The bee venom melittin induces lysis of arthropod granular cells and inhibits activation of the prophenoloxidase-activating system

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The bee venom melittin induced degranulation and lysis of isolated granular cells from freshwater crayfish at a concentration of 3.5 μM. Melittin also efficiently blocked β1,3-glucan activation of the prophenoloxidase-activating system present in the blood cells of arthropods. Therefore, it is suggested that melittin in the bee venom may function as a toxin towards parasitic or predatory insects on bees and the bee community.

Arthropod immunity *Prophenoloxidase* *Arthropod blood-cell lysis*

1. INTRODUCTION

The effects of a sting from the honey bee (*Apis mellifera*) are well known [1]. The protein components of bee venom are melittin (50% dry wt), phospholipases A (12%), apamin (3%), 'mast cell degranulating factor' (3%) and hyaluronidase (3%) [2,3]. Melittin is suggested to be responsible for the acute effects and pain in animal skin after a bee sting [1] and is a 26-amino-acid amphiphilic peptide which interacts with phospholipids in a voltage-dependent manner [4]. Some studies have been done on both in vivo and in vitro effects of melittin in insects [5,6]. Acetylcholinesterase of *Drosophila* was found to be noncompetitively inhibited by melittin and Mitchell et al. [5] suggested that this toxin also disrupted the membranes in general in the insect haemolymph.

Recently we have provided evidence that the so-called prophenoloxidase activating (proPO) system present in the blood cells of arthropods (insects and crustaceans) is an important recognition system in these invertebrates [7,8]. We have likened this recognition system to vertebrate complement, since the proPO system in arthropod haemolymph is specifically activated by β1,3-glucans [9–11], bacterial cell walls [12,13] or lipopolysaccharides [14]. Furthermore, the proPO system has been shown to exert several of the biological functions of vertebrate complement such as to produce opsonic [15–17], fungistatic [18] and lytic and degranulating factors [19].

In a recent article, Prince et al. [1] proposed that the acutely painful melittin is aimed at mammalian predators on the bee community, and that the other constituents of the bee venom such as apamin, phospholipases, hyaluronidase and the catecholamines are toxic to other arthropods such as predatory wasps and the parasitic wax moths. The haemocyte types of crustaceans and insects are very similar [20] and activation of the proPO system in arthropods appears to be more or less identical [20] and since no studies have been done on the effects of melittin on arthropod blood cells or the proPO system, we report here that melittin lyses crayfish granular cells and prevents activation of the proPO system.

2. MATERIALS AND METHODS

The crayfish *Pacifastacus leniusculus* were maintained and housed as described [4]. The granular cells of crayfish haemolymph were isolated according to Söderhäll and Smith [21]

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with a slight modification as in [19]. Monolayers of the isolated granular cells were prepared as described [19].

The effect of melittin on the granular cell monolayers was studied by adding different concentrations of melittin (150 μl) dissolved in crayfish saline, CFS [19], to the monolayers, incubating the cells with melittin usually for 5 min at 20°C and then immediately fixing the cells with 10% neutralized formalin. The degree of degranulation and lysis was then estimated under phase contrast optics. At least 300 cells and 3 replicates were run for each experiment. The effect of certain drugs, known to inhibit degranulation of granular cells [20], was tested by first incubating the cells with the drug for 15 min at 20°C prior to the addition of melittin. A crude proPO system from the haemocytes was made as detailed in Söderhäll and Häll [14] and phenoloxidase activity was measured spectrophotometrically by recording the formation of dopachrome from L-dihydroxyphenylalanine, L-dopa [14].

Activation of the proPO system by laminaran, a β1,3-glucan (Calbiochem), was as described in [14]; after preincubating 50 μl laminaran (1 mg/ml) or 50 μl melittin (0.5 mg/ml) with 50 μl crude proPO system for 15 min at 20°C 50 μl L-dopa (3 g/l) was added. The reaction was followed usually for 20 min at 20°C. The effect of melittin on β1,3-glucan activation of the proPO system was studied by preincubating 50 μl melittin (0.5 mg/ml), 50 μl laminaran (1 mg/ml) and 50 μl crude proPO system for 15 min at 20°C after which the enzyme reaction was initiated by adding 50 μl L-dopa. Controls in which melittin or laminaran were substituted with water were always run in parallel.

Chemicals were obtained as follows: SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid, disodium salt, Fluka, Buchs; calmidazolium R24571, Janssen Pharmaceutica, Beerse, Belgium; laminaran, Calbiochem, La Jolla, CA; percoll, Pharmacia, Uppsala; melittin and phospholipase, Sigma. All other chemicals were of the highest grade commercially available.

3. RESULTS

To ensure that melittin was responsible for degranulating and lysing the granular cell monolayers, phospholipase A in a concentration of 30 units was added to each cell monolayer, since the commercial preparation of melittin contains at most ~20 units phospholipase A per mg solid. The final concentration of phospholipase A on the monolayers in the melittin experiments therefore never exceeded 2 units. Phospholipase A at a concentration of 30 units had no effect on the granular cells.

