Biochimica et Biophysica Acta, 985 (1989) 106–110
Elsevier

BBAMEM 70623

BBA Report

A ^{1}H-NMR study of the influence of *n*-alcohols on the stoichiometry of melittin-induced permeability of phospholipid vesicles

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(Received 14 April 1989)

Key words: Phospholipid; *n*-Alcohol; Melittin; Permeability; NMR, Vesicular membrane

At concentrations of 5 mM, the *n*-alcohols were seen to produce inhibition of melittin-mediated transport of Pr^{3+} and an active complex of four melittin molecules per unit. In contrast, at 50 mM, the *n*-alcohols produced a strongly synergistic interaction and a melittin stoichiometry of 1. However, at both concentrations, the same pattern of dependence on *n*-alcohol chain-length in the relative rates of transport was observed. The results are discussed in terms of the membrane perturbations involved and the current understanding of melittin interactions with lipid bilayers.

Melittin is a polypeptide that represents about 50% of bee venom (by dry weight). It consists of 26 amino acids starting with predominantly hydrophobic amino acids in the N-terminal part and finishing with six charged and hydrophilic residues in the C-terminal part. It possesses two major roles: (1) the lysis of natural and artificial lipid membranes; and (2) the potentiation of the activity of phospholipase A_2 found in bee venom. Melittin–bilayer interactions are currently serving as a useful model for protein–lipid interactions in membranes [1–3]. Further, *n*-alcohol–membrane interactions are used as a model for general and local anaesthetic mechanisms [4]. Therefore a study which combines these should give considerable insight into the interaction mechanisms of membrane-active compounds in general. It is still not clear, also, whether melittin produces its permeability effects by perturbing the structural organization of bilayer membrane lipids with a monomeric stoichiometry or inducing discrete pores through which ions may diffuse with a tetrameric stoichiometry [5–8]. We have therefore studied the effects of a series of *n*-alcohols on melittin-induced permeability in an attempt to deduce possible melittin-lipid and *n*-alcohol–lipid interaction mechanisms.

Synthetic dipalmitoylphosphatidylcholine (DPPC), melittin and all the *n*-alcohols (spectroscopic grade where available) were purchased from Sigma, U.K. Deuterium oxide, ^2H_2O (99.8% Gold Label), was purchased from Aldrich, U.K., and praesodymium chloride from Lancaster Synthesis. All other chemicals were analytical grade or equivalent.

Small, unilamellar DPPC vesicles were prepared by sonication of lipid in ^2H_2O as previously described [9] to give a phospholipid concentration of 10 mg/ml. Following sonication for the brief periods required (5–10 min), TLC and ^{31}P-NMR spectra show that the vesicles are not degraded and in particular no lysoPC is formed [10]. The ^{1}H-NMR spectra were recorded on a Jeol FX90Q multinuclear NMR spectrometer operating at 90 MHz. Typically 40 pulse sequences were used (π–τ–$\pi/2$) with a pulse interval of 1 s to minimize the $^2HO^1H$ signal. The 10-mm NMR tubes contained 1 ml of vesicle dispersion confined by a vortex plug and capped. Melittin was introduced by pipetting a known volume of a stock solution in ^2H_2O into 1 ml of the vesicular dispersion. A known volume of *n*-alcohol was introduced by pipetting into 1 ml of the vesicular dispersion to give the appropriate concentration. The samples were then incubated for 30 min at 60 °C (above the phase transition temperature of the lipid) and all spectra were run at 60 °C. Transport was initiated by adding the required quantity of a stock solution of $PrCl_3$ in ^2H_2O to give an extravesicular Pr^{3+} concentration of 5 mM. The transport process was followed by monitoring the time-dependent shift of the inside (I) signal as described below [11]. The presence of 5 mM Pr^{3+} has been shown to inhibit phospholipase A_2 activity [10,12],

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which normally requires Ca^{2+} for enzymatic activation, so that the transport observed is not due to phospholipase attack of the vesicles. The stoichiometry of melittin was elucidated by obtaining the transport rates with different concentrations of melittin. Plotting the log rate against the log (melittin concentration) produces a straight line of which the gradient is equal to the stiochiometry of the transporting species. The stoichiometry of melittin in the presence of low (5 mM) and high (50 mM) concentrations of hexan-1-ol was also investigated. The recorded rates were the average obtained from two or more separate samples of vesicles of the same composition.

