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Leakage of internal markers from erythrocytes and lipid vesicles induced by melittin, gramicidin S and alamethicin: a comparative study

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The membrane-disruptive capacities of melittin, derivatised melittins, alamethicin and gramicidin S have been compared for the human erythrocyte membrane and lipid vesicles of three different compositions (phosphatidylcholine, 85% phosphatidylcholine / 15% phosphatidylserine, and a lipid analogue of the outer leaflet of the human erythrocyte membrane). The sensitivity to ionic strength, divalent metal ions and polylysine of release of fluorescent markers from liposomes and of haemoglobin from intact erythrocytes has been assayed. Acetyl melittin was found to he more effective than melittin in lysing phosphatidylcholine and phosphatidylcholine / phosphatidylserine vesicles, somewhat less effective in the lipid analogue and markedly less effective in lysing erythrocytes. Succinyl melittin was non-haemolytic, but was able to lyse lipid vesicles at a high concentration. Ca^{2+} inhibited melittin haemolysis at high ionic strength (150 mM NaCl), but produced a more complex response of stimulation followed by inhibition at low ionic strength. In lipid vesicles, Ca^{2+} either stimulated melittin lysis or was ineffective. Zn^{2+} exerted effects similar to Ca^{2+} with lipid vesicles at approx. 10-fold lower concentration except that a weak inhibition was observed for the erythrocyte membrane lipid analogue at high ionic strength. Polylysine strongly inhibited haemolysis by melittin at low ionic strength, but was ineffective or stimulatory in lipid vesicle lysis. High phosphate concentration also inhibited melittin haemolysis, but again no corresponding effect could he found in any of the lipid vesicle systems. These disparities between effects of melittin on erythrocytes and lipid vesicles support the proposal that melittin-protein interactions are of consequence to its haemolytic action. Similar experiments were performed with gramicidin S and alamethicin in order to compare their lytic properties with those of melittin. It was found that each lysin exhibited its own individual pattern of sensitivity to lipid composition, ionic strength and inhibition by cations. It thus appears likely that the detailed molecular interactions responsible for lysis are significantly different for each of these three agents.

Introduction

Melittin is a 26-amino-acid basic peptide (Fig. 1), commonly held to permeabilise biological membranes via a non-specific disruption of the lipid component. That such a phenomenon can occur is not an issue, as a number of studies have shown melittin-induced leakage from a variety of model membranes comprised solely of lipid [1–3]. However, studies have also shown that, for

Melittin

H_2N-Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile
-Lys-Arg-Lys-Arg-Gln-Gln$CONH_2$

Alamethicin

Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Phol

Gramicidin S

cyclo-(-Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro-)

Fig. 1. Amino-acid sequences of the three lysins used in the present study (Orn = ornithine, Aib = α-aminoisobutyric acid, Phol = phenylalaninol).

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Abbreviations: RBC, red blood cell; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin.

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more complex membranes containing integral proteins, a further effect is melittin-induced aggregation of such proteins within the plane of the membrane. This has been demonstrated for band 3 in erythrocyte ghosts [4–6], and for bacteriorhodopsin in a reconstituted system [7]. Furthermore, the relative band 3 aggregating abilities of melittin derivatives have been shown to correspond with their haemolytic potencies (melittin > acetyl melittin > succinyl melittin) [5]. As a consequence, it has been questioned as to whether a purely lipid-based mechanism of disruption is relevant to lytic concentrations of melittin added to biological membranes. Various perturbations of erythrocyte membrane proteins have previously been implicated in the formation of relatively non-specific leakage pathways. Cross-linking with diamide [8,9] or treatment with organic mercurials [10,11] has this result; Pooler has proposed band 3 dimers as the target in photohaemolysis [12], and aggregates of the tryptic hydrophobic segment of glycophorin increase the bilayer permeability of lipid vesicles relative to the situation in which monomers are present [13].

In this present study, we have sought to make a detailed comparison of the release of entrapped dye from large unilamellar vesicles of varying lipid compositions with the haemolysis of erythrocytes. In each case, the sensitivity of induced leakage to changes in the experimental conditions has been assayed for melittin and two other 'pore' forming peptides, gramicidin S and alamethicin. These peptides have occasionally been compared to melittin on the basis of structural and behavioural similarities. Both can act as non-specific lysins and alamethicin, like melittin, has been shown to form voltage-dependent leakage pathways in black lipid membranes [14,15]. Melittin can be thought of as comprising two sections containing residues 1–19 and 20–26, respectively, although both are required for haemolysis [16]. The 1–19 section has a hydrophobicity index greater than some membrane spanning sections of integral proteins, but also shares with alamethicin the capacity to form an amphiphilic α-helical structure, i.e., a sidedness in the lateral distribution of hydrophobic and hydrophilic residues along the helix axis [17,18]. The 20–26 section is hydrophilic and basic, conferring a longitudinal amphiphilicity on the complete outstretched molecule. This stuctural feature is thought to also be important for gramicidin S, wherein if a folded structure is adopted, the two ornithine residues can unite to form a hydrophilic headgroup on the end of an eight residue hydrophobic moiety [19].

The roots of these experiments also lie in previous studies of melittin-induced lysis, which have shown inhibition of haemolysis due to the presence of divalent metal ions or of high phosphate concentrations [20,21]. Knowledge of the relative efficacies of these 'inhibitors' in pure lipid systems, including an analogue of the erythrocyte outer leaflet, helps clarify the contributions of lipid mediated leakage pathways. In experiments more directly descended from the hypothesis of protein aggregation mediated leakage, the effects of the presence of a non-lytic aggregator of band 3, polylysine [6], have also been investigated.

