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MASTOPARAN, A WASP VENOM, AND MELITTIN, A BEE VENOM, ENHANCE PHAGOCYTOSIS IN MOUSE PERITONEAL MACROPHAGES

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To characterize the direct effects of wasp and bee venom peptides on mouse peritoneal macrophages, the effects of mastoparan and melittin on phagocytosis were examined. Mastoparan induced an enhancement of phagocytosis in a dose-dependnt manner up to $2x10^{-6}$M. Melittin enhanced phagocytosis at lower concentrations, but the dose-response curve was bell-shaped. Even in Ca^{2+}-and Mg^{2+}-free solutions containing EGTA, which reduced extracellular Ca^{2+}, and BAPTA, which reduced intracellular free Ca^{2+}, phagocytosis was enhanced by mastoparan. The results of the present study suggest that the venom peptides, mastoparan and melittin, activate macrophage function.

Key words: macrophage / phagocytosis / mastoparan /melittin

Venoms of stinging insects of the order Hymenoptera, such as bees and wasps, contain numerous biologically active substances, including amines (histamine, serotonin, dopamine and noradrenaline), enzymes (phospholipase, hyaluronidase and histidine decarboxylase) and polypeptides such as mastoparan, melittin and apamin (1). These venom components may act synergistically to produce the pain and inflammation at a site of a sting and sometimes induce lethal anaphylactic shock which can occur in sensitized individuals. Mastoparan (M.W. 1479) is a tetradecapeptide purified from a wasp venom (*Vespula lewisii*) and is named for its ability to stimulate histamine release from rat peritoneal mast cells (2). Melittin (M.W. 2840) is a 26-residue peptide from honeybee (*Apis melifera*) venom, comprising 50% of the dry weight of the venom (1). Both mastoparan and melittin are amphiphilic polypeptides (3).

Macrophages play a key role in inflammation and host defense against infection and neoplasia (4). Their importance derives from their ubiquitous presence in normal and inflamed tissues, their potential to become activated in response to appropriate stimuli, and their repertoire of secretory products. The phagocytic and digestive capacities are the most representative functions of macrophages and are also key responses in activating the immune system.

Because mastoparan and melittin induce degranulation in tissue mast cells (1,2), these peptides may affect inflammation and anaphylactic shock. Furthermore, those peptides may modulate activity of other cell types in the immune system, because they activates phospholipase C and phospholipase A_2 in mast cells, polymorphonuclear leukocytes, hepatocytes and PC 12 cells (3,5,6,7,8). Effect of mastoparan and melittin on macrophages has not been fully evaluated; limited paper was reported dealing effects of unidentified honey bee venom on interleukin production and mitogenic responses in adherent splenocytes (9). Therefore, we tried to clarify direct effects of mastoparan and melittin on macrophages in the present study. Effects of those peptides on phagocytosis including attachment and ingestion in mouse peritoneal macrophages were examined using fluorescent latex particles and flow cytometry as previously described (10,11) since phagocytosis is one of most representative function of macrophages. In addition, effect of extracellular and intracellular Ca^{2+} on the macrophage phagocytic modulation by the peptides was investigated.

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MATERIALS AND METHODS

Preparation of cells

Macrophages were prepared by the method of Gallily and Feldman (12). The peritoneal cells were harvested by intraperitoneal lavage with Hanks' salt solution from BALB/cA Jcl mice (Nihon Clea, Tokyo) of either sex (10-25 weeks old) 5days after intraperitoneal injection of 4ml thioglycollate medium, and were washed 3 times by centrifugation. The cells were plated onto dishes (Falcon 3001, Becton Dickinson, Oxnard, CA) at a density of $5x10^5$ cells per dish and cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100μg /ml streptomycin and 100 units/ml penicillin G in a CO_2 incubator at 37℃. Macrophages were allowed to adhere for 2 hrs in the CO_2 incubator, washed twice with cold Dulbecco's phosphate buffer solution (PBS), and cultured in the RPMI 1640 medium described above. The phagocytic assay was conducted as early as possible until 10 days after cells were plated onto culture dishes. Though phagocytic activities are variable dependent on days of cultures, days of culture does not impair the evaluation of phagocytosis by the present method up to 10 days (10).

