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Divalent cation-sensitive pores formed by natural and synthetic melittin and by Triton X-100

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Leakage of ions and low-molecular-weight metabolites from Lettre cells is induced by synthetic melittin, as effectively as by melittin isolated from bee venom; in each case leakage is inhibited by Ca^{2+}, Zn^{2+} or H^+. Inhibition of leakage by divalent cations is reversible in that Lettre cells incubated with melittin (or with Triton X-100) in the presence of inhibitory amounts of Zn^{2+}, when freed of Zn^{2+} by EGTA or by centrifugation, begin to leak (in Zn^{2+}-sensitive manner). Electrorotation of Lettre cells is altered by melittin, compatible with membrane permeabilization; melittin plus Zn^{2+} does not alter electrorotation until Zn^{2+} (and unbound melittin) are removed. Melittin or Triton X-100 added to calcein-loaded liposomes induces leakage of calcein; divalent cations inhibit. Energy transfer between liposome-associated melittin and 2-, 7- or 12-(9-anthroyloxy)stearate (AS) is maximal with 12-AS; addition of Zn^{2+} has little effect. Circular dichroism spectra of melittin plus liposomes are unaffected by Zn^{2+}. These results show that the formation of divalent cation-sensitive pores is not dependent on the presence of endogenous membrane proteins and that the action of divalent cations is not by displacement of melittin (or Triton) from the lipid bilayer.

Introduction

The haemolytic polypeptide melittin is the cause of the pain associated with bee stings [1]. Its relatively simple structure (a cationic polypeptide of 26 amino acids [2]) coupled with the fact that it induces the formation of pores when added to vesicles [3] or bilayers [4] consisting solely of phospholipids, has made it a favourite model for studying the interactions of pore-forming proteins with membranes (e.g., see Ref. 5). As yet, though, the suggestion that the wedge-shaped structures [6,7] formed when melittin interacts with membranes are able to rearrange to form protein-lined aqueous channels [8] similar to those seen with *Staphylococcus aureus* α-toxin [9,10] or the membrane-attack complex of complement [11,12], is by no means proven. Moreover, the detergent Triton X-100, at sublytic concentrations, forms pores in cells that functionally resemble those formed by melittin in several regards [13]. In this case, formation of protein-lined channels cannot account for the leakiness of cells, unless the detergent induces redistribution of endogenous membrane proteins. The lesions induced by various pore-formers are inhibited, characteristically, by divalent cations [13] or by protons [14]. Bee venom from which melittin is isolated contains additional components including phospholipase A_2 [15], which may contribute to the pore-forming action, and Zn^{2+} or H^+ may inhibit this enzyme, which is known to require Ca^{2+} for maximal activity [16]. Here we show that the lesions induced in Lettre cells by synthetic melittin [17] are as sensitive to Ca^{2+}, Zn^{2+} or H^+ as those induced by melittin isolated from natural sources.

The nature of the protective effect of divalent cations (or protons) on melittin- or Triton-induced lesions is not clear. One possibility is that divalent cations (which are unlikely to bind either to melittin or to Triton) displace melittin and triton from the surface of susceptible cells. The present demonstration, that potentially functional lesions can be induced in Lettre cells by

Abbreviations: 2-, 7- or 12-AS, 2-, 7- or 12-(9-anthroyloxy)stearic acid; CD, circular dichroism; DMSO, dimethyl sulphoxide.

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melittin or Triton in the presence of inhibitory concentrations of Zn^{2+}, rules this out.

The action of divalent cations on melittin-induced lesions can be assessed directly in studies of the electrorotation of cells [18,19]. Cells or vesicles rotate when placed in an appropriate electric field; if the insulating properties of the bounding membrane are breached by a pore-forming agent, optimum field frequency for rotation increases [20]. Here we show that treatment of Lettre cells with melittin alters electrorotation in just such manner, that this effect of melittin can be prevented by Zn^{2+}, and that removal of Zn^{2+} restores the altered electrorotation.

In cells melittin or Triton may cause endogenous proteins to aggregate in such a way as to form a pore [21,22], and divalent cations could interfere with that process. Here we report the ability of melittin and Triton to form divalent cation-sensitive lesions in purely lipidic systems (liposomes); in the case of melittin we have recorded: (i) the ability of its Trp-19 to undergo resonance energy transfer with anthracene-derivatives [23] by measuring the effectiveness of transfer to an anthroyl group anchored at position 2, 7 or 12 of a stearic acid molecule; and (ii) circular dichroism (CD) spectra in the presence and absence of phospholipid and Zn^{2+}. The results show that the lesions formed by melittin in liposomes can indeed be prevented by divalent cations without displacement of melittin from the liposome. The characteristic CD spectrum of lipid-associated melittin [24] is unaffected by the presence of Zn^{2+}. Some of these results have been presented in brief at meetings [25,26].

Methods and Materials

Leakage from cells

Lettre cells grown intraperitoneally as an ascitic suspension in Swiss white mice, were harvested in 150 mM NaCl, 5 mM KCl, 5 mM Hepes, 1 mM $MgSO_4$, pH adjusted to 7.4 at 22°C with NaOH (Hepes-buffered saline, HBS) containing heparin and washed and resuspended in a similar medium without heparin. 20% v/v cells in HBS were incubated at 37°C with [^{3}H]choline for 30–45 min in order to label the intracellular phosphorylcholine pool [27]. Labelled cells were washed with HBS and stored as 20% v/v suspensions in HBS at room temperature prior to use. Labelled cells ($(3–5)\cdot 10^6$/ml) were incubated in HBS, or a similar medium in which phosphate/citrate buffers (1 : 20 dilution of McIlvaine buffers [28]) replaced Hepes, with melittin or Triton in the absence or presence of $CaCl_2$ or $ZnSO_4$ at the pH specified. Leakage of monovalent cations [29] or phosphoryl[^{3}H]choline [30] was assessed after pelleting cells through oil. On occasion, cells with melittin or Triton were incubated in the presence of an inhibiting concentration of Zn^{2+} and leakage was assessed after pelleting cells and resuspending them in fresh medium in the absence of additional melittin or Zn^{2+}.

