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Effect of melittin upon cellular and lysosomal membranes*

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MELITTIN is a basic polypeptide which constitutes over 50 per cent of the dry weight of crude bee venom. The toxin is of interest because its primary structure (Fig. 1) has been directly related to its activity

Gly-ile-gly-ala-val-leu-lys-val-leu-thr-thr-gly-leu-pro-ala-leu-ile-ser-trp-lys-arg-lys-arg-gluNH$_2$-glu(NH$_2$)$_2$

p

FIG. 1. Primary structure of melittin.[2]

upon biomembranes.[1–4] Since one end of the molecule contains amino acids which are relatively hydrophilic (positions 21–26) while the rest are relatively hydrophobic (positions 1–20), melittin may be considered a biological amphipath. Indeed, we have found that melittin, like other amphipathic molecules, is capable of interacting with artificial lipid membranes arranged either as monolayers or as lamellar bilayers (liposomes).[5] The direct disruption of lipid structures by melittin may account for its well established hemolytic and inflammatory properties, particularly melittin's capacity to induce cutaneous necrosis after intradermal injection (reviewed by Habermann[1]). Since lysosomes appear to mediate inflammation induced by a number of membrane-active agents such as bacterial toxins,[6] polyene antibiotics,[7] croton oil, etc.,[8] we studied the effects of melittin upon leukocytes and lysosomes.

Rabbit polymorphonuclear leukocytes were obtained from glycogen-induced peritoneal exudates and studied by methods previously described in detail.[9] Cells were suspended in Eagle's minimum essential medium (Grand Island Biologicals) and 20% fetal calf serum, permitted to settle for 30 min on glass slides, and gently washed free of unattached cells and debris. Thereafter, either melittin (Prof. E. Habermann, Giessen) in isotonic (0·9%) saline, or saline alone, was added in equal volumes (0·15 ml). Cells and additives were incubated for 15–20 min, air-dried, and stained with tetrachrome. After 15 min of incubation, control leukocytes (Fig. 2A) appeared relatively normal with intact, multilobulate nuclei, prominent heterophile granules and sharp cell borders. When exposed to melittin (10^{-6} M) for 15 min, the cells maintained their sharp cell boundaries (Fig. 2B). However, the distinct leukocyte granules could no longer be discerned and vacuoles appeared in many cells. The

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multilobulated nuclei remained intact, but appeared displaced to the periphery by homogeneous masses of eosinophilic material, in some of which discrete vacuoles could be seen. After exposure to higher concentrations of melittin (10^{-5} M), the cells appeared considerably damaged. Cell boundaries became indistinct, cytoplasmic fragments and smudges of destroyed cell remnants were observed. Some cells and nuclei were pyknotic, while others were swollen. Few, if any, normal cells remained (Fig. 2C). However, in contrast to their appearance after exposure to lower concentrations of melittin, a considerable number of the cells appeared to contain intact granules. This suggested that, whereas low concentrations of melittin affected leukocyte granules without visibly compromising the cell membrane, higher concentrations directly lysed the cells by disrupting their outer membrane. We have previously reported that lysolecithin produced similar effects (cell disruption without preliminary degranulation) in polymorphonuclear leukocytes, and suggested that various membrane-disruptive agents might have differential effects upon cellular and subcellular membranes.[9]

