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Voltage-dependent interaction of the peptaibol antibiotic zervamicin II with phospholipid vesicles

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Abstract The effect of a transmembrane potential on ion channel formation by zervamicin II (ZER-II) was studied in a vesicular model system. The dissipation of diffusion potential caused by addition of ZER-II to small phosphatidylcholine vesicles was monitored using fluorescent (Safranine T) and optical (Oxonol VI) probes. *Cis*-positive potentials facilitated channel formation, while at *cis*-negative potentials, ion fluxes were inhibited. A potential-independent behavior of ZER-II was observed at high peptide concentrations, most likely due to its membrane modifying property.

Key words: Ion channel; Transmembrane potential; Phosphatidylcholine vesicle

1. Introduction

Zervamicins (ZER), the secondary metabolic products of the fungus *Emericellopsis salmosynnemata*, are generally considered to belong to the family of voltage-gated ion channel forming peptaibols [12]. These channels are thought to be formed by self-assembly of amphoteric molecules to an oligomeric cluster of transmembrane helices with the hydrophilic atoms exposed inside the cavity of the channel. Compared to alamethicin (ALM), the most intensively investigated representative peptaibol antibiotic, the non-ionic ZER (ZER-II) received relatively little attention, in spite of that it might be a better model to study the mechanism of ion channel formation by molecules having a higher proportion and a more regular distribution of hydrophilic residues:

ZER-IC, the minor product (10%) of *E. salmosynnemata*:
Ac-Trp-Ile-*Glu*-Iva-Ile-*Thr*-Aib-Leu-Aib-*Hyp*-*Gln*-Aib-*Hyp*-Aib-Pro-Phl

ZER-IIA, the major product (45%):
Ac-Trp-Ile-*Gln*-Aib-Ile-*Thr*-Aib-Leu-Aib-*Hyp*-*Gln*-Aib-*Hyp*-Aib-Pro-Phl

ZER-IIB, the major product (45%):
Ac-Trp-Ile-*Gln*-Iva-Ile-*Thr*-Aib-Leu-Aib-*Hyp*-*Gln*-Aib-*Hyp*-Aib-Pro-Phl

ALM:
Ac-Aib-Pro-Aib-Ala-Aib-Ala-*Gln*-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-*Glu*-*Gln*-Phl

The voltage activation of ZERs was studied by conductance measurements of planar bilayers of which one side (*cis*-side) was exposed to the peptide solution [2]. For non-ionic peptaibols like ZER-IIA and ZER-IIB (the structures of these hexadecapeptides vary slightly at position 4), only *cis*-positive potentials induce channel formation that closely resembles the macroscopic conductances observed for ALM that carries a negative charge at the C-terminal end of the molecule [11]. The observed asymmetric current-voltage relationship might be explained by assuming specific binding of the N-terminal end of the molecule to the membrane, followed by (i) insertion of the whole macro-dipolar (or charged) molecule into the hydrophobic pocket of the membrane (driven by electrostatic forces induced by the transmembrane electric field) and (ii) aggregation of the amphoteric helices parallel to the external electric field. This hypothesis has been confirmed by conductance measurements using ZER-IC, containing a negatively charged residue near the N-terminus. In contrast to ALM (having a negative charge at the C-terminus), ZER-IC is activated only by *cis*-negative potentials [2]. For full understanding of the process of ZER-induced pore formation at the atomic level, it is necessary to obtain information in situ about the nature of the voltage-dependent step in ion channel formation. A very efficient approach to experimental elucidation of structure–function relationships is one based on non-invasive spectroscopic techniques. For example, solid state ^{15}N-NMR spectroscopy could provide valuable structural information about the orientation of molecules in oriented phospholipid membranes [3] and for this reason, we started a program to label ZER-II (a 1:1 mixture of ZER-IIA and ZER-IIB) with stable isotopes [6,9]. In order to get information about the structure of the molecular cluster, we have developed a new pulsed EPR spectroscopic technique (the so-called PELDOR technique) [8]. However, up to so far, methods to investigate ion channel formation at the atomic level in the presence of a membrane potential are still lacking.

