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Basal $cPLA_2$ Phosphorylation is Sufficient for Ca^{2+}-Induced Full Activation of $cPLA_2$ in A549 Epithelial Cells

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Abstract The release of [^{3}H] arachidonic acid (AA) and its connection with the triggering of the MAP kinase cascade were studied in the human A549 epithelial cell line upon stimulation with thapsigargin. Thapsigargin can increase AA release along with the increase of intracellular calcium concentration, phosphorylation, and activation of extracellular regulated kinase (ERK) and cytosolic phospholipase A_2 ($cPLA_2$). Both ERK and $cPLA_2$ phosphorylation in response to thapsigargin were inhibited by PD 98059, a specific inhibitor of MAP kinase kinase of the ERK group (MEK), and EGTA. $cPLA_2$ phosphorylation was not affected by Ro 31-8220 (an inhibitor of all PKC isoforms) or LY 379196 (a PKCβ selective inhibitor), while both of them indeed attenuated ERK activation. On the other hand, rottlerin (the selective PKCδ inhibitor), SB 203580 (the selective p38 MAPK inhibitor), and wortmannin (the PI 3-kinase inhibitor) can affect neither $cPLA_2$ nor ERK phosphorylation. In A549 cells, PKC activator PMA cannot increase either the basal or thapsigargin-induced ^{3}H-AA release, while it can induce the phosphorylation of ERK and $cPLA_2$. The PMA-induced ERK phosphorylation was inhibited by Ro 31-8220, LY 379196, rottlerin, and PD 98059, but unaffected by SB 203580 and wortmannin. Moreover, the phosphorylation by PMA was non-additive with that of thapsigargin. This implies that intracellular Ca^{2+} level is the key factor for induction of $cPLA_2$ activity and thapsigargin-elicited ERK activation itself is substantially sufficient for $cPLA_2$ activation upon intracellular Ca^{2+} increase. J. Cell. Biochem. 79:601–609, 2000.

Key words: $cPLA_2$; ERK; PKC; PI 3-kinase; AA release

Phospholipase A_2 (PLA_2) is the rate-limiting enzyme regulating the release of arachidonic acid (AA) from the sn-2 position of glycerol phospholipids in membrane of most cell types. AA functions as a precursor of several eicosanoids, including prostaglandins, thromboxanes, and leukotrienes, all of which play important roles in numerous physiological and pathophysiological processes [Zhang et al., 1999]. The intracellular PLA_2s can be classified mainly into three subfamilies according to localization, sequence homology, and biochemical characteristics [Dennis, 1994, 1997; Leslie, 1997; Tischfield, 1997]. The most abundant subfamily is secretory PLA_2, which is characterized by a low molecular mass, the presence of at least five disulfide bridges, activation via extracellular secretion and a requirement for calcium in the mM range [Cupillard et al., 1997]. Cytosolic PLA_2 ($cPLA_2$), on the other hand, displays a constitutive and ubiquitous expression, and is activated by uM calcium concentration achieved in the cytoplasm of cells stimulated with various agonists [Leslie, 1997]. In contrast, calcium-independent PLA_2, despite an identical subcellular localization, differs from $cPLA_2$ by the absolute lack of calcium requirement for its enzymatic activity [Balsinde and Dennis, 1997].

To date, two kinds of post-translational regulation is thought attributable for $cPLA_2$ activation. The first regulation involves the activation of the Ca^{2+}-dependent lipid binding domain in response to increased intracellular calcium [Clark et al., 1991; Nalefski et al.,

Abbreviations used: AA, arachidonic acid; (c)PLA_2, (cytosolic) phospholipase A_2; ERK, extracellular regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAP kinase kinase of the ERK group; PKC, protein kinase C; PI 3-kinase, phosphoinositol 3-OH-kinase; PMA, phorbol 12-myristate 13-acetate.

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1994], which leads enzyme translocation from the cytosol to the nuclear envelope and endoplasmic reticulum, allowing $cPLA_2$ access to its arachidonyl-containing phospholipid substrate [Channon and Leslie, 1990; Paglin et al., 1993; Nalefski et al., 1994; Schievella et al., 1995]. $cPLA_2$ activity is also increased by the phosphorylation of p42 mitogen-activated protein kinase (MAPK) consensus site, in response to stimulation of cells with cytokines [Lin et al., 1992, 1993; Nemenoff et al., 1993]. Although the consensus primary sequence of the other two MAPKs c-Jun N-terminal kinase (JNK) [Nishio et al., 1996; Hernandez et al., 1997] and p38 MAPK [Waterman and Sha'afi, 1995; Kramer et al., 1996] also appear in $cPLA_2$, their functional roles still are not so clear.

