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Involvement of protein kinase C pathway in UVC-stimulated phospholipase D2 activity in Vero 76 cells

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Abbreviations: BAPTA/AM, 1,2-Bis (2-aminophenoxy) ethane-N,N, N',N'-tetraacetic acid tetrakis (acetoxymethyl ester); LOOH, lipid hydroperoxide; PA, phosphatidic acid; PAL, palmitic acid; PBt, phosphatidylbutanol; PKC, protein kinase C; PLD, phospholipase D; ROS, reactive oxygen species

Abstract

Phospholipase D (PLD) activity is known to be related to oxidant-induced cellular signaling and membrane disturbance. Previously, an induction of PLD activity in various cell lines by X-ray irradiation was observed. In this study, we examined the effect of UVC radiation on the PLD activity in Vero 76 cells. At a dose of 10 kJ/m^2 of UVC irradiation, the PLD activity was stimulated approximately 10-fold over the basal activity. This UVC-induced PLD activity was found to be dependent on the presence of extracellular calcium and was inhibited by catalase as well as amifostine - an intracellular thiol antioxidant. Pretreatments with Ro32-0432 - a selective inhibitor of protein kinase C (PKC) - and downregulation of PKC by preincubation of phorbol 12-myristate 13-acetate significantly inhibited the UVC-induced PLD activity. UVC-stimulated PLD activity was observed only in murine PLD2 (mPLD2)-transfected Vero 76 cells and not in human PLD1 (hPLD1)-transfected cells. Transient incorporation of PKC with mPLD2 and the phosphorylation of mPLD2 by α and β forms of PKC by UVC irradiation were observed. These results suggest that the UVC-stimulated PLD activity in Vero 76 cells is mediated through transient phosphorylation of PLD2 by the translocation of PKC to PLD2.

Keywords: phospholipase D; protein kinase C; reactive oxygen species; ultraviolet rays

Introduction

UVC - 100 nm~280 nm - is known to produce superoxide radicals, resulting in hydrogen peroxide generation. The hydrogen peroxide-derived hydroxyl radical (HO-) reacts with cellular proteins and membrane lipids that provoke various physiological cellular responses (Girotti, 1998). The prominent targets of oxidant attack are unsaturated phospholipids, glycolipids, and cholesterol in cell membranes and other cellular organelles. These attacks result in lipid hydroperoxidation that disturbs the membrane structures and induces various physiological disorders (Halliwell and Gutteridge, 1989). Physiological events such as detoxification processes, apoptosis, or necrotic cell death are dependent on the amount of lipid hydroperoxide (LOOH) present. At a relatively low dose of LOOH, oxidative injury may trigger a series of cytoprotective responses, e.g., induction of GSH and antioxidant proteins such as glutathione peroxidase, catalase, or heme oxygenase (Girotti, 2001). At a moderate dose of LOOH, peroxide induces apoptosis, which is characterized by cell shrinkage and the internucleosomal fragment of nuclear DNA. At a high dose of LOOH, the oxidative stress triggers membrane lysis associated with gross structural damage and random DNA fragmentation.

Oxidant-induced lipid signalings are usually associated with several enzymes such as phospholipase A2, phosphoinositide phospholipase C, and phospholipase D in various types of cells (Weigel *et al.,* 1997; Shen *et al.,* 2001). Phospholipase D activation, in particular, has been reported to be an important cellular signaling process, which leads to the generation of phosphatidic acid as a potential second messenger (Rydzewska *et al.,* 1996). There exist two mammalian PLD isoforms (PLD1 and PLD2) that occur as splice variants (Frohman *et al.,* 1999). PLD1 appears to be localized in the Golgi or perinuclear vesicular structure (Liscovitch *et al.,* 1999; Freyberg *et al.,* 2001; Choi *et al.,* 2002; Jang *et al.,* 2004), whereas PLD2 appears to be associated with the plasma membrane (Choi *et al.,* 2002; Exton, 2002; Sarri *et al.,* 2003). PLD1 is known to be activated by protein kinase C (PKC), ADP-ribo-

sylation factor, RalA, and RhoA in the presence of phosphatidylinositol 4, 5-bisphosphate. However, it has been reported that PLD2 is not activated by PLD1 activators (Colley *et al.,* 1997). In vivo activity of PLD2 is activated by factors that cause membrane disturbance, such as oleate (Sarri *et al.,* 2003) and mastoparan (Chahdi *et al.,* 2003).

It has been reported that the PLD activity that is stimulated by extracellular treatment of reactive oxygen species (ROS) is dependent on various protein kinase pathways in several cell lines. For instance, p38 MAPK (mitogen-activated protein kinase) and PKC mediated the activation of PLD by pervanadate and diperoxovanadate (Natarajan *et al.,* 1996; Min *et al.,* 2002; Kim *et al.,* 2003). In astrocytes and fibroblasts, H_2O_2-induced PLD1 stimulation was inhibited by PKC inhibitors and was almost completely suppressed by PKC downregulation (Min *et al.,* 1998; Servitja *et al.,* 2000). However, in other cell lines such as PC12 and the vascular smooth muscle cells, which exhibit only PLD2 activity, PLD activation was shown to be independent of PKC activity (Ito *et al.,* 1997; Taher *et al.,* 1998). On the other hand, in the lymphocytic leukemic L1210 cells and the vascular smooth muscle cells, H_2O_2-induced PLD stimulation was reported to be dependent on PKC (Lee *et al.,* 2000; Kim *et al.,* 2004). Therefore, the involvement of PKC in ROS-induced PLD activation remains controversial.