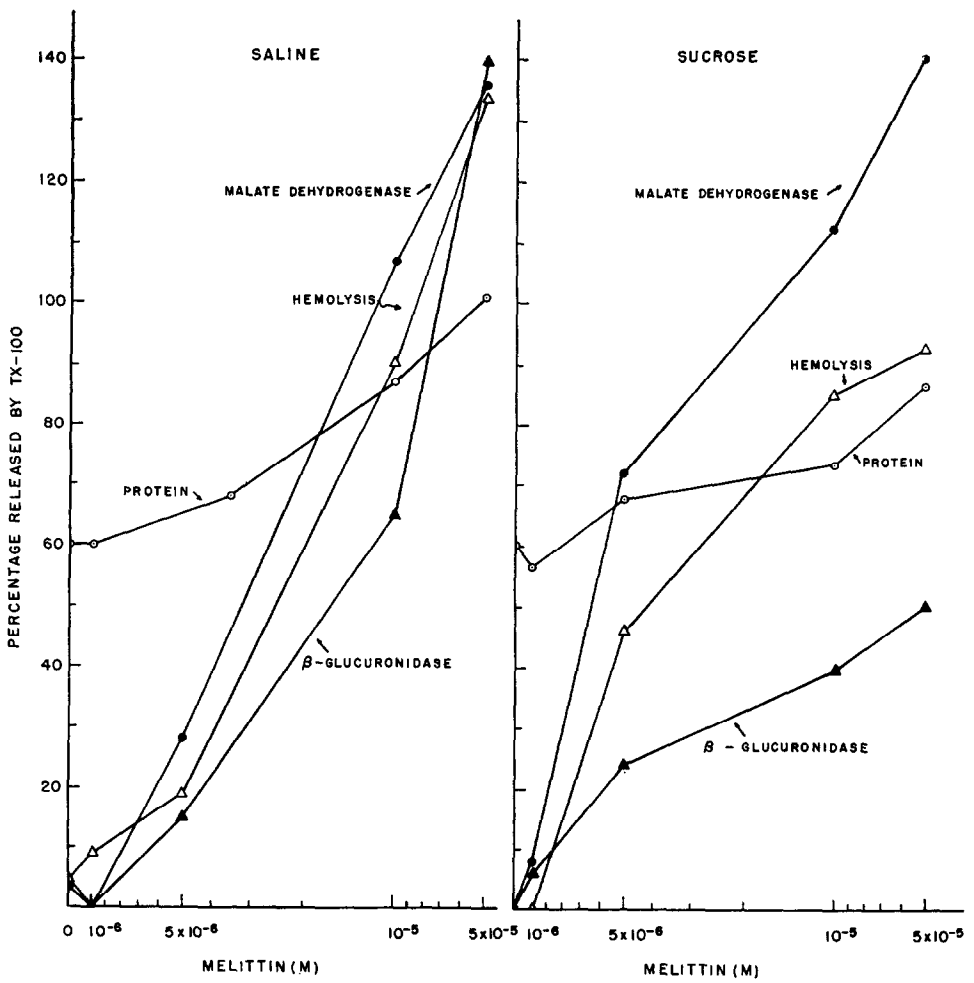

FIG. 3. Release of enzymes and protein into suspending medium from rabbit heterophiles after 15 min of exposure to melittin in saline (left) and sucrose, 0·25 M (right). Hemolysis = release of hemoglobin from erythrocytes. Data are expressed as percentage released by 0·1% v/v Triton X-100. Absolute values: β-glucuronidase, 2·55 μg phenolphthalein/100 μg protein/hr; acid phosphatase, 2·81 μmole mg protein/hr; malate dehydrogenase, 6·1 μg/$NADH_2$ oxidized/mg protein/min.

To test this hypothesis with melittin, we incubated leukocytes with the polypeptide and determined the release of various marker enzymes and proteins. For such experiments, 5×10^6 rabbit heterophiles were suspended in either isotonic saline or 0·25 M sucrose (1·0 ml) and were incubated for 15 min at 37° with increasing concentrations of melittin (in 0·1 ml saline or sucrose). After 15 min of incubation the cells were centrifuged at 20,000 *g* for 20 min in a Servall Superspeed centrifuge and the supernatant was analyzed for the activity of malate dehyodrgenase, β-glucuronidase and protein by micromethods described elsewhere.[10] Each of the leukocyte preparations had been permitted to contain some erythrocytes (approximately 10^4) and the hemoglobin released from these was determined by its absorbance at 540 mμ. As in previous work,[6, 8] Triton X-100 (0·1 %, v/v), a nonionic detergent, was added to aliquots of leukocytes and erythrocytes; the amounts of protein, hemoglobin and enzyme activity were expressed as a percentage of that released by detergent.