In addition to conductance measurements on planar lipid membranes, the lipid vesicles represent another model system to study the process by which membrane-active agents insert into the lipid bilayer. To elucidate the effect of the potential sign, the vesicular system is particularly convenient, as a required positive or negative transmembrane potential can be easily installed with a K^+ gradient in the presence of valinomycin (VAL). For ALM, the results on channel formation in egg phosphatidylcholine (PC) vesicles are comparable with those in planar bilayers [16,17,5]. In the present paper, the results on the behavior of ZER in polarized small PC vesicles are described.

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2. Materials and methods

Isolation of ZERs was performed according to [6] by methanol extraction of the biomass produced by *E. salmosynnemata*. The extract was purified by gel filtration on a Sephadex LH-20 column (eluent: methanol) followed by high performance liquid chromatography separation on a RP-C_{18} column (gradient elution: 50% aqueous methanol–100% methanol) and resulting in a mixture of ZER-IIA and IIB that was further used without separation.

For the small vesicle preparation, a common procedure was used. Lipid film obtained by evaporation of solvent from chloroform/methanol solution of egg L-α-PC (Sigma) was further dried for 30 min at 40°C in vacuo and deaerated aqueous KCl solution was added to a final lipid concentration of 10–25 mg/ml. The dispersion was sonicated with a titanium tip sonificator in an ice bath under a continuous flow of nitrogen gas. The solution was centrifuged at $30\,000\times g$ for 30 min and the supernatant (kept as a stock solution at 4°C) was used within a few days. The lipid concentration was determined by an enzymatic colorimetric method using a commercial available reagent kit (PL MPR2; Boehringer Mannheim).

To produce a K^+ ion gradient across a membrane, the stock solution of vesicles was diluted 100-fold into the appropriate electrolyte solution (for instance a sodium chloride solution, see Section 3), keeping the osmolarity constant. In several preparations, a transmembrane K^+ gradient was created by passing the vesicle solution through a Bio-Gel P-6 (Bio-Rad) column that was preliminary equilibrated with the desired electrolyte solution. The transmembrane potential was developed by adding a small aliquot of VAL in DMSO to a final concentration of 1–2 μM, depending on the lipid concentration. ZER was also added from a stock solution in DMSO. Control experiments showed that the used amounts of DMSO alone do not have any effect. The optical probes Safranine T (Riedel-de-Haën AG, Seelze-Hannover) and Oxonol YI (Molecular Probes) were used as aqueous solutions.

Fluorescence measurements were made on a Spex Fluorolog 2 spectrofluorimeter. Optical spectra were measured with a Perkin-Elmer Lambda 900 UV/VIS/NIR spectrometer.

3. Results and discussion

For vesicles with a K^+ concentration gradient across a membrane, the formation of a transmembrane potential ($\Delta\psi$) is possible by addition of the potassium-specific ionophore VAL to PC vesicles. The magnitude of $\Delta\psi$ can be calculated with the Nernst equation:

$$\Delta\psi = RT/F * \ln([K^+]_{ext}/[K^+]_{int})$$

If $[K^+]_{int} > [K^+]_{ext}$, the potential is positive outside the vesicular membrane, while for the opposite case ($[K^+]_{int} < [K^+]_{ext}$), the potential outside is negative.

