Vitamin E potentiates arachidonate release and phospholipase A_2 activity in rat heart myoblastic cells

Khai TRAN*, Jason T. WONG*, Edmund LEE*, Alvin C. CHAN† and Patrick C. CHOY*‡

*Department of Biochemistry and Molecular Biology, University of Manitoba, Winnipeg, Manitoba, Canada R3E 0W3, and †Department of Biochemistry, University of Ottawa, Ottawa, Canada K1H 8M5

Cytosolic phospholipase A_2 ($cPLA_2$) selectively catalyses the release of arachidonic acid from the *sn*-2 position of glycerophospholipids to produce prostaglandins and leukotrienes. In this study, vitamin E enrichment of rat heart myoblastic H9c2 cells caused an increase in the release of arachidonate during ionophore (A23187) stimulation. PLA_2 activity in the cytosolic fraction was also enhanced but enzyme activity in the particulate fraction was not affected by this treatment. Immunoblotting analysis with a polyclonal anti-$cPLA_2$ antibody showed an increased level of the enzyme in vitamin E-treated cells. Direct incorporation of vitamin E into lipid vesicles in the assay mixture resulted in modulation of enzyme activity in a biphasic manner. Pretreatment of cells with phorbol 12-myristate 13-acetate, a known activator of protein kinase C, synergistically potentiated the ionophore-induced arachidonate release in both the control and vitamin E-treated cells. However, vitamin E treatment by itself did not affect the protein kinase C activity, indicating that the vitamin E-induced activation of $cPLA_2$ was independent of the protein kinase C cascade. Collectively, these results suggest that vitamin E potentiates arachidonate release through the direct and/or indirect modulation of $cPLA_2$ activity.

INTRODUCTION

The central role of arachidonic acid for eicosanoid production in mammalian cells is well documented [1]. Arachidonic acid is also an important membrane component which is required for the maintenance of cellular viability under normal and pathological conditions. The vast majority of the available arachidonic acid is stored in acylated form within membrane phospholipids, and the free acid is released from phospholipid molecules via the hydrolytic action of phospholipase A_2 (PLA_2). PLA_2 in mammalian tissues is composed of three distinct enzymes with different primary structures, subcellular localizations and calcium requirements [2]. The first group is commonly known as the secretory (s-form) PLA_2 which has a molecular mass of 14 kDa and requires extracellular (millimolar) concentrations of Ca^{2+} for maximum activation [3]. The s-form of the enzyme does not display any specificity towards the acyl group at the *sn*-2 position. The second group is commonly known as the cytosolic PLA_2 ($cPLA_2$) which has a molecular mass of 85 kDa [4,5]. The enzyme is located primarily in the cytosol and its activity can be detected at low intracellular (micromolar) Ca^{2+} concentrations. The c-form of the enzyme is activated by phosphorylation followed by the translocation of the phosphorylated enzyme to the membrane in a Ca^{2+}-dependent manner [5–7]. The enzyme displays a high degree of preference for arachidonoyl groups at the *sn*-2 position [5,8] and also possesses lysophospholipase [9,10] and transacylase activity [11]. The third type of PLA_2 (h-type) is found exclusively in cardiac tissues, has a molecular mass of 45 kDa, and displays limited selectivity towards specific acyl groups [12]. With the exception of secretory organs, the cytosolic enzyme represents the major type of PLA_2 for the cellular release of free arachidonic acid.

The naturally occurring form of vitamin E (*RRR*-α-tocopherol) is a key membrane-bound antioxidant in mammalian cells. It is known as a chain-breaking antioxidant due to its ability to terminate the chain reaction of the lipid peroxidation [13]. Beyond its antioxidative property, vitamin E has been shown to be involved in the regulation of certain enzymes associated with signal transduction processes across biological membranes. For example, Azzi and his group have shown that *RRR*-α-tocopherol can inhibit protein kinase C and the proliferation of smooth-muscle cells in a manner independent of its antioxidant property [14,15]. We have recently demonstrated that enrichment of endothelial cells with *RRR*-α-tocopherol activates diacylglycerol kinase [16] and the CoA-independent acyltransferase [17].

The role of vitamin E in the regulation of eicosanoid biosynthesis has been well documented [18,19]. In vitamin E-treated animal models, we and others have reported the reduced formation of thromboxane [20], 5-hydroxyeicosatetraeinoic acid [21], and prostaglandin E_2 [22] in stimulated platelets, neutrophils and macrophages, respectively. Since the release of arachidonate by PLA_2 is the rate-limiting step in eicosanoid biosynthesis, the inhibition of PLA_2 activity [23–25] is generally regarded as the mechanism for the attenuation of eicosanoid production. Alternatively, vitamin E has been shown to stimulate eicosanoid production in some other cell types. For example, the enrichment of cultured endothelial cells with vitamin E caused an increase in arachidonic acid release [26] and prostaglandin I_2 synthesis [27]. The apparent discrepancy on the action of vitamin E between cell types epitomizes the limitation of each study and illustrates the fragmented nature of our understanding on the role of vitamin E in the regulation of arachidonic acid release in mammalian cells.

The present study is therefore designed to extend our understanding of the role of vitamin E in the regulation of arachidonic

Abbreviations used: $cPLA_2$, cytosolic phospholipase A_2; PMA, phorbol 12-myristate 13-acetate; HELSS, E-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2-H-pyran-2-one; $AACOCF_3$, arachidonoyl trifluoromethane; DME, Dulbecco's modified Eagle's.

