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Electron microscopic observation of the aggregation of membrane proteins in human erythrocyte by melittin

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Human erythrocytes and erythrocyte ghost membranes were treated with native and modified melittins, up to 250 nmol/mg membrane protein. Native melittin induced aggregation of intramembranous particles (IMPs, observed by freeze-fracture electron microscopy), and created large, smooth bilayer areas devoid of IMP. The degree of IMP aggregation increased with increasing concentration of melittin, corresponding to hemolysis results. Membrane ghosts were slightly more susceptible to IMP aggregation than membranes on intact cells. The potency of inducing IMP aggregation was ranked in the order of: native melittin > acetylated melittin > succinylated melittin = 0. The concentration range of melittin which caused IMP aggregation corresponded to that which caused the immobilization of band 3 proteins as detected by measurement of rotational mobility by transient dichroism (Dufton et al. (1984) Eur. J. Biophys. 11, 17–24). Because both IMP aggregation and band 3 protein immobilization decreased with decreasing positive charge of the melittins used, the nature of melittin–protein interaction is likely to be at least in part electrostatic in the case of human erythrocyte membranes. Possible roles of IMP aggregation and the consequent creation of 'exposed' bilayer areas in the cytotoxic reaction of melittins are discussed.

I. Introduction

Melittin, a major constituent of bee venom, is a relatively simple polypeptide which has been subject to extensive biophysical studies. Melittin has 26 residues and different secondary tertiary and quaternary structures depending upon the physical environment [1–4]. In monomeric form, melittin can form an amphipathic helix which is thought to enable it to partition into the lipid bilayer [5]. The result of melittin–barrier interaction leads to the breakdown of the permeability barrier at sufficiently high melittin/lipid ratio [4,6–10]. This is generally believed to be the mechanism whereby melittin causes cell lysis. However, in addition to its ability to disrupt lipid bilayers, it has been found that lytic concentrations of melittin dramatically reduce the rotational mobility of band 3 protein in erythrocyte membranes [11,12] and of bacteriorhodopsin in lipid vesicles [13]. It was proposed that melittin promotes protein aggregation through electrostatic interactions of its cationic C-terminal section with charged residues on the membrane protein. Recent studies reveal that the hydrophobic N-terminal section of melittin is also important in conferring protein-aggregating properties which are not shared by purely hydrophilic polyvalent cationic species such as polylysine [14].

The importance of melittin–membrane protein interaction was further supported by the studies using modified melittins [11]. Native melittin was modified by acetylation and succinylation to alter the electric charge of the molecules [15]. Succinylation converts the net charge from +6 to −2, whilst acetylation reduced the net charge from +6 to +2. Changes in the overall charges would be expected to alter the interaction of melittin with charged glycoproteins or proteins in the membrane. The study of hemolysis as well as of immobilization of band 3 proteins in erythrocyte membranes and of bacteriorhodopsin in lipid vesicles, showed a hierarchy of decreasing potencies as the overall positive charge was decreased, in the order of native melittin > acytyl melittin > succinyl melittin = 0 [11,13]. The experiments with bacteriorhodopsin provide good evidence that immobilization of membrane proteins is a result of direct melittin–protein interactions, rather than an indirect consequence of melittin–lipid interactions [13]. The observed correlation between hemolysis and

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protein immobilization suggests that such melittin–protein interactions may play a role in melittin's lytic properties.

Although protein aggregation is the most probable explanation of the loss of rotational mobility by melittin, it is important to confirm this by an independent technique. In this study, we use freeze-fracture electron microscopy to provide visual evidence of melittin-induced protein aggregation in erythrocyte membranes. The dependency of observed aggregation on melittin concentration and modification is found to be comparable to that causing immobilization of band 3 protein and hemolysis.

II. Materials and Methods

(A) Purification of melittin and preparation of melittin derivatives

Native melittin, low in phospholipase A_2 activity, was generously provided by Prof. R.C. Hider. Succinyl- and acetyl-melittin derivatives were prepared as described previously [11] using methods based on those of Habermann and Kowallek [15].

