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Biochemical and Biophysical Research Communications 301 (2003) 13–16
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Mastoparan induces Ca^{2+}-independent cortical granule exocytosis in sea urchin eggs

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Received 13 December 2002

Abstract

In most species, cortical granule exocytosis is characteristic of egg activation by sperm. It is a Ca^{2+}-mediated event which results in elevation of the vitelline coat to block permanently the polyspermy at fertilization. We examined the effect of mastoparan, an activator of G-proteins, on the sea urchin egg activation. Mastoparan was able to induce, in a concentration-dependent manner, the egg cortical granule exocytosis; mastoparan-17, an inactive analogue of mastoparan, had no effect. Mastoparan, but not sperm, induced cortical granule exocytosis in eggs preloaded with BAPTA, a Ca^{2+} chelator. In isolated egg cortical lawns, which are vitelline layers and membrane fragments with endogenously docked cortical granules, mastoparan induced cortical granule fusion in a Ca^{2+}-independent manner. By contrast, mastoparan-17 did not trigger fusion. We conclude that in sea urchin eggs mastoparan stimulates exocytosis at a Ca^{2+}-independent late site of the signaling pathway that culminates in cortical granule discharge.
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Keywords: Ca^{2+} signaling; Cortical granule; Egg; Exocytosis; Fertilization; Mastoparan; Sea urchin

A universal characteristic of egg activation at fertilization is an increase in cytosolic free Ca^{2+} within the egg. Among other processes, Ca^{2+} triggers exocytosis of cortical granules (CG) to prevent polyspermy at fertilization [1,2]. Upon exocytosis, CG release their content into the perivitelline space to convert the vitelline coat in an impenetrable barrier to supernumerary sperm. In sea urchin eggs, inositol 1,4,5-trisphosphate (IP_3) is the primary mediator of intracellular Ca^{2+} release at fertilization (see [3] for review). However, eggs from sea urchins and from several species also respond to other Ca^{2+}-mobilizing molecules such as cyclic ADP-ribose, cyclic GMP, and nicotinic acid adenine dinucleotide phosphate [4–6]. The molecular events that occur downstream the Ca^{2+} signal and culminate in CG exocytosis are not completely understood. Cortical lawns consisting of vitelline layer and plasma membrane fragments with endogenously docked CG have been widely used as a simplified system to study Ca^{2+}-triggered fusion of CG since they retain the molecular machinery for docking, Ca^{2+} sensing, and CG-plasma membrane fusion [7–11]. This machinery includes VAMP, syntaxin, and SNAP-25 proteins [9–11] which presumably are complexed to modulate Ca^{2+} sensitivity of CG fusion in the sea urchin egg [12]. G-proteins have also been involved in exocytosis of secretory vesicles from several species [13–15]. Mastoparan, an amphiphilic tetradecapeptide from wasp venom, interacts with the amino terminus of the α-subunits of G-proteins to stimulate guanine nucleotide exchange [16,17], promoting exocytosis in adrenal chromaffin cells [18], in platelets [15], and in pancreatic β-cells [19], among others. In adrenal chromaffin cells and in insulin-secreting β-cells mastoparan stimulates exocytosis independently of increases in intracellular Ca^{2+} concentration; in the case of pancreatic cells, the mastoparan effect is mediated by the G-protein Cdc42 [20]. We studied here the action of mastoparan on CG exocytosis by sea urchin eggs and on

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doi:10.1016/S0006-291X(02)02979-0

CG-plasma membrane fusion using cortical lawns isolated from unfertilized eggs.

Materials and methods

Gamete collection and reagents. Gametes from the sea urchins *Strongylocentrotus purpuratus* and *Lytechinus pictus* were collected as indicated in [21], stored on ice, and protected from light until use. Salts to prepare complete seawater were from InstantOcean. Mastoparan (VDWKKIGQHILSVL), mastoparan-17 (INLKAKAALAKKLL), 1,2-bis(2-Aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis/acetoxymethylester) (BAPTA/AM), leupeptin, antipain, cholera toxin, pertussis toxin, and NAD were from Sigma. C3 exoenzyme from *Clostridium botulinum* was from Upstate Biotechnology. All other reagents were of analytical grade.