‡ To whom correspondence should be addressed.

acid release and PLA_2 activity. Using rat heart myoblastic (H9c2) cells as a model, vitamin E was found to potentiate the ionophore-induced release of arachidonic acid. Vitamin E appears to enhance the arachidonic acid release in H9c2 cells via the activation of the $cPLA_2$ by both direct and indirect mechanisms.

MATERIALS AND METHODS

Materials

[5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (210 Ci/mmol), 1-stearoyl-2-[1-^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine (55 mCi/mmol), protein kinase C enzyme assay kit and [γ-^{32}P]ATP (30 Ci/mmol) were purchased from Amersham Canada Ltd. (Oakville, Ontario, Canada). Calcium ionophore A23187, phorbol 12-myristate 13-acetate (PMA), and all cell culture media and reagents were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). HELSS [E-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2-H-pyran-2-one] was obtained from Biomol Inc. (Plymouth Meeting, PA, U.S.A.) and arachidonoyl trifluoromethane ($AACOCF_3$) was purchased from Calbiochem-Novabiochem Co. (La Jolla, CA, U.S.A.). All lipid standards were obtained from Serdary Research Laboratory (London, Ontario, Canada). TLC plates (silica gel G) were products of Fisher Scientific Co. (Edmonton, Alberta, Canada). All other chemicals and solvents (reagent- or HPLC-grade) and culture plasticware were obtained from the Canlab Division of Baxter Co. (Edmonton, Alberta, Canada). *RRR*-α-Tocopherol was a gift from the Vitamin E Research Information Services (La Grange, IL, U.S.A.). The term vitamin E used herein refers to *RRR*-α-tocopherol.

Culture of H9c2 cells

Rat myoblastic H9c2 cells were obtained from the American Type Culture Collection and cultured as described previously [28]. Cells were grown in Dulbecco's modified Eagle's (DME) medium supplemented with 10% (v/v) heat-inactivated newborn-calf serum, 100 units/ml penicillin G, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B, at 37 °C in an atmosphere of humidified air/5% CO_2. When the cell cultures reached 80% confluency, each dish was subcultured at a 1:5 ratio. After 4 days of incubation, 80–85% confluency was obtained. Cells grown in 60-mm-diam. dishes were used for arachidonic acid release experiments, and cells grown in 150-mm-diam. dishes were used for the preparation of subcellular fractions and the subsequent PLA_2 assay.

Preparation of medium containing vitamin E and radiolabelled arachidonic acid

Vitamin E-enriched medium containing 0.8 μCi/ml of radiolabelled arachidonic acid and 1% (v/v) newborn-calf serum was prepared as described previously [26]. Aliquots of [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (210–217 Ci/mmol) in ethanol were pipetted into sterile plastic tubes and the solvent was evaporated to dryness under N_2 gas. Appropriate amounts of *RRR*-α-tocopherol dissolved in DMSO were added to reconstitute the arachidonic acid. Newborn-calf serum was then added to the mixture which was then vortexed vigorously and incubated at 37 °C in the dark for 15 min. DME medium containing antibiotics was added and the mixture was further incubated for 15 min before being added to the culture dishes. The amount of DMSO added was 0.2% of the final volume. Control medium contained the same amount of DMSO as in the vitamin E-enriched medium. Cells were incubated with vitamin E and radiolabelled arachidonic acid for 20 h prior to experimental studies.

Calcium ionophore stimulation and measurement of arachidonic acid release

After incubation with radiolabelled arachidonic acid and the indicated concentrations of vitamin E for 20 h, cells were rinsed three times with warm Hepes-buffered saline (pH 7.4) containing 10 mM Hepes, 140 mM NaCl, 4 mM KCl, 5.5 mM glucose, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.2% BSA. Cells were stabilized with the buffer for 15 min in a 37 °C incubator and then challenged with calcium ionophore A23187 for 10 min or as otherwise indicated. In some experiments, cells were incubated with PMA for 10 min prior to A2318 stimulation. In other experiments involving $cPLA_2$ inhibitors, cells were preincubated with $AACOCF_3$ (1 min) or HELSS (15 min) before stimulation with A23187. After 10 min of stimulation, the incubation solution was collected and acidified with acetic acid. The mixture was centrifuged for 5 min at 800 ***g*** and an aliquot was used for lipid extraction by the method of Bligh and Dyer [29] using chloroform/methanol/water (4:2:3, by vol.). Unlabelled arachidonic acid (50 μg) was added to the extraction mixture as fatty acid carrier. The lower organic phase was collected, the solvent was evaporated to dryness, and the free fatty acid was resolved by TLC with a solvent system consisting of hexane/diethyl ether/acetic acid (70:30:1, by vol.). The fatty acid band was identified with iodine vapour and its radioactivity was determined by liquid scintillation counting.

Subcellular fractionation

Cells grown in 150-mm-diam. dishes were used for subcellular fractionation. When cell cultures reached approx. 90% confluency, the cells were rinsed three times with ice-cold PBS and were removed from the dish in the same buffer. The cells were sedimented by centrifugation and then resuspended in 1 ml of buffer A (10 mM Hepes, pH 7.4, 0.25 M sucrose, 1 mM EDTA and 1 mM dithiothreitol). The cell suspension was homogenized with a glass douncer (type A) in an ice-cold water bath for 5 strokes. The cell homogenate was centrifuged for 5 min at 800 ***g***, and the resulting supernatant was centrifuged at 100000 ***g*** for 60 min to obtain the cytosolic and microsomal fractions. The microsomes were suspended in the homogenizing buffer, and an aliquot containing 0.05–0.10 mg of protein from each fraction was used for the PLA_2 assay.

