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Effects of the wasp venom peptide, mastoparan, on GTP hydrolysis in rat brain membranes

[1]Y. Odagaki, N. Nishi & T. Koyama

Department of Psychiatry, Hokkaido University School of Medicine, North 15, West 7, Sapporo 060, Japan

1 The effects of mastoparan, a wasp venom toxin, on GTP hydrolyzing activity were examined in rat brain membranes.

2 Mastoparan inhibited the low-affinity GTPase activity, defined as the amount of $^{32}P_i$ released from 0.3 μM [γ-^{32}P]-GTP in the presence of 100 μM unlabelled GTP, in a concentration-dependent manner. This inhibitory effect of mastoparan on low-affinity GTPase activity was diminished by increasing concentrations of UDP and was completely attenuated at 20 mM, indicating that activation of nucleoside diphosphokinase (NDPK) is inolved in the phenomenon.

3 In the presence of 20 mM UDP, mastoparan stimulated the high-affinity GTPase activity by increasing the V_{max} value without affecting the apparent K_M for GTP. Mastoparan-stimulated high-affinity GTPase activity was apparent at concentrations higher than 1 μM, in a concentration-dependent manner, but without saturation even at 100 μM.

4 Mastoparan-induced high-affinity GTPase activity showed a characteristic sensitivity to $MgCl_2$, quite different from that seen in L-glutamate-stimulated activity, a representative of receptor-mediated G-protein activation.

5 There appeared to be a simple additive interaction between mastoparan- and L-glutamate-stimulated high-affinity GTPase activities, indicting that distinct pools of G-proteins are involved in receptor-independent and receptor-mediated G-protein activation.

6 These results suggest that G-proteins in brain membranes are functionally altered by mastoparan through multiple mechanisms of action and that the mastoparan-induced, direct G-protein activating process lacks a synergistic or antagonistic interaction with an agonist-induced, receptor-mediated activation of G-proteins.

Keywords: Mastoparan; G-proteins; GTPase activity; nucleoside diphosphokinase; metabotropic glutamate receptor

Introduction

Mastoparan, a 14 amino acid polypeptide from wasp venom, has been shown to activate pertussis toxin-sensitive G-proteins, such as G_i and G_o, in a receptor-independent manner (Higashijima *et al*., 1988; 1990; Weingarten *et al*., 1990; Tomita *et al*., 1991). This type of direct activation of G-proteins has also been obtained with other cationic amphiphilic peptides, such as substance P and the snythetic polyamine compound 48/80 (for review, see Mousli *et al*., 1990). Besides the direct action on heterotrimeric G-proteins, mastoparan shows pleiotropic effects through multiple potential mechanisms of action, e.g., interaction with low molecular mass guanosine 5′-triphosphate (GTP)-binding proteins *rho*/*rac* (Koch *et al*., 1991), stimulation of bovine brain nucleotidase with a molecular mass of 87 kDa (Denker *et al*., 1991), activation of guanylyl cyclase (Song *et al*., 1993), blockade of the activity of calmodulin-stimulated phosphodiesterase (Barnette *et al*., 1983), activation of phospholipase A_2 (Argiolas & Pisano, 1983), inhibition of phospholipase C-catalyzed phosphoinositide hydrolysis (Wojcikiewicz & Nahorski, 1989; Nakahata *et al*., 1990), membrane-perturbing effect resulting in the inhibition of protein kinase C, Na^+/K^+-ATPase and calcium-activated phospholipase C (Raynor *et al*., 1992; Danilenko *et al*., 1993), and activation of nucleoside diphosphokinase (NDPK; NTP nucleoside diphosphate phosphotransferase; EC 2.7.4.6) (Kikkawa *et al*., 1992; Klinker *et al*., 1994; 1996; Kowluru *et al*., 1995).

The effects of mastoparan on G-proteins have been analysed by studying the GTPase of, and guanosine 5′-[γ-thio]-triphosphate (GTPγS) binding to, purified G-proteins reconstituted in phospholipid vesicles (Higashijima *et al*., 1988; 1990; Weingarten *et al*., 1990; Tomita *et al*., 1991; Klinker *et al*., 1994) or membranes prepared from various cultured cells such as HL-60 leukaemic cells (Klinker *et al*., 1994; 1996). On the other hand, there have been few studies focusing on the effects of mastoparan on G-protein function in membrane preparations derived from living animals. Mastoparan-stimulated high-affinity GTPase (EC 3.6.1.-) activity has been described in guinea-pig bronchial membranes (Rhoden & Douglas, 1994) and mouse periaqueductal grey matter membranes (Martinez-Pena *et al*., 1995). We have recently developed a method of measuring the high-affinity GTPase activity of G-proteins coupled with some neurotransmitter receptors in rat brain membranes (Odagaki & Fuxe, 1997). In the present study, we have adapted this technique to investigate the effects of mastoparan on GTP hydrolyzing activity in rat brain membranes. In some experiments, 20 mM uridine 5′-diphosphate (UDP) was included in an assay mixutre to inhibit the stimulant effects of the venom on NDPK, through which part of its modulatory effect on GTP hydrolysis could be attributed (Klinker *et al*., 1994; 1996). Furthermore, L-glutamate-stimulated high-affinity GTPase activity derived from metabotropic glutamate receptor-coupled G-proteins (Odagaki *et al*., 1996) was determined, in parallel with the mastoparan-induced GTP hydrolysis, to compare the effects of mastoparan with receptor-coupled G-protein activation.

