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ORIGINAL PAPER

Kinetic models for peptide-induced leakage from vesicles and cells

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Abstract In this article analytical expressions for peptide-induced membrane leakage are presented. Two different models for time-dependent leakage have been developed. In the first, the leakage is assumed to be coupled by pores formed by the peptides. In the second model the peptide is assumed to induce a stress/perturbation in the membrane, and in order to reduce the stress, rearrangements in the membrane are induced. The leakage is coupled to these rearrangements, and when equilibrium is achieved no more leakage occurs. From the kinetic models simple fitting routines have been developed involving only two fitting parameters, and these have been used to fit experimental data for two prion protein-derived peptides as well as the honey bee toxin melittin in both vesicles and erythrocytes with good results. The fitted parameters provide both a quantitative and a qualitative basis for interpreting the experimental results. In addition a model for the peptide concentration-dependent leakage is presented, which was used to fit experimental data for leakage induced by the prion protein-derived peptides. The models presented in this article are compared with other models for peptide-induced membrane leakage.

Keywords Membrane · Leakage · Vesicle · Hemolysis · Kinetics · Peptide

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Abbreviations

mPrpP	Mouse prion protein peptide
bPrPp	Bovine prion protein peptide
POPC	Palmityl-oleoyl-phosphatidylcholine
POPG	Palmityl-oleoyl-phosphatidylglycerol
hRBC	Human red blood cells

Introduction

Peptide-induced membrane leakage experiments have lately become a standard experiment for investigating the membrane interaction and potential toxicity of peptides. The leakage may be investigated either in cells, e.g., hemolysis, or from vesicles with entrapped reporter molecules (Weinstein et al. 1977). The membrane disrupting properties of peptides, related to the toxicity in biological systems, such as that observed for the antimicrobial peptides (Dempsey 1990; Matsuzaki et al. 1997; Huang 2000; Ambroggio et al. 2005) and the amyloid aggregate forming β-amyloid peptide from the Amyloid Precursor Protein (Lashuel et al. 2002; Ambroggio et al. 2005), can directly be related to the induced leakage. It is therefore important to have appropriate models that can describe the leakage.

Originally the leakage was assumed to be coupled to holes (pores) in the membrane formed by the peptides (Schwarz and Robert 1990). In this case, it is expected that as time approaches infinity the concentration of particles inside and outside the membrane equilibrate. This behavior is observed for some, but not all, peptides. Schwarz and coworkers introduced the concept ''graded'' leakage (Schwarz and Robert 1992),

meaning that the transmembrane concentrations of reporter molecules do not equilibrate at long experimental times. However, as time approaches infinity, even graded leakage will equilibrate the concentrations.

When a peptide binds to a membrane several correlated peptide–lipid rearrangements may occur. The peptide may transfer from a peripheral to a transverse state (Hunt et al. 1997), or translocate the membrane altogether (Matsuzaki et al. 1995; Terrone et al. 2003). Peptide structure, dynamics and lateral diffusion are also expected to change (Andersson et al. 2004; Andersson and Mäler 2002). The presence of a peptide also affects the lipids; increased lipid flip/flop rate is induced by certain peptides (Matsuzaki et al. 1996; Pokorny and Almeida 2004), the peptide might make the membrane thinner (Chen et al. 2003), the lateral diffusion may be changed (Orädd and Lindblom 2004) and the transmembrane pressure might be altered (Heerklotz 2001; van den Brink-van der Laan et al. 2004), all kinetically changing the state of the membrane. If the leakage of entrapped reporter molecules is correlated with a kinetic process of any of the above-mentioned types, i.e., with the changing states of the membrane, the leakage is expected to decrease as the peptide reaches a membrane-bound equilibrium state. Experimentally it is in some instances found that the concentrations of reporter molecules on the inside and outside of the membrane do not equilibrate as time goes towards infinity (Matsuzaki et al. 1995; Pokorny and Almeida 2004; Magzoub et al. 2005; Kobayashi et al. 2000) and the coupled change of membrane states may account for these observations.

In this study we present two kinetic models for peptide-induced membrane leakage applicable to both model membranes and cells. In the first model we assume that the peptides form a hole (or a pore) in the membrane, and that the hole fluctuates between an open and a closed state. This model is similar to what has been presented by Schwarz and Robert (Schwarz and Robert 1992). In the second model the leakage is coupled to a peptide-induced rearrangement process in the membrane that decays in amplitude with time. The two models are complementary in the sense that they describe two physically distinct situations and the fitted parameters in both cases have solid physical interpretations, in terms of peptide rearrangement kinetics and the flow of reporter molecules over the membrane.

For certain peptides, the leakage induced in the membrane does not equilibrate the difference in concentration of reporter molecules over the membrane, even at high peptide concentrations. A model for concentration-dependent leakage is presented, which is related to the second situation described above. It explains these observations in terms of a saturation of the peptide concentration in the membrane. The models are used to explain experimental leakage data for two prion protein-derived peptides, the N-terminal residues 1–28 of the mouse and the N-terminal residues 1–30 of the bovine prion protein, as well as for the honey bee venom melittin. Experimental leakage data from both vesicles and erythrocytes were interpreted successfully. The models have the advantage that no particular leaky states have to be specified, and can thus be used in several situations. We show, however, that the models can be used to describe the specific molecular mechanism behind leakage caused by two prion protein-derived peptides.

Theory

In this section, two kinetic models for the time-dependent peptide-induced membrane leakage and one model for the peptide concentration-dependence are presented. A brief summary of the models is provided here, while a more thorough derivation can be found in Appendices 1 and 2. The second model is later combined with the assumption that a saturation of peptide may occur in the membrane and a model for leakage as a function of peptide concentration is then derived. These models share the common assumption that the initial binding of peptides to the membrane is a fast process compared with the actual leakage. This is a reasonable assumption since the typical leakage in model membranes and cells occurs on the minute time-scale compared with the typical time-scale for membrane binding, which occurs on the sub-second timescale (Sekharam et al. 1991; Schwarz et al. 1987).

Passive diffusion over a membrane

According to Fick's first law the flow of particles (J) due to translational diffusion is given by:

$$J = -D\frac{\partial \phi}{\partial x}, \tag{1}$$

where D is the diffusion coefficient, φ is the concentration and x is the position. Assuming that the diffusion occurs from one side to another over a membrane through a peptide-induced hole, Eq. 1 can be reformulated as (Schwarz and Robert 1990):

$$J = \frac{\mathrm{d}i(t)}{\mathrm{d}t} = -U(i(t) - o(t)), \tag{2}$$

where $i(t)$ is the concentration of diffusing particles on the inside of the membrane and $o(t)$ is the concentration on the outside. U is a proportionality constant related to the size of the peptide pore and the diffusion coefficient of the particle.

Model 1: pore formation

In the first model, the peptides are assumed to form holes/pores, through which the reporter molecules can escape. These pores can either be open or closed (Fig. 1). At the beginning of the leakage experiment, all pores are assumed to be closed, and thus no leakage can occur. The number of open pores gradually increases with time until an equilibrium state is achieved. Thus, the leakage is initially slow, but after a while the rate increases due to the opening of pores. When the concentration on the inside of the membrane compartment is equal to the concentration on the outside, the net flow of reporter molecules stops. The opening and closing of the pore/hole is modeled as a differential equation, where the fraction of peptide in the open state is defined as $\alpha(t)$ (Fig. 1).

