Mastoparan promotes exocytosis and increases intracellular cyclic AMP in human platelets

Evidence for the existence of a G_c-like mechanism of secretion

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Recent studies have shown that mastoparan, an amphiphilic peptide derived from wasp venom, accelerates guanine nucleotide exchange and GTPase activity of purified GTP-binding proteins. In the present study we have examined the functional consequences of exposure of intact human platelets to mastoparan. Mastoparan promoted rapid (≤ 1 min) dose-dependent increases in 5-hydroxy[¹⁴C]tryptamine and β -thromboglobulin release from dense-granule and α -granule populations respectively. The exocytotic response did not result from a lytic effect of mastoparan and occurred in the complete absence of platelet shape change and aggregation. Liberation of [³H]arachidonate and increases in cytosolic [Ca^{2+}] (detected with fura 2) were not observed in platelets stimulated with mastoparan. Similarly, in platelets preloaded with [³H]inositol during reversible electroporation, mastoparan did not cause the accumulation of [³H]inositol phosphates. Mastoparan-induced secretion was unaffected by preincubation with either the protein kinase C inhibitor staurosporine (10 nM–10 μ M) or prostacyclin (PGI_2 ; 100 ng/ml) and was not accompanied by phosphorylation of the 45 kDa protein kinase C substrate or the 20 kDa protein normally associated with platelet activation. The G-protein inhibitor guanosine 5'-[β -thio]diphosphate (GDP[S]; 1 mM) attenuated the secretion induced by mastoparan in both intact and saponin-permeabilized platelets. Encapsulation of GDP[S] during reversible permeabilization inhibited mastoparan-induced secretion, providing evidence for an intracellular action of GDP[S]. In all these studies thrombin (0.05–0.2 unit/ml) elicited characteristic responses, and thrombin-induced secretion was inhibited by staurosporine, PGI_2 and GDP[S]. Mastoparan also increased intra-platelet cyclic AMP in a dose-dependent manner. Mastoparan and PGI_2 increased ³²P incorporation into a protein of approx. 24 kDa, whereas phosphorylation of a 50 kDa substrate was only seen in PGI_2 -stimulated platelets. These results indicate that mastoparan promotes secretion by a mechanism which does not involve stimulation of phospholipase C and suggest that the secretory event may result either from a direct fusogenic action of mastoparan and/or from stimulation of the putative exocytosis-linked G-protein, G_c.

INTRODUCTION

The amphiphilic tetradecapeptide mastoparan (Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH₂) is a component of wasp venom which has been reported to exhibit diverse pharmacological effects on several biochemical processes in a variety of cell types. These include the activation *in vitro* of phospholipase A₂ [1] and the ability to antagonize calmodulin activity [2]. Current interest in this compound has arisen from its recently described effects on purified GTP-binding proteins (G-proteins), where mastoparan has been shown to accelerate both nucleotide exchange and the subsequent GTP hydrolysis [3,4]. It has therefore been proposed that the cellular effects of mastoparan, and other amphiphilic peptides [5], are due to an ability to mimic G-protein-linked agonist-liganded receptors [3].

There have been several reports of the effects of mastoparan in intact and permeabilized cells, and the peptide has been shown to promote exocytosis of histamine from mast cells [6], 5-hydroxytryptamine (5-HT) from platelets [7,8], prolactin from anterior pituitary cells [9], insulin from pancreatic islets [10] and catecholamines from adrenal chromaffin cells [11]. However, there are conflicting reports concerning the nature of the biochemical mechanisms associated with these secretory responses. For example, it has been clearly demonstrated that mastoparan-induced secretion of histamine from peritoneal mast cells occurs

via a pertussis-toxin-sensitive G-protein-dependent activation of phospholipase C (PLC) [5,6], whereas catecholamine release from mastoparan-stimulated chromaffin cells occurs as a consequence of cell lysis [12]. In addition, mastoparan can attenuate receptor-mediated PLC activation, apparently both by affecting a pertussis-toxin-insensitive G-protein linked to phosphoinositide-specific PLC [13] and by a direct interaction with membrane-associated polyphosphoinositides [14]. The evidence drawn from studies at the cellular level therefore suggests that mastoparan directly or indirectly modulates PLC activity.

There is currently substantial evidence from platelet studies to support G-protein involvement in the receptor-mediated stimulation of phosphatidylinositol 4,5-bisphosphate hydrolysis by PLC (reviewed in [15,16]). In the present study, we have investigated the functional consequences of exposing human platelets to mastoparan in an attempt to define its mode of action, and to examine its usefulness as a specific probe of G-protein-dependent events involved in the platelet activation cascade. We show that mastoparan induces secretion of platelet granule constituents, but that this secretory event is not linked to mobilization of intracellular Ca^{2+} or to phosphorylation of the 45 and 20 kDa proteins normally associated with platelet activation. In addition, mastoparan increases intra-platelet cyclic AMP and results in the parallel phosphorylation of a protein with an apparent molecular mass of approx. 24 kDa. To our

Abbreviations used: 5-HT, 5-hydroxytryptamine; PLC, phospholipase C; BTG, β -thromboglobulin; PGI_2 , prostacyclin (prostaglandin I₂); [cAMP]_i, intracellular cyclic AMP concn., GDP[S], guanosine 5'-[β -thio]diphosphate.

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knowledge, mastoparan represents the first agent that increases cyclic AMP and simultaneously causes granule secretion in human platelets.

