

MELITTIN, the predominant fraction of bee venom proteins, was studied in an experimental model of human neutrophil granulocytes to reveal its influence on eicosanoid release, metabolism and receptor function in relation to intracellular calcium metabolism. Melittin ( $2 \mu\text{mol/l}$ ) was as potent as the calcium ionophore A23187 ( $10 \mu\text{mol/l}$ ) for activation of 5-lipoxygenase, releasing arachidonate only from phosphatidyl-choline and phosphatidyl-ethanolamine of cellular membranes, as judged from the decreases in radioactivity by 15.4% and 30.5%, respectively. The mechanism responsible for the release of arachidonate from cellular membranes is closely coupled to cellular calcium metabolism, and melittin was found to promote calcium entry through receptor gated calcium channels, probably due to an activation of phospholipase  $A_2$ . Furthermore, a down-regulation of leukotriene  $B_4$  receptors was seen. The maximal number of binding sites per cell was reduced from a median of 1520 to 950 with melittin ( $1 \mu\text{mol/l}$ ). The study has revealed some factors important for the inflammatory mechanisms mediated by melittin.

**Key words:** Arachidonic acid, Calcium, Cytosol, Ionophores, Leukotrienes, Melittin, Neutrophils, *N*-formylmethionine-leucyl-phenylalanine, Phospholipids, Receptors

## Arachidonic acid and calcium metabolism in melittin stimulated neutrophils

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### Introduction

Melittin is a polypeptide toxin which constitutes more than 50% of bee venom proteins.<sup>1</sup> It has been claimed to release endogenous arachidonic acid (AA) from cultured cells and to increase formation of prostanoids through activation of membrane bound enzymes.<sup>2–4</sup> Further, melittin was found to be able to stimulate exogenous non-incorporated AA metabolism in human polymorphonuclear neutrophils (PMN),<sup>5</sup> and recent research has dealt with the interaction between melittin and cellular membranes.<sup>6</sup>

The aims of the present work were: (1) to assess if melittin was a stimulator of endogenous AA metabolism in purified human PMNs; (2) to compare its potency with that of the calcium ionophore A23187; (3) to reveal where in the phospholipid pool AA was mobilized by melittin challenge; (4) to evaluate its influence on cellular calcium metabolism; and finally (5) to investigate its possible action on surface leukotriene  $B_4$  receptors.

### Materials and Methods

In six experiments neutrophils were isolated from EDTA-blood ( $0.2 \text{ mmol/l}$ ), with a recovery of 45%, and a purity of more than 95%, by: (1) methylcellulose (0.8%) sedimentation of erythrocytes; (2) washing and gradient centrifugation

of 'buffy coat' leukocytes according to Böyum;<sup>7</sup> and finally (3) hypotonic lysis of residual erythrocytes. Incorporation of  $1\text{-}^{14}\text{C-AA}$  ( $37 \times 10^3 \text{ Bq/ml}$ ,  $2.2 \times 10^9 \text{ Bq/mmol}$ ) (Amersham International, UK) with labelling of intracellular pools of phospholipids proceeded for 5 h at  $37^\circ\text{C}$  under 5% carbon dioxide and 95% atmospheric air in RPMI 1640 ( $5 \times 10^6$  cells) (5% autologous serum).<sup>8</sup> After removal of excess extracellular AA by washing, the cells were challenged with melittin ( $0.05\text{--}10 \mu\text{mol/l}$ ) for various lengths of time (Sigma Chemical Co., St Louis, MO, USA) or calcium ionophore A23187 (Calbiochem, La Jolla, CA, USA) ( $10 \mu\text{mol/l}$ ) for 15 min, which was found to be optimal from previous experiments.<sup>8</sup> Released eicosanoids were extracted with dichloromethane:methanol, 2:1; separated by thin layer chromatography, developing solvents 1: (supernatants), chloroform:methanol:acetic acid:water, 90:9:1:0.65; 2: (total cell suspensions), dichloromethane:methanol:2-propanol, 0.25% KCl:ethylacetate, 30:9:25:6:18; and quantified by autoradiography and laser densitometry.<sup>8</sup>

