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Direct Inhibition of *in Vitro* PLD Activity by 4-(2-Aminoethyl)-Benzenesulfonyl Fluoride

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While conducting a purification protocol of phospholipase D (PLD) from human granulocytes, we observed that PLD activity was inhibited by a commonly-used protease inhibitor cocktail. Of the six inhibitors present in the cocktail, the serine protease inhibitor, 4-(2-aminoethyl)-benezensulfonyl fluoride (AEBSF), was found to be the sole inhibitor of PLD. AEBSF caused a loss of neutrophil and purified plant PLD activities *in vitro,* but not in intact cells at the concentrations used, nor did it affect the related phospholipases A_2 and C, that were utilized as specificity controls. The compound $AEBSNH_2$, which has the fluoride replaced by an $-NH_2$ group, failed to affect PLD activity as did other compounds structurally related to AEBSF with known protease inhibitory capabilities. Finally, basal- and agonist-stimulated PLD activity was inhibited in phosphatidylcholine-specific anti-PLD immunoprecipitates (IC_{50} = 75 μM). These results suggest that AEBSF, in an effect probably unrelated to its anti-proteolytic ability, directly interferes with PLD enzymatic activity, making it a significant compound to begin analyzing the role of PLD in mammalian cell signaling.

Key Words: **PLD; neutrophils; protease inhibitor; signal transduction.**

Abbreviations used: PLD, phospholipase D; PC, phosphatidylcholine; PA, phosphatidic acid; PEt, phosphatidylethanol; PBu, phosphatidylbutanol; PIP_2, phosphatidylinositol-4,5-bis-phosphate; PC8, dioctanoyl phosphatidylcholine; PIC, protease inhibitor cocktail; E-64, trans-epoxysuccinyl-1-leucylamido-(4-guanidino) butane; AEBSF, 4-(2-aminoethyl)-benezensulfonyl fluoride; $AEBSNH_2$, 4-(2-aminoethyl)-benzene-sulfonamide; TLCK, tosyl-lysine chloromethyl ketone (*N*-[5-amino-1-(chloroacetyl)pentyl]-4-methyl-benzenesulfonamide); TPCK, tosyl-phenylalanine chloromethyl ketone (*N*-[3-chloro-2-oxo-1-phenylmethyl)propyl]-4-methyl-benzenesulfonamide); pTF, *p*-toluenesulfonyl fluoride; pTCl, *p*-toluenesulfonyl chloride; PMA, phorbol, 12-myristate, 13-acetate; MAPK, mitogen-activated protein kinase; FMLP, fMet-Leu-Phe.

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Phospholipase D catalyzes the hydrolysis of phosphatidylcholine into choline and phosphatidic acid. The wide range of fates that phosphatidic acid (PA) has, enables it to become a significant second messenger and ties phospholipase D to many important cellular events including: mitogenesis, cell signaling, synthesis of superoxide radicals, inflammation, exocytosis, membrane delivery, vesicle budding, phagocytosis, and apoptosis [reviewed 1–11].

There are many pathways involved in PLD signaling with many upstream and downstream regulators. Some of the proposed regulators are the protein tyrosine kinases [1, 6–8, 10] and the mitogen-activated protein kinases (MAPKs), which may act as upstream and/or downstream regulators of PLD [12–16]. Both of these types of kinases are very abundant in the human neutrophil and could likely facilitate PLD activation. A number of inhibitors of PLD activity have been described; however, they generally do not act directly on PLD itself. Rather, they cause inhibition by interfering with molecules that are required for PLD activation, such as the small GTP-binding proteins [17–19, 21], namely ARF and/or Rho, PIP_2 [17, 21], tyrosine phosphorylation [20, 22, 23] or lipid repression [26].

We have uncovered that neutrophil PLD activity is inhibited in the presence of a commonly-used protease inhibitor cocktail. The common and unexplainable loss of PLD activity during protein purification observed by various authors, might be explained if a certain component of the protease mix used in cell disruption and column chromatography buffers were to inhibit PLD activity when it is later assayed from eluates. The effect that each individual protease inhibitor in the cocktail had on PLD activity was analyzed separately in order to determine which specific inhibitor or inhibitors were responsible for the observed decrease in PLD activities. The serine protease inhibitor 4-(2-aminoethyl)-benzenesulfonyl fluoride, AEBSF, was

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found to be the only inhibitor of neutrophil PLD activity and the effect was exerted directly on the enzyme as demonstrated in three cell-free systems: cell sonicates, purified plant enzyme, and anti-PLD immunoprecipitates. Although the IC_{50} for the latter *in vitro* enzymatic assays was 75 μM, AEBSF only represents a starting point for structural modification that could potentially evolve into a much-needed specific inhibitor for studying PLD signaling.