Materials and Methods

Materials

L-α-Phosphatidylcholine (from frozen egg yolk), L-α-phosphatidylserine (from bovine brain), sphingomyelin (from bovine brain) and cholesterol were all obtained from Sigma, as were alamethicin, gramicidin S, 5(6)-carboxyfluorescein, calcein and polylysine ($M_r = 4000$). Melittin free of phospholipase A_2 activity was provided by Prof. R.C. Hider (King's College, University of London). Acetyl and succinyl melittins were prepared as previously described [22,5]. High-performance liquid chromatography elution profiles showed no contamination of these derivatives by native melittin. $CaCl_2$ and $ZnCl_2$ were of the highest grade available. All melittin concentrations were determined using a molar extinction coefficient of 5600 cm^{-1} at 280 nm.

Haemolysis assay

Blood (O^+, obtained by venipuncture) was washed three times in either 5PB or 5 mM Hepes buffer (pH 7.5), both containing 150 mM KCl and 1 mM EDTA. Packed cells were resuspended to 1% (v/v) in the buffer relevant to a particular experiment. 1 ml of red blood cell suspension was rapidly combined with 1 ml of buffer containing the specified lysin concentrations. After the given incubation time samples were spun down for 20 s in a microfuge (MSE), the supernatant was collected and its optical absorbance at 578 nm was determined relative to suitable controls. The percentage haemolysis was calculated from a comparison with the optical density of a fully lysed sample. When the effects of a second agent were being studied, this was pre-incubated in the red blood cell suspension. Lysin : phospholipid mole ratios were calculated by counting cells with a Coulter counter and converting to a phospholipid concentration on the basis of data reported by Shiga et al. [23].

Encapsulation of dye into large unilamellar vesicles

Large unilamellar vesicles were prepared by an extrusion technique [24]. Dried lipid films were hydrated with 10 mM Tris buffer solution (pH 7.4), containing 50 mM calcein, 5 mM EDTA, except for divalent metal ion experiments, for which it contained 50 mM 5(6)-carboxyfluorescein, with EDTA omitted. The resulting multilamellar vesicles were freeze-thawed five times. The vesicles were then placed in a device (Lipex Biomembranes, Vancouver, B.C.) which enabled their ex-

trusion through polycarbonate filters with 100 nm pore size (Nucleopore, Pleasanton, CA). They were injected into a central chamber and nitrogen pressures of 100–500 lb/in^2 were applied. The extrusion procedure was carried out ten times resulting in homogeneous sets of unilamellar vesicles (approx. 100 nm in diameter, as shown by light scattering analysis). Unentrapped calcein was removed by passing the vesicles down a PD-10 column (Sephadex, G25) equilibrated with iso-osmotic buffer. All experiments were performed at least twice, the reproducibility was similar to that for lipid vesicle experiments described in the following section.

Fluorescence dequenching assays

All fluorescence experiments were performed at 37 ° C using a Baird Nova spectrofluorimeter. The release of dye from vesicles was monitored using excitation and emission wavelengths of 490 nm and 520 nm, respectively. An increase in fluorescence intensity is observed as the dye escapes the self-quenching concentrations within the vesicles. The intensity value for 100% lysis was determined after the addition of Triton X-100, which releases all entrapped marker. Vesicles were suspended to a typical concentration of 10 μM lipid in 10 mM Tris buffer (pH 7.4), containing either 130 mM KCl or 225 mM sucrose to osmotically balance entrapped dye. Lysin was then added by injection of a stock solution and rapidly mixed. The fluorescence intensity was then followed for 7.5 min. In order to determine the phospholipid to lysin ratio required for 50% vesicle lysis, measurements were performed at different final lysin concentrations. All experiments were performed at least twice, the reproducibility of the 50% point was better than $\pm$5% (lysin concentration). In experiments to study the inhibitory effects of divalent cations and polylysine, the amount of lysin was chosen to produce between 50–80% lysis in the absence of the putative inhibitor. In all cases the % lysis was measured as a function of inhibitor concentration. Where the data are presented in tabular form, the results for representative concentrations were taken from these plots. All experiments were performed at least twice, the % lysis produced under a given set of conditions was reproducible to within $\pm$3. For simplicity, we use the term 'lysis' to refer to the release of dye from lipid vesicles, although the exact pathway is poorly characterised (see also Discussion).

Results

Vesicle lysis and haemolysis

Table I summarises data from basic leakage experiments performed on all the membrane systems utilised in this study. Immediately we have a wealth of intriguing observations. The particular robustness of the RBC and of its outer leaflet lipid analogue is striking. We found that the presence or absence of 5% PE within the analogue membrane makes no substantive difference to its susceptibility (data not shown). For leakage induced from PC and PC/PS membranes, we observe an entire spectrum of dependencies on the ionic strength, ranging from melittin and its derivatives, which are more effective at high ionic strength, through alamethicin which, is constant, to gramicidin S, which is less effective. Surprisingly, acetyl melittin is more effective than melittin for these membranes, particularly when PS is included, but the opposite is true for the RBC and its analogue. Fig. 2 shows more comprehensive data illustrating the melittin concentration dependence for disruption of each system. Choice of buffer made no difference to the lysin concentration required for 50% lysis; nor did the omittance of sucrose from the low salt solution, when this was checked for PC and analogue vesicles.

Fig. 3 shows the time course of leakage over 7.5 min, for each lipid system. The kinetics are similar for gramicidin S and melittin at an effective concentration in a given membrane system. For alamethicin, the kinetics are markedly different and appear to be independent of lipid composition. For the PC/PS system only, a delay (10–15 s for melittin, 30 s for gramicidin S) before the onset of leakage, is observed. We found melittin

TABLE I

Phospholipid-to-lysin ratios obtained for 50% release of assay component

Analogue composition is 50% cholesterol, 25% PC, 20% SM, 5% PE. Incubation time was 7.5 min at 37 ° C, buffer was 5 mM phosphate (pH 7.4), with either 150 mM salt or 290 mM sucrose for haemolysis experiments, for vesicle experiments 10 mM Tris was used with either 130 mM salt or 280 mM sucrose. Phospholipid concentration was 5 μM for membranes containing cholesterol, otherwise 10 μM.