Assay for phagocytosis

To measure phagocytic activity, ingestion of fluorescein isothiocyanate (FITC)-labeled latex particles was analyzed by flow cytometry, the same method as previously described (11). Fluorescent latex bead 2μm in diameter, no. 18338, Polysciences, Warrington, PA) solutions were sonicated for 3 min. Macrophage monolayers were incubated in the presence or the absence of mastoparan or melittin with the latex beads at a density of 10^7 beads per dish for 30-50 min in normal external solution containing 140 mM NaCl, 5 mM KCl,

2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.5. The cell monolayers were washed six times with cold PBS to eliminate uningested and freely floating beads. Each dish was then incubated in 1 ml PBS containing 0.25% trypsin for 3 hrs at 37℃. The cell suspension was transferred to plastic tubes (Falcon 2008). Flow cytometric analyses were performed by using a FACStar (Becton Dickinson Immunocytometry System, Mountain View, CA; excitation wavelength, 488 nm; collected scattering wavelength from 515 to 545 nm; room temperature) to measure the number of particles per cell and total number of beads attached and ingested. The percentage of phagocytic cells was defined as the percentage of macrophages that ingested one or more particles. The phagocytic index, defined as the average number of particles ingested per macrophage, was calculated by dividing the total number of ingested beads by the total number of macrophages (10,000 cells).

Materials

Mastoparan and melittin were purchased from Peptide Institute Inc. Osaka; trypsin was from Difco Lab., Detroit, MI; penicillin G and ethylene glycol bis (b-aminoethylether)-N,N,N',N',-tetraacetic acid (EGTA) were from Sigma, St. Louis, MO; [Bis-(o-aminophenoxy)ethane-N,N,N',N'-tetaacetic acid tetra (acetoxymethyl) ester] (BAPTA-AM) was from Wako, Osaka; [N-(2-hydroxyethyl) piperazine-N'-3-propane sulfonic acid] (HEPES) was from Dojin, Kumamoto, Japan; streptomycin was from Meiji Seika, Tokyo; fetal bovine serum was from Boehringer Mannheim GmbH, Mannheim; thioglycollate, PBS, Hanks and RPMI 1640 medium were from Nissui Pharmac., Tokyo.

Statistics

Results were expressed as mean ± standard deviation (SD) or standard error of mean (SE). Statistical analyses were performed by the paired Student's t-test in a dose-response curve. Analysis of variance (ANOVA) was employed and post hoc Bonferroni-Dunn test (multiple t-test) was used to determine statistical significance for effect of mastoparan in Ca^{2+}-free solution. Differences were considered to be significant at $P<0.05$, $P<0.025$ and $P<0.01$.

RESULTS

Flow cytometric analysis of phagocytosis

Fig. 1a1 is a contour map of phagocytic cells showing relative forward scattering (FSC) versus relative fluorescence intensity (FL). The left cluster represents the population that ingests no beads and the right cluster represents the bead-ingesting macrophages. The distribution of cell numbers versus FL intensity from Fig. 1a1 is shown in Fig. 1a2. Figs. 1b1 and 1b2 are the contour map and histogram, respectively, in the presence of mastoparan, similar to the left. Comparing Fig. 1a1 with Fig. 1b1, the area and contour height of the non-ingesting population were decreased and those of the bead-ingesting population were increased. As shown in Fig. 1c, mastoparan decreased the number of non-ingesting cells and the number of cells which ingested one or more beads was increased, indicating that mastoparan enhanced the phagocytosis of latex particles in mouse peritoneal macrophages. Similar to mastoparan, melittin also enhanced the phagocytosis, as shown in Fig. 2. Specific activity of mastoparan (14 amino acids) and melittin (26 amino acids) might be supported by results that apamin (18 amino acids), human endothelin-1 (21 amino acids), mouse vasoactive intestinal contractor (21 amino acids) had no such effect on phagocytic activity.