Identification of the radioactive spots was performed by co-chromatography with pure standards of phospholipids (Sigma Chemical Co.), 5-hydroxyeicosatetraenoic acid (5-HETE), leukotriene  $B_4$  ( $\text{LTB}_4$ ) (Paesel GmbH, Frankfurt am Main, Germany, and 12-hydroxy-heptadecatrienoic acid (HHT) (Upjohn Company, Kalamazoo, MI, USA). Further identification was performed by high-

performance liquid chromatography (HPLC) as earlier described.<sup>8</sup> The intra-assay coefficient of variation for release of AA metabolites was approximately 15%.<sup>8</sup>

The viability of the PMNs were assessed by the trypan blue exclusion technique. In six separate experiments PMNs ( $5 \times 10^6/\text{ml}$ ) were incubated with Fura-2/am (Sigma Chemical Co.) ( $2 \mu\text{mol/l}$ ) for 15 min. The cells were centrifuged, washed twice, and resuspended. Fluorescence was recorded with a Hitachi 4000 fluorescence spectrophotometer. For the calibration of Fura-2 fluorescence as a function of  $(\text{Ca}^{2+})_i$ , we used digitonin (10 mg/ml) to obtain a maximum fluorescence signal,  $F_{\text{max}}$ , followed by the addition of EGTA for determination of  $F_{\text{min}}$ . Intermediate values for  $\text{Ca}^{2+}$  corresponding to an intracellular Fura-2 fluorescence,  $F$ , were calculated by the equation:  $\text{Ca}^{2+} = Kd (F - F_{\text{min}}/F_{\text{max}} - F)$ , assuming an effective  $Kd$  of  $224 \text{ nmol/l}$ .<sup>9</sup> *N*-formylmethionine-leucyl-phenylalanine (fMLP) (Sigma Chemical Co.) was used in the concentration range  $10 \times 10^{-9} - 10^{-6} \text{ mol/l}$ .

In separate receptor studies duplicate suspensions of PMNs ( $10^7/\text{ml}$ ) were incubated with radioactive  $^3\text{H-LTB}_4$  (specific activity  $6.3-8.5 \times 10^3 \text{ GBq/mmol}$ , Amersham International, UK),  $0.1 \text{ nmol/l} - 2.5 \text{ nmol/l}$  at  $4^\circ\text{C}$  for 60 min. Following incubation, the cells were rapidly centrifuged through a precooled oil phase.<sup>9</sup> The tips of the tubes were cut off, and cell bound radioactivity was determined in a tracer analytical scintillation counter with an automatic quench correction. Nonspecific binding was determined by adding a 1000-fold excess of non-radioactive  $\text{LTB}_4$  (Paesel GmbH, Germany).<sup>10</sup>

In specific experiments,  $1 \mu\text{mol/l}$  melittin was added to the cell suspensions at  $37^\circ\text{C}$  for 5 min. The cells were then rapidly cooled to  $4^\circ\text{C}$  and binding experiments were done. For estimation of the dissociation constant ( $Kd$ ) and receptor number per cell ( $B_{\text{max}}$ ) a Scatchard plot was applied.<sup>11</sup>

## Results

*Potential sources of arachidonic acid:* Optimal conditions for AA release and metabolism were  $2 \mu\text{mol/l}$  melittin (Table 1) for 10 min (Fig. 1), which resulted in the median release of  $415 \text{ Bq}/5 \times 10^6$  PMNs (range 289–618) compared to  $670 \text{ Bq}/5 \times 10^6$  PMNs (range 260–990) for A23187 ( $p < 0.05$ ).

