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An Essential Role for Phospholipase D in the Activation of Protein Kinase C and Degranulation in Mast Cells

Ze Peng and Michael A. Beaven

Activation of phospholipase D (PLD) and protein kinase C (PKC) as well as calcium mobilization are essential signals for degranulation of mast cells. However, the exact role of PLD in degranulation remains undefined. In this study we have tested the hypothesis that the PLD product, phosphatidic acid, and diacylglycerides generated therefrom might promote activation of PKC.

Studies were conducted in two rodent mast cell lines that were stimulated with Ag via FcεRI and a pharmacologic agent, thapsigargin. Diversion of production of phosphatidic acid to phosphatidylbutanol (the transphosphatidylation reaction) by addition of 1-butanol suppressed both the translocation of diacylglyceride-dependent isoforms of PKC to the membrane and degranulation. Tertiary-butanol, which is not a substrate for the transphosphatidylation, had a minimal effect on PKC translocation and degranulation, and 1-butanol itself had no effect on PKC translocation when PKC was stimulated directly with phorbol ester, 12-O-tetradecanoylphorbol-13-acetate. Also, in cells transfected with small inhibitory RNAs directed against PLD1 and PLD2, activation of PLD, generation of diacylglycerides, translocation of PKC, and degranulation were all suppressed. Phorbol ester, which did not stimulate degranulation by itself, restored degranulation when used in combination with thapsigargin whether PLD function was disrupted with 1-butanol or the small inhibitory RNAs. However, degranulation was not restored when cells were costimulated with Ag and phorbol ester. These results suggested that the production of phosphatidic acid by PLD facilitates activation of PKC and, in turn, degranulation, although additional PLD-dependent processes appear to be critical for Ag-mediated degranulation. The Journal of Immunology, 2005, 174: 5201–5208.

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tivation of phospholipase D (PLD) and protein kinase C (PKC) as well as calcium mobilization are essential signals for release of preformed inflammatory mediators in granules from mast cells (1–5). However, the exact role of PLD in degranulation remains undefined. PLD is thought to regulate a variety of membrane-related processes, including vesicle transport, membrane reorganization, membrane budding, and endocytosis (6–8). The formation of phosphatidic acid from phosphatidylcholine by PLD and the rapid conversion of phosphatidic acid to other biologically active molecules, such as lysophosphatidic acid and sn-1,2-diacylglyceride (DAG), are thought to facilitate various biological and signaling events within the cell (9). A controversial proposal is that activation of PLD might reinforce and sustain the activation of DAG-dependent isoforms of PKC (10, 11) through the conversion of PLD-generated phosphatidic acid to DAG by phosphatidate phosphohydrolase (7, 12). Such a role for PLD has been disputed on the basis of differences in the acyl substituents of DAGs derived from phosphatidylcholine via PLD and DAGs derived from phosphoinositides via PLC (13, 14). At least in some cell systems, the activation of PKC best correlates with the increase in levels of polyunsaturated DAGs, which were derived primarily from phosphoinositides (15–18). Also, polyunsaturated DAGs appear to be better activators of PKC than less unsaturated species, although virtually all species of DAGs are capable of activating PKC in vitro (19, 20). The issue of whether PLD-derived DAG contributes to PKC activation, however, remains unresolved (reviewed in Ref. 9).

A unique and widely exploited property of PLD is that in the presence of modest concentrations of primary alcohols, PLD preferentially catalyzes the transphosphatidylation of the alcohol to favor production of the relatively metabolically inert phosphatidyl-alkanol instead of phosphatidic acid (21, 22). This reaction is used to assay PLD activity and identify downstream targets of PLD-derived phosphatidic acid. Tertiary alcohols are poor substrates for transphosphatidylation and can thus serve as controls to assess nonspecific actions of the alcohols.