Determination of PLA_2 activity

Enzyme activity was determined by the release of radiolabelled arachidonic acid from the *sn*-2 position of 1-stearoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine. The assay mixture contained 50 mM Tris/HCl (pH 8.0), 100 mM NaCl, 1 mg/ml BSA, 5 mM $CaCl_2$, 1 mM dithiothreitol and 2.45 μM 1-stearoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine (60000 d.p.m./assay) in a final volume of 0.2 ml. The substrate was prepared by evaporating the solvent containing the required amount of 1-stearoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine to dryness under N_2 gas. The dried phospholipid substrate was resuspended in Tris/HCl buffer containing NaCl, and phosphatidylcholine vesicles were formed by placing the mixture in a water-bath sonicator at 0 °C for 30 min. In experiments that required the presence of vitamin E in the phosphatidylcholine vesicles, a known amount of vitamin E dissolved in ethanol was mixed with the radiolabelled substrate, and the

solvent was evaporated prior to sonication with the buffer. The enzyme reaction was initiated by the addition of the enzyme preparation from either the cytosolic or microsomal fraction, and the reaction was allowed to proceed at 37 °C for 30 min. The reaction was quenched by the addition of 1.0 ml of chloroform/methanol (2:1, v/v), followed by 0.05 ml of saturated NaCl and 0.25 ml of water. The radiolabelled arachidonic acid released was separated from the substrate by TLC, and radioactivity was determined by scintillation counting.

Determination of protein kinase C activity

Cells were homogenized in buffer B (50 mM Tris/HCl, pH 7.5, 5 mM EDTA, 10 mM EGTA, 0.25 M sucrose, 0.3 % 2-mercaptoethanol, 10 μM benzamidine, 50 μg/ml PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin). The homogenates were centrifuged at 1500 ***g*** for 10 min and the resulting supernatant was subjected to centrifugation at 100000 ***g*** for 45 min. The soluble and particulate fractions from the centrifugation were used for subsequent enzyme assays. Protein kinase C activity was determined in an assay system consisting of 4 mM calcium acetate, 2 mol % phosphatidylserine, 6 μg/ml PMA, 225 μM peptide substrate, 7.5 mM dithiothreitol, 35 μM ATP and 11 mM magnesium acetate in a total volume of 75 μl of 50 mM Tris/HCl (pH 7.5) with 0.05 % NaN_3. The reaction, which contained 15–30 μg of proteins from the cytosolic or particulate fractions, was initiated by the addition of 0.2 μCi of [γ-^{32}P]ATP. After incubation for 15 min at 25 °C, the reaction was terminated by the addition of 100 μl of an acidic quenching reagent. An aliquot of the reaction mixture was spotted on to a piece of peptide-binding paper (RPN77) which was then washed thoroughly with 5 % acetic acid. The radioactivity of ^{32}P-labelled protein bound to the membrane was determined by liquid scintillation counting. The phosphorylation of cellular proteins in the absence of the peptide substrate was subtracted from the data for the calculation of protein kinase C activity.

Immunoblotting analyses

Cells were incubated with the indicated concentrations of vitamin E for 20 h. Samples (50 mg) of protein from each cell lysate were subjected to SDS/7.5 %-PAGE. The protein fractions from the gel were transferred to a nitrocellulose membrane and subsequently allowed to react with a polyclonal anti-cPLA$_2$ antibody. The membrane was then exposed to goat anti-(rabbit IgG) antibodies which were coupled with horseradish peroxidase for colour development.

Other analytical procedures

Protein content was determined by the method of Lowry et al. [30] using BSA as standard. Lipid extraction, cell numbers and the quantification of total cellular vitamin E were determined as described previously [26]. Phospholipid fractions were separated from the total lipid extract by TLC with a solvent containing chloroform/methanol/acetic acid/water (85:15:10:3, by vol.). Lipid phosphorus was determined by the method of Rouser et al. [31]. Student's *t*-test was used for statistical analysis of data.

RESULTS

Vitamin E enhances A23187-induced arachidonic acid release

The presence of vitamin E in the incubation media did not affect the total cellular uptake or incorporation of radiolabelled arachidonate into the major phospholipids and neutral lipids in H9c2 cells (Table 1). However, when these cells were stimulated with A23187, vitamin E enrichment caused a significant increase in arachidonate release. The data in Figure 1 clearly demonstrate that, irrespective of different times of stimulation or doses of A23187, vitamin E-enriched cells consistently displayed a 2-fold increase in arachidonate release.

To demonstrate that the cellular level of vitamin E was elevated during incubation, the vitamin E content, as well as total phospholipid contents, in H9c2 cells was determined. As shown in Table 2, the endogenous level of vitamin E in H9c2 cells was not detectable. However, a linear relationship was established between the cellular vitamin E content and the concentration of vitamin E (25–100 μM) in the medium after a 20 h incubation. Vitamin E treatment did not affect cell numbers, total protein and phospholipid levels. The ratio of vitamin E to phospholipid was roughly estimated to be 1:1450 (0.07 mol %), 1:690 (0.15 mol %) and 1:350 (0.29 mol %) in cells treated with 25, 50 and 100 μM vitamin E, respectively.