Leakage of calcein from liposomes

Liposomes containing calcein were prepared by sonicating 12.5 mg lipid/ml in 80 mM calcein, 50 mM NaCl, pH adjusted to 7 with NaOH. Either asolectin (Sigma) purified as described by Kagawa and Racker [31] or a 1/1 (w/w) mixture of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC; Avanti Polar Lipids) and cholesterol (Calbiochem) was used to form calcein-containing liposomes. The sonicate was applied to a column of Sephadex G50 and eluted with 160 mM NaCl, 10 mM Hepes, pH adjusted to 7 with NaOH; the fractions used for leakage experiments contained more than 80% entrapped calcein. Fractionated liposomes (5–10 μg lipid/ml) were incubated in the elution buffer at room temperature. Calcein fluorescence was excited at 490 nm (10 nm slitwidth) and monitored at 520 nm (10 nm slitwidth) with a Perkin Elmer MPF-44A spectrofluorimeter; maximal leakage at the end of each experiment was assessed by lysis with 0.025% Triton X-100 (final concentration).

Electrorotation

Experimental. ^{3}H-labelled cells (approx. $5\cdot 10^6$/ml) were incubated in HBS at 37°C for 3 min without or with melittin in the absence or presence of 0.1 mM $ZnSO_4$. Pelleted cells ($300\times g\times 3$ min, MSE Chilspin) were resuspended in 280 mM inositol containing sufficient Hepes buffer (added from 0.1 M Hepes, pH adjusted to 7 with KOH) and, when required, 0.1 mM $ZnSO_4$, to give a conductivity close to 30 μS/cm (resistivity 33.3 kΩ · cm). Rotation was carried out at room temperature (21–25°C) in a four-electrode chamber [19,20]. For each cell the in-chamber conductivity was read from a conductometer attached to two of the electrodes, and the cell diameter read from an ocular micrometer on the microscope (a 100 × oil-immersion objective and interference contrast were used).

f_c measurement and its evaluation. We measured that frequency (the f_c) of rotating field that gave fastest rotation. The measurement used a switched contra-rotating field [19,32] of strength 120 V/cm. In some cases rotation speed in response to a field of frequency f_c and strength 68 V/cm was also measured.

The f_c (in kHz) is inversely proportional ($2\pi\cdot\tau = 1/f_c$) to the cell membrane-charging time constant (τ, in ms), which increases with the resistivities of the fluids inside and outside the cell membrane (ρ_i and ρ_a in kΩ · cm) and with the cell radius a (cm). In terms of the surface properties of the cell:

$$f_c\cdot C_m\cdot\pi = G_m/2+[a(2\rho_i+\rho_a)]^{-1}+K_s/a^2 \qquad (1)$$

where C_m (in $\mu F/cm^2$), G_m (in mS/cm^2) are the area-specific membrane capacity and conductivity, respectively, and K_s (in mS) is the surface conductance (Eqn. 1 is Eqn. 11 of Ref. 19 generalised to include the case that ρ_i may be significant compared to ρ_a, see also Ref. 33). As melittin permeabilises cells, it should affect G_m rather than K_s. We therefore neglect K_s in the following discussion of melittin-induced changes in f_c. The dependence of f_c on cell size (see Fig. 5 below) also indicates that K_s has at most a minor influence on the f_c values.

As long as the internal resistivity, ρ_i, of the cells remains much lower than that of the medium, ρ_a, then the factor $2\rho_i + \rho_a$ remains constant (approx. ρ_a); if the cell radius remains constant, the increase ΔG_m that caused an observed increase Δf_c can be deduced from:

$$\Delta G_m = 2\pi \cdot C_m \cdot \Delta f_c \quad (2)$$

Should melittin cause so much ion loss to the rotation medium that ρ_i approaches ρ_a, then Eqn. 1 shows that f_c will be lower than otherwise produced by a given G_m, so that Eqn. 2 will underestimate G_m. In extreme cases ($\rho_i = \rho_a$), the f_c may even be lower than that of control cells. However, this effect is unlikely to cause damaged cells to be mistaken for unaffected cells, because heavily permeabilised membrane systems show very weak rotation [20].

There is a further reason why Eqn. 2 may underestimate an increase in G_m caused by melittin. The large dipole moment of the peptide bond [34] should cause membrane-dissolved peptides to raise the permittivity, and therefore the C_m, of the membrane. However, even if C_m were increased many-fold, the decrease in the f_c values would be small compared to the large f_c increase seen in treated cells.

Energy transfer measurements

Liposomes without calcein were prepared by sonicating POPC (10 mg/ml) in 160 mM NaCl, 10 mM Hepes, pH adjusted to 7 with NaOH. Sonicated liposomes (0.5 mg/ml) were incubated in a similar medium without or with 2-, 7- or 12-(9-anthroyloxy)stearic acid (2-AS, 7-AS, 12-AS), or with both melittin and 2-, 7- or 12-AS. Fluorescence was excited at 280 nm (10 nm slitwidth) and emission spectra (10 nm slitwidth) were recorded in the ratio mode using a Perkin Elmer MPF-44A spectrofluorimeter. Emission intensity was quantitated by cutting and weighing traced spectra. 2-, 7- or 12-AS intensities were normalised by comparing fluorescence at 440 nm excited at 370 nm. This procedure corrects for differential binding of 2-, 7- or 12-AS to liposomes in the absence or presence of melittin. In no case did the correction factor exceed 10%. Energy transfer is defined as the relative loss of melittin fluorescence produced by the presence of 2-, 7- or 12-AS or the (numerically equal) relative gain of 2-, 7- or 12-AS fluorescence in the presence of melittin, expressed as percentage of the total melittin and 2-, 7- or 12-AS fluorescence.

Circular dichroism measurements

CD spectra were recorded at room temperature with a Jasco J600 spectropolarimeter.

Materials

Melittin prepared from bee venom [35], essentially free of phospholipase, was donated by Dr. R.C. Hider, King's College, London. Synthetic melittin [17] was donated by Dr. M. Tosteson, Department of Physiology and Biophysics, Harvard Medical School. Unless stated otherwise, melittin from bee venom was used throughout. Triton X-100 was from BDH. [^{3}H]Choline was from Amersham International, Bucks. 2-, 7- and 12-AS were from Molecular Probes, Eugene, OR, U.S.A.

