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Phospholipases D1 and D2 Regulate Different Phases of Exocytosis in Mast Cells

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The rat mast cell line RBL-2H3 contains both phospholipase D (PLD)1 and PLD2. Previous studies with this cell line indicated that expressed PLD1 and PLD2 are both strongly activated by stimulants of secretion. We now show by use of PLDs tagged with enhanced green fluorescent protein that PLD1, which is largely associated with secretory granules, redistributes to the plasma membrane in stimulated cells by processes reminiscent of exocytosis and fusion of granules with the plasma membrane. These processes and secretion of granules are suppressed by expression of a catalytically inactive mutant of PLD1 or by the presence of 50 mM 1-butanol but not *tert*-butanol, an indication that these events are dependent on the catalytic activity of PLD1. Of note, cholera toxin induces translocation of PLD1-labeled granules to the plasma membrane but not fusion of granules with plasma membrane or secretion. Subsequent stimulation of calcium influx with Ag or thapsigargin leads to rapid redistribution of PLD1 to the plasma membrane and accelerated secretion. Also of note, PLD1 is recycled from plasma membrane back to granules within 4 h of stimulation. PLD2, in contrast, is largely confined to the plasma membrane, but it too participates in the secretory process, because expression of catalytically inactive PLD2 also blocks secretion. These data indicate a two-step process: translocation of granules to the cell periphery, regulated by granule-associated PLD1, and a calcium-dependent fusion of granules with the plasma membrane, regulated by plasma membrane-associated PLD2 and possibly PLD1. ***The Journal of Immunology*****, 2002, 168: 5682–5689.**

Phospholipase (PLD)[3] is activated in stimulated mast cells where it is thought to play an essential role in secretion of granules (1, 2). Activated PLD catalyzes the hydrolysis of phosphatidylcholine to form phosphatidic acid, which can be converted in turn to diglycerides by the action of phosphatidate hydrolase. PLD is thought to promote sustained activation of protein kinase (PK)C through the generation of phosphatidic acid and possibly diacylglycerides, both of which stimulate PKC (3). PLD could thus promote secretion, because activation of PKC along with an increase in cytosolic free calcium are essential signals for secretion in mast cells (4). In addition, phosphatidic acid may play a more direct role in facilitating fusion of the perigranule and plasma membranes, as has been proposed for neuroendocrine cells (5) and neutrophils (6).

Several studies demonstrate a close relationship between secretion and PLD activation in the rat mast cell line RBL-2H3. For example, treatment of these cells with cholera toxin enhances PLD activation and secretion to the same extent without affecting the activation of other PLs (1). Also, inhibitors of PKs that regulate PLD activity suppress both PLD activation and secretion similarly (7). Finally, primary alcohols that divert production of phosphatidic acid by PLD to phosphatidylalcohol (referred to as a transphosphatidylation reaction) suppress secretion as well (1, 2). The transphosphatidylation reaction is used to assay PLD activity in vivo (see *Materials and Methods*) because, unlike phosphatidic acid, the phosphatidylalcohol is produced specifically by PLD, is relatively inert as a signaling lipid, and accumulates within the cell (8).

Two mammalian isoforms have been cloned, namely PLD1 and PLD2 (9–11). PLD1 can be activated by several mechanisms. These include activation by small GTPases (12–16), Rho kinase (17), Ca^{2+}/calmodulin-dependent (CaM) kinase II (18), and PKC in a catalytically dependent and independent manner (12, 19, 20). For example, recombinant PLD1 is stimulated in vitro in the presence of phosphatidylinositol 4,5-bisphosphate by ADP-ribosylation factor (ARF), Rho proteins (i.e., RhoA, Rac1, and Cdc42), and PKCα by direct interaction with this PLD (12–16). PLD2 also requires phosphatidylinositol 4,5-bisphosphate for expression of activity, but unlike PLD1 this activity is not affected by the small GTPases or PKCα (10), although modest stimulation by ARF has been reported by some workers (21, 22).

PLD is activated in isolated mast cells (23) and cultured mast cell lines such as the RBL-2H3 cell (1, 24, 25) by receptor ligands (23, 26), calcium ionophores (24), thapsigargin (1, 27), and PMA (24) to cause sustained production of phosphatidic acid and diglycerides (23–25). PLD is responsible for much of the increase in diglycerides in stimulated mast cells (23). The secretory response to Ag is lost on permeabilizing mast cells but can be fully restored by provision of ARF1 or phosphatidylinositol transfer protein, both of which increase levels of phosphatidylinositol 4,5-bisphosphate and restore the PLD and secretory responses to Ag (2). RBL-2H3 cells express message for PLD1b (one of the two variant forms of PLD1) and, to a much greater extent, PLD2 (A. Vaid,

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[2] Address correspondence and reprint requests to Dr. Michael A. Beaven, National Institutes of Health, Room 8N109, Building 10, Bethesda, MD 20892-1760. E-mail address: beavenm@nhlbi.nih.gov

[3] Abbreviations used in this paper: PLD, phospholipase D; ARF, ADP-ribosylation factor; CaM, calmodulin/Ca^{2+} dependent; $[Ca^{2+}]_i$, concentration of cytosolic free calcium; EGFP, enhanced green fluorescent protein; PK, protein kinase.

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P. M. Holbrook, and M. A. Beaven, unpublished data). Subcellular fractionation studies indicate that both granule and plasma membrane fractions contain PLD activity (A. Chahdi, P. F. Fraundorfer, and M. A. Beaven, unpublished data). Although the type of PLD associated with these fractions is unclear, expressed enhanced green fluorescent protein (EGFP)-tagged PLD1 localizes primarily with the secretory granule (28) and endogenous PLD2 protein is located primarily in plasma membrane fractions (A. Chahdi, P. F. Fraundorfer, and M. A. Beaven, unpublished data). Our studies with expressed EGFP-tagged PLD1 and PLD2 also indicate that both isoforms are activated by the PLD stimulants noted above and that activation is markedly enhanced by prior treatment of RBL-2H3 cells with cholera toxin (7).

