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

D. Baranes, F.T. Liu* and E. Razin+

*Institute of Biochemistry, Hebrew University Medical School, PO Box 1172, Jerusalem 91010, Israel* and **Department of Immunology, The Medical Biology Institute, La Jolla, CA 92037, USA*

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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.

+ To whom correspondence should be addressed

*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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40 $\mu$Ci *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 $\mu$l 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 $\mu$g mouse monoclonal anti-DNP IgE [1] washed, suspended in 500 $\mu$l 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

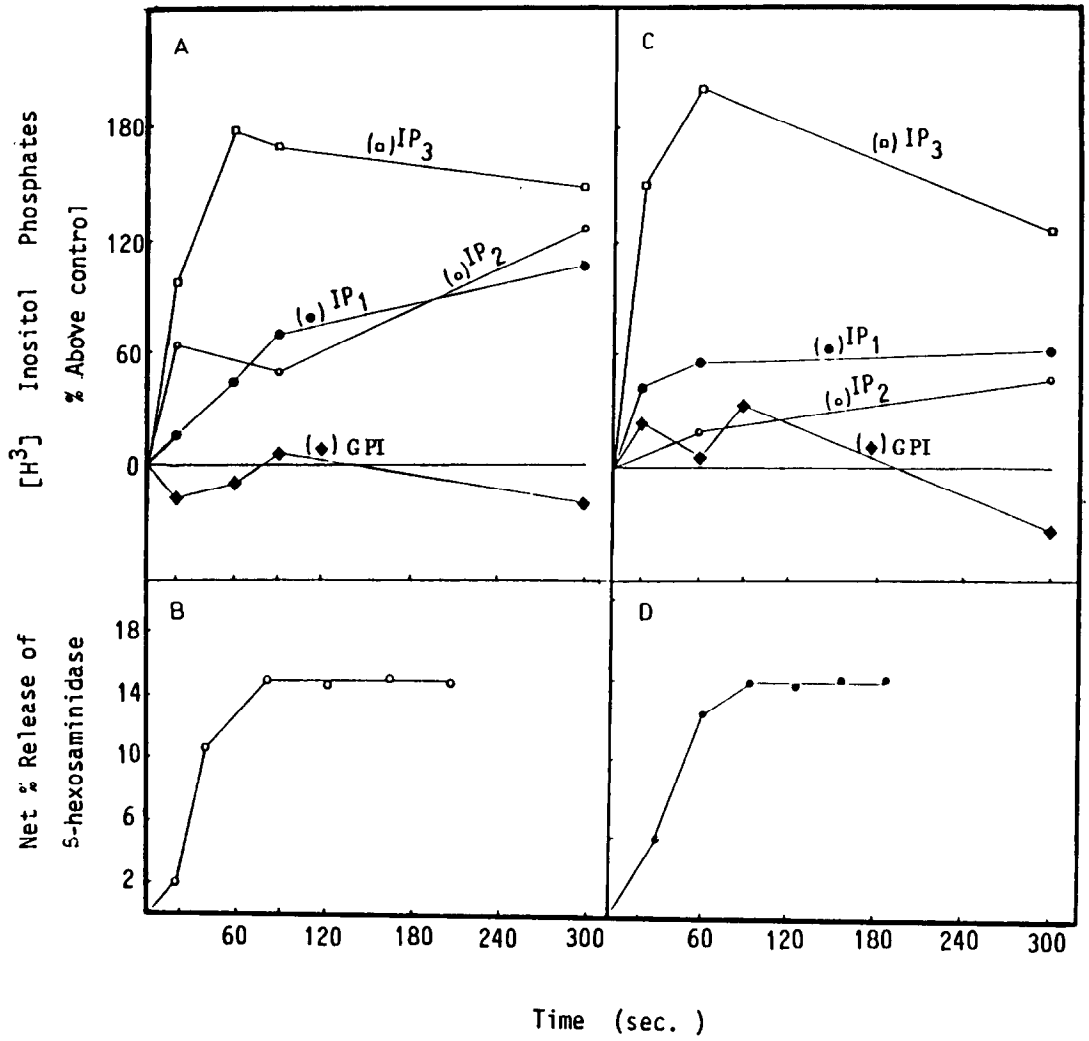


Fig.1. Kinetics of antigen or thrombin-induced [$^3$H]IP formation in [$^3$H]inositol-labelled cultured mouse E-MC (A,C) as compared to their effect on degranulation (B,D). (A,B) IgE-sensitized E-MC challenged with antigen; (C,D) thrombin (0.5 U/$10^6$ cells) stimulated E-MC. The data in panels A and 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

