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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. Beaven1

Activation of phospholipase D (PLD)2 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 FceRI 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.
An initial spike in DAG levels has been attributed to hydrolysis of phosphatidylinositol 1,4-bisphosphate by PLC, and a second sustained phase has been attributed to hydrolysis of phosphatidylcholine 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 (PKCa, -b1, -b2, and -g) and novel calcium-independent (PKCδ, -θ, -ɛ, and -η) isoforms of PKC are dependent on phosphatidyserine and DAG for activation (33, 34). The atypical PKC isoforms (PKCζ, -λ, and -ż) are activated by phosphatidyserine, but not by DAG or Ca2⁺. The PKC isoforms can only be activated when primed for activation by phosphorylation of the activation loop by phosphoinositide-dependent protein kinase 1 (PDK1) 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 elevated 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 via FceRI 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 PLD2 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, baccharbol, 2-methyl-2-propanol (tertary-butanol), 2-ME, p-nitrophosphoryl-N-acetyl-β-o-glucosaminide, diethylenetriamine-penta-acetic acid, n-octyl-β-D-glucoside, imidazole, and Triton X-100 from Sigma-Aldrich; normal butyl alcohol (1-butanol) from Mallinckrodt; thapsigargin from LC Laboratories; PMA from AlexisBiochemicals; PKCα, -β1, -δ, -ε, and -η from Calbiochem; phosphatidylinositol 1,4-bisphosphate from Nitto/PerkinElmer; phosphatidylinositol 1,4,5-trisphosphate from Calbiochem; synthetic 1,2-dioleylglycerol, 1,2-dioleyl-sn-glycero-3-phosphobutanol, and bovine cardioplin from Avanti Polar Lipids; [γ-32P]ATP from Amersham Biosciences; mAbs against PKCa, PKCB, PKCδ, PKCE, and PKCε from BD Transduction Laboratories; polyclonal Abs against phospho-p60 (Ser398), phospho-PLCδ (Thr308), and phospho-PDK1 (Ser380) from Cell Signaling Technology; HRP-conjugated goat anti-mouse IgG from Jackson Immunoresearch; and Escherichia coli diacylglycerol kinase from Calbiochem. DNP-specific IgE and the BMMC line were supplied by Dr. J. Rivera (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). BMMC is a transformed IL-3-dependent cell line that arose spontaneously from BMMC-deriven Ly5⁻⁺ C57BL/6 mice and was used as a control for BMMC obtained from Ly5⁺ C57BL/6 mice (37, 38). This cell line exhibits robust responses to Ag, which include the activation of PLCγ1/2, sphingosine kinase, and mobilization of intracellular Ca2⁺ (Z. Peng, unpublished observations) in addition to the activation of PLD and PKC, degranulation (this paper), and cytokine production (39).

Cell culture and stimulation

BMMC were cultured in suspension in complete growth medium (1 mM sodium pyruvate, 100 μM nonessential amino acids, 2 mM l-glutamine, 10 μM anti-ME, and 10% FCS supplemented with 10% WEHI/3-D-conditioned medium). RBL-2H3 cells were maintained as adherent cultures in MEM with Eagle’s salts, supplemented with glutamine, antibiotics, and 15% FCS in a humidified atmosphere of 5% CO₂ at 37°C.

Except where stated otherwise, BMMC or RBL-2H3 cells were incubated overnight in six-well-plate clusters (2 × 10⁶ cells/2 ml/well) with 0.5 μg/ml DNP-specific IgE in the growth medium described above. Cells were washed twice and repropidionized with a glucose-saline/PIPES buffer (23). The suspensions of BMMC were separated and washed by centrifugation at 250 × g for 5 min at room temperature, whereas adherent RBL-2H3 cells were washed directly in the culture plates. 1-Butanol or tertary-butanol was added where indicated, and the cells were incubated for 10 min at 37°C before addition of stimulants. Cells were stimulated with 50 mM DNP-BSA, 300 nM thapsigargin, or 20 nM PMA for 5 min, and assays were performed thereafter.