(B) Treatment of intact erythrocytes and erythrocyte ghosts with melittin and its derivatives

Human erythrocytes were collected from donors and used immediately. The whole blood was washed in Hanks' balanced salt solution (BSS, NaCl 125 mM, KCl, 5 mM, $CaCl_2$ 3.8 mM, $MgCl_2$ 2.5 mM, Tris 5 mM at pH 7.4) after the serum and buffy coat were removed. Erythrocyte ghost membranes were prepared according to the previously described method [16]. After the final hemolysis and wash, the ghost membranes were suspended at a concentration of 5 mg of membrane protein per ml. The intact cell samples were also resuspended to an equivalent number of cells (10^{10} cells per ml). 0.02 ml of each sample was then diluted in 0.1 ml of BSS. Stock solutions of melittin and its acetyl-derivative were made in BSS to a concentration of 2.5 mM. Since succinyl-melittin is only sparing soluble in water, the stock solution for this derivative was made in 6 M guanidine chloride. Between 1 and 10 μl of the melittin solution was added to the buffer prior to the addition of erythrocyte or erythrocyte ghost suspensions, and the mixtures were incubated at 37° C for 5 min. The samples were then placed at 4° C and frozen immediately for electron microscopy.

(C) Electron microscopy

0.1 μl of the sample was placed between two thin copper holders for freeze-fracture preparation. The specimens sandwiched between the two copper plates were rapidly submerged into liquid propane without cryoprotectant, using an accelerator. The frozen samples were freeze-fractured in a modified Polaron freeze-fracture module with Cressington E beam evaporators. The replicas were examined in an Hitachi H-600 electron microscope.

III. Results

The freeze-fracture morphology of intact erythrocyte membranes is shown in Fig. 1a. The intramembranous particles (IMPs) in untreated erythrocyte membrane are irregularly distributed on the fracture surface. The distribution and the density of these intramembranous particles are similar to those reported previously [17,18]. The IMPs are believed to represent dimers of band 3 proteins and glycophorin complex.

When erythrocytes were treated with 25 nmol melittin/mg membrane protein, the IMP on the fracture plane became aggregated, leaving small areas of particle-denuded bilayer. The degree of aggregation was not extensive, but was apparent by visual examination (Fig. 1b). When the melittin concentration was increased to 50 nmol/mg protein, more extensive particle aggregation took place. The particle denuded areas became larger, adjoining neighboring smooth areas (see for instance Fig. 2a). As the melittin concentration was increased to 125 nmol/mg protein, the membrane showed extensive aggregation of IMP, together with large areas of membrane entirely free of IMPs (similar to Fig. 1e). At 250 nmol/mg protein, the erythrocytes were extensively disrupted. The remaining membrane or membrane fragments in the sample appeared as small vesicles with sparingly distributed IMPs, together with protein loaded bilayer pieces and hemoglobin in the buffer (Fig. 1c).

The effects of melittin on erythrocyte membrane ghosts were similar to those on intact erythrocytes. Fig. 1d shows a ghost membrane treated with 25 nmol melittin/mg protein. The degree of particle aggregation was slightly more extensive than that shown in similarly treated erythrocytes (Fig. 1b). Fig. 1e shows ghost membranes treated with 125 nmol melittin/mg protein. At this concentration, the ghost membranes are extensively damaged. Areas with extremely high numbers of IMPs were found next to areas with no IMP. At 250 nmol/mg protein, the ghost membrane was all but disintegrated into vesicles with no visible IMPs, and remnants of membranes that were packed with IMPs adjoining these smooth vesicles (Fig. 1f). In many areas, internal and external spaces of the ghost membranes cannot be distinguished, yet membranes from a single ghost tend to stay together and away from neighboring membrane aggregates, presumably from a different cell ghost.