Egg activation. In a typical experiment, eggs (400 cells) were incubated in 60 μl seawater at 16 °C in the absence or presence of various concentrations of mastoparan (5 mM stock solution prepared in distilled water). For fertilization, eggs were mixed with 5 μl sperm (diluted 1:16 in seawater) and incubated as above. When required, eggs were first incubated with 1 mM BAPTA/AM for 1 h, followed by sperm or mastoparan addition. Three minutes after mixing with sperm or mastoparan, eggs were immediately observed by phase-contrast microscopy (Carl Zeiss Axioskop) for determining the percentage of eggs showing the characteristic fertilization membrane.

Preparation of sea urchin egg cortical lawns. Cortical lawns are fragments of the egg surface consisting of vitelline layer and plasma membrane in which CG remain intact and firmly attached [7,8]. Egg cortices were prepared according to the procedure described by Haggerty and Jackson [7]. Briefly, eggs were kept on ice and gently homogenized in isolation medium (220 mM K-gluconate, 5 mM EGTA, 500 mM glycine, 10 mM NaCl, 5 mM $MgCl_2$, 5 μg/ml antipain, 5 μg/ml leupeptin, and 2 mM DTT, pH 6.8). After three washes by centrifugation (800*g*, 2 min), the cortical lawns were finally resuspended in fusion medium (400 mM KCl, 2 mM EGTA, 1 mM GTP, and 20 mM Tris–HCl, pH 7.0) and maintained at 4 °C until used in fusion assays. In this buffer, CG display a low extent of Ca^{2+}-dependent fusion allowing the analysis of mastoparan effect.

In vitro membrane fusion assay. The loss of refractility of CG as they fuse can be followed by measuring the decrease in absorbance at 400–450 nm [7,8]. To assess CG fusion in cortical lawns we used the following procedure: aliquots of egg cortices were dispensed into 96-well microtiter plate to give an A_{450} of 1.0 U. Changes in absorbance as a function of time were measured immediately after the addition of an equal volume of either distilled water (control), Ca^{2+} or mastoparan; simultaneous additions of Ca^{2+} and mastoparan were also tested. Light scattering was recorded at intervals and the extent of fusion was calculated as $\Delta OD/OD_{initial}$, correcting for a background by adding 1% Triton X-100 in distilled water. The free Ca^{2+} concentrations used in these experiments were calculated using a computer program [22]. Under our conditions, 5–100 μM free Ca^{2+} was sufficient to stimulate CG fusion. The results described in this work were essentially identical using eggs from either *S. purpuratus* or *L. pictus*.

Results and discussion

The elevation of the vitelline layer of sea urchin egg is the first morphological sign that results from the Ca^{2+}-dependent CG exocytosis at fertilization. We studied whether mastoparan was able to induce egg activation. Fig. 1A shows that mastoparan stimulated CG exocytosis in a concentration-dependent manner. The percentage of eggs activated by mastoparan was similar to that activated by sperm. Mastoparan-17, which is an inactive analogue of mastoparan [14,20], failed to activate the eggs, indicating that mastoparan activates a physiological step in the mechanism of exocytosis. The morphological change accompanying egg activation by mastoparan was similar to that elicited by sperm (Fig. 1B). These results indicate that mastoparan acts in a crucial step of the egg signaling pathway activated by sperm.

