LYSOLECITHIN INHIBITS AN ACTION OF BEE VENOM PHOSPHOLIPASE A_2 IN ERYTHROCYTE MEMBRANE

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1. Introduction

Bee venom phospholipase A_2 does not alter the permeability of erythrocyte membranes unless exogenous long-chain fatty acids are also present [1]. The acids that are effective are also direct activators of the enzyme, but this may not explain their action here since enzyme activated by treatment with decanoic anhydride [2] does not enhance membrane leakiness. An alternative possibility is that the exogenous fatty acid brings the membrane close to a threshold level for high permeability that may be exceeded when further lytic products are formed. The kinetics of the leakage process indicate a complex underlying mechanism. Briefly, leakiness increases very rapidly after addition of either fatty acid or enzyme to cells that have been incubated with the other component, but then falls and, depending on the concentration of reagents may rise again and is then accompanied by lysis. The initial phase of rapid leakage is subject to a number of possible experimental artefacts (of mixing or of cell heterogeneity) which make interpretation difficult, but it is very strongly inhibited by lysolecithin and as this effect is unlikely to be influenced by the same artefacts it is of more immediate interest.

The enzyme can be activated (20–50-fold) by both fatty acid and lysolecithin [2,3] reaction products and, after making an appropriate correction for activation, an underlying competitive and weak product inhibition is observed. No conditions have been found where either product is as strong an inhibitor as the action of lysolecithin outlined above would suggest.

2. Materials and methods

Lysolecithin, fatty acids and rabbit erythrocytes were prepared as described in an earlier paper [1]. The erythrocytes were stored at 0°C as a 50% by volume suspension in saline solution. Purified bee venom phospholipase A_2 [4] was a gift from Dr R. Shipolini (University College London) and was stored at 0°C in light-proof bottles as a 500 μg per ml solution. Leakage of electrolyte from erythrocytes was followed by conductimetry in an isotonic sucrose solution buffered with 10mM morpholino propane sulphonate /Na^+ (MOPS/Na^+), pH 7.4 at 37°C and in 1.5 ml reaction cells [1]. Phospholipase A_2 activity was measured by conductimetry [5], using water soluble substrates prepared by acylating purified egg lysolecithin with hexanoic or octanoic anhydrides [6]. Erythrocyte lysis was determined by filtering the suspension and determining the absorbance of the filtrate at 4200 Å.

3. Results

The dose–response curves for pre-lytic leakage of electrolyte from erythrocytes induced by long-chain fatty acids and long-chain saturated lysolecithin show a threshold level above which leakage is very rapid. Below this threshold the curves have a significant difference; the response to fatty acids increases gradually whilst that to lysolecithin is biphasic with an early increase followed by a plateau level until the threshold is reached (fig.1).

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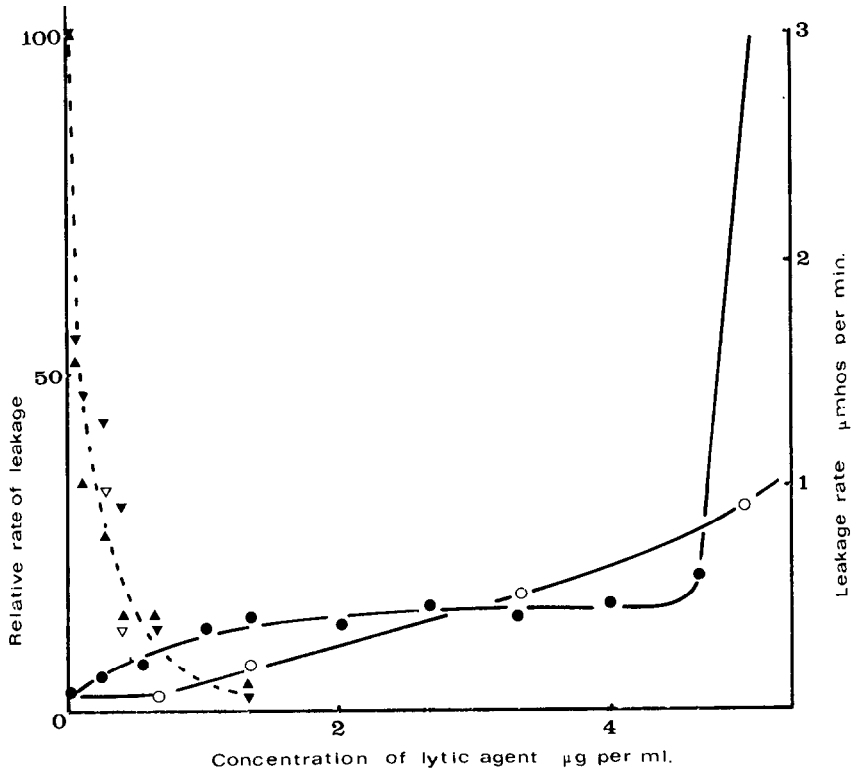

