ALBUMIN MEDIATES LYSIS OF ERYTHROCYTES BY BEE VENOM PHOSPHOLIPASE A_2 ACTIVATED WITH OLEOYL IMIDAZOLIDE

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1. Introduction

In a study of the activation of bee venom phospholipase A_2 by long-chain fatty acids we showed that the enzyme was irreversibly activated by treatment with reactive derivatives of these acids [1,2]. This indicated that fatty acids were allosteric activators of the enzyme and that the binding site contains a nucleophilic residue which can form a covalent linkage to an acyl group.

Activity measurements were made using a defined chemical assay, the hydrolysis of dioleoyl phosphatidyl choline, but under non-physiological conditions in the presence of a dilute organic solvent [3]. Chemically modified enzyme was tested in a sensitive variant of the erythrocyte lysis assay for phospholipase A_2 involving conductimetric determination of electrolyte release from the cells [4,5], but initial results failed to show any increase in enzymic activity. Subsequent work showed that acyl imidazolides were more potent activators than other fatty-acid derivatives and they produced less extensive, but presumably more specific modification of the protein than other reagents [6]. When enzyme, activated by oleoyl imidazolide, was tested in the erythrocyte leakage assay the results proved positive and formed the basis for a new and sensitive assay of covalent activation of venom phospholipase A enzymes.

2. Materials and methods

Purified phospholipase A_2 from *Apis mellifica* was a gift from Dr R. Shipolini [7] (University College London). Bovine serum albumin fraction V (essentially fatty acid-free) was from Sigma Chemical Co. Oleoyl imidazolide was prepared from oleic acid and carbonyl diimidazole (both from Sigma Chemical Co.) as in [8], but using methylene dichloride as solvent. Fresh rabbit blood was obtained as described elsewhere using heparin anticoagulant, washed 4 times by pelleting in isotonic saline and stored at 0°C as a suspension of 20% haematocrit. The kinetic conductivity apparatus was essentially as in [4], but using a modified 2 ml reaction cell.

3. Results

The rate of leakage of internal electrolyte from rabbit erythrocytes is potentiated by bee venom phospholipase A_2 but the effect is quite low even when the amount of enzyme is a significant fraction of the total weight of cell membrane (i.e., 5 μg enzyme treating 5 μl packed cells, which contain ~25 μg membrane phospholipids gives a 2–4 fold increase in basal leakage rates).

When the enzyme is pre-incubated with oleoyl imidazolide by the procedure which gives maximum enhancement of catalytic activity for hydrolysis of dioleoyl phosphatidyl choline in 20% *n*-propanol medium [6], its effect on erythrocyte leakage is enhanced (fig.1a). The response is, however, limited corresponding to efflux of a rather low proportion of total cell electrolyte. Control experiments in which the cells are pretreated with oleoyl imidazolide or its hydrolysis product oleic acid also show an enhanced response to the enzyme (fig.1b,c) but this effect is smaller than given by pretreatment of the enzyme with oleoyl imidazolide although the margin for discrimination is rather low. The assay is therefore insensitive and not sufficiently specific to be a general

