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A comparative study of band 3 aggregation in erythrocyte membranes by melittin and other cationic agents

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The technique of laser flash-induced transient dichroism has been used to measure the rotational diffusion of eosin-labelled band 3 proteins in erythrocyte ghosts. A retardation in the mobility of band 3, measured subsequent to the addition of a variety of polyvalent cationic species, has been interpreted to reflect aggregation or 'clustering' of the protein in the plane of the membrane. A comparative study is reported between three such aggregators: melittin, polylysine and Zn^{2+}, wherein their respective abilities to induce aggregation have been measured under varying conditions. Unlike that for melittin, band 3 aggregation by polylysine and Zn^{2+} is shown to be sensitive to proteolytic degradation of the membrane and to the ionic strength of the surrounding medium. Studies with fragments of melittin derived from its chymotryptic cleavage show the hydrophilic C-terminal 20–26 section to possess independent aggregating ability, but also the requirement of the 1–19 hydrophobic section to be attached in order to prevent reversibility by high ionic strength buffers. Melittin is also shown to have a unique ability to aggregate bacteriorhodopsin reconstituted into DMPC vesicles, which is partially retained by its 1–19 but not by its 20–26 fragment.

Introduction

Integral membrane proteins of erythrocytes can be aggregated by a wide variety of positively charged agents under certain conditions [1–3]. This can be detected by electron microscopy as clustering of intramembranous particles in the freeze-fracture face [1,4], or as here, by rotational mobility measurements of the eosin-labelled anion transporter, band 3, using laser flash-induced transient dichroism [2,5].

We have been particularly interested in such aggregation induced by the bee venom peptide melittin in relation to the mechanism of its haemolytic action. Previous work has shown a correspondence in the haemolytic potency of derivatised melittins with that for aggregation of band 3 [5]. In this study we focus on the mechanism of melittin-induced band 3 aggregation itself, and compare it to that of other non-lytic aggregators, Zn^{2+} and polylysine, under selectively modified conditions. Separate studies have shown melittin to be also capable of aggregating bacteriorhodopsin in a reconstituted membrane system [6]. We have used this system to further highlight the distinctive versatility of melittin's aggregating properties.

The primary structure of melittin is given in Fig. 1. It is a basic peptide thought to possess substantial α-helicity when bound to a membrane-like surface. Helical wheel plots and crystallographic determination have shown the predicted regions of α-helix to be amphiphilic within the section incorporating residues 1–20 [7], which adjoins the very hydrophilic C-terminal section. We have investigated the contributions of these two distinct regions to the band 3 aggregating properties of the whole peptide by comparing the effects of two fragments comprised of residues 1–19 and 20–26, which are products of the chymotryptic cleavage of melittin.

Abbreviations: TPCK, tosyl phenylalanine chloromethyl ketone; PMSF, phenylmethylsulfonyl fluoride; DMPC, dimyristoylphosphatidylcholine.

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NH_3^+-GLY-ILE-GLY-ALA-VAL-LEU-$\overset{+}{L}$YS-VAL-LEU-THR-THR-GLY-LEU-PRO-ALA-LEU-ILE-SER-TRP-ILE-$\overset{+}{L}$YS-$\overset{+}{A}$RG-$\overset{+}{L}$YS-$\overset{+}{A}$RG-GLN-GLN-$CONH_2$

Fig. 1. Amino acid sequence of melittin. Charges are as indicated at pH 7.4, and uncharged hydrophilic residues are underlined.

Materials and Methods

Materials

Melittin, low in phospholipase A_2 activity prepared from whole bee venom was donated by Prof. R.C. Hider (King's College, London); this was used throughout except for the preparation of fragments in which melittin from Sigma was used. Polylysine (average $M_r = 3600$) was from Sigma, $ZnCl_2$ (gold label) from Aldrich, and eosin-5-maleimide from Molecular Probes Inc. Chymotrypsin (type VII) was from Sigma, TPCK-treated trypsin was the gift of Prof. K.T. Douglas (University of Manchester). Outdated blood (O^+) was obtained from the local hospital.