Agent	100% PC		85% PC-15% PS		Analogue		RBCs	
	sucrose	KCl	sucrose	KCl	sucrose	KCl	sucrose	NaCl
Melittin	150	300	40	150	20	22	23	16
Acetyl melittin	240	340	325	400	16	12	11	1.8
Succinyl melittin	5.8	22	7.4	13	0.1	0.1	0	0
Alamethicin	75	75	75	75	<1	<1	1.5	2
Gramicidin S	20	7.5	10	8	3	<1	3	1.5

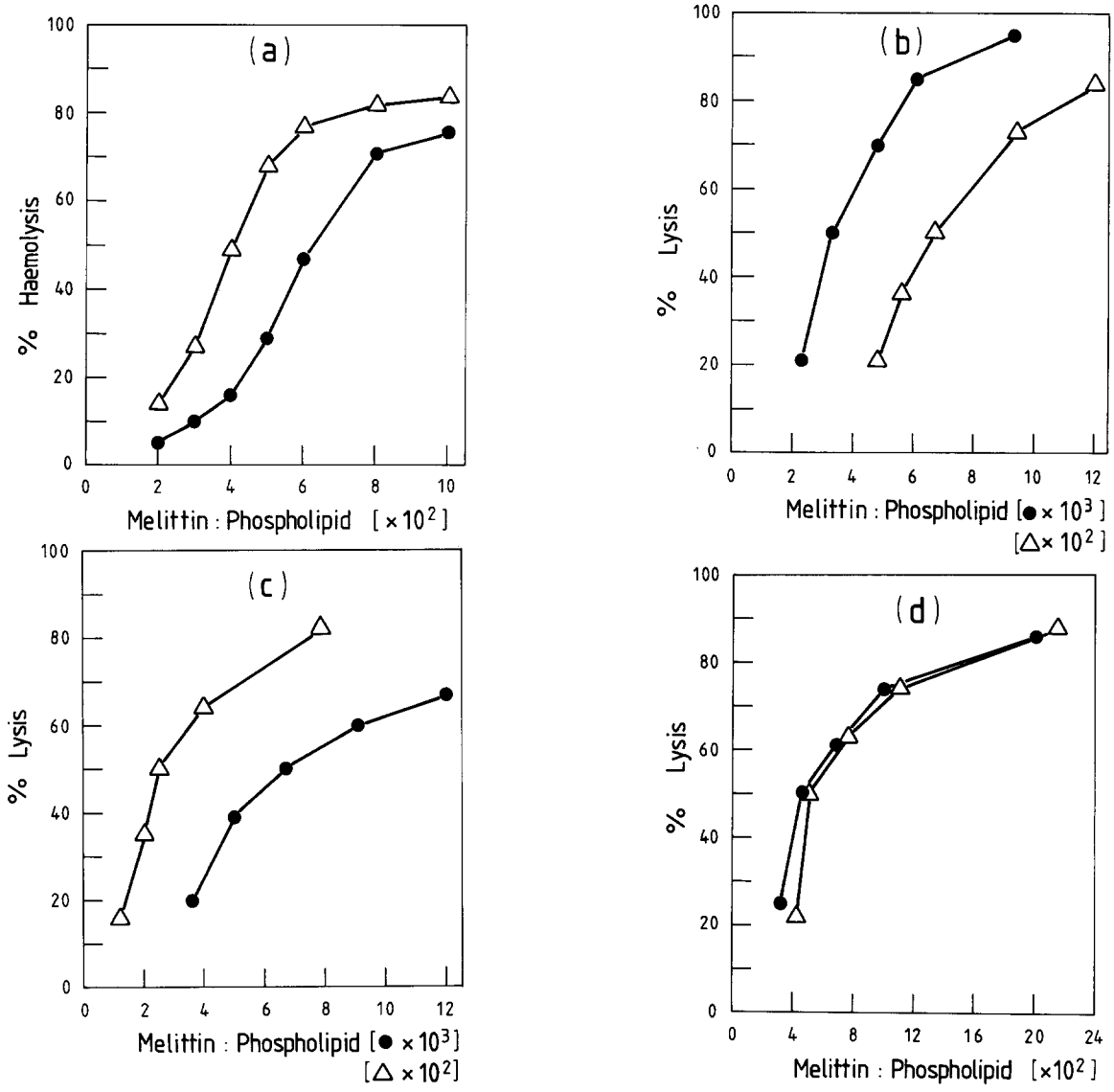
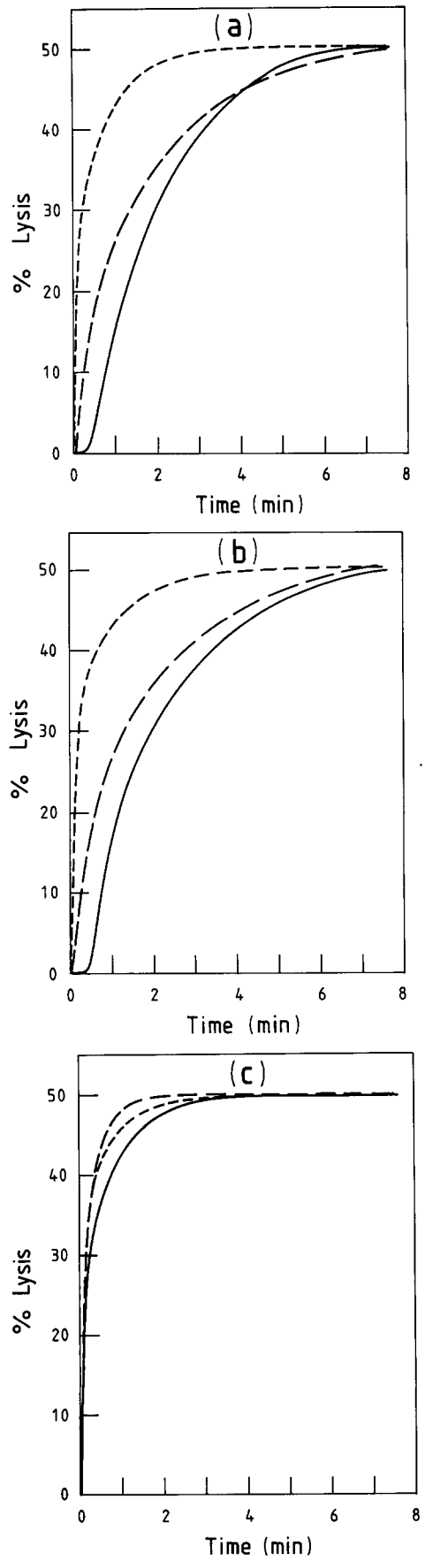


