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Involvement of Phosphatidate Phosphohydrolase in Arachidonic Acid Mobilization in Human Amnionic WISH Cells*

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Prostaglandins are known to play a central role in the initiation of labor in humans, and amnionic cells constitute a major source of these compounds. Prostaglandin synthesis and release by amnion cells in response to hormones and ligands takes place after a characteristic 4–5 h lag. However, we report herein that free arachidonic acid (AA), the metabolic precursor of prostaglandins, can be induced at much shorter times (1 h) in human amnionic WISH cells by phorbol 12-myristate 13-acetate (PMA) through activation of protein kinase Cα (PKCα). WISH cells were found to possess both cytosolic group IV phospholipase A_2 (cPLA$_2$) and Group VI Ca^{2+}-independent phospholipase A_2 (iPLA$_2$). Of these, the cPLA$_2$ was found to be the likely mediator of AA mobilization in PMA-activated WISH cells. PMA also activates phospholipase D (PLD) in these cells and ethanol, a compound that inhibits PLD-mediated phosphatidic acid (PA) formation, blocked AA release. Moreover, prevention of PA dephosphorylation by the PA phosphohydrolase inhibitors propranolol and bromoenol lactone, resulted in inhibition of AA release by PMA-treated WISH cells. Collectively, these data suggest that activation of cPLA$_2$ and attendant AA release by phorbol esters in WISH cells requires prior generation of DAG by phosphatidate phosphohydrolase.

Phospholipase A_2 (PLA$_2$)[1] constitutes a key regulatory step in the production of prostaglandins (PGs) because it catalyzes the release of arachidonic acid (AA) from the sn-2 position of phospholipids, making the fatty acid accessible to PG synthases. At present, ten different PLA$_2$ groups have been identified (1–3). Those include five groups of small secreted PLA$_2$s, which show millimolar requirements for Ca^{2+} (Groups I, II, III, V, and X), and two groups of intracellular, high molecular weight enzymes (Groups IV and VI). Group IV PLA$_2$, or cPLA$_2$, is Ca^{2+}-dependent and a highly regulated enzyme (4); whereas, Group VI PLA$_2$, or iPLA$_2$, is Ca^{2+}-independent (5). At present it is not known whether Group VI iPLA$_2$ is subjected to post-translational regulation (5). Among these PLA$_2$s, Groups II, V, and IV have been shown to be the responsible enzymes for prostaglandin generation in different systems (6–8). On the other hand, Group VI PLA$_2$ has been implicated in basal fatty acid remodeling reactions (5, 9).

PGs, especially PGE_2 and $PGF_{2\alpha}$, are thought to play a central role in the initiation of spantaneous labor in humans by mediating physiological effects such as uterine contractions (10) and cervical softening and effacement (11). The human amnion has the capacity of producing PGE_2, and it is known that changes in this capacity occur in association with parturition (12). Thus, numerous studies have focused on PG production by amnionic cells, mostly at the level of PG synthase enzymes (13–15). Surprisingly however, the study of PLA$_2$ in amnion cells has received much less attention. Myatt and coworkers (16, 17) recently documented the enhancement of cPLA$_2$ protein by interleukin (IL)-1β in amnionic WISH cells after an 8-h treatment, which correlates with PGE_2 production under those conditions. These studies were conducted at late stages of activation (several hours). Unfortunately, no information is available on the events that occur immediately after amnionic cell activation (*i.e.* up to 1 h). In the current study, we have investigated the signaling mechanisms that operate at the early stages of WISH cell activation and lead to increased PLA$_2$ activity and concomitant AA release.

MATERIALS AND METHODS

Materials—Human WISH cells (established amnion cell line) were obtained from the American Type Culture Collection (Rockville, MD). Iscove's modified Dulbecco's medium (endotoxin <0.05 ng/ml) was from BioWhittaker (Walkersville, MD). Fetal bovine serum was from Hyclone Labs. (Logan, UT). Trypsin/EDTA solution was purchased from Irvine Scientific (Santa Ana, CA). (5,6,8,9,11,12,1,15-^{3}H)Arachidonic acid (specific activity 100 Ci/mmol), (9,10-^{3}H)palmitic acid (specific activity 43.3 Ci/mmol), and 1-palmitoyl-2-[^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine (specific activity 55 mCi/mmol) were obtained from NEN Life Science Products (Boston, MA). 1-Palmitoyl-2-[^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine (specific activity 59 mCi/mmol). Bromoenol lactone (BEL) was from Biomol (Plymouth Meeting, PA). Group VI iPLA$_2$ antiserum was generously provided by Dr. Simon Jones (Genetics Institute, Cambridge, MA). Group IV cPLA$_2$ antibodies were kindly provided by Dr. Ruth Kramer (Lilly Research Laboratories, Indianapolis, IN). The sPLA$_2$ inhibitor LY311727 was kindly provided by Dr. Edward Mihelich (Lilly Research Laboratories). Rabbit polyclonal anti-ERK-2 that recognizes p42 and p44 MAPKs were a generous gift from Dr. Alan Saltiel (Parke-Davis, Ann Arbor, MI). Methyl arachidonyl fluorophosphonate (MAFP) was from Cayman (Ann Arbor, MI). Antibodies against PKCα, PKCβ, and PKCϵ and the polyconal anti-Raf-1 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against PKCγ, PKCδ, and PKCζ were purchased from Calbiochem.

Cell Culture—WISH cells (18) were maintained in Iscove's modified Dulbecco's medium suplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere at 90% air and 10% CO_2. The cells were subcultured twice weekly by trypsinization and, when used for experiments, were seeded into 24-well (2×10^5 cells/well, NUNC) or 12-well plates (5×10^5 cells/well, Corning Inc.), or 100 × 20-mm dishes (2.5×10^6 cells/dish,

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[1] The abbreviations used are: PLA$_2$, phospholipase A_2; AA, arachidonic acid; BEL, bromoenol lactone; cPLA$_2$, cytosolic PLA$_2$; DAG, diacylglycerol; iPLA$_2$, Ca^{2+}-independent PLA$_2$; MAFP, methyl arachidonyl fluorophosphonate; MAPK, mitogen-activated protein kinase; PA, phosphatidic acid; PAP, phosphatidate phosphohydrolase; PKC, protein kinase C; PLD, phospholipase D; PG, prostaglandin; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride; PEt, phosphatidylethanol.

Falcon). After a 2-day growth, the cells, at 90% confluency, were rinsed with serum-free medium and incubated for 1–2 h before stimulation.

