

Mastoparan, a Novel Mitogen for Swiss 3T3 Cells, Stimulates Pertussis Toxin-sensitive Arachidonic Acid Release without Inositol Phosphate Accumulation

Joan Gil, Theresa Higgins, and Enrique Rozengurt

Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, United Kingdom

Abstract. Mastoparan, a basic tetradecapeptide isolated from wasp venom, is a novel mitogen for Swiss 3T3 cells. This peptide induced DNA synthesis in synergy with insulin in a concentration-dependent manner; half-maximum and maximum responses were achieved at 14 and 17 μM , respectively. Mastoparan also stimulated DNA synthesis in the presence of other growth promoting factors including bombesin, insulin-like growth factor-1, and platelet-derived growth factor. The synergistic mitogenic stimulation by mastoparan can be dissociated from activation of phospholipase C. Mastoparan did not stimulate phosphoinositide breakdown, Ca^{2+} mobilization or protein kinase C-mediated phosphorylation of a major cellular substrate or transmodulation of the epidermal growth

factor receptor. In contrast, mastoparan stimulated arachidonic acid release, prostaglandin E_2 production, and enhanced cAMP accumulation in the presence of forskolin. These responses were inhibited by prior treatment with pertussis toxin. Hence, mastoparan stimulates arachidonic acid release via a pertussis toxin-sensitive G protein in Swiss 3T3 cells. Arachidonic acid, like mastoparan, stimulated DNA synthesis in the presence of insulin. The ability of mastoparan to stimulate mitogenesis was reduced by pertussis toxin treatment. These results demonstrate, for the first time, that mastoparan stimulates reinitiation of DNA synthesis in Swiss 3T3 cells and indicate that this peptide may be a useful probe to elucidate signal transduction mechanisms in mitogenesis.

ELUCLIDATION of the molecular mechanisms leading to cell proliferation requires the identification of the signal transduction pathways that, when activated, induce a mitogenic response. In this respect cultured fibroblasts, such as murine 3T3 cells, have emerged as a model system. These cells cease to proliferate when they deplete the medium of its growth promoting activity and enter a quiescent or nondividing state. However, such cells remain viable and can be stimulated to reinitiate DNA synthesis and cell division either by replenishing the medium with fresh serum or by the addition of polypeptide growth factors, neuropeptides and pharmacological agents in serum-free medium. Extensive analysis of early signaling events using this model has revealed the existence of multiple signal transduction pathways that in synergy initiate a proliferative response (Rozengurt, 1986). The discovery of additional mitogens can provide novel approaches to explore cellular signaling pathways leading to cell proliferation.

Mastoparan, an amphiphilic tetradecapeptide isolated from wasp venom, stimulates exocytosis in a variety of target cells

including mast (Hirai et al., 1979), chromaffin (Kuroda et al., 1980), pituitary (Camoratto and Gradison, 1985; Kurihara et al., 1986), and pancreatic (Yokokawa et al., 1989) cells. The early events in the action of mastoparan include the breakdown of phosphoinositides and the mobilization of Ca^{2+} from internal stores (Okano et al., 1985; Perianin and Snyderman, 1989; Tohkin et al., 1990). The mechanism by which mastoparan activates these signaling pathways does not involve specific membrane receptors (Aridor et al., 1990). Instead, mastoparan acts directly on G proteins reconstituted into phospholipid vesicles to stimulate guanine nucleotide exchange and GTP hydrolysis by a mechanism similar to that used by surface receptors (Higashijima et al., 1988, 1990; Mousli et al., 1990; Weingarten et al., 1990). This receptor-like activity of mastoparan could provide a novel probe to activate G proteins in intact cells. Indeed, some of the cellular and molecular effects of mastoparan can be blocked by pertussis toxin (Aridor et al., 1990; Higashijima et al., 1987; Mousli et al., 1989; Saito et al., 1987; Yokokawa et al., 1989), which ADP-ribosylates and functionally inactivates a family of G proteins including transducin, G_i , and G_o (Gilman, 1987). In addition, mastoparan has been previously shown to interact with phospholipids (Higashijima et al.

J. Gil's present address is Unitat de Bioquímica, Facultat d'Odontologia, Universitat de Barcelona, 08907 Hospitalet de Llobregat, Spain.

1983) and to directly stimulate phospholipase A₂ (PLA₂)¹ activity leading to the generation of arachidonic acid (Argiolas and Pisano, 1983). Some of the early signals elicited by mastoparan in secretory cells, e.g., phosphoinositide breakdown, Ca²⁺ mobilization, and arachidonic acid release, have also been implicated in triggering cell proliferation by mitogenic neuropeptides and polypeptide growth factors in quiescent cells (Rozenfurt, 1986). As yet, however, it is not known which signal transduction pathways are activated by mastoparan in quiescent cells nor whether this peptide can act as a mitogen for these cells.

In the present study we report that mastoparan, at micromolar concentrations, stimulates reinitiation of DNA synthesis in Swiss 3T3 cells acting synergistically with insulin and other growth promoting factors. Before the stimulation of DNA synthesis, mastoparan increases pertussis toxin-sensitive arachidonic acid release in the absence of inositol phosphate accumulation. Thus, mastoparan provides a novel probe to elucidate signal transduction pathways in the mitogenic response.

