Thrombin and IgE antigen induce formation of inositol phosphates by mouse E-mast cells

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Received 28 June 1986, revised version received 30 July 1986

Stimulation of murine chondroitin sulfate E containing mast cells (E-MC) in vitro either by thrombin or immunologically resulted in a rapid formation of inositol phosphates (IPs). Increase in all of the three IPs (IP_1, IP_2 and IP_3) could be detected 20 s after stimulation. The depletion of Ca^{2+} from the medium resulted in more than 80% reduction in β-hexosaminidase release from either thrombin or IgE antigen stimulated cells. However, both thrombin and IgE antigen increased the formation of IP_3 under these conditions independent of the presence of extracellular Ca^{2+}.

(E-mast cell) Inositol phosphate

1. INTRODUCTION

The murine cultured chondroitin sulfate E containing mast cells (E-MC) have been shown to degranulate upon challenge with physiological stimulus such as IgE antigen or thrombin [1]. The immunological activation secretion response of cultured E-MC by IgE antigen was found to be similar to that induced by thrombin in the release of preformed mediators such as histamine and β-hexosaminidase [1] and transmembrane activation of adenylate cyclase [2]. However, in contrast to the IgE-mediated activation, stimulation of E-MC by physiological concentration of thrombin did not result in the oxidation of arachidonic acid through the 5-lipoxygenase pathway [1]. Measurement of calcium fluxes employing $^{45}Ca^{2+}$ as a tracer has revealed that the process of degranulation in cultured E-MC triggered by either one of the physiological stimuli is accompanied by an increased uptake of $^{45}Ca^{2+}$ [3].

Activation of rat heparin-containing mast cells (H-MC) with a specific stimulus such as IgE antigen, calcium ionophore A23187 or compound 48/80 is accompanied by enhanced metabolism of PI [4] and in the de novo synthesis of PI [5,6]. More recently it has been shown that in rat basophilic leukemia (RBL) cell line, PI and its phosphorylated derivatives are rapidly broken down after immunological stimulation [7].

Here, rapid formation of IPs was observed in cultured mouse E-MC challenged either by thrombin or immunologically.

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Abbreviations: GPI, glycerophosphatidylinositol; IgE, immunoglobulin E; IPs, inositol phosphates; IP_2, inositol 1,4-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; PI, phosphatidylinositol; TG, Tyrode's buffer

2. EXPERIMENTAL

2.1. *[^{3}H]Inositol labelling of cultured mouse E-MC*

Mouse E-MC were grown and differentiated by culturing mouse bone marrow cells as in [1].

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00145793/86/$3.50

40 μCi *myo*-[2-^{3}H]inositol (16.5 Ci/mmol, New England Nuclear, Boston, MA) was added to each set of culture containing 20 × 10^6 cells in 15 ml culture medium. After 17 h at 37°C the cells were washed with TG containing 1 mM Ca^{2+}, 0.3 mM Mg^{2+} and 0.05% gelatin.

Duplicate samples of 4 × 10^6 cells were suspended in 500 μl TG with or without 1.5 mM LiCl and activated by incubation for specified time intervals at 37°C with defined concentrations of bovine thrombin. Alternatively, duplicate samples of 4 × 10^6 washed and labelled cells were sensitized by incubation for 1 h with 20 μg mouse monoclonal anti-DNP IgE [1] washed, suspended in 500 μl TG with or without 1.5 mM LiCl and challenged with 300 ng DNP-BSA [1]. In other sets of experiments duplicate samples of [^{3}H]inositol-labelled 4 × 10^6 cells prepared as above were challenged by IgE antigen or 0.5 U thrombin for 20 or 60 s in calcium-free TG containing 2 mM EGTA. Reactions were

