Mastoparan may activate GTP hydrolysis by G_i-proteins in HL-60 membranes indirectly through interaction with nucleoside diphosphate kinase

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The wasp venom, mastoparan (MP), activates reconstituted pertussis toxin (PTX)-sensitive G-proteins in a receptor-independent manner. We studied the effects of MP and its analogue, mastoparan 7 (MP 7), on G-protein activation in HL-60 cells and a reconstituted system and on nucleoside diphosphate kinase (NDPK)-catalysed GTP formation. MP activated high-affinity GTP hydrolysis in HL-60 membranes with an EC_{50} of 1–2 μM and a maximum at 10 μM. Unlike the effects of the formyl peptide receptor agonist, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMet-Leu-Phe), on GTPase, those of MP were only partially PTX-sensitive. MP-induced rises in cytosolic Ca^{2+} concentration and superoxide-anion formation in intact HL-60 cells were also only incompletely PTX-sensitive. *N*-Ethylmaleimide inhibited MP-stimulated GTP hydrolysis to a greater extent than that stimulated by fMet-Leu-Phe. Unlike the latter, MP did not enhance incorporation of GTP azidoanilide into, and cholera toxin-catalysed ADP-ribosylation of, G_i-protein α-subunits in HL-60 membranes. By contrast to fMet-Leu-Phe, MP did not or only weakly stimulated binding of guanosine 5′-[γ-thio]triphosphate to G_i-protein α-subunits. MP 7 was considerably more effective than MP at activating the GTPase of reconstituted G_i/G_o-proteins, whereas in HL-60 membranes, MP and MP 7 were similarly effective. MP and MP 7 were similarly effective at activating [^{3}H]GTP formation from [^{3}H]GDP and GTP in HL-60 membranes and by NDPK purified from bovine liver mitochondria. Our data suggest the following: (1) MP activates G_i-proteins in HL-60 cells, but (2) the venom does not simply mimic receptor activation. (3) MP and MP 7 may activate GTP hydrolysis in HL-60 membranes indirectly through interaction with NDPK. (4) MP 7 is a more effective direct activator of PTX-sensitive G-proteins than MP, whereas with regard to NDPK, MP and MP 7 are similarly effective.

INTRODUCTION

The wasp venom, mastoparan (MP), is a cationic-amphiphilic tetradecapeptide which activates the GTPase of, and guanosine 5′-[γ-thio]triphosphate (GTP[γS]) binding to, reconstituted G_i- and G_o-proteins in a receptor-independent manner (Higashijima et al., 1988, 1990; Weingarten et al., 1990; Tomita et al., 1991). Like the effects of receptor agonists, pertussis toxin (PTX) inhibits those of MP on G_i/G_o-proteins (Higashijima et al., 1988; Weingarten et al., 1990; Tomita et al., 1991). However, there is increasing evidence to support the assumption that MP does not simply mimic receptor activation as the venom shows pleiotropic effects (Nakahata et al., 1990; Joyce-Brady et al., 1991; Gusovsky et al., 1991; Danilenko et al., 1993). For example, in addition to G-proteins, MP activates small GTP-binding proteins (Koch et al., 1991), an ATP- and GTP-degrading nucleotidase (Denker et al., 1991) and guanylyl cyclase (Song et al., 1993). Furthermore, inhibitory effects of MP on protein kinase C and Na^+/K^+-ATPase have been reported (Raynor et al., 1992; Danilenko et al., 1993). Moreover, MP activates nucleoside diphosphate kinase (NDPK) (Kikkawa et al., 1992), an SH-group-containing and Mg^{2+}-dependent enzyme which catalyses the phosphorylation of various NDPs to NTPs and which is inhibited by NDP at high concentrations through formation of an abortive enzyme/NDP complex (Parks and Agarwal, 1973). Most intriguingly, NDPK-catalysed trans-phosphorylation reactions are involved in G-protein activation (Otero, 1990; Lacombe and Jakobs, 1992).

Unlike reconstituted G-proteins, little is known about G-protein activation by MP *in situ*. Additionally, it is unknown whether there is a functional link between MP-induced G-protein- and NDPK activation. Therefore, we studied the effects of MP and the MP analogue, MP 7, on activation of G-proteins in HL-60 cells and a reconstituted system and on NDPK. For comparison, we also studied the effects of the formyl peptide receptor agonist, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMet-Leu-Phe), in HL-60 cells. We report here on differential G-protein activation by MP and fMet-Leu-Phe and present evidence to forward the notion that MP and MP 7 activate G_i-proteins in HL-60 membranes indirectly through interaction with NDPK.

MATERIALS AND METHODS

Materials

MP and MP 7 were from Saxon Biochemicals (Hannover, Germany). Stock solutions of MP and MP 7 (1 mM each) and dilutions were prepared in 1 mM sodium acetate, pH 5.0, and stored at −20 °C. NDPK (80 units/mg, activity measured at 25 °C using dTDP and ATP as substrates) purified from bovine liver mitochondria according to Glaze and Wadkins (1967) was purchased from Boehringer Mannheim (Mannheim, Germany).

Abbreviations used: $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; CTX, chlorea toxin; GTP[γS], guanosine 5′-[γ-thio]triphosphate; fMet-Leu-Phe, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; MP, mastoparan (INLKALAALAKKIL); MP 7, mastoparan 7 (INLKALAALAKALL); NDPK, nucleoside diphosphate kinase; NEM, *N*-ethylmaleimide; O_2^-, superoxide anion; PTX, pertussis toxin; DTT, dithiothreitol.

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Sources of other materials have been described elsewhere (Seifert et al., 1988, 1989, 1992, 1994; Klinker et al., 1993).