Results

Leakage induced by synthetic melittin is inhibited by divalent cations and protons

Synthetic melittin is as active as melittin isolated from natural sources at inducing the leakage of cations and phosphorylated metabolites across the plasma membrane of Lettre cells. Thus, $3 \cdot 10^{-7}$ M and 10^{-6} M synthetic melittin induce leakage of cations, and 10^{-6} M induces leakage of phosphoryl[^{3}H]choline as well (Fig. 1); this may be compared with extensive cation leakage at $5 \cdot 10^{-7}$ M, and extensive phosphoryl[^{3}H] choline leakage at 10^{-6} M melittin isolated from bee

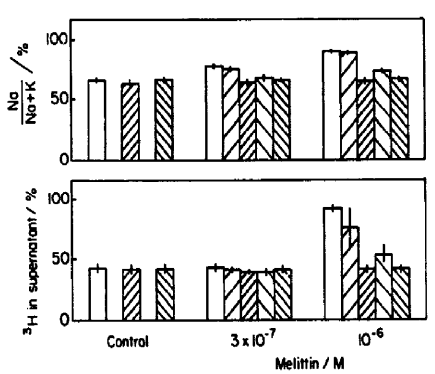

Fig. 1. Inhibition by Ca^{2+} and Zn^{2+} of leakage from Lettre cells induced by synthetic melittin. $5 \cdot 10^6$ Lettre cells/ml, prelabelled with [^{3}H]choline, were incubated in HBS at 37°C in the absence (control) or presence of melittin without (□) or with 10^{-3} M $CaCl_2$ (▨), or 10^{-2} M $CaCl_2$ (▨), or 10^{-4} M $ZnSO_4$ (▧), or 10^{-3} M $ZnSO_4$ (▧). Leakage of ^{3}H and intracellular cation content were determined in quadruplicate over a 13 min time course after pelleting aliquots of the cell suspensions through oil. Bars indicate the standard deviation of the quadruplicate determinations.

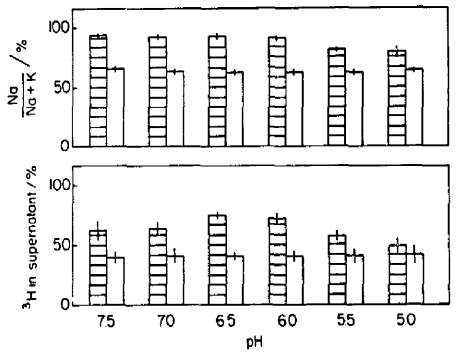

Fig. 2. Inhibition by H^+ of leakage of Lettre cells induced by synthetic melittin. $5 \cdot 10^6$ Lettre cells/ml, prelabelled with [^{3}H]choline, were incubated in isotonic saline buffered with phosphate/citrate at 37 °C without (□) or with (▤) $3 \cdot 10^{-7}$ M melittin. Leakage of ^{3}H and intracellular cation content were determined in triplicate over a 12 min time-course after pelleting aliquots of the cell suspensions through oil. Bars indicate the standard deviation of the triplicate determinations.

venom [13]. $5 \cdot 10^{-7}$ M melittin from bee venom (Fig. 3) induces extensive phosphoryl[^{3}H]choline leakage when incubated with reduced numbers of Lettre cells. We conclude that the potency of synthetic melittin is similar to that of melittin from bee venom. Leakage induced by the latter is inhibited by divalent cations and protons [13,14]. Figs. 1 and 2 show that leakage induced by synthetic melittin is similarly sensitive to inhibition by divalent cations and protons, the efficacy being $H^+ > Zn^{2+} > Ca^{2+}$ for synthetic, as for natural melittin (cf. Figs. 1 and 2 with panel B of the Appendix to Ref. 14).

Inhibition by divalent cations is reversible

Lettre cells exposed to melittin in the presence of an inhibitory concentration of divalent cation begin to leak phosphorylated metabolites if EGTA is added. Fig. 3, panel A illustrates such an experiment. The result indicates that inhibition of leakage by Zn^{2+} is reversible. However, it could be argued that Zn^{2+} acts to displace melittin from the surface of Lettre cells, and that in the absence of Zn^{2+} (achieved by chelation with EGTA), melittin rebinds to cells. In the experiment shown in Fig. 3, panel B, in which the cells are removed from the Zn^{2+} by centrifugation, leakage is again initiated. Since in this case any melittin displaced by the Zn^{2+} is removed as well, the result indicates that Zn^{2+} does not displace melittin, but rather prevents leakage through an existing pore. That Zn^{2+} can block a preformed pore is indicated by the fact that if cells that have been pre-treated with melittin and Zn^{2+} are suspended with Zn^{2+}, leakage is inhibited (Fig. 3, panel B). Subsequent addition of EGTA to such cells (i.e., with no free melittin present) initiates leakage.

Triton X-100 at sublytic concentration, like melittin, induces leakage from Lettre cells that is inhibited by divalent cations [13]. In this case, also, inhibition is reversible, since it can be initiated by removal of Zn^{2+} through chelation with EGTA (Fig. 4, panel A) or by centrifugation of cells (Fig. 4, panel B).

Electrorotation of Lettre cells is altered by melittin in a divalent cation-sensitive fashion

The consequences of melittin damage that should be detectable using electrorotation are an increase in membrane permeability and, possibly, a loss of cellular ionic content (to the low ionic strength rotation medium).

