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POSTTRANSLATIONAL ISOPRENYLATION OF RHO PROTEIN IS A PREREQUISITE FOR ITS INTERACTION WITH MASTOPARAN AND OTHER AMPHIPHILIC AGENTS

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SUMMARY: The amphiphilic agents melittin, compound 48/80 and mastoparan inhibit ADP-ribosylation of porcine brain rho protein by *Clostridium botulinum* exoenzyme C3. However, ADP-ribosylation of recombinant rhoA expressed in *E.coli* was not inhibited by these agents. Accordingly, steady state GTP hydrolysis by recombinant rhoA was not stimulated by mastoparan, whereas GTP hydrolysis by porcine brain rho was stimulated 2.5-fold in the presence of this wasp venom. After microinjection of recombinant rhoA into *Xenopus laevis* oocytes the inhibitory effect of mastoparan on C3 ADP-ribosylation was restored. The data suggest that the amphiphilic agents tested are only active at the posttranslationally processed form of rho and that they exert their effects via the C-terminal end.

The rhoA, B, C proteins belong to the family of the small GTP-binding proteins with a molecular mass of 20 to 25 kDa (1, 2). They have GTP-binding and hydrolyzing properties and are posttranslationally modified by geranylgeranylation, cleavage of the carboxy-terminal three amino acids and subsequent methylation of the C-terminal cysteine (3). Among the small GTP-binding proteins they are unique insofar as they are ADP-ribosylated by *Clostridium botulinum* exoenzyme C3 (4-6). Accordingly, this bacterial exoenzyme has been used to investigate the functional and structural properties of the rho proteins. Microinjection of constitutively active Val-14 rhoA into mouse fibroblasts (7) or *Xenopus laevis* oocytes (8) causes morphological alterations which can be reversed by ADP-ribosylation of activated rho, indicating the involvement of rho proteins in the organization of the cytoskeleton.

Recently, it has been demonstrated that the vasp venom mastoparan and other amphiphilic agents directly activate heterotrimeric G proteins without receptor interaction (9, 10). Subsequently, the activation of small GTP-binding rho proteins by mastoparan, compound 48/80, melittin and compound 48/80 was shown (11, 12). Here

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we report that the amphiphilic agents melittin, compound 48/80 and mastoparan inhibit ADP-ribosylation and increase GTP hydrolysis only of the posttranslationally modified form of rho.

MATERIALS AND METHODS

Materials: Rho protein from porcine brain cytosol (6), recombinant rhoA protein (7, 8) and *Clostridium botulinum* exoenzyme C3 (13) were purified as described. Melittin was a gift from Dr. E. Habermann (Gießen, F.R.G.). Mastoparan and compound 48/80 were purchased from Sigma (Deisenhofen, F.R.G.). [^{14}C]isopentenyl pyrophosphate was obtained from Amersham (Braunschweig, F.R.G.), [α-^{32}P]NAD and [γ-^{32}P]GTP were obtained from NEN (Dreieich, F.R.G.) and all nucleotides from Boehringer (Mannheim, F.R.G.). All other chemicals were obtained from commercial sources.

ADP-ribosylation assay: ADP-ribosylation assays were essentially performed as described (13, 14). The reaction medium contained mastoparan in the concentrations given, purified porcine brain rho protein (3.5 μg/ml) or recombinant rhoA protein (5 μg/ml), buffer A (1 mM EDTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM PMSF and 50 mM triethanolamine-HCl (pH 7.4), 30 μM GTPγS, 0.1 μM [α-^{32}P]NAD (0.2 μCi/ml) in a total volume of 50 μl. The reaction was initiated by the addition of C3 ADP-ribosyltransferase (0.25 μg/ml). After incubation for 10 min at 30°C the reaction was stopped by addition of 1 ml trichloroacetic acid (300 mg/ml). Proteins were collected onto nitrocellulose filters. The filters were washed ten times with 1.5 ml of 60 mg/ml trichloroacetic 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.

