Dual effects of mastoparan on intracellular free Ca^{2+} concentrations in human astrocytoma cells

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1 The effect of mastoparan, a wasp venom toxin, on intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was examined in human astrocytoma cells. Mastoparan inhibited $[\text{Ca}^{2+}]_i$ induced by carbachol (100 μM) in a concentration-dependent manner in the absence of extracellular Ca^{2+} , consistent with our previous results showing that mastoparan inhibits phosphoinositide hydrolysis in human astrocytoma cells.

2 In contrast, mastoparan itself increased $[\text{Ca}^{2+}]_i$ and augmented carbachol-induced increase in the $[\text{Ca}^{2+}]_i$ in the presence of extracellular Ca^{2+} , suggesting that mastoparan elicited Ca^{2+} influx from the extracellular medium. The increase appeared to be maximum at extracellular Ca^{2+} concentrations of 0.1–0.2 mM. The higher concentrations of extracellular Ca^{2+} depressed the influx.

3 Pertussis toxin did not affect mastoparan-induced inhibition of $[\text{Ca}^{2+}]_i$ in the absence of extracellular Ca^{2+} , consistent with the previous results that pertussis toxin did not affect mastoparan-induced inhibition of phosphoinositide hydrolysis.

4 Pertussis toxin augmented mastoparan-induced increase in $[\text{Ca}^{2+}]_i$ in the presence of extracellular Ca^{2+} , suggesting that pertussis toxin substrate(s) seems to be inhibitory for Ca^{2+} influx induced by mastoparan.

5 Verapamil, nifedipine and diltiazem (each 10 μM), L-type Ca^{2+} antagonists, did not affect mastoparan-induced Ca^{2+} influx. However, verapamil (10 μM) slightly inhibited the increase in $[\text{Ca}^{2+}]_i$ induced by carbachol in the presence of mastoparan.

6 The results obtained in the present study indicate that mastoparan has two opposite effects on $[\text{Ca}^{2+}]_i$ in human astrocytoma cells and possibly has at least two sites of action.

Keywords: Mastoparan; calcium ions; phospholipase C; inositol phosphate; calcium channel; pertussis toxin

Introduction

Mastoparan is a wasp venom toxin with a structure of 14 amino-acids. It is well known that mastoparan liberates histamine from mast cells (Hirai *et al.*, 1979), a release that is mediated via an activation of phosphatidylinositol 4,5-bisphosphate (PIP_2)-specific phospholipase C (Okano *et al.*, 1985), resulting in an accumulation of inositol 1,4,5-trisphosphate (IP_3) and intracellular Ca^{2+} mobilization. The effect of mastoparan on mast cells disappears after treatment of the cells with pertussis toxin, suggesting that mastoparan activates pertussis toxin-sensitive G-protein, such as G_i and G_o (Higashijima *et al.*, 1987). Mastoparan has been reported to increase GTPase activity and ^{35}S -GTPyS binding of purified G_o or G_i in phospholipid vesicles (Higashijima *et al.*, 1988). Recent lines of evidence suggest that mastoparan directly interacts with a G-protein in a manner similar to an activated receptor by agonists (Higashijima *et al.*, 1990; Tomita *et al.*, 1991). The interacting site of mastoparan with G-protein is assumed to be around the 4th cysteine residue from the C-terminal of pertussis toxin-sensitive G-protein (Higashijima *et al.*, 1990).