Table 1 Incorporation of [^{3}H]arachidonic acid into major phospholipids and neutral lipids of H9c2 cells

Cells were incubated with [^{3}H]arachidonic acid in the absence or presence of 50 μM vitamin E for 20 h. Cellular lipids were separated by TLC as described in the Materials and methods section. Data are expressed as means ± S.D. of four separate experiments.

	Radioactivity (d.p.m. × 10^{-4}/dish)	
	Control	Vitamin E
Phosphatidylinositol	4.32 ± 0.11	4.36 ± 0.37
Phosphatidylcholine	5.30 ± 0.27	5.30 ± 0.23
Phosphatidylethanolamine	14.37 ± 0.36	13.27 ± 0.65
Phosphatidic acid	0.44 ± 0.10	0.37 ± 0.07
Diacylglycerol	0.19 ± 0.03	0.18 ± 0.07
Triacylglycerol	0.04 ± 0.01	0.03 ± 0.01
Free fatty acid	0.11 ± 0.02	0.10 ± 0.01
Total uptake	33.00 ± 1.40	32.44 ± 0.86

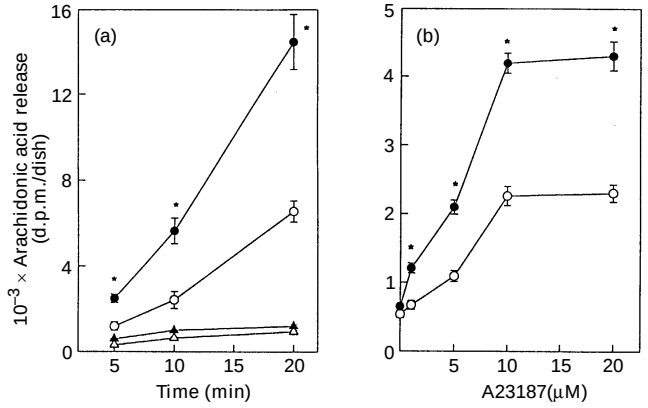

Figure 1 Effect of vitamin E on arachidonic acid release in A23187-stimulated H9c2 cells

Cells were incubated with [^{3}H]arachidonic acid in the absence (○,△) or presence (●,▲) of 50 μM vitamin E for 20 h. In (**A**), cells were treated with (○,●) or without (△,▲) 10 μM A23187 for the indicated times. In (**B**), cells were stimulated with 0–20 μM A23187 for 10 min. The arachidonic acid release was quantified as described in the Materials and methods section. Each data point represents the mean ± S.D. of three separate experiments. * indicates $P < 0.05$ when compared with controls.

Table 2 Cellular vitamin E and total phospholipid levels in H9c2 cells

Cells were incubated with medium supplemented with 0, 25, 50, 100 μM vitamin E for 20 h. Cellular vitamin E (α-tocopherol) levels were determined by HPLC and total cellular phospholipids were quantified by phosphorus determination as described in the Materials and methods section. Data are expressed as means $\pm$ S.D. of three separate experiments. Abbreviation: ND, not detected.

Vitamin E treatment (μM)	Cellular vitamin E (nmol/10^6 cells)	Total phospholipid (nmol/10^6 cells)	Vitamin E:phospholipid ratio
0	ND	79.5 $\pm$ 5.1	0
25	0.05 $\pm$ 0.01	72.8 $\pm$ 3.2	1:1450
50	0.11 $\pm$ 0.01	76.0 $\pm$ 2.4	1:690
100	0.20 $\pm$ 0.03	70.1 $\pm$ 1.0	1:350

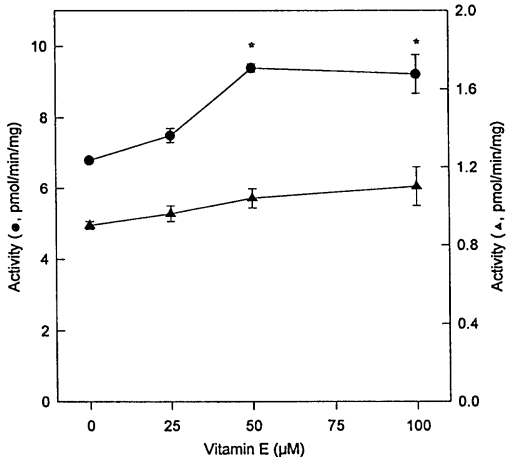

Figure 2 PLA_2 activities in the soluble and particulate fractions of cells treated with various concentrations of vitamin E

Cells were incubated with 0–100 μM vitamin E for 20 h. PLA_2 activities in the soluble (●) and particulate (▲) fractions were determined. Each data point represents mean $\pm$ S.D. of triplicate determinations in three separate experiments. * indicates $P < 0.05$ when compared with controls.

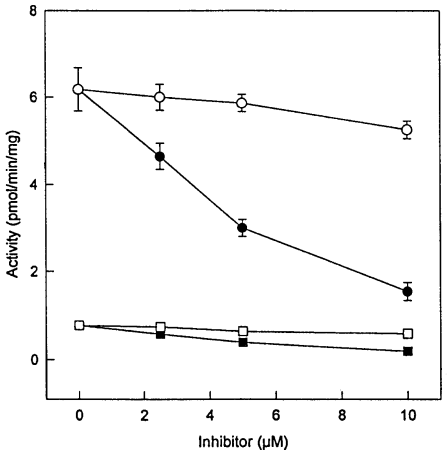

Figure 3 Inhibition of PLA_2 activities in the soluble and particulate fractions by specific inhibitors

PLA_2 activities in the soluble (○,●) and particulate (□,■) fractions were determined by the hydrolysis of L-α-1-stearoyl-2-[^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine vesicles prepared in the presence of the indicated concentrations of $AACOCF_3$ (●,■) or HELSS (○,□). Each data point represents the mean $\pm$ S.D. in three separate experiments.

