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SELECTIVE SOLUBILIZATION BY MELITTIN OF GLYCOPHORIN A AND ACETYLCHOLINESTERASE FROM HUMAN ERYTHROCYTE GHOSTS

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Melittin, the main basic and hydrophobic peptide of bee venom, has been used for solubilizing membrane components of the human erythrocyte ghost. Up to 1.0 mM, it does not extract any phospholipid. Between 0.1 and 1.0 mM, it solubilizes partially glycophorin A and acetylcholinesterase. When the membrane is first degraded by phospholipase A_2, the solubilization of both proteins by melittin is total, and 48% of the phospholipids are removed, mainly as lysoproducts, whereas phospholipase A_2, by itself, has no solubilizing properties. In its melittin-solubilized state, acetylcholinesterase is in a dimeric form and displays a slow time-dependent irreversible inactivation. Triton X-100 at 1.0% (v/v) interrupts the inactivation. We suggest that melittin binds to the hydrophobic site of acetylcholinesterase which anchors it in the lipid bilayer.

Introduction

Melittin, an amphiphilic peptide from bee venom, affects various membrane structures. At concentrations ranging from 1.0 nM to 1.0 μM, it penetrates planar lipid bilayers [1] and it orients itself in a transbilayer conformation when a voltage is applied across the membrane [2]. In addition, evidence was provided that melittin binds to hydrophobic surfaces in an α-helical conformation in which all hydrophobic residues are placed to one side of the helix [3–7]. In solution, melittin, as a monomer, can assume a flexible extended conformation, or, as a tetramer, an α-helical structure [8,9]. In micromolar concentrations, melittin lyses artificial [10] and biological membranes [10–14]. Electron microscopy shows that membranes lysed by melittin display both lamellar and amorphous structures, without totally disrupting the bilayer structure [10]. Melittin also modulates various membrane-associated proteins either by perturbing the lipid bilayers or by directly interacting with them [15–19].

Direct interactions between melittin and membrane proteins have not yet been sufficiently documented for the two following reasons: (a) most purified membrane proteins still contain bound lipids, which makes it difficult to decide whether a modulation due to melittin is caused by its interaction with the lipids or the protein; (b) contamination of melittin by a phospholipase A_2 present in the bee venom would induce the formation of lysophospholipids that also could affect the protein. This is of special importance since melittin itself activates phospholipase A_2 [20,21]. This cumulative effect has been described in erythrocyte lysis [22] and in membrane protein solubilization [23].

We studied the effect of melittin totally devoid

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of phospholipase A_2 activity [24] on human erythrocyte membranes at concentrations far above the lytic amount ($\gg 10^{-6}$ M). We then compared its effects to those obtained with exogenously added bee venom phospholipase A_2. Under those conditions, two integral membrane proteins were solubilized, namely glycophorin A, the major integral sialoglycoprotein which spans the bilayer, and acetylcholinesterase, a non-spanning, outside-facing, intrinsic protein. Since the latter needs hydrophobic interactions to warrant catalytic activity [25,26] and since it is available as a pure, detergent-free, protein [27], we further studied acetylcholinesterase-melittin interactions using both the pure enzyme and that present in extracts from melittin-treated ghosts.

Materials and Methods

Materials

Melittin free of phospholipse A_2 was prepared as described elsewhere [24]. Acetylcholinesterase from human erythrocyte membranes was purified into a detergent-free form according to Brodbeck et al. [27]. Human erythrocyte ghosts were prepared from fresh blood according to Dodge et al. [28] and used within 48 h. Bee venom phospholipase A_2 was purchased from Sigma, and assayed according to Nieuwenhuizen [29]. Sodium [^{3}H]borohydride (254 mCi/mmol) was obtained from Amersham International (U.K.); fluorographic enhancer 'En^{3}Hance' was from New England Nuclear, and Instagel was from Packard.

Methods

^{3}H-labeling of ghosts. This was achieved by the general method of Gahmberg and Andersson [30] modified in the following way: incompletely washed packed ghosts were used as starting material, and the remaining hemoglobin was removed during the subsequent washing procedure. All operations were carried out in 7.14 mM sodium phosphate buffer/1.0 mM EDTA (pH 7.4). Oxidation was performed for 10 min at 0°C in the presence of 2.0 mM sodium metaperiodate and reduction of 1 ml packed ghosts was achieved with 30 μl of an NaB^3H_4 solution (0.48 mCi in 10 mM NaOH). Nonincorporated radioactive material was eliminated by three consecutive washes.

