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Generalised bilayer perturbation from peptide helix dimerisation at membrane surfaces: vesicle lysis induced by disulphide-dimerised melittin analogues

Jiro Takei[1], Attila Remenyi[2], Christopher E. Dempsey*

Biochemistry Department and Centre for Molecular Recognition, University of Bristol, Bristol BS8 1TD, UK

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Abstract The effects of covalent dimerisation of melittin by disulphide formation in cysteine-substitution analogues, (melittin K23C)$_2$ and (melittin K23Q,Q25C)$_2$, on the kinetics of pore formation in phosphatidylcholine small unilamellar vesicles was measured under low ionic strength conditions. The initial rate of melittin-induced pore formation increased with the square of the peptide concentration, whereas both disulphide-dimerised melittin analogues showed a first-order dependence of pore formation rates on peptide concentration. These results indicate that peptide dimerisation is rate-limiting for pore formation under these conditions. A model for a generalised bilayer perturbation resulting from the self-association of a pair of peptide helices at the membrane surface is proposed which may have implications for a number of biological processes that involve the interaction of helical polypeptides with membranes.

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Key words: Peptide antibiotic; Sec-independent; Magainin; Colicin; Membrane protein

1. Introduction

Melittin [1,2] is an example of a class of lytic peptides from microorganisms and animal venoms that adopt amphipathic helical conformations on binding to membrane surfaces resulting, ultimately, in lysis [3,4]. The nature of the membrane perturbation induced by these peptides is of interest in view of the potential for design of selective antibiotics [5,6]. Although many of the peptides form voltage-gated ion channels at low concentrations in planar bilayers [7], the lytic pore is not necessarily the ion channel state, and in many cases the peptide concentration required for vesicle or cell lysis is considerably higher than that required to support voltage-dependent ion conductance [8]. Recently, compelling models for magainin- and melittin-induced vesicle lysis have been proposed based on observations that lysis is associated with peptide internalisation and lipid flip-flop [9,10]. In these models, pores comprise bundles of transmembrane (TM)-oriented peptide with interspersed lipids (providing a pathway for lipid flip-flop) that form transiently as the build-up of peptide in the outer bilayer leaflet is relieved by redistribution of peptide and lipid between the outer and inner bilayer leaflets.

Events that trigger the formation of the lytic pore are not known at the molecular level. In general, no evidence for significant peptide self-association is observed at equilibrium (melittin [11]; cecropin P [12]; magainin [13]) although the observation of greater than first-order dependence of pore formation rates on peptide concentration may indicate that transient peptide self-association might trigger the lytic event in some cases [14,15]; see also [16,17]. Alternatively, Huang and colleagues have shown that the bilayer perturbation induced by partitioning of peptide into the interfacial region should increase as the square of the peptide concentration [18], and this might underlie the higher-order dependence of pore formation rates on peptide concentration sometimes observed. In this report we show that disulphide dimerisation of cysteine-containing melittin analogues can convert bimolecular lysis kinetics into unimolecular kinetics, indicating that peptide dimerisation per se is rate-limiting for melittin-induced lysis under some conditions. We propose a generalised model for perturbation of a phospholipid bilayer resulting from peptide helix dimerisation in the interfacial region that might have wider implications for the insertion of amphipathic or hydrophobic helical polypeptides into membranes.

2. Materials and methods

Melittin was purified from bee venom as described previously [19]. The synthesis, purification, disulphide dimerisation and characterisation of the cysteine-containing peptides used in this study have been described [20]. Dioleoyl-phosphatidylcholine (DOPC) and egg phosphatidylcholine (egg PC) were from Lipid Products (Nutfield, UK) and carboxyfluorescein (CF) was from Sigma Chemical Co. Small unilamellar vesicles (SUV) loaded with CF were prepared by hydrating vacuum-dried lipids in assay buffer (10 mM Tris-HCl, pH 8.0) containing CF (50 mM), followed by sonication on ice until the solution was optically clear. Vesicles containing encapsulated CF were separated from external CF by gel filtration in assay buffer.