The kinetics of the opening dynamics is given by the following differential equation:

$$\frac{\mathrm{d}\alpha(t)}{\mathrm{d}t} = k_{\mathrm{on}}(1-\alpha(t)) - k_{\mathrm{off}}\alpha(t), \tag{3}$$

where k_{on} and k_{off} are defined in Fig. 1. Keeping Eq. 2 in mind, it is reasonable to assume that the change in concentration of reporter molecules on the inside ($i(t)$) is assumed to be proportional to the concentration of peptides in the open state according to:

$$\frac{\mathrm{d}i(t)}{\mathrm{d}t} = X(k_{\mathrm{in}}o(t) - k_{\mathrm{out}}i(t))(\alpha(t)[\mathrm{peptide}]), \tag{4}$$

where k_{in} and k_{out} are defined in Fig. 1, $o(t)$ is the concentration of reporter molecules on the outside, [peptide] is the concentration of peptides bound to the membrane. The flow of molecules is theoretically described by Fick's laws of diffusion (Eqs. 1, 2), and in the case of flow through a membrane this is proportional to the concentration difference over the membrane. In this treatment the flow is modulated by the presence of a peptide, and the constant X reflects the size of the perturbation caused by the peptide. Since the peptide is assumed to cause pores, the flow can be assumed to be related to the size of the pore. Furthermore one can safely assume that $k_{\mathrm{in}} \approx k_{\mathrm{out}}$. It is useful to define the relative amount of reporter molecules on the inside as $q(t) = n_{\mathrm{in}}(t)/(n_{\mathrm{in}}(t) + n_{\mathrm{out}}(t))$, and at the same time we define, $z = k_{\mathrm{in}}(V_{\mathrm{in}}/V_{\mathrm{out}})$. By assuming that the relative volumes of the vesicles are unchanged (which is reasonable as long as the vesicles are intact) the change in $q(t)$ with time becomes:

$$\frac{\mathrm{d}q(t)}{\mathrm{d}t} = X(z(1-q(t)) - k_{\mathrm{out}}q(t))\alpha(t)[\mathrm{peptide}]. \tag{5}$$

By solving this equation we can derive a relationship between the fraction of reporter molecules on the inside of the vesicle, $q(t)$, and k_{on}, k_{off}, k_{in} and k_{out} (see Fig. 1).

The observed peptide-induced leakage of reporter molecules is commonly presented in terms of a percentage of the maximum leakage, related to $q(t)$, induced as determined by for instance a membrane disruptive detergent. In order to fit the experimental data, the following equation is obtained:

$$\frac{I_{\mathrm{obs}}(t)}{I_{\mathrm{max}}} = 1 - \exp\left(\frac{J_1}{v_{\mathrm{fluct}}^2}(1 - v_{\mathrm{fluct}}t - \mathrm{e}^{-v_{\mathrm{fluct}}t})\right), \tag{6}$$

where $I_{\mathrm{obs}}(t)$ is the measured signal of reporter molecules for the investigated peptide and $I_{\mathrm{max}}(t)$ is the maximum leakage (obtained by the addition of a membrane disruptive agent, e.g., Triton X-100). Thus we arrive at a relatively simple equation with only two fitting parameters: $J_1 = k((V_{\mathrm{in}} + V_{\mathrm{out}})/V_{\mathrm{out}})k_{\mathrm{on}}\,X[\mathrm{peptide}]$ where $k = k_{\mathrm{in}} = k_{\mathrm{out}}$, and $v_{\mathrm{fluct}} = k_{\mathrm{on}} + k_{\mathrm{off}}$. The parameter v_{fluct} is the rate with which the pore fluctuates between the open and the closed state. The interpretation of the parameter J_1 is more complicated, and depends both on the pore kinetics and the transmembrane flow. The ratio J_1/v_{fluct}, however, reflects the flow of reporter molecules over the membrane. In order to obtain a measure of the induced leakage per membrane-bound peptide, the concentration of membrane-bound peptide [peptide], needs to be estimated. This can be done by measuring leakage as a function of peptide concentration, as will be outlined later.

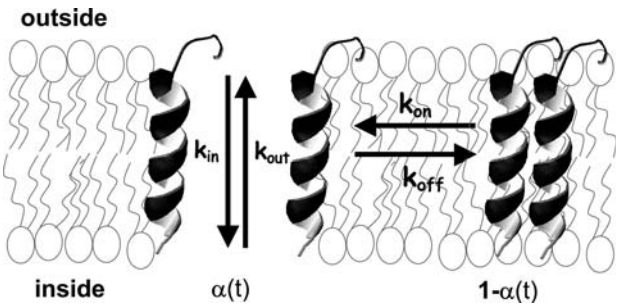

Fig. 1 In model 1 the diffusion of reporter molecules out of the membrane compartment is assumed to occur through peptide induced holes (or pores) in the membrane. The pores fluctuate between an open ($\alpha(t)$) and a closed state ($1 - \alpha(t)$)

Model 2: peptide-induced membrane stress/perturbation

In the second model a stress/perturbation is induced in the membrane by the peptide. The stress is initially high, but gradually relaxes as rearrangements in the membrane are induced. The leakage is caused by a non-equilibrium situation, such as that immediately after addition of peptide, and the amount of leakage induced by this process is proportional to how much rearrangement that is induced by the peptide. When the peptide-membrane system approaches equilibrium the rearrangements stops and the leakage therefore also stops, and this may occur before the concentration equilibrium of reporter molecules between the inside and outside of vesicles has been reached. This corresponds to having two states in the peptide-membrane system, and a conversion between the peptide in state 1 and 2 occurs (Fig. 2). We do not explicitly define the two states, as the model is applicable to any process that may occur when a peptide interacts with the membrane.

The kinetics of the conversion of the peptide from state 1 to state 2 is modeled as a differential equation. The fraction of peptide in the first state is defined as $\beta(t)$ while it is $1 - \beta(t)$ in state 2 (Fig. 2):

$$\frac{\mathrm{d}\beta(t)}{\mathrm{d}t} = ((1-\beta(t))k_2 - \beta(t)k_1), \tag{7}$$

where k_1 and k_2 are defined in Fig. 2. The leakage of reporter molecules is described analogously to model 1, except that the flow of reporter molecules is proportional to the rate of conversion between state 1 and 2, and thus to $\mathrm{d}\beta(t)/\mathrm{d}t$, which gives us:

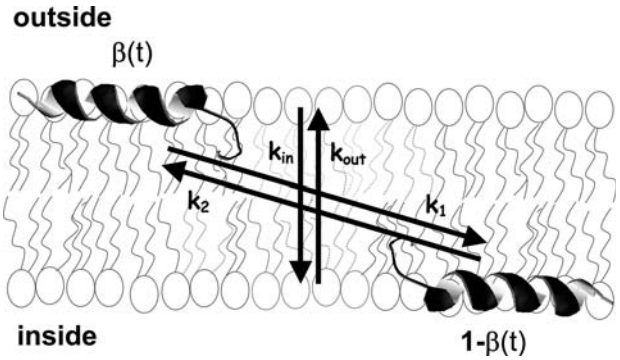

Fig. 2 In model 2 the diffusion of reporter molecules out of the membrane compartment is coupled to rearrangements between two states, 1 ($\beta(t)$) and 2 ($1 - \beta(t)$), in the membrane induced by the peptide. These rearrangements allow reporter molecules to leak out. A hypothetical situation is shown, in which the leakage is assumed to be coupled to the translocation of the peptide over the membrane

$$\frac{\mathrm{d}q(t)}{\mathrm{d}t} = -Y(z(1-q(t)) - k_{\mathrm{out}}q(t))\left(\frac{\mathrm{d}\beta(t)}{\mathrm{d}t}[\mathrm{peptide}]\right), \tag{8}$$

where Y is the analog of the constant X in model 1 and is thus a measure of the stress/perturbation induced flow caused by the peptide. In analogy with model 1 we can find a solution to Eq. 8, and with the definitions: $J_2 = k((V_{\mathrm{in}} + V_{\mathrm{out}})/V_{\mathrm{out}})(k_1/(k_1 + k_2))Y[\mathrm{peptide}]$, where $k = k_{\mathrm{in}} = k_{\mathrm{out}}$, and $v_{\mathrm{relax}} = (k_1 + k_2)$ we obtain:

$$\frac{I_{\mathrm{obs}}(t)}{I_{\mathrm{max}}} = 1 - \exp(J_2(\mathrm{e}^{-v_{\mathrm{relax}}t} - 1)) \tag{9}$$

where, again, $I_{\mathrm{obs}}(t)$ is the observed data signal and I_{max} is the maximum leakage. J_2 is related to the rate of flow of reporter molecules, while v_{relax} is related to the rate of the membrane reorganization process leading to the non-leaking equilibrium state. The amount of reporter molecules on the inside of the membrane compartment as $t \rightarrow \infty$ clearly depends on the coupled kinetics of the flow of reporter molecules and the peptide undergoing conversion between the two states. Thus, model 2 can, in contrast to model 1, explain the observation that certain peptides induce a leakage that stops before the transmembrane concentration gradient of reporter molecules is equilibrated. As for model 1, to obtain the leakage per membrane-bound peptide one needs to calculate the concentration of membrane-bound peptides, [peptide].