Materials and Methods

Cell Culture

Stock cultures of Swiss 3T3 cells were propagated as described previously (Rozenfurt and Sinnott-Smith, 1983). For experimental purposes, cells were subcultured in 33- or 90-mm Nunc dishes with Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum and incubated in a humidified atmosphere of 10% CO₂, 90% air at 37°C until they became confluent and quiescent (6–8 d).

[³H]Thymidine Incorporation Assay

Determinations of DNA synthesis were performed as previously described (Dicker and Rozenfurt, 1980). Briefly, cultures were washed twice with DMEM and incubated in DMEM/Waymouth's medium (1:1[vol/vol]) containing [³H]thymidine (1 μCi/ml; 1 μM) and various additions as described in the experiments. After 40 h, the cultures were washed twice with PBS and incubated in 5% trichloroacetic acid for 30 min at 4°C. Trichloroacetic acid was then removed and the cultures washed twice with ethanol and extracted in 1 ml of 2% Na₂CO₃, 0.1 M NaOH, 1% SDS. Incorporation was determined by scintillation counting in 10 ml of picrofluor.

Autoradiography of Labeled Nuclei

Labeled nuclei were determined by autoradiography as described previously (Dicker and Rozenfurt, 1980). Briefly, cultures were washed twice with DMEM and incubated in 2 ml of DMEM/Waymouth's medium (1:1[vol/vol]) containing [³H]thymidine (5 μCi/ml) and the indicated factors. After 40 h the medium was removed and the cultures were fixed in formol/saline (1:9[vol/vol]). The cultures were washed twice with Tris-saline, pH 7.4 at 4°C, fixed twice (for 5 and 2 min) with 5% trichloroacetic acid at 4°C, and washed three times with 70% ethanol. Autoradiographic film was put on the cultures and developed after a 1-wk exposure. After staining with Giemsa, the labeled nuclei were counted.

Determination of Total Inositol Phosphates

Confluent and quiescent cells grown on 90-mm dishes were labeled with 5 μCi/ml of D-myo-[³H]inositol for 24 h at 37°C in conditioned medium. The cells were washed twice with DMEM preincubated for 20 min in DMEM and then for 10 min in DMEM containing 20 mM LiCl. Mastoparan or bombesin were then added and the cells incubated for the indicated time. The incubations were stopped by rapidly replacing the medium with ice-cold 3% perchloric acid. After 30 min at 4°C, the acid soluble extracts

were collected and neutralized (pH 7–8) with 2 M K₂CO₃, containing 10 mM EDTA and 100 mM Hepes. Samples were analyzed for their content of total inositol phosphates by anion exchange chromatography using a Bio-Rad AGIX8 resin (Bio-Rad Laboratories, Richmond, CA) and elution with 1 M ammonium formate/0.1 M formic acid was performed as previously described (Berridge et al., 1983; Nånberg and Rozenfurt, 1988).

80 K Phosphorylation

Confluent and quiescent cells grown on 35-mm dishes were washed twice in phosphate-free DMEM and incubated for 5 h in 1 ml of the same medium containing 200 μCi ³²P_i. Peptides were then added for various times. The reaction was stopped by removing the medium and rapidly washing the culture twice with TBS. The cells were immediately extracted with 100 μl of a solution containing 100 mM Tris-HCl, pH 6.9, 0.5% Triton X-100, 2 mM EGTA, 10 mM sodium fluoride, 50 μM PMSF, 3.5 μg/ml aprotinin for 5 min at room temperature. The extract was heated at 100°C for 5 min, centrifuged, and the supernatant was then mixed with an equal volume of 2× SDS sample buffer. One-dimensional SDS-PAGE was performed using 7.5% (wt/vol) acrylamide and 0.1% (wt/vol) SDS (Laemmli, 1970).

⁴⁵Ca²⁺ Efflux

Quiescent cultures of Swiss 3T3 cells were equilibrated with ⁴⁵Ca²⁺ by incubating them for 14–16 h in conditioned medium containing 5 μCi/ml ⁴⁵Ca²⁺. Mastoparan or bombesin were added to the cultures and after 30 min of incubation at 37°C the extracellular medium was removed and the cells rapidly washed seven times with DMEM containing 3 mM EGTA. Cellular ⁴⁵Ca²⁺ was extracted with 0.1 N NaOH, 2% Na₂CO₃, 1% SDS, and determined by scintillation counting.

¹²⁵I-EGF Binding Assay

Quiescent 3T3 cells were washed twice with DMEM at 37°C and incubated in a mixture of 1:1 DMEM and Waymouth's medium containing 50 mM Hepes, pH 7.0 at 37°C and factors as indicated. After 1 h, cells were then incubated at 4°C in PBS (pH 7.2) containing 25 mM Hepes and 0.5 ng/ml (110,000 cpm/ng) ¹²⁵I-EGF. After 2 h, cells were washed rapidly with PBS, extracted with 0.1 M NaOH containing 2% Na₂CO₃ and 1% SDS, and cell-associated radioactivity was determined in a gamma counter. Nonspecific binding was determined as cell-associated radioactivity in the presence of a 250-fold excess of unlabeled EGF as previously described (Zachary et al., 1986).

Arachidonic Acid Release

Confluent and quiescent cells in 33-mm dishes were labeled for 14–16 h with 1 μCi/ml [³H]arachidonic acid in conditioned medium. The cells were then washed 3 times with DMEM and incubated at 37°C for 30 min in 1 ml DMEM/Waymouth (1:1) containing mastoparan or bombesin as indicated. The medium was collected and centrifuged for 5 min at 2,000 g to remove contaminating cellular debris and the level of radioactivity measured by scintillation counting.