MATERIALS AND METHODS

Materials. 1,2-Dioctanoyl-sn-glycero-3-phosphocholine (PC8), L-α-phosphatidyl-choline, β-arachidonoyl-γ-stearoyl (arachidonoyl-PC), purified cabbage PLD, bee venom PLA_2, pancreatic PLA_2 and silica gel (70-230 mesh, 60 Å pore), the protease inhibitor cocktail (P-2714), AEBSF, EDTA, bestatin, E-64, leupeptin, aprotinin, PMA and FMLP were obtained from Sigma (St. Louis, MO); *Bacillus cereus* PLC was from Boehringer Mannheim (Indianapolis, IN); n-[1-^{3}H] butanol (5 Ci/mmol) and L-α-phosphatidylcholine, β-arachidonoyl [^{3}H]-γ-stearoyl PC (200 Ci/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO); L-3-phosphatidylcholine, 1-stearoyl-2-[1-^{14}C]arachidonyl-3-PC (114 mCi/mmol) was from Amersham Life Science (Arlington Heights, IL); 1,2-dioleoyl-*sn*-glycero-3-phosphoethanol (PEt) and 1,2-dioleoyl-sn-glycero-3-phosphobutanol (PBu) standards were obtained from Avanti Polar Lipids (Alabaster, AL); LK6D silica gel 60 Å TLC plates were from Whatman (Clifton, NJ); Scintiverse II scintillation cocktail was purchased from Fisher (Pittsburgh, PA); $AEBSNH_2$, TLCK and TPCK were from RBI (Natick, MA); pTF and pTC1 were from Aldrich (Milwaukee, WI); electrophoresis and Bradford protein assay chemicals were from Bio-Rad Laboratories (Richmond, CA); Immobilon PVDF membranes were from Millipore (Bedford, MA). Anti-phosphotyrosine (PY-20 clone) affinity purified monoclonal antibody and anti-p42-MAPK monoclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-PC specific PLD1 and anti-PC specific PLD2, N-terminal, affinity purified antibodies were from QCB (Camarillo, CA). Enhanced chemiluminescence (ECL) Western blotting detection reagents were obtained from Amersham Pharmacia Biotech (Piscataway, NJ).

Isolation of human peripheral blood neutrophils. The isolation procedure was based on English and Anderson [27], with some minor modifications. Between 50–55 ml of blood were collected from the antecubital vein of healthy individuals in a 60-ml syringe containing 6 ml of sodium citrate as anticoagulant. Blood was mixed with 15 ml of 6% dextran, allowed to settle, and the plasma and buffy coat were removed and spun down at 800*g* for 5 min. The pellet was resuspended in 35 ml of saline and centrifuged at 800*g* for 15 min at 10°C in a Ficoll-Histopaque discontinuing gradient. Neutrophils were recovered and contaminating erythrocytes were lysed by hypotonic shock. Cells were spun again and the pellet was flash frozen in dry ice and ethanol and stored at −70°C until later use. Since no heparin was used as an anticoagulant, but sodium citrate instead, the possibility of activation during isolation is extremely small. No neutrophil aggregation (i.e., the hallmark for neutrophil activation) was observed during this isolation procedure.

Preparation of cell sonicates and addition of protease inhibitors. Flash-frozen cells were allowed to thaw on ice and 5 mM HEPES pH 8.0 (hypotonic sonication buffer) was added in differing concentrations based upon pellet size. The cells were resuspended and sonicated on ice (three cycles, 10 sec each). The sonicated cells were then placed on ice for 10 min to promote further lysis. Protein concentration was determined as previously described by Bradford [28] and the sonicates were kept on ice until used in PLD activity assays, or frozen immediately after stimulation for activity assays or SDS–PAGE.

Measurement of PLD and other phospholipases. Phospholipase D activity was directly measured *in vitro* by utilizing the transphosphatidylation reaction with short-chain PC (PC8) and [^{3}H]butanol exogenous substrates as previously described by Davis *et al.* [29] with only minor modifications. 50 μl of cell sonicates (0.8–1.2 mg/ml protein) were added to 1.5 ml-Eppendorf tubes containing 50 μl of the following assay mix: 4.5 mM phospholipid, 75 mM HEPES, pH 7.9, and 4.7 μCi [^{3}H]butanol. The resulting PLD reaction (final volume 100 μl) was incubated for 15 min at 30°C. The reactions were stopped by adding 0.3 ml ice-cold chloroform/methanol (1:2) and 70 μl of 1% perchloric acid. The tubes were then vortexed and 100 μl of 1% perchloric acid and 100 μl of chloroform were added. The tubes were again vortexed and centrifuged before aspirating the upper phase. The lower phase was washed once with 100 μl 1% perchloric acid and an aliquot of 125 μl was removed to be dried for thin-layer chromatography (TLC). TLC lanes were scraped and dissolved in 3 ml of Scintiverse I scintillation cocktail and counted by scintillation spectrometry.

Phospholipase A_2 activity was measured *in vitro* by the production of radiolabeled arachidonic acid from an exogenous substrate as described by Van den Bosch *et al.* [30] with minor modifications. Briefly, samples (neutrophil sonicates or purified PLA_2) were added to 1.5 ml-Eppendorf tubes containing the following final reaction conditions (in 100 μl): 0.25 M Tris–HCl, pH 8.5, 10 mM $CaCl_2$, 12 nmol arachidonoyl-PC and 0.2 μCi [^{3}H]arachidonoyl-PC, and incubated for 20 min at 30°C. The reactions were stopped by adding 0.5 ml Dole's extraction medium (2-propanol, *n*-heptane, 1 N H_2SO_4; 40:10:1, v/v), 0.4 ml *n*-heptane and 0.3 ml H_2O. The upper phase was chromatographed over a 240-mg, 70-230 mesh, silica gel column and a 0.6-ml diethyl ether wash was collected directly into scintillation vials and counted by scintillation spectrometry.

Phospholipase C activity was measured *in vitro* by the production of radiolabeled diglycerides from an exogenous substrate as described by Smith and Waite [31] with some modifications. Samples (cell sonicates or purified PLC) were added to 1.5 ml-Eppendorf tubes containing the following final reaction conditions (in 100 μl): 15 mM Tris–HCl, pH 7.4, 30 mM NaCl, 1.5 mM $CaCl_2$, 50 nCi L-3-phosphatidylcholine, 1-stearoyl-2-[1-^{14}C]arachidonyl-3-PC, 12 nmol distearoyl-PC and 1 mM sodium deoxycholate, and incubated for 10 min at 30°C. Lipids were separated by TLC in benzene/chloroform/methanol (80:15:3.25; v/v/v) that separates DAG subspecies (Rf $\sim$ 0.4) from PA (Rf = 0), triacylglycerides (Rf $\sim$ 0.7) and other neutral lipids (Rf $\sim$ 0.9). Zones comigrating with authentic standards (C14:0/C14:0-DAG; C16:0/C18:0-DAG) were scraped and counted.

