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Membrane destabilization assay based on potassium release from liposomes

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Abstract

Inorganic ions are highly suitable markers for monitoring release of the inner content of liposomes. In the present study, a potassium (K^+) selective electrode was used to evaluate the rate of K^+ release from large unilamellar vesicles (LUV). The developed method is highly sensitive, reproducible and inexpensive. Since the K^+ ion is smaller than other markers conventionally used, the method described is more sensitive than one of the standard methods that uses ANTS/DPX. In addition, the method allows us to expand the set of molecules used as inner content markers to a lower size range. The experimental protocol we described contains improvements on the method of Breukink et al. (Biochemistry, 36 (1997) 6968). Our developed method was applied to compare the destabilizing activities of two amphipathic peptides of natural origin (Melittin and HIV env seg I, 827–851) and of two artificial peptides (Hels 7:11 and 9:9) synthesized de novo by Kiyota et al. (Biochemistry, 35 (1996) 13196). The tested peptides released 20% of the liposomal K^+ in 1 min at peptide-to-lipid ratio of a few mmol per mol of total lipids (LUV sized to 0.2 μm, molar composition is POPC:POPS:Chol 2:2:1).

Keywords: Liposome; Potassium release; Melittin; Membrane–peptide interaction; PEG-coated liposome

1. Introduction

The release of liposomal inner content allows evaluation of membrane destabilizing activity by different compounds. Conventionally, fluorescent dyes (e.g. ANTS/DPX, Calcein/Co^{2+}, Calcein, or Fluorescein at self-quenching concentrations, and FITC-dextran) are used as markers of inner content release [1–5].

In contrast to fluorescent markers, the ubiquity of inorganic ions Cl^- and K^+ allowed us to avoid preliminary loading of the liposomes with high concentrations of fluorescent dyes. The first use the inorganic ions as markers for internal liposomal volume

Abbreviations: ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; DPX, *p*-xylylenebis (pyridinium bromide); FITC, fluorescein-5-isothiocyanate; HIV, human immunodeficiency virus; octyl glucoside, *n*-octyl β-D-glucopyranoside; rTX100, reduced Triton X-100; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (sodium salt); Chol, cholesterol; PEG2k-PE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol) 2000]; Rh-PE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl); NBD-PE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl); LUV, large unilamellar liposomes

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was done by Loew et al. [6]. Changes in K^+ diffusion potential mediated by valinomycin were monitored using the voltage-sensitive fluorescent dye dis-C2-(5). The method was used to study pore formation in multilayer liposomes. This method was not widely accepted possibly because of the complex data analysis required [6].

Shai and colleagues [7] monitored the release of ions Tl^+ and IO_3^- to evaluate the membrane activity of pardaxin and its analogs. Pulse polarography was used to monitor the ion release. The conclusion obtained in these studies indicated that the size of an ion and not its charge is essential for membrane permeability in the presence of the membrane-active peptides.

Later, Gruber et al. [8] successfully used chloride ions to evaluate the internal volume of liposomes. This group overcame a set of technical pitfalls and developed a more applicable method for the evaluation of internal liposomal volume using Cl^- anions as markers. Furthermore, Breukink et al. [9,10] used the K^+ ions release to evaluate destabilizing activity of the antimicrobial drug nisin, but the details of the method were omitted [9].

In the present study, we developed and applied the method to quantitate of the rate of K^+ release from large unilamellar liposomes. The conventional membrane-active peptide Melittin was used to develop the method. The developed protocols were used to compare the destabilizing activities of amphiphilic peptides of viral and artificial origin.

A number of amphiphilic peptides show membrane-destabilizing activities (reviewed in e.g. [11]). The peptide corresponding to the amphiphilic cytoplasmic fragment of the envelope protein from HIV seg I (HIV-1 gp41, fragment 827–851) has one of the highest amphiphilicity indices among proteins analyzed [12]. We chose to test this peptide in our assay. The membrane effects of Melittin and HIV seg I were compared with the effects of the two amphiphilic peptides (Hel 7:11 and Hel 9:9) designed de novo by Kiyota et al. [13].

