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Protein tyrosine phosphatase activity enhancement is induced upon Fc_ε receptor activation of mast cells

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Abstract

Immunological stimulation of rat mucosal type mast cells (line RBL-2H3) by clustering the type I Fc_ε receptor ($Fc_\varepsilon RI$) causes a fast and transient tyrosine phosphorylation of several proteins. This implied the involvement of both, protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPases) in that process. In order to identify the PTPases involved in these very early steps coupling $Fc_\varepsilon RI$ stimulus to cell response, we undertook the purification and characterization of PTPases present in RBL-2H3 cells. In one of the cells' membranal fractions, a PTPase activity was found to be enhanced 2- to 3-fold upon cell stimulation by $Fc_\varepsilon RI$ clustering. Characterization of this activity implies its involvement in control of the $Fc_\varepsilon RI$ signalling cascade.

Key words: RBL-2H3 line; Immunological signal transduction; Rat mucosal mast cell

1. Introduction

Protein tyrosine phosphorylation is a key element in cellular signal transduction cascades and is therefore a highly regulated process [1–3]. It is controlled by the specific action of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPases), [1,4,5]. While the diverse types of PTKs have been extensively characterized for their regulation mechanisms and physiological substrates, considerably less is known about these aspects pertinent to PTPases. Two distinct general families of PTPases have so far been identified: cytosolic and transmembranal. One of the better studied PTPases is the transmembranal CD45 [6,7], a hematopoietic-cell specific molecule [8,9]. CD45 is apparently essential for the very early signalling events generated via the T-cell receptor [10–12]. It has been shown that CD45 dephosphorylates the carboxy-terminal phosphotyrosine (Tyr^{505}) of the PTK lck – a member of the src-family and thereby activates it [13].

CD45 has also been shown to activate by dephosphorylation another member of the src-family; fyn, also involved in the signal transduction of T-cells [14]. This suggested an activating function of CD45 and positions it at one of the earliest steps in the stimulus-response coupling cascade of T-cells. However, it is still unknown how the activity of CD45 is regulated. It has been shown that it undergoes phosphorylation on tyrosine residues by an unknown PTK upon T-cell stimulation [15] yet no influence of this modification on the CD45 PTPase activity could be established. Recently, a decrease in the CD45 PTPase activity was reported upon increasing intracellular free Ca^{2+}-ion concentration, induced by ionomycin [15].

Mast cells serve as a paradigm for investigating immunological stimulus–response coupling cascades initiated by clustering antigen receptors, e.g. the type 1 Fc receptor for IgE ($Fc_\varepsilon RI$). This immunological stimulation leads to the association of the src-family member PTKs lyn or yes with the $Fc_\varepsilon RI$ and their activation [16]. As a consequence, several cellular proteins undergo transient phosphorylation of their tyrosine residues. Among these are two of the $Fc_\varepsilon RI$ subunits namely the β- and γ-chains [17] phospholipase Cγ1 (PLCγ1) [18,19] and a 72-kDa protein (pp72) [20,21] that has been recently identified to be the tyrosine kinase $p72^{syk}$ [22]. This phosphorylation is very transient and depends on $Fc_\varepsilon RI$ clustering as its abrogation by addition of an excess of monovalent hapten was found to accelerate rapid dephosphorylation of these proteins [17–19]. This indicates that these processes are highly regulated and involve both the action of PTKs and PTPases.

In order to try and resolve the PTPase(s) involved in the early $Fc_\varepsilon RI$ signalling steps in mast cells we studied PTPase activities present in RBL-2H3 cells and report here the partial purification and characterization of a PTPase that exhibits marked activity enhancement upon $Fc_\varepsilon RI$ clustering.

2. Materials and methods

2.1. Reagents and cell-culture media

O-Phospho-L-tyrosine, *O*-phospho-L-serine, *O*-phospho-L-threonine, 4-nitrophenyl phosphate (pNPP), Triton X-100, phenyl arsine oxide, sodium orthovanadate, phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin and dithiotreitol were all obtained from Sigma Chemical Co. St. Louis, MO, USA. Powdered culture media as well as fetal calf serum were purchased from Gibco, Grand Island, NY, USA. Penicillin–streptomycin mixture was from Bio-lab, Jerusalem, Israel. DE-52 was from Whatman, Maidstone, Kent, UK. Thapsigargin was

