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-I, 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, 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 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., 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, Gₐ, and Gₛ (Gilman, 1987). In addition, mastoparan has been previously shown to interact with phospholipids (Higashijima et al.
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 AG1X8 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; Näsberg 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 μCi $^{32}$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 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).

**$^{35}$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 μCi/ml $^{45}$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.

**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, the cells were then incubated at 4°C in PBS (pH 7.2) containing 25 mM Hepes and 0.5 mg/ml (31,000 cpm/ng) $^{125}$I-EGF. After 2 h, cells were washed 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 33-mm dishes were labeled for 14–16 h with 1 μCi/ml $^{3}$H]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 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 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-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, 0.035% 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 2% Na$_2$CO$_3$, 0.1 M NaOH, and 1% SDS and determined by scintillation counting.
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.

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-eis recombinant PDGF, recombinant insulin-like growth factor 1 (IGF-I), 125I-EGF, [3H]thymidine, [3H]inositol, [32p]Pi (carrier free), n,n-my o[3H]inositol, 4CaCl2, [5, 6, 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 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. Cumulative [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 μM and the maximum effect was achieved at 17 μM. 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 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 Sinet-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; Nântor 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) Mastoparan does not stimulate the phosphorylation of the Mr 80,000 protein kinase C substrate (80 K). Quiescent cells labeled with 32P were treated with mastoparan (+ 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 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 μM 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 μM, did not alter 125I-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 125I-EGF

<table>
<thead>
<tr>
<th>Addition</th>
<th>EGF binding (percent of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Bombesin (6 nM)</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>Mastoparan (15 μM)</td>
<td>102 ± 7</td>
</tr>
<tr>
<td>Mastoparan (17 μM)</td>
<td>98 ± 8</td>
</tr>
<tr>
<td>Mastoparan (20 μM)</td>
<td>94 ± 14</td>
</tr>
</tbody>
</table>

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 125I-EGF to intact cells was measured as described in Materials and Methods. The results are expressed as a percentage of the control and correspond to the mean ± SEM of six independent experiments.

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

<table>
<thead>
<tr>
<th>Additions</th>
<th>PDBu pretreatment</th>
<th>DNA synthesis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>-</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>+</td>
<td>8 ± 1</td>
<td></td>
</tr>
<tr>
<td>Insulin + mastoparan</td>
<td>-</td>
<td>55 ± 9</td>
</tr>
<tr>
<td>+</td>
<td>61 ± 5</td>
<td></td>
</tr>
<tr>
<td>Insulin + PDBu</td>
<td>-</td>
<td>93 ± 2</td>
</tr>
<tr>
<td>+</td>
<td>12 ± 1</td>
<td></td>
</tr>
</tbody>
</table>

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 [3H]thymidine incorporation in the presence of 1 μg/ml insulin and either 17 μM 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 ± 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 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 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.

Arachidonic Acid Release as a Mitogenic Signal in Mastoparan Action

Stimulation of a proliferative response in 3T3 cells requires 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

<table>
<thead>
<tr>
<th>Addition</th>
<th>cAMP (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td>Mastoparan</td>
<td>4.3 ± 0.1</td>
</tr>
<tr>
<td>IBMX</td>
<td>5.1 ± 0.6</td>
</tr>
<tr>
<td>Mastoparan + IBMX</td>
<td>12.7 ± 1.1</td>
</tr>
</tbody>
</table>

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.

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

<table>
<thead>
<tr>
<th>Addition</th>
<th>Indomethacin (1 μM)</th>
<th>PGE2 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>Mastoparan</td>
<td>-</td>
<td>22.0 ± 2.0</td>
</tr>
<tr>
<td>None</td>
<td>+</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Mastoparan</td>
<td>+</td>
<td>2.6 ± 0.2</td>
</tr>
</tbody>
</table>

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.

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, bombesins (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 (Uij, 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...
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 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 µg/ml [3H]thymidine, and various concentrations of arachidonic acid, either in the absence (open circles) or in the presence of 10 ng/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 µg/ml [3H]thymidine, 1 µg/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 acid 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.

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 (Miller 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 PGF2α 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., G3 and Gq) 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 polyphosphoinositol-specific phospholipase C (Okano et al., 1985; Peri-anin and Snyderman, 1989; Tokhin 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 neuroepetides 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. Further...
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 (Mehet 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 [H³]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 (U1, 1990) suggest that in 3T3 cells mastoparan stimulates PLA₂ activity via an entirely different mechanism. Treatment with pertussis toxin of 3T3 cells markedly attenuates 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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References