In this paper, we have taken advantage of the presence of both PLD1 and PLD2 in RBL-2H3 cells to determine the role of each isoform in the secretory process. Specifically, we have investigated the disposition of EGFP-tagged PLDs during cell stimulation and show that, while PLD1 is essential for the translocation of granules to the plasma membrane, PLD2 is essential for fusion of the granules to the plasma membrane and secretion.

Materials and Methods

Reagents

Thapsigargin, PMA, and Ro31-7549 were purchased from LC Laboratories (Woburn, MA); KN-92 and KN-93 were purchased from Calbiochem (La Jolla, CA); H-89 was from Alexis Biochemicals (San Diego, CA); GTP and GTPγS were from Boehringer Mannheim (Indianapolis, IN); radiolabeled compounds were from DuPont-NEN (Boston, MA); cholera toxin was from List Biologicals (Campbell, CA); anti-rat mast cell protease II Ab was from Moredun Animal Health (Midlothian, U.K.); cell culture reagents were from Life Technologies (Rockville, MD); Tris-glycine polyacrylamide gels were from NOVEX (San Diego, CA). DNP-BSA and DNP-specific monoclonal IgE were purchased from Sigma-Aldrich (St. Louis, MO). The Ab against the FcεRIIα subunit was kindly supplied by Dr. J. Rivera (National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD).

Cell culture and experimental conditions

RBL-2H3 cells were grown as monolayers in MEM with Earle's salts, supplemented with glutamine, antibiotics, and 15% FBS. For each experiment cells were harvested by trypsinization, transferred to 24-well (2×10^5 cells/0.4 ml/well) cluster plates (29), and incubated overnight in complete growth medium with 25 ng/ml DNP-specific IgE to achieve 100% occupancy of FcεRI. Where indicated, 1 μg/ml cholera toxin was added to cell cultures 4 h before the experiment. Cultures were washed and the required buffered solution was added (0.2 ml/well). Experiments on intact cells were performed in a PIPES-buffered medium (25 mM PIPES (pH 7.2), 159 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 5.6 mM glucose, and 0.1% fatty acid-free fraction V from bovine serum) or in calcium-free PIPES-buffered medium (as above except 0.1 mM EGTA replaced 1 mM $CaCl_2$). Unless stated otherwise, cultures were incubated for 30 min with or without inhibitors before addition of stimulants for 15 min. Also, the concentrations of inhibitors and stimulants used were those found to produce maximal effects, although a suboptimal dose (3 ng/ml) of Ag was used in some experiments as noted.

Assay of PLD by the transphosphatidylation assay and inhibition of phosphatidic acid formation by use of butanol

RBL-2H3 cells were incubated overnight with DNP-specific IgE in 24-well plates and, when required, exposed to cholera toxin as described above. [^{3}H]Myristic acid (2 μCi/ml) was added for the final 90 min of incubation to label cellular phospholipids. Cells were then incubated in the PIPES-buffered medium in the presence of 1% ethanol for 10 min before stimulation. Under these conditions, [^{3}H]phosphatidylethanol is formed by a PLD-specific transphosphatidylation reaction and accumulates within the cell (30). Radiolabeled phosphatidic acid and phosphatidylethanol were isolated by solvent extraction and quantified by TLC as described previously (26). The total amount of [^{3}H]phosphatidylethanol and [^{3}H]phosphatidic acid was expressed as a percentage of total [^{3}H]phospholipid in Triton X-100 (Sigma-Aldrich) extracts of unstimulated cultures.

In experiments where butanol was used to suppress phosphatidic acid formation by PLD, 50 mM 1-butanol was added to cultures in place of ethanol. *tert*-Butanol, which is not a substrate for transphosphatidylation, was added to additional cultures as a control. [^{3}H]Phosphatidylbutanol and [^{3}H]phosphatidic acid were assayed as described above.

Construction of plasmids and techniques for transient transfection

Full-length cDNA were excised from hemagglutinin-tagged plasmids by *Sma*I and *Hpa*I for PLD1b and PLD1bK898R and *Sma*I and *Xba*I for PLD2 and PLDK758R and subcloned into a pEGFP-C expression vector (Clontech Laboratories, Palo Alto, CA). The sequences (Fig. 1*A*) were confirmed by DNA sequence analysis. Cells were transiently transfected with each DNA preparation (25 μg/2×10^7 cells) by electroporation (Gene Pulser, 960 μF, 250 V; Bio-Rad, Hercules, CA). Successful transfection was confirmed by fluorescence microscopy and Western blotting. Cells were used within 24 h of transfection. All expressed PLDs were EGFP fusion proteins of PLD1b, PLD2, PLD1bK898R, and PLD2K758R, even when referred to in the text without the EGFP designation.

Confocal microscopy

RBL-2H3 cells were transfected with EGFP vector or the EGFP-tagged PLDs by electroporation as described above and then suspended in complete growth medium before transfer to Lab-Tek chambered coverslips (Nalge Nunc International, Naperville, IL). The chambered coverslips were then incubated overnight (16 h) at 37°C. Stimulants and other reagents were added directly to the chambers for the times indicated in the text. The cultures were then washed three times with PBS immediately before microscopic examination. For double labeling of cells, all incubations were performed at room temperature and samples were washed three times with PBS as indicated. Cultures were fixed with 4% formaldehyde in PBS for 10 min, washed, permeabilized with 0.5% Triton X-100 for 15 min, and then washed before incubation for 1 h with a blocking reagent, 1% BSA in PBS. The samples were then incubated for 2 h with a solution of the primary Ab in 1% BSA in PBS and, after washing, with the secondary Ab conjugated with rhodamine for a further 45 min. After washing, samples were mounted using the Prolonged Antifade kit (Molecular Probes, Eugene, OR).

