Characterization of a mastoparan-stimulated nucleotidase from bovine brain

Bradley M. DENKER,*§ Paul TEMPST† and Eva J. NEER‡

*Renal and ‡Cardiology Divisions, Brigham and Women's Hospital and Harvard Medical School,

and †Howard Hughes Medical Institute and Department of Genetics, Harvard Medical School, Boston, MA 02115, U.S.A.

Mastoparan is a 14-amino-acid peptide that stimulates secretion from several cell types. Secretion can be partially blocked by pertussis toxin and may be mediated by guanine-nucleotide-binding proteins (G-proteins). Mastoparan can act directly on G-proteins, probably at the hormone receptor-binding site, to stimulate guanosine 5'-[γ -thio]triphosphate binding and GTPase activities of pertussis-toxin substrates G_o and G_i [Higashijima, Uzu, Nakajima & Ross (1988) *J. Biol. Chem.* **263**, 6491–6494]. We now describe a nucleotidase from bovine brain that is not a known G-protein whose GTPase and ATPase activities are stimulated by mastoparan. This nucleotidase hydrolyses ATP faster than GTP, but has similar affinities for both (0.4 μ M). Mastoparan maximally stimulates both ATPase and GTPase activities by about 8-fold after insertion of the protein into phospholipid vesicles, but does not affect the EC_{50} (concentration at which half the maximal effect is observed) for ATP and GTP. The EC_{50} for mastoparan stimulation of GTPase and ATPase is 6 and 12 μ M respectively. The native molecular mass of the partially purified mastoparan-stimulated nucleotidase is 87 kDa. This nucleotidase may be another receptor-activated enzyme, and its identification may be useful for understanding mastoparan-stimulated processes.

INTRODUCTION

Mastoparan is a 14-amino-acid polypeptide in wasp venom that causes secretion of histamine from mast cells, 5-hydroxytryptamine from platelets, catecholamines from chromaffin cells, and prolactin from the anterior pituitary (Hirai *et al.*, 1979; Kuroda *et al.*, 1980; Kurihara *et al.*, 1986). These secretory processes can be partly inhibited by pertussis toxin and may be mediated by guanine-nucleotide-binding proteins (G-proteins) (Okano *et al.*, 1985; Higashijima *et al.*, 1987; Wallace & Carter, 1989). Mastoparan can act on pure G-proteins to stimulate directly GTP[S] binding and GTPase activities of the pertussis-toxin substrates G_o and G_i (Higashijima *et al.*, 1988, 1990).

To date, heterotrimeric G-proteins are the only proteins known to have mastoparan-stimulated GTPase activity. In the course of studies to analyse the mastoparan-stimulated GTPase activity of the bovine brain G-protein G_o , we discovered another GTPase that is clearly not a G-protein, but that is significantly stimulated by mastoparan. Although our initial identification of this protein was based on its GTPase activity, it is even more active with ATP as the substrate. We now report the partial purification and characterization of this mastoparan-stimulated brain nucleotidase. This nucleotidase is not a known heterotrimeric G-protein, but the effects of mastoparan on this nucleotidase are similar to those reported for G_o and G_i . It may have a regulatory role in some of the mastoparan-stimulated cellular processes that have been described.

MATERIALS AND METHODS

Partial purification of mastoparan-stimulated nucleotidase

Fresh bovine brain cortex (80 g; from a local slaughter-house) was homogenized in 400 ml of buffer A (50 mM-Tris, pH 8.0,

75 mM-sucrose, 1 mM-EDTA, 6 mM-MgCl₂, 1 μ g of soybean and lima-bean trypsin inhibitors/ml, 3 mM-benzamide, 1 mM-dithiothreitol). All reagents in buffer A were from Sigma. The homogenate was centrifuged at 10000 *g* for 20 min, and the pellet resuspended in 350 ml of buffer A and re-centrifuged at 10000 *g* for 20 min. The second pellet was resuspended in 130 ml of buffer A and frozen at -70°C in 10 ml portions. Bovine brain membranes (20 ml; 450 mg of protein) in buffer A were solubilized in 1% Triton X-100 (Fisher) and 1 μ M-GDP[S] (Sigma) for 90 min at 4°C . The solution was centrifuged at 30000 *g* for 70 min, and 30 ml of the supernatant (160 mg of protein) was applied to a 2.5 cm $\times$ 200 cm Ultrogel ACA-34 (LKB) column equilibrated with buffer A at 45 ml/h in 0.3% Triton X-100 and 1 μ M-GDP[S]. Fractions were analysed by SDS/PAGE; proteins with Stokes radii of 5–7 nm were pooled (total volume 90 ml). Of this, 85 ml was applied to a 100 ml DEAE-cellulose (Whatman) column equilibrated with buffer A and eluted with a 600 ml linear 0–0.2 M NaCl gradient in buffer A at 30 ml/h. Mastoparan-stimulated GTPase activity was eluted in two peaks, one at approx. 0.04 M-NaCl (76–104 ml; DEAE I), and the other at about 0.1 M-NaCl with known G-proteins (300–340 ml; DEAE II). A 2 ml portion of DEAE I was concentrated 6-fold, and 100 μ g of protein in 0.2 ml was applied to a linear 5–20% sucrose gradient in buffer A for further separation. All procedures were performed at 4°C .

