Activation of human neutrophils by mastoparan

Reorganization of the cytoskeleton, formation of phosphatidylinositol 3,4,5-trisphosphate, secretion, up-regulation of complement receptor type 3 and superoxide anion production are stimulated by mastoparan

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In human neutrophils, mastoparan induced rapid F-actin polymerization which was followed by a slow and sustained depolymerization to below the initial F-actin content. Incubation of neutrophils with pertussis toxin inhibited mastoparan-stimulated actin polymerization; however it did not prevent sustained depolymerization of F-actin. Analyses of phospholipids performed in parallel revealed that mastoparan stimulated rapid formation of phosphatidylinositol 3,4,5-trisphosphate (PIP₃) and consumption of phosphatidylinositol 4,5-bisphosphate (PIP₂). Pertussis toxin treatment blocked mastoparan-induced formation of PIP₃. Furthermore, mastoparan stimulated the release of *N*-acetylglucosaminidase from primary granules. Cytochalasin B enhanced mastoparan-stimulated secretion. Mastoparan triggered superoxide radical production in a cytochalasin B-sensitive manner and induced complement type 3 receptor (CR3) up-regulation.

INTRODUCTION

Stimulation of neutrophils by chemoattractants induces a series of co-ordinate cell responses such as migration, phosphatidylinositol (PI) 3-kinase activation, F-actin polymerization, receptor up-regulation, degranulation and superoxide anion production [1,2]. Specific ligand–receptor interactions cause activation of G-proteins by catalysing the release of bound GDP and subsequent binding of GTP [3]. Activated G-proteins interfere with effector proteins such as phospholipase C to transmit and amplify chemical signals [4].

Mastoparan, a tetradecapeptide purified from wasp venom [5], is a potent stimulator of histamine release from mast cells and of insulin release from pancreatic islets [6,7]. Originally, mastoparan was considered as an activator of phospholipase A₂ [8]. However, Higashijima *et al.* [9] and Weingarten *et al.* [10] demonstrated direct activation of G-proteins by mastoparan.

Perianin & Snyderman [11] reported that mastoparan increased intracellular Ca²⁺ in neutrophils by two different mechanisms. One pathway was similar to that induced by chemoattractant receptors, whereas the second pathway was insensitive to pertussis toxin and required extracellular Ca²⁺. In the present paper we demonstrate that mastoparan stimulates several cell responses in neutrophils and compare these effects with those of *N*-formyl peptide-induced cell activation.

MATERIALS AND METHODS

Materials

N-Formyl-methionyl-leucyl-phenylalanine (fMetLeuPhe), superoxide dismutase, pertussis toxin, cytochalasin B, *p*-nitrophenyl-*N*-acetyl-D-glucosaminide, phosphatidylinositides, lysophosphatidylcholine and cytochrome *c* were obtained from Sigma

(Deisenhofen, Germany). Dextran and Ficoll–Hypaque were purchased from Pharmacia Fine Chemicals (Freiburg, Germany), mastoparan from Bissendorf (Hannover, Germany), [³²P]P_i from Amersham–Buchler (Braunschweig, Germany), 7-nitrobenz-2-oxa-1,3-diazol-(NBD)-phalloidin from Molecular Probes Inc. (Junction City, OR, U.S.A.), and anti-Leu-15 (anti-CD 11b) from Becton–Dickinson (Heidelberg, Germany).

Preparation of human neutrophils

Anticoagulated blood from healthy human donors was sedimented for 45 min with 6% dextran 70. The upper phase containing the white-cell-enriched plasma was layered on to Ficoll–Hypaque and centrifuged for 30 min at 400 *g* and 4 °C. Red blood cells were lysed and neutrophils were washed and resuspended in buffer containing 137 mM-NaCl, 5 mM-KCl, 1 mM-MgCl₂, 1.5 mM-glucose, 1.5 mM-CaCl₂, 10 mM-Hepes, pH 7.45 [12]. Viability of isolated and mastoparan-stimulated neutrophils was tested by Trypan Blue exclusion. Furthermore, mastoparan up to a concentration of 35 μM did not induce the release of lactate dehydrogenase.

F-actin measurements

Measurements of F-actin formation were performed as described [13]. Briefly, after stimulation, samples of cell suspension were withdrawn at the indicated time points and fixed with formaldehyde. Thereafter, neutrophils were permeabilized with lysophosphatidylcholine, and F-actin was stained with NBD-phalloidin. The intensity of fluorescence of F-actin was quantified using a flow cytometer (FACScan; Becton–Dickinson).