MATERIALS AND METHODS

Materials

Mastoparan (INLKALAALAKKIL) was synthesized and purified by reverse-phase h.p.l.c. The purified peptide was dissolved in 0.9% NaCl and appropriately diluted from a stock solution of 2 mg/ml. *myo*-[³H]inositol (108 Ci/mmol), 5-hydroxy[¹⁴C]tryptamine ([¹⁴C]5-HT; 57 Ci/mmol), [³H]adenine (20 Ci/mmol), [³H]arachidonic acid (100 Ci/mmol) and the β -thromboglobulin (BTG) radioimmunoassay kit were obtained from Amersham International, Amersham, Bucks., U.K. [³²P]_i (1000 mCi/mmol) was purchased from ICN Biochemicals, High Wycombe, Bucks., U.K. Fura 2-AM and bovine thrombin were obtained respectively from Molecular Probes, Eugene, OR, U.S.A., and Parke-Davis, Detroit, MI, U.S.A. Chemicals for gel electrophoresis were from Bio-Rad, Hemel Hempstead, Herts., U.K., all other chemicals were of reagent grade and purchased from either Sigma Chemical Co. or BDH, both of Poole, Dorset, U.K.

Preparation of labelled washed platelet suspensions

Human blood was drawn from healthy volunteers, who had denied taking medication for at least 10 days, into 3.2% (w/v) trisodium citrate (1 vol.) by forearm venepuncture and was centrifuged at 800 *g* for 15 min at room temperature to yield platelet-rich plasma as described previously [17]. In some experiments the platelet-rich plasma was preincubated with [¹⁴C]5-HT (0.1 μ Ci/ml) for 20 min at 37 °C (see below). In all experiments, platelet-rich plasma was subsequently acidified to pH 6.5 with 0.1 M-citric acid, centrifuged at 1500 *g* for 10 min, and the pellet was resuspended in a pH 6.5 buffer consisting of 36 mM-citric acid, 103 mM-NaCl, 5 mM-KCl, 60 mM-prostacyclin (PGI₂) and 5 mM-D-glucose. In some experiments, resuspended platelets (3 $\times$ 10⁸ cells/ml) were dual-labelled with [¹⁴C]5-HT (0.1 μ Ci/ml; 30 min, 37 °C) and [³H]arachidonate (1 μ Ci/ml; 90 min) to allow simultaneous quantification of dense-granule secretion and arachidonate release.

For the measurement of protein phosphorylation, platelets (1 $\times$ 10⁹ cells/ml) were loaded with ³²P by incubation with 0.25 mCi of carrier-free [³²P]_i/ml for 90 min at 37 °C and washed twice by centrifugation (1500 *g*, 10 min). No prelabelling procedure was required for experiments involving measurement of either BTG release or intracellular cyclic AMP concentrations ([cAMP]).

Platelets were finally resuspended in a Hepes-Tyrode buffer (pH 7.4) composed of 10 mM-Hepes, 145 mM-NaCl, 5 mM-KCl, 1 mM-MgSO₄, 1 mM-CaCl₂ and 5 mM-D-glucose. All platelet suspensions were treated at this stage with indomethacin (10 μ M) to avoid the effects of endogenously generated prostaglandin endoperoxides/thromboxane A₂; platelets prelabelled with [³H]arachidonate were additionally treated with the combined lipoxygenase/cyclo-oxygenase inhibitor phenidone (250 μ M final concn.).

Reversible electroporation: labelling of platelet phosphoinositides with [³H]inositol and entrapment of guanosine 5'-[β -thio]diphosphate (GDP[S])

This procedure followed that described previously in detail by Authi *et al.* [18]. Briefly, platelet-rich plasma was incubated with [¹⁴C]5-HT (0.1 μ Ci/ml for 20 min at 37 °C) and subsequently acidified to pH 6.5 as described above. After centrifugation (1500 *g*, 10 min) the resulting platelet pellet was resuspended in

a glutamate-based permeabilizing buffer containing 15–20 μ Ci of *myo*-[³H]inositol/ml. Cells were electroporated at 7 kV/cm (7 discharges), allowed to reseal for 2 h at 37 °C and, after centrifugation (1500 *g*, 10 min), were finally resuspended in a Ca²⁺-free Tyrode buffer (pH 7.4). Platelet counts were 6 $\times$ 10⁸/ml for experiments involving inositol phosphate analysis and 3 $\times$ 10⁸/ml for those in which [¹⁴C]5-HT secretion was monitored. The efficiency of the permeabilization procedure was confirmed by the complete (100%) release of [³H]adenine metabolites achieved in platelets prelabelled in PRP with 10 μ Ci of [³H]adenine/ml for 1 h at 37 °C (results not shown). In the same experiments, the release of [³H]adenine metabolites was used to monitor possible changes in membrane leakiness in mastoparan-stimulated platelets (see the Results section).

In additional experiments, a similar procedure was adopted to examine the effects of entrapment of GDP[S] during reversible electroporation on mastoparan-induced [¹⁴C]5-HT secretion. GDP[S] (1 mM final concn.) or vehicle was added to the platelet suspension 15 min before permeabilization by electric discharge, and the platelets were subsequently allowed to reseal for 45 min at 37 °C. After centrifugation and resuspension in Tyrode buffer devoid of GDP[S], mastoparan-induced secretion was monitored as described below. In a parallel control experiment in which platelets were treated identically with GDP[S] or vehicle, no permeabilization was performed.