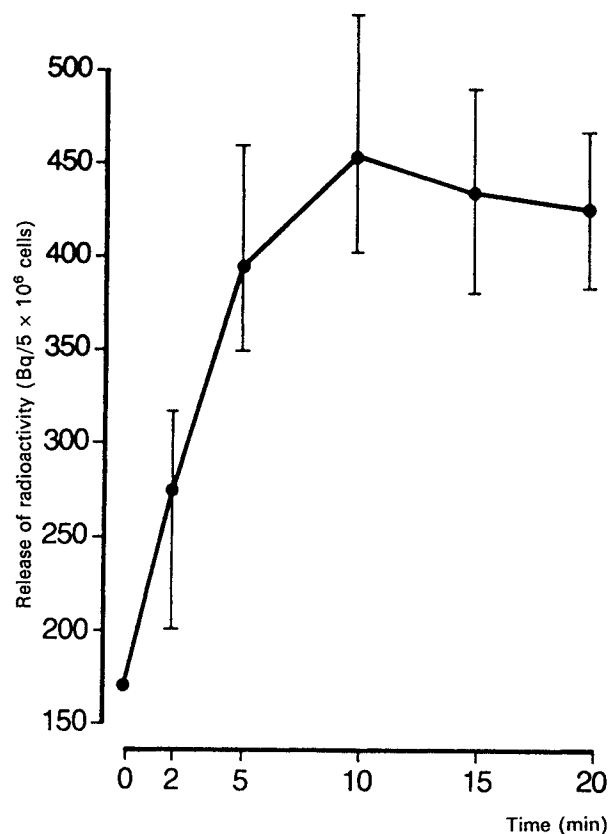


FIG. 1. Time course experiments for stimulation of  $1\text{-}^{14}\text{C}$ -arachidonic acid labelled neutrophils with melittin ( $2 \mu\text{mol/l}$ ). Values are given as medians with ranges for six experiments.

The radioactivity, when challenged with melittin ( $2 \mu\text{mol/l}$ , 10 min) or A23187 ( $10 \mu\text{mol/l}$ , 15 min), was distributed on eicosanoids and unmetabolized AA, as seen in Table 2. No significant differences occurred.

The substrate for formation of most of the AA following melittin stimulation was mobilized from phosphatidyl-choline (PC) and phosphatidyl-ethanolamine (PE), owing to the relative decreases in median radioactivity by 15.4% ( $p < 0.01$ ), and 30.5% ( $p < 0.01$ ), respectively (Fig. 2). No significant changes were found for phosphatidylinositol (PI) or phosphatidyl-serine (PS).

*Calcium mobilization:* Addition of melittin to PMNs caused a dose-related increase in intracellular  $\text{Ca}^{2+}$  ( $\text{Ca}^{2+})_i$  concentrations (Fig. 3). This rise was

**Table 1.** Dose response for release of radioactivity (eicosanoids and unmetabolized arachidonic acid) by melittin (15 min stimulation period) ( $n = 6$ ). Values for optimal stimulation was taken to 100% (equal to  $415 \text{ Bq}/5 \times 10^6$  PMNs). Medians with 95% confidence limits on medians are given

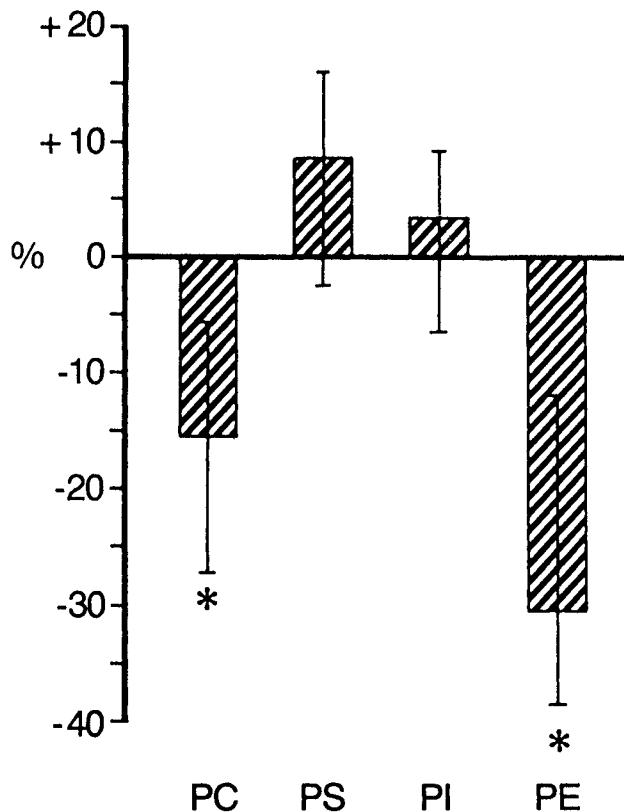
Concentration of melittin ( $\mu\text{mol/l}$ )	0.05	0.1	0.5	1	2	5	10
Radioactivity	46 (41–49)	48 (46–51)	52 (49–55)	77 (72–80)	100 (93–107)	68 (64–71)	74 (69–77)