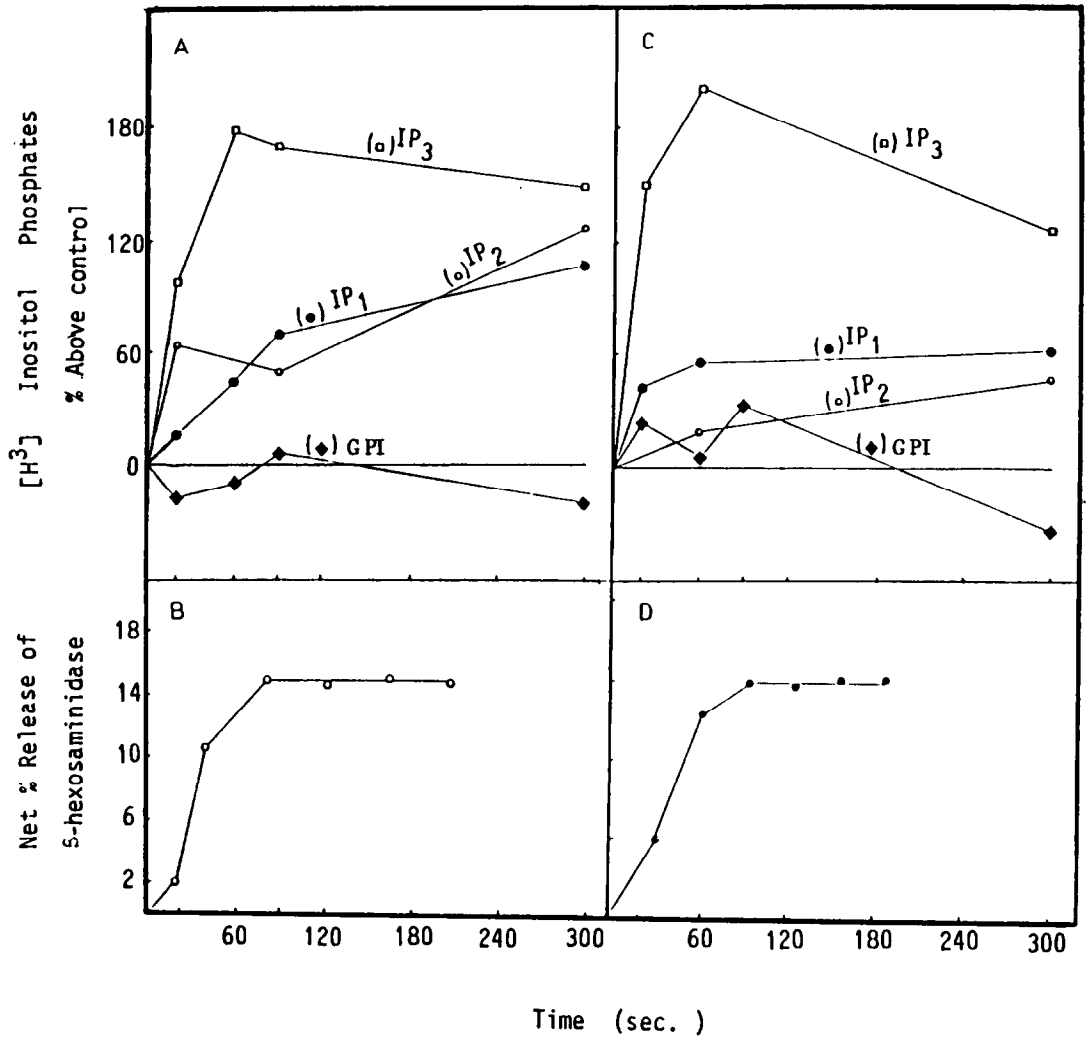

Fig.1. Kinetics of antigen or thrombin-induced [^{3}H]IP formation in [^{3}H]inositol-labelled cultured mouse E-MC (A,C) as compared to their effect on degranulation (B,D). (A,B) IgE-sensitized E-MC challenged with antigen; (C,D) thrombin (0.5 U/10^6 cells) stimulated E-MC. The data in panels A and C represent the percent change in accumulation of [^{3}H]IPs and [^{3}H]GPI in stimulated as compared to unstimulated cells. Each point represents the mean of 4 separate experiments whereas the SE is <30% in A, and <5% in B, D. The p value for each point was $0.001 < p < 0.01$.

terminated by the addition of 2 ml chloroform-methanol (2:1) to each sample. The solutions were vortex-mixed and the organic and aqueous phases separated by centrifugation (500 × *g* for 5 min). The upper layers were separated and mixed with 2 ml H_2O and analyzed as below.