As positive potentials were reported to induce ZER channel formation, the vesicles with a high internal K^+ concentration were investigated at first. To monitor a transmembrane potential, which is positive outside, several fluorescent dyes were used in the literature, including the cationic Safranine type [15–17]. To test the potential response of Safranine T, a *cis*-positive transmembrane potential was generated by diluting a stock suspension of vesicles ($[KCl]_{int} = 200$ mM) into KCl–NaCl solutions with different concentrations of KCl and keeping $[KCl]_{ext} + [NaCl]_{ext} = 200$ mM. Each curve of Fig. 1 shows the changes in fluorescence intensity of Safranine T caused by a number of sequential additions. Safranine binding to lipid vesicles (first arrow) results in a small increase of fluorescence which is independent on the value of the K^+ gradient (curves a–d). Subsequent addition of VAL (second arrow) causes a sharp jump of fluorescence followed by a slower phase during which a stationary level is achieved. The potential-induced

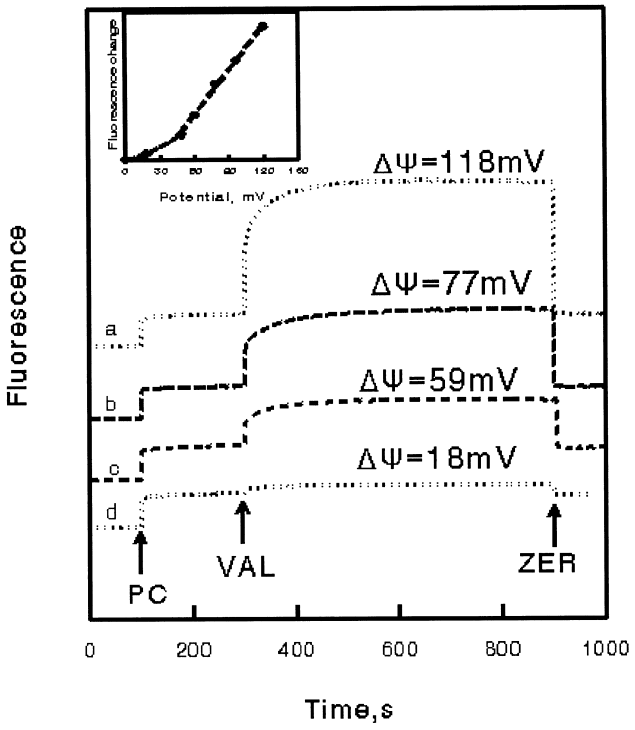

Fig. 1. Time-dependence of Safranine T fluorescence ($\lambda_{ex} = 520$ nm, $\lambda_{em} = 572$ nm). At times indicated by arrows, the following additions were made to 2 ml of Safranine T aqueous solution: PC, VAL and ZER. Egg PC vesicles (20 μl) loaded with $[KCl]_{int} = 200$ mM and 5 mM sodium phosphate (pH 7.0). The resulting external concentrations were: $[KCl]_{ext}$: 2 mM (a), 10 mM (b), 20 mM (c), 100 mM (d); [NaCl]: 198 mM (a), 190 mM (b), 180 mM (c), 100 mM (d). All solutions also contained 5 mM sodium phosphate (pH 7.0). VAL (20 μl in DMSO): the resulting values of $\Delta\psi$ are indicated. ZER (20 μl in DMSO). The final concentrations of components are: Safranine 1 μM, VAL 2 μM, ZER 1.7 μM, PC 0.15 mg/ml. Inset: The dependence of Safranine T VAL-induced fluorescence enhancement on a potential (positive outside) across the egg PC vesicular membrane.

Safranine fluorescence enhancement is due to accumulation of the lipophilic cationic dye at the negatively charged interior of the vesicular membrane [15]. At a small concentration of VAL, the fluorescence remains stable for a long time, what proves that no ion leakage occurs through the vesicular membrane. The magnitude of VAL-induced increase of Safranine fluorescence is a function of $\Delta\psi$ with a linear dependence in the range 50–120 mV (Fig. 1, inset). A similar potential response was reported in the literature [15] for Safranine O in the presence of PC-cholesterol vesicles.

At all transmembrane potentials (positive outside), the addition of ZER (Fig. 1, third arrow) leads to a sharp drop of Safranine fluorescence due to dissipation of $\Delta\psi$ caused by sodium ion influx via the formed ion channels. Note that at the applied ZER concentrations (1–2 μM), the fluorescence drops to the initial level within a few seconds, demonstrating that installed $\Delta\psi$ is completely abolished. However, at lower ZER concentrations (< 0.5 μM), the decay of fluorescence is much slower (because a lower number of ion channels are formed) and after ca. 30 min, the curve levels off at a significant higher value (Fig. 2).