Incubation of ghosts with melittin. Packed ghosts, about 5.0 mg protein/ml, were diluted 10-fold in 0.1 M Tris-HCl (pH 7.4), containing 1.0 mM EDTA or 10 mM $CaCl_2$ when phospholipase A_2 treatment was applied. Unless otherwise stated, incubations were performed for 30 min at 37°C. When Ca^{2+} was added, it was complexed at the end of the incubation by the addition of 0.2 M EDTA (pH 7.4) (1/10 of the incubation volume). Solubilized material was assessed after centrifugation at 4°C either in a Beckman Airfuge (178 000 $\times g$, 35 min) for volumes smaller than 0.2 ml, or in a Beckman L8-80 ultracentrifuge (200 000 $\times g$, 30 min) for larger volumes. In some experiments, ghosts were sequentially incubated with phospholipase A_2 and melittin. The phospholipase-treated ghosts were centrifuged (30 000 $\times g$, 40 min, 4°C), and the pellet was washed once with the incubation buffer containing 1.0 mM EDTA. The pellet, resuspended in the original volume of buffer, was further treated with melittin, as described above.

Phospholipid analysis. Pellets were resuspended by strong magnetic stirring in 1.0 ml distilled water. Lipids were extracted according to Reed et al. [31] and kept in 0.2 ml chloroform/methanol (1 : 1). A 50 μl aliquot was removed for total phosphorus determination [32], and the remaining material was analyzed by two-dimensional thin-layer chromatography according to Broekhuyse [33]. Spots revealed by iodine vapor were scrapped off the plate and analyzed for their phosphorus content [32].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis. This was performed with the modified buffer system of Laemmli [34] applied on a gradient of acrylamide. Slabs of 150 × 150 × 1.5 mm were used. The running gel, 130 mm, consisted of a linear, 12 to 25%, gradient from top to bottom. The ratio of methylenebisacrylamide to acrylamide was 0.55% in the 12% solution and 0.8% in the 25% one. The stacking gel, 10 mm, contained 5.5% total acrylamide, and the ratio of methylenebisacrylamide to acrylamide was 5%. Samples, about 50 μl, were mixed first with 5.0 μl of 0.1 M Tris-HCl/10 mM EDTA/10% SDS/50% sucrose/0.5 mg · ml^{-1} bromophenol blue adjusted to pH 8.0, then with 2.5 μl β-mercaptoethanol and then kept for 5 min at 100°C. Electrophoresis was

performed at a constant current of 20 mA until the dye penetrated the running gel; then the current was increased to 60 mA until the dye reached the bottom. The gels were stained with Coomassie blue G 250. For fluorography, they were treated with the autoradiographic enhancer 'En^{3}Hance', according to the supplier's specifications. Exposures were generally performed for 48 and 96 h. Densitometric scanning of the films was conducted on a Joyce microdensitometer and planimetric integration on a Numonic integrator.

Scintillation counting. Samples (generally 50 μl) were mixed with 2.0 ml of Instagel into 3.0 ml polypropylene tubes and directly counted in a Beckman LS 9000 counter. Gel slices were soaked for 72 h in 2.0 ml of Instagel at room temperature and then counted.

Acetylcholinesterase assay. This was performed at 25°C according to Ellman et al. [35] in 0.1 M sodium phosphate buffer (pH 7.4) containing 0.05% (v/v) Triton X-100. When melittin was present in the samples, they were preequilibrated with Triton X-100 for 1 h at room temperature in 3.0 ml assay solution devoided of acetylthiocholine. Thereafter, 10 μl of 0.3 M acetylthiocholine were added, and the activity was measured.