In addition, the membrane destabilizing activities of these peptides were also tested in liposomes containing 5 mol% of PEG2k-PE. The steric protection of liposomes was previously shown to cause significant decrease in the immune response to systemic liposome administration [14,15]. The effect of the liposomal surface PEGylation on the access of the membrane for the fusogenic peptides was evaluated by the K^+ release assay.

Preliminary results were presented elsewhere [16].

2. Materials and methods

2.1. Materials

Melittin, ethyl ether, octyl glucoside, Triton X-100 and reduced Triton X-100 were purchased from Sigma (MO); inorganic salts, from Mallickrodt AR, (KN), HEPES, from USB (OH). POPC, POPS, cholesterol, NBD-PE, and Rh-PE were purchased from Avanti Polar Lipids (AL). HIV seg I peptide was synthesized at the Norris Comprehensive Cancer Center Microchemical Core Facility (University of Southern California, CA). Hels 7:11 and 9:9 were synthesized by QCB (Quality Controlled Biochemicals, MA). The sequences of the peptides are shown in Fig. 1. HIV seg I peptide is poorly soluble in an aqueous solutions. It was diluted and stored in dimethylsulfoxide at −20°C. The peptide concentrations were monitored by UV absorption of tryptophan or tyrosine.

The quality of water is essential for sensitivity of K^+-release method. We used Milli Q Plus water ($R \sim 18$ MOhm). Aqueous solutions contained 100 mM NaCl (Na^+-solution) or 100 mM KCl (K^+-solution) and were supplemented with 2 mM dithiothreitol (to prevent peptide oxidation) and 5 mM HEPES, pH 7.4. All assays were conducted at a room temperature.

For the fluorescent dye-release assay, the solution of 12.5 mM ANTS, 45 mM DPX, and 20 mM HEPES adjusted to pH 7.4 was used instead of K^+-solution.

2.2. Liposome preparation

Lipids were mixed in chloroform at a preset molar ratio. We used mainly a mixture of POPC:POPS:Chol 2:2:1 (standard mixture). The lipid composition was supplemented with fluorescent marker Rh-PE (0.5 mol%) to monitor lipid content by light absorption at 530 nm or with NBD-PE (0.7 mol%) to evaluate the amount of lipids exposed on the outside

of the liposomes. 5 mol% of PEG2k-PE was added in the lipid mixture to form PEG-coated liposomes.

The large unilamellar liposomes were prepared by reverse-phase evaporation followed by extrusion of the initial liposomes through 0.2-μm polycarbonate filter using the mini extruder (Avanti Polar Lipids) [17]. The liposome suspension was centrifuged at 14 000 rpm for 10 min (Eppendorf 5415 C) to remove traces of multilamellar liposomes. The exchange of an external medium of the prepared liposomes for Na^+-solution was done by gel filtration on PD-10 columns (Pharmacia, NJ). The supernatant (1 ml) was loaded and ~1.5 ml of the brightly red washed suspension of the liposomes was harvested from the column.

2.3. Monitoring of unilamellarity of the liposomes

The method developed by McIntyre et al. [18] was used to evaluate unilamellarity of the liposomes. Reduction of NBD-PE by dithionite ($Na_2S_2O_4$) was monitored to evaluate the amount of NBD-PE molecules in outer leaflets of the liposomes. The relative amount of the fluorescent dye was measured using LS-50B spectrofluorimeter (Perkin-Elmer; Ex, 460; Em, 530; slits, 5 nm).

2.4. Setup for K^+ release assay

A sample solution (250–300 μl) was placed in a well of a 96-well plate and stirred constantly by a magnet bar. The concentration of K^+ in aqueous solution ($[K]_{out}$) was measured by MI-442 potassium electrode (Microelectrodes, NH). The reference electrode, SDR-2 (World Precision Instruments, FL) or a homemade Ag/AgCl electrode was connected to the well via agarose bridge filled with Na^+-solution. An electrode voltage was measured by the pH meter 430 (Corning, NY) and recorded by BD112 chart recorder (Kipp and Zonen, Netherlands) as $V_{out}(t)$.