Fig. 2. Dependence of lysis/haemolysis on melittin:phospholipid molar ratio. (a) Haemolysis of RBCs in 280 mM sucrose (△) and 150 mM NaCl (●). (b)–(d) Lysis of lipid vesicles in 225 mM sucrose (△) and 130 mM KCl (●). (b) 100% PC; (c) 85% PC, 15% PS; (d) RBC analogue. Other details as in Table I.

haemolysis of RBC's to be reduced when the experiment was carried out in high phosphate buffer (Fig. 4a). With all liposome systems, the opposite was observed, as illustrated for the outer leaflet analogue in Fig. 4b.

Divalent cation effects

Figs. 5a and 5b illustrate the sensitivity of the disruptive effects of melittin on lipid vesicles to the presence of Ca^{2+} and Zn^{2+} ions respectively. The effect of these ions on haemolysis by melittin is shown in Figs. 5c and 5d. Zn^{2+} was not tested at concentrations above 100 μM for RBC's in 150 mM NaCl and above 20 μM for RBCs in sucrose because it was found to precipitate haemoglobin at higher concentrations. At low ionic strength, divalent metal ions enhance melittin-induced leakage from PC and PC/PS vesicles, whilst the RBC analogue is unaffected. Ca^{2+} at low ionic strength initially stimulates melittin haemolysis, but subsequently inhibits as the concentration is increased. Zn^{2+} stimulates haemolysis up to the maximum concentration tested. At high ionic strength, enhancement of melittin-induced lysis of lipid vesicles is abolished, except in the Ca^{2+}, PC/PS case, and a weak inhibition is observed in the Zn^{2+}, RBC analogue instance. Ca^{2+} and Zn^{2+} both inhibit melittin-induced haemolysis at high ionic strength. Tables II and III summarise the results of similar experiments with gramicidin S and alamethicin. For gramicidin S, inhibitory effects of varying degrees can be observed in all lipid systems, being most pronounced at low ionic strength, particularly in the case of



RBC's and their analogue. Zn^{2+} and polylysine inhibitory effects are reduced by inclusion of PS in PC vesicles. Slight enhancements are observed for the RBC analogue with alamethicin, which otherwise is not much affected by Ca^{2+} and Zn^{2+} except in the case of PC/PS at low ionic strength, where an inhibition is observed. The picture we obtain from haemolysis experiments is somewhat puzzling: Zn^{2+} appears to inhibit haemolysis by all three lysins in NaCl but not in sucrose (data not shown), whereas Ca^{2+} inhibits melittin in NaCl only but alamethicin and gramicidin S in sucrose only.

Polylysine effects

Fig. 6 shows that polylysine enhances melittin-induced lysis of all lipid-only systems, but uniquely at low ionic strength it inhibits melittin haemolysis of RBCs. The effects of polylysine on lysis by gramicidin S and alamethicin are shown in Table IV. As for Zn^{2+}, inhibition of gramicidin S induced leakage is observed at low ionic strength in all systems, although markedly less so for PC/PS. At high ionic strength the reverse is observed, i.e., polylysine enhances leakage. Alamethicin action upon lipid-only systems is little affected, but slightly enhanced in some cases. Its action on RBCs is strongly inhibited at low and less so at high ionic strength. Fig. 6 also shows that the inhibition of melittin-induced haemolysis can be overridden at sufficiently high lysin concentration.

Discussion

We have used two different assays for permeabilisation of erythrocytes and leakage from LUV's. To what extent do they correspond? At first consideration, the release of a macromolecule such as haemoglobin by relatively small peptides may appear less likely than calcein. However, haemolysis is most likely the result of a colloid osmotic mechanism, whereby permeabilisation of the erythrocyte membrane to ions and small molecules results in cell swelling and its eventual rupture [25]. Lesions sufficiently large to release calcein in lipid vesicles will thus also cause haemolysis if they occur in the RBC membrane. The essential common feature of the two assays is that they are ultimately reflecting permeabilisation of the membrane to ions or small molecules.

We have previously shown that lytic concentrations of melittin aggregate integral membrane proteins in the

Fig. 3. Time course of induced lysis for lipid vesicles by (a) melittin, (b) gramicidin S (c) alamethicin. Concentrations of each lysin, which produced 50% lysis after 7.5 min, were incubated with the following lipid vesicle systems: 100% PC (long dashes), 85% PC, 15% PS (solid line), RBC analogue (short dashes). All vesicles were in 130 mM KCl at 37 °C.