Cell culture and membrane preparation

HL-60 cells were differentiated with dibutyryl cAMP (Seifert et al., 1989). HL-60-membranes and -cytosol were prepared as described (Seifert and Schultz, 1987; Klinker et al., 1993). Before experiments, HL-60 membranes (100–500 μg of protein) were suspended in 1.5 ml of 10 mM triethanolamine/HCl, pH 7.4, and were centrifuged for 10 min at 30000 *g* at 4 °C to remove residual cytosolic NDPK and cytosolic proteins involved in NADPH oxidase activation. Thereafter, membranes were resuspended at various concentrations in the above buffer and were immediately used for experiments. PTX (100 ng/ml) or its carrier (control) were added to cell cultures 24 h before membrane preparation or measurement of $[Ca^{2+}]_i$ or O_2^- formation. Under these conditions, virtually all G_i-protein α-subunits were ADP-ribosylated (data not shown).

Purification and reconstitution of G_i/G_o proteins

Heterotrimeric G_i/G_o-proteins were purified from bovine brain membranes (Nürnberg et al., 1994). The preparation of G_i/G_o-proteins (purity > 90%) contained predominantly G_{o1}, significant amounts of G_{o2} and another, as yet unidentified, G_o-subtype ('G_{o3}'), G_{i1} and G_{i2} and traces of G_{i3}. Reconstitution of G_i/G_o-proteins into phospholipid vesicles was performed as described by Hagelüken et al. (1994).

GTPase assay

High-affinity GTP hydrolysis was determined as described (Klinker et al., 1993). Reaction mixtures (100 μl) contained HL-60 membranes (3.0–7.0 μg of protein/tube), 0.5 μM [γ-^{32}P]GTP (0.1 μCi/tube), 0.5 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM ATP, 1 mM adenosine 5′-[β,γ-imido]triphosphate, 5 mM creatine phosphate, 40 μg of creatine kinase, 1 mM dithiothreitol (DTT) and 0.2% (w/v) BSA in 50 mM triethanolamine/HCl, pH 7.4. For experiments with the reconstituted system, reaction mixtures (100 μl) contained 0.4–0.6 pmoles of G_i/G_o-proteins and 50 nM [γ-^{32}P]GTP. The other conditions were as described above.

GTP[γS] binding assay in HL-60 membranes

[^{35}S]GTP[γS] binding was assessed as described (Seifert et al., 1994). Reaction mixtures (100 μl) contained HL-60 membranes (3.0–5.0 μg of protein/tube), 0.4 nM [^{35}S]GTP[γS] (50 nCi/tube), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 150 mM NaCl, 0.5 μM GDP and 0.2% (w/v) BSA in 50 mM triethanolamine/HCl, pH 7.4. Reactions were conducted for 30 min at 25 °C. In some experiments, [^{35}S]GTP[γS] binding was determined for 60 min at 0 °C as described by Wieland et al. (1992). In these experiments, GDP and NaCl were omitted from the reaction mixtures.

Assay for photolabelling of membrane proteins

HL-60 membranes (50 μg of protein in a total volume of 60 μl) were incubated at 30 °C in a buffer containing 0.1 mM EDTA, 5 mM $MgCl_2$, 1 mM benzamidine, 10 μM GDP and 30 mM Hepes/NaOH, pH 7.4. Following exposure to solvent (control), MP (10 μM) or fMet-Leu-Phe (10 μM) for 3 min, samples were incubated for another 3 min with 10 nM [α-^{32}P]GTP azidoanilide (1 μCi/tube). Stopping of reactions and irradiation of samples were performed as described by Offermanns et al., 1991.

Assay for CTX-catalysed ADP-ribosylation of membrane proteins

Reaction mixtures (50 μl) contained HL-60 membranes (50 μg of protein/tube), 3 μM [^{32}P]NAD (5 μCi/tube), 3 mM $MgCl_2$, 1 mM ATP, 10 mM thymidine, 0.2% (w/v) BSA, 2 μg of activated cholera toxin (CTX) and 0.1 M potassium phosphate, pH 7.4. Reactions were conducted for 60 min at 30 °C.

Assay for [^{3}H]GTP formation

For determination of [^{3}H]GTP formation in HL-60 membranes, reaction mixtures (50 μl) contained 0.5–1.0 μg of membrane protein/tube, 0.5 μM [^{3}H]GDP (1 μCi/tube), NTPs at various concentrations, 0.5 mM $MgCl_2$, 0.1 mM EGTA, 1 mM adenosine 5′-[β,γ-imido]triphosphate, 5 mM creatine phosphate, 40 μg of creatine kinase, 1 mM DTT and 0.2% (w/v) BSA in 50 mM triethanolamine/HCl, pH 7.4. Reactions were conducted for 10 min at 25 °C. Stopping of reactions, separation of nucleotides by t.l.c. and nucleotide elution from t.l.c. plates were performed as described (Seifert et al., 1988). For determination of [^{3}H]GTP-formation by the purified enzyme, reaction mixtures (50 μl) contained 0.1 munits of NDPK from bovine liver mitochondria, 0.5 μM [^{3}H]GDP (1 μCi/tube), 10 μM GTP and 5 mM $MgCl_2$ in 50 mM triethanolamine/HCl, pH 7.4. Reactions were conducted for 10 min at 25 °C.

Miscellaneous

Protein was determined according to Lowry et al. (1951). [^{3}H]fMet-Leu-Phe binding was determined according to Wenzel-Seifert and Seifert (1993). Determination of the activities of Na^+/K^+-ATPase and Mg^{2+}-ATPase in HL-60 membranes was performed as described (Klinker et al., 1993). [γ-^{32}P]GTP was prepared as described (Walseth et al., 1991). [α-^{32}P]GTP azidoanilide was prepared according to Offermanns et al. (1991). [^{32}P]NAD was synthesized according to Cassel and Pfeuffer (1978). SDS/PAGE and autoradiography were performed as described by Rosenthal et al. (1986). Treatment of HL-60 membranes with *N*-ethylmaleimide (NEM) was performed according to Klinker et al. (1993). $[Ca^{2+}]_i$ was determined using the fluorescent dye, fura-2, as described (Seifert et al., 1992). O_2^- formation in intact HL-60 cells and in a cell-free system was determined as described (Seifert et al., 1988, 1989).