Fig.1. The effect of lysolecithin (•) and oleic acid (○) on the rate of leakage of electrolyte from erythrocytes (approx. 0.33% by vol) suspended in isotonic sucrose medium buffered with 10 mM morpholinopropane sulphonate/Na^+, pH 7.4 at 37°C. Also the effect of lysolecithin on the leakiness of cells after treatment with oleic acid and bee venom phospholipase A_2. (▲▽) 1.33 µg per ml of oleic acid and 3.3 µg and 16.5 µg per ml of enzyme respectively. (▼) 2.67 µg per ml of oleic acid with 3.3 µg of enzyme.

The present experiments are possible because fatty acids synergise the action of the enzyme at concentrations below the leakage threshold. When cells are treated with low levels of lysolecithin in addition to fatty acid, the initial phase of the response to the enzyme is greatly reduced although subsequent leakage occurs at a higher rate and the cells lyse rather earlier than those without lysolecithin (figs.2,3). The early leakage response provides a method for studying the distribution of fatty acid, enzyme and lysolecithin between cells and solution and also the rates at which they are exchanged between cells. In particular it provides a very

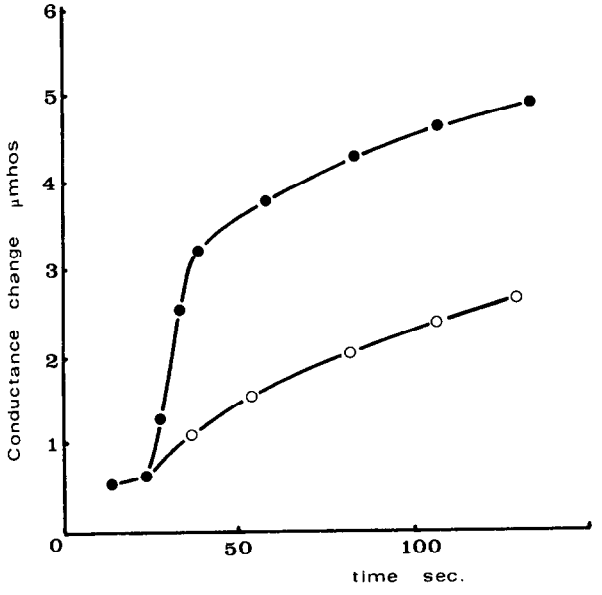

Fig.2. The initial phase of electrolyte leakage from erythrocytes treated with oleic acid (2.0 µg per ml) and bee venom phospholipase (33 ng per ml) under the conditions of fig.1 in the absence (•), and presence (○) of 1.33 µg per ml of lysolecithin.

