Role of a membrane-associated serine esterase in the oxidant activation of phospholipase A₂ by t-butyl hydroperoxide

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Exposure of bovine pulmonary-arterial endothelial cells to the oxidant lipid t-butyl hydroperoxide (t-Bu-OOH) increases cell-membrane-associated phospholipase A₂ (PLA₂) activity and stimulates arachidonic acid (AA) release. To test the hypothesis that a membrane-associated serine esterase plays an important role in activating PLA₂, the present study was undertaken. In addition to increasing PLA₂ activity and AA release, t-Bu-OOH also enhances the activity of a membrane-associated serine esterase that cleaves the synthetic substrate *N*α-*p*-tosyl-L-arginine methyl ester (TAME). Changes in the activity of this membrane-bound serine esterase correlate directly with changes in the activity of PLA₂. Serine esterase inhibitors such as phenylmethanesulphonyl fluoride, di-isopropyl fluorophosphate

and α₁-proteinase inhibitor, and TAME, a synthetic substrate for serine esterase, prevent the increase in serine esterase activity, PLA₂ activity and AA release caused by t-Bu-OOH. Pretreatment of the endothelial cells with the antioxidant vitamin E prevents t-Bu-OOH-induced stimulation of AA release and the cell-membrane-associated serine esterase and PLA₂ activities. Adding t-Bu-OOH or the serine esterase trypsin to the endothelial-cell membrane fraction also significantly augments PLA₂ activity, implying that these treatments activate latent PLA₂. These results suggest that t-Bu-OOH stimulates a membrane-associated serine esterase that plays a crucial role in activating PLA₂ and releasing AA.

INTRODUCTION

Activation of phospholipase A₂ (PLA₂) with subsequent release of arachidonic acid (AA) is an important physiological and pathophysiological event. Since a serine esterase activates pancreatic phospholipase A₂, which is secreted as an inactive pro-enzyme (De Haas et al., 1968), it has been proposed that a serine esterase may be involved in regulating the activity of intracellular PLA₂. Previous reports provide support for this concept, because exposure of neutrophils and smooth-muscle cells to serine esterases may increase PLA₂ activity (Kawaguchi and Yasuda, 1988; Camussi et al., 1989). In addition, serine esterase inhibitors may prevent the increase in PLA₂ activity and AA release caused in platelets by agonists such as platelet-activating factor, thrombin and the prostaglandin H₂ analogue U46619 (Sugatani et al., 1987).

Because the oxidant lipid hydroperoxide t-butyl hydroperoxide (t-Bu-OOH) increases AA release from bovine pulmonary-arterial endothelial cells by activating phospholipase A₂ (Chakraborti et al., 1989), we used this model to test the hypothesis that a membrane-associated serine esterase plays a role in the activation of PLA₂. We asked the following questions: does t-Bu-OOH stimulate serine esterase activity in the endothelial-cell membrane? do changes in membrane serine esterase activity correlate with changes in PLA₂ activity? do serine esterase inhibitors prevent the increase in PLA₂ activity and AA release resulting from t-Bu-OOH? and does adding t-Bu-OOH or the serine esterase trypsin increase PLA₂ activity in the endothelial-cell membrane fraction? Our results indicate that a

membrane-associated serine esterase plays a crucial role in the activation of PLA₂ by the oxidant t-Bu-OOH.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) was obtained from Whittaker Bioproducts (Walkersville, MD, U.S.A.). Fetal-calf serum, L-glutamine, non-essential amino acids, gentamycin sulphate and PBS without Ca²⁺ and Mg²⁺ were purchased from GIBCO (Grand Island, NY, U.S.A.). The trypsin used to passage the cells was obtained from GIBCO. t-Bu-OOH, di-isopropyl fluorophosphate (DFP), phenylmethanesulphonyl fluoride (PMSF), *N*α-*p*-tosyl-L-arginine methyl ester (TAME), α₁-proteinase inhibitor (α₁-PI), elastase, fatty-acid-free BSA and unlabelled phosphatidylcholine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Type XIII trypsin (Sigma; from bovine pancreas) that had been treated with tosylphenylalanylchloromethane was used in the experiments with the endothelial-cell membrane fraction. [1-¹⁴C]AA and the phospholipid 1-stearoyl-2-[1-¹⁴C]arachidonoyl-*sn*-glycero-3-phosphocholine were obtained from New England Nuclear (Wilmington, DE, U.S.A.). All other chemicals were of analytical grade.