With respect to the role of PLD in mast cell degranulation, production of phosphatidic acid and degranulation are suppressed by primary alcohols (3, 5, 23, 24). In addition, overexpression of tagged PLD1 and PLD2, the two known mammalian isoforms of PLD (25, 26), in the RBL-2H3 mast cell line indicates that PLD1 associates with granule membranes and intracellular vesicles, whereas PLD2 associates with the plasma membrane (3, 23, 27). Both isoforms are activated upon Ag stimulation, and the expression of a catalytically inactive mutant of PLD1 blocks migration of granules to the cell periphery, as does 1-butanol. The expression of a catalytically inactive mutant of PLD2 blocks degranulation (5, 23). Both isoforms thus appear to regulate distinct phases of degranulation by virtue of their different locations within the cell. Pharmacologic studies have also indicated that PLD activation and degranulation are closely correlated under a wide variety of experimental conditions in RBL-2H3 cells (2, 5, 24, 28, 29).

With respect to the potential link between PLD and PKC activation, the hydrolysis of phosphatidylcholine by PLD is the major source of DAG in stimulated mast cells (28, 30, 31). After Ag stimulation, the levels of DAG increase in a biphasic manner (28,
32). An initial spike in DAG levels has been attributed to hydrolysis of phosphatidylisotol 1,4-bisphosphate by PLC, and a second sustained phase has been attributed to hydrolysis of phosphatidylinositol by PLD (32). This second phase is associated with sustained activation of PLD and PKC (28). The temporal relationships of these events have led to the conclusion that PLD-mediated production of DAGs and the associated sustained activation of PKC are obligatory for mast cell degranulation (28).

PKC is a family of phospholipid-dependent serine-threonine kinases that are subdivided into three categories (33, 34). The classical calcium-dependent (PKCα, -β1, -β2, and -γ) and novel calcium-independent (PKCδ, -θ, -ε, and -η) isoforms of PKC are dependent on phosphatidylinosine and DAG for activation (33, 34). The atypical PKC isoforms (PKCζ, -λ, and -ζ) are activated by phosphatidylinosine, but not by DAG or Ca2+. The PKC isoforms can only be activated when primed for activation by phosphorylation of the activation loop by phosphorylase-dependent protein kinase 1 (PKD1) and autophosphorylation of the C terminus of PKC (35, 36). PKCs thus phosphorylated can then translocate from cytosol to cell membrane in response to activating ligands and elevate cytosolic Ca2+.

As part of an investigation of the mechanisms by which PLD regulates mast cell degranulation, we have attempted to resolve the issue of whether PLD is required for the activation of DAG-dependent PKC isoforms in mast cells. Studies were conducted with a transformed mouse bone marrow-derived mast cell line (BMMC) and RBL-2H3 cells. Cells were stimulated with Ag for FcεRI and thapsigargin. Previous studies had shown that thapsigargin, like Ag, mediates degranulation through activation of PLD and PKC in addition to elevation of intracellular Ca2+ (2, 23). We found that 1-butanol and small inhibitory RNAs (siRNAs) directed against PLD1 and -2 inhibited the activation of PKC and degranulation. However, these inhibitory effects could be bypassed by direct activation of PKC with PMA.

Materials and Methods

Materials

Reagents were obtained from the following sources: culture reagents from Invitrogen Life Technologies; DNP-BSA, carbachol, 2-methyl-2-propanol (tertiary-butanol), 2-ME, p-nitrophenyl-N-acetyl-β-D-glucosaminide, diethylenetriamine-penta-acetic acid, n-octyl-β-D-glucoside, imidazole, and Triton X-100 from Sigma-Aldrich; normal butyl alcohol (1-butanol) from Mallinkrodt; thapsigargin from LC Laboratories; PMA from Alexis Biotechnologies; 2-ME, and 10% FCS, supplemented with 10% Wehi 3BD-conditioned medium from BD Transduction Laboratories; polyclonal Abs against phospho-PKC (Ser241) from Cell Signaling Technology; HRP-conjugated goat anti-rabbit and anti-mouse IgG from Oncogene; and mouse FcεRI and thapsigargin. Previous studies had shown that thapsigargin, like Ag, mediates degranulation through activation of PLD and PKC in addition to elevation of intracellular Ca2+ (2, 23). We found that 1-butanol and small inhibitory RNAs (siRNAs) directed against PLD1 and -2 inhibited the activation of PKC and degranulation. However, these inhibitory effects could be bypassed by direct activation of PKC with PMA.