In general, the ghost membranes were more susceptible to the effect of melittin of the same concentration as compared to intact erythrocytes. The membrane of the ghosts was not sealed, therefore both sides of the ghost membrane were accessible to melittin. This is contrary

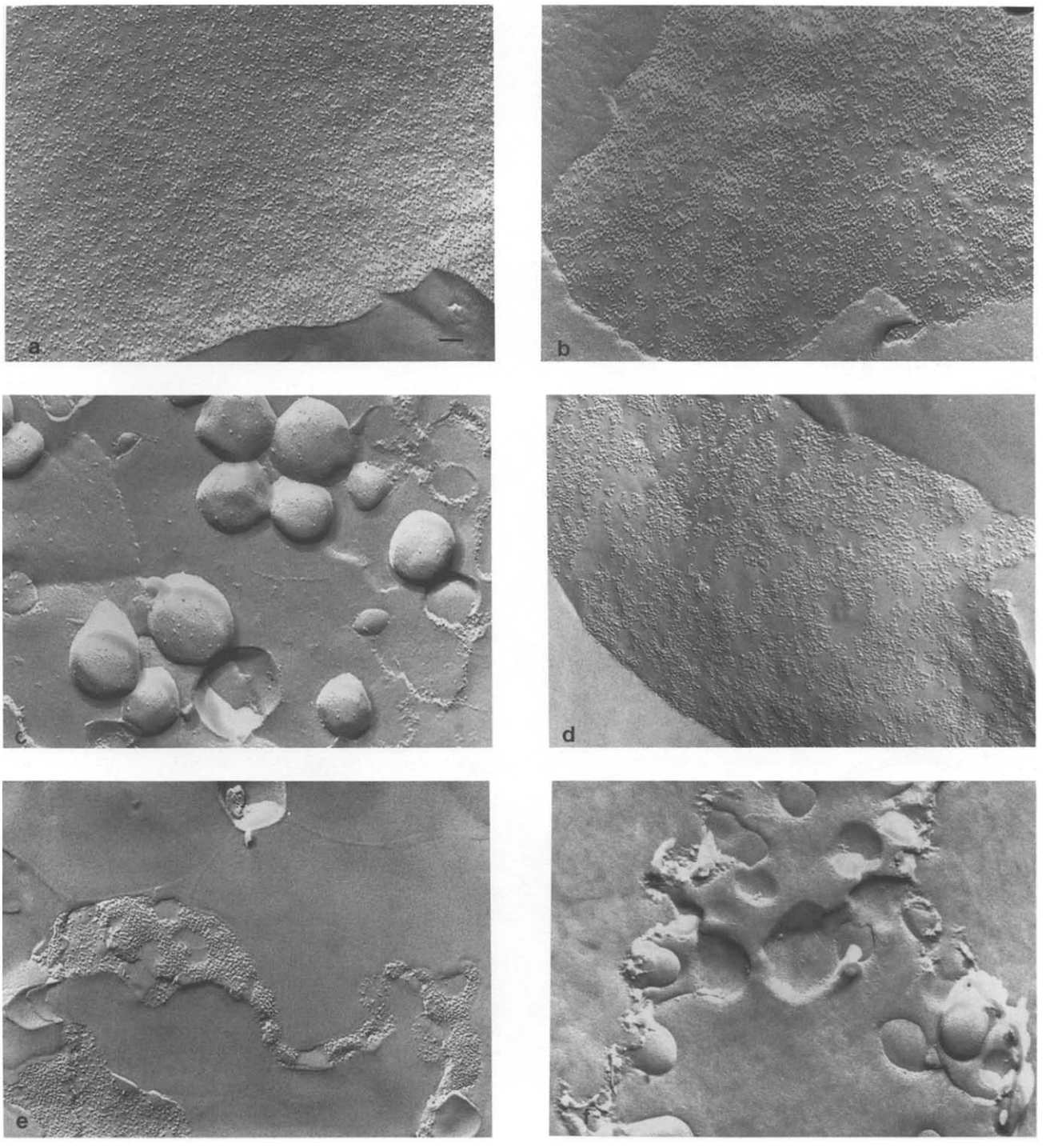

Fig. 1. Freeze-fracture electron micrographs of human erythrocytes (a–c) and erythrocyte ghost membranes (d–f), treated with native melittin at concentrations (a) 0, (b) 25, (c) 250, (d) 25, (e) 125, (f) 250 nmol/mg membrane protein. Treatment is for 30 min at 37 °C. Bar = 100 nm.

to intact cells where melittin has access to the membrane from one side, namely the outside only.