All confocal images were obtained using a Zeiss LSM410 confocal laser scanning microscope and a Fluar ×100 objective (1.3 numerical aperture; Zeiss, Oberkochen, Germany). EGFP fluorescence images were acquired by use of the 488-nm excitation line of an argon laser, a LP 505-nm emission filter, and a pinhole set to produce a 1.25-μm slice. For transmitted light images, the 543-nm line of a HeNe laser was used. The images were collected at a size of $10^3 \times 10^3$ pixels and a data depth of 8 bits. Custom-written programs in the IDL programming language (Research Systems, Boulder, CO) were used for morphometric analysis. These programs evaluated the average distance of all fluorescent pixels from the nearest outer edge of the cell perimeter. This program may underestimate actual distance of remaining granules when these are obscured by reuptake of EGFP-tagged PLD into Golgi (see *Results*).

Presentation of results

Individual experiments were performed with three to four cultures for each data point. The data are presented as the mean ± SEM from three or more separate experiments as indicated. Statistical significance was determined by use of the SigmaPlot 2000 (Jandel Scientific, Corte Madera, CA).

Results

Distribution of EGFP-tagged PLDs in unstimulated and stimulated cells

EGFP-tagged PLDs (Fig. 1*A*) were expressed in RBL-2H3 cells by electroporation. Consistent with the extent of expression of endogenous mRNA for PLD1 and PLD2, EGFP-PLD2 was expressed more efficiently (~7-fold by densitometric measurement) than EGFP-PLD1 (Fig. 1*B*). Examination of the cells by phase contrast and confocal microscopy revealed that PLD1 was localized with intracellular structures and that PLD2 was localized in the vicinity of the plasma membrane (Fig. 1*C*). As shown in Fig. 1*D*, EGFP-PLD1 colocalized with the granule marker, rat mast cell protease II (31), although some granules were not associated with the EGFP label. PLD2, in contrast, colocalized with the α subunit of FcεRI in the plasma membrane. These data confirm previous findings that EGFP-PLD1 is associated with secretory granules in RBL-2H3

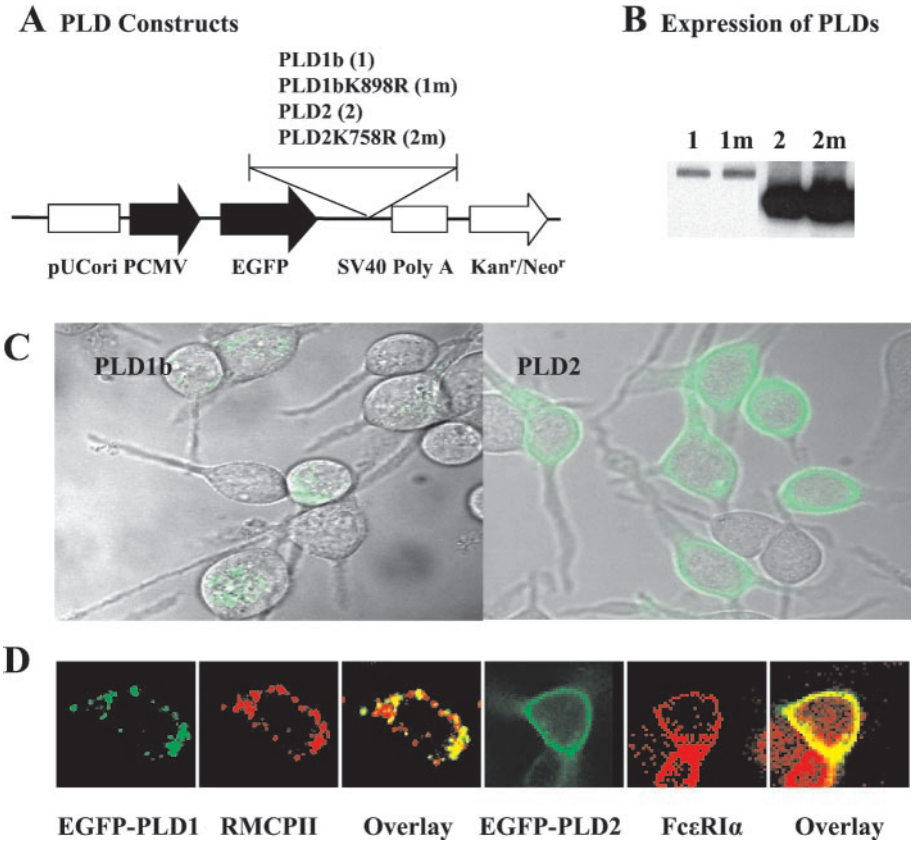

FIGURE 1. Structures of EGFP-PLD plasmids and intracellular localization of EGFP-PLD1 and EGFP-PLD2 after introduction of plasmids into RBL-2H3 cells by electroporation. *A*, Constructs of the four plasmids used in this study. These constructs encode for EGFP-tagged PLD1b, PLD2, and the catalytically inactive forms of these PLDs, namely PLD1bK898R and PLD2K758R. *B*, Expression of wild-type EGFP-tagged PLD1b (1), PLD2 (2), and mutant forms (1m and 2m) as revealed by Western blots prepared from whole cell lysates. *C*, Intracellular distribution of EGFP-tagged PLD1b (referred to hereafter as PLD1) and PLD2. The histograms show selected fields of view by phase contrast and fluorescence microscopy. Magnification, ×100. *C*, Representative histograms of cells after counterstaining with rhodamine-labeled Abs against rat mast cell protease II (RMCPII), a granule marker, or the α subunit of FcεRI (FcεRIα), a marker for the plasma membrane.

cells (28) and show for the first time that EGFP-PLD2 is expressed exclusively on the plasma membrane in these cells. In addition, stimulation of cells with Ag or thapsigargin resulted in translocation of most if not all PLD1-labeled structures to the cell periphery and the relocation of PLD1 to the plasma membrane, probably as a consequence of fusion of the labeled structures with the plasma membrane (Fig. 2). The doses of stimulants used in this experiment produced maximal secretion of another granule marker, hexosaminidase (~40% release, data not shown).

PMA stimulates PLD, but, unlike Ag or thapsigargin, it does not induce an increase in the concentration of free cytosolic calcium ($[Ca^{2+}]_i$) or secretion (7, 32). PMA induced some redistribution of the PLD1-labeled granules toward the periphery but failed to induce their fusion with the plasma membrane (Fig. 2) and secretion (data not shown). As seen in Fig. 2, these structures remained intact and there was little, if any, transfer of EGFP-PLD1 to the plasma membrane.

