MELOTTIN, 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 µmol/l) was as potent as the calcium ionophore A23187 (10 µmol/l) for activation of 5-lypoxigenase, 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 A2. Furthermore, a down-regulation of leukotriene B4 receptors was seen. The maximal number of binding sites per cell was reduced from a median of 1520 to 950 with melittin (1 µmol/l). The study has revealed some factors important for the inflammatory mechanisms mediated by melittin.

**Keywords:** Arachidonic acid, Calcium, Cytosol, Ionophores, Leukotrienes, Melittin, Neutrophils, N-formylmethionin-leucyl-phenylalanine, Phospholipids, Receptors

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**Introduction**

Melittin is a polypeptide toxin which constitutes more than 50% of bee venom proteins. 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. Further, melittin was found to be able to stimulate exogenous non-incorporated AA metabolism in human polymorphonuclear neutrophils (PMN), and recent research has dealt with the interaction between melittin and cellular membranes.

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 B4 receptors.

**Materials and Methods**

In six experiments neutrophils were isolated from EDTA-blood (0.2 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; and finally (3) hypotonic lysis of residual erythrocytes. Incorporation of 14C- AA (37 × 10^6 Bq/ml, 2.2 × 10^9 Bq/mmol) (Amersham International, UK) with labelling of intracellular pools of phospholipids proceeded for 5 h at 37°C under 5% carbon dioxide and 95% atmospheric air in RPMI 1640 (5 × 10^6 cells) (5% autologus serum). After removal of excess extracellular AA by washing, the cells were challenged with melittin (0.05–10 µmol/l) for various lengths of time (Sigma Chemical Co., St Louis, MO, USA) or calcium ionophore A23187 (Calbiochem, La Jolla, CA, USA) (10 µmol/l) for 15 min, which was found to be optimal from previous experiments. 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.

Identification of the radioactive spots was performed by co-chromatography with pure standards of phospholipids (Sigma Chemical Co.), 5-hydroxyecosatetraenoic acid (5-HETE), leukotriene B4 (LTB4) (Paesel Gmbh, Frankfurt am Main, Germany, and 12-hydroxy-heptadecatrienoic acid (HHT) (Upjohn Company, Kalamazoo, MI, USA). Further identification was performed by high-

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**Arachidonic acid and calcium metabolism in melittin stimulated neutrophils**

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

The viability of the PMNs were assessed by the trypan blue exclusion technique. In six separate experiments PMNs (5 x 10⁶/ml) were incubated with Fura-2/am (Sigma Chemical Co.) (2 μmol/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 (Ca²⁺), we used digitonin (10 mg/ml) to obtain a maximum fluorescence signal, Fmax, followed by the addition of EGTA for determination of Fmin. Intermediate values for Ca²⁺ corresponding to an intracellular Fura-2 fluorescence, Fi, were calculated by the equation: Ca²⁺ = Kd (Fi - Fmin/Fmax - Fmin). Assuming an effective Kd of 224 nmol/l, N-formylmethionine-leucyl-phenylalanine (FMLP) (Sigma Chemical Co.) was used in the concentration range 10 x 10⁻⁹–10⁻⁶ mol/l.

In separate receptor studies duplicate suspensions of PMNs (10⁷/ml) were incubated with radioactive ³H-LTB₄ (specific activity 6.3–8.5 x 10⁵ Bq/mm, Amersham International, UK), 0.1 nmol/l–2.5 nmol/l at 4°C for 60 min. Following incubation, the cells were rapidly centrifuged through a precooled oil phase. 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 LTB₄ (Paesel Gmbh, Germany).

In specific experiments, 1 μmol/l melittin was added to the cell suspensions at 37°C for 5 min. The cells were then rapidly cooled to 4°C and binding experiments were done. For estimation of the dissociation constant (Kd) and receptor number per cell (Bmax) a Scatchard plot was applied.

**Results**

**Potential sources of arachidonic acid:** Optimal conditions for AA release and metabolism were 2 μmol/l melittin (Table 1) for 10 min (Fig. 1), which resulted in the median release of 415 Bq/5 x 10⁶ PMNs (range 289–618) compared to 670 Bq/5 x 10⁶ PMNs (range 260–990) for A23187 (p < 0.05).

The radioactivity, when challenged with melittin (2 μmol/l, 10 min) or A23187 (10 μmol/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 Ca²⁺ (Ca²⁺)i concentrations (Fig. 3). This rise was

| Concentration of melittin (μmol/l) | 0.05 | 0.1 | 0.5 | 1 | 2 | 5 | 10 |
|-----------------------------------|------|-----|-----|---|---|---|----|
| Radioactivity                     | 46   | 48  | 52  | 77 | 100 | 68 | 74 |
|                                  | (41–49) | (46–51) | (49–55) | (72–80) | (93–107) | (64–71) | (69–77) |

**FIG. 1.** Time course experiments for stimulation of 1⁴C-arachidonic acid labelled neutrophils with melittin (2 μmol/l). Values are given as medians with ranges for six experiments.
Table 2. Percentage distribution of radioactivity on eicosanoids and unmetabolized arachidonic acid after stimulation with melittin (2 μmol/l, 10 min) or A23187 (10 μmol/l, 15 min). (n = 6). Medians and ranges are given.

| Arachidonic acid | 5-HETE | LTB₄ | HHT |
|------------------|--------|------|-----|
| Melittin         | 68.1   | 13.5 | 5.8 | 1.7 |
| (63.7–76.7)      | (11.3–14.7) | (1.5–6.8) | (1.4–2.4) |
| A23187           | 67.0   | 14.9 | 5.3 | 2.2 |
| (56.2–80.4)      | (9.5–19.4) | (2.8–9.6) | (1.0–5.3) |

abolished in Ca²⁺-free media, suggesting that a melittin-induced increase in ([Ca²⁺])ₙ was mediated by an increase of the plasma membrane permeability to Ca²⁺.