For SDS-polyacrylamide gel electrophoresis (SDS-PAGE) 100 μM mastoparan, 100 μg/ml compound 48/80 and 100 μM melittin were added and incubation was terminated by addition of sample buffer for SDS-PAGE as described (15). Gels (12.5%) were stained and destained and subjected to autoradiography for 12 hours.

Xenopus oocyte lysate (20 μl) was [^{32}P]ADP-ribosylated (30 min at 37°C) in buffer A in the absence and presence of 100 μM mastoparan, 1 μg/ml ADP-ribosyltransferase C3, 0.1 μM [α-^{32}P]NAD (5 μCi/ml) in a total volume of 100 μl. The reaction was stopped by addition of 1 ml trichloroacetic acid (300 mg/ml). The sample was centrifuged for 10 min at 12,000 g, the pellet was washed with ether, dissolved in 20 μl sample buffer and subjected to 15% SDS-PAGE and autoradiography for 72 h.

GTP hydrolysis assay: The GTP hydrolysis assay was performed as described (16, 17). The reaction medium contained 50 mM triethanolamine-HCl (pH 7.4), 1 mM dithiothreitol, 0.9 mM $MgCl_2$, 1 mM EDTA, 0.1 mg/ml bovine serum albumin, 0.5 mM dimyristoyl L-α-phosphatidylcholine, 15 nM [γ-^{32}P]GTP (0.5 μCi/ml) and recombinant rhoA (0.1 μg) or purified porcine brain rho protein (0.7 μg) in a total volume of 0.1 ml. Incubations were performed in the absence or presence of 100 μM mastoparan at 30°C for 20 min. The reaction was discontinued by addition of 0.5 ml ice-cold sodium phosphate buffer (20 mM, pH 7.0) with 50 mg/ml charcoal. Samples were centrifuged for 10 min (12,000 g) at 4°C and subsequently 0.4 ml of the supernatant was transferred into scintillation vials for counting of radioactivity.

In vitro isoprenylation of recombinant rhoA: Recombinant rhoA preparation (1 μg) was incubated (1 h, 37°C) in buffer A with 10 mM thymidine, 0.4 mM GDP and

0.1 mM NAD in the absence and presence of 80 μg/ml ADP-ribosyltransferase C3 in a total volume of 20 μl.

Xenopus oocyte lysate preparation and *in vitro* isoprenylation were performed as described (18). In brief, 100 *Xenopus* oocytes were lysed by trituration in 50 μl of buffer B (10 mM Tris hydrochloride, pH 7.45; 5 mM dithiothreitol, 4 mM EDTA) and the cytoplasmic fraction was obtained by centrifugation. For *in vitro* isoprenylation cytoplasm (50 μg of protein) was incubated for 2h at 37°C in 50 mM Tris hydrochloride (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.5% Triton X-100, 1 μg of recombinant rhoA preparation preincubated in the absence and presence of ADP-ribosyltransferase C3 and [^{14}C]isopentenyl pyrophosphate (55 mCi/mmol). After addition of 1 ml trichloroacetic acid (300 mg/ml) the sample was centrifuged (10 min, 12,000 g), washed with ether, dissolved in 25 μl of sample buffer and separated on a 15% SDS-polyacrylamide gel containing 4 M urea for better separation of recombinant rhoA and ADP-ribosylated recombinant rhoA. The dried gel was subjected to autoradiography for 5 days at -70°C.