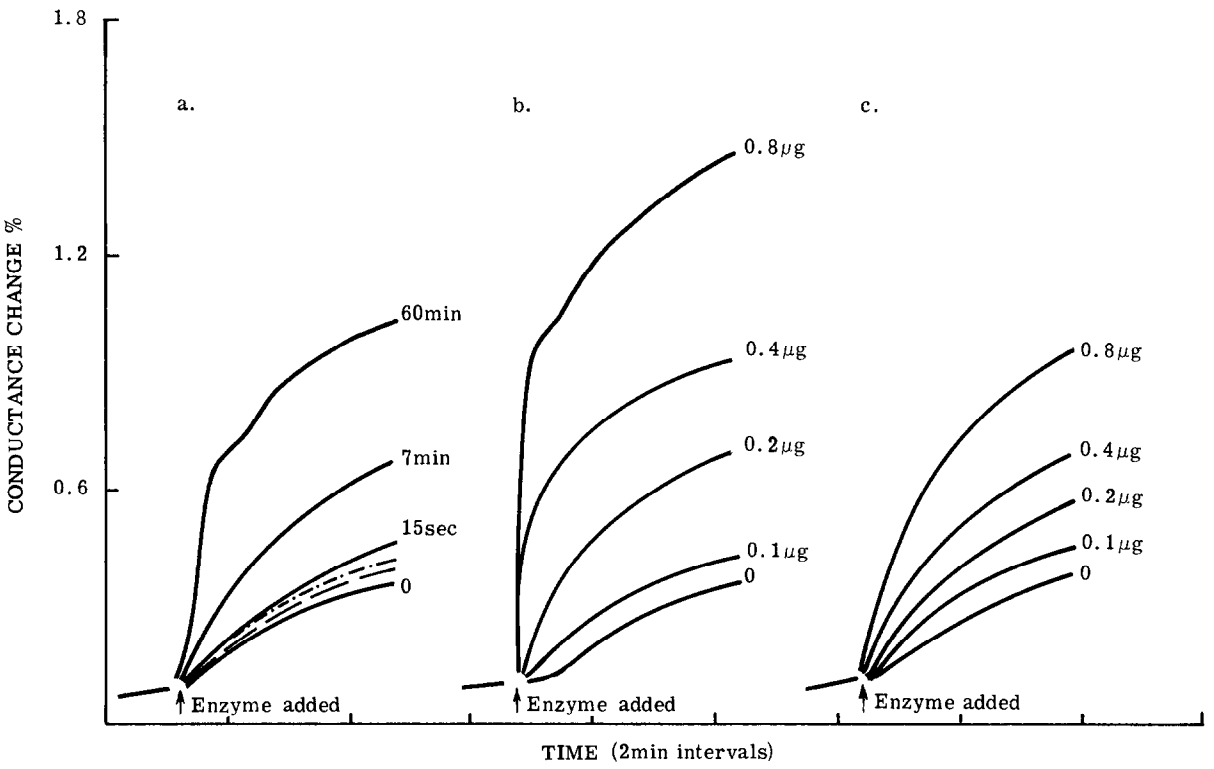

Fig.1. Leakage of rabbit erythrocytes induced by bee venom phospholipase A_2 either preincubated with oleoyl midiazolide or acting in the presence of free oleoyl imidazolide or oleic acid. Rabbit erythrocytes were incubated at 0.4% haematocrit in 2 ml isotonic sucrose solution buffered with Hepes/Na^+ (10 mM NaOH) (pH 7.4) 37°C and electrolyte leakage from the cells monitored by continuous measurement of electrical conductance change, and the effect of addition of 1.25 μg/ml of bee venom phospholipase A_2 recorded. (Total efflux of electrolyte from the cells gave a conductance range of 2.25%.)

(a) The enzyme was preincubated in 100 μl suspension buffer at 37°C at 2.5 mg protein/ml. Samples (1 μl) were withdrawn for assays both before and at measured times after addition of 0.5 μl solution of oleoyl imidazolide in acetone (2% w/v) (——). Curves (–·–·–, – – –) show the effect on the leakage response of addition of 0.1 μg/ml of oleic acid and oleoyl imidazolide, respectively, before addition of 1.25 μg/ml of untreated enzyme.

(b) Erythrocytes were incubated as in fig.1(a) but in the presence of various concentrations of oleic acid, and the effect of addition of 1.25 μg/ml of untreated phospholipase A_2 recorded.

(c) As in fig.1(b) but the cells were preincubated with oleoyl imidazolide.

method for studying the covalent activation of the enzyme.

A variant of the assay was then tested in which fatty acid-free albumin was added to the medium to sequester free fatty-acids. Fatty acid-free albumin greatly enhanced the lytic activity of venom phospholipase A enzymes without increasing the rate of hydrolysis of membrane phospholipids [8,9]. The effect was correlated to extraction of hydrolysis products from the cell membrane and we expected that this action would lead to catastrophic breakdown of the membrane only after extensive hydrolysis of phospholipids. In practice fatty acid-free albumin enhanced the rate of leakage of cell electrolyte virtually from the time of addition of enzyme and totally eliminated synergism of the response to the enzyme by free oleoyl imidazolide or oleic acid (fig.2a). This assay therefore provided high sensitivity to phospholipase activity together with the possibility of high selectivity towards the effects of covalent activation. When the activation procedure was repeated using the modified assay, a large and progressive acti-