The characteristics of the calcium signal (time courses of changes in ([Ca²⁺])ₙ generated upon activation of phospholipase A₂ (PLA₂): (melittin) and phospholipase C (PLC): (LTB₄ and fMLP) of human PMNs are shown in Fig. 4. As can be seen from this figure the Ca²⁺ response pattern is similar after addition of LTB₄ or fMLP. This response is transient, as the rise in ([Ca²⁺])ₙ in the bulk cytosol is due to a release of Ca²⁺ from intracellular stores. With melittin, however, the Ca²⁺ rise shows a sustained phase which is due to influx of Ca²⁺ 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 Ca²⁺-free medium (Fig. 5). When extracellular Ca²⁺ was removed, the melittin-induced Ca²⁺-influx was inhibited (Fig. 5). However, in a Ca²⁺-free medium, LTB₄ and fMLP still result in a transient rise in free ([Ca²⁺])ₙ (data not shown).

However, in a Ca²⁺-free medium, if melittin is added before LTB₄, the increase in cytosolic free calcium elicited by LTB₄ is completely inhibited (Fig. 5). To obtain a rise in cytosolic free Ca²⁺, a higher concentration (50-fold) of LTB₄ is needed. Further, the Ca²⁺ rise (Ca²⁺ release from

FIG. 2. Change in relative distribution of arachidonic acid in the different phospholipid fractions after stimulation with melittin (2 μmol/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 Ca²⁺ ([Ca²⁺])) in human neutrophil granulocytes by the stimulation with melittin.

FIG. 4. Time-course changes in fura-2 fluorescence and ([Ca²⁺])ₙ in human PMNs following addition of LTB₄ (3 × 10⁻¹⁰ mol/l), fMLP (0.1 × 10⁻⁶ mol/l), and melittin (3.5 × 10⁻⁶ mol/l). Typical trace of five experiments.
intracellular stores) induced by fMLP is unaffected by melittin.

To investigate whether inhibition of the $\text{Ca}^{2+}$ signal by LT$\text{B}_4$ paralleled a decrease in the number or affinity of LT$\text{B}_4$ receptors, receptor ligand binding assay experiments were performed.

Figure 6 demonstrates specific binding (a) of LT$\text{B}_4$ and the corresponding Scatchard plot, and (b) when PMNs were incubated with $^3\text{H}$-LT$\text{B}_4$ 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 LT$\text{B}_4$ binding sites per cell ($B_{\text{max}}$) 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 LT$\text{B}_4$, in human PMNs which are of importance for inflammatory reactions.$^{12,13}$ Its potency regarding phospholipases 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,$^{14}$ melittin and A23187 were equipotent, as evaluated from the relative distribution of the eicosanoids LT$\text{B}_4$ and 5-HETE. The characteristic responses of the PMNs to LT$\text{B}_4$, 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.$^{15-17}$ 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,$^{18}$ appears to involve cellular calcium.$^{19}$ Increase in ($\text{Ca}^{2+}$)$_i$ could be effected either by PLC mediated IP$_3$ accumulation, or by $\text{Ca}^{2+}$ influx through receptor operated (voltage independent) $\text{Ca}^{2+}$ 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...
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Exogenous AA,\textsuperscript{5} and an ability to alter membrane permeability to calcium has been further suggested.\textsuperscript{5} The calcium antagonists, verapamil and nifedipine, at concentrations known to block classical voltage dependent calcium channels, had no effect on the Ca\textsuperscript{2+} influx induced by melittin, possibly due to receptor operated Ca\textsuperscript{2+} influx.\textsuperscript{9} This is contrary to apamin, another toxin from bee venom, which affects the calcium channel function.\textsuperscript{6} The possibility that melittin interferes with calcium influx via secondary messengers (i.e. IP\textsubscript{3}) is out of question, since there was no increase in (Ca\textsuperscript{2+}), 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.\textsuperscript{19} It has been described earlier, that AA is mobilized in vivo via PI, PE, PC as the three sources of AA.\textsuperscript{19}

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\textsubscript{4}, but not to fMLP.\textsuperscript{20} However, in this respect melittin has earlier been found to be a PKC inhibitor.\textsuperscript{21} Therefore melittin may act indirectly as an activator of PKC through AA release.\textsuperscript{23} The latter (AA) is known to induce diacylglycerol (DAG) generation, which then activates PKC.\textsuperscript{23} 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\textsubscript{4} receptors found in the present study, as explained above.\textsuperscript{20,23}

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\textsuperscript{7} 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\textsuperscript{2+} entry through receptor gated Ca\textsuperscript{2+}-channels, probably due to a direct activation of phospholipase A\textsubscript{2}. Receptor studies indicate that melittin further affects the total number of LTB\textsubscript{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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