Data reproducibility

Data shown in Figures 1, 3 and 4 are the means of assay quadruplicates; SD values were generally less than 5% of the means. Similar results were obtained in at least three independent experiments. The autoradiographs shown in Figures 2a and b are representative of at least three independent experiments. Data shown in Figures 5 and 6 represent typical original tracings from individual experiments. Similar results were obtained in at least three experiments with different preparations of HL-60 cells. Data shown in Table 1 are the means ± SD of assay quadruplicates. Similar results were obtained in at least three independent experiments.

RESULTS

In order to exclude the possibility that MP binds to formyl peptide receptors, we first studied modulation of fMet-Leu-Phe-binding by MP in HL-60 membranes. Binding of [^{3}H]fMet-Leu-Phe at 3 nM was 610 ± 21 fmol mg^{-1} (mean ± S.D., $n = 3$). MP (10 μM) had no effect on agonist binding (data not shown).

Concentration/response curves for the effects of MP and fMet-Leu-Phe on high-affinity GTP hydrolysis in HL-60 mem-

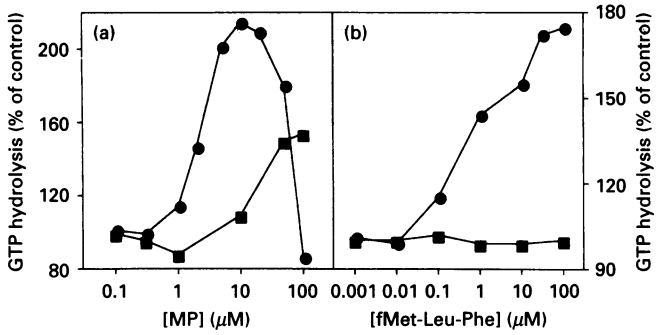

Figure 1 Concentration/response curves for MP and fMet-Leu-Phe on high-affinity GTP hydrolysis in HL-60 membranes: effect of PTX

High-affinity GTPase activity in HL-60 membranes was determined as described in Materials and methods in the presence of MP (**a**) or fMet-Leu-Phe (**b**) at the indicated concentrations. Treatment of HL-60 cells with carrier (control) or PTX was performed as described in Materials and methods. Control membranes (●); membranes from PTX-treated cells (■). Basal GTP hydrolysis rates in membranes from carrier- and PTX-treated cells were 17.2 ± 0.4 pmol·min^{-1} mg^{-1} and 10.0 ± 0.2 pmol·min^{-1} mg^{-1}, respectively.

branes are shown in Figure 1. In control membranes, MP activated GTP hydrolysis in a biphasic manner (EC_{50} = 1–2 μM, maximum at 10 μM). At higher concentrations, the stimulatory effect of MP was lost and MP (100 μM) reduced GTP hydrolysis slightly below basal values. In membranes from PTX-treated cells, MP activated GTPase with an EC_{50} of 15 μM. In marked contrast to control membranes, the effect of MP in membranes from PTX-treated cells reached a plateau at 30–100 μM. In the experiment shown, fMet-Leu-Phe activated GTPase in control membranes with an EC_{50} of 0.45 μM and a maximum at 30–100 μM. In experiments with other membrane preparations, stimulation of GTPase by fMet-Leu-Phe reached a maximum already at 10 μM (Wenzel-Seifert and Seifert, 1993). In agreement with recent data (Klinker et al., 1993; Seifert et al., 1994), fMet-Leu-Phe had no effect on GTP hydrolysis in membranes from PTX-treated cells (see Figure 1), indicative for activation of G_i-proteins. As is the case for fMet-Leu-Phe (Klinker et al., 1993), MP increased $V_{max.}$ of GTP hydrolysis without affecting K_m (data not shown).

NEM alkylates G_i-protein α-subunits, and thereby uncouples formyl peptide receptors from G-proteins in a manner similar to that of PTX-catalysed ADP-ribosylation (Seifert et al., 1994). Pretreatment of HL-60 membranes with NEM reduced the stimulatory effect of fMet-Leu-Phe on GTPase by about 40% (Seifert et al., 1994). NEM reduced the stimulatory effect of MP (10 μM) on GTP hydrolysis by 85% (data not shown).

The effects of MP on Na^+/K^+-ATPase and Mg^{2+}-ATPase were assessed. The activity of these enzymes in HL-60 membranes amounted to 0.14 ± 0.01 μmol·min^{-1} mg^{-1} and 0.25 ± 0.03 μmol·min^{-1} mg^{-1} (means ± S.D., $n = 3$), respectively. MP (10 μM) inhibited Na^+/K^+-ATPase and Mg^{2+}-ATPase by 21% and 36%, respectively (data not shown).

We also compared the effects of fMet-Leu-Phe and MP on GTP[γS] binding. When GTP[γS] binding was determined at 25 °C, the stimulatory effects of fMet-Leu-Phe and MP (10 μM each) were 149% and 39%, respectively (Table 1). MP at 3 μM increased GTP[γS] binding by 16% and MP at 1 μM was ineffective (data not shown). The stimulatory effect of MP (10 μM) on GTP[γS] binding was completely PTX-sensitive (see Table 1). When GTP[γS] binding was assessed at 0 °C, fMet-Leu-Phe (10 μM) still enhanced nucleotide association to membranes by 30%, whereas MP at 100 μM was without stimulatory effect (data not shown).

