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ACTION OF PHOSPHOLIPASES ON THE CYTOPLASMIC MEMBRANE OF *ESCHERICHIA COLI*

STIMULATION BY MELITTIN*

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SUMMARY

The emission maximum of the single tryptophan residue of melittin was measured in the presence of phosphatidylethanolamine liposomes and *Escherichia coli* cytoplasmic membranes. In both cases, the fluorescence maximum was shifted to shorter wavelengths indicating a transfer of the indole ring to an apolar environment.

E. coli membranes were labelled in position 2 of their phospholipids with [^{14}C]oleic acid. These membranes were used for measuring the activity of an endogenous phospholipase A_2. A slow hydrolysis is observed, which can be accelerated by adding melittin. The extent of the stimulation depends on the molar ratio of melittin to membrane phospholipid. Under suitable conditions, the initial rate of hydrolysis is six to seven times higher in the presence than in the absence of melittin.

The action of the phospholipase A_2 from bee venom is also stimulated by melittin. An identical stimulation was observed with either *E. coli* membranes or pure phosphatidylethanolamine liposomes as substrate.

INTRODUCTION

Melittin, the main constituent of bee venom, is known to be a membrane-active peptide. It was discovered through its potent cell-disrupting activity, which can be measured conveniently, e.g. as lysis of red blood cells [1]. Sessa et al. [2] demonstrated that melittin causes release of marker ions from phosphatidylcholine liposomes, suggesting that, in natural membranes, the target of melittin is also phospholipids. Experiments using spin labelling [3, 4] and infrared spectra [4] also indicate a distinct melittin-phospholipid interaction. The hydrophobic nature of part of this interaction has been demonstrated by fluorescence measurements [5], which

* Some of these results have been presented at the 18th International Conference on the Biochemistry of Lipids (Graz, July 1975).

show that the single tryptophan residue, and presumably other hydrophobic regions of melittin, penetrates the apolar interior of a lipid bilayer.

The interaction of melittin with phospholipids appears to cause a synergistic effect on the activity of another venom component; phospholipase A_2 [6, 7]. We have shown earlier that a prerequisite for the stimulation of phospholipase A_2 activity is the binding of melittin to phosphatidylcholine liposomes [7]. These studies have now been extended using a natural membrane as substrate. Melittin forms a complex with *Escherichia coli* cytoplasmic membranes, which in turn stimulates the activity of an endogenous as well as an added phospholipase A_2.

MATERIALS AND METHODS

(1) *Bee venom components*

Experiments with radioactive phospholipids as substrate have shown that melittin previously prepared by us, as well as all commercially available samples we have tested, still contain traces of bee venom phospholipase A_2. The following method describes the preparation of phospholipase-free melittin. 1 g lyophilized bee venom (Firma Mack, Illertissen) was dissolved in 10 ml water, 75 mg sodium sulphite were added and the pH adjusted to 8.0 with ammonia. This treatment reduces the disulfide bonds present in bee venom phospholipase A_2 [8] while melittin, which does not contain cysteine [9], remains unaffected. After standing at room temperature for 10 min, the solution was centrifuged and the supernatant extracted twice with 5 ml *n*-butanol. The combined extracts were dried, redissolved in 2 ml water and applied to a column (40×0.8 cm) of Dowex 1×2 in the bicarbonate form. Melittin was eluted with water and the eluate lyophylized.

The sample of synthetic melittin was a present of Dr. Lübke (Schering AG, Berlin). Highly purified bee venom phospholipase A_2 was a gift of Dr. R. A. Shipolini (London).

(2) *Preparation and analysis of E. coli cytoplasmic membranes*

E. coli, strain K-1060, was grown in 4 l batch cultures using a medium described by Vogel and Bonner [10] with glycerol as carbon source. To each culture, 20 mg of oleic acid and 1 mCi [1-^{14}C]oleic acid (The Radiochemical Centre, Amersham) were added. The cells were harvested at the end of the log phase.

Membranes were isolated following the procedure of Kaback [11] and purified by isopycnic sucrose gradient centrifugation [12]. A typical membrane preparation contained 110 mg protein/100 mg phospholipids with a specific activity of 1.43 Ci/mol phospholipids.

The distribution of the labelled fatty acid was determined by extensive hydrolysis of the membranes with bee venom (0.05 mg bee venom/mg phospholipid, incubated for 3 h). Lipid fractions from the digest and from untreated membranes were applied to thin-layer silica gel sheets (Polygram Sil G, Macherey-Nagel) and developed with $CHCl_3/CH_3OH/H_2O$, 96:36:6 (v/v). Strips containing compounds detected by I_2 vapour were cut out and the radioactivity measured in a liquid scintillation counter.