In order to compare the effects of native melittin with those caused by melittin derivatives with altered electric charges, intact erythrocytes were treated with native as well as with acetyl and succinyl melittin of the same concentrations. Fig. 2a shows a cell treated with 50 nmol of native melittin/mg protein. There are visible IMP aggregations as described previously. When the intact erythrocytes were treated with the same concentration of acetyl melittin, the degree of IMP aggregation is only slightly different from that in the control

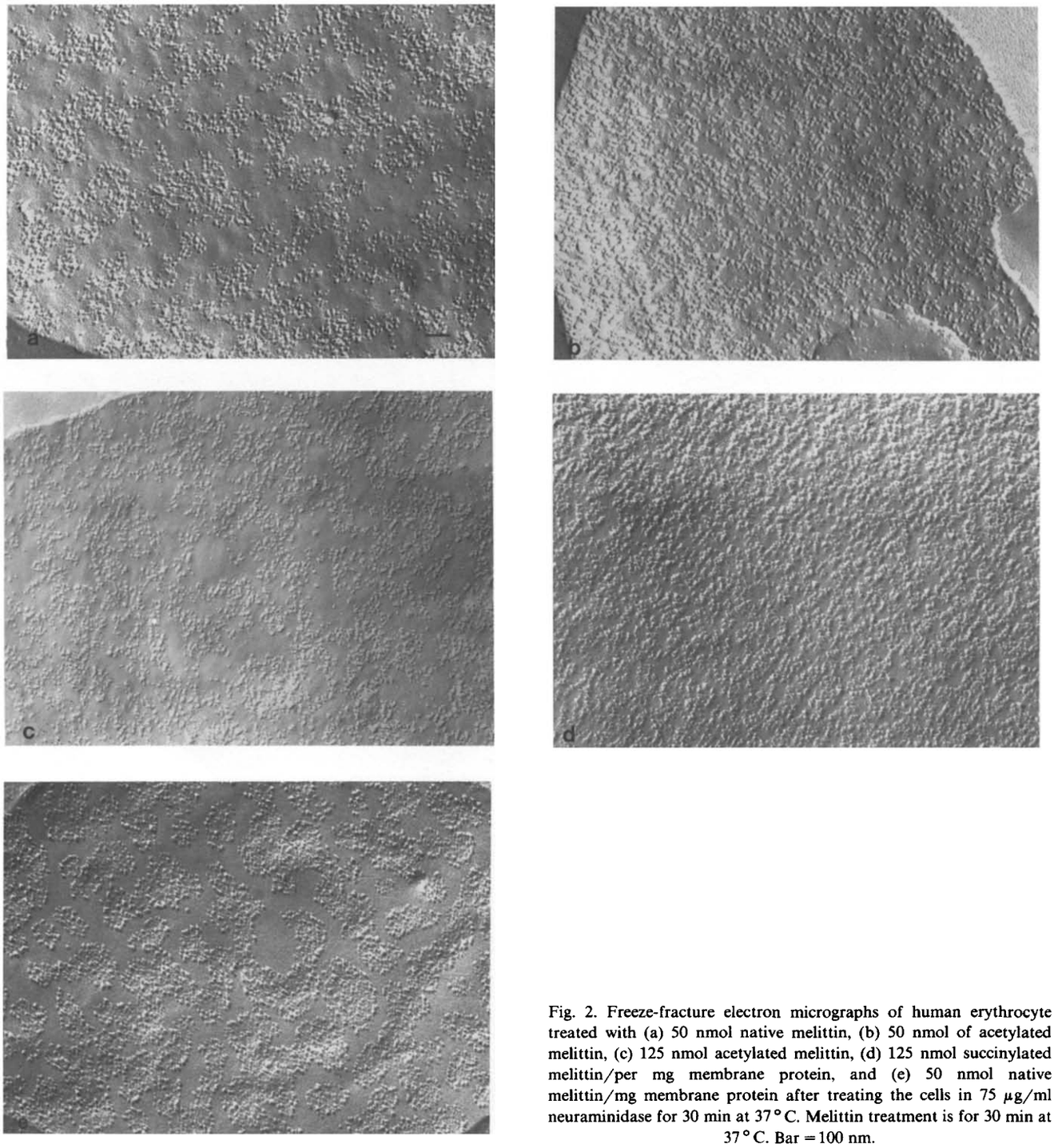

Fig. 2. Freeze-fracture electron micrographs of human erythrocyte treated with (a) 50 nmol native melittin, (b) 50 nmol of acetylated melittin, (c) 125 nmol acetylated melittin, (d) 125 nmol succinylated melittin/per mg membrane protein, and (e) 50 nmol native melittin/mg membrane protein after treating the cells in 75 μg/ml neuraminidase for 30 min at 37 °C. Melittin treatment is for 30 min at 37 °C. Bar = 100 nm.

cell. There is no change in IMP size (Fig. 2b). Without the aid of computerized analysis [17,18], the aggregation of IMPs in the membrane of treated cells cannot be differentiated with certainty from that in control cells. It requires 125 nmol acetyl melittin/mg protein to produce particle aggregation comparable to that shown in Fig. 2a. The result of treatment with 125 nmol acetyl melittin/mg protein is shown in Fig. 2c. At concentrations of 125 nmol succinyl melittin/mg protein, the treated erythrocytes showed no signs of IMP aggregation (Fig. 2d). The morphology of cells treated with succinyl melittin was not different from an untreated control cell, up to this molar concentration of succinyl melittin. This is in stark contrast to native melittin, which at this concentration would have caused extensive IMP aggregation similar to that shown in Fig. 1e. Free

succinyl-melittin was not detectable by absorption spectroscopy in the supernatant after centrifuging the cells, indicating that its inactivity is not due to an inability to bind. Thus, the potency to induce IMP aggregation has the order: native melittin > acetyl melittin > succinyl melittin = 0. This is the same order of potency as that reported for hemolysis as well as that reported for band 3 protein immobilization over the same melittin concentration range [11].