Preparation of eosin labelled ghosts

Eosin-labelled ghosts in a variety of final forms were required, each derived from the same initial procedure. Band 3 of intact erythrocytes was selectively labelled as previously described [8] by incubation with eosin-5-maleimide for 45 min at room temperature. Ghosts were prepared from these cells by hypotonic lysis using 20–30 volumes of 5 mM phosphate buffer, 1 mM EDTA (pH 7.5) at 4° C. Four washes (24000 × *g*, 10 min) provided the basic ghost material.

Trypsin-treated ghosts, from which the 43 kDa cytoplasmic domain of band 3 is released [9,10], were prepared by incubation of ghosts (3.5 mg of membrane protein/ml) with an equal volume of 5 mM phosphate buffer containing 2 μg/ml of trypsin for one hour at room temperature. They were then washed three times in 5 mM phosphate buffer containing 0.4 mM PMSF.

Spectrin/actin depleted ghosts were prepared as described by Tyler et al. [11]. Ghosts were incubated in 30 volumes of low ionic strength buffer containing 0.3 mM phosphate, 0.2 mM EDTA, 0.2 mM PMSF (pH 7.5) for 30 min at 37° C. This releases about 99% of actin and > 90% of spectrin, which are removed by centrifuging at 24000 × *g* for 30 min. The ghosts were collected after two washes in 5 mM phosphate buffer. Stripped ghosts, further depleted of ankyrin and band 4.1, were prepared by incubation of spectrin/actin depleted ghosts in 5 mM phosphate buffer, containing 1 M KCl for 30 min at 37° C [11]. Ghosts were collected after two washes in 5 mM phosphate buffer.

Preparation of melittin fragments

Cleavage of melittin was effected using the method of Mackler et al. [12]. 45 mg of melittin was dissolved in 3 ml of water containing 0.4 mg of TPCK-treated chymotrypsin and the pH was adjusted to 8.5. The solution was incubated at 28° C for 30 min, frozen and then thawed. The dense precipitate thus formed was collected by centrifugation at 10000 × *g* for 5 min and was subsequently washed twice with 10 ml of distilled water. The initial supernatant, rich in 20–26 fragment, was passed down a 1 × 30 cm Sephadex G-10 column equilibrated with 2% acetic acid. Fractions corresponding to the 20–26 fragment, retained relative to melittin, were collected and lyophilised.

Melittin contamination (< 5%) of the precipitated 1–19 fragment was removed by ion-exchanged chromatography. The sample was first dissolved in 2 ml of buffer containing 50 mM ammonium acetate, 4 M urea (pH 4.5) and passed down a 1.5 × 5 cm column containing Whatmans CM-52 ion exchange resin equilibrated with the same buffer. Under these conditions the 1–19 fragment elutes, whilst melittin remains bound to the column [13]. The eluant was passed down a 0.75 × 25 cm Sephadex G-10 column equilibrated with 20% acetic acid and then lyophilised.

The absence of melittin from either purified fragment was checked using high-performance liquid chromatography (Water Associates) and conditions under which melittin is known to elute [14]. Amino acid analysis (Locarte Instruments) was performed on each fragment, and in the case of the 20–26 fragment, used to assess the concentration derived from a given weight using norleucine as an internal standard. The concentrations of both melittin and the 1–19 fragment were determined by absorption, using a molar absorption coefficient of 5600 cm^{-1} at 280 nm [14].

Reconstitution of bacteriorhodopsin into lipid vesicles

Bacteriorhodopsin was prepared from *Halobacterium halobium* and reconstituted into dimyristoylphosphatidylcholine (DMPC) vesicles as previously described [15]. The lipid/protein mole ratio of the vesicles used in this study was approx. 50 : 1.

Rotational diffusion measurements

The laser flash-induced transient dichroism technique has been described in detail elsewhere [16]. Briefly, ghosts were added to peptide or metal ions dispersed in buffer to a typical concentration of 0.8 mg of membrane protein/ml. Except for the experiments on ordinary ghosts to determine the effects of increasing ion concentrations, solutions contained 66% (w/v) glycerol, in order to obviate consideration of vesicle tumbling and to alleviate scattering effects. Experiments involving Zn^{2+} utilised 10 mM Hepes buffer rather than 5 mM phosphate buffer; control experiments show no difference between these two buffer systems for melittin and polylysine. The rotational diffusion of band 3 was measured by observing transient dichroism of ground state depletion signals, arising from the excitation of the probe into the triplet state by a linearly polarised light pulse from a Nd-Yg laser (JK Lasers Ltd.). Excitation was at 532 nm and absorbance changes were recorded at 515 nm for light polarised parallel and perpendicular relative to the exciting flash. The signals were collected and averaged by a Datalab DL 102A signal averager.