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obtained from Calbiochem Corp., La Jolla, CA, USA. Bovine serum albumin (BSA) was derivatized with an average of eleven 2,4 dinitrophenyl residues (DNP_{11}-BSA) in our laboratory from BSA (fraction V) (Sigma Chemical Co.) and 1-fluoro,2,4,-dinitrophenyl-benzene (DNP). DNP-specific IgE class mAb A_2 [25] was grown as ascites in mice. Rat mucosal mast cells, subline RBL-2H3 [26] were originally obtained from Dr. R. Siraganian (NIH, Bethesda, MD). They were maintained in Dulbecco's modified EAGLE's medium (DMEM), supplemented with 10% FCS, 2 mM glutamine and antibiotics in a humidified atmosphere with 7% CO_2 at 37°C. Cells from confluent cultures were detached and harvested by incubation with 10 mM EDTA in medium for 15 min.

2.2. Preparation of subcellular fractions

RBL-2H3 cells (3×10^8) were detached and harvested as indicated above. The following procedure was carried out on ice, unless indicated otherwise: The cells were washed in cold Tyrode's buffer (130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 10 mM HEPES, 0.1% BSA, pH 7.4) and were swollen by treatment with the hypotonic homogenization buffer (25 mM Tris, 2% glycerol, 2 mM DTT, 1 mM PMSF, 10 μg of aprotinin/ml, 1 μg of pepstatin/ml) at 3×10^8 cells in 3 ml for 15 min. Following that, the cells were homogenized by 40 strokes in a Dounce homogenizer. Nuclei and unbroken cells were removed by centrifugation ($10^3 \times g$, 5 min, 4°C). The supernatant was centrifuged ($10^5 \times g$, 60 min, 5°C) in order to separate the soluble fractions. The pellet was then treated with 3 ml of homogenization buffer to which Triton X-100 was added (1% final concentration). The resultant suspension was recentrifuged as before, but only for 20 min. The supernatant was taken as membranal fraction.

2.3. Partial purification of PTPases

Cytosolic and membranal fractions were each fractionated on DE-52 columns (2 ml volume in Bio-Rd Poly-Prep 0.8 × 4 cm columns) which were equilibrated in homogenization buffer. The cytosolic and membranal fractions were each loaded on the columns. The columns were washed with 2 ml homogenization buffer and proteins were then eluted by a step-wise increase in ionic strength of the eluting homogenization buffer (0.06, 0.12, 0.25 and 0.5 M NaCl). Three ml of each salt concentration were used and 1 ml fractions were collected.

2.4. Assays for PTPase activity

Each eluted fraction was assayed for its PTPase activity using, with some modifications the protocol originally described by Mustelin et al. [25]: Briefly, 50 μl of the fraction and 50 μl of the substrate (5 mM *O*-phosphotyrosine in 1 M sodium acetate, pH 6.0) were mixed and incubated for 30 min at 37°C. The reaction was stopped by addition of 150 μl of 25% (w/v) TCA and 50 μl BSA (10 mg/ml). The precipitated proteins were removed by centrifugation and the supernatants were used for measurement of the produced inorganic phosphate. To this end 100 μl of the supernatant were transferred to 96-well microtiter plates and to each well 100 μl of Reagent C (1 vol of 6 N sulfuric acid, 1 vol of distilled water, 1 vol of 2,5% ammonium molybdate, 1 vol of 10% ascorbic acid) were added, mixed well and incubated for 30 min at 37°C. The absorbance of this solution at 620 nm was measured against the blank by an ELISA plate reader. The control expressing the basal hydrolysis rate of the substrate contained 50 μl of homogenization buffer instead of an eluted fraction. PTPase activity is presented as μmol inorganic phosphate produced per min per mg protein present in the sample. In some assays *O*-phosphothreonine or *O*-phosphoserine were employed as substrates using the same protocol. The basal hydrolysis values were for: *O*-phosphotyrosine 8×10^{-4} mM P_i/min, *O*-phosphoserine 5.8×10^{-3} mM P_i/min and *O*-phosphothreonine 2.4×10^{-4} mM P_i/min. These values and the background values were subtracted.