Previously, we investigated the effects of irradiation on PLD activity in various cultured cells and discovered that the PLD activity of Vero 76 (monkey kidney endothelial) cells was extremely sensitive to oxidative stress that was induced by irradiation (Kim *et al.,* 1997). In this report, PLD in the Vero 76 cells was further characterized by UVC irradiation, and a potential correlation between PLD activation and intracellular ROS was explored. The UVC-stimulated PLD activity is effectively inhibited by antioxidants and is quite sensitive to the expressed PLD2 isoform. We also demonstrated the transient phosphorylation of PLD2 by PKC and that PKC interacts with the transfected PLD2 isoform.

Materials and Methods

Materials

[$^{9,10\text{-}3}$H(N)]palmitic acid ([^{3}H]PAL) was purchased from Dupont NEN (Boston, MA). [^{3}H] dipalmitoyl phosphatidylcholine (DPPC) was kindly prepared by S. J. Lee (Seoul National University, Seoul, Korea). Catalase, amifostine, PD098059, SB203581, wortmanin, genistein, Ro32-0432, GF109203X, phobol 12-myristate 13-acetate, anti P-ser antibody, BAPTA/AM, and Z-VAD-fmk were purchased from Sigma (St. Louis, MO). The cell culture medium, Dulbecco's modified Eagle's medium (DMEM), Lipofectamine and PLUS Reagent were purchased from Invitrogen (Carlsbad, CA). 2′-7′-Dichlorodihydrofluorescein diacetate (H2DCFDA) was purchased from Microprobe (Eugene, OR). Anti-PLD1 antibody and anti-PLD2 antibody were purchased from Biosource (Camarillo, CA). Anti-P-thr, anti-P-tyr, anti-HA antibody, anti-PKC (MC5), anti-PKC α (A-3), and anti-PKC β1 (E-3) antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Precoated TLC gel (silica gel 60 F_{254}) was purchased from Merck (Darmstadt, Germany). Human PLD1b (hPLD1b) and mouse PLD2 (mPLD2) cDNAs subcloned into a pCGN vector was kindly provided by Dr. Sue Goo Rhee (National Institutes of Health, Bethesda, MD). Phosphatidylbutanol (PBt) was prepared from phosphatidylcholine (PC) by using PLD from *Streptomyces sp.* according to the previously described method (Jung *et al.,* 1989).

Cell culture, isotope labeling, and UVC irradiation

Vero 76 (monkey kidney endothelial) cells were grown in HEPES- (20 mM) buffered DMEM medium supplemented with 10% (v/v) FBS. The confluent cells were labeled with [^{3}H]PAL (1.0-2.0 μCi/ml) for 3 h in a serum-free DMEM medium containing 0.3% (w/v) fatty acid-free bovine serum albumin. After washing with phosphate-buffered saline (PBS), the cells were preserved with the serum-free DMEM medium containing inhibitors and incubated for 30 min at 37°C. Cells overlaid with serum-free DMEM were exposed to an indicated dose of UVC with a UVC 500 UV crosslinker (Hoefer, Austria).

Assay of PLD activity

The PLD activity in intact cells was determined by measuring PBt, the unique transphosphatidylation product of PLD in the presence of n-butanol, as previously described (Lee *et al.,* 1998). [^{3}H]PAL-labeled cells were resuspended in the assay medium supplemented with 0.3% n-butanol. After UVC irradiation at 37°C, the [^{3}H]PBt that was produced was separated on a TLC plate by using a solvent system that comprised ethylacetate/isooctane/acetic acid/water (13/2/3/10) and counted in a liquid scintillation counter (Wallac 1409; Turku, Finland).

For the PLD assay with cell lysates, the cell pellet was disrupted by using a microprobe-type ultrasonicator (Danbury, CT) in PBS-containing protease inhibitor cocktails (Boehringer Manheim, Germany), and cell lysates were prepared by harvesting the supernatant of microcentrifugation at 10,000 rpm for 3 min. ARF-dependent PLD activity was essentially assayed according to the method described by

Brown *et al.*, (1993) with slight modifications. A 20-μl aliquot of mixed lipid vesicles (PE/PIP_2/egg PC, molar ratio 16/1.4/1) with 0.2 μCi of [^{3}H]DPPC was added to 50 μl of the assay solution containing 50 mM HEPES (pH 7.4), 3 mM EGTA, 80 mM KCl, 1 mM DTT, 3.0 mM $MgCl_2$, and 2.0 mM $CaCl_2$. Reaction mixtures also contained 100 μM GTPγS, 1 μM ARF, 0.3% n-butanol, and 30 μg of the cell lysates; this was incubated for 60 min at 37°C. The separation and measurement of [^{3}H] PBt were performed by the method described above.

Transfection of PLD vector

Transient expression of hPLD1b and mPLD2 cDNAs to Vero 76 cells was performed using lipofectamine PLUS in accordance with the manufacturer's instructions. Subconfluent Vero 76 cells that were maintained at room temperature for 30 min in 1 ml of serum-free DMEM medium were previously treated with DNA complex containing 1 μg of DNA, 6 μl of PLUS Reagent, and 4 μl of lipofectamine. After incubation for 4 h, 1 ml of fresh culture medium containing 20% FBS was added. The PLD in Vero 76 cells was assayed and blotted after 24 h.