Methods

Membrane preparation

Male Sprague-Dawley rats weighing 200–250 g were decapitated and their brains were quickly removed. The cerebral

[1] Author for correspondence.

cortices, hippocampi and striata were dissected on ice and homogenized in 5 ml of ice-cold TED buffer (5 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4) containing 10% (w/v) sucrose with a motor-driven Teflon/glass tissue grinder (20 strokes). All the following centrifuge procedures were performed at 0–4°C. Each homogenate was centrifuged at 1,000 *g* for 10 min and the supernatant was decanted to another centrifuge tube, which was kept on ice. The pellet was vortexed in 5 ml of TED/sucrose buffer and was centrifuged at 1,000 *g* for 10 min. The combined supernatant was centrifuged at 9,000 *g* for 20 min and the pellet was resuspended in 10 ml of TED buffer followed by another centrifugation at 9,000 *g* for 20 min. The pellet was resuspended in 10 ml of TED buffer and kept on ice for 30 min. The suspension was centrifuged at 35,000 *g* for 10 min and the resulting pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.4) to produce the homogenate with a protein concentration range of 1.6–3.2 mg ml^{-1}. The homogenate was divided into 150 μl aliquots in plastic tubes, frozen quickly on fine-grained dry ice and stored at $-70 \sim -80$°C until use.

The protein concentration was measured by the method of Lowry *et al*. (1951) with BSA as a standard.

Measurement of GTP hydrolysis

GTP hydrolysis activity was assayed by measuring the radioactivity of the $^{32}P_i$ released from [γ-^{32}P]-GTP derived from an enzymatic conversion of GTP to GDP and P_i by the incubated membranes. In brief, the thawed membranes were diluted with 50 mM Tris-HCl buffer (pH 7.4) to a one tenth concentration (160–320 μg protein ml^{-1} and 25 μl aliquots of the membranes corresponding to 4–8 μg protein were incubated at 30°C for 15 min in the reaction mixture (final volume 100 μl), which contained the following constituents: 50 mM Tris-HCl (pH 7.4), 0.1 or 0.3 μM [γ-^{32}P]-GTP, 2 mM (or indicated concentrations of) $MgCl_2$, 0.5 mM ATP, 0.5 mM adenylylimidodiphodphate, 5 mM phosphocreatine, creatine phosphokinase (50 ml^{-1}), BSA (50 μg), 0.1 mM EDTA, 0.2 mM EGTA, 0.2 mM dithiothreitol, 0.5 mM cyclic AMP, 0.5 mM 3-isobutyl-1-methylxanthine and 100 mM NaCl. The low-affinity GTP hydrolysis was determined in the test tubes which contained 100 μM unlabelled GTP in addition to the above-mentioned constituents. The enzyme reaction was terminated by transferring the tubes to an ice bath followed by the addition of 500 u l of 20 mM phosphoric acid (pH 2.5) containing 5% (w/v) activated charcoal. The tubes were kept chilled for about 30 min and centrifuged at 13,000 *g* for 10 min. An aliquot (200 μl) from the supernatant fraction was pipetted onto the solid scintillator, Ready Cap (Beckman; Fullerton, CA, U.S.A.). After the samples had been dried overnight, the radioactivity (c.p.m.) of each sample was counted for 5 min by a liquid scintillation spectrometer. The GTP hydrolyzing activity was expressed as pmol released $^{32}P_i$ mg^{-1} protein 15 min^{-1}. High-affinity GTPase activity was routinely determined by subtracting the low-affinity GTP hydrolysis, defined as the released $^{32}P_i$ in the presence of 100 μM unlabelled GTP, from the total GTP hydrolyzing activity.

Data analysis

Unless otherwise indicated, all results are presented as mean ± s.e.mean of the indicated number of separate experiments, each performed at least in duplicate. In the GTP dilution experiment in the presence of 20 mM UDP, the high-affinity GTP hydrolyzing activity was calculated at each GTP concentration and the K_M and V_{max} values were determined by computer-assisted non-linear regression analysis with the values of GTP concentrations of 0.1–1.0 μM. L-Glutamate-induced increase in high-affinity GTPase activity above the basal value was analysed by computer-assisted non-linear regression programme originally designed to determine K_M and V_{max} for enzyme reactions in accordance with the Michaelis-Menton equation. Statistical analysis was performed by using Student's paired two-tailed *t* test with a *P* value less than 0.05 considered significant.

Materials

[γ-^{32}P]-GTP (30 Ci $mmol^{-1}$) was purchased from Du Pont NEN Research Products (Boston, MA, U.S.A.). Mastoparan and L-glutamate were obtained from Research Biochemical Int. (Natick, MA, U.S.A.) and Kanto Chemical Co. (Tokyo, Japan), respectively. All other chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Results

In the cerebral cortical membranes, mastoparan (1–100 μM) increased the amount of $^{32}P_i$ released from 0.3 μM [γ-^{32}P]-GTP in a concentration-dependent manner (Figure 1). The total GTP hydrolyzing activity was significantly augemented by mastoparan at 10 μM and higher concentrations ($n=4$, $P<0.01$, Student's paired two-tailed *t* test adjusted by the Bonferroni procedure). A similar increase was observed in both the hippocampal and striatal membranes, though the differences between the total GTP hydrolyzing activity in the presence of mastoparan and the respective control value did not reach statistical significance at any concentration. On the other hand, low-affinity GTPase activity, defined as the GTP hydrolysis in the presence of 100 μM GTP, was inhibited dose-dependently by mastoparan (Figure 1). The inhibitory effect of mastoparan on the low-affinity GTPase activity was statistically significant at 1 μM and higher concentrations in the cerebral cortex ($n=4$, $P<0.01$, Student's paired two-tailed *t* test adjusted by the Bonferroni procedure) and at 10 μM and higher concentrations in the hippocampus and striatum ($n=3$, $P<0.01$, Student's paired two-tailed *t* test adjusted by the Bonferroni procedure).