3. Results

3.1. Calibration of the K^+ electrode

Before the start of an experiment, the K^+-electrode was calibrated by the addition of defined amounts of KCl stock solution into the well to a final concentration of about 100 μM KCl ($[K]_0$). The electrode voltage obtained (V_0) was used as the reference point for the determination of $[K]_{out}(t)$ during experiment:

$$[K]_{out}(t) = [K]_0 \exp((V_{out}(t) - V_0)/\varsigma) \quad (1)$$

where ς is the constant, determined from a calibration curve $V(\ln([K]_{out}))$ and is equal to 25.4 mV for an ideal electrode at room temperature.

In order to correctly transform a K^+ electrode reading into the concentration of K^+ ions in the measuring well, the properties of three different K^+ electrodes MI-442 were checked. All of them had linear dependence of $V(\ln[K])$ over a wide range of added concentrations of KCl in distilled water (10 μM to 10 mM K^+, not shown). The slopes of these dependencies, ς in Eq. 1 were close to the theoretical one.

To simplify the analysis of the experimental data, the deviations from Eq. 1 in Na^+-solution at low concentrations of added K^+ were interpreted as admixtures of K^+ in the Na^+-solution $[K]_{min}$. The ratio $[K_{min}]/[NaCl]$ in the aqueous solution used (~3 μM/100 mM) is close to the value labeled by the manufacturer for K^+ ion admixtures in NaCl batch used (0.002%).

The control assays showed negligible effects of octyl glucoside and dimethylformamide (in the concentrations used) on the electrode readings.

Thus, K^+ electrode provides a correct evaluation of K^+ concentrations in the measuring well.

3.2. Monitoring of liposome characteristics

Using the above methods for liposome preparation, we succeeded in obtaining stable suspension of LUV loaded with K^+ ions as a marker. In contrast to the previous method [8], in our assays, losses of liposomes during gel filtration were negligible. Namely, trace contamination of the gel column by the red-stained lipids was minor and 85–95% of the initially added lipid dye was evaluated in the harvested liposome solution by light absorption at 560 nm. At the same time, less than 5 μM K^+ ions (from 100 mM initial level) were found in the freshly harvested suspension.

During storage at 5°C, the washed liposomes slowly lost their inner potassium. This effect was

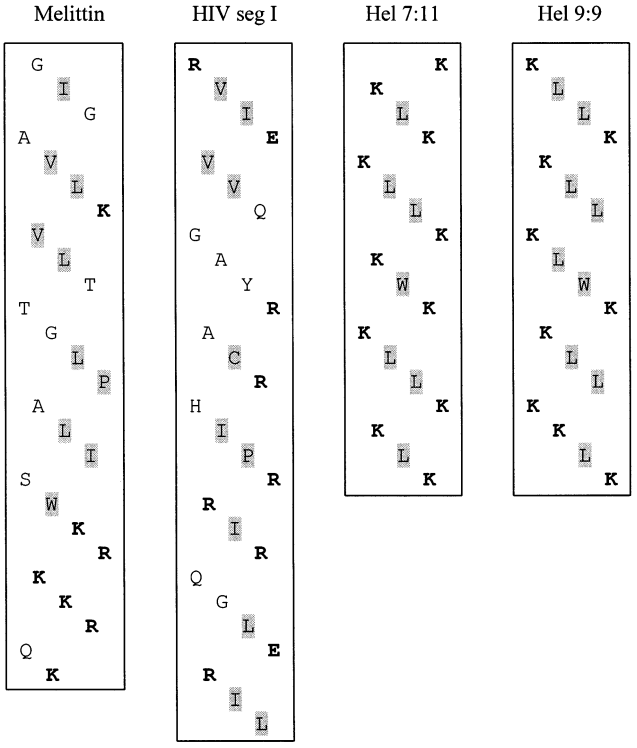

Fig. 1. The amphiphilic structure of the peptides tested. Highlighted letters represent hydrophobic residues; bold are charged residues.

more pronounced for the liposomes containing no cholesterol. Thus, 20% cholesterol was used in our standard lipid composition. As a result, about 2% of inner K^+ ions were lost from the liposomes of the standard composition following an overnight storage (right curve in Fig. 2, no added peptide).