Measurement of aggregation, arachidonate release, protein phosphorylation and granule secretion

Platelet aggregation was measured in 300 μ l volumes of platelet suspension by recording the extent of light transmission in an Aggregometer II four-channel aggregometer (Daiichi Kagaku Co., Kyoto, Japan). Measurements in these suspensions of [³H]arachidonate release, secretion of [¹⁴C]5-HT from dense granules and release of BTG from α -granules were carried out essentially as described previously [17,19]. Briefly, [¹⁴C]5-HT and [³H]arachidonate release were quantified by stopping the incubation at a specified time after addition of stimulant with ice-cold EDTA (16 mM) plus 1% formaldehyde. After centrifugation (10000 *g*, 3 min), release into the platelet supernatant was assessed by liquid-scintillation counting. An identical procedure was followed for BTG measurement, except that platelet supernatants were analysed by radioimmunoassay. For the protein-phosphorylation studies, phosphoproteins were separated as previously reported [17], except that separation was carried out on gradient gels (7–20%) and dried gels were autoradiographed at –80 °C by using Fuji RX X-ray film.

Saponin studies

Permeabilization of washed platelets with saponin was carried out as described by Authi *et al.* [20]. An optimal saponin concentration (10–14 μ g/ml) for each individual experiment was chosen which caused maximal permeabilization of the plasma membrane but did not promote greater than 20% [¹⁴C]5-HT secretion and less than a 20% increase in light transmission over a 4 min exposure period [20]. Mastoparan and GDP[S] additions were made as described in the Results section.

Measurement of intracellular Ca²⁺ concentrations ([Ca²⁺]_i) by using fura 2-AM

Platelets were preloaded with 2 μ M-fura 2-AM (45 min, 37 °C) in buffer A (pH 7.4), consisting of 10 mM-Hepes, 140 mM-NaCl, 5 mM-KCl, 1 mM-MgSO₄, 10 mM-D-glucose and 1 μ M-PGI₂. Cells were subsequently centrifuged at 1000 *g* for 15 min, washed with a pH 6.5 buffer (36 mM-citric acid, 10 mM-EDTA, 5 mM-D-glucose, 5 mM-KCl, 90 mM-NaCl) and resuspended in buffer A without PGI₂ at 3 $\times$ 10⁸ platelets/ml. Fluorescence measurements

were carried out on 0.5 or 1.0 ml samples of platelet suspension in the presence of either 1 mM- Ca^{2+} or 1 mM-EGTA with a Perkin-Elmer LS5 luminescence spectrometer, with excitation and emission wavelengths of 340 and 500 nm respectively. In some experiments, excitation at 360 nm was also monitored (see the Results section). Intracellular $[\text{Ca}^{2+}]$ was calculated by the method of Gryniewicz *et al.* [21].

Analysis of [^3H]inositol phosphates

Production of [^3H]inositol phosphates was assessed in the presence of 10 mM-LiCl in permeabilized/resealed platelets ($6 \times 10^8/\text{ml}$) [18] and quantified by the column-separation procedure for inositol phosphates originally reported by Downes & Wusteman [22]. Radioactive peaks eluted from the Dowex formate columns were identified by using [^3H]inositol phosphate standards (Amersham International). [^{14}C]5-HT secretion was monitored in parallel in samples of the [^3H]inositol/[^{14}C]5-HT-loaded resealed platelets, and in non-permeabilized control platelets incubated under the same conditions but in buffer devoid of [^3H]inositol.

Determination of [cAMP]_i

[cAMP]_i was determined in samples (100 μl) of indomethacin-treated platelets [$(3-4) \times 10^8$ cells/ml] incubated, with stirring, in the presence of the phosphodiesterase inhibitor theophylline (600 μM) and the agent(s) of interest. Reactions were terminated by addition of an equal volume of ethanol (70 $^\circ\text{C}$), and the samples were centrifuged (2000 g , 15 min, 4 $^\circ\text{C}$) after a 30 min period on ice. Supernatants were dispensed into separate Eppendorf tubes, evaporated to dryness in a Savant Speed Vac SVC 100 (Stratech Scientific Ltd.), and extracts were resuspended in 140 μl of assay buffer (50 mM-Tris/HCl, 4 mM-EDTA, pH 7.0). Cyclic AMP was determined in duplicate 50 μl portions of sample by using the protein-binding assay originally described by Gilman [23]. Results are expressed as pmol of cyclic AMP/ 10^8 platelets.

Statistical analysis

All experiments were performed on platelets from at least two different donors. Unless otherwise stated, data are given as means $\pm$ S.E.M. of 3-5 experiments with 2-4 observations per experiment. Statistical analysis was performed by Student's paired or unpaired *t* test as appropriate, and means were considered significantly different when $P < 0.05$.

RESULTS

Effects of mastoparan on aggregation, granule secretion, arachidonate release and Ca^{2+} mobilization

When examined over a wide concentration range (1-50 μM) and over time periods of up to 10 min, mastoparan induced no detectable shape change or aggregation, as determined by conventional aggregometry. Transmission electron micrographs of glutaraldehyde-fixed platelet suspensions indicated the absence of micro-aggregate formation in the presence of mastoparan and revealed no obvious differences in the appearance of control and mastoparan-treated platelets (results not shown). In contrast, exposure of platelets to mastoparan promoted dose-dependent increases in the secretion of [^{14}C]5-HT and BTG from dense-granule and α -granule populations respectively (Fig. 1). Dense-granule secretion reached a peak at approx. 30 μM -mastoparan, whereas near-maximal release of BTG occurred at lower doses (1-10 μM). Release of BTG and [^{14}C]5-HT at all mastoparan concentrations tested was maximal within a 1 min stimulation period and was not altered significantly with extended incubation

times of up to 5 min. Thus 30 μM -mastoparan gave $38 \pm 3\%$ and $33 \pm 3\%$ [^{14}C]5-HT secretion with exposure times of 1 min (see Fig. 1) and 4-5 min respectively (means $\pm$ S.E.M., $n = 3$). Mastoparan-induced secretion from both granule populations was not an artifact of centrifugation, since platelet suspensions were fixed with EDTA/formaldehyde (see the Materials and methods section) before the centrifugation step.