PLA_2 Assays—Ca^{2+}-dependent PLA_2 assay was conducted as described by Kramer *et al.* (20), with slight modifications. Briefly, aliquots of WISH cell homogenates were incubated for 30 min at 37 °C in 2 mM $CaCl_2$, 50 mM Hepes, pH 7.4, and sonicated liposomes consisting of 2 μM 1-palmitoyl-2-[^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine and 1 μM 1,2-dioleoyl-*sn*-glycerol. Products were analyzed by thin-layer chromatography using the system *n*-hexane/diethyl ether/acetic acid (70:30:1). To measure Ca^{2+}-independent PLA_2 activity, aliquots of WISH cell homogenates were incubated for 30 min at 37 °C in 100 mM Hepes, pH 7.5, 5 mM EDTA, 0.8 mM ATP, 400 μM Triton X-100, 100 mM 1-palmitoyl-2-[^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine, in a final volume of 500 μl. The substrate was used in the form of mixed micelles of Triton X-100/phospholipid at a molar ratio 4:1, obtained by a combination of heating, vortex mixing, and water bath sonication (21). Products were analyzed by thin-layer chtomatography using the same system described above.

[^{3}H]AA Release—Radiolabeling of the cells with [^{3}H]AA was achieved by including 0.5 μCi [^{3}H]/10^6 cells in the culture medium 20 h before stimulation. Cells were stimulated with PMA (25–50 ng/ml) for different periods of time in the presence of 1 mg/ml bovine serum albumin (fatty acid-free). The supernatants were removed and cleared of detached cells by centrifugation, and radioactivity was counted by liquid scintillation. When inhibitors were used, they were added to the cells 30 min before PMA was added to the medium.

PKC Activity—A Promega kit (PKC assay system, V5910) was used for this purpose, and the manufacturer instructions were followed. Briefly, the cells were washed with phosphate-buffered saline, resuspended in 0.5 ml of extraction buffer (25 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 0.5 mM PMSF) at 4 °C, and homogenized using a Dounce homogenizer. Lysates were centrifuged in a microcentrifuge for 5 min at 4 °C, and supernatants were passed through a 1-ml column of DEAE cellulose pre-equilibrated with the extraction buffer. PKC was extracted by using the extraction buffer plus 200 mM NaCl. PKC activity was then measured with a biotinylated peptide substrate of PKC that binds to Streptavidin-disks. PKC was assayed in 25 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 0.3 mg/ml phosphatidylserine, 30 μg/ml DAG, 25 μM EGTA, 400 μM $CaCl_2$, and [γ-^{32}P]ATP (5000 pmol, 100–200 cpm/pmol). Reactions were run with and without phospholipids and stopped by adding 7.5 M guanidine-HCl. Aliquots from the reactions were spotted in streptavidin-disks and washed with 1 M NaCl, and radioactivity was quantified by scintillation counting.

PKC Translocation Assays—Experiments were carried out as described elsewhere (20). Briefly, cells were plated in 100-mm dishes. Control and PMA-stimulated WISH cells were washed with phosphate-buffered saline and homogenized with a Dounce homogenizer in a buffer consisting of 20 mM Tris-HCl, 2 mM EDTA, 10 mM EGTA, 1 mM PMSF, 20 μM leupeptin, 20 μM aprotinin, and 0.1% β-mercaptoethanol, pH 7.5. Homogenates were centrifuged at 500 × *g* for 5 min at 4 °C. The resulting supernatant was centrifuged at 100,000 × *g* for 1 h at 4 °C to separate soluble and membrane fractions. Membranes were washed with buffer, resuspended, and sonicated. After protein quantification, 100 μg were separated by SDS-polyacrylamide gel electrophoresis (10% gel) and transferred to Immobilon-P membrane (Millipore). Western blotting analysis was performed by using specific antibodies against PKC isoforms.

PLD Activation—The cells were labeled with [^{3}H]palmitic acid (3 μCi/10^6 cells) for 20 h, and the stimulations were carried out in the presence of 1% ethanol. At the end of the reactions, total lipids were extracted (21, 22) and phosphatidylethanol (PEt), a specific product of PLD activity, was resolved from cellular lipids by thin-layer chromatography on silica-gel G plates (Whatman), using the upper phase of a system consisting of ethyl acetate/isooctane/acetic acid/water (13:2:3:10, v/v/v/v). The lipids were identified by comparison with authentic standards run in the same plate and visualized by iodine vapors. Radioactivity was determined by liquid scintillation counting.

DAG Production—Cells were labeled overnight with either [^{3}H]palmitic acid (3 μCi/10^6 cells) or [^{3}H]arachidonic acid (0.5 μCi/10^6 cells), washed, and were incubated with inhibitors for 30 min prior to stimulation with 25–50 ng/ml PMA. At the indicated times, supernatants were removed, cell monolayers were scraped, and total lipids were extracted (22). For separation of DAG, lipids were separated by thin-layer chromatography with *n*-hexane/diethyl ether/water (70:30:1, v/v/v). The plates were run twice in this system if monoacylglycerol determination was required as well. Radioactivity in DAG and monoacylglycerol was determined by liquid scintillation counting.

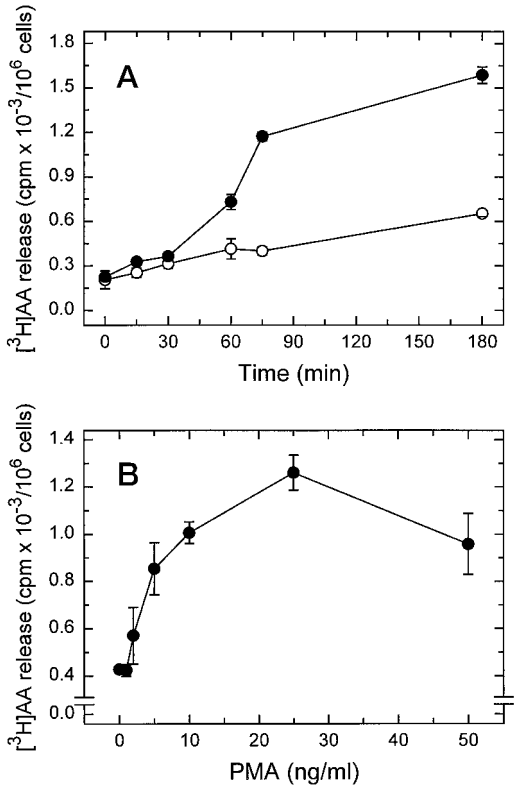

FIG. 1. **AA release by PMA in WISH cells.** [^{3}H]AA-labeled cells were stimulated by PMA (*closed circles*) or vehicle (*open circles*). Supernatants were taken at different time points, and radioactivity was quantified by liquid scintillation counting. *A*, time course (25 ng/ml PMA); *B*, dose-response (75 min). Data are the results from three separate experiments, each carried out in triplicate. Values are shown as means ± S.E.

