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Interaction of mastoparan with the low molecular mass GTP-binding proteins rho/rac

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Mastoparan, which has been shown to active G proteins [1], inhibits the ADP-ribosylation of 20 kDa human platelet membrane proteins catalyzed by *Clostridium botulinum* exoenzyme C3 half-maximally and maximally (90%) at 20 and 100 μM concentrations, respectively Inhibition of ADP-ribosylation was enhanced by GTP-γS Mastoparan increased GTP hydrolysis by porcine brain rho protein and stimulated GTP binding in a concentration dependent manner The data suggest that mastoparan not only interacts with heterotrimeric G proteins but also with low molecular mass GTP-binding proteins of the rho/rac family

Mastoparan, Small GTP-binding proteins, rho, rac, *Clostridium botulinum* exoenzyme C3, ADP-ribosylation

1. INTRODUCTION

A family of heterotrimeric GTP-binding proteins, the G proteins G_s, G_i, G_o and G_t, are involved in the regulation of transmembrane signal transduction The G proteins operate by utilizing a guanine nucleotide-binding and -hydrolyzing cycle Whereas ligand receptor interaction causes a GDP/GTP exchange at the α-subunit of the G protein and subsequent activation, the active state is terminated by hydrolysis of the bound GTP by an inherent GTPase activity [2–5] These G proteins are substrates of cholera and pertussis toxins which ADP-ribosylate the α-subunits of the regulatory proteins, thereby inhibiting or facilitating signal transduction processes [6,7]

A second family of smaller proteins (M_r 20 000–25 000) with GTP-binding and GTPase activities has been identified with the ras proteins as its best-studied members [8,9]. Recently, it has been shown that the GTP-binding proteins rhoA, B, C and rac 1, 2 are substrates of *Clostridium botulinum* ADP-ribosyltransferase C3 [10–16] So far the precise functions of rho and rac proteins are unclear It has been suggested that the rho proteins are directly or indirectly involved in the regulation of the structure of the cytoskeleton [15,17]

Mastoparan is an amphiphilic tetradecapeptide toxin from wasp venom which causes mast cell degranulation [18] Various biological activities like interaction with calmodulin [19,20] and activation of phospholipase A_2 [21] have been ascribed to the peptide Recently, it has been reported that mastoparan activates G proteins directly without receptor interaction [1,22] It appears that this compound promotes the GDP/GTP exchange at G proteins in a way which is comparable with that of ligand occupied receptors Here we report that mastoparan also interacts with the low molecular mass GTP-binding proteins rho/rac, thereby inhibiting the ADP-ribosylation of the proteins by *Clostridium botulinum* exoenzyme C3

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2 MATERIALS AND METHODS

2 1 *Materials*

Clostridium botulinum exoenzyme C3 [23], *Clostridium perfringens* iota toxin [24] and rho protein from porcine brain cytosol [13] (80% purity by SDS-PAGE, with contaminants in the high molecular weight range) were purified as described Human platelet membranes were isolated as described previously [25] Pertussis toxin was donated by Dr P Gierschik (Heidelberg, Germany) Mastoparan was obtained from Sigma (Deisenhofen, Germany) or Bachem (Heidelberg, Germany) Identical results were obtained with both preparations [α-^{32}P]NAD, [^{3}H]GTP, and [γ-^{32}P]GTP were purchased from NEN (Dreieich, Germany) and all nucleotides from Boehringer (Mannheim, Germany) All other chemicals were from commercial sources

2 2 *ADP-ribosylation assay*

ADP-ribosylation assays were carried out essentially as described [23,26] The reaction medium contained mastoparan in the concentrations given, platelet membranes (protein concentration 0 5 2 mg/ml), buffer A (1 mM EDTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM PMSF, 50 mM triethanolamine-HCl (pH 7 4)), 20 nM [α-^{32}P]NAD (5 μCi/ml) in a total volume of 50 μl The reaction was initiated by the addition of C3 ADP-ribosyltransferase (25 ng) After incubation for 10 min at 30°C the reaction was stopped by addition of 1 ml trichloroacetic acid (0 3 g/ml) Proteins were collected onto nitrocellulose filters The filters were washed ten times with 1 5 ml of 6% trichloro-

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acetic acid and placed in scintillation fluid for counting of retained radioactivity The filter blank in the absence of botulinum ADP-ribosyltransferase C3 was 0 1–0 2% of added [α-^{32}P]NAD and was subtracted from retained radioactivity