In vitro and intact-cell enzymatic assays. The standard procedure used in this paper for PLD, referred to as "*in vitro*" assay involved neutrophil cells that were flash frozen upon isolation from human blood, thawed, sonicated immediately and left on ice until AEBSF was added, to either the assay mix or to the cell sonicates (but not to both), with a similar outcome, at the time of assaying enzymatic activity. For an "intact cell" stimulation, freshly isolated neutrophils (1×10^7 cells/ml cells) were pretreated with AEBSF for 15 min and then stimulated with either PMA (50 ng/ml) for 5 min or with fMet-Leu-Phe (100 μM) in presence of cytochalasin B (0.1 μg/ml) for 3 min. The cells were spun down (13,000*g*, 15 sec) to remove inhibitor (unless otherwise indicated) and pellets were flash frozen. Later, the cells were thawed, sonicated and assayed for activity.

Immunoprecipitation and Western blotting. For immunoprecipitation, total cell sonicates were incubated with the immunoprecipitating anti-PLD antibodies [32]. PC-specific anti-PLD antibodies

TABLE 1

Protease Inhibition Cocktail (PIC) Composition

	PIC Concentration			
	2×	1×	0.5×	0.25×
AEBSF (mM)	2	1	0.5	0.25
EDTA (mM)	1	0.5	0.25	0.12
Bestatin (μM)	130	65	32.5	16.25
E-64 (μM)	1.4	0.7	0.35	0.17
Leupeptin (μM)	1	0.5	0.25	0.12
Aprotinin (μM)	0.3	0.15	0.075	0.037

Note. The PIC lyophilized powder (1 bottle) was dissolved in 1 ml of H_2O to prepare a 100× stock from which aliquots were taken to achieve the indicated dilutions in the PLD *in vitro* assay, as described under Materials and Methods.

were conjugated overnight with goat anti-rabbit IgG linked to agarose beads in a conjugation buffer (10 mM HEPES, pH 7.3, 1 mM EGTA, 0.1 mM PMSF, 0.21 mM sodium orthovanadate, 1 mM ammonium molybdate, 1.2 mM diisopropyl fluorophosphate (DFP), 10 mM *p*-nitro-phenyl-phosphate (PNPP), 0.5% Triton X-100, and 0.5 100 μg/ml each of leupeptin, aprotinin, and pepstatin A). Conjugates were thoroughly mixed (1:1; v/v) with cell sonicates for 4-h (antibody final concentration, 3 μg/ml). Immune complexes were recovered by centrifugation and washed with buffer A (100 mM Tris–HCl, pH 7.4, 400 mM LiCl) and two times with buffer B (10 mM Tris–HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA). Immune complexes were resuspended in a final volume of 60 μl with sonication buffer and assayed for PLD or with 60 μl of sonication buffer for subsequent PLD assays under the same conditions described above. Controls were run with immunoprecipitates carried in the absence of primary antibodies and resulting background radioactive counts were subtracted from test results.

RESULTS

Effects of Protease Inhibitor Cocktail (PIC) on Neutrophil PLD Activity in Vitro

After noticing a great loss of enzyme activity along the different steps of protein purification, we suspected that the column buffers might contain an inhibitor. We investigated whether a protease inhibitor cocktail (PIC) (a mixture of water-soluble inhibitors with broad specificity for Ser, Cys, Asp and metallo-proteases; see Table 1 for composition]) routinely included in protein purification had an effect on PLD activity *in vitro*. Thus, we incorporated PIC directly into the PLD activity assay described under Methods and we observed that it indeed inhibited the phospholipase activity at the two pH's (in HEPES buffer) measured (Fig. 1A) although slightly more at the more basic pH. Next, we carried out activity assays using HEPES (pH 8.0, optimal) and a range of PIC concentrations. The data presented in Fig. 1B shows that addition of the protease inhibitor cocktail is inhibitory to PLD activity in a concentration-dependent manner, with 0.5 × PIC concentration resulting in ~50% inhibition of PLD activity.

Determination of the Specific Compound(s) Responsible for PLD Inhibition

In order to determine the specific protease inhibitor(s) responsible for inhibiting PLD activity, we assayed PLD in the presence of each of the six individual components of the cocktail at varying concentrations:

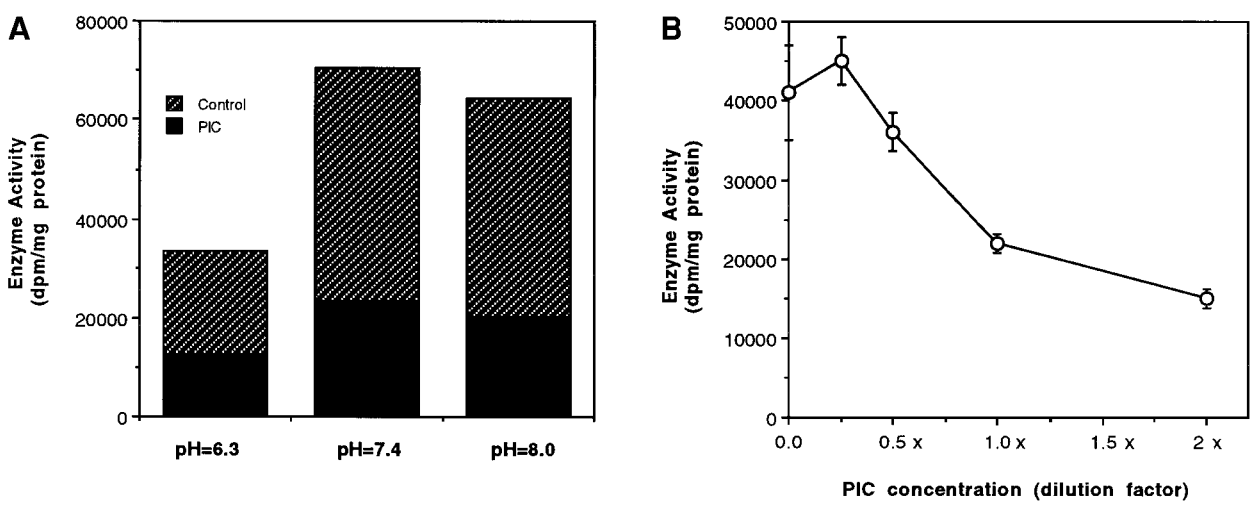


FIG. 1. Effects of PIC on neutrophil PLD activity *in vitro*. (A) PLD activity assays were carried out in HEPES buffer at the indicated pHs with (solid bars) or without (hatched bars) PIC. (B) Various concentrations of PIC were incorporated into the assay mix as indicated and activity assays were conducted as described (see Table 1 for concentration of individual inhibitors at the indicated dilutions). These data represent the average ± SEM of four separate experiments each done in duplicate.

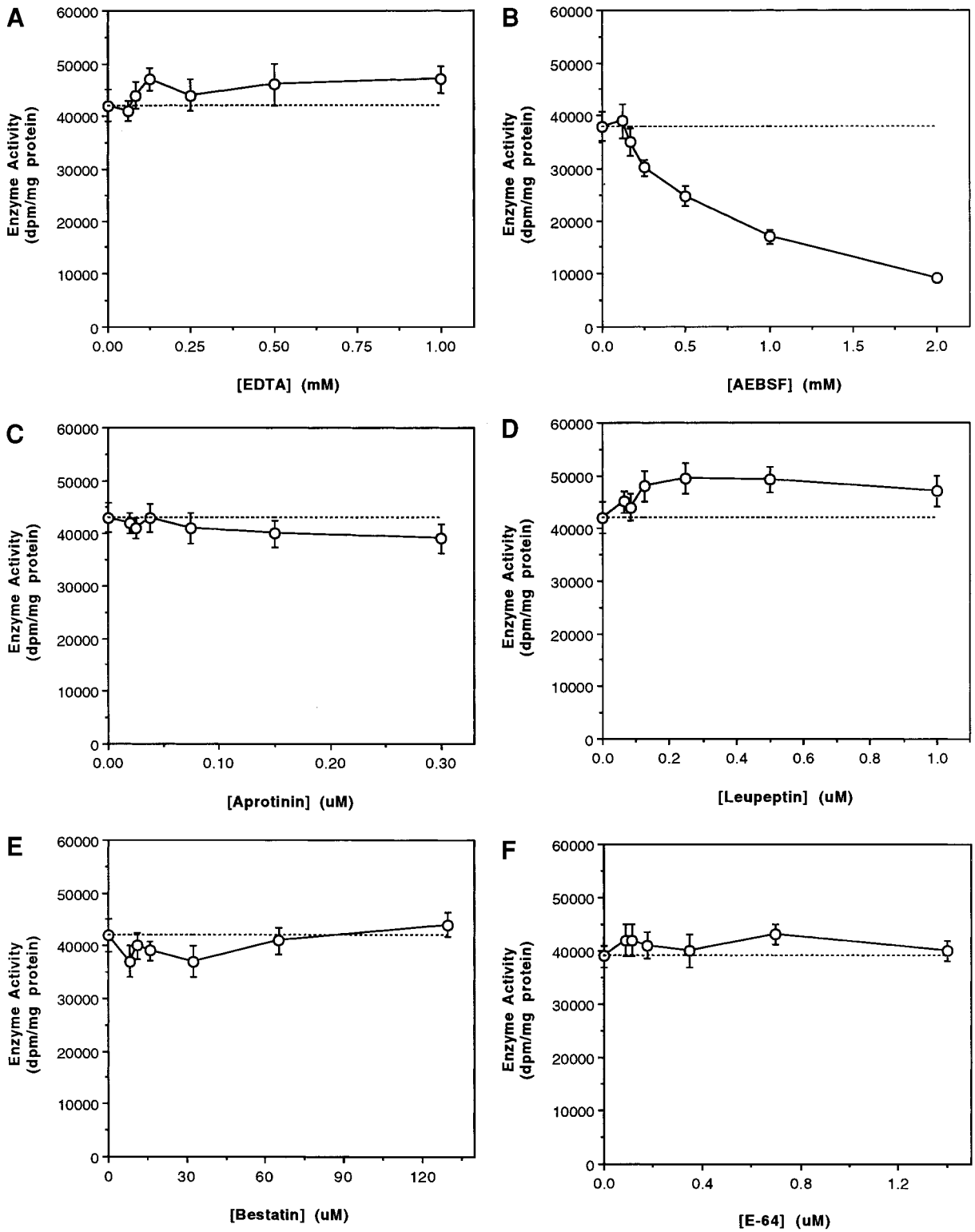


FIG. 2. Effects of each individual PIC protease inhibitor on *in vitro* neutrophil PLD activity. PLD activity was assayed separately in the presence of each individual protease inhibitor contained within the cocktail: EDTA, AEBSF, aprotinin, leupeptin, bestatin, and E-64. Various concentrations (1/8× through 2× PIC concentration) of each inhibitor were incorporated into the assay mix, assayed, and compared to controls as shown. The data represent the average ± SEM of four separate experiments each done in duplicate. Horizontal dotted lines have been drawn at control values.