Experiments on bacteriorhodopsin which utilised absorption depletion of the intrinsic chromophore, retinal, employed the same procedure except that absorption changes were recorded at 565 nm. Bacteriorhodopsin measurements were made at 30 °C as in Ref. 6, whereat the membrane lipid is above its phase transition temperature.

Data were analysed by calculating the absorption anisotropy $r(t)$, given by

$$r(t) = \frac{A_{\parallel}(t) - A_{\perp}(t)}{A_{\parallel}(t) + 2A_{\perp}(t)} \quad (1)$$

where $A_{\parallel}(t)$ and $A_{\perp}(t)$ represent absorbance changes at time t after the flash for light polarised parallel and perpendicular relative to the polarisation of the exciting flash. The experimental decay curves were normally fitted to a double exponential equation

$$r(t) = r_1 \exp(-t/T_1) + r_2 \exp(-t/T_2) + r_3 \quad (2)$$

where T_1 and T_2 are not exact relaxation times, but may give a rough estimate for faster and slower rotating components. The interpretation of these decay curves is discussed in detail elsewhere [8,10]. Some variation in the apparent value of the anisotropy at $t = 0$ can arise from the curve-fitting procedure or from instrumental factors.

Results

Band 3 aggregation in normal ghosts

Fig. 2 shows absorption anisotropy decays arising from eosin-labelled band 3 in ghost membranes, subjected to increasing concentrations of Zn^{2+}. The decay reflects the rotational motion of band 3 protein, which probably occurs only about an axis perpendicular to the plane of the membrane. As Zn^{2+} concentration increases, the curves obtained demonstrate successive retardations in the rate of decay, thereby implying a decrease in rotational motion. A similar trend is also

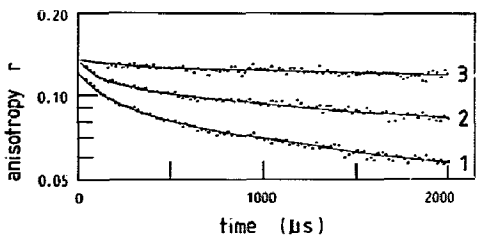

Fig. 2. Anisotropy decay data for eosin-labelled band 3 in erythrocyte ghosts upon incubation with (1) 0 mM, (2) 0.04 mM and (3) 0.1 mM $ZnCl_2$. Measurements were at 37 °C in a medium containing 10 mM Hepes, 66% (w/v) glycerol (pH 7.5).

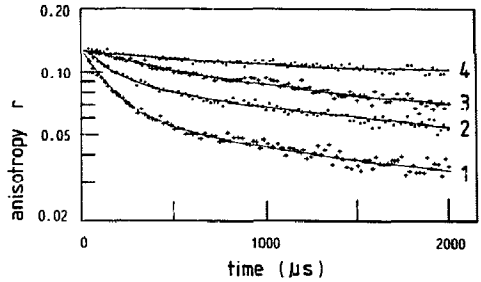

Fig. 3. Anisotropy decay data for normal (●) and trypsin-treated (+) ghosts at 37 °C in 5 mM phosphate buffer, 1 mM EDTA (pH 7.5), containing 66% (w/v) glycerol. Curves (1) and (2) are controls, (3) and (4) are data after addition of melittin (50 μg) to 500 μl of ghost suspension.

observed for melittin [5] and polylysine [2] over specific concentration ranges.

It was noted that the concentration of Zn^{2+} and polylysine required to immobilise band 3 caused a substantial increase in turbidity of the ghost suspensions. Such effects with melittin only became significant at much higher concentrations. Although ghosts from outdated blood were routinely used for these experiments, no systematic differences were detected when ghosts were prepared from fresh blood.