Concentration-dependence

For certain peptides it has been observed that the induced membrane leakage, as a function of peptide concentration, grows towards a non-maximum leakage asymptote. Here, a model is presented that accounts for the asymptotic behavior in terms of peptide saturation of the membrane. We will assume that the peptide can reside in two states: free in solution ($A(t)$) and bound to the membrane ($B(t)$). The partitioning of a peptide within a membrane is described by the following relation:

$$X = \frac{B(t)}{C} = K_0 A(t), \tag{10}$$

where X is the molar ratio of bound peptide compared with the total concentration of the lipids (C) and K_0 is the binding constant (Seelig 2004). Assuming that the lipid concentration is fixed, the kinetics of this binding process is described by:

$$\frac{dB(t)}{dt} = k_{bind}CA(t) - k_{diss}B(t), \quad (11)$$

where k_{bind} and k_{diss} refers to the peptide-membrane binding and dissociation rates, respectively (defining $K_0 = k_{bind}/k_{diss}$). This relation is true if the concentration of the lipids is in a large excess. However, when the peptide-to-lipid concentration increases the membrane becomes saturated with peptides due to steric/packing effects. Assuming that the maximum peptide concentration that the membrane can allow for is B_0, the binding of the peptide to the membrane is governed by the following differential equation, which is a generalization of Eq. 11:

$$\frac{dB(t)}{dt} = k_{bind}CA(t)^m \frac{(B_0 - B(t))^n}{B_0^n} - k_{diss}B(t), \quad (12)$$

where $B(t) < B_0$ and k_{bind} and k_{diss} refers to the peptide-membrane binding and dissociation rates respectively and m and n are cooperativity factors (Hills' coefficients). The exponent m reflects cooperativity for peptide oligomerization in solution and the factor n reflects the cooperativity of oligomerization in the membrane. When the peptide concentration is much lower than the saturation in the membrane ($B \ll B_0$) and in the absence of cooperativity ($m = n = 1$), Eq. 12 reduces to Eq. 11. The effects of saturation is in this presentation assumed to be caused by steric/packing effects when the membrane accommodates the peptide. However, other mechanisms also could cause a concentration-dependent saturation, e.g., electrostatics. By implementing the Gouy–Chapman theory within the present framework, the effect of electrostatics could be accounted for in more detail. However, in the present treatment the effect of electrostatics is interpreted as an apparent dissociation constant of the peptide binding to the membrane (Seelig 2004).

Since the membrane-binding kinetics in all models presented in this study is assumed to be much faster than the leakage kinetics, we will leave this equation and focus on the equilibrium condition (at long times), for which we have $dB(t)/dt = 0$. Focusing on the simple case with no cooperativity ($m = n = 1$) and defining $B(t \to \infty) = B$ we get:

$$B = 0.5\left[[\text{total}] + B_0 + K - \sqrt{([\text{total}] + B_0 + K)^2 - 4[\text{total}]B_0}\right], \quad (13)$$

where the total concentration of peptide is given by [total] = $A(t) + B(t)$ and the definition $K = \frac{B_0}{C}\frac{k_{diss}}{k_{bind}}$ has been introduced. It can be shown that $B \to B_0$ as the total peptide concentration approaches infinity. For cases with cooperativity in the membrane binding, one obtains expressions for which numerical solutions may be required.

It is important to examine the assumptions that need to be made when coupling the concentration-dependence model to the previous time-dependent models. Formally, the peptide can reside in at least three states: free in solution and two different membrane-bound states. At least in theory the time- and concentration-dependence of these states can be solved as a set of three coupled differential equations. Since Eq. 13 is non-linear, this system of equations may require a numerical solution. If, however, the binding process of the peptide to the membrane is much faster compared to the leakage kinetics, as was implied in both models 1 and 2, Eq. 13 can be combined with either of the two time-dependent models (Eq. 6 or Eq. 9) to model the concentration dependence. It is, however, preferable if the concentration dependence is monitored at long times, and thus in practice at equilibrium. Here we will only use model 2, but fitting to model 1 would only require minor alterations to the discussion below. It should be pointed out that for model 1 no concentration-dependent leakage is expected as time approaches infinity.

At a given time, ideally as $t \to \infty$, Eq. 9 can be used to give:

$$\frac{I_{obs}([\text{peptide}])}{I_{max}} = (1 - \exp(-J_{conc}[\text{peptide}])), \quad (14)$$

where $J_{conc} = J_2(1 - e^{-\nu_{relax}t})/[\text{peptide}]$. By noting that B in Eq. 13 equals [peptide] in Eq. 14, these equations may be combined:

$$\frac{I_{obs}([\text{total}])}{I_{max}} = 1 - \exp\left(-0.5J_{conc}\left([\text{total}] + B_0 + K - \sqrt{([\text{total}] + B_0 + K)^2 - 4[\text{total}]B_0}\right)\right) \quad (15)$$

In this equation we have three parameters: J_{conc}, B_0 and K. B_0 is the maximum peptide concentration that the membrane allows for, J_{conc} is the leakage induced per membrane-bound peptide and K is the dissociation constant scaled by B_0/C. It is interesting to note that K is directly proportional to half of the maximum leakage, as is shown in Appendix 3.

Materials and methods

Sample preparation

POPC was purchased from Avanti Polar Lipids (Alabaster, AL, USA), Calcein was purchased from Molecular Probes (the Netherlands) and Triton X-100 was purchased from Sigma (St. Louis, USA). Large unilamellar phospholipid vesicles were prepared by dispersing the lipids at desired concentration in 50 mM phosphate buffer. The solution was freeze-thawed-cycled six times and then passed 20 times through a 0.1 μm pore-size polycarbonate filter. Calcein loaded vesicles were prepared as above but in the presence of desired concentration of calcein. Calcein not entrapped in the vesicles was removed on a Sephadex-G25 column.

Fluorescence

The fluorescence experiments were performed at 25°C on a Horiba Jobin Yvon Flourolog-3 spectrometer using the DataMax software (version 2.20). A 2 mm quartz cuvette was used. The fluorescence intensity was measured at 514 nm (excitation at 490 nm). The self-quenching of calcein as a function of concentration was estimated from concentration dependent fluorescence measurements. The data were fitted to the equation $I = \beta\,(1–10^{-\varepsilon C})e^{-kC}$, where I is the fluorescence intensity β is a proportionality factor, ε is the molar extinction coefficient, k is the related to the self quenching, and C is the calcein concentration (Memoli et al. 1999) When fitted to the experimental data the following values were obtained: $\beta = 202$, $\varepsilon = 47{,}750$, and $k = 13{,}900$. In order to determine the maximum amount of leakage, the fluorescence emission intensity from vesicles, containing 75 mM calcein, lysed by 10 % (w/v) Triton X-100 was measured as a function of dilution with buffer. The errors were estimated from repeating the experiment three times.