Phospholipid-vesicle preparation

This was done by the method of Higashijima *et al.* (1988). A 4–8 μ g sample of total protein in buffer A was diluted 1:10 into 0.5 ml of buffer B (50 mM-NaHepes, pH 8.0, 6 mM-MgCl₂, 1 mM-EDTA, 1 mM-dithiothreitol) and mixed with 0.1 ml of buffer B plus 0.17% L- α -dioleoyl phosphatidylcholine (Avanti Polar Lipids, Pelham, AL, U.S.A.) and 0.84% cholate (Sigma). The suspension was held at 0°C for 1 h and chromatographed on a 7 ml column of ACA-34 equilibrated in buffer B.

Abbreviations used: GTP[S], guanosine 5'-[γ -thio]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; G-protein, guanine-nucleotide-binding protein; α_i , α subunit of G_i class of G proteins; EC_{50} , concentration at which half the maximal effect is observed.

§ To whom correspondence should be addressed.

ATPase and GTPase activity

The standard 100 μ l reaction mixture contained 0.1–1.0 μ g of protein in buffer B, 1 μ M-ATP or -GTP (Sigma) and [γ - ^{32} P]ATP or [γ - ^{32} P]GTP (Amersham) to give a specific radioactivity of 100–2000 c.p.m./pmol depending on the requirements of the assay. Samples were incubated for 20 min at 30 °C, and 0.5 ml of 5% (w/v) trichloroacetic acid in 50 mM-Tris was added to terminate the reaction, followed by 1 ml of 10% (w/v) charcoal (Norit A; Fisher) in 5% trichloroacetic acid. Samples were centrifuged at 3000 g for 5 min, and 1 ml of clear supernatant was counted for radioactivity in an LKB 1219 Rackbeta liquid-scintillation counter. All samples were assayed in duplicate, and the blank was 2–10% of the total radioactivity in the assay, depending on the lot of radioactive GTP or ATP. ATPase and GTPase activities are expressed as pmol of phosphate per 20 min per μ g of total protein.

Physical properties of mastoparan-stimulated nucleotidase

The sedimentation coefficient and $\bar{v}$ were determined as previously described (Denker & Neer, 1991), by using 5–20% sucrose gradients in H₂O or ²H₂O made in buffer A with 0.3% Triton X-100 and internally calibrated with BSA ($s_{20,w}$ 4.31 S; $\bar{v}$ 0.732 cm³/g), carbonic anhydrase ($s_{20,w}$ 2.85 S; $\bar{v}$ 0.735 cm³/g) and catalase ($s_{20,w}$ 11.3 S; $\bar{v}$ 0.730 cm³/g). The Stokes radius was determined by gel filtration on Ultrogel ACA-34 (LKB) columns equilibrated with buffer A/0.3% Triton X-100 and internally calibrated with β -galactosidase (6.9 nm), catalase (5.2 nm), BSA (3.7 nm) and ovalbumin (2.7 nm).

Protein determination

Protein was determined by the method of Lowry *et al.* (1951) as modified by Bailey (1967).

SDS/PAGE

Analysis of proteins was routinely performed on 11%-acrylamide gels prepared as described by Laemmli (1970), and stained with Coomassie Blue or AgNO₃ (Merril *et al.*, 1981).

Mastoparan

Peptide sequence INLKALAALAKKIL; 99% pure from Sigma.