In order to analyse the effects of mastoparan on GTP hydrolysis in more detail, isotopic dilution experiments with 0.1 μM [γ-^{32}P]-GTP and various concentrations of unlabelled GTP were carried out in the absence and presence of 30 μM

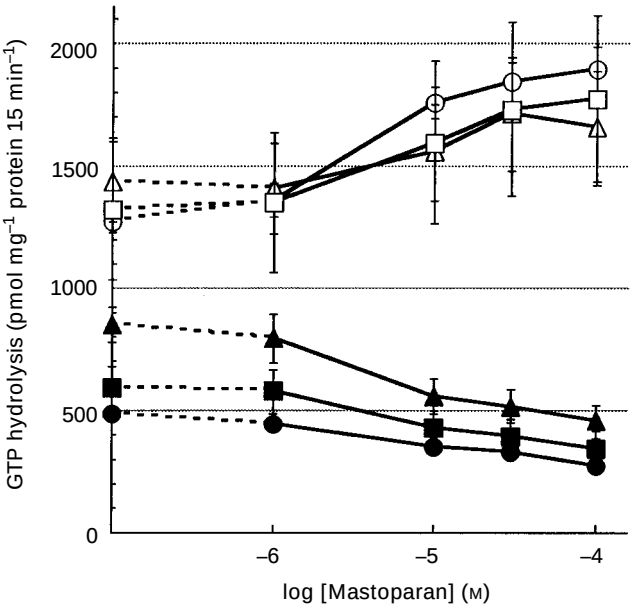

Figure 1 Effect of mastoparan on GTP hydrolysis in rat brain membranes. Hydrolysis of 0.3 μM [γ^{32}P]-GTP was determined after incubation of cortical (○, ●), hippocampal (△, ▲), and striatal (□, ■) membranes at 30°C for 15 min with increasing concentrations of mastoparan in the absence (○, △, □) and presence (●, ▲, ■) of 100 μM unlabelled GTP. Each point represents the mean, and vertical lines show s.e.mean, of 3–4 separate experiments, each performed in duplicate.

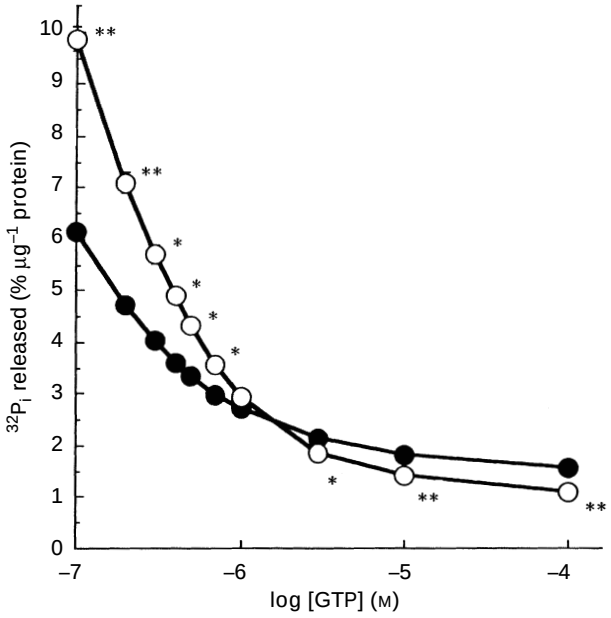

Figure 2 Effect of mastoparan on the hydrolysis of [γ-^{32}P]-GTP (0.1 μM) in the presence of various concentrations of unlabelled GTP in rat cerebral cortical membranes. The $^{32}P_i$ released from 0.1 μM [γ-^{32}P]-GTP in the absence (●) and presence (○) of 30 μM mastoparan is expressed as percentage of radioactivity of added [γ-^{32}P]-GTP μg^{-1} membrane protein at various concentrations of GTP (0.1 μM [γ-^{32}P]-GTP plus unlabelled GTP) indicated on the abscissa scale. Each point represents the mean, and vertical lines show s.e.mean of four separate experiments, each performed in duplicate. $^*P<0.05$, $^{**}P<0.01$; as compared with the respective value in the absence of mastoparan (Student's paired two-tailed t test adjusted by the Bonferroni procedure).

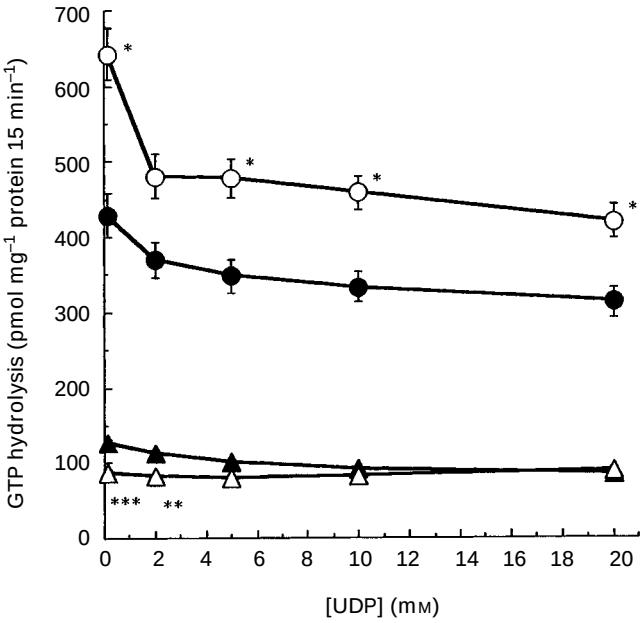

Figure 3 Effect of UDP on mastoparan-induced alterations in GTP hydrolysis in rat cerebral cortical membranes. Hydrolysis of 0.1 μM [γ-^{32}P]-GTP was determined in the absence (●, ▲) and presence (○, △) of 30 μM mastoparan at various concentrations of UDP indicated on the abscissa scale. Low-affinity GTP hydrolyzing activity was determined in the presence of 100 μM unlabelled GTP (▲, △). Each point represents the mean, and vertical lines show s.e.mean, of three separate experiments, each performed in duplicate. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$; as compared with the respective value in the absence of mastoparan (Student's paired two-tailed t test adjusted by the Bonferroni procedure).