Data fitting

To test the models presented here, leakage data for entrapped calcein in vesicles, both as a function of time and peptide concentration, was used. For the hemolysis, only leakage data as a function of time was used (K. Oglecka, P. Lundberg, M. Magzoub, L.E.G. Eriksson, Ü. Langel,A. Gräslund 2006, in preparation). The peptides used were melittin, the N-terminal residues 1–28 of the mouse prion protein (mPrPp), and the N-terminal residues 1–30 of the bovine prion protein (bPrPp) as described elsewhere (Magzoub et al. 2005). The maximum amount of leakage in these experiments was defined by the leakage induced by adding the detergent Triton X-100, leading to an equilibrium distribution of reporter molecules. The only peptide that induced leakage corresponding to, or approaching, this maximum amount of leakage was melittin. For this peptide we therefore fitted both model 1 (Eq. 6) and model 2 (Eq. 9), but for the other peptides we used only model 2, (Fig. 3) The fitting routines as well as the error analysis were made using MATLAB version 7.0.1 (The MathWorks, Natick, MA, USA).

Results

In order to test the models developed in this paper, we fitted them to existing experimental data for the honey bee venom melittin, with the sequence GIGAVLKVLTTGLPALISWIKRKRQQ, and two peptides corresponding to the N-terminal residues 1–28 of the mouse prion protein (mPrPp, MANLGYWLLALFVTMWTDVGLCKKRPP), and the N-terminal residues

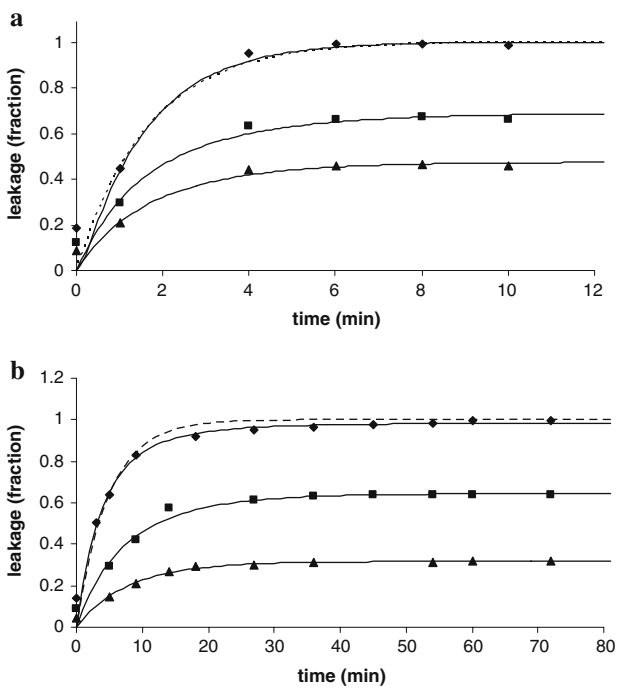

Fig. 3 Time-dependent leakage induced by the peptides melittin (*diamonds*), mPrPp (*squares*) and bPrPp (*triangles*). In **a** the leakage of calcein from 30% negatively charged vesicles is shown and in **b** leakage in erythrocytes is shown. Model 2 was fitted to data for all peptides (*solid line*) and model 1 also used for melittin data (*dashed lines*). The experimental data was provided by Magzoub and coworkers (Magzoub et al. 2005; K. Oglecka, P. Lundberg, M. Magzoub, L.E.G. Eriksson, Ü. Langel,A. Gräslund 2006, manuscript in preparation)

1–30 of the bovine prion protein (bPrPp, MVKSKIGSWILVLFVAMWSDVGLCKKR PKP).

The time-dependent leakage from neutral POPC and 30% negatively charged POPC/POPG vesicles was measured at a peptide concentration of 12.5 and 250 μM lipid concentration (Magzoub et al. 2005). Time-dependent hemolysis was also investigated. In short, the hRBCs from fresh blood were washed three times with buffer (150 mM NaCl, 0.1 mM EDTA, 20 mM Tris; pH 7.4) by centrifugation (10 min, 3,000 rpm, 10°C) and re-suspension in the same buffer. The hemolysis was induced by 30 μM prion peptides or 2 μM melittin. The details will be published elsewhere (K. Oglecka, P. Lundberg, M. Magzoub, L.E.G. Eriksson, Ü. Langel,A. Gräslund 2006, in preparation).

The concentration-dependent leakage of calcein from neutral and 30% negatively charged POPC/POPG vesicles was measured 5 min after the addition of peptide and the data were fitted by the concentration model combined with the time-dependence in model 2.

Time-dependent leakage

The time-dependent leakage data for melittin and the two prion protein-derived peptides, together with the fits are shown in Fig. 3. It is evident that model 2 can be used to fit both vesicle leakage (Fig. 3a) and hemolysis (Fig. 3b) data for all three peptides with a good result. For the prion protein-derived peptides, model 2 provides an explanation for the observation that the concentration difference across the membrane of the reporter molecules does not equilibrate at long times. The fitted parameters for these peptides are collected in Table 1. For the leakage in partially acidic vesicles induced by mPrPp and bPrPp, we find that the parameter J_2, related to the flow of calcein over the membrane, is a factor of 1.9 times larger for mPrPp, which indicates that this peptide induces a larger stress/perturbation over the membrane. The ratio between the v_{relax} parameter seen for the two peptides is 0.83 indicating that bPrPp approaches an equilibrium state in the membrane faster than mPrPp.

The rate of hemolysis induced by the prion protein-derived peptides is significantly slower compared to calcein leakage in vesicles. This is reflected in the fitting parameter v_{relax}, which is approximately six times lower compared to the fitted parameters obtained from the vesicle data. The ratio of the v_{relax} values for the two peptides is 0.84, indicating that the relative rate of approaching equilibrium for the peptide in the membrane is the same as in vesicles. By examining the ratio of J_2 for the two peptides in the case of hemolysis, it is 2.6. Thus, mPrPp is more efficient than bPrPp in causing leakage also in erythrocytes and even more so than what is observed in vesicles. Thus, mPrPp creates a larger stress/perturbation in the membrane as compared to bPrPp.

Melittin-induced leakage is both qualitatively and quantitatively different. The melittin-induced vesicle leakage as well as the hemolysis could very well be fitted using model 2, but model 1 was also seen to be sufficient to fit the vesicle data, and gave a reasonable fit of the hemolysis data. A major problem in distinguishing between the two models is that the resolution of the experimental data allows no estimate of the rate of the lag-phase, needed for model 1 (when the pores start to open). This causes the rate of opening-closing to be extremely high. The leakage approaches 1 as $t \rightarrow \infty$ meaning that J_2 approaches infinity in model 2. Thus, even though the models qualitatively can fit experimental data, the fitted rates are unreasonable. Model 2 is therefore not well-suited for fitting leakage that approaches 100%.

Since the opening-closing dynamics of a putative melittin pore is difficult to fit to the data using model 1, a simpler model can be used. By assuming that there is only an open state of the peptide, Eq. 5 can be recast in the form:

$$\frac{\mathrm{d}q(t)}{\mathrm{d}t} = Z(z(1-q(t)) - k_{\text{out}}q(t))[\text{peptide}], \qquad (16)$$

Table 1 Parameters of model 2 (Eq. 9) fitted to time-dependent leakage induced by the prion protein-derived peptides from mouse (mPrPp) and cow (bPrPp)

System	Peptide	J_2	v_{relax} (min^{-1})	$L^{\text{a}}_{\text{max}}$	$t_{0.5}$ (min)[b]
Vesicles	mPrPp	1.2 ± 0.4	0.4 ± 0.3	0.70	1.2
Vesicles	bPrPp	0.6 ± 0.1	0.5 ± 0.3	0.47	1.2
Erythrocytes	mPrPp	1.0 ± 0.1	0.092 ± 0.03	0.63	5.2
Erythrocytes	bPrPp	0.38 ± 0.03	0.11 ± 0.03	0.32	5.5

The leakage was monitored in 30% negatively charged POPC/PG vesicles and erythrocytes

[a] calculated from J_2 using Eq. 18

[b] calculated from J_2 and v_{relax} using Eq. 39

where Z is a proportionality constant, related to X. From this a simple formula can be derived for the leakage as:

$$\frac{I_{\text{obs}}(t)}{I_{\max}} = 1 - \exp(-tJ_0), \tag{17}$$

where J_0 is the induced flow-rate of leakage of reporter molecules. By using Eq. 17 rather than Eq. 6 to fit the melittin-induced leakage the same quality of fit was obtained, but with only one fitting parameter. For the leakage induced by melittin in partially acidic vesicles $J_0 = 0.60\ \text{min}^{-1}$ and for the hemolysis $J_0 = 0.21\ \text{min}^{-1}$. Thus, the leakage in the vesicles is roughly three times faster compared to the leakage in erythrocytes.