Density gradient centrifugations. Linear gradients, 5 to 30% sucrose in 0.1 M Tris-HCl (pH 7.4) /1.0 mM EDTA contained either Triton X-100 (0.1% (v/v)) or melittin. Samples up to 150 μl were layered on the gradient, and centrifugations were performed during 16 h in a SW 40 swinging bucket rotor at 38000 rpm and 4°C. Fractions of 250 μl were collected from the bottom at 4°C. The standards used were: horse heart myoglobin (2.04 S), human erythrocyte acetylcholinesterase (6.5 S) and bovine liver catalase (11.2 S). Myoglobin was assessed spetrophotometrically at 410 nm after a 10-fold dilution. Catalase was measured according to the supplier's specification and acetylcholinesterase as described above.

Results and Discussion

Degradation and solubilization of membrane phospholipids

Ghosts were incubated 30 min with melittin (1.0 mM) and/or phospholipase A_2 (0.7 I.U./ml). The insoluble material present in the pellet fraction was analyzed for phospholipids. As shown in Table I, melittin does not promote any degradation of phospholipids even in the presence of Ca^{2+}, thus ruling out any contamination with phospholipase A_2. Melittin is also unable to

TABLE I

PHOSPHOLIPID CONTENT OF GHOSTS

0.5-ml packed ghosts were incubated in the presence of phospholipase A_2 and/or melittin. They were sedimented, and their phospholipid content was extracted and assessed. The major classes of phospholipids are expressed as percentage of total phosphorus.

	Control	0.7 I.U./ml phospholipase	1 mM melittin +0.7 I.U./ml phospholipase	1 mM melittin	Data from Ref. 33
Total phospholipids [a] (μM):	10.3±0.9	11.3±1.1	4.9±0.4	13.7± 3.2	
(nmol):	51.5±4.5	56.5±5.5	24.5±2.0	68.5±16.0	
Major classes of phospholipids (%)					
Sphingomyelin	25.6	30.0	46.8	25.9	25.8
Phosphatidylcholine	31.4	0.7	0.4	31.2	28.3
Lysophosphatidylcholine	–	24.5	30.6	–	1.4
Phosphatidylethanolamine	27.5	0.9	–	32.5	26.7
Lysophosphatidylethanolamine	–	19.0	19.7	–	–
Phosphatidylserine	13.2	6.7	2.5	9.7	12.7
Lysophosphatidylserine	–	–	–	–	–

[a] Total phosphorus extracted corrected for material remaining at the origin of the chromatography.

solubilize any phospholipid. This demonstrates that this peptide does not act on biological membranes like a conventional detergent. As was previously mentioned by others [37], the phospholipase A_2, although it degrades most of the major phospholipids, does not disrupt the bilayer structure of the membrane, and lysoproducts are not released in the medium. If phospholipase A_2 and melittin are applied successively or simultaneously, then a loss of 48% of the lipid-bound phosphorus is observed, indicating a solubilization of lysoproducts. In all experiments, further incubation up to 3 h does not change the results.

Solubilization of proteins

Fig. 1 shows that melittin alone, or together with phospholipase A_2, causes a partial solubilization of the proteins stained as a double faint band in the region of band 4.1. The solubilization of band 6 (glyceraldehyde-3-phosphate dehydrogenase) observed even in the controls, can be attributed to the enhancement in ionic strength encountered by the ghosts in the incubation buffer [38]. The other major proteins are not solubilized. Melittin is observed as a broad spot at the bottom of the gel. The phospholipase A_2 alone does not promote any protein solubilization.

The effect of melittin and/or phospholipase A_2 on ghosts membrane sialoglycoproteins was studied using the periodate-borohydride method of Gahmberg and Anderson [29]. This allowed us to label selectively the proteins displaying a high sialic acid content, i.e., the glycophorin family, leaving the other glycoproteins almost unlabeled. 12% of the total labeling is bound to the glycolipid fraction (data not shown) and is washed off the gels during the processing for fluorography. Melittin induces a concentration-dependent solubilization of sialoglycoproteins (Fig. 2A). When the ghosts are first treated with phospholipase A_2, the consecutive solubilization by melittin is much more effective (Fig. 2B). Under these latter conditions, a maximal effect is already noticed at 0.15 mM melittin. Identical results are observed after simultaneous incubation with phospholipase and melittin.