Detection of PLD1 and PLD2 mRNAs by RT-PCR

Total cellular RNA was isolated from RBL-2H3 cells using an RNA iso- lation kit (RNAeasy; Qiagen) and was reversed-transcribed with the Script First-Strand Synthesis Kit (Invitrogen Life Technologies) according to the manufacturer’s protocol. The primers used were as follows: rat PLD1: sense primer, 5'-GGT AGG AGT GTC AAG CGG CTC ACC-3'; antisense primer, 5'-GCC AAA ACC TAG TCT CCC CAT GGA-3'; rat PLD2: sense primer, 5'-ATG ACT GTA ACC CAG ACG GCA CTC-3'; antisense primer, 5'-CAT CTC ATG GAA TGG TGG CCC TTT-3'; rat GAPDH: sense primer, 5'-GGT GAC TCT ACT GGC GTC TTC-3'; antisense primer, 5'-CCA AGG CTG TGG GCA AGG TCA-3'. The reaction mixture was denatured at 94°C for 2 min, then exposed to 29–34 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s, followed by an extension of 8 min at 72°C. RT-PCR for GAPDH was performed as a control. The PCR products were separated on 2% agarose gels in 1× TBE buffer and visualized with ethidium bromide. All PCR amplifications were performed at least three times with multiple sets of experimental RNAs.

Measurement of β-hexosaminidase release

β-Hexosaminidase was measured in medium and cell lysates (in 0.1% Triton X-100) by a colorimetric assay (40). Aliquots (10 μl) of samples were incubated with 10 μl of 1 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide at 37°C in 0.1 M sodium citrate buffer (pH 4.5) for 1 h. The product, p-nitrophenol, was converted to the chromophore, p-nitrophenate, by ad- dition of 250 μl of a 0.1 M Na2CO3/0.1 M NaHCO3 buffer. Absorbance was read at 405 nm in an ELISA reader. Results are reported as the percent of intracellular β-hexosaminidase that was released into the me- dium after correction for spontaneous release.

Measurement of [3H]phosphatidic acid, [3H]phosphatidylbutanol, and diacylglycerides

For measurement of production of [3H]phosphatidic acid and [3H]phosphatidylbutanol, cells were labeled with [3H]myristic acid. βMMMC and RBL-2H3 cells were incubated first with IgE overnight, then in fresh growth medium with 2 μCi/ml [3H]myristic acid for 90 min. The medium was replaced with glucose-saline/PIPES buffer as described above. Cultures were incubated in the absence or the presence of 50 mM 1-butanol or tertiary-butanol for 10 min before addition of stimulants. The reaction was terminated 5 min later by addition of a mixture of chloroform, methanol,
and 4 N HCl (50/100/1, v/v/v). Radiolabeled phospholipids were extracted after addition of unlabeled phosphatidic acid and phosphatidylbutanol (20 μg of each), then separated by TLC for assay of radioactivity exactly as previously described (29). The amount of [3H]phosphatidic acid or [3H]phosphatidylbutanol was expressed as a percentage of the total [3H]phospholipid extracted from nonstimulated cells. These values were converted for corrected values obtained with nonstimulated cells (>0.18% for [3H]phosphatidic acid and >0.08% for [3H]phosphatidylbutanol). For the assay of PLD activity, the values were the percentage of cellular [3H]lipids converted to [3H]phosphatidylbutanol in the presence of 1-butanol.

The total amount of DAG was determined by conversion to [14C]phosphatidic acid according to the procedure used by Preiss et al. (41) with minor modifications as described previously (29). In this procedure, extracted DAGs were incubated with E. coli DAG kinase, and [γ-32P]ATP and [14C]phosphatidic acid thus formed were extracted and quantitated by TLC. Synthetic sn-1,2 dioleoylglycerol was used to prepare standard solutions for calibration.