Concentration-dependence

The concentration-dependent leakage caused by the two prion protein-derived peptides was modeled using Eq. 15 (Fig. 4). The model was used successfully to describe leakage in both zwitterionic and partly negatively charged vesicles. In Table 2 the fitted parameters for concentration-dependent leakage in both zwitterionic (POPC, Fig. 4a) and partially negatively charged (POPC/PG, Fig. 4b) vesicles are presented. The most interesting parameters are perhaps the scaled dissociation constant K and the maximum binding concentration of the peptide B_0.

The apparent scaled dissociation constant (K) is very similar for the two peptides in neutral vesicles, whereas the maximum concentration in the membrane is a factor 1.2 larger for mPrPp. The K-value for mPrPp is lower in the partially acidic vesicle than in zwitterionic vesicles. For bPrPp, on the other hand, K is larger in partially acidic as compared to zwitterionic vesicles. This is somewhat unexpected since bPrPp has two more positive charges compared with mPrPp (Magzoub et al. 2005) and would therefore be expected to bind tighter to a charged membrane. The results thus indicate that the electrostatics is not the dominating contribution to the peptide-membrane interaction for bPrPp.

When investigating the maximum membrane-bound peptide concentration, B_0, a larger amount of mPrPp than bPrPp is seen in both types of membranes. By using the values of K and B_0 obtained for the partially negatively charged vesicles, we can calculate the apparent concentration of bound peptide in the time-dependent experiments using Eq. 13. The concentration of mPrPp in the membrane was calculated to be 11.4 μM, while for bPrPp it is 7.4 μM. From this we can estimate the leakage caused per peptide in the membrane by simply dividing the values of J_2 obtained in the time-dependent leakage with the peptide concentration. The J_2/B-ratio for mPrPp is 0.11 and 0.08 for bPrPp. Thus, both peptides cause similar amount of leakage per peptide.

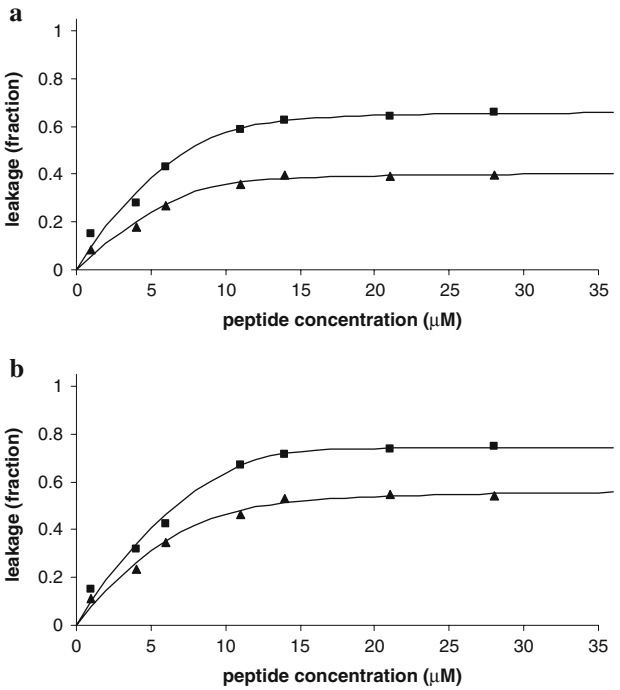

Fig. 4 Concentration-dependent leakage induced after 5 min incubation by the peptides mPrPp (*squares*) and bPrPp (*triangles*). The leakage was monitored in **a** zwitterionic POPC vesicles and **b** 30% negatively charged POPC/PG vesicles. The experimental data was provided by Magzoub and coworkers (Magzoub et al. 2005)

From the fitted values, the half-maximum leakage caused by peptides can be obtained (see Appendix 3), and the values for these parameters have been calculated and are included in Tables 1 and 2. One striking observation is that in all the different systems and for both concentration- and time-dependence the values for half-maximum leakage are similar for both prion protein-derived peptides. This is in contrast to the maximum leakage, which is generally higher for mPrPp. This shows that the leakage process for the two peptides has effectively the same rate, but that mPrPp causes a larger perturbation in the membrane.

It has been argued in the literature that the fluorescence signal in leakage experiments not always directly reflects the actual leakage (Schwarz and Arbuzova 1995; Ladokhin et al. 1995). One reason is the nonlinear concentration dependence of the fluorescence signal. Another reason is the signal contribution from both the inside and the outside of the vesicles. In order to account for such effects the fluorescence signal

Table 2 Parameters of the concentration model (Eq. 15) fitted to concentration-dependent leakage induced after 5 min incubation with the prion-protein-derived peptides from mouse (mPrPp) and cow (bPrPp)

Vesicle type	Peptide	B_0 (μM)	K (μM)	J_{conc} (μM^{-1})	$C_{0.5}$(μM)[a]
POPC	mPrPp	10.0 ± 1.5	0.6 ± 0.9	0.11 ± 0.02	4.0
POPC	bPrPp	8.3 ± 1.5	0.6 ± 0.8	0.063 ± 0.01	4.0
POPC/PG	mPrPp	13.0 ± 1.8	0.16 ± 0.6	0.11 ± 0.02	4.4
POPC/PG	bPrPp	9.4 ± 1.9	1.0 ± 1.4	0.09 ± 0.02	4.4

The leakage was monitored in zwitterionic (POPC) and 30% negatively charged POPC/POPG vesicles

[a] calculated from B_0, K and J_{conc} using Eq. 38

of calcein was measured as a function of concentration, in the presence of LUVs (Fig. 5). The result of this experiment was compared to the fluorescence signal obtained by lysing vesicles with entrapped calcein with the detergent Triton X-100. By diluting this sample with small amounts of buffer a linear relationship between the decrease in fluorescence signal and dilution was obtained. From these experiments it is possible to confirm that at the studied concentrations no self-quenching occurs outside the vesicles, and we determined the final concentration of calcein to 24 μM in the leakage experiment. The diameter of the LUVs used is ~100 nm, and at the current lipid concentration, the volume fraction of the inside of the vesicles is ~0.05% (Walde and Ichikawa 2001). Thus, the fluorescence signal observed in the leakage experiments is approximately linearly dependent on calcein concentration, and is completely dominated by the outside contribution.

It is interesting to note that, if the leakage can be described by model 2, the parameter J_2 can be derived from the data without a fitting procedure. If L_{max} is the maximum leakage induced by the peptide, Eq. 9 can be reformulated, and we get:

$$J_2 = -\ln|1 - L_{max}|. \tag{18}$$

Thus, by estimating the maximum leakage level, information on the stress/perturbation caused by the peptide on the membrane is gained in a simple way.

Error and stability analysis

Since no error estimates were available for the experimental data points used in the fits, errors were estimated from the deviation of the data from the fitted curve, weighted by the Jacobian matrix from the fitting procedure. It is important to emphasize that these errors are generated under the assumption that the models are able to correctly describe the experimental data.

In order to investigate the stability and error propagation in the presented models, the models were fitted to simulated data. The data was generated from the models, with an increasing error, which was assumed to be normal-distributed around the mean-value, and the size was defined as the standard deviation. By incrementing the number of data points it was found that four data points within a dynamical range (<95% of maximum leakage) was sufficient to obtain good fits for high-precision data (error typically below 5% for the time-dependent models). During the error-propagation simulations eight data points were used, with 4 points within the dynamical range. The total number of iterations used in the simulations was 5,000.

