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# Requirement of Calcium Influx for Hydrolytic Action of Membrane Phospholipids by Cytosolic Phospholipase $A_2$ Rather than Mitogen-Activated Protein Kinase Activation in FcεRI-Stimulated Rat Peritoneal Mast Cells[1]

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**The mechanism by which cytosolic phospholipase $A_2$ ($cPLA_2$) is not responsible for eicosanoid production in rat peritoneal mast cells upon antigen stimulation [Ishimoto *et al.* (1996) *J. Biochem.* 120, 616-623] was investigated in the mast cells stimulated by cross-linking of the IgE receptor or with thapsigargin. Stimulation with thapsigargin, but not with antigen, resulted in apparent lysophosphatidylcholine (lysoPC) formation. Antigen stimulation significantly increased the activities of mitogen-activated protein (MAP) kinase and $cPLA_2$. These activities were further potentiated by phorbol ester. The antigen elicited a rapid and transient increase in intracellular $Ca^{2+}$ concentration, while thapsigargin produced a slow and sustained increase. Furthermore, a combination of antigen and thapsigargin rapidly increased and prolonged the intracellular $Ca^{2+}$ concentration. Under these conditions, lysoPC was apparently generated, whereas it was not in response to antigen alone. These results suggest that a prolonged increase in the intracellular $Ca^{2+}$ concentration is required for $cPLA_2$ to associate with membranes, thus leading to hydrolysis of membrane phospholipids by the enzyme.**

**Key words: cytosolic phospholipase $A_2$, intracellular calcium concentration, lysophosphatidylcholine, mast cell, mitogen-activated protein kinase.**

Arachidonic acid is liberated from membrane phospholipids in a variety of cells by the hydrolytic action of phospholipase $A_2$ ($PLA_2$) upon stimulation with physiological agonists. The release of arachidonic acid, which is the limiting step for eicosanoid production, is thought to be achieved mainly by 85-kDa cytosolic $PLA_2$ ($cPLA_2$), since the enzyme has a preference for arachidonic acid in the *sn*-2 position of phospholipids (*1-3*). Studies suggest that the $cPLA_2$ is phosphorylated by mitogen-activated protein (MAP) kinase, its activity is increased, then it is translocated from the cytosol to membranes to release arachidonic acid in an intracellular $Ca^{2+}$-dependent manner upon stimulation (*1-3*). In cultured mast cells, an increase in $cPLA_2$ phosphorylation by MAP kinase correlates with arachidonic acid release as well as eicosanoid production in response to cross-linking of the IgE receptor (FcεRI), suggesting that this cascade is involved in the hydrolytic action of $cPLA_2$ on membrane phospholipids (*4-8*). However, these findings do not show that the $cPLA_2$ actually hydrolyzes membrane phospholipids to liberate arachidonic acid in whole cells.

We provided evidence indicating that more than half of the total arachidonic acid is derived from diacylglycerol (DAG) lipase action on DAG, which is generated *via* the sequential actions of phospholipase D (PLD) and phosphatidate phosphohydrolase in rat peritoneal mast cells stimulated with calcium ionophore (*9, 10*). Other investigators have also reported that the PLD pathway is involved in arachidonic acid release as well as eicosanoid production in IgE receptor- or c-*kit* ligand-stimulated mast cells (*11, 12*). In addition, we found that arachidonic acid liberation and prostaglandin $D_2$ formation were fully dependent on the PLD pathway upon stimulation with cross-linking of the IgE receptor in mast cells (*13*). This finding indicates that $PLA_2$ activation is not associated with arachidonic acid liberation or eicosanoid production upon stimulation with antigen.

In the present study, we tried to elucidate the mechanism underlying the lack of involvement of $PLA_2$ in hydrolyzing membrane phospholipids intracellularly. From this view point, we focused on IgE receptor-stimulated activation of MAP kinase-$cPLA_2$ pathway and intracellular $Ca^{2+}$ behavior in rat peritoneal mast cells in comparison with thapsigargin stimulation.

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Abbreviations: $cPLA_2$, 85 kDa cytosolic phospholipase $A_2$; DAG, diacylglycerol; DNP-BSA, dinitrophenol-bovine serum albumin conjugate; FcεRI, high-affinity Fc receptor for IgE; lysoPC, lysophosphatidylcholine; lysoPS, lysophosphatidylserine; MAP, mitogen-activated protein; $PLA_2$, phospholipase $A_2$; PLD, phospholipase D; PMA, 4β-phorbol 12-myristate 13-acetate.