The ^{1}H-NMR spectrum of DPPC vesicles (10 mg/ml) at 60 °C shows high-resolution signals arising from the lipid acyl chains (H), the terminal methyl groups of the acyl chains (M) and the choline headgroups. On adjusting the extravesicular concentration to 5 mM Pr^3, the spectrum shown in Fig. 1a is obtained. Separate signals are seen originating from the extravesicular choline headgroups (O) and the intravesicular headgroups (I). The separation of the headgroup resonances is due to a pseudocontact dipolar interaction of the paramagnetic $Pr^{3+}_{(aq)}$ which is in rapid exchange between the ^2H_2O and phosphate sites on the choline headgroups in the outer monolayer [13]. The mean ratio of the areas O : I = 1.7, obtained by integrating the two peaks indicates that vesicles of average diameter 34 nm have been formed [14]. In the absence of melittin the signal from the inner choline headgroups remains unaffected for up to several

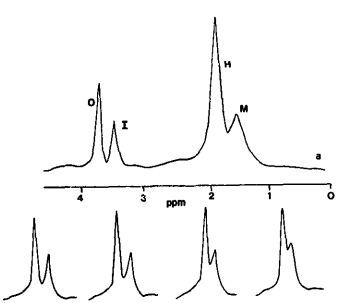

Fig. 1.(a) ^{1}H-NMR spectrum of DPPC vesicles (10 mg/ml) run at 60 °C in the presence of 5 mM Pr^{3+} showing the separate signals from the inner (I) and outer (O) choline headgroups, the lipid acyl chains (H) and the terminal methyl groups (M). (b–e) Spectra showing the result of transport of the Pr^{3+} from the outer to the inner vesicle environment by melittin (40 μg/ml) at times after addition of 5 mM Pr^{3+}. Spectra taken at (b) 17 min, (c) 146 min, (d) 1614 min and (e) 3239 min. All shifts are shown with respect to external TMS.

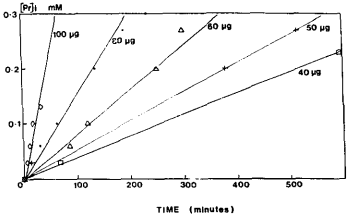

Fig. 2. Increase in the intravesicular concentration of Pr^{3+}, $[Pr]_i$, as a function of time using different concentrations of melittin with DPPC vesicles (10 mg/ml).

days after the addition of the Pr^{3+} to the external medium, indicating that the vesicles remain impermeable to Pr^{3+}. However, in the presence of melittin, a time-dependent downfield shift of the inner choline headgroup signal towards that of the outer choline headgroup signal is observed. This occurs as the melittin transports Pr^{3+} ions uniformly for all the vesicles from the outer to the inner environment (Fig. 1b–e)). In order to convert experimentally observed shifts into an intravesicular concentration of Pr^{3+}, a calibration graph is necessary. This calibration graph (not shown) is obtained by sonicating known concentrations of Pr^{3+} into separate vesicle preparations and then adjusting the extravesicular concentration to 5 mM Pr^{3+}. At each different intravesicular concentration of Pr^{3+} ions the shift of signal I is measured [11].

Increasing the concentration of melittin resulted in increased rates of Pr^{3+} transport (Fig. 2). A plot of the log rates of Pr^{3+} transport (mM Pr^{3+}/min) obtained with various melittin concentrations against the log of the melittin concentrations gave a straight line of gradient 3.8 (Fig. 3a). Similar experiments gave a gradient of 3.7 in the presence of 5 mM hexan-1-ol (Fig. 3b) and a gradient of 1.2 in the presence of 50 mM hexan-1-ol (Fig. 3c).