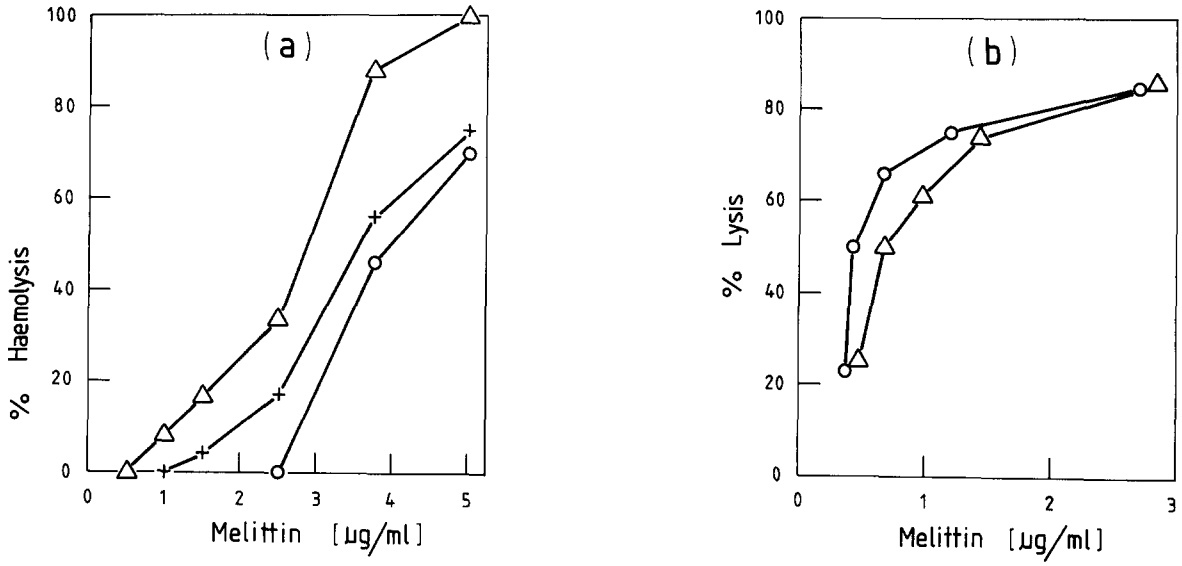


Fig. 4. Effects of phosphate on melittin-induced lysis and haemolysis. (a) RBCs in 150 mM NaCl (△), 250 mM sodium phosphate (+) and 500 mM sodium phosphate (○) pH 7.4. (b) RBC lipid analogue in 130 mM KCl (△) and 500 mM potassium phosphate (○) pH 7.4.

TABLE II

Effect of calcium on percentage lysis

The amount of lysin added was chosen to give lysis in the 50–80% range in the absence of calcium. Incubation time was 7.5 min at 37°C. Buffers, phospholipid concentration and analogue composition were as stated in the legend to Table I. The data are taken from plots of % lysis versus calcium concentration similar to that shown for melittin in Fig. 5a. Each experiment was performed twice with different samples, the reproducibility of the % lysis for a given Ca^{2+} concentration was better than ±3.

Agent	% Lysis							
	100% PC		85% PC-15% PS		Analogue		RBCs	
	sucrose	KCl	sucrose	KCl	sucrose	KCl	sucrose	NaCl
Gramicidin S	77	68	63	68	70	64	82	59
Gramicidin S + 10 mM Ca^{2+}	12	64	28	48	16	52	3	64
Alamethicin	48	50	46	43	52	54	87	69
Alamethicin + 5 mM Ca^{2+}	48	50	28	46	55	60	9	66

TABLE III

Effect of zinc on percentage lysis

The amount of lysin added was chosen to give lysis in the range 50–80% in the absence of zinc. Incubation time was 7.5 min at 37°C. Buffers, phospholipid concentration and analogue composition were as stated in the legend to Table I. The data are taken from plots of % lysis versus zinc concentration similar to that shown for melittin in Fig. 5b. Each experiment was performed twice with different samples, the reproducibility of the percentage lysis for a given Zn^{2+} concentration was better than ±3.

Agent	% Lysis					
	100% PC		85% PC-15% PS		Analogue	
	sucrose	KCl	sucrose	KCl	sucrose	KCl
Gramicidin S	73	72	73	77	77	78
Gramicidin S + 1 mM Zn^{2+}	9	35	40	45	25	60
Gramicidin S + 2 mM Zn^{2+}	8	34	40	45	25	57
Alamethicin	43	47	43	45	50	53
Alamethicin + 1 mM Zn^{2+}	45	48	33	50	55	62
Alamethicin + 2 mM Zn^{2+}	45	49	30	50	55	62

