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Mast cell activation by pedicellarial toxin of sea urchin, *Toxopneustes pileolus*

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Pedicellarial toxin, partially purified from the sea urchin *Toxopneustes pileolus*, dose-dependently and time-dependently caused histamine release from rat peritoneal mast cells. Pedicellarial toxin induced a rapid initial rise in $[Ca^{2+}]_i$ within several seconds which was followed by a further slower increase of $[Ca^{2+}]_i$ (second rise). The toxin induced a dose-dependent formation of inositol 1,4,5-triphosphate (IP3) as well as the histamine release in mast cells. Furthermore, the toxin stimulated phosphoinositide-specific phospholipase C (PI-PLC) activity in mast cell membranes. 2-Nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbamate (NCDC), a PLC inhibitor, inhibited the activation of PI-PCL induced by pedicellarial toxin. Cholera toxin inhibited pedicellarial toxin-induced histamine release, whereas pretreatment of pertussis toxin failed to inhibit it. These results suggest that pedicellarial toxin from *T. pileolus* activates PI-PCL and the stimulation of PI turnover may lead to the release of IP3 into the cytoplasm, resulting in histamine release from rat mast cells.

Pedicellarial toxin, Histamine release; Mast cell; IP3; PI-PLC

1. INTRODUCTION

The globiferous pedicellariae of the toxopneustid sea urchins, *Toxopneustes pileolus* and *Tripneustes gratilla* contain toxic substances [1–5]. The pharmacological properties of the pedicellarial venom of the sea urchin *T. pileolus* have been studied in some tissues [6,7]. We have previously demonstrated that an extract from the pedicellariae of *T. pileolus* caused a release of histamine from rat peritoneal mast cells [8]. More recently, we reported also that a toxic substance (P-II fraction), fractionated from the pedicellariae of *T. pileolus* caused histamine release from mast cells and the release appeared to be sensitive to the glycolytic pathway [9].

The activation of mast cells by cross-linking of the high affinity IgE-Fc receptor (FcεRI) induces the secretion of a variety of performed biological active substances that mediate allergic and inflammatory responses [10]. The biochemical events involved in the triggering of histamine release suggested that the bridging of IgE receptors on mast cells results in activation of various membrane associated enzymes [11,12], mobilization of intracellular calcium and enhancement of the hydrolysis of phosphoinositide [13].

The present study was undertaken to evaluate the mode of action of pedicellarial toxin, partially purified from *T. pileolus*, on rat peritoneal mast cells and in particular, to examine the involvement of phospholipase C activation in the secretory process.

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2. MATERIALS AND METHODS

2.1. *Partial purification of pedicellarial toxin*

Pedicellarial toxin was partially purified from the globiferous pedicellariae of the sea urchin *T. pileolus* as reported previously [14]. Briefly, the crude extract from the pedicellariae was subjected to the gel filtration of a Sephadex G-200 column (2.6 × 65 cm) equilibrated with 0.15 M NaCl solution. The active fraction (P-II fraction) was dissolved in 0.02 M Tris-HCl buffer (pH 7.4) containing 0.4 M NaCl and applied on a concanavalin A-Sepharose column (1 × 10 cm) equilibrated with the same buffer. The sample was eluted, and unretained protein fractions were obtained as Pre-Con A fraction In the present experiments, Pre-Con A fraction was pooled and further fractionated in a column (2.6 × 60 cm) of Sephadex G-75 using 0.15 M NaCl solution. The active fraction was recovered into second protein peak (F-II fraction). The biological activity (U) of F-II fraction was bioassayed for the isometric contraction of longitudinal muscle of the isolated guinea-pig ileum in an oxygenized Krebs–Ringer bicarbonate at 37°C, by the modified method of Kimura et al. [14] Total protein of F-II fraction was 10.26 mg and its specific activity was 9,000 U/mg protein and this fraction was used as pedicellarial toxin in the present experiment.