Subcellular fractionation

Subcellular fractionation of the Vero 76 cell lysates was carried out as described by Hu and Exton with slight modifications. After UVC irradiation, 100-mm plates of Vero 76 cells were washed twice with ice-cold PBS and then harvested using lysis buffer (25 mM HEPES, pH 7.2, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and protease inhibitor mixture) with a rubber policeman. After a 10-s sonication, the cell lysates were first centrifuged at 500 × *g* for 10 min to remove the unbroken cells. The supernatant was then centrifuged at 120,000 × *g* for 45 min at 4°C with a Beckmann ultracentrifuge to separate the cytosolic and crude membrane fractions.

Measurement of intracellular reactive oxygen species

ROS produced in the intracellular space of the Vero 76 cells was quantized by measuring the fluorescence of 2′-7′-dichlorodihydrofluorescin - an intracellular localized ROS sensor. The Vero 76 cells were incubated with HBSS buffer containing 20 μM 2′-7′-dichlorodihydrofluorescin diacetate (H2DCFDA) for 20 min at 37°C. The cells were washed twice with HBSS to separate the free H2DCFDA and then resupplied with serum-free DMEM for UVC irradiation. After UVC irradiation, fluorescence changes were analyzed by Zeiss fluorescence microscopy and quantized with an SFM 25 Spectro fluorometer (Kontron, Italy). The excitation wavelength was 520 nm, and the emission wavelength was 604 nm.

Immunoprecipitation and Western blotting

Vero 76 cells that were cultured in a 100-mm plate were transfected as previously described. The cells were washed twice with ice-cold PBS buffer and harvested using immunoprecipitation buffer containing 20 mM Tris, 137 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% Triton X-100, 2 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM PMSF, and 1 mM vanadate. The cell suspension was sonicated for 10 s and then centrifuged at 10,000 rpm for 5 min in order to pellet the detergent-insoluble fraction. One milligram of the supernatant was incubated overnight with 2 μg of anti-PLD2 antibody or anti-HA antibody in the presence of 20 μl of protein A/G beads. The immunoprecipitates were washed three times with ice-cold DPBS and resuspended with the Laemmli sampling buffer. The samples were analyzed by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blotted with 5% skimmed milk in TTBS solution, incubated with a primary antibody, and then with an HRP-conjugated antibody or an AP-conjugated antibody. The bands were detected using an enhanced chemiluminescence kit (Pierce, Rockford, IL).

Results

Activation of PLD by UVC irradiation

We investigated the effect of UVC on the activation of PLD in Vero 76 cells. Cultured cells overlaid with serum-free DMEM were exposed to various doses of UVC and incubated for 30 min at 37°C. As shown in Figure 1A, exposure of Vero 76 cells to 5 kJ/m^2~20 kJ/m^2 resulted in an increase greater than 10-fold of the intact cell PLD activity, whereas at doses above 20 kJ/m^2, the induction of PLD activity was reduced.

The PLD activity stimulated by UVC irradiation (10 kJ/m^2) was assayed at 5-min intervals in order to characterize time dependence (Figure 1B). This PLD activity was increased within 5 min after 10 kJ/m^2 of UVC irradiation. Major stimulation of PLD activity occurred during the second period of 5–10 min. The activity level then gradually decreased to the basal activity after approximately 30 min. Cumulative 5 min-interval PLD activity was replotted as time-dependent PLD activity (Figure 1B inset). The cumulative quantity of [^{3}H] PBt in Vero 76 cells during the 30-min incubation period reached approximately 1% of the total lipids. This amount is similar to that of the PLD activity shown in Figure 1A with the same dose of UVC during the 30-min incubation period.