Bacterial phospholipases(s) were characterized by incubating the membrane

with melittin (1 mol peptide per 100 mol phospholipid) for 3 h and the distribution of label was analyzed as above.

(3) *Isolation of labelled E. coli phosphatidylethanolamine*

The supernatants obtained during the membrane preparation were lyophilized, and from these residues total lipids were extracted [13] and fractionated by silica gel chromatography [14]. Two fractions containing about 95 % phosphatidylethanolamine and some phosphatidylglycerol were collected. These fractions with specific activities of 2.05 and 2.81 Ci/mol were used for the experiments with liposomes.

(4) *Phospholipase assay*

The reaction mixture had the following composition: 10 μl 0.1 M Tris · HCl (pH 8.0), 10 μl 0.001 M EDTA, 10 μl 0.04 M $CaCl_2$, 10 μl 0.01 M KCl, phospholipase and/or melittin and water to a total volume of 70 μl. The reaction was started by adding 30–60 nmol (membrane) phospholipid in 10 μl water. Samples were incubated at 37 °C and the hydrolysis was terminated by adding 350 μl of $CHCl_3$/CH_3OH/CH_3COOH, 1:2:0.5 (v/v). After a further addition of 100 μl $CHCl_3$ and 50 μl H_2O, the phases were allowed to seperate [13]. The chloroform phase was concentrated in a stream of N_2 and applied, together with unlabelled oleic acid as carrier, to silica gel thin-layer sheets. Free fatty acids were separated from phosphatides by developing with light petroleum/ether/acetic acid, 70:30:1 (v/v). Strips were cut out and the amount of radioactivity in the respective fractions was measured. In all experiments, zero-time blanks were subtracted. These were usually about 1 % of the total label.

(5) *Fluorescence measurements*

Fluorescence spectra were measured on a Perkin-Elmer MPF-3L fluorimeter as described previously [5].

RESULTS

(1) *Fluorimetric measurements*

E. coli membranes contain phophatidylethanolamine as the main phospholipid. In a first series of experiments we determined whether melittin and this phospholipid interacted in the same way as melittin and phosphatidylcholine [5]. Upon addition of *E. coli* or egg phosphatidylethanolamine liposomes to a melittin solution, a blue-shift in the emission maximum of the single tryptophan residue takes place.

A similar change of the tryptophan fluorescence occurs after adding melittin to a suspension of *E. coli* membranes. Fig. 1 shows the emission spectra of the individual components, their superposition and the spectrum of the mixture. The latter spectrum has a maximum shifted to shorter wavelengths compared with the melittin and the addition spectrum. This indicates a hydrophobic interaction of melittin with a complex biomembrane, probably with its phospholipid components. Spectral shifts have previously been observed when melittin was added to a suspension of erythrocyte ghosts [5].

(2) *Characterisation of the labelled E. coli membrane*

E. coli K-1060 is a mutant deficient in the biosynthesis and degradation of

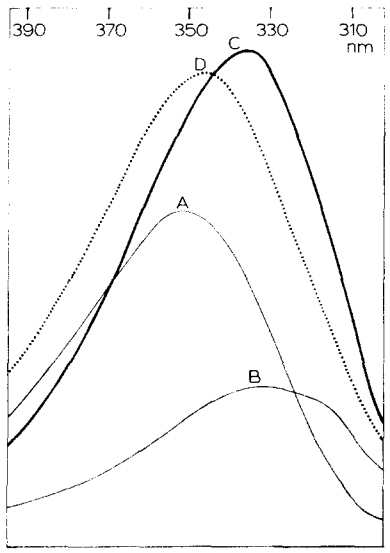

Fig. 1. Fluorescence spectrum of melittin in the absence and presence of *E. coli* cytoplasmic membranes. Melittin (8 nmol) and membranes (equivalent to 80 nmol phospholipid) were incubated in 3 ml buffer [5]. (A) Melittin; (B) membranes; (C) mixture of melittin and membranes; (D) addition spectrum A+B.

unsaturated fatty acids. Cytoplasmic membranes from this strain, grown in the presence of [^{14}C]oleic acid, were extensively digested with bee venom, which contains as the only lipolytic enzyme a phospholipase A_2 [1]. Fractionation of the digest shows that more than 95 % of the radioactivity is found with the free fatty acids and virtually none with the lysophosphatides (see Table I). As expected the [^{14}C]oleic acid was almost exclusively incorporated into position two of the phospholipids. Identical results have been obtained with phosphatidylethanolamine isolated from labelled membranes (data not shown).