RESULTS

Mastoparan-stimulated nucleotidase is not a G-protein

During the course of studying mastoparan-stimulated GTPase activity of partially purified preparations of G-proteins, we discovered that another protein distinct from the G-proteins also had this activity. Both mastoparan-stimulated GTPase activities are eluted together on gel-filtration chromatography of Triton-solubilized crude brain membranes. They are separated on subsequent DEAE-cellulose chromatography, with one peak (DEAE I) eluted at 0.04 M-NaCl and the other proteins (DEAE II) at 0.1 M-NaCl. The G-proteins are eluted in the latter peak, and a comparison of the proteins in these two peaks is shown in Fig. 1. Although two faint bands similar in molecular mass to G-proteins are seen in peak DEAE I, two lines of evidence show that these are probably not G-proteins and that the nucleotidase activity in this fraction is not due to known G-proteins. First, we could detect no pertussis-toxin-sensitive G-protein in DEAE I. $\beta\gamma$ subunits of G-proteins (purified from bovine brain) were added to DEAE I, and the sample was treated with [^{32} P]NAD⁺ and pertussis toxin. No radiolabel was incorporated into the

proteins in DEAE I (results not shown). Second, the mastoparan-stimulated enzyme in DEAE I is more active with ATP than with GTP as substrate (see below). There was no ATPase activity detected in the G-protein fraction (DEAE II), and known G-proteins do not utilize ATP as substrate (Cassel & Selinger, 1976).

The major 43 kDa band in DEAE I (Fig. 1) was identified as glutamine synthetase (EC 6.3.1.2). Proteins in DEAE I (Fig. 1) were transferred to nitrocellulose and the 43 kDa band was excised and digested with trypsin, and the peptides were separated by h.p.l.c. as described by Tempst *et al.* (1990). Two peptides were sequenced, and the protein was identified unequivocally as glutamine synthetase. This is a 16 S octamer, which was separated from the mastoparan-stimulated nucleotidase activity by sucrose-density-gradient centrifugation (see below). The glutamine synthetase peak had no detectable ATPase activity under the assay conditions.

Mastoparan-stimulated ATPase and GTPase activities co-sediment on sucrose gradients

The sample containing mastoparan-stimulated nucleotidase was further fractionated to try to separate the mastoparan-stimulated ATPase from the GTPase activity. The proteins in DEAE I (Fig. 1) were separated on a linear 5–20% sucrose gradient and stained with AgNO₃ (Fig. 2). All the ATPase and GTPase activity sedimented together in a single peak which coincided with the mastoparan-stimulated ATPase and GTPase activities. The ATPase activities are shown in Fig. 2(a). These

Fig. 1. Comparison of DEAE I and DEAE II fractions

Samples from the steps of purification as outlined in the Materials and methods section were analysed by SDS/PAGE (11% gels) and stained with Coomassie Blue. Lane 1, molecular-mass markers. Lane 2, 20 μ g of Triton X-100-solubilized membranes. Lane 3, 40 μ g of DEAE I peak. Lane 4, 12 μ g of DEAE II peak. The DEAE I peak was concentrated 6-fold before analysis. The arrows on DEAE II indicate α_0 (39 kDa) and β (36 kDa) subunits as determined by Western blotting with antibodies specific for α_0 and β (results not shown). The major band at 43 kDa in DEAE I is glutamine synthetase (see the Results section).

Fig. 2. Separation of mastoparan-stimulated ATPase on sucrose density gradient

(b) A 100 μ g sample of DEAE I peak was applied to a linear 5–20% sucrose gradient and centrifuged at 370000 g for 9 h as described in the Materials and methods section. Fractions (200 μ l) were collected, and 40 μ l portions of each were analysed by SDS/PAGE (11% gels) and stained with AgNO_3 . The final lane represents the fraction at 3.8 ml. (a) Portions (100 μ l) from indicated fractions were inserted into phospholipid vesicles as described in the Materials and methods section and assayed for ATPase activity in the absence (●) and presence (○) of 10 μ M-mastoparan.

results support the conclusion that both the ATPase and GTPase activities arise from a single protein.

Mastoparan effects on GTPase and ATPase activities

Although the nucleotidase in DEAE I had both GTPase and ATPase activity in solution, neither activity could be stimulated by mastoparan unless the protein was inserted into phospholipid vesicles. The mastoparan-stimulated GTPase of G-proteins also requires insertion into a phospholipid vesicle (Higashijima *et al.*, 1988). Figs. 3(a) and 3(b) show the dependence of basal and mastoparan-stimulated nucleotidase activity on ATP and GTP concentrations. Table 1 summarizes the EC_{50} and velocity data for ATP and GTP with and without mastoparan. The affinity for GTP and ATP appears to be the same, with an EC_{50} of about 0.4 μ M for both. The addition of mastoparan has no apparent effect on the affinity of the enzyme for substrate. Both ATPase and GTPase activities are stimulated

about 4-fold in the presence of 10 μ M-mastoparan, but the unstimulated activity is 2–3 times higher for ATP than for GTP (Table 1).