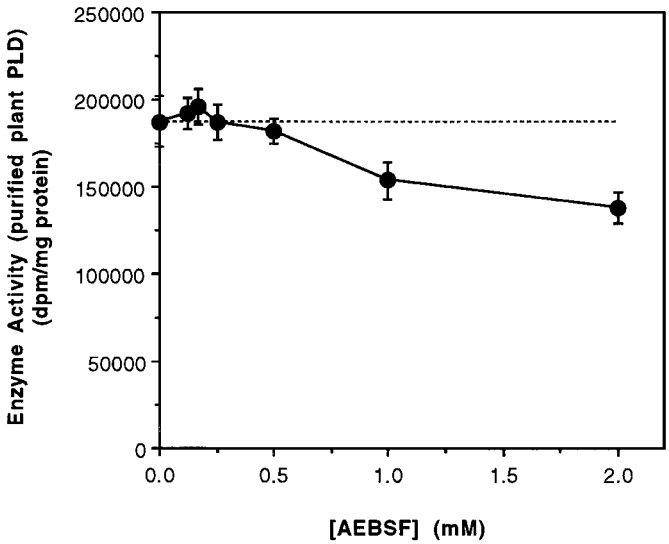


FIG. 3. Effects of various concentrations of AEBSF on purified cabbage PLD *in vitro*. Various concentrations of AEBSF were incorporated into the assay mix and activity assays were conducted using purified cabbage PLD as the enzyme source. The data represent the average ± SEM of three separate experiments each done in duplicate. The horizontal dotted line has been drawn at control values.

EDTA (0–2 mM), AEBSF (0–4 mM), aprotinin (0–0.5 μM), leupeptin (0–2 μM), bestatin (0–260 μM), or E-64 (0–2.8 μM). AEBSF was identified as the sole inhibitor of PLD activity, having an IC_{50} of ~50 μM (Fig. 2). Conversely, EDTA and leupeptin showed no inhibition, but a slight stimulatory effect. This suggests that these protease inhibitors are perhaps somewhat protective of PLD activity.

Purified Plant PLD

Similar activity assays were conducted *in vitro* with purified cabbage phospholipase D in place of the neutrophil sonicates. This was done in order to examine the effects of AEBSF on PLD in the absence of any serine proteases and other molecules that are usually present in unpurified samples. Figure 3 shows that this purified cabbage PLD is also inhibited by AEBSF, but to a lesser (~25%) extent than neutrophil PLD (75%). It also indicates that AEBSF can inhibit PLD by a direct mechanism.

AEBSF on in Vitro and on Intact-Cell PLD Activities

AEBSF at various concentrations was included in the *in vitro* PLD enzyme assay mixture. Figure 4 indicates that AEBSF exerts its inhibitory effect at short times (~20% activity loss at 0.5 min) and that 250 μM is needed for statistically significant differences in activity. A different set of experiments was also undertaken where freshly isolated, intact, neutrophils were pretreated with 500 μM AEBSF for 15 min and then stimulated with PMA or FMLP. In the first set (Fig. 5, left), cells were washed free off the inhibitor after stimulation. AEBSF did not inhibit basal or agonist-stimulated PLD activity. When AEBSF was present at the time of cell sonication (Fig. 5, middle), basal and agonist-stimulated PLD activities were lessened by ~60%. Further, when AEBSF was present during cell sonication and at the time of PLD activity measurement (Fig. 5, right), the inhibition reached ~85%. Thus, the inhibitory effect of AEBSF is observed at the tested concentrations in the *in vitro* setting but not in intact cells. A lack of effect in whole-cell neutrophils was observed on two common routes of signal transduction: tyrosine phosphorylation and p42-MAPK (Fig. 6).

AEBSF on PLD Immunocomplexes

To ascertain if AEBSF would inhibit PLD in a more enriched sample than whole cell sonicates, we performed immunoprecipitation with specific anti-PLD antibodies and then assaying the immunocomplex agarose beads for PLD activity in the presence or absence of AEBSF. The results presented in Fig. 7 indicate that PLD activity was strongly inhibited by AEBSF *in vitro*. A calculated IC_{50} of ~75 μM in this enriched sample preparation was a value close to one order of magnitude higher than that observed for whole cell sonicates (Fig. 2).

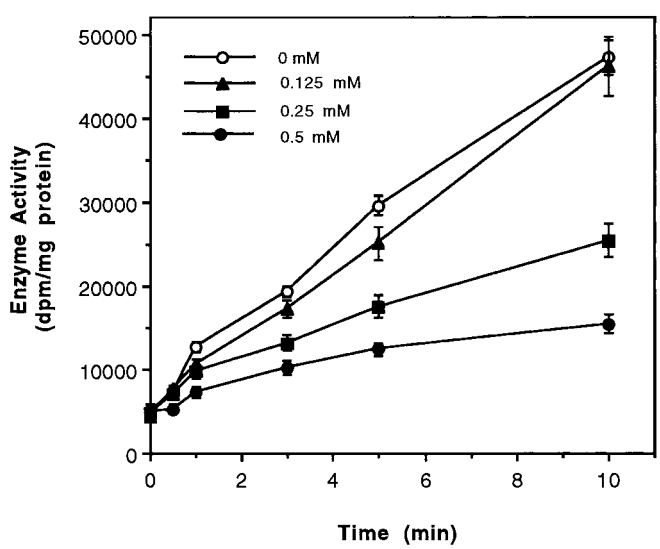


FIG. 4. Time course/dose dependency of AEBSF. Differing concentrations of AEBSF were added to cell sonicates at the time of enzyme assay and incubated at 30°C for the times shown. After the indicated time, the sonicates were removed from incubation and assayed for activity. For reliable comparisons, each sample was incubated for exactly 20 min in the activity assay. The results are the average of three separate experiments. The data represent the average ± SEM of three separate experiments.