Leakage of encapsulated CF was measured from the increase in fluorescence intensity (excitation and emission wavelength 490 and 520 nm, respectively), resulting from the reduction of fluorescence self-quenching as the dye is diluted into the extravesicular space [21]. Dye efflux was initiated by adding an aliquot (20 μl) of vesicles (to give a final lipid concentration of 52 μM) to a stirred peptide solution in a fluorescence cuvette. The fluorescence resulting from complete dilution of all encapsulated CF was determined after addition of an aliquot of vesicles into a 0.25% Triton X-100 solution in assay buffer.

2.1. Kinetic analysis

The time course for the peptide-induced fluorescence increase was analysed according to the treatment of Schwarz et al. [15] in terms of

*Corresponding author. Fax: (44) (117) 928 8274.
E-mail: c.dempsey@bristol.ac.uk

[1]Present address: Laboratory of Biochemistry, National Cancer Institutes, National Institutes of Health, Bethesda, MD 20892, USA.

[2]Present address: EMBL, Gene Expression Program, 69117 Heidelberg, Germany.

Abbreviations: DOPC, dioleoylphosphatidylcholine; egg PC, egg phosphatidylcholine; (mlK23C)$_2$, K23C-melittin disulphide dimer; (mlK23Q,Q25C)$_2$, K23Q,Q25C-melittin disulphide dimer; SUV, small unilamellar vesicle

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the rate of pore formation. No assumption is made about the structure of the peptide-induced pore other than as a pathway for dye efflux. The average number of pores per vesicle, p, open during the time t ($0 \rightarrow t$) is defined by the following formula:

$$p(t) = \int_0^t v_p dt \quad (1)$$

where $v_p = n \cdot dr_p/dt$ (r_p = molar ratio of pores per lipid and n = number of lipids per vesicle). Under conditions where a vesicle is depleted upon opening of the first dye efflux pathway ('all or nothing' kinetics), $p(t)$ can be determined directly from the experimental fluorescence data as follows:

$p(t) = -\ln E(t)$, where $E(t) = (F_\infty - F)/(F_\infty - F_0)$ and F, F_0 and F_∞ are, respectively, the fluorescence intensities at time t, at $t = 0$, and upon full dilution of dye into the extravesicular medium (determined after vesicle disruption with Triton X-100).

Since the rate of dye efflux (and v_p) decreases from an initial fast rate (v_{po}) to a smaller, steady-state rate (v_{pi}), contributions from each kinetic phase are incorporated into equation involving a time constant, k:

$$v_p = v_{pi} + (v_{po} - v_{pi})e^{-kt} \quad (2)$$

and (from Eq. 1):

$$p(t) = v_{pi} \cdot t + (v_{po} - v_{pi}) \cdot [(1 - e^{-kt})/k]. \quad (3)$$

Fits of the experimental data to Eq. 3 are illustrated in the Figures. The degree to which dye efflux obeys 'all or nothing' or 'graded' kinetics has not been determined (preliminary results indicate that under the experimental conditions described, release by $(mlK23Q,Q25C)_2$ obeys (close to) 'all or nothing' kinetics whereas melittin- or $(mlK23C)_2$-induced release is 'graded'). Therefore, we concentrate on the initial rate of dye efflux (characterised by v_{po} in Eqs. 2 and 3 above) since extraction of this rate is minimally affected by inaccuracies due to incomplete quenching by residual unreleased dye in partially lysed vesicles.

