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# Phosphoinositide hydrolysis in permeabilized SH-SY5Y human neuroblastoma cells is inhibited by mastoparan

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The effects of mastoparan on phospholipase C-catalysed phosphoinositide hydrolysis were examined in [$^3$H]inositol-labelled human neuroblastoma SH-SY5Y cells. [$^3$H]Inositol phosphate formation in intact cells was not altered by 20 $\mu$M mastoparan. In contrast, [$^3$H]inositol phosphate formation in electrically permeabilized cells stimulated with guanosine 5'-[$\gamma$-thio]triphosphate and/or carbachol was inhibited by mastoparan with half-maximal effects at approx. 3 $\mu$M. The peptide was much less effective in inhibiting stimulatory effects of $Ca^{2+}$. Similar but less potent inhibitory effects were observed with the cations, neomycin and spermine, indicating that direct interaction of mastoparan with polyphosphoinositides might account for its inhibitory effects on inositol phosphate formation.

Mastoparan; Phosphoinositide hydrolysis; G-protein; Muscarinic receptor; (SH-SY5Y cell)

## 1. INTRODUCTION

The peptide mastoparan (Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-$NH_2$) is a component of wasp (*Vespula lewisii*) venom that exhibits a range of biological activities that include stimulation of secretion from a variety of cell types [1] and activation of phospholipase $A_2$ [2]. As it is amphiphilic, mastoparan interacts strongly with phospholipids and assumes an $\alpha$-helical conformation upon binding to phosphatidylcholine vesicles [3]. Interest in the actions of mastoparan has been stimulated recently by findings that the peptide enhances GTP-analogue binding to and GTPase activity of guanine nucleotide-binding regulatory proteins [4].

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*Abbreviations:* GTP[S], guanosine 5'-[$\gamma$-thio]triphosphate; G-protein, guanine nucleotide-binding regulatory protein; $PIP_2$, phosphatidylinositol 4,5-bisphosphate; IP(s), inositol phosphate(s)

Phosphoinositides including $PIP_2$ are pivotal to cell function as hydrolysis of $PIP_2$ by phospholipase C is stimulated by binding of agonists to a variety of receptors and leads to the formation of the intracellular second messengers inositol, 1,4,5-trisphosphate and diacylglycerol [5,6]. The location of $PIP_2$ within the plasma membrane and its importance as a precursor of second messengers make this phospholipid and the proteins involved in its hydrolysis possible and important targets for mastoparan. Thus, using SH-SY5Y human neuroblastoma cells [7] that express muscarinic receptors linked to phospholipase C [8,9], we have examined the effects of mastoparan on phosphoinositide hydrolysis.

## 2. EXPERIMENTAL

SH-SY5Y cells were cultured in 175 cm$^2$ flasks as described [8] and were radiolabelled by incubation of 5-day-old cultures for 2 days with 4 $\mu$Ci [$^3$H]inositol/ml of culture medium. Labelled cells were mechanically removed from culture flasks, were washed twice in $Ca^{2+}$/$Mg^{2+}$-free phosphate-buffered saline and were finally resuspended in ice-cold 'cytosol-like' buffer (120 mM KCl, 20 mM Na Hepes, 6 mM $MgCl_2$, 2 mM $KH_2PO_4$, 0.1 mM EGTA, 2 mM $Na_2ATP$, 0.5 mg/ml bovine