Cell culture

Bovine pulmonary-arterial endothelial cells were obtained from the American Tissue Culture Collection (A.T.C.C. 209) (Rockville, MD, U.S.A.) and studied between passages 17 and 25, as

Abbreviations used: t-Bu-OOH, t-butyl hydroperoxide; PLA₂, phospholipase A₂; AA, arachidonic acid; DFP, di-isopropyl fluorophosphate; TAME, *N*α-*p*-tosyl-L-arginine methyl ester; PMSF, phenylmethanesulphonyl fluoride; α₁-PI, α₁-proteinase inhibitor; DMEM, Dulbecco's modified Eagle's medium.

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previously described (Chakraborti et al., 1989). Cells were maintained in DMEM supplemented with 20% fetal-calf serum, L-glutamine and non-essential amino acids. Cells were subcultured after treatment with 0.25% trypsin. All experiments were performed on confluent monolayers and in serum-free DMEM supplemented with fatty-acid-free BSA (1 mg/ml).

Preparation of cell membrane fraction

The endothelial-cell plasma-membrane fraction was isolated by the method of Neville (1968) with some modifications. Briefly, the endothelial cells grown in T-150 flasks were washed twice with ice-cold PBS. The cells were then scraped from the flasks and suspended in ice-cold homogenizing medium containing 0.25 M sucrose and 5 mM Tris/HCl buffer (pH 7.4) and homogenized by using a Dounce homogenizer with a tight-fitting pestle. The homogenate was then strained through cheesecloth and successively centrifuged at 13 000 *g* for 30 min and at 80 000 *g* for 1 h at 4 °C. The 80 000 *g* pellet was suspended in 2.5 ml of 10% (w/v) sucrose containing 10 mM Tris/HCl (pH 7.4) and was layered on a discontinuous gradient consisting of 40% (w/v) and 20% (w/v) sucrose both containing 10 mM Tris/HCl (pH 7.4). The gradient was centrifuged at 100 000 *g* for 2 h at 4 °C in a Spinco LSW 27 rotor (Beckman). The fraction collected at the 20%/40%-sucrose interface was diluted with 10 mM Tris/HCl (pH 7.4) and was used as the cell-membrane fraction. Protein concentrations were assayed as described by Lowry et al. (1951).

Assay of 5'-nucleotidase and acid phosphatase activities in the membrane fraction

Release of P_i from 5'-AMP, an index of 5'-nucleotidase activity, was measured by the method of Chen et al. (1956). Acid phosphatase activity was determined at pH 5.5 with *p*-nitrophenyl phosphate as the substrate (Fleischer and Packer, 1974).

Measurement of [14 C]AA release

Cells grown on 6-well plates were washed twice with PBS and incubated for 20 h with [14 C]AA (2 μ Ci/well). After incubation, the supernatant was removed and the cells were washed twice with PBS. To measure t-Bu-OOH-mediated AA release, cells were exposed for 15 min to 300 μ M t-Bu-OOH in serum-free DMEM supplemented with 1 mg/ml fatty-acid-free BSA. [14 C]AA release was determined as previously described (Chakraborti et al., 1989).

Assay of PLA₂ activity *in vitro*

Endothelial monolayers were washed twice with PBS (pH 7.2). The cells were then removed in PBS with a rubber policeman and the cell membrane fraction was isolated as described above. A 20 μ l portion of the membrane preparation (2–3 mg of protein) was added to 30 μ l of the reaction mixture which contained (final concns.): Tris buffer (100 μ M), NaCl (100 mM), deoxycholate (1 mM), and the phospholipid L-3-phosphatidylcholine, i.e. 1-stearoyl-2-[1- 14 C]arachidonoyl-*sn*-glycero-3-phosphocholine (10 μ M). PLA₂ activity was assayed at pH 9.0, which is the optimal pH when phosphatidylcholine is used as the substrate (Clark et al., 1986).