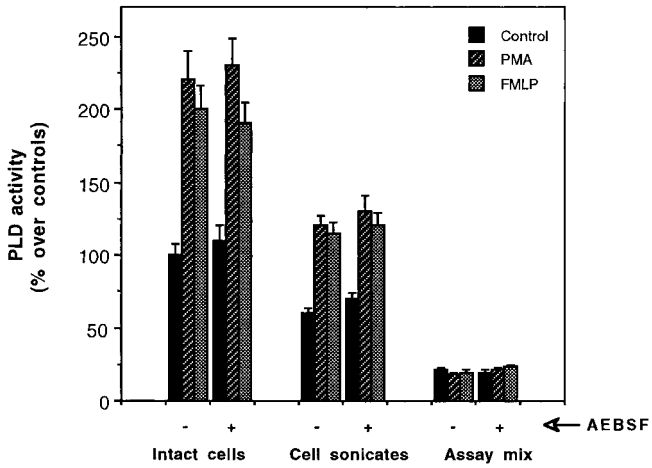


FIG. 5. Effects of AEBSF on neutrophil PLD activity in intact cells and *in vitro*. Neutrophils (1×10^7 cells/ml) were pretreated with 500 μM AEBSF for 6 min at 37°C. Following this incubation, some cells were stimulated with PMA or FMLP for 5 min (hatched bars) or left untreated (solid bars). After stimulation, cells were divided in three sets and spun down (13,000*g*, 15 sec) to remove the inhibitor, the supernatant was then decanted and the pellets were flash frozen. The first set (left group of bars) was sonicated in the absence of AEBSF and the second (middle group of bars) and third (right group of bars) sets were sonicated in its presence. Aliquots of sonicates were assayed for PLD activity in the standard assay mix that further included AEBSF only for the third set. These experiments are the average ± SEM of three separate experiments each done in duplicate.

Effect on Other Phospholipases

We next examined the effect of AEBSF on related PC-specific phospholipases, namely, PLA_2 and PLC. As reported in Table 2 no appreciable inhibition was observed on PLA_2 from neutrophil sonicates or purified enzyme from either bee venom or from bovine pancreas. Similarly no inhibition was present on PLC activity from either neutrophil or purified enzyme from *Bacillus cereus*. Thus, the observed inhibitory effect of AEBSF is specific for PLD activity.

Structural Analogues of AEBSF

Finally we aimed to investigate what part of the structure in the AEBSF molecule was responsible for inhibition. Out of the several structural analogues that were considered (Table 3), PLD inhibition was most potent in AEBSF (Table 4). Substitution of the fluoride group for $-NH_2$ abrogated the inhibitory effect. The *p*-aminoethyl group was also important to confer an effect. Quantitatively, the inhibitory potency was: AEBSF $\gg$ TLCK $>$ pTF $>$ pTCl $\gg$ TPCK = $AEBSNH_2$.

DISCUSSION

It has been known for a long time that the presence of protease inhibitors is a requirement for protein purification. Without them, uncontrolled proteolysis would occur and result in considerable damage to cellular proteins and an abundant loss in enzyme functionality. In our search for an appropriate concentration of inhibitors to use for mammalian PLD purification, we found that a commonly used protease inhibitor cocktail (PIC) resulted in a concentration-dependent inhibition of neutrophil PLD activity from column purification eluates. Only a 4 times dilution (0.25×) of the original concentrate allowed for maintenance of higher protein levels by preventing proteolysis and preserving PLD activity. We report that AEBSF was highly inhibitory while the other protease inhibitors in the cocktail, at the manufacturer's recom-