MAPK and PLA_2 Immunoblotting Studies—Cells were serum-starved for 24 h, preincubated with 100 μM propranolol for 30 min, and stimulated with 25–50 ng/ml PMA for 1 h. Cells were washed and then lysed in a buffer consisting of 1 mM Hepes, 0.5% Triton X-100, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin at 4 °C. Protein was quantified, and a 100-μg aliquot was analyzed by Western blot under conditions previously described (23), with antibodies against ERK-2 that recognizes both p42 and p44 MAPKs or against PLA_2 isoforms.

RESULTS

Phospholipase A_2 Activation in Human WISH Cells—One of the best established systems for the study of lipid mediators in amnionic cells is the human-derived cell line WISH (24). This cell line was established from altered colonies appearing in a subculture of a primary monolayer of amnion cells (18, 19). WISH cells produce large amounts of PGs after prolonged exposure to phorbol esters (18 h) (13, 20, 25). To characterize the steps in the regulation of PG production that occur during the early stages of WISH cell activation, we measured [^{3}H]AA release in these cells after incubation with 50 ng/ml PMA for different time periods (Fig. 1*A*). After a time lag of approximately 30 min, significant release of [^{3}H]AA was observed at 60 min, reaching a plateau at about 75 min. Typically, a 2–5-fold increase over basal unstimulated release was detected at an optimal PMA concentration of 25 ng/ml (Fig. 1*B*).

As a first approach, *in vitro* measurements of PLA_2 activity in homogenates from stimulated *versus* unstimulated cells were conducted to identify the phospholipase A_2 involved in PMA-stimulated AA release. As shown in Fig. 2*A*, WISH cell homogenates exhibited both Ca^{2+}-dependent and -independent PLA_2 activity. Interestingly, the Ca^{2+}-dependent PLA_2 activity was increased by a little bit less than 2-fold in homogenates

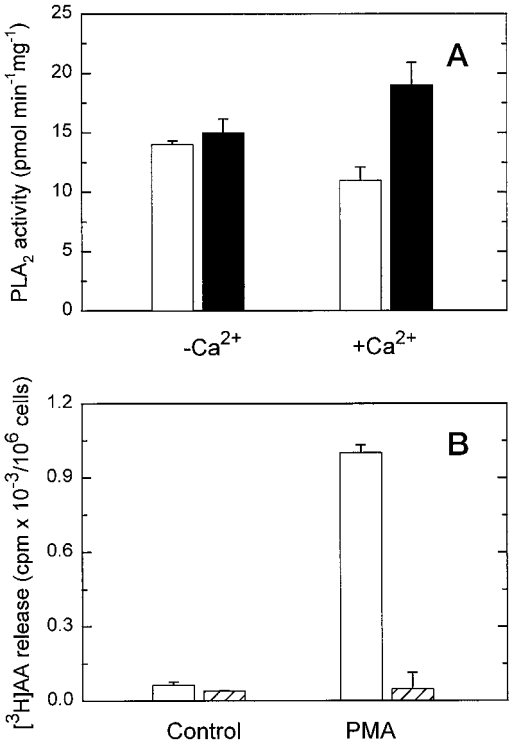

FIG. 2. **AA release in WISH cells.** *A*, PLA_2 activity in homogenates from untreated (*open bars*) or PMA-treated (*solid bars*) WISH cells. Homogenates were prepared as described in the text, and PLA_2 activity was measured in the absence or presence of calcium in the assay mix as indicated. *B*, effect of EGTA on AA release. [^{3}H]AA-labeled cells were either not stimulated (*Control*) or stimulated with 25 ng/ml of PMA in the absence (*open bars*) or presence (*stripped bars*) of 2 mM EGTA in the incubation medium. Experiments were performed in triplicate, and the data are shown as means ± S.E.

from PMA-treated cells as compared with control unstimulated cell homogenates; whereas, Ca^{2+}-independent PLA_2 activity did not change (Fig. 2*A*). These data suggest that the PLA_2 mediating PMA-induced AA mobilization is Ca^{2+}-dependent. In keeping with these data, PMA-induced AA release by PMA was inhibited by the presence of 2 mM EGTA in the incubation medium, (Fig. 2*B*), demonstrating that Ca^{2+} is an important regulatory element in this system. We did not detect any increase in $iPLA_2$ protein content in WISH cells or in the membrane fraction after 1 h of treatment with PMA (data not shown).

There are two types of Ca^{2+}-dependent PLA_2 in mammalian cells, *i.e.* the secretory enzymes ($sPLA_2$s) and the cytosolic Group IV PLA_2 ($cPLA_2$). An easy method for distinguishing them is to use specific inhibitors for each of these enzymes, as we have done previously in macrophages (26). Thus the effect of MAFP and LY311727, specific inhibitors of both $cPLA_2$ and $sPLA_2$, respectively, on PMA-stimulated AA release was analyzed. As shown in Fig. 3, MAFP strongly inhibited PMA-induced AA release, suggesting involvement of the $cPLA_2$. On the other hand, LY311727, at concentrations up to 50 μM, was totally unable to affect the response (data not shown), ruling out a role for $sPLA_2$ in PMA-induced AA release in WISH cells. Consistent with the latter, using reverse transcriptase-polymerase chain reaction, we have not detected significant levels of mRNA for either Group II or Group V $sPLA_2$s in WISH cells, whether resting or treated with PMA.[2] However, both the Group IV $cPLA_2$ and the Group VI $iPLA_2$ were easily detectable by immunoblot (see below).

[2] B. Johansen and E. A. Dennis, unpublished data.

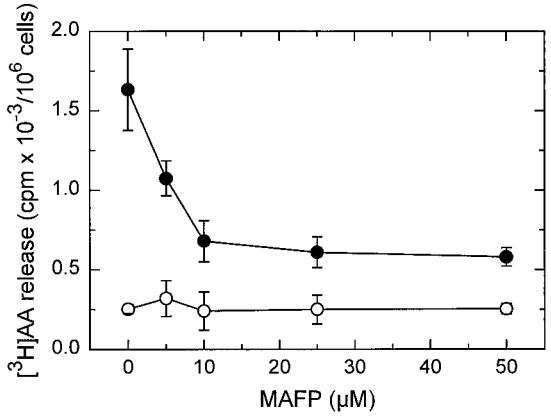

FIG. 3. **Effect of MAFP on AA release.** The cells were pretreated with different concentrations of MAFP for 30 min and then stimulated with 25 ng/ml PMA (*open circles*) or vehicle (*closed circles*) for 75 min. Radioactivity in supernatants was quantified by liquid scintillation counting. Experiments were performed in triplicate and the data are shown as means ± S.E.