The phospholipid 1-stearoyl-2-[1- 14 C]arachidonoyl-*sn*-glycero-3-phosphocholine was evaporated under N₂, resuspended in

deoxycholate (5 mM), sonicated in a water-bath sonicator, and added to the assay mixture in a volume of 10 μ l. The reaction mixture was allowed to incubate for 1 h at 37 °C, and then the reaction was stopped by addition of 50 μ l of chloroform/methanol (2:1, v/v), followed by addition of 50 μ l of chloroform and 50 μ l of 4 M KCl. This mixture was centrifuged at 12 000 *g* in a microfuge to separate the organic and aqueous phases. The organic phase was removed, applied to a silica-gel t.l.c. plate (Analtech, Newark, DE, U.S.A.), and chromatographed in chloroform/methanol/acetic acid/water (25:15:4:2, by vol.) to separate the phosphatidylcholine from the major product produced by phospholipase A₂ activity, i.e. lysophospholipid. Authentic standards were chromatographed with the lipid extract to locate the compounds of interest, and these components were scraped from the plate and counted by liquid-scintillation spectroscopy. PLA₂ activity was quantified as the release of the free fatty acid from phosphatidylcholine. Unlabelled phosphatidylcholine was added to the labelled phosphatidylcholine to give a specific radioactivity of 510 mCi/mmol (Clark et al., 1986).

Effect of serine esterase inhibitors and vitamin E

To determine the effect of the serine esterase inhibitors on basal and t-Bu-OOH-stimulated AA release and PLA₂ activity, cells were pretreated with PMSF (1 mM), DFP (1 mM) and α_1 -PI (1 mg/ml) for 20 min before adding t-Bu-OOH. To investigate the effect of vitamin E pretreatment, the endothelial cells were pretreated with vitamin E (1 mM) for 20 min before adding t-Bu-OOH (300 μ M).

Effect of trypsin and t-Bu-OOH on PLA₂ activity in the endothelial-cell membrane fraction

Bovine pulmonary-arterial endothelial cells grown in T-150 flasks were washed twice with PBS (pH 7.2). The cells were then removed with a rubber policeman, and the cell membrane fraction was isolated by the procedure described above. The membrane fraction was then treated with trypsin (1 μ g/ml) for 15 min at 37 °C, and PLA₂ activity was measured as described above. To determine whether serine esterase inhibitors would block the increase in PLA₂ activity caused by trypsin, the endothelial-cell membrane fraction was pretreated for 20 min with PMSF (1 mM), DFP (1 mM) and α_1 -PI (1 mg/ml) before adding trypsin. To test whether or not t-Bu-OOH augments PLA₂ activity, the cell membrane fraction was treated with t-Bu-OOH (300 μ M) alone or combined with trypsin (1 μ g/ml).

Determination of endothelial-cell membrane-associated serine esterase activity

Serine esterase activity was assessed by determining the hydrolysis of the synthetic substrate TAME, by the method of Hummel (1959). Briefly, 300 μ l of the substrate (37.9 mg/10 ml of water) was added to 2.4 ml of 0.05 M Tris buffer containing 10 mM CaCl₂ (pH 8.5). The reaction was initiated by adding 50 μ g of the endothelial-cell membrane fraction, giving a final reaction volume of 3 ml. Hydrolysis was measured as an increase in A_{247} . A blank without enzyme activity was included to monitor for spontaneous hydrolysis of the substrate. To determine the effect of t-Bu-OOH on serine esterase activity, the endothelial cells were exposed to t-Bu-OOH (300 μ M) for 15 min. The membrane fraction was isolated and serine esterase activity was determined as described above. To determine the effect of the serine esterase inhibitors PMSF (1 mM), DFP (1 mM) and α_1 -PI (1 mg/ml) on the increase in serine esterase activity caused by t-Bu-OOH, cells were

pretreated with the above inhibitors for 20 min before addition of t-Bu-OOH.

Comparison of the endothelial-cell membrane-associated serine esterase with elastase and cathepsin G

Azocasein degradation

Cleavage of azocasein was determined by the method of Starkey and Barrett (1976). Portions (50 µg) of elastase, cathepsin G or endothelial-cell membrane were added to 0.5 ml of an azocasein solution (6% in 0.1 M Tris, pH 7.0), and the final volume was adjusted to 1.0 ml by addition of 0.1 M Tris. This reaction mixture was incubated at 37 °C for 90 min, precipitated with 3 ml of cold 5% trichloroacetic acid, and then centrifuged at 200 *g* for 10 min. The supernatant was removed and the A₄₁₀ was measured.

