Purification and Characterization of a Cytosolic, 42-kDa and Ca\(^{2+}\)-dependent Phospholipase A\(_2\) from Bovine Red Blood Cells

ITS INVOLVEMENT IN Ca\(^{2+}\)-DEPENDENT RELEASE OF ARACHIDONIC ACID FROM MAMMALIAN RED BLOOD CELLS*

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Hae Sook Shin‡**, Mi-Reyoung Chint‡, Jung Sun Kim‡, Jin-Ho Chung‡, Chung-Kyu Ryu‡, Sung Yun Jung‡, and Dae Kyong Kim‡

From the ‡Department of Environmental & Health Chemistry, College of Pharmacy, Chung-Ang University, Dongjak-Ku, Seoul 156-756, the §College of Pharmacy, Seoul National University, Kwanak-Ku, Seoul 151-742, and the ¶Department of Pharmaceutical Analysis, College of Pharmacy, Ewha Womans University, Seodaemun-Ku, Seoul 120-750, South Korea

It has become evident that a Ca\(^{2+}\)-dependent release of arachidonic acid (AA) and subsequent formation of bioactive lipid mediators such as prostaglandins and leukotrienes in red blood cells (RBCs) can modify physiological functions of neighboring RBCs and platelets. Here we identified a novel type of cytosolic PLA2 in bovine and human RBCs and purified it to apparent homogeneity with a 14,000-fold purification. The purified enzyme, termed rPLA2, has a molecular mass of 42 kDa and reveals biochemical properties similar to group IV cPLA2, but shows different profiles from cPLA2 in several column chromatographies. Moreover, rPLA2 did not react with any of anti-cPLA2 and anti-sPLA2 antibodies and was identified as an unknown protein in matrix-assisted laser desorption/ionization time-of-flight mass spectrometric analysis. Divalent metal ions tested exhibited similar effects between rPLA2 and cPLA2, whereas mercurials inhibited cPLA2 but had no effect on rPLA2. Antibody against the 42-kDa protein not only precipitated the rPLA2 activity, but also reacted with the 42-kDa protein from bovine and human RBCs in immunoblot analysis. The 42-kDa protein band was selectively detected in murine fetal liver cells known as a type of progenitor cells of RBCs. It was found that EA4, a derivative of quinone newly developed as an inhibitor for rPLA2, inhibited a Ca\(^{2+}\) ionophore-induced AA release from human and bovine RBCs, indicating that this enzyme is responsible for the Ca\(^{2+}\)-dependent AA release from mammalian RBCs. Finally, erythroid progenitor cell assay utilizing dianimobenzidine staining of hemoglobinized fetal liver cells showed that rPLA2, detectable in erythroid cells was down-regulated when differentiated to non-erythroid cells. Together, our results suggest that the 42-kDa rPLA2 identified as a novel form of Ca\(^{2+}\)-dependent PLA2 may play an important role in hemostasis, thrombosis, and/or erythropoiesis through the Ca\(^{2+}\)-dependent release of AA.

Evidence is accumulating that suggests that red blood cells (RBCs) can play an active role in hemostasis and thrombosis by markedly enhancing platelets aggregation in vitro induced by Ca\(^{2+}\) ionophore (1, 2), collagen (1–4), thrombin (1, 2), and shear stress (5), where platelet serotonin release, arachidonic acid (AA) production, and eicosanoid formation were also observed. It has been further demonstrated that collagen-stimulated platelets aggregate three times more effectively and discharge seven times more ADP in the presence of RBCs than in their absence (1, 6). Thus, RBCs amplify platelet activation in vitro, a phenomenon that may be related to the known clinical participation of RBCs in pathophysiological responses of platelets. However, at present the RBC-derived diffusible chemical mediators remain to be clarified.