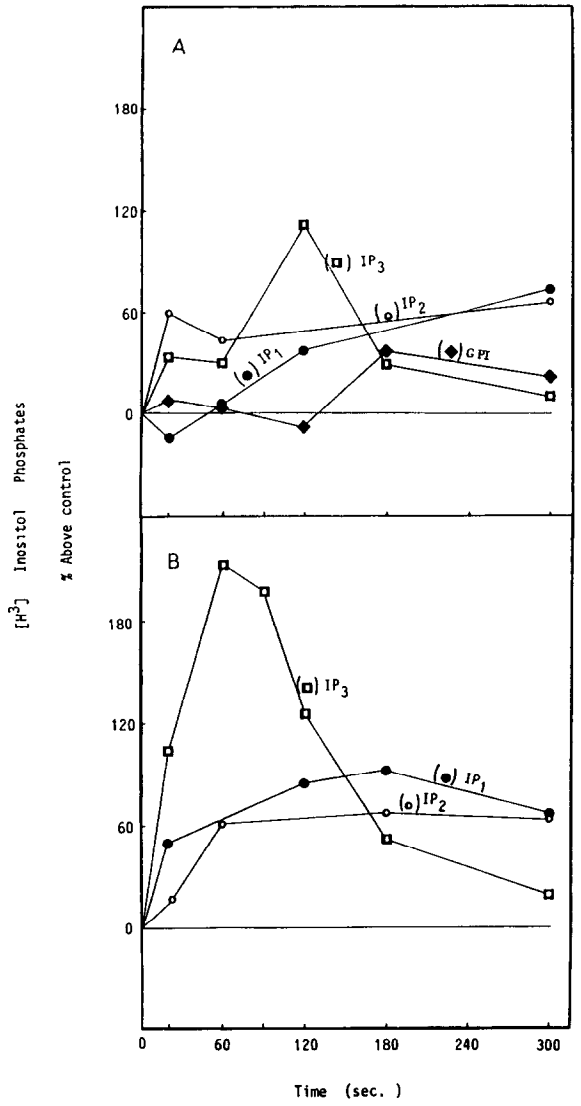


Fig.2. Kinetics of PI breakdown in E-MC stimulated by either IgE antigen (A) or thrombin (B) in the presence of 1.5 mM $Li^+$. Each point represents the mean of 4 separate experiments where the SE is <30% and the $p$ value for each point was $0.001 < p < 0.01$.

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

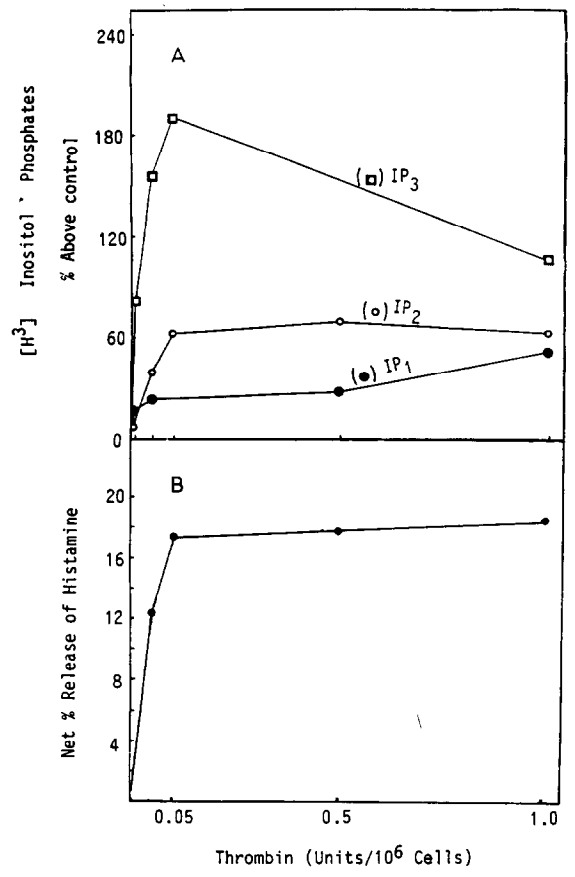


Fig.3. The effect of thrombin concentration on IP formation (A), and histamine release (B) from E-MC stimulated for 60 s. Each point represents the mean of 4 separate experiments where the SE is <30% in A and <5% in B. The $p$ value for each point is $0.001 < p < 0.01$.

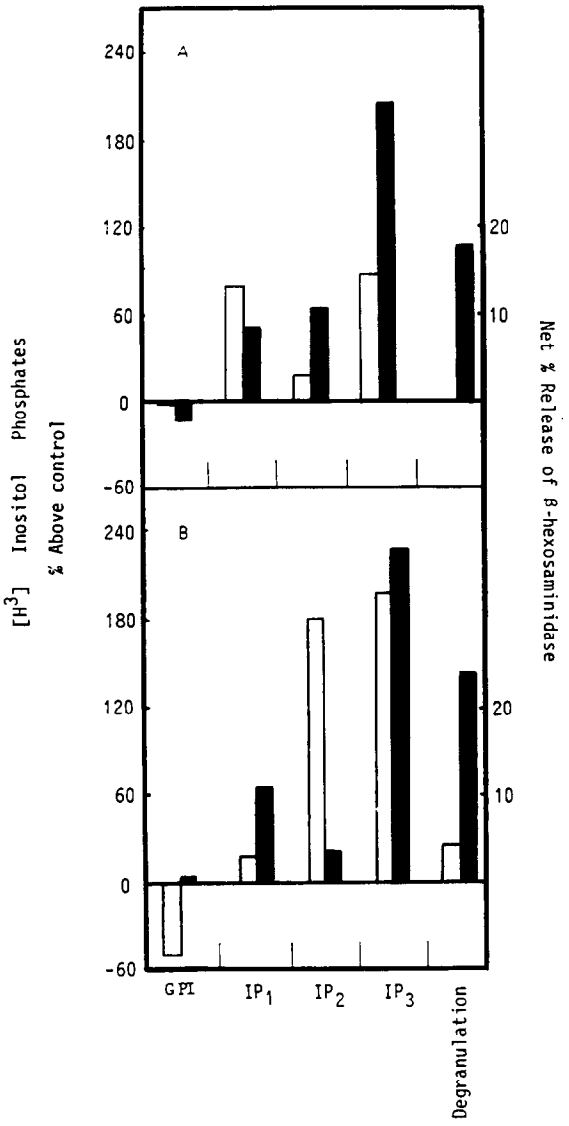


Fig.4. Effect of extracellular $Ca^{2+}$ on the [$^3$H]PI breakdown in [$^3$H]inositol-labelled E-MC stimulated by either IgE-antigen (A) or thrombin (B). PI metabolites were measured 60 s after the stimulation. Empty bars represent E-MC triggered in $Ca^{2+}$-free medium in the presence of 2 mM EGTA. 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 $\beta$-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 $\beta$-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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