The EGFP-tagged catalytically inactive mutant of PLD1 (PLD1K898R) also associated with granule-like structures (Fig. 2). However, in cells expressing this mutant there was no apparent translocation of these granules after cell stimulation. Therefore, the catalytic activity of PLD1 is necessary for translocation of granules, and expression of PLD1K898R blocks this translocation.

In contrast to EGFP-PLD1, wild-type EGFP-PLD2 and its catalytically inactive mutant, PLD2K758R, were expressed on the plasma membrane. No marked changes in this distribution occurred whether cells were stimulated with Ag, thapsigargin, or PMA.

Recycling of PLD1 to secretory granules after cell stimulation

The translocation and fusion of EGFP-PLD1-labeled granules with the plasma membrane was evident 5 min after addition of Ag (Fig. 3*A*). Few intact labeled granules remained after 15 min. In accord with these results, release of hexosaminidase was apparent by 5 min and largely complete by 15 min (data not shown). At 15 min almost all of the EGFP label was located in the plasma membrane. Thereafter, EGFP begun to appear in extranuclear (possibly perinuclear) structures (i.e., 30- and 60-min time points in Fig. 3). By 240 min the label had mostly disappeared from the plasma membrane and was now again associated with granule-like structures.

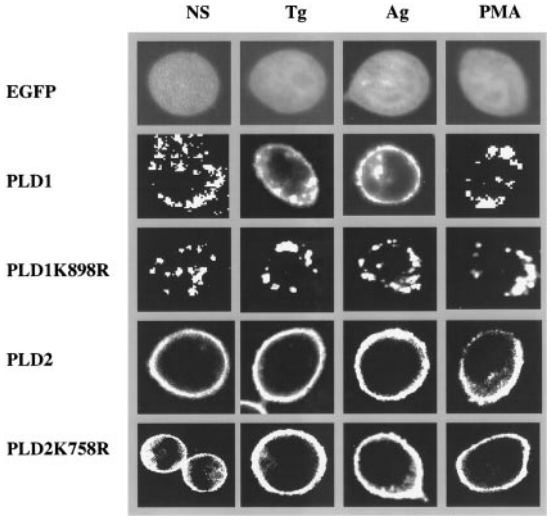

FIGURE 2. Intracellular distribution of EGFP label after transfection of cells with vector for EGFP (vector) and EGFP-tagged PLD1, PLD1K898R, PLD2, and PLD2K758R. RBL-2H3 cells were transfected by electroporation and then left unstimulated (NS) or stimulated with 25 ng/ml DNP-BSA (Ag), 300 nM thapsigargin (Tg), or 50 nM PMA for 15 min for examination by confocal microscopy. Representative cells are shown.

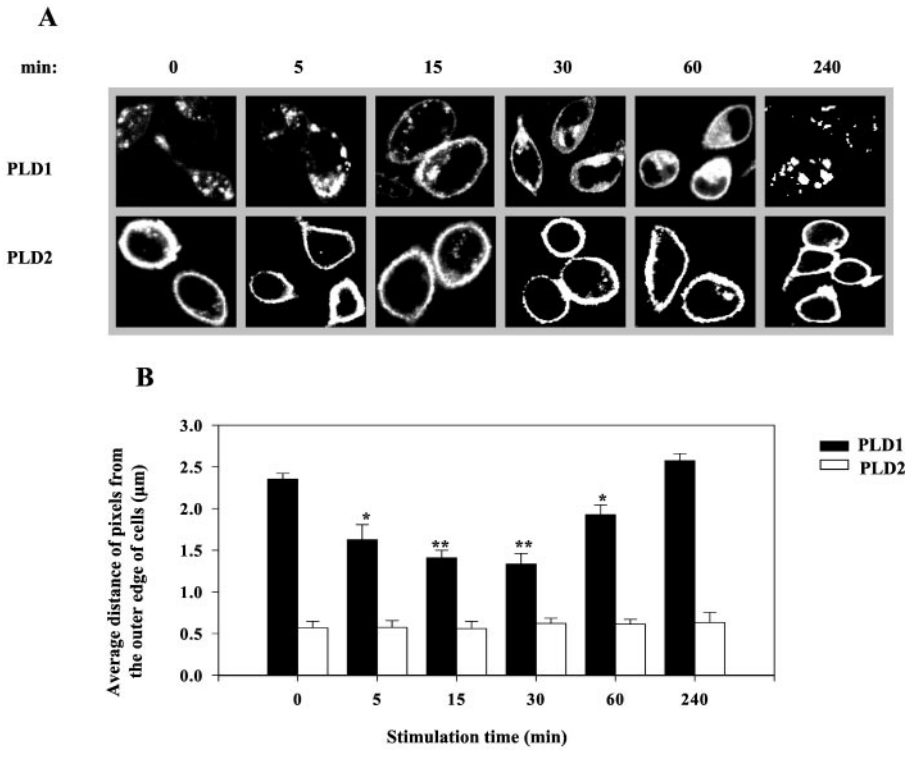

FIGURE 3. Time course of changes in intracellular localization of EGFP-tagged PLD1 and PLD2 in Ag-stimulated cells. RBL-2H3 cells were transfected by electroporation with the EGFP vector, EGFP-tagged PLD1 or PLD2 and stimulated with 25 ng/ml DNP-BSA for the indicated times for determination of location of the tagged PLDs by confocal microscopy. *A*, Photomicrographs from a representative experiment. *B*, Data (mean ± SEM) obtained from morphometric analysis of three experiments (five representative cells each).

These results indicated that PLD1 did not remain in the plasma membrane but was recycled to residual or newly matured granules. As noted previously, there was no apparent change in the disposition of PLD2 during the course of stimulation (Fig. 3*A*). The movement of granule-associated PLD1 to the cell membrane and the reassociation of PLD1 with granules was verified by morphometric analysis of data from several experiments (Fig. 3*B*).

