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Phospholipase D1 as a Key Enzyme for Decidualization in Human Endometrial Stromal Cells[1]

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ABSTRACT

Using primary cell cultures of human endometrial stromal cells (ES cells), we investigated the role of phospholipase D (PLD) in 8-Br-cAMP-induced decidualization, which involves morphological and biological differentiation processes. When treated with 0.5 mM 8-Br-cAMP for 12 days, ES cells were transformed into a decidualized morphology and produced significant amounts of prolactin (PRL) and insulin-like growth factor-binding protein 1 (IGFBP1). Simultaneously, the activity and expression levels of PLD1 increased. In addition, removal of 8-Br-cAMP from decidualized ES cells restored the undifferentiated state, and this was accompanied by decreases in PLD1 promoter activity and PLD1 expression. Overexpression of dominant negative (DN)-*PLD1* inhibited the morphological changes induced by 0.5 mM 8-Br-cAMP, whereas PLD1 overexpression induced morphological changes in the absence of 0.5 mM 8-Br-cAMP treatment. Moreover, knockdown of PLD1 by siRNA and blockage of PLD by treatment with 0.3% 1-butanol decreased *PRL/IGFBP1* mRNA expression, whereas *PLD1* overexpression increased *PRL/IGFBP1* mRNA expression. Treatment of ES cells with phosphatidic acid (PA) for 3 days induced *PRL* mRNA expression and morphological changes, which implies that PA is an end-product of PLD activation-induced decidualization. In addition, pretreatment of ES cells with mepacrine decreased *PRL/IGFBP1* expression and inhibited morphological change, whereas pretreatment with propranolol caused no changes, as compared to cAMP-treated cells, which suggests that PA induces decidualization through phospholipase A2 (PLA2G1B). Taken together, these results suggest that PLD1 regulates 8-Br-cAMP-induced decidualization through PLA2G1B, and that PLD1 upregulation is essential for the decidualization of ES cells.

8-Br-cAMP, developmental biology, decidualization, human endometrial stromal cell, phospholipase

INTRODUCTION

A satisfactory reproductive outcome requires synchronous growth and maturation of the endometrium throughout the menstrual cycle. The decidualization of human endometrial stromal cells (ES cells), which develop in the late secretory stage of the menstrual cycle, is characterized by morphological and functional differentiation. ES cells around blood vessels begin to differentiate into a larger and rounder decidual phenotype on the 23rd day in women with a regular 28-day menstrual cycle [1]. Following implantation, decidualization persists and extends throughout the endometrium, leading to the formation of pregnancy deciduas. This morphological change is accompanied by the expression of several factors, such as prolactin (PRL) and insulin-like growth factor-binding protein 1(IGFBP1) [2–5]. These factors are correlated with decidualization and in turn, act as regulators of both decidualization and trophoblast functions. Thus, decidualization is necessary for normal embryo implantation and the maintenance of pregnancy [6].

There is abundant clinical and experimental evidence in support of a critical role for progesterone in maintaining the decidual phenotype. However, recent reports have suggested that the expression of decidua-specific genes may not be transcriptionally regulated by the activated progesterone receptor (PR) [7]. Furthermore, progesterone is a very weak inducer of the decidual phenotype in primary ES cells [1], and initiation of the decidual process requires both a high level of intracellular cAMP and sustained activation of the protein kinase A (PKA) pathway [8, 9]. Therefore, our present knowledge regarding the intracellular signaling pathways for decidualization is preliminary at best.

Over the past two decades, it has been extensively documented that phospholipase D (PLD) plays an essential role in the signal transduction of mammalian cells [10]. PLD catalyzes the hydrolysis of phospholipids at the terminal phosphodiester bond, thereby producing phosphatidic acid (PA) and releasing the free polar head group. PA can also be metabolically converted to diacylglycerol (DAG) by phosphatic acid phosphatase type 2A (PPAP2A), and to lyso-PA or arachidonic acid by phospholipase A2 (PLA2G1B). Both of these factors serve as second messengers that contribute to the effects of PLD. Recently, two PLD isozymes (PLD1 and PLD2) have been cloned and characterized from mammalian cells [11, 12]. PLD1 is activated by protein kinase C (PKC) and small GTP-binding proteins, such as ADP-ribosylation factor (ARF)-, and Ral- and Rho-family GTPases. In contrast, PLD2 activity is known to be constitutively active in vitro and its activity is elevated by fatty acid, although it is not affected by GTPases or PKC-α. Both PLD isoforms require phosphatidylinositol-4,5-bisphosphate (PIP2) as a cofactor for their activities [11, 12]. PLD appears to play a significant role in a broad range of physiological processes, including both short-term (e.g., secretion and superoxide generation) as well as long-term (e.g., cell proliferation, differentiation, and apopto-

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sis) responses. Recently, a role for PLD in cell differentiation has been proposed; Marjou et al. have reported that the activity and expression of PLD increase with maturation and differentiation in human myeloid cells [13], up-regulation of *PLD1* [14] and *PLD2* [15] mRNA has been reported in differentiated HL60 cells, and regulated transcription of *PLD1* and/or *PLD2* has been observed in C6 glioma cells [16] and differentiating keratinocytes [17]. Taken together, these reports suggest a functional role for PLD in differentiation. Despite a large body of data that suggests that PLD is a pivotal factor in differentiation, it is not known whether PLD has a role in the differentiation of the endometrium.

In the present study, we investigated the role of PLD in 8-Br-cAMP-induced decidualization of human endometrial stromal cells in vitro. To the best of our knowledge, this is the first evidence that PLD1 is a regulator for decidualization, and that PLD1 activation may contribute to embryo implantation and maintenance of pregnancy in human endometrial stromal cells.

MATERIALS AND METHODS

Materials

Materials for tissue culture, such as fetal bovine serum (FBS), penicillin, streptomycin, and Dulbecco modified Eagle medium (DMEM, low glucose), were obtained from Gibco-BRL (Gaithersburg, MD), and 8-Br-cAMP was from Sigma (St. Louis, MO). [^{3}H]-palmitic acid was obtained from DuPont-New England Nuclear (Boston, MA). The following antibodies were as follows: anti-ACTB (Cell Signaling), anti-GFP monoclonal antibody (Roche, Indianapolis, IN), and a polyclonal antibody that recognizes both PLD1 and PLD2 (provided by Dr. D.S. Min, Pusan National University, Korea). L-α-PBt was obtained from Avanti Polar Lipid Inc. (Alabaster, AL), and the silica gel 60A plates for TLC were purchased from Whatman (Clifton, NJ). All other chemical reagents were of analytical grade.