On the other hand, 1321N1 human astrocytoma cells express muscarinic cholinergic receptors (Masters *et al.*, 1985), H_1 -histamine (Nakahata *et al.*, 1986), bradykinin (Hepler *et al.*, 1987), thromboxane A_2 (Nakahata *et al.*, 1989) and endothelin (Yanai *et al.*, 1992) receptors, which are coupled to PIP_2 -specific phospholipase C mediated via a pertussis toxin-insensitive G-protein. We have shown that mastoparan inhibits phosphoinositide hydrolysis in human astrocytoma

cells (Nakahata *et al.*, 1990), although it stimulates phosphoinositide hydrolysis in mast cells (Higashijima *et al.*, 1987). The results are consistent with the reports by Wojcikiewicz & Nahorski (1989) and Gusovsky *et al.* (1991) that mastoparan inhibits phosphoinositide hydrolysis in permeabilized SH-SY5Y and HL-60 cells, respectively. Mastoparan-induced inhibition of phosphoinositide hydrolysis in human astrocytoma cells was resistant to pertussis toxin treatment (Nakahata *et al.*, 1990), indicating that the action of mastoparan may not be mediated via a pertussis toxin-sensitive G-protein.

In the present study, we analyzed intracellular free Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) using fura-II for examining whether mastoparan inhibits an increase in $[\text{Ca}^{2+}]_i$ induced by carbachol in human astrocytoma cells. The results obtained suggest that mastoparan has two opposite actions on $[\text{Ca}^{2+}]_i$ in human astrocytoma cells; one is a decrease in $[\text{Ca}^{2+}]_i$ that supports the inhibitory effect of mastoparan on phosphoinositide hydrolysis, the other is an increase in $[\text{Ca}^{2+}]_i$ which might be due to some Ca^{2+} -channel opening.

Methods

Cell culture

Human astrocytoma cells (1321N1) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% foetal bovine serum, 50 units ml^{-1} of penicillin and 50 $\mu\text{g ml}^{-1}$ of streptomycin in a 37°C humidified incubator in an atmosphere of 95% O_2 and 5% CO_2 .

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Measurement of intracellular free Ca^{2+} concentrations with fura II

Human astrocytoma cells cultured on a 150 mm dish were washed three times with a modified Tyrode solution (composition, mM: NaCl 137, KCl 2.7, MgCl_2 1.0, CaCl_2 0.18, glucose 5.6, HEPES 10, pH 7.4). The cells were freed from the dish by treatment with 0.1% collagenase and 1.0% bovine serum albumin (BSA) in 10 ml of the modified Tyrode solution for 15 min at 37°C. Cells liberated from the dish were collected into a 50 ml tube and centrifuged at 250 g for 1 min. Cells were washed once with 10 ml of the modified Tyrode solution. The cells (10^6 – $5 \times 10^6 \text{ ml}^{-1}$) were treated with 1 μM fura-2-AM at 37°C for 15 min and then were centrifuged at 250 g for 1 min in order to remove the remaining fura-2-AM, and washed twice with the modified Tyrode solution. The cells were suspended in the modified Tyrode solution in the concentrations of 10^6 – $5 \times 10^6 \text{ ml}^{-1}$, and 1–2 ml of the cell suspension were used for fura 2 assay. Fluorescence of fura 2 at 510 nm by excitation waves at 340 and 380 nm was monitored simultaneously by a spectrofluorometer (Hitachi, F-2000). The maximum ratio of fluorescence at 510 nm by the excitation wave of 340 nm to that by 380 nm was obtained in the presence of 0.1% triton X-100, and the minimum fluorescence ratio was obtained in the presence of 2 mM EGTA. Free calcium concentrations were calculated by using the K_d of fura II to Ca^{2+} ions as 224 nM.

Materials

Foetal bovine serum was obtained from Cell Culture Laboratory (Cleveland, OH, U.S.A.). Dulbecco's modified Eagle's medium (DMEM) was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan). Mastoparan was from Peptide Institute (Osaka, Japan). Fura 2-AM was from Dojindo (Kumamoto, Japan). Carbachol, verapamil, diltiazem and nifedipine was from Sigma (St. Louis, MO, U.S.A.), Pertussis toxin was purchased from Funakoshi (Tokyo, Japan). EGTA was from Nakari Chemicals (Kyoto, Japan). Triton X-100 was from Wako Pure Chemicals (Tokyo, Japan). Collagenase was obtained from Warthington Biochemical Corp. (Freehold, NJ, U.S.A.). Other chemicals and drugs were of reagent grade or highest quality available.