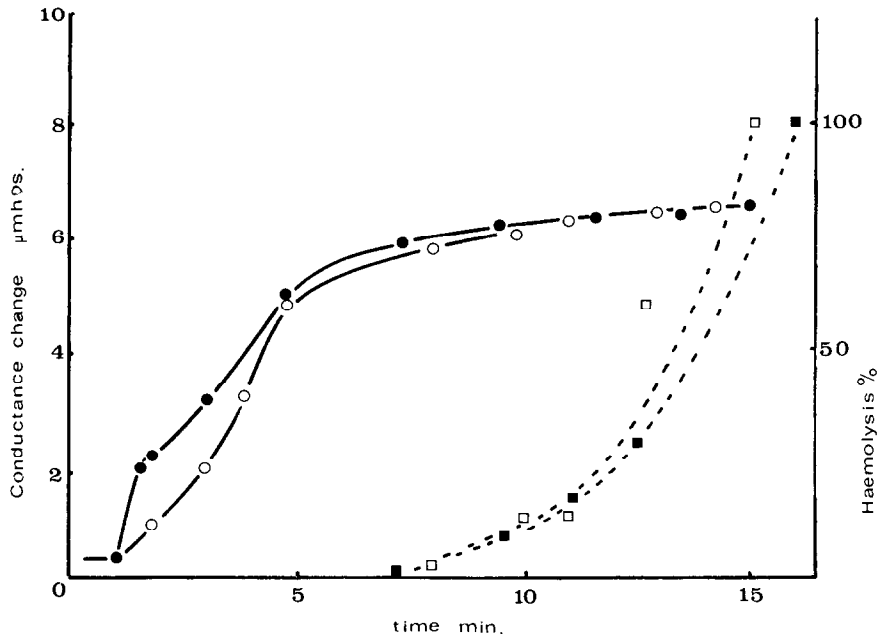

Fig.3. Electrolyte leakage and lysis curves for erythrocytes treated as in fig.2, but in a 6 ml conductivity cell. 100 μl samples were withdrawn, added to 2 ml of isotonic sucrose, centrifuged and the absorbance determined at 4200 Å(■□). The solutions contained oleic acid 3.3 μg per ml, bee venom phsopholipase A_2 33 ng per ml both with (○□) and without (●■) lysolecithin at 1.33 μg per ml.