Further studies were carried out to determine the mechanism(s) responsible for mastoparan-induced exocytosis. Platelets pre-loaded with fura 2 were used to ascertain whether secretion caused by mastoparan was associated with increases in cytosolic Ca^{2+} . Mastoparan (1-30 μM) caused no apparent change in the fluorescence of fura 2-loaded platelets in either the presence (1 mM) or the absence (1 mM-EGTA) of extracellular Ca^{2+} (Fig. 2). In parallel incubations, thrombin (0.2 unit/ml) increased intracellular Ca^{2+} to between 600 and 800 nM above resting levels. Similar results were obtained in thrombin-stimulated platelets in the absence of extracellular Ca^{2+} , except that the peak Ca^{2+} returned more rapidly to basal levels. In addition, mastoparan caused no decrease in the fluorescence monitored at an excitation wavelength of 360 nm, an event which is associated with increased membrane leakiness (e.g. [24]) (Fig. 2).

Experiments were also performed to examine whether mastoparan promoted the release of adenine nucleotides from the cytosol. In platelets in which the cytosolic ATP pool was labelled by preincubation with [^3H]adenine (90 min; 10 $\mu\text{Ci}/\text{ml}$), mastoparan (1-30 μM) caused no release of [^3H]adenine metabolites into the platelet supernatant.

The effects of the protein kinase inhibitor staurosporine were examined to determine the role of protein kinase activation in mastoparan-induced secretion (Table 1). Preincubation for 1 min with 50 nM-staurosporine, a concentration previously shown to inhibit maximally phorbol myristate acetate-induced phosphorylation of the 45 kDa protein kinase C substrate [25] and potentially to decrease exocytosis in response to a range of stimulatory platelet agonists [26,27], caused no decrease in [^{14}C]5-HT secretion induced by mastoparan. Moreover, at staurosporine concentrations of 1 μM and above, at which myosin light-chain kinase [26] and the cyclic nucleotide-dependent kinases [28] are also inhibited, mastoparan-induced dense-granule secretion remained completely unaffected (Table 1), whereas the effect of thrombin

Fig. 1. Dose-related effects of mastoparan on platelet [^{14}C]5-HT secretion and BTG release

Unlabelled or [^{14}C]5-HT-labelled platelets were incubated with mastoparan or saline vehicle, the reaction was terminated after 1 min, and the level of either [^{14}C]5-HT (●) or BTG (■) in the platelet supernatant was determined as described in the Materials and methods section. Basal release values for [^{14}C]5-HT and BTG were $1.8 \pm 0.7\%$ and $3.7 \pm 0.8\%$ respectively. Data are means $\pm$ S.E.M. from 6 (5-HT) and 3 (BTG) experiments.

Fig. 2. Effects of mastoparan and thrombin on Ca^{2+} mobilization in fura 2-loaded platelets

Platelets were preloaded with fura 2 as described in the Materials and methods section. The representative traces are shown from a single experiment taken from a series of three performed on platelets from different donors. Upper traces show the effect of $30 \mu\text{M}$ -mastoparan (Mast) on the fluorescence of fura 2-loaded platelets monitored at excitation wavelengths of 340 and 360 nm in the presence of extracellular Ca^{2+} . The lower trace depicts the effect of thrombin (0.2 unit/ml), which elevated intracellular $[\text{Ca}^{2+}]$ in this experiment to 680 nm above a resting level of 95 nm.

Table 1. Effects of pretreatment with staurosporine on thrombin- and mastoparan-induced dense-granule secretion

Platelets prelabelled with $[^{14}\text{C}]\text{5-HT}$ were preincubated (1 min) with various concentrations of staurosporine (SS) before challenge with mastoparan (1 min) or thrombin (3 min). Control incubations received an equivalent volume of ethanol vehicle. Reactions were terminated and the $[^{14}\text{C}]\text{5-HT}$ released into the platelet supernatant was determined as described in the Materials and methods section. Staurosporine alone (4 min) did not promote $[^{14}\text{C}]\text{5-HT}$ secretion. The mastoparan data are from two experiments with triplicate determinations in each. The results with thrombin are from two further experiments: * $P < 0.05$ versus thrombin control.

Incubation conditions	$[^{14}\text{C}]\text{5-HT}$ release (% of total platelet content)
Resting	1.2 ± 0.7
Mastoparan ($30 \mu\text{M}$)	25.9 ± 2.1
10 nM-SS + mastoparan	22.4 ± 1.0
50 nM-SS + mastoparan	24.5 ± 2.6
1 μM -SS + mastoparan	28.8 ± 0.5
10 μM -SS + mastoparan	29.9 ± 5.9
Thrombin (0.2 unit/ml)	66.4 ± 2.6
50 nM-SS + thrombin	$33.4 \pm 1.4^*$
1 μM -SS + thrombin	$3.3 \pm 1.0^*$

Table 2. Effects of prostacyclin on thrombin- and mastoparan-induced $[^{14}\text{C}]\text{5-HT}$ secretion

$[^{14}\text{C}]\text{5-HT}$ -labelled platelets were incubated (37°C), with stirring, with PGI_2 alone (4 min), thrombin or mastoparan at the indicated concentrations. The effect of PGI_2 was examined by preincubation for 1 min before addition of thrombin (3 min) or mastoparan (3 min). Control incubations received an equivalent volume of vehicle before exposure to the agonist. Reactions were terminated and $[^{14}\text{C}]\text{5-HT}$ released into the platelet supernatant was determined as outlined in the Materials and methods section. Data (means $\pm$ S.E.M.) are from three experiments with three observations per treatment in each: * $P < 0.01$ versus thrombin control.