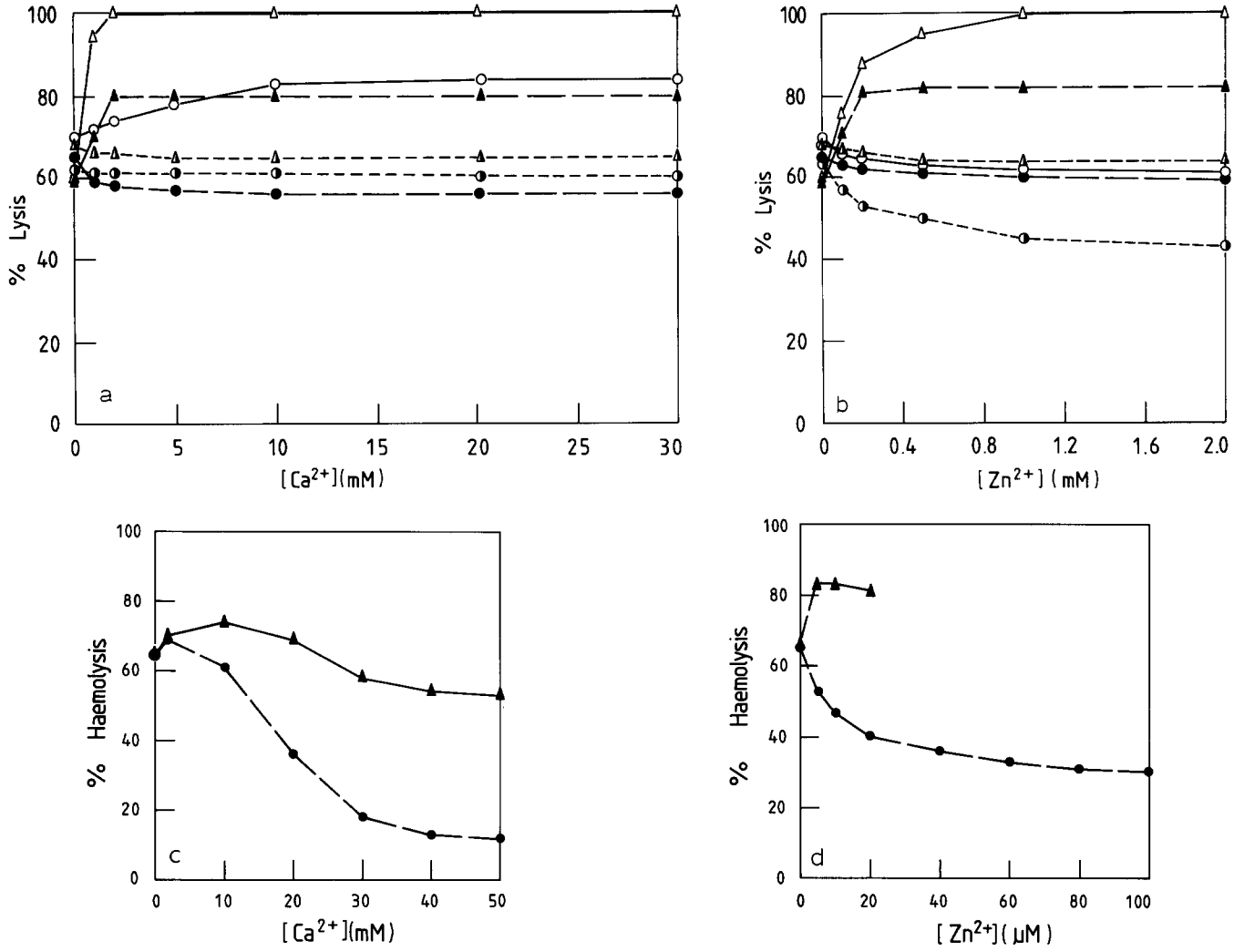


Fig. 5. Effects of divalent cations on vesicle lysis and haemolysis by melittin. (a) Ca^{2+}, (●) vesicles in 130 mM KCl; (△) vesicles in 280 mM sucrose. Vesicle compositions were: 100% PC (long dashes), 85% PC, 15% PS (solid line), RBC lipid analogue (short dashes). (b) Zn^{2+} effect on vesicle lysis. Symbols as in (a). (c) Ca^{2+} inhibition of haemolysis for RBCs in 150 mM NaCl (●) or 280 mM sucrose (△). Data points correspond to % release of haemoglobin from 0.5% (v/v) RBC suspension at 37°C after 7.5 min incubation with melittin. (d) Zn^{2+} inhibition of haemolysis; symbols and conditions as in (c).

TABLE IV

Effect of polylysine on percentage lysis

The amount of lysin added was chosen to give lysis in the 50–80% range in the absence of polylysine. Incubation time was 7.5 min at 37°C. Buffers, phospholipid concentration and analogue composition were as stated in the legend to Table I. The data are taken from plots of % lysis versus polylysine concentration similar to that shown for melittin in Fig. 6. Each experiment was performed twice with different samples, the reproducibility of the % lysis for a given polylysine concentration was better than ±3.

Agent	% Lysis							
	100% PC		85% PC-15% PS		Analogue		RBCs	
	sucrose	KCl	sucrose	KCl	sucrose	KCl	sucrose	NaCl
Gramicidin S	60	80	70	72	73	57	53	63
Gramicidin S + 0.5 μg polylysine	41	81	58	72	37	60	–	–
Gramicidin S + 5 μg polylysine	18	82	50	74	18	62	42	63
Gramicidin S + 50 μg polylysine	14	82	45	75	15	63	9	57
Alamethicin	68	72	65	63	60	61	55	69
Alamethicin + 0.5 μg polylysine	68	78	73	70	59	61	–	–
Alamethicin + 5 μg polylysine	71	82	76	70	58	61	61	71
Alamethicin + 50 μg polylysine	72	84	78	70	56	61	6	67