It has previously been shown that glycerol up to 70% (w/v) has no effect on band 3 rotation in ghosts, presumably because the dominant viscous drag is in the lipid bilayer [8]. In the present study it was noted that glycerol may slightly increase the effectiveness of a given concentration of aggregator but to the same degree for each agent investigated.

Effect of removal of cytoskeletal proteins

Incubation of ghosts in low-salt/high-salt media resulted in the release of cytoskeletal proteins as previously described [11]. Rotational diffusion experiments with ghosts depleted of spectin and actin or spectrin actin, ankyrin and band 4.1 revealed only very minor variations from control ghosts in the extent of retardation of band 3 induced by relevant concentrations of melittin, Zn^{2+} and polylysine (data not shown).

Effect of trypsin treatment

Fig. 3 shows that the anisotropy decay curve for trypsin-treated ghosts exhibits a marked increase in band 3 rotational mobility compared to normal ghosts, as previously described by Nigg and Cherry [10]. Melittin induced immobilisation of band 3 was found to be least sensitive to this modification. Fig. 3 shows anisotropy decay curves from normal and trypsin-treated ghosts after addition of the same amount of melittin. Full band 3 immobilisation was achieved for trypsin-treated ghosts using a concentration of melittin not

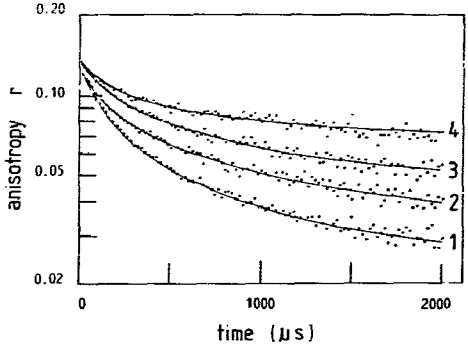

Fig. 4. Anisotropy decay data for eosin-labelled band 3 in trypsin-treated ghosts upon incubation with: (1) 0 mM, (2) 0.08 mM, (3) 0.13 mM, and (4) 0.26 mM $ZnCl_2$. Measurements were at 37 °C in a medium containing 10 mM Hepes, 66% (w/v) glycerol (pH 7.5). Compare with Fig. 2.

greater than 20% in excess of that required for the same effect on normal ghosts (not shown in Fig. 3).

Both polylysine- and Zn^{2+}-induced immobilisation of band 3 were affected to much greater extents by the trypsin treatment. In neither case could full immobilisation be achieved. For polylysine, increased turbidity of the sample due to ghost aggregation did not allow measurements at concentrations greater than twice that required for full immobilisation in normal ghosts, but for Zn^{2+} no further immobilisation than that obtained with polylysine was obtained, even at five times the normal concentration. Fig. 4 shows a typical data set.

Effect of ionic strength

The effect of ionic strength on band 3 immobilisation was investigated by measuring anisotropy decays either in 5 mM and 110 mM phosphate buffer or in 5 mM Hepes buffer with or without 150 mM NaCl. It was found that the immobilising effects of both Zn^{2+} and polylysine seen in 5 mM Hepes buffer were abolished

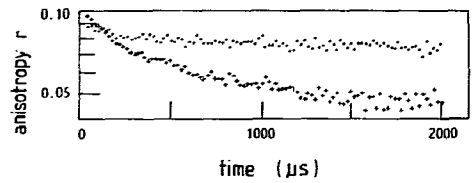

Fig. 6. Reversibility of band 3 aggregation by melittin 20–26 fragment with elevated salt concentrations. Equal amounts of 20–26 fragment added to equal concentrations of ghosts suspended in 5 mM phosphate buffer (●) and in 5 mM phosphate buffer + 150 mM NaCl (+) at 37 °C. Amino acid analysis indicates the amount of 20–26 fragment added (in 500 μl) to derive from approx. 0.11 mg of melittin.

by the higher ionic strength. The effect was independent of whether ionic strength was increased before or after addition of the aggregating agent. In contrast, immobilisation of band 3 by melittin was unaffected by the ionic strength of the medium over the range investigated. Typical results for polylysine and melittin are shown in Fig. 5.