PTPase activity was also assayed by measuring the hydrolysis of pNPP monitoring the absorption changes due to the formation of *p*-nitrophenol. The reaction mixture consisted of 50 μl of 10 mM pNPP in 1 mM EDTA, 2.5 mM DTT, 0.1% Triton X-100, 50 mM Bis-Tris, pH 6.0 and 50 μl of fraction and was incubated at 37°C for 30 min. The reaction was terminated by the addition of 100 μl of NaOH (250 mM). The color was measured at 405 nm by an ELISA plate reader. The control expressing the basal hydrolysis of the substrate only contained the homogenization buffer instead of an eluted fraction sample.

2.5. β-Hexoseaminidase activity assay

The secretory response of RBL-2H3 cells was monitored by measuring the activity of the granule-stored enzyme β-hexoseaminidase appearing in the cells' supernatants as described previously [26]. Briefly, cells (20×10^6) were suspended in medium with excess of monoclonal DNP-specific IgE (1 μl of ascites/10^6 cells) to saturate the $Fc_\epsilon RIs$ and were plated in 96-well plates at 2×10^5 cells/well. On the following day, monolayers were washed in Tyrode's buffer and incubated for 45 min at 37°C with a range of different DNP_{11}-BSA concentrations. Following the incubation, 20 μl samples of the supernatants were transferred to a different plate and 50 μl of substrate solution (1.3 mg/ml *p*-nitrophenyl-*N*-acetyl-β-D-glucosamine in 0.1 M citrate, pH 4.5) were added. The plates were incubated for 1 h at 37°C. The reaction was stopped with 150 μl of 0.2 M glycine, pH 10.7. The color formed due to the substrate hydrolysis was measured at 405 nm in an ELISA plate reader. Results are reported as percent of the total β-hexoseaminidase content of the cells (determined by lysis of control cells by 1% Triton X-100) after subtracting the spontaneous release observed in the absence of secretagogue. Each assay was performed in triplicate.

3. Results

3.1. Partial purification of protein tyrosine phosphatases of RBL-2H3 cells

Both cytosolic and membranal fractions of RBL-2H3 cells were prepared and screened for their PTPase activity. To this end, RBL-2H3 cells (3×10^8) were harvested, homogenized and the homogenate was separated into cytosolic and membrane fractions as described above. In both fractions, considerable phosphatase activities were observed. Both were further characterized following anion- exchange chromatography on DE-52 columns using step-wise elution with a range of NaCl concentrations (0.06 M–0.5 M NaCl in 0.025 M Tris, 2% glycerol, pH 7.4). In the cytosolic fractions, 3 peaks of activity were reproducibly observed (data not shown). Three main peaks of activity were resolved in the membranal

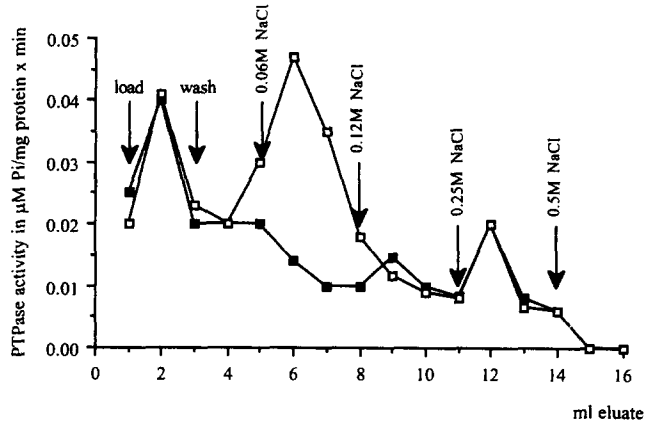

Fig. 1. Elution profile of PTPase activity: The membranal fraction of the cells homogenate was loaded onto a DE-52 column equilibrated in the homogenization buffer and eluted with different NaCl concentrations (indicated in the figure) in the same buffer. One ml fractions were collected. Each fraction was tested for its PTPase activity. Samples from two different cell preparations were tested; resting (-■-) and antigen stimulated (-□-). This figure represents a typical elution profile, observed in more than 7 independent experiments.

fraction (Fig. 1), one was observed immediately in the run-through of the column, the next peak eluted at 0.06 M NaCl, and the third eluted at 0.25 M NaCl concentration. Fig. 1 represents a typical elution profile of PTPase activity of the membranal fraction that was observed in 7 independent experiments.