[^{32}P]ADP-ribosylation of large and small GTP-binding proteins and of actin by pertussis toxin, C3 ADP-ribosyltransferase and iota toxin, respectively, was performed under modified conditions Platelet membranes (protein concentration 1 mg/ml) were preincubated without and with 50 μM mastoparan in incubation medium containing buffer A, 0 002% Lubrol PX, 0 5 mM ATP and 0 1 mM GTP for 5 min at 37°C ADP-ribosylation (1 h, 37°C) was initiated by addition of 20 μg/ml pertussis toxin (previously activated for 10 min at 37°C with 20 mM dithiothreitol and 0 2 mM ATP), 2 μg/ml iota toxin or 1 5 μg/ml botulinum ADP-ribosyltransferase C3, respectively, and of 20 nM [α-^{32}P]NAD (5 μCi/ml) in a total volume of 100 μl

For SDS-polyacrylamide gel electrophoresis (SDS-PAGE) incubation was terminated by addition of sample buffer for SDS-PAGE according to Laemmli [27] Gels (12 5%) were stained and destained and subjected to autoradiography for 12 h

2 3 *GTP-hydrolysis assay*

The time course of GTP hydrolysis was determined as described [28,29] The reaction medium consisted of 50 mM triethanolamine-HCl (pH 7 4), 1 mM dithiothreitol, 1 mM EDTA, 0 1 mg/ml bovine serum albumin, 2 mM free $MgCl_2$, 15 nM [γ-^{32}P]GTP (0 5 μCi/ml), 30 μM mastoparan and 4 μg/ml rho protein Incubations were performed at 30°C and 50 μl aliquots were removed at the indicated times The reaction was terminated by addition of 0 5 ml ice-cold sodium phosphate buffer (20 mM, pH 7 0) containing 50 mg/ml charcoal After centrifugation for 10 min (12 000×g) at 4°C, 0 4 ml of the supernatant was transferred into scintillation vials for subsequent counting of radioactivity

2 4 *GTP-binding assay*

GTP-binding to the rho protein preparation was determined as described [30] The binding buffer contained 50 mM triethanolamine-HCl (pH 7 4), 1 mM dithiothreitol 2 mM $MgCl_2$, 1 mM EDTA, 50 mM NaCl, 0 1 mg/ml bovine serum albumin, 1 mM PMSF, 12 nM [^{3}H]GTP (0 5 μCi/ml), 8 μg/ml rho protein and mastoparan in the given concentrations in a total volume of 100 μl For termination of the reaction 1 ml of washing-buffer (50 mM Tris-HCl, pH 7 4, 50 mM NaCl, 5 mM $MgCl_2$) was added and samples were filtered immediately Filters were washed 4 times with 4 ml of washing-buffer and placed in scintillation fluid for counting of retained radioactivity Because [^{3}H]GTP is partly hydrolyzed to [^{3}H]GDP in this assay, the amount of radioactive guanine nucleotide bound will be referred to as 'bound [^{3}H]GDP/GTP'

