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

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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, Ca²⁺ 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 E₂ 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.

Elucidation 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, neuropetides 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 Ca²⁺ 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., 1989; 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, G₁, and G₂ (Gilman, 1987). In addition, mastoparan has been previously shown to interact with phospholipids (Higashijima et al.};
1983) and to directly stimulate phospholipase A$_2$ (PLA$_2$) 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, Ca$^{2+}$ mobilization, and arachidonic acid release, have also been implicated in triggering cell proliferation by mitogenic neuropeptides and polypeptide growth factors in quiescent cells (Rozengurt, 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 (Rozengurt and Sinnett-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% CO$_2$, 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 Rozengurt, 1980). Briefly, cultures were washed twice with DMEM and incubated in DMEM/Waymouth’s medium (1:1 [vol/vol]) containing [3H]thymidine (1 mM/ml; 1 PM) and various additions as described in the experiments. After 40 h, the cultures were washed twice with PBS and incubated in 5% trichloracetic acid for 30 min at 4°C. Trichloracetic acid was then removed and the cultures washed twice with ethanol and extracted in 1 ml of 2% Na$_2$CO$_3$, 0.1M NaOH, 1% SDS. Incorporation was determined by scintillation counting in 10 ml of picofluor.

**Autoradiography of Labeled Nuclei**

Labeled nuclei were determined by autoradiography as described previously (Dicker and Rozengurt, 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 PM/ml) and the indicated factors. After 40 h the medium was removed and the cells 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% trichloracetic 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 PM of t-[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 K$_2$CO$_3$, containing 10 mM EDTA and 100 mM Hepes. Samples were analyzed for their content of total inositol phosphates by anion exchange chromatography using Bio-Rad AG 1X8 resin (Bio-Rad Laboratories, Richmond, CA) and elution with 1 M ammonium formate/0.1 M formic acid as performed previously described (Berridge et al., 1983; Nöhrberg and Rozengurt, 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 PM of $^{32}$P$_1$. 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 mL of a solution containing 100 mM Tris-HCl, pH 6.9, 0.5% Triton X-100, 2 mM EGTA, 10 mM sodium fluoride, 50 PM PMSF, 3.5 g/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).

**Ca$^{2+}$ Efflux**

Quiescent cultures of Swiss 3T3 cells were equilibrated with $^{45}$Ca$^{2+}$ by incubating them for 14-16 h in conditioned medium containing 5 PM of $^{45}$Ca$^{2+}$. 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 $^{45}$Ca$^{2+}$ was extracted with 0.1 M NaOH, 2% Na$_2$CO$_3$, 1% SDS, and determined by scintillation counting.

**121I-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, the cells were then incubated at 4°C in PBS (pH 7.2) containing 25 mM Hepes and 0.5 mg/ml (110,000 cpm/mg) $^{125}$I-EGF. After 2 h, cells were washed twice with PBS, extracted with 0.1 M NaOH containing 2% Na$_2$CO$_3$ 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 35-mm dishes were labeled for 14-16 h with 1 PM of [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 2000 g to remove contaminating cellular debris and the level of radioactivity measured by scintillation counting.

**Measurement of Prostaglandin E$_2$ (PGE$_2$) 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 siliconised glassware. Measurements of prostaglandin E$_2$ (PGE$_2$) were performed by radioimmunoassay using a $^{125}$I-PGE$_2$ assay system. Aliquots of samples were diluted in assay buffer containing 0.9% NaCl, 0.01 M EDTA, 0.3% bovine $\gamma$-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-PGE$_2$ antibody using $^{125}$I-PGE$_2$ 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 times with DMEM and incubated at 37°C for 4 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 siliconised glassware. Measurements of cAMP were performed by radioimmunoassay using a $^{125}$I-cAMP assay system. Aliquots of samples were diluted in assay buffer containing 0.9% NaCl, 0.01 M EDTA, 0.3% bovine $\gamma$-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-cAMP antibody using $^{125}$I-cAMP 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.
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 µl 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.

Results

Mastoparan and pertussis toxin were obtained from Peninsula Laborato-
ries, 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-41 recombinant PDGF, recombinant insulin-like growth factor 1 (IGF-I), 
125I-EGF, [3H]thymidine, [3H]inositol, [3H]arachidonic acid, and cAMP radioimmunoas-
say kit were purchased from Amersham International (Amersham, UK). 
125I-EGF, radioimmunoassay system was from New England Nuclear (Boston, MA). Bicinchoninic acid reagent was from Pierce Chemical Co. (Rock-
ford, IL). Forskolin was from Calbiochem-Behring Corp. (San Diego, CA). Dowex AGI-X8 (formate form) was obtained from Bio-Rad Laboratories, Richmond, CA). All other materials were of the purest grade commercially available.