Isolation and Culture of ES Cells

Human endometria were obtained by hysterectomy from 20 normally cycling pre-menopausal women, aged 35 to 44 years, who underwent surgery for non-endometrial abnormalities at Hanyang University Hospital between January 2004 and June 2006. Samples were collected under protocols approved by the Institutional Review Board of Hanyang University Hospital. Written informed consent was obtained from all participants. A portion of each endometrial specimen obtained was examined histologically. ES cells were isolated as described previously [18]. Briefly, tissue samples were collected in DMEM that contained 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10% (vol/vol) fetal calf serum. After cleaning and trimming to remove blood clots and mucus, specimens were minced to fragments of less than 1 mm in size under a laminar flow hood, and then digested at 37°C for 60 min with 0.25% (wt/vol) collagenase I (Sigma) and DNase (Sigma). The cell suspension was filtered twice through a 40-μm sieve (BD Falcon). After enzymatic digestion, most of the ES cells were present as single cells or small aggregates, and they were purified. The purity of the ES cells obtained by this method was usually >90%, as determined by immunocytochemical staining of vimentin (stromal cell marker). The purified ES cells were washed, and the number of viable cells was counted by trypan blue exclusion. The viability of the isolated cells was at least 90% in each experiment. The ES cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air with 10 ml DMEM that was supplemented with 10% (vol/vol) FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

In Vitro Decidualization of Human ES Cells

ES cells were cultured to confluence in 100-mm culture dishes. To induce decidualization in vitro, the cells were then cultured with 0.5 mM 8-Br cAMP, which is a decidualization inducer, for 3–12 days. The culture medium was changed every three days with continuous supplementation with 8-Br-cAMP. Phase contrast microscopy was used to verify morphological changes associated with differentiation in response to 8-Br-cAMP.

Determination of PLD Activity

PLD activity was determined as previously described by measuring [^{3}H]-PBt produced in [^{3}H]-palmitic acid-labeled cells via PLD-catalyzed transphosphatidylation [19]. Briefly, ES cells cultured in 6-well plates were metabolically labeled with 1 μCi/ml of [^{3}H]-palmitate for 24 h. The cells were then pretreated with 0.3% (vol/vol) 1-butanol for 15 min, and were quickly washed with ice-cold PBS and suspended in ice-cold methanol. Lipids were extracted according to the method of Bligh and Dyer [20], and [^{3}H]-PBt was separated from other phospholipids by thin layer chromatography on Silica G-60 plates, using a solvent system of ethyl acetate/iso-octane/acetic acid/water (26:4:6:20, v/v). The regions that corresponded to the authentic PBt bands were identified using 0.002% (wt/vol) primulin in 80% (vol/vol) acetone, scraped, and then counted using a scintillation counter.

Western Blot Analysis

ES cells were scraped into ice-cold phosphate-buffered saline (PBS) and harvested by microcentrifugation. The cells were then resuspended in buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1% [vol/vol] Triton X-100, 1 mM PMSF, 1 mM Na_3VO_4) and disrupted by sonication. Proteins were resolved by 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked for 1 h with TTBS (Tris-buffered saline with 0.01% [vol/vol] Tween 20) that contained 5% (wt/vol) non-fat dry skim milk, and then incubated for an additional 1 h with the primary antibodies (1 μg/ml). Unbound primary antibodies were removed by three washes (10 min each) with TTBS. The blots were then incubated with horse radish peroxidase (HRP)-conjugated secondary antibody (1:2000; New England Biolabs, Beverly, MA), and specific bands were detected by ECL (Amersham Pharmacia Biotech).

Immunocytochemistry

For immunocytochemical tests, ES cells were plated on a glass coverslip that was coated with fibronectin. Cells were fixed with 4% (wt/vol) paraformaldehyde/0.15% (wt/vol) picric acid in PBS, and then incubated overnight with primary antibody at 4°C. The anti-green fluorescent protein (GFP) monoclonal antibody was used at 1:400 (Roche Molecular Biochemical, Basel, Switzerland). For detection of the primary antibody, fluorescently labeled (Cy3) secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) were used according to the specifications of the manufacturer. Cells were mounted in Vectashield with DAPI (mounting medium for fluorescence analysis; Vector Laboratories, Burlingame, CA) and photographed using a fluorescent microscope (Nikon).

Construction of the Expression Vector

Lipase-inactive mutant *rPLD1* cDNA was provided by J.H. Exton (Vanderbilt University, Nashville, TN). Briefly, the N-terminal Xpress-tagged *rPLD1* was created by PCR amplification of the coding region that corresponds to amino acid residues 1 to 1036, and subcloned into the *Kpn*I/*Xba*I sites of the polylinker region in the pcDNA3.1 vector. *rPLD1-V5* was also generated by PCR amplification, followed by subcloning into the *Hin*dIII/*Xba*I site within the same frame as the C-terminal V5 tag of the pcDNA3.1/V5-HisA vector. The *Hin*dIII/*Kpn*I fragment of the C-terminal-tagged *rPLD1* was subcloned into the polylinker region of the pEGFP-C1 vector, to generate the N-terminal EGFP-tagged *rPLD1-V5*. ES cells grown for 4–6 days were transfected using the Nucleofector Kit (Amaxa), according to the manufacturers protocol. Transfection controls were carried out with the same amount of vector. Twenty-four hours after transfection, the cells were differentiated by treatment with 0.5 mM 8-Br-cAMP for the indicated day. Western blot analysis for the expression of plasmid DNA was used as an internal control, to determine the transfection efficiency.

Small Interfering RNA Sequences

The single siRNA for PLD1 was purchased from Dharmacon Research Inc. (Lafayette, CO) and the second siRNA for human PLD1 was a kind gift from Sung Ho Ryu (Pohang University of Science and Technology, Korea) [21]. A BLAST search of all siRNA sequences revealed no significant homology to any other sequences in the database. Then, 200 nM of the siRNA duplex was transiently transfected into human ES cells using the Nucleofector kit. Transfection controls were carried out with the same amount of EGFP-expressing vector. Twenty-four hours after transfection, the cells were differentiated by treatment with 0.5 mM 8-Br-cAMP for an additional 2 days in vitro.