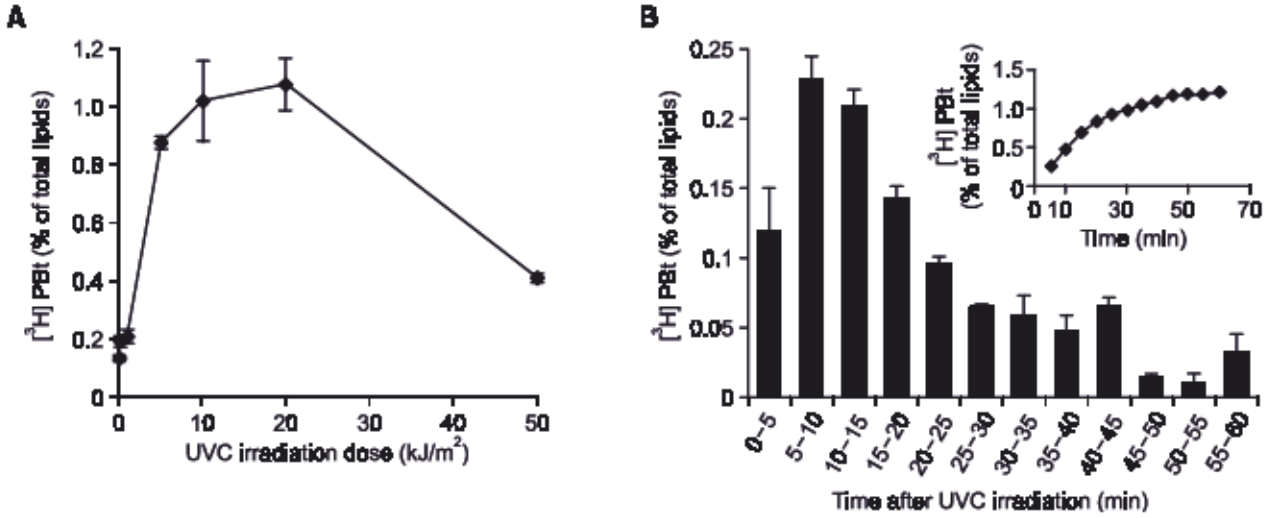

Figure 1. Effect of UVC on the PLD activity in Vero 76 cells. (A) [^{3}H] PAL-labeled Vero 76 cells were irradiated with the indicated dose of UVC and incubated for 30 min at 37°C in the presence of 0.3% of *n*-butanol. (B) PLD activity of Vero 76 cells irradiated with 10 kJ/m^2 UVC, followed by incubation at the indicated time intervals. The 5-min interval PLD activity was obtained after subtraction of the basal activity. (inset) The 5-min interval PLD activity was plotted cumulatively. The radioactivity incorporated into PBt was measured as described under Materials and Methods. The data presented are the mean ± SD in triplicate.

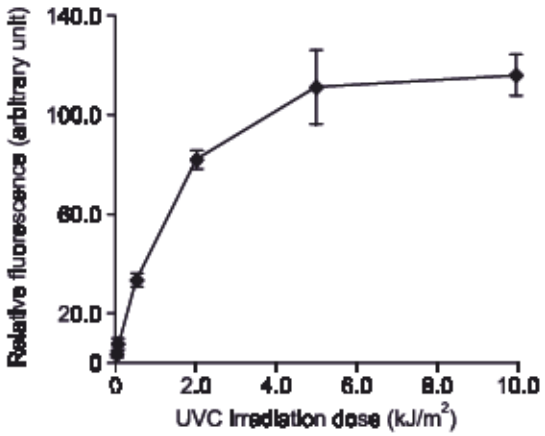

Figure 2. Oxidative species induced by UVC irradiation in Vero 76 cells. Cells labeled with 2'-7'-dichlorodihydro-fluorescein diacetate (20 μM) were irradiated with the indicated dose (kJ/m^2) of UVC. After 5 min, fluorescence emitted by the oxidative species was quantized with a spectrofluorometer. The excitation wavelength was 520 nm, and the emission wavelength was 604 nm.

Increase in intracellular ROS by UVC irradiation

In an effort to identify the oxidative effect induced by UVC irradiation on Vero 76 cells, we incubated the cells with H2DCFDA - an intracellular ROS sensor (Figure 2). The relative fluorescence determined after incubation for 5 min indicated an excessive increase in the ROS in intact cells by UVC irradiation. Even at low and moderate doses of UVC irradiation (0.5-2.0 kJ/m^2), which are insufficient to induce a large increase in UVC-stimulated PLD activity in Figure 1A, the fluorescence increased to approximately 22-fold of the basal fluorescence. The slope of fluorescence becomes attenuated at a dose of 2.0-5.0 kJ/m^2 and appears to be almost saturated at 5.0-10.0 kJ/m^2. The intracellular ROS observed by H2DCFDA had increased to 32-fold of the basal ROS by a 10.0-kJ/m^2 dose of UVC irradiation.

Effect of calcium chelator and antioxidant

In order to study the role of calcium and ROS in UVC-stimulated PLD activity in Vero 76 cells, we examined the effects of EGTA - an extracellular calcium chelator - and catalase - an antioxidant - on UVC-stimulated PLD. BAPTA/AM and amifostine, an intracellular calcium chelator and a membrane permeable antioxidant, respectively, were also treated before UVC irradiation. As shown in Figure 3, both extracellular and intracellular calcium chelators as well as the antioxidant inhibited UVC-stimulated PLD activity. However, the extracellular effectors, namely, EGTA and catalase, markedly inhibited the PLD activity near the basal level, whereas BAPTA/AM and amifostine only partially inhibited the stimulated PLD activity by 26.3% and 62.4%, respectively.

Effects of protein kinase inhibitors on the UVC-stimulated PLD activity

In order to identify the kinase-dependent signaling pathway, we examined PD098059, SB203581, wortmannin, genistein, and R032-0432 and GF109203X that inhibit mitogen-activated protein kinase kinase