Data analysis

The results obtained in separate experiments were expressed as mean $\pm$ s.e., and a significant difference ($P < 0.05$) was determined with Student's *t*-test.

Results

A resting level of $[\text{Ca}^{2+}]_i$ in fura 2-loaded human astrocytoma cells was 50–100 nM in the presence of extracellular Ca^{2+} ions. In the absence of extracellular Ca^{2+} ions, i.e. in the presence of 2 mM EGTA, the resting level of $[\text{Ca}^{2+}]_i$ was approximately 30 to 50 nM. Carbachol (100 μM) increased $[\text{Ca}^{2+}]_i$ in the absence of extracellular Ca^{2+} ions, as shown in Figure 1. The results indicate that carbachol could mobilize intracellular Ca^{2+} ions from intracellular storage sites, consistent with an activation of phosphoinositide hydrolysis and IP_3 accumulation by carbachol, previously described by Masters *et al.* (1985).

Mastoparan (30 μM) potently inhibited carbachol-induced increase in $[\text{Ca}^{2+}]_i$ (Figure 1). Mastoparan also inhibited the histamine (100 μM)-induced increase in $[\text{Ca}^{2+}]_i$ (data not shown). Figure 2 shows the concentration-inhibition curve for mastoparan on the carbachol-induced increase in $[\text{Ca}^{2+}]_i$. The inhibition curve for mastoparan is similar to that for mastoparan on carbachol-induced phosphoinositide hydrolysis (Nakahata *et al.*, 1990). On the other hand, mas-

Figure 1 Effect of mastoparan on intracellular free Ca^{2+} concentrations in the absence of extracellular Ca^{2+} ions. The cells loaded with fura 2 were suspended in the modified Tyrode solution. EGTA (2 mM final concentration) was added 225 s before addition of carbachol (CCh, 100 μM). Mastoparan (30 μM) was added 100 s before addition of carbachol. Mastoparan inhibited carbachol-induced increase in $[\text{Ca}^{2+}]_i$.

Figure 2 Concentration-dependent inhibition by mastoparan of carbachol-induced increase in intracellular free Ca^{2+} concentrations in the presence of 2 mM EGTA. Experiments were performed as in Figure 1, except that the concentrations of mastoparan were varied. Each point represents the mean value $\pm$ s.e. of six separate experiments. Abscissa scale: concentration of mastoparan (μM). Ordinate scale: intracellular free Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$). (○) Mastoparan alone; (●) carbachol (100 μM) in the presence of various concentrations of mastoparan.

toparan itself (30 μM) increased $[\text{Ca}^{2+}]_i$ in the presence of extracellular Ca^{2+} ions (Figure 3). Interestingly, mastoparan augmented carbachol-induced increase in $[\text{Ca}^{2+}]_i$ in the presence of extracellular Ca^{2+} ions (Figure 3). In the presence of extracellular Ca^{2+} ions, mastoparan concentration-dependently increased $[\text{Ca}^{2+}]_i$, and also augmented the carbachol-induced increase in $[\text{Ca}^{2+}]_i$ (Figure 4). The effect of mastoparan on $[\text{Ca}^{2+}]_i$ in the presence of extracellular Ca^{2+} ions was completely different from its effect in the absence of extracellular Ca^{2+} ions, suggesting that mastoparan has at least two sites of action on $[\text{Ca}^{2+}]_i$. As there is a possibility that mastoparan could activate Ca^{2+} -influx from extracellular space across plasma membranes, we examined whether extra-

cellular Ca^{2+} ion concentrations ($[Ca^{2+}]_i$) could regulate mastoparan-induced Ca^{2+} mobilization or not (Figure 5). Mastoparan ($30 \mu M$) alone increase $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} ions with a peak in the presence of 0.1 to 0.2 mM $[Ca^{2+}]_o$. Higher concentrations of $[Ca^{2+}]_o$ did not

Figure 3 Effect of mastoparan on intracellular free Ca^{2+} concentrations in the presence of extracellular Ca^{2+} ions. The cells loaded with fura 2 were suspended in the modified Tyrode solution containing 0.18 mM $CaCl_2$. Mastoparan ($30 \mu M$) was added 175 s before addition of carbachol (CCh, $100 \mu M$).