Haemoglobin degradation

Haemoglobin degradation was determined by a modification of the method originally described by Anson (1940). Portions (50 µg) of elastase, cathepsin G or the endothelial-cell membrane were incubated with 1% non-denatured haemoglobin in 0.01 M PBS (pH 7.2) at 37 °C for 20 h. The suspension was then precipitated with 5% trichloroacetic acid and pelleted by centrifugation at 10000 *g* for 10 min. The A₂₈₀ of the supernatant was measured.

Cleavage of synthetic peptides

Synthetic chromogenic peptide substrates were used to characterize the endothelial-cell membrane-associated serine esterase. We used Suc-Ala₃-pNA and Suc-Ala₂-Val-pNA as substrates for elastase and Suc-Ala₂-Pro-Phe-pNA as a substrate for chymotrypsin-like enzymes (Suc, succinyl; pNA, *p*-nitroanilide). To assess the activity of the serine esterases for these substrates, we used with some modifications the method described by Barrett (1981). Briefly, 50 µl of the substrate (1 µg/ml of dimethyl sulphoxide) was added to 0.1 M Tris/HCl buffer (pH 7.0). The reaction was initiated by adding 50 µg of elastase, cathepsin G or the endothelial-cell membrane fraction. The final reaction volume was adjusted to 3 ml. Cleavage was measured by the increase in A₄₁₀.

Statistical analysis

Data were analysed by unpaired *t* test, repeated-measures analysis of variance, and the Duncan multiple-range test for group comparisons (Snedecor and Cochran, 1980). Statistical significance was assumed when *P* < 0.05.

RESULTS

In bovine pulmonary-arterial endothelial cells, the oxidant lipid hydroperoxide t-Bu-OOH increases the activity of a membrane-associated serine esterase, the activity of PLA₂, and the release of [¹⁴C]AA (Table 1). Pretreatment of the endothelial cells with PMSF, DFP and α₁-PI decreases serine esterase activity, PLA₂ activity and AA release under basal conditions (Table 1). These inhibitors also prevent the increase in serine esterase activity, PLA₂ activity and AA release caused by t-Bu-OOH (Table 1). Changes in the activity of the membrane-bound serine esterase correlate directly with changes in the activity of membrane PLA₂

(Figure 1). Pretreatment of the cells with vitamin E also prevents the t-Bu-OOH-induced increase in serine esterase activity, PLA₂ activity and AA release (Table 1).

We characterized the membrane fraction by measuring at different steps in the preparation process the activity of acid phosphatase, a lysosomal enzyme, and the activity of 5'-nucleotidase activity, an endothelial plasma-membrane marker (Ketis et al., 1986) (Table 2). Compared with the crude membrane pellet, the membrane fraction has a 16-fold decrease in specific activity of acid phosphatase and a 12-fold increase in specific activity of the plasma-membrane marker 5'-nucleotidase.

The endothelial-cell membrane-associated serine esterase appears distinct from elastase, cathepsin G or chymotrypsin. For

Table 1 Effect of the oxidant t-Bu-OOH and serine esterase inhibitors on membrane-associated serine esterase and PLA₂ activities and AA release from bovine pulmonary-arterial endothelial cells

Results are means ± S.E.M. (*n* = 4): **P* < 0.01, ***P* < 0.05 compared with appropriate control; †*P* < 0.01 compared with t-Bu-OOH.

Treatment	Serine esterase activity (change in A ₂₄₇ /min per mg)	PLA ₂ activity (pmol of AA/min per mg of protein)	[¹⁴ C]AA release (c.p.m./10 ⁵ cells)
Control	0.54 ± 0.08	3.28 ± 0.28	468 ± 22
t-Bu-OOH (300 µM)	3.94 ± 0.42*	9.18 ± 0.72*	1154 ± 53*
PMSF (1 mM)	0.22 ± 0.03*	2.42 ± 0.16**	395 ± 19**
PMSF (1 mM) + t-Bu-OOH (300 µM)	0.82 ± 0.11†	3.54 ± 0.33†	520 ± 28†
DFP (1 mM)	0.28 ± 0.04*	2.33 ± 0.19**	382 ± 21**
DFP (1 mM) + t-Bu-OOH (300 µM)	0.71 ± 0.12†	3.50 ± 0.28†	514 ± 32†
α ₁ -PI (1 mg/ml)	0.31 ± 0.05**	2.40 ± 0.17**	388 ± 18**
α ₁ -PI (1 mg/ml) + t-Bu-OOH (300 µM)	0.92 ± 0.16†	3.72 ± 0.31†	531 ± 36†
Vitamin E (1 mM)	0.46 ± 0.05	3.12 ± 0.23	438 ± 24
Vitamin E (1 mM) + t-Bu-OOH (300 µM)	0.66 ± 0.07†	3.44 ± 0.38†	503 ± 27†