The inhibitor of endoplasmic reticulum Ca^{2+}-ATPase, thapsigargin, can increase intracellular Ca^{2+} level ($[Ca^{2+}]i$) in a sustained manner by depletion of intracellular Ca^{2+} stores and subsequently stimulating the capacitative Ca^{2+} channel [Takemura et al., 1989]. Due to $[Ca^{2+}]i$ increasing ability, thapsigargin has been shown to elicit $cPLA_2$ activation and AA release in several cell types, although the significance of kinase-mediating thapsigargin action might vary. In human polymorphonuclear neutrophils, the ERK cascade but not protein kinase C (PKC) is involved in thapsigargin-induced activation and phosphorylation of $cPLA_2$ [Zhang et al., 1999]. In RAW 264.7 macrophages, the PKC but not ERK cascade involves in the AA response of thapsigargin [Lin and Chen, 1998a]. In murine resident peritoneal macrophages, thapsigargin can induce a rise in $[Ca^{2+}]i$, however, did not induce appreciable AA release [Lloret et al., 1995]. In bovine pulmonary artery endothelial cells, thapsigargin can induce both $[Ca^{2+}]i$ increase and AA release, while without causing $cPLA_2$ phosphorylation [Chen et al., 1999].

Airway epithelial cell is a physiological barrier resistant to foreign substance in the environment and regulates inflammatory airway events [Jacoby et al., 1995]. While stimulating pulmonary A549 epithelial cells can produce different inflammatory mediators, such as interleukin-8 and interleukin-6 [Arnold et al., 1994], the regulatory pathways accounted for $cPLA_2$ activation has not been investigated. In addition, it is unique that A549 cells at resting state has already expressed high activity of cyclooxygenase-2 and released a large amount of PGE_2 [Asano et al., 1996]. Thus it is interesting to investigate the $cPLA_2$ regulation in this cell type.

MATERIALS AND METHODS

Materials

Cell culture medium and its supplements were obtained from Gibco BRL (Grand Island, NY). [^{3}H]AA (100 Ci/mmol) was purchased from New England Nuclear (Boston, MA). The $cPLA_2$ antibody, horseradish peroxidase-conjugated goat anti-mouse and sheep anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PD 98059 was from RBI (Natick, MA). Ro 31-8220, Go 6976, and SB 203580 were purchased from Calbiochem (La Jolla, CA). LY 379196 was a generous gift from Eli Lilly (Indianapolis, IN). Rottlerin was obtained from Biomol Research Laboratories Inc. (Plymouth Meeting, PA). The antibody specific to phosphorylated ERK was obtained from New England BioLabs (Beverly, MA). Phorbol 12-myristate 13-acetate (PMA), wortmannin, LY 294002, Fura-II/AM, thapsigargin, BAPTA/AM, EGTA were obtained from Sigma (St. Louis, MO). All the materials for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad Laboratories (Hercules, CA).

Cell Culture

A549 cells obtained from American Type Culture Collection were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, in a humidified atmosphere of 95% air and 5% CO_2.

[^{3}H] AA Release

As described previously [Chen et al., 1999], cells in 24-well plates (approximately 3×10^5 cells/well) were labeled with 0.3 μCi/ml [^{3}H]AA in DMEM overnight. Cells were then washed three times with serum-free DMEM and incubated in medium containing 0.5% fatty acid-free bovine serum albumin for 20 min before stimulation with thapsigargin for different intervals as indicated. At the end of the incubation, the medium was removed and centrifuged at 250*g* for 5 min to remove floating cells. The radioactivity in the supernatant was then measured.

Western Blotting

Cells grown in 35 mm dishes were quiescent for three days and treated with stimuli as indicated. Then cells were washed three times with ice-cold PBS and the plates were immediately placed on ice to stop the reactions. The cells were scraped from the plates in lysis buffer (20 mM Tris-HCl, 1 mM $MgCl_2$, 125 mM NaCl, 1% Triton, 1 mM p-methylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 50 mM NaF, 25 mM β-glycerophosphate, 100 μM sodium vanadate, pH 7.5), transferred to microfuge tubes and standed on ice for 30 min, then centrifuged 14000 rpm for 30 min. The supernatant were transferred to microfuge tubes. The protein levels in cell lysates were assayed by the Bradford method. Equal amounts (80 μg) of cell lysates were treated with 5× Laemmli buffer, boiled for 5 min, and subjected to 10% SDS-PAGE. The resolved proteins were transferred to nitrocellulose membranes (Amersham Pharmacia Biotech) and blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween-20 (TBST) overnight at 4°C. After three washes with TBST, the membrane was incubated with either cPLA$_2$ or phosphorylated ERK-specific primary antibody, each diluted in TBST, for 2 h. After further washes, the blots were incubated with horseradish peroxidase-conjugated immunoglobulin G for 1 h. After further washing, the blots were processed for visualization using enhanced chemiluminescence system (Amersham Pharmacia Biotech), following the manufacturer's instructions. Densitometrical analyses were performed on a Molecular Dynamics densitometer.