Materials

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Mastoparan Induced DNA Synthesis in Synergy with Other Mitogens

Initial experiments revealed that addition of 10–20 µM mastoparan to quiescent cultures of Swiss 3T3 cells induced marked morphological changes and cytotoxic effects at the higher concentrations (25–30 µM). 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. Cumu-

![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 µCi/ml [3H]thymidine and various concentrations of mastoparan, in the absence (open circles) or in the presence (closed circles) of insulin at 1 µg/ml. After 40 h DNA synthesis was assessed by measuring the [3H]thymidine incorporated into acid precipitable material. Each point is the mean ± SEM of eight determinations from four independent experiments expressed as a percentage of the incorporation induced by 10% FBS (4.5–6 × 10^6 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 µCi/ml [3H]thymidine and various concentrations of mastoparan in the presence of insulin at 1 µg/ml. 10% fetal bovine serum produced 95% labeled nuclei. The values are the mean ± SEM of 10 different microscopic fields from 2 different dishes.

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 µM 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-I, 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 (Rozengurt and Sinnert-Smith, 1983) and vasopressin (Rozengurt 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; Nørh and Rozengurt, 1988). Ca2+ mobilization leading to a decrease in cellular Ca2+ content and sustained activation of protein kinase C (Zachary et al.,
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 μCi/ml myo-[3H]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) Mastopaxan does not stimulate the phosphorylation of the Mr 80,000 protein kinase C substrate (80 K). Quiescent cells labeled with 32Pi were treated with mastopaxan (+ 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 45Ca2+ efflux from quiescent cultures of 3T3 cells. Quiescent 3T3 cells, preincubated with 45Ca2+ 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 ± 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; SinneR-Smith et al., 1990; Coffer 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 45Ca2+-labeled cells. Similar results were obtained when the cells were incubated with 17 μM 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...
presence of 100 ng/ml pertussis toxin. Medium was removed and stored at 4°C and PGF₂ was measured at the indicated concentrations for 1 h. After 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) PGF₂ release. Quiescent cells were preincubated in DMEM/Waymouth 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 3 h. At this time the medium was removed and counted directly to monitor arachidonic acid release. (C) cAMP accumulation. Quiescent cells were preincubated in DMEM/Waymouth either in the absence (open bars) or presence (closed bars) of mastoparan 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.

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. 4A 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 PGF₂. As shown in Fig. 4B, mastoparan caused a marked and dose-dependent release of PGF₂ 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. 4C). The cyclooxygenase inhibitor indomethacin blocked mastoparan-stimulated production of PGF₂ (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 (Uli, 1990). A salient feature shown in Fig. 4 is that arachidonic acid release, PGF₂ 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 acid may be a mitogenic signal in 3T3 cells.
acid, like mastoparan, will act synergistically with insulin to
lin. The experiments presented in Figs. 5 and 6 were de-
induce mitogenesis in 3T3 ceils; (b) pertussis toxin treatment
potentiate DNA synthesis induced by mastoparan and insu-
Effect of pertussis toxin pretreatment on mastoparan stimulation of
DNA synthesis. Cultures were preincubated in DMEM/Way-
mouth 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 medi-
un and the incubation continued in DMEM/Way-
mouth medium containing 1 μCi/ml [3H]thymidine, 1 μg/ml insu-
lin, and different concentrations of mastoparan either in the ab-
ence (open circles) or in the presence (closed circles) of 100 ng/ml
pertussis toxin. After 40 h DNA synthesis was assessed by measur-
ing the incorporation of [3H]thymidine into precipitable material
described in Materials and Methods. The results represent the
mean of two different dishes of one representative experiment ex-
pressed as a percentage of the incorporation induced by 10% FBS.

Cultures of Swiss 3T3 cells were exposed to various concen-
trations 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 sy-
nergized with insulin to stimulate DNA synthesis (Fig. 5 A).
As reported recently (Millar and Rozengurt, 1990), arachi-
donic 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 PGF; 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 con-
centrations of mastoparan in the presence of insulin. These
potentiating effects were markedly reduced by prior treat-
ment 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 (Hi-
rai et al., 1979; Kuroda et al., 1980; Camoratto and Grandi-
on, 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 in-
terest because this peptide may mimic the receptor do-
main(s) that directly activates G proteins either in reconsti-
tuted phospholipid vesicles or in intact cells (Higashijima
et al., 1988, 1990; Moussli et al., 1990; Weingarten et al.,
1990). It is known that mastoparan regulates the activity of
purified G proteins (e.g., Gs and Gt) over a narrow range
of concentration (Higashijima et al., 1990). In the present
study we showed that mastoparan stimulation of DNA syn-
thesis in 3T3 cells was steeply dose-dependent. The coopera-
tivity of these dose responses suggests that several molecules
of mastoparan bound to phospholipid bilayers may be re-
quired to activate G protein activity either in vitro or in intact
cells.

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