The effect of melittin on the granular cells was then investigated. After incubation of cells with different concentrations of melittin for 5–10 min and then assaying cell viability by trypan blue exclusion, it was found that melittin first rapidly degranulated the cells which was followed by a subsequent lysis. On these monolayers of granular cells melittin was active in a concentration of 1.6 μg per coverslip (3.5 μM) (fig.1). Of the compounds tested only the anion channel blocker SITS partially prevented degranulation and subsequent lysis of the granular cells in the presence of melittin, whereas calmidazolium was without effect (table 1). If melittin was incubated with a haemocyte lysate containing the proPO system and then an elicitor of prophenoloxidase activation, a β1,3-glucan was added, no or only slight induction of phenoloxidase activity was observed and the inhibition was more than 80% (table 2). Melittin was efficient in blocking proPO activation by laminaran at 35 μM. That melittin inhibited the conversion of the proenzyme to active phenolox-

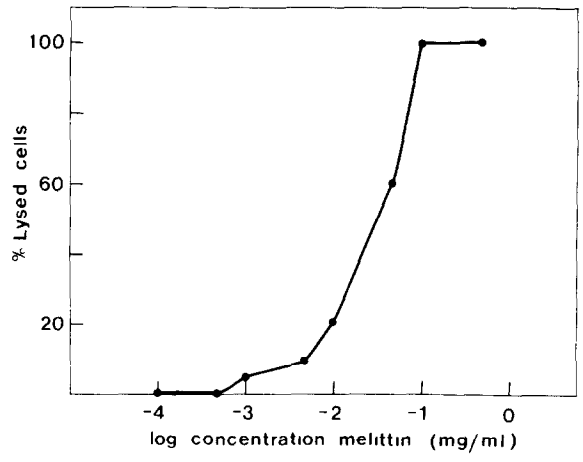

Fig.1. Concentration-dependent lysis of isolated crayfish granular cells by melittin.

Table 1

Effect of calmidazolium and SITS on melittin-induced degranulation and lysis of crayfish granular cells

Treatment of granular cell monolayer	Degranulated and lysed cells (%)
Crayfish saline	1.5 ± 1.7 (26)
Crayfish saline + melittin (16 μg)	99.4 ± 1.1 (25)
SITS (1 mM) + melittin (16 μg)	23.6 ± 17.3 (9)
Calmidazolium (10 μM) + melittin (16 μg)	91.1 ± 8.4 (7)

Values are means ± SD and in parentheses the number of experiments

Table 2

Effect of melittin on proPO activation by a β1,3-glucan, laminaran

Treatment of the proPO system	Phenoloxidase activity (ΔA_{490}/min per mg protein)
Control, H_2O	0.55 ± 0.06 (5)
Melittin (0.16 mg/ml) + laminaran (0.33 mg/ml)	0.96 ± 0.08 (5)
Melittin (0.16 mg/ml)	0.52 ± 0.10 (5)
Laminaran (0.33 mg/ml)	2.93 ± 0.31 (6)

idase and not merely phenoloxidase activity is shown by the following experiment. A proPO system preactivated with laminaran and assayed for phenoloxidase activity was incubated with melittin (0.16 mg/ml) for 10 min at 20°C, but no reduction in phenoloxidase activity was observed.

4. DISCUSSION

Melittin induced rapid degranulation and lysis of isolated crayfish granular cells. The mechanism by which this occurs cannot be elucidated from the present results. Terwilliger et al. [22] have suggested that melittin causes membrane breakage by partial penetration of the toxin into the membrane and Tosteson and Tosteson [4] demonstrated that melittin formed channels in lecithin bilayers which were more permeable to anions than to cations. Bhakoo et al. [23] found that cod and sheep erythrocytes lysed in the presence of melittin at similar concentrations of the toxins as for crayfish granular cells. The anion channel blocking agent, SITS, delayed lysis of the granular cells (table 1) and this may be consistent with the results of Tosteson and Tosteson [4] that the pores formed by melittin in membranes are more permeable to anions.

Melittin also nearly completely inhibited activation of prophenoloxidase by the β1,3-glucan elicitor, laminaran. This means that this toxin has dual functions in arthropod haemolymph, first to lyse the granular cells, which contain most of the proPO system [19] and, second, to prevent activation of the complement-like system the proPO cascade by microbial polysaccharides. The first function is probably not very lethal to the arthropods, since it is possible to deplete the haemocyte number nearly totally in crustaceans [24] and totally in the primitive arthropod *Limulus polyphemus* (Levin, personal communication). Therefore, it may be a correct statement by Prince et al. [1] to conclude that other substances than melittin are aimed at other predatory arthropods, but on the other hand since melittin very efficiently inhibited activation of prophenoloxidase, a component of a recognition system in arthropods, shows that melittin is a toxin for arthropods.

Stoltz and Cook [25] recently showed that parasitic wasps injecting their eggs into a host insect also inject a substance which prevents activation of prophenoloxidase. It is therefore tempting to suggest that the compound associated with egg-laying by parasitic wasps into their host insects may well be melittin or a similar compound. This speculative proposal, however, remains to be demonstrated.

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