Measurement of $[Ca^{2+}]i$

Cells grown on glass slides were incubated with fura-II/AM (3 μM) and pluronic F-127 (0.02% v/v) in DEME at 37°C for 45 min. Fluorescence was monitored on a PTI M-series spectrofluorometer with dual excitation wavelengths of 340 nm and 380 nm and an emission wavelength of 510 nm. $[Ca^{2+}]i$ was calculated as described by Grynkiewicz et al. [1985].

Statistical Analysis

Each experiment was performed in duplicate, and data represent the mean ± S.E.M. from at least three independent experiments. $P < 0.05$ was considered significant by evaluation of the data with Student's *t*-test. The error bar was omitted when it was within the symbol representing the mean value.

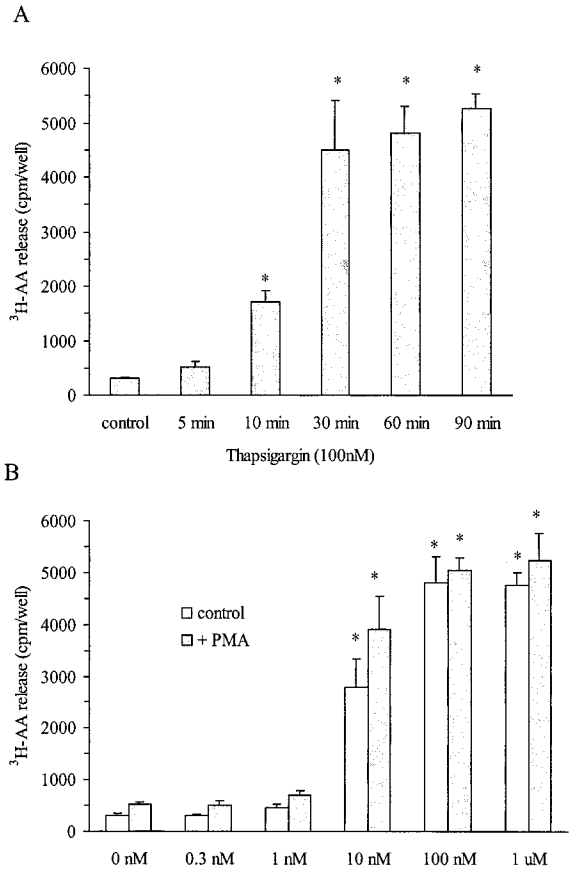

Fig. 1. Concentration- and time-dependent stimulation of AA release by thapsigargin. **A**: A549 cells were treated with 100 nM thapsigargin for different time intervals (5 min–1.5 h) and then ^{3}H-AA release into the medium was measured. **B**: Cells were treated with 0.3 nM–1 μM thapsigargin for 1 h either in the absence or presence of 1 μM PMA. The data represent the mean ± S.E.M. from three independent experiments. *$P < 0.05$ as compared to the control response without thapsigargin stimulation.

RESULTS

$[Ca^{2+}]i$ Increase Plays a Major Role in Thapsigargin-Induced AA Release

To elicit Ca^{2+}-dependent cPLA$_2$ activation, A549 cells were treated with 100 nM thapsigargin for different intervals. As shown in Figure 1A, thapsigargin elicits a time-dependent increase in AA release, with the onset of 10 min and maximum at 30 min. The thapsigargin response also exhibited a concentration-dependent manner. Within 1 h incubation, thapsigargin at 10 nM began to increase AA

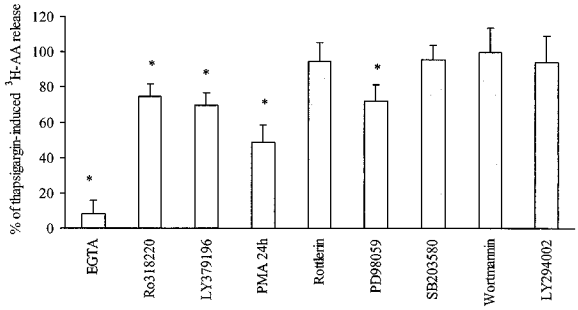

Fig. 2. Effects of pharmacological manipulation on thapsigargin-induced ^{3}H-AA release. Cells following ^{3}H-AA labeling were pretreated with EGTA (10 mM), Ro 31-8220 (3 μM), LY 379196 (30 nM), rottlerin (10 μM), PD 98059 (50 μM), SB 203580 (3 μM), wortmannin (300 nM), or LY294002 (10 μM) for 40 min or PMA (1 μM) for 24 h, and then stimulated with 100 nM thapsigargin for 1 h. The data represent the mean ± S.E.M. from three independent experiments. *$P < 0.05$ as compared to the control response without drug pretreatment.