Luciferase Promoter Assay

The PLD1 reporter plasmid was constructed with the human PLD1 promoter fragment cloned upstream of the luciferase cDNA in the pGL3-Basic

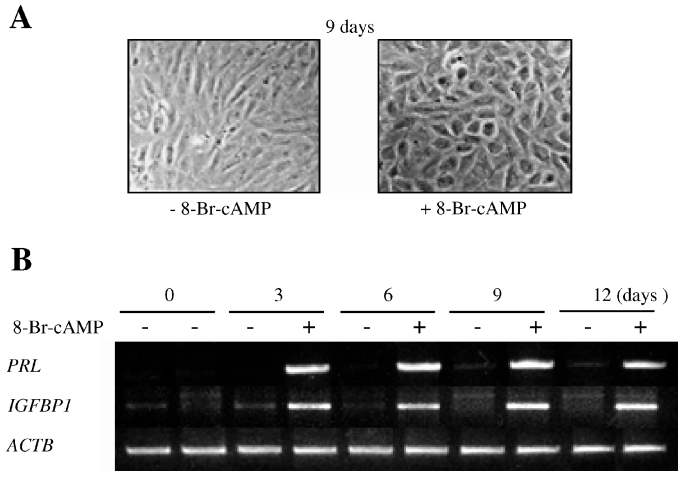

FIG. 1. Effects of decidualization on cell morphology and *PRL/IGFBP1* mRNA expression in human ES cells. **A**) ES cells were cultured in the absence or presence of 0.5 mM 8-Br-cAMP for 9 days (original magnification ×200). **B**) ES cells were either undifferentiated or differentiated into decidual cells for the indicated periods of time in the presence of 8-Br-cAMP. RNA was isolated from ES cells, and RT-PCR was used to analyze the levels of *PRL* and *IGFBP1* mRNA.

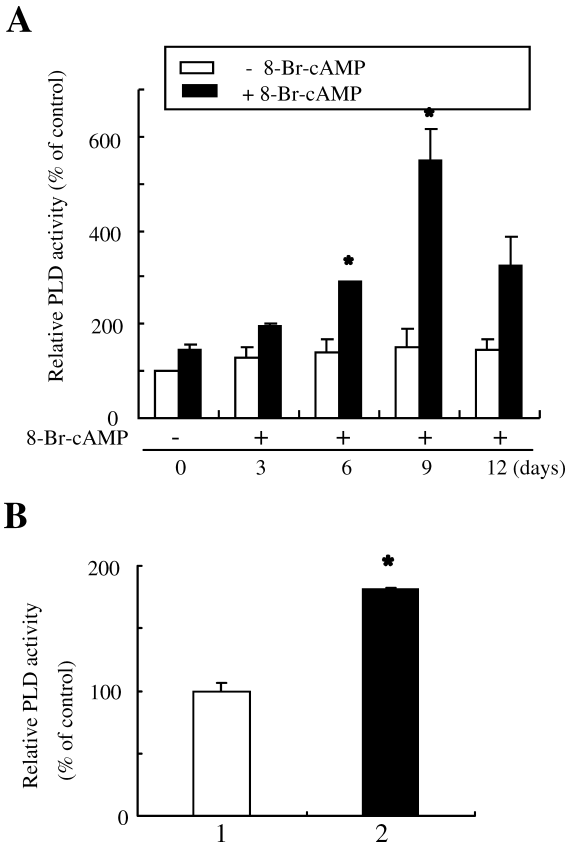

FIG. 2. Changes in the PLD activities of ES cells during decidualization. **A**) ES cells (3×10^5 cells/well) were cultured with 8-Br-cAMP for the indicated time periods and then labeled with 1 μCi/ml [^{3}H]-palmitic acid. PLD activities were determined by estimating the formation of [^{3}H]-PBt in the presence of 1-butanol. The results represent the mean ± SD of four separate experiments. **B**) ES cells (3×10^5 cells/well) isolated from a nonpregnant uterus and a 5-mo-pregnant uterus were labeled with 1 μCi/ml [^{3}H]-palmitic acid. Lane 1: ES cells from the nonpregnant uterus; lane 2: ES cells from the 5-mo-pregnant uterus.

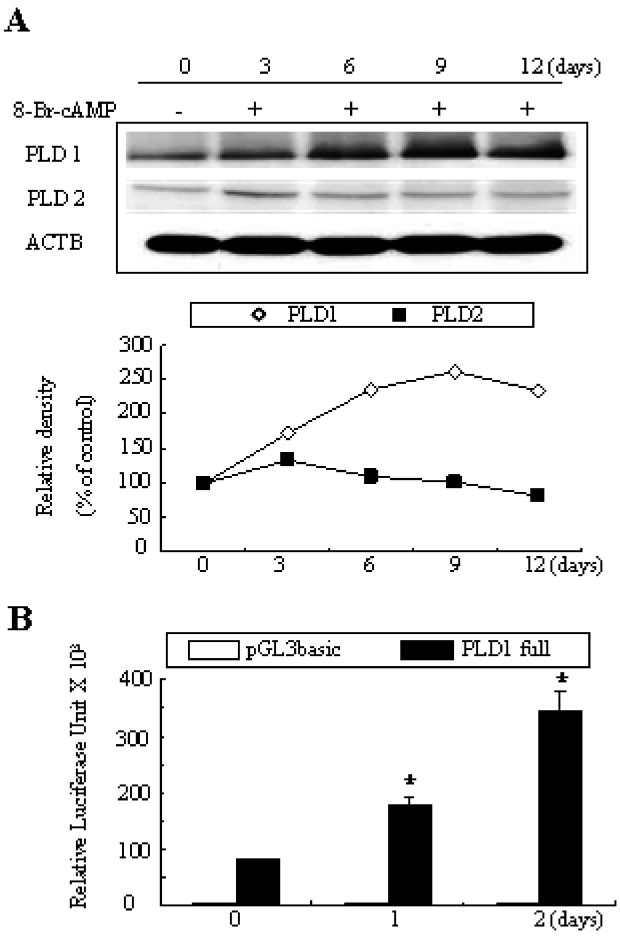

FIG. 3. Changes in the PLD protein expression levels and PLD1 promoter activities of ES cells upon decidualization. **A**) ES cells were differentiated into decidual cells for different periods of time in the presence of 8-Br-cAMP. The PLD1 and PLD2 proteins were detected in cellular extracts from ES cells. The amount of each protein sample used for PAGE was 30 μg per lane. Band intensity was quantified using the QuantityOne software (Bio-Rad). **B**) ES cells were transfected transiently with either the promoterless vector (pGL3-Basic) or the PLD1 reporter plasmid (pGL3-PLD1p-1865/+9) and pCMV-β-gal. Luciferase activity was measured in triplicate after 48 h of treatment.

vector (Promega). ES cells were transfected transiently with pGL3-Basic, the promoterless vector or the PLD1 reporter plasmid (pGL3-PLD1$_p$-1865/+9) using the Nucleofector Kit. To correct for the variance of transfection efficiency in each assay, the pCMV-β-gal vector was cointroduced. One day after transfection, the cells were treated with 0.5 mM 8-Br-cAMP for 2 days, to induce decidualization. Gene expression was analyzed using a firefly luciferase assay system (BD-Pharmingen) and a microplate luminometer (Berthold). The relative firefly luciferase activity was defined as the luciferase activity normalized for β-galactosidase activity in each corresponding cell lysate.