For model 1 the relative error in the fitted parameter J_1 is approximately twice the error of the simulated data, whereas the error for v_{fluct} is a factor five larger than the input error (Fig. 6a). This indicates that the error in the data is moderately increased by the model. The higher degree of error propagation for v_{fluct} is due to the fast opening and closing dynamics of the pores, which is not well defined by only four data points in the dynamical range. The trend in error propagation in model 2 is similar to the one observed in model 1 (Fig. 6b) but here the error in data is suppressed. The parameter J_2 has a fitted error of approximately one fourth of the input error and the error in v_{relax} is roughly half of the input error. Thus the model is robust when fitted to data containing a random error. For the concentration model J_{conc} has similar error propagation as the J_1 and J_2 parameters, while the input errors for B_0 and K are increased (Fig. 6c). This shows that in order to obtain quantitative information concentration-dependent leakage, data with high precision is needed, or more data points.

The error propagation of model 1 described in Fig. 6a, is likely to explain why no reasonable parameters were obtained for the melittin time-dependent data. In the concentration model the membrane-binding parameters, K and B_0, have larger errors compared

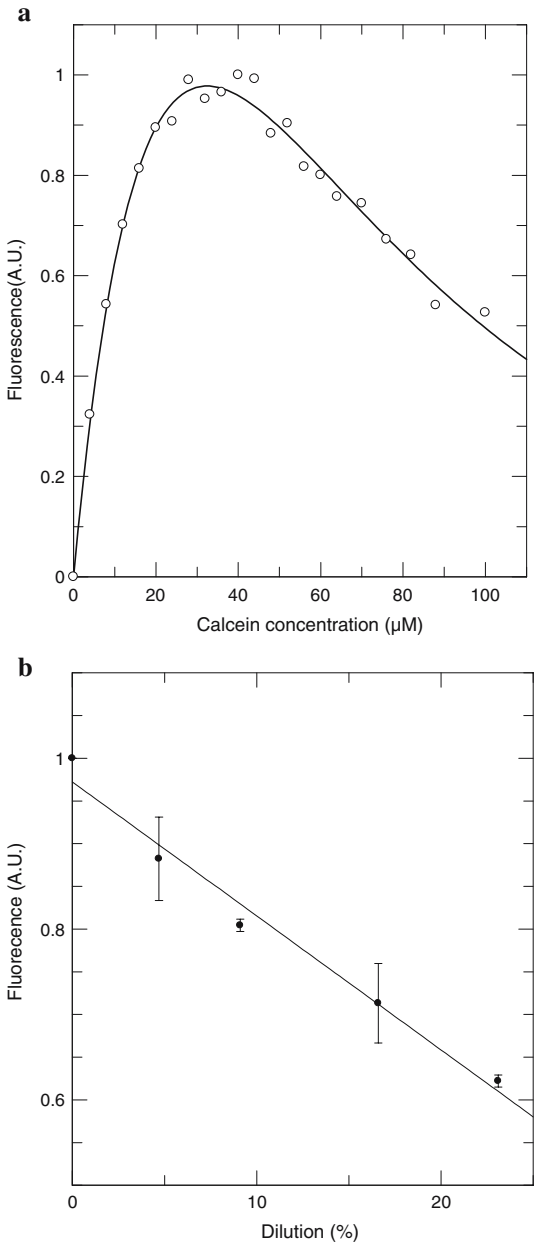

Fig. 5 The self-quenching of calcein depicted as a function of concentration. In **a** the fluorescence emission intensity is plotted against calcein concentration in the presence of POPC LUV vesicles (250 μM POPC, 50 mM phosphate buffer pH 7). In **b** the fluorescence emission intensity from vesicles, containing 75 mM calcein, lysed by 10% (w/v) Triton X-100 is plotted as a function of dilution with buffer

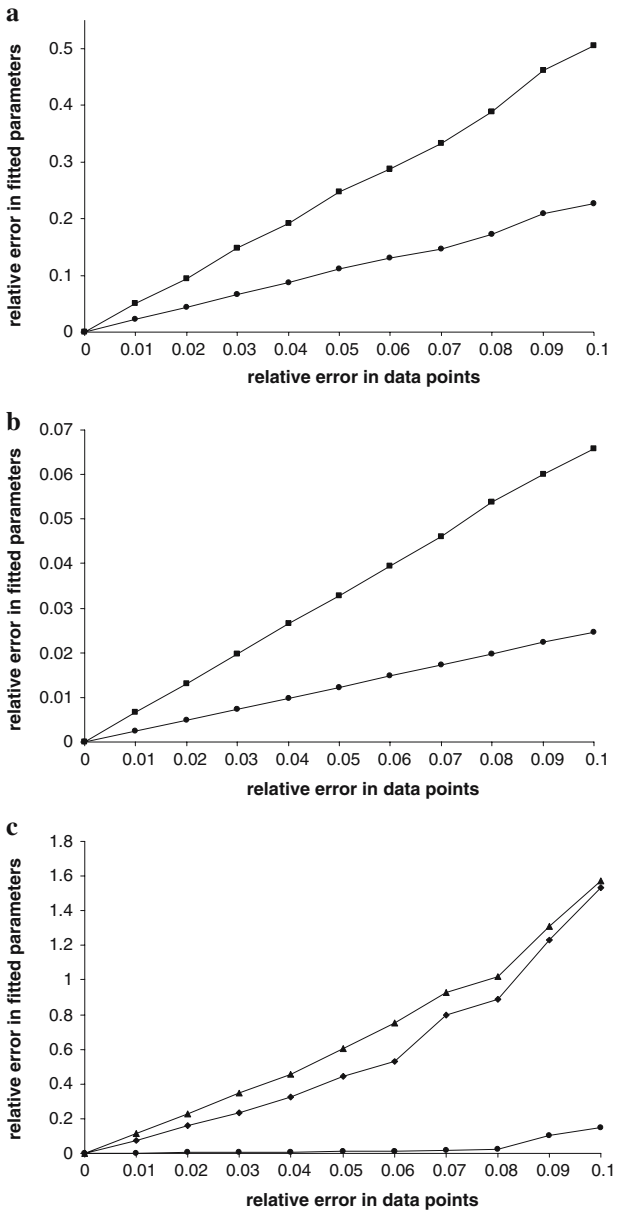

Fig. 6 Simulated propagation of error from data points to fitted parameters in **a** model 1 (Eq. 6), **b** model 2 (Eq. 9) and **c** the concentration model (Eq. 15). In **a** the fitted parameters are J_1 (*circles*) v_{fluct} (*squares*). In **b** the fitted parameters are J_2 (*circles*) and v_{relax} (*squares*). In **c** the fitted parameters are J_{conc} (*circles*), K (*diamonds*) and B_0 (*triangles*)

with J_{conc}, which is expected from the simulations (Fig. 6c).

Discussion

Several models for peptide-induced membrane leakage have been presented in the literature. Especially Schwarz and co-workers have developed models with increasing complexity (Schwarz and Robert 1990, 1992; Schwarz and Arbuzova 1995; Schwarz and Rex 1998). The underlying assumption of these models is that the leakage is caused by pores formed by the peptides. In their first model, Schwarz and Robert developed an expression for the flow though an open pore by integrating Fick's first law of diffusion with respect to time, related to Eqs. 16 and 17 in this presentation (Schwarz

and Robert 1990). This theory was later refined, and opening and closing dynamics of the pores were included (Schwarz and Robert 1992). In this formulation a distinction between ''all-or-none'' leakage and ''graded'' leakage is made. All-or-none leakage is obtained if the time of the experiment is much longer compared to the efflux kinetics and if the open pores have long lifetimes. The graded leakage is obtained if the pore lifetime is shorter compared to the average efflux time, and if the time of the experiment is on the same time scale or shorter than the pore lifetime. This second formulation is made in terms of lifetimes instead of reaction rates, but otherwise it is analogous to model 1 in the present investigation. Both models share the observation that as time approaches infinity the concentration of reporter molecules should equilibrate.