Table 1 Effect of PTX on MP- and fMet-Leu-Phe-stimulated GTP[γS] binding in HL-60 membranes

Pretreatment of HL-60 cells with PTX or carrier (control) was performed as described in Materials and methods. GTP[γS] binding in HL-60 membranes was determined as described in Materials and methods. The concentration of MP and fMet-Leu-Phe was 10 μM each.

	GTP[γS] binding (pmol mg^{-1})	
Addition	Control	PTX
Solvent (control)	1.27 ± 0.10	0.99 ± 0.05
fMet-Leu-Phe	3.16 ± 0.17	1.02 ± 0.03
MP	1.77 ± 0.11	0.75 ± 0.04

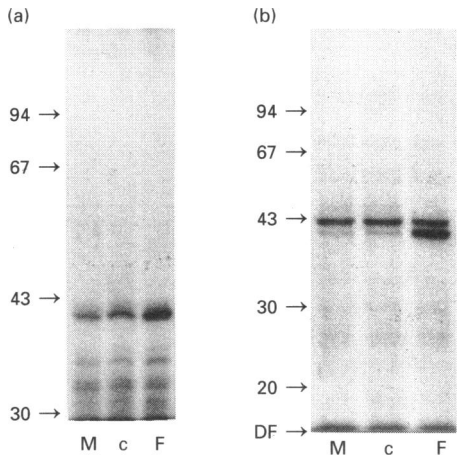

Figure 2 Effects of MP and fMet-Leu-Phe on incorporation of GTP azidoanilide into, and CTX-catalysed ADP-ribosylation of, G_i-protein α-subunits in HL-60 membranes

(**a**) Photolabelling was performed as described in Materials and methods. The autoradiogram of an SDS gel containing 4 M urea and 9% (m/v) acrylamide is shown. Lane 1, MP (10 μM) (M); lane 2, solvent (control) (c); lane 3, fMet-Leu-Phe (10 μM) (F). (**b**) ADP-ribosylation was performed as described in Materials and methods. The autoradiogram of an SDS gel containing 10% (m/v) acrylamide is shown. Lane 1, MP (10 μM) (M); lane 2, solvent (control) (c); lane 3, fMet-Leu-Phe (10 μM) (F). Numbers on the left, molecular masses of marker proteins (kDa). DF, dye front.

Labelling of G_i-protein α-subunits in HL-60 membranes by the photoreactive GTP analogue, GTP azidoanilide, was studied. As has been shown recently (Klinker et al., 1993; Seifert et al., 1994), fMet-Leu-Phe increased incorporation of GTP azidoanilide into a 40 kDa protein, corresponding to the α-subunit of G_{i2} (Figure 2a). fMet-Leu-Phe also stimulated CTX-catalysed ADP-ribosylation of $G_{i\alpha 2}$ in HL-60 membranes (Figure 2b) (Klinker et al., 1993; Seifert et al., 1994). Unlike fMet-Leu-Phe, MP had no stimulatory effect on photolabelling and CTX-catalysed ADP-ribosylation of $G_{i\alpha 2}$.

We assessed the effects of MP and MP 7 on GTP hydrolysis in HL-60 membranes and by G_i/G_o-proteins. In the reconstituted system, MP showed only relatively small stimulatory effects on GTPase, and even at a concentration as high as 100 μM the effect of MP was not yet saturated (Figure 3). MP 7 was similarly potent and as effective as MP at increasing GTP hydrolysis in HL-60 membranes. In marked contrast, MP 7 was several-fold

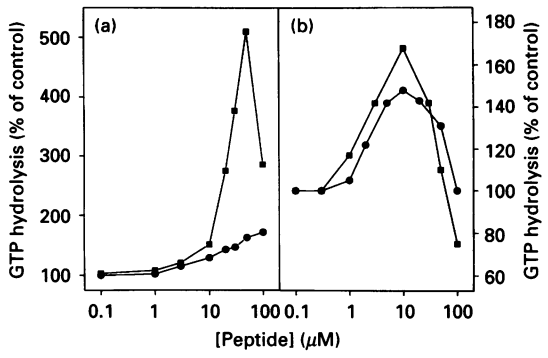

Figure 3 Concentration/response curves for MP and MP 7 on high-affinity GTP hydrolysis by reconstituted G_i/G_o-proteins and in HL-60 membranes

High-affinity GTPase activity of a reconstituted mixture of G_i/G_o-proteins (**a**) and in HL-60 membranes (**b**) was determined in the presence of MP (●) or MP 7 (■) at various concentrations as described in Materials and methods. Basal GTP turnover of reconstituted G_i/G_o-proteins was 0.085 min^{-1}. Basal GTP hydrolysis rate in HL-60 membranes was 17.4 ± 0.5 pmol · min^{-1} mg^{-1}.

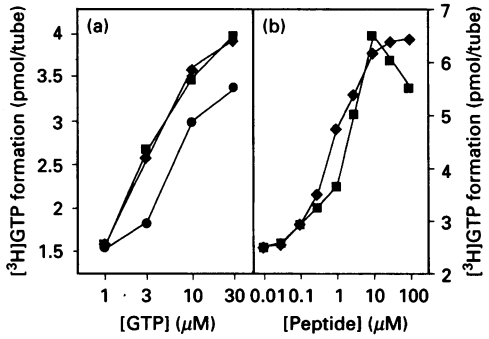

Figure 4 Effects of MP and MP 7 on [³H]GTP formation in HL-60 membranes and by NDPK purified from bovine liver mitochondria

[³H]GTP formation in HL-60 membranes (**a**) and by purified NDPK (**b**) was determined as described in Materials and methods. (**a**) [³H]GTP formation in HL-60 membranes was determined in the presence of 0.5 μM [³H]GDP and GTP at various concentrations. Basal [³H]GTP formation (●); [³H]GTP formation in the presence of MP (10 μM) (■) or MP 7 (10 μM) (◆). (**b**) [³H]GTP formation by purified NDPK was determined in the presence of 0.5 μM [³H]GDP, 10 μM GTP and MP (■) or MP 7 (◆) at various concentrations.

more effective than MP at stimulating the GTPase of G_i/G_o-proteins. In addition, the stimulatory effect of MP 7 reached a maximum at 30 μM. Higashijima et al. (1990) also found that MP 7 is a much more effective activator of reconstituted PTX-sensitive G-proteins than MP.