Whether in saline or sucrose, leukocytes released much of their total protein into the medium (Fig. 3), but leaked relatively little malate dehydrogenase (a marker for mitochondrial damage) or β-glucuronidase (a marker of lysosomal disruption). Nor was hemoglobin released from erythrocytes. However, when exposed to increasing amounts of melittin in saline, leukocytes released progressively more protein and enzymes; hemolysis was also evident. This suggested that melittin disrupted not only the outer membranes of leukocytes and erythrocytes, but also those of mitochondria and lysosomes within leukocytes. Furthermore, we studied the effects of melittin upon cells suspended in sucrose, reasoning that the presence of a relatively nonpermeant sugar should at least partially protect cells against lysis induced by entry of water into cells rendered permeable to small ions. However, at low concentrations of melittin (5×10^{-6} M), release of mitochondrial and lysosomal markers was, if anything, more marked in sucrose than in saline, and hemolysis was enhanced. At higher concentrations of melittin in sucrose ($>10^{-5}$ M), release of proteins, hemoglobin and β-glucuronidase was less than when the cells were in saline. The two sets of experiments (morphological and biochemical) suggest that high concentrations of melittin disrupt cell membranes (leukocytes and erythrocytes) both by a direct action and by an indirect mechanism involving osmotic swelling and rupture. Thus cells exposed to 10^{-5} melittin would become disrupted before the polypeptide had reached a sufficient concentration at, or in, lysosomes. In contrast, at lower concentrations ($<5 \times 10^{-6}$ M), melittin, disrupted mitochondria, lysosomes and erythrocytes without inducing gross injury to the cell membranes of leukocytes. At these concentrations, relatively small amounts of protein were released from leukocytes (compared to β-glucuronidase or malate dehydrogenase) and the outer membranes appeared intact by light microscopy.

To determine whether lysosomes differed from mitochondria in their susceptibility to melittin *in vitro*, we isolated large-granule fractions rich in these organelles from rabbit liver in 0·25 M sucrose as previously described.[6, 8] Briefly, twice-washed fractions sedimenting between 800 *g* (10 min) and 20,000 *g* (20 min) were incubated with melittin or bradykinin (Sandoz) in sucrose. After 30 min of incubation at 37°, the activity of enzymes released into clear supernatants (20,000 *g*, 20 min) was determined (Fig. 4). With increasing amounts of melittin, release of β-glucuronidase, acid phosphatase and malate dehydrogenase was considerably increased. Melittin was compared to the basic, non-amphipathic polypeptide, bradykinin (5×10^{-5} M). Release of acid phosphatase, β-glucuronidase or malate dehydrogenase was not augmented from granules exposed to bradykinin. Although β-glucuronidase was solubilized to a greater extent than malate dehydrogenase, acid phosphatase was not, a finding which is in keeping with the greater affinity for membrane residues of the latter enzyme.[11] These experiments did not suggest a differential susceptibility of lysosomal (rather than mitochondrial) membranes to melittin. We found it impractical to add melittin to large-granule fractions of rabbit liver or intact leukocytes, and to measure the release of enzymes at varying time intervals. This was difficult because melittin appeared to act upon the cells or organelles during the course of removing particulates, even at 4°. Thus by the time granules or cells had been spun at 20,000 *g* for 10–20 min nearly maximum amounts of enzymes had already been released. Therefore all experiments were performed at a fixed time interval, with only the concentration of melittin varied. Under such circumstances, we could obtain no satisfactory proof that melittin preferentially interacted with the membranes of lysosomes rather than with other biological structures.

Nevertheless, the differential effects upon leukocytes of high s. low concentrations of melittin suggest a possible mechanism for selective injury to lysosomes within cells. Lysosomes, by virtue of their role in the vacuolar system of cells, take up and concentrate agents from the medium.[12] Thus toxins or drugs, present in sublytic concentrations at the cell surface, can become internalized in

secondary lysosomes where concentrations can be achieved which suffice to affect their membranes or contents.[13] This mechanism can be invoked to explain both the actions of substances which labilize (silica suspensions, streptolysin S) and those which stabilize (chloroquine) lysosomes.[13, 14] Unfortunately, since these experiments did not measure redistribution of hydrolases within leukocytes after exposure to melittin, it remains unclear whether melittin acted selectively on any structure within the cell.