## MATERIALS AND METHODS

*Materials*—Bovine serum albumin (BSA, fraction V), thapsigargin and 4$\beta$-phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Chemical (St. Louis, MO). *p*-(Amidinophenyl)methanesulfonyl fluoride (APMSF) and DTT were from Wako Pure Chemical Industries (Osaka). Heparin was from Novo Nordisk (Denmark) and leupeptin was from Peptide Institute (Osaka). Lysophosphatidylserine (lysoPS) was from Avanti Polar Lipids (Alabaster, AL) and monoclonal anti-2,4-dinitrophenyl mouse immunoglobulin E (anti-DNP IgE) was from BioMakor (Israel). Fura 2 penta-acetoxymethyl ester (fura 2-AM) was from Dohjin Chemical (Kumamoto). Triacsin C was from Kyowa Medex (Tokyo). [$^3$H]Choline chloride (79.2 Ci/mmol) and 1-palmitoyl-2-[$^{14}$C]arachidonoyl-*sn*-glycero-3-phosphoethanolamine (57 mCi/mmol) were purchased from New England Nuclear (Boston, MA). Mitogen-activated protein (MAP) kinase assay kit was obtained from Amersham (Buckinghamshire, UK). Dinitrophenol-bovine serum albumin conjugate (DNP-BSA) used as the antigen was prepared as described previously (*14*). Other reagents were obtained from commercial sources.

*Mast Cells*—Mast cells were isolated and purified from the peritoneal cavity of Wistar rats as described previously (*10*). The purified mast cells were suspended in medium composed of 150 mM NaCl, 3.7 mM KCl, 3 mM $Na_2HPO_4$, 3.5 mM $KH_2PO_4$, 6 mM glucose, 1 mg/ml BSA, 1 mg/ml gelatin, and 10 U/ml heparin (pH 6.8).

*Labeling and Sensitization of Mast Cells*—Purified mast cells ($5\times10^6$ cells/ml) were incubated with [$^3$H]choline chloride (40 $\mu$Ci/ml) in the presence of anti-DNP IgE (1 $\mu$g/ml) at 37°C for 2 h. The cells were washed three times with the medium described above, then suspended in the same medium without heparin.

*Measurement of Lysophosphatidylcholine Levels*—The [$^3$H]choline-labeled mast cells or the cells sensitized with anti-DNP IgE ($5\times10^5$ cells/ml) were stimulated with various agents and the reaction was terminated by adding a sevenfold volume of ice-cold chloroform/methanol/HCl (200 : 200 : 1, v/v/v) and a threefold volume of 0.1 M KCl plus 5 mM EGTA. Lipids in the mixture were extracted and separated by thin layer chromatography on a Silica Gel G plate (Merck, Germany) using chloroform/methanol/water (65 : 35 : 6, v/v/v) as the developing system. Authentic lysophosphatidylcholine (lysoPC) was co-chromatographed and visualized by exposing the plate to iodine vapor. The area corresponding to lysoPC was scraped into scintillation vials and the radioactivity was determined by liquid scintillation counting.

*Assay of cPLA$_2$ and MAP Kinase Activity*—Mast cells ($5\times10^6$ cells/ml) sensitized with anti-DNP IgE were stimulated with various agents and the reaction was terminated by adding a one-third volume of 400 mM Tris-HCl (pH 8.5) containing 11 mM EGTA. The mixture was centrifuged at $600\times g$ for 5 min at 4°C. The pellet was suspended in 100 mM Tris-HCl (pH 8.5) containing 2 mM EGTA, 2 mM $Na_3VO_4$, 100 $\mu$M APMSF, 100 $\mu$M leupeptin, and 20 mM $\beta$-glycerophosphate, and then lysed by $N_2$ cavitation. The lysate was centrifuged at $100{,}000\times g$ for 1 h at 4°C. To measure cPLA$_2$ activity, the supernatant was mixed with 5 mM DTT for 10 min, then incubated with 4 $\mu$M 1-palmitoyl-2-[$^{14}$C]arachidonoyl-*sn*-glycero-3-phosphoethanolamine in the presence of 2 mM $CaCl_2$, 2 mM $Na_3VO_4$, and 100 mM Tris-HCl (pH 8.5) at 37°C for 30 min. After the reaction had been stopped, the released [$^{14}$C]arachidonic acid was extracted and determined as described by Sundaram *et al.* (*15*). MAP kinase activity was assayed in the supernatant using a commercial assay kit. The protein concentration in the supernatant was determined by means of a commercial assay kit using BSA as the standard.