Inner content (captured volume) of liposomes is the essential parameter of the liposomal suspension, which determines amplitude of K^+ changes during its release. This parameter was measured using K^+ electrode as is shown below. Liposomes before extrusion had a captured volume $\sim$3 times more than after extrusion through 0.2 μm filter. However, size distribution of non-extruded liposomes was worse: a mean liposome diameter 0.25 μm was evaluated, but greater liposomes having diameter up to 3 μm were observed by the fluorescent microscope. To suppress the effects of giant liposomes from the tail of their size distribution and, thus, improve reproducibility of the method, we used extruded liposomes in our assay.

3.3. Determination of inner volume of liposomes

The inner content of liposomes is the parameter of the liposomal suspension that is essential for the sensitivity of the method. Total amount of K^+ ions, released by a detergent from the liposomes let us evaluate their inner volume:

$$V_{in} = ([K^+]_{released} * V_{sample} - K_{corr})/[K^+]_{ini} \quad (2)$$

where $[K^+]_{released}$ is the change of K^+ concentration measured by K^+ electrode; K_{corr} is the correction for the charge of lipids as it is described below; $[K^+]_{ini}$ is the initial concentration of K^+ ions in the bulk of the liposome inner volume. We suppose that the last value is equal to the concentration of K^+ ions in the solution used for the liposome preparation (100 mM).

In addition to K^+ ions present in the bulk volume inside liposomes, the inner volume contains K^+ ions in the membrane-adjacent layer, which compensates the negative charge of the lipid molecules facing inside of the liposome. An aqueous solution used for liposome preparation contained no divalent ions. Hence, in accordance with the electroneutrality principle, the amount of K^+ ions compensating negative charge of lipids is equal to the amount of the charged lipid molecules facing the inside liposome. When the liposome dispersion contains C_l lipids and the share of negatively charged lipids is equal to Q, then for the unilamellar liposomes the total amount of the charged lipid molecules facing inside liposomes is close to $C_l * Q/2$. To evaluate the bulk volume, this value was subtracted from the total amount of K^+ ions released (see Eq. 2).

The inner volume of liposomes was normalized to the mole amount of the total lipid used $V_{in}/([lipid] * V_{sample})$.

As an example of such evaluation the assay presented in Fig. 2 may be used. The K^+ ions, totally released from the liposomal suspension (1 mM total lipids) by the detergent, increase in concentration outside liposomes to 0.55 mM. Taking into account that 40% of the lipids in the mixture are charged, it means that 0.2 mM of K^+ ions compensate the charge of the lipids facing inside liposomes. Hence, 0.35 mM of K^+ ions is released from the bulk solution inside the liposomes. Taking the original concentration of KCl inside liposomes being equal to

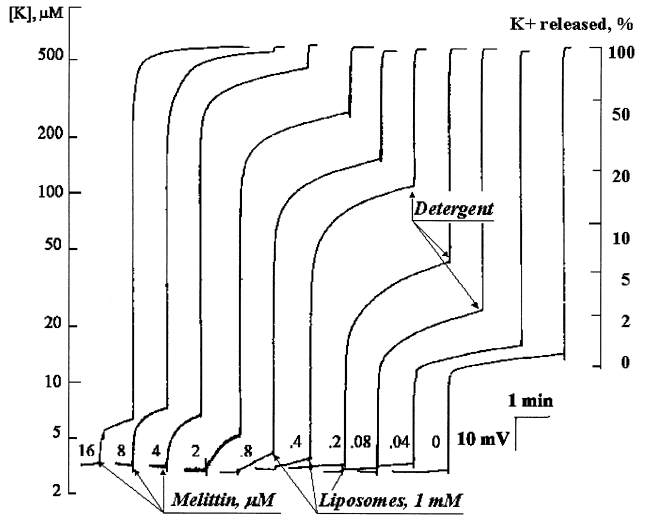

Fig. 2. The records of the potassium release from the large unilamellar liposomes induced by Melittin. The liposomes were made from the mixture of POPC:POPS:Chol 2:2:1. First, Melittin was added to Na^+-solution at the concentration shown above the curve. After 1 min of incubation, the liposomes were added. To release K^+ ions from the liposomes totally, detergent octyl glucoside was added to a final concentration of 0.7 vol%.

100 mM one obtains that the captured volume in this sample is equal to 3.5 l/mol. For different batches of liposomes averaged value of the captured volume was 2.5 ± 1.0 l/mol.