We studied the effects of MP on [³H]GTP formation from [³H]GDP and GTP in HL-60 membranes under conditions which were very similar to those employed for measurement of GTP hydrolysis (see Materials and methods). Using a fixed concentration of [³H]GDP (0.5 μM), the addition of GTP at increasing concentrations resulted in a progressive increase in [³H]GTP formation in HL-60 membranes (Figure 4a). In the presence of GTP above 1 μM, MP and MP 7 (10 μM each) increased [³H]GTP formation up to 44%. Both peptides were similarly effective at increasing [³H]GTP-formation. [³H]GTP formation in HL-60 membranes was abolished by UDP (10 mM) and by the addition of EDTA (10 mM) (data not shown). In the presence of ATP, ITP, CTP and UTP (10 μM each), MP (10 μM)

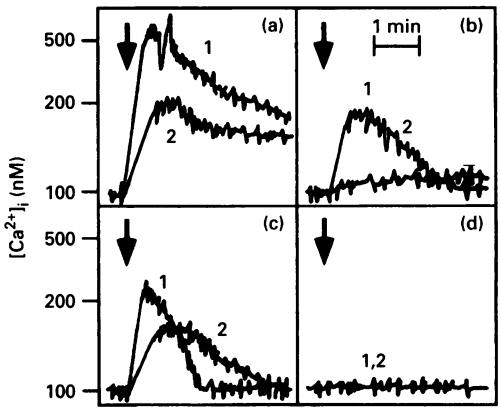

Figure 5 Effect of PTX on MP- and fMet-Leu-Phe-induced increases in $[Ca^{2+}]_i$ in HL-60 cells

Determinations of $[Ca^{2+}]_i$ were performed as described in Materials and methods. The increases in $[Ca^{2+}]_i$ induced by MP and fMet-Leu-Phe in the presence of 1 mM extracellular $CaCl_2$ (**a** and **b**) or 1 mM extracellular EGTA (**c** and **d**) were assessed. Treatment with carrier (control) (**a** and **c**) or PTX (**b** and **d**) was performed as described in Materials and methods. Trace 1, fMet-Leu-Phe (1 μM); trace 2, MP (3 μM). Arrows indicate the addition of stimuli. Superimposed original fluorescence tracings are shown.

increased [³H]GTP formation by up to 30% (data not shown). These findings show that MP-induced stimulation of [³H]GTP formation in HL-60 membranes was mediated through NDPK (Parks and Agarwal, 1973; Seifert et al., 1988; Otero, 1990).

For comparison, we also assessed the effects of MP and MP 7 on NDPK purified from bovine liver mitochondria. MP was an effective activator of [³H]GTP formation from [³H]GDP and GTP by purified NDPK, i.e., the venom increased enzyme activity by up to 150% (Figure 4b). The EC_{50} of MP was about 2 μM and a maximum was reached at 10–100 μM. MP 7 activated purified NDPK with an EC_{50} of 0.75 μM and a maximum at 10–100 μM. By analogy to HL-60 membranes, MP and MP 7 were similarly effective at activating [³H]GTP formation by the purified kinase.

Activation of G_i-proteins by fMet-Leu-Phe in HL-60 cells results in increases in $[Ca^{2+}]_i$ and in stimulation of the O_2^--forming NADPH oxidase (Seifert et al., 1989, 1992, 1994). In the presence of extracellular Ca^{2+}, MP (10 μM) and fMet-Leu-Phe (1 μM) effectively increased $[Ca^{2+}]_i$ in HL-60 cells (Figure 5a). In the absence of extracellular Ca^{2+}, the MP- and fMet-Leu-Phe-induced increases in $[Ca^{2+}]_i$ were considerably shorter and smaller than those in the presence of extracellular Ca^{2+} (compare Figure 5a and c). These findings indicate that MP and fMet-Leu-Phe increased $[Ca^{2+}]_i$ mainly through Ca^{2+} influx from the extracellular space. As is the case for fMet-Leu-Phe (Seifert et al., 1992), MP-induced Ca^{2+} influx is mediated through non-selective cation channels as the imidazole, 1-{β-[3-(4-methoxyphenyl)-propoxy]-4-methoxyphenethyl}-1*H*-imidazole hydrochloride (SK&F 96365) (30 μM), substantially reduced the stimulatory effect of MP (data not shown). In PTX-pretreated cells, the stimulatory effects of MP and fMet-Leu-Phe on Ca^{2+} mobilization were abolished, whereas inhibition of Ca^{2+} influx was incomplete (compare Figure 5a and b and Figure 5c and d).