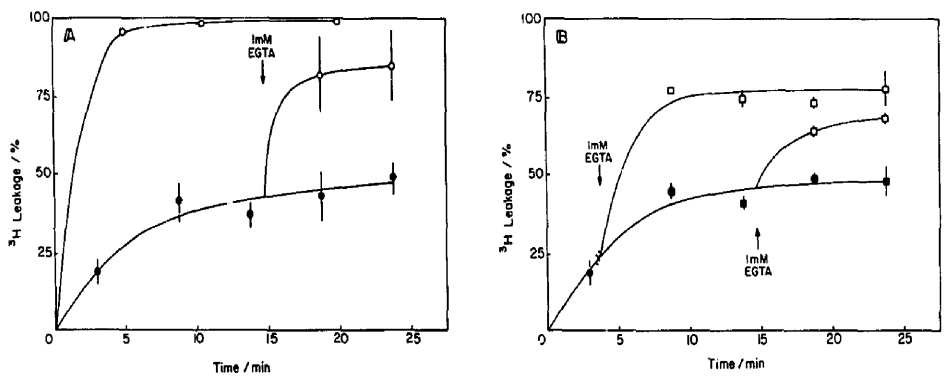

Fig. 3. Reversal of Zn-inhibited, melittin-induced leakage of ^{3}H from Lettre cells. (A) $4 \cdot 10^6$ Lettre cells/ml, prelabelled with [^{3}H]choline, were incubated in HBS at 37 °C containing $5 \cdot 10^{-7}$ M melittin in the absence (○) or presence (●) of $3 \cdot 10^{-5}$ M $ZnSO_4$. EGTA, at the final concentration indicated, was added to a portion of the Zn-treated cells after 15 min. (B) $4 \cdot 10^6$ Lettre cells/ml, prelabelled with [^{3}H]choline, were incubated in HBS containing $3 \cdot 10^{-5}$ M $ZnSO_4$ and $5 \cdot 10^{-7}$ M melittin for 3 min at 37 °C. The cells were pelleted and resuspended in a similar medium in the absence of melittin with (□) or without (■) EGTA at the final concentration indicated. EGTA was added at the time of resuspension or 10 min later. Leakage was measured after pelleting the cells and has been scaled such that leakage from control cells not treated with melittin (30–50% of ^{3}H in supernatant) has a value of 0. Bars indicate the range of duplicate observations.

Increase of membrane conductivity will shorten the membrane-charging time constant, and, therefore, increase the optimum field frequency required for rotation (the f_c, see Eqns. 1 and 2). Fig. 5 shows that the f_c values of some of the cells treated with $(0.33–1) \cdot 10^{-6}$ M melittin are indeed much higher than control f_c values. This holds over the whole range of cell sizes, but note that melittin-treated cells were somewhat larger than controls, presumably due to cell swelling [30]. The scatter of the treated cell f_c values is very large, reflecting a comparable variability of individual cell sensitivity to this range of melittin concentrations.

The increase in f_c caused by melittin occurred at concentrations above a well-defined threshold [30], the absolute level of which varied somewhat between batches of cells. Table I shows data obtained from a single preparation of cells on 1 day: Cells treated with $5.5 \cdot 10^{-7}$ M melittin showed no significant increase in f_c compared with controls; however, $1.1 \cdot 10^{-6}$ M melittin gave large increases in both mean value and scatter of the f_c values (cf Fig. 5). On another occasion (Table II), $3.3 \cdot 10^{-7}$ M melittin was sufficient to increase the mean f_c of Lettre cells from 6.3 to 12.8 kHz; however, no such increase was observed if the cells were treated with melittin in the presence of 0.1 mM $ZnSO_4$ or if the treated cells were assessed in a rotation medium containing 0.1 mM $ZnSO_4$. Rotation of control cells (treated with DMSO without melittin) was not significantly affected by 0.1 mM $ZnSO_4$.

The changes in f_c run broadly parallel, with respect to concentration-dependence and to sensitivity to Zn^{2+}, to the changes in membrane potential, cation and metabolite leakage induced by melittin (Table II). The slightly different thresholds for these differing manifestations of membrane damage allow deductions to be made as to the cause of the large increase in G_m implied by the changes in f_c.

The concentrations of melittin applied to the treated cells in Fig. 5 gave membrane depolarisations > 90%, leakage of cellular cations of 50–80% and phosphoryl [^{3}H]choline leakage of 12–80%. The data in Tables I and II indicate that extensive depolarisation and cation leakage can occur even when the melittin concentration is insufficient to cause large changes in f_c. We conclude that the f_c increase is caused by a large G_m increase associated with the existence of 'holes' large enough to permit the passage of phosphorylcholine, and that the scatter in the f_c data (Fig. 5, Tables I and II) reflects a large scatter in the leakage of phosphorylcholine (with mean values as low as 12%) at a threshold melittin dosage.

The maximum melittin-induced increase in f_c was 18 kHz (Fig. 5). Use of Eqn. 2 shows that this implies that increases in G_m of up to 300 mS/cm^2 occurred. The membrane capacitance is required for this calculation: measurements of Lettre cell f_c at several conductivities (Arnold, Bashford, Pasternak and Zimmermann, unpublished data) gave a mean C_m of 2.75 μF/cm^2. Strictly, Eqn. 2 is applicable only when the cell radius stays constant. However, the small increase in cell radius after melittin treatment (see Fig. 5) is insignificant compared to the large f_c increases considered here.

The rotation of treated cells, even those with very high f_c values, appeared to be as fast (0.5–0.8 rev/s) as that of control cells (0.6–0.7 rev/s). However, rotation speed measurements are relatively inaccurate [19,32], so