Construction and transient transfection of siRNA plasmids

The siRNA constructs were made using the siRNA Expression Cassette kit (Ambion) and contained a mouse U6 promoter element adjacent to a hairpin siRNA oligonucleotide template and an RNA polymerase terminator. The manufacturer’s website program was used to design the siRNA oligonucleotide templates to target PLD1 and PLD2 genes. The cassette was inserted into a pcR 4-TOPO expression vector (Invitrogen Life Technologies) for transfection into TOP10 E. coli and subsequent selection of positive clones. Plasmids were purified, and their DNA sequences were confirmed. RBL-2H3 cells were transiently transfected with the above plasmids along with a vector (pd2ZEFP-N1; BD Clontech) that encoded yellow fluorescent protein in the ratio of 5:1 by electroporation (Gene Pulser; 250 μF, 250 V; Bio-Rad). Transfected cells were selected for the yellow fluorescent protein label by cell sorting. Cells were used within 24 h of transfection.

The siRNA constructs for PLD1 and -2 were tested for effects on cellular PLD activity and Ag-induced degranulation in RBL-2H3 cells. Previous work had shown that degranulation is dependent on the presence of both isoforms (23). One construct against PLD1 and one against PLD2 possessed marked inhibitory activity when expressed in cells, whereas all other constructs were inactive. The two constructs were targeted for the nucleotide segment: 5’-GCTCGATTATTCGACCAAA-3’ (nt positions 1576–1594) of the PLD1 mRNA and 5’-GTGCTTGGAACATAAGGCTGTTG-3’ (nt positions 4014–4032) of the PLD2 mRNA.

Detection of PLD1 and PLD2 mRNAs by RT-PCR

Total cellular RNA was isolated from RBL-2H3 cells using an RNAiso Plus (RNaseasy; Qiagen) kit, which was reversed-transcribed with the First-Strand Synthesis System (Invitrogen Life Technologies) according to the manufacturer’s protocol. The primers used were as follows: rat PLD1: sense primer, 5’-GGT GGC AGT GTC AAG CGG GTT GCC ACC-3’; antisense primer, 5’-GCC AAA ACC TAG TAT CTC CAT CCC GAG-3’; rat PLD2: sense primer, 5’-ATG ACT GTA ACC CAG AGC GCA CTC-3’; antisense primer, 5’-CAG CTC ATG AAA TGG TCG GAA TTT-3’; and rat GAPDH: sense primer, 5’-GGT GAG TCT ACT GGC TGC 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-nitrophosphoryl-N-acetyl-β-o-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 addition of 250 μl of 0.1 M Na₂CO₃/0.1 M NaHCO₃ buffer. Absorbance was read at 405 nm in an ELISA reader. Results are reported as the percentage of intracellular β-hexosaminidase that was released into the medium after correction for spontaneous release.

Measurement of [1H] Phosphatidic acid, [1H]Phosphatidilbutanol, and diacylglycerides

For measurement of production of [1H]Phosphatidic acid and [1H]Phosphatidilbutanol, cells were labeled with [1H]myristic acid. BMMC and RBL-2H3 cells were incubated first with IgE overnight, then in fresh growth medium with 2 μCi/ml [1H]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 tertary-butanol for 10 min before addition of stimulants. The reaction was terminated 5 min later by addition of a mixture of chloroform, methanol,
Measurement of release of the granule marker, of the indicated concentrations of 1-butanol or thapsigargin-induced degranulation in tBMMC. IgE-primed cells were stimulated for 20 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.

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 corrected for 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 [32P]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 [32P]phosphatidic acid thus formed were extracted and quantitated by TLC. Synthetic α,β,γ-trioleoylphosphatidylcholine 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 re-suspended in 200 μl of homogenization buffer (20 mM Tris-HCl (pH 7.5), 2 mM DTT, 1 mM EGTA, 2 mM EDTA, 1 mM PMSF, 5 mM 4-nitrophenylphosphate, 20 μg/ml aprotinin, and 20 μg/ml leupeptin). The cells were disrupted by brief sonication. 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 pelleted 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 tBMMC. 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 [3H]myristate-labeled tBMMC, 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 there are 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 2](https://www.jimmunol.org)
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 50 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 PKCε and PKCd. Ag induced minimal translocation of PKCε and PKCd, 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 PKCε 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 autophosphorylation 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 PKCd (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).
PMA can reverse the inhibitory effects of 1-butanol on thapsigargin, but not to Ag, in cells treated with 1-butanol or siRNAs. Provision of PMA enhances degranulation in response to thapsigargin (Tg) for 5 min in the absence or the presence of 50 mM 1-butanol. Immunoblots were prepared from whole cell lysates and probed for phosphorylated PKC (Ser\(^{\text{Thr}}\)), phospho-pn-PKC, PKC\(_{\alpha}\) (Thr\(^{\text{Ser}}\)), and PKD1 (Ser\(^{\text{Thr}}\)) with phosphospecific Abs. The blots are representative of results from three separate experiments.