Rex and Schwarz (1998) have presented a model where the leakage caused by melittin is related to transient states giving rise to a non-equal transmembrane concentration of reporter molecules. This model is qualitatively similar to the present model 2, but there are important differences. The transient leaky states in the previous formulation are intermediates on the pathway to a regular pore in the membrane. In other words, even though the initial leakage occurs through relatively short-lived rate-limiting states, the transmembrane concentrations of reporter molecules will equilibrate by leakage through pores as time approaches infinity.

Model 2, on the other hand, predicts a situation where the transmembrane concentration difference of reporter molecules does not equilibrate, even at very long times, as observed for instance for the two prion protein-derived peptides. This situation occurs when the leakage is caused by a perturbation of the membrane caused by the peptide. As a response to the perturbation the peptide–lipid system reorganizes, which causes the leakage to stop as the system approaches equilibrium. The model involves two distinct physical states and two fitting parameters. These two fitting parameters are related to general features of leakage such as the speed of how fast the reporter molecules diffuse through the membrane and how fast the membrane-peptide system reorganizes after the peptide is added. Since no specific leaky states need to be defined, model 2 has an inherent generality with respect to the molecular mechanism causing the leakage.

This does not mean that it is not possible to link the model with a detailed mechanism. In the present case, this model is consistent with the observation that bPrPp is a transmembrane α-helix under equilibrium conditions (Biverståhl et al. 2004). The peptide first associates with the membrane, and upon rearrangements towards a transmembrane configuration, it causes leakage. This leakage stops after an equilibrium situation has been achieved. The fitted parameters, J_2 and v_{relax}, for the time-dependent leakage can in this case be interpreted as the rate of flow of reporter molecules, and the rate of the rearrangement process towards a transmembrane location of the peptide.

Lately, expanded models for leakage have been presented by Pokorny and coworkers (Pokorny and Almeida 2004; Pokorny et al. 2002). Their setup is formally more similar to model 1 presented here, compared with the original formulation by Schwarz and coworkers. The large numbers of states presented in these models make the system difficult to solve analytically and numerical solutions are necessary.

An important question that is addressed by the present study is what physical information that can be extracted from leakage experiments, related to the biological function of the investigated peptide. For certain peptides, like antimicrobial peptides, it is believed that the induction of leakage is actually the major biological function of the peptide (Matsuzaki 1999). For other peptides the leakage is something that should be avoided, for instance in identifying and designing so-called cell-penetrating peptides (CPPs) (Magzoub and Gräslund 2004). These peptides are thought to be able to translocate biological membranes, and transport, e.g., drugs and reporter molecules into cells in an efficient manner. Peptides with different effect on membranes have been shown to locate differently in the membrane (Biverståhl et al. 2004; Bárány-Wallje et al. 2004; Lindberg et al. 2003) and the toxicity and leakage properties seem correlated with the location in the membrane. It is, for instance, not unreasonable to assume that a peripherically attached peptide induces a very different stress in the membrane compared with an integral transmembrane peptide. This has interesting implications, since most integral peptides probably attach peripherically to the membrane at the first encounter.

The possibility to estimate how much leakage a peptide causes compared to the time it takes for the peptide/membrane system to reach equilibrium provides a nice example of the applicability of model 2. Assuming that the process that causes leakage is coupled to peptide translocation (Fig. 2) (which is crucial for the CPPs), model 2 can thus give the speed of translocation (v_{relax}) together with the membrane distortion (the leakage of reporter molecules, related to J_2). By designing several peptides and comparing the

leakage properties one can easily estimate peptide translocation rates in combination with the lowest cost of perturbing the homeostasis of the cell.

In conclusion we find that the present models can describe peptide-membrane interactions that lead both to maximum-leakage, e.g., melittin, and sub-maximum leakage, e.g., the prion protein-derived peptides. The fitted parameters provide insights into how fast the peptide-membrane system approaches equilibrium, and how large membrane perturbation is caused by the peptide. These models thus provide a solid physical basis for understanding peptide-induced leakage experiments.

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Appendix 1. Model 1: pore formation

The kinetics of the opening process is given by the following differential equation:

$$\frac{\mathrm{d}\alpha(t)}{\mathrm{d}t} = k_{\mathrm{on}}(1-\alpha(t)) - k_{\mathrm{off}}\alpha(t), \tag{19}$$

where k_{on} and k_{off} are defined in Fig. 1. By using the boundary condition that all of the peptide is in the closed state at the beginning of the experiment, we get:

$$\alpha(t) = \frac{k_{\mathrm{on}}}{k_{\mathrm{on}}+k_{\mathrm{off}}}\left(1-\mathrm{e}^{-(k_{\mathrm{on}}+k_{\mathrm{off}})t}\right) \tag{20}$$

The change in concentration of reporter molecules on the inside ($i(t)$) is assumed to be proportional to the concentration of peptides in the open state, (o(t)):

$$\frac{\mathrm{d}i(t)}{\mathrm{d}t} = (k_{\mathrm{in}}o(t) - k_{\mathrm{out}}i(t))X(\alpha(t)[\mathrm{peptide}]), \tag{21}$$

where k_{in} and k_{out} are defined in Fig. 1, [peptide] is the concentration of peptides bound to the membrane. The proportionality constant X is related to the size of the perturbation caused by the peptide. The concentration of reporter molecules inside and outside is defined as $i(t) = n_{\mathrm{in}}(t)/V_{\mathrm{in}}$ and $o(t) = n_{\mathrm{out}}(t)/V_{\mathrm{out}}$, where $n_{\mathrm{in}}(t)$ and $n_{\mathrm{out}}(t)$ are the molar amounts of reporter molecules on the in- and outside, and V_{in} and V_{out} are the respective volumes. It is useful to define the relative amount of reporter molecules on the inside as $q(t) = n_{\mathrm{in}}(t)/(n_{\mathrm{in}}(t) + n_{\mathrm{out}}(t))$, and at the same time we define, $z = k_{\mathrm{in}}(V_{\mathrm{in}}/V_{\mathrm{out}})$. By assuming that the relative volumes of the vesicles are unchanged (which is reasonable as long as the vesicles are intact) the change in $q(t)$ with time becomes:

$$\frac{\mathrm{d}q(t)}{\mathrm{d}t} = (z(1-q(t)) - k_{\mathrm{out}}q(t))X\alpha(t)[\mathrm{peptide}]. \tag{22}$$

By noting that the direction of the flow of reporter molecules requires that $\mathrm{d}q/\mathrm{d}t < 0$, and by noting that all reporter molecules are on the inside at $t = 0$ ($q(0) = 1$) we get:

$$q(t) = \left(z + k_{\mathrm{out}}\exp\left[(z+k_{\mathrm{out}})\frac{k_{\mathrm{on}}}{(k_{\mathrm{on}}+k_{\mathrm{off}})^2}X[\mathrm{peptide}] \times \left(1 - t(k_{\mathrm{on}}+k_{\mathrm{off}}) - \mathrm{e}^{-(k_{\mathrm{on}}+k_{\mathrm{off}})t}\right)\right]\right)/(z+k_{\mathrm{out}}). \tag{23}$$

It is now useful to see what happens as time approaches infinity. Since $k_{\mathrm{in}} \approx k_{\mathrm{out}}$ we get:

$$q(t\to\infty) = \frac{V_{\mathrm{in}}}{V_{\mathrm{in}}+V_{\mathrm{out}}}. \tag{24}$$

Thus, the fraction of molecules on the inside is simply proportional to the vesicle volume fraction. By reformulating Eq. 23 and using $k_{\mathrm{in}} = k_{\mathrm{out}} = k$, we get:

$$q(t) = \left(V_{\mathrm{in}} + V_{\mathrm{out}}\exp\left[k\left(\frac{V_{\mathrm{in}}+V_{\mathrm{out}}}{V_{\mathrm{out}}}\right)\frac{k_{\mathrm{on}}}{(k_{\mathrm{on}}+k_{\mathrm{off}})^2}X[\mathrm{peptide}]\left(1 - t(k_{\mathrm{on}}+k_{\mathrm{off}}) - \mathrm{e}^{-(k_{\mathrm{on}}+k_{\mathrm{off}})t}\right)\right]\right)/(V_{\mathrm{in}}+V_{\mathrm{out}}). \tag{25}$$