Lipid analysis

Phosphatidylinositol 4,5-bisphosphate (PIP₂) and phosphatidylinositol 3,4,5-trisphosphate (PIP₃) were analysed according

Abbreviations used: PI, phosphatidylinositol; PIP₃, PI 3,4,5-trisphosphate; PIP₂, PI 4,5-bisphosphate; IP₃, inositol 1,4,5-trisphosphate; fMetLeuPhe, formyl-methionyl-leucyl-phenylalanine; CR3, complement type 3 receptor; NBD, 7-nitrobenz-2-oxa-1,3-diazol.

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to the method described by Traynor-Kaplan *et al.* [2]. Neutrophils were labelled with [32 P]P_i for 180 min. Cells were washed, resuspended and stimulated. The stimulation reaction was stopped at the indicated time points with a solution of chloroform/methanol (1:2, v/v). Extraction of phospholipids was performed as described [2]).

Superoxide radical production

Superoxide radical production was performed at 37 °C and measured as superoxide dismutase-inhibitable reduction of cytochrome *c* at 550 nm, according to [12].

Secretion of *N*-acetylglucosaminidase

Neutrophils were stimulated for 5 min at 37 °C and then centrifuged. Thereafter, the supernatant was exposed to *N*-acetylglucosaminidase for 30 min, and the reaction was stopped with 0.4 M-glycine and read at 405 nm according to [12].

Up-regulation of complement receptor type III (CR3) in whole blood

Analysis of expressed CR3 was performed with phycoerythrin-conjugated anti-(human CR3) (anti-CD 11b; anti-Leu-15). Whole blood was stimulated for 90 s, and the reaction was stopped by dilution of the sample 100-fold with ice-cold buffer. Samples were incubated for 40 min on ice with anti-Leu-15 and analysed with the flow cytometer by gating neutrophils on forward scatter and side scatter, as described earlier [14,15]. Specific binding was calculated by subtracting the mean channel number of fluorescence in the presence of a 10-fold excess of unlabelled anti-Leu-15 from the mean channel number in the absence of unlabelled anti-Leu-15. Up-regulation of CR3 induced by mastoparan was verified also in isolated neutrophils.

RESULTS

Dose-response effect of mastoparan actin polymerization and depolymerization and measurement of stained fluorescence

Control neutrophils comprised a single population of NBD-phalloidin-stained fluorescent cells with a mean channel number of 75 (Fig. 1a). After stimulation of neutrophils with mastoparan (35 µg/ml) for 10 s, the fluorescence increased by about 74 %, i.e. the mean channel number was 129. However, prolonged stimulation of cells with mastoparan decreased the fluorescence to below the initial basal level by about 25 %.

The time- and concentration-dependence of actin polymerization and depolymerization in response to mastoparan is shown in Fig. 1(b). High concentrations of mastoparan stimulated a rapid formation of F-actin, followed by a sustained and slow depolymerization of F-actin to below initial values. On stimulation with lower concentrations of mastoparan only the first phase of F-actin polymerization was observed, and no depolymerization to below basal levels was detectable.

Influence of pertussis toxin pretreatment on mastoparan- and fMetLeuPhe-stimulated actin polymerization and depolymerization

It is known that pertussis toxin catalyses the ADP-ribosylation of G-proteins, most likely G₁₂ and G₁₃, in neutrophils [16], thereby uncoupling the G-protein from chemoattractant receptors [17] and inhibiting activation of the G-protein by mastoparan [10]. As shown in Fig. 2, pertussis toxin treatment completely inhibited fMetLeuPhe-stimulated F-actin formation. Furthermore, the rapid polymerization response induced by

mastoparan was also blocked. In contrast, the slow and sustained depolymerization phase was not affected by pertussis toxin treatment.