Isolation of membrane fraction

Stimulated and nonstimulated cultures were washed twice with cold PBS. The cells were harvested by centrifugation (250 × g for 5 min) and resuspended in 200 μl of homogenization buffer (20 mM Tris-HCl (pH 7.5), 2 mM DTT, 1 mM EDTA, 1 mM PMSF, 5 mM 4-nitrophenylphosphate, 20 μg/ml apronitin, and 20 μg/ml leupeptin). The cells were disrupted by brief sonification. Nuclei and unbroken cells were pelleted by centrifugation at 700 × g for 10 min. Nuclei-free supernatant fractions were centrifuged at 100,000 × g at 4°C for 1 h. The pellet fraction was solubilized in 100 μl of the homogenization buffer to which 0.5% Triton X-100 had been added. Samples were kept on ice for 10 min. Samples were then centrifuged at 12,000 × g for 15 min at 4°C to obtain a clarified soluble membrane fraction.

**SDS-PAGE and immunoblotting**

Proteins in whole-lysates or the soluble membrane fraction were separated by SDS-PAGE on 8% Tris-glycine gels, then transferred to nitrocellulose membranes. Blots were incubated with blocking buffer (0.05% Tween 20 and 5% skimmed milk in TBS) for 1 h before overnight incubation at 4°C with the indicated primary Abs. For detection of phosphorylated PKC isoforms and PDK1, 5% BSA was substituted for skimmed milk in the blocking buffer. Blots were washed three times and incubated for 1 h at room temperature with the secondary Ab. Immunoreactive bands were visualized by the ECL system (Amersham Biosciences) according to recommended procedures.

**Results**

**Suppression of production of phosphatidic acid and degranulation by 1-butanol**

The effects of 1-butanol and tertiary-butanol on degranulation were examined first in rBMCC. 1-Butanol, but much less so tertiary-butanol, suppressed degranulation in a concentration-dependent manner when cells were stimulated with either Ag or thapsigargin (Fig. 1). Similar results were observed in RBL-2H3 cells (data not shown). In [14C]myristate-labeled rBMCC, the suppression of degranulation by 1-butanol (Fig. 2A) was associated with decreased levels of [3H]phosphatidic acid (Fig. 2B) and increased levels of [3H]phosphatidylbutanol (Fig. 2C). In this set of experiments, tertiary-butanol had only modest effects on degranulation (Fig. 2A) and levels of [3H]phosphatidic acid (Fig. 2B), nor was tertiary-butanol converted to [3H]phosphatidylbutanol (Fig. 2C). The foregoing experiments indicated that the differences between the effects of primary and tertiary butanol on the production of phosphatidic acid and degranulation were relative rather than absolute, but that a reasonable discrimination between the effects of the two alcohols could be obtained using 50 mM butanol.

**FIGURE 1.** 1-Butanol, but not tertiary-butanol, inhibits Ag- and thapsigargin-induced degranulation in rBMCC. IgE-primed cells were stimulated with 50 ng/ml Ag or 300 nM thapsigargin for 5 min in the presence of the indicated concentrations of 1-butanol or tertiary-butanol for measurement of release of the granule marker, β-hexosaminidase. Values are the mean ± SEM from six experiments and are expressed as the percentage of intracellular β-hexosaminidase released into the medium after correction for spontaneous release (~3%). The asterisks indicate significant inhibition of release with 1-butanol compared with release from tertiary-butanol-treated cells: *, p < 0.05; **, p < 0.01.