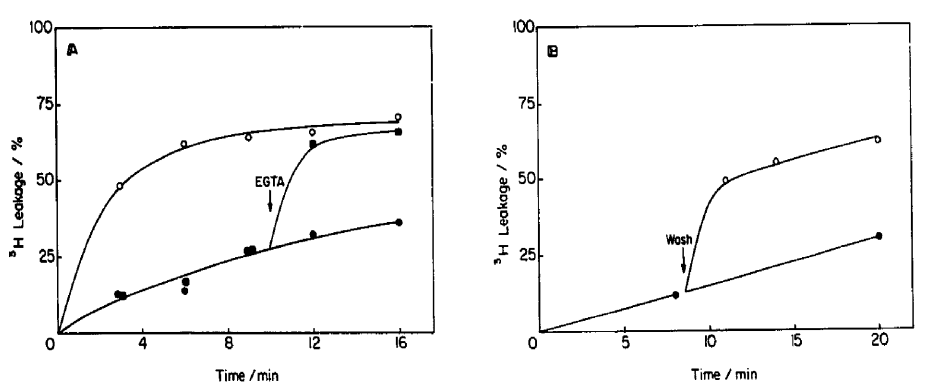

Fig. 4. Reversal of Zn-inhibited Triton-induced leakage of ^{3}H from Lettre cells. (A) $5 \cdot 10^6$ Lettre cells/ml, prelabelled with [^{3}H]choline were incubated in HBS containing 0.1% albumin at 37°C with 0.005% (w/w) Triton X-100 in the absence (○) or presence (●, ■) of $4 \cdot 10^{-4}$ M $ZnSO_4$; after 10 min 10^{-2} M EGTA (final concentration) was added to one of the incubations containing $ZnSO_4$ (■) as indicated. (B) $3 \cdot 10^6$ Lettre cells/ml, prelabelled with [^{3}H]choline were incubated in HBS containing 0.1% albumin at 37°C with 0.005% (w/w) Triton X-100 and 10^{-4} M $ZnSO_4$. After 8 minutes the cells were spun and the pellet resuspended in fresh HBS containing 0.1% albumin and further incubated at 37°C in the absence (○) or presence (●) of 10^{-4} M $ZnSO_4$. Leakage of ^{3}H was measured after pelleting the cells and has been scaled such that leakage from control cells not treated with Triton (20–40% of ^{3}H in the supernatant) has a value of 0. Data taken from a single set of experiments that is representative of three other, similar experiments.

much so that these data are not conclusive proof that the internal resistivity (ρ_i) of melittin-treated cells is still very low. The consequence of increased ρ_i would be that the above G_m value might be an underestimate. However, even 300 mS/cm^2 is a much higher membrane conductivity than that measurable for the membranes of healthy cells [34]. We suspect that this may be the maximum measurable in this system. This would be the case if higher values of G_m actually occur, but are the consequence of such large 'holes' in the membrane that serious loss of electrolyte (in the rotation medium) and a drastic increase in cytoplasmic resistivity occur. The end effect of large holes would be a depressed f_c and a slowed rotation (see discussion of Eqn. 2 above), and these were indeed observed in some cells.

The electrical data allow a rough estimation of the physical extent of membrane damage. The G_m value of 300 mS/cm^2 is equivalent, in a membrane with a thickness of insulating material of 5 nm, to a mean conductivity of 0.15 μS/cm. If the conductance is due only to pores with the same conductivity as the outside medium (30 μS/cm), the pores occupy 0.5% of the membrane area.

The electrorotation experiments also indicate that inhibition of melittin-induced damage by Zn^{2+} can be reversible. The results presented in Table III show that cells pre-treated with melittin exhibit an increased f_c in comparison to those treated with melittin plus 0.1 mM $ZnSO_4$ (cf. Table II). However, the cells treated with melittin and Zn^{2+} gradually attain the high levels of f_c typical of melittin-treated cells when they are suspended in medium which lacks both melittin and Zn^{2+}. One interpretation of this result is that Zn^{2+} dissociates from melittin-induced pores which then revert to the open state (in the absence of extracellular melittin). Similar experiments (data not shown) show that removal of Zn^{2+} with 10 mM EGTA greatly potentiates the reappearance of melittin-induced damage of cells pre-treated with melittin and Zn^{2+}.

TABLE II

The effect of Zn^{2+} during or after melittin treatment of Lettre cells: Comparison of rotational f_c with depolarisation and leakage of intracellular cations and phosphoryl[^{3}H]choline

Procedure was as for Table I, except that the conductivity (27–35 μS/cm) of the 0.28 M inositol rotation medium plus K^+/Hepes (pH 7.2) was adjusted with or without 10^{-4} M Zn^{2+}. The depolarisation in $3.3 \cdot 10^{-7}$ M melittin must be interpolated from the data pairs given which were determined in incubations with 2.2 and $4.4 \cdot 10^{-7}$ M melittin with or without Zn^{2+}.

	Control incubation (1% DMSO)	$3.3 \cdot 10^{-7}$ M melittin	
		no Zn^{2+} during incubation	10^{-4} M Zn^{2+} during incubation
Rotation f_c/kHz in control medium	6.3 ± 2.0 (14)	12.8 ± 7.3 (12)	6.6 ± 1.2 (16)
Rotation f_c/kHz in medium containing 10^{-4} M Zn^{2+}	7.6 ± 3.1 (15)	7.7 ± 2.8 (14)	7.8 ± 3.8 (14)
Depolarisation	< 5%	72–92%	15–34%
Cellular cation leakage	0%	51%	12%
Phosphoryl[^{3}H]-choline leakage	5%	12%	10%

Leakage from phospholipid vesicles is inhibited by divalent cations and protons

Unilamellar vesicles (liposomes) made either of asolectin or of a mixture of palmitoyloleoylphosphati-

TABLE I

The effect of treating Lettre cells with melittin on their rotational f_c: Comparison with depolarisation and leakage of intracellular cations and phosphoryl[^{3}H]choline

Lettre cells were incubated at 37 °C in HBS; aliquots were taken before and 3 min after the addition of melittin (melittin 1 and 2) or an equivalent volume of DMSO (control 1 and 2) for determination of cellular cation and phosphoryl[^{3}H]choline leakage. Cellular cation leakage was calculated from the relative Na^+ content of cells (namely $Na^+/(Na^+ + K^+)$) and scaled such that 0% represents the value found before addition of melittin or DMSO (0.3–0.5) and 100% represents the value for cells in which the intracellular and extracellular pools of Na^+ and K^+ have completely equilibrated (0.97 in HBS); phosphoryl[^{3}H]choline leakage at 3 min was scaled such that 0% represents ^{3}H in the supernatant before the addition of melittin or DMSO (25–40%) and 100% indicates that all ^{3}H is in the supernatant. Membrane potential was assessed in parallel incubations using oxonol-V [52] as indicator; depolarisation is quoted as the change in signal 3 min after addition of melittin or DMSO expressed as a % of the total change found after the subsequent addition of 10^{-6} M gramicidin (sufficient completely to depolarise cells). After the 3 min incubation the remaining cells were pelleted and resuspended in 0.28 M inositol containing sufficient K^+/Hepes (pH 7) to give a conductivity of 27–35 μS/cm for assessment of electrorotation. Values of f_c are mean ± S.D. with number of cells in parentheses. All f_c values have been normalised to cells of 5.8 μm radius measured at 30 μS/cm conductivity.