The above calculations were done supposing that liposomes are unilamellar and, hence, half of the lipids faced inside the liposomes. In the case of multilamellar liposomes, the fraction of charged lipids inside liposomes is higher and the correction is more complex. The NBD-reduction assay showed that about half of the liposomal lipids ($46 \pm 2\%$) are exposed outside, i.e. the liposomes are unilamellar (data are not shown).

3.4. Selection of a detergent for the K^+ release assay

Our preliminary assays indicated that Triton X-100 affects the readings of a potassium microelectrode as has been previously observed [8]. To select a detergent that does not cause artifacts in data acquisition, we conducted a comparative analysis of different detergents (Triton X-100, deoxycholic acid, dodecyl maltoside, CHAPS, octyl glucoside) and their effects on the electrode and liposomes. As a result, the octyl glucoside was chosen for the K^+ release assay. After washing off the electrode by 0.7 vol% of octyl glucoside, the readings of the K^+ electrode was stable and it was sensitive to less than 1 μM K^+ concentration, which was about one order of magnitude better than in a solution of Triton X-100. In addition, the octyl glucoside detergent releases K^+ ions from liposomes faster than other detergents tested. As can be seen from the Fig. 2, the release takes less than 10 s. The detergent is easily washed off from the measuring cell by aqueous solutions.

Detergent *N*,*N*-dimethyldodecylamine *N*-oxide was also successfully used instead of Triton X-100 in the K^+-release assay [9,10].

3.5. Calculation of the rate of K^+ release $R(t)$

The rate of potassium release $R(t)$ was calculated from the values $[K]_{out}(t)$ (obtained from Eq. 1) as follows: a background level of K^+ ions ($[K]_{bk}$) is a sum of $[K]_{min}$ and the increments of potassium ions added with a peptide solution (first steps on traces in Fig. 2) and liposomal suspension (the last trace in Fig. 2). The last value is the release of potassium ions from liposomes during its storage. The value of $R(t)$ is calculated as

$$R(t) = ([K]_{out}(t) - [K]_{bk})/([K]_{max} - [K]_{bk}) * 100\%/t \quad (3)$$

where $[K]_{max}$ is the maximal level of K^+ in the presence of detergent when all inner K^+ is released.

The rate of inner marker release from liposomes decreases with time faster than in an exponential process (see e.g. [5,19]). The pattern of the time course and its reproducibility depend strongly on the protocol of the experiment (see below). Our data obtained with the improved protocol is also a non-exponential process (not shown). Schwarz and Robert presented proof [20] that pore formation determines the extent of the marker release and the pattern of time course release depends on the size distribution of the liposome used. Larger liposomes have a higher probability to form pores (due to a larger surface) and contribute more to the marker efflux (due to a larger inner volume) in the initial time period.

It is the reason why in our assay we chose not the

initial rate of efflux, but an arbitrary value, percent of inner K^+ ions released during the first minute after liposome addition R(1 min) as the measure of the peptide activity.

For comparison of the destabilizing activities of different peptides, the concentration inducing 20% release during the first minute of the assay (C_{20}) was found by interpolation using two close peptide concentrations. The simple program implemented with Microsoft Excel significantly simplifies all the above calculations.

3.6. Protocol of the K^+ release assay

To quantitatively compare the destabilization activities of the different peptides we developed a standard protocol, which gives well-reproducible results. Preliminary experiments with Melittin showed that the time course and the reproducibility of the inner marker release depend strongly on the order in which the peptide and liposomes were added into the test chamber.

Mixing equal volumes of peptide and liposomes was previously recommended [21]. We used a similar protocol when the peptide was first diluted in the measuring chamber. After 1 min incubation, the liposome suspension was added (1:4 by volume). Concomitant dilution of both the lipid and peptide was taken into account in further calculations.

One more important feature of this protocol is the ability to evaluate probable effects of peptides on the K^+ electrode and to assess potassium admixtures in the peptide sample before the experiment (first step in traces of Fig. 2). At the end of the K^+-release assay, 30% octyl glucoside solution was added to a final concentration of 0.7 vol%. It induced complete inner K^+ release providing the value of 100% level of potassium ions, $[K]_{max}$.