mastoparan. As shown in Figure 2, the radioactivity of $^{32}P_i$ released from 0.1 μM [γ-^{32}P]-GTP was augmented by 30 μM mastoparan at GTP concentrations lower than 1 μM, but inhibited at 3 μM and higher concentrations of GTP. As a result, the two GTP dilution curves crossed at a GTP concentration of 1–2 μM, giving a pattern quite different from that in the case of agonist-induced, receptor-mediated activation of GTP hydrolysis in brain membranes (Odagaki & Fuxe, 1995a, b, c). Similar results were also obtained in the hippocampal membranes, although the crossing of the two curves occurred at a lower GTP concentration of around 0.6 μM (data not shown).

Since mastoparan has been shown to alter GTP hydrolysis not only by mimicking receptor activation but also indirectly through interaction with NDPK (Klinker *et al*., 1994; 1996), the possible involvement of NDPK in the effects of mastoparan on GTP hydrolysis in rat brain membranes was then investigated. UDP, which has been demonstrated to inhibit NDPK activity by forming an abortive complex (Kimura & Shimada, 1983; Kowluru & Metz, 1994), attenuated the effect of mastoparan on low-affinity GTP hydrolyzing activity in a concentration-dependent manner (Figure 3). Even in the presence of 20 mM UDP, a condition in which the effect of mastoparan on the low-affinity GTPase was completely attenuated ($n=3$, $P>0.05$, Student's paired two-tailed t test adjusted by the Bonferroni procedure), mastoparan (30 μM)-induced stimulation of the total GTP hydrolyzing activity was still retained ($P<0.05$), indicating that there is a high-affinity GTPase activity which can be activated by mastoparan other than via an interaction with NDPK (Figure 3). Indeed, GTP dilution experiments performed in the presence of 20 mM UDP showed that mastoparan (30 μM) stimulated the high-affinity GTPase activity without affecting the low-affinity GTP hydrolyzing activity (Figure 4a). Kinetic analysis of the high-affinity GTPase activity (Figure 4b) revealed that the addition of 30 μM mastoparan significantly increased the V_{max} value (670 ± 26 and 1190 ± 48 pmol mg^{-1} protein 15 min^{-1} in the absence and presence of 30 μM mastoparan, respectively; $n=4$, $P<0.001$) without changing the apparent K_M (0.19 μM in both cases). In the presence of 20 mM UDP, mastoparan stimulated the high-affinity GTPase activity in a concentration-dependent fashion in all three brain regions (Figure 5). The EC_{50} values for mastoparan could not be determined, because the stimulating effect of mastoparan did not reach saturation even at the highest concentration examined, i.e., 100 μM.

The sensitivity to Mg^{2+} was then compared between mastoparan- and L-glutamate-induced GTP hydrolyzing activites. As demonstrated in Figure 6a, the high-affinity GTPase activity was markedly stimulated by 30 μM mastoparan even in the absence of $MgCl_2$ ($n=4$, $P<0.01$, Student's paired two-tailed t test adjusted by the Bonferroni procedure), while the activating effect of mastoparan diminished as the concentrations of $MgCl_2$ increased. In contrast, L-glutamate-stimulated high-affinity GTPase activity was negligible in the absence of $MgCl_2$ ($n=4$, $P>0.05$), but became apparent in the presence of millimolar concentrations of $MgCl_2$ (Figure 6b).

Lastly, the possible interaction between mastoparan- and L-glutamate-stimulated high-affinity GTPase activities was investigated. In the presence of 20 mM UDP and 2 mM $MgCl_2$, 100 μM mastoparan doubled the high-affinity GTPase activity as demonstrated before (Figure 7, inset). Even in the presence of 100 μM mastoparan, L-glutamate still stimulated the high-affinity GTPase activity in the cortical membranes in a concentration-dependent manner and to the same extent as in the absence of mastoparan (Figure 7). Negative logarithmic values of EC_{50} (5.46 ± 0.27 and 5.53 ± 0.14 in the absence and presence of 100 μM mastoparan, respectively; $n=3$, $P>0.05$, Student's paired two-tailed t test) as well as maximal increases in high-affinity GTPase activity above the respective basal values (70.7 ± 2.9 and 89.0 ± 16.0 pmol mg^{-1} in the absence

and presence of 100 μM mastoparan, respectively; $P>0.05$) were not statistically different between the two conditions.