Table 3 Inhibition of arachidonic acid release in A23187-stimulated cells by $cPLA_2$ inhibitor

Cells were incubated with [^{3}H]arachidonic acid in the absence or presence of 50 μM vitamin E for 20 h. Subsequently, the cells were treated with 10 μM $AACOCF_3$ for 1 min or 10 μM HELSS for 15 min prior to stimulation with 10 μM A23187 for 5 min. The labelled arachidonic acid release was isolated by TLC and quantified by scintillation counting. Data are expressed as means $\pm$ S.D. of three separate experiments.

	Arachidonic acid release (d.p.m. $\times 10^{-3}$/dish)	
Treatment	Control	Vitamin E
None	2.80 $\pm$ 0.45	4.43 $\pm$ 0.42
$AACOCF_3$	1.33 $\pm$ 0.18	2.80 $\pm$ 0.10
HELSS	2.50 $\pm$ 0.23	4.30 $\pm$ 0.15

Vitamin E enhances the activity of PLA_2

To test the hypothesis that the vitamin E-enhanced arachidonate release is mediated by PLA_2, the activity of this enzyme was determined in the soluble and particulate fractions of the H9c2 cells enriched with vitamin E. The enzyme activity was assayed in the presence of 5 mM calcium under reducing conditions with L-α-1-stearoyl-2-[^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine as the substrate. The assay condition was designed to favour the detection of the c-type enzyme. Figure 2 shows that PLA_2 activity was significantly increased in the soluble fraction of cells incubated with 50 μM or higher vitamin E. In contrast, no significant difference in enzyme activity could be detected in the particulate fraction. Our results indicate that the enhanced arachidonic acid release in H9c2 cells enriched with vitamin E was caused in part by an elevation of the $cPLA_2$ activity.

To determine the contribution of the s-type (low-molecular-mass) enzyme to the total PLA_2 activity, the cytosol from H9c2 cells was subjected to ultrafiltration by an Amicon Centriflo CF25 membrane system. Over 85 % of the total enzyme activity was retained by the membrane and less than 5 % of the enzyme activity was found in the filtrate. The increase in calcium concentration and/or removal of the reducing conditions in the assay did not significantly increase the enzyme activity in the filtrate. Since the CF25 membrane has a molecular mass cut-off of 25 kDa, our results indicate that the vast majority of the PLA_2 in the cytosol has a molecular mass larger than 25 kDa, and imply that the c-type form is the principal type of enzyme in the cytosol. In a separate set of experiments, the effects of inhibitors to different types of PLA_2 were determined. In the presence of 10 μM $AACOCF_3$, a specific inhibitor of the c-type enzyme [32,33], over 70 % of the enzyme activity in the soluble fraction and 50 % of the enzyme activity in the particulate fraction were inhibited (Figure 3). In contrast, HELSS, a haloenol lactone known as the 'suicidal' inhibitor for myocardial PLA_2 [34], failed to inhibit the enzyme activity in both fractions (Figure 3). When the cytosol was titrated with excess polyclonal anti-$cPLA_2$ antibody, at least 75 % of the enzyme activity was inhibited (results not shown). When H9c2 cells were preincubated with $AACOCF_3$ prior to A23187 stimulation, arachidonic acid release was attenuated, and the degree of attenuation was similar between control and vitamin E-treated cells (Table 3). Pretreatment with

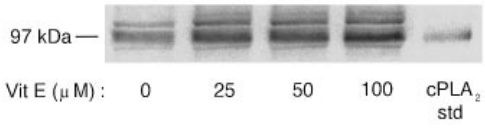

Figure 4 Immunoblots of cPLA$_2$ from cells treated with different concentrations of vitamin E

Cells were incubated with vitamin E for 20 h. The cell lysates, containing 50 μg of protein, were subjected to SDS/PAGE (7.5%). The protein bands in the gel were transferred to a nitrocellulose sheet and treated with a polyclonal anti-cPLA$_2$ antibody. Protein band(s) containing cPLA$_2$ was detected by a coupled peroxidase colour development system.

HELSS had no effect on the rate of arachidonic acid release. Taken together, these results indicate that the vast majority of PLA$_2$ activity in H9c2 cells appears to be of the 85 kDa c-type. Our results also support the notion that the increase in arachidonate release was mediated via activation of the cPLA$_2$.

Effect of vitamin E on the c-PLA$_2$ level measured by immunoblot

The vitamin E-induced enhancement of cPLA$_2$ activity could be caused by the modulation of enzyme activity and/or by an increase in enzyme protein. To delineate the mechanism for the elevated PLA$_2$ activity, quantitative determination of enzyme proteins was conducted by Western blotting analysis. Cell lysates obtained from the control and vitamin E-treated cells were analysed by SDS/7.5%-PAGE, after which proteins in the gel were transferred to a cellulose membrane and allowed to react with a polyclonal anti-cPLA$_2$ antibody. As shown in Figure 4, the cPLA$_2$ level was 2.3 ± 0.3-fold higher in cells treated with 25–100 μM vitamin E than in the untreated cells. This result clearly shows that vitamin E caused an up-regulation of the enzyme level, which might contribute to the observed increase in enzyme activity.