Figure 4 Concentration-dependency of mastoparan-induced increase in intracellular free Ca^{2+} ion concentrations in the presence of extracellular Ca^{2+} ions. Experiments were performed as in Figure 3, except that concentrations of mastoparan were varied. (O) $[Ca^{2+}]_i$ before addition of mastoparan; (Δ) $[Ca^{2+}]_i$ after addition of mastoparan; (●) $[Ca^{2+}]_i$ after addition of carbachol ($100 \mu M$) and mastoparan. Abscissa scale: concentration of mastoparan (μM). Ordinate scale: intracellular free Ca^{2+} concentrations. Each point represents the mean value $\pm$ s.e. of four separate experiments.

potentiate, but rather inhibited, mastoparan-induced increase in $[Ca^{2+}]_i$, indicating that the plasma membrane of astrocytoma cells was still intact after addition of mastoparan. The carbachol-induced increase in $[Ca^{2+}]_i$ in the presence of mastoparan ($30 \mu M$) was not changed in various $[Ca^{2+}]_o$. Because mastoparan did not increase $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} ions, mastoparan is assumed to mobilize Ca^{2+} ions from extracellular space.

Pertussis toxin ADP-ribosylated G_i or G_o protein; then signal transduction involved in G_i or G_o was inhibited by treatment of the cells with pertussis toxin (Ui, 1984). Mastoparan-induced inhibition of the increase in $[Ca^{2+}]_i$ induced by carbachol was not affected by pertussis toxin treatment in the absence of extracellular Ca^{2+} ions (Figure 6), consistent with the previous results that pertussis toxin

Figure 5 Extracellular Ca^{2+} dependency of mastoparan-induced increase in intracellular Ca^{2+} concentration. Fura 2-loaded cells were incubated in the indicated extracellular Ca^{2+} concentrations or 2 mM EGTA, then $30 \mu M$ mastoparan was added to the medium. After the response reached a steady-state, $100 \mu M$ carbachol was added to the medium. (O) $[Ca^{2+}]_i$ before addition of mastoparan; (Δ) $[Ca^{2+}]_i$ after addition of mastoparan; (●) $[Ca^{2+}]_i$ after addition of carbachol and mastoparan. Note that the peak increase in $[Ca^{2+}]_i$ by mastoparan appeared between 0.1 and 0.2 mM extracellular Ca^{2+} concentration. Each point represents the mean value $\pm$ s.e. of three separate experiments.

Figure 6 Effect of pertussis toxin (PT) on intracellular free Ca^{2+} concentrations induced by carbachol and mastoparan in the absence of extracellular Ca^{2+} . Cells were treated with (a) or without (b) pertussis toxin (100 ng ml^{-1}) for 18 h. Intracellular free Ca^{2+} concentrations were monitored as described in Figure 1. Open column: control; hatched column: carbachol (CCh, $100 \mu M$); stippled column: mastoparan (MP, $30 \mu M$); cross hatched column: carbachol (CCh, $100 \mu M$) after mastoparan (MP, $30 \mu M$). Each point represents the mean value $\pm$ s.e. of four separate experiments. Note that the inhibitory effect of mastoparan was unaffected by pertussis toxin treatment.