Figure 1 Changes in membrane-associated serine esterase activity correlate directly with changes in membrane-associated PLA₂ activity

Data were obtained from the experiments shown in Table 1.

Table 2 Specific activity of 5'-nucleotidase and acid phosphatase in membrane fractions isolated from bovine pulmonary-arterial endothelial cells

5'-Nucleotidase activity is expressed as μmol of phosphate/min per mg of protein. Acid phosphatase activity is expressed as μmol of *p*-nitrophenol/min per mg of protein. Values are presented as means $\pm$ S.E.M. ($n = 4$). The values in parentheses indicate the activity as a percentage of that of the 13000 *g* pellet (values of the 13000 *g* pellet are set at 100%).

Fraction	5'-Nucleotidase activity	Acid phosphatase activity
13000 <i>g</i> pellet	0.035 ± 0.005	0.758 ± 0.1
80000 <i>g</i> pellet	0.276 ± 0.04 (788%)	0.206 ± 0.03 (37%)
Membrane fraction	0.454 ± 0.09 (1297%)	0.045 ± 0.008 (6%)

Table 3 Characteristics of bovine pulmonary-arterial endothelial cell membrane-associated serine esterase compared with elastase and cathepsin G

The activity of each serine esterase is expressed compared with the activity of the enzyme that is most reactive with each substrate. The activity of the most reactive enzyme was defined as 100%. Values shown are percentage activities (means $\pm$ S.E.M.; $n = 4$).

Substrate	Endothelial-cell membrane-associated serine esterase	Elastase	Cathepsin G
Azocasein	0	55 ± 6	100
Haemoglobin	0	100	52 ± 8
Synthetic peptides			
Suc-Ala ₃ -pNA	0	100	0
Suc-Ala ₂ -Val-pNA	0	100	0
Suc-Ala ₂ -Pro-Phe-pNA	0	0	100
TAME	100	0	0

Table 4 Effect of trypsin and t-Bu-OOH on membrane-associated PLA₂ activity in pulmonary endothelial cells

Results are means $\pm$ S.E.M. ($n = 4$); * $P < 0.01$ compared with control; † $P < 0.01$ compared with trypsin or t-Bu-OOH alone.

Treatment	PLA ₂ activity (pmol of AA/min per mg of protein)	Change (% versus control)
Control	3.16 ± 0.32	
Trypsin (1 $\mu\text{g}/\text{ml}$)	$8.47 \pm 0.68^*$	+168
PMSF (1 mM)	2.64 ± 0.26	-16
Trypsin (1 $\mu\text{g}/\text{ml}$) + PMSF (1 mM)	$3.89 \pm 0.37^\dagger$	+23
DFP (1 mM)	2.84 ± 0.22	-10
Trypsin (1 $\mu\text{g}/\text{ml}$) + DFP (1 mM)	$3.54 \pm 0.28^\dagger$	+12
α_1 -PI (1 mg/ml)	3.02 ± 0.25	-4
Trypsin (1 $\mu\text{g}/\text{ml}$) + α_1 -PI (1 mg/ml)	$4.15 \pm 0.42^\dagger$	+31
t-Bu-OOH (300 μM)	$8.02 \pm 0.54^*$	+154
Trypsin (1 $\mu\text{g}/\text{ml}$) + t-Bu-OOH (300 μM)	$11.48 \pm 0.86^*$	+263

example, it differs from elastase and cathepsin G in its inability to degrade azocasein and haemoglobin (Table 3). In addition, this serine esterase does not cleave synthetic substrates for elastase, such as Suc-Ala₃-pNA and Suc-Ala₂-Val-pNA, or a substrate for chymotrypsin-like enzymes, Suc-Ala₂-Pro-Phe-pNA (Table 3). In contrast, TAME is a synthetic substrate for the endothelial-cell membrane-associated serine esterase, but not for elastase or cathepsin G (Table 3).