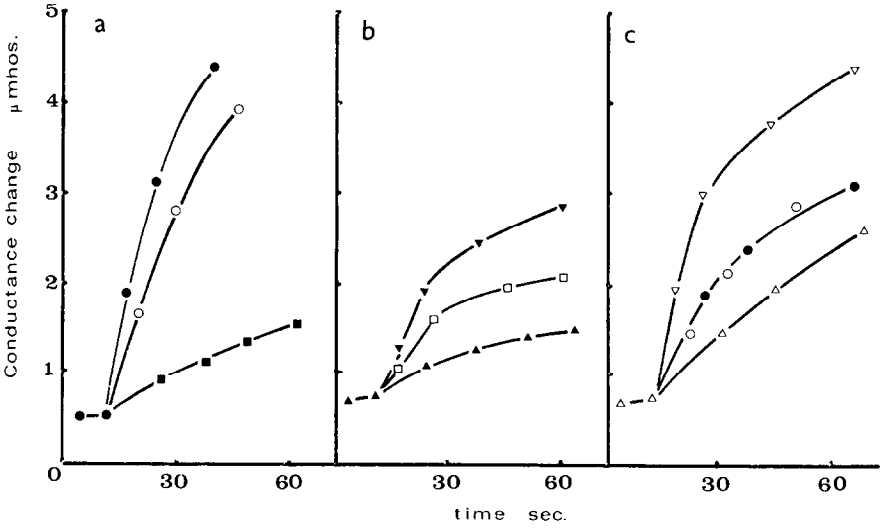

Fig.4. The absorbtion of bee venom phospholipase A_2, oleic acid and lysolecithin by erythrocytes. (a) Bee venom phospholipase A_2 (10 μg) was added to an isotonic sucrose medium both with (○) and without (●) erythrocytes and 100 μl withdrawn for assay using the 2-hexanoyl derivative of egg lysolecithin as substrate. (Lawrence and Moores, submitted for publication (●) Control. (○) Cell supernatant. The solution containing erythrocytes was filtered and 100 μl of the supernatant solution assayed (■). (b) Erythrocyte suspensions containing 0.67 (▲) and 1.33 (▼) μg per ml of oleic acid were treated with 3.3 ng per ml of bee venom phospholipase A_2. Similarly, cells treated with 1.33 μg of oleic acid per ml were mixed with an equal volume of untreated cells and 3.3 ng per ml of enzyme added (□). (c) Erythrocytes incubated without (▽) and with (○), 0.67 and (△) 1.33 μg per ml of lysolecithin were treated with oleic acid 2 μg per ml and enzyme 3.3 μg per ml. Cells were also suspended in a medium from which cells incubated with 3.3 μg of lysolecithin per ml had been removed by centrifugation and then treated with oleic acid and enzyme as above (●).

sensitive quantitative test for lysolecithin. Experiments in which cells are treated with a reagent, centrifuged and the amount remaining in solution assayed, show that the enzyme is almost completely bound to the cells whilst approximately 25% of lysolecithin remains in solution [7]. Similarly by treating cells with one reagent and then adding untreated cells it can be shown that fatty acid does not exchange between cells whilst the enzyme exchanges at a significant rate (fig.4). Lysolecithin must exchange very rapidly and should reach an equivalent concentration in all cells under the conditions used here.

Direct measurement of the amount of membrane phospholipid hydrolysed in the early response indicate that it lies within the margin of error for the total phospholipid determination. Nevertheless enzymic activity is probably involved because the action is inhibited by EDTA and restored by calcium. Because the amount hydrolysed is small it is even more difficult to determine directly whether or not hydrolysis is inhibited by lysolecithin. In the second state of the response leading to lysis, phospholipid degradation can be demonstrated (Lawrence and Drainas, unpublished work) and is not inhibited by lysolecithin.

Cells treated with low levels of enzyme may show a very small initial response, but are desensitised to larger amounts of enzyme.

4. Discussion

The initial response of fatty acid treated erythrocytes to bee venom phospholipase A_2 (and to the enzymes from other sources) might arise from different susceptibilities of parts of the surface of a single cell, or from different susceptibilities within the cell population. In either case the action of lysolecithin can only have two explanations: either it inhibits the enzyme, or else it inhibits the action of lytic products on membrane leakiness. The only experimental evidence that the former is correct is that the degree of inhibition is proportional to enzyme concentration, fig.1. This is however not a sensitive test because the response curve is relatively flat. Against this there is no experimental evidence, or experimental precedent, for such inhibition. The strongest evidence that lysolecithin might act at the level of membrane organisation is the correlation between inhibition and the pre-lytic leakage response. We postulated elsewhere (Lawrence, Moores and Steel [1]) that lysolecithin in low concentration cannot pass through the membrane and produces a limited effect on membrane leakiness because it only perturbs the outer lamella of the bilayer structure (seen in fig.1 for concentrations between approx. 1.5 μg and 4.5 μg per ml). There is, however, evidence that lysolecithin promotes reorganisation of membrane components [8] and it is thus possible that the increased leakiness observed is a result of the aggregation of membrane proteins to give a less effective hydrophobic barrier than the native structure. Reorganisation of this type could change the way in which the enzyme attacks the membrane or the way in which lytic products affect leakiness. The system is further complicated because lysolecithin, produced by the reaction, should have an autoinhibitory effect. If, however, the action of lysolecithin is to be understood in terms of inhibition of enzymic activity a further explanation must be sought for the decrease in response to products already formed. Explanations in terms of cell heterogeneity do not account for desensitisation by low enzyme levels and it seems most likely that the initial response is determined by the rate of production of lytic agents rather than the concentrations reached.

Acknowledgement

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