After treatment with both agents, the sialoglycoproteins were analyzed on sodium dodecyl sulfate-gel electrophoresis. Densitometric scans of the fluorographs show that most of the ^{3}H-labeled

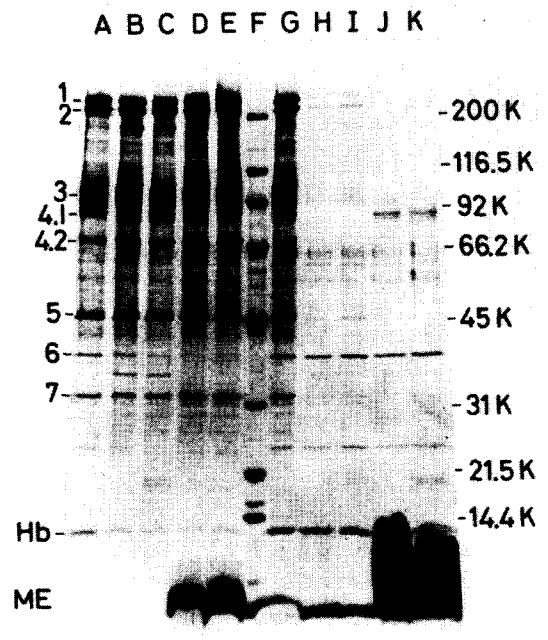

Fig. 1. Sodium dodecyl sulfate gradient-gel electrophoresis of ghost proteins stained with Coomassie blue. Incubation of ghosts with phospholipase A_2 and/or melittin was performed as described in Materials and Methods. The pellets and supernatants were analyzed on polyacrylamide gradient-gels. Ghost proteins are identified in the left margin according to the nomenclature of Steck [36]. Hb, hemoglobin; ME, melittin. Lanes A to E plus G, ghosts; lanes H to K, supernatants; lanes A and G, controls before incubation; lanes B and H, controls after incubation; lanes C and I, samples treated with phospholipase (1 I.U./ml); lanes D and J, samples treated with melittin (1.0 mM); lanes E and K, samples treated with both melittin (1.0 mM) and phospholipase (1.0 I.U./ml); lane F, standards (myosin, 200 kDa; β-galactosidase, 116.5 kDa; phosphorylase B, 92 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa).

sialoglycoproteins are solubilized at 0.1 mM melittin (Fig. 3) and that the solubilized proteins migrate in the band I. (We identified the sialoglycoprotein bands on electrophoresis according to the nomenclature used by Steck for periodic acid-Schiff stained proteins [36], using roman numerals.) This band comigrates with the solubilized 4.1 band observed after Coomassie blue staining. A concomitant decrease in the sedimentable band I and

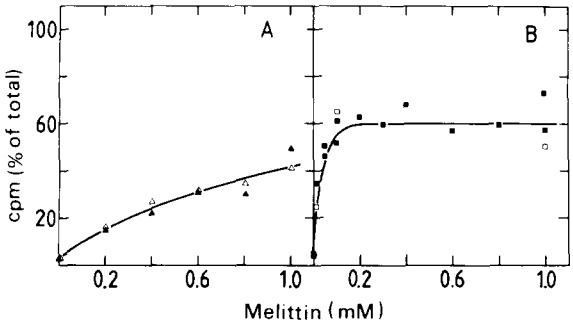

Fig. 2. Solubilization by melittin of ghost sialoglycoproteins. ^{3}H-labeled ghosts were incubated in the presence of increasing concentrations of melittin and centrifuged. Each point represents the counts found in the supernatant. Total counts were at least 15000 cpm (100% value). (A) Ghosts incubated with melittin in the presence of either 10 mM $CaCl_2$ (▲) or 1.0 mM EDTA (△). (B) treatment of ghosts with phospholipase A_2 (1.0 I.U./ml) preceding (■) or simultaneous with (□) melittin addition.

II is observed, and, within experimental errors, the sum of the peak areas corresponding to pellet and supernatant remains constant. Since bands I and II contain glycophorin A dimer and monomer, respectively [38], our results indicate a dimerization of glycophorin A during its solubilization. Such an aggregation should not be interpreted as a direct effect of melittin, and may occur later after the solubilization step. Band II and III proteins are only partially solubilized. Since they contain glycophorin B [38], we conclude that melittin and phospholipase A_2 partially solubilize glycophorin B, whereas glycophorin A is totally solubilized. The data on the band IV protein are difficult to interprete, due to the low proportion of this material.