*Measurement of Intracellular $Ca^{2+}$ Concentrations*—Mast cells ($5\times10^5$ cells/ml) sensitized with anti-DNP IgE were loaded with 2 $\mu$M fura 2-AM at 37°C for 30 min, washed twice with the medium described above, then suspended in the same medium without heparin. The fura 2-loaded mast cells ($5\times10^5$ cells/ml) were stimulated with DNP-BSA plus lysoPS and/or thapsigargin for 20 s prior to the addition of $CaCl_2$ at 37°C. The fluorescence of the suspension was continuously monitored in a spectrofluorometer (F-2000; Hitachi) with excitation at 340 nm and 380 nm, and emission at 500 nm. The reaction was stopped by adding 0.3% Triton X-100 to obtain maximal fluorescence. Thereafter, the minimum fluorescence was measured after adding 2 mM Tris-HCl (pH 8.5) containing 5 mM EGTA. The intracellular $Ca^{2+}$ concentration was calculated as described previously (*16*).

## RESULTS

*Thapsigargin- or Antigen-Induced LysoPC Formation*—We examined the effect of the extracellular $Ca^{2+}$ concentration on the hydrolytic action of PLA$_2$ on membrane phospholipids upon stimulation with thapsigargin or antigen. This was assayed as lysoPC formation as a result of PLA$_2$ hydrolytic action, because arachidonic acid release is also catalyzed by DAG lipase from DAG produced *via* the sequential actions of PLD-phosphatidate phosphohydrolase (*10, 13*). Stimulation of [$^3$H]choline-labeled mast cells with thapsigargin caused remarkable lysoPC formation dose-dependently upon the extracellular $Ca^{2+}$ concentration, whereas stimulation with antigen did not at any $Ca^{2+}$ concentration tested (Fig. 1). In similar experiments with [$^3$H]ethanolamine-labeled mast cells, thapsigargin stimulation produced a significant amount of lysophosphatidylethanolamine, while antigen stimulation did not (data not shown). These results indicate that thapsigargin-induced intracellular $Ca^{2+}$ mobilization is involved in the modulation of PLA$_2$ association with membrane phospholipids.

*Increase in MAP Kinase and cPLA$_2$ Activity upon Stimulation*—To examine whether the lack of hydrolyzing activity of PLA$_2$ in response to antigen is due to an insufficient increase in PLA$_2$ activity, we measured MAP kinase and cPLA$_2$ activities upon stimulation, since cPLA$_2$ activity is regulated by phosphorylation by MAP kinase. When the mast cells were stimulated with antigen, MAP kinase activity significantly increased. Since PMA increases MAP kinase activity (*17*), we examined the additive effect of PMA on antigen-induced MAP kinase activation. Stimulation with antigen and PMA in combination further increased MAP kinase activity, which correlated well with the increase in cPLA$_2$ activity (Fig. 2). However, significant lysoPC formation was not detected upon stimulation with antigen or the combination of antigen and PMA, despite the increased cPLA$_2$ activity (Fig. 3).

*Intracellular $Ca^{2+}$ Mobilization*—The association of $cPLA_2$ with membranes is essential for the enzyme to hydrolyze membrane phospholipids. The translocation of the enzyme from cytosol to membrane is correlated with the increase in intracellular $Ca^{2+}$ concentration in response to stimuli (*1, 2*). As shown in Fig. 1, lysoPC formation was induced by thapsigargin but not by antigen, suggesting that intracellular $Ca^{2+}$ responds differently to thapsigargin and antigen. Therefore, we measured the intracellular $Ca^{2+}$ concentration under the stimulation in fura 2-loaded mast cells. As shown in Fig. 4, antigen gave a rapid and transient increase in intracellular $Ca^{2+}$, whereas thapsigargin induced a slow and sustained increase. Furthermore, simultaneous stimulation with antigen and thapsigargin resulted in a rapid and prolonged increase in the intracellular $Ca^{2+}$ concentration, and the concentration was greater than that induced by either stimulus alone.