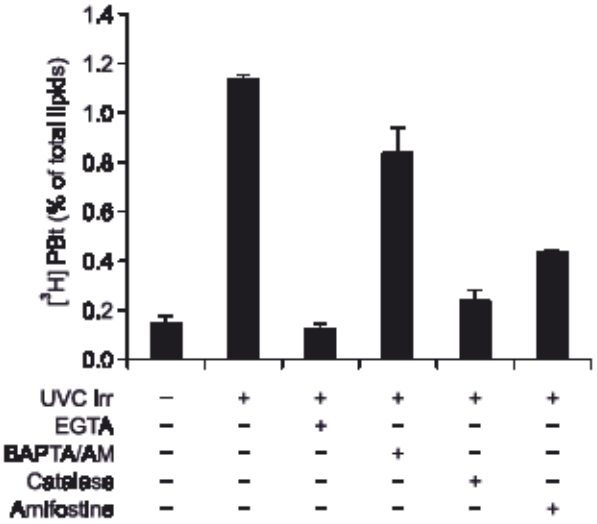

Figure 3. Effects of an antioxidant and a Ca^{2+} chelator on the UVC-stimulated PLD activity in Vero 76 cells. [^{3}H] PAL-labeled cells were preincubated with EGTA (2.5 mM, for 5 min), BAPTA/AM (100 μM, for 30 min), catalase (10,000 U/ml, for 5 min), or amifostine (0.5 mg/ml, for 24 h) and then incubated for 30 min after UVC irradiation (10 kJ/m^2) in the presence of 0.3% of n-butanol. The radioactivity incorporated into PBt was measured as described under Materials and Methods. The data presented are the mean ± SD in triplicate.

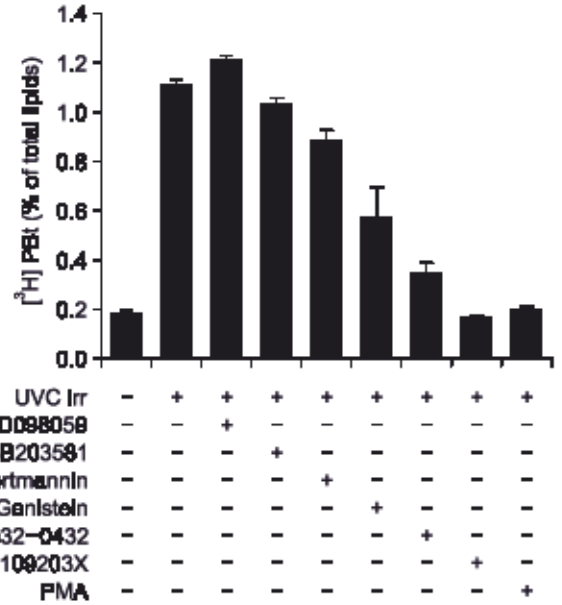

Figure 4. Effects of kinase inhibitors on the UVC-stimulated PLD activity in Vero 76 cells. [^{3}H] PAL-labeled cells were preincubated with PD098059 (10 μM), SB203581 (10 μM), wortmanin (100 nM), genistein (100 μM), Ro32-0432 (100 nM), and GF109203X (10 μM) for 30 min and then incubated for 30 min after UVC irradiation (10 kJ/m^2) in the presence of 0.3% of n-butanol. For PMA pretreatment, [^{3}H] PAL-labeled cells were preincubated with PMA (200 nM) for 18 h. The radioactivity incorporated into PBt was measured as described under Materials and Methods. The data presented are the mean ± SD in triplicate.

(MAPKK), P38 mitogen-activated protein kinase (P38MAPK), phosphatidyl inositol-3 kinase (PI3K), protein tyrosine kinase, and protein kinase C, respectively. As shown in Figure 4, PD098059 and SB203581 did not significantly inhibit PLD activity. Wortmanin and genistein partially inhibited the UVC-stimulated PLD activity. However, the PKC inhibitors GF109203X and Ro32-0432 appeared to inhibit the PLD activity to the basal level. This effect of PKC inhibitors was confirmed by the downregulation of PKC with phorbol ester (van Blitterswijk *et al.*, 1991). The accumulation of PBt by UVC irradiation was almost eliminated by the preincubation of 100 nM PMA for 18 h. This study on the inhibitor suggests that PKC plays a key role in UVC-stimulated PLD activity in Vero 76 cells.

Effect of UVC irradiation on the PLD isoforms transfected in Vero 76 cells

The PLDs in Vero 76 cells were blotted with anti-PLD1 and anti-PLD2 antibodies (Figure 5A). Endogenous PLDs in Vero 76 cells were not detected clearly; however, the Vero 76 cells that were transfected by human PLD1b (hPLD1b) and murine PLD2 (mPLD2) showed strong PLD bands. In vitro PLD activity of Vero 76 cells by using [^{3}H] DPPC was significantly increased by the transfection of hPLD1b and mPLD2 (Figure 5B).

In order to identify the subspecies of PLD that respond to UVC irradiation, we examined the effect of UVC irradiation on the hPLD1b- and mPLD2- expressed Vero 76 cells (Figure 5C). The intact cell PLD activity of the PLD2-expressed cells greatly increased at a dose of 0.5–3.0 kJ/m^2 UVC irradiation, while mPLD2 activity increased moderately to 2% when compared with 1.2% of untransfected Vero 76 cells at 10 kJ/m^2 UVC irradiation. However, hPLD1-expressed cells did not surpass the untransfected UVC-stimulated PLD activity. The mPLD2-transfected cells were sensitive to UVC irradiation, whereas the hPLD1-expressed cells appeared to be unaffected by UVC.