2.2. *Assay of [^{3}H]IPs*

The assay of [^{3}H]IPs was performed as described in [7]. The methanol/water solutions were applied to 3.5 × 0.5 cm columns containing 1 ml anion-exchange resin (Dowex 1-X8, 20–50 mesh, Bio-Rad, CA), converted to the formate form by extensive washing with 1 N formic acid. Inositol, GPI, inositol 1-phosphate (IP_1), IP_2 and IP_3 were sequentially eluted with water, 3 ml (inositol), with 5 ml of 60 mM ammonium formate and 5 mM sodium tetraborate (GPI); with 7 ml of 200 mM ammonium formate and 100 mM formic acid (IP_1); 400 mM ammonium formate and 100 mM formic acid (IP_2); and 1 M ammonium formate and 100 mM formic acid, respectively (IP_3). The eluates were collected and determined for their ^{3}H content by liquid scintillation counting. Unlabelled *myo*-inositol, IP_1, IP_2 and IP_3 (Sigma) were used as markers for the column. 95% of the unlabelled IPs were recovered from the column after elution.

Total [^{3}H]inositol lipids were estimated by mixing the chloroform phase of the cell extracts with 3 ml chloroform-methanol (2:1). The solution was washed twice with 2.5 ml methanol containing 1 mM KCl and 10 mM *myo*-inositol and after removal of the solvent by evaporation at 22°C its ^{3}H content was determined by liquid scintillation counting.

2.3. *Degranulation assays of E-MC*

Duplicate samples of 1 × 10^6 cells were suspended in 500 μl TG or calcium-free TG containing 2 mM EGTA and incubated for a specified time at 37°C with or without defined concentrations of thrombin. Alternatively, 1 × 10^6 IgE-sensitized cells in TG or calcium-free TG containing 2 mM EGTA were challenged with 75 ng DNP-BSA [1]. The cells were centrifuged at 400 × *g* at 22°C, and the supernatants and pellets were assayed for either histamine [2] or β-hexoaminidase [8] and the net percentage release of the preformed mediators was calculated [8].

3. RESULTS

Stimulation of [^{3}H]inositol-labelled E-MC either by thrombin or immunologically resulted in a rapid formation of radioactive IP_1, IP_2 and IP_3 (fig.1). After 17 h incubation, 4 × 10^6 E-MC were found to have incorporated 69000 ± 7900 dpm (mean ± SE, n = 4) of *myo*-[^{3}H]inositol into