*$Ca^{2+}$-Enhanced LysoPC Formation*—The effect of the thapsigargin-induced $Ca^{2+}$ response on the absence of hydrolytic ability of $PLA_2$ in response to antigen (Fig. 3) was investigated. As shown in Fig. 5, stimulation with antigen in the presence of thapsigargin significantly increased lysoPC generation, which did not occur in the presence of antigen alone (Fig. 3). Simultaneous stimulation with antigen and PMA induced a further increase in

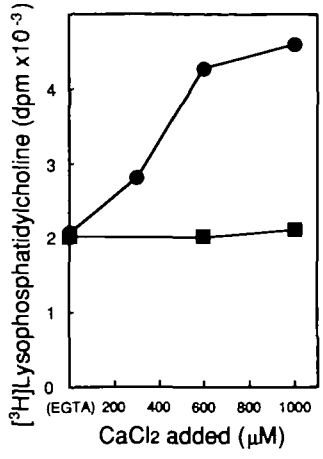


Fig. 1. **Effect of extracellular $Ca^{2+}$ concentrations on lysophosphatidylcholine formation induced by DNP-BSA or thapsigargin.** [³H]Choline-labeled mast cells were stimulated with 10 $\mu$g/ml DNP-BSA plus 5 $\mu$g/ml lysoPS (■) or 0.3 $\mu$M thapsigargin (●) in the presence of 2 mM EGTA or for 20 s prior to the addition of various concentrations of $CaCl_2$, and incubated at 37°C for 5 min. Lysophosphatidylcholine formation was determined as described under "MATERIALS AND METHODS." Each point represents the mean of duplicate experiments.

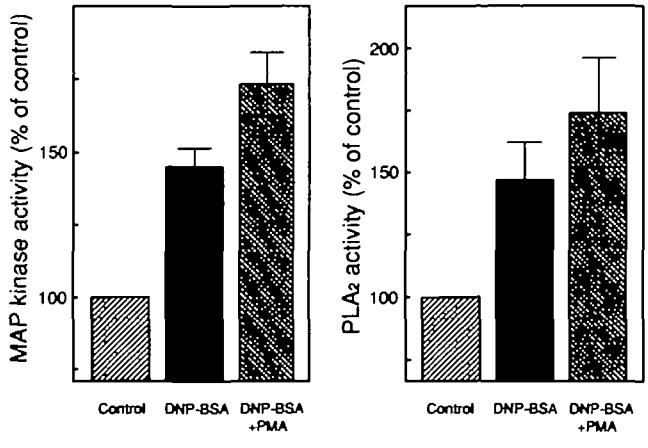


Fig. 2. **Increases in MAP kinase and cytosolic $PLA_2$ activity upon stimulation.** Mast cells were pretreated with or without 10 nM PMA at 37°C for 1 min, then stimulated with 10 $\mu$g/ml DNP-BSA plus 5 $\mu$g/ml lysoPS for 20 s prior to the addition of $CaCl_2$ to a final concentration of 1 mM. The MAP kinase and cytosolic $PLA_2$ activity in the supernatant of the cell lysate were determined 5 min after addition of $CaCl_2$, as described under "MATERIALS AND METHODS." Each bar represents the mean±SE of three separate experiments.

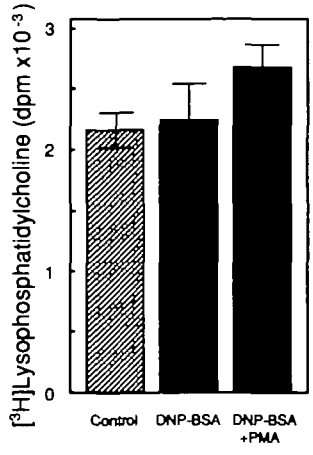


Fig. 3. **Effect of PMA on DNP-BSA-induced lysophosphatidylcholine formation.** [³H]Choline-labeled mast cells were stimulated under the same conditions as described in the legend to Fig. 2. Lysophosphatidylcholine formation was determined as described under "MATERIALS AND METHODS." Each bar represents the mean±SE of three separate experiments.