2.2. *Preparation of mast cells*

Rat peritoneal mast cells were obtained from male Wistar rats

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(200–300 g) [15] and mast cells were purified using a method of Németh and Röhlich [16]

2.3. *Assay of histamine*

The mast cell suspensions in Tyrode–HEPES solution (pH 7 4) were incubated in duplicate at 37°C for 5 min before the addition of an appropriate concentration of various agents. Tyrode–HEPES solution contained 124 mM NaCl, 4 0 mM KCl, 1 0 mM $CaCl_2$, 10 mM $NaHCO_3$, 5.6 mM glucose, 0 64 mM NaH_2PO_4, 0 5 mM $MgSO_4$, 5 0 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES), 50 mg of bovine serum albumin (BSA) per liter. BSA was omitted from the solution in experiments on histamine release. At intervals, the reaction was stopped by the addition of 1 mM Tris–EDTA buffer. The Tris EDTA buffer contained 25 mM Tris, 120 mM NaCl, 5 mM KCl, 1 mM EDTA and 20 mg human serum albumin per liter. Then, the cells were separated from the released histamine by centrifugation at $1300 \times g$ for 10 min at 4°C. Residual histamine remaining in the cells was released by distrupting the cells with 100% trichloric acid (TCA), and centrifugation at $1500 \times g$ for 15 min at 4°C. Histamine content was determined fluorometrically [17]. The amount of histamine released was calculated as percentage of the total histamine present in the control suspension.

2.4. *Measurement of intracellular calcium concentration*

Fluoresense was recorded using a fluorimeter (model 650-40 Fluorescence 100, Hitachi) with a temperature-controlled cuvette and a magnetically driven stirrer. Purified mast cells (1×10^6 cells/ml) were incubated at 37°C for 10 min with 100 μM Quin-2/AM 2-[(2-amino-5-methylphenoxy)methyl]-6-methoxy-8-aminoquinoline-*N,N,N,N*-tetraacetic acid, tetraacetoxymethyl ester in Tyrode–HEPES solution. The cell suspension was diluted 1:10 with Tyrode-HEPES solution, left to stand for 60 min, and then washed twice with complete solution Samples of the cell suspension (1×10^5 cells/ml) were placed in the cuvette described above, and agents were with a microsyringe directly into the cuvette, without interrupting the recording. The cell suspension was incubated at 37°C for 5 min and challenged with pedicellarial toxin. Fluorescence excitation and emission wavelengths were 339 nm and 492 nm, respectively. The concentration of intracellular Ca^{2+} was calculated by the method of Tsien et al. [18]

2.5. *Determination of IP3*

Measurment of IP3 was carried out using a commercially available kit (Amersham) and the manufacturer's protocol. Briefly, mast cells were extracted with chloroform/methanol (1:2) on ice for 10 min. Methanol fractions containing phosphorylated inositols were lysophilized and mixed with bovine adrenal IP3-binding proteins in the presence of limiting amount of tracer D-myo-[^{3}H]inositol 1,4,5-triphosphate The mixtures were centrifuged at $2000 \times g$ for 10 min and radioactivity bound to IP3-binding protein was measured in a β-scintilation counter.

2.6. *Measurement of PI-PCL activity*

The activity of PI-PLC in rat mast cell membrane fraction was measured as described by Mustelin et al. [19]. Purified mast cells were suspended in Tyrode- HEPES solution. After washing, mast cells were resuspended in 1 ml lysis buffer containing 25 mM Tris (pH 7.5), 25 mM sucrose, 0.1 mM EGTA and 5 mM $MgCl_2$. The cells were homogenized by sonication and the samples were centrifuged at $1000 \times g$ to sediment the nuclei. The post-nuclear supernatant was centrifuged at $10,000 \times g$ for 30 min. The cytosolic fraction was recovered and the sedimentable membrane pellet was resuspended in lysis buffer. Protein was determined by the method of Bradford [20]. Membrane preparation of mast cells was assayed in a final volume of 50 μl containing 20 mM sodium phosphate (pH 6 8), 40 mM KCl, 1 mM sodium pyrophosphate, 0.65% octylglucoside, 0.4 mM EGTA, 0 8 mM $CaCl_2$, aprotinin (10 μg/ml), leupeptin (10 μg/ml) and 0 2 mM [^{3}H]phosphatidylinositol-4,5-biphosphate (5 μCi/ml). The mixture was incubated at 37°C for 15 min and the reaction was stopped by adding 100 μl of 1% bovine serum albumin, followed by 500 μl of 10% TCA The precipitate was removed by centrifugation and the radioactivity present in 0 5 ml of the supernatant was determined. The counts from a control incubation with no enzyme (buffer only, 284 ± 13 cpm) was subtracted from all values