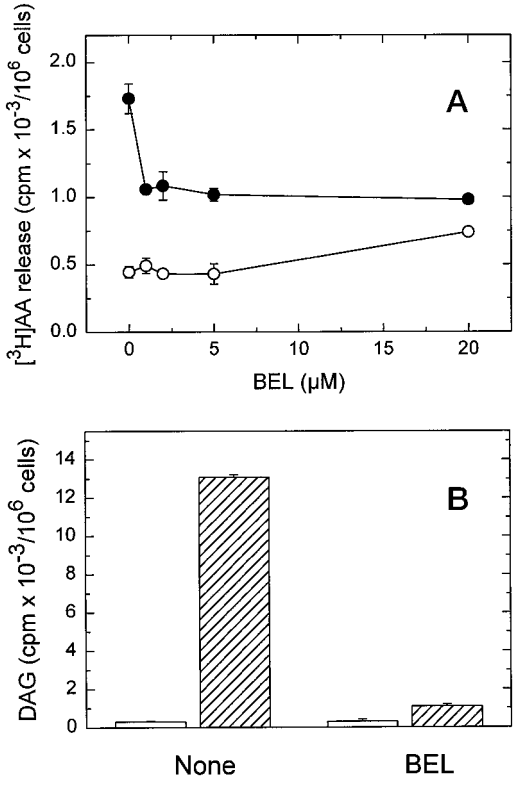

FIG. 4. *A*, effect of BEL on AA release. *B*, effect of BEL (5 μM) on DAG levels. The cells were pretreated with different concentrations of BEL for 30 min and then stimulated with 25 ng/ml PMA (*filled bars*) or vehicle (*white circles*) for 75 min. Free AA and DAG were quantified as described under "Materials and Methods." AA release was measured in cells prelabeled with [^{3}H]AA. DAG was measured in cells prelabeled with [^{3}H]palmitic acid.

PAP Is Involved in PMA-induced AA Release in WISH Cells—The effect of BEL on PMA-induced AA release was examined, and the results are shown in Fig. 4*A*. BEL was previously identified as a potent $iPLA_2$ inhibitor (5), but more recent results have demonstrated its lack of specificity for $iPLA_2$ in cells, as BEL also potently inhibits another key enzyme in lipid metabolism, *i.e.* the Mg^{2+}-dependent PA phosphohydrolase (PAP) (27). In fact, the inhibitory effect of BEL on PMA-induced AA release shown in Fig. 4*A* cannot be attributed to $iPLA_2$ inhibition on the basis of the results presented in Fig.

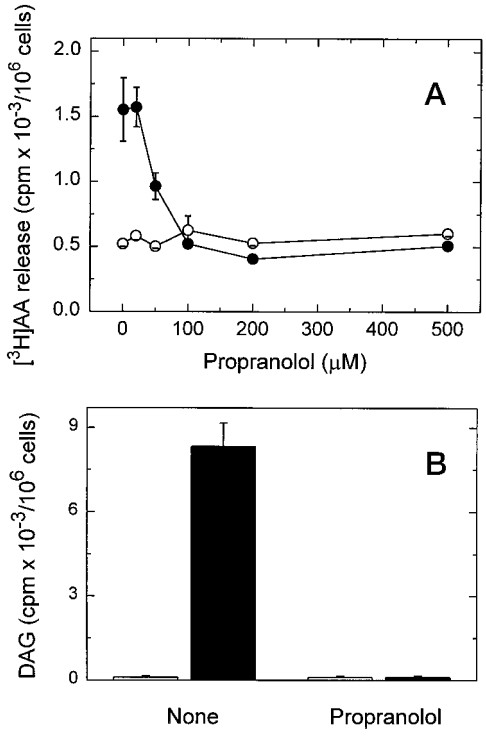

FIG. 5. *A*, effect of propranolol on AA release. *B*, effect of propranolol (100 μM) on DAG levels. The cells were pretreated with different concentrations of propranolol for 30 min and then stimulated with 25 ng/ml PMA (*closed circles*) or vehicle (*open circles*) for 75 min. Free AA and DAG were quantified as described under "Materials and Methods." AA release was measured in cells prelabeled with [^{3}H]AA. DAG was measured in cells prelabeled with [^{3}H]palmitic acid.

2*A*, which show that the iPLA$_2$ activity does not change upon PMA treatment while the cPLA$_2$ activity does. Moreover, BEL inhibited the PMA-induced DAG production in cells labeled with [^{3}H]palmitic acid (Fig. 4*B*), indicating that BEL is indeed inhibiting the PAP. Thus, the possibility arises that the BEL effect on AA release is due to PAP inhibition. To investigate this possibility, we employed propranolol, a well established PAP inhibitor. Analogous to BEL, propranolol appreciably inhibited the PMA-induced [^{3}H]AA release (Fig. 5*A*) and [^{3}H]palmitate-labeled DAG production (Fig. 5*B*).

Phospholipase D Involvement in PMA-induced AA Mobilization—One major route for the production of the PA to be used by PAP is the PLD-mediated hydrolysis of phospholipids (28). Fig. 6*A* shows that, in the presence of ethanol, PMA induced the time-dependent accumulation of PEt in WISH cells, reflecting PLD activation. PEt is a specific product of PLD action in the presence of ethanol. Accumulation of PEt was detected at much earlier time points than AA release (*i.e.* 15 min), suggesting the possibility that products of PLD may be implicated in cPLA$_2$ activation and attendant AA release. Should this be the case, one would expect that addition of exogenous PLD to the WISH cells would mimic the activating effect of PMA on AA release. Fig. 6, *B* and *C*, shows that treatment of the WISH cells with exogenous PLD produced a time- and dose-dependent release of [^{3}H]AA. PLD activation by PMA was unaffected by BEL, confirming that PLD is upstream of the BEL-sensitive step, *i.e.* the PAP (data not shown).