	Control 1 (1% (v/v) DMSO)	Control 2 (2% (v/v) DMSO)	Melittin 1 ($5.5 \cdot 10^{-7}$ M, 1% (v/v) MDSO)	Melittin 2 ($1.1 \cdot 10^{-6}$ M, 2% (v/v) DMSO)
Rotation f_c/kHz	5.9 ± 1.6 (15)	5.5 ± 1.3 (14)	0.8 ± 2.1 (6)	12.8 ± 6.1 (12)
Depolarisation	< 5%	< 5%	93%	95%
Cellular cation leakage	< 5%	< 5%	72%	83%
Phosphoryl[^{3}H]choline leakage	< 10%	< 10%	10%	80%

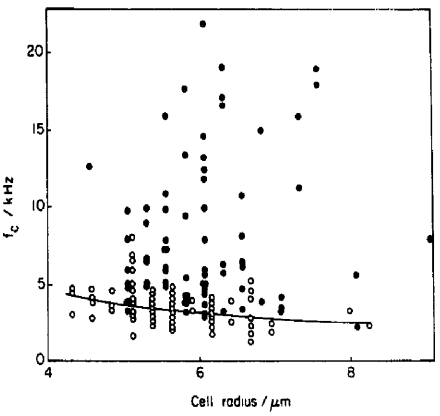

Fig. 5. The effect of melittin-treatment on the electrorotation of Lettre cells. $5 \cdot 10^6$ Lettre cells/ml were incubated in HBS at 37°C for 3 min in the absence (○) or presence of $(0.33–1.1) \cdot 10^{-6}$ M melittin (●); melittin treatment yielded more than 90% membrane depolarization, 50–80% intracellular cation leakage and 12–80% phosphoryl-[^{3}H]choline leakage. Cells were spun, the pellets were resuspended in 280 mM inositol containing sufficient K^+/Hepes (pH 7) to give a conductivity in the range 25–40 μs/cm and electrorotation monitored at room temperature (21–25°C). Results are shown for 90 untreated cells and 74 melittin-treated cells (superposition obscures a number of data points) from five separate incubations with two different cell preparations; data have been normalised to a medium conductivity of 30 μS/cm and, for the purposes of presentation, the radius values of all treated cells have been reduced by 0.13 μm. The mean ± S.D. cell radii were: controls 5.5 ± 0.7 μm, melittin-treated 6.1 ± 0.8 μm. The high f_c of some melittin-treated cells corresponds to short membrane relaxation time-constants, caused by increases in membrane conductivity (see Eqn. 2 in Methods and Materials). Eqn. 1 predicts a decrease in f_c with increasing radius which is only apparent in the case of untreated cells. A linear regression between f_c and $1/a$ (suggested by the inverse term in Eqn. 1) gave parameters leading to the continuous curve indicated. Regression of f_c on $1/a^2$ gave a poorer correlation (data not shown), indicating that the effect of K_s (Eqn. 1) on the f_c was small.

dylcholine (POPC):cholesterol, and pre-loaded with calcein, leak calcein when treated with melittin (Fig. 6). Leakage is inhibited by divalent cations, the efficacy being $Cd^{2+} > Zn^{2+} > Ca^{2+} > Mg^{2+}$ (Table IV), just as with intact cells, except for the greater efficacy of Cd^{2+} in liposomes compared with cells [14]. Low pH and low ionic strength inhibit leakage, as they do in intact cells [13,14].

Treatment of liposomes with low (i.e., sublytic) concentrations of Triton stimulates calcein leakage. Reducing the pH inhibits leakage; a similar effect of H^+ on Triton-induced conductivity across phospholipid bilayers has previously been reported [37]. Cd^{2+} inhibits leakage in the millimolar range, other divalent cations inhibit but only at 10 mM or above (Table IV).

TABLE III

The effect of Zn^{2+} in the incubation medium on the rotation f_c measured at given times after transfer of melittin-treated Lettre cells to rotation medium

Procedure was as for Table I, except that 0.3 M mannitol replaced 0.28 M inositol in the rotation medium.

Time in rotation medium (min)	f_c (kHz)	
	Pre-incubation with $5 \cdot 10^{-7}$ M melittin	Pre-incubation with $5 \cdot 10^{-7}$ M melittin and $2 \cdot 10^{-4}$ M $ZnSO_4$
1	12.8 ± 5.9 (3)	6.8 ± 0.5 (3)
5	12.6 ± 5.1 (3)	9.1 ± 3.6 (3)
9	16.4 ± 7.2 (3)	11.7 ± 6.6 (3)
13	18.1 ± 3.0 (3)	13.0 ± 2.0 (3)
18	15.1 ± 8.9 (3)	14.1 ± 8.2 (2)
All cells	14.8 ± 5.7 (21)	11.2 ± 5.6 (17)

Energy transfer between melittin and liposomes containing anthroyl stearate

The fluorescence of 12-AS, incorporated into POPC liposomes, at 440 nm (excited at 280 nm) is increased in the presence of melittin, while the fluorescence of melittin at 330 nm (due to its sole Trp at position 19 [2]) is correspondingly decreased. The emission maximum of melittin in the presence of POPC liposomes is at 332 nm, whereas in purely aqueous media the maximum is at 350 nm [23]. Energy transfer between melittin and AS is likely to be due to a melittin–AS interaction

TABLE IV

Sensitivity of leakage from liposomes induced by melittin or Triton to divalent cations, protons and low ionic strength

Calcein-containing liposomes (5–10 μg lipid/ml) prepared from POPC/cholesterol (1:1, w/w) or asolectin were incubated in 0.16 M NaCl, 0.01 M Hepes (pH 7, adjusted with NaOH) at 22°C in the absence or presence of differing concentrations of $CdSO_4$, $ZnSO_4$, $CaCl_2$ or $MgSO_4$, or at differing pH values. For low ionic strength experiments NaCl was replaced by 0.3 M mannitol. In the absence of divalent cations melittin (10^{-7} M) induced 40 ± 10% leakage, and Triton X-100 (0.001%) 30 ± 5% leakage, of entrapped calcein from liposomes. The values obtained with both types of liposome have been averaged and are expressed ± S.D. where appropriate.