Measurement of Prostaglandin E₂ (PGE₂) Release

Cultures were washed twice with DMEM and incubated at 37°C for 1 h in the required conditions. After this time the medium was removed and stored at 4°C. All vessels used were made of polypropylene or silicized glassware. Measurements of prostaglandin E₂ (PGE₂) were performed by radioimmunoassay using a ¹²⁵I-PGE₂ assay system. Aliquots of samples were diluted in assay buffer containing 0.9% NaCl, 0.01 M EDTA, 0.3% bovine γ-globulin, 0.005% Triton X-100, 0.05% sodium azide, 25 mM phosphate buffer, pH 6.8. The samples were then bound to a rabbit anti-PGE₂ antibody using ¹²⁵I-PGE₂ as a competitive tracer for 16 h at 4°C. After this time the immune complexes were precipitated by the addition of 16% polyethylene glycol, 0.05% sodium azide, and 50 mM phosphate buffer, pH 6.8 for 30 min at 4°C. Samples were centrifuged for 30 min at 2,000 g and the supernatants removed. The resulting pellets were counted in a gamma counter (Beckman Instruments Inc., Palo Alto, CA). Additions of growth factors to the medium had no effect on the radioimmunoassay.

Measurement of cAMP

Confluent and quiescent cells grown in 33-mm dishes were washed three

1. Abbreviations used in this paper: IGF-1, insulin-like growth factor 1; PDBu, phorbol 12,13-dibutyrate; PGE₂, prostaglandin E₂; PLA₂, phospholipase A₂.

times in DMEM and incubated at 37°C for 1 h in 2 ml of the same medium in the presence of various factors. The medium was then rapidly replaced with 200 μ l of 0.1 M HCl and incubated for 20 min at 4°C. The solution was then diluted into 50 mM sodium acetate buffer, pH 5.8 and cAMP levels were determined using a commercial radioimmunoassay kit. The precipitated protein, remaining on the dish, was dissolved in 1 ml of 2% Na₂CO₃, 0.1 M NaOH, and measured using the bicinchoninic acid reagent.

Materials

Mastoparan and pertussis toxin were obtained from Peninsula Laboratories, Inc. (Belmont, CA). Bombesin, EGF, bovine insulin, and phorbol-12,13-dibutyrate (PDBu) were from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum was from Gibco Laboratories (Grand Island, NY). C-sis recombinant PDGF, recombinant insulin-like growth factor 1 (IGF-1), ¹²⁵I-EGF, [³H]thymidine, [³²P]P_i (carrier free), DR-myo [³H]inositol, ⁴⁵CaCl₂, [⁵6, 8, 9, 11, 12, 14, 15-³H]arachidonic acid, and cAMP radioimmunoassay kit were purchased from Amersham International (Amersham, UK). ¹²⁵I-PGE₂ radioimmunoassay system was from New England Nuclear (Boston, MA). Bicinchoninic acid reagent was from Pierce Chemical Co. (Rockford, IL). Forskolin was from Calbiochem-Behring Corp. (San Diego, CA). Dowex AG1-X8 (formate form) was obtained from Bio-Rad Laboratories, Richmond, CA). All other materials were of the purest grade commercially available.

Results

Mastoparan Induced DNA Synthesis in Synergy with Other Mitogens

Initial experiments revealed that addition of 10–20 μ M mastoparan to quiescent cultures of Swiss 3T3 cells induced marked morphological changes and cytotoxic effects at the higher concentrations (25–30 μ M). To determine whether mastoparan can act as a mitogen for these cells, confluent and quiescent cultures were incubated in medium containing insulin and increasing concentrations of mastoparan. Cumulative

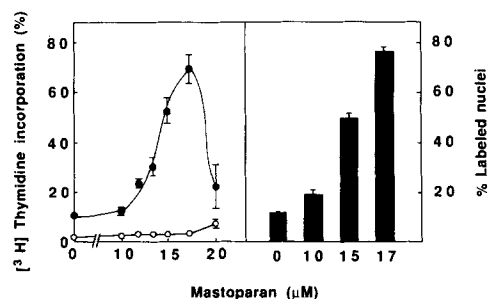


Figure 1. (Left) Dose response for the stimulation of DNA synthesis by mastoparan. Confluent and quiescent cultures of Swiss 3T3 cells were washed and incubated at 37°C in 2 ml DMEM/Waymouth medium containing 1 μ Ci/ml [³H]thymidine and various concentrations of mastoparan, in the absence (open circles) or in the presence (closed circles) of insulin at 1 μ g/ml. After 40 h DNA synthesis was assessed by measuring the [³H]thymidine incorporated into acid precipitable material. Each point is the mean \pm SEM of eight determinations from four independent experiments expressed as a percentage of the incorporation induced by 10% FBS (4.5–6 \times 10⁵ cpm). (Right) Effect of mastoparan on DNA synthesis assessed by autoradiography. Cells were incubated at 37°C in 2 ml DMEM/Waymouth medium containing 5 μ Ci/ml [³H]thymidine and various concentrations of mastoparan in the presence of insulin at 1 μ g/ml. 10% fetal bovine serum produced 95% labeled nuclei. The values are the mean \pm SEM of 10 different microscopic fields from 2 different dishes.