**FIGURE 2.** Suppression of degranulation by 1-butanol is associated with the production of phosphatidylbutanol instead of phosphatidic acid. IgE-primed rBMCC were labeled with [3H]myristate for 90 min. Cells were stimulated with 50 ng/ml Ag or 300 nM thapsigargin (Tg) for 5 min in the absence or the presence of 50 mM 1-butanol or tertiary-butanol for measurement of β-hexosaminidase release (A) or of the production of [3H]phosphatidic acid (B), or [3H]phosphatidylbutanol (C). Values are the mean ± SEM from six experiments and are expressed as the percent release of intracellular β-hexosaminidase or as a percentage of the total intracellular [3H]-labeled lipids recovered as [3H]phosphatidic acid or [3H]phosphatidylbutanol after correction for values in nonstimulated cells (~3% β-hexosaminidase, ~0.2% [3H]phosphatidic acid, and ~0.08% [3H]phosphatidylbutanol). Asterisks indicate a significant decrease in response compared with controls (first column in each panel): *, p < 0.05; **, p < 0.01.
Suppression of translocation of PKC isoforms by 1-butanol

To investigate the possible effects of butanol on PKC activation, cells were stimulated with Ag or thapsigargin in the absence or the presence of 1 mM 1-butanol or tertiary-butanol. Immunoblotting of the cell membrane fraction revealed an increase in the amounts of membrane-associated phosphorylated PKC (phospho-pan PKC) after stimulation, and this increase was suppressed by 1-butanol and less so by tertiary-butanol. As was the case for degranulation, this suppression was dependent on concentration, and optimal discrimination was achieved with 50 mM 1-butanol and tertiary-butanol (data not shown). Examination of the effects of 50 mM butanol on individual isoforms of PKC indicated that Ag and thapsigargin induced translocation of phospho-pan PKC, PKCα, PKCβ, PKCδ, and PKCθ, which was suppressed by 1-butanol and, to a lesser extent, by tertiary-butanol. Typical immunoblots are shown in Fig. 3, and quantitative data for all experiments are shown in Fig. 4. The butanols had no effect on the basal levels of membrane-associated PKC in nonstimulated cells (data not shown). The exceptions to this pattern were PKCe and PKCi. Ag induced minimal translocation of PKCe and PKCi, and this translocation was not significantly affected by the butanols. Thapsigargin failed to induce translocation of either isoform, although a significant decrease in the association of PKCe with the membrane fraction was apparent in the presence of 1-butanol. The butanols and stimulants had virtually the same effects in RBL-2H3 cells (data not shown).

Phosphorylation of PKC is not suppressed by 1-butanol

The reduced association of phosphorylated PKC isoforms could be due to suppression of priming phosphorylations of PKC in addition to translocation of PKC. Therefore, the extent of PKC phosphorylation was examined in whole-cell lysates by use of Abs that specifically recognized PKC phosphorylated at Ser660, an auto-phosphorylation site, and PKCδ phosphorylated at Thr505, a site phosphorylated by PDK1 in the activation loop (35, 36). The immunoblots revealed no change in the extent of these phosphorylations in response to Ag or thapsigargin in the absence or the presence of 1-butanol (Fig. 5). In addition, the autophosphorylation of PDK1 on Ser241, which is necessary for PDK1 activity (42), was unaffected. The mechanisms for phosphorylation of PKC thus appeared to be intact in the presence of 1-butanol.

PMA-induced translocation of PKC is not suppressed by 1-butanol

To examine the effects of butanol on PKC itself, bBMMC were stimulated with 20 nM PMA to directly activate DAG-dependent conventional and novel isoforms of PKC. PMA induced translocation of all PKC isoforms tested, except for the DAG-insensitive PKCθ (Figs. 6 and 7). These responses were equally apparent in cells exposed to 1-butanol and tertiary-butanol. Therefore, butanol did not impair direct activation of the PKC isoforms by PMA. Similar results were obtained in studies with RBL-2H3 cells (data not shown).
Suppression of activation of PLD, production of DAGs, and translocation of PKC by siRNAs directed against PLD1 and PLD2

Transfection of RBL-2H3 cells with PLD1 siRNA reduced the expression of mRNA for PLD1 and PLD2, whereas transfection of cells with PLD2 siRNA reduced the expression of only PLD2 mRNA (Fig. 8A). We were unable to detect changes in the expression of PLD protein because of the lack of reliable high affinity Abs that specifically detect PLD 1 or PLD2 (9). Transfection with either siRNA blocked Ag-induced activation of PLD (Fig. 8B); increases in DAGs (Fig. 8C); translocation of phospho-pan-PKC, PKCa, and PKCθ to the cell membrane fraction (Fig. 8D); and degranulation (Fig. 8E). Neither siRNA impaired phosphorylation of PKC at Ser660 (Fig. 8D, lower blot). In these experiments, Ag stimulation resulted in a comparable increases in PLD activity and levels of DAGs (40–100%).