Autodigestion of the *E. coli* membrane yields a more complex pattern (Table I).

TABLE I

DISTRIBUTION OF [^{14}C]OLEIC ACID IN THE LIPID FRACTIONS OF THE CYTOPLASMIC MEMBRANE OF *E. COLI*

Radioactive membranes were incubated with bee venom or with melittin only (autodigestion). The percentage of label present in the different chromatographic fractions is shown (details are described in Methods).

	Origin	Lyso-phosphatides	Phosphatidyl-glycerol	Phosphatidyl-ethanolamine	Free fatty acids*
Control	2	0	2	86	10
Bee venom digest	< 1	< 1	3	2	94
Autodigestion	< 1	13	2	39	45

* All label, migrating ahead of phosphatidylethanolamine.

After incubating the membranes for 3 h with melittin, which stimulates the endogenous enzyme(s), the hydrolysis was still incomplete. Of the radioactive products, about 75 % was free fatty acid and 25 % lysophospholipid. Assuming that no transacylation takes place and since all [^{14}C]oleic acid is found in position two, this means that an A_1-type phospholipase is also present. This is in agreement with earlier results of Albright et al. [15]. In our subsequent experiments we have measured only the amount of free oleic acid. This reflects mainly the activity of the phospholipase A_2, although some of the labelled fatty acid may be liberated through the sequential action of phospholipase A_1 and lysophospholipase A_2, which are both present in *E. coli* membranes [15].

(3) *Stimulation of membrane phospholipase A_2 by melittin*

On incubating cytoplasmic membranes from *E. coli* with Ca^{2+} at pH 8.0,

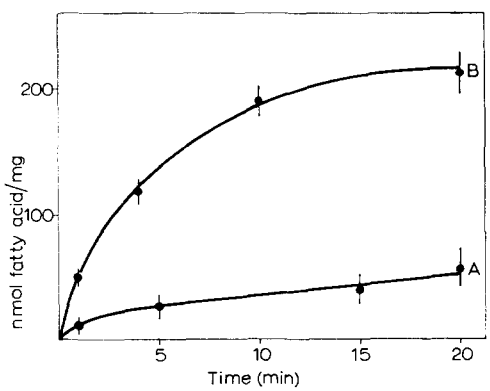

Fig. 2. Activity of the membrane phospholipase A_2. Cytoplasmic membranes (40 nmol membrane phospholipid) were incubated alone or in presence of melittin (0.13 nmol). (A) Membranes alone; (B) membranes plus melittin.

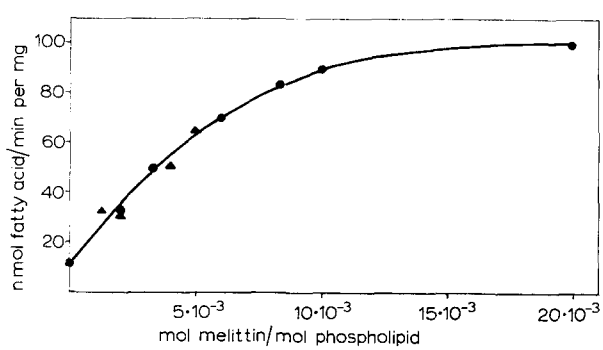

Fig. 3. Initial rate of hydrolysis by membrane phospholipase A_2 at different melittin to phospholipid ratios. Cytoplasmic membranes (30–60 nmol phospholipid) were incubated in the presence of varying amounts of melittin. Values obtained with natural (●) and synthetic (▲) melittin are shown.

free fatty acids are slowly liberated (Fig. 2). When melittin is added, the rate of hydrolysis increases considerably (Fig. 2, curve B).

The degree of stimulation is melittin-dependent, and this is illustrated in Fig. 3. Several values obtained with synthetic melittin coincide with those of the natural peptide. The increase in initial rate of phospholipid hydrolysis is about 2-fold at a ratio of 1 mol peptide to 700 mol membrane phospholipid. The curve reaches a plateau at a ratio of about 1:100 corresponding to a 6- to 7-fold stimulation over the activity of the membrane enzyme in the absence of melittin.