Production of phosphatidic acid by PLD1 is necessary for translocation of granules to the plasma membrane

Another indication that movement of granules to the cell periphery was dependent on PLD1 activity came from studies with 1-butanol which, as noted earlier, diverts production of phosphatidic acid by PLD to phosphatidylbutanol. *tert*-butanol (2-methyl-2-propanol), which is not a substrate for this transphosphatidylation reaction, was used as a control reagent (for examples, see Refs. 33 and 34). The presence of 50 mM 1-butanol suppressed the translocation of EGFP-labeled granules (Fig. 4*A*, with morphometric data shown in Fig. 4*B*) and secretion of hexosaminidase (Fig. 4*C*), possibly through a decreased production of [^{3}H]phosphatidic acid (~60% reduction, data not shown). However, *tert*-butanol had minimal or no discernable effect on these responses. The inhibitory effects of 1-butanol on translocation were consistently observed in all experiments but were contrary to an earlier report (35) for reasons that are unclear.

Translocation and fusion of granules with plasma membrane are regulated differently

We next examined stimulants of PLD, such as PMA and cholera toxin, that do not elicit an increase in $[Ca^{2+}]_i$ (32, 36) or secretion (7) in RBL-2H3 cells. Interestingly, both PMA (see Fig. 2) and more so cholera toxin (Fig. 5*A*, with morphometric data shown in Fig. 5*B*) induced movement of granules to the cell periphery. Treatment with cholera toxin induced movement of granules to the cell periphery without apparent fusion of granules with the plasma membrane (Fig. 5*A*, second panel), and 1-butanol retards this movement (Fig. 5*B*, third panel). However, additional studies revealed that treatment with cholera toxin substantially enhanced the extent of fusion of granules with the plasma membrane (Fig. 5*A*,

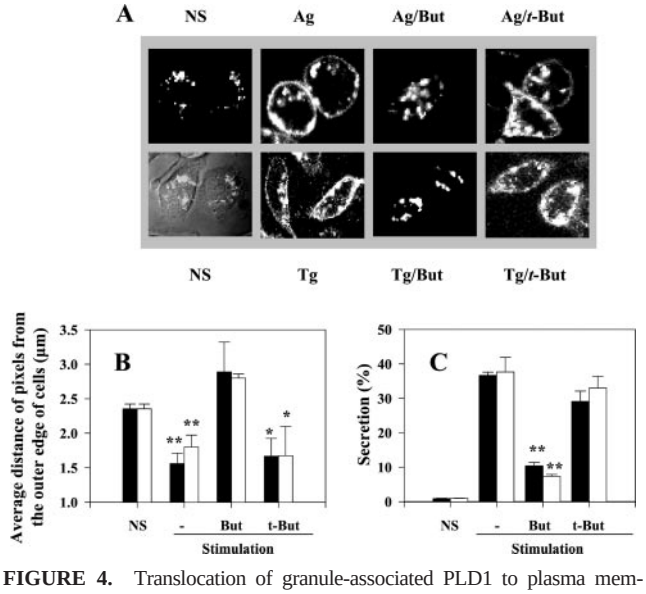

FIGURE 4. Translocation of granule-associated PLD1 to plasma membrane in stimulated cells and inhibition of this translocation with 1-butanol. RBL-2H3 cells transfected with EGFP-tagged PLD1 were stimulated with 25 ng/ml DNP-BSA (Ag) or 300 nM thapsigargin (Tg) for 15 min in the absence or presence of 50 mM 1-butanol (But) or *tert*-butanol (*t*-But). Cells were then examined by confocal microscopy and subjected to morphometric analysis. Location of the EGFP-tagged PLD1 for a typical experiment (*A*) and the calculated distance of granules from plasma membrane for three separate experiments (five cells each) (*B*) are shown. Secretion of the granule marker, hexosaminidase, in the same experiments is also shown (*C*). Filled and open bars indicate values for DNP-BSA- and thapsigargin-stimulated cells, respectively. Values are the mean ± SEM of values from the three experiments and the asterisks indicate significant difference (*, $p < 0.05$; **, $p < 0.01$) compared with nonstimulated (*B*) or stimulated (*C*) cells. NS, Nonstimulated cells.