RNA Extraction and Reverse Transcription-PCR (RT-PCR) Analysis

Total cellular RNA was prepared using Tri Reagent (Molecular Reagent Center, Cincinnati, OH), according to the recommendations of the manufacturer. The Superscript Kit (Invitrogen) was used for cDNA synthesis. PCR reactions were carried out according to standard protocols. Initially, the optimal $MgCl_2$ concentrations and cycle numbers for a linear amplification range were determined. The primer sequences (forward and reverse) were as follows: *ACTB* (5′-CCCAGGCACCAGGGCGTGATC-3′ and 5′-TCAAACAT-GATCTGGGTCAT-3′); *PRL* (5′-GCCCCCTTGCCCATCTGTCC-3′ and 5′-AGAAGCCGTTTGGTTTGCTCC-3′); *IGFBP1* (5′-TGCTGCAGAGG-CAGGGAGCCC-3′ and 5′-AGGGATCCTCTTCCCATTCCA-3′); *hPLD1* (5′-ACTCTGTCCAAAGTTAACATGTCACTG-3′ and 5′-GGCTTTGTAC-

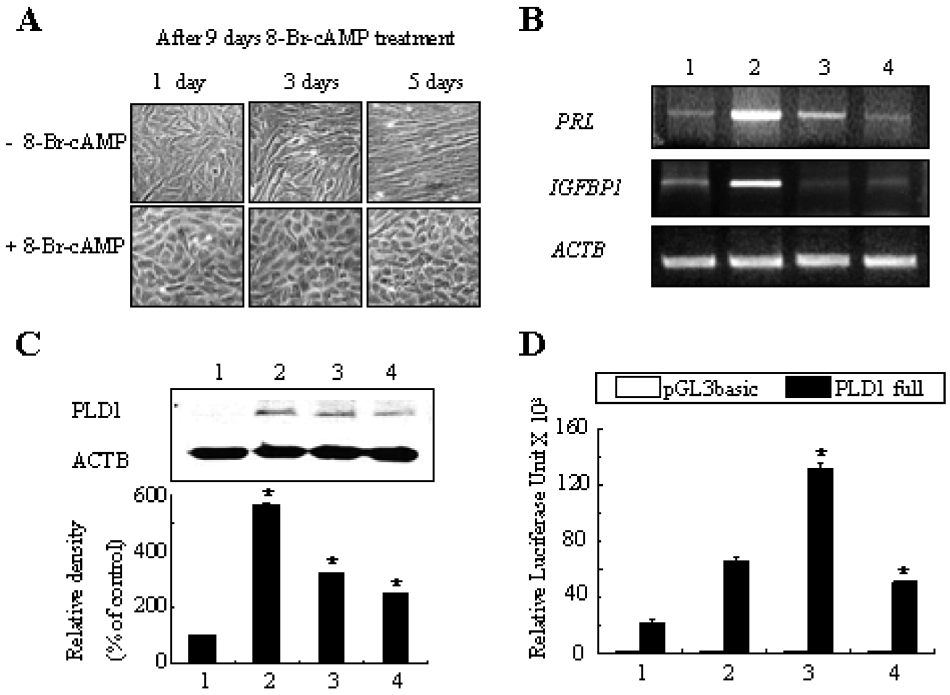

FIG. 4. Effects of 8-Br-cAMP withdrawal on morphology (A), *PRL/IGFBP1* mRNA expression (B), PLD1 protein expression (C), and PLD1 promoter activity (D) in ES cells decidualized by 8-Br-cAMP. ES cells were differentiated into decidual cells for 9 days in the presence of 8-Br-cAMP, followed by 8-Br-cAMP withdrawal. **A**) After withdrawal for the indicated periods, the ES cells were photographed under a microscope (original magnification ×200). RNA and protein were isolated from the ES cells. The levels of *PRL* and *IGFBP1* mRNA were analyzed by RT-PCR (**B**) and the levels of PLD1 were analyzed by Western blot analysis (**C**). Lane 1: Day 0; lane 2: after 9 days of 8-Br-cAMP treatment; lane 3: after 9 days of 8-Br-cAMP treatment and 8-Br-cAMP withdrawal for 3 days; lane 4: after 9 days of 8-Br-cAMP treatment and 8-Br-cAMP withdrawal for 6 days. **D**) ES cells were transfected transiently with either the promoterless vector (pGL3-Basic) or the PLD1 reporter plasmid (pGL3-PLD1p-1865/+9) and pCMV-β-gal. One day after transfection, the cells were treated with 8-Br-cAMP for 24 h and then cultured with or without 8-Br-cAMP for an additional 24 h. Luciferase activity was measured in triplicate after 48 h of treatment. Lane 1: Day 0; lane 2: after 1 day of 8-Br-cAMP treatment; lane 3 : after 2 days of 8-Br-cAMP treatment; lane 4: after 1 day of 8-Br-cAMP treatment and 8-Br-cAMP withdrawal for 1 day.

TTGAGCAGCTCTCT-3′). The RT-PCR products were analyzed using an ethidium bromide-containing agarose gel.

Statistical Analysis

All experiments were performed at least three times, and the data were analyzed using one-way ANOVA with statistical significance set at the level of $P < 0.05$.

RESULTS

Culture and Decidualization of Human Endometrial Stromal Cells

ES cells were separated from epithelial cells by digesting the tissue fragments with collagenase, as described above. The cells, which were spindle-shaped when plated, reached confluence after 3–6 days of culture, and retained a fibroblast-like appearance. Decidualization was then induced by incubating subconfluent cells in medium that contained 0.5 mM 8-Br-cAMP and replacing the supernatant every 3 days with fresh culture medium containing 0.5 mM 8-Br-cAMP. After growing in the presence of 0.5 mM 8-Br-cAMP for 6 days, the cells were transformed into large polygonal cells with enlarged nuclei and increased amounts of cytoplasm, resembling decidual cells in vivo (Fig. 1A). As the cells proceeded to differentiation, their borders became less distinct because of extracellular matrix formation. With further differentiation, the cells began to form aggregates and increased in size and number. These morphological features were maintained throughout the culture periods. To confirm in vitro decidualization, the levels of *PRL* and *IGFBP1* expression were determined by RT-PCR, since these genes show increased expression during decidualization [22]. As shown in Figure 1B, treatment with 8-Br-cAMP increased *PRL* and *IGFBP1* mRNA expression within 3 days, and the increased expression was maintained throughout the culture period. Therefore, these results suggest that *PRL* and *IGFBP1* can be used as decidualization markers.

