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Mastoparan-induced phosphatidylcholine hydrolysis by phospholipase D activation in human astrocytoma cells

Kouichi Mizuno, [1]Norimichi Nakahata & Yasushi Ohizumi

Department of Pharmaceutical Molecular Biology, Faculty of Pharmaceutical Sciences, Tohoku University, Aoba, Aramaki, Aoba-ku, Sendai 980, Japan

1 The effect of mastoparan on phosphatidylcholine hydrolysis was examined in 1321N1 human astrocytoma cells. Mastoparan (3-30 μM) caused an accumulation of diacylglycerol (DG) and phosphatidic acid (PA) accompanied by choline release in a concentration- and time-dependent manner.

2 In the presence of 2% n-butanol, mastoparan (3-100 μM) induced phosphatidylbutanol (PBut) accumulation in a concentration- and time-dependent manner, suggesting that mastoparan activates phospholipase D (PLD). Propranolol (30-300 μM), a phosphatidate phosphohydrolase inhibitor, inhibited DG accumulation induced by mastoparan, supporting this idea.

3 Depletion of extracellular free calcium ion did not alter the effect of mastoparan on PLD activity.

4 A protein kinase C (PKC) inhibitor, calphostin C (1 μM), did not inhibit mastoparan-induced PLD activation but the ability of mastoparan to stimulate phospholipase D activity was decreased in the PKC down regulated cells.

5 PLD activity stimulated by mastoparan was not prevented by pretreatment of the cells with pertussis toxin (PT) or C3 ADP-ribosyltransferase. Furthermore, guanine nucleotides did not affect PLD activity stimulation by mastoparan in membrane preparations.

6 Mastoparan stimulated PLD in several cell lines such as RBL-2H3, RBL-1, HL-60, P388, endothelial cells, as well as 1321N1 human astrocytoma cells.

7 These results suggest that mastoparan induces phosphatidylcholine (PC) hydrolysis by activation of PLD, not by activation of phosphatidylcholine-specific phospholipase C (PC-PLC); mastoparan-induced PLD activation is not mediated by G proteins.

Keywords: Mastoparan; phospholipase D; phosphatidylcholine; G protein

Introduction

Mastoparan is a versatile tetradecapeptide purified from wasp venom (Hirai *et al.*, 1979) which stimulates phospholipase A_2 (Argiolas & Pisano, 1983), phosphoinositide-specific phospholipase C (Okano *et al.*, 1985; Gusovsky *et al.*, 1991) and nucleoside diphosphate kinase (Kikkawa *et al.*, 1992) and inhibits calmodulin-dependent enzymes by binding competitively to calmodulin and protein kinase C (PKC) in phosphatidylserine vesicles (Raynor *et al.*, 1991). Mastoparan also acts as a strong secretagogue in many cell lines and tissues including mast cells (Hirai *et al.*, 1979; Okano *et al.*, 1985), platelets (Ozaki *et al.*, 1990) and pancreatic islets (Yokokawa *et al.*, 1989). In some of these cell lines (Okano *et al.*, 1985) and in other cell lines (Perianin & Snyderman, 1989), mastoparan induces phosphatidylinositol turnover and increases the intracellular free calcium ion concentration. It is well known that mastoparan mimics hormone receptor activity and stimulates the guanine nucleotide exchange reaction of the α-subunit of heterotrimeric G protein Gi/Go family (Higashijima *et al.*, 1988; 1990; Higashijima & Ross, 1991).

Phosphatidylcholine (PC) is the principal phospholipid in mammalian tissue. Besides its role as a structural component of cellular membranes, the metabolism of PC generates second messengers for signal transduction in response to extracellular stimuli (Exton, 1994). Hydrolysis of PC by phosphatidylchoine-specific-phospholipase C (PC-PLC) produces diacylglycerol (DG) and phosphocholine, while hydrolysis of PC by phospholipase D (PLD) produces phosphatidic acid (PA) and choline. DG is known as an activator of PKC (Nishizuka, 1992) and PA potentially serves as a mediator for a number of cellular functions (Moolenaar *et al.*, 1986; Yu *et al.*, 1988). The observed fusiogenic ability of PA is consistent with the suggestion that PLD is involved in secretory responses (Exton, 1994). The products of PC hydrolysis by either PC-PLC or PLD can be interconverted through the actions of DG kinase and phosphatidate phosphohydrolase. Thus, PA also can be a precursor for the production of DG.