The most straightforward explanation of the latter observation is that channels are formed only in a fraction of the total

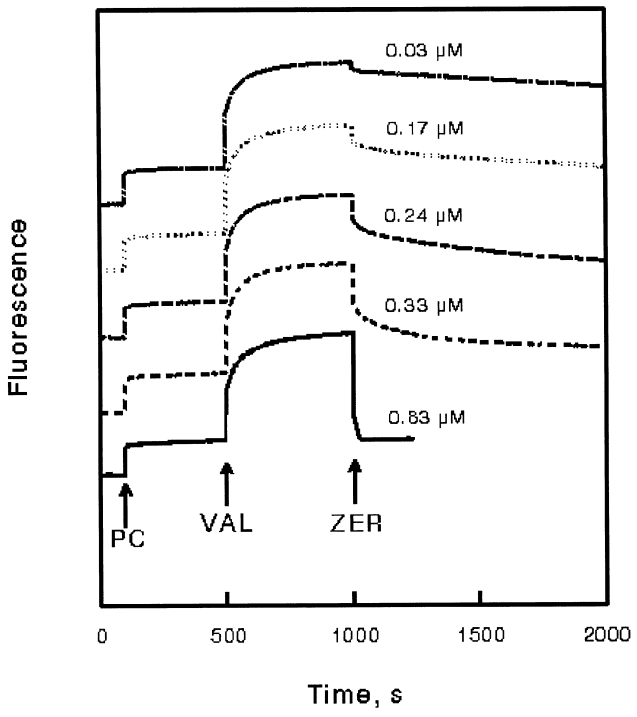

Fig. 2. Dissipation of a transmembrane potential ($[KCl]_{int} = 200$ mM, $[KCl]_{ext} = 6$ mM, $[NaCl]_{int} = 194$ mM, $\Delta\psi = 90$ mV, positive outside) caused by addition of different concentrations of ZER. Concentrations: 0.15 mg/ml (PC), 1 μM (Safranine), 2 μM (VAL).

available number of vesicles. In terms of the barrel stave theory [7] wherein ion channels are assumed to be formed by association of a minimum number (4–8) of peptaibol molecules [12], it means that at a low ZER concentration, only a fraction of vesicles contains the minimum number of molecules that is needed to form a channel. On the other hand, the number of peptaibol molecules that is bound to the remaining vesicles is less than the critical number needed to form a channel and for this reason, the VAL-induced potential cannot become dissipated.

Another reason for incomplete dissipation of $\Delta\psi$ by a voltage-dependent peptide, like ZER, may be that channels get closed by reaching a so-called threshold potential, i.e. the minimum potential needed to form a channel. Although a concentration-dependent threshold potential cannot be excluded, this view seems to be in contradiction with the information obtained from the former experiment that has been carried out with a high ZER concentration (Fig. 1), where the peptaibol is shown to induce formation of ion channels in the potential range of 18–118 mV, while the remaining values of $\Delta\psi$ in Fig. 2 correspond to 30–80 mV.

The experiments performed so far prove that ZER forms ion channels in a PC membrane, in spite that it is not clear if this process is potential-dependent. It might be that channels are formed even in the absence of any potential. In order to elucidate this question, the results of the following two parallel experiments should be compared (Fig. 3). When 0.12 μM ZER solution is added to a vesicular solution having a KCl concentration gradient across the membrane, about 2 h prior