The effects of MP and fMet-Leu-Phe of O_2^--formation in HL-60 cells are shown in Figure 6. By comparison to fMet-Leu-Phe at a maximally stimulatory concentration (1 μM), MP (10 μM) was substantially less effective at activating O_2^--formation. However, the effect of MP at higher concentrations could not be studied as the venom lysed cells (data not shown). Cytochalasin

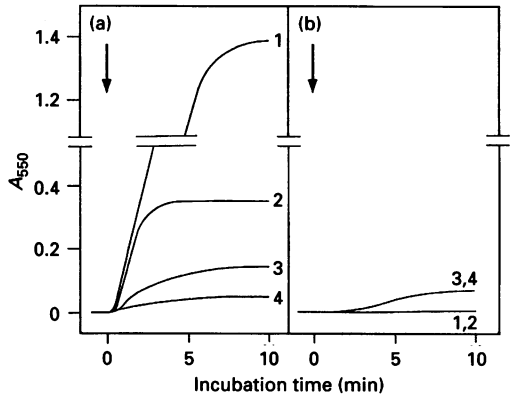

Figure 6 Effects of PTX on MP- and fMet-Leu-Phe-induced O_2^--formation in HL-60 cells

Determination of O_2^--formation was performed as described in Materials and methods. O_2^--formation was stimulated by MP (10 μM) or fMet-Leu-Phe (1 μM). In some experiments, cytochalasin B (1 μg/ml) was added to cells 3 min before stimuli. Treatment with carrier (control) (**a**) or PTX (**b**) was performed as described in Materials and methods. Trace 1, fMet-Leu-Phe with cytochalasin B; trace 2, fMet-Leu-Phe alone; trace 3, MP with cytochalasin B; trace 4, MP alone. Arrows indicate the addition of stimuli. Superimposed original spectrophotometric tracings are shown.

B, which prevents actin polymerization, potentiated MP- and fMet-Leu-Phe-induced O_2^- formation. PTX abolished O_2^--formation caused by fMet-Leu-Phe in the absence and presence of cytochalasin B (compare traces 1 and 2 in Figure 6a and b). PTX reduced the extent of O_2^--formation induced by MP (10 μM) in the presence of cytochalasin B by 55% and further delayed the onset of O_2^--formation (compare trace 3 in Figure 6a and b). PTX prolonged the onset of O_2^--formation induced by MP in the absence of cytochalasin B without affecting the extent of O_2^--formation (compare trace 4 in Figure 6a and b).

Finally, we studied the effects of arachidonic acid and MP on O_2^- formation in a cell-free system consisting of HL-60 membranes and HL-60 cytosol. Arachidonic acid (160 μM) induced the formation of 6.5 ± 0.4 nmol of O_2^- min^{-1} mg^{-1} (mean $\pm$ S.D., $n = 3$). GTP[γS] (10 μM) potentiated this O_2^--formation by about three-fold (data not shown). In contrast, MP (0.1–100 μM) did not activate O_2^--formation in the cell-free system, regardless of whether GTP[γS] (0.01–10 μM) or GTP (0.1–10 μM) were present or not (data not shown). Rather, MP (10 μM) inhibited O_2^--formation induced by arachidonic acid in the absence or presence of GTP[γS] by about 30–40% (data not shown).

DISCUSSION

MP activates G_i-proteins in HL-60 cells as PTX and NEM reduced the stimulatory effects of the venom on GTP hydrolysis in membranes (see Figure 1). Additionally, the stimulations of GTP[γS] binding, increases in $[Ca^{2+}]_i$ and O_2^--formation induced by MP were also at least partially PTX-sensitive (see Figures 5 and 6 and Table 1). MP activates G_i-proteins independently of formyl peptide receptors as the venom did not affect fMet-Leu-Phe binding. As CTX-catalysed ADP-ribosylation of G_i-protein α-subunits strongly depends on agonist-occupation of formyl peptide receptors (see Figure 2b), the lack of interaction of MP with these receptors could account for its failure to enhance this reaction. Figure 7 summarizes the interactions of MP and formyl peptide receptors with G_i-proteins in HL-60 membranes.

Interestingly, parts of the stimulatory effects of MP on GTP hydrolysis, $[Ca^{2+}]_i$ and O_2^--formation were PTX-insensitive (see Figures 1, 5 and 6). Perianin and Snyderman (1989) and Norgauer et al. (1992) found that in human neutrophils, some of the stimulatory effects of MP are PTX-insensitive, too. As ADP-ribosylation of G_i-protein α-subunits in HL-60 cells was virtually complete (see Materials and methods), it is unlikely that the substantial GTP hydrolysis in HL-60 membranes caused by MP at high concentrations (30–100 μM) was due to activation of unmodified G_i-proteins. Moreover, stimulation by MP of a nucleotidase (Denker et al., 1991) cannot explain our findings as MP reduced nucleotidase activity in HL-60 membranes.

The β-adrenoceptor agonist, isoproterenol, which leads to activation of the stimulatory G-protein of adenylyl cyclase, G_s, does not stimulate GTPase in HL-60 membranes although functional β-adrenoceptors are present in this system (Seifert et al., 1989; Hagelüken et al., 1994). In addition, a part of fMet-Leu-Phe-induced Ca^{2+} influx is mediated through PTX-insensitive G-proteins, presumably through a member of the G_q-family (see Figure 5) (Wu et al., 1993a), but the formyl peptide did not show stimulatory effects on GTPase in membranes from PTX-treated cells (see Figure 1). These data suggest that the concentration and/or GDP/GTP exchange rates of G_s- and G_q-proteins in HL-60 membranes are too low to be detected in the GTPase assay. Accordingly, the PTX-insensitive stimulation of GTP hydrolysis caused by MP is unlikely to be attributable to these G-proteins (see Figure 1).