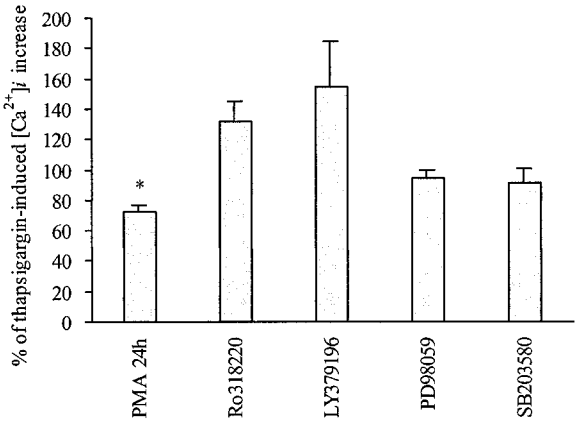

Fig. 3. Effects of pharmacological manipulation on thapsigargin-induced increase in [Ca^{2+}]i. Cells loaded with fura-II/AM were pretreated with Ro 31-8220 (3 μM), LY379196 (30 nM), PD98059 (50 μM), or SB203580 (3 μM) for 40 min. In the experiment of PMA, cells were pretreated with PMA (1 μM) for 24 h prior to the addition of fura-II/AM. After these pretreatment, thapsigargin (1 μM)-induced [Ca^{2+}]i response was measured by fluorometry as described in Materials and Methods. *$P < 0.05$ as compared to the control response without drug pretreatment.

release and exhibited its maximum effect of approximate 15-fold at 100 nM (Fig. 1B).

To understand the relative contribution of [Ca^{2+}]i and several protein kinases in thapsigargin-induced AA release, cells were pretreated with EGTA or protein kinase inhibitors. As shown in Figure 2, cells in serum-free DMEM containing 10 mM EGTA cannot response to thapsigargin for AA release. Ro 31-8220 (3 μM, an inhibitor of all PKC isoforms), LY 379196 (30 nM, a selective PKCβ inhibitor) [Lin and Chen, 1998b], and long-term PMA treatment (1 μM, 24 h), which caused PKC down-regulation, inhibited AA response of thapsigargin by 25 ± 7% (n = 4), 31 ± 7% (n = 4), and 52 ± 10% (n = 4), respectively. The selective MAPK kinase (MEK) inhibitor PD 98059 (50 μM) slightly inhibited thapsigargin response by 28 ± 9% (n = 7). On the contrary, PKCδ inhibitor rottlerin (10 μM), p38 MAPK inhibitor SB 203580 (3 μM), and phosphoinositol 3-OH-kinase (PI 3-kinase) inhibitors wortmannin (1 μM) and LY 294002 (10 μM) failed to change thapsigargin-induced AA release (Fig. 2). These results suggest that [Ca^{2+}]i increase by thapsigargin is almost sufficient for the maximal activation of AA release in A549 epithelial cells and that ERK-dependent signaling pathway plays a minor role in this event.

Since [Ca^{2+}]i level is a crucial factor for $cPLA_2$ activity, we further performed experiments to explore whether the inhibitory effects of Ro 31-8220, LY 379196, PD 98059, and PMA on AA response ascribed from their influences on thapsigargin-elicited [Ca^{2+}]i increase. Within 10 min treatment, thapsigargin at 1 μM caused a rapid increase of [Ca^{2+}]i from the basal level of 203.1 ± 21.5 nM to the peak response of 2029.6 ± 343.6 nM (n = 5; data not shown). The results revealed that the four protein kinase inhibitors, Ro 31-8220, LY 379196, PD 98059, and SB203580, had no significant effects on [Ca^{2+}]i rise in response to thapsigargin. On the contrary long-term PMA treatment caused 28 ± 4% (n = 3) inhibition of thapsigargin-induced [Ca^{2+}]i increase (Fig. 3).

Blockade of ERK but not PKC Activation Reduced $cPLA_2$ Phosphorylation

Since ERK- and/or PKC-dependent $cPLA_2$ phosphorylation are involved in $cPLA_2$ activity, we further analyzed these mechanisms by directly measuring the phosphorylation extents of ERK and $cPLA_2$. As shown in Figure 4A, thapsigargin caused a time- and concentration-dependent phosphorylation of ERK. Thapsigargin-induced ERK1 and ERK2 phosphorylation were abolished by the presence of EGTA, PD 98059, and inhibited by the presence of Ro 31-8220 and LY 379196. On the contrary, rottlerin did not affect thapsigargin-induced ERK phosphorylation.