Analysis of 32 P-labelled phospholipids in human neutrophils and influence of pertussis toxin on fMetLeuPhe- and mastoparan-stimulated phospholipid metabolism

It has been reported that stimulation of neutrophils with *N*-formyl-peptide induces a decrease in PIP₂ and an increase in PIP₃ [18]. The decrease in PIP₂ is generally thought to be caused primarily by activation of phospholipase C, which hydrolyses PIP₂ to inositol 1,4,5-trisphosphate (IP₃) and 1,2-diaclyglycerol [4]. Moreover, activated PI 3-kinase phosphorylates PIP₂ in the D-3 position of the inositol ring to form PIP₃ [2]. As demonstrated in Figs. 3(a) and 3(b), mastoparan induced an increase in [32 P]PIP₃ and consumption of [32 P]PIP₂, similar to fMetLeuPhe. Analyses of the phospholipids revealed that the formation of [32 P]PIP₃ stimulated by mastoparan decayed rapidly towards basal values. Consumption of [32 P]PIP₂ was slower and more sustained than changes in [32 P]PIP₃. Stimulation with lower concentrations of mastoparan (4 µM) caused attenuated changes in the metabolism

Fig. 1. Distribution and dose-dependence of F-actin formation in mastoparan-stimulated neutrophils

(a) Human neutrophils were stimulated with 35 µM-mastoparan, and samples of neutrophils were fixed after 0 (A), 10 (B) or 240 (C) s, stained with NBD-phalloidin as described in the Materials and methods section and compared with control cells. Data are representative of one experiment performed five times in duplicate. (b) Neutrophils were stimulated with 35 µM- (◆), 10 µM- (●) or 4 µM- (■) mastoparan. Samples of cells were fixed at the indicated time points, stained and fluorescence was measured by flow cytometry. Data are means ± S.D. of a representative experiment performed three times.

Fig. 2. Influence of pertussis toxin on F-actin in mastoparan-stimulated neutrophils

Neutrophils were stimulated with 100 nM-fMetLeuPhe (◆, ◇) or 35 μM-mastoparan (●, ○) with (◇, ○) or without (◆, ●) preincubation with pertussis toxin (10 μg/ml) for 3 h. Aliquots of cells were fixed at the indicated time points, stained and analysed. Data represent means ± S.D. of a representative experiment performed twice with similar results.

Fig. 3. Time course of ^{32}P labelling of phospholipids

Changes in ^{32}P -labelled phospholipids after stimulation with 35 μM- (●) or 4 μM- (■) mastoparan were analysed. The individual data points represent the incorporation of ^{32}P into PIP_2 (a) and PIP_3 (b), relative to the ^{32}P incorporation into total phospholipids, plotted versus time. Data are means ± S.D. of a representative experiment repeated twice.

of the phospholipids (Figs. 3a and 3b). Furthermore, pertussis toxin treatment inhibited *N*-formyl-peptide-induced phospholipid metabolism and abolished mastoparan-stimulated [^{32}P]PIP₃ formation (Table 1).

Table 1. Effect of pertussis toxin on fMetLeuPhe and mastoparan-stimulated PI 3-kinase activity

Neutrophils were incubated with or without 10 μg of pertussis toxin (PT)/ml for 3 h and the relative distribution of ^{32}P -labelled phospholipids was analysed after stimulation with 100 nM-fMetLeuPhe or 35 μM-mastoparan for 10 s. Data are means ± S.D. of the incorporation of ^{32}P into PIP₃ relative to that into total phospholipids.

Stimulus	PT	Relative incorporation into PIP ₃ (% of total phospholipids)
None	—	0.56 ± 0.08
None	+	0.61 ± 0.14
fMetLeuPhe	—	2.22 ± 0.06
fMetLeuPhe	+	0.64 ± 0.24
Mastoparan	—	1.70 ± 0.33
Mastoparan	+	0.64 ± 0.04

Mastoparan-stimulated secretion of primary granules, CR3 up-regulation and superoxide production

Mastoparan is a strong stimulator of secretion in mast cells and pancreatic islets [6,7]. Whereas chemoattractants in the absence of cytochalasin B are weak stimuli of secretion of primary granules, mastoparan induced secretion of *N*-acetylglucosaminidase in a concentration-dependent manner (Fig. 4a). Additionally, cytochalasin B potentiated mastoparan-triggered secretion. Similarly, as has been shown for various chemoattractants [14,15], mastoparan up-regulated CR3 in a concentration-dependent manner in the whole blood (Fig. 4b). The experiment was performed with whole blood to avoid alterations in the number of expressed CR3 due to isolation procedures. However, up-regulation of CR3 by mastoparan was also verified in isolated neutrophils (M. Eberle & J. Norgauer, unpublished work).

Furthermore, superoxide anion production was stimulated by mastoparan in a concentration-dependent manner. In the absence of cytochalasin B, the fMetLeuPhe-stimulated superoxide anion production was greater than that induced by mastoparan. The presence of cytochalasin B potentiated superoxide production induced by both peptides (Fig. 4c).