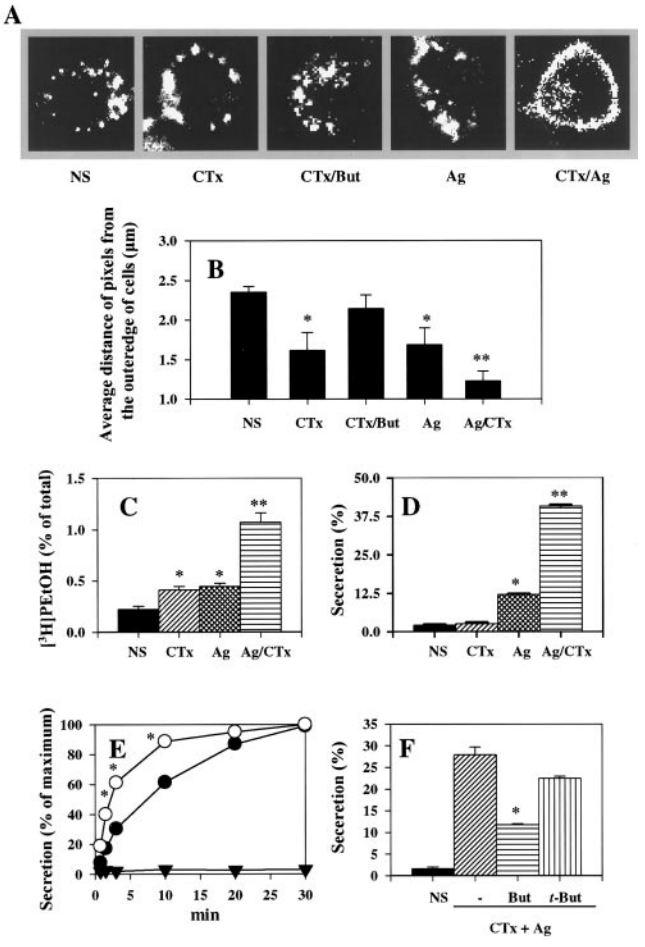

FIGURE 5. Treatment with cholera toxin induces movement of granule-associated EGFP-PLD1 to cell periphery and facilitates secretion of granules in response to Ag stimulation. [^{3}H]Myristate-labeled RBL-2H3 cells were transfected with EGFP-tagged PLD1 and incubated in the absence or presence of 1 μg/ml cholera toxin for 4 h (CTx) with or without 50 mM 1-butanol (But) before addition of vehicle (NS) or 3 ng/ml DNP-BSA (Ag) for an additional 5 min. Cells were then examined by confocal microscopy and subjected to morphometric analysis. Location of the EGFP-tagged PLD1 for a typical experiment (*A*) and the calculated distance of granules from plasma membrane for three separate experiments (five cells each) (*B*) are shown. In the same experiments production of [^{3}H]phosphatidylethanol (*C*), a measure of PLD activity, and secretion of hexosaminidase (*D*) were also measured. *E*, The time course of secretion, expressed as a percentage of maximal response (○, Ag plus cholera toxin; ●, Ag; ▼, cholera toxin). The effects of 1- and *tert*-butanol on secretion in cholera toxin-treated cells were determined in three additional experiments (*F*). The data show mean ± SEM except where values for SEM fall within the data points. Significant differences in values are indicated by asterisks (*, $p < 0.05$; **, $p < 0.01$).

final panel), activation of PLD (Fig. 5*C*), and secretion (Fig. 5*D*) when the cells were subsequently stimulated with a low dose of Ag (3 ng/ml). Cholera toxin by itself did not induce secretion (Fig. 5*D*). Cholera toxin treatment enhanced not only the extent of secretion (i.e., as in Fig. 5*D*) but also the rate of the secretory response to low-dose Ag (Fig. 5*E*). This accelerated response was attenuated in the presence of 1-butanol but not in the presence of *tert*-butanol (Fig. 5*F*). Studies with a minimal stimulatory dose (75 nM) of thapsigargin produced virtually identical results to those shown in Fig. 5 (data not shown). These results suggested that, after treatment cholera toxin, granules were poised for rapid fusion with the plasma membrane once $[Ca^{2+}]_i$ increased upon addition of Ag or thapsigargin.

Experiments were undertaken to identify PKs that might regulate translocation and fusion of granules with the cell membrane. Ag-induced translocation of granules was suppressed by H89 and KN93, which inhibit PKA and CaM kinase II, respectively (Fig. 6*A*). However, the PKC inhibitor, Ro31-7549, had unexpected effects. Although Ro31-7549 blocked fusion of PLD1-labeled granules with the plasma membrane (Fig. 6*A*), effects on translocation were unclear because this inhibitor perturbed intracellular structure in both stimulated and, to a lesser extent, unstimulated cells (data not shown). We found no previous reports of disruption of intracellular structure by PKC inhibitors. All three kinase inhibitors suppressed secretion (Fig. 6*B*).

To focus on fusion events, cells were first primed with cholera toxin or vehicle as a control and then stimulated with Ag in the presence of the kinase inhibitors. Inhibitors were tested at concentrations that caused half maximal (Fig. 7*A*) and maximal (Fig. 7*B*) inhibition of secretion. Secretion was still blocked by all three kinase inhibitors in cholera toxin-treated cells. Nevertheless, cholera toxin-treated cells were more resistant than control cells to the inhibitory effects of KN93, especially with the high dose of this inhibitor (Fig. 7*B*). This increased resistance was verified by additional experiments with various doses of KN93 (Fig. 7, *C* and *D*). Similar results were obtained in studies with thapsigargin (data not shown). These studies indicated that all three PKs participated at some stage of the secretory process and, possibly, that CaM kinase II plays a less prominent role than PKC and PKA in the final fusion events.

A role for PLD2 in the fusion events

The preceding findings strongly indicated that PLD1 was essential for translocation of granules to the cell periphery before their eventual fusion with the plasma membrane. We next investigated whether expressed PLD2 participates in the secretory process, because its location in the plasma membrane might facilitate membrane fusion. The secretory response to thapsigargin was enhanced

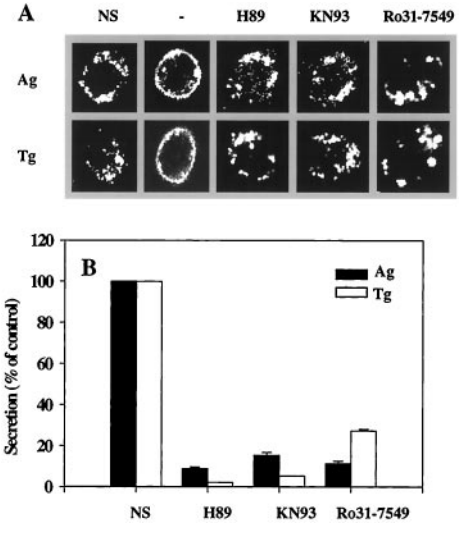

FIGURE 6. Effects of inhibitors of serine/threonine PKs on translocation and secretion of PLD1-labeled granules in Ag-stimulated cells. Vehicle (NS) or inhibitor (30 μM H-89, 50 μM KN-93, or 10 μM Ro31-7549) were added to cultures of RBL-2H3 cells 10 min before addition of 25 ng/ml DNP-BSA (Ag). The localization of EGFP-tagged PLD1 by confocal microscopy (*A*) and secretion of hexosaminidase (*B*) were determined 15 min thereafter. Values in *B* were mean ± SEM of three experiments.