Direct effect of vitamin E on PLA$_2$ activity

In addition to the net increase in enzyme protein, the ability of vitamin E to modulate PLA$_2$ by direct or indirect mechanisms was also explored. Since vitamin E could not be readily dissolved in the assay mixture, its direct effect on PLA$_2$ activity was studied by incorporating this vitamin into the substrate phosphatidylcholine vesicles. As depicted in Figure 5, the addition of vitamin E into the substrate vesicles directly stimulated enzyme activity in the cytosolic (Figure 5A) and particulate (Figure 5B) fractions when the vitamin E to phospholipid ratio was below 1:250 (0.5 mol%). Maximum stimulation of enzyme activity was observed for both subcellular fractions when the ratio of vitamin E to phospholipid was 1:500 (0.2 mol%). However, high vitamin E to phospholipid ratios ($\geqslant$ 1 mol%) inhibited PLA$_2$ activity. The vitamin E to phospholipid ratios which caused enhancement of enzyme activity appear to be similar to those found in the H9c2 cells during vitamin E incubation (Table 2).

Effect of vitamin E and PMA on protein kinase C activity

The indirect effect of vitamin E in modulating PLA$_2$ activity was next explored. PMA, a known activator of protein kinase C, has been shown to cause an increase in PLA$_2$ activity in stimulated macrophages [35], glomerular mesangial cells [36] and Chinese hamster ovary cells [37]. Therefore, this agent was employed as a probe to delineate the mechanism which might involve the indirect activation of PLA$_2$. Table 4 shows that when cells labelled with arachidonate were stimulated with PMA alone, there was no detectable change in the amount of arachidonate release compared with the controls. However, a 2-fold increase in arachidonic acid release was detected when the cells were incubated with PMA for 10 min prior to A2318 stimulation. The vitamin E-induced enhancement of arachidonate release was once again observed in cells treated with A23187 alone. The stimulation of arachidonic acid release by PMA preincubation was reminiscent of the ability of vitamin E to enhance the release of arachidonic acid. To determine whether the actions of PMA and vitamin E were mediated via a common mechanism, the possible effect of protein kinase C in enhancing PLA$_2$ activity was explored. The cells were enriched with vitamin E for 20 h, after which time they were incubated with PMA or A23187 for 10 min, and protein kinase C activity was determined in the soluble and particulate fractions. Table 5 shows that in untreated cells, protein kinase C was found mostly in the cytosol. PMA treatment caused a shift of protein kinase C activity from the cytosol to the particulate fraction. In contrast, stimulation of

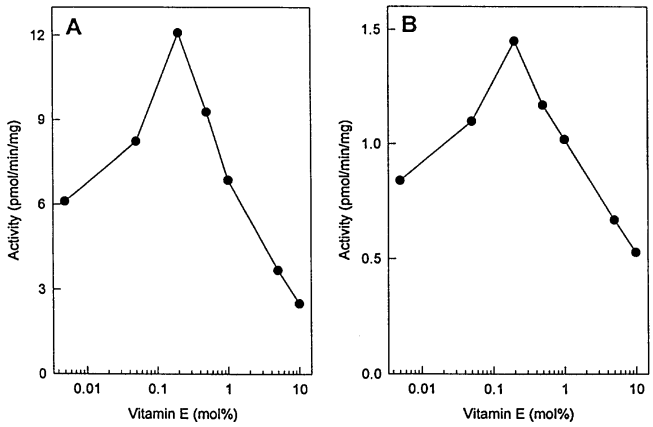

Figure 5 Direct effect of vitamin E on PLA$_2$ activities

L-α-1-Stearoyl-2-[^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine vesicles were prepared in the presence of vitamin E. PLA$_2$ activities in the soluble (**A**) and particulate (**B**) fractions were determined. Each data point represents the mean of duplicate determinations in two separate experiments.

Table 4 Effect of PMA and vitamin E on A23187-induced arachidonic acid release

Cells were incubated with [^{3}H]arachidonic acid in the absence (control) or presence (vitamin E) of 50 μM vitamin E for 20 h. Cells were then stimulated with 10 μM A23187 for 10 min, 100 nM PMA for 10 min, or 100 nM PMA for 10 min followed by 10 μM A23187 for 10 min and the arachidonic acid release was determined. Data are expressed as means $\pm$ S.D. of three separate experiments.

	Arachidonic acid release (d.p.m. $\times 10^{-3}$/dish)	
Treatment	Control	Vitamin E
None	0.59 ± 0.02	0.71 ± 0.01
PMA	0.60 ± 0.01	1.06 ± 0.05
A23187	3.29 ± 0.12	6.12 ± 0.18
PMA/A23187	6.70 ± 0.60	11.47 ± 0.59

Table 5 Protein kinase C activity in stimulated cells preincubated with or without vitamin E

Cells were incubated with (+) or without (−) 50 μM vitamin E for 20 h and then stimulated with 100 nM PMA or 10 μM A23187 for 10 min. Subsequently, subcellular fractions were obtained and protein kinase C activity was determined as described in the Materials and methods section. Data are means ± S.D. of three separate experiments.