3.2. Enhancement of PTPase activity upon immunological stimulation of RBL-2H3 cells.

In order to investigate whether the PTPase activities are affected by the $Fc_\varepsilon RI$ mediated stimulation of the RBL-2H3 cells, the cells (3×10^8) were harvested and its $Fc_\varepsilon RI$ saturated by incubation with a DNP-specific monoclonal IgE for 2 h. One portion (1.5×10^8) of the cells was then stimulated for 1 min with an optimal dose of antigen (100 ng DNP_{11}-BSA/ml), while the other portion was left as a control. Both cell samples were then separately homogenized, the homogenates were fractionated on DE-52 columns and the fractions examined for their PTPase activity as described above. Whereas no difference could be observed between resting and activated cell samples in the three peaks of PTPase activity derived from the cytosolic fraction, a significant (up to 3-fold) enhancement was observed in the activity of the second membranal peak derived from stimulated cells as compared to that monitored in the same peak derived from resting cells (Fig. 1).

3.3. PTPase activity depends on the degree of immunological stimulation

In order to examine whether the enhancement of the PTPase activity correlates with the secretory response to immunological stimulation of the cells, antigen dose dependence of both processes were measured in parallel.

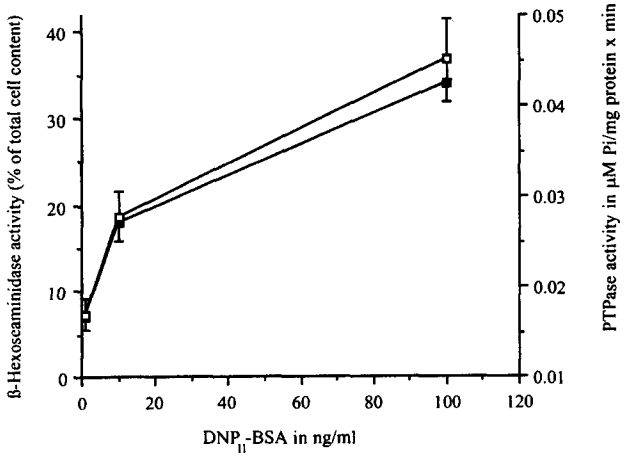

Fig. 2. Correlation between the dose–response to antigen stimulation of the PTPase activity and mediator release from RBL-2H3 cells. PTPase activity present in peak II of the membranal fraction (–□–) was assayed as described with *O*-phosphotyrosine as a substrate. Mediator release (–■–) was assayed by the activity of secreted β-hexoseaminidase. The measured β-hexoseaminidase activity is reported as percent of the total enzymatic activity content of the cells after subtraction of spontaneous release of the enzyme.

The PTPase activity of membranal peak II exhibited a clear dependence on the concentrations of the stimulating antigen (Fig. 2). At low antigen concentrations (1ng DNP_{11}-BSA/ml) the activity was hardly increased while at 10 ng/ml it was already doubled and at 100 ng/ml the activity was nearly tripled as compared to that observed in this fraction derived from resting cells. These findings matched the antigen dose dependence of the secretory response as monitored by the secreted activity of the granule-stored β-hexoseaminidase activity (Fig. 2).

3.4. Dependence of the PTPase activity enhancement on extracellular Ca^{2+} ions

In order to try and localize where in the $Fc_\varepsilon RI$ coupling cascade the PTPase activity enhancement takes place, the stimulation of RBL-2H3 cells was performed in the absence of extracellular Ca^{2+}. Under such condition no elevation of the PTPase activity present in the membranal peak II could be observed upon $Fc_\varepsilon RI$ clustering when compared to that present in the same peak obtained from resting cells.

3.5. Influence of increase in intracellular free Ca^{2+} ions on PTPase activity

Since the observed PTPase activity depends on the presence of extracellular Ca^{2+}-ions, we examined whether it can be affected by a rise in the concentration of free cytosolic Ca^{2+}-ion ($[Ca^{2+}]_i$). We employed two reagents known to artificially increase $[Ca^{2+}]_i$: Thapsigargin (TG) and ionomycin. The cells were incubated with an optimal dose of Thapsigargin (2 μM) [27] or ionomycin (1 μM) for the indicated time periods, were then homogenized and fractionated as described above. In no case could an increase in the PTPase activity of the membranal peak II be observed compared to that activity measured in the same peak derived from untreated cells.