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serum albumin, pH 7.5, and p$Ca^{2+}$ 6.9; 1.6 ml per flask of cells). Aliquots (0.8 ml) of this suspension were exposed to discharges of a 3 $\mu$F capacitor (field strength, 3.75 kV/cm; time constant, 0.1 ms) spaced by 2 s intervals. The aliquots were then diluted to 2 ml and 100 $\mu$l portions were dispensed into ice-cold polypropylene tubes containing reagents to give 200 $\mu$l of a mixture composed of 'cytosol-like' buffer with 3 mM $Na_2ATP$, 0.75 mg/ml bovine serum albumin and appropriate stimuli. All experimental incubations were for 5 min at 37°C and were terminated by addition of 1 ml methanol/1 M HCl (2:1), 0.1 ml water and 1 ml chloroform. The samples were then thoroughly mixed and centrifuged (1700 × g for 10 min) and 1 ml of upper phases were neutralized, diluted and applied to Dowex AG 1-X8 resin (200–400 mesh, formate form) and [$^3$H]inositol phosphates ([$^3$H]IPs) were eluted with 3 ml of 1.0 M ammonium formate/0.1 M formic acid [10]. Background radioactivity (that present in unincubated samples) was unaffected by the stimuli used and in all experiments was subtracted from radioactivity recovered after experimental incubations. All data shown are mean ± SE of 3 independent experiments. Approx. 70000 dpm of [$^3$H]lipid per tube was present at the start of incubations.

SH-SY5Y cells were a gift from Dr J.L. Biedler, Sloan-Kettering Institute, USA. [$^3$H]Inositol (D-*myo*-[2-$^3$H(N)]-inositol, approx. 15 Ci/mmol) was obtained from NEN; guanosine 5'-[$\gamma$-thio]triphosphate (GTP[S]) was from Boehringer Mannheim Biochemicals; and synthetic mastoparan, carbachol, neomycin, $Na_2ATP$ and spermine were from Sigma.

## 3. RESULTS

The muscarinic agonist carbachol (1 mM), the hydrolysis-resistant GTP analogue GTP[S] (0.1 mM) and $Ca^{2+}$ were employed as stimuli of phospholipase C activity. It was necessary to permeabilize cells before stimulatory effects of $Ca^{2+}$ and GTP[S] were apparent (not shown). Mastoparan (20 $\mu$M) did not affect basal (not shown) or stimulated [$^3$H]IP levels in intact cells (fig.1) but inhibited the effects of carbachol/GTP[S] after 3 or more discharges (fig.1). The total inhibition by mastoparan of the effects of carbachol/GTP[S] after 9 or 12 discharges (fig.1) correlates with the inability of cells treated in this manner to exclude trypan blue or to retain [$^3$H]IPs in the intracellular compartment (not shown) and indicates that the cells have been fully permeabilized. The somewhat surprising increase in stimulated [$^3$H]IP levels caused by exposure to 3–9 discharges (fig.1) was not seen in parallel experiments conducted in the presence of $Li^+$ (4 mM), which inhibits inositol phosphate phosphatases [11,12] and which enhanced stimulated [$^3$H]IP accumulation by 75% only in intact cells (not shown). Apparently, therefore, only in intact cells were [$^3$H]IPs actively dephosphorylated.

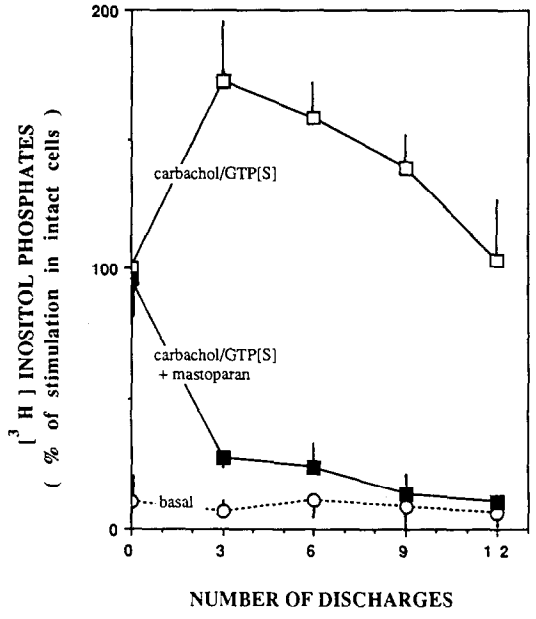


Fig.1. Effects of electroporation on responsiveness to mastoparan. [$^3$H]Inositol-labelled cells in suspension were exposed to 0, 3, 6, 9 or 12 discharges and were incubated at 37°C for 5 min with either no further addition (○) or with 1 mM carbachol/0.1 mM GTP[S] in the absence (□) or presence (■) of 20 $\mu$M mastoparan. [$^3$H]IP formation was normalized to that (2910 ± 290 dpm) obtained in carbachol/GTP[S]-stimulated intact cells.