Activation of PLD by hormones and other extracellular stimuli has been shown in a variety of intact cells. It has been reported that calcium ions (Halenda & Rehm, 1990; Huang *et al.*, 1991), PKC (Liscovitch & Amsterdam, 1989; Balboa *et al.*, 1994), G proteins (Van Der Meulen & Haslam, 1990; Kusner *et al.*, 1993) and tyrosine phosphorylation (Bourgoin & Grinstein, 1992) regulate PC hydrolysis by PLD. However, the mechanisms for regulation of PLD activity remain unknown.

In the present study, we examined the effect of mastoparan on PC hydrolysis in 1321N1 human astrocytoma cells. The results obtained suggest that mastoparan is an activator of PLD.

Methods

Cell culture

1321N1 human astrocytoma cells were grown in Dulbecco's modified Eagle's medium with 5% foetal bovine serum, 50 u ml^{-1} of penicillin and 50 μg ml^{-1} streptomycin at 37°C in an atmosphere of 5% CO_2 in air. RBL-2H3, RBL-1, HL-60, P388 and endothelial cells were grown in RMPI1640 with 10% foetal bovine serum, 50 u ml^{-1} of penicillin and 50 μg ml^{-1} streptomycin at 37°C in an atmosphere of 5% CO_2 in air. For experiments, the cells which were grown on culture plates were seeded at a density of 10^5 cells/well in a 12-well culture plate and were used after culturing for 3-4 days.

[1] Author for correspondence.

Measurements of PLD activity in intact cells

To determine the PLD activity in intact cells, the cellular phospholipid pool was labelled by incubating cells in growth medium containing [^{3}H]-palmitic acid 10 μCi ml^{-1} at 37°C for 18–22 h in an atmosphere of 5% CO_2 in air. To examine the effects of ADP-ribosylation catalysed by PT or C3 ADP-ribosyltransferase 200 ng ml^{-1} of PT or 1 μg ml^{-1} C3 ADP-ribosyltransferase were included in the medium during the labelling. After washed three times with Eagle's minimal essential medium containing 10 mM HEPES (pH 7.35), the prelabelled cells were preincubated at 37°C for 10 min in Eagle's Minimal Essential medium containing 10 mM HEPES (pH 7.35) in the presence or absence of 2% n-butanol. Then, the reagents were added to the medium, and the reactions were terminated by addition of 1 ml of methanol/1N HCl (10/1). Lipids were extracted by the method of Bligh & Dyer (1959). Authentic PBut, PA and DG as standards were added to each sample. The extracted lipids were separated on LK5D silica gel (Whatman Inc.) with the organic layer of ethyl acetate/isooctane/water/acetic acid (11/5/10/2) and then detected with iodine vapour. DG was separated with hexane/ether/ethyl acetate (25/75/1). Spots corresponding to PBut, PA and DG were scraped off and their radioactivities were quantified by liquid scintillation counting.

Measurement of PLD activity in crude membrane preparations

1321N1 human astrocytoma cells in 150 mm-diameter dishes were labelled with [^{3}H]-palmitic acid 20 μCi ml^{-1}, at 37°C for 18–22 h in an atmosphere of 5% CO_2 in air. The prelabelled cells were lysed on ice for 15 min with 2 mM EDTA/10 mM HEPES (pH 7.0). Crude membranes were obtained by centrifugation at 10,000 *g* for 10 min at 4°C and washed twice with 2 mM EDTA/10 mM HEPES (pH 7.0) and twice with 2 mM EGTA/10 mM HEPES (pH 7.0). Crude membranes were incubated at 37°C for 12 min in 200 μl of medium of the following composition (mM): KCl 110, NaCl 10, KH_2PO_4 1, $MgCl_2$ 0.4, EGTA 1, $CaCl_2$ 0.2 (free concentration of 0.1 μM), ATP 0.1 and HEPES 20. The reactions were terminated by addition of 1 ml of methanol/1N HCl (10/1). Lipids were analysed by the same method described above.

Measurement of [^{3}H]-choline release

The cellular phospholipid pool was labelled by incubating 1321N1 cells in growth medium containing [^{3}H]-choline 5 μCi ml^{-1} at 37°C for 18–22 h in an atmosphere of 5% CO_2 in air. After washed three times with Eagle's minimal essential medium containing 10 mM HEPES (pH 7.35), the prelabelled cells were preincubated at 37°C for 10 min in Eagle's minimal essential medium containing 10 mM HEPES (pH 7.35). Then, the reagents were added to the medium for incubation, after which [^{3}H]-choline release was measured in the medium.