Changes in PLD Activity and Expression Levels during Decidualization

To examine the role of PLD in decidualization, we first measured PLD activity after treatment of ES cells with 8-Br-cAMP to induce decidualization. As 8-Br-cAMP-induced decidualization progressed, PLD activity was increased by up to 550% (Fig. 2A). However, in the absence of 8-Br-cAMP, the morphology and PLD activity of the ES cells did not change during the same time period (data not shown). To confirm PLD activation during in vivo decidualization, we investigated the levels of PLD activity in ES cells isolated from a 5-mo-pregnant uterus, and found that the basal PLD activity in these ES cells was increased to 200% compared to ES cells isolated from a nonpregnant uterus (Fig. 2B). These results demonstrate that PLD activity is upregulated during in vivo and in vitro decidualization.

Next, we investigated whether this increase in PLD activity was due to increased biosynthesis of PLD protein. As shown in Figure 3A, the level of PLD1 protein increased gradually following 8-Br-cAMP treatment, peaking after 9 days (up to 260% higher than the control cells), whereas PLD1 expression in the absence of 8-Br-cAMP did not change during the

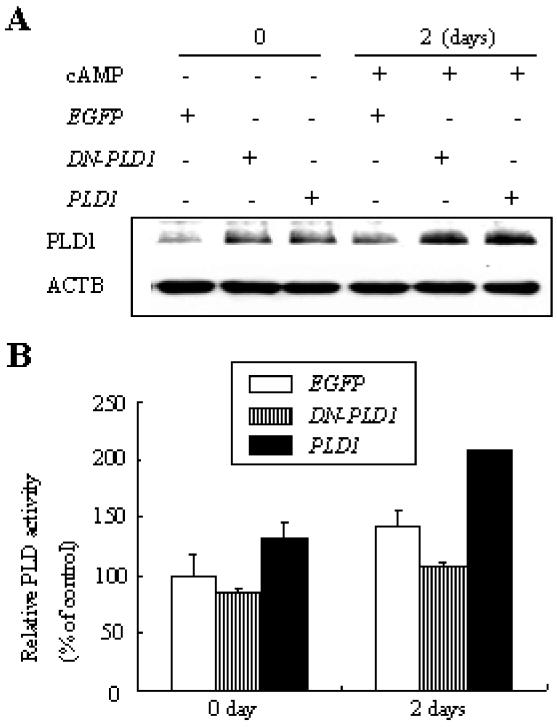

FIG. 5. Changes in PLD1 protein expression and PLD activity after transient transfection of the *EGFP, EGFP-dominant-negative (DN)-PLD1,* and *EGFP-PLD1* plasmids into ES cells. ES cells were transfected with 4 μg of *EGFP, DN-PLD1* or *PLD1* plasmid DNA. One day after transfection, the ES cells were differentiated for 2 days in the presence of 8-Br-cAMP. **A**) Protein was isolated from the ES cells, and Western blotting was performed, to analyze the PLD1 levels and to validate transfection efficiency. **B**) Transfected ES cells were labeled with [^{3}H]-palmitic acid. PLD activity was measured via [^{3}H]-PBt formation using the method described in *Materials and Methods.*

same time period (data not shown). In contrast, the level of PLD2 protein remained relatively constant during 8-Br-cAMP-induced decidualization. To confirm further the increase in PLD1 expression, we examined the effect of decidualization on PLD1 promoter activity. Thus, we transfected ES cells with the luciferase reporter gene under the control of the full-length PLD1 promoter and pCMV-β-gal gene. After 24 h, the cells were treated with 8-Br-cAMP for an additional 2 days, and luciferase was assayed. Figure 3B shows that treatment of the cells with 8-Br-cAMP markedly enhanced PLD1 promoter activity by up to 8.4 fold, which demonstrates that the increased level of PLD1 was due to the activation of the PLD1 promoter. Therefore, the observed correlation between decidualization and PLD1 upregulation in ES cells indicates that PLD1 is involved in the decidualization of ES cells.

Effects of 8-Br-cAMP Withdrawal on PLD1 Expression and Morphology

Upon withdrawal of 8-Br-cAMP stimulation, decidualized ES cells are restored to an undifferentiated phenotype and cease to express differentiation markers, such as PRL and IGFBP1 [5]. Therefore, to verify that the upregulation of PLD1 was a downstream event of 8-Br-cAMP stimulation, we withdrew 8-Br-cAMP from the cultures of fully decidualized ES cells after treatment with 8-Br-cAMP for 9 days. Microscopic examination revealed that an undifferentiated morphology appeared 5 days after 8-Br-cAMP withdrawal (Fig. 4A). In addition, RT-PCR analysis showed that the mRNA expression levels of *PRL* and *IGFBP1* decreased according to the timing of 8-Br-cAMP withdrawal (Fig. 4B). Western blot analysis revealed that PLD1 protein expression decreased concomitant with withdrawal of 8-Br-cAMP for 6 days (Fig. 4C). We also investigated whether PLD1 protein expression changed in response to 8-Br-cAMP withdrawal. As shown in Figure 4D, when ES cells were treated with 8-Br-cAMP for 2 days, PLD1 promoter activity was increased up to 8-fold compared to the control cells. However, after treatment with 8-Br-cAMP for 1 day and 8-Br-cAMP withdrawal for 1 day, PLD1 promoter activity decreased by 70%, compared to cells that were treated with 8-Br-cAMP for 2 days. This result suggests the coupling of PLD1 expression with decidualization. Taken together, these results confirm that the level of PLD1 is related to decidualization, and that PLD1 is a decidualization marker in ES cells.

Effects of DN-PLD1 *and* WT-PLD1 *Overexpression on Decidualization*

In order to confirm the effect of PLD activity on decidualization, we transfected ES cells with the dominant-negative (DN)-*PLD1* or *PLD1* expression plasmid. Thus, *DN-PLD1* and *PLD1* DNA was inserted into the EGFP vector, and the recombinant plasmid was electroporated into ES cells. As shown in Figure 5A, ES cells were successfully transfected with *DN-PLD1* or *PLD1* by electroporation. Furthermore, the overexpression of exogenous *PLD1* increased the basal PLD activity 1 day after transfection, whereas the overexpression of *DN-PLD1* decreased PLD activity (Fig. 5B). During 8-Br-cAMP-induced decidualization, the PLD activity of the *PLD1*-overexpressing cells was significantly higher than that of the EGFP-transfected cells. However, the PLD activity of the *DN-PLD1*-transfected cells decreased to the basal level.