In order to test the influence of the negatively charged sialic acid in the band 3-glycophorin complex of the effect of the positively charged native melittin, erythrocytes were treated with 75 μg/ml neuraminidase at 37° C for 30 min to remove the sialic acid moiety of the complex prior to melittin treatment. An example is shown in Fig. 2e, which is a neuraminidase-treated sample otherwise identical to that in Fig. 2a. The results are indistinguishable from those without neuraminidase treatment, at all levels of melittin used. Neuraminidase treatment was also found to influence neither hemolysis nor protein immobilization by melittin.

Discussion

Melittin has long been recognized as a membrane-disrupting agent [4,6–10]. The mechanism of membrane disruption is generally believed to involve the partition of the amphiphilic helix of melittin into the lipid bilayer [5]. Various models for melittin's action have been proposed including perturbation of the bilayer stability by a 'wedge effect' of melittin monomers [4] and formation of ion channels by melittin oligomers [19,20]. Although the particular conformation of melittin responsible for bilayer disruption remains unresolved, there is no doubt that melittin can induce leakage of contents from pure lipid vesicles and at sufficiently high concentration may break the bilayer into micellar or discoid particles [21,22]. On the other hand, melittin has also been shown to perturb the arrangement of membrane proteins as evidenced by a loss of their rotational mobility [11,13]. The present study was carried out in order to further establish the nature of melittin–membrane protein interactions.

The freeze-fracture electron micrographs shown in Fig. 1 provide a morphological evidence of melittin-induced IMP aggregation and membrane disruption in erythrocytes. These experiments were carried out with similar melittin concentrations to those used in the previous hemolysis and band 3 protein immobilization experiments. The onset of extensive membrane disruption corresponds to the onset of hemolysis. The IMP of erythrocytes have been shown to represent the band 3 protein-glycophorin complex. Since the rotational immobilization of the latter and the aggregation of IMP is observed at the same melittin concentration range, there is little doubt that the aggregation of band 3 protein-glycophorin complexes is the cause for the impedence of rotation. The aggregation precedes the extensive disruption of the membrane.