Microinjection into *Xenopus* oocytes: Stage VI oocytes from *Xenopus laevis* were selected after collagenase incubation of the ovarian tissue as described (19) and kept in Barth's medium (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$ and 10 mM Hepes, pH 7.8) at 16-19°C overnight. One hour after microinjection of vehicle (50 nl of 10 mM Tris, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF with 10 ng of bovine serum albumin) and of recombinant rhoA protein (10 ng in 50 nl of vehicle), respectively, into each of fifty oocytes, progesterone (10 μM) was added for one hour. Thereafter, progesterone was removed and after one further hour of incubation (19°C) oocytes were homogenized in 0.1 ml of 0.32 M sucrose with 1 mM PMSF, centrifuged for 10 min at 1,000 g and the cytoplasmic fraction between lipid and yolk layer was removed and centrifuged for 20 min at 12,000 g. The pellets were resuspended in 80 μl of 0.32 M sucrose with 1 mM PMSF for the C3 [^{32}P]ADP-ribosylation assay as described above.

Protein concentration: Protein concentrations were determined as described (20) with bovine serum albumin as standard.

RESULTS

Fig. 1a shows that the amphiphilic agents melittin, compound 48/80 and mastoparan inhibit ADP-ribosylation of porcine brain rho protein by *C. botulinum* exoenzyme C3. On the contrary, no effect of these agents on ADP-ribosylation of recombinant rhoA was observed. Also, C3 ADP-ribosylation of porcine brain rho protein is inhibited in a concentration dependent manner by mastoparan. Half maximal and maximal (90%) inhibition of C3 ADP-riboslyation were observed at 4.4 and 34 μM concentrations of mastoparan, respectively. However, little influence of mastoparan on C3 ADP-ribosylation of recombinant rhoA was found (Fig. 1b). Even at 200 μM mastoparan the ADP-ribosylation of recombinant rhoA was not significantly reduced.

Furthermore, the effect of mastoparan on GTP hydrolysis of both types of rho protein preparations was studied. In comparison to control values mastoparan did not increase GTP hydrolysis by recombinant rhoA (Fig. 2). However, GTP hydrolysis by porcine brain rho protein was increased 2.5-fold in the presence of mastoparan.

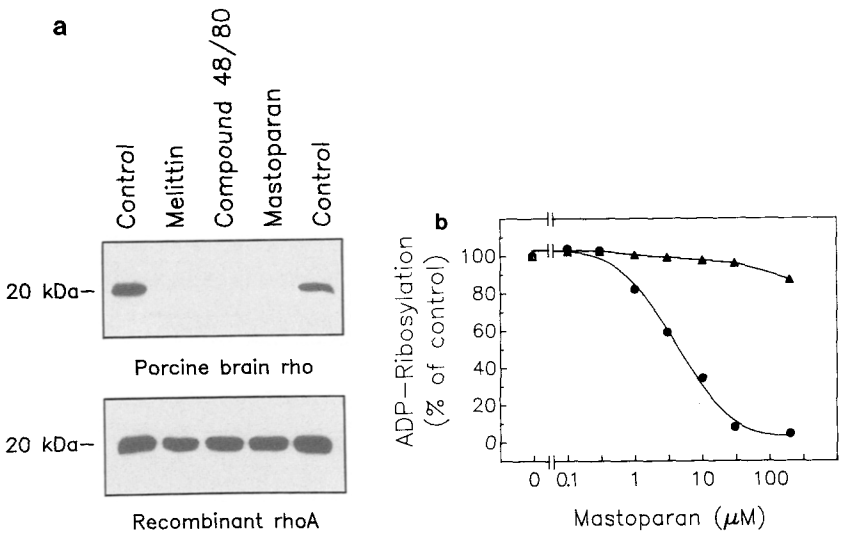


Figure 1. Comparison of the effect of various amphiphilic agents on [^{32}P]ADP-ribosylation of recombinant rhoA or porcine brain rho protein. (a) Rho proteins were [^{32}P]ADP-ribosylated with *C. botulinum* C3 ADP-ribosyltransferase in the absence and presence of melittin (100 μM), mastoparan (100 μM) or compound 48/80 (100 μg/ml) as described in the materials and methods section. The autoradiogram of the SDS-polyacrylamide gel is shown with the molecular mass marker on the left.
(b) Concentration dependence of the effect of mastoparan on [^{32}P]ADP-ribosylation of recombinant rhoA and porcine brain rho. Porcine brain rho (●) and recombinant rhoA (▲) were C3 [^{32}P]ADP-ribosylated without and with increasing concentrations of mastoparan and the radioactivity of labeled proteins was measured by the filter method as described. In the absence of mastoparan incorporation of [^{32}P]ADP-ribose into recombinant rhoA and porcine brain rho was 3957 and 8175 dpm/10 min, respectively. Data are mean values of duplicates and are representative for two experiments.