To test further the hypothesis that a serine esterase can activate intracellular PLA₂, we treated the endothelial-cell membrane fraction with trypsin. Trypsin markedly stimulates membrane-associated PLA₂ activity, and serine proteinase inhibitors prevent the increase in PLA₂ activity caused by trypsin (Table 4). The oxidant t-Bu-OOH also strikingly increases PLA₂ activity in the endothelial-cell membrane fraction (Table 4). Combined treatment of the endothelial-cell membrane fraction with t-Bu-OOH and trypsin further augments PLA₂ activity compared with the effect of trypsin or t-Bu-OOH alone (Table 4).

DISCUSSION

Given the potency of the metabolic products of AA, the activity of intracellular PLA₂ must be tightly regulated. The biochemical mechanisms involved in the regulation of intracellular PLA₂, however, are poorly understood. Since pancreatic phospholipase A is secreted in an inactive form, which is then activated by serine esterases, it has been suggested that serine esterases may also play a role in regulating intracellular PLA₂ activity. Previous studies have found, for example, that exogenous serine esterases may stimulate AA release from smooth-muscle cells and neutrophils by increasing PLA₂ activity (Camussi et al., 1989; Kawaguchi and Yasuda, 1988). In addition, serine esterase inhibitors appear to prevent the increase in PLA₂ activity caused by platelet-activating factor or tumour necrosis factor (Camussi et al., 1989; Sugatani et al., 1987). These previous reports provide indirect evidence that a serine esterase may participate in activating PLA₂. To test further the hypothesis that a membrane-associated serine esterase plays an important role in regulating intracellular PLA₂, we used the oxidant lipid t-Bu-OOH, which we have previously shown stimulates AA release in bovine pulmonary-arterial endothelial cells by increasing PLA₂ (Chakraborti et al., 1989).

Several lines of evidence indicate that a serine esterase plays an important role in the increased AA release and PLA₂ activity caused by the oxidant t-Bu-OOH. First, we have previously shown that bovine pulmonary-artery endothelial cells contain a membrane-associated serine esterase (Chakraborti and Michael, 1991). The endothelial-cell membrane-associated serine esterase has an M_r of 29000, which is similar to that of other members of the serine esterase family (Chakraborti and Michael, 1991). The identity of this serine esterase is unknown, but it has characteristics that distinguish it from elastase and cathepsin G (Table 3). Secondly, t-Bu-OOH not only augments PLA₂ activity and AA release, but also increases the activity of a membrane-associated serine esterase (Table 1). t-Bu-OOH appears to produce these effects via an oxidant species, because pretreatment of the cells with vitamin E prevents the increase in serine esterase activity, PLA₂ activity and AA release (Table 1). Thirdly, changes in the activity of the membrane-associated serine esterase correlate directly with changes in the activity of membrane PLA₂ (Figure 1). Fourthly, serine esterase inhibitors decrease basal serine esterase activity, PLA₂ activity and AA release (Table 1). Fifthly, these inhibitors also prevent the t-Bu-OOH-mediated increase in serine esterase activity, PLA₂ activity and AA release. Sixthly, adding trypsin to an endothelial-cell membrane fraction

markedly increases PLA₂ activity, indicating that a serine esterase can activate latent PLA₂ activity in endothelial cells (Table 4). Seventhly, t-Bu-OOH also increases PLA₂ activity in an endothelial-cell membrane fraction, implying that this oxidant also converts inactive PLA₂ into an active form (Table 4).

In summary, our results indicate that a membrane-associated serine esterase appears to play an important role in regulating PLA₂ and that one mechanism for the increase in PLA₂ activity caused by the oxidant t-Bu-OOH is probably an increase in the activity of this serine esterase. The identity of this serine esterase is currently under investigation. How the oxidant t-Bu-OOH increases the activity of the serine esterase, or how the serine esterase contributes to the activation of PLA₂, is currently unknown. Conceivably, the serine esterase may regulate PLA₂ activity directly or via an effect on other regulators of PLA₂, such as intracellular Ca²⁺, protein kinase C, PLA₂-activating protein (Clark et al., 1987, 1988), or lipocortin.

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