Solubilization of the membrane acetylcholinesterase

The solubilizing effect of melittin was tested on acetylcholinesterase, an integral protein of the membrane. Fig. 4 shows that melittin solubilizes this enzyme in the same range of concentrations as those that are effective for the solubilization of the sialoglycoproteins. However it exhibits a different concentration dependency. Below 0.2 mM melittin, the release of acetylcholinesterase is inferior to 5%, whereas under those conditions, 28% of the glycophorin A is in the soluble fraction. When higher concentrations of melittin are used, the

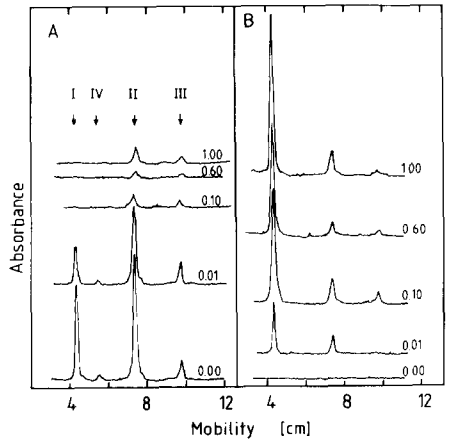

Fig. 3. Densitometric scans of fluorographs obtained after sodium dodecyl sulfate gradient-gel electrophoreses of ^{3}H-labeled ghost sialoglycoproteins. ^{3}H-labeled membranes were first treated by phospholipase A_2 (1 I.U./ml), then incubated with increasing concentrations of melittin and centrifuged. (A) Pellets; (B) supernatants. The gels were exposed for 48 h. Arabic numbers indicate the millimolar concentration of melittin; roman numbers relate to the protein pattern defined by the periodic acid-Schiff nomenclature of Steck [36].

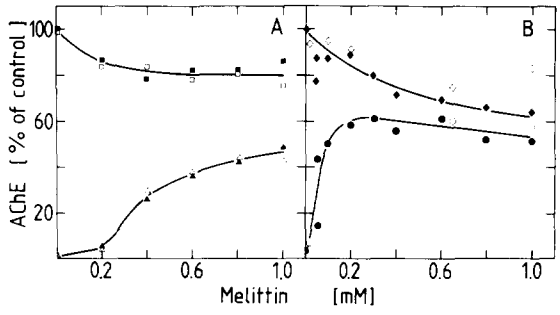

Fig. 4. Ghost acetylcholinesterase solubilization by melittin. Ghosts were incubated with increasing concentrations of melittin, centrifuged and assayed for acetylcholinesterase activity in the presence of 0.05% (v/v) Triton X-100. Values are expressed as the percentages of the activity of the samples devoid of melittin. (A) Ghosts incubated with melittin in the presence of either 10 mM $CaCl_2$ (■, ▲) or 0.1 mM EDTA (□, △). Activity in the supernatant, △ and ▲. Activity in the ghost suspension, □ and ■. (B) Ghosts incubated with melittin after treatment with phospholipase A_2 (1 I.U./ml). Activity in the supernatant, ○ and ●. Activity in the ghost suspension, ◇ and ◆. Closed symbols (● and ◆) are related to sequential treatment by phospholipase A_2 and melittin; open symbols (○ and ◇) refer to simultaneous treatment with these two reagents.

acetylcholinesterase solubilization is more pronounced and becomes comparable to that of glycophorin. This irregular behavior may be due to the fact that melittin has to reach a critical concentration within the lipid bilayer in order to destabilize the interaction of the enzyme with its hydrophobic surrounding, may it be lipids or proteins. The absence of parallelism between the extraction curve of those proteins suggests that no interaction exists between them in the membrane. The solubilization of the acetylcholinesterase is enhanced when the membrane is first digested with phospholipase A_2 (Fig. 4B). A maximal solubilization is reached at 0.2 mM melittin. Those experiments also show that the total enzyme activity decreases along with its solubilization, the soluble form being unstable.