Hegner,[15] however, in studies of white cells performed while these experiments were in progress obtained morphologic evidence that leukocyte lysosomes were an early target for low concentrations

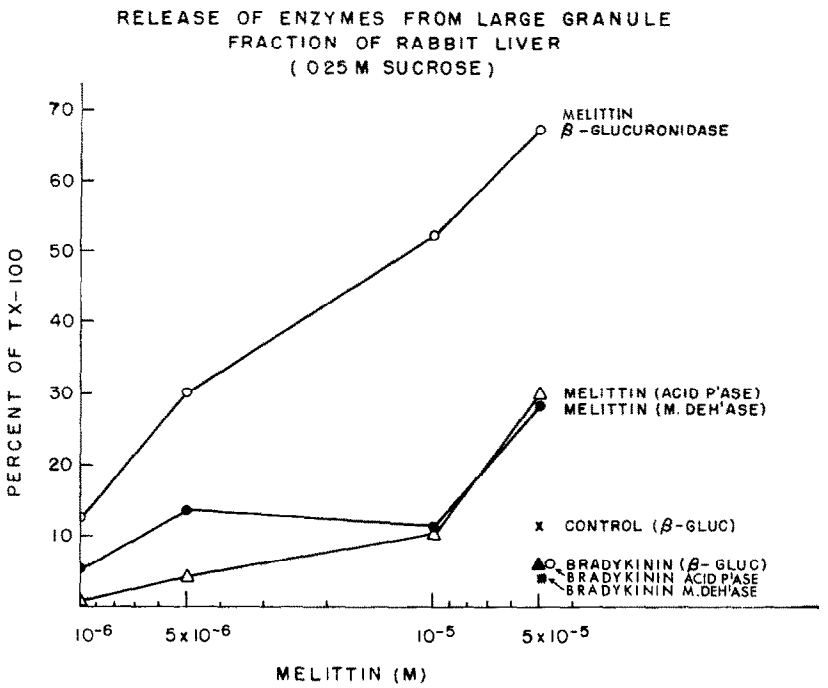

FIG. 4. Release of β-glucuronidase and acid phosphatase from lysosomes and of malate dehydrogenase from mitochonria after 30 min of incubation of a large-granule fraction from rabbit liver with varying concentrations of melittin or bradykinin. Control = 0·25 M sucrose. Data are expressed as percentage released by 0·1% (v/v) Triton X-100. Absolute values: β-glucuronidase, 142 μg phenolphthalein/100 mg protein/hr; acid phosphatase, 6·12 μmoles P_i/mg protein/hr; malate dehydrogenase, 30·0 μmoles $NADH_2$ oxidized/mg protein/min.

of melittin. At $3{\cdot}5 \times 10^{-6}$ M, melittin caused dissolution of lysosomal membranes as observed in the electron microscope; the granules discharged their contents into large cytoplasmic vacuoles at a time when the cell membrane seemed intact. By using techniques similar to those outlined above, Hegner also found that melittin disrupted isolated leukocyte lysosomes. Furthermore he determined the time-course of enzyme release from intact polymorphs and found that a cytoplasmic marker enzyme, lactate dehydrogenase, appeared first, followed by cathepsin (from lysosomes) and then by glutamate-pyruvate transaminase (from mitochondria). None of these experiments provides evidence for a primary effect of melittin upon lysosomes in living cells, since the polypeptide acts equally well upon all membrane-bounded structures *in vitro*. Nevertheless, together with the extensive studies of Habermann[1-4] and Hegner,[15] these data suggest that bee venom contains a polypeptide which disrupts biomembranes by virtue of its unique hydrophobic/hydrophilic structure.