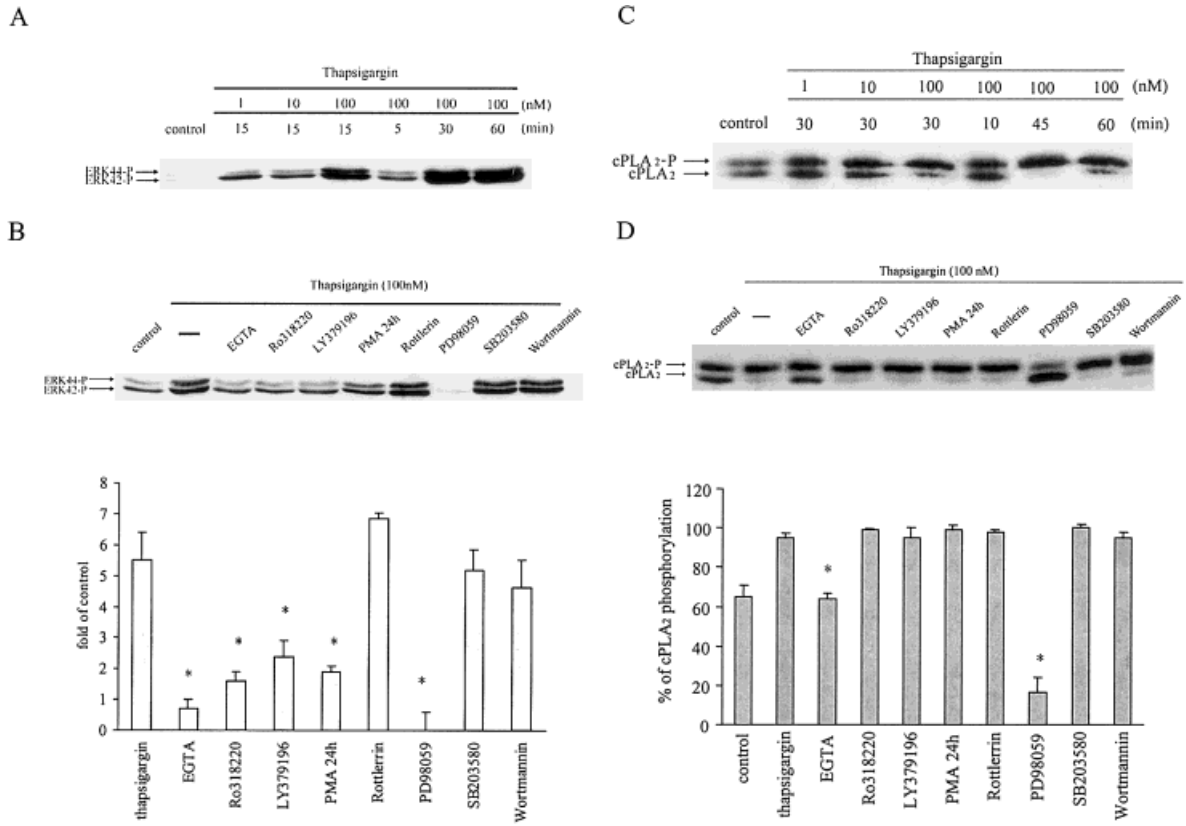

Fig. 4. Effects of pharmacological manipulation on thapsigargin-induced ERK and cPLA$_2$ phosphorylation. **A, C**: Quiescent cells were treated with thapsigargin at concentration indicated for different intervals, then immunoreactivity of phosphorylated ERK and cPLA$_2$ were determined. **B, D**: Quiescent cells pretreated with EGTA (10 mM), Ro 31-8220 (3 μM), LY 379196 (30 nM), rottlerin (10 μM), PD 98059 (50 μM), SB 203580 (3 μM), for 40 min or PMA (1 μM) for 24 h were stimulated with 100 nM thapsigargin for 15 min, and then cell lysates were prepared for measuring the immunoreactivity of phosphorylated ERK and cPLA$_2$. Imunoreactive bands were quantitated by densitometry. Identical patterns were observed in three separate experiments. *$P < 0.05$ as compared to the control response without drug pretreatment.

Cells long-term pretreated with PMA (1 μM) for 24 h also exhibited a weak response for ERK phosphorylation after thapsigargin stimulation (Fig. 4B). Rottlerin, SB 203580, and wortmannin, however, cannot affect thapsigargin-induced ERK phosphorylation.

With respect to cPLA$_2$, its phosphorylated form is evident by a slower mobility than the unphosphorylated form on SDS-PAGE. We found that cPLA$_2$ in A549 cells even quiescent as long as three days is composed of unphosphorylated and phosphorylated form at almost equal ratio. Thapsigargin at 1 or 10 nM cannot induce cPLA$_2$ phosphorylation within 30 min incubation, while apparent mobility shift was observed by 30 min treatment with 100 nM thapsigargin. At 45 min, complete mobility shift of cPLA$_2$ was achieved and it was gradually recovered at 60 min (Fig. 4C).