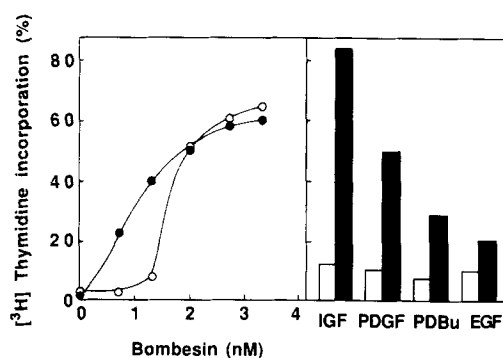


Figure 2. Stimulation of DNA synthesis by mastoparan in the presence of various growth factors. (Left) Dose-response curve for the stimulation of DNA synthesis by bombesin in the absence (open circles) or in the presence (closed circles) of 15 μ M mastoparan. Each value is the mean of two determinations from one representative experiment. (Right) Effect of mastoparan on IGF-1, PDGF, PDBu, and EGF stimulation of DNA synthesis. Cells were incubated with different factors either in the absence (open bars) or in the presence (closed bars) of 15 μ M mastoparan. The concentrations of IGF-1, PDGF, PDBu, and EGF were 20, 10, 100, and 10 ng/ml, respectively. Each value is the mean of two determinations from one representative experiment. Similar results were obtained in two other independent experiments.

relative [³H]thymidine incorporation was measured after 40 h of incubation. Fig. 1 (left), shows that mastoparan caused a striking stimulation of DNA synthesis, in a narrow concentration range. Half of the maximum response was obtained at a concentration of 14 μ M and the maximum effect was achieved at 17 μ M. Mastoparan added without insulin did not induce any significant [³H]thymidine incorporation in Swiss 3T3 cells (Fig. 1, left).

To assess whether mastoparan and insulin interact synergistically in eliciting initiation of DNA replication rather than in changing the specific activity of the [³H]thymidine precursor pool, quiescent cultures of Swiss 3T3 cells were treated with various concentrations of mastoparan in the presence of insulin and incorporation of [³H]thymidine into DNA was quantified by autoradiography of labeled nuclei. As shown in Fig. 1 (right), mastoparan caused a marked enhancement of the labeling index (from 10% in cultures treated with insulin alone to 78% in cultures treated with insulin and 17 μ M mastoparan). Thus, mastoparan and insulin synergistically enhance the proportion of cells that enter into DNA synthesis.

Mastoparan also stimulated DNA synthesis in the presence of other growth promoting factors including bombesin (Fig. 2, left), IGF-1, PDGF, PDBu, or EGF (Fig. 2, right). The results shown in Figs. 1 and 2 demonstrate that mastoparan promotes synergistic stimulation of DNA synthesis.

Mastoparan Does Not Stimulate Phosphoinositide Breakdown, Ca²⁺ Mobilization, or Protein Kinase C

The mitogenic neuropeptides bombesin (Rozenfurt and Sinent-Smith, 1983) and vasopressin (Rozenfurt et al., 1979) bind to distinct receptors on 3T3 cells and induce rapid production of inositol phosphates (Heslop et al., 1986; Lopez-Rivas et al., 1987; Nånberg and Rozenfurt, 1988), Ca²⁺ mobilization leading to a decrease in cellular Ca²⁺ content and sustained activation of protein kinase C (Zachary et al.,

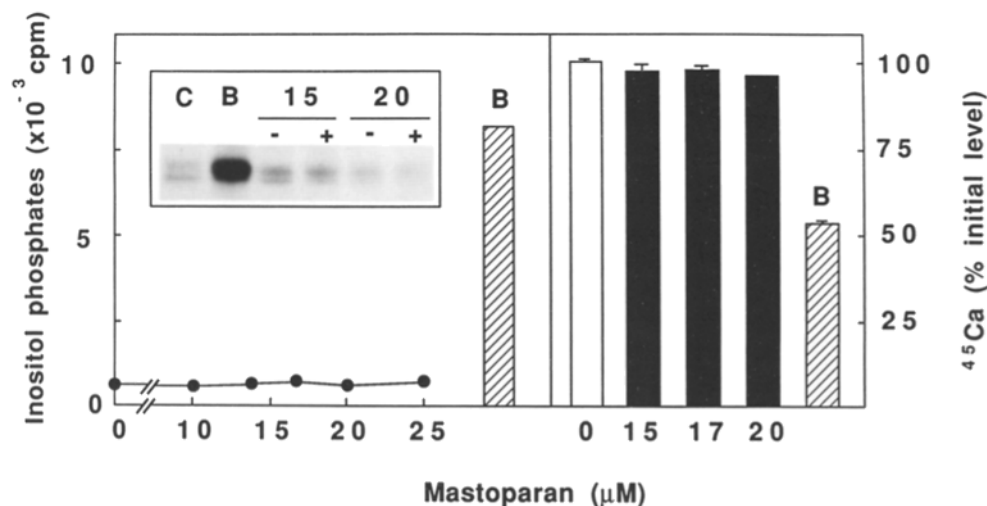


Figure 3. Mastoparan does not stimulate phospholipase C. (*Left*) Mastoparan does not increase the formation of inositol phosphates in Swiss 3T3 cells. Quiescent cells grown on 90-mm dishes were loaded with 1 μ Ci/ml myo- 3 H]inositol and preincubated for 20 min with 20 mM LiCl in DMEM, and then incubated for 10 min with different concentrations of mastoparan or 6 nM bombesin (*B*, *cross-hatched bars*). The acid-soluble inositol phosphates were determined as described in Materials and Methods. The results correspond to one representative experiment. Similar