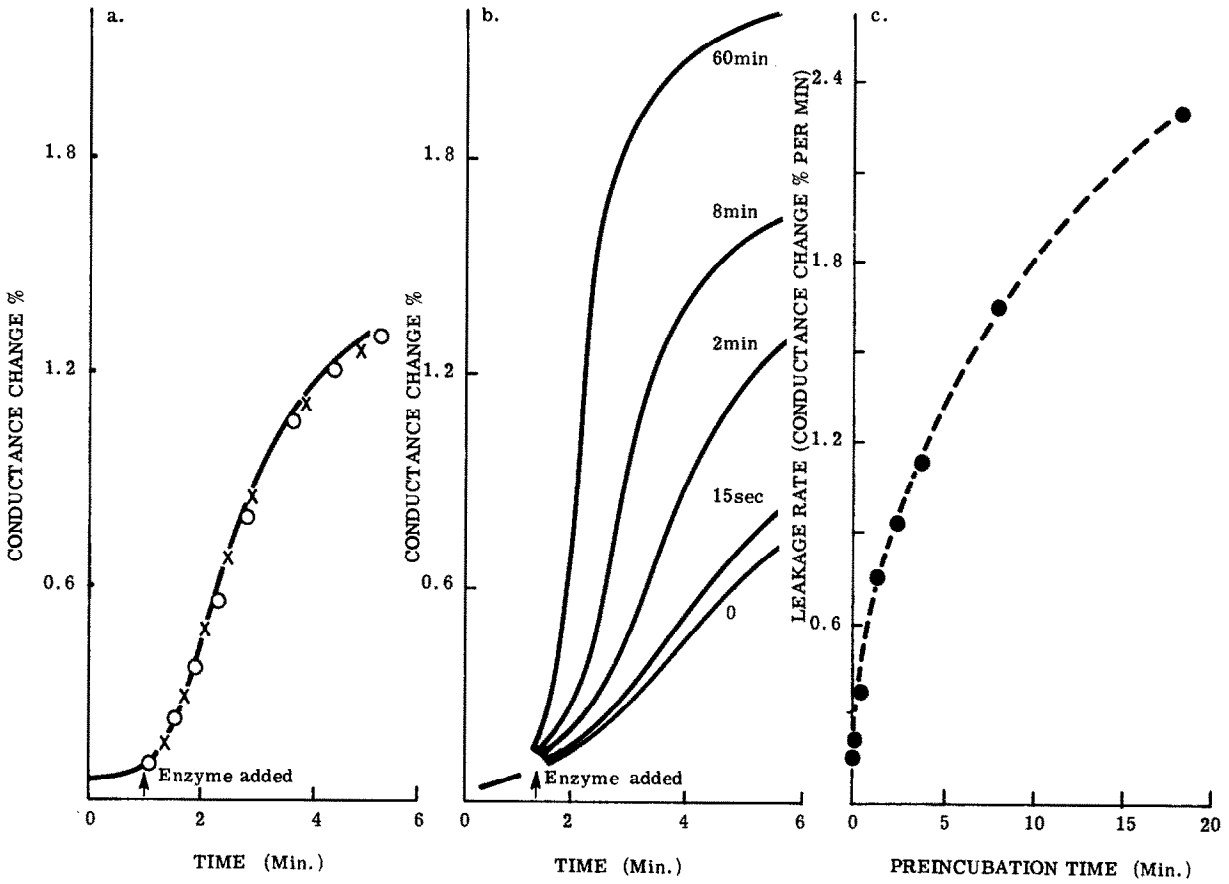

Fig.2. The effect of fatty acid free albumin on the leakage response of rabbit erythrocytes to bee venom phospholipase A_2. Erythrocyte leakage was measured under the conditions of fig.1 but with 1 mg/ml of fatty acid free albumin added to all cell suspensions.

(a) The effect of bee venom phospholipase A_2 (2.5 μg/ml) on erythrocyte leakage in the presence of fatty acid albumin (1 mg/ml) also with oleic acid (0.4 μg/ml) (○), or oleoyl imidazolide (0.4 μg/ml (X).

(b) Bee venom phospholipase A_2 was treated with oleoyl imidazolide under conditions of fig.1(b) and 1 μl samples withdrawn for assay in fig.1(a), but in the presence of fatty acid free albumin (1 mg/ml).

(c) Bee venom phospholipase A_2 was activated and assayed as in fig.2(b) and maximum leakage rates were determined from the individual progress curves (●).

vation effect was observed (fig.2b,c) and <1% of this activation could be attributed to direct interaction of enzyme with free activator or oleic acid within the cell membrane.

Although this assay approaches the use of physiological conditions, the medium is not at physiological ionic strength. Activation was therefore assayed using measurement of cell lysis in an isotonic saline medium. Under these conditions, the lytic power of the enzyme appeared to be >100-fold that of the untreated control, although the presence of fatty acid-free albumin was essential for expression of this activity (fig.3).