**Table 2.** Percentage distribution of radioactivity on eicosanoids and unmetabolized arachidonic acid after stimulation with melittin (2  $\mu\text{mol/l}$ , 10 min) or A23187 (10  $\mu\text{mol/l}$ , 15 min). ( $n = 6$ ). Medians and ranges are given

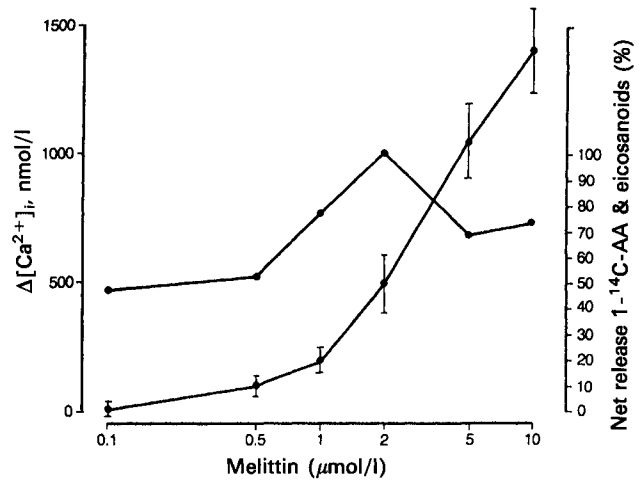
	Arachidonic acid	5-HETE	LTB <sub>4</sub>	HHT
Melittin	68.1 (63.7–76.7)	13.5 (11.3–14.7)	5.8 (1.5–6.8)	1.7 (1.4–2.4)
A23187	67.0 (56.2–80.4)	14.9 (9.5–19.4)	5.3 (2.8–9.6)	2.2 (1.0–5.3)

abolished in  $\text{Ca}^{2+}$ -free media, suggesting that a melittin-induced increase in  $(\text{Ca}^{2+})_i$  was mediated by an increase of the plasma membrane permeability to  $\text{Ca}^{2+}$ .

The characteristics of the calcium signal (time courses of changes in  $(\text{Ca}^{2+})_i$ ) generated upon activation of phospholipase A<sub>2</sub> (PLA<sub>2</sub>):(melittin) and phospholipase C (PLC):(LTB<sub>4</sub> and fMLP) of human PMNs are shown in Fig. 4. As can be seen from this figure the  $\text{Ca}^{2+}$  response pattern is similar after addition of LTB<sub>4</sub> or fMLP. This response is transient, as the rise in  $(\text{Ca}^{2+})_i$  in the bulk cytosol is due to a release of  $\text{Ca}^{2+}$  from intracellular stores. With melittin, however, the