3. RESULTS

Pedicellarial toxin induced histamine release from rat peritoneal mast cells in a dose-dependent manner (Fig. 1). The toxin-induced histamine release was evoked with the concentrations higher than 20 μg/ml and reached a maximum at a concentration of 1000 μg/ml. At 20, 80 and 400 μg/ml the histamine release induced by pedicellarial toxin at the incubation for 15 min was 6 ± 1%, 14 ± 3% and 29 ± 4%, respectively. The toxin, at its optimal concentration of 400 μg/ml, produced a time-dependent release of histamine from the mast cells. The histamine release was essentially complete within 15 min (data not shown).

Purified mast cells were challenged with 80 μg/ml of pedicellarial toxin. The toxin induced a rapid increase in $[Ca^{2+}]_i$ within several seconds and the maximal increase was about 170 nM (data not shown). As shown in Fig. 2, at 20, 40, 80, 200 and 400 μg/ml, the initial increase in $[Ca^{2+}]_i$ induced by pedicellarial toxin was 40 ± 4, 69 ± 4, 168 ± 4, 173 ± 5 and 186 ± 7 nM, respectively.

There are several mechanisms involved in the regulation of intracellular Ca^{2+}, some which function to control Ca^{2+} entry into the cell through the plasma membrane, and others which release Ca^{2+} from an intracellular pool by the second messenger IP3 [21]. Therefore, we examined the effect of pedicellarial toxin on IP3

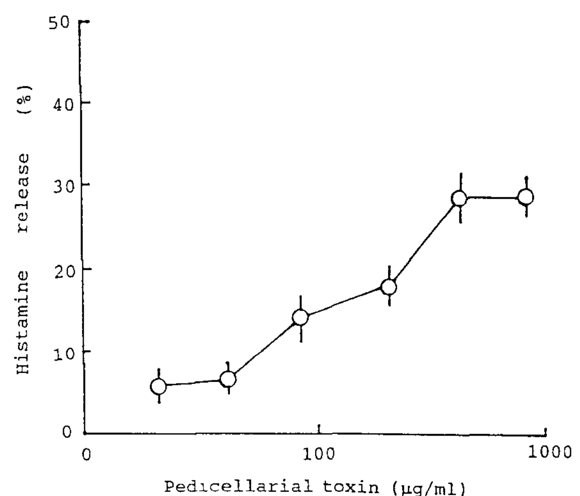

Fig. 1. Dose–response curve for the histamine release from mast cells induced by pedicellarial toxin. The mast cells (5×10^5 cells/ml) were incubated with pedicellarial toxin for 10 min Spontaneous histamine release from mast cells was 5.3 ± 0 3%, and this value was subtracted from each experimental value. Each point represents the mean of 5 experiments and vertical bars indicate S.E.M.

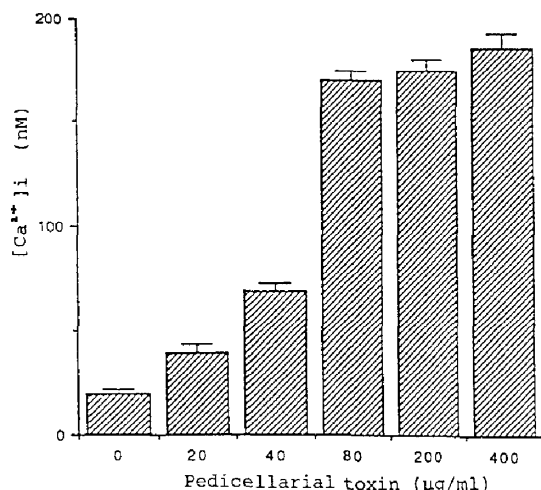

Fig. 2. Effect of pedicellarial toxin on $[Ca^{2+}]_i$ increase in mast cells. The mast cells (1×10^5 cells/ml) were incubated at 37°C for 5 min and challenged with various concentrations of pedicellarial toxin. The increase in $[Ca^{2+}]_i$ in the cells was measured as described in section 2. Each point represents the mean of 5 experiments and vertical bars indicate S.E.M.