Incubation conditions	$[^{14}\text{C}]\text{5-HT}$ secretion (% of total platelet content)
Resting	1.6 ± 0.6
PGI_2 (100 ng/ml)	1.1 ± 0.8
Thrombin (0.02 unit/ml)	45.9 ± 12.8
Mastoparan ($30 \mu\text{M}$)	24.3 ± 0.4
PGI_2 + thrombin	$3.5 \pm 0.6^*$
PGI_2 + mastoparan	29.7 ± 0.8

on dense-granule release was attenuated (50 and 96 % inhibition at 50 nM- and $1 \mu\text{M}$ -staurosporine respectively).

In platelets prelabelled with $[^3\text{H}]\text{arachidonate}$, mastoparan caused no significant liberation of arachidonic acid above that measured in vehicle-treated platelets at any of the concentrations examined: control $0.9 \pm 1.0\%$, $30 \mu\text{M}$ -mastoparan $1.4 \pm 0.3\%$ of total platelet $[^3\text{H}]\text{arachidonate}$ content (means $\pm$ S.E.M., $n = 4$). In parallel incubations, thrombin (1.0 unit/ml) promoted $15.0 \pm 1.4\%$ release of arachidonic acid.

Effects of prostacyclin on dense-granule secretion induced by thrombin and mastoparan

The results from secretion experiments performed under conditions identical with those described above are given in Table 2. A 1 min pre-exposure period to PGI_2 (100 ng/ml), which did not promote $[^{14}\text{C}]\text{5-HT}$ release on its own, had no measurable effect on dense-granule secretion induced by $30 \mu\text{M}$ -mastoparan, but potently inhibited thrombin (0.2 unit/ml)-induced $[^{14}\text{C}]\text{5-HT}$ secretion by approx. 95%.

Absence of phosphoinositide hydrolysis in mastoparan-stimulated platelets

In order to determine whether mastoparan stimulated PLC activity, the effects of the peptide and of thrombin on inositol phosphate formation were examined in platelets prelabelled with $[^3\text{H}]\text{inositol}$ during reversible electroporation. When compared directly with non-permeabilized control cells, permeabilized/resealed platelets exhibited only slightly decreased $[^{14}\text{C}]\text{5-HT}$ -secretory responses to 0.2 unit of thrombin/ml ($76 \pm 1\%$ versus $54 \pm 7\%$) and virtually identical responses to $30 \mu\text{M}$ -mastoparan ($38 \pm 10\%$ versus $40 \pm 11\%$; means $\pm$ S.E.M., $n = 3$). As shown in Table 3, stimulation (1 min) of platelet suspensions with mastoparan caused no detectable elevation in the levels of $[^3\text{H}]\text{inositol}$ phosphates, whereas a 15 s challenge with thrombin (0.2 unit/ml) enhanced the accumulation of both $[^3\text{H}]\text{InsP}_2$ and $[^3\text{H}]\text{InsP}_3$ (Table 3).

Effect of GDP[S] on mastoparan-induced $[^{14}\text{C}]\text{5-HT}$ secretion in saponin-permeabilized platelets

The studies described above suggest that the secretion elicited by mastoparan cannot be accounted for by elevated PLC activity, by increased Ca^{2+} mobilization or by liberation of arachidonic

Table 3. Effects of mastoparan and thrombin on inositol phosphate formation in platelets preloaded with [³H]inositol during reversible electroporation

Platelets were preloaded with [³H]inositol during reversible electroporation (see the Materials and methods section), and samples (500 μ l) of platelet suspensions were incubated (37 °C) with either mastoparan (1 min) or thrombin (15 s) at the indicated concentrations. Resting platelets received vehicle alone. Reactions were terminated with 400 μ l of 15 % (w/v) trichloroacetic acid, and neutralized aqueous extracts were analysed for [³H]inositol phosphates. Data are presented as means $\pm$ S.E.M. from three experiments performed on platelets from different donors.

Incubation conditions	Radioactivity (c.p.m.)	
	[³ H]InsP ₂	[³ H]InsP ₃
Resting	1893 $\pm$ 543	507 $\pm$ 106
Mastoparan (10 μ M)	1781 $\pm$ 463	439 $\pm$ 144
Mastoparan (30 μ M)	1728 $\pm$ 783	360 $\pm$ 48
Thrombin (0.2 unit/ml)	4391 $\pm$ 277	657 $\pm$ 136

Table 4. Effect of GDP[S] on mastoparan-induced dense-granule secretion in saponin-permeabilized platelets

Samples (300 μ l) of [¹⁴C]5-HT-labelled platelets were incubated (37 °C) with either saponin (2 min) or mastoparan at the indicated concentrations. Mastoparan-induced [¹⁴C]5-HT release in permeabilized platelets was monitored by addition of saponin 1 min before challenge with mastoparan; reactions were terminated 1 min later. GDP[S] was added 30 s before addition of saponin, or 90 s before addition of mastoparan in those incubations where saponin was omitted. GDP[S] caused no detectable [¹⁴C]5-HT secretion when added alone. Data are means $\pm$ S.E.M. of two experiments performed on platelets from different donors with triplicate or quadruplicate determinations per experiment: **P* < 0.02 versus mastoparan alone; ***P* < 0.05 versus saponin + mastoparan.