Next, we examined the transfected cells under a fluorescence microscope, to examine the effect of gene transfection on cell morphology. The transfected cells were easily visible under the fluorescence microscope owing to their green color. One day after transfection, the control and *DN-PLD1* transfectants showed very few cells with morphological changes, whereas 23% of the *PLD1*-transfected cells exhibited altered morphology (Fig. 6, A and B), which suggests that PLD activity induces morphological changes. Subsequently, the transfected cells were cultured with or without 0.5 mM 8-Br-cAMP. As shown in Figure 6B, the percentage of cells with the decidualized morphology significantly increased by 72% after treatment of the control cells with 8-Br-cAMP for 9 days, whereas only 23.8% of the cells changed in the absence of 8-Br-cAMP treatment. On the other hand, the *DN-PLD1* transfectants showed very few morphological changes in the presence of 8-Br-cAMP for 9 days, and only 20% of the cells were transformed into the decidualized morphology. Accordingly, these results indicate that morphological change is inhibited by the blockage of PLD1 activation. When we cultured the *PLD1*-transfected cells in the presence of 8-Br-cAMP, they were rapidly transformed into the decidualized morphology (Fig. 6B). Treatment with 8-Br-cAMP for 3 days changed 72% of the *PLD1*-transfected cells into the decidualized morphology, whereas only 31% of the control cells were altered. This morphological change was markedly potentiated by *PLD1* overexpression, which increased up to 87% with 8-Br-cAMP treatment for 9 days (Fig. 6B). Moreover, 73% of the *PLD1*-transfected cells were transformed into large polygonal cells in the absence of 8-Br-cAMP for 9 days. In order to ascertain further the effect of PLD

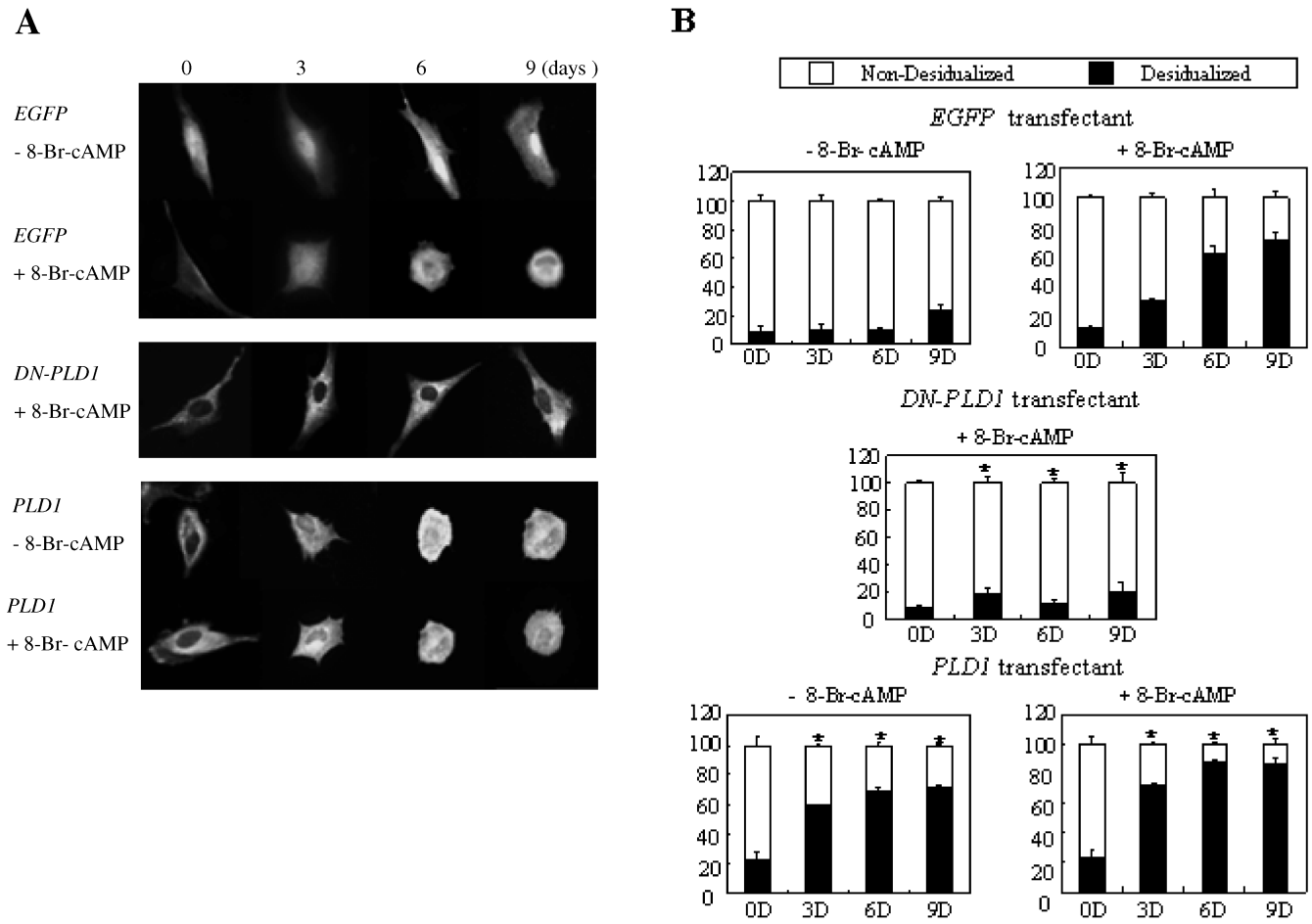

FIG. 6. Changes in morphology after transient transfection of *EGFP, EGFP-DN-PLD1,* and *EGFP-PLD1* plasmids into ES cells. **A**) Comparison of the morphologies of the *EGFP* vector-, *EGFP-DN PLD1*-, and *EGFP-PLD1*-transfected cells. One day after transfection, ES cells were differentiated by 8-Br-cAMP treatment for the indicated period of time. Transfected cells are defined by their green color under the fluorescence microscope after immunostaining with EGFP (original magnification ×400). **B**) The percentage of transfected cells with decidualized morphology. EGFP-positive cells from randomly selected areas of at least five cultures from three independent experiments were photographed.

activity on decidualization, we determined the expression levels of *PRL* and *IGFBP1*, which are known decidualization markers. One day after transfection of the cells with siRNA directed against PLD1 or with *PLD1*, the cells were treated with 8-Br-cAMP for 2 days, and the *PRL* and *IGFBP1* mRNA levels were then examined by RT-PCR. As shown in Figure 7, A and B, transfection with PLD1 siRNA decreased *PRL* and *IGFBP1* expression, i.e., transfection with PLD1 siRNA(1) decreased *PRL* expression by 47% and *IGFBP1* expression by 44%, and transfection with PLD1 siRNA(2) decreased *PRL* by 46% and *IGFBP1* expression by 44%. However, transfection with PLD1 upregulated *PRL* expression by 212% and *IGFBP1* expression by 210% (Fig. 7, C and D). Moreover, pretreatment for 18 h with 0.3% 1-butanol, which is a PLD inhibitor, completely abolished *PRL* and *IGFBP1* expression, whereas pretreatment with 0.3% 2-butanol for 18 h did not induce any change (Fig. 7E). These results suggest that PLD1 upregulation is important for 8-Br-cAMP-induced decidualization.