DISCUSSION

It appears that the reorganization of the cortical F-actin network is a prelude and/or a prerequisite of some cell responses such as chemotaxis, phagocytosis and degranulation [1,19]. Several findings indicate that the signal transduction pathway of receptor-mediated actin polymerization involves G-protein activation, but is not the consequence of metabolic products formed by phospholipase C [20]. G-protein involvement has been deduced from studies with pertussis toxin [1] on intact leucocytes and with guanosine 5-[γ-thio]triphosphate on permeabilized cells [21]. It has been proposed that mastoparan, which apparently activates G-proteins without receptor interaction, might be a useful tool for studies of G-protein-dependent processes. Since mastoparan acts on intact cells, complications due to permeabilization of cells by detergents or electrical discharge are avoided [6,7,11]. Here we report that mastoparan induced a biphasic F-actin response and PIP₃ formation in neutrophils. Rapid polymerization of F-actin was followed by a sustained depolymerization to below the initial F-actin level. Furthermore, treatment of neutrophils with pertussis toxin blocked the fMetLeuPhe- and mastoparan-induced polymerization of actin, but did not inhibit the sustained and slow F-actin depolymerization.

Fig. 4. Secretion of *N*-acetylglucosaminidase (a), CR 3 up-regulation (b) and superoxide anion production (c) stimulated by mastoparan

Isolated neutrophils were preincubated without (●) or with (■) cytochalasin B (2 $\mu\text{g}/\text{ml}$) and stimulated with the indicated concentrations of mastoparan for 5 min at 37 °C, and the release of *N*-acetylglucosaminidase (a) and superoxide anion production (c) were measured. Furthermore, whole blood was stimulated with mastoparan for 90 s and bound anti-Leu-15 was analysed as described in the Materials and methods section (b). Data are means $\pm$ S.D. ($n = 3$).

ation provoked by mastoparan, indicating distinct mechanisms. Similarly, Perianin & Snyderman [11] showed that mastoparan increased IP_3 and cytosolic Ca^{2+} by two different mechanisms in neutrophils. One pathway was pertussis toxin-sensitive, whereas the other depended on extracellular Ca^{2+} and was insensitive to pertussis toxin [11]. Pertussis toxin ADP-ribosylates G_{i2} and G_{i3} in neutrophils [16], thereby uncoupling chemoattractant receptors [17]. Moreover, it has been demonstrated that ADP-ribosylation blocked mastoparan-induced activation of G-proteins [10]. Thus the ability of pertussis toxin to inhibit mastoparan-induced actin polymerization and formation of PIP_3 suggests that direct G-protein activation is involved in these effects. Since quinacrine and bromphenacyl bromide, both inhibitors of phospholipase A_2 [22], were without effect on the mastoparan-induced polymerization, activation of phospholipase A_2 by mastoparan is unlikely.

It is generally thought that actin-binding proteins such as gelsolin and profilin are involved in the cellular regulation of polymerization and depolymerization of actin [19]. The activities of these actin-binding proteins are apparently regulated by Ca^{2+} and/or polyphosphoinositides [23–25]. The recent report by Eberle *et al.* [18] suggests that PIP_3 might trigger fMetLeuPhe-stimulated F-actin polymerization. Here we show that mastoparan also induced short-lived PIP_3 formation which was paralleled by F-actin polymerization. Perianin & Snyderman [11] demonstrated that mastoparan causes a pertussis toxin-insensitive slow increase in intracellular Ca^{2+} . Furthermore, studies performed with permeabilized leucocytes [26] suggest that an increase in cellular Ca^{2+} is involved in the depolymerization of F-actin. Thus one can speculate that a delayed increase in Ca^{2+} is responsible for the delayed actin depolymerization observed with mastoparan.

Similar to chemoattractants such as fMetLeuPhe, mastoparan stimulated secretion in neutrophils. Secretion was partially sensitive to pertussis toxin and to maptam, a Ca^{2+} chelator accessible to cells (J. Norgauer, M. Eberle, & K. Aktories, unpublished work). Barrowman *et al.* [27] speculated that G-proteins and intracellular Ca^{2+} might mediate the secretory response in neutrophils. Since bromphenacyl bromide, a phospholipase A_2 blocker [22], did not prevent mastoparan-induced secretion (results not shown), it is highly unlikely that this mastoparan effect is caused by activation of phospholipase A_2 . Thus either elevated intracellular Ca^{2+} or direct activation of a G-protein might induce secretion.