(4) *Stimulation of bee venom phospholipase A_2 by melittin*

Besides melittin, phospholipase A_2 is a prominent component of bee venom [1, 8]. The synergistic action of these two venom constituents in haemolysis and degradation of phosphatidylcholine liposomes has been described [6, 7]. Table II illustrates the effect of melittin on the activity of venom phospholipase using either *E. coli* cytoplasmic membranes or liposomes as substrate. The initial rate of hydrolysis by the membrane enzyme and its stimulation by melittin are shown in lines A and C. Addition of venom phospholipase to the membrane suspension yields a definite increase in the respective rates, in both the absence and the presence of melittin (lines B and D of Table II). Because of the low amount of venom enzyme used for these experiments (molar enzyme-substrate ratio about $1:10^6$) we assume that the activities of endogenous and venom enzyme are additive. The net increase due to the latter enzyme can then be calculated. In the experiment shown in Table II, the small quantity of melittin (1:300) causes an approximately 2-fold stimulation of the activity of the venom enzyme. Using the same experimental conditions, the activity of venom enzyme on phosphatidylethanolamine liposomes was tested. Labelled oleic acid was liberated at a rate similar to the net rate observed with intact membranes. Moreover, the addition of an equal amount of melittin also leads to a stimulation by a factor of

TABLE II

EFFECT OF MELITTIN ON THE ACTIVITY OF BEE VENOM PHOSPHOLIPASE A_2

E. coli cytoplasmic membranes were used in Experiment I and phosphatidylethanolamine liposomes in Experiment II, each containing 56 nmol phospholipid. To these suspensions, melittin (0.19 nmol) and/or bee venom phospholipase A_2 (0.5 ng) was added. The percent of total label present in the fatty acid fraction was measured after incubating for 2 min. The mean values from three such experiments are shown in the table.

Experiment	Additions	% hydrolysis per min
I A	–	0.7
B	bee enzyme	2.75
C	melittin	2.2
D	bee enzyme + melittin	6.0
B–A	(net effect of bee enzyme)	2.05
D–C	(net effect of bee enzyme in presence of melittin)	3.8
II E	bee enzyme	1.85
F	bee enzyme + melittin	3.4

about 2 (lines E and F of Table II). The effect of melittin on the activity of venom phospholipase seems to be independent of the complexity of the substrate.

DISCUSSION

The interaction of polypeptides with lipids has received increasing attention in recent years [16–19]. The system melittin-phospholipid seems to be a simple prototype for such an interaction. In its primary sequence [9], this peptide contains an asymmetric arrangement of residues with apolar and basic side chains, and this arrangement has been rigidly conserved during evolution inspite of sequence differences found in the melittin of different honey bees [20].

The interaction of melittin with phospholipids has been measured directly by various physicochemical techniques [3–5], or indirectly through its effect on the activity of phospholipases [6, 7]. The present paper extends the latter type of measurements. Using the cytoplasmic membrane of *E. coli* K-1060 as substrate the activity of an endogenous phospholipase A_2 and the stimulation of its activity by melittin could be measured. A 6- to 7-fold increase of the rate of hydrolysis could be observed at a ratio of about 1 mol melittin per 100 mol phospholipid. This is a much lower ratio than is required, for example, for the detergent Triton X-100 [21]. It is noteworthy that the maximal stimulation is already observed at a melittin concentration which does not yet cause lysis of erythrocytes [22].

In a second set of experiments, the enzymatic hydrolysis of a biological membrane or pure phosphatidylethanolamine liposome by bee venom phospholipase A_2 was studied. The stimulatory effect of melittin with either substrate was virtually identical; a surprising result in view of the complexity of a biomembrane. Apparently, in this case the action of melittin is based solely on its interaction with phospholipids.

The following picture emerges from these and earlier data.

(1) Melittin readily interacts with different types of phospholipids, whereby a partial transfer of the molecule into the apolar interior of the lipid bilayer takes place; for this interaction it seems to be irrelevant whether the phospholipids are part of a complex biomembrane or a simple liposome.

(2) As a consequence of this interaction an as yet unknown parameter of the lipid bilayer is changed, which results in an increased susceptibility towards phospholipases. With both membranes and liposomes, the effect is dependent on the amount of melittin and a maximal enzymatic hydrolysis is observed at melittin-phospholipid ratios of about 1:100. This indicates that the state of a certain number of phospholipid molecules in the vicinity of the bound peptide is changed. Obviously, much further physicochemical information will be needed to allow a more detailed description of the melittin-phospholipid interaction and its consequences.

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