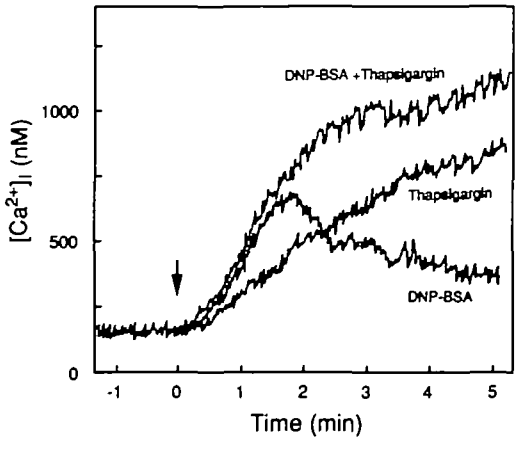


Fig. 4. **Increase in intracellular $Ca^{2+}$ concentration upon stimulation.** Mast cells were stimulated with 10 $\mu$g/ml DNP-BSA plus 5 $\mu$g/ml lysoPS and/or 0.3 $\mu$M thapsigargin at 37°C, then $CaCl_2$ was added to a final concentration of 1 mM to the reaction mixture at time 0 min (arrow). The traces are representative of duplicate experiments.

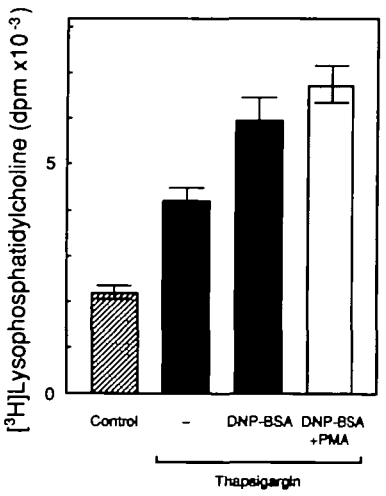


Fig. 5. **Effect of thapsigargin on DNP-BSA-induced lysophosphatidylcholine formation.** [$^3$H]Choline-labeled mast cells were stimulated under the same conditions as described in the legend to Fig. 2 in the presence or absence of 0.3 $\mu$M thapsigargin. Lysophosphatidylcholine formation was determined as described under "MATERIALS AND METHODS." Each bar represents the mean ± SE of three separate experiments.

lysoPC generation in the presence of thapsigargin (Fig. 5). This enhancement by PMA may reflect PMA-induced potentiation of MAP kinase followed by an increase in $cPLA_2$ activation in response to antigen (Fig. 2). These results suggest that the prolonged increase in intracellular $Ca^{2+}$ concentration is involved in the association of $cPLA_2$ with membranes, where it hydrolyzes phospholipids.

## DISCUSSION

Receptor-stimulated $cPLA_2$ activation is regulated by MAP kinase-dependent phosphorylation and the intracellular $Ca^{2+}$-dependent association with membrane phospholipids, resulting in the release of arachidonic acid in a variety of cells (*1-3*). However, we provided evidence that in rat peritoneal mast cells, the sequential pathway of PLD-phosphatidate phosphohydrolase-DAG lipase plays a critical role in arachidonic acid release as well as prostaglandin $D_2$ formation upon stimulation by cross-linking of the IgE receptor, suggesting that $cPLA_2$ contributes little to the arachidonic acid cascade (*10, 13*). The purpose of the present study was to elucidate the mechanism underlying the lack of hydrolytic action of $cPLA_2$ on cell membrane phospholipids.

We showed that stimulation of mast cells with antigen or the combination of antigen and PMA increased $cPLA_2$ activity in parallel with MAP kinase activation (Fig. 2), but did not significantly increase lysoPC generation, which is an indicator of $PLA_2$ hydrolytic activity (Fig. 3). These results suggest that the phosphorylation and activation of $cPLA_2$ associated with MAP kinase activation is essential, but not sufficient for $cPLA_2$ to hydrolyze membrane phospholipids. Similar evidence is available that increasing activity of $cPLA_2$ associated with phosphorylation by MAP kinase does not necessarily correlate with arachidonic acid mobilization in neutrophils (*18, 19*), platelets (*20*), or macrophages (*21*).