The effect of a series of *n*-alcohols on the rate of permeability induced by melittin was also investigated. Fig. 4 is a bar graph of the transport rates of Pr^{3+} obtained with melittin (40 μg) in the presence of 5 mM of *n*-alcohols where $n = 4$–12. C represents a control experiment and it is seen therefore that all the *n*-alcohols exhibit inhibition of melittin-induced permeability. Fig. 5 is a bar graph of the transport rates of Pr^{3+} obtained with melittin (40 μg) in the presence of 50 mM of *n*-alcohols where $n = 4$–12. Alcohols of $n = 4$–11 caused promotion of melittin-induced permeability, whereas alcohols with $n > 12$ caused inhibition. Interestingly, similar patterns in the transport rates obtained with low and high alcohol concentrations are seen.

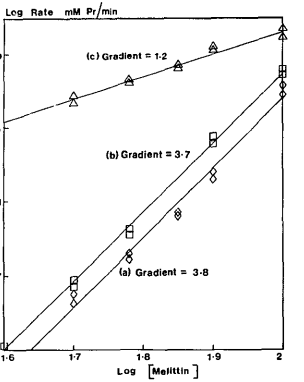

Fig. 3. Plot of log rate (mM Pr^{3+}/min) against log [melittin] for (a) DPPC vesicles (10 mg/ml), (b) DPPC vesicles dispersions (10 mg/ml) containing 5 mM hexan-1-ol, (c) DPPC vesicle dispersions (10 mg/ml) containing 50 mM hexan-1-ol.

Hexan-1-ol is seen to be the most effective of the alcohols at low and high concentrations. This is followed by a gradual decrease in transport rates as the *n*-alcohol chain-length is increased. The *n*-alcohols do not produce any transport in the absence of melittin.

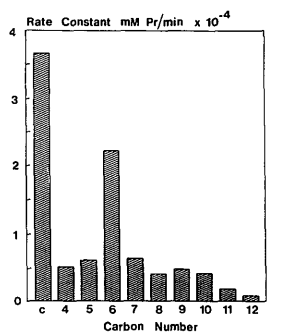

Fig. 4. Bar graph showing rates of Pr^{3+} transport by melittin (40 μg) into DPPC vesicles (10 mg/ml) in the presence of various *n*-alcohols at a 5 mM concentration. C represents control experiments (40 μg/ml).

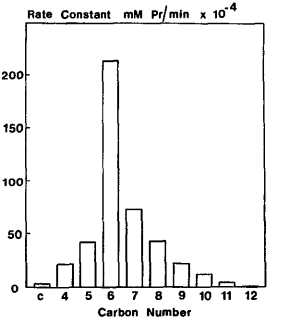

Fig. 5. Bar graph showing rates of Pr^{3+} transport by melittin (40 μg) into DPPC vesicles (10 mg/ml) in the presence of various *n*-alcohols at a 50 mM concentration. C represents control experiments (40 μg/ml).

The stoichiometry calculations indicate that both monomeric (in the presence of high hexan-1-ol concentrations) and tetrameric (in the absence of any alcohol and in the presence of low hexan-1-ol concentrations) are present and both forms interact with small, unilamellar DPPC vesicles to produce permeability in the bilayer [5–7]. The difference in rates at a alcohol concentration of 50 mM compared to that at 5 mM is approx. 50-fold. Part of this increase will be due to the effective 4-fold increase in concentration of pore-forming structures, $(melittin)_4 \rightarrow 4(melittin)_1$. However, the remaining increase is due to synergistic interaction between the melittin and the alcohols up to $n = 12$, when slight inhibition occurs (Fig. 5).

It is still uncertain whether melittin interacts with biological membranes in the monomeric or tetrameric form to produce permeability [7]. The variability of results in the literature would suggest that the species causing the permeability depends on the conditions and composition of the bilayer. The stoichiometry value of 3.8 obtained above (Fig. 3a) for melittin alone indicates that it acts as a pore-forming tetramer in DPPC vesicular bilayers. The idea of a tetramer of identical subunits was reported by Tosteson and Tosteson [15]. The hydrophilic sides of the helices would orient themselves towards each other, thereby producing a pore capable of allowing ion passage, whereas the hydrophobic sides of the helices would be in contact with the acyl chains of the phospholipids.