Properties of melittin fragments

At concentrations whereat melittin produces complete immobilisation of band 3 the 1–19 fragment produced no measurable retarding effect. In contrast the 20–26 fragment was found to immobilise band 3 over a relevant concentration range (Fig. 6). This immobilisation was found to be reversible upon addition of 150 mM NaCl.

Effects upon bacteriorhodopsin reconstituted in DMPC vesicles

Addition of polylysine or Zn^{2+} to suspensions of bacteriorhodopsin-DMPC vesicles increased turbidity, presumably due to vesicle aggregation, but no retardation in the bacteriorhodopsin anisotropy decay was observed even at low ionic strength. The concentrations of Zn^{2+} (0.5 mM) and polylysine (1 mg/ml) were many times that which would have been required for im-

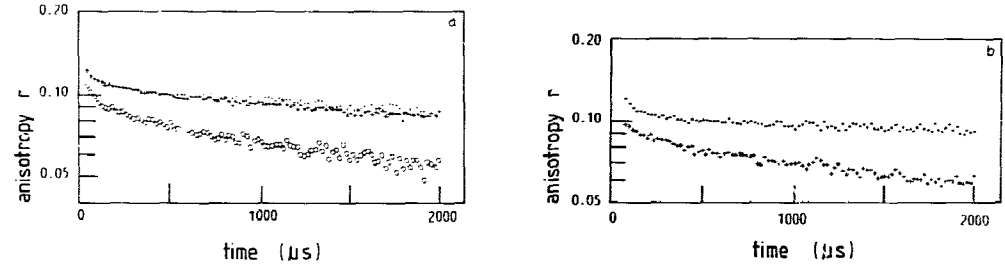

Fig. 5. Reversibility of band 3 aggregation by elevated salt concentrations: comparison between (a) melittin and (b) polylysine. (a) shows data points for control ghosts (○) incubated in 110 mM Na_2HPO_4 (pH 7.5) at 37 °C and for ghosts with (0.1 mg/ml) of melittin added, in the same buffer (+) and in 5 mM phosphate buffer (●). (b) shows the effects of 0.15 mg/ml of polylysine added in 110 mM Na_2HPO_4 (+) and 5 mM phosphate buffer (●). The polylysine/5 mM phosphate buffer curve is distorted by a scattering artifact at times less than 100 μs. The control is the same as for (a) and is virtually identical with the lower curve.

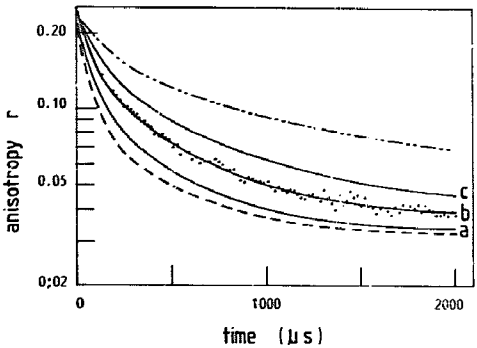

Fig. 7. Anisotropy decay curves for reconstituted bacteriorhodopsin in DMPC vesicles compared for different concentrations of melittin 1–19 fragment and with melittin itself. Measurements were at 30 °C in 0.1 M sodium acetate buffer (pH 5.0) containing 0.02% sodium azide. Bacteriorhodopsin concentration was 10 μM and the lipid/protein mole ratio was approx. 50. The dashed line represents the control curve, solid lines represent control plus (a) 10 μM, (b) 20 μM (c) 30 μM melittin 1–19 fragment, the upper line (- - - - -) represents a control plus 10 μM melittin. Typical signal to noise is illustrated in curve (b).

mobilisation if their powers to do so relative to melittin had been the same as for band 3. The same was true for the melittin 20–26 fragment; the 1–19 fragment, however, did display activity which approached a similar magnitude at three times the melittin concentration (Fig. 7).