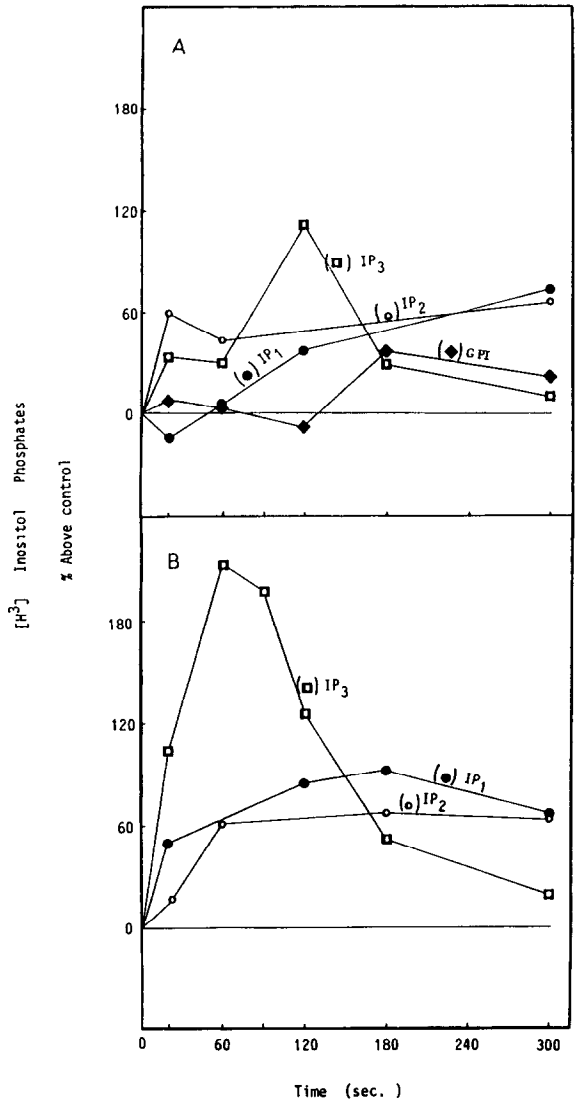

Fig.2. Kinetics of PI breakdown in E-MC stimulated by either IgE antigen (A) or thrombin (B) in the presence of 1.5 mM Li^+. Each point represents the mean of 4 separate experiments where the SE is <30% and the p value for each point was $0.001 < p < 0.01$.

phospholipids. The amount of [^{3}H]inositol extracted as free inositol composed 18.6% ± 1.8 (mean ± SE, $n = 4$) of the total dpm incorporated. The IPs, which were extracted after the cells were triggered by either one of the stimuli represented 4.2% of the total dpm incorporated into the phospholipids. The increase in IP_3 plateaued after 60 s in cells triggered by either one of the stimuli, whereas IP_1 and IP_2 plateaued after 60 s in cells triggered with thrombin and continued to rise throughout the first 5 min in cells stimulated by IgE antigen.

To determine whether lithium, a known inhibitor of the IP_1 phosphatase [9] could affect the

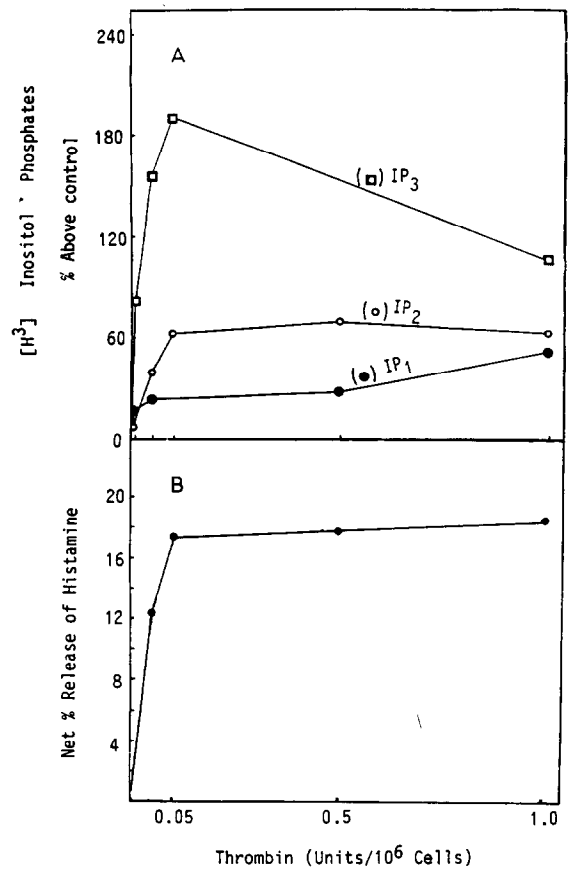

Fig.3. The effect of thrombin concentration on IP formation (A), and histamine release (B) from E-MC stimulated for 60 s. Each point represents the mean of 4 separate experiments where the SE is <30% in A and <5% in B. The p value for each point is $0.001 < p < 0.01$.