Incubation conditions	[¹⁴ C]5-HT release (% of total platelet content)
Resting	1.8 $\pm$ 1.3
GDP[S] + mastoparan	15.1 $\pm$ 0.1*
Mastoparan (30 μ M)	26.7 $\pm$ 1.1
Saponin (12 μ g/ml)	9.9 $\pm$ 1.1
Saponin + mastoparan	51.7 $\pm$ 2.3
GDP[S] (1 mM) + saponin + mastoparan	42.6 $\pm$ 2.1**
GDP[S] + saponin	7.7 $\pm$ 1.0

acid. In a number of recent studies, however, it has been shown that the actions of mastoparan in intact and permeabilized cells can be attributed, at least in part, to direct interaction with G-protein(s) [5,6,13]. In an attempt to determine the involvement of G-proteins in the secretory response to mastoparan, we examined the effect of GDP[S] on mastoparan-induced [¹⁴C]5-HT secretion in saponin-permeabilized platelets (Table 4). Previous studies have demonstrated that GDP[S] completely abolishes GTP[S]-induced dense-granule secretion under these conditions [29]. As noted previously, saponin induced a small basal release of [¹⁴C]5-HT. Addition of mastoparan to platelets pre-exposed to saponin resulted in a slightly greater than additive effect on [¹⁴C]5-HT secretion, which was attenuated when GDP[S] was added to the suspension 30 s before addition of saponin. In a total of six experiments, the GDP[S]-induced inhibition of secretion ranged

Fig. 3. Changes in [cAMP]_i in mastoparan- and PGI₂-stimulated platelets

Samples (100 μ l) of platelet suspension [(3–4) $\times$ 10⁸/ml] were incubated with either PGI₂ (5–100 ng/ml; 2 min) or mastoparan (MP; 1–30 μ M; 1 min), or exposed to PGI₂ for 1 min before addition of mastoparan (1 min). Reactions were terminated and [cAMP]_i was determined as described in the Materials and methods section. All incubations were carried out in the presence of 600 μ M-theophylline. Results are given as means $\pm$ S.E.M. from 2 experiments with 2–3 observations in each.

between 15 and 53 %. However, a comparable decrease in mastoparan-induced secretion was observed when platelets were exposed to GDP[S] in the absence of saponin, suggesting the possibility of both intracellular and extracellular actions of GDP[S] (Table 4). An equimolar concentration of GDP[S] inhibited thrombin (0.1 unit/ml)-induced secretion by 60.5 $\pm$ 2.0 %.

Inhibition of mastoparan-induced [¹⁴C]5-HT release in platelets permeabilized and resealed in the absence or presence of GDP[S]

Experiments were performed in which GDP[S] was encapsulated into platelets to examine further a possible intracellular action of GDP[S] in modulating mastoparan-induced secretion. Entrapment of GDP[S] (1 mM final concn.) during reversible electroporation and subsequent exposure to mastoparan (30 μ M, 1 min) significantly inhibited [¹⁴C]5-HT secretion as compared with that obtained in platelets exposed to vehicle alone during the permeabilization procedure (control 50 $\pm$ 1 %; GDP[S]-treated 36 $\pm$ 2 %; *P* < 0.05). In a parallel experiment, in which platelets were not subjected to permeabilization but otherwise treated identically, control and GDP[S]-treated platelets showed similar secretory responses when challenged with mastoparan (control 33 $\pm$ 1 %; GDP[S]-treated 38 $\pm$ 1 %).

Changes in [cAMP]_i in mastoparan-stimulated platelets

It has recently been reported that mastoparan potently enhances the GTP-binding/GTPase activity of purified G_i, with considerably less effect on that associated with the adenylate-cyclase-stimulatory G-protein, G_s [3]. The possibility that mastoparan may lead to changes in platelet adenylate cyclase activity was examined by monitoring its effect on [cAMP]_i. Fig. 3 depicts the effects of mastoparan, PGI₂ and combinations of the two agents on platelet [cAMP]_i measured in the presence of the phosphodiesterase inhibitor theophylline (600 μ M). Mastoparan (20 μ M) did not affect the cyclic-AMP-binding assay, as judged by its inability to affect the standard curve (results not shown). The results clearly show that incubation of platelet suspensions with mastoparan (1–30 μ M) for 1 min elicited dose-dependent increases in [cAMP]_i. These increases were similar to those induced by PGI₂ (5–100 ng/ml), a powerful inhibitor of platelet responsiveness which causes a marked elevation in [cAMP]_i via

Fig. 4. Stimulatory effects of mastoparan and PGI₂ on protein phosphorylation

[³²P]P_i-labelled platelets were incubated for 1 min with vehicle, mastoparan (MP) or PGI₂ at the indicated concentrations. After termination of the reactions, proteins were separated by SDS/PAGE and phosphoproteins were assessed by autoradiography. Autoradiographs from two separate experiments are shown and are representative of seven similar experiments performed on platelets from different donors. In panel (a) the lanes represent: a, resting platelets; b, +30 μM-mastoparan.

activation of G_s [15,16]. Moreover, when [cAMP]_i was raised by prior stimulation (1 min) with PGI₂, a subsequent incubation with mastoparan (1–30 μM) was unable to decrease cyclic AMP, an effect which gives an indirect estimate of G_i activation. Furthermore, combinations of mastoparan and PGI₂ led to approximately additive effects on cellular [cAMP] (Fig. 3). Thrombin (0.2 unit/ml) and NaF (30 mM), both known to activate G_i [15], decreased PGI₂ (100 ng/ml)-stimulated [cAMP]_i by 55±11 and 72±17% respectively (means±S.E.M., *n* = 3). Mastoparan obtained from Sigma had less potent effects on intra-platelet [cAMP], raising values to between 6 and 12 pmol/10⁸ platelets at concentrations of 30–60 μM (results not shown).