In order to obtain a simple fitting model with parameters that have physical meaning, we can define $J_1 = k((V_{\mathrm{in}} + V_{\mathrm{out}})/V_{\mathrm{out}})k_{\mathrm{on}}X[\mathrm{peptide}]$ and $v_{\mathrm{fluct}} = k_{\mathrm{on}} + k_{\mathrm{off}}$, and with these definitions Eq. 25 becomes:

$$q(t) = \left(V_{\mathrm{in}} + V_{\mathrm{out}}\exp\left[\frac{J_1}{v_{\mathrm{fluct}}^2}(1 - v_{\mathrm{fluct}}t - \mathrm{e}^{-v_{\mathrm{fluct}}t})\right]\right)/(V_{\mathrm{in}}+V_{\mathrm{out}}). \tag{26}$$

Since $q(t)$ is the fraction of reporter molecules on the inside of the membrane, and the experimental observable in most cases is proportional to the

amount on the outside, we are really interested in (1–$q(t)$), and from this we obtain:

$$\frac{I_{\mathrm{obs}}(t)}{I_{\max}} = 1 - \exp\left[\frac{J_1}{v_{\mathrm{fluct}}^2}(1 - v_{\mathrm{fluct}}t - \mathrm{e}^{-v_{\mathrm{fluct}}t})\right], \tag{27}$$

where $I_{\mathrm{obs}}(t)$ is the measured signal of reporter molecules for the investigated peptide and $I_{\max}(t)$ is the maximum leakage.

Appendix 2. Model 2: peptide-induced membrane stress/perturbation

In the second model, the leakage of reporter molecules is coupled to the reorganization of the membrane/peptide system as a response to a stress/perturbation induced by the peptide, related to two states, 1 and 2. The fraction of peptide in the first state is defined as $\beta(t)$ (Fig. 2). The kinetics of the conversion of the peptide from state 1 to state 2 is modeled as a differential equation:

$$\frac{\mathrm{d}\beta(t)}{\mathrm{d}t} = ((1 - \beta(t))k_2 - \beta(t)k_1), \tag{28}$$

where k_1 and k_2 are defined as in Fig. 2. The solution of this equation is analogous with model 1, with the boundary condition that all of the peptide should reside in state 1 at time zero ($\beta(0) = 1$). Using this condition we get:

$$\beta(t) = \frac{1}{k_1 + k_2}\left(k_2 + k_1\mathrm{e}^{-(k_1+k_2)t}\right). \tag{29}$$

The leakage of reporter molecules is proportional to the conversion between state 1 to state 2, and thus to $\mathrm{d}\beta\ (t)/\mathrm{d}t$, which gives us:

$$\frac{\mathrm{d}q(t)}{\mathrm{d}t} = -Y(z(1 - q(t)) - k_{\mathrm{out}}q(t))\left(\frac{\mathrm{d}\beta(t)}{\mathrm{d}t}[\mathrm{peptide}]\right), \tag{30}$$

where Y is the analog of the constant X in model 1. As for model 1, the direction of flow of reporter molecules requires that $\mathrm{d}q(t)/\mathrm{d}t < 0$. By using the boundary condition that all reporter molecules are inside the membrane compartment at $t = 0$, $q(0) = 1$, we get:

$$q(t) = \left[z + k_{\mathrm{out}}\exp\left((z + k_{\mathrm{out}})\frac{k_1}{(k_1 + k_2)}Y[\mathrm{peptide}]\left(\mathrm{e}^{-(k_1+k_2)t} - 1\right)\right)\right]/(z + k_{\mathrm{out}}). \tag{31}$$

In analogy with Eq. 24 it is again useful to examine what happens as time approaches infinity:

$$q(t \to \infty) = \left[z + k_{\mathrm{out}}\exp\left(-(z + k_{\mathrm{out}})\frac{k_1}{(k_1 + k_2)}Y[\mathrm{peptide}]\right)\right]/(z + k_{\mathrm{out}}). \tag{32}$$

As in model 1, it is also reasonable to assume that $k_{\mathrm{in}} = k_{\mathrm{out}} = k$ and Eq. 32 can be reformulated using this assumption:

$$q(t) = \left[V_{\mathrm{in}} + V_{\mathrm{out}}\exp\left(k\left(\frac{V_{\mathrm{in}}}{V_{\mathrm{out}}} + 1\right)\frac{k_1}{(k_1 + k_2)}Y[\mathrm{peptide}]\left(\mathrm{e}^{-(k_1+k_2)t} - 1\right)\right)\right]/(V_{\mathrm{in}} + V_{\mathrm{out}}). \tag{33}$$

To obtain a simple equation we define $J_2 = k((V_{\mathrm{in}} + V_{\mathrm{out}})/V_{\mathrm{out}})(k_1/(k_1 + k_2))Y[\mathrm{peptide}]$ and $v_{\mathrm{relax}} = (k_1 + k_2)$. With these definitions we get:

$$q(t) = [V_{\mathrm{in}} + V_{\mathrm{out}}\exp(J_2(\mathrm{e}^{-v_{\mathrm{relax}}t} - 1))]/(V_{\mathrm{in}} + V_{\mathrm{out}}). \tag{34}$$

Thus, again, we have a relatively simple equation with only two fitting parameters: J_2 and v_{relax}. In order to compare with experimental data, we obtain:

$$\frac{I_{\mathrm{obs}}(t)}{I_{\max}} = 1 - \exp(J_2(\mathrm{e}^{-v_{\mathrm{relax}}t} - 1)), \tag{35}$$

where, $I_{\mathrm{obs}}(t)$ is the observed data signal and $I_{\max}$ is the maximum leakage.

Appendix 3. Half-maximum leakage

The relation describing the concentration-dependent leakage was given in Eq. 15. As the total peptide concentration approaches infinity, we get:

$$\frac{I_{\mathrm{obs}}([\mathrm{total}] \to \infty)}{I_{\max}} = 1 - \exp(-J_{\mathrm{conc}}B_0). \tag{36}$$

From this equation we note that the asymptotic values of the concentration-dependent leakage depend on B_0 and J_{conc}, but not K. The half-maximum leakage (HMLC) (at infinite peptide concentration) for concentration-dependent leakage is defined as HMLC = (1 – exp(– J_{conc} B_0))/2. By combining this expression with Eq. 15, we can calculate the concentration ($C_{0.5}$) where HMLC is induced:

$$\frac{1-\exp\left(-J_{\mathrm{conc}}B_0\right)}{2}=1-\exp\left(-\frac{1}{2}J_{\mathrm{conc}}\left[C_{0.5}+B_0+K-\sqrt{\left(C_{0.5}+B_0+K\right)^2-4C_{0.5}B_0}\right]\right). \quad (37)$$

By solving this equation with respect to $C_{0.5}$ we get:

$$C_{0.5}=\frac{uB_0+uK-u^2}{B_0-u}, \quad (38)$$

where $u = -ln\,|\,0.5(1+\exp(-J_{\mathrm{conc}}B_0))\,|/J_{\mathrm{conc}}$. Thus $C_{0.5}$ and K are linearly related.

The half maximum leakage (HMLT) as a function of time-dependence (model 2) is defined as HMLT = $(1-\exp(-J_2))/2$. Defining the time where half-maximum leakage is obtained as $t_{0.5}$, we get from Eq. 35:

$$t_{0.5}=\frac{\ln\left|J_2/(J_2+v)\right|}{v_{\mathrm{relax}}}, \quad (39)$$

where $v = ln\,|\,0.5(1+\exp(-J_2))|$. Thus $t_{0.5}$ is inversely proportional to v_{relax}. Since v_{relax} is the sum of the rates k_1 and k_2, $1/v_{\mathrm{relax}}$ is the correlation time for the conversion from state 1 to state 2 in the peptide.

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