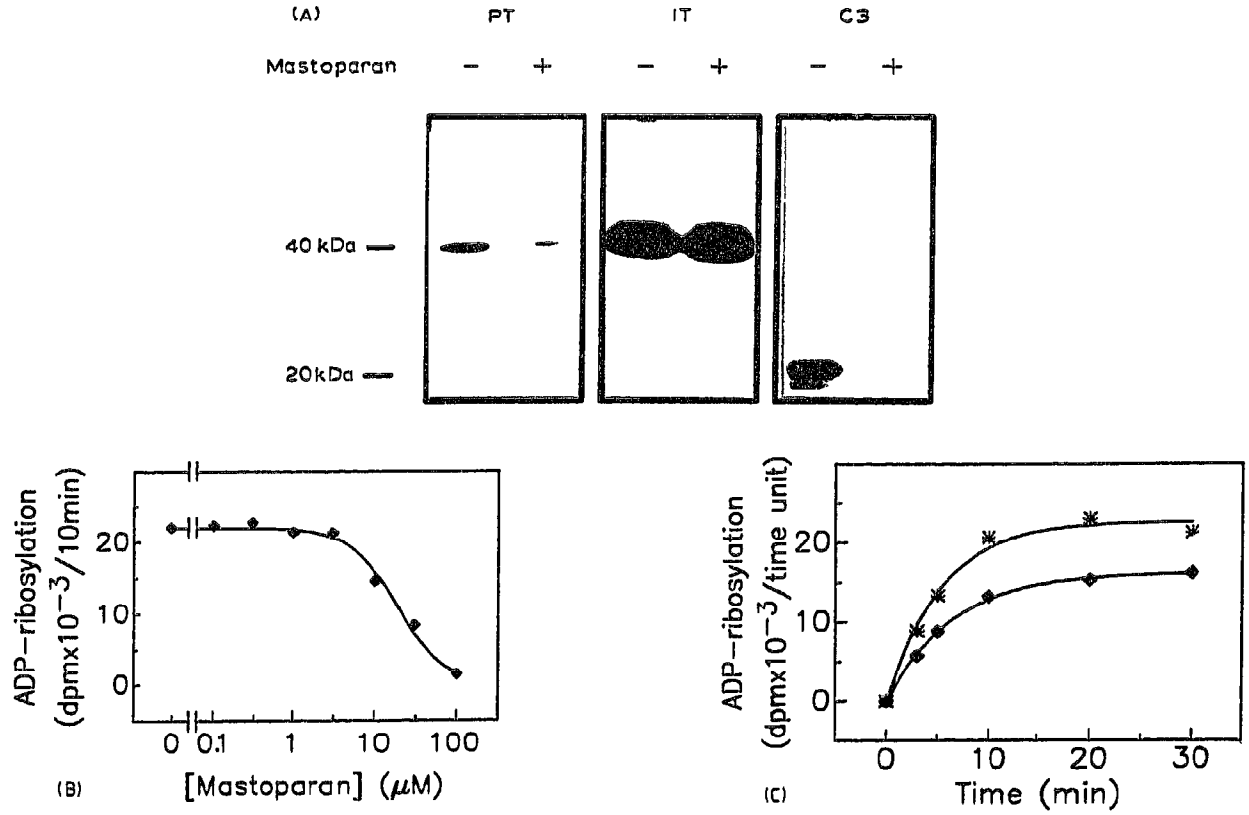

Fig 1 (A) Comparison of the effect of mastoparan on [^{32}P]ADP-ribosylation of various human platelet membrane proteins Small and large GTP-binding proteins as well as actin were [^{32}P]ADP-ribosylated with pertussis toxin (20 μg/ml), iota toxin (2 μg/ml) and C3 ADP-ribosyltransferase (1 5 μg/ml) in the absence and presence of 50 μM mastoparan as described in section 2 The autoradiogram, by which the labeled proteins were visualized after SDS-PAGE, is shown with the molecular mass markers on the left (B) Concentration dependence of the inhibition of C3-induced [^{32}P]ADP-ribosylation of human platelet membrane proteins by mastoparan Platelet membrane proteins were [^{32}P]ADP-ribosylated by C3 without and with increasing concentrations of mastoparan (0 1–100 μM) The radioactivity of labeled proteins was measured by the filter method as described Data are mean values of triplicates and are representative for 3 experiments (C) Influence of mastoparan on the time course of C3-induced [^{32}P]ADP-ribosylation of human platelet membrane proteins Human platelet membrane proteins were [^{32}P]ADP-ribosylated by C3 without (*) and with (•) 3 μM mastoparan Aliquots (50 μl) were removed at the times indicated and radioactivity of labeled proteins was determined as described in section 2 Data are mean values of duplicates and are representative for 3 experiments

2.5 *Protein concentration*

Protein concentrations were determined according to Bradford [31] with bovine serum albumin as standard

3 RESULTS

It has been shown by Higashijima and coworkers [1,22] that mastoparan activates the G_i and G_o proteins by increasing the binding of GTP to the α-subunit of the GTP-binding proteins On the other hand, activated G proteins (G_i, G_t) are reportedly poor substrates of pertussis toxin [32]. Fig. 1 shows that in agreement with those reports mastoparan inhibited the pertussis toxin-induced ADP-ribosylation of a 40 kDa G protein in human platelet membranes. While the ADP-ribosylation of actin by *Clostridium perfringens* iota toxin was not affected, mastoparan blocked the ADP-ribosylation of 20 kDa proteins catalyzed by *C botulinum* exoenzyme C3 in platelet membranes (Fig 1A) with half-maximal and maximal inhibition (90%) at 20 and 100 μM mastoparan, respectively (Fig. 1B). The time course of ADP-ribosylation of human platelet membrane rho/rac proteins was apparently not changed in the presence of 3 μM mastoparan, however, maximal ADP-ribosylation was reduced by about 30% (Fig 1C)

