# ENHANCEMENT OF BEE VENOM PHOSPHOLIPASE $A_2$ ACTIVITY BY MELITTIN, DIRECT LYTIC FACTOR FROM COBRA VENOM AND POLYMYXIN B*

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## 1. Introduction

The peptide melittin and the enzyme phospholipase $A_2$, two main constituents of bee venom, show a marked synergism in their action on cell membranes. Under conditions where neither of the two substances has any deleterious effect, their combined action readily leads to the lysis of e.g. erythrocytes [1]. We have recently observed that melittin forms a complex with lecithins provided the lipid is in the liquid–crystalline state [2]. This hydrophobic binding of melittin to phospholipids may be an intermediate step in the action of bee venom on cell membranes and thus be of relevance for explaining the synergistic action of venom components. If this argument is correct, one would expect that melittin stimulates the action of phospholipase only when complex formation between the peptide and the phospholipid can occur.

In this report we present our results on the action of purified phospholipase $A_2$ from bee venom on lecithin liposomes in the presence and absence of melittin. It could be shown that melittin stimulates the enzymatic hydrolysis of egg lecithin up to about 5-fold, while similar effects could not be observed with dipalmitoyllecithin. Previous experiments have shown that at the assay temperature melittin interacts with the former but not with the latter type of phospholipid. Starting from these findings, we have tested additional peptides known to interact with membranes: these were two lytic factors from snake venom [1,3] and the bacterial toxin polymyxin B [4]. These compounds were found to resemble melittin in that they increase the initial rate of hydrolysis of lecithin by phospholipase, and these stimulatory effects were also dependent on the fluidity of the phospholipid.

## 2. Materials and methods

Egg lecithin was purchased from Lipid Products (Nutfield, England), 1,2 dipalmitoyl-DL-3-glyceryl-phosphorylcholine from Koch Light (England) and polymyxin B from Sigma (USA). Melittin was purified from lyophilized bee venom as described earlier [5]. Direct lytic factors from the venom of the Indian cobra *(Naja naja)* and the African Ringhals cobra *(Haemachatus haemachates)* were generous gifts of Dr. W. Vogt and Dr. D. Eaker, respectively. We are also indebted to Dr. R. A. Shipolini for the sample of highly purified bee venom phospholipase $A_2$, which was used throughout this study.

Liposomes were prepared from the dried lipids which were subsequently swollen for several hours in water and finally sonicated for 15 min at maximal power with a Branson B-12 Sonifier. The hydrolysis of lecithins by phospholipase $A_2$ was measured by titrating the liberated fatty acids with 0.01 N NaOH in an autotitrator (Radiometer Copenhagen). All components were adjusted to pH 8.0 and the reaction started by adding substrate. The rate of hydrolysis was followed at pH 8.0, the pH optimum of the enzyme [6]. Additional details are given in the legends of the figures.

* This paper is dedicated to Prof. Dr. O. Hoffmann-Ostenhof on the occasion of his 60th birthday.

## 3. Results

The action of bee venom phospholipase $A_2$ on liposomes prepared from egg lecithin is shown in fig. 1A. In the presence of melittin (upper curve) a marked increase in the initial rate and the extent of hydrolysis can be observed. In the parallel experiment with dipalmitoyllecithin, a phospholipid which does not interact with melittin at room temperature, no stimulatory effect could be detected (fig. 1B). For these experiments, melittin and phospholipase $A_2$ were used in a weight ratio of 10:1 which is about the ratio found in bee venom [7].

The stimulation observed with egg lecithin has been investigated further by varying the molar ratio of melittin to phospholipid. In fig. 2 the initial rate of hydrolysis is plotted versus the relative amount of melittin, with all other reaction components being held constant. Already small amounts of melittin (melittin to lipid ratio of 1:2500) stimulate the reaction to a measurable extent. This effect increases with the amount of melittin added until at a melittin to lipid ratio of 1:150 a plateau is reached which corresponds to about a 5-fold stimulation over the reaction without melittin. Working with large amounts of melittin we also noted that our preparations of this peptide still contain traces of phospholipase (see curve A of fig. 2). We did not succeed in our attempts to further purify the peptide and tests with several commercial preparations of melittin have shown that these too contain varying amounts of phospholipase $A_2$. The impurity present in melittin does, however, not interfere with the interpretation of our results.