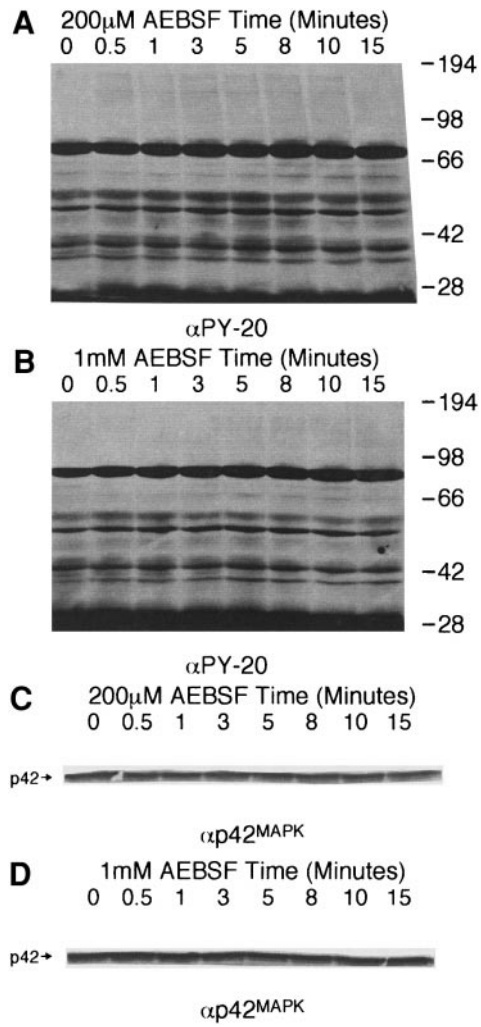


FIG. 6. Effects of AEBSF on neutrophil PY-proteins and p42-MAPK. Western blots were prepared from whole cell sonicates after neutrophils were stimulated with either 200 μM (A) or 1 mM AEBSF (B) for the indicated lengths of time. (C, D) Represent the same blots after they were stripped of anti-phosphotyrosine antibodies and re-probed with anti-p42-MAPK (ERK2) antibodies. These results are representative of two different experiments.

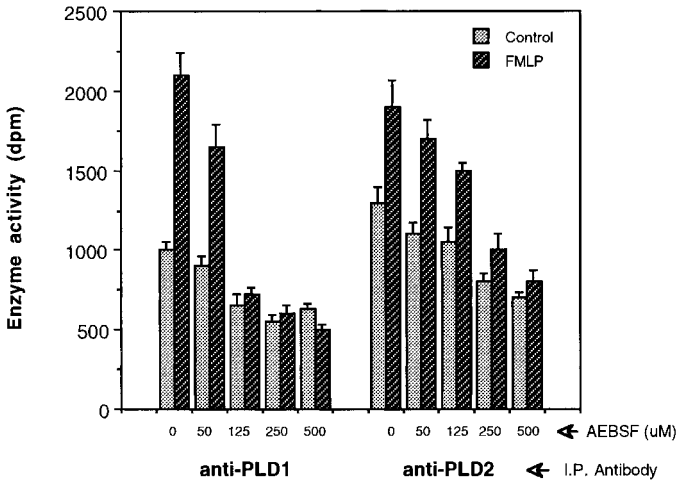
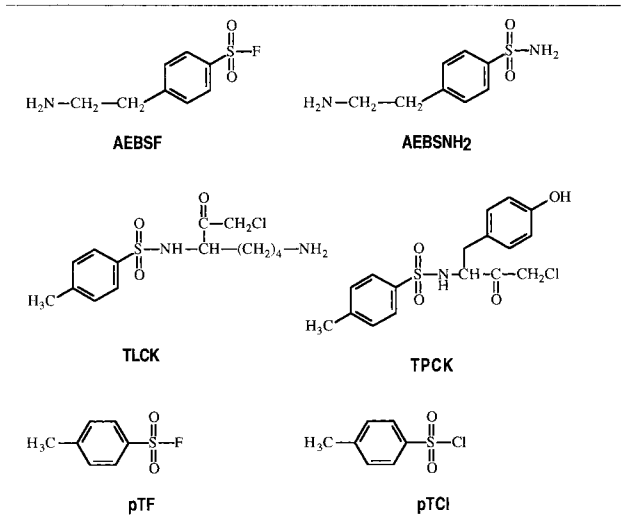


FIG. 7. Effect of AEBSF on PLD activity in immunocomplexes. Neutrophil cells were subjected to stimulation with FMLP for 3 min, washed flash-frozen, and sonicated. The cell sonicates (approximately 1.6 mg protein/ml) were subjected to immunopreciptation with either 3 μg/ml anti-PLD1 or anti-PLD2 affinity-purified polyclonal antibodies. Immunocomplex agarose beads were recovered by centrifugation and assayed for *in vitro* PLD activity with [^{3}H]butanol as elaborated under Materials and Methods in the presence or the absence of the indicated concentrations of AEBSF. Immunoprecipitation data presented are the average ± SEM of four separate experiments.

TABLE 3

Chemical Structures of AEBSF Analogs Used in This Study



mended concentrations, showed little or no significant effects on PLD activity. It is worth noting that AEBSF working concentrations [EC_{50} = 500 μM in the *in vitro* studies (Fig. 2) and 75 μM for the enzyme-enriched/immunoprecipitation setting (Fig. 7)] are relatively low to achieve an effect on intact cells (Fig. 5). In previous studies, authors have used 2 mM or 200 μM for a 24-hour incubation to achieve AEBSF-induced inhibition on the NADPH oxidative burst [24, 33–35].

As for the exact mechanism by which a serine esterase inhibitor exerts its effects on PLD, several points should be considered. First, does it work on an upstream mechanism that regulates PLD in the process of cell signaling or does it directly affect the enzyme? For the former, superoxide release has been shown to be prevented and/or inhibited by AEBSF in various systems such as macrophages and monocytes [35]. A mechanism was proposed in which the enhanced functions of priming for superoxide release could be regulated by proteolysis by serine proteases, although the target protein of the proteases was unknown. Others have proposed the possibility of such a proteolytic mechanism for PLD activation in response to inhibitor. Kessels *et al.* described the inhibitor of chymotrypsin-like proteases, zLYCK, in human neutrophils with fMLP-induced PLD [21]. Also, Lukowski *et al.* suggest

TABLE 2

Effect of AEBSF Inhibitor on Other Phospholipases

	PLA_2 activity			PLC activity	
	Neutrophil	Bee venom (60 units)	Pancreas (3 units)	Neutrophil	B. cereus (100 units)
Control	874 ± 55	17603 ± 1046	3416 ± 212	1066 ± 66	72879 ± 4030
100 μM	890 ± 70	19537 ± 957	3088 ± 249	1190 ± 79	68715 ± 3641
250 μM	851 ± 59	17204 ± 938	3509 ± 199	1392 ± 100	74933 ± 4819
500 μM	803 ± 65	18365 ± 1224	4127 ± 260	1122 ± 91	77456 ± 5111