**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-pn-PKC, PKC\(_{\alpha}\), and PKC\(_{\theta}\) to the cell membrane fraction (Fig. 8D); and degranulation (Fig. 8E). Neither siRNA impaired phosphorylation of PKC at Ser\(^{\text{Thr}}\) (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 \(t\)BMMC (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 \(t\)BMMC 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) stimulants (Fig. 9). The significant exception was the DAG-insensitive PKC\(_{\alpha}\), 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 PKD1 (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 PLC\(_{\gamma}\), the production of inositol 1,4,5-trisphosphate, or the increase in intracellular Ca\(^{2+}\) that precedes degranulation in Ag-stimulated \(t\)BMMC (Z. Peng, unpublished observations). In fact, both 1-butanol and the siRNAs accelerate the initial increase in cytosolic Ca\(^{2+}\) 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 A\(_{1}\) 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 Ca\(^{2+}\) (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),

**FIGURE 5.** 1-Butanol does not affect the phosphorylation of PKC and PDK-1. IgE-primed BMMC 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. Immunoblots were prepared from plasma membrane fractions as described in Fig. 3. Typical blots from one of three experiments are shown.

**FIGURE 6.** 1-Butanol does not inhibit translocation of PKC isoforms in \(t\)BMMC stimulated with PMA. Cells were exposed to vehicle or 20 nM PMA for 5 min in the absence or the presence of 50 mM 1-butanol or tertiary-butanol. Immunoblots were prepared from plasma membrane fractions as described in Fig. 3. Typical blots from one of three experiments are shown.
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\textsuperscript{2+}/ATPases that regulate Ca\textsuperscript{2+} reuptake into Ca\textsuperscript{2+} stores in the endoplasmic reticulum.

FIGURE 8. The siRNAs directed against PLD1 and PLD2 suppress PLD activation, PKC translocation, and degranulation in RBL-2H3 cells. RBL-2H3 cells that had been primed with IgE and transiently transfected with the siRNAs or empty vector (EV) were either left unstimulated or stimulated with 20 ng/ml Ag for 5 min. Levels of PLD1 and PLD2 mRNA were determined by RT-PCR (A). [\textsuperscript{3}H]Myristate-labeled cells were used to assay PLD activity by measurement of formation of [\textsuperscript{3}H]phosphatidylbutanol (expressed as a percentage of the total \textsuperscript{3}H-labeled lipids) in the presence of 1-butanol (B). The increase in levels of DAGs was determined by enzymatic conversion of DAGs to [\textsuperscript{32}P]phosphate-labeled phosphatidic acid with [\gamma-\textsuperscript{32}P]ATP (C). Translocation of phosphorylated PKC, PKCo, and PKC\beta was determined by electrophoretic separation of membrane proteins and immunoblotting as described in previous figures (D). Degranulation was assessed by measurement of the percentage of intracellular β-hexosaminidase that was released into the medium (E). A and D, Representative blots from three experiments; B, C, and E, mean ± SEM from three separate experiments. Asterisks indicate a significant decrease compared with responses of cells transfected with empty vector: **, p < 0.01.
FIGURE 9. PMA enhances thapsigargin-induced, but not Ag-induced, degranulation in RBL-2H3 cells exposed to 1-butanol or transfected with PLD siRNAs. IgE-primed RBL-2H3 cells were not stimulated (N.S.) or were stimulated for 5 min with 50 ng/ml Ag or 300 nM thapsigargin (Tg), alone or in combination with 20 nM PMA as indicated. Cells were exposed to 1-butanol for 10 min before stimulation or were previously transfected with empty vector (EV), PLD1 siRNA (siPLD1), or PLD2 siRNA (siPLD2). Values indicate the percentage of intracellular β-hexosaminidase that was released into the medium and are the mean ± SEM from six experiments. Significant enhancement of release when cells were stimulated with the combination of thapsigargin and PMA is indicated by asterisks: **, p < 0.01.

The resulting depletion of the Ca2+ stores leads to influx of extracellular Ca2+ by mechanisms that sense the depletion status of these stores and a sustained increase in cytosolic Ca2+. These effects are apparent at nanomolar concentrations of thapsigargin in RBL-2H3 cells (44). At higher concentrations (>30 nM), thapsigargin also stimulates activation of PLD and degranulation in a highly correlative 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 Ca2+ 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 Ca2+ mobilization in Ag-stimulated cells (46, 47), whereas it has no effect on Ca2+ 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.

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