	Protein kinase C activity (pmol/min per mg)			
	Particulate fraction		Soluble fraction	
Treatment Vitamin E ...	−	+	−	+
None	8 ± 2	14 ± 5	45 ± 3	48 ± 6
PMA	63 ± 8	69 ± 6	11 ± 2	14 ± 2
A23187	5 ± 1	4 ± 1	55 ± 7	57 ± 5

cells with A23187 did not cause any apparent translocation of protein kinase C activity. No significant change in protein kinase C activity or distribution was observed following vitamin E enrichment. Collectively, these results show that vitamin E has no ability to activate or translocate protein kinase C. Hence, the mechanism for the enhancement of PLA_2 activity and the increase in arachidonate release may be different between vitamin E and PMA.

DISCUSSION

The central role of arachidonic acid release in the production of eicosanoids in mammalian cells is well documented [1]. Among the different types of PLA_2 that have been identified thus far, the c-type, which can be up-regulated in response to inflammatory cytokines or various growth factors, is now considered as the key intracellular enzyme which directs arachidonic acid release in stimulated cells [38]. The present study demonstrated this c-type enzyme is regulated by vitamin E in myoblastic H9c2 cells. Since this enzyme also possesses lysophospholipase and transacylase activities [9–11] and can thus be viewed as a complete enzyme system for the catabolism of membrane phospholipids, the physiological consequence of its activation is expected to lie beyond merely the synthesis of eicosanoids.

The regulation of vitamin E on the particulate PLA_2 remains undefined. Enzyme activity was not significantly increased in the particulate fractions from cells incubated with vitamin E, but enhancement of enzyme activity was observed when vitamin E was incorporated into substrate vesicles. It is possible that enzyme activity in the particulate fraction was also stimulated by vitamin E, but the presence of endogenous phospholipids would make it difficult to detect such an activation with a labelled exogenous substrate.

Vitamin E appears to modulate the intracellular PLA_2 activity in H9c2 cells by at least two mechanisms. First, direct incorporation of this vitamin into substrate vesicles was shown to enhance the rate of substrate hydrolysis by $cPLA_2$, with a maximum increase in activity when the molar ratio of vitamin E to phospholipid reached 0.2 mol%. We have shown that this concentration could be achieved through vitamin E supplementation. However, the precise distribution and actual concentration of vitamin E in biomembranes have not been well defined. The possibility of having a very high vitamin E to membrane phospholipid ratio (i.e. > 0.5 mol%) which could be inhibitory to the enzyme activity within a particular region of the membrane cannot be ruled out.

Secondly, the modulation of $cPLA_2$ level and/or activity by vitamin E was also demonstrated in this study. Cells treated with vitamin E showed a higher PLA_2 activity which was partly attributed to an elevation of the enzyme protein. However, the change in enzyme protein (about 2-fold) was not commensurate with the increase in enzyme activity (about 30–40%). The discrepancy could be explained by the recognition of the antibodies to all forms of the enzyme with different degrees of activity. Although the mechanism by which vitamin E elicited a higher amount of enzyme is not known, its action in up-regulating the rate of enzyme synthesis at the transcription or translation level remains a distinct possibility. Indeed, it is becoming increasingly apparent that vitamin E may play a role in the regulation of gene expression through profound effects on the activities of transcription factors NF-κB in Jurkat T cells [39] and AP-1 in smooth-muscle cells [40]. Vitamin E was also found to inhibit monocyte adhesion to endothelial cells by altering the expression of E-selectin on the surface of endothelial cells [41].

Beyond its ability to increase the protein level of PLA_2, vitamin E may modulate the activity of the enzyme in an indirect manner. Numerous studies have suggested that phosphorylation may stimulate PLA_2 activity by 2–3-fold [5–7]. In the present study, we have confirmed that cells 'primed' with PMA prior to A2318 stimulation released more arachidonic acid. However, vitamin E did not alter the protein kinase C activity in cells stimulated with A23187 or PMA. Thus, it appears that the vitamin E-enhanced PLA_2 activity observed in our study is independent of protein kinase C. Our result is not in agreement with the work of Azzi and his co-workers [14,15] which showed that protein kinase C activity was inhibited by vitamin E in smooth-muscle cells. This discrepancy can be explained by the cell-specific action of vitamin E which has no effect on protein kinase C activity in other cell types [14].

In summary, our study clearly shows that vitamin E caused an increase in the $cPLA_2$ activity and the subsequent release of arachidonic acid in H9c2 cells. This study also demonstrates the significant role of vitamin E in the regulation of eicosanoid biosynthesis at the level of substrate release during agonist stimulation.

We thank Drs. J. L. Knopf and L.-L. Lin, Genetics Institute, U.S.A., for the gift of anti-$cPLA_2$ polyclonal antibodies. This work was supported by the Heart and Stroke Foundation.