Effects of mastoparan on protein phosphorylation

These were studied in platelets prelabelled with [³²P]P_i. When examined over exposure periods ranging between 10 s and 10 min, no phosphorylation of either the major 45 kDa protein kinase C substrate or the 20 kDa protein could be detected in the presence of mastoparan (1–30 μM). Representative data for platelets exposed to mastoparan (1–30 μM) for 1 min are included in Figs. 4(a) and 4(b). However, in parallel with our ability to detect increased cyclic AMP levels, mastoparan appeared to enhance the incorporation of ³²P into a protein of approx. 24 kDa (Figs. 4a and 4b). In additional experiments in which the effects of mastoparan were compared directly with those of PGI₂ (Fig. 4b), both agents enhanced phosphorylation of a 24 kDa protein (lanes d, e, g and h), whereas elevated ³²P incorporation into a 50 kDa protein was seen only in the presence of PGI₂ (lanes g and h).

DISCUSSION

There is growing evidence drawn from studies in several cell types that mastoparan can affect cellular function through direct or indirect interaction with membrane-associated G-proteins; as such, this agent has potential value as a biochemical tool for probing G-protein-dependent processes. However, the mechanisms through which cellular activation is achieved appear to differ considerably between cell types. In intact rat peritoneal mast cells, mastoparan transiently stimulates PLC-catalysed phosphatidylinositol 4,5-bisphosphate hydrolysis, and inositol phosphate formation, through a pertussis-toxin-sensitive mechanism [6]. These findings strongly suggest the involvement of a G-protein(s) in the stimulatory action of mastoparan on these cells and substantiate the studies *in vitro* by Higashijima *et al.* [3,4] in which mastoparan was suggested to interact directly with, and enhance the activity of, the pertussis-toxin-sensitive G-proteins G_i and G_o. In contrast, mastoparan has also been reported to exert both stimulatory and inhibitory effects on PLC which are not modulated by pertussis-toxin pre-treatment [30,13,14], raising the possibility that mastoparan can additionally interact with pertussis-toxin-insensitive substrates and/or that this agent can affect cellular processes by a mechanism(s) unrelated to its direct action on G-protein function.

In the present study, we describe the effects and possible mechanism of action of mastoparan on intact human platelets. During the course of these studies, Ozaki *et al.* [8] demonstrated that extended (10 min) incubation periods with mastoparan induced moderate (20%) secretion of 5-HT from intact human platelets, but did not promote either the release of arachidonate

or an elevation of cytosolic $[Ca^{2+}]$. In the present study, mastoparan at an equivalent concentration caused a greater level of dense-granule release (25–45%), which was complete within 1 min of exposure and which was accompanied by the release of α -granule constituents. This secretory response occurred in the complete absence of either shape change or aggregation and, in agreement with Ozaki *et al.* [8], was observed under conditions where no liberation of arachidonic acid or increases in cytosolic $[Ca^{2+}]$ were detected. The absence of mastoparan-stimulated arachidonate release suggests that the actions of this agent in intact platelets do not include direct or indirect activation of phospholipase A_2 , as has been demonstrated *in vitro* [1].

The present studies have also shown that exposure of platelets to mastoparan neither stimulated inositol phosphate formation nor enhanced phosphorylation of the 45 and 20 kDa proteins normally associated with platelet activation [15]. These results suggest that the mechanism whereby mastoparan elicits exocytosis is not dependent upon changes in PLC activity. Since most physiological platelet stimulants (e.g. thrombin, thromboxane A_2) elevate PLC activity, secretion in response to these agonists has been shown without exception to be either decreased or abolished, both by agents which inhibit protein kinase C, and by PGI_2 , which decreases platelet activation, at least in part, by a cyclic AMP-mediated inhibition of PLC [15,16]. A PLC-independent mechanism of exocytosis is therefore further supported by the observation that mastoparan-induced secretion was insensitive to both prostacyclin and the protein kinase C inhibitor staurosporine. In addition, micromolar concentrations of staurosporine inhibit myosin light-chain kinase [26], the cyclic nucleotide-dependent kinases [28] and some tyrosine kinases [31], suggesting that the secretory response to mastoparan is also unrelated to the activation of these enzymes. Furthermore, secretion was not due to cell lysis, since mastoparan caused no release of $[^3H]$ adenine and its metabolites from prelabelled platelets and did not result in a decreased fluorescence of fura 2-loaded platelets monitored at 360 nm. A mechanism of secretion involving activation of phospholipase D is also unlikely to contribute to the secretory activity of mastoparan, since recent evidence suggests that stimulation of human platelet phospholipase D occurs primarily as a consequence of PLC activation [32,33]. From our findings, it seems reasonable to postulate that mastoparan promotes exocytosis by at least two possible mechanisms. First, mastoparan may exert a direct fusogenic action resulting from insertion of its hydrophobic domain into the plasma membrane; this effect may or may not be dependent on its subsequent interaction with G-protein(s). Secondly, and equally tenable, secretion may result from stimulation of the putative G-protein G_e , which is linked directly to exocytosis in a number of cell types and which has been reported to be responsible for the secretory response observed in the absence of PLC activation and Ca^{2+} mobilization [34,35]. Although there have been a number of platelet studies describing the occurrence of receptor-mediated exocytosis in the absence of PLC activity [36–38], there is currently little evidence to support the involvement of a G_e -like protein in the regulation of secretion from platelet granules. However, recent studies in electrically permeabilized human platelets have shown that GTP[S] can promote Ca^{2+} - and PLC-independent secretion from both dense-granule and α -granule populations [39,40]. In the present study GDP[S] consistently inhibited mastoparan-induced dense-granule secretion in both intact and saponin-permeabilized platelets, suggesting that this effect could be attributed to an extracellular action of GDP[S]. Nevertheless, this does not exclude the possibility that GDP[S] may affect mastoparan-induced secretion in part via an intracellular action. Indeed, in further experiments, mastoparan-induced secretion was diminished in platelets perme-

abilized and resealed in the presence of GDP[S] compared with permeabilized/resealed control platelets. Since this inhibitory effect was absent in platelets treated identically with GDP[S] but not permeabilized, the results provide some evidence for a possible intracellular action of GDP[S] in modulating mastoparan-induced secretion. In this respect, it has recently been proposed that cationic mast-cell secretagogues, including compound 48/80 and mastoparan, induce secretion in these cells by activating a G-protein which operates distal to PLC and which is located close to the exocytotic site [41].