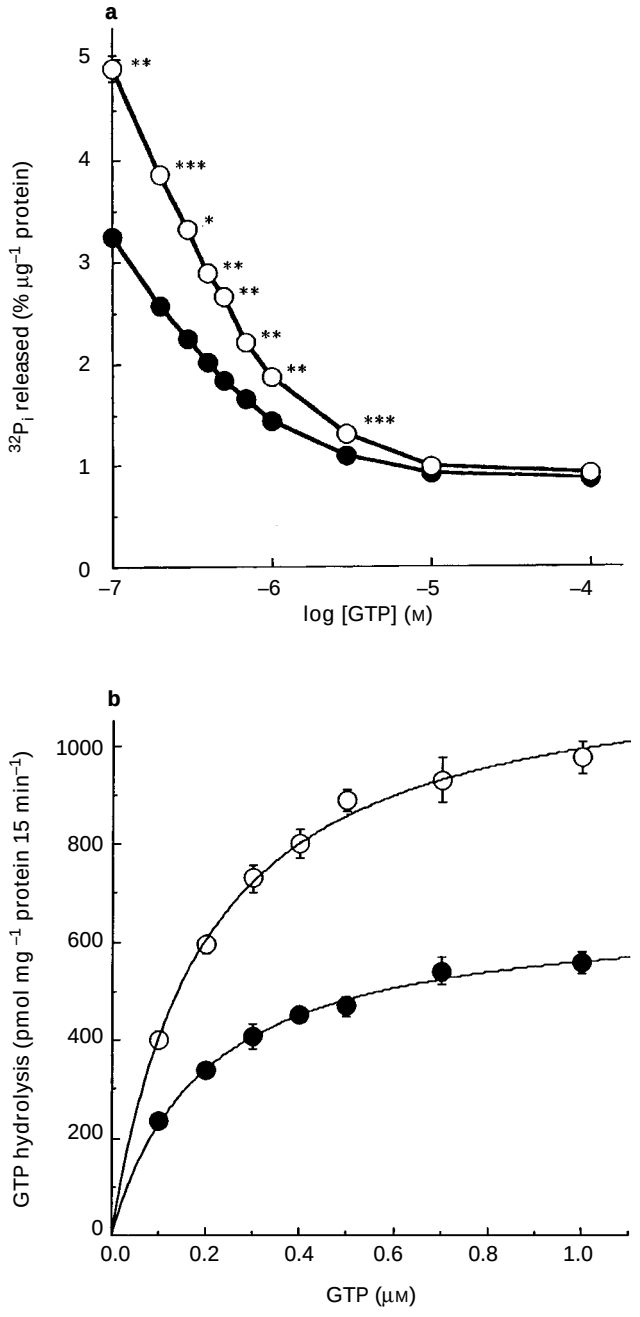

Figure 4 Effect of mastoparan in the presence of 20 mM UDP on the hydrolysis of [γ-^{32}P]-GTP (0.1 μM) in the presence of various concentrations of unlabelled GTP in rat cerebral cortical membranes. (a) $^{32}P_i$ released from 0.1 μM [γ-^{32}P]-GTP in the absence (●) and presence (○) of 30 μM mastoparan in the presence of 20 mM UDP is expressed as percentage of radioactivity of added [γ-^{32}P]-GTP μg^{-1} membrane protein at various concentrations of GTP (0.1 μM [γ-^{32}P]-GTP plus unlabelled GTP) indicated on the abscissa scale. Each point represents the mean, and vertical lines show s.e.mean of four separate experiments, each performed in duplicate. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$; as compared with the respective value in the absence of mastoparan (Student's paired two-tailed t test adjusted by the Bonferroni procedure). (b) High-affinity GTPase activity at various concentrations of GTP in the absence (●) and presence (○) of 30 μM mastoparan. High-affinity GTP hydrolyzing activity was calculated for various concentrations (0.1–1.0 μM) of GTP (0.1 μM [γ-^{32}P]-GTP plus unlabelled GTP) from the data presented in (a). Each point represents the mean, and vertical lines show s.e.mean, of four separate experiments, each performed in duplicate.

Discussion

In the present study, GTP hydrolysis was determined in rat brain membranes in the absence and presence of UDP. In the absence of UDP, mastoparan inhibited the low-affinity GTPase activity defined in the presence of 100 μM GTP in a concentration-dependent manner. As a result, the isotope dilution curves for the radioactivity of $^{32}P_i$ released from 0.1 μM [γ-^{32}P]-GTP in the absence and presence of 30 μM mastoparan crossed at a GTP concentration of 1–2 μM in cerebral cortical membranes and at about 0.6 μM GTP in hippocampal membranes, respectively. As the activation of G-protein coupled receptors by an agonist has been shown to evoke stimulation of high-affinity GTPase activity but not, so far, to alter low-affinity GTP hydrolysis (Odagaki & Fuxe, 1995a, b, c), the inhibitory effect of mastoparan on low-affinity GTPase activity was assumed to derive from mechanisms other than the well-known direct action of mastoparan on pertussis toxin-sensitive G-proteins (G_i and/or G_o) induced by mimicking the agonist-activated receptors coupled with the heterotrimeric G-proteins (Higashijima *et al*., 1988; 1990; Weingarten *et al*., 1990; Tomita *et al*., 1991). Under such a condition, the real effect of mastoparan on high-affinity GTPase activity as a direct activator of G_i/G_o seemed difficult to evaluate unless the unexpected alteration of low-affinity GTPase activity by mastoparan could be negated.