3.6. Characterization of the PTPase activity of membranal peak II

3.6.1. Substrate specificity. To further characterize the observed PTPase activity present in membranal peak II (resting and stimulated), its specificity was studied by measuring hydrolysis of other phosphate esters in addition to *O*-phospho-L-tyrosine, namely *O*-phospho-L-threonine, *O*-phospho-L-serine and *p*-nitrophenyl phosphate (pNPP). As shown in Fig. 3, neither *O*-phospho-L-threonine nor *O*-phospho-L-serine were found to undergo significant hydrolysis above basal levels, while both *O*-phospho-L-tyrosine and *p*-nitrophenyl phosphate were hydrolysed.

3.6.2. pH dependence of the PTPase activity. The PTPase activity of the membranal peak II was assayed across the 4.0 to 9.0 pH range. It displays a pH optimum ranging from pH 5.0 to pH 6.0, with significant drop of

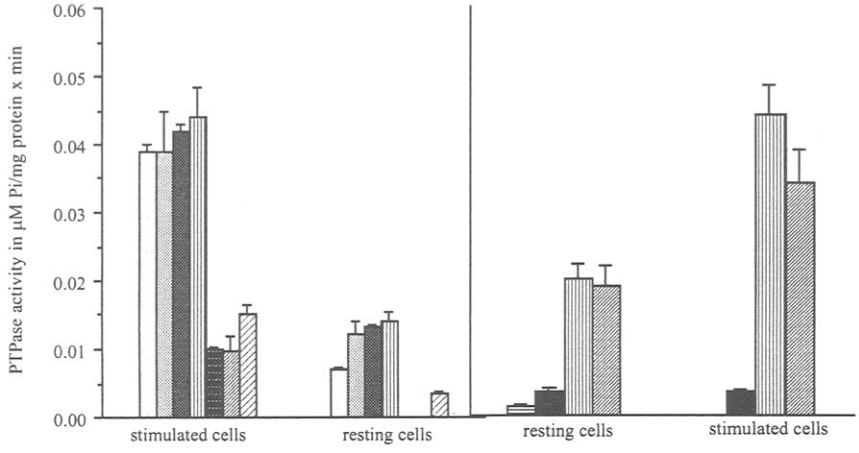

Fig. 3. Characterization of the PTPase activity present in membranal peak II. The right panel shows the substrate specificity of the activity present in this peak assayed utilizing the following phosphate esters, *O*-phosphotyrosine (vertical stripes), *O*-phosphoserine (black bars), *O*-phosphothreonine (horizontal stripes) and pNPP (cross-hatched bars). All phosphate esters were tested at a 5 mM concentration in 0.5 M sodium acetate pH 6.0. The left panel shows the PTPase activity present in membranal peak II in the presence of each of the two PTPase inhibitors; sodium orthovanadate and phenylarsine oxide (PAO). To this end, the samples were incubated as before but in the presence of the respective inhibitor at the indicated concentration. For the inhibition studies involving PAO, DTT was omitted from both the control as well as from the sample containing PAO for the time of the assay only. Sodium orthovandate: (200 μM, black stippled bar; 100 μM, narrow cross-hatch; 10 μM, wide cross-hatch), PAO: (50 μM, white bars; 10 μM, light-shaded bars; 1 μM, dark-shaded bars). Control experiments were carried out without additon of any inhibitor (vertical stripes).

activity at pH < 4.5 and practically no activity at pH > 8.5.

3.6.3. Kinetics of PTPase activity. We next examined whether the PTPase activity of membranal peak II also follows Michaelis–Menten kinetics. Hydrolysis of *O*-phosphotyrosine and pNPP was assayed over a range of concentrations at pH 6.0. For both substrates, saturation kinetics were observed with K_m of 10 mM and 6 mM for pNPP and *O*-phosphotyrosine, respectively. The V_{max} values were 0.044 μM of released P_i/mg protein × min and 0.034 μM of released P_i/mg protein × min for *O*-phosphotyrosine and pNPP, respectively.

3.6.4. Effect of PTPase inhibitors. The phosphatase activity of membranal peak II was further characterized by studies using two widely known PTPase inhibitors, vanadate ions and phenyl arsine oxide (PAO): as shown in Fig. 3, the PTPase activity present in samples prepared from both resting as well as from $Fc_\varepsilon RI$ stimulated cells was abolished already by relatively low concentrations of vanadate ions (at 10 μM Vanadate only 33% of activity of the stimulated sample and 25% of the resting control remained). The other PTPase inhibitor, PAO caused only a slight reduction in the activity even at higher concentrations (90% of activity at 50 μM PAO for the activated sample and 50% at the same concentration for the resting control).