Comparing the effects observed with the different lecithins it appears that interaction of melittin with the phospholipid is a prerequisite for the enhanced hydrolysis. The possibility that phospholipase $A_2$ and melittin form a complex which is more active than the enzyme alone has also been considered. Preliminary attempts to detect such a complex in the ultracentrifuge have failed. Furthermore, the action of melittin in our assays might conceivably be due to the fact, that this peptide interacts with lysolecithins or free fatty acids and may thus release product inhibition. Added lysolecithin or oleic acid well above the amounts pres-

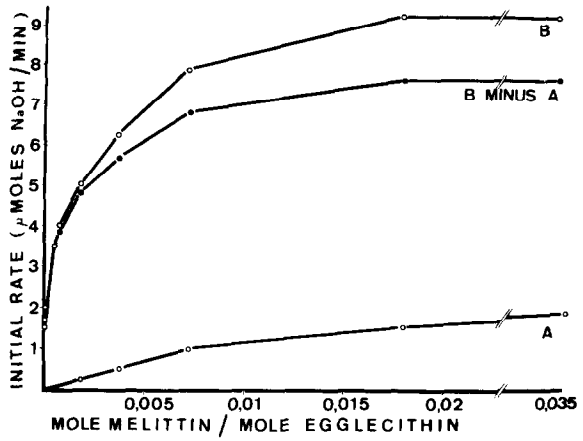


Fig. 2. Initial rate of egg lecithin hydrolysis by phospholipase at different melittin to lipid ratios. Conditions as in fig. 1. Curve A: Control without enzyme. The slow hydrolysis is due to traces of phospholipase present in our melittin preparations. Curve B: Hydrolysis in the presence of 20 μg enzyme. The difference between curves B and A yields the actual stimulation of the initial rate due to melittin. The data represent mean values of a series of four experiments.

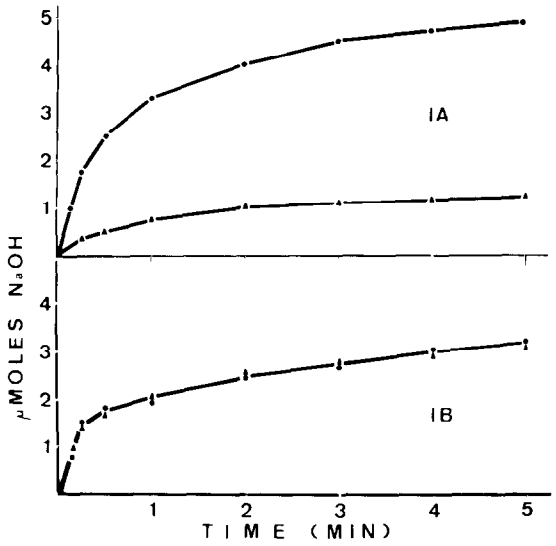


Fig. 1. Hydrolysis of lecithins by bee venom phospholipase $A_2$ in the presence and absence of melittin. Conditions: 10 μmoles lecithin, 20 μg enzyme, in 5 ml of 4 mM $CaCl_2$ adjusted to pH 8.0 with NaOH. The reaction was monitored by measuring the consumption of NaOH, which is equivalent to the amount of fatty acid liberated by enzymatic hydrolysis, versus time at room temperature. The substrate was egg lecithin in fig. 1A and dipalmitoyllecithin in fig. 1B. (–▲–) no melittin, (–●–) plus 200 μg (about 0.07 μmoles) melittin.

ent in the early phase of the reaction did, however, not influence the stimulatory action of melittin. Moreover, it has previously been shown [2] that the melittin–lecithin complex is tighter than the one with lysolecithin.

Using the same assay system we have also tested peptides isolated from the venom of two cobra species, which like melittin have been characterized as direct lytic factors. Both compounds could be shown to stimulate the reaction catalyzed by bee venom phospholipase $A_2$. Again higher rate of hydrolysis was observed only with egg lecithin as substrate (see fig. 3), but not with dipalmitoyllecithin (data not shown). On a molar basis both factors were about as effective as melittin in their ability to stimulate the phospholipase reaction.

Finally, a peptide from an entirely different source was tested: polymyxin B produced by the bacterium *Bacillus polymyxa.* Like melittin, polymyxin B has been shown to interact with phospholipids [4]. From the data shown in fig. 3 it is evident that this peptide too stimulates the action of the bee venom enzyme on ovolecithin liposomes, while this could not be observed with dipalmitoyllecithin.

## 4. Discussion

In this paper we show that lytic peptides isolated from diverse biological sources apparently act via similar mechanism on phospholipid liposomes. Melittin from bee venom, the direct lytic factors from two cobra venoms and the bacterial peptide polymyxin B all stimulate the action of bee venom phospholipase $A_2$ on liposomes prepared from egg lecithin. It is remarkable that, on a molar basis, the different compounds were found to be about equally effective.