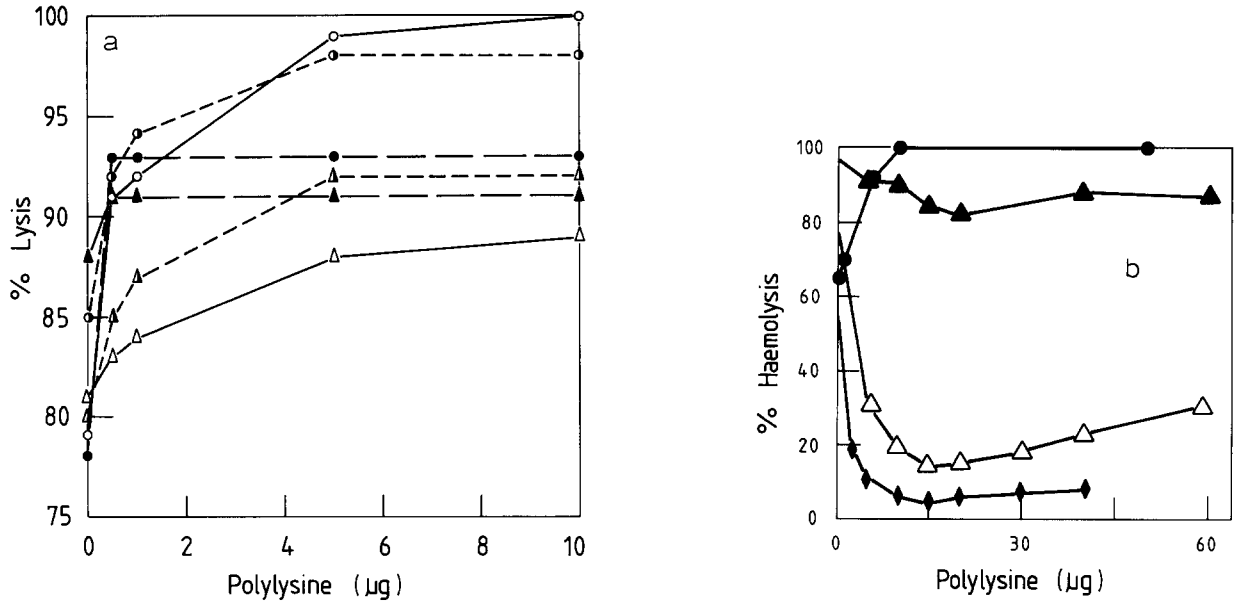


Fig. 6. Effects of polylysine on vesicle lysis and haemolysis by melittin. (a) Polylysine effects on vesicle lysis, (●) vesicles in 130 mM KCl, (△) vesicles in 280 mM sucrose. Vesicle compositions were 100% PC (long dashes), 85% PC, 15% PS (solid line), RBC lipid analogue (short dashes). (b) Polylysine inhibition of haemolysis. Data points correspond to % release of haemoglobin from 0.5% (v/v) RBC suspension, after 7.5 min incubation at 37°C. The three curves, (△, ▲, ▽) correspond to different amounts of addded melittin, which were chosen to produce different amounts of haemolysis in 280 mM sucrose. (●) corresponds to a parallel experiment in 150 mM NaCl.

erythrocyte membrane. From model system studies, it seems likely that aggregation is a consequence of melittin–protein interactions [6,7]. If such interactions occur, it might be expected that they will produce quantitative or even mechanistic differences in melittin's lytic action on RBCs, when compared with pure lipid membrane systems. A motivation of the present study was to look for such differences. This issue is addressed in the ensuing section dealing with melittin. The lytic properties of gramicidin S and alamethicin have their own intrinsic interest and are discussed separately.

Melittin

From Table I, it is apparent that RBCs and their outer leaflet lipid analogue are much less susceptible to lysis than the simpler lipid systems (PC and PC/PS), which have often been used as models in the study of melittin's action [1–3,26]. Further studies, not reported here, implicate cholesterol as the component which confers resistance on the RBC analogue relative to other systems.

The inhibition at high phosphate concentration of RBC haemolysis by melittin (Fig. 4) has been observed previously and attributed to melittin's oligomeric properties in solution, i.e., phosphate promotes tetramer over monomer [21]. However, high phosphate concentrations fail to inhibit melittin lysis of any of the lipid vesicle systems investigated here; in fact, an enhancement is observed in most cases. This may simply be a further consequence of the effect of high ionic strength in these systems as observed with KCl relative to sucrose containing medium. The difference between lipid vesicles and RBCs with respect to phosphate inhibition indicates the requirement of further factors in the RBC membrane.

The results discussed so far might be taken to indicate no role or a protective one for proteins in the RBC membrane. Melittin could be inactive when bound to membrane proteins. This would provide a possible explanation of the effect of phosphate upon haemolysis. Whether or not a sufficient concentration of melittin in potentially lytic conformation is reached would depend on the relative kinetics of tetramer dissociation followed by membrane insertion and of melittin-protein association in the membrane. This effect could be overcome by increasing the melittin concentration, as is observed experimentally (Fig. 4). The idea that melittin may adopt an inactive conformation with time is supported by the observation of Tosteson et al. that sublytic melittin concentrations cause a transient ion leakage [25].

The above hypothesis of inactivation by protein binding is, however, not supported by the experiments with melittin derivatives. Previous experiments showed that acetyl melittin is less effective than native melittin in aggregating membrane proteins [5]. This leads to the expectation that acetyl melittin would be the more effective in promoting haemolysis, especially as acetyl melittin is more effective in PC and PC/PS vesicles and only somewhat less so in RBC analogue vesicles. In fact, the reverse is observed, even after allowing for the relatively lower efficiency of acetyl melittin in lysing the

RBC analogue vesicles. Similarly, succinyl melittin has a weak lytic effect on lipid vesicles but is non-haemolytic, even though it has no interaction with membrane proteins as judged by aggregation phenomena [5]. The reversal in ionic strength dependence that we see between liposomes and erythrocytes may also be considered along similar lines. Low ionic strength conditions, which favour haemolysis, would be expected to favour electrostatic interaction with negatively charged integral proteins (although this cannot be discriminated for melittin, using the rotational diffusion aggregation assay [6]). The promotion of liposome lysis by higher ionic strengths may be due to decreased charge repulsion leading to an enhancement of melittin aggregate formation.

Inhibition experiments with divalent metal cations and with polylysine are of particular interest in comparing the effects of melittin on RBCs with lipid vesicles. Pasternak and co-workers have previously shown that divalent cations, including Ca^{2+} and Zn^{2+}, can inhibit 'pores' formed by a variety of agents in cell membranes [20]. The inhibitory action of divalent cations appears to involve negatively charged groups located at the extracellular side of the plasma membrane [27]. The present experiments with Ca^{2+} and Zn^{2+} performed in 150 mM NaCl are in agreement with previous studies [20,27] except that somewhat higher concentrations of Zn^{2+} are required to produce 50% inhibition. However, our observations of the effects of Ca^{2+} and Zn^{2+} at low ionic strength, and of polylysine, which have not previously been reported, reveal that the effects of these cationic agents are complex. Ca^{2+} and Zn^{2+} stimulate haemolysis at low concentrations in a sucrose medium, although Ca^{2+} at least becomes inhibitory as the concentration is further increased. In contrast polylysine inhibits haemolysis by melittin in sucrose but is stimulatory in 150 mM NaCl.