An inverse in the order of additions, when a peptide is added to a liposomal solution is not advisable [20]. In our assays, in this case K^+ release had a stepwise character and was not reproducible. As was mentioned by Schwarz and Roberts [20], these effects presumably are due to transient high concentration of the peptide in the area of its addition. First, the liposomes in the concentrated-peptide solution area rapidly lose their content, then the peptide is captured by these liposomes. Thus, to release the inner

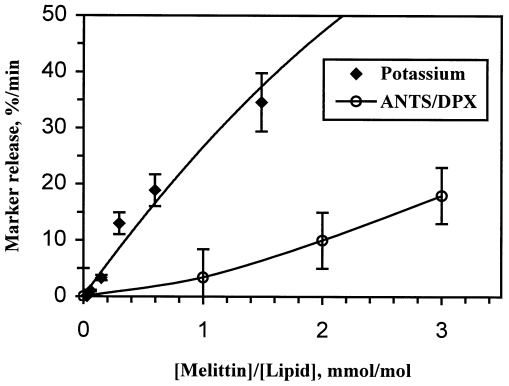

Fig. 3. The efflux of the inner markers from liposomes induced by Mellitin at different concentrations. The potassium (upper curve) or ANTS/DPX (lower curve) release from the liposomes. The percent of the inner content released during the first minute after the liposome addition is shown as a function of Melittin concentration in liposomes. Liposome concentration in the suspension was 1 mM in the K^+-release assay and 0.1 mM in the ANTS/DPX-release assay.

content of other liposomes, the fusogenic peptide molecules should be redistributed between the liposomes that captured the peptide and peptide-free liposomes. For the peptides with high affinity to a liposomal surface, this process takes considerable time. For Melittin, this time delay is about 15 min.

The experimental records representing the K^+-release assay are shown in Fig. 2. A set of time courses of the $V_{out}(t)$ at different concentrations of Melittin in the aqueous solution is presented. The corresponding peptide concentrations are shown above the records in the descending order. The left axis shows corresponding concentrations of K^+ ions in the measuring well. The right axis presents the percent of K^+ ions released during the assay.

Thus, the protocol used allowed us to obtain reproducible results in the K^+ release assay. Similar protocol was used in the ANTS/DPX release assay.

3.7. Comparison of the methods for the release of K^+ ions and ANTS/DPX

To compare sensitivities of the developed method and a conventional fluorescent method, the K^+-release assay was conducted in parallel with the ANTS/DPX release assay [1]. The same amount of liposomes (0.25 μmol) was used per each assay.

The rates of the inner content release rise up with

Melittin concentration for both methods (Fig. 3). Notably, significantly lower concentrations of Melittin induce similar percent of leakage in the K^+ release assay than in the ANTS/DPX method. An additional advantage of the method is that, due to the logarithmic scale of concentrations measured by K^+ electrode, the K^+ release method has a better sensitivity in the range of low release rates.

Thus, the comparison of the two methods showed that the K^+ release assay is $\sim$5 times more sensitive (as per peptide concentration) than the ANTS/DPX assay. This result supports the conclusion of Shai et al. [7] that the size of a probe molecule determines the rate of peptide-induced release of a marker from liposomes.

3.8. Measurement of the membrane destabilizing activities for different peptides by the K^+ release assay

The method developed was used to compare membrane activities for peptides of both viral and artificial origin. All of the four peptides chosen have a propensity to form an amphiphilic α-helix [12,13]; however, the destabilizing activities of different peptides, as evaluated by the K^+ release method, are different.

The summary of the K^+ release assays for the four peptides tested are shown in Fig. 4. Melittin induces K^+ release 20% min^{-1} at the concentration of about 0.7 μM in the liposomal suspension (1 mM of the lipids in the measuring well). The HIV seg I induces similar release rate at 3.8 μM of the peptide. However, the synthetic amphiphilic helices tested induce the same release rate at higher concentrations of the peptides 15–20 μM.