Provision of PMA enhances degranulation in response to thapsigargin, but not to Ag, in cells treated with 1-butanol or siRNAs

Previous studies have shown that direct stimulation of PKC with PMA can reverse the inhibitory effects of 1-butanol on thapsigargin-stimulated degranulation in RBL-2H3 cells (2) and tBMMC (our unpublished observations). As an extension of these observations, we investigated whether Ag-induced degranulation could be rescued by direct stimulation of PKC with PMA in RBL-2H3 cells after exposure to 1-butanol or the PLD siRNAs. However, the suppression of Ag-induced degranulation by 1-butanol (Fig. 9A), anti-PLD1 siRNA (Fig. 9C), and anti-PLD2 siRNA (Fig. 9D) was not reversed by costimulation of cells with PMA and Ag. In contrast, PMA reversed the inhibitory effects of 1-butanol (Fig. 9E), anti-PLD1 siRNA (Fig. 9G), and anti-PLD2 siRNA (Fig. 9H) on thapsigargin-induced degranulation. In mock-transfected cells, PMA had no effect on Ag-stimulated degranulation (Fig. 9B), but it potentiated thapsigargin-stimulated degranulation, which is consistent with previous studies (5). This potentiating action of PMA was still apparent in the siRNA-transfected cells (i.e., Fig. 9, G and H).

Discussion

The evidence that PLD regulates degranulation has come exclusively from studies with RBL-2H3 cells (2–4, 23). These studies showed that exposure to primary, but not tertiary, alcohols or the expression of catalytically inactive mutants of PLD1 and PLD2 suppressed degranulation and production of phosphatidic acid. The present study extends these findings by demonstrating that in tBMMC as well as RBL-2H3 cells, 1-butanol and siRNAs directed against PLD1 and PLD2 blocked translocation of DAG-dependent forms of PKC (Figs. 4 and 8) in addition to inhibiting activation of PLD (Figs. 2 and 8) and degranulation (Figs. 1 and 8) when cells were stimulated with physiologic (i.e., Ag) or pharmacologic (i.e., thapsigargin) stimuli (Fig. 9). The significant exception was the DAG-insensitive PKCi, which showed little or no response to stimulation or the presence of butanol. These observations in two mast cell lines support the idea that activation of DAG-dependent isoforms of PKC and degranulation are linked to activation of PLD.

1-Butanol and the siRNAs probably acted indirectly by preventing the formation of PKC-activating ligands such as DAG (Fig. 8C) as a result of suppression of the formation of phosphatidic acid via PLD (Fig. 2B). The direct activation of PKC by PMA (Figs. 6 and 7) and the priming phosphorylation of PKC isoforms by PDK1 (Figs. 5 and 8) were not impaired by 1-butanol or the siRNAs. With respect to other PKC-activating signals, 1-butanol disrupted translocation of both calcium-dependent and calcium-independent forms of PKC (Figs. 4 and 7) to suggest that the calcium signal is probably not a factor. Moreover, ongoing studies have shown that neither 1-butanol nor the siRNAs impair the activation of PLCγ, the production of inositol 1,4,5-trisphosphate, or the increase in intracellular Ca2+ that precedes degranulation in Ag-stimulated tBMMC (Z. Peng, unpublished observations). In fact, both 1-butanol and the siRNAs accelerate the initial increase in cytosolic Ca2+ in cells stimulated with either Ag or thapsigargin.