Although the reason why activated $cPLA_2$ is not responsible for hydrolyzing phospholipids in rat peritoneal mast cells is still not clear, the elevated intracellular $Ca^{2+}$ concentration, especially the prolonged increase in the concentration evoked by stimuli such as thapsigargin or calcium ionophore, is essential for the association of $PLA_2$ with membranes in mast and other cells (*22-26*). Therefore, we examined whether the lack of phospholipid hydrolyzing activity of $cPLA_2$ is due to an insufficient rise or sustainment of intracellular $Ca^{2+}$ concentration under antigen stimulation. We used thapsigargin, because it induces depletion of intracellular $Ca^{2+}$ stores by inhibiting endoplasmic reticulum $Ca^{2+}$-ATPase, thus stimulating the influx of external $Ca^{2+}$ (*27, 28*). Activation of mast cells with antigen led to a rapid and transient increase in intracellular $Ca^{2+}$ concentration with a peak after 1-2 min, whereas stimulation with thapsigargin led to a gradual and sustained increase (Fig. 4). The apparent hydrolyzing activity of $cPLA_2$, assessed by lysoPC generation, was detected in response to thapsigargin, but not to antigen (Fig. 1). These results suggest that a sustained rather than transient increase in intracellular $Ca^{2+}$ is important for the association of $cPLA_2$ with membranes. Simultaneous stimulation with antigen and thapsigargin induced a rapid and prolonged increase in the intracellular $Ca^{2+}$ concentration (Fig. 4), and lysoPC generation, which was not induced by antigen alone (Fig. 5). Therefore, it is suggested that the antigen-induced transient increase in intracellular $Ca^{2+}$ is not sufficient to evoke the hydrolyzing activity of $cPLA_2$ on membrane phospholipids, although antigen-stimulation induces $cPLA_2$ activation *via* phosphorylation by MAP kinase (Fig. 2).

Further efforts are needed to determine why a prolonged, but not a transient, increase in intracellular $Ca^{2+}$ is required for $cPLA_2$ to hydrolyze membrane phospholipids. However, Glover *et al.* (*29*) found using immuno-gold electron microscopy that stimulation of RBL 2H3.1 cells with antigen resulted in the translocation of only 20-30% of $cPLA_2$ from the cytosol to the nuclear envelope, whereas that with calcium ionophore resulted in the translocation of most (more than 80%) of the $PLA_2$. Furthermore, most of the $PLA_2$ bound to the envelope was reduced after a 30-min exposure to antigen, while the $PLA_2$ bound remained on the envelope after stimulation with calcium ionophore. On the basis of these findings we propose that sustained elevation of intracellular $Ca^{2+}$ maintains the association of a large amount of $cPLA_2$ with membranes for a sufficient time for it to exhibit its hydrolytic activity, whereas a transient increase in intracellular $Ca^{2+}$ fails to do so.

Since purified $cPLA_2$ has lysophospholipase activity (*30-32*), the lysoPC generated by $cPLA_2$ hydrolytic action may be simultaneously hydrolyzed by the catalytic action of lysophospholipase of the $cPLA_2$, thus being undetectable in response to antigen. However, the lysophospholipase activity of the $cPLA_2$ is reportedly $Ca^{2+}$-independent against micellar substrates but $Ca^{2+}$-dependent against membranous substrates (*32*). We detected a significant amount of lysoPC when simultaneous stimulation with antigen and thapsigargin mobilized the intracellular $Ca^{2+}$ concentration to a greater extent than either alone. Therefore, we suggest that the lysophospholipase activity of $cPLA_2$ participates little, if at all, under our experimental conditions.

To assess the possibility that lysoPC formed in response

to antigen is rapidly reacylated, and thus undetectable, mast cells were stimulated with antigen in the presence of triacsin C, which is known to inhibit long chain acyl-CoA synthetase (*33*), to inhibit the reacylation system of lysoPC in the cells. The result showed, however, that almost no lysoPC formation was detected even in the presence of triacsin C upon stimulation with antigen (data not shown), suggesting that the rapid reacylation is not involved in the lack of lysoPC formation under antigen stimulation.

In conclusion, we suggest that stimulation of rat peritoneal mast cells by cross-linking of the IgE receptor increases $cPLA_2$ activity *via* MAP kinase activation, but cannot elicit the hydrolyzing activity of $cPLA_2$. Furthermore, we suggest that a prolonged increase in the intracellular $Ca^{2+}$ concentration is required for $cPLA_2$ to hydrolyze membrane phospholipids, and that the transient increase in the concentration induced by antigen stimulation is not sufficient to evoke the hydrolytic action of the enzyme.

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