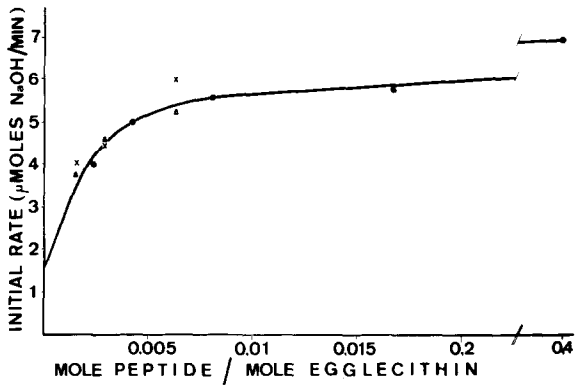


Fig. 3. Initial rate of egg lecithin hydrolysis by bee venom phospholipase $A_2$: Stimulation by direct lytic factor from cobra venom and by polymyxin B. (–x–) direct lytic factor from *Naja naja* venom, (–△–) direct lytic factor from *Haemachatus haemachates* venom, (–●–) polymyxin B. Molecular weights were taken as 7000 for the lytic factors and as 1200 for polymyxin B. In the absence of bee venom phospholipase no hydrolysis could be detected.

The fluidity of the phospholipid appears to be a critical factor in all instances, since no stimulatory effects could be observed when dipalmitoyllecithin was used as a substrate. At room temperature, this lipid is in a crystalline state, while that of egg lecithin is described as liquid–crystalline [8]. In case of melittin, this dependence on the fluidity can be interpreted on the basis of our earlier observations [2]. Formation of a complex between melittin and phospholipid, as measured by changes in the fluorescence of the single tryptophan residue, could be observed only above the temperature where transition from the crystalline to the liquid–crystalline state takes place. Complex formation thus appears to be closely linked with the ability to enhance the action of phospholipase. It is tempting to apply this interpretation to the other peptides as well and postulate specific interactions with phospholipids, which would in turn facilitate the attack by phospholipase. Indeed, polymyxin B has been shown to interact with lecithin [4].

Other interpretations for these results would seem to be less likely. For instance, the fact that peptides of widely differing structure yield such similar effects argues strongly against specific peptide–enzyme interactions. The proposal by Vogt et al. [1] that the direct lytic factor reacts primarily with membrane proteins through formation of disulfide bridges is clearly not applicable to melittin and polymyxin B, since these are both devoid of sulfur-containing amino acids. The fact that the factors from snake venom are also active in our assay with liposomes containing no proteins argues against such a mechanism. We prefer the interpretation that peptide–phospholipid interaction is primarily responsible for the effects observed with liposomes and probably with cell membranes as well.

Considering the role of such peptides in nature, two main aspects of their action may be distinguished: 1) Through interaction with the phospholipids of cell membranes the organization of these biological struc-

tures will be disturbed which can in turn lead to e.g. release of biogenic amines, cell lysis etc. 2) Due to this interaction a membrane becomes more susceptible to the action of exogenous or endogenous (unpublished results) phospholipases. In the presence of such an enzyme, lysophospholipids are thus rapidly generated. Peptides with these properties have apparently been 'discovered' several times during evolution, with melittin, direct lytic factor and polymyxin B being just three out of a larger number of such compounds (see for instance ref. [9]). Yet, inspite of the structural differences, a common mechanism of action may exist.

## References

[1] Vogt, W., Patzer, P., Lege, L., Oldigs, H.-D. and Wille, G. (1970) Naunyn-Schmiedebergs Arch. Pharmak. 265, 442–454.
[2] Mollay, C. and Kreil, G. (1973) Biochim. Biophys. Acta 316, 196–203.
[3] Fryklund, L. and Eaker, D. (1973) Biochemistry 12, 661–667.
[4] Pache, W., Chapman, D. and Hillaby, R. (1972) Biochim. Biophys. Acta 255, 358–364.
[5] Kreil, G. and Kreil-Kiss, G. (1967) Biochem. Biophys. Res. Commun. 27, 275–280.
[6] Shipolini, R. A., Callewaert, G. L., Cottrell, R. C., Doonan, S., Vernon, C. A. and Banks, B. E. C. (1971) Eur. J. Biochem. 20, 459–468.
[7] Habermann, E. (1972) Science 177, 314–322.
[8] Ladbrooke, B. D., Williams, R. M. and Chapman, D. (1968) Biochim. Biophys. Acta 150, 333–340.
[9] Hessinger, D. A. and Lenhoff, H. M. (1973) Biochem. Biophys. Res. Commun. 53, 475–481.