PKC interaction with PLD transfected in Vero 76 cells

Figures 4 and 5 suggest that PKC and PLD2 were responsible for the UVC-induced PLD activation observed in Vero 76 cells. The PKC inhibitors G109203X and Ro32-0432 have been evaluated to be inhibitors of the conventional PKC family. Ro32-0432, in particular, showed a significant selectivity for PKC α and PKC β (Wilkinson *et al.*, 1993). Therefore, we examined the interaction between PLD and PKC by using monoclonal PKC (MC5 clone) antibody specific for α, β and γ isoforms. Cell lysates transfected

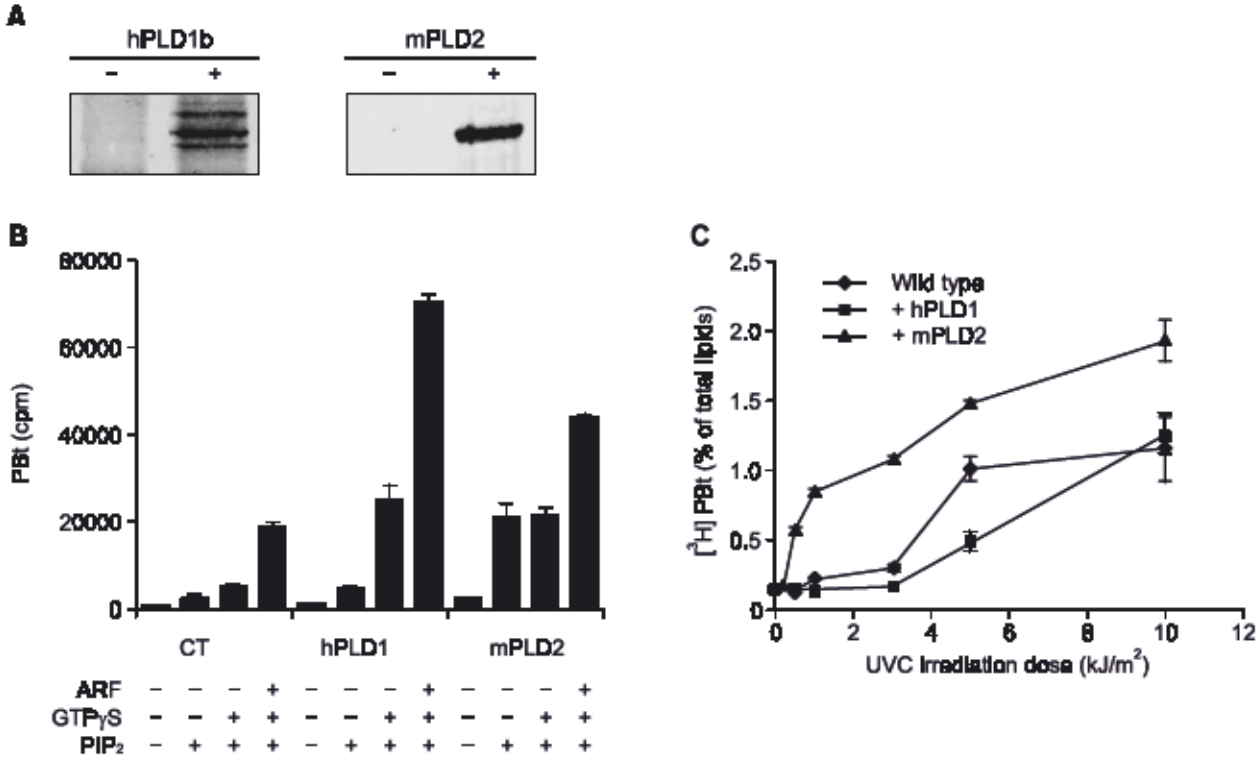

Figure 5. Effect of transfection of PLD isoforms in Vero 76 cells. Western blotting and ARF-dependent PLD assay were carried out with cell lysates prepared from the wild-type (-), hPLD1b, or mPLD2-transfected(+) Vero 76 cells. (A) Cell lysates (50 μg) were separated and blotted with anti-PLD1 and anti-PLD2 antibodies. (B) Cell lysates were incubated in the presence and absence of 12 μM PIP2, 100 μM GTPγS, and 1 μM ARF at 37°C for 1 h. (C) Vero 76 cells that were transfected with hPLD1b or mPLD2 were radiolabeled, irradiated with the indicated dose of UVC, and then incubated for 30 min in the presence of 0.3% of n-butanol. The radioactivity incorporated into PBt was measured as described under Materials and Methods. The data presented are the mean ± SD in triplicate.

with hPLD1 or mPLD2 were immunoprecipitated with PLD1 or PLD2 antibody, respectively, and immunoblotted with MC5 clone antibody (Figure 6A). The level of PKC coimmunoprecipitated with transfected mPLD2 dramatically increased after 10 min upon UVC irradiation, whereas the level of PKC with hPLD1 minimally increased in the same time period. This time course of maximum mPLD2 incorporation with PKC appeared to be similar to the second period of incubation interval (5-10 min), which produced a major PLD stimulation (Figure 1B).