Effects of Treatment with PA and Pretreatment with Mepacrine or Propranolol on Decidualization

To confirm the role of PLD1 in decidualization, we treated the cells with phosphatidic acid (PA), which is one of the enzymatic products of PLD. When treated with the indicated concentration of PA for 3 days, both *PRL* expression (Fig. 8A) and morphological changes (Fig. 8B) were induced, which implies that PA produced by PLD activation induces decidualization. PLD-induced PA is usually degraded to DAG by phosphatidic acid phosphatase (PPAP2A) or to LPA/AA by PLA2G1B [10]. In order to determine how PA regulates decidualization, we pretreated the cells with propranolol, PPAP2A inhibitor, or mepacrine, PLA2G1B inhibitor for 1 h. As shown in Figure 8, C and D, pretreatment with propranolol for 1 h had no effect on *PRL* and *IGFBP1* expression or morphological change. However, pretreatment with mepacrine for 1 h decreased *PRL* and *IGFBP1* expression as well as morphological change, which suggests that PLD1 induces decidualization through PLA2G1B.

DISCUSSION

Decidualization is a process during which endometrial cells undergo dramatic changes in their biochemical properties and morphological appearance. However, intracellular signaling pathways leading to decidualization are still far from clear. In the present study, we tried to determine the contribution of PLD to 8-Br-cAMP-induced decidualization.

The significance of PLD activity in cell maturation and differentiation has been suggested, although the role of PLD in

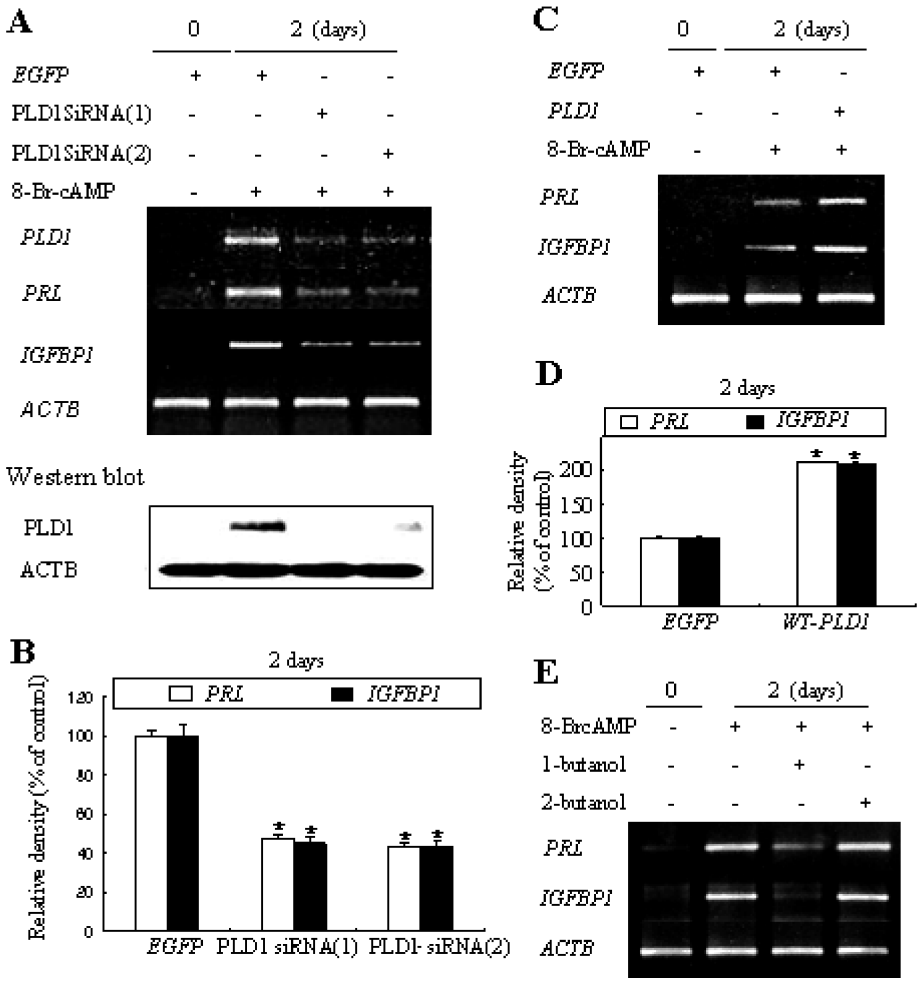

FIG. 7. Effects of transfection of PLD1 siRNA and *PLD1*, as well as pretreatment with 0.3% 1-butanol on *PRL*/*IGFBP1* mRNA expression. ES cells were transfected with 4 μg each of PLD1 siRNA (**A**) or *PLD1* plasmid DNA vector (**C**). One day after transfection, the ES cells were differentiated with 8-Br-cAMP for 2 days, and RNA and protein were isolated. **B**, **D**) Band intensities were quantified using the QuantityOne software (Bio-Rad). **E**) ES cells were pretreated with 0.3% 1-butanol or 2-butanol for 18 h, and then differentiated with 8-Br-cAMP for 3 days.

the differentiation processes remains speculative. In the present study, the levels of PLD activity as well as the expression levels of PLD1 increased in ES cells as 8-Br-cAMP-induced decidualization progressed. Moreover, the PLD activity of ES cells from 5-mo-pregnant deciduas was upregulated about 2-fold compared to that in ES cells from nonpregnant deciduas, which indicates that PLD activity is upregulated during in vivo decidualization. Recently, Brar et al. [23] observed by microarray analysis that *PLD1* was induced 3.6-fold during decidualization, which supports our finding that *PLD1* mRNA was increased under decidualization conditions. Based on the following experimental data, we propose that PLD activation plays a pivotal role in decidualization. First, withdrawal of 8-Br-cAMP from the culture medium after 9 days decreased PLD1 promoter activity and PLD1 protein expression, and this was accompanied by a morphological change whereby the original cell morphology was restored. Second, *DN-PLD1* transfection into ES cells almost completely inhibited the appearance of the decidualized morphology induced by 8-Br-cAMP. However, overexpression of *PLD1* induced rapid change in the cells to the decidualized morphology in the absence of 8-Br-cAMP. Third, PLD1 blockage by PLD1 siRNA and 0.3% 1-butanol decreased the *PRL* and *IGFBP1* mRNA expression levels, whereas *PLD1* overexpression increased these levels. Fourth, PA, which is an enzymatic product by PLD, induced morphological change and *PRL* expression. Taken together, our results strongly support the notion that PLD1 activation and increased PLD1 expression are correlated with decidualization.