Small GTP-binding proteins of the rho/rac family, which are not substrates for PTX, are activated by MP as well (Koch et al., 1991), and rac-2 and rap-1A are involved in NADPH oxidase

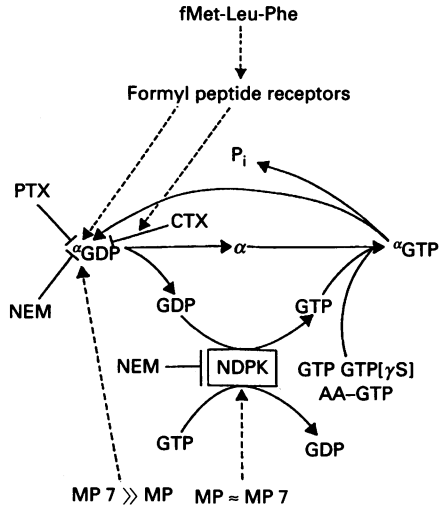

Figure 7 Model of formyl peptide receptor-mediated and receptor-independent G_i-protein activation in HL-60 membranes

G_i-proteins in HL-60 membranes may be activated through two different mechanisms, i.e., through agonist-occupied chemoattractant receptors such as formyl peptide receptors, and in a receptor-independent manner. The latter mechanism can be further divided into direct and indirect receptor-independent G-protein activation. In the case of direct receptor-independent G-protein activation, cationic-amphiphilic substances such as MP and MP 7 interact with α-subunits. In the case of indirect receptor-independent G-protein activation, cationic-amphiphilic substances interact with NDPK. MP 7 is considerably more effective than MP at activating PTX-sensitive G-proteins directly, whereas both peptides are similarly effective at activating NDPK. An important difference between receptor-mediated and direct receptor-independent G-protein activation on one hand and indirect receptor-independent G-protein activation on the other hand is that, in case of the latter mechanism, only GTP-hydrolysis is stimulated but not other parameters of G-protein activation such as GTP[γS] binding. For further explanations, see Discussion.

activation (Knaus et al., 1992; Ligeti et al., 1993; Sawai et al., 1993). These data would be compatible with our finding that MP-induced O_2^--formation in intact HL-60 cells was relatively resistant to inhibition by PTX (see Figure 6). However, rac-2 is cytosolic and translocates to the plasma membrane only in the presence of arachidonic acid (Sawai et al., 1993). Note that we assessed GTP hydrolysis in the absence of arachidonic acid and in washed membranes. Recently, Ligeti et al. (1993) reported that there is a close correlation between arachidonic acid-induced activation of the GTPase of rap-1A and NADPH oxidase-catalysed O_2^-formation in a cell-free system of HL-60 cells. We observed that MP, unlike arachidonic acid, did not activate O_2^--formation in the cell-free system, but the venom was rather inhibitory. Thus, it is unlikely that the PTX-insensitive part of MP-stimulated GTP hydrolysis in HL-60 membranes is due to activation of rac-2 and rap-1A. Another possibility to explain the PTX-insensitive effects of MP in intact neutrophils and HL-60 cells and in HL-60 membranes is discussed below.

We found that MP is an effective activator of GTPase in HL-60 membranes but only weakly effective or even ineffective with regard to GTP[γS] binding and incorporation of GTP azidoanilide into G_i-protein α-subunits (see Figures 1 and 2 and Table 1). By contrast, MP is similarly effective at activating GTP hydrolysis of, and GTP[γS] binding to, reconstituted G_i/G_o-proteins (Tomita et al., 1991). Interestingly, cationic-amphiphilic lipopeptides also do not activate photolabelling of G_i-protein α-subunits in HL-60 membranes (Klinker et al., 1993) and their stimulatory effects in neutrophils and HL-60 cells are, like those of MP, partially or even completely PTX-insensitive (Seifert et al., 1990; Offermanns et al., 1992). In addition, the stimulatory effects of substance P, which also activates G-proteins in a receptor-independent manner (Mousli et al., 1990) in human neutrophils are partially PTX-insensitive as well (Serra et al., 1988). Moreover, the β-adrenoceptor antagonist, propranolol, and the local anaesthetic, tetracaine, are more effective activators of GTP hydrolysis than of GTP[γS] binding in HL-60 membranes (Hagelüken et al., 1994). Furthermore, Wu et al. (1993b) reported that compound 48/80 activates high-affinity GTPase but not GTP[γS] binding in rat mast-cell homogenates. Similar to MP, compound 48/80 effectively stimulates GTP[γS] binding to, and GTP hydrolysis by, reconstituted G_i/G_o-proteins (Tomita et al., 1991). Finally, *in situ*, MP, MP 7, β-adrenoceptor antagonists and local anaesthetics are more potent activators of GTPase than in reconstituted systems (see Figure 3) (Hagelüken et al., 1994). In contrast to cationic-amphiphilic substances, fMet-Leu-Phe is similarly effective at activating GTP hydrolysis, GTP[γS] binding and photolabelling (see Figures 1 and 2 and Table 1) (Klinker et al., 1993; Seifert et al., 1994). Taken together, all these findings indicate that cationic-amphiphilic substances on one hand and agonist-occupied receptors on the other activate G-proteins through different mechanisms and/or induce different G-protein activation states. The results obtained by other authors support the view that MP does not simply mimic receptor activation (Nakahata et al., 1990; Joyce-Brady et al., 1991; Gusovsky et al., 1991; Danilenko et al., 1993).

The findings discussed above raise the important question inasmuch as a mechanism distinct from direct interaction with G-protein α-subunits contributes to the stimulatory effects of cationic-amphiphilic substances in general and, in particular, MP and MP 7, on GTP hydrolysis *in situ*. Interaction of MP and MP 7 with NDPK could explain the apparent discrepancies in their effectivenesses in the various assays assessing G-protein activation in membranes and the partial PTX-insensitivity of their effects. According to our model, which is illustrated in Figure 7, NDPK in HL-60 membranes catalyses the phosphorylation of GDP (either added exogenously or generated endogenously by the GTPase of G-proteins) to GTP, GTP being also the phosphoryl group donor (see Figure 4). We propose that MP and MP 7 enhances this transphosphorylation and that the newly formed GTP is channelled to, and subsequently hydrolysed by, the GTPase of G_i-protein α-subunits. GTP[γS] and GTP azidoanilide at the very low concentrations employed in our experiments (see Materials and methods) cannot serve as thiophosphoryl group- and phosphoryl group donors, respectively, for NDPK. They can bind to G-protein α-subunits only via the classical nucleotide-exchange reaction. Thus, NDPK activators would be expected not to enhance binding of GTP[γS] and GTP azidoanilide to G-proteins. The experimental data obtained with MP in HL-60 membranes are in accordance with this expectation (see Figures 1 and 2 and Table 1).