A third, indirect strategy to *inhibit* the effect of the PAP activity is to use ethanol. By forming PEt instead of PA via phospholipase D, this alcohol depletes the substrate for PAP, thereby impairing DAG production by this route. The overall effect is thus the same as if PAP was directly inhibited. Consistent with the data with BEL and propranolol, ethanol induced a dose-dependent decrease in the PMA-induced AA release (Fig. 7). Collectively, the use of three distinct approaches to inhibit PAP activity have yielded the same result, thus

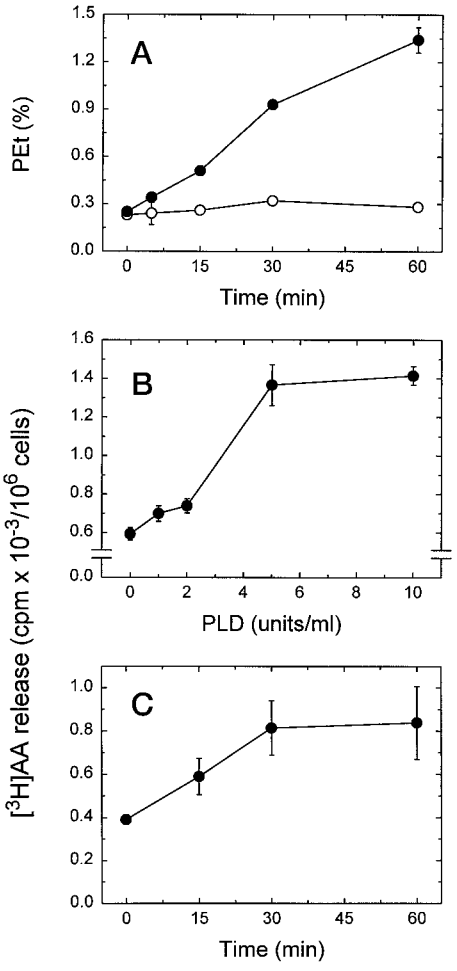

FIG. 6. **PLD activation in WISH cells.** *A*, [^{3}H]palmitic acid-labeled cells were stimulated with 25 ng/ml PMA (*closed circles*) or vehicle (*open circles*) in the presence of 1% ethanol for different periods of time. Lipids were extracted and separated by thin-layer chromatography, and radioactivity present in PEt was quantified by liquid scintillation counting. Data are given as percentage of radioactivity in PEt with respect to total phospholipid radioactivity. *B* and *D*, effect of purified PLD on AA release in WISH cells. [^{3}H]AA-labeled cells were treated with different concentrations of *Streptomyces chromofuscus* purified PLD for 45 min (*B*) or with 5 units/ml PLD for different periods of time (*C*). Supernatants were taken, and radioactivity was quantified. Experiments were carried out in triplicate, and data are shown as means ± S.E.

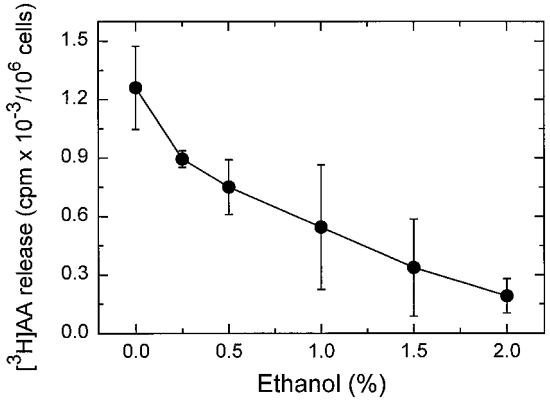

FIG. 7. **Effect of ethanol on AA release.** The cells were stimulated in the presence of the indicated ethanol concentrations, and AA was quantitated as described under "Materials and Methods."

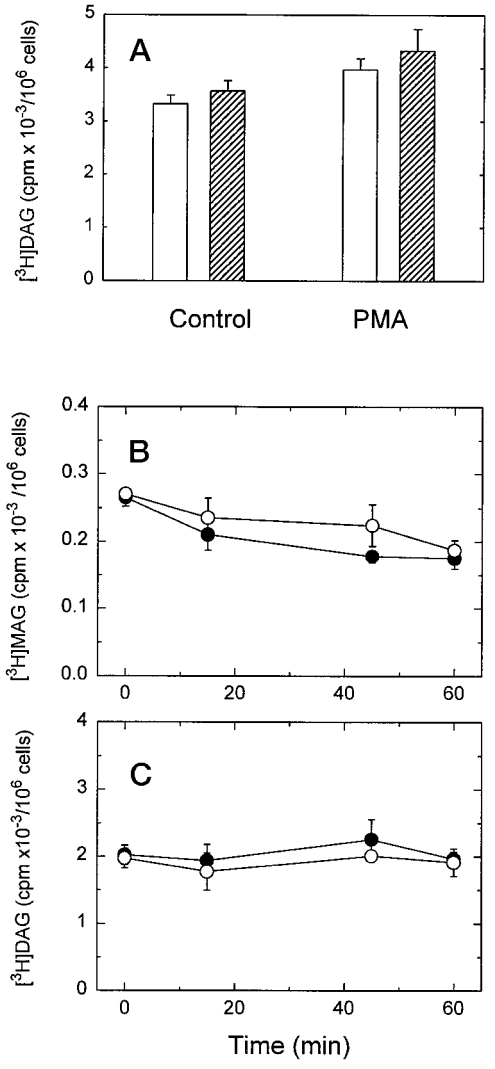

FIG. 8. *A*, [^{3}H]AA-containing DAG levels in control and PMA-activated cells in the absence (*open bars*) or presence (*stripped bars*) of 50 μM RHC80267. Time-course of production of [^{3}H]AA-containing monoacylglycerol (*MAG*) (*B*) and diacylglycerol (*C*) in control (*open circles*) and PMA-activated (*closed circles*) cells.