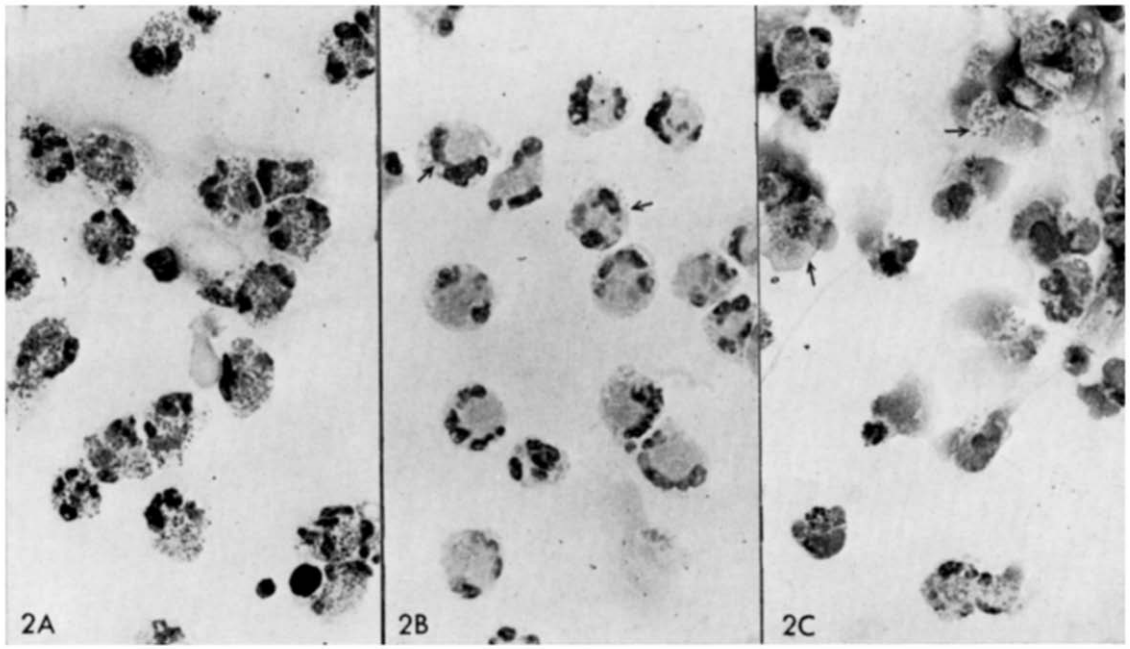

FIG. 2. (A) Rabbit heterophiles incubated 15 min with saline, air-dried, and strained with tetrachrome. (B) Rabbit heterophiles incubated 15 min with 10^{-6} M melittin. Distinct granularity has disappeared in almost all cells. Cytoplasm is filled with homogeneous material; arrows point to cells with vacuoles. (C) Rabbit heterophiles incubated 15 min with 10^{-5} M melittin. Most cell outlines are smudged and cytoplasmic strands are seen. Arrows point to ruptured or deformed cells, the intracellular granules of which are still intact (× 250, tetrachrome).

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Staphylococcal alpha toxin induced ionic transport and permeability changes in frog skin

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STAPHYLOCOCCAL alpha toxin (ST), one of the most active staphylococcal products changes and eventually damages membranes of erythrocytes,[1] thrombocytes[2] and mast cells.[3] The frog skin was chosen as a further model to study the membrane effects of the toxin. The technique of Ussing and Zerahn[4] that we used, enables to determine the changes of ionic transport and of permeability of amphibian membranes by measurement of electrophysiological parameters. The potential difference (PD) and short-circuit current (SCC) were checked. 119 mM Ringer's solution bathed the inside of the skin (corium), whereas 20 mM NaCl bathed the outside (epidermis). The replacement of Na^+ by K^+ on the outside of the untreated skin led to the loss of PD and SCC,[5] as only Na^+ is actively transported from the outside across the skin. Unpurified ST was added to the inside.