Dose-dependency of phagocytic enhancement by mastoparan and melittin

As shown in Fig. 3, mastoparan enhanced phagocytosis of latex particles in a dose-dependent manner. The threshold concentration of mastoparan may be close to 10^{-3}M. The enhancement of phagocytosis at 10^{-6}M was 187.6 ± 10.5% (mean ± standard error of mean,

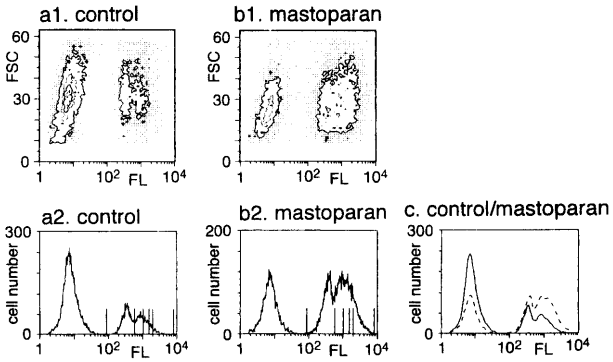

Fig. 1. Effects of mastoparan on flow cytometric profiles. (a1) A contour plot based on forward scattering (FSC) and relative fluorescence (FL) intensity. Each dot shows area at a cell density of 1 to 4 cells per unit square. Contour lines indicate increases in cell densities every 15 cells. (a2) Histogram of cells based upon FL intensities from a1. Non-ingested, 1 bead-ingested and 2 beads-ingested cell populations are the first, second and third peaks from the left, respectively. (b1) A contour plot in the presence of 10^{-6}M mastoparan. (b2) Histogram from b1. (c) Histograms from (a2) and (b2) were smoothed and then superimposed. Note that the broken line is above the continuous line in the higher FL intensity, indicating that the number of bead-ingested cells is increased by mastoparan.

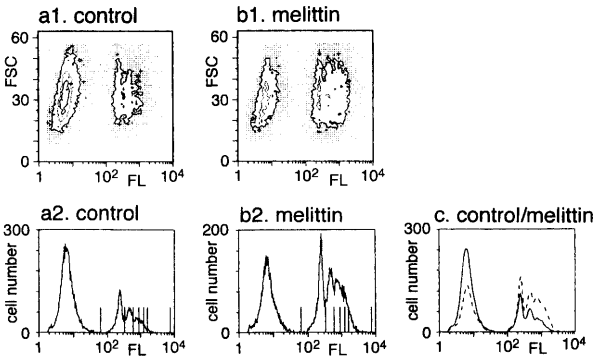

Fig. 2. Effects of melittin on flow cytometric profiles. (a1) A control contour plot based on FSC and FL intensity. (a2) Histogram based upon FL intensities from a1. (b1) A contour plot in the presence of melittin (10^{-7}M). (b2) Histogram from b1. (c) Histograms from (a2) and (b2). Note that the number of bead-ingested cells with the higher FL intensity is increased by melittin.

SE, n=6) for percentage of phagocytic cells and 246.5 ± 20.3% (mean ± SE, n=6) for the phagocytic index, compared with unstimulated control phagocytosis. In contrast, melittin enhanced phagocytosis at even lower concentrations. The threshold concentration may be 2x1 0^{-9}M. However, at the higher concentration the enhancement disappeared, as shown in Fig. 3. Therefore, dose-response curve is bell-shaped. The maximum enhancement of phagocytosis by melittin at 10^{-7}M was 194.8 ± 11.0% (mean ±SE, n=6) for the percentage of phagocytic cells and 231.6 ± 21.8% (mean ± SE, n=6) for the phagocytic index, compared with unstimulated control phagocytosis.