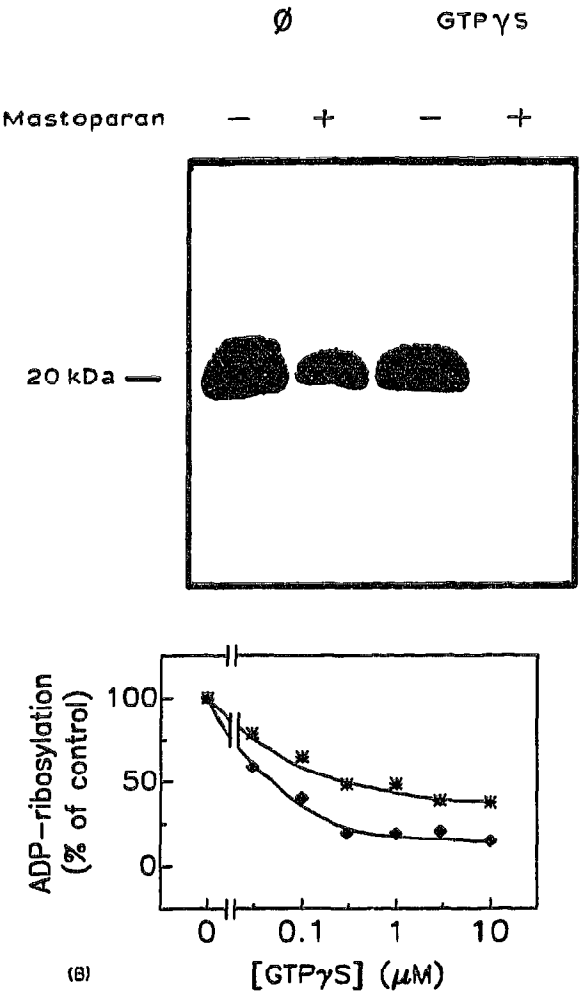

Fig 2 (A) Influence of GTPγS on the effect of mastoparan on C3-induced [^{32}P]ADP-ribosylation of platelet membrane proteins Platelet proteins were [^{32}P]ADP-ribosylated by C3 without and with 30 μM mastoparan in the absence or presence of 1 μM GTPγS The autoradiogram of the SDS-PAGE analysis of the labeled proteins is shown (B) Inhibitory effects of GTPγS and mastoparan on C3-induced [^{32}P]ADP-ribosylation of human platelet membrane proteins Platelet membranes were ADP-ribosylated without (*) or with (•) 10 μM mastoparan in the presence of increasing concentrations of GTPγS The radioactivity incorporated was determined by the filter method Inhibition of GTPγS is given in percent of control (absence of GTPγS) Control values of incorporation of ADP-ribose in the absence and presence of mastoparan were 109 000 and 81 000 dpm/10 min, respectively. Data are mean values of duplicates and are representative for 2 experiments.

It has been reported previously that GTPγS inhibits the C3-induced ADP-ribosylation of platelet membrane proteins. Fig. 2A shows that mastoparan (10 μM) increased the inhibitory effect by GTPγS. In the absence of mastoparan, GTPγS decreased ADP-ribosylation maximally by about 60% (Fig. 2B) When mastoparan (10 μM) was added in the absence of GTPγS, the incorporation of [^{32}P]ADP-ribose was decreased from 109 000 to 81 000 dpm/10 min Under this condition, the maximal inhibitory effect of GTPγS increased to about 85%, while the half-maximal effect of GTPγS (0 05 μM and 0.03 μM without and with mastoparan, respectively) was almost not changed GDP or GDPβS did neither increase the inhibitory effect of mastoparan nor were these nucleotides able to prevent the mastoparan-induced inhibition even at high concentrations (not shown).

Since mastoparan was shown to stimulate the GTPase cycle of G proteins, we were prompted to study whether this holds true also for the small GTP-binding protein rho which serves as substrate of C3. At first we confirmed that masoparan also inhibited the C3-induced ADP-ribosylation of rho protein purified from porcine brain (not shown). The influence of mastoparan on the GTP hydrolysis catalyzed by the rho protein is

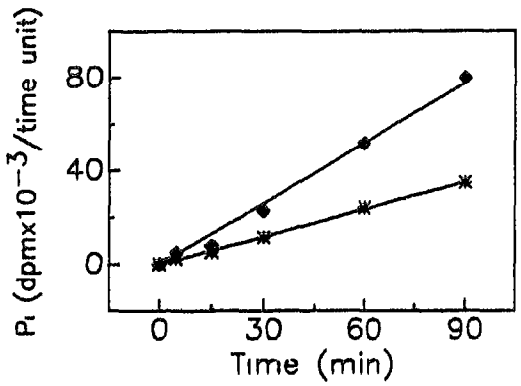

Fig 3 Time-dependent effect of mastoparan on GTP hydrolysis Release of orthophosphate from [γ-^{32}P]GTP by porcine brain rho was determined in the absence (*) and presence of 30 μM mastoparan (•) Aliquots (50 μl) were removed at the indicated times and further processed as described