Ozaki *et al.* [8] have reported that mastoparan-induced 5-HT secretion from digitonin-permeabilized platelets is pertussis-toxin-sensitive. However, mastoparan clearly does not activate PLC in intact platelets, suggesting that a G-protein(s) other than the putative G_p is involved in this response. Whether G_e is a pertussis-toxin substrate is unclear at present. However, based on the observation that mastoparan-induced histamine release in mast cells occurs in the absence of PLC stimulation and is attenuated by pertussis toxin, Aridor *et al.* [41] have proposed that G_e may be a member of the G_i G-protein family. Similarly, a pertussis-toxin-sensitive G-protein seems to play an inhibitory role in modulating the final stages of the exocytotic process in adrenal chromaffin cells [42]. Clearly, further studies to evaluate the mechanism of mastoparan-induced secretion in human platelets are required.

The reported ability of mastoparan to stimulate the activities of purified G_i and G_o [3] prompted us to examine the effects of the peptide on $[cAMP]_i$. The activation of G_i by physiological platelet stimuli such as thrombin and adrenaline can readily be demonstrated by the ability of these agents to decrease PGI_2 -stimulated $[cAMP]_i$ [43]. However, in experiments designed to examine a possible activation of G_i by mastoparan, we observed no inhibitory effect of the peptide on intra-platelet $[cAMP]_i$ elevated by prior exposure to PGI_2 . In fact, mastoparan itself increased $[cAMP]_i$, and the elevation of $[cAMP]_i$ achieved in the presence of both mastoparan and PGI_2 was consistently greater than with either agent alone. These results therefore argue against a direct effect of mastoparan on G_i . The increased $[cAMP]_i$ induced by mastoparan would appear to be due neither to inhibition of cyclic AMP phosphodiesterase activity nor to interference of mastoparan with the cyclic AMP assay procedure. The results are more likely to reflect either a direct interaction of mastoparan with adenylate cyclase or preferential stimulation of the adenylate-cyclase-stimulatory G-protein, G_s . Despite its apparent preference for purified G_i , mastoparan has been reported to enhance adenylate cyclase activity in S49 lymphoma-cell plasma membranes [3], implying that this preference is not necessarily reflected in a similar specificity under more physiological conditions.

The additive effects of mastoparan and PGI_2 on $[cAMP]_i$ may indicate the existence of multiple adenylate cyclase pools in platelets, although a more detailed examination of the time- and dose-dependent changes in $[cAMP]_i$ is required. In this respect it is known that human platelets express an additional pool of this enzyme, which appears to be distinct from that associated with the plasma membrane [44,45].

PGI_2 , through its activation of cyclic-AMP-dependent protein kinase (protein kinase A), has been shown to increase the phosphorylation of several platelet proteins, including those with approximate molecular masses of 22, 24, 39, 50, 68, 120 and 240 kDa [46,47]. The 22 and 24 kDa proteins have recently been identified as rap1B [48] and the β -chain of glycoprotein Ib (GPIb) [49] respectively. In the present study, the rise in $[cAMP]_i$ elicited by mastoparan was reflected in the increased incorporation of ^{32}P into a 24 kDa protein. Further studies are required to determine the identity of this protein. An earlier

study showed delayed phosphorylation of a 27 kDa protein in thrombin-stimulated platelets [50]. This substrate is an unlikely candidate for the phosphoprotein seen in mastoparan-stimulated cells, since the latter clearly co-migrated with the 24 kDa protein phosphorylated by PGI₂ in the same platelet suspensions. In contrast, exposure of platelets to mastoparan did not result in phosphorylation of a 50 kDa protein under conditions where PGI₂ clearly enhanced phosphorylation of this substrate. Thus mastoparan-induced [cAMP]_i elevation may result in incomplete activation of protein kinase A, perhaps suggesting compartmentalization of the cyclic AMP generated. Although the effects of cyclic AMP are believed to be mediated by protein kinase A stimulation, a recent report has proposed that intra-platelet cyclic AMP may have additional anti-platelet effects which are unrelated to the activation of protein kinase A [46]. In addition, our preliminary findings have demonstrated an inhibitory effect of mastoparan on receptor-mediated platelet activation (C. P. D. Wheeler-Jones, T. Saermark, V. V. Kakkar & K. S. Authi, unpublished work), results which are consistent with the suppressive actions of other cyclic-AMP-elevating agents on platelet reactivity.

In summary, we have shown that mastoparan can simultaneously promote granule secretion and raise [cAMP]_i in intact human platelets. The exocytotic response to mastoparan does not involve changes in the classical intracellular mediators known to modulate exocytosis, suggesting that mastoparan-induced secretion could result either from a direct fusogenic action and/or from stimulation of the putative exocytosis-linked G-protein G_e. These findings may indicate the existence in platelets of a secretory pathway which is independent of PLC stimulation. The physiological relevance of this pathway to receptor-mediated platelet activation remains to be determined.

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