inserted into the bilayer, implying that it lowers the energy of the toxin in the bound state.

Four our case, modification of thickness, electrostatic phenomena, and fatty acid acyl-chain ordering of the lipid bilayer (see review Cevc 1990) by the additives, and on the other hand, determinants of protein insertion into lipid bilayers (Ben-Tal et al. 1996) could be important. In fact, a lowered membrane thickness seems to be very likely because the concentrations of alcohols that we used were actually those producing bilayer interdigitation (Yamazaki et al. 1992; Zeng et al. 1993; Yamazaki et al. 1994; Vierl et al. 1994; Löbbecke et al. 1995). The decline of $(F/F_0)_{max}$ at higher concentrations of alcohols, seen in Fig. 6, derives from the enhanced polarity of the tryptophan environment (Lakowicz 1983) and not from reduced toxin insertion as inferred from hemolysis and calcein release experiments. The fluorescence quenching is consistent with a location of the tryptophans in the glycerol backbone region of the lipid membrane, that, as a consequence of interdigitation, becomes more rich in alcohols and water (Brasseur et al. 1985; Barry and Gawrisch 1994; Vierl et al. 1994). In membranes, quenching or dequenching of fluorescence produced by alcohols has already been observed using lipid soluble fluorescence probes confined to the interphase region (Rottenberg 1992), and it has been interpreted as a consequence of lipid interdigitation (Löbbecke et al. 1995). Moreover, we found maxima of the tryptophan fluorescence (see Fig. 6) at around 2 M methanol, 500 mM ethanol, 300 mM n-propanol, 200 mM n-butanol, 50 mM n-pentanol, i.e. the concentrations very similar to those reported as inducing complete interdigitation in DPPC-vesicles (Löbbecke et al. 1995).

The membrane energetic barrier accounted for by membrane electrostatic potential(s) (Cevc 1990) may also be influenced by alcohols via lowering of the actual dielectric constant of the lipid interphase and hydrophobic core. At present, the contribution of particular membrane associated electrostatic potentials, such as surface, hydration, transmembrane, and dipole potential, as well as of those accounted for by electrostatic properties of the toxin itself are unknown. In fact, reduction of the effective membrane dipole potential by anesthetics, due to the lowered membrane dielectric constant and dipolar screening of the membrane dipoles, has been reported (Qin et al. 1995). The impact of the membrane surface potential is very likely of minor importance as EqTx II activity is not sensitive to ionic strength (Maček, unpublished data), neither was the transmembrane potential significant for toxin insertion into planar lipid membranes (Belmonte et al. 1994). It is most likely, therefore, that the main contribution to the change of free energy of the toxin insertion into lipid bilayer arises from a solvation free energy as suggested for a model α-helical peptide (for details see Ben-Tal et al. 1996). It accounts for electrostatic contributions resulting from changes in the solvent dielectric constant as well as for van der Waals and solvent structure effects.

On the basis of the difference in action between saturated palmitic acid and unsaturated palmitoleic acid, it appears that lipid acyl-chain ordering might also modulate toxin insertion and/or membrane penetration. Unlike with alcohols, unsaturated palmitoleic acid (see Fig. 7) does not exhibit the bimodal effect on the toxin intrinsic fluorescence while it accelerates permeabilization. This is consistent with the fact that only short chain amphiphilic molecules, for example n-alcohols up to 6–8 carbons long, induce membrane lipid interdigitation. The effects of palmitic acid seen in Fig. 7 and their correlation with the low permeabilization of SUV and hemolysis could reflect reduced toxin associated with lipids. Therefore, we suggest that palmitic fatty acid when partitioned into the POPC bilayer or erythrocyte membrane strengthens the cohesion of the hydrophobic core while the unsaturated one, in contrast, perturbs lipid chain packing. These phenomena account for the variation in lateral membrane pressure opposing the insertion of the polypeptide chain. The same modulating effects of partitioned fatty acids, obviously correlated with the main transition temperature of the targeted vesicle lipids, has been very recently reported for melittin interaction with POPC and DPPC vesicles (Benachir and Lafleur 1996; Monette and Lafleur 1996).

In conclusion, our experiments demonstrate that additives soluble in lipid membranes, such as n-alcohols and fatty acids, may markedly modulate the activity of a pore-forming toxin by thinning of the lipid bilayer, and by modulating lipid acyl-chain packing. Also, it is implicit that varying the transmembrane profile of dielectric constant by partitioned additives may influence protein insertion. Yet, one should be aware of the interdependence of the described membrane parameters, in particular if membrane (Cevc 1990) or protein (Eisenberg 1996) associated electrostatic phenomena are under consideration. The experimental results also suggest that lipid interdigitation may occur in cell membranes, at least in vitro.

Our findings may have a wider significance for other pore-forming toxins as we have already shown in Table 1 or in general for the mechanism of membrane transloca-

tion of proteins, and of viral fusion. Moreover, they suggest one possible reason for the observed synergism operating in some pathological situations between pore-forming toxins and phospholipases which liberate free fatty acids.

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