Materials

Mastoparan was obtained from Peptide Institute Inc. (Osaka, Japan). Pertussis toxin (PT), 12-O-tetradecanoylphorbol-13-O-acetate (TPA), DG and PA were from Funakoshi Inc. (Tokyo, Japan). [^{3}H]-choline was from American Radiochemicals (St. Louis, MO, U.S.A.). [^{3}H]-palmitic acid was from Amersham Japan (Tokyo, Japan). Foetal bovine serum was from Bioserum (Victoria, Australia). C3 ADP-ribosyltransferase was a gift from Prof. S. Narumiya, Department of Pharmacology, Kyoto University Faculty of Medicine, Japan. RBL-2H3 and HL-60 were gifts from the Japanese Cancer Research Resources Bank. Phosphatidylbutanol (PBut) was produced from soybean PC using crude cabbage PLD by the method of Yang *et al.* (1967). The sources of other reagents were described previously (Nakahata *et al.*, 1994).

Data analysis

The results obtained in separate experiments were expressed as mean ± s.e.mean.

Results

The effects of mastoparan on cellular [^{3}H]-DG and [^{3}H]-PA levels were examined in [^{3}H]-palmitic acid pre-labelled 1321N1 human astrocytoma cells. In the prelabelled 1321N1 cells, mastoparan increased [^{3}H]-DG and [^{3}H]-PA levels in a time-dependent manner (Figure 1). To determine the origin of [^{3}H]-DG and [^{3}H]-PA, we measured [^{3}H]-choline release in [^{3}H]-choline-prelabelled 1321N1 cells. Mastoparan also caused concentration- and time-dependent [^{3}H]-choline release in a similar manner to the increase in [^{3}H]-DG and [^{3}H]-PA (Figure 2). These results suggest that mastoparan stimulates PC hydrolysis, resulting in accumulation of DG and PA in 1321N1 cells.

To determine whether mastoparan hydrolyzes PC by activation of PC-PLC or PLD, the effect of mastoparan on PC hydrolysis was examined in the presence of 2% n-butanol. In [^{3}H]-palmitic acid prelabelled cells, butanol addition decreased [^{3}H]-DG and [^{3}H]-PA levels induced by mastoparan (data not shown) and an accumulation of [^{3}H]-PBut. The accumulation

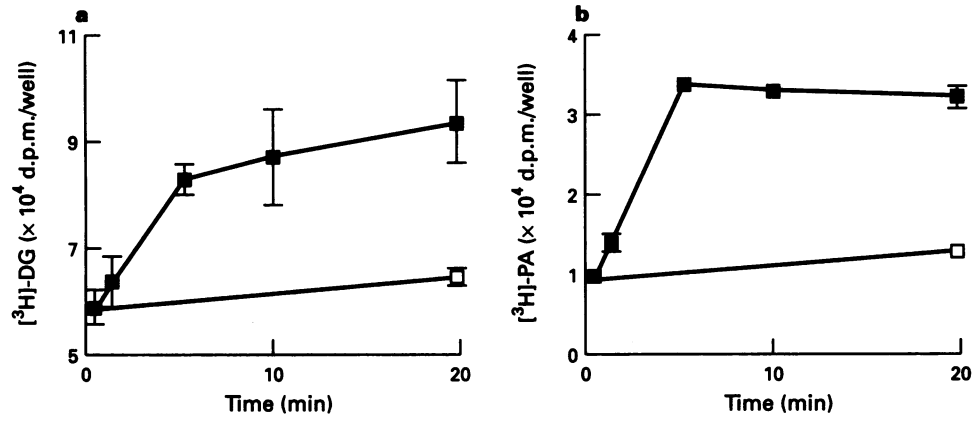

Figure 1 Time course of mastoparan effect on the accumulation of [^{3}H]-diacylglycerol ([^{3}H]-DG) and [^{3}H]-phosphatidic acid ([^{3}H]-PA) in 1321N1 human astrocytoma cells. 1321N1 human astrocytoma cells prelabelled with [^{3}H]-palmitic acid were incubated with (□) or without (■) 30 μM mastoparan at 37°C for the indicated time. After the reaction was terminated, [^{3}H]-DG (a) and [^{3}H]-PA (b) were determined. Data represent the mean ± s.e.mean of three independent experiments.

of [^{3}H]-PBut induced by mastoparan was concentration- and time-dependent (Figure 3). [^{3}H]-PBut accumulation was detectable at a concentration of 5 μM mastoparan and reached a maximum at 30 μM mastoparan. [^{3}H]-PBut accumulation induced by 30 μM mastoparan reached a plateau within 15 min (Figure 3). This is slower than that induced by receptor agonists such as carbachol (Martinson *et al.*, 1990). From these results, mastoparan activates PLD resulting in PC hydrolysis.