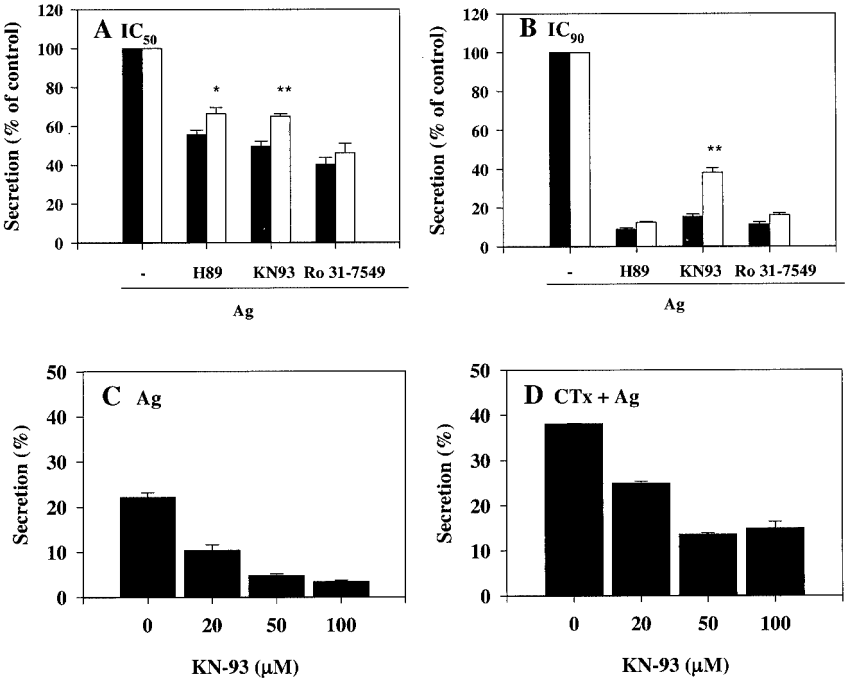

FIGURE 7. Suppression of Ag-induced secretion by inhibitors of serine/threonine PKs in untreated and cholera toxin-treated cells. *A* and *B*, RBL-2H3 cells were incubated in the absence (filled bars) or presence (open bars) of 1 μg/ml cholera toxin for 4 h. Inhibitors were added to cultures at concentrations that caused 50 (*A*) or 90% (*B*) inhibition of secretion 10 min before addition of Ag (10 ng/ml DNP-BSA). The concentrations of inhibitors were as follows: 10 and 30 μM H-89, 20 and 50 μM KN-93, and 3 and 10 μM Ro31-7549. *C* and *D*, Cells were incubated in the absence (*C*) or presence of cholera toxin (*D*) as described above. The indicated concentrations of KN-93 were added before addition of Ag. Secretion was measured 15 min later. Values are mean ± SEM from three separate experiments. For *A* and *B*, values are expressed as a percentage of response to Ag alone (22.1 ± 1.1 and 38.1 ± 0.2% secretion in *A* and *B*, respectively) and asterisks indicate significant differences between untreated and cholera toxin-treated cells (*, $p < 0.05$; **, $p < 0.01$).

by expression of either EGFP-tagged PLD1 or PLD2 and suppressed by expression of the catalytically inactive mutants of PLD1 and PLD2 (Fig. 8*A*). Similar but more modest effects on secretion were observed in Ag-stimulated cells (Fig. 8*B*). Analysis by flow cytometry indicated that 20–30% of cells expressed EGFP-tagged PLDs. Experiments with cells selected for expression of the EGFP-tagged PLDs (5) were unsuccessful because of poor responses to Ag after the protracted selection procedure.

Discussion

As previously noted, studies with intact and permeabilized RBL-2H3 cells strongly suggest that activation of PLD (1, 2, 7), along with the activation of PKC and a rise in $[Ca^{2+}]_i$ (4), provide essential signals for secretion in these cells. Nevertheless, secretion occurs only when the activation of PLD and PKC is accompanied by sustained increases in $[Ca^{2+}]_i$. For example, activation of PLD, and presumably PKC, by PMA fails to induce secretion (Ref. 7 and this paper). Also, secretion is not observed when RBL-2H3 cells are stimulated via adenosine A_3 receptors, which promote sustained activation of PLD but only transient release of calcium ions from intracellular stores (26). Thapsigargin and Ag, in contrast, provoke a sustained increase in $[Ca^{2+}]_i$, activation of PLD, and secretion (1, 7). Moreover, the activation of PLD by thapsigargin correlates exactly with secretion under a variety of experimental manipulations (1).

In this paper we show that both isoforms of expressed PLD can participate in the secretory process and may do so at different steps, namely PLD1 in the movement of secretory granules to the periphery and PLD2 (and possibly PLD1) in the fusion of granules with the plasma membrane. The evidence for these conclusions is the following: 1) PLD1 is expressed predominantly on secretory granules and PLD2 on plasma membrane; 2) both PLD isoforms probably participate in the secretory process, as expression of the catalytically inactive form of either PLD1 or PLD2 suppresses thapsigargin-induced and, to a lesser extent, Ag-induced secretion; and 3) a role for PLD1 in the movement of granules to the cell periphery is suggested by the suppression of this phase of secretion in cells expressing the catalytically inactive form of PLD1. The location of PLD2 implies that its role is restricted to the plasma membrane, where it presumably facilitates docking or fusion of the granule with the plasma membrane. However, the data do not exclude a role for PLD1 as well as PLD2 in these fusion events.

It would appear that Ag-induced secretion is less dependent on PLD than thapsigargin-induced secretion (Fig. 8). The reason for this is unknown, but, unlike Ag, thapsigargin weakly stimulates PLC (1), and activation of PKC by thapsigargin (Z. Peng and M. A. Beaven, unpublished observations) may depend primarily on PLD-mediated generation of PKC activators. However, PLD could regulate secretory events other than PKC, as will be discussed later.