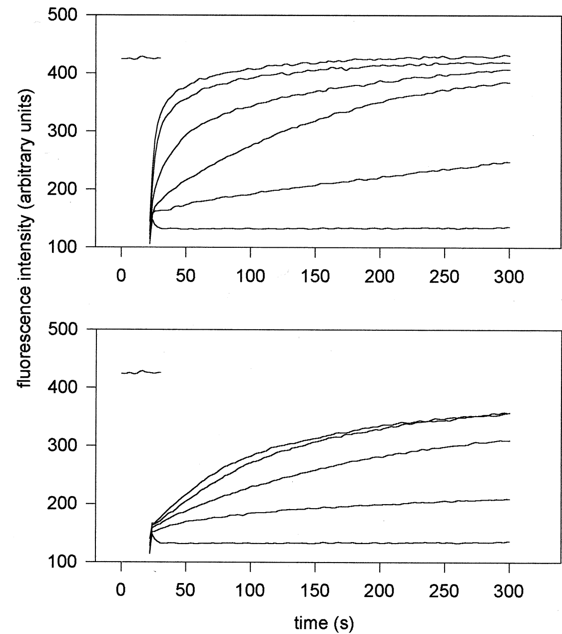

Fig. 1. Time-dependent increase in fluorescence resulting from decreased self-quenching of CF upon addition of CF-loaded DOPC SUV to solutions of (a) melittin at concentrations of 0, 0.5, 0.6, 0.75, 1 and 1.25 μM (bottom to top) and (b) $(mlK23C)_2$ at concentrations of 0, 0.25, 0.5, 1.0 and 1.25 μM (bottom to top). Vesicles were added 20 s after starting the traces. The fluorescence intensity resulting from complete release of CF upon disruption of vesicle with Triton X-100 is shown in the upper left trace.

3. Results

Fig. 1 illustrates the time course of the fluorescence increase due to the release of internalised CF induced by binding of melittin (top) and $(mlK23C)_2$ to DOPC SUV. In Fig. 2a,b these data are converted into the time course for apparent pore openings according to Eq. 3. For each peptide the dye release follows biphasic kinetics, with an initial fast phase of dye release relaxing to a slow steady-state rate. Also shown in Fig. 2 are the fits of the experimental data to Eq. 3.

Fig. 2 also illustrates data from a second dye release experiment in which the rates of pore formation induced by melittin and $(mlK23Q,Q25C)_2$ in egg PC SUV are compared (Fig. 2c,d). In the case of the melittin dimer cross-linked at residue 25, the dye release kinetics are almost purely monophasic

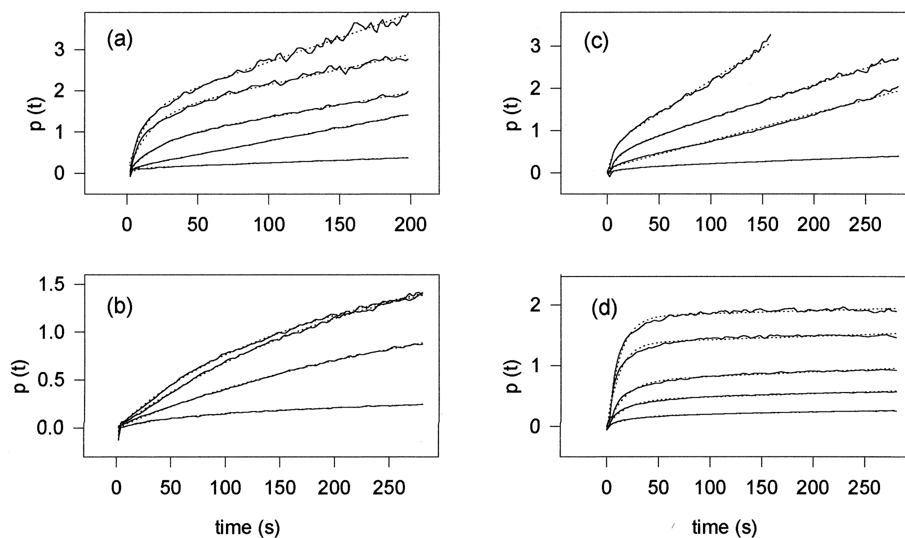

Fig. 2. Time dependence of apparent pore formation, determined as described in Section 2, induced by (a) melittin, (b) $(mlK23C)_2$ in DOPC SUV, determined from the dye efflux data in Fig. 1. The peptide concentrations are as described in Fig. 1 (excluding the zero peptide concentration curve). c and d: Pore formation from sets of dye release data (not shown) in egg PC SUV induced by melittin (c) at peptide concentrations of 0.125, 0.3, 0.5 and 0.62 μM (bottom to top), and (d) $(mlK23Q,Q25C)_2$ at peptide concentrations of 0.085, 0.16, 0.32, 0.42 and 0.63 μM (bottom to top). In all panels, the dotted lines illustrate fits of the experimental data to Eq. 3.