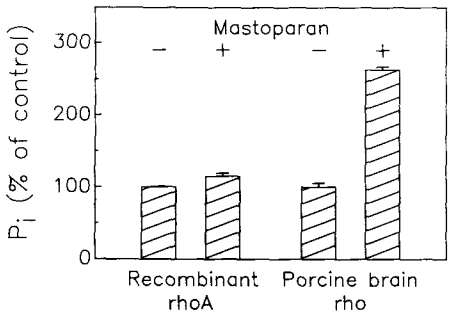
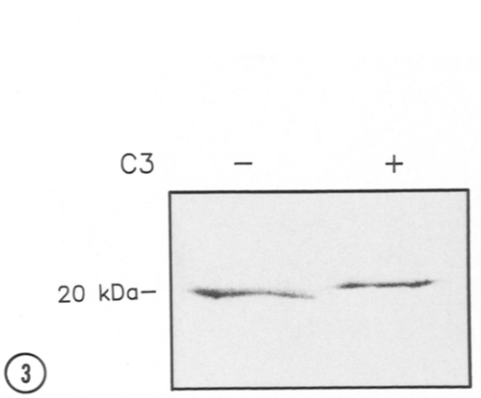


Figure 2. Comparison of the effect of mastoparan on GTP hydrolysis by recombinant rhoA or porcine brain rho protein. GTP hydrolyis by the two rho proteins was measured in the absence or presence of 100 μM mastoparan. Without mastoparan the release of inorganic phosphate was 4767 and 4683 dpm/20 min for recombinant rhoA and porcine brain rho, respectively. Data are mean values of triplicates and are representative of two experiments.



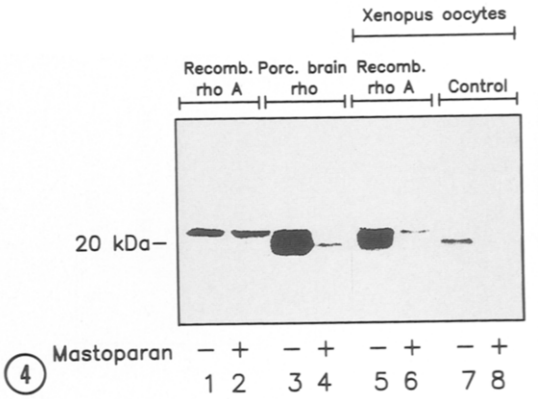


Figure 3. *In vitro* isoprenylation of recombinant rhoA. Recombinant rhoA without and with incorporated ADP-ribose was incubated with [^{14}C]isopentenyl pyrophosphate and *Xenopus* oocyte lysate for 2h as described. The autoradiogram of the SDS-polyacrylamide gel with 4 M urea is shown with the molecular mass marker on the left.

Figure 4. Inhibition of C3 [^{32}P]ADP-ribosylation of recombinant rhoA protein by mastoparan after microinjection into *Xenopus* oocytes. The effect of mastoparan on [^{32}P]ADP-ribosylation of recombinant rhoA protein alone (lanes 1 and 2), on porcine brain rho protein (lanes 3 and 4), on recombinant rhoA protein microinjected into *Xenopus* oocytes (lanes 5 and 6) and on *Xenopus* oocytes lysate (lanes 7 and 8) are shown. The autoradiogram of the SDS-PAGE analysis of the labeled proteins is depicted.