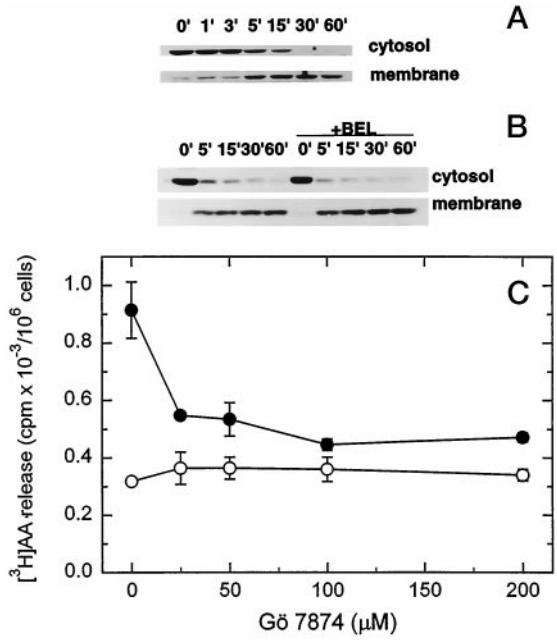

FIG. 9. **PKCα translocation in PMA-treated WISH cells.** *A*, cells were stimulated with 25 ng/ml PMA for different periods of time. *B*, cells were pretreated with 25 μM BEL for 30 min before stimulation with PMA. Cytosolic and membrane fractions were obtained by centrifugation as described under "Materials and Methods." Protein was analyzed by Western blot using antibodies against PKCα. *C*, [^{3}H]AA-labeled cells were preincubated with different concentrations of Gö7874 for 30 min and stimulated with 25 ng/ml PMA (*closed circles*) or vehicle (*open circles*) for 75 min. Radioactivity in the supernatants was quantified by liquid scintillation counting. Experiments were carried out in triplicate, and the data are shown as means ± S.E.

underscoring the critical role that PAP plays in the chain of events leading to cPLA$_2$ activation by PMA and, hence, to AA release in WISH cells.

The possibility that PAP-derived DAG is serving itself as a substrate for the AA release via DAG lipase was first investigated by using the DAG lipase specific inhibitor RHC80267. This compound, at concentrations up to 100 μM, did not affect the PMA-induced AA release (not shown). However, the inhibitor slightly raised both basal and activated DAG levels in [^{3}H]AA labeled-cells (Fig. 8*A*), indicating that the drug indeed prevents DAG breakdown. Examination of the time course of accumulation of DAG and MAG in [^{3}H]AA-labeled cells did not reveal any significant variation in the levels of these two metabolites up to 60 min after PMA addition, a time point at which AA release is well underway (cf. Figs. 1*A* and 8, *B* and *C*).

Involvement of PKCα in PMA-induced AA Mobilization—Activation of PKC, particularly the α isoform, has previously been shown to constitute a major route for PLD activation in a wide variety of cell types (22, 28, 29). WISH cells express PKCα, ϵ, δ, and ζ.[3] Of them, only PKCα was translocated to the membrane fraction after cellular activation with PMA (Fig. 9*A*). The translocation took place very early, being observed at 5 min and disappearing completely from the cytosolic fraction after 30 min of stimulation. To assess whether or not PKCα translocation to the membrane fraction was mediated by PAP-derived DAG, experiments were conducted in the presence of BEL. BEL affected neither PKCα binding to the membrane (Fig. 9*B*) nor PKC activity, as measured *in vitro* using a commercial kit (PKC assay system V5910, Promega) (data not shown). Like BEL, propranolol did not have any effect on PKCα translocation (data not shown). Involvement of PKCα in PMA-induced AA release was confirmed by the use of the inhibitor Gö7874, specific for Ca^{2+}-dependent isoforms, which inhibited [^{3}H]AA release (Fig. 9*C*).

PMA-induced Phosphorylation Cascades in WISH Cells—PMA-induced signaling events have been shown to include activation of p42/p44^{MAPK} downstream of PKCα (30). In keeping with this notion, PMA was able to induce a mobility shift on SDS-polyacrylamide gel electrophoresis, indicating phosphorylation and activation of these kinases. cPLA$_2$, which in some instances lies downstream of p42/p44^{MAPK} (4), also experienced a mobility shift after PMA treatment (Fig. 10). Interestingly, after BEL or propranolol treatment, conditions that decrease AA release, the MAPK and cPLA$_2$ mobility shifts were not prevented (Fig. 10). In fact, even in the absence of PMA, both inhibitors were able to induce a cPLA$_2$ mobility shift. Moreover, neither BEL nor propranolol affected the intrinsic activity of the cPLA$_2$ as measured in homogenates from PMA-treated cells (26; data not shown). These data indicate that inhibition of AA mobilization by PAP blockers is not due to inhibition of the signaling mechanism through which the cPLA$_2$ increases its intrinsic specific activity, *i.e.* phosphorylation by MAPKs.

[3] M. A. Balboa and E. A. Dennis, unpublished data.

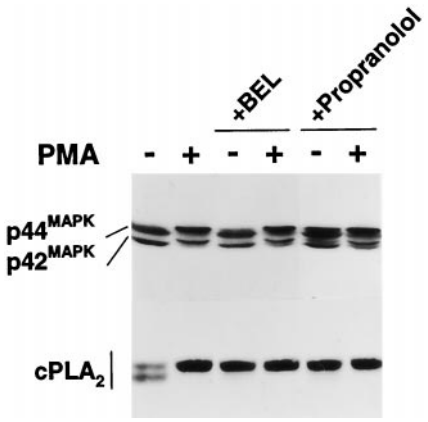

FIG. 10. **Phosphorylation of p42^{MAPK}, p44^{MAPK}, and cPLA$_2$.** Cells were stimulated with 25 ng/ml PMA in the presence or absence of 100 μM propranolol or 25 μM BEL for 1 h. Protein was quantified and analyzed by Western blot with specific antibodies against p42/p44^{MAPK} (ERK-2) and against cPLA$_2$.

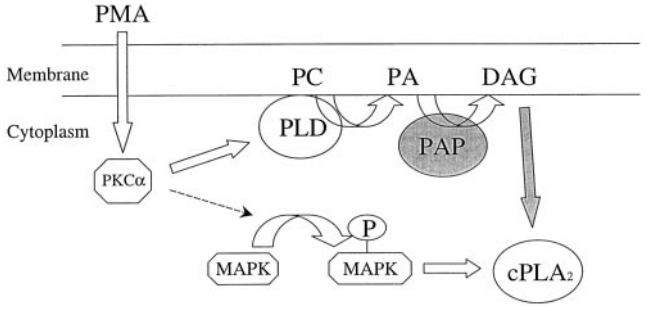

FIG. 11. **Proposed mechanism for AA release in PMA-activated WISH cells**.

DISCUSSION

Very little is known about how free AA levels are regulated in the amnion, the PLA$_2$s responsible for such a regulation, and the molecular mechanisms involved. In the present study, we have uncovered phosphatidate phosphohydrolase as a novel regulatory element within the signaling cascade that results in cPLA$_2$ activation and AA release during the early stages of activation of the amnionic-like cell line, WISH.