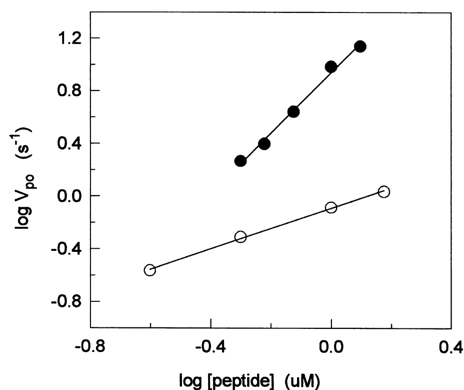

Fig. 3. Concentration dependence of the initial pore formation rate (v_{po}; Eq. 3) in DOPC SUV induced by melittin (●) and $(mlK23C)_2$ (○) determined from the fits shown in Fig. 2.

with only a small contribution from a second, steady-state phase of dye release. This near-monophasic kinetics in $(mlK23Q,Q25C)_2$ is also observed in dye release from DOPC SUV (not shown).

The relaxation of the rapid, initial phase of dye release results from the decrease in bilayer perturbation (a consequence of peptide accumulation in the outer bilayer leaflet), as peptide is transferred to the inner bilayer leaflet during transient pore formation [10]. The second, steady-state release is due to transient pore formation at equilibrium (i.e. after the peptide and lipid have equilibrated between the inner and outer bilayer leaflets [10]). The absence of a significant second phase in dye release induced by $(mlK23Q,Q25C)_2$ probably indicates that essentially complete release of encapsulated CF occurs from a single pore opening (so-called all or nothing release); i.e. the lifetime of the pore is greater than the time required for diffusion of the internal vesicle contents through the pore. As described previously [20], $(mlK23Q,Q25C)_2$ was designed to stabilise ion channel states; i.e. the disulphide lies on the polar face of the amphipathic helix where it is potentially accommodated in the 'barrel stave' array thought to underlie the conducting channel [7,22]. To the extent that the lytic pore involves an associated array of transmembrane oriented helices, such an arrangement may also be stabilised by dimerisation in the mlK23Q,Q25C analogue (see [20] for a description of the geometry of the melittin cysteine dimers).

Table 1
Concentration dependence of the rate-limiting step in the initial phase of peptide-induced pore formation (v_{po}) in PC SUV

Peptide	DOPC	Egg PC
Melittin	2.3 ± 0.2	1.7 ± 0.3
$ml(K23C)_2$	0.8 ± 0.1	1.1 ± 0.1
$ml(K23Q,Q25C)_2$	n.d.	0.9 ± 0.2

Fig. 3 illustrates the relationship between peptide concentration and the apparent pore formation rate for melittin and $(mlK23C)_2$. The rate of pore formation increases linearly with the concentration of $(mlK23C)_2$ whereas the rate of pore formation in DOPC vesicles has a second-order dependence on melittin concentration under these conditions (Table 1). Similarly, the dimerisation of melittin in the $(mlK23Q,Q25C)_2$ analogue converts a near-second-order dependence of the apparent pore formation on peptide concentration into a first-order dependence (Table 1). Note that rate-limiting dimerisation is not always apparent in melittin-induced lysis (compare, for example, [2,14,15,23]), and applies in the present study under low ionic strength conditions, where contributions to membrane binding resulting from salt- and pH-dependent self-association of melittin in solution are minimised.

4. Discussion

The initial rate of pore formation in DOPC or egg PC SUV increases with the square of the melittin concentration at pH 8 (low ionic strength) whereas pore formation rates increase linearly with the concentration of disulphide-dimerised analogues, $(mlK23C)_2$ and $(mlK23Q,Q25C)_2$. These observations indicate that association of a pair of melittin monomers (either two melittin molecules or monomers within a disulphide-linked pair) is rate-limiting for pore formation associated with the initial stage of dye efflux from PC SUV under these conditions. Since it is known that upon initial binding to the membrane surface, melittin adopts a helical conformation [24] (extending to residue 22 in the native peptide [25,26]), which lies parallel to the membrane surface [27], the rate-lim-