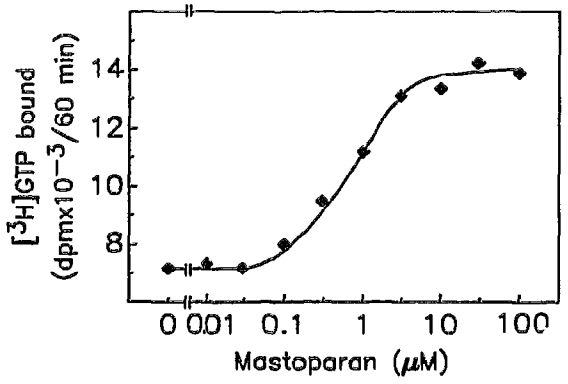

Fig 4 Concentration dependent effect of mastoparan on [^{3}H]GDP/GTP-binding to bovine brain rho protein [^{3}H]GDP/GTP was allowed to bind for 60 min at 30°C to the rho preparation The radioactivity bound to the rho protein was determined by the filter method as described Data are mean values of triplicates and are representative of 3 experiments

depicted in Fig 3 Mastoparan (30 μM) increased the release of orthophosphate about 2 3-fold after 90 min The time course of GTP hydrolysis was linear for at least 90 min in the presence and absence of mastoparan Furthermore, Fig 4 shows that mastoparan stimulated the binding of [^{3}H]GDP/GTP to the rho protein in a concentration dependent manner. A similar mastoparan effect on binding of [^{3}H]GDP/GTP was observed in the presence of 0 5 mM dimyristoyl L-α-phosphatidylcholine (data not shown) Stimulation of GTP/GDP-binding was observed at rather low concentrations of mastoparan with half-maximal and maximal effects at about 0 7 μM and 10 μM, respectively.

4. DISCUSSION

Mastoparan has been shown to activate G proteins [1,22] This is most likely the reason why mastoparan inhibited the ADP-ribosylation of about 40 kDa proteins by pertussis toxin, because it is known that the activated G protein is a poor substrate of the toxin [32] Mastoparan also inhibited the *C botulinum* exoenzyme C3-induced ADP-ribosylation of proteins of approximately 20 kDa. Several findings reported in this communication indicate that this effect of the histamine liberator mastoparan is caused by its interaction with the low molecular mass GTP-binding proteins of the rho/rac family which are substrates of C3 First, it has been reported that the ADP-ribosylation by C3 is regulated by guanine nucleotides [33]. Similarly as observed for the pertussis toxin-induced ADP-ribosylation, GTPγS inhibits the C3-induced ADP-ribosylation of 20 kDa proteins in human platelet membranes [33] Accordingly, the inhibitory effect of mastoparan on ADP-ribosylation increased with GTPγS. Mastoparan did not unspecifically block ADP-ribosylation reaction because the modification of platelet membrane actin by *C perfringens* iota toxin was not impaired Importantly, mastoparan stimulated GTP-binding and GTP hydrolysis catalyzed by rho protein Most likely, these mastoparan effects on rho/rac proteins are not simply caused by stabilisation of the GTP-binding proteins against inactivation or denaturation because even without the tetradecapeptide the purified rho protein elicited a linear GTP-hydrolyzing activity for at least 90 min Concentrations of mastoparan necessary for inhibition of ADP-ribosylation were higher than for stimulation of nucleotide binding Thus, it appears that the nucleotide binding activity of the rho protein is more sensitive towards mastoparan than its ability to serve as substrate for C3

Recently, it has been reported that the C3-induced ADP-ribosylation of about 20–25 kDa proteins in rod outer segment membranes is regulated via the activated light receptor rhodopsin in a manner similar to that of pertussis toxin-catalyzed ADP-ribosylation of transducin [34] Therefore, it has been suggested that also the small GTP-binding proteins which are substrates of C3 somehow interact with G protein-coupled receptors Some effects of mastoparan on intact cells have been ascribed to the ability of the peptide to activate G proteins, particularly G_i and G_o [35,36] Because some of these effects are pertussis toxin-insensitive, we speculate that low molecular mass GTP-binding proteins might be involved in some of these processes

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