Since acetylcholinesterase exists in different states of aggregation, the molecular forms of the melittin-induced solubilized enzyme were studied, using purified human erythrocyte acetylcholinesterase. The enzyme was incubated for 30 min at 37°C in the presence of 1.0 mM melittin and analyzed on gradients of sucrose containing fixed concentrations of melittin throughout the gradient (Fig. 5). At high concentrations of melittin (over 0.1 mM) the enzyme sediments at 8 S as a single species. This indicates that melittin dissociates the high-molecular-weight aggregates of the enzyme preparation [27]. The decrease of the melittin concentration to 0.01 mM in the gradient reveals a partial aggregation (main peak at about 11 S; a tetrameric form of the enzyme would sediment at 10 to 11 S [44]) with a significant loss of activity. Triton X-100 in the gradient changes the 8 S value due to melittin into 6.5 S, a value which corresponds to that of the disulfide-linked dimer [26]. From those results we conclude that acetylcholinesterase is solubilized in a dimeric state with additional melittin molecules bound, stabilizing the enzyme. When the acetylcholinesterase is directly solubilized from the membrane by melittin and loaded onto the gradients, similar results are obtained, although the resolution and homogeneities are lower, due to low enzyme recoveries and possible interactions with contaminating membrane constituents.

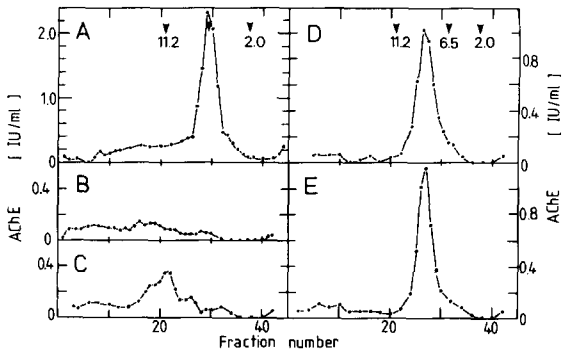

Fig. 5. Sucrose density gradient centrifugation of purified acetylcholinesterase. The enzyme was incubated for 30 min at 37°C in 0.1 M Tris-HCl/1.0 mM EDTA (pH 7.4) in the presence of 1.0 mM melittin and 2.5 to 2.8 I.U. were applied onto 5 to 30% sucrose gradients containing (A) 0.1% (v/v) Triton X-100; (B) buffer only; (C) 0.01 mM melittin; (D) 0.1 mM melittin; (E) 0.5 mM melittin. Arrows indicate the positions of the standards in 0.1% (v/v) Triton X-100: catalase, 11.2 S; pure erythrocyte membrane acetylcholinesterase, 6.5 S; horse heart myoglobin, 2.0 S.

Effect of melittin on the acetylcholinesterase activity

Since melittin extraction decreases the activity of acetylcholinesterase, we studied further this modulation, using the purified enzyme. In preliminary experiments, we observed that melittin inhibits slightly and reversibly the acetylcholinesterase, and that dilution of the sample into an excess of Triton X-100 reactivates the enzyme within several minutes (data not shown). Accordingly, in order to look at the irreversible inactivation, we took aliquots of the melittin-acetylcholinesterase incubate at the indicated time and diluted it in Triton X-100, as described in Materials and Methods. Under these conditions the detergent prevents any further inactivation and relieves the reversible inhibition. At 37°C, a time-dependent inactivation is observed in the presence of 1.0 mM melittin with an apparent half-inactivation time of 80 min (Fig. 6). The kinetics of inactivation exhibit a significant deviation from a first-order law and is independent of the melittin concentration within the range of 0.01 to 1.0 mM. This inactivation is, however, strongly dependent on the temperature, and no significant loss of activity can be observed at 4°C during several hours (data not shown).

The addition of 0.1% Triton to an incubation

medium containing 1.0 mM melittin does not affect the inactivation rate. However, when the detergent is raised to 1.0%, the inactivation is immediately interrupted (Fig. 6). The acetylcholinesterase, in the presence of 0.1% Triton X-100, with or without melittin at low concentrations (less than 0.1 mM), remains fully active. Those experiments may be interpreted in the following way. The inactivation is caused by the dissociation of melittin from the hydrophobic anchoring site of the enzyme. It is well known that this site must be occupied by amphipathic compounds in order to sustain the catalytic activity, and that the removal of the amphiphile induces an inactivation within seconds [25,26]. In this case, it seems that the inactivation rate of the enzyme not interacting with melittin is much greater than its association rate with melittin. Triton X-100 prevents the denaturation by replacing rapidly melittin on the same site.