To what extent are these effects of cationic agents on melittin-induced haemolysis reproduced in lipid vesicles? The stimulatory effects of polylysine in high ionic strength and of Ca^{2+} and Zn^{2+} in low ionic strength media are observed with vesicles as well as with RBCs and are especially pronounced when negatively charged PS is present. Although PS is not significantly present in the outer leaflet of the RBC membrane, there are negatively charged glycolipids and glycoproteins which could provide appropriate binding sites. In contrast, the inhibition of melittin haemolysis by Ca^{2+} and Zn^{2+} at high and by polylysine at low ionic strength has no counterpart in the lipid systems. The one exception, inhibition by Zn^{2+} of melittin-lysis of RBC analogue vesicles occurs at 100-times higher Zn^{2+} concentration than the corresponding effect in RBCs.

Because of the complexity of the erythrocyte membrane, it is difficult to interpret these findings in detail. In broad terms, the data suggest that there are at least two classes of anionic binding sites for melittin and the cationic inhibitors. For one class of sites, melittin binding does not promote lysis and hence competitition by cationic agents is stimulatory. The second class of sites are involved in the haemolytic mechanism, so that competition for these sites is inhibitory. The failure to find corresponding inhibitory effects with lipid vesicles of various compositions strongly suggest that this second class of sites is associated with membrane proteins. The relative weakness of acetyl melittin in inducing haemolysis is consistent with inferior binding to these sites. It is perhaps significant that the cationic inhibitors share with melittin the ability to aggregate integral membrane proteins [6], and also that acetyl melittin has a reduced ability to do so relative to melittin [5]. Protein aggregation could play a role in haemolysis if the membrane protein clusters are leaky. Binding of cationic agents to membrane proteins could then interfere with this process. In fact, if leakage were to occur exclusively during the process of clustering, the phosphate inhibition data could be reconciled within this framework, whilst retaining the 'relative rates' hypothesis, discussed above. The data in Fig. 6 show that polylysine inhibition is saturable, which in the above interpretation occurs at a concentration where a lipid-only mechanism becomes effective.

The above interpretation clearly leaves a number of questions unanswered. In particular, it is not clear why the stimulatory and inhibitory effects of polylysine should have the reverse dependence on ionic strength to Ca^{2+} and Zn^{2+}. A detailed understanding will require identification of the binding sites in question. We are currently investigating the action of melittin on lipid vesicles containing reconstituted band 3 proteins in order to attempt to clarify some of the issues raised by the present work.

It is worth noting that the incorporation of 15% PS into PC vesicles makes them less susceptible to lysis by melittin. This contrasts with the finding that melittin binds more strongly to anionic than to zwitterionic lipids, as would be expected from melittin's positive charge [29]. These observations are compatible with the suggestion that negatively charged lipids may provide non-lytic binding sites for melittin. Dempsey et al. [30] report that the presence of dimyristoylphosphatidylserine stabilises dimyristoylphosphatidylcholine bilayers with respect to melittin-induced micellisation. This effect occurs at very high melittin concentration (lipid : melittin $\approx$ 4) and its relationship with lytic phenomena is unclear [31].

Gramicidin S and alamethicin

Katsu et al. [32] have demonstrated parallels between haemolysis induced by gramicidin S and melittin. We find that the ionic strength dependence of divalent cation inhibition of haemolysis is opposite for the two

peptides. Moreover, with each lipid system, gramicidin S displays an opposite dependence on ionic strength to that of melittin and can uniquely (for the three lysins studied) be inhibited by cationic species. Thus, in contrast to melittin, we demonstrate no contradictions between determinants of liposome and erythrocyte disruption for gramicidin S. The simplest assumption is that they are the same in each case. Indeed, the concept that haemolysis can be explained entirely by lipid–lysin interaction appears to be much better supported for gramicidin S than for melittin.

Although alamethicin is normally thought of as a voltage dependent channel former, studies have shown that, at a sufficiently high level of incorporation, a trans-negative voltage is not necessary [33]. In contrast to melittin and gramicidin S, alamethicin-induced lysis is insensitive to ionic strength up to 150 mM KCl and to the incorporation of PS in PC vesicles. This suggests that ionic interactions are of less significance for this agent. The liposome data demonstrate a particular sensitivity to cholesterol and also the promotion of divalent metal ion inhibition by PS, i.e., the opposite to that which is observed with gramicidin S. This latter effect illustrates the protean nature of these observed inhibitions, dependent upon membrane, lysin and cationic species employed.

Concluding remarks

Studies of the mode of action of lytic peptides may be relevant to the cytotoxic effects of complement [20,34]. In an intriguing study, Esser and co-workers observe that anti-melittin antibodies inhibit complement lysis [34]. The many observations that lysins act on pure lipid membrane systems has encouraged the view that their disruption of cell membranes can be understood solely in terms of their interaction with the lipid bilayer. The present studies reveal that, at least in the case of melittin, there are a number of discrepancies between liposome and erythrocyte lysis, which require explanation. Correlation of melittin's haemolytic properties and the influence of cationic agents with aggregation phenomena provide a body of circumstantial evidence for direct protein involvement in disruption of cell membranes. The lytic properties of alamethicin and gramicidin S, whilst having some features in common with melittin are sufficiently individual to suggest significant differences in the molecular basis of their action.

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