Figure 4D showed that consistent to ERK phosphorylation, thapsigargin-induced cPLA$_2$ shift was reversed by EGTA and PD 98059, but resistant to rottlerin, SB 203580, and wortmannin. Whereas intriguingly we found that the two PKC inhibitors Ro 31-8220 and LY 379196 as well as the chronic treatment with PMA for 24 h failed to change cPLA$_2$ mobility. These results indicate that thapsigargin-induced cPLA$_2$ phosphorylation majorly ascribes to the PKC-dependent ERK activation.

PMA Elicited ERK and cPLA$_2$ Phosphorylation but Cannot Potentiate AA Release

Since PKC-dependent enhancement of cPLA$_2$ activity indirectly through ERK-mediated cPLA$_2$ phosphorylation has been evident in other cell types, we herein evaluated the effects of PMA in A549 cells. We found that

PMA alone at 1 μM slightly increased basal AA release by 67 ± 15% (n = 7) within 90 min. Nonetheless, PMA co-addition with thapsigargin (0.3 nM–1 μM) cannot further enhance thapsigargin response (Fig. 1B).

To explore this unusual AA phenomenon observed for PMA in A549 cells possibly coming from the ineffectiveness of PMA on ERK and in turn $cPLA_2$ phosphorylation, we directly assessed PMA action in these two aspects. However, in contrast to its inability in increasing AA release, PMA (1 μM) treatment for 20 min markedly increased ERK phosphorylation and $cPLA_2$ phosphorylation (Fig. 5). We found that the ERK phosphorylation by PMA was unaffected by the presence of SB 203580, while inhibited by PD 98059, Ro 31-8220, LY 379196, and rottlerin (Fig. 5A,B). Consistently, the $cPLA_2$ phosphorylation by PMA was inhibited by PD 98059, Ro 31-8220, and long-term PMA pretreatment, but unaffected by SB 203580. All these findings revealed that the ineffectiveness of PMA on AA production is dissociated from its stimulatory action on ERK and $cPLA_2$ phosphorylation and that $[Ca^{2+}]i$ increase is essential for the enzyme activity of $cPLA_2$.

In this context, we further examined the effects of PMA on thapsigargin-induced ERK and $cPLA_2$ phosphorylation. The results indicated that both the ERK and $cPLA_2$ phosphorylation by thapsigargin and PMA are non-additive (Fig. 6). Thus, these results strengthen the notion that $cPLA_2$ phosphorylation plays a minor role in $cPLA_2$ regulation.

JNK Activation did not Involve in $cPLA_2$ Phosphorylation

In order to understand the inconsistent actions of Ro 31-8220 and LY 379196 on ERK and $cPLA_2$ phosphorylation (Fig. 4) ascribing from ERK-independent $cPLA_2$ phosphorylation by these two PKC inhibitors, we tested the possibility of JNK involvement. Indeed JNK activation has been associated with AA release and/or $cPLA_2$ phosphorylation by adrenoceptor, thrombin, okadaic acid, and zymosan stimulation [Nishio et al., 1996; Hernandez et al., 1997]. Moreover, Ro 31-8220 was shown to nonspecifically inhibit the expression of MAP kinase phosphatase-1 and consequently activate JNK [Beltman et al., 1996]. To assess this issue, we directly measure the effects of PKC inhibitors on JNK phosphorylation as an index for enzyme activation. We found that Ro 31-8220 and LY 379196 cannot induce JNK phosphorylation, while anisomycin and UV-irradiation can (data not shown). Furthermore, anisomycin itself cannot induce AA release or affect the AA response of thapsigargin (data not shown), ruling out the regulatory role of JNK in A549 $cPLA_2$ activation.