Considering the inhibitory effects of EGTA and catalase as observed in Figure 3, translocation of PKC by an interaction with mPLD2 would be prevented in the absence of extracellular calcium or by the elimination of ROS from the plasma membrane. Figure 6B illustrates the localization of PKC α and PKC β through a Western blot analysis with anti-PKC α- or PKC β-specific antibody. After UVC irradiation, we separated the membrane fraction from the cytosolic fraction by ultracentrifugation. In the control cells, PKC α and PKC β were found to be located in the cytosolic fraction; however, PKC α and PKC β were translocated into the membrane fraction by UVC irradiation (Figure 6B). However, when EGTA or catalase was added to the reaction media, UVC-induced translocation of PKCs to the membrane fraction was prevented. On the other hand, Ro32-0432 - the PKC inhibitor - did not prohibit the translocation of PKC α and PKC β but blocked the UVC-stimulated PLD activation, probably blocking the PKC phosphorylation of PLD2 by Ro32-0432 (Figure 4). From these observations, we can postulate that PKC α and PKC β are translocated to the membrane fraction by excessive oxidative stress in the presence of extracellular calcium. This enables PKC interaction with PLD2 in order to stimulate PLD2 activity via phosphorylation of the enzyme.

Phosphorylation of PLD2 by UVC irradiation on mPLD2

PKC is known as a multifunctional protein kinase that phosphorylates serine and threonine residues in many target proteins. Generally, serine and threonine residues of PLD2 are known to be phosphor-

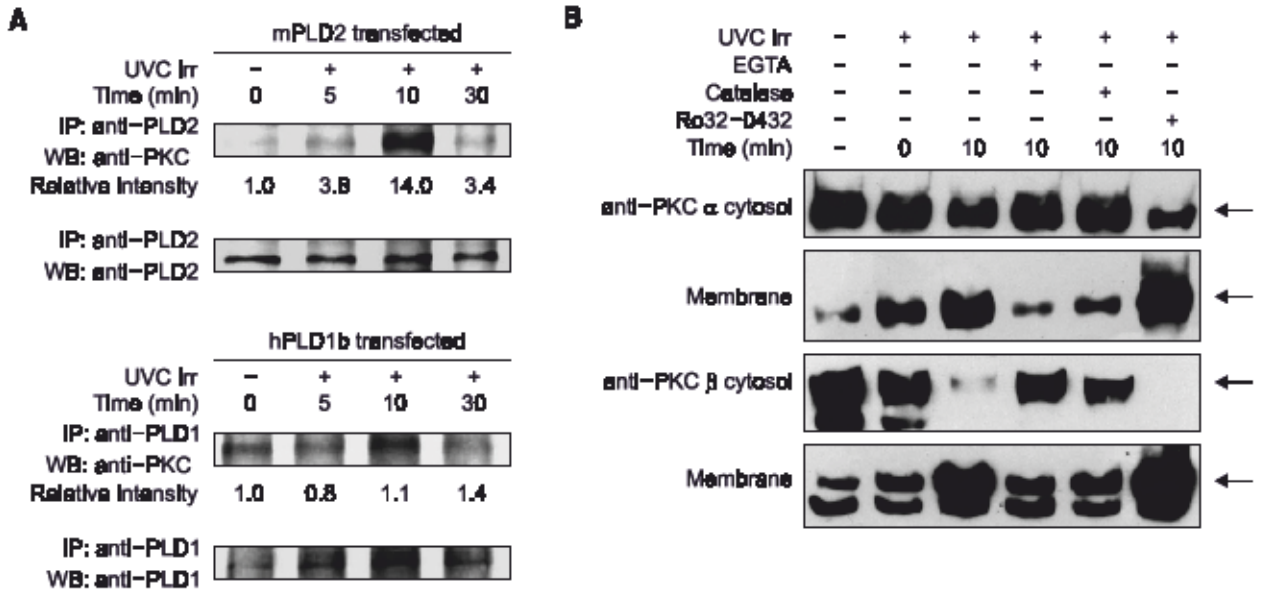

Figure 6. Translocation of PKC after UVC irradiation in PLD-transfected Vero 76 cells. (A) Vero 76 cells transfected with hPLD1b or mPLD2 were incubated for the indicated time period after UVC irradiation. PKC that was associated with PLD1 and PLD2 was immunoprecipitated by anti-PLD1 and anti-PLD2, respectively, and blotted with anti-PKC (MC5). (B) Vero 76 cells transfected with mPLD2 were preincubated with EGTA (2.5 mM, for 5 min), catalase (10,000 U/ml, for 5 min), or GF109203X (10 μM, for 30 min) and incubated for the indicated time period after UVC irradiation (10 kJ/m^2). The cytosolic and membrane fractions were separated as described under Materials and Methods. The amount of PKC α and β located in the membrane and cytosolic fractions were determined by Western blotting using the anti-PKC α or β antibody.

ylated and dephosphorylated by the physiological state of the cells (Chen and Exton, 2004). Since PKC transiently interacted with PLD2 and activated PLD activity after UVC irradiation, detection of the phosphorylation of the serine and threonine residues in PLD2 could be verified. Vero 76 cells transfected with the mPLD2 gene tagged to a hemagglutin (HA) epitope were lysed at the indicated time after UVC irradiation. This cell homogenate was then immunoprecipitated with anti-HA antibody and blotted by anti-phosphoserine- or anti-phosphothreonine-specific antibody. The time-dependent phosphorylation of PLD2 is presented in Figure 7. After UVC irradiation, the basal level of phosphorylation of mPLD2 appeared to show a marked increase at approximately 5 min. However, the phosphorylation level decreased to the basal level within 30 min. This transient phosphorylation could be compared with the time courses of PLD stimulation (Figure 1B) and PKC incorporation with mPLD2 (Figure 6A). The time-dependent phosphorylation of the serine and threonine residues in mPLD2 indicates that the phosphorylation state is the activated state of PLD induced by UVC irradiation.