At a hexan-1-ol concentration of 5 mM, a stoichiometry of 3.7 is obtained (Fig. 3b) suggesting that melittin remains in its tetrameric form. It is seen from Fig. 4

that in the presence of 5 mM *n*-alcohols, strong inhibition of melittin-induced permeability is observed. This inhibition can result from an alteration to the hydrophilic nature of the tetrameric pore by the presence of the *n*-alcohols thereby restricting ion transport, or a shift in the monomer ⇌ tetramer equilibrium in the bilayer towards a non-permeable monomer form. The similarity of the inhibition of pore-mediated transport by general anaesthetics, which we have previously reported [16,17], would indicate the former as the most likely effect, especially since increasing the concentration of alcohols in steps (results not shown) gradually switches the effect from inhibition to promotion.

Melittin evidently further reverts to its monomeric form in the presence of 50 mM hexan-1-ol (obtained from the stoichiometry of 1.2 in Fig. 3c).

The increase in hexan-1-ol concentration must therefore affect the equilibrium that exists between the tetrameric and monomeric forms of melittin [18].

Three current models of the mode of monomeric melittin insertion into a lipid bilayer have been proposed [19–21]. Terwilliger and co-workers [20] proposed a model for the lytic activity of melittin. The monomer is bound at the surface of the membrane in such a way that the axis of the helix between residues 13 and 26 is oriented parallel to the membrane surface. The N-terminal part would partially penetrate into the membrane. Since the melittin enters only into one half of the vesicle bilayer, the surface area of this half would increase relative to that of the other side. Membrane curvature would therefore increase and may assist in the formation of lipid pores which would be stabilized by the melittin leading to ion permeability. This is consistent with the explanation of lateral compressibility fluctuations causing increased membrane permeability at the phase transition temperature of lipids (T_c) [22].

The presence of higher alcohol concentrations (Fig. 5) would lead to an increase in the hydrophobic nature of the membrane, causing greater melittin penetration and therefore leading to increased permeability by the 'wedge-like' action of the monomers [20].

The observations that (1) inhibition occurs at 5 mM alcohols with a stoichiometry of $(melittin)_4$ while promotion occurs at 50 mM alcohols with a stoichiometry of $(melittin)_1$ and (2) the overall pattern of activity as n varies is the same at 5 mM and 50 mM alcohol concentration are not consistent with the alcohols' affecting only the equilibrium between the aqueous micelles and the membrane bound melittin. Rather, specific interaction of the *n*-alcohols with the bilayer must occur in a chain-length dependent manner. The stoichiometry change from 4 to 1 suggests that the alcohols affect the equilibrium between the active states $(melittin)_4$ and $(melittin)_1$ in the bilayer with inhibition of the tetrameric micelles as channels and promotion of the $(melittin)_1$ perturbation.

The similarity in pattern of *n*-alcohol chain-length effect at both concentrations can then be rationalized as follows. The observation that hexan-1-ol is the most effective in producing melittin-induced permeability at both concentrations is consistent with our previous work [23] on the effect of *n*-alcohols on vesicular permeability induced at T_c and with the semi-empirical analysis of Brasseur et al. for the interaction of *n*-alcohols with DPPC [24]. When $n = 5–8$, alcohol–alcohol clustering occurs in the lipid headgroup region. So at $n = 6$ (hexan-1-ol) the alcohol molecules form clusters and (at 5 mM) are removed from interferring with the $(melittin)_4$ tetramer pores. As the concentration of alcohols increases, the monomeric 'wedge' form of melittin becomes favoured and structural perturbation of the bilayer is then maximal when the melittin interacts with clusters of hexan-1-ol molecules (Fig. 5). Increasing the *n*-alcohol chain-length results in an identical or greater interaction energy between the alcohol and lipid molecules leading to a more ordered alcohol–lipid organization [24]. This would restrict the insertion of melittin into the bilayer and, at $n = 12$, inhibition of permeability is observed (Fig. 5). This corresponds to the commonly observed 'cut-off' in anaesthetic potency of the *n*-alcohols, although recent discussion of this 'cut-off' has been with reference to the interaction of the alcohols directly with proteins [25].

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