The effect of the phosphatidate phosphohydrolase inhibitor, propranolol, on accumulation of [^{3}H]-DG and [^{3}H]-PA induced by mastoparan was also examined. Propranolol concentration-dependently reduced mastoparan-induced [^{3}H]-DG accumulation and increased the [^{3}H]-PA level (Figure 4). Although it is reported that propranolol inhibits not only phosphatidate phosphohydrolase but also PKC (Sozzani *et al.*, 1992), the effect of propranolol on [^{3}H]-DG and [^{3}H]-PA accumulation may be due to the phosphatidate phosphohydrolase inhibition. These results also suggest that mastoparan activates PLD.

It is known that PLD activity is dependent on extracellular calcium ions in some cases (Kanaho *et al.*, 1992). Thus, we examined the effect of mastoparan on PLD activity in the absence of extracellular free calcium ions. As shown in Figure 5, extracellular free calcium ion depletion by 4 mM EGTA did not inhibit mastoparan-induced PLD activation.

To investigate the relationship between mastoparan-induced PLD activation and PKC, the effect of PKC down-regulation on PLD activation induced by mastoparan was examined. In 1321N1 cells treated with 1 μM TPA during prelabelling, the accumulation of [^{3}H]-PBut induced by mastoparan was reduced to 35% of that in untreated cells (Table 1). Under the same conditions, TPA-induced PBut accumulation was completely inhibited. However, a selective PKC inhibitor, calphostin C, did not affect the accumulation of [^{3}H]-PBut stimulated by mastoparan, although calphostin C inhibited TPA-induced [^{3}H]-PBut accumulation (Table 1). Moreover, the non-selective protein kinase inhibitor, staurosporine, also did not affect the accumulation of [^{3}H]-PBut stimulated by mastoparan (Table 1).

Because of the ability of mastoparan to activate G proteins, we examined the mastoparan-induced PLD activation in the cells pretreated with PT or C3 ADP-ribosyltransferase. As shown in Table 1, mastoparan-induced PLD activation was not inhibited by the cells pretreated with PT or C3 ADP-ribosyltransferase. Moreover, [^{3}H]-PBut accumulation induced by mastoparan was enhanced by pretreatment of the cells with PT. From these results, it seems that mastoparan activates PLD neither via the heterotrimeric G protein G_i/G_o family nor by the small molecular weight G protein rho/ras family.

To examine the effects of guanine nucleotides on mastoparan-induced PLD activation, we used crude membrane preparations. In these preparations, mastoparan induced [^{3}H]-PBut accumulation at the same concentrations as those required in intact cells (Figure 6). Although it has been reported

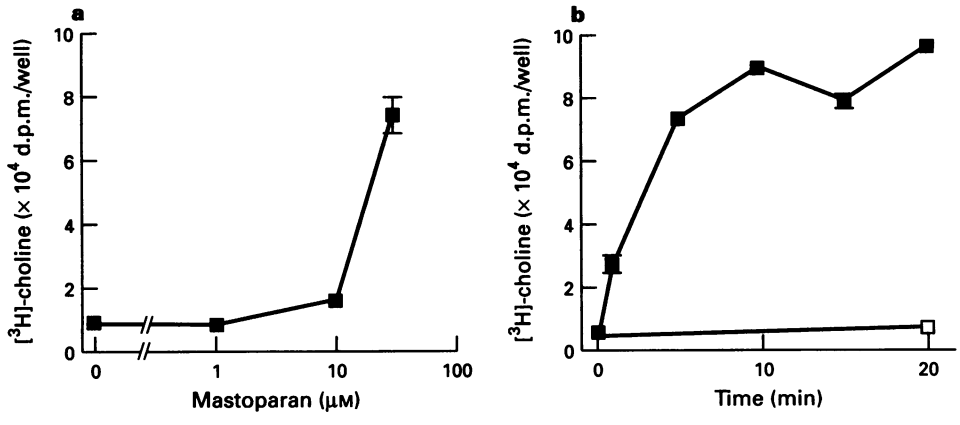

Figure 2 Effect of mastoparan on [^{3}H]-choline releases in 1321N1 human astrocytoma cells. 1321N1 human actrocytoma cells prelaballed with [^{3}H]-choline were incubated with the indicated concentrations of mastoparan at 37°C for 10 min (a) or incubated with (□) or without (■) 30 μM mastoparan at 37°C for the indicated time (b). After the reaction was terminated, [^{3}H]-choline release was determined. Data represent the mean ± s.e.mean of three independent experiments.