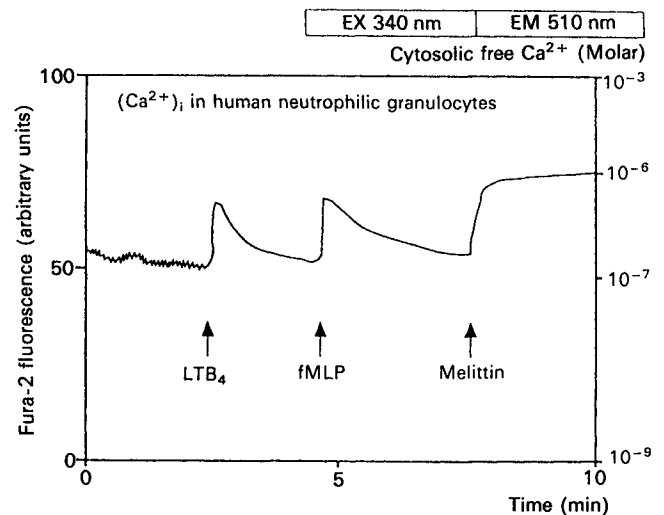
**FIG. 2.** Change in relative distribution of arachidonic acid in the different phospholipid fractions after stimulation with melittin (2  $\mu\text{mol/l}$ , 10 min). Phosphatidyl-choline (PC), phosphatidyl-serine (PS), phosphatidyl-inositol (PI), and phosphatidyl-ethanolamine (PE). Values are given as medians with ranges (bars) for six experiments. \* $p < 0.01$ .



**FIG. 3.** Release of radioactivity (eicosanoids and unmetabolized arachidonic acid) (●—●) (median values) and change in intracellular free  $\text{Ca}^{2+}$  [ $(\text{Ca}^{2+})_i$ ] (○—○) (median values and ranges) in human neutrophil granulocytes by the stimulation with melittin.

$\text{Ca}^{2+}$  rise shows a sustained phase which is due to influx of  $\text{Ca}^{2+}$  across the plasma membrane, and the effect is sustained as long as melittin is present in the medium. Validation of these results is supported by the experiments performed in a  $\text{Ca}^{2+}$ -free medium (Fig. 5). When extracellular  $\text{Ca}^{2+}$  was removed, the melittin-induced  $\text{Ca}^{2+}$ -influx was inhibited (Fig. 5). However, in a  $\text{Ca}^{2+}$ -free medium, LTB<sub>4</sub> and fMLP still result in a transient rise in free  $(\text{Ca}^{2+})_i$  (data not shown).

However, in a  $\text{Ca}^{2+}$ -free medium, if melittin is added before LTB<sub>4</sub>, the increase in cytosolic free calcium elicited by LTB<sub>4</sub> is completely inhibited (Fig. 5). To obtain a rise in cytosolic free  $\text{Ca}^{2+}$ , a higher concentration (50-fold) of LTB<sub>4</sub> is needed. Further, the  $\text{Ca}^{2+}$  rise ( $\text{Ca}^{2+}$  release from



**FIG. 4.** Time-course changes in fura-2 fluorescence and  $(\text{Ca}^{2+})_i$  in human PMNs following addition of LTB<sub>4</sub> ( $3 \times 10^{-10}$  mol/l), fMLP ( $0.1 \times 10^{-6}$  mol/l), and melittin ( $3.5 \times 10^{-6}$  mol/l). Typical trace of five experiments.

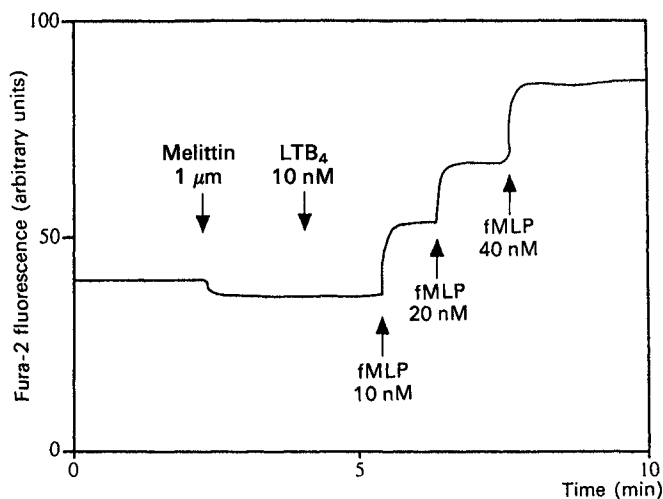


FIG. 5. Influence of melittin (1 μmol/l) on  $(Ca^{2+})_i$  after stimulation of human PMNs with  $LTB_4$  (10 nmol/l) or fMLP (10–40 nmol/l) in calcium free medium.

intracellular stores) induced by fMLP is unaffected by melittin.