Dependency of Ca^{2+}

The effect of Ca^{2+}on phagocytosis was examined, as shown in Fig. 4. When extracellular $Ca^{2+}$was eliminated by adding 1 mM EGTA to Ca^{2+}-and Mg^{2+}-free solution and intracellular free Ca^{2+} was ligated by adding 25μM BAPTA-AM simultaneously to the solution, unstimulated phagocytosis was reduced to 30.3% (the percentage of phagocytic cells) and 22.1% (the phagocytic index) of control phagocytosis in the normal solution. However, the enhancement of percentage of phagocytic cells in the normal solution and the divalent-deficient solution was 149.2 and 394.9%, respectively. The enhancements of the phagocytic index in the normal and the divalent-deficient solutions were 176.0 and 533.1%, respectively. These data indicate that non-stimulated phagocytic activity is reduced in the divalent deficient solution, but the magnitude of the mastoparan-induced enhancement in divalent cation-free solution is larger than that in the normal solution.

DISCUSSION

Though mastoparan and melittin are known as venoms which may stimulate inflammation at the site of a sting and the effects on mast cells have been studied (1,2,5,6), the direct effects of the peptides on macrophages have not been reported. By using a flow cytometric assay, the present study revealed for the first time that mastoparan and melittin strongly enhanced the phagocytosis of polystyrene microspheres by mouse peritoneal macrophages ranging at concentrations from 10^{-9} to 10^{-6}M.

Mastoparan appears to cause activation of phospholipase A_2 (PLA_2), leading to arachidonic acid (AA) release from rat peritoneal mast cells and human fibroblasts (5) and activation of phospholipase C (PLC), leading to phosphoinositide (PI) breakdown in rat peritoneal mast cells (6). Melittin causes activation of PLA_2 and release of AA in rat peritoneal cells (5). Mastoparan and melittin directly activate G proteins (13,14). Thus, both mastoparan and melittin could activate phospholipases. This activation may induce the enhancement of phagocytosis, because activation of phospholipases may lead to an increase in intracellular Ca^{2+} concentration which might be related to an enhancement of phagocytosis in macrophages. In fact, an increase of the cytosolic free Ca^{2+} concentration via an elevation of inositol 1,4,5-trisphosphate (IP_3) by mastoparan was reported in human polymorphonuclear leukocytes (7) and rat hepatocytes (8). In rat pheochromocytoma PC12 cells, mastoparan and melittin induce calcium influx, PI breakdown and AA release (3). The following two facts suggest that an increase in intracellular Ca^{2+} concentration may be related to an enhancement of macrophage phagocytosis. 1) Platelet activating factor (PAF) stimulates PI breakdown, inducing inositol trisphosphate production and eventually an increase in intracellular Ca^{2+} in mouse peritoneal macrophages (15). We have reported that phagocytosis of mouse peritoneal macrophages is enhanced by PAF (10). 2) The calcium ionophore A23187 (10^{-7}M) enhanced phagocytosis of latex beads in rat peritoneal macrophages (16).

The percentage of phagocytic cells and the phagocytic index of unstimulated phagocytosis in Ca^{2+}- and Mg^{2+}-free solutions in the presence of EGTA and BAPTA-AM were reduced to 30.3% and 22.1% of control the per-