The fact that Triton X-100 must be far above its critical micellar concentration (0.02%) in order to protect the enzyme in the presence of high concentration of melittin, is due to the high affinity of melittin for detergents [40,41]. When the molar ratio of Triton X-100 to melittin is close to 1, most of the molecules of detergent bind to the peptide and there is not enough free detergent for stabilizing the acetylcholinesterase (at 0.1% (v/v) Triton X-100 and 1.0 mM melittin, the average molar ratio of these two substances is about 1.5.).

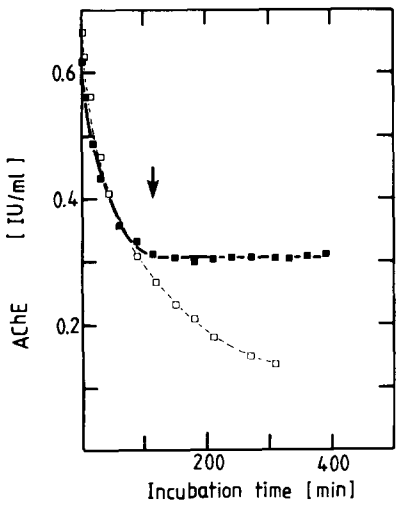

Fig. 6. Time-course of the melittin-induced inactivation of acetylcholinesterase. Purified acetylcholinesterase was diluted to 60 I.U./ml in 0.1 M Tris-HCl (pH 7.4)/1 mM EDTA/1 mM melittin and preincubated (30 min, 37°C). At zero time it was diluted 100-fold within the same buffer containing either 1 mM melittin (□) or 1 mM melittin plus 0.1% (v/v) Triton X-100 (■). At the time point indicated by the arrow, Triton X-100 was increased to a final concentration of 1.0% (v/v). In order to measure only the irreversible inactivation, the enzyme activity was assayed after 60 min preequilibration in 0.05% (v/v) Triton X-100.

Although the binding of melittin is likely to be hydrophobic, electrostatic interactions cannot be excluded, since acetylcholinesterase has a rather acidic isoelectric point [42]. To the present knowledge, neither acetylcholinesterase nor glycophorin A do show any interaction with the cytoskeleton, whereas the other membrane proteins herein studied are associated with the spectrin-actin network [43]. The release of both of those proteins may indicate that melittin perturbs the membrane without affecting the architecture of the cytoskeleton nor extracting any lipid into micelles, and allows the release of those proteins that are free to diffuse within the plane of the membrane. Its mechanism of action is still unknown and may involve both a complexation of the lipids within the membrane-releasing proteins and a direct interaction with those proteins. From this study, it follows that melittin, used as a solubilizing agent, displays more selective properties than a conventional detergent.

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References

1 Schoch, P. and Sargent, D.F. (1980) Biochim. Biophys. Acta 602, 234–247
2 Kempf, C., Klausner, R.D., Weinstein, J.N., Van Renswoude, J., Pincus M. and Blumenthal, R. (1982) J. Biol. Chem. 257, 2469–2476