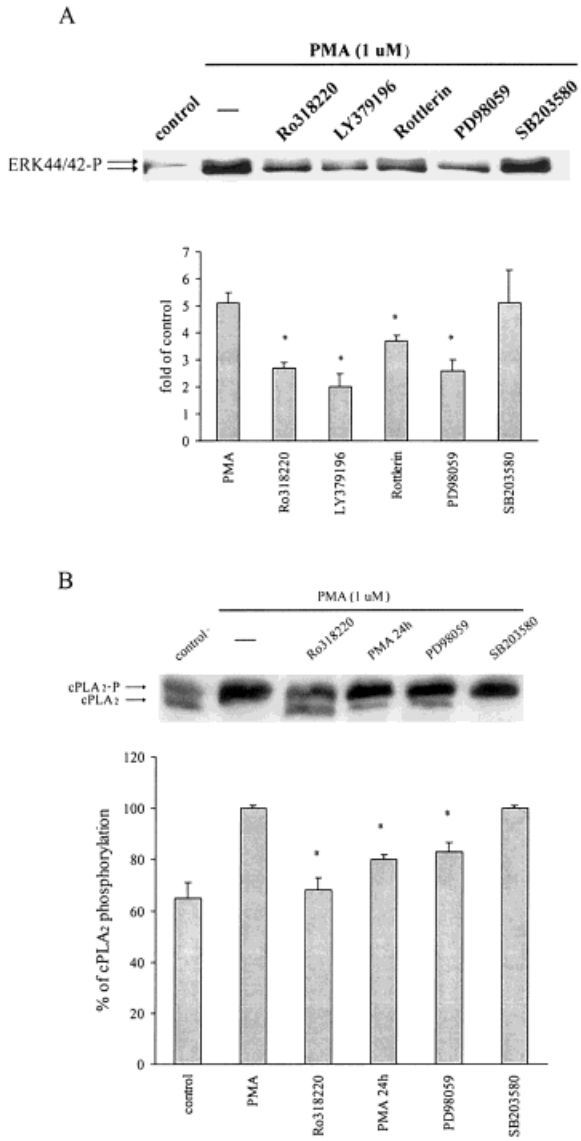

Fig. 5. Effects of pharmacological manipulation on PMA-induced ERK and $cPLA_2$ phosphorylation. Quiescent cells pretreated with Ro 31-8220 (3 μM), LY 379196 (30 nM), rottlerin (10 μM), PD 98059 (50 μM), SB 203580 (3 μM) for 40 min, or PMA (1 μM) for 24 h were stimulated with 1 μM PMA for 15 min, and then cell lysates were prepared for measuring the immunoreactivity of phosphorylated ERK (**A**) and $cPLA_2$ (**B**). Immunoreactive bands were quantitated by densitometry, Identical patterns were observed in three separate experiments. $^*P < 0.05$ as compared to the control response without drug pretreatment.

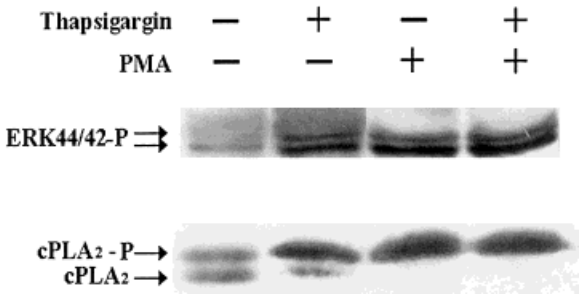

Fig. 6. Non-additivity of thapsigargin- and PMA-induced ERK and cPLA$_2$ phosphorylation. Quiescent cells were treated with 100 nM thapsigargin, 1 μM PMA or both for 15 min, and then cell lysates were prepared for measuring the immunoreactivity of phosphorylated ERK and cPLA$_2$.

DISCUSSION

To date, the mechanisms involved in the regulation of cPLA$_2$ are still not completely understood. In spite of this, all the previous studies have strengthened the indispensable factor of intracellular calcium for cPLA$_2$ activation. Our study here also has underlined the critical role of [Ca^{2+}]i increase in the activation of cPLA$_2$ and further delineate the contribution of cPLA$_2$ phosphorylation in this event in airway epithelial cells.

All the results in this study suggest that [Ca^{2+}]i increase by thapsigargin is almost sufficient for the maximal activation of cPLA$_2$ and AA release in A549 epithelial cells and that ERK-dependent cPLA$_2$ phosphorylation plays a minor role in cPLA$_2$ regulation. The reason for this suggestion is accounted by the following findings. Firstly, it is interesting to find that about half amount of cPLA$_2$ protein in cells under 3-day quiescence, based on the different mobility but corresponding immunoreactivity at resting state, is phosphorylated. Secondly, the AA response of thapsigargin was only inhibited by 28% under condition that the induced ERK and cPLA$_2$ phosphorylation were abolished by PD 98059 (Figs. 2 and 4), implying the minor role of ERK-dependent cPLA$_2$ regulation in A549 cells. Thirdly, the ERK-dependent cPLA$_2$ caused by PMA is not associated with AA release (Figs. 1 and 5). Thus, these results suggest that perhaps the basal amount of cPLA$_2$ phosphorylation is substantially enough for Ca^{2+}-mediated maximal cPLA$_2$ activation.