Figure 7 Effect of pertussis toxin on intracellular free Ca^{2+} concentrations induced by carbachol and mastoparan in the presence of extracellular free Ca^{2+} ions. Cells were treated with (a) or without (b) pertussis toxin (100 ng ml^{-1}) for 18 h. Intracellular free Ca^{2+} concentrations were monitored as described in Figure 3. Open column: control; hatched column: carbachol (CCh, $100 \mu\text{M}$); stippled column: mastoparan (MP, $30 \mu\text{M}$); cross hatched column: carbachol (CCh, $100 \mu\text{M}$) after mastoparan (MP, $30 \mu\text{M}$). Each point represents the mean value $\pm$ s.e. of four separate experiments. *Significant difference between the response of the cells with pertussis toxin treatment and the response of the cells without pertussis toxin treatment. Note that pertussis toxin augmented mastoparan-induced increase in $[\text{Ca}^{2+}]_i$.

Figure 8 Effects of Ca^{2+} antagonists on mastoparan-induced increase in free Ca^{2+} concentrations. Cells loaded with fura 2-AM were preincubated with verapamil (Ver, $10 \mu\text{M}$), diltiazem (Dil, $10 \mu\text{M}$), nifedipine (Nif, $10 \mu\text{M}$) or vehicle for 5 min; then the mastoparan-induced increase in $[\text{Ca}^{2+}]_i$ was observed. After the response reached a steady-state, carbachol (CCh, $100 \mu\text{M}$) was added. Open column: control; stippled column: mastoparan (MP, $30 \mu\text{M}$); cross-hatched column: carbachol (CCh, $100 \mu\text{M}$) after mastoparan (MP, $30 \mu\text{M}$). Each point represents the mean value $\pm$ s.e. of three separate experiments. Note that carbachol-induced increase in $[\text{Ca}^{2+}]_i$ in the presence of mastoparan was slightly inhibited by verapamil.

did not modify the inhibitory action on carbachol-induced phosphoinositide hydrolysis (Nakahata *et al.*, 1990). In the presence of extracellular Ca^{2+} ions, the mastoparan-induced increase in $[\text{Ca}^{2+}]_i$ was augmented by treatment of the cells with pertussis toxin (Figure 7). The results suggest that a pertussis toxin substrate may inhibit Ca^{2+} influx from extracellular space.

As there is a possibility that the mastoparan-induced increase in $[\text{Ca}^{2+}]_i$ may be dependent on influx of Ca^{2+} ions through a Ca -channel, the effects of Ca -channel antagonists

on mastoparan-induced increase in $[\text{Ca}^{2+}]_i$ were examined (Figure 8). Verapamil, diltiazem and nifedipine at a concentration of $10 \mu\text{M}$ did not affect mastoparan-induced increase in $[\text{Ca}^{2+}]_i$, suggesting that Ca^{2+} -influx by mastoparan might not be due to the opening of L-type Ca^{2+} -channels. On the other hand, carbachol-induced Ca^{2+} -influx in the presence of mastoparan was somewhat reduced by $10 \mu\text{M}$ verapamil (Figure 8), although verapamil did not affect the carbachol-induced increase in $[\text{Ca}^{2+}]_i$ (data not shown). The results suggest that in the presence of mastoparan, carbachol is able to stimulate the opening of some Ca^{2+} -channel.

Discussion

The present study demonstrated that mastoparan has two opposite effects on $[\text{Ca}^{2+}]_i$ in human astrocytoma cells; one is an inhibitory action that is explained by the inhibition of PIP_2 -specific phospholipase C and a decreased accumulation of IP_3 (Nakahata *et al.*, 1990); the other is Ca^{2+} influx from extracellular space. Ca^{2+} influx from extracellular space induced by mastoparan was not inhibited by treatment of the cells with pertussis toxin, indicating that there is no involvement of pertussis toxin-sensitive G-protein in mastoparan-induced Ca^{2+} influx. Interestingly, pertussis toxin rather potentiated Ca^{2+} influx induced by mastoparan. Therefore, pertussis toxin-sensitive G-protein may inhibit the Ca^{2+} influx induced by mastoparan in human astrocytoma cells, instead of an activation of influx. Pertussis toxin did not abolish mastoparan-induced inhibition of an increase in $[\text{Ca}^{2+}]_i$ induced by carbachol in the absence of extracellular Ca^{2+} ions. It is suggested that one of the target proteins of mastoparan was a G-protein α -subunit (Higashijima & Ross, 1991). Recent lines of evidence indicated that a small molecular G-protein (substrate for botulinum toxin C3 exoenzyme, rho/rac) was affected by mastoparan (Koch *et al.*, 1991), although the G-proteins activated by mastoparan are usually considered as pertussis toxin-substrates (Higashijima & Ross, 1991).