REFERENCES

1 Smith, W. L. (1989) Biochem. J. **259**, 315–324
2 Mayer, R. J. and Marshall, L. A. (1993) FASEB J. **7**, 339–348
3 Kudo, I., Murakami, M., Hara, S. and Inoue, K. (1993) Biochim. Biophys. Acta **117**, 217–231
4 Sharp, J. D., White, D. L., Chiou, G., Goodson, T., Gamboa, G. C., McClure, D., Burgett, S., Hoskins, J., Skattrud, P. L., Sportsman, J. R., Becker, G. W., Kang, L. H., Roberts, E. F. and Kramer, R. M. (1991) J. Biol. Chem. **266**, 14850–14853
5 Clark, J. D., Lin, L.-L., Krig, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N. and Knopf, J. L. (1991) Cell **65**, 1043–1051
6 Lin, L.-L., Wartmann, M., Lin, A. Y., Knopf, J. L., Seth, A. and Davis, R. J. (1993) Cell **72**, 269–278
7 Channon, J. T. and Leslie, C. C. (1990) J. Biol. Chem. **265**, 5409–5413
8 Leslie, C. C., Voelker, D. R., Channon, J. Y., Wall, M. M. and Zelarney, P. T. (1988) Biochim. Biophys. Acta **963**, 476–492
9 Leslie, C. C. (1991) J. Biol. Chem. **266**, 11366–11371
10 de Carvalho, M. G. S., Garritano, J. and Leslie, C. C. (1995) J. Biol. Chem. **270**, 20439–20446
11 Reynolds, L. J., Hughes, L. L., Louis, A. I., Kramer, R. M. and Dennis, E. A. (1993) Biochim. Biophys. Acta **1167**, 272–280
12 Gross, R. W. (1992) Cardiovascular Med. **2**, 115–121
13 Machlin, L. J. (1991) in Handbook of Vitamins (Machlin, L. J., ed.), pp. 99–144, Marcel Dekker, New York

14 Boscoboinik, D., Szewczyk, A., Hensey, C. and Azzi, A. (1991) J. Biol. Chem. **266**, 6188–6194
15 Tasinato, A., Boscoboinik, D., Bartoli, G.-M., Maroni, P. and Azzi, A. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 12190–12194
16 Tran, K., Proulx, P. R. and Chan, A. C. (1994) Biochim. Biophys. Acta **1212**, 193–202
17 Tran, K., D'Angelo, A. F., Choy, P. C. and Chan, A. C. (1994) Biochem. J. **298**, 115–119
18 Chan, A. C. (1993) in Vitamin E, its Usefulness in Health and in Curing Diseases (Mino, M., Nakamura, H., Diplock, A. T. and Kayden, H. J., eds.), pp. 197–207, Japan Sci. Soc. Press, Tokyo/S.kargre, Basel
19 Traber, M. B. and Packer, L. (1995) Am. J. Clin. Nutr. **62** (Suppl.), 1501s–1509s
20 Hamelin, S.-St. and Chan, A. C. (1983) Lipids **18**, 267–269
21 Chan, A. C., Tran, K., Pyke, D. D. and Powell, W. S. (1989) Biochim. Biophys. Acta **1005**, 265–269
22 Sakamoto, W., Fujie, K., Handa, H., Nishihara, J. and Mino, M. (1991) Biochim. Biophys. Acta **1074**, 296–300
23 Douglas, C. E., Chan, A. C. and Choy, P. C. (1986) Biochim. Biophys. Acta **876**, 639–645
24 Cao, Y.-Z., O, K., Choy, P. C. and Chan, A. C. (1987) Biochem. J. **247**, 135–140
25 Sakamoto, W., Fujie, K., Nishihara, J. and Handa, H. (1993) Biochim. Biophys. Acta **1170**, 296–300
26 Tran, K. and Chan, A. C. (1988) Biochim. Biophys. Acta **963**, 468–475
27 Tran, K. and Chan, A. C. (1990) Biochim. Biophys. Acta **1043**, 189–197
28 Tran, K., Man, R. Y. K. and Choy, P. C. (1995) Biochim. Biophys. Acta **1259**, 283–290
29 Bligh, E. G. and Dyer, W. Y. (1959) Can. J. Biochem. **37**, 911–917
30 Lowry, O. H., Rosebrough, M. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
31 Rouser, G., Slakotos, A. N. and Fleischer, S. (1966) Lipids **1**, 85–86
32 Street, I. P., Lin, H.-K., Laliberté, F., Ghomashchi, F., Wang, Z., Perrier, H., Tremblay, N. M., Huang, Z., Weech, P. K. and Gelb, M. H. (1993) Biochemistry **32**, 5935–5940
33 Riendeau, D., Guay, J., Weech, P. K., Laliberté, F., Yergey, J., Li, C., Desmarais, S., Perrier, H., Liu, S., Nicoll-Griffith, D. and Street, I. P. (1994) J. Biol. Chem. **269**, 15619–15624
34 Hazen, S. L., Zupan, L. A., Weiss, R. H., Getman, D. P. and Gross, R. W. (1991) J. Biol. Chem. **266**, 7227–7232
35 Phannkuche, H., Kaever, V., Gemsa, D. and Resch, K. (1989) Biochem. J. **260**, 471–478
36 Bonventre, J. V., Gronich, J. H. and Nemenoff, R. A. (1990) J. Biol. Chem. **265**, 4934–4938
37 Lin, L.-L., Lin, A. Y. and Knopf, J. L. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 6147–6151
38 Kramer, R. M. (1994) in Signal-Activated Phospholipases (Liscovitch, M., ed.), pp. 13–30, R. G. Landes, Co., Austin
39 Suzuki, Y. J. and Packer, L. (1993) Biochem. Biophys. Res. Commun. **193**, 277–283
40 Azzi, A., Boscoboinik, D., Marilley, D., Özer, N. K., Stäuble, B. and Tasinato, A. (1995) Am. J. Clin. Nutr. **62** (Suppl.), 1337s–1346s
41 Faruqi, R., de la Motle, C. and Di Corleto, P. E. (1994) J. Clin. Invest. **94**, 592–600

Received 18 March 1996/5 June 1996; accepted 19 June 1996