The effect of varying mastoparan concentration on the ATPase activity is shown in Fig. 4. The EC_{50} with ATP is 12 ± 4 μ M-mastoparan ($n = 4$), and the EC_{50} for GTP is 6 ± 0.5 μ M mastoparan ($n = 4$; results not shown). We do not consider that the difference in the EC_{50} for mastoparan between ATPase and GTPase is significant. At saturating mastoparan concentrations, the maximal degree of stimulation was about 8-fold for both ATP and GTP. The degree of stimulation of the nucleotidase by mastoparan and the EC_{50} for mastoparan are similar to those reported for G_o and G_i (Higashijima *et al.*, 1988).

Size of the mastoparan-stimulated nucleotidase

The physical properties of the mastoparan-stimulated nucleotidase were further characterized by measurement of Stokes radius, partial specific volume and $s_{20,w}$, as summarized in Table

Fig. 3. Mastoparan-stimulated ATPase and GTPase activity

(a) Vesicles containing 5 µg of protein in 2.5 ml were prepared as previously described. ATPase activity was measured with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described in the Materials and methods section. The range of concentrations of ATP was 0.1–5.0 µM in the absence (●) and presence (○) of 10 µM-mastoparan. Activity is expressed as pmol of P_i released/20 min per µg of total protein. (b) Vesicles containing 5 µg of protein/2.5 ml were prepared as previously described and GTPase activity was measured with $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ at concentrations of GTP of 0.1–5.0 µM in the absence (●) and presence (○) of 10 µM-mastoparan.

Table 1. Kinetic parameters of mastoparan-stimulated nucleotidase

Substrate*	EC_{50} (µM)	V_{max} †	Stimulation (fold) ‡
ATP(–)	0.4 ± 0.1	42 ± 10	1
ATP(+)	0.4 ± 0.1	146 ± 3	3.7 ± 0.8
GTP(–)	0.4 ± 0.2	16 ± 2	1
GTP(+)	0.5 ± 0.1	72 ± 18	4.3 ± 0.8

* ATP or GTP in the absence of mastoparan (–), or in the presence of 10 µM mastoparan (+). Mastoparan values are from proteins inserted into phospholipid vesicles as described in the Materials and methods section [$n = 4$ for all conditions except ATP(–), where $n = 3$].

† V_{max} is maximal velocity, in pmol of phosphate/20 min per µg of protein at 10 µM-mastoparan.

‡ Stimulation is calculated from pairwise comparisons of activity with or without mastoparan in the same assay.

2. The molecular mass of this protein is about 87 kDa, as determined from the hydrodynamic data. The identification of this enzyme in a detergent-solubilized membrane preparation, and the fact that it is slightly more hydrophobic ($\bar{v} = 0.77$ ml/g) than an average protein ($\bar{v} = 0.74$ ml/g) suggests that it may be a membrane-associated protein.

We do not yet know the subunit composition of the enzyme. SDS/PAGE analysis of fractions from the sucrose density gradient (Fig. 2) shows no visible bands at 87 kDa. However,

Fig. 4. Effect of mastoparan concentration on ATPase activity

Vesicles were prepared as previously described and ATPase activity was measured in the presence of 1 µM-ATP. Mastoparan concentrations ranged from 0.1 to 100 µM. Results are means $\pm$ S.E.M. for four individual experiments done in duplicate. The EC_{50} is 12 ± 4 µM.

Table 2. Physical properties of mastoparan-stimulated nucleotidase

The values are calculated from all possible paired combinations of data from H_2O and $^2\text{H}_2\text{O}$ gradients, and the results given are means $\pm$ S.E.M. Each number in parentheses is the sum of experiments in H_2O and $^2\text{H}_2\text{O}$. The values of $\bar{v}$ were calculated from H_2O and $^2\text{H}_2\text{O}$ gradients by the method of Sadler (1979).

Parameter	
Partial specific volume, $\bar{v}$ (ml/g)	0.77 ± 0.04 (8)
Sedimentation coefficient, $s_{20,w}$ (S)	3.6 ± 0.2 (4)
Stokes radius, a (nm)	5.8 ± 0.3 (4)
M_r (protein plus detergent)*	104 000
	(97 100–112 000)†
Triton X-100 bound (mg/mg of protein)‡	0.17
M_r (protein only)	87 000
	(81 000–93 000)

* Molecular mass of protein and detergent, calculated from the following equation:

$$M_r = \frac{6\pi N \eta_{20,w}}{1 - (\bar{v} \rho_{20,w})} a s_{20,w}$$

where N is Avogadro's number, $\eta_{20,w}$ is the viscosity of water at 20 °C and $\rho_{20,w}$ is the density of water at 20 °C.