Mastoparan itself increased $[\text{Ca}^{2+}]_i$ (Figure 2). Mastoparan is a amphiphilic peptide and exists in plasma membranes by interacting with phospholipids (Higashijima *et al.*, 1983); when it is applied to cells, there is a possibility that mastoparan-induced increase in $[\text{Ca}^{2+}]_i$ by fura 2 assay is merely an exclusion of fura 2 from inside the cells to outside. In fact, Tanimura *et al.* (1991) demonstrated the presence of lactate dehydrogenase (LDH) in the extracellular space after treatment of rat acinar cells with mastoparan, suggesting that mastoparan could change the membrane permeability for proteins or small molecules. However, this is not the case in the present experiments, because higher concentrations of extracellular Ca^{2+} ions did not augment mastoparan-induced increase in fura-2 fluorescence. Furthermore, Yule & Williams (1991) demonstrated that mastoparan induces oscillations of cytosolic Ca^{2+} in rat pancreatic acinar cells, showing that mastoparan could increase cytosolic Ca^{2+} without exclusion of fura 2.

The carbachol-induced increase in $[\text{Ca}^{2+}]_i$ after mastoparan in the presence of extracellular Ca^{2+} ions, was inhibited by $10 \mu\text{M}$ verapamil. As diltiazem and nifedipine did not inhibit the increase, the Ca^{2+} channel involved may not be of the L-type. Since verapamil did not inhibit carbachol-induced increase in $[\text{Ca}^{2+}]_i$ (data not shown), mastoparan may change the state of the cell membranes so that some Ca^{2+} -channel is active. But the situation is abnormal, because the increased level of $[\text{Ca}^{2+}]_i$ is too high.

Mastoparan is believed to activate G-protein (Higashijima & Ross, 1991). The present study showed two different effects of mastoparan on $[\text{Ca}^{2+}]_i$. If both effects of mastoparan involve G-protein(s), these G-protein(s) could not be pertussis toxin-substrates in human astrocytoma cells. In fact, mastoparan activates $\text{GTP}\gamma\text{S}$ binding in membrane preparations derived from human astrocytoma cells after treatment

with pertussis toxin (Nakahata *et al.*, unpublished observations). Although mastoparan may directly activate G-protein(s), it has been shown to activate or inhibit several biological systems but not G-protein(s). Kikkawa *et al.* (1992) reported that mastoparan activates nucleoside diphosphate kinase, which in turn activates G-protein(s). Raynor *et al.* (1991) reported that mastoparan inhibits protein kinase C, Ca/CaM kinase II, Na-K ATPase and the Na pump. It is interesting that phosphoinositide 3,4,5-trisphosphate is accumulated by mastoparan in human neutrophils (Norgauer *et al.*, 1992), and mastoparan activates arachidonic acid release without inositol phosphate accumulation in Swiss 3T3 cells (Gil *et al.*, 1991). Denker *et al.* (1991) reported that mastoparan activates nucleotidase of which the molecular

mass is 85 kDa from bovine brain. The accumulated evidence suggests that there might be cell specificity and multi-functional effects in the action of mastoparan.

The present results indicate that mastoparan has two opposite effects on $[Ca^{2+}]_i$ in human astrocytoma cells, and hence that mastoparan possibly has at least two sites of action for regulation of $[Ca^{2+}]_i$ in human astrocytoma cells.

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