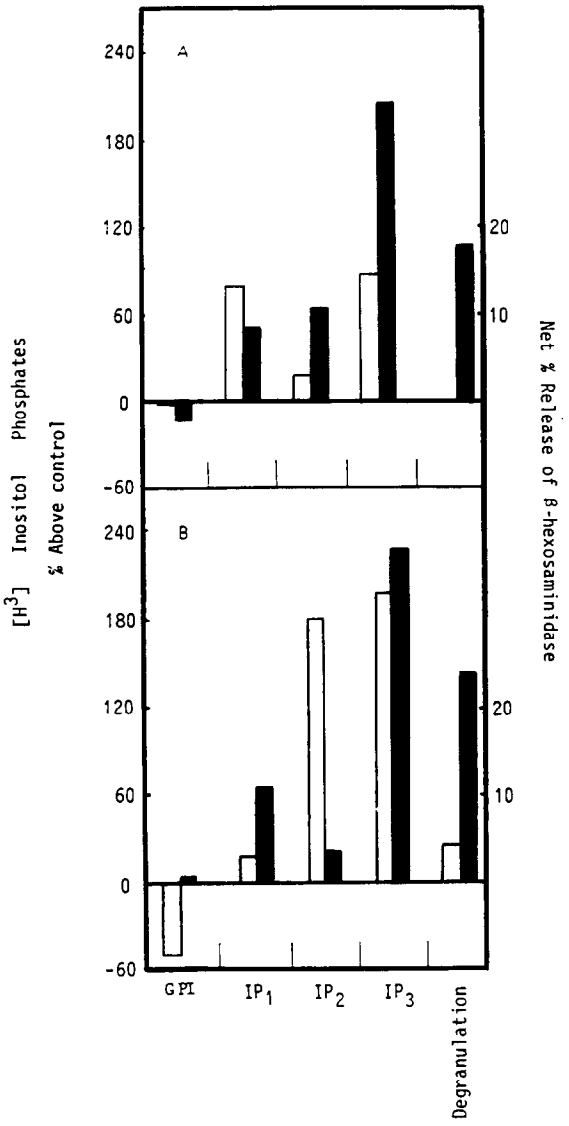

Fig.4. Effect of extracellular Ca^{2+} on the [^{3}H]PI breakdown in [^{3}H]inositol-labelled E-MC stimulated by either IgE-antigen (A) or thrombin (B). PI metabolites were measured 60 s after the stimulation. Empty bars represent E-MC triggered in Ca^{2+}-free medium in the presence of 2 mM EGTA. Full bars represent cells stimulated in the presence of 1 mM Ca^{2+}. The value for degranulation after IgE antigen or thrombin stimulation in the presence of calcium was 18 and 24%, respectively, whereas in the absence of Ca^{2+} the values were 0 and 4%. Each column represents the mean of 3 separate experiments where the SE is <30% for the PI metabolites and <5% for degranulation. The p value for each point was $0.001 < p < 0.01$.

pattern of IP formation, E-MC were stimulated in the presence of lithium. A sharp decrease in [^{3}H]IP_3 was observed after 60 s in thrombin-stimulated E-MC in the presence of 1.5 mM Li^+ (fig.2). Lithium did not affect the secretion response in E-MC by either one of the stimuli (not shown).

The formation of all three [^{3}H]IPs by thrombin was concentration-dependent and correlated with the concentration-response curve for degranulation (fig.3).

To determine the role of extracellular Ca^{2+} in the PI breakdown following cell stimulation, E-MC were challenged by either one of the stimuli in calcium-free medium in the presence of 2 mM EGTA. The depletion of Ca^{2+} from the medium resulted in more than 80% reduction of β-hexosaminidase release from either IgE antigen or thrombin-stimulated cells. However, both thrombin and IgE antigen caused increased formation of [^{3}H]IP_3 under these conditions (fig.4).

4. DISCUSSION

The uncoupling between Ca^{2+} influx and PI breakdown (fig.4) was found to be consistent with the report regarding antigen-stimulated RBL [7] and surprising in view of the sensitivity of phospholipase C to Ca^{2+} concentration [10,11]. The formation of IPs in the E-MC activated in the absence of extracellular Ca^{2+} may reflect an activation of an as yet unidentified phospholipase C which acts at low cytosolic Ca^{2+} concentrations. However, the formation of some IPs was unaltered and some decreased, dependent on the stimulus used, suggesting a more complex situation.

The release of histamine and β-hexosaminidase from the activated E-MC was not paralleled by that of IP_3 accumulation (figs 1,3). However, the data do not allow us to conclude yet whether the breakdown of PI plays an essential role in the biochemical events leading to degranulation in E-MC.

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