BEL has recently been used as a tool to investigate whether the iPLA$_2$ has a role in AA mobilization in different cell types, as this inhibitor possesses over 1000-fold selectivity for the iPLA$_2$ among other PLA$_2$ forms (31). However, BEL also inhibits the magnesium-dependent PAP (26, 32). In P388D$_1$ cells, the IC$_{50}$ for inhibition by BEL of the PAP is 8 μM, *i.e.* almost identical to that for inhibition of iPLA$_2$ in the same cells (21). We have found that BEL appreciably blunts AA release in activated WISH cells; however, it blunts DAG production as well, demonstrating that the drug is affecting the PAP in addition to any effect on the iPLA$_2$. Moreover, the inhibitory effects of BEL on AA release herein reported appear to be a consequence of PAP inhibition, since blockage of this enzyme by two other unrelated strategies, *i.e.* (i) direct inhibition of the enzyme by propranolol and (ii) PAP substrate depletion by ethanol, gave the same inhibitory effect on AA release. Moreover, unlike the cPLA$_2$, the iPLA$_2$ specific activity does not increase after cell stimulation. Our recent attempts at inhibiting iPLA$_2$ expression by using antisense mRNA technology in WISH cells, similar to those succesfully used in P388D$_1$ macrophages (33), have failed to detect any effect on AA release, which reinforces the notion that the iPLA$_2$ is not an effector in this process.[4] It should be noted, however, that unlike P388D$_1$ cells, the WISH cells express very high levels of iPLA$_2$ protein, as judged by immunoblot analysis (results not shown).

Collectively, the aforementioned results constitute, to the best of our knowledge, the first evidence implicating PAP and the metabolite it produces, DAG, in the regulation of AA mobilization. Since PAP is usually coupled to PLD, as is the case in WISH cells as well, and PLD uses phosphatidylcholine as a preferred substrate, it seems logical to assume that the DAG involved in cPLA$_2$ activation in WISH cells arises mainly from phosphatidylcholine.

Besides the identification of PAP as an important regulator of the AA release response in WISH cells, another striking feature of the current work is the finding that none of the PAP-inhibition strategies used resulted in alteration of the intrinsic activity of the cPLA$_2$, as measured by both phosphorylation and *in vitro* activity. This finding raises interesting questions as to the role of DAG in cPLA$_2$ activation in WISH cells. The currently accepted paradigm of cPLA$_2$ activation by stimuli considers the involvement of two different signaling branches that converge at the cPLA$_2$ itself (4). The first one is a phosphorylation cascade that culminates in the phosphorylation of the cPLA$_2$ and serves to increase the intrinsic activity of the enzyme. Both the nature of the kinase involved as well as the site of phosphorylation remain controversial (4). The second branch for cPLA$_2$ signaling involves the translocation of the enzyme from the cytosol to the membrane, where its substrate is localized. This translocation, which does not modulate the cPLA$_2$ activity itself, is currently believed to be mediated by increased Ca^{2+} availability although other factors may also be involved, especially in the case of stimuli like PMA which do not promote Ca^{2+} increases (34, 35). The two pathways for cPLA$_2$ activation are independent of each other, but both appear to be required for proper cPLA$_2$ activation and subsequent AA release (4).

Because PAP is not involved in regulating the cPLA$_2$ phosphorylation cascade, it appears logical to suggest that PAP is involved in regulating binding of the cPLA$_2$ to the membrane. DAG is long known to cause perturbations in membrane bilayers, rendering them susceptible to PLA$_2$ attack (36). DAG accumulation in membranes has the effect of spreading apart the phospholipid headgroups, thereby making the glycerol backbone more accessible to the PLA$_2$. Indeed, one of the most commonly utilized methods for detecting cPLA$_2$ activity is based on this principle (20). Thus, at the same time the specific activity of the cPLA$_2$ is increased as a result of its enhanced phosphorylation, local accumulations of DAG in the membrane allow for an appropriate substrate presentation for the enzyme. When the cPLA$_2$ translocates to the membrane as a a result of increased Ca^{2+} availability and/or other factors, the enzyme will find optimal conditions to initiate AA release. The key role for DAG in this process is strengthened by the observation that, in the presence of BEL or propranolol, both p42/p44^{MAPK} and cPLA$_2$ become phosphorylated normally in WISH cells but no AA release is induced.

DAG is regarded as a universal activator of PKCs. However it is highly unlikely that the PAP-derived DAG is playing such a role in PMA-induced AA release in WISH cells because, in this system, the only PKC that becomes activated is the α isoform, which is the one that PMA directly activates. PMA-induced PKCα is most likely the upstream event that triggers DAG production by activating the PLD, which in turn generates the PA substrate for the PAP. Therefore, despite the presence in WISH cells of the DAG-activable isoforms δ and ϵ, none of them become activated after DAG levels increase. This is consistent with our suggestion that the PAP-derived DAG serves a structural, not messenger,

[4] M. A. Balboa, J. Balsinde, and E. A. Dennis, unpublished data.

role for AA release in PMA-activated WISH cells. In agreement with this view is the recent work by Pettitt *et al.* (37) in activated endothelial cells. These investigators provided compelling evidence that only the DAG which derives from inositol lipids, *i.e.* the one that PMA mimics in our WISH cell system, is able to activate PKC; whereas, the DAG arising from the PLD/PAP pathway does not serve a messenger role but rather a structural/metabolic role (37).

An interesting but yet unresolved question regarding AA metabolism by amnionic WISH cells relates to the fact that apreciable free AA mobilization is already detectable after a 30-min cell challenge, whereas PGE_2 is released only after 4–6 h of stimulation with the phorbol ester (13). It has been suggested that COX-2 is the enzyme responsible for PG production and that its synthesis by PMA-activated WISH cells requires at least 4 h (13). Thus the question arises as to why free AA is produced much before it can be metabolized to PGE_2. It seems likely that at short times, the free AA may act as a signaler rather than an intermediary metabolite. As a matter of fact, it has been suggested that exogenous AA up-regulates the expression of COX-2 in uterine stromal cells (38). The induction is not due to conversion of AA to prostaglandin by COX-1 because it occurs even in the presence of aspirin, a well known inhibitor of COX activity. Studies are currently underway in our laboratory to investigate this intriguing possibility.