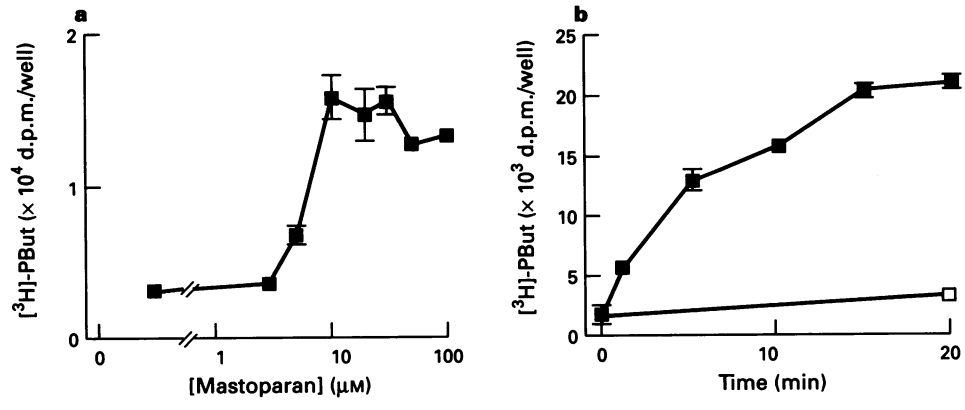

Figure 3 Effect of mastoparan on [^{3}H]-phosphatidylbutanol ([^{3}H]-PBut) accumulation in 1321N1 human astrocytoma cells. 1321N1 human astrocytoma cells prelabelled with [^{3}H]-palmitic acids were incubated with 2% butanol and the indicated concentrations of mastoparan at 37°C for 10 min (a) or incubated with 2% butanol and 30 μM mastoparan (■) or vehicle (□) at 37°C for the indicated time (b). After the reaction was terminated, [^{3}H]-PBut was determined. Data represent the mean ± s.e.mean of three independent experiments.

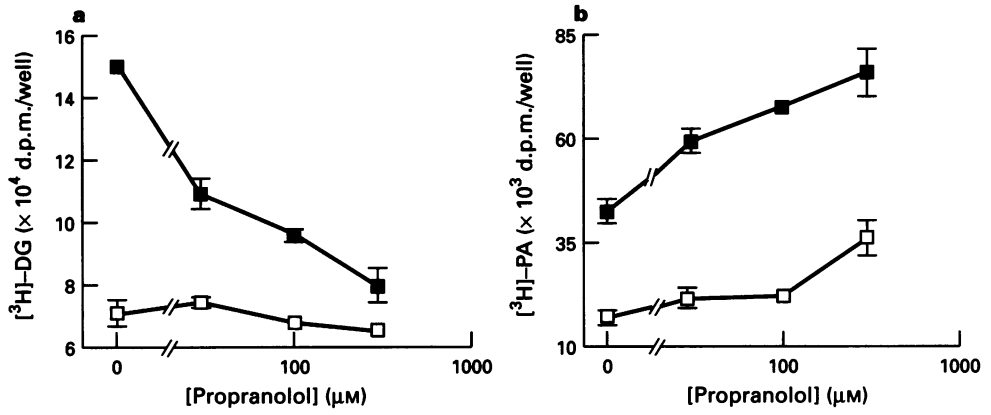

Figure 4 Effect of propranolol on accumulation of [^{3}H]-diacylglycerol ([^{3}H]-DG) and [^{3}H]-phosphatidic acid ([^{3}H]-PA) induced by mastoparan in 1321N1 human astrocytoma cells. 1321N1 human astrocytoma cells prelabelled with [^{3}H]-palmitic acids were incubated with the indicated concentrations of propranolol at 37°C for 10 min, supplemented with (■) or without (□) 30 μM mastoparan. After the reaction was terminated, [^{3}H]-DG (a) and [^{3}H]-PA (b) were determined. Data represent the mean ± s.e.mean of three independent experiments.