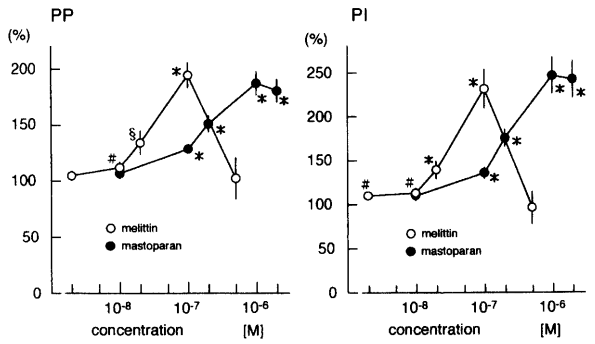

Fig. 3. Concentration-response curves showing the enhancement of phagocytosis in peritoneal macrophages by mastoparan (closed circles) and melittin (open circles). (#), (§) and (*) indicate significant differences between control and effects of various peptides ($P<0.05$, $P<0.025$ and $P<0.01$). Data are mean ± standard error of means (S.E., n=6). The left percentage of phagocytic cells and the right phagocytic index graphs are represented as the percentage of control value (100%) in the absence of the peptides.

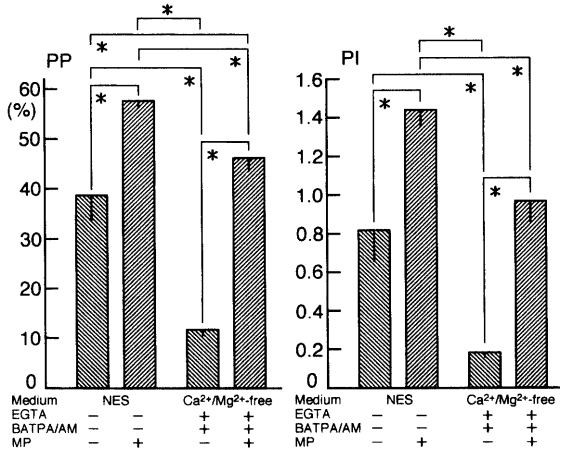

Fig. 4. Effect of Ca^{2+} on the enhancement of phagocytosis induced by mastoparan (10^{-6}M). Asterisks indicate significant differences between each pair ($P<0.01$). Data are the means ± S.D. (n=4). In each graph, the left pair are the control and mastoparan-induced phagocytosis in the normal external solution. The right pair are non-stimulated and mastoparan-enhanced phagocytosis in Ca^{2+}-and Mg^{2+}-free solution containing 1mM EGTA and 25μM BAPTA-AM. NES,Ca^{2+}-Mg^{2+}-free and MP are the normal external solution, Ca^{2+} and Mg^{2+}-free solution and mastoparan, respectively.

centage of phagocytic cells and the phagocytic index in the normal solution, respectively, as shown in Fig. 4. Therefore approximately 70-80% of the phagocytosis in the normal solution is dependent on extracellular Ca^{2+} and Mg^{2+} and intracellular free Ca^{2+}. This result suggests that phagocytosis in the present study consists mainly of divalent cation dependent- and in part divalent cation-independent phagocytosis. This is consistent with the report that non-opsonized phagocytosis is reduced by elimination of intracellular and extracellular Ca^{2+} (17). The magnitudes (394.9 and 533.1%) of the enhancement in the percentage of phagocytic cells and the phagocytic index by mastoparan in EGTA and BAPTA-AM containing Ca^{2+}- and Mg^{2+}-free solution were larger than those (149.2 and 176.0%) in the normal solution. This can be explained by the following two possibility, that is, 1) mastoparan mainly enhances the divalent cation-independent phagocytosis or 2) mastoparan stimulates Ca^{2+} release from intracellular store site or stimulates release of other phagocytic stimulating factors from arachidonic acid metabolism, resulting the present phagocytic enhancement. Concerning effects of BAPTA, it not only suppresses an increase in intracellular free-Ca^{2+} by some stimulators but also induces a translocation and inactivation of protein kinase C (PKC) in liver macrophages (18). Because activation of phagocytic activity in macrophages by neuropeptide Y and peptide YY is associated with stimulation of PKC (19), inactivation of PKC may induce suppression of phagocytosis. Therefore, we cannot exclude the possibility that BAPTA reduces control phagocytosis and mastoparan stimulates PKC activity, leading to the phagocytic enhancement.

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