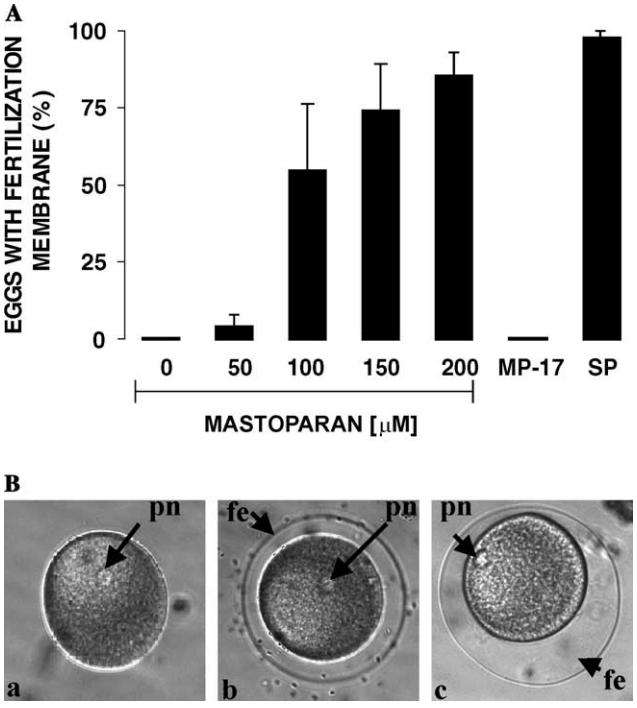

Fig. 1. Mastoparan activates sea urchin eggs. (A) *L. pictus* sea urchin eggs were incubated for 3 min in seawater containing various concentrations of mastoparan or 100 μM of the inactive analogue mastoparan 17 (MP-17). Sperm (SP) addition is the control to verify physiological activation of eggs. Cortical granule exocytosis was evaluated by determining the percentage of eggs with fertilization envelope. (B) Phase-contrast pictures show activation by mastoparan (c) and by sperm (b); a non-activated egg is also shown (a). pn, pronucleus; fe, fertilization envelope. Results represent means ± SD of four different batches of eggs.

During fertilization, sperm induce in eggs an intracellular Ca^{2+} mobilization, which is prerequisite for cortical granule exocytosis [3]. It is possible to prevent Ca^{2+} increases in cells by using the membrane-permeant, Ca^{2+} chelator BAPTA/AM [23]. If mastoparan mobilizes Ca^{2+} in eggs, then loading the cells with BAPTA/AM before mastoparan addition should result in inhibition of CG exocytosis. Table 1 shows that 1 mM BAPTA prevented the eggs from activating in response to sperm (control). Unlike sperm, mastoparan was able to stimulate CG exocytosis in BAPTA-loaded eggs. Therefore, mastoparan induces CG exocytosis through a mechanism that bypasses the increase in $[Ca^{2+}]_i$ revealing the existence of a late, Ca^{2+}-independent step in the signaling pathway which results in CG fusion.

Table 1
Mastoparan induces cortical granule exocytosis in BAPTA-loaded eggs

Condition	Eggs with fertilization envelope (%)
Eggs	0
Eggs + sperm (control)	92 ± 5
Eggs + 100 μM mastoparan	88 ± 7
Eggs preloaded with BAPTA + sperm	4 ± 1
Eggs preloaded with BAPTA + 100 μM mastoparan	84 ± 6

Where indicated, eggs were preincubated for 30 min at 16 °C with 1 mM BAPTA/AM. Eggs were observed under the microscope 3 min after addition of either sperm or mastoparan and the percentage of cells showing the fertilization membrane was recorded. Values represent means ± SD of three independent experiments.

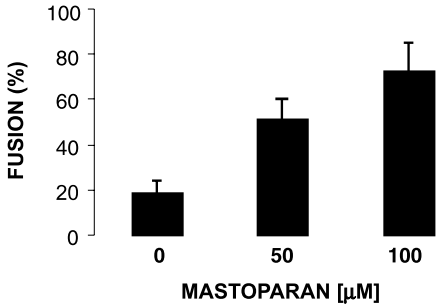

Fig. 3. In vitro cortical granule fusion triggered by mastoparan is Ca^{2+}-independent. Fusion was measured in egg cortices as in Fig. 2, except that the Ca^{2+}-free medium was supplemented with 5 mM EGTA. Results shown are means ± SD of three independent determinations.

A cell-free system consisting of isolated cortical lawns (already described in Materials and methods) is able to support Ca^{2+}-dependent membrane fusion [7,8]. These cortical surfaces have been used to study in vitro the mechanisms involved in fertilization–CG exocytosis coupling. It is known that the buffer in which cortical lawns are resuspended influences the extent of Ca^{2+} sensitivity of fusion [8,24]. In our case, egg cortices were prepared with a low extent of Ca^{2+}-dependent CG fusion in order to observe the possible in vitro effect of mastoparan. As noted in Fig. 2A, the extent of Ca^{2+}-dependent CG fusion was lower than 25% within the first 7 min. Fig. 2A shows that simultaneous addition of 100 μM Ca^{2+} and mastoparan increased by 80% the percentage of fusion with respect to Ca^{2+} alone. In agreement with the result shown in Fig. 1A, it is also observed that mastoparan-17 did not stimulate any fusion increase beyond the values observed for Ca^{2+} alone. Additionally, Fig. 2B shows that the in vitro effect of mastoparan on CG fusion was concentration-dependent.