In conclusion, this study has shown that PAP plays an important role in the regulation of $cPLA_2$, possibly by facilitating interaction of the enzyme with its substrate and not by increasing the specific enzyme activity. Our data suggest the mechanism for AA mobilization in PMA-activated WISH cells depicted in Fig. 11. According to this model, the phorbol ester activates PKCα which, in turn, activates PLD. The PLD gives rise to PA that will be converted to DAG by PAP. The DAG produced by this pathway will act to allow a good substrate presentation for the $cPLA_2$. Parallel to but independent of this sequence of events, PKCα and perhaps PA as well (39) act to activate the MAP kinase pathway, which leads to the phosphorylation of $cPLA_2$. When these two signaling routes are turned on, the $cPLA_2$ will start hydrolyzing phospholipids, resulting in the early generation of free AA.

REFERENCES

1. Dennis, E. A. (1994) *J. Biol. Chem.* **269,** 13057–13060
2. Dennis, E. A. (1997) *Trends. Biochem. Sci.* **22,** 1–2
3. Cupillard, L., Koumanov, K., Mattei, M.-G., Lazdunski, M., and Lambeau, G. (1997) *J. Biol. Chem.* **272,** 15745–15752
4. Leslie, C. C. (1997) *J. Biol. Chem.* **272,** 16709–16712
5. Balsinde, J., and Dennis, E. A. (1997) *J. Biol. Chem.* **272,** 16069–16072
6. Barbour, S. E., and Dennis, E. A. (1993) *J. Biol. Chem.* **268,** 21875–21882
7. Balboa, M. A., Balsinde, J., Winstead, M. V., Tischfield, J. A., and Dennis E. A. (1996) *J. Biol. Chem.* **271,** 32381–32384
8. Reddy, S. T., Winstead, M. V., Tischfield, J. A., and Hersman H. R. (1997) *J. Biol. Chem.* **272,** 13591–13596
9. Balsinde, J., Bianco, I. D., Ackerman, E. J., Conde-Frieboes, K., and Dennis, E. A. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 8527–8765
10. Embrey, M. P. (1971) *Ann. N. Y. Acad. Sci.* **180,** 518–523
11. Calder, A. A. (1979) in *Practical Applications of Prostaglandins and their Synthesis Inhibitors* (Karim, S. M. M., ed) pp. 301–318, MTP Press, Lancaster, England
12. Okazaki, T., Casey, M. L., Okita, J. R., MacDonald, P. C., and Johnson, J. M. (1981) *Am. J Obstet. Gynecol.* **139,** 373–381
13. Hulkower, K. I., Otis, E. R., Li, J., Ennis, B. W., Cugier, D. J., Bell, R. L., Carter, G. W., and Glaser, K. B. (1997) *J. Pharmacol. Exp. Ther.* **280,** 1065–1074
14. Perkins, D. J., and Kniss, D. A. (1997) *Biochem. J.* **321,** 677–681
15. Albert, T. J., Su, H.-C., Zimmerman, P. D., Iams, J. D., and Kniss D. A. (1994) *Prostaglandins* **48,** 401–416
16. Xue, S., Brockman, D. E., Slater, D. M., and Myatt, L. (1995) *Prostaglandins* **49,** 351–369
17. Xue, S., Slatter, D. M., Bennett, P. R., and Myatt, L. (1996) *Prostaglandins* **51,** 107–124
18. Hayflick, L. (1961) *Exp. Cell Res.* **23,** 14–20
19. Kniss, D. A., Mershon, J., Su, H.-C., Sonek, J., Fertel, R. H., Wasman, M., Iams, J. D., and Gabbe, S. G. (1990) *Am. J. Obstet. Gynecol.* **163,** 1883–1890
20. Kramer, R. M., Roberts, E. F., Manetta, J., and Putnam, J. E. (1991) *J. Biol. Chem.* **266,** 5268–5272
21. Balboa, M. A., Balsinde, J., Dennis, E. A., and Insel, P. A. (1995) *J. Biol. Chem.* **270,** 11738–11740
22. Balboa, M. A., Firestein, B. L., Godson, C. A, Bell, K. S., and Insel, P. A. (1994) *J. Biol. Chem.* **269,** 10511–10516
23. Balsinde, J., Balboa, M. A., Insel, P. A., and Dennis, E. A. (1997) *Biochem. J.* **321,** 805–809
24. Frenkel, R. A., and Johnston, J. M. (1992) *J. Biol. Chem.* **267,** 19186–19191
25. Harris, A. N., Perlman, M., Schiller, S. L., Romero, R., and Mitchell, M. D. (1988) *Am. J. Obstet. Gynecol.* **159,** 1385–1389
26. Balsinde, J., and Dennis, E. A. (1996) *J. Biol. Chem.* **271,** 6758–6765
27. Balsinde, J., and Dennis, E. A. (1996) *J. Biol. Chem.* **271,** 31937–31941
28. Exton, J. H. (1997) *J. Biol. Chem.* **272,** 15579–15582
29. Nishizuka, Y. (1995) *FASEB J.* **9,** 484–496
30. Nishida, E., and Gotoh, Y. (1993) *Trends Biochem. Sci.* **18,** 128–131
31. Hazen, S. L., Zupan, L. A., Weiss, R. H., Getman, D. P., and Gross, R. W. (1991) *J. Biol. Chem* **266,** 7227–7232
32. Dillon, D. A, Chen, X., Zeimetz, G. M., Wu, W. I., Waggoner, D. W., Dewald, J., Brindley, D. N., and Carman, G. M. (1997) *J. Biol. Chem.* **272,** 10361–10366
33. Balsinde, J., Balboa, M. A., and Dennis, E. A. (1997) *J. Biol. Chem.* **272,** 29317–29321
34. Fernández, B., Balboa, M. A., Solís-Herruzo, J. A., and Balsinde, J. (1994) *J. Biol. Chem.* **269,** 26711–26716
35. Mosior, M., Six, D. A., and Dennis, E. A. (1998) *J. Biol. Chem.* **273,** 2184–2191
36. Dawson, R. M. C., Irvine, R. F., Bray, J., and Quinn, P. J. (1984) *Biochem. Biophys. Res. Commun.* **125,** 836–842
37. Pettitt, T. R., Martin, A., Horton, T., Liossis, C., Lord, J. M., and Wakelam, M. J. O. (1997) *J. Biol. Chem.* **272,** 17354–17359
38. Prigent-Tessier, A., Pageaux, J.-F., Fayard, J.-M., Lagarde, M., Laugier, C., and Cohen, H. (1996) *Eur. J. Biochem.* **241,** 872–878
39. Ghosh, S., Strum, J. C., and Bell, R. M. (1997) *FASEB J.* **11,** 45–50

Involvement of Phosphatidate Phosphohydrolase in Arachidonic Acid Mobilization in Human Amnionic WISH Cells

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