Since the protein immobilization experiment was done on ghost membranes due to experimental requirements of the transient dichroism measurements, we also performed similar morphological experiments using erythrocyte ghosts in parallel to experiments using intact cells. Although the control (untreated) cells and ghosts show similar morphology, ghost membranes are more susceptible to melittin disruption than intact cells. This is to be expected, since the ghosts we use are unsealed, thereby providing accessibility of melittin from both sides. Furthermore, the cytoskeleton structures are believed to be somewhat disrupted during the ghost preparation procedure. Lateral movements of IMP are known to be impeded by the spectrin/actin network [23]. If this network is disrupted during the ghost preparation, then the IMPs would be more free to move, therefore more susceptible to melittin-induced rearrangement. The differences between ghosts and cells, however, are only quantitative. It only requires a slightly higher concentration of melittin to produce the same results in cells as that in ghost membranes.

The aggregation of IMPs may be caused by a direct melittin–protein interaction, or indirectly by a mechanism whereby the melittin would partition into the lipid bilayer and cause extensive areas of smooth bilayers devoid of band 3-glycophorin complex, i.e. IMPs. The latter mechanism would lead to the exclusion of IMP from areas occupied by melittin which is too small to be seen as IMP. While this morphological study cannot distinguish these two mechanisms, it is unlikely that sufficient amounts of melittin would have partitioned into the bilayer to force IMP aggregation by volume exclusion. Melittin can easily fit between the native distribution of IMPs without causing them to aggregate, even if exclusion is considered to be the main driving force of IMP aggregation.

A major argument for the direct melittin–band 3-glycophorin complex interaction is the differential effect of melittin and its derivatives. The differential effect does not seem to be a result of a differential binding or partition of melittin into the bilayer [24]. However, the overall charge of melittin at pH 6 or less has been changed by acetylation and succination from +6 to −2. If there is electrostatic interaction between melittin and the charged proteins or lipids of the erythrocyte membrane, this modification would reflect on the potency of the melittin derivatives to cause IMP aggregation. This is in fact shown to be the case (Fig. 2). The acetylated melittin is much less potent than the native melittin, while the succinylated melittin has no effect at all. The electrostatic interaction does not depend on the sialic acid moiety of the protein, since neuraminidase treatments have no effect on either IMP aggregation or

hemolysis. Since succinylated melittin only dissolves sparingly in water, we needed to dissolve it in 6 molar guanidinium chloride. It is therefore necessary to do a control experiment with guanidinium chloride alone. The results show that 6 molar guanidinium chloride has no effect on particle aggregation (not shown). Moreover, when guanidinium chloride was used together with native melittin, the potency of the latter was unaffected. The lack of potency of succinyl melittin is therefore a property from the modification alone. The same order of potency and the occurrence of IMP distribution at the concentration range of these melittins agree entirely with the previously published results on hemolysis as well as on band 3 protein immobilization. The exact correspondence gives strong support that the measurement of band 3 protein immobilization and the aggregation of IMP by these melittins are one and the same phenomenon.

There are several factors that can cause IMP aggregation in erythrocyte membranes. Among them are low pH, low ionic strength, and the disruption and precipitation of spectrin [16,18]. It has been shown that melittin-induced loss of rotational mobility of band 3 is unaffected by the removal of cytoskeletal proteins, indicating that they are not primarily involved in the aggregation mechanism [14]. However, a relatively small degree of aggregation is required to produce immobilization on the time scale of the transient dichroism measurements. The extensive IMP aggregation seen in the freeze-fracture electron micrographs at higher melittin concentrations may imply that there is also disruption of the attachment of IMP to the underlying spectrin-actin network, which would permit redistribution over large distances.

The observation that the ability of melittin and its derivatives to aggregate IMPs correlates with their hemolytic potency, either alone or in synergism with phospholipase A_2 [11], provides circumstantial evidence that melittin–protein interactions play a role in the lytic mechanism. At the present time, however, a mechanism whereby protein aggregation causes hemolysis has not been demonstrated and indeed such aggregation is not a sufficient condition since agents such as polylysine and concanavalin A [25] can promote aggregation with no accompanying hemolysis. On the other hand, significant differences between melittin and polylysine in their mode of aggregation of membrane proteins have recently been detected [14].