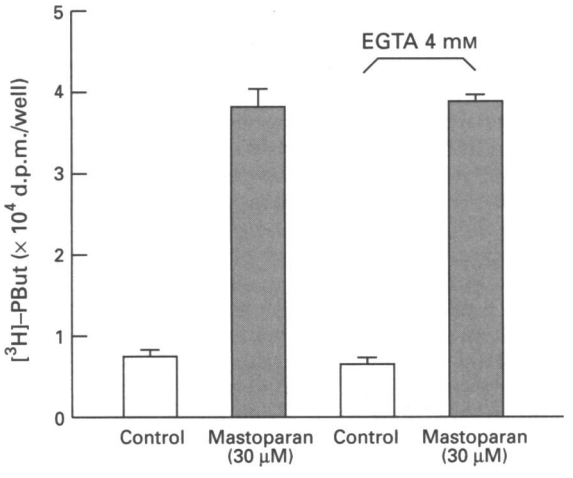

Figure 5 Effect of extracellular calcium ion depletion on [^{3}H]-phosphatidylbutanol ([^{3}H]-PBut) accumulation induced by mastoparan in 1321N1 human astrocytoma cells. 1321N1 human astrocytoma cells prelabelled with [^{3}H]-palmitic acids were incubated with 2% butanol and 30 μM mastoparan at 37°C for 10 min in the presence or absence of 4 mM EGTA. After the reaction was terminated, [^{3}H]-PBut was determined. Data represent the mean ± s.e.mean of three independent experiments.

Table 1 The relation of G protein and protein kinase C (PKC) to mastoparan-induced phospholipase D (PLD) activation

a *Phosphatidylbutanol* (d.p.m./well)

	Pertussis toxin (PT)-treatment	
	−	+
Control	12769.89 ± 607.25	12646.54 ± 939.39
Mastoparan	31232.48 ± 1282.67	39767.89 ± 1107.09
	C3-treatment	
	−	+
Control	2257.68 ± 218.27	2274.15 ± 508.03
Mastoparan	10793.35 ± 851.28	10953.63 ± 292.46

b *Phosphatidylbutanol* (d.p.m./well)

	PKC down regulation	
	−	+
Control	2257.68 ± 218.27	1896.66 ± 103.19
Mastoparan	10793.35 ± 851.28	3724.42 ± 469.59
TPA	8932.51 ± 300.33	1951.54 ± 255.33
	Calphostin C	
	−	+
Control	4748.40 ± 113.70	4145.75 ± 120.08
Mastoparan	22032.71 ± 1010.73	23253.00 ± 290.88
TPA	20816.83 ± 493.36	4829.02 ± 299.59
	Staurosporine	
	−	+
Control	2201.98 ± 819.25	2203.88 ± 191.15
Mastoparan	12968.92 ± 224.04	13939.17 ± 320.04
TPA	8532.78 ± 300.84	2807.93 ± 118.24

(**a**) For ADP ribosylation experiments, 1321N1 human astrocytoma cells were treated with or without 200 ng ml^{-1} PT or 1 μg ml^{-1} C3 during prelabelling with [^{3}H]-palmitic acid, then incubated with 2% butanol and 30 μM mastoparan at 37°C for 10 min. (**b**) For PKC downregulation experiments 1321N1 human astrocytoma cells were treated with or without 1 μM TPA during prelabelling with [^{3}H]-palmitic acid, then incubated with 2% butanol, with 2% butanol and 30 μM mastoparan or with 2% butanol and 100 nM TPA at 37°C for 10 min. For PKC inhibitor experiments, 1321N1 human astrocytoma cells prelabelled with [^{3}H]-palmitic acids were incubated with 2% butanol, with 2% butanol and 30 μM mastoparan or with 2% butanol and 100 nM TPA in the presence or absence of 1 μM calphostin C or 1 μM staurosporine at 37°C for 10 min. After the reaction was terminated, [^{3}H]-PBut was determined. Data represent the mean ± s.e.mean of three independent experiments.

that 10 μM guanosine 5'-3-O-(thio)triphosphate (GTPγS) can induce PLD activation in membrane preparations or permeabilized cells (Van Der Meulen & Haslam, 1990; Kusner *et al.*, 1993); 10 μM GTPγS alone did not induce an accumulation of [^{3}H]-PBut in our crude membrane preparation. Synergism between GTPγS and mastoparan was not observed, and 1 mM guanosine 5'-2-O-(thio)diphosphate (GDPγS) did not inhibit mastoparan-induced PLD activation. These results suggest that mastroparan does not mediate G proteins for PLD activation.

To identify the mechanism of mastoparan action on PLD, we examined the effect of mastoparan on PLD activity extracted from bovine brain membranes with a solution of higher ionic strength and on partially purified soluble PLD of cabbage. In both cases, mastoparan stimulated PLD activity (data not shown).

Finally, we examined the effects of mastoparan on PLD activation in various cell lines such as RBL-2H3, RBL-1, HL-60, P388 and endothelial cells. In all cell lines we tested, mastoparan stimulated PLD. These results suggest that the ability of mastoparan to activate PLD is common in animal cells and is not limited to 1321N1 human astrocytoma cells (Table 2).