4. Discussion

Phosphorylation of specific tyrosyl residues of proteins is a widespread control mechanism employed at crucial steps in cellular signal transduction. Thus PTPases have a functional role central in the circuitry of signalling cascades as for example illustrated by their specific regulatory involvement in T-cell activation, where the PTPase CD45 is essential [8–10]: CD45 has been shown to dephosphorylate the regulatory Tyr^{505} of lck [11], thus activating this src-family member PTK and initiating the signalling cascade in T-cells [12].

We wish to resolve the role of PTPases in the initial phase of $Fc_\varepsilon RI$ stimulus-secretion coupling cascade of mast cells. To this end, PTPase activity present in different fractions of the mast cell model system, i.e. the RBL-2H3 cell line was investigated in resting and $Fc_\varepsilon RI$ activated state. We found that the PTPase activity present in one membranal fraction of the activated cells exhibits significant elevation compared to that of the same fraction isolated from resting, control cells. We further observed that the described PTPase activity is specific to aromatic phosphate esters as illustrated by hydrolysis of phosphotyrosine and *p*-nitrophenylphosphate whereas no activity towards phosphoserine and phosphothreonine was observed. Further studies with established inhibitors of alkaline phosphatase (Bromotetramizole) and of serine/threonine phosphatase (okadaic acid) were carried out and in both cases no inhibition of the phosphatase activity of the membranal peak II could be resolved. The high K_m is probably due to the use of these low molecular weight substrates rather than of physiological high molecular weight ones. Similar specific activities where observed for other PTPases [29–32].

Two PTPase inhibitors were employed earlier in trying to identify the site of action of PTPases in the $Fc_\varepsilon RI$

signal transduction. Adamczewski et al. [33] have described the inhibitory action of PAO on the generation of second messengers (e.g. inositol phosphates, Ca^{2+} ions) and of the eventual secretory response of RBL-2H3 cells to $Fc_\varepsilon RI$ clustering. Whereas the tyrosine phosphorylation levels of the $Fc_\varepsilon RI$ subunits and of several other proteins were found to be unaffected, that of PLCγ_1 was markedly reduced. This was interpreted to suggest that at least two distinct PTPases are involved in the $Fc_\varepsilon RI$ coupling cascade of these cells, one being PAO sensitive, while the other one is not. Zick and Sagi-Eisenberg [34] showed that treatment of the same cells with hydrogen-peroxide and vanadate ions has quite a different effect. Namely, the stimulation of PLCγ_1 activity and the secretion of mediators without $Fc_\varepsilon RI$ clustering. Our present results show that the $Fc_\varepsilon RI$ mediated enhancement of a PTPase activity is strongly inhibited by vanadate ions while PAO does not affect it significantly. This inhibition pattern suggests that the enhanced PTPase activity might be due to one of the enzymes described by Adamczewski et al. and by Zick and Sagi-Eisenberg, namely a PAO-insensitive and vanadate ion-sensitive activity.

In order to get an insight in to where this PTPase activity is involved in the coupling cascade, we performed the above described two sets of experiments; first the cells were stimulated in the absence of nominal extracellular Ca^{2+}-ions and no enhancement was observed. Thus extracellular Ca^{2+} is required for the $Fc_\varepsilon RI$ mediated activity enhancement Second, we examined whether an increase in $[Ca^{2+}]_i$, artificially induced by thapsigargin or ionomycin, would affect the activity of the isolated peak II PTPase. No difference in that activity could be resolved by either of the two agents. Thus, elevation of $[Ca^{2+}]_i$ does not, by itself, activate the PTPase present in membranal peak II. Hence the activity enhancement could either be initiated upstream to steps causing the calcium signal, or the increase in $[Ca^{2+}]_i$ alone is not sufficient for causing it and other biochemical events initiated by the $Fc_\varepsilon RI$ are required for the PTPase activation.

We conclude that the here described PTPase activity enhancement is likely to have a control function in the $Fc_\varepsilon RI$ signal transduction cascade of RBL-2H3 cells. Whether a PTPase causes the activation of the src PTK phosphorylation pathway analogous to that described for CD45 in T-cells or it is involved in a desensitization process requires further studies.

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