Other observations also support the idea that PLD-derived DAG can activate PKC. As noted previously, the activation of PKC in Ag-stimulated RBL-2H3 cells correlates with the increase in DAG that is associated with the activation of PLD and not with the activation of PLC (28). A similar scenario is apparent when RBL-2H3 cells are stimulated through adenosine A3 receptors. Such stimulation results in sustained activation of PLD, generation of DAG, and activation of PKC, but in only transient PLC-mediated increases in inositol 1,4,5-trisphosphate and cytosolic Ca2+ (29). The PLD-related responses, including the activation of PKC, are sustained well beyond the time when PLC-mediated events have subsided to basal levels. It should be noted also that thapsigargin elicits minimal phosphoinositide hydrolysis in RBL-2H3 cells (2).
yet it appears to be as capable as Ag in stimulating PLD (Fig. 2), translocation of PKC (Fig. 4), and degranulation (Fig. 1).

Previous work has shown that both PLD1 and PLD2 regulate degranulation, that is, PLD1 in the migration of granules to the cell periphery and PLD2 in the fusion of granules with the plasma membrane (23). However, it is uncertain from the present work whether both isoforms regulate PKC activity, because PLD1 siRNA suppressed levels of mRNA for both PLDs. Nevertheless, PLD2 siRNA appeared to selectively suppress the expression of PLD2 mRNA. Therefore, the inhibitory effects of this siRNA on PLD activity (Fig. 9B) and PKC translocation (Fig. 9D) suggest that PLD2 is a major factor in the enhancement of total PLD activity and PKC translocation in stimulated RBL-2H3 cells.

The present findings also provide additional information about the mechanism by which thapsigargin causes degranulation of mast cells. Past studies with inhibitors have suggested that thapsigargin-stimulated degranulation is dependent on PKC as well as PLD and a calcium signal (2). The present findings confirm that thapsigargin stimulation results in activation of calcium-dependent and -independent forms of PKC in two mast cell lines. The primary action of thapsigargin is the inhibition of Ca\(^{2+}\)/ATPases that regulate Ca\(^{2+}\)/H\(^{+}\)-ATPases to regulate Ca\(^{2+}\) stores in the endoplasmic reticulum.
The depletion of the Ca$^{2+}$ store leads to influx of extracellular Ca$^{2+}$ by mechanisms that sense the depletion status of these stores and a sustained increase in cytosolic Ca$^{2+}$. These effects are apparent at nanomolar concentrations of thapsigargin in RBL-2H3 cells. At higher concentrations (>30 nM), thapsigargin also stimulates PLD and degranulation in a highly correlated manner (2). Presumably, the activation of PLD and, as a consequence, the activation of PKC and degranulation are secondary to the relatively rapid and substantial increases in cytosolic Ca$^{2+}$ observed with high concentrations of thapsigargin.

Additional evidence that PLD-dependent PKC regulates thapsigargin-induced degranulation is the restoration of degranulation by PMA in cells treated with 1-butanol or siRNAs. However, PMA does not restore degranulation in Ag-stimulated cells. One reason could be that PKC has both positive and negative regulatory actions in Ag-stimulated RBL-2H3 cells (45). Thus, PMA markedly suppresses Ca$^{2+}$ mobilization in Ag-stimulated cells (46, 47), whereas it has no effect on Ca$^{2+}$ mobilization in thapsigargin-stimulated cells (44). It is also possible that PLD regulates other Ag-mediated signals in addition to PKC that do not operate in thapsigargin-stimulated cells. PLD-derived phosphatidic acid is known to interact with a number of intracellular signaling molecules, although the physiologic significance of many of these interactions is unclear (9). Although PLD appears to regulate degranulation through PKC, this may not be the exclusive mechanism by which PLD regulates degranulation in Ag-stimulated cells.

Disclosures

The authors have no financial conflict of interest.

References


30. Kennerly, D. A. 1990. Phosphatidylcholine is a quantitatively more important source of increased 1,2-diacylglycerol than is phosphatidylinositol in mast cells. J. Immunol. 144:3912.