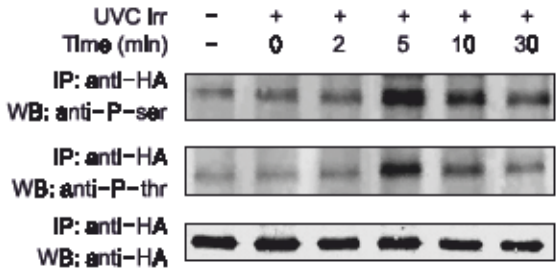

Figure 7. Transient phosphorylation of the transfected PLD2 after UVC irradiation. Cells transfected with mPLD2 DNA containing hemagglutin (HA) epitope were incubated for the indicated time after UVC irradiation (10 kJ/m^2). The cell lysates were harvested and immunoprecipitated with anti-HA and blotted with anti-phospho-Ser and anti-phospho-Thr. The relative amount of phosphorylation of mPLD2 was determined by Western blotting.

Discussion

In this study, we observed marked stimulation of PLD activity in Vero 76 cells by UVC irradiation (Figure 1). This stimulated PLD activity required significant amount of ROS produced by a 10 kJ/m^2 dose of UVC irradiation (Figure 2). The UVC-stimulated PLD was greatly dependent on the presence of extracellular calcium and was effectively inhibited by catalase (Figure 3). However, BAPTA/AM - an intracellular calcium chelator - and amifostine - an intracellular antioxidant - only partially inhibited the stimulated PLD activity. The influx of calcium into the

intracellular space was not important since preincubation of a calcium ionophore, A23187, as well as several calcium channel inhibitors such as flunarizine and neomycin did not alter the UVC-stimulated PLD activity in Vero 76 cells (data not shown). Similarly, PLD activity stimulated by hydroperoxide (Lee *et al.,* 2000; Servitja *et al.,* 2000) or pervanadate (Oh *et al.,* 2000) has been reported to be dependent on extracellular calcium. On the other hand, other stimulants of PLD activity, such as phorbol ester (Huang *et al.,* 1992), α-thrombin (Garcia *et al.,* 1992), or oleate (Lee *et al.,* 1998), are not dependent on extracellular calcium.

The PLD activity stimulated by UVC was effectively inhibited by the protein kinase C inhibitors, namely, Ro32-0432 and G109203X (Figure 4). This PKC dependence was further confirmed by the downregulation of PKC. Other protein kinase inhibitors, such as wortmanin and genistein, exerted only slight inhibition on the UVC-stimulated PLD in the Vero 76 cells. Some PLD activities were reported to be dependent on tyrosine kinases, including P38 mitogen-activated protein kinase in cell lines such as bovine pulmonary artery endothelial cells (Natarajan *et al.,* 2001) and PC12 cells (Banno *et al.,* 2001).

The UVC upregulation of PLD2 activity in Vero 76 cells that were transfected with mPLD2 strongly suggests that endogenous PLD activity stimulated by UVC irradiation is probably the isoform of PLD2 (Figure 5). In particular, the exhibited mPLD2 activity increased its sensitivity toward UVC irradiation at relatively low doses in a range of 0.5–3.0 kJ/m^2, whereas hPLD1 did not exhibit sensitivity toward any doses of UVC irradiation (Figure 5C).

The study of incorporation of PKC into the transfected mPLD2 revealed that PKC was transiently incorporated with mPLD2 at approximately 10 min and dissociated to a basal level after 30 min (Figure 6A). As shown in Figure 6B, PKC α and PKC β were translocated upon UVC irradiation in the presence of calcium ions. Despite the translocation of PKC, blocking the UVC-stimulated PLD activity by preincubation of Ro32-0432 demonstrates the importance of the phosphorylation status of mPLD2. A study on the time-dependent phosphorylation of mPLD2 (Figure 7) revealed similar time courses of PKC incorporation and mPLD2 activation. The time required for maximum activation of endogenous PLD activity by UVC (Figure 1B) may be compared with the time courses of mPLD2 activation and translocation. Recently, Hu and Exton reported a study of the phosphorylation of PLD1 stimulated by PMA compared with the status of time-dependent PLD1 activity and PKC α binding (Hu and Exton, 2003). They observed that PLD1 activity was maximized within 5 min and decreased to the basal level within 30 min. However, the phosphorylation of threonine residues in PLD1 and the incorporation of PKC were found to be interconnected, and they were phosphorylated even after 30 min. This indicates that the phosphorylation status did not coincide with the transient stimulation of the PLD1 activity. In contrast to the PMA-induced PLD1 activation, the UVC-stimulated mPLD2 activity in Vero 76 cells appears to coincide with the phosphorylation of PLD2 with the transient translocation of PKC to the membrane fraction. This relationship demonstrates the phosphorylation-dependent activation of mPLD2 by PKC. These data indicate that PLD2 activity in Vero 76 cells is selectively stimulated by UVC, and the phosphorylation status of PLD2 appears to coincide with the time course of the translocation of PKC (α and β) to the membrane fraction. Thus, our results demonstrate the involvement of the PKC pathway in the UVC-stimulated PLD activity in Vero 76 cells.

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