Discussion

The agents melittin, Zn^{2+} and polylysine all share the ability to immobilise band 3 in a low ionic strength medium. Such effects can be achieved by increasing the radius of the rotating species via protein aggregation [17]. Freeze-fracture electron microscopic studies have supported this interpretation; in the case of melittin, clustering of intramembranous particles is clearly seen after exposure of erythrocytes to relevant concentrations of the peptide (Ref. 4; Hui, S., Roswell Park Memorial Institute, Buffalo, unpublished data). Elgsaeter et al. [1] attributed such clustering induced by divalent metal ions and polylysine to a co-precipitation of integral protein with spectrin, which was demonstrated to be precipitated by these agents under similar conditions. However, our experiments with spectrin/actin-depleted ghosts and stripped ghosts, revealed only very minor variations in the extent of retardation induced by relevant concentrations of our chosen agents. Thus aggregation is largely unaffected by removal of the major cytoskeletal proteins, indicating that they are unlikely to be the primary cause of the effect.

Further experiments performed in the present study of the aggregating effects of polylysine and Zn^{2+} have shown common sensitivities to the conditions, often divergent from that of melittin. The salt-dependent reversibility of band 3 aggregation by polylysine or Zn^{2+} strongly suggests its origin to be due to electrostatic interaction, either with band 3 itself or with a membrane component closely associated with it. The highly acidic nature of the band 3 cytoplasmic domain [18] makes it a possible site of electrostatic interactions with the agents under study. Trypsin-treated ghosts allow us to study its importance, as they lack this domain together with an area of sialic acid derived negative charge from the extracellular side of glycophorin [5]. In these modified ghosts we indeed observe a reduced efficiency of aggregation by polylysine and Zn^{2+}. However, a significant residual susceptibility is retained, indicating that some areas of negative charge probably remain after the trypsin treatment.

If seen in isolation, the unimpaired aggregation of band 3 by melittin, in a high ionic strength medium or in trypsin-treated ghosts, could well be taken to indicate the involvement of a fundamentally different mechanism. Previous results showing a decrease in melittin's aggregating potency upon derivatisation of its amino groups [5], and most strikingly the present study with melittin fragments, imply otherwise. These latter experiments implicate the hydrophilic and very positive (+4) 20–26 C-terminal end of melittin as the promoter of band 3 aggregation, the 'polylysine-like' salt-dependent reversibility of this effect suggests both an electrostatic mechanism, and a crucial role for the amphiphilic 1–19 section in conferring on the whole peptide an ability to induce aggregation under saline conditions.

The explanation for this is probably allied to the ability of the 1–19 section to confine the 20–26 section of melittin close to the membrane surface via hydrophobic interactions. Once ionic conditions favouring release of the aggregating agent are realised the concentrations of polylysine and Zn^{2+} will vastly decrease as they reach a new equilibrium position with the bulk medium, whereas anchorage of melittin to the membrane prevents such a redistribution taking place. It should also be borne in mind that release into three dimensions is entropically more favourable than into the two dimensions provided by the membrane surface. Whether actual electrostatic cross linking occurs, or if just the intercession of these agents and consequent negation of electrostatic repulsion between integral protein units is sufficient for the aggregation process, is unresolved. However, in this context, it is interesting to note that low pH (< 5), which presumably leads to neutralisation of negative charge from some surface protein residues, also results in the aggregation of band 3 [19].

For polylysine, Zn^{2+} and the melittin 20–26 fragment we observed turbidity increase concomitant with band 3 aggregation, indicating aggregation of the ghosts.

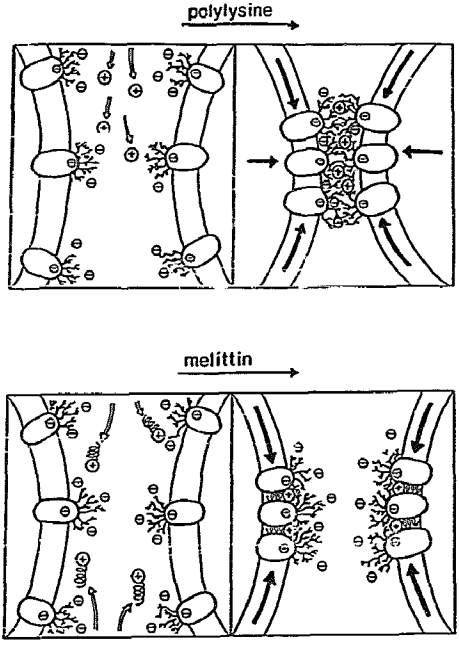

Fig. 8. Schematic representation of possible means of integral protein aggregation by polylysine and melittin, highlighting the greater potential for cell-cell cross linking in the former case by virtue of predominant interactions with charged groups further removed from the membrane surface. The helical section of melittin is drawn perpendicular to the membrane only to emphasise its anchoring role.