With respect to the non-effectiveness of PMA to potentiate AA release, it is assumed that either the basal or thapsigargin-induced cPLA$_2$ phosphorylation has played its permissive role in enzyme activity upon stimulation and membrane translocation by increasing [Ca^{2+}]i. The results that PKC activator PMA-elicited complete phosphorylation of ERK and cPLA$_2$ are additive to thapsigargin responses indicate the presence of the latter assumption, i.e., thapsigargin-induced cPLA$_2$ phosphorylation has already played its permissive role in enzyme activity, and thereby PMA cannot further enhance thapsigargin-induced AA release. This phenomenon is unique from those observed in different cell types, such as in endothelial cells [Chen et al., 1999] and macrophages [Lin and Chen, 1998a,b; Ambs et al., 1995] where full cPLA$_2$ activity is achieved by co-stimulation with Ca^{2+}-elevating agent and PMA. Apparently one reason for the potentiation effect is ascribed to the inability of Ca^{2+}-elevating agent itself to induce ERK and cPLA$_2$ phosphorylation in these cells [Chen et al., 1999; Ambs et al., 1995]. Thus, the outcome of PKC- and/or ERK-dependent cPLA$_2$ phosphorylation depends on the nature of cell types. Our results using pharmacological inhibitors, respectively selective for PKCβ (LY 379196) and PKCδ (rottlerin), further indicate that in A549 cells PKC at least isoforms of β and δ activation by PMA, and isoform β activation by thapsigargin mediate ERK phosphorylation.

With respect to the signaling molecule for cPLA$_2$ phosphorylation, ERK-independent mechanisms have been suggested. Previous studies have shown that cPLA$_2$ has lots of important phosphorylation sites with different function [Waterman and Sha'afi, 1995; Borsch-Haubold et al., 1995, 1998; Kramer et al., 1995; de Carvalho et al., 1996; Waterman et al., 1996]. Ser 505 is a phosphorylation site of ERK and possesses positive regulation on catalytic function [Lin et al., 1993]. In addition to Ser 505 as a key site in controlling cPLA$_2$ activity, phosphorylation of cPLA$_2$ at sites by protein kinases other than ERK may also involve in enzyme regulation. For example, p38 MAPK and JNK are the candidate kinases in this aspect [Waterman et al., 1995; Hernandez et al., 1997; Kramer et al., 1996; Syrbu et al., 1999]. Herein, we rule out the possible involvement of both kinases in cPLA$_2$ phosphorylation and AA release in response to thapsigargin. With respect to p38 MAPK, we showed that SB 203580, a highly selective inhibitor of the p38

MAPK activity, does not affect both responses. To exclude the involvement of JNK comes from the non-effectiveness of the potent JNK stimulus anisomycin to stimulate AA release and affect thapsigargin-induced AA release.

In this study, except concerning the results of Ro 31-8220 and LY379196, there is an approximately good correlation between ERK-dependent $cPLA_2$ phosphorylation and AA release by treatment with PD 98059 and EGTA. With respect to the non-correlation between the two phenomena for PKC inhibitors, i.e., the non-effectiveness of Ro 31-8220 and LY 379196 on thapsigargin-induced $cPLA_2$ phosphorylation in contrast to their partial inhibition on ERK phosphorylation and AA release in response to thapsigargin, two possibilities are proposed as followings. First, the inhibitory degree on ERK phosphorylation by Ro 31-8220 is not so great enough to overcome the higher phosphorylation level of $cPLA_2$ at resting state. Secondly, there exists a phosphorylation-independent enzyme activity of $cPLA_2$. Previous studies have indicated similar phenomenon by demonstrating the dissociative effects of SB 203580 and PD 98059 on stimuli-induced AA release and $cPLA_2$ phosphorylation [Kramer et al., 1995; Syrbu et al., 1999]. In this aspect, it is also impossible that both PKC inhibitors inhibit the rise of $[Ca^{2+}]i$ in response to thapsigargin, which in turn leads to the reduced AA release . Moreover, we used the pharmacological approach to test the possibility of PI 3-kinase, which although has not been implicated in $cPLA_2$ activation, was reported to involve in ERK activation in response to some stimuli [Hawes et al., 1996; Conway et al., 1999; Rakhit et al., 1999]. Present results showed that wortmannin failed to affect thapsigargin response in AA release, ERK and $cPLA_2$ phosphorylation, thus we conclude that PI 3-kinase and its dependent signaling kinases, such as 3-phosphoinositide-dependent protein kinase and protein kinase B cannot be involved. Except the evidence based on the PI 3-kinase inhibitor wortmannin, SB203580 also strengthened this notion, since the latter which has recently been shown to inhibit 3-phosphoinositide-depedent protein kinase [Lali et al., 2000], has no effects on ERK and $cPLA_2$ phosphorylation caused by thapsigargin. Thus, the determination of the kinase(s) that mediate the phosphorylation of $cPLA_2$ in human A549 epithelial cells remains to be examined.

In conclusion, we conclude that A549 cells at resting express a high extent of $cPLA_2$ phosphorylation which is substantially sufficient for the maximal $cPLA_2$ activation upon intracellular Ca^{2+} increase.

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