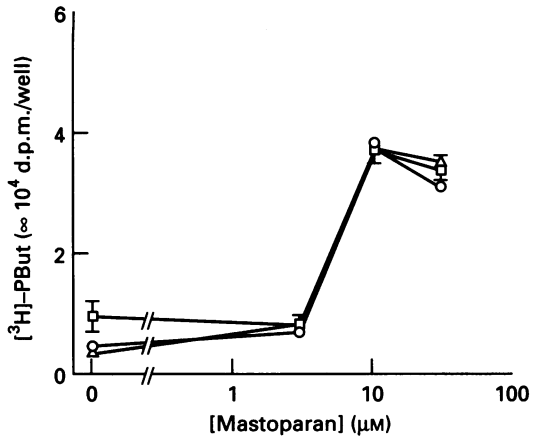

Figure 6 Effects of guanine nucleotides on [^{3}H]-phosphatidylbutanol ([^{3}H]-PBut) accumulation induced by mastoparan in crude membrane preparations of 1321N1 human astrocytoma cells. Crude membranes were prepared by centrifugation of 1321N1 cell lysate treated by hypotonic buffer at 10,000g for 10 min. Crude membranes were incubated with 2% butanol and the indicated concentrations of mastoparan supplemented with no other drugs (○), 10 μM GTPγS (△) or 1 mM GDPβS (□) at 37°C for 12 min. After the reaction was terminated, [^{3}H]-PBut was determined. Data represent the mean ± s.e.mean of three independent experiments.

Discussion

This paper is the first report to demonstrate that mastoparan is a strong activator of PLD resulting in PC hydrolysis. It has been shown that vesicle fusion requires activation of PLD, which generates PA from PC. The action of PLD can potentially alter the surface milieu of membranes by producing negatively charged phospholipids from neutral PC. It is very interesting that mastoparan, which is a strong secretagogue, can activate PLD. Although it has been thought that mastoparan induces histamine release from mast cells by activation of phosphoinositide-specific phospholipase C, mediated via PT-sensitive G protein, resulting in an accumulation of inositol 1,4,5-trisphosphate and intracellular calcium mobilization, mastoparan stimulates exocytosis in a calcium-independent manner in some cases. Mastoparan induced 5-hydroxytryptamine release from intact human platelets without an elevation of intracellular free calcium ion concentration (Ozaki *et al.*, 1990). In contrast to mast cells, mastoparan inhibits phosphoinositide hydrolysis and intracellular calcium mobilization induced by agonist in the absence of extracellular free calcium ions in 1321N1 human astrocytoma cells (Nakahata *et al.*,1990; 1994). It has been substantiated that the stimulatory effects of mastoparan are mediated via G protein activation because of the inhibitory action of PT on mastoparan-induced cellular functions. However, it is reported that mastoparan-stimulated exocytosis is not inhibited by pretreatment of the cells with PT in some cell lines including the RINm5F β-cell line (Komatsu *et al.*, 1993). Moreover, insulin release from RINm5F cells induced by mastoparan increased after treatment of the cells with PT. It is possible that mastoparan stimulates cell function by activating PLD in some of these cases.

It has been reported that mastoparan increases GTPase activity and [^{35}S]-GTPγS binding of G_i/G_o in phospholipid vesicles (Higashijima *et al.*, 1988; 1990). A cross-linking study revealed a binding site for mastoparan on the amino terminus of the α subunit of the heterotrimeric G protein G_i/G_o family (Higashijima & Ross, 1991). It has been also reported that mastoparan inhibits the ADP-ribosylation of 20 kDa human platelet membrane proteins catalyzed by C3 ADP-ribosyltransferase (Koch *et al.*,1991; 1992). This result suggests that mastoparan interacts not only with the heterotrimeric G protein G_i/G_o family but also with the small molecular weight G protein rho/rac family. On the other hand, there is much evidence that PLD activity is regulated by G proteins (Van Der Meulen & Haslam, 1990; Kusner *et al.*, 1993), and that ADP-ribosylation catalyzed by PT inhibits agonist-induced PLD activation in neutrophils (Kanaho *et al.*, 1991; 1992). It has been also reported that PLD is activated by a membrane-associated rho family, small molecular weight G protein because of inhibition of GTPγS-stimulated PLD activity by rho GDP dissociation inhibitor and stimulation of GTPγS-stimulated PLD activity by small molecular weight G protein GDP dissociation stimulator in neutrophils (Bowman *et al.*, 1993). However, in 1321N1 human astrocytoma cells pretreated with PT or C3 ADP-ribosyltransferase, mastoparan retained the ability to stimulate PLD activity. Moreover, pretreatment of the cells with PT enhanced mastoparan-induced PLD activation. Recently, Malcolm *et al.* (1994) reported that Rho A activates PLD in rat plasma membranes treated with glutathione S-transferase fused Rho GDP dissociation inhibitor. However, in our crude membrane preparations, guanine nucleotides did not modulate mastoparan-induced PLD activation. These results suggest that mastoparan stimulates PLD by a mechanism not mediated via G protein activation, such as G_i, Rho and ADP-ribosylation factor which is identified as a cytosolic factor-enhanced PLD activity (Brown *et al.*, 1993; Cockcroft *et al.*, 1994).