results were obtained in two other independent experiments. (*Inset*) Mastoparan does not stimulate the phosphorylation of the *M*, 80,000 protein kinase C substrate (80 K). Quiescent cells labeled with 32 P_i were treated with mastoparan (\pm insulin) or 6 nM bombesin (*B*) for 10 min. The incubation was terminated and the samples analyzed by SDS-PAGE as described in Materials and Methods. (*Right*) Mastoparan has no effect on 45 Ca²⁺ efflux from quiescent cultures of 3T3 cells. Quiescent 3T3 cells, preincubated with 45 Ca²⁺ were incubated with different concentrations of mastoparan (*closed bars*) or 6 nM bombesin (*cross-hatched bar*) for 10 min in the presence of the isotope. The radioactive medium was aspirated and the cultures were rapidly washed seven times with 2 ml of 3 mM EGTA-containing DMEM at 37°C. The radioactivity left in the cells was extracted and measured as described in Materials and Methods. The values represent the mean \pm SEM of four to five determinations from two independent experiments.

1986; Rodriguez-Pena and Rozengurt, 1986) via a G protein-linked transduction pathway (Erusalimsky et al., 1988; Erusalimsky and Rozengurt, 1989; Sinnett-Smith et al., 1990; Coffey et al., 1990). Mastoparan is known to stimulate Ins(1,4,5)P₃ synthesis and Ca²⁺ mobilization in certain target cells (Okano et al., 1985; Perianin and Snyderman, 1989; Tohkin et al., 1990), presumably through direct activation of a G protein coupled to phospholipase C. Hence, we determined whether mastoparan-induced mitogenesis was mediated by a similar signaling pathway. Fig. 3 shows that mastoparan, at concentrations that induced mitogenesis, neither increased inositol phosphate production nor caused Ca²⁺ mobilization from intracellular stores, as measured in 45 Ca²⁺-labeled cells. Similar results were obtained when the cells were incubated with 17 μ M mastoparan for 0.5, 1, 2, or 4 h (results not shown).

We next examined whether mastoparan treatment increases the phosphorylation of the 80 K protein, a major substrate

of protein kinase C in 3T3 cells (Rozengurt et al., 1983; Rodriguez-Pena and Rozengurt, 1986a; Erusalimsky et al., 1988). Mastoparan added for 10 min either in the absence or presence of insulin failed to increase 80 K phosphorylation (Fig. 3, *inset*). Similar results were obtained when the cells were incubated with mastoparan at 17 μ M for 30 min (results not shown). Activation of protein kinase C is known to reduce the affinity of the EGF receptor for its ligand (Zachary et al., 1986). Table I shows that mastoparan, at concentrations up to 20 μ M, did not alter 125 I-EGF binding to Swiss 3T3 cells. In contrast, under identical experimental conditions, bombesin stimulated inositol phosphate accumulation, decreased Ca²⁺ content, promoted 80 K phosphorylation, and

Table I. Effect of Various Concentrations of Mastoparan on the Binding of 125 I-EGF

Addition	EGF binding (percent of control)
None	100
Bombesin (6 nM)	19 \pm 2
Mastoparan (15 μ M)	102 \pm 7
Mastoparan (17 μ M)	98 \pm 8
Mastoparan (20 μ M)	94 \pm 14

Quiescent Swiss 3T3 cells were incubated for 1 h at 37°C in binding medium containing bombesin (6 nM) or various concentrations of mastoparan. After this time, the binding of 125 I-EGF to intact cells was measured as described in Materials and Methods. The results are expressed as percentage of the control and correspond to the mean \pm SEM of six independent experiments.

Table II. Protein Kinase C Downregulation Does Not Affect Mastoparan-induced DNA Synthesis

Additions	PDBu pretreatment	DNA synthesis %
Insulin	-	6 \pm 1
	+	8 \pm 1
Insulin + mastoparan	-	55 \pm 9
	+	61 \pm 5
Insulin + PDBu	-	93 \pm 2
	+	12 \pm 1

Quiescent cells were incubated for 40 h in their own conditioned medium, in the absence or presence of PDBu (400 ng/ml). At this time, cells were washed with DMEM and incubated as described in Materials and Methods for 3 H]thymidine incorporation in the presence of 1 μ g/ml insulin and either 17 μ M mastoparan or 100 ng/ml PDBu. Results are expressed as a percentage of the incorporation induced by 10% fetal bovine serum and correspond to the means \pm SEM of three independent experiments with incubations in duplicate.