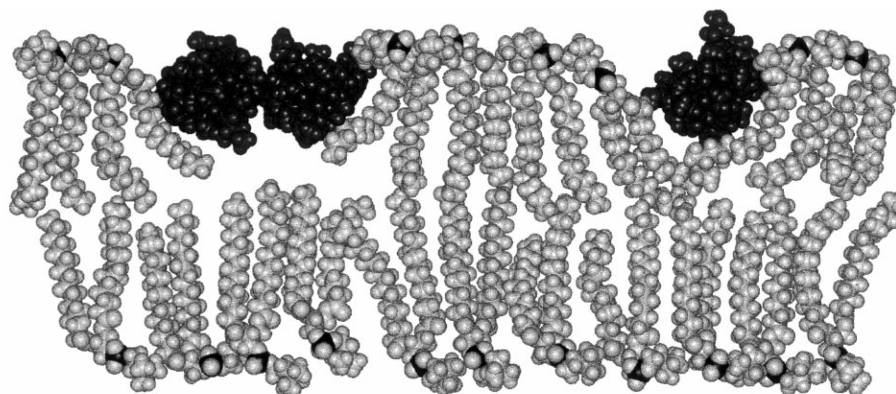

Fig. 4. Cross-section of a DMPC bilayer model containing a melittin monomer (right) or dimer (left) in the interfacial region. DMPC molecules were arranged on a lattice with spacing equivalent to that found in hydrated bilayer membranes. The molecules were randomised by molecular dynamics simulation and the peptides were inserted by replacing lipid molecules followed by further dynamics simulation. The peptides were constructed as ideal α-helices with extended conformations from residue 22 [25,26], and were placed in the interfacial region of the bilayer such that the non-polar surface of the amphipathic helices contacted the lipid acyl chains while the polar helix surfaces remained in contact with the 'aqueous phase' or with polar lipid groups in the interfacial region (see [29]).

iting dimerisation event is likely to be association of a pair of surface helices. These helical associates are parallel (rather than anti-parallel) at least for the disulphide-linked pairs. Since studies with spin-labelled, or fluorescently labelled, melittins have failed to identify a significant population of self-associated, vesicle-bound peptide under equilibrium conditions [11,28], the dimerisation underlying pore formation in the present study is likely to be a transient phenomenon consistent with its role as a rate-limiting event in pore formation.

We propose that dimerisation of hydrophobic or amphipathic helical peptides at the membrane surface *should* result in a significant bilayer perturbation resulting from basic structural features of peptide helices and membrane lipids in a single bilayer leaflet. As illustrated in Fig. 4, the cross-section of a peptide helix is accommodated in the interfacial region of a bilayer leaflet [29] since the acyl chains of membrane-forming phospholipids are long enough to 'solvate' the hydrophobic regions of the helix (see also [30]). This occurs with an increase in disorder of lipids in the peptide-containing leaflet and a thinning of the membrane, each of which has been characterised experimentally for interfacially located amphipathic helical peptides in bilayers (e.g. [18]). However, expulsion of lipids from the interface of a pair of associated surface helices (whether parallel or anti-parallel) results in a cavity below the helix pair that cannot be filled by lipids in the same leaflet (Fig. 4). Either the helix pair will sink towards the inner bilayer leaflet or lipids in the inner leaflet will move into the membrane to fill the gap, and each of these possibilities might trigger translocation of lipids and peptide across the bilayer. Since the mismatch between surface helix dimer and membrane lipid acyl chain length is a property of the general structural and geometric features of peptide helices and the common membrane forming lipids, the perturbation induced by surface dimerisation is likely to be largely independent of the amino acid sequence as long as the peptide has suitable amphipathic or hydrophobic properties that promote surface helix formation. In addition to its possible role in the lytic properties of amphipathic peptides, the perturbation resulting from surface helix association might be important for other biological phenomena that involve interaction of helical peptides with membranes. These include, for example, the sec-independent insertion of helical hairpin segments of membrane proteins into membranes, which requires interaction between pairs of membrane-bound hydrophobic or amphipathic helices [31], and the membrane insertion of 'helical hairpin' segments of bacterial toxins [32].

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