Agent	Concentration required for 50% inhibition of leakage (M)					Leakage at 0.01 *I* as % of leakage at 0.16 *I*
	Cd^{2+}	Zn^{2+}	Ca^{2+}	Mg^{2+}	H^+	
Melittin	$1.5 \cdot 10^{-5}$	$9.0 \pm 3 \cdot 10^{-4}$	$2.9 \pm 0.5 \cdot 10^{-3}$	$1.6 \pm 0.8 \cdot 10^{-2}$	$3.2 \cdot 10^{-7}$	52 ± 31
Triton X-100	$4.0 \cdot 10^{-3}$	$4.0 \pm 1.3 \cdot 10^{-2}$	$3.0 \pm 0.4 \cdot 10^{-2}$	$6.1 \pm 2.4 \cdot 10^{-2}$	$6.3 \cdot 10^{-7}$	32 ± 20

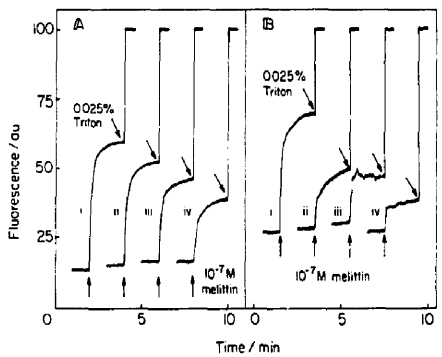

Fig. 6. Leakage of calcein from liposomes. Calcein-containing liposomes (5–10 μg lipid/ml) prepared from POPC/cholesterol (panel A) or asolectin (panel B) were incubated in 0.16 M NaCl, 0.01 M Hepes (pH 7, adjusted with NaOH) at 22°C without (traces i) or with $MgSO_4$ (traces ii) or $CaCl_2$ (traces iii) or $ZnSO_4$ (traces iv); the concentrations were as follows: panel A, 10^{-3} M $MgSO_4$, 10^{-3} M $CaCl_2$, 10^{-3} M $ZnSO_4$; panel B, 10^{-2} M $MgSO_4$, 10^{-3} M $CaCl_2$, 10^{-4} M $ZnSO_4$. Melittin and Triton X-100 (to give 100% leakage) at the final concentration indicated were added as shown by the arrows. Fluorescence at 520 nm was excited at 490 nm with excitation and emission slitwidths of 10 nm.

TABLE V

Energy transfer between melittin and (9-anthroyloxy)stearates in liposomes

Liposomes (0.5 mg/ml) prepared from POPC (without cholesterol) were incubated in 0.16 M NaCl, 0.01 M Hepes (pH 7, adjusted with NaOH) at 24°C with $5 \cdot 10^{-6}$ M melittin and $5 \cdot 10^{-6}$ M 2-, 7- or 12-AS in the presence or absence of $CdSO_4$ or $ZnSO_4$ as indicated. Fluorescence was excited at 280 nm with 10 nm excitation and emission slitwidths and emission spectra were recorded in the ratio mode. Energy transfer was calculated from the loss of fluorescence at 330 nm (due to melittin alone) and the equivalent gain at 440 nm (due to AS alone) and is expressed as the percentage of the total fluorescence (mean ± S.D. (number of experiments)). Energy transfer in the absence of liposomes is difficult to quantitate accurately as 2-, 7- or 12-AS fluorescence is very low; with melittin and 12-AS an upper limit of 8% energy transfer was found in the absence of liposomes under the conditions employed here.

	Melittin–AS energy transfer (%)
2-[9-Anthroyloxy]stearate	23.23 ± 2.40 (4)
$+10^{-2}$ M $ZnSO_4$	10.25 (2)
7-[9-Anthroyloxy]stearate	22.40 ± 2.23 (4)
$+10^{-2}$ M $ZnSO_4$	13.80 (2)
12-[9-Anthroyloxy]stearate	26.91 ± 2.34 (8)
$+10^{-3}$ M $CdSO_4$	22.85 (2)
$+10^{-2}$ M $ZnSO_4$	19.68 ± 2.95 (6)

within the lipid bilayer, since 7-AS and 2-AS, in which the anthroyl group is anchored successively nearer the carboxyl, (i.e., polar), end of the stearic acid molecule show somewhat less energy transfer (Table V). Addition of Cd^{2+} or Zn^{2+} to AS-containing liposomes, before or after addition of melittin, does not alter the position of the melittin or the AS fluorescence emission spectra (data not shown). The energy transfer between melittin and 12-AS is only slightly reduced by 10^{-3} M Cd^{2+} or 10^{-2} M Zn^{2+} (Table V); the effect of Zn^{2+} is greater with 2-AS and 7-AS, compatible with an effect on melittin bound at the head group region of the bilayer.

CD experiments with melittin and liposomes

The circular dichroism spectrum of melittin associated with phospholipid differs considerably from that of melittin in water [24]. In Fig. 7 the CD of melittin in

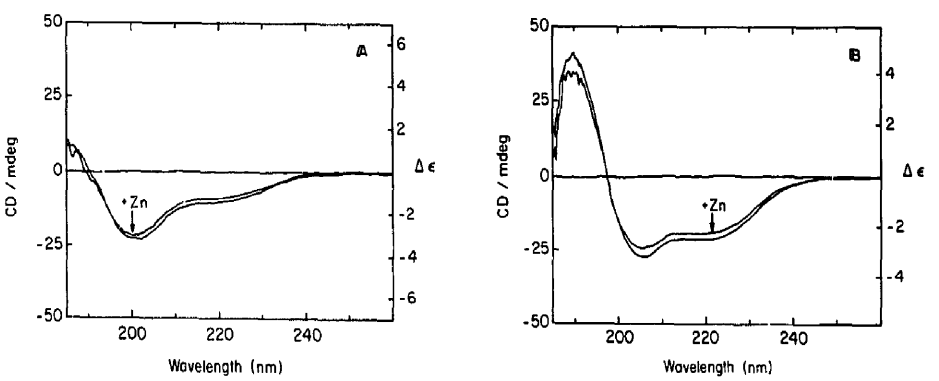

Fig. 7. Circular dichroism of melittin in water and in liposomes. (A) 0.52 mg melittin/ml in water before and after (trace labelled + Zn) the addition of $2.35 \cdot 10^{-3}$ M $ZnCl_2$. (B) 0.52 mg melittin/ml in water with 2.25 mg dipalmitoylphosphatidylcholine/ml and 10% (v/v) ethanol before and after (trace labelled + Zn) the addition of $2.13 \cdot 10^{-3}$ M $ZnCl_2$.

water without (panel A) or with phospholipid (panel B) is shown in the absence of presence of $ZnCl_2$. In neither case does the presence of Zn^{2+} significantly change the CD of melittin.