Mastoparan inhibited the stimulatory effects of carbachol and/or GTP[S] in permeabilized cells maximally by >80% with half-maximal effects at approx. 3 $\mu$M, but did not alter basal [$^3$H]IP levels (fig.2). In contrast, 20 $\mu$M mastoparan inhibited the effects of 0.2 mM and 0.5 mM $Ca^{2+}$ by only 33% and 23%, respectively (fig.2). These effects reflected changes in the rate of polyphosphoinositide hydrolysis since $PIP_2$ levels were reduced by carbachol/GTP[S] and by $Ca^{2+}$, mastoparan (20 $\mu$M) reversed only the reduction caused by carbachol/GTP[S], and the majority of [$^3$H]IPs formed in stimulated cells were derived from polyphosphoinositide rather than phosphatidylinositol hydrolysis (not shown). The polyamine spermine and the antibiotic neomycin also inhibited carbachol/GTP[S]-stimulated [$^3$H]IP formation (with half-maximal effects at approx. 1.5 mM and 0.3 mM, respectively) and again were less potent inhibitors of the effects of $Ca^{2+}$ (fig.3).

The inhibitory effects of mastoparan were

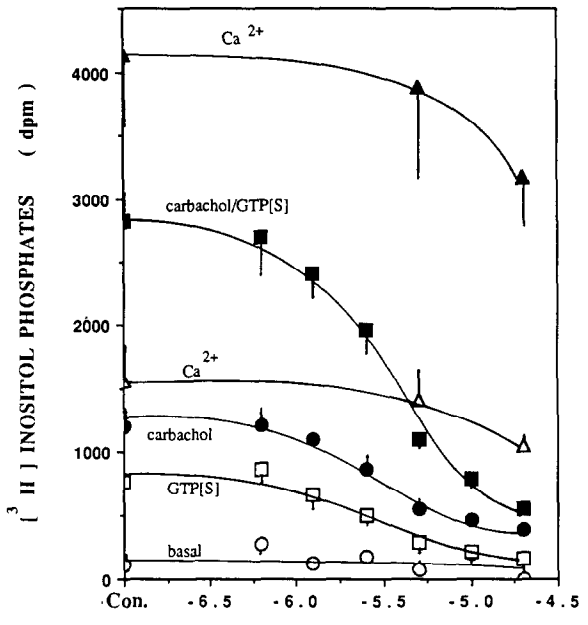


Fig.2. Dose-dependence of inhibitory effects of mastoparan. [$^3$H]Inositol-labelled cells that had been exposed to 12 discharges were incubated for 5 min at 37°C with either no further addition (○), 0.1 mM GTP[S] (□), 1 mM carbachol (●), 0.1 mM GTP[S]/1 mM carbachol (■), 0.2 mM $Ca^{2+}$ (△) or 0.5 mM $Ca^{2+}$ (▲) and a range of mastoparan concentrations.

destroyed by preincubation of the peptide with trypsin, which will cleave the peptide at its lysine residues (not shown). Thus, it is the intact peptide that possesses the inhibitory activity.

## 4. DISCUSSION

Stimulation of [$^3$H]IP production by GTP[S], the synergy between carbachol and GTP[S] when these agents were co-incubated, and additional studies in permeabilized SH-SY5Y cells on the specificity of nucleotide effects on [$^3$H]IP production [13] indicate that a G-protein, that has been termed Gp [14,15], is involved in coupling the muscarinic receptors of SY-SY5Y cells to increased polyphosphoinositide hydrolysis.