Since the first study by Kikkawa *et al*. (1992) on the stimulating effect of mastoparan on NDPK activity, accumulating data have suggested that this indirect effect is involved in the action of mastoparan upon GTP hydrolysis in HL-60 and various cell membranes (Klinker *et al*., 1994; 1996) and that NDPK plays some role in G-protein-mediated intracellular signal transduction systems (for reviews, see Otero, 1990; Lacombe & Jakobs, 1992; Klinker *et al*., 1995). We, therefore, investigated a possible involvement of NDPK activity in the effect of mastoparan on GTP hydrolysis in rat brain membranes. UDP attenuated mastoparan-induced inhibition of

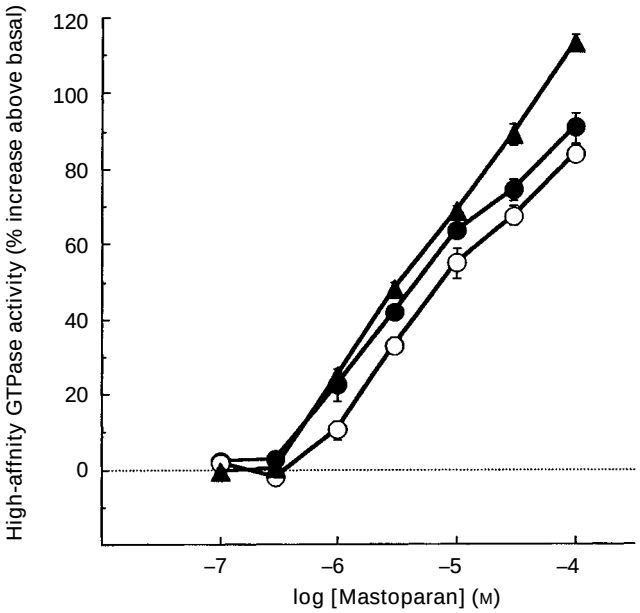

Figure 5 Mastoparan-induced stimulation of high-affinity GTPase activity in the presence of 20 mM UDP in cerebral cortical (●), hippocampal (○) and striatal (▲) membranes. High-affinity GTPase activity in the presence of 20 mM UDP was defined as the difference between the amounts of $^{32}P_i$ released from 0.3 μM [γ-^{32}P]-GTP in the absence and presence of 100 μM GTP, and the mastoparan-induced activation was expressed as % increase above the respective basal activities (475 ± 31, 377 ± 27 and 318 ± 8 pmol mg^{-1} protein 15 min^{-1} for cerebral cortical, hippocampal and striatal membranes, respectively). Each point represents the mean, and vertical lines show s.e.mean of 4–5 separate experiments, each performed in duplicate.

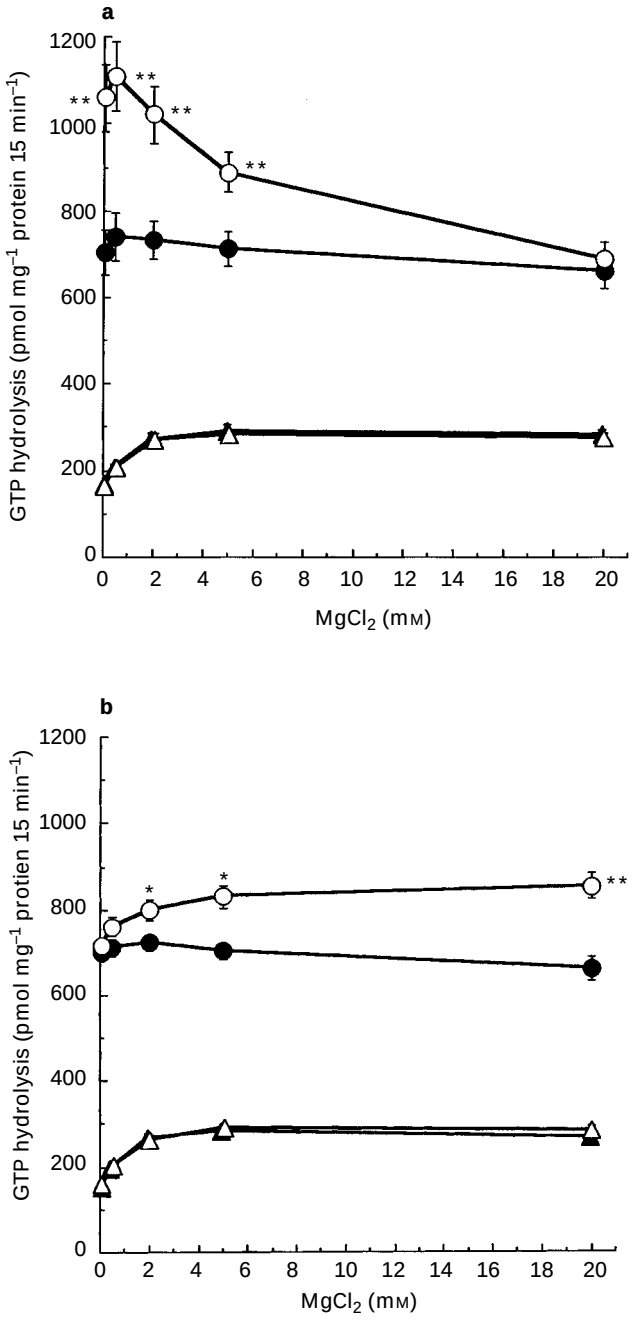

Figure 6 Effects of $MgCl_2$ on mastoparan- and L-glutamate-induced alterations in GTP hydrolysis in rat cerebral cortical membranes. (a) Hydrolysis of 0.3 μM [γ-^{32}P]-GTP in the presence of 20 mM UDP was determined in the absence (●, ▲) and presence (○, △) of 30 μM mastoparan at various concentrations of $MgCl_2$ indicated on the abscissa scale. (b) Hydrolysis of 0.3 μM [γ-^{32}P]-GTP in the presence of 20 mM UDP was determined in the absence (●, ▲) and presence (○, △) of 1 mM L-glutamate at various concentrations of $MgCl_2$ indicated on the abscissa scale. In (a) and (b) low-affinity GTP hydrolyzing activity was determined in the presence of 100 μM unlabelled GTP (▲, △). Each point represents the mean, and vertical lines show s.e.mean, of four separate experiments, each performed in duplicate. $^*P<0.05$, $^{**}P<0.01$; as compared with the respective value in the absence of mastoparan (Student's paired two-tailed t test adjusted by the Bonferroni procedure).