As NDPK is not a substrate for PTX, stimulation by cationic-amphiphilic substances of the kinase could circumvent, at least in part, the inhibitory effect of ADP-ribosylation on G_i-protein activation (see Figure 7). In fact, the stimulatory effects of MP and certain other cationic-amphiphilic substances are partially PTX-insensitive (see Figures 1, 5 and 6) (Serra et al., 1988; Perianin and Snyderman, 1989; Seifert et al., 1990; Norgauer et al., 1992; Offermanns et al., 1992).

The stimulatory effects of MP on GTPase were more sensitive to inhibition by NEM than those of fMet-Leu-Phe (Seifert et al., 1994). Interestingly, not only G_i-protein α-subunits possess SH-groups which are sensitive to covalent modification by alkylating substances, but also NDPK (Parks and Agarwal, 1973). Thus, in case of MP-stimulated GTP hydrolysis, NEM may impair the function of two involved proteins, whereas in the case of fMet-Leu-Phe-stimulated GTP-hydrolysis the function of only one protein is impaired (see Figure 7).

In order to obtain more direct evidence of the involvement of NDPK in G-protein activation by MP *in situ*, we studied the effects of MP on GTP formation in HL-60 membranes. In fact, we found MP to enhance [^{3}H]GTP formation from [^{3}H]GDP and GTP, provided that the latter nucleotide was employed at concentrations above 1 μM (see Figure 4a). Our failure to detect a stimulatory effect of MP on [^{3}H]GTP formation with GTP at lower concentrations, i.e., concentrations routinely employed in the GTPase assay (see Materials and methods) could be explained by rapid channelling of [^{3}H]GTP to, and cleavage by, the GTPase of G-proteins to [^{3}H]GDP and P_i, thus masking a stimulatory effect of the venom on the kinase (see Figure 7). In order to avoid interference of NDPK activity measurements with GTPase reactions, we additionally studied the effects of MP on NDPK purified from bovine liver mitochondria. As anticipated from our model (see Figure 7), MP was indeed more effective at increasing [^{3}H]GTP formation by the purified enzyme than in HL-60 membranes (see Figure 4).

In HL-60 membranes, MP and MP 7 are similarly potent and effective activators of GTP hydrolysis, whereas in a reconstituted system MP 7 is considerably more potent and effective than MP (see Figure 3). In addition, the potencies of MP and MP 7 to activate GTPase *in situ* are considerably higher than to activate the GTPase of G_i/G_o-proteins (see Figure 3) (Higashijima et al., 1988, 1990; Tomita et al., 1991). Thus, if NDPK mediates GTPase activation by MP and MP 7 *in situ*, both peptides are expected to activate NDPK-catalysed [^{3}H]GTP formation with similar effectiveness. This was, in fact, true, both with respect to HL-60 membranes and purified NDPK (see Figure 4). Moreover, the potencies of MP and MP 7 to activate GTP hydrolysis in HL-60 membranes on one hand and [^{3}H]GTP formation by purified NDPK on the other are similar (see Figures 3 and 4). Interestingly, differential effects of MP and MP analogues on the activity

of various proteins have also been reported by Raynor et al. (1992) and Danilenko et al. (1993). In agreement with our data, Kikkawa et al. (1992) reported that MP is a more potent activator of NDPK purified from rat liver than of reconstituted G_o-proteins. Taken together, MP 7 is a much more effective and potent direct activator of PTX-sensitive G-proteins than MP, whereas with regard to NDPK, both peptides show similar activity (see Figure 7). By analogy with the results obtained with MP and MP 7, the structure–activity relationships and potencies of β-adrenoceptor antagonists and local anaesthetics for GTPase activation in HL-60 membranes on one hand and reconstituted G_i/G_o-proteins on the other are different (Hagelüken et al., 1994). Future studies will have to answer the question of whether activation of NDPK by these substances in membranes accounts for the above differences.

GDP release from α-subunits is considered to be a rate-limiting step in G-protein activation by receptor agonists (Wieland et al., 1992). From our data we raise the hypothesis that the availability of GTP, at least in the case of MP and MP 7, is involved in G-protein activation, too. Some functional and/or structural barriers may control and restrict the access of GTP to G-protein α-subunits. Interestingly, NDPK-catalysed transphosphorylation of GDP to GTP takes place in the presence of an NTP-regenerating system (see Materials and methods and Figure 4). Resistance to abolishment by creatine kinase and creatine phosphate of certain NDPK-catalysed transphosphorylation reactions in HL-60 membranes had also been observed in our previous study (Seifert et al., 1988). Additionally, not only G_i-proteins but also NDPK, which can be activated by MP, is extracted from HL-60 membranes by sodium cholate and *n*-octylglucoside (J. F. Klinker and R. Seifert, unpublished work). From all these findings it may be envisaged that NDPK-catalysed transphosphorylation reactions in HL-60 membranes, leading to the formation of GTP from GDP and GTP, take place in a deeply buried domain of the plasma membrane, possibly in the vicinity of the guanine-nucleotide-binding pocket of α-subunits.

In conclusion, we have shown that MP activates G_i-proteins in HL-60 cells and that the venom and fMet-Leu-Phe do so through different mechanisms. NDPK-catalysed phosphorylation of GDP to GTP by GTP may contribute to the stimulatory effects of the venom on high-affinity GPTase in HL-60 membranes. Indirect receptor-independent GTPase stimulation by MP may be a novel mechanism of G-protein activation

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