To investigate whether inhibition of the  $Ca^{2+}$  signal by  $LTB_4$  paralleled a decrease in the number or affinity of  $LTB_4$  receptors, receptor ligand binding assay experiments were performed.

Figure 6 demonstrates specific binding (a) of  $LTB_4$  and the corresponding Scatchard plot, and (b) when PMNs were incubated with  $^3H$ - $LTB_4$  0.1–2.5 nmol/l. The nonspecific binding was linear with increasing ligand concentration. From the Scatchard plot it can be derived that the  $K_d$  is approximately the same, 0.95 nmol/l and 1.05 nmol/l in cell suspensions with and without melittin (1 μmol/l), respectively. However, melittin significantly reduced the maximal number of  $LTB_4$  binding sites per cell ( $B_{max}$ ) from 1520 to 950 under identical experimental conditions.

The viability of the PMNs after challenge with melittin or A23187 in the concentration ranges used was more than 97% as assessed by the trypan blue exclusion technique.

## Discussion

The data demonstrate that the bee venom polypeptide, melittin, is a potent stimulator of endogenous AA metabolism to mono- and di-hydroxy products, including  $LTB_4$ , in human PMNs which are of importance for inflammatory reactions.<sup>12,13</sup> Its potency regarding phospholipase stimulation in human PMNs is about 2/3 that of A23187, which is assumed to produce a maximal stimulation of phospholipases and synthesis of leukotrienes in response to calcium influx. However, regarding 5-lipoxygenase stimulation, an enzyme which has been shown to exhibit an absolute requirement of calcium ions,<sup>14</sup> melittin and A23187 were equipotent, as evaluated from the

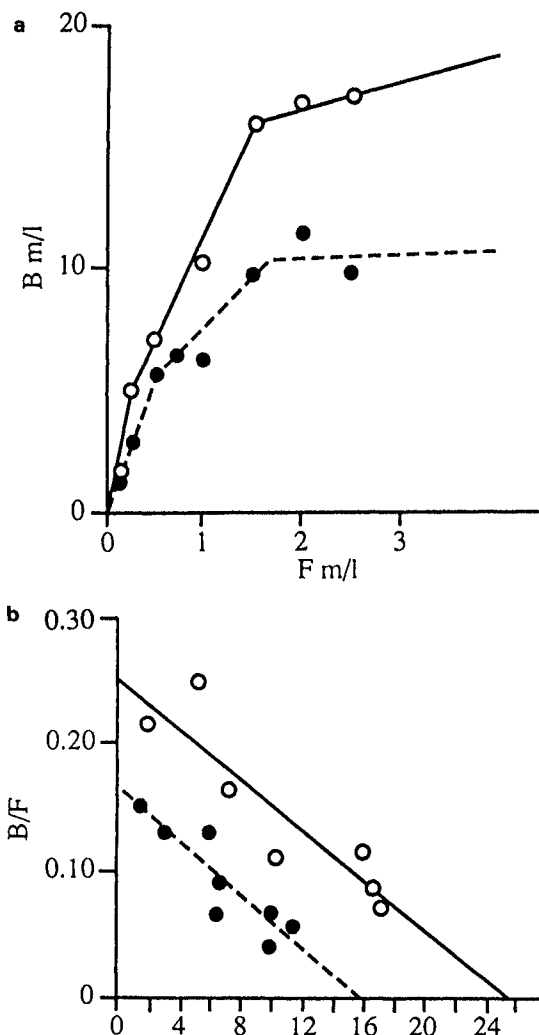


FIG. 6. Specific binding (a) and Scatchard plot (b) of neutrophils ( $10^7$  ml) incubated with  $^3H$ - $LTB_4$  (0.1–2.5 nmol/l) with (●): melittin (1 μmol/l) or (O) without melittin.

relative distribution of the eicosanoids  $LTB_4$  and 5-HETE. The characteristic responses of the PMNs to  $LTB_4$ , resemble those of the primary stimulus, fMLP, in the ability to promote a rapid accumulation of inositol trisphosphate ( $IP_3$ ) and calcium mobilization through the phospholipase C system.<sup>15–17</sup> Further, melittin stimulates cyclooxygenase, as evaluated from the production of HHT, with a similar potency as found for A23187.