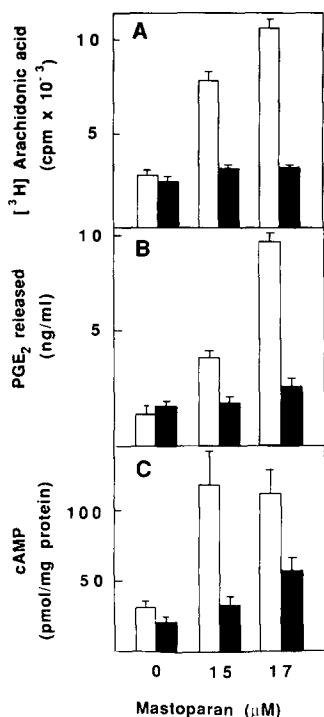


Figure 4. Effect of mastoparan on arachidonic acid release, PGE₂ production, and cAMP accumulation in control and pertussis toxin-treated cells. (A) Arachidonic acid release. Quiescent cells were labeled for 16 h with 1 μCi/ml [³H]arachidonic acid preincubated in DMEM/Waymouth medium either in the absence (*open bars*) or presence (*closed bars*) of 100 ng/ml pertussis toxin for 3 h. At this time the cells were incubated with fresh medium containing mastoparan at the indicated concentrations for 30 min. After this time the medium was removed and centrifuged for 5 min at 2,000 g to remove contaminating cellular debris and the supernatant was counted directly to monitor arachidonic acid release. (B) PGE₂ release. Quiescent cells were preincubated in DMEM/Waymouth medium either in the absence (*open bars*) or

presence of 100 ng/ml pertussis toxin (*closed bars*) for 3 h. At this time the cells were incubated with fresh medium containing mastoparan at the indicated concentrations for 1 h. After this time the medium was removed and stored at 4°C and PGE₂ was measured as described in Materials and Methods. (C) cAMP accumulation. Cultures were preincubated in DMEM/Waymouth medium containing either 100 ng/ml pertussis toxin (*closed bars*) or no addition (*open bars*) for 3 h. At this time the cells were incubated with fresh medium containing various concentrations of mastoparan in the presence of 25 μM forskolin. After 1 h the cellular content of cAMP was measured. The values correspond to the mean ± SEM of three determinations from one representative experiment.

inhibited ¹²⁵I-EGF binding in parallel cultures (Fig. 3; Table I). Furthermore, down regulation of protein kinase C by prolonged exposure to PDBu (Rodriguez-Pena and Rozengurt, 1984) did not prevent the mitogenic effect of mastoparan in the presence of insulin (Table II). Collectively, these results indicate that the mitogenic activity of mastoparan can be dissociated from activation of phospholipase C.

Table III. Effect of Mastoparan on cAMP Accumulation in the Presence of IBMX

Addition	cAMP (pmol/mg protein)
None	3.6 ± 0.3
Mastoparan	4.3 ± 0.1
IBMX	5.1 ± 0.6
Mastoparan + IBMX	12.7 ± 1.1

Quiescent Swiss 3T3 cells were incubated for 1 h at 37°C in DMEM containing mastoparan (17 μM), 50 μM IBMX, or both. After this time the cellular content of cAMP was measured as described in Materials and Methods. The cAMP content of cultures incubated for 4 h (instead of 1 h) without or with mastoparan was 4.9 ± 0.3 and 5.4 ± 0.5, respectively. The results correspond to the mean ± SEM of three determinations.

Mastoparan Stimulates Arachidonic Acid Release

It is known that mastoparan stimulates PLA₂ activity from a variety of sources presumably by rendering the substrate more susceptible to PLA₂ action (Argiolas and Pisano, 1983). We therefore examined the effect of mastoparan on arachidonic acid release in 3T3 cells.

Fig. 4 A shows that mastoparan induces a dose-dependent release of radioactivity into the medium from quiescent cultures prelabeled with [³H]arachidonic acid. Since arachidonic acid is a major precursor for the synthesis of E-type prostaglandins, we examined the effect of mastoparan on the production of PGE₂. As shown in Fig. 4 B, mastoparan caused a marked and dose-dependent release of PGE₂ from quiescent cultures of Swiss 3T3 cells.

In previous studies, bombesin (Millar and Rozengurt, 1988) and PDGF (Rozengurt et al., 1983b) have been shown to enhance cAMP accumulation via a cyclooxygenase-dependent pathway. Although mastoparan did not increase the basal level of cellular cAMP, mastoparan enhanced cAMP accumulation in the presence of either the phosphodiesterase inhibitor IBMX (Table III) or the diterpene forskolin, a direct activator of adenylate cyclase (Fig. 4 C). The cyclooxygenase inhibitor indomethacin blocked mastoparan-stimulated production of PGE₂ (Table IV) and inhibited the enhancement of cAMP accumulation by the peptide in the presence of forskolin (results not shown).

Pertussis toxin is known to ADP-ribosylate G proteins thereby blocking their function in receptor-mediated signal transduction (Ui, 1990). A salient feature shown in Fig. 4 is that arachidonic acid release, PGE₂ output, and enhancement of cAMP accumulation induced by mastoparan were inhibited markedly by prior treatment (3 h) with 100 ng/ml pertussis toxin. These findings strongly suggest that mastoparan increases arachidonic acid release via a pertussis toxin-sensitive G protein.

Arachidonic Acid Release as a Mitogenic Signal in Mastoparan Action

Stimulation of a proliferative response in 3T3 cells requires the synergistic interaction of multiple distinct signaling pathways (Rozengurt, 1986). The preceding results raise the possibility that stimulation of arachidonic acid release by mastoparan may constitute a synergistic mitogenic signal. This possibility predicts that: (a) externally applied arachidonic

Table IV. Effect of Indomethacin on Mastoparan-induced PGE₂ Release

Addition	Indomethacin (1 μM)	PGE ₂ ng/ml
None	–	1.6 ± 0.3
Mastoparan	–	22.0 ± 2.0
None	+	1.3 ± 0.1
Mastoparan	+	2.6 ± 0.2

Quiescent Swiss 3T3 cells were incubated for 1 h at 37°C in DMEM or DMEM plus 20 μM mastoparan in the absence (–) or presence (+) of 1 μM indomethacin. After this time, PGE₂ release was measured as described in Materials and Methods. The values correspond to the mean ± SEM of three determinations.