Mastoparan also stimulated CR3 up-regulation and superoxide radical production. Interestingly, cytochalasin B potentiated mastoparan-stimulated superoxide radical production. This observation corroborates previous findings by Norgauer *et al.* [13] that additional superoxide anion production in cytochalasin B- or botulinum C2 toxin-treated neutrophils might be a post-receptor effect.

In summary, we have demonstrated that, in neutrophils, mastoparan stimulates reorganization of cytoskeleton, PI 3-kinase, secretion, receptor up-regulation and superoxide anion production. Thus mastoparan might be a useful tool with which to study G-protein-dependent processes in leucocytes.

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REFERENCES

1. Omann, G. M., Allen, R. A., Bokoch, G. M., Traynor, A. E. & Sklar, L. A. (1987) *Physiol. Rev.* **67**, 285–322
2. Traynor-Kaplan, A. E., Harris, A. L., Thompson, B. L., Taylor, P. & Sklar, L. A. (1989) *J. Biol. Chem.* **264**, 15669–15673
3. Gilman, A. G. (1987) *Annu. Rev. Biochem.* **56**, 615–650
4. Berridge, M. J. & Irvine, R. F. (1989) *Nature (London)* **341**, 197–205
5. Hirai, Y., Yasuhara, T., Yoshida, H., Nakajima, T., Fujino, M. & Kitada, C. (1979) *Chem. Pharm. Bull.* **27**, 1942–1944
6. Aridor, M., Traub, L. & Sagi-Eisenberg, R. (1990) *J. Cell Biol.* **111**, 909–917
7. Yokokawa, N., Komatsu, M., Takeda, T., Aizawa, T. & Yamada, T. (1989) *Biochem. Biophys. Res. Commun.* **158**, 712–716
8. Argiolas, A. & Pisano, J. J. (1983) *J. Biol. Chem.* **258**, 13697–13702
9. Higashijima, T., Burnier, J. & Ross, E. M. (1990) *J. Biol. Chem.* **265**, 14176–14186
10. Weingarten, R., Ransnäs, L., Müller, H., Sklar, L. A. & Bokoch, G. M. (1990) *J. Biol. Chem.* **265**, 11044–11049
11. Perianin, A. & Snyderman, R. (1989) *J. Immunol.* **143**, 1669–1673
12. Norgauer, J., Kownatzki, E., Seifert, R. & Aktories, K. (1988) *J. Clin. Invest.* **82**, 1376–1382
13. Norgauer, J., Just, I., Aktories, K. & Sklar, L. A. (1989) *J. Cell Biol.* **109**, 1133–1140

14. Norgauer, J., Eberle, M., Fay, S. P., Lemke, H.-D. & Sklar, L. A. (1991) *J. Immunol.* **146**, 975–980
15. Lemke, H.-D. & Sklar, L. (1989) *FASEB J.* **3**, A1086
16. Gierschik, P., Sidiropoulos, D. & Jakobs, K. H. (1989) *J. Biol. Chem.* **264**, 21470–21473
17. Sklar, L. A., Bokoch, G. M., Button, D. & Smolen, J. E. (1987) *J. Biol. Chem.* **262**, 135–139
18. Eberle, M., Traynor-Kaplan, A. E., Sklar, L. A. & Norgauer, J. (1990) *J. Biol. Chem.* **265**, 16725–16728
19. Stossel, T. P. (1989) *J. Biol. Chem.* **264**, 18261–18264
20. Bengtsson, T., Rundquist, I., Stendahl, O., Wymann, M. P. & Andersson, T. (1988) *J. Biol. Chem.* **263**, 17385–17389
21. Therrien S. & Naccache P. H. (1989) *J. Cell Biol.* **109**, 1125–1132
22. Henderson, L. M., Chappell, J. B. & Jones, O. T. W. (1989) *Biochem. J.* **264**, 249–255
23. Janmey, P. A. & Stossel, T. P. (1987) *Nature (London)* **325**, 362–364
24. Janmey, P. A., Iida, K., Yin, H. L. & Stossel, T. P. (1987) *J. Biol. Chem.* **262**, 12228–12236
25. Howard, T., Chaponnier, C., Yin, H. & Stossel, T. (1990) *J. Cell Biol.* **110**, 1983–1991
26. Downey, G. P., Chan, C. K., Trudel, S. & Grinstein, S. (1990) *J. Cell Biol.* **110**, 1975–1983
27. Barrowman, M. M., Cockcroft, S. & Gomperts, B. D. (1986) *Nature (London)* **319**, 504–507

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