Discussion

The present experiments demonstrate three points in regard to the pore-forming ability of melittin and Triton. First, the sensitivity to divalent cations [13] and protons [14] of melittin-induced pores in Lettre cells is not due to an effect of traces of phospholipase A (or of *N*-formyl melittin [2]) in the melittin preparation. That is leakage of ions and phosphoryl[^{3}H]choline induced by chemically synthesized unformylated melittin [17] is as sensitive to inhibition by Ca^{2+}, Zn^{2+} or H^+ as is melittin from natural sources (Figs. 1 and 2).

Second, the action of divalent cations is not to compete with melittin for binding and/or insertion into the plasma membrane: for Lettre cells incubated with melittin in the presence of an inhibitory concentration of Zn^{2+} begin to leak phosphoryl[^{3}H]choline (Fig. 3), and to alter their electrorotational characteristics with an increased permeability (Tables I and II), if Zn^{2+} and melittin are removed. This result is compatible with our previous demonstration [25] that the amount of melittin bound to Lettre cells is the same whether Ca^{2+} is present or not. The inhibitory action of Zn^{2+} on Triton-induced leakage [13,38] is likewise reversible (Fig. 4), and therefore not due to the prevention of binding or insertion of Triton by Zn^{2+}. Since neither melittin [2] nor Triton contains any anionic sites, it is unlikely that approx. 10^{-4} M Zn^{2+} or approx. 10^{-3} M Ca^{2+} binds directly to these agents. Hence, the inhibitory role of divalent cations on leakage induced by melittin or Triton is presumably via a direct effect on the membrane lesion itself [25]. The ability of Zn^{2+} to restore the electrorotation of Lettre cells pretreated with melittin to control values (Table II) indicates that divalent cations can close open pores in cell membranes. This conclusion is compatible with the observation that divalent cations inhibit leakage induced by other pore-formers in a reversible manner also [13,39,40], and that divalent cations exert a reversible 'gating' effect on pores induced in phospholipid bilayers by *S. aureus* α-toxin [13,14,41] or cytolysin from cytotoxic lymphocytes [40].

The third point concerns the nature of the lesion itself. Since melittin is able to induce divalent cation-sensitive leakage from purely lipidic vesicles (Fig. 6; Table IV), it is clear that a clustering of endogenous proteins by melittin to form an aqueous pore [21,22] is not a prerequisite for sensitivity to Ca^{2+} or Zn^{2+}. On the contrary, energy transfer experiments with AS (Table V) and CD measurements (Fig. 7) confirm previous results that indicate considerable penetration of melittin into the bilayer [23,42]. Furthermore, such penetration, indicated by energy transfer to 12-AS (Table V) appears to be relatively little affected by the presence of Zn^{2+} or Cd^{2+}. On the other hand, energy transfer from melittin to 2- or 7-AS, where the anthroyl group approaches the headgroup region of the bilayer [43] is markedly reduced by Zn^{2+} (Table V). This result is in accord with experiments with deuterated lipids [42,44] which indicate that melittin interacts strongly with the headgroup region of the lipid bilayer, as do other cationic amphiphiles [45] and divalent cations such as Ca^{2+} [46,47]. The essentially electrostatic interaction of melittin with the surface of lipid bilayers is likely to be competitive with divalent cations, but may not be directly relevant to pore-formation. We, therefore, propose that a small proportion of the melittin associated with liposomes – perhaps no more than a few percent – interacts with the hydrocarbon region of the bilayer as suggested previously [8], and that this interaction leads to the induction of pores: the lifetime, or extent of 'open' configuration, of such pores – i.e., the ability to promote leakage of water soluble ions and molecules across the membrane – is subtly modulated by divalent cations, whereas the formation of pores is not affected. This model would likewise explain the pore-forming ability of melittin in cells, as well as the sensitivity of such pores to divalent cations.

Triton-induced leakage from liposomes is inhibited only at such high concentrations of Zn^{2+} or Ca^{2+} (Table IV) that their action is unlikely to be the result of binding to some membrane component(s); not surprisingly, there is little discrimination between Zn^{2+}, Ca^{2+} or Mg^{2+}. Rather, it probably reflects a non-specific 'screening' of surface charge [40] as has been observed for the gramicidin channel [49,50] also. It is possible that Triton-induced leakage does not reflect the induction of pores so much as solubilization of liposomes (which in the case of proteins such as *S. aureus* α-toxin or *C. perfringens* Θ-toxin cannot be the case, since there is specificity with regard to the approximate size of the pore [51]). Although low pH or low ionic strength do inhibit leakage from Triton-treated liposomes, it appears that significant divalent cation sensitivity may require the presence of (endogenous) membrane proteins, and that calcein leakage from liposomes may not be a good model for Triton-induced leakage from Triton-treated cells.

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Since the submission of this paper we have become aware of the paper by Portlock et al. [53]. The apparently conflicting results, so far as the divalent cation-sensitivity of melittin-induced leakage from liposomes is concerned, may be due to differences in vesicle composition and preparation. Certainly we both observe reduction of leakage by low ionic strength (compare Table IV (this paper) with Table I of Portlock et al.). Also, in agreement with Portlock et al. we find no inhibition of melittin-induced leakage from cells (in 0.15 M NaCl) by polylysine (M_r 4000); higher molecular weight polylysine induces leakage from Lettre cells on its own (see Ref. 14, this paper), which may in part explain the stimulation seen by Portlock et al. (Fig. 6).

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