Stimulation of  $PLA_2$  as well as PLC, which are essential for AA metabolism and which have been demonstrated in PMN membranes,<sup>18</sup> appears to involve cellular calcium.<sup>19</sup> Increase in  $(Ca^{2+})_i$  could be effected either by PLC mediated  $IP_3$  accumulation, or by  $Ca^{2+}$  influx through receptor operated (voltage independent)  $Ca^{2+}$  channels. If calcium dependent phospholipases are regulators of AA release, then agents that stimulate  $PLA_2$  might be expected to increase calcium availability. Melittin has earlier been described to stimulate phospholipase  $A_2$  in human leukocytes in the presence of

exogenous AA,<sup>5</sup> and an ability to alter membrane permeability to calcium has been further suggested.<sup>5</sup> The calcium antagonists, verapamil and nifedipine, at concentrations known to block classical voltage dependent calcium channels, had no effect on the  $Ca^{2+}$  influx induced by melittin, possibly due to receptor operated  $Ca^{2+}$  influx.<sup>9</sup> This is contrary to apamin, another toxin from bee venom, which affects the calcium channel function.<sup>6</sup> The possibility that melittin interferes with calcium influx via secondary messengers (i.e.  $IP_3$ ) is out of question, since there was no increase in  $(Ca^{2+})_i$  in calcium-free media. Further, melittin only released AA from PC and PE and not via the classical way, which involves PI, PE, PC as the three sources of AA.<sup>19</sup> It has been described earlier, that AA is mobilized from PC, PE, and PI using the calcium ionophore A23187.<sup>8</sup>

The present investigation has demonstrated that melittin increases the plasma permeability to calcium, and that cytosolic free  $Ca^{2+}$  is closely coupled to melittin induced activation of  $PLA_2$ . Further, melittin affects PMN surface  $LTB_4$  receptors, as it significantly reduces the maximal number of  $LTB_4$  binding sites per cell ( $B_{max}$ ). One possible explanation is that melittin generates  $LTB_4$  from PMNs.<sup>5</sup>  $LTB_4$  is then exported to the extracellular medium where it subsequently acts as a receptor agonist on the PMN surfaces with a resulting down-regulation of  $LTB_4$  receptors. Another explanation is that activators of protein kinase C (PKC), an important component of the signal transduction pathway in human PMNs, cause cells to become unresponsive to  $LTB_4$ , but not to fMLP.<sup>20</sup> However, in this respect melittin has earlier been found to be a PKC inhibitor.<sup>21</sup> Therefore melittin may act indirectly as an activator of PKC through AA release.<sup>22</sup> The latter (AA) is known to induce diacylglycerol (DAG) generation, which then activates PKC.<sup>23</sup> Since the production of AA is high following addition of melittin, we believe that bee venom via DAG is an indirect PKC-activator. This could explain the deactivation and the decrease in the expression of  $LTB_4$  receptors found in the present study, as explained above.<sup>20,23</sup>

In conclusion, melittin is a potent challenger of 5-lipoxygenase AA metabolism in human PMNs. Its potency regarding phospholipases stimulation is about 2/3 that of A23187 which is assumed to produce a maximal stimulation in response to calcium influx. Further, melittin mobilizes AA from PC and PE, whereas PI and PS seem to be unaffected, and melittin promotes  $Ca^{2+}$  entry through receptor gated  $Ca^{2+}$ -channels, probably due to a direct activation of phospholipase  $A_2$ . Receptor studies indicate that melittin further affects the total number of  $LTB_4$ -receptors, either by a down-regulatory mechanism or via the PKC

system. The present data provide evidence for the complicated mechanism of melittin, and its sensitivity to arachidonate metabolism, cellular eicosanoid receptors and intracellular calcium suggests that these factors may play a role for the inflammation mediated by melittin.

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