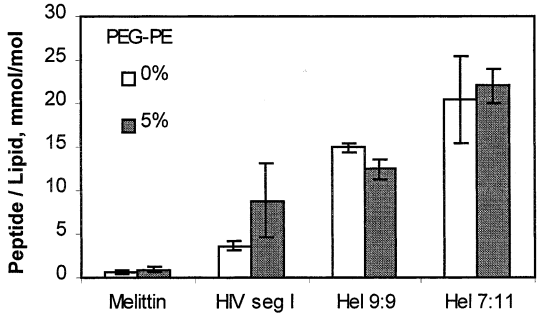

Fig. 4. Comparison of the destabilizing activities for different peptides. The peptide concentrations inducing 20% K^+ release during the first minute of the interaction are presented. Hollow bars correspond to liposomes with the same composition as in Fig. 2, the shaded bars correspond to liposomes with 5 mol% of PEG2k-PE.

3.9. The K^+ release from the PEG-coated liposomes

It is well established that lipid membranes coated by grafted PEG molecules are shielded from the immune system [22]. We tested whether 5% PEG-PE in the lipid composition may prevent the liposome-destabilizing effects of the different membrane active peptides.

The results of comparing destabilizing activities of the peptides on non-coated and PEG-coated liposomes are presented in Fig. 4. The concentrations of Melittin and the artificial amphiphilic (Hels) peptides that induced the same level of K^+ release (20% min^{-1}) were comparable for the coated and non-coated formulations of liposomes. The peptide HIV seg I was measured to have two times lower activity in PEG-coated liposomes compared to that in non-coated liposomes. However, the difference between the peptide's activities on the two types of liposomes is statistically non-significant.

This result was unexpected. Theoretical consideration had shown that 4.5 mol% of PEGylated lipids form 'weak overlap' regime [23], i.e. most of the membrane surface is shielded. Nevertheless, 5 mol% of PEG-PE in liposome composition does not prevent liposomal membranes from destabilization due to the activity of the amphiphilic peptides.

4. Discussion

4.1. The K^+-release assay

Among different solutes used as a marker for liposomal leakage assays, univalent ions seem the most promising. They are physiologically relevant, small and have very low affinity for liposomal surface. Due to high selectivity of the potassium electrode, minor concentrations of K^+ ion, at a level less than 1 μM, can be detected. This allowed us to measure very low rates of K^+ release (a few percent per minute) from the liposomes taken at moderate concen-

tration (~1 mM). Lower concentrations ~250 μM may be used for routine measurements with an accuracy of about 2%. Further decrease of lipid concentration may cause intensification of undesirable effects such as peptide adsorption on the walls of the measuring well [5].

The possible disadvantage of the protocol developed is the requirement to dissolve the peptide under study in the aqueous solution as a first step. The poorly soluble amphiphilic peptides added into an aqueous solution start to aggregate and this aggregation may be irreversible. To minimize this effect, we used low concentrations of the peptides in aqueous solution (20 μM and less). In addition, the peptides were diluted in the media for a short 1-min time just to obtain an even peptide solution and to check the effects of the peptide on the electrode. Such effects did not present problems for the water-soluble peptides (Melittin, Hel 9:9, and Hel 7:11). Moreover, dilution of these peptides before the assay shifts the equilibrium in the aggregation reaction to the side of monomer formation.

The K^+-release assay may be used also to monitor properties of liposomes. For the liposomal suspension extruded through 0.2 μm polycarbonate filter, the captured volume corrected for lipid charge inside liposomes (see Section 3.2 for detail), is equal to 2.5 μl/μmol, as it was evaluated from the amount of K^+ ions inside the liposomes. This value corresponds to the data of Mayer et al. [25] obtained for liposomes prepared by a freeze–thaw method. According to the analysis conducted by Perkins et al. [26], this value corresponds well to the non-spherical vesicles with the wall thickness of 4 nm and the diameter of 0.2 μm. The diameter of liposomes used in our experiments is indeed 0.20 μm (light scattering assay done by Dr. S. Potekhin, Institute of Protein Research, Russian Academy of Science; data not shown).

4.2. Comparison of the developed K^+ release method with other methods

In comparison with fluorescent methods, K^+ release assay is simple, inexpensive and even more sensitive (Fig. 3) assay. The most important feature of this method is the use of a smaller marker (compared to fluorescent probes), a K^+ ion, for the evaluation of defects in liposomal membrane. Thus, this method expands the set of markers into the lower size range.