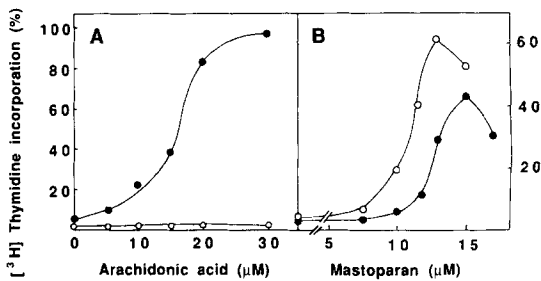


Figure 5. (A) Dose response for the stimulation of DNA synthesis by arachidonic acid. Confluent and quiescent cultures of these cells were exposed to 2 ml of DMEM/Waymouth medium containing 0.1% fatty acid-free BSA, 1 μ Ci/ml [³H]thymidine, and various concentrations of arachidonic acid, either in the absence (*open circles*) or in the presence of 1 μ g/ml insulin (*closed circles*). (B) Effect of pertussis toxin pretreatment on mastoparan stimulation of DNA synthesis. Cultures were preincubated in DMEM/Waymouth medium either in the absence (*open circles*) or in the presence of 100 ng/ml pertussis toxin (*closed circles*) for 3 h. At this time medium was removed and the incubation continued in DMEM/Waymouth medium containing 1 μ Ci/ml [³H]thymidine, 1 μ g/ml insulin, and different concentrations of mastoparan either in the absence (*open circles*) or in the presence (*closed circles*) of 100 ng/ml pertussis toxin. After 40 h DNA synthesis was assessed by measuring the incorporation of [³H]thymidine into precipitable material as described in Materials and Methods. The results represent the mean of two different dishes of one representative experiment expressed as a percentage of the incorporation induced by 10% FBS.

acid, like mastoparan, will act synergistically with insulin to induce mitogenesis in 3T3 cells; (b) pertussis toxin treatment should reduce the mitogenic response induced by mastoparan; and (c) agents that induce cAMP accumulation in the presence of mastoparan, such as IBMX or forskolin, should potentiate DNA synthesis induced by mastoparan and insulin. The experiments presented in Figs. 5 and 6 were designed to test the predictions.

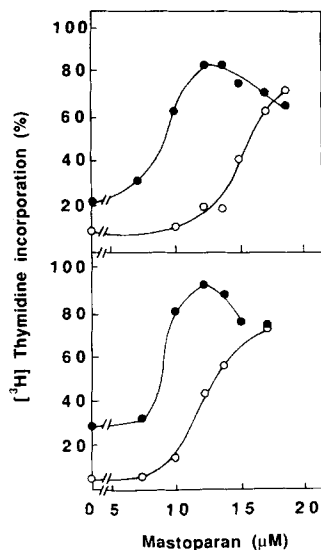


Figure 6. Effect of forskolin or IBMX on mastoparan stimulation of DNA synthesis in Swiss 3T3 cells. Confluent and quiescent cultures of these cells were exposed to 2 ml of DMEM/Waymouth medium containing 1 μ Ci/ml [³H]thymidine, 1 μ g/ml insulin, and various concentrations of mastoparan either in the absence (*open circles*) or presence of forskolin at 5 μ M (*closed circles, top*) or IBMX at 50 μ M (*closed circles, bottom*). After 40 h DNA synthesis was assessed by measuring the level of [³H]thymidine incorporated into acid precipitable material. Each point is the mean of two determinations from one representative experiment expressed as a percentage of the incorporation induced by 10% FBS.

Cultures of Swiss 3T3 cells were exposed to various concentrations of arachidonic acid in the absence or presence of insulin and assayed for [³H]thymidine incorporation as an indicator of DNA synthesis. Arachidonic acid potently synergized with insulin to stimulate DNA synthesis (Fig. 5 A). As reported recently (Millar and Rozengurt, 1990), arachidonic acid did not induce DNA synthesis in the absence of other factors. Furthermore, the ability of mastoparan to stimulate [³H]thymidine incorporation in the presence of insulin was markedly reduced by pertussis toxin treatment, especially at lower concentrations of peptide (Fig. 5 B).

Mastoparan stimulates PGE₂ release and, in the presence of forskolin or a phosphodiesterase inhibitor, elevates cAMP levels which is known as a mitogenic signal for Swiss 3T3 cells (Rozengurt et al., 1981, 1983; Rozengurt, 1986). Fig. 6 shows that addition of either forskolin or IBMX strikingly potentiated the induction of DNA synthesis by various concentrations of mastoparan in the presence of insulin. These potentiating effects were markedly reduced by prior treatment with pertussis toxin (results not shown).

Discussion

The present results demonstrate that mastoparan is a novel mitogen for Swiss 3T3 cells. The peptide, at micromolar concentrations, stimulates reinitiation of DNA synthesis only in synergistic combinations, particularly with insulin and IGF-1. Furthermore, mastoparan potentiates the mitogenic response elicited by bombesin or PDGF. Since its isolation from wasp venom as a mast cell degranulating factor (Hirai et al., 1979), mastoparan has been shown to induce short-term exocytosis in numerous secretory cell types (Hirai et al., 1979; Kuroda et al., 1980; Camoratto and Grandison, 1985; Kurihara et al., 1986; Yokokawa et al., 1989). To our knowledge, this is the first time that mastoparan has been shown to stimulate reinitiation of DNA synthesis in any target cell.