4. Discussion

Bee venom phospholipase A_2 is activated by free fatty acids, but in the complex substrate system provided by a cell membrane, it is difficult to separate the allosteric effects of these compounds from their

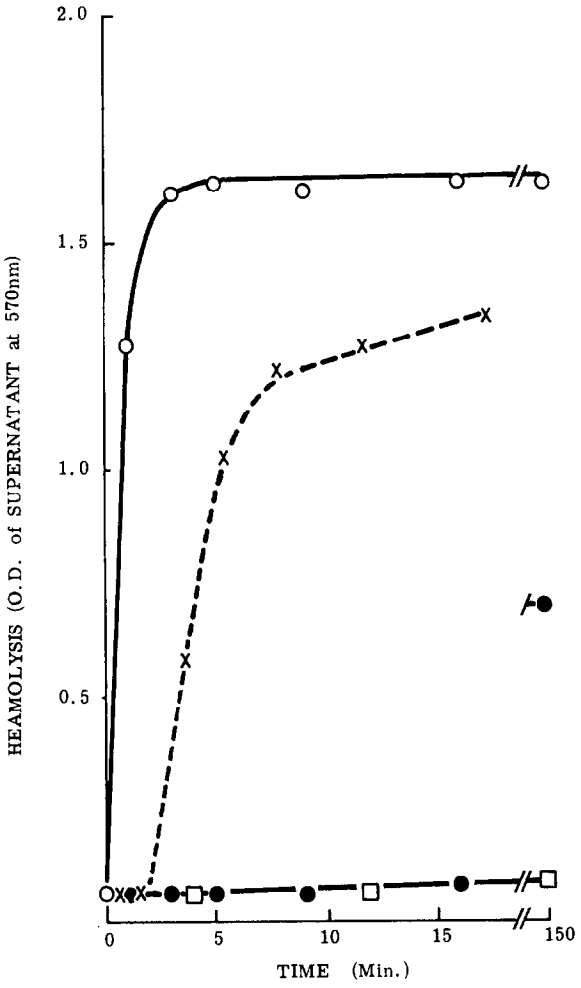

Fig.3. Lysis of rabbit erythrocytes by activated bee venom phospholipase A_2. Erythrocytes were suspended in 14 ml isotonic NaCl solution buffered with Hepes/Na^+ (10 mM NaOH) (pH 7.4) at 37°C at 4% (v/v) either in the presence (○, x, •) or absence (□) of 1 mg bovine serum albumin. Bee venom phospholipase (1.25 μg/ml (○, •, □) or 0.125 μg/ml (X)), either untreated (□, •) or activated for 2 h under the conditions of fig.2b (○, x) was added and 2 ml samples withdrawn, centrifuged and the A_{577} of the supernatant solution measured.

indirect action on enzymic activity produced by modification of the membrane structure. The preparation of covalently activated enzyme which is not susceptible to further allosteric activation by free fatty acids allows the biological effects of the components of activation to be distinguished and investigated. Other phospholipase A_2 enzymes are fatty acid activated [10,11] and it is not known whether the same mechanisms operate or whether the techniques used to separate free activator and fatty acids from the protein (solvent extraction or gel filtration in media containing dilute organic solvents) would work in these cases. Therefore it is extremely valuable to have an assay method which is absolutely specific for the effects of covalent modification of the enzyme and the erythrocyte leakage or lysis assays using fatty acid free albumin to absorb fatty acids give this specificity with the bonus of enhanced sensitivity.

These results give the first demonstration that covalent activation of bee venom phospholipase is effective under conditions approaching those found in vivo and that it enhances the presumed biological function of the enzyme, the destruction of membranes. These assay methods do not distinguish the effects of increased catalytic activity from increased ability of the enzyme to couple substrate hydrolysis to membrane destruction and this issue can only be resolved by direct measurement of substrate hydrolysis. Circumstantial evidence that enzyme most highly activated by the unambiguous activity assay is also most activated in the cell leakage assay suggests that increased catalytic activity is the dominant factor. Different venom phospholipase A_2 enzymes do, however, appear to vary in their relative lysis/hydrolysis potency and the precise mode of production or release of reaction products may be significant. In this respect, the action of albumin is extremely interesting. Albumin was shown not to activate phospholipases and was more effective when added to cells after termination of phospholipase action by addition of EDTA [8,9]. The erythrocyte assay which gives a continuous response allows the effect of albumin addition to be studied when cells are pretreated with phospholipid degradation products. The results show that the interaction of albumin with fatty acids originally sequestered by cell membrane is extremely damaging to the membrane but that the effect of albumin on the response of cells to phospholipase A_2 may not be entirely explained by this mechanism (A. J. L., unpublished).

In summary, the measurement of erythrocyte lysis in the presence of fatty acid-free albumin provides a highly sensitive method for measuring covalent activation of bee venom phospholipase A_2 which does not require a specialised apparatus or materials and conductimetric assay of electrolyte leakage from the cells allows the basic mechanisms to be investigated.

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