It is known that PLD activity is dependent on extracellular calcium ions in some cases (Kanaho *et al.*, 1992). However, extracellular free calcium ion depletion by 4 mM EGTA did not inhibit mastoparan-induced PLD activation in 1321N1 human astrocytoma cells. From this result, mastoparan-induced PLD activation is not dependent on extracellular free calcium ions. Furthermore, PLD activity stimulated by mastoparan is not mediated via intracellular free calcium ion concentration elevation.

Although calphostin C, a selective PKC inhibitor (by binding to the phorbol ester binding site of PKC) did not inhibit mastoparan-induced PLD activation, PKC down-reg-

Table 2 Effects of mastoparan on phospholipase D (PLD) activity in various cell lines

Cell	*Butanol*	*Phosphatidylbutanol* (d.p.m./well) *Control*	*Mastoparan*
1321N1	2%	2257.68 ± 218.27	10793.35 ± 851.28
RBL-2H3	2%	1654.51 ± 103.98	5355.80 ± 233.72
Endothelial cells	2%	6069.60 ± 490.56	19007.25 ± 2164.17
		Phosphatidylbutanol (d.p.m./1 × 10^6 cells) *Control*	*Mastoparan*
HL-60	0.2%	1600.27 ± 25.58	14004.43 ± 54.55
RBL-1	0.2%	1808.95 ± 85.67	3237.56 ± 209.49
P388	0.2%	2022.39 ± 67.72	8575.19 ± 208.40

Various cell lines were incubated with the indicated concentrations of butanol and 30 μM mastoparan at 37°C for 10 min. After the reaction was terminated, [^{3}H]-phosphatidylbutanol ([^{3}H]-PBut) formed was determined. Data represent the means ± s.e.mean of three independent experiments.

ulation partially inhibited mastoparan-induced PLD activation. However, mastoparan did not increase protein phosphorylation levels in 1321N1 cells (Mizuno *et al.*, unpublished observation). In fact, it has been reported that mastoparan inhibits PKC (Raynor *et al.*, 1991). From these results, mastoparan does not activate PLD by protein phosphorylation via PKC. It might be due to the disappearance of positive feedback regulations by a certain PKC isozyme that mastoparan has less potency on PLD in PKC down-regulated cells. Moreover, mastoparan does not activate PLD by protein phosphorylation, because 1 μM staurosporine, which binds to the ATP binding site of protein kinases and inhibits broad range of protein kinases including PKC and a certain tyrosine kinase (Fallon, 1990), did not affect mastoparan-induced PLD activation. In addition to these protein kinase inhibitors, we tested the effect of 100 μM genistein, a selective protein tyrosin kinase inhibitor, on PLD activation induced by mastoparan. Genistein also did not inhibit mastoparan-induced PLD activation (Mizuno *et al.*, unpublished obeservation).

We examined the effect of mastoparan on PLD in various cell lines such as RBL-2H3, RBL-1, HL-60 and P388 endothelial cells as well as 1321N1 cells. In all cell lines tested, mastoparan could induce PLD activation (Table 1) suggesting that mastoparan is a general activator of PLD in animal cells.

Mastoparan also stimulated PLD activity extracted from bovine brain membranes and partially purified soluble PLD of cabbage. It is reported that anionic amphipathic substances stimulated purified soluble PLD of cabbage and cationic amphipathic substances inhibit its activity (Dawson & Hemington, 1967). Mastoparan is a basic amphipathic peptide. Thus, this property was not correlated with the effect of mastoparan on PLD. One possible mechanism is that mastoparan interacts with PLD directly. Another possible mechanism is that mastoparan interacts with a protein or phospholipid that activates PLD subsequently. Okamura & Yamahita (1994) have purified PLD from a mammalian source so that the precise mechanism of mastoparan action on PLD will be clear. In conclusion, mastoparan stimulates PLD in 1321N1 human astrocytoma cells and various cell lines, and as such it is a useful tool.

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