Such effects with melittin only became significant at concentrations greater than that required for the full retardation of the band 3 anisotropy decay. We think that this may indicate a greater freedom in the former agents to interact with areas of negative charge further removed from the bilayer surface than can melittin, thereby enabling dual interaction with apposed membranes. This is indicated schematically in Fig. 8. The outer negative charge, although not directly interacting with melittin may in fact shield it from interaction with more than one membrane, and in the extreme case also from the buffered medium which would be relevant to its insensitivity to salt.

It might be argued that protein aggregation occurs as a result of some modification in the organisation of the lipid component in the membrane. Whilst this cannot be completely discounted, it can be ruled out for melittin with the reconstituted bacteriorhodopsin system, where it was found that the extent of aggregation was determined by the melittin/protein ratio and not by the melittin/lipid ratio [6]. For polylysine and Zn^{2+} induced aggregation of band 3, the sensitivity to trypsin treatment of the membrane also indicates the primary involvement of protein.

In contrast to band 3 in erythrocytes, the possession of positive polyvalency does not ensure the ability to aggregate reconstituted bacteriorhodopsin even under low salt conditions. Although the 1–19 fragment does possess independent aggregating ability, its reduced potency relative to intact melittin suggests a role for the 20–26 component, if it can be directed to an appropriate part of the membrane.

In Table I, we have collected together results obtained in the present study with data for other cationic agents accumulated in our laboratory or previously reported in the literature. Although data are incomplete for some agents, we propose as a working hypothesis that such agents can be divided into two categories. The first category, which might be termed 'polylysine-like'

TABLE I

A range of agents found to aggregate band 3 in erythrocyte ghosts

C_{max} is a rough estimate of the concentrations of agent required to produce full retardation of anisotropy decay under normal experimental conditions (membrane protein concentrations, 0.6–0.8 mg/ml). Underlined references refer to electron microscope studies. n.d., not determined.

Agent	Number of amino acids	Charge at pH 7.4	C_{max}	Hydrophobic component	Reversibility by 150 mM NaCl	References
Melittin	26	+6	0.1 mg/ml	✓	×	4, 5
Acetyl melittin	26	+2	0.15 mg/ml	✓	n.d.	5
p25 mitochondrial presequence peptide	25	+6	0.1 mg/ml	✓	×	20
Polylmyxin B	10	+5	0.2 mg/ml	✓	×	21
Gramicidin S	10	+2	0.1 mg/ml	✓	n.d.	4
Polylysine	M_r = 4000		0.15 mg/ml	×	✓	1, 2
Melittin 20–26 fragment	7	+4	0.25 mg/ml	×	✓	
Mast cell degranulating peptide	22	+8	0.1 mg/ml	×	✓	21
Zn^{2+}	–	+2	0.1 mM	–	✓	22
Ca^{2+}	–	+2	10 mM	–	✓	1, 22
Mg^{2+}	–	+2	20 mM	–	✓	1, 22
Glyceraldehyde-3-phosphate dehydrogenase	M_r = 35000	+ve	0.4 mg/ml	×	✓	23, 24

consists of hydrophilic agents which bind to superficial anionic groups only at low ionic strength. The second 'melittin-like' category consists of more versatile agents with a hydrophobic moiety which anchors them to lipid bilayers resulting in interactions with anionic groups taking place close to the membrane surface. Unlike the first category, these agents remain effective at physiological ionic strength. Hydrophobic anchorage could thus provide a general means of expression of positive charge otherwise denied to foreign agents at biological membrane surfaces under physiological conditions, which may frequently result in aggregation of integral membrane proteins. A further noteworthy difference between the two categories is that only agents with a hydrophobic moiety are haemolytic. Whether or not this property is related to their protein aggregating ability is currently under further investigation.

Acknowledgements

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