† Range values were calculated by using 1 S.E.M. above and below each parameter.

‡ Calculated by using $\bar{v}_{\text{protein}} = 0.735$ and $\bar{v}_{\text{Triton}} = 0.94$ (Steele *et al.*, 1978).

there are other proteins in the 50–60 kDa range that appear to form a peak with the ATPase activity. At this degree of purification, it is not possible to identify which protein band is the nucleotidase, and the enzyme activity could come from a protein which is not seen on the silver-stained gel. Attempts to identify the enzyme by covalent modification with the photoactivatable analogue 8-azido-ATP were unsuccessful.

DISCUSSION

In these studies we report an additional enzyme from bovine brain that is stimulated by mastoparan. Although the EC_{50} for GTP and mastoparan of the brain nucleotidase is similar to that for heterotrimeric G-proteins, the 3-fold greater velocity with ATP than with GTP as a substrate clearly distinguishes it from typical signal-transducing G-proteins.

The mastoparan-stimulated ATPase and GTPase activity that we describe and the mastoparan-stimulated GTPase activity of G_o are similar in several ways. Both proteins need to be inserted into phospholipid vesicles for stimulation to occur. Most biological effects of mastoparan have required a phospholipid environment, possibly owing to its amphipathic amino acid sequence. Like the GTPase of G-proteins, this nucleotidase has apparent affinities for substrate in the $0.5\text{ }\mu\text{M}$ range, and is stimulated by a similar range of mastoparan concentration. The EC_{50} for mastoparan stimulation is similar to the G-proteins ($6\text{ }\mu\text{M}$ for GTPase and $12\text{ }\mu\text{M}$ for ATPase of this nucleotidase, compared with $5\text{--}10\text{ }\mu\text{M}$ for G_o ; Higashijima *et al.*, 1990). Like the G-protein α -subunits, the brain nucleotidase is slightly hydrophobic ($\bar{v} = 0.77$), which suggests that it may be membrane-associated.

Mastoparan has been used to stimulate a variety of physiological processes in primary and cultured cell preparations. Mastoparan stimulates 5-hydroxytryptamine release from platelets, $InsP_3$ accumulation in hepatocytes and MDCK cells, histamine secretion from rat peritoneal mast cells, and insulin secretion from islet cells (Ozaki *et al.*, 1990; Tohkin *et al.*, 1990; Eng & Lo, 1990; Mousli *et al.*, 1989; Yokokawa *et al.*, 1989). In these systems mastoparan caused an increase in Ca^{2+} . In some cells Ca^{2+} release could be partially inhibited by pertussis toxin, suggesting that it may be mediated through a G-protein. However, in other cells mastoparan inhibited phospholipase C, possibly through a pertussis-toxin-insensitive G-protein (Nakahata *et al.*, 1990; Wojcikiewicz & Nahorski, 1989). In neutrophils, mastoparan stimulates Ca^{2+} release through two different pathways. The first pathway appears to be through activation of phospholipase C and can be blocked with pertussis toxin. The second pathway requires extracellular Ca^{2+} and is insensitive to pertussis toxin (Perianin & Snyderman, 1989). These disparate findings suggest that mastoparan may be acting through multiple pathways. Mastoparan is known to stimulate phospholipase A_2 and to interact with calmodulin (Argiolas & Pisano, 1983; Yazawa *et al.*, 1987). The interaction of mastoparan with several proteins, as well as the nucleotidase described here, may be important in the signal-transduction pathways leading to secretion.

Higashijima *et al.* (1988, 1990) have proposed that mastoparan may interact with G-proteins at a receptor-binding domain in a manner similar to that of hormone receptors. There is some similarity of charge between the α -helix formed by mastoparan in phospholipid vesicles and the cytoplasmic domains of G-protein-coupled receptors (Dohlman *et al.*, 1987; Higashijima *et al.*, 1983; Wakamatsu *et al.*, 1983). Based on antibody studies of α_i , the C-terminus of the α subunit is thought to be the site of interaction between mastoparan and the α subunit (Weingarten *et al.*, 1990). Identification of the nucleotidase described here will be useful for further understanding of mastoparan-stimulated processes, and raises the possibility that this nucleotidase may be another receptor-activated enzyme which may share some similarities with the C-terminus of G-protein α subunits.

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