An alternative view is that membrane proteins play a protective role by binding melittin and preventing its harmful interaction with the lipid bilayer. Conceivably higher concentrations of melittin disrupt the smooth areas of lipid bilayer which are a consequence of protein aggregation. Synergism with phospholipase A_2 [24] may also be explained by the exposure of smooth bilayer areas which are more accessible to the enzyme than in the unperturbed membrane. Melittin may thus truly act as a front agent in the cytotoxic reaction of bee venom.

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References

1 Talbot, J.C., Dufourcq, J., De Bony, J., Faucum, J.F. and Lussan, C. (1979) FEBS Lett. 102, 191–193.
2 Quay, S.C. and Condie, C.C. (1983) Biochemistry 22, 695–700.
3 Brown, L.R., Lauterwein, J. and Wuthrich, K. (1980) Biochim. Biophys. Acta 622, 231–244.
4 Dawson, C.R., Drake, A.F., Helliwell, J. and Hider, R.C. (1978) Biochim. Biophys. Acta 510, 75–86.
5 Terwilliger, T.C., Weissman, L. and Eisenberg, D. (1982) Biophys. J. 37, 353–361.
6 Habermann, E. (1972) Science 177, 314–322.
7 Sessa, G., Freer, J.H., Colacicco, G. and Weismann, G. (1969) J. Biol. Chem. 244, 3575–3582.
8 De Grado, W.F., Musso, G.F., Lieber, M., Kaiser, E.T. and Kezdy, F.J. (1982) Biophys. J. 37, 329–338.
9 Tosteson, M.T., Holmes, S.J., Razin, M. and Tosteson, D.C. (1985) J. Membr. Biol. 87, 35–44.
10 Kemp, C., Klausner, R.D., Weinstein, J.N., Renswonde, J., Van Pincus, M. and Blummenthal, R. (1982) J. Biol. Chem. 257, 2469–2476.
11 Dufton, M.J., Hider, R.C. and Cherry, R.J. (1984) Eur. Biophys. J. 11, 17–24.
12 Clague, M.J. and Cherry, R.J. (1988) Biochem. J. 252, 791–794.
13 Hu, K.-S., Dufton, M.J., Morrison, I.E.G. and Cherry, R.J. (1985) Biochim. Biophys. Acta 816, 358–364.
14 Clague, M.J. and Cherry, R.J. (1989) Biochim. Biophys. Acta 980, 93–99.
15 Habermann, E. and Kowallek, H. (1970) Hoppe-Seyler's Z. Physiol. Chem. 351, 884–890.
16 Hui, S.W., Stewart, C.M., Carpenter, M.P. and Stewart, T.P. (1980) J. Cell Biol. 85, 283–291.
17 Pearsons, R.P., Hui, S.W. and Stewart, T.P. (1979) Biochim. Biophys. Acta 557, 265–282.
18 Hui, S.W. and Frank, J. (1985) J. Microsc. 137, 293–303.
19 Tosteston, M.T. and Tosteson, D.C. (1981) Biophys. J. 36, 109–116.
20 Vogel, H. and Jahnig, F. (1986) Biophys. J. 50, 573–582.
21 Dempsey, C.E. and Watts, A. (1987) Biochemistry 26, 5803–5811.
22 Dufourcq, J., Faucon, J.-F., Fourche, G., Dasseux, J.-L., Le Maire, M. and Gulik-Krzywicki, T. (1986) Biochim. Biophys. Acta 859, 33–48.
23 Sheetz, M.P., Schindler, M. and Koppel, D.E. (1980) Nature (London) 285, 510–512.
24 Yunes, R., Goldhammer, A.R., Garner, W.K. and Cordes, E.H. (1977) Arch. Biochem. Biophys. 183, 105–112.
25 Huang, S.K. and Hui, S.W. (1986) Biochim. Biophys. Acta 860, 539–548.