generation. Fig. 3 shows kinetics of IP3 generation induced by pedicellarial toxin (20–400 μg/ml). The toxin at a concentration of 40 μg/ml induced a rapid increase in IP3 generation in mast cells, the maximal value was 4.9 ± 0.4 pmol/10^7 cells after 20 s. The levels decreased to 0.5 ± 0.02 pmol/10^7 cells after 50 s. At 20, 80, and 400 μg/ml, the toxin-induced IP3 generation at 20 s was 1.7 ± 0.6, 5.6 ± 0.7 and 6.0 ± 0.9 pmol/10^7 cells, respectively.

The above results suggest that pedicellarial toxin-induced generation of IP3 is due to a mechanism which mediates PI-PLC activation in mast cells. Thus, we examined the effect of pedicellarial toxin on the activity of PI-PLC in the membrane fraction (Fig. 4). At 20, 80 and 200 μg/ml, the activity of PI-PLC induced by the toxin was 39 ± 4, 73 ± 6 and 75 ± 6 pmol/IP3, respectively. Moreover, the observed effect of the toxin could result from either direct or indirect activation of PI-PLC. To investigate this point, we made use of 2-nitro-4-carboxyphenyl-*N*,*N*-diphenyl-carbamate (NCDC), an inhibitor of phospholipase C. At 100 μM, NCDC produced half-maximum inhibition of PI-PLC activation induced by pedicellarial toxin (Fig. 4).

Pertussis toxin (PT) and cholera toxin (CT) have been found to inhibit phospholipase C activation [22–24]. We examined whether PT or CT inhibits histamine release from mast cells induced by pedicellarial toxin. The cells were preincubated with PT or CT at a concentration of 10 μg/ml at 37°C for 3 h, and then PT- and CT-treated cells were incubated with the toxin (400 μg/ml) for 15 min. PT failed to inhibit the toxin-induced histamine release, whereas CT inhibited the histamine release to 68% of control (data not shown).

4. DISCUSSION

The present study is the first report that pedicellarial toxin from *T. pileolus* causes the activation of PI-PLC and generates IP3 in dose-dependent manner, and then induces histamine release from rat mast cells.

Mast cells release inflammatory mediators through the reaction of cell-bound IgE antibodies with multivalent antigen [10] and through various IgE-independent stimuli [25]. The cellular events that take place following stimulation of mast cells include breakdown of inositol phospholipids, transient increase of the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$, generation of arachidonic acid, and increase in cyclic nucleotides [26]. In the present study, pedicellarial toxin induced a rapid initial increase in $[Ca^{2+}]_i$ within several seconds. It has been suggested that the initial rise in $[Ca^{2+}]_i$, due to Ca^{2+} mobilization, correlates with the histamine release promoted by the various secretagogues [27]. Thus, the response to the toxin appears to be due to the release of Ca^{2+} from internal stores in mast cells.

Rapid increase in the generation of IP3 was observed with 20 s after addition of pedicellarial toxin. The generation of IP3 and the rise in $[Ca^{2+}]_i$ in mast cells activated by the toxin showed a close correlation. Recent evidence demonstrates that IP3 stimulates Ca^{2+} release from the intracellular stores in mast cells [28].

It seems that the inositol phospholipid cascade requires to activate Ca^{2+} release. Therefore, we examined the activation of PI-PLC by pedicellarial toxin in mast cell membrane fraction. The toxin produced the activation of PI-PLC at the same concentration range similar