Since mastoparan stimulated in eggs a Ca^{2+}-independent CG exocytosis (Table 1), the experiments shown in Fig. 3 were conducted to resolve whether the in vitro stimulatory effect of mastoparan depended on Ca^{2+}. As observed, mastoparan was able to trigger fusion in the nominal absence of Ca^{2+}. On the other hand, isolated CG can fuse in the presence of micromolar Ca^{2+} in the mode CG–CG [9–11,25,26]. We observed that mastoparan also triggered CG–CG fusion (not shown).Therefore, the results of Fig. 3 and Table 1 argue that mastoparan is acting at a late, Ca^{2+}-independent site in CG exocytosis signaling. The Ca^{2+}-independent effect displayed by mastoparan has also been documented in other cells such as chromaffin cells and insulin-secreting cells [18,19].

It has been proposed that mastoparan mimics the G-protein-binding domain on receptors and binds to the α-subunits of G_s and G_i/G_o at their receptor-binding sites [16]. Additionally, the α-subunits of G_s and G_i/G_o can be ADP-ribosylated by cholera and pertussis toxins, respectively, and this modification alters its function. In an attempt to identify the putative G-protein that serves as target for mastoparan action, we used the in vitro fusion assay to test the effect of ADP-ribosylation by bacterial toxins on mastoparan-induced CG fusion. In egg cortices, cholera toxin ADP-ribosylated a 48 kDa protein suggesting the presence of G_s while pertussis toxin ADP-ribosylated a 42 kDa protein which could be

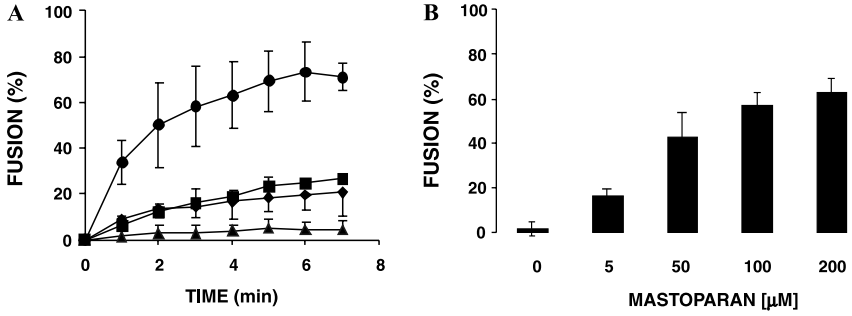

Fig. 2. Mastoparan stimulates in vitro cortical granule fusion. (A) Egg cortices from *L. pictus* sea urchin eggs were incubated in fusion medium containing: (▲) no Ca^{2+} and 5 mM EGTA, (◆) 100 μM free Ca^{2+}, (●) 100 μM free Ca^{2+} plus 100 μM mastoparan, or (■) 100 μM free Ca^{2+} plus 100 μM mastoparan-17. (B) In vitro fusion was recorded after 6 min of incubation of egg cortices in fusion medium with different mastoparan concentrations. Fusion was measured by a turbidimetric assay as described in Materials and methods. Results are means ± SD of five independent determinations made with cortical lawns prepared from different egg batches.

G_i or G_o (results not shown). Neither cholera or pertussis toxins affected mastoparan-induced CG fusion (data not shown), suggesting that G_s, G_i, and G_o are not involved. However, we cannot eliminate the possibility that a heterotrimeric G-protein, insensitive to ADP-ribosylation by cholera or pertussis toxins, participates in the mechanism of CG exocytosis. On the other hand, there is evidence to suggest that mastoparan can also activate some small G-proteins, specifically Rho [27] and Cdc42 [20]. The small G-proteins RhoA [21] and Rab3 [28] have been identified in sea urchin CG. The exotoxin C3, which inactivates RhoA by ADP-ribosylation, had no effect on the extent of fusion induced by mastoparan in egg cortices (not shown), suggesting that RhoA is not involved. Rab3 could be the candidate for mastoparan action since it participates in CG fusion [28]. It is plausible, however, that mastoparan effects on CG fusion might not be mediated by a G-protein.

In summary, the results presented here indicated that a Ca^{2+} sensing site in the fusion machinery of sea urchin egg cortical granules is overcome by mastoparan action and suggest that a G-protein is involved in the mechanism of cortical granule discharge.

Acknowledgments

We are grateful to Sergio Arias and Mario Pedraza for critical comments on the manuscript. This investigation was supported by CONACyT (28196N) and University of Guanajuato to J.G.S. A scholarship to T.I.G. was from CONACyT and CONCyTEG.

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