Note. Phospholipase activities were measured *in vitro* against an appropriate exogenous substrate (see Materials and Methods) in the presence or absence of the indicated concentrations of AEBSF. Results shown are free [^{3}H]-arachidonic acid dpm (PLA_2 activity) or free [^{14}C]-diacylglycerol dpm (PLC activity). "Neutrophil" refers to 50 μl of neutrophil cell sonicates (0.8–1.2 mg protein/ml). Original stocks of purified phospholipases were: PLA_2 from been venom, 600–1800 units/mg protein at pH 8.9; PLA_2 from bovine pancreas, 25–75 units/mg protein at pH 8.0; PLC from *Bacillus cereus*, 2000 units/mg protein at pH 6.6–8.0. Values presented in the table are the mean ± SEM of three independent experiments performed in quadruplicate.

TABLE 4

Effect of Structural Analogs of AEBSF on PLD Activity

	Analog Concentration (μM)			
	0	100	250	500
AEBSF	31981 ± 1599	29110 ± 1892	25757 ± 1152	17127 ± 1198
$AEBSNH_2$	30252 ± 1510	30192 ± 1721	28829 ± 1960	28446 ± 2270
TLCK	29756 ± 2289	28473 ± 2063	27012 ± 1160	24589 ± 1707
TPCK	27397 ± 1917	28796 ± 2015	31122 ± 2391	30239 ± 2116
pTF	34103 ± 1364	33721 ± 1783	30740 ± 1816	29345 ± 1404
pTCl	31493 ± 1420	29981 ± 2341	30184 ± 12475	29028 ± 2177

Note. Neutrophil PLD activity ([^{3}H]butanol dpm/mg protein) was measured *in vitro* in the presence or absence of the indicated concentrations of AEBSF and 5 structural analogs. To normalize the data (due to the fact that some compounds were water soluble and others were water insoluble), all 6 were dissolved as concentrated stocks in DMSO and added to the PLD reaction mixture (see Materials and Methods). The final concentration of DMSO was fixed at 0.5 μl/100 μl in each case (DMSO vehicle alone added to 0 μM column). Values are the mean ± SEM of four independent experiments performed in duplicate.

that the protein fodrin could be involved in regulation of cytoskeletal proteins and PLD by a proteolytic mechanism [25] or by blocking access of PLD to PIP_2 [17, 36]. Inhibition of PLD activity has also been described in the presence of Rho-GDP dissociation inhibitor [18], and presqualene diphosphate (PSDP) is the first lipidic substance of intracellular origin that shows a direct inhibition of PLD and LTB_4- and lipoxin A_4-stimulated superoxide release [26].

However, it is our belief that AEBSF works directly on the enzyme rather than on an upstream mechanism that regulates PLD, based on the following: (i) AEBSF inhibits PLD *in vitro* in a dose- and time-dependent manner, and at the concentrations used, does not affect intact cells, whether they were resting or stimulated with classical neutrophil agonists; (ii) AEBSF inhibited purified plant PLD; (iii) AEBSF inhibited immunoprecipitated PLD (i.e., a high-enriched enzyme preparation). However, it can not be excluded at this time the possibility that AEBSF inhibits PLD activity by altering an upstream regulator (a serine protease) of PLD that had been coimmunoprecipitated by the antibody. This possibility seems unlikely since immunocomplexes treated with a glycine-based buffer (pH = 2) and neutralization to pH = 7.4, to dissociate the antigen-antibody complex still showed the same pattern of activity and/or activity inhibition as those shown in Fig. 7.

If a direct effect of AEBSF on PLD is to be contemplated, it remains to be learned the exact mechanism (i.e., catalytic or allosteric site) by which a serine esterase inhibitor exerts its effects on a phospholipase. Chemical alterations on the molecule will undoubtedly aid in answering this question. The study using the 5 structural analogues (Tables 3 and 4) revealed several facts regarding the chemical properties needed to elicit PLD inhibition. The fluoride atom is necessary for its PLD inhibitory effect, as demonstrated by the lack of effectiveness by $AEBNH_2$. Some marginal inhibition of PLD was observed with the chloromethyl ketone TLCK, but not with TPCK, that has an extra hydrophobic benzene ring. Some inhibition was observed with pTF indicating that the *p*-aminoethyl moiety is also key for the PLD inhibitory effect perhaps by making a nucleophilic attack to the substrate more likely. However, the observation that $AEBSNH_2$ (which is also inactive in inhibiting proteolysis) resulted in an absence of PLD inhibition, does not mean that any protease inhibitor will be a PLD inhibitor. In fact, leupeptin, aprotinin, bestatin, E-64, and the structural analogues (that are also protease inhibitors) TLCK, TPCK, pTF and pTCl had marginal or no effect on PLD, which led us to hypothesize that AEBSF might inhibit PLD without involving its antiproteolytic activity *per se.* In this vein, AEBSF has been described previously as inhibiting activation of macrophages by inhibition of signal transduction rather than inhibition of proteases [37].

Finally, a systematic study of this compound allowed us to conclude that AEBSF was unable to exert any measurable effect on related phospholipases, namely PLA_2 and PLC (Table 2), further establishing its possible usefulness as a specific inhibitor of PLD. The synthesis of a compound based on the AEBSF structure that would act *in vitro* at nM concentrations would make this inhibitor useful to explore a putative role of PLD in cell signaling in an investigator's particular system of study.

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