The mechanism(s) of action of mastoparan is attracting interest because this peptide may mimic the receptor domain(s) that directly activates G proteins either in reconstituted phospholipid vesicles or in intact cells (Higashijima et al., 1988, 1990; Mousli et al., 1990; Weingarten et al., 1990). It is known that mastoparan regulates the activity of purified G proteins (e.g., G₀ and G_i) over a narrow range of concentration (Higashijima et al., 1990). In the present study we showed that mastoparan stimulation of DNA synthesis in 3T3 cells was steeply dose-dependent. The cooperativity of these dose responses suggests that several molecules of mastoparan bound to phospholipid bilayers may be required to activate G protein activity either in vitro or in intact cells.

In certain cell types, mastoparan stimulates polyphosphoinositide-specific phospholipase C (Okano et al., 1985; Perianin and Snyderman, 1989; Tohkin et al., 1990), leading to Ca²⁺ mobilization and activation of protein kinase C, a pathway known to be regulated by a G protein and involved in mitogenic signaling by neuropeptides and growth factors (Rozengurt, 1986). The results presented here demonstrate that this is not the case in Swiss 3T3 cells as judged by measurements of inositol phosphate accumulation, Ca²⁺ mobilization, and protein kinase C-mediated phosphorylation of the 80 K substrate and EGF receptor transmodulation. Fur-

thermore, downregulation of protein kinase C that blocks mitogenic signaling via this pathway (Rodriguez-Pena and Rozengurt, 1984; Rozengurt et al., 1984) did not prevent the mitogenic response initiated by mastoparan. From these results we conclude that mastoparan stimulation of DNA synthesis can be dissociated from phospholipase C activation and consequently, that the peptide stimulates a different signaling pathway.

In the present study we demonstrate that mastoparan induces a striking release of arachidonic acid and its cyclooxygenase metabolite PGE₂ into the medium. Recent evidence has indicated that the liberation of arachidonic acid and its conversion to eicosanoids constitutes one of the early mitogenic signals induced by bombesin in 3T3 cells (Millar and Rozengurt, 1990). It is also noteworthy that the PDGF AA and PDGF BB homodimers that, like bombesin, stimulate reinitiation of DNA synthesis in the absence of other growth promoting factors induce a large and sustained release of arachidonic acid in Swiss 3T3 cells (Mehmet et al., 1990). The results presented here with mastoparan also support the conclusion that arachidonic acid release contributes to mitogenic signal transduction. However, since the inhibition of arachidonic acid release by pertussis toxin treatment was more pronounced than the inhibition of [³H]thymidine incorporation, we cannot exclude the possibility that mastoparan induces cellular DNA synthesis through additional signaling pathways.

Arachidonic acid may be liberated as a result of either direct action of PLA₂ or by indirect routes involving the release of arachidonic acid from diacylglycerol by diglyceride lipase. Arachidonic acid-containing diacylglycerol is one of the most potent physiological activators of protein kinase C (Nishizuka, 1986). Here we showed that mastoparan stimulates arachidonic acid release without causing concomitant inositol phosphate accumulation or activation of protein kinase C. In contrast, vasopressin which stimulates phospholipase C-mediated phosphoinositide breakdown, diacylglycerol formation, and activates protein kinase C does not cause sustained stimulation of arachidonic acid release from Swiss 3T3 cells (Millar and Rozengurt, 1990). Hence, arachidonic acid release can be separated from diacylglycerol formation in these cells. We conclude that mastoparan stimulates arachidonic acid release from 3T3 cells through activation of PLA₂.

Previous studies demonstrated that mastoparan stimulates the activity of purified PLA₂ reconstituted into phospholipid vesicles, presumably interacting with the phospholipids and rendering them more susceptible to PLA₂ action (Argiolas and Pisano, 1983). Our results using pertussis toxin that catalyses ADP-ribosylation of the α subunits of G proteins (Ui, 1990) suggest that in 3T3 cells mastoparan stimulates PLA₂ activity via an entirely different mechanism. Treatment with pertussis toxin of 3T3 cells markedly attenuated the ability of mastoparan to stimulate arachidonic acid release, PGE₂ production, and enhancement of cAMP accumulation. These findings indicate that mastoparan stimulates arachidonic acid release via a pertussis toxin-sensitive G protein, rather than by interaction with cellular phospholipids. In contrast, pertussis toxin does not interfere with phospholipase C activation by either bombesin (Zachary et al., 1987) or vasopressin (Erusalimsky and Rozengurt, 1989) in Swiss 3T3 cells. Recently, the possibility has been raised

that PLA₂ is directly regulated by a pertussis toxin-sensitive G protein and that separate G proteins transduce receptor-mediated activation of PLA₂ and phospholipase C (Axelrod, 1990). In this context, the results presented here suggest that mastoparan selectively stimulates a pertussis toxin-sensitive G protein that regulates PLA₂ activity in intact 3T3 cells. In conclusion, mastoparan should be a useful probe to elucidate G-protein coupled signal transduction pathways in the mitogenic response.

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