3 Drake, A.F. and Hider, R.C. (1979) Biochim. Biophys. Acta 555, 371–373
4 Brown, L.R. and Wüthrich, K. (1981) Biochim. Biophys. Acta 647, 95–111
5 Brown, L.R., Braun, W., Kumar, A. and Wüthrich, K. (1982) Biophys. J. 37, 319–328
6 Terwilliger, T.C. and Eisenberg, D. (1982) J. Biol. Chem. 257, 6016–6022
7 Terwilliger, T.C., Weissman, L. and Eisenberg, D. (1982) Biophys. J. 37, 353–361
8 Lauterwein, J., Brown, L.R. and Wüthrich, K. (1980) Biochim. Biophys. Acta 622, 219–230
9 Brown, L.R., Lauterwein, J. and Wüthrich, K. (1980) Biochim. Biophys. Acta 622, 231–244
10 Sessa, G., Freek, J.H., Colacicco, G., and Weissman, G. (1969) J. Biol. Chem. 244, 3575–3582
11 Habermann, E. and Zeuner, G. (1971) Naunyn-Schmiedeberg's Arch. Pharmacol. 270, 1–9
12 Weissmann, G., Hirschhorn, R. and Krakauer, K. (1969) Biochem. Pharmacol. 18, 1771–1775
13 Fredholm, B. and Haegemark, O. (1967) Acta Physiol. Scand. 71, 357–367
14 Pik, J.R. and Lawford, H.G. (1980) Can. J. Biochem. 58, 996–1003
15 Shimada, H., Terayama, H., Fujjiwara, A. and Yasumasu, I. (1982) Develop. Growth Differ. 24, 7–16
16 Lad, P.L., and Shier, W.T. (1980) Arch. Biochem. Biophys. 204, 418–424
17 Lin, S.Y.S., Huang, M.C., Tseng, W.C. and Lee, C.Y. (1975) Naunyn-Schmiedberg Arch. Pharmacol. 287, 349–358
18 Lad, P.J. and White, A.A. (1979) Biochim. Biophys. Acta 570, 198–209
19 Bishop, D.G. and Kenrick, J.R. (1980) Biochem. Biophys. Res. Commun. 97, 1082–1090
20 Yunes, R., Goldhammer, A.R., Garmer, W.K. and Cordes, E.H. (1977) Arch. Biochem. Biophys. 183, 105–112
21 Mollay, C. and Kreil, G. (1974) FEBS Lett. 46, 141–144
22 Vogt, W., Patzer, P., Lege, L., Oldigs, H.D. and Wille, G. (1970) Naunyn-Schmiedeberg's Arch. Pharmacol. 265, 442–454
23 Rüegg, U.T., Cuénoud, S., Fulpius, B. and Simon, E.J. (1982) Biochim. Biophys. Acta 685, 241–248
24 Maulet, Y., Brodbeck, U. and Fulpius, B.W. (1982) Anal. Biochem. 127, 61–67
25 Frenkel, E.J., Roelofsen, B., Brodbeck, U., Van Deenen, L.L.M., and Ott, P. (1980) Eur. J. Biochem. 109, 377–382
26 Wiedmer, T., Di Francesco, C. and Brodbeck, U. (1979) Eur. J. Biochem. 102, 59–64
27 Brodbeck, U., Gentinetta, R. and Ott, P. (1981) in Membrane proteins (Azzi, A. et al., eds.), pp. 85–96, Springer-Verlag, Berlin, New York
28 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1973) Arch. Biochem. Biophys. 100, 119–130
29 Nieuwenhuisen, W., Kunze, H. and De Haas, G.H. (1974) Methods Enzymol. 32, 147–154
30 Gahmberg, C.G., and Andersson, L.C., (1977) J. Biol. Chem. 252, 5888–5894
31 Reed, C.F., Swisher, S.N., Marinetti, G.V. and Eden, E.G. (1960) J. Lab. Clin. Med. 56, 281–289
32 Rouser, G., Fleischer, S. and Yamamoto, A. (1970) Lipids 5, 494–496
33 Broekhuyse, R.M. (1969) Clin. Chim. Acta 23, 457–461
34 Laemmli, U.K. and Favre, M. (1973) J. Mol. Biol. 80, 575–599
35 Ellman, G.L., Courtney, D.K., Andres, V. and Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88–95
36 Steck, T.L. (1974) J. Cell Biol. 62, 1–19
37 Van Meer, G., De Kruijff, B., Op den Kamp, J.A.F. and Van Deenen, L.L.M. (1980) Biochim. Biophys. Acta 596, 1–9
38 Kliman, H.J. and Steck, T.L. (1980) J. Biol. Chem. 255, 6314–6321
39 Marchesi, V.T. (1979) Seminars Hematol. 16, 3–20
40 Knöppel, E., Eisenberg, F. and Wickner, W. (1979) Biochemistry 18, 4177–4181
41 Maulet, Y. and Cox, J. (1983) Biochemistry 22, 5680–5686
42 Ott, P., Jenny, B. and Brodbeck, U. (1975) Eur. J. Biochem. 57, 469–480
43 Lux, S.E. (1979) Seminars Hematol. 16, 21–51