Fig. 3 shows that after incubation of recombinant rhoA with *Xenopus* oocyte lysate and [^{14}C]isopentenyl pyrophosphate, a precursor for polyisoprenylation, 20 kDa proteins are labeled. Furthermore, the molecular weight of the labeled protein, previously ADP-ribosylated by C3, was increased. No difference in the amount of radioactive material incorporated in the nonribosylated and ribosylated 20 kDa proteins was observed. Radioactive material was not incorporated into 20 kDa proteins when the *Xenopus* oocyte lysate was omitted from the reaction mixture (data not shown).

In order to study whether the posttranslational modification of recombinant rhoA is necessary for the interaction with the amphiphilic agents, we microinjected rhoA into intact *Xenopus* oocytes. As can be seen in Fig. 4 (lanes 5 and 6) [^{32}P]ADP-ribosylation of recombinant rhoA protein by mastoparan was inhibited after microinjection of recombinant rhoA into *Xenopus* oocytes. On the contrary, ADP-ribosylation of recombinant rhoA alone is not altered by mastoparan (lanes 1 and 2). For control, the inhibitory effect of mastoparan on [^{32}P]ADP-ribosylation of porcine brain rho (lanes 3 and 4) and *Xenopus* oocyte lysate (lanes 7 and 8) is shown.

DISCUSSION

Recently, we have shown that the amphiphilic agents mastoparan, mast cell degranulating peptide, melittin and compound 48/80 inhibit C3 ADP-ribosylation of

human platelet membrane rho and of rho protein purified from porcine brain cytosol (11, 12). Here we demonstrate that they are inactive on recombinant rhoA.

Because inhibition of ADP-ribosylation of rho protein by the amphiphilic agents was accompanied by an increase in steady state GTPase activity, we compared the effect of mastoparan on GTP hydrolysis by both rho protein preparations. In accordance with the results obtained for inhibition of C3-induced ADP-ribosylation, mastoparan was only active on porcine brain rho. The reason for the different effects of mastoparan on both rho protein preparations might be due to the fact that recombinant rhoA is not posttranslationally modified.

Recently, we have shown that the membrane translocation of microinjected rho protein in *Xenopus* oocytes is inhibited by lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (21), suggesting that rho protein is a substrate for isoprenylation in the oocyte (8). Using isopentenyl pyrophosphate, a precursor of polyisoprenylation, we present direct evidence that recombinant rhoA is poly-isoprenylated in *Xenopus* oocytes. Furthermore, we show that ADP-ribosylated rho is also a well-accepted substrate for polyisoprenylation. This finding largely exclude the possibility that the recently reported biological inactivation of rho by C3 (7, 8) is caused by inhibition of polyisoprenylation.

After microinjection of recombinant rhoA into *Xenopus* oocytes the inhibitory effect of mastoparan on C3-induced ADP-ribosylation was reestablished. Thus, it is conceivable that the respective *Xenopus* oocyte enzymes have posttranslationally processed the microinjected rhoA and have rendered it sensitive towards mastoparan. In fact, a geranylgeranyltransferase for rhoA was described, which was shown to be responsible for polyisoprenylation in mammalian cells (22).

In conclusion, this data point to the importance of the carboxyl terminus for the interaction of mastoparan with rho proteins. Similarly, at the α subunits of heterotrimeric G proteins mastoparan exerts its activating effects at the carboxyl terminus (23), and this part of the α subunit is important for heterotrimeric G protein activation by receptors (24). Recently, a cytosolic protein has been described which stimulates the guanine nucleotide exchange of small GTP-binding rho protein (25). However, it was only active on the posttranslationally processed form of rhoA. Thus, it is concluded that like the guanine nucleotide exchange factors the amphiphilic agents tested exert their effects at the carboxyl terminus. However, it remains open whether isoprenylation, cleavage of the carboxy-terminal three amino acids or methylation or all three modifications of rho are necessary, so that the amphiphilic agents or the guanine nucleotide exchange factors can exert their effects

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