In contrast to findings that mastoparan transiently stimulates $PIP_2$ hydrolysis and IP formation in intact mast cells [16], the peptide did not affect polyphosphoinositide hydrolysis in intact SH-SY5Y cells and was inhibitory if it had access to the cell interior. Two lines of evidence indicate that the intracellular site with which mastoparan interacts might be $PIP_2$ and that such an interaction inhibits $PIP_2$ hydrolysis. Firstly, mastoparan interacts with phospholipids [1,3] and because it contains 3

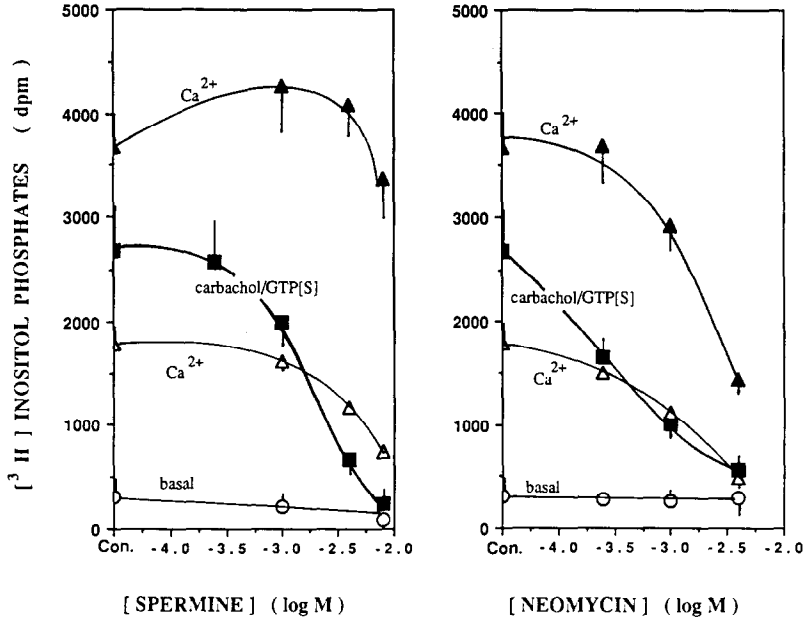


Fig.3. Dose-dependence of inhibitory effects of spermine and neomycin. [$^3$H]Inositol-labelled cells that had been exposed to 12 discharges were incubated as in fig.2 with a range of spermine or neomycin concentrations.

positively charged lysine residues at neutral pH it should interact particularly strongly with a polyanion like $PIP_2$. Secondly, the cations spermine and neomycin both inhibit the effects of carbachol/GTP[S] in permeabilized cells. Both compounds bind to $PIP_2$ [17,18] and inhibit phospholipase C-catalysed $PIP_2$ hydrolysis [19,20]. Apart from the greater potency of mastoparan, the inhibitory characteristics of mastoparan, neomycin and spermine were similar indicating that they may share a common mechanism of action. Competition between binding of these cations and $Ca^{2+}$ to $PIP_2$ [17,18,20] may explain the relatively low potency of inhibition by mastoparan, neomycin and spermine of $Ca^{2+}$-stimulated [$^3$H]IP formation. Finally, an action of mastoparan akin to the activating effect of the peptide on purified G-proteins reconstituted in phospholipid vesicles [4] is unlikely to subserve the inhibitory effects of the peptide in permeabilized SH-SY5Y cells, since if Gp was similarly activated by mastoparan then an increase in [$^3$H]IP formation would be expected.

It is interesting to note that mastoparan mimics some of the effects of G-protein $\beta\gamma$-subunits. Both $\beta\gamma$ and mastoparan stimulate phospholipase $A_2$ activity [21,2] and inhibit phospholipase C activity ([22] and this study). Thus, these agents may share a common mechanism of action that is perhaps related to the ability of both mastoparan [1,3] and $\beta\gamma$ [23] to interact strongly with phospholipid membranes.

In conclusion, mastoparan is a potent inhibitor of phospholipase C activity in permeabilized cells. While this may not be relevant to its biological function [1], mastoparan appears to interfere with the sequence of events initiated by receptor and/or Gp activation that lead to increased $PIP_2$ hydrolysis and may, therefore, serve as a valuable tool in investigations of this signal transduction system.

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