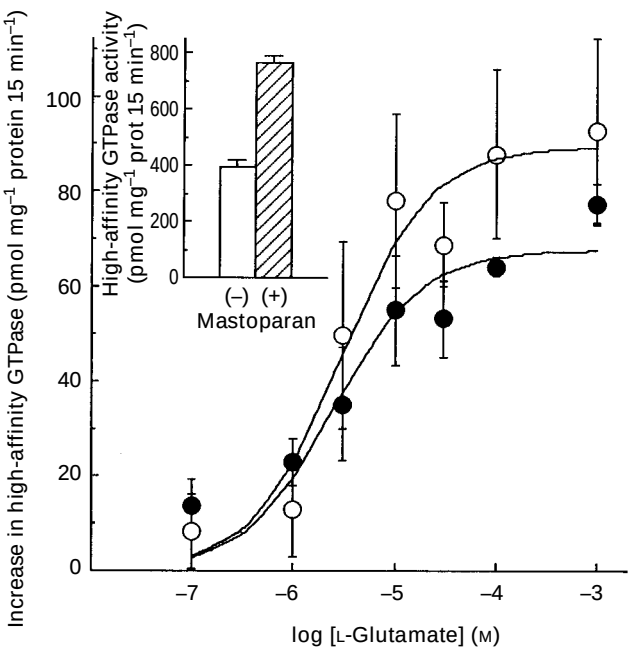

Figure 7 Lack of interaction between mastoparan- and L-glutamate-induced high-affinity GTPase activities in rat cerebral cortical membranes. High-affinity GTPase activity in the presence of 20 mM UDP was defined as the difference between the amounts of $^{32}P_i$ released from 0.3 μM [γ-^{32}P]-GTP in the absence and presence of 100 μM GTP, and the L-glutamate-induced increase in high-affinity GTPase activity above the respective basal activities (indicated in the inset: open column, in the absence of mastoparan; hatched column, in the presence of 100 μM mastoparan) was indicated for various concentrations of L-glutamate in the absence (●) and in the presence (○) of 100 μM mastoparan. Each point represents the mean, and vertical lines show s.e.mean, of three separate experiments, each performed in duplicate.

low-affinity GTPase activity in a concentration-dependent manner, with complete attenuation at 20 mM. It has been shown that UDP inhibits NDPK activity by forming an abortive, enzyme-nucleoside diphosphate complex, and that almost complete inhibition of NDPK is achieved by UDP 10–20 mM (Kimura & Shimada, 1983; Kowluru & Metz, 1994). Hence, mastoparan-induced inhibitory effect on low-affinity GTPase activity, which has been shown to be abolished by 20 mM UDP, is attributable to the activation of NDPK by mastoparan. Although the precise molecular mechanism for this phenomenon is unclear, mastoparan-activated NDPK may convert GDP bound to the low-affinity GTPase(s) to GTP, which inhibits the accessibility of radiolabelled GTP added immediately before incubation to the low-affinity GTPase(s), resulting in the reduction of $^{32}P_i$ released from [γ-^{32}P]-GTP.

Even in the presence of 20 mM UDP, under which condition the inhibitory effect of mastoparan on the low-affinity GTPase activity was completely cancelled, the amount of $^{32}P_i$ released from 0.1 μM [γ-^{32}P]-GTP in the absence of 100 μM unlabelled GTP was still stimualted by mastoparan, indicating that mastoparan stimulated high-affinity GTPase activity in rat brain membranes probably through the well-known direct activation of pertussis toxin-sensitive G-proteins (Higashijima *et al*., 1988; 1990; Weingarten *et al*., 1990; Tomita *et al*., 1991). When NDPK activity was completely inhibited, further detailed kinetic analysis showed that mastoparan augemented the V_{max} without affecting the apparent K_M for GTP. This means that mastoparan increases the capacity of the G-proteins to catalyze the conversion of GTP to GDP without altering the affinity of the G-proteins for GTP, by mimicking the action of agonist-activated receptors coupled with G-proteins. However, in the brain membranes, the kinetic analysis of the agonist-induced high-affinity GTPase activity often reveals that the addition of an agonist results in the augmentation of V_{max} as well as slight increase in K_M for GTP (Odagaki & Fuxe, 1995a, b, c). The reason for this discrepancy is currently unclear. The stimulating effects of mastoparan on high-affinity GTPase activity occur at

concentrations higher than 1 μM, consistent with previous concentration-response relationships obtained for mastoparan-induced activation of purified G-proteins and of G-proteins in various cell membranes (Higashijima *et al*., 1988; 1990; Tomita *et al*., 1991; Kikkawa *et al*., 1992; Klinker *et al*., 1994; Rhoden & Douglas, 1994; Martinez-Pena *et al*., 1995).

While concentration-dependent, mastoparan-induced high-affinity GTPase stimulation seemed not to saturate even at 100 μM. We were unable to investigate the effect of mastoparan at higher concentrations due to its restricted availability. It is possible that some nonspecific or indirect effects of mastoparan resulting in alterations in GTP hydrolyzing activity is partly involved in mastoparan-induced activation of high-affinity GTPase in rat brain membranes, especially at higher concentrations of mastoparan. Of the many effects of mastoparan described so far (see the Introduction), the involvement of cytosolic low molecular mass GTP-binding proteins (Koch *et al*., 1991) is unlikely, because well-washed membranes were used in the present study. Nucleotidase with a molecular mass of 87 kDa, which was shown to be stimulated by mastoparan in bovine brain (Denker *et al*., 1991), is assumed to be negligible under the assay condition used (ie the solution contains 0.5 mM ATP) even if present in rat brain.