Gruber et al. [8] successfully used Cl^--electrode for the evaluation of the intravesicular volume of liposomes. They obtained worse results with K^+-electrode for the following reasons. First, the Triton X-100 that was used to achieve complete release of the liposomal content affected the electrode readings. Then, the electrode was unstable with time and demanded thorough washing after each measurement.

We have avoided these problems using octyl glucoside instead of Triton X-100. The former detergent is an active liposome solubilizing agent [24]. Our data obtained by K^+-release assay indicate that octyl glucoside is the best detergent for the assay among the ones studied. It has only minor effects on the electrode, induces fast release of K^+ from the liposomes, and may be washed off easily by an aqueous solution. Moreover, octyl glucoside purifies the surface of the electrode, increasing its stability and sensitivity. The sensitivity of the K^+ electrode in our assay was about 10 times higher than Cl^- electrode in the previously published report [8].

In recent publications [9,10], the K^+ release assay was used to evaluate destabilizing activity of the antimicrobial peptide nisin. The authors obtained even lower background levels of K^+ ions (0.4 μM; E.J. Breukink, personal communication). The lipid concentration used was lower than in our assay. However, due to the smaller sample volume, we use a similar amount of liposomes per assay (~200 μmol). The use of the small well with higher concentration of both the lipid and destabilizing peptide has an advantage when highly hydrophobic peptides are tested. In this case, artifacts that originate by sorption of the peptide on the walls of the well are decreased.

Thus, the K^+ release method developed here has pronounced advantages in comparison with another methods used.

4.3. The electrogenic nature of ion flux in liposomal vesicles

A surprising result was reported by Breukink et al. [9], that nisin-induced rate of K^+ release from liposomes was very low in comparison with the rate of carboxyfluorescein release. These data contradict the

results obtained both by us and by Shai et al. [7]. Breukink et al. [9] attributed such an observation to an anion selectivity of the nisin-induced pore. We may suggest another explanation.

The likely reason may be due to a peculiarity of an ion release from the liposomal vesicles: the release is possible when at least two ions are permeable and the net flux of the electric charges transferred by the ions is equal to zero. In this case, the rate of the marker-ion release from the vesicles is determined by the ion with the worst permeability. In another case, when only one ion is permeable, as it was shown using Valinomicin [9], only a small portion of the permeating ions will be released. Due to the electrogenic nature of the ion flux, the concomitant increase of the membrane voltage prevents the further efflux of K^+ ions.

From this point of view, the obtained level of the nisin-induced K^+ release [9] was low, not because of a low permeability for a K^+ ions, but because of a low permeability for the counter ion (aqueous solutions contained sulfate anions on the inside and choline cations on the outside of liposomes, the proton level is too low to compensate for the efflux of 200 mM K^+ ions). In the CF-release experiment, efflux of the dye was better because of a high permeability of the opposite ion-K^+ (or Cl^- from outside). The role of Valinomicin in this case is possibly the facilitation of K^+ transport that indeed rises up the coupled efflux of CF ions.

In our assay, we had no such pitfalls. The counter ions for K^+ efflux were Na^+ ions outside of the liposomes and/or Cl^- ions inside the liposomes. If the values of the permeability coefficients of the peptide-induced pores for the marker ion and the counter-ion are comparable, free release of the marker takes place.

As it was asserted by Schwarz and Robert [20], the kinetics of the pore formation, but not the kinetics of the marker release determines the rate of the marker efflux from liposomes. This conclusion was based on evaluations of the rate of the marker release made with the assumption of free diffusion of the marker. However, if the marker efflux is restricted by the very low permeability of the counter ion, the rate of ion release may be crucial to the marker efflux.

Thus, the question on electrogenic nature of the ionic transport through liposomes is complex. It demands thorough analysis and further experiments to be solved.

4.4. Conclusion

The developed K^+ release assay is a simple, effective method to evaluate membrane destabilizing activities for different peptides. The use of the K^+ ion as a marker has some advantages and supplements conventional methods for qualitative evaluation of liposomal membrane stability. This method should be easily applicable for the evaluation of other membrane-active substances (detergents, organic solvents, channel-forming compounds etc.).

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