Multiple lines of evidence suggest that a PKA signal transduction pathway mediates the action of cAMP to induce decidualization [8, 9, 24–26]. The cAMP signal to the nucleus is transduced almost exclusively by the binding of cAMP to the R subunit of PKA and subsequent phosphorylation of nuclear target proteins by released and translocated C subunits [27]. During decidualization, elevated levels of cellular cAMP lead to elevated kinase activity and decreased R/C subunit ratio for PKA. In addition, the endometrial PKA system does not desensitize to the chronic stimulus. Therefore, the cAMP/PKA pathway may contribute to decidualization-induced gene expression. Brar et al. [23] have used by microarrays to identify 281 genes that are dynamically regulated in decidual ES cells during in vitro decidualization that is induced by cAMP. They have also determined that cAMP induces several genes that are involved in the phosphoinositide signal transduction pathway (including *PLD1*) during decidualization, and they suggest that PLD1 plays a role in decidualization. In the present study, treatment of ES cells with 8-Br-cAMP upregulated PLD1 expression and PLD activity during decidualization, and treatment with PA for 3 days induced *PRL* expression and morphological change without 8-Br-cAMP treatment. Moreover, PLA2G1B blockage by pretreatment with mepacrine resulted in decreased *PRL* and *IGFBP1* expression and morphological change. These data suggest that PA produced by PLD1 activation induces decidualization through PLA2G1B. PLA2G1B hydrolyzes the ester bonds of fatty acids at the sn-2 position of phospholipids, producing free fatty acids and lysophospholipids [28]. When the fatty acid is

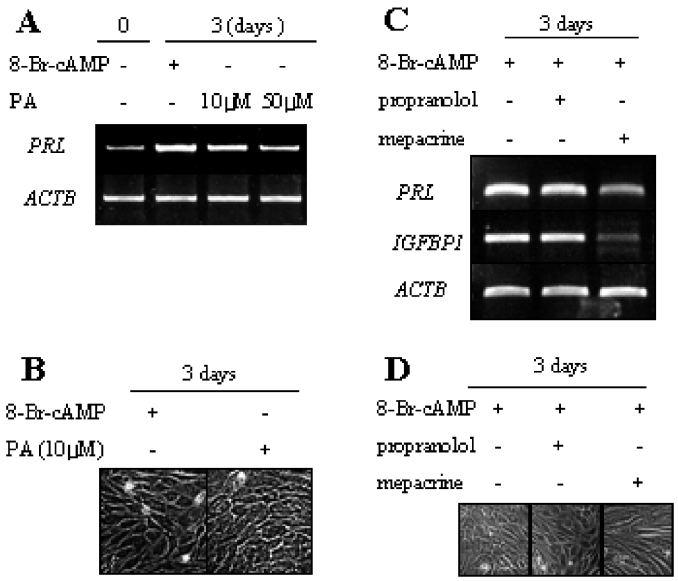

FIG. 8. Effects of treatment with PA and pretreatment with propranolol or mepacrine on decidualization. **A**) ES cells were treated with the indicated concentrations of PA in the absence of 8-Br-cAMP for 3 days, and RNA was isolated from the cells and analyzed for *PRL* mRNA by RT-PCR. **B**) The cells were photographed under a microscope (original magnification ×200). ES cells were pretreated with propranolol or mepacrine for 1 h and then treated with 8-Br-cAMP for 3 days. **C**) RNA was isolated from the ES cells and analyzed for *PRL* and *IGFBP1* mRNAs by RT-PCR. **D**) ES cells were photographed under a microscope (original magnification ×200).

arachidonic acid, prostaglandin is generated, which acts as a multiple intracellular effector during decidualization. Kurusu et al. [29] have reported that arachidonyl trifluoromethyl ketone (ATK), which is a PLA2G1B inhibitor, causes dose-dependent inhibition of decidualization, and that PGE2 and prostacyclin have a weak effect in restoring ATK-inhibited decidualization. Therefore, they suggest that PLA2G1B induces decidualization through the generation of bioactive lipid mediators, such as prostaglandin (PG.). The importance of PGs in reproduction is evidenced by the multiple reproductive failures in mice when PG synthase 2 (PTGS2; COX-2) genes are disrupted [30]. Therefore, we suggest that PLD1 plays a role in the supply of bioactive lipid mediators, such as PG through PLA2G1B.

Rearrangement of the cytoskeleton is also important for morphological change during decidualization. Reorganization of stress fibers near the cell periphery has been reported in human ES cell that express the temperature-sensitive simian virus 40 large T antigen [31] and in a mouse decidualization model in vitro [32]. Furthermore, changes in the actin-based cytoskeleton and loss of focal adhesions within the decidualization-associated multicellular nodules have been reported [5]. Therefore, it is of a great interest to study whether the process of decidualization involves a protein that potentially regulates the focal adhesion assembly and organization of the actin cytoskeleton. Based on reports that PLD-dependent cytoskeletal rearrangements may play a pivotal role, it is tempting to suggest that PLD affects the actin cytoskeleton during decidualization. Iyer and Kusner [33] have reported that PLD activity is associated with the detergent-insoluble cytoskeleton. In support of this finding, Cross et al. have shown that actin stress fiber formation and PA accumulation in aortic endothelial cells can be inhibited by 1-butanol but not by 2-butanol [34]. In addition, Kam and Exton [35] have implicated PLD in the cross-linking of actin filaments mediated by α-actinin. In agreement with this hypothesis, we observed that PLD upregulation induced morphological change in a PLD1-dependent manner, as shown by the selective inhibition conferred by *DN-PLD1* transfection. Given the above considerations, it is highly likely that PLD1 is an essential factor for decidualization through its involvement in the reorganization of the cytoskeleton.

In conclusion, we have shown that PLD1 activity is increased during decidualization, and that PLD1 activation regulates the decidualization-specific phenotype through PLA2G1B. These characteristics suggest that PLD1 is one of the important regulators during decidualization, and that its activity affects successful implantation. Further studies to elucidate the signaling events that lead to PLD expression and eventual decidualization signaling events will broaden our understanding of the molecular mechanisms underlying decidualization and implantation.

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