If PLD1 regulates translocation of granules and PLD2, alone or in combination with PLD1, regulates fusion of translocated granules with the plasma membrane, the activation of these two isoforms must then be coordinated to maintain effective coupling

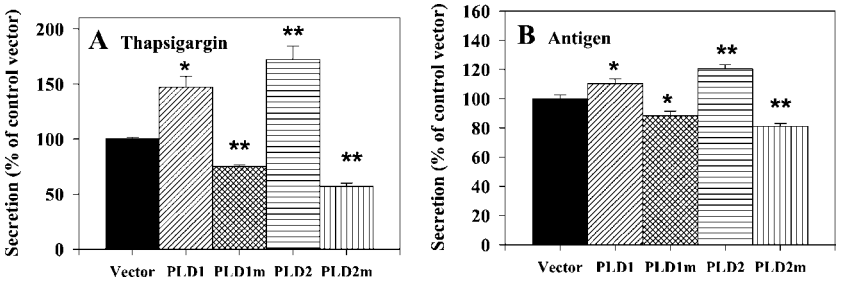

FIGURE 8. Expression of catalytically inactive mutants of PLD suppresses exocytosis of secretory granules. RBL-2H3 cells were transfected with the gene for vector, EGFP-tagged PLD1, PLD1K898R, PLD2, or PLD2K758R and stimulated with 25 ng/ml DNP-BSA (Ag) or 300 nM thapsigargin (Tg) for 15 min for determination of secretion of hexosaminidase. Data are mean ± SEM of values from seven experiments and asterisks indicate significant differences in values (*, $p < 0.05$; **, $p < 0.01$).

of the two events. Both PLD1 and PLD2 when expressed in RBL-2H3 cells respond to the same external stimulants (7). They are both activated in a synergistic manner by any combination of PMA, thapsigargin, and cholera toxin and by Ag (7). These and other studies with inhibitors in RBL-2H3 cells suggest that both expressed isoforms of PLD as well as endogenous PLD may be regulated by PKC, CaM kinase II, and PKA, although it is unknown whether these kinases regulate PLD directly or indirectly. The fact that expressed PLD1 and PLD2 respond in tandem to the same stimulants means that either both isoforms are directly regulated by the same stimulatory signals or activation of PLD1 leads to activation of PLD2 as granules begin to fuse with the plasma membrane. PLD2 requires phosphatidylinositol 4,5-bisphosphate for its activity (10), but unlike PLD1 no other regulatory factor has been described to date for PLD2 (37). Phosphatidylinositol 4,5-bisphosphate production is increased following PLD activation (2, 38, 39), probably as a consequence of stimulation of type I phosphatidylinositol 4 phosphate 5-kinase (40) by the PLD product, phosphatidic acid. Thus, one possible scenario to account for the coactivation of both isoforms of PLD is that, once granules and their associated PLD1 begin to fuse with the plasma membrane, PLD1-stimulated phosphatidylinositol 4,5-bisphosphate production leads to activation of PLD2 in the plasma membrane. This scenario extends the notion of a positive feedback loop between phosphatidylinositol 4 phosphate 5-kinase and PLD1 (39) to include PLD2.

Further studies are necessary to elucidate the exact roles of the PKs and calcium at different steps of the secretory process in mast cells. The studies with inhibitors suggest that in Ag-stimulated cells PKA, PKC, and CaM kinase II all participate at one or more stages of the secretory process and that translocation of PLD1-labeled granules is regulated by PKA and CaM kinase II. The role of PKC in the latter process is uncertain. Nevertheless, the ability of cholera toxin, and possibly PMA, to induce movement of granules to the cell periphery suggests that this process can be mediated via a single kinase and can occur regardless of changes in $[Ca^{2+}]_i$. Therefore, there may be some redundancy in the regulation of PLD and secretion by the various PKs.

Many studies of exocytosis have focused on the interaction of phosphatidylinositol 4,5-bisphosphate and PKs with proteins such as synaptotagmin and the calcium-dependent activator protein for secretion that are thought to promote docking and fusion of granules with the plasma membrane. PKC, CaM kinase II, and cAMP-dependent kinases have been implicated in the regulation of recruitment of secretory granules in various types of secretory cells (reviewed in Ref. 41). Phosphatidylinositol 4,5-bisphosphate is required for successful exocytosis of granules in neuroendocrine and chromaffin cells, where it may recruit and regulate the interaction of the calcium sensor, synaptotagmin (42), and the calcium-dependent activator protein for secretion with the fusion apparatus (43). The connections of PLD to these events have not been determined, but regulation of exocytosis and PLD by the same PKs and the stimulation of phosphatidylinositol 4,5-bisphosphate synthesis via PLD point to possible connections. Other possible connections include recruitment and activation of essential components to membranes by virtue of the charged nature of phosphatidic acid (44) and the promotion of membrane fusion by phosphatidic acid itself. PLA_2-mediated fusion of neutrophil membranes is augmented by phosphatidic acid and, of relevance to this study, stimulation of intact neutrophils leads to activation of PLD in granules as well as plasma membrane (6).

Although we believe from our data that the two isoforms of PLD act cooperatively in coordinating translocation and fusion events in RBL-2H3 cells, it is likely that the exact role of PLD in secretion may vary from one cell type to another. A recent report, also based on studies with expressed PLDs, indicates an essential role for PLD1 but not PLD2 in fusion events in chromaffin cells (5). In these cells, PLD2 was not detectable and PLD1 was located primarily in the plasma membrane.

An interesting detail in the present studies is the recycling of PLD1 from the plasma membrane to granules. The phenomenon points to possible reuse of this enzyme by the cell. It incidentally reassured us that EGFP-PLD1 was not initially expressed at an inappropriate site within the cell (45). Another interesting detail is that only a fraction of rat mast cell protease II-positive granules acquired PLD1 (Fig. 1*C*), yet the majority of PLD1-labeled granules appeared to be secreted in all cells. A consistent feature of RBL-H3 cells is that secretion of other granule constituents such as histamine, 5-hydroxytryptamine, and hexosaminidase rarely exceeds 50% of that originally present in the cells (for examples, see Refs. 4, 29, and 32). This is probably due to incomplete secretion of granules from individual cells, as almost all cells (~90%) secrete granules in response to Ag (Fig. 5 in Ref. 46). Therefore, it is possible that only granules associated with PLD1 are competent to secrete, but this remains to be determined.

In summary, PLD1 in RBL-2H3 cells appears to play an essential role in translocation of secretory vesicles/granules to the plasma membrane through recruitment of newly synthesized and recycled PLD1, although the molecular details of how this is accomplished requires further study. In addition, PLD1 and PLD2 appear to serve distinct but complementary roles in secretion when both are expressed within the cell.

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