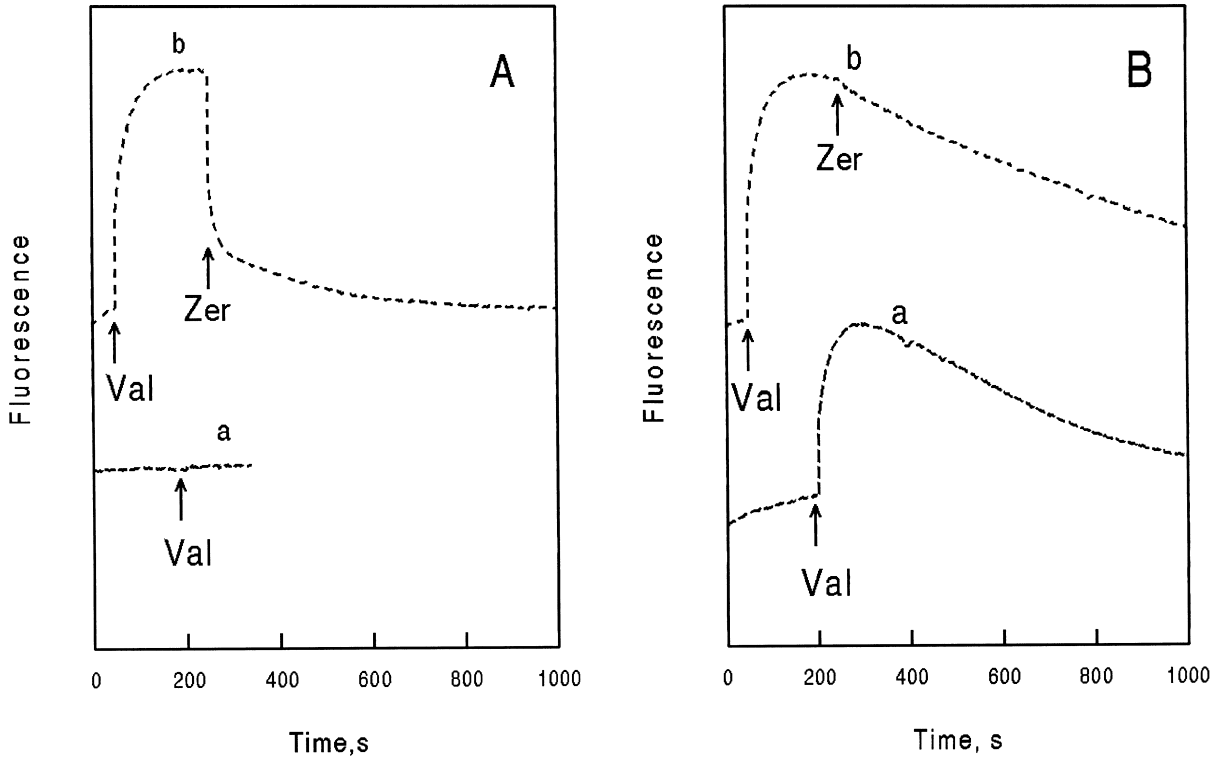

Fig. 3. Time-dependence of Safranine T fluorescence. PC vesicles with a K^+ gradient ($[KCl]_{int} = 100$ mM, $[KCl]_{ext} = 1$ mM, $[NaCl]_{ext} = 99$ mM) were treated with ZER, 2 h before adding VAL (curves a). ZER was added to PC vesicles with $\Delta\psi = 120$ mV preliminary installed with VAL (curves b). Concentrations of ZER: 0.12 μM (A), 0.01 μM (B). The final concentrations of components are: Safranine 1 μM, VAL 1 μM, PC 0.05 mg/ml. It should be noted that the more effective action of ZER as shown by curve Ab at a concentration similar to one of the curves shown in Fig. 2 is due to the lower lipid content and a higher value of installed transmembrane potential.