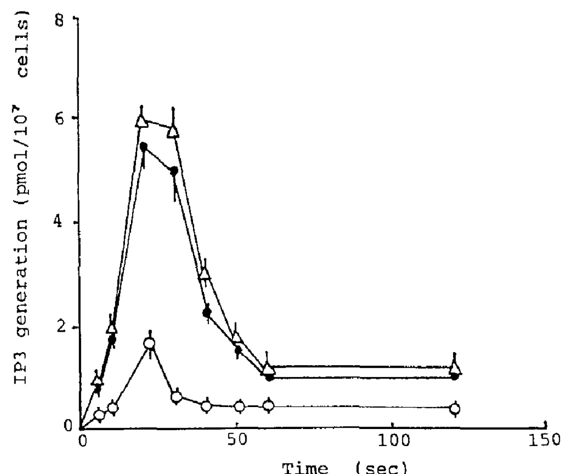

Fig. 3. Kinetics of pedicellarial toxin-induced generation of IP3. The mast cells (1 × 10 cells/ml) were incubated at 37°C for 5 min and challenged with various concentrations of pedicellarial toxin. IP3 was analyzed as described in section 2. The generation of IP3 in control mast cells was 0.3 ± 0.05 pmol/10 cells and this value was subtracted from experimental values. Each point represents the mean of 5 experiments and vertical bar indicate S.E.M. ○, 20 μg/ml; ●, 80 μg/ml; △, 400 μg/ml.

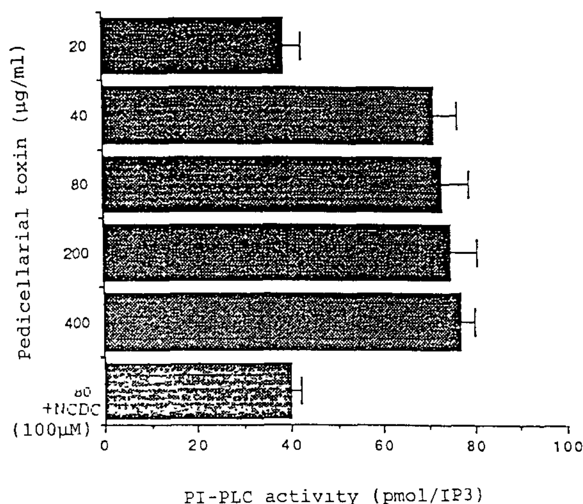

Fig. 4. Effect of pedicellarial toxin on the enzymatic activity of PI-PLC and inhibition of PI-PLC activity by NCDC. The cells (1 × 10 cells/ml) were disrupted by sonication and pellet and cytosol fractions were assessed for PLC activity by the procedures described in section 2. Abscissa represents pmoles IP3 formed by hydrolysis of [^{3}H]-phosphatidylinositol 4,5-bisphosphate. Each point represents the mean of 5 experiments and vertival bars indicate S.E.M.

to that for histamine release. Furthermore, the stimulatory effect of the toxin on the activity of PI-PLC was inhibited by NCDC. We previously demonstrated that NCDC inhibited histamine release, the initial rise in $[Ca^{2+}]_i$ and the generation of IP3 in mast cells activated by anti-IgE [29].

CT caused the inhibition of the toxin-induced histamine release, whereas PT treatment failed to inhibit the toxin-induced histamine release from mast cells. The histamine release induced by the toxin could not be caused by cell surface–receptor interaction, since it might be caused by a direct interaction of the CT-activated GTP-binding proteins with PLC. In some cells, CT was shown to inhibit receptor-mediated activation of phospholipase C [30,31]. Our data suggest that in rat mast cells CT might activate GTP-binding proteins, which are involved in regulation of pedicellarial toxin-induced activation of phospholipase C.

The pedicellarial toxin showed at least three protein bands (mol. wt. = 17–30 kDa) by SDS-polyacrylamide gel electrophoresis. We have currently isolated a protein component in the toxin by high performance liquid chromatography and it showed a molecular weight of 18 kDa (unpublished observation). The characterization of the 18 kDa component is under investigation.

From these results, we suggest that pedicellarial toxin activates PI-PLC and stimulation of PI turnover may lead to the release of IP3 into the cytoplasm, and then IP3 may trigger mobilization of intracellular Ca^{2+}, resulting in histamine release from rat mast cells. PI-PLC activation may be a prerequisite for histamine release from rat mast cells induced by pedicellarial toxin. Pedicellarial toxin and purified pedicellarial toxin from *T. pileolus* should be useful in studies on the functional connection between the G-protein involved and PI-PLC that regulates histamine release from mast cells.

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