Despite the likelihood that mastoparan stimulates high-affinity GTPase activity in rat brain membranes by activating pertussis toxin-sensitive G-proteins directly in a manner similar to an agonist activated receptor (Higashijima *et al*., 1988; 1990; Weingarten *et al*., 1990; Tomita *et al*., 1991), mastoparan- and receptor-mediated activations of high-affinity GTPase showed an entirely different sensitivity to $MgCl_2$ and there appeared to be a simple additive interaction between these two responses. Mastoparan-induced high-affinity GTPase was apparent even in the absence of $MgCl_2$ whereas L-glutamate-stimulated high-affinity GTPase activity, which has been shown to be mediated through metabotropic glutamate receptors (Odagaki *et al*., 1996) and was chosen to represent receptor-mediated G-protein activation, was barely detectable in the absence of $MgCl_2$. Mastoparan-stimulated high-affinity GTPase activity in the absence of $MgCl_2$ is most likely indicative of the presence of a trace of Mg^{2+}, as GTPase activity of purified G-proteins was shown to be activated by mastoparan preferentially at very low Mg^{2+} concentrations (Higashijima *et al*., 1988; 1990). Their study on purified pertussis toxin-sensitive G-proteins (Higashijima *et al*., 1988; 1990) also revealed that the efficiency of G-protein activation by mastoparan is rather inhibited at Mg^{2+} concentrations higher than 1 mM, consistent with our own data on rat brain membranes in the present study. On the other hand, L-glutamate-stimulated high-affinity GTPase activity through metabotropic glutamate receptors required millimolar concentrations of $MgCl_2$ to be apparent. Magnesium is an important cation involved in receptor-mediated G-protein coupled signal transduction systems at multiple sites, and the strict requirement of millimolar concentrations of Mg^{2+} has also been demonstrated in receptor-mediated stimulation of high-affinity GTPase activity of G-proteins in our previous studies (Odagaki & Fuxe, 1995b, c).

The interaction between mastoparan- and L-glutamate-stimulated high-affinity GTPase activities appeared to be simply additive, not synergistic or antagonistic. L-Glutamate-stimulated high-affinity GTPase activity in rat cerebral cortical membranes is, as suggested in our previous study (Odagaki *et al*., 1996), attributable to the activation of the G-proteins coupled with group II metabotropic glutamate receptors mediating adenylyl cyclase inhibition (Pin & Duvoisin, 1995), thus indicating that the G_i responsible for adenylyl cyclase inhibition is involved in L-glutamate-induced high-affinity GTPase activity. As mastoparan has been shown to activate both G_i and G_o (Higashijima *et al*., 1988; 1990; Weingarten *et al*., 1990; Tomita *et al*., 1991), we hypothesized that activation of a receptor coupled to G_i could not stimulate the high-affinity GTPase activity of G-proteins any more if G_i and G_o are fully activated by mastoparan. The experimental results on the additivity between L-glutamate- and mastoparan-induced high-affinity GTPase activities do not support this hypothesis. This may be explained by the abundant existence of G-proteins, in particular G_o, in the CNS. It has been shown that the average ratio of α subunits of G_s, G_i and G_o in rat brain is 1 : 3 : 7, by use of competitive enzyme-linked immunosorbent assay (ELISA) techniques (Lesch *et al*., 1991). As the stimulating effect of mastoparan on purified G_o has been shown to be somewhat more efficacious than that on purified G_i (Higashijima *et al*., 1988), only a part of the abundant G_o might be activated by mastoparan even at the highest concentration, leaving mostly G_i and the rest of G_o to be in a state that is potentially able to respond to another stimulant. The lack of saturability even at the highest concentration of mastoparan examined (100 μM) supports this notion. Alternatively, receptor-coupled G_i may be distinct from the mastoparan-sensitive G-proteins, regardless of the assumption that mastoparan mimics the agonist-activated receptors coupled with pertussis toxin-sensitive G-proteins (Higashijima *et al*., 1988; 1990; Weingarten *et al*., 1990; Tomita *et al*., 1991). Further studies are necessary to examine the implications of receptor-independent and receptor-mediated activation of G-proteins in the brain.

This is the first study to focus on the effects of mastoparan on GTP hydrolyzing activity of G-proteins in brain membranes. As expected from previous studies with purified G-proteins (Higashijima *et al*., 1988; 1990; Weingarten *et al*., 1990; Tomita *et al*., 1991), mastoparan stimulated the high-affinity GTPase activity of G-proteins in a direct and receptor-independent manner. In addition, it appeared that mastoparan stimulated NDPK activity resulting in a complicated modification of GTP hydrolyzing activity. It is of great interest to know how mastoparan affects the function of heterotrimeric G-proteins and subsequently G-protein coupled signal transduction systems *in vivo*. Furthermore, investigation of the mechanisms of action of mastoparan might lead to the development of novel therapeutically relevant agents which bypass neurotransmitter receptors and act directly on G-protein coupled signalling.

In conclusion, the present study has demonstrated the modulatory effects of mastoparan on GTP hydrolysis in rat brain. The inhibiting effect of the venom on the low-affinity GTPase activity was shown to be due to the activation of NDPK. The stimulating effect of mastoparan on the high-affinity GTPase activity occurs in a manner different from that of the receptor-coupled G-protein activation, probably through direct activation of pertussis toxin-sensitive G-proteins. However, the receptor-mediated G-protein activation was shown to occur independently, regardless of the presence of mastoparan, indicating the lack of interaction between the receptor-independent and receptor-mediated G-protein activating effects.

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