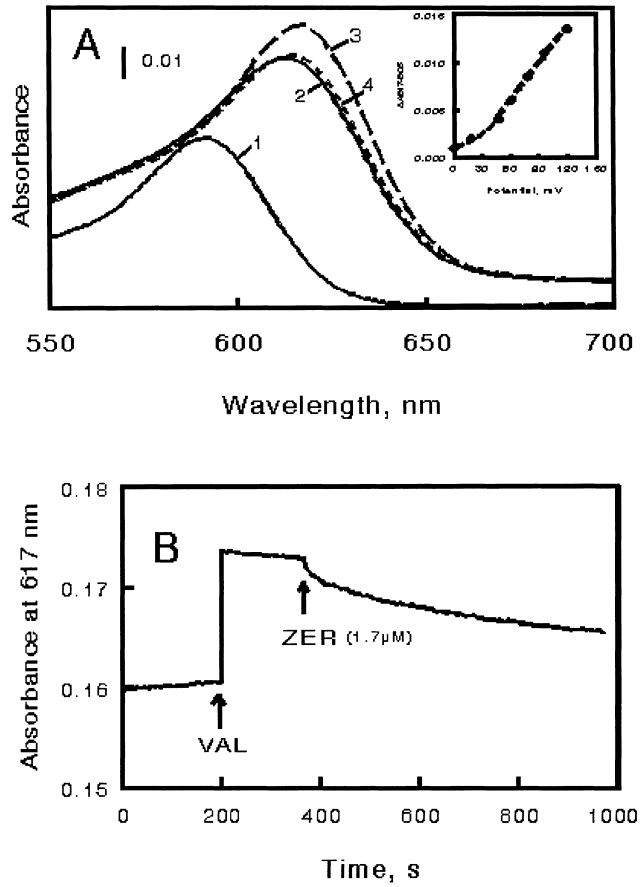

Fig. 4. (A) Absorbance spectra of Oxonol YI (~1 μM) in water (100 mM KCl, 100 mM NaCl) (1). After sequential addition to 2 ml of this solution of 20 μl PC vesicles (loaded with 2 mM KCl and 198 mM NaCl) (2), after addition of 20 μl of VAL in DMSO ($\Delta\psi$= 100 mV, negative outside) (3), 10 min after addition of 20 μl of ZER in DMSO (4). The final concentrations were: VAL 2 μM, ZER 1.7 μM, PC 0.17 mg/ml. Inset: Oxonol YI absorbance changes as a function of a transmembrane potential negative outside. Absorbances were measured ~10 s after adding VAL. (B) Time-dependence of Oxonol YI absorbance. At times indicated by arrows, the following additions were made to 2 ml of PC vesicular solution (PC 0.17 mg/ml, $[KCl]_{int}$ = 2 mM, $[NaCl]_{int}$ = 198 mM, $[KCl]_{ext}$ = 200 mM): Val: VAL (20 μl in DMSO), the resulting value $\Delta\psi$= 118 mV. ZER: ZER (20 μl in DMSO). The final concentrations of components were 2 μM (VAL), 1.7 μM (ZER).

to VAL, no rise of Safranine fluorescence is observed (Fig. 3A, curve a), while complete dissipation of potential occurs when ZER is added after the K^+ ionophore (Fig. 3A, curve b).

The result may be explained in two different ways: (i) no potential is required for channel formation and (ii) the process of channel formation is too fast to be detected at the applied ZER concentration. In order to get more insight into this process, the next experiment has been performed at a lower peptide concentration (0.01 μM), where the decay of fluorescence is expected to be much slower. Curve a of Fig. 3B shows that upon addition of ZER prior to K^+ ionophore, the fluorescence increases after addition of VAL to a level which is smaller than for untreated vesicles (Fig. 3B, curve b). Thus, even in the absence of a transmembrane potential, in a fraction of vesicles, some ion leakage may occur due to membrane modifying properties of ZER as has been reported for other (short) peptaibols [1]. However, in the remaining intact fraction, the potential installed by addition of VAL is not stable (like in the experiments shown in Fig. 1). The decrease of fluorescence (Fig. 3B, curve a), representing dissipation of $\Delta\Psi$, is almost the same as when the peptaibol is added after the K^+ ionophore (Fig. 3B, curve b). Thus, for low concentrations of ZER, the installation of $\Delta\Psi$ leads to formation of new ion channels.

To study the possibility of ZER channel formation at *cis*-negative potentials, vesicles with a low internal concentration of K^+ ion (2 mM) were prepared. The external concentration of K^+ was varied in the range 2–200 mM, keeping the isotonicity constant by addition of NaCl. Monitoring of the transmembrane potential (negative outside) was performed with the anionic optical probe Oxonol YI [14,13]. Oxonol binding to PC vesicles leads to a spectral shift to a longer wavelength (592→613 nm) (Fig. 4A). The subsequent addition of VAL causes a further red shift (613→617 nm) with a simultaneous increase of absorbance. The changes in absorbance at 617 nm are linearly proportional to a transmembrane potential (positive inside the vesicular membrane) in the range 40–120 mV (Fig. 4A, inset).

The addition of ZER to vesicles with a potential installed across a membrane (negative at the side of added peptide) gradually reverses the spectral shift with a final spectrum close to that observed before addition of the K^+ ionophore (Fig. 4A). Thus, for a *cis*-negative transmembrane potential (negative at the ZER-containing side), dissipation of $\Delta\psi$ is also observed. However, under similar conditions (in respect to peptide and lipid concentrations), the dissipation of the *cis*-negative potential caused by ZER is much slower compared to its action at *cis*-positive values of $\Delta\psi$. The time-dependence measurements of Oxonol absorbance changes caused by subsequent addition of VAL and ZER (Fig. 4B) can be compared with the data on Safranine fluorescence changes (Figs. 1 and 2). While for positive outside potentials at ZER concentrations above ~0.5 μM, $\Delta\psi$ was completely abolished within a few seconds, for a *cis*-negative potential (118 mV), the potential drop was not finished even after ~10 min. In fact, ZER channel formation is even inhibited by *cis*-negative potentials compared to the situation when no potential is installed at all. Indeed, under similar conditions at such high concentrations of ZER (1.7 μM), the time required for channel formation at $\Delta\psi\rightarrow 0$ is within a few seconds (Fig. 1), while at all negative $\Delta\psi$ values, the potential drop takes place in a minutes time scale.

The obtained data can explain the results on ZER-induced conductivity of diphytanoyl PC planar membrane where the current was observed only at *cis*-positive potentials [2]. Much slower interaction of ZER with lipid membrane at *cis*-negative potentials may be the reason why short time (~10 s), negative potential pulses were not effective. Thus, the obtained results support the idea on a potential-dependent nature of ZER interaction with a lipid membrane. The channel formation is stimulated by a positive potential from the side of added peptide and suppressed by a negative outside $\Delta\psi$. At high concentrations of added ZER, no potential is required for channel functioning. The obtained results may be well understood in terms of peptide water bilayer partition facilitated by the electric field installed across a membrane [10]. While for high concentrations of ZER in a fraction of vesicles, ion leakage occurs by the membrane modifying property even

without any potential, for lower amounts of ZER, the installation of $\Delta\Psi$ causes the additional penetration of peptide into the lipid phase followed by channel formation. Indeed, the potential-independent ion fluxes were also observed in the egg PC-cholesterol vesicular system for ALM at $\sim$2.5 μM [17] and in dioleoylphosphatidylcholine vesicles at 3.3 μM ALM [17]. Recent (not yet published) solid state ^{15}N-NMR experiments with (^{15}N-Aib)$_5$-ZER bound to planar oriented 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine membranes with different degrees of hydration indicate orientational distributions (with a large proportion of an in-plane orientation) of ZER molecules [4]. Thus, in the absence of a transmembrane potential, no stable transmembrane helices are observed and it might be that under this condition, ZER does not form channels but acts as a ion carrier.

If one assumes that the voltage-dependent step in channel formation is insertion of a peptide into the lipid bilayer [10], then, the results of the present study show that ZER incorporation into the lipid phase is promoted by potentials positive from the side of added peptide. Assuming that ZER-II helix represents a dipole with partial positive and negative charges at the N- and C-terminus, respectively [12], the penetration of the peptide N-terminal end inside the membrane is more effective for channel formations.

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