In this context, several studies have suggested that, when RBCs are stimulated by the Ca\(^{2+}\) ionophore A23187 (7) and shear stress (8), the cells by themselves release AA from membrane phospholipids possibly by the action of phospholipase A\(_2\) (PLA2). Although the released AA is subsequently metabolized to eicosanoids such as 12-hydroxyeicosatetraenoic acid (12-HETE), prostaglandin E\(_1\) and E\(_2\) in the cells, it is also suggested that the AA may be captured by nearby platelets and metabolically converted into prothrombotic thromboxane A\(_2\) (1, 7). Furthermore, it is known that lipoygenase metabolites of AA stimulated K\(^+\) efflux during regulatory volume decrease by RBCs (9) and erythropoiesis (10), and prostaglandin E\(_2\) inhibited RBC volume regulation (11) and filterability (11, 12). These results suggest a crucial role of these RBC-derived bioactive chemical mediators such as AA and its metabolites in pathophysiology of neighboring platelets or RBCs in the microcirculation and thus prompted us to focus on a RBC form of PLA2.

In the last several decades, many types of mammalian PLA2s have been identified, purified and characterized from a number of non-erythroid cells (13–16). In contrast, over 30 years ago, since Paysant et al. detected PLA2 activity in RBC membranes

The abbreviations used are: RBCs, red blood cells; MFL, murine fetal liver; PLA\(_2\), phospholipase A\(_2\); cPLA\(_2\), group IV cytosolic PLA\(_2\); sPLA\(_2\), secretory group II PLA\(_2\); AA, arachidonic acid; 2-[1\(^{-14}C\)] AA-GPC, 1-stearoyl-2-[1\(^{-14}C\)] arachidonyl-sn-glycerol-3-phosphocholine; HETE, hydroxyeicosatetraenoic acid; rPLA\(_2\), the purified cytosolic RBC PLA\(_2\); MEM, minimum essential medium; DAB, 3,3'-diaminobenzidine; EPO, erythropoietin; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; HPLC, high performance liquid chromatography; BSA, bovine serum albumin; EA4, 7-chloro-6-(4-diethylaminophenyl)-5,8-quinoledinedione; TP1, 2-(3,5-di-tert-butyl-4-hydroxyphenyl)-3-chloro-1,4-naphthalele di-one; CFU-E, colony-forming unit erythroid cells.

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‡ To whom correspondence should be addressed: Dept. of Environmental & Health Chemistry, College of Pharmacy, Chung-Ang University, 221 Hukusuk-Dong, Dongjak-Ku, Seoul 156-756, South Korea, Tel.: 82-2-820-5610; Fax: 82-2-816-7338; E-mail: dkkim@cau.ac.kr.

** Current address: Renal Division, Medical service, Massachusetts General Hospital East and Department of Medicine, Harvard Medical School, Charlestown, MA 02129; E-mail: hsshin64@hotmail.com.

† The abbreviations used are: RBCs, red blood cells; MFL, murine fetal liver; PLA2, phospholipase A2; cPLA2, group IV cytosolic PLA2; sPLA2, secretory group II PLA2; AA, arachidonic acid; 2-[1\(^{-14}C\)] AA-GPC, 1-stearoyl-2-[1\(^{-14}C\)] arachidonyl-sn-glycerol-3-phosphocholine; HETE, hydroxyeicosatetraenoic acid; rPLA2, the purified cytosolic RBC PLA2; MEM, minimum essential medium; DAB, 3,3'-diaminobenzidine; EPO, erythropoietin; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; HPLC, high performance liquid chromatography; BSA, bovine serum albumin; EA4, 7-chloro-6-(4-diethylaminophenyl)-5,8-quinoledinedione; TP1, 2-(3,5-di-tert-butyl-4-hydroxyphenyl)-3-chloro-1,4-naphthalele di-one; CFU-E, colony-forming unit erythroid cells.
from rat (17) and human (18) and Kramer et al. described the purification of a Ca$^{2+}$-dependent 18.5-kDa PLA$_2$ from sheep RBC membranes (19), the RBC form of PLA$_2$ has been poorly studied. Moreover, since Adachi et al. detected a Ca$^{2+}$-independent cytosolic PLA$_2$ preferentially hydrolyzing phosphatidylethanolamine to phosphatidylcholine in chicken RBCs (20), no cytosolic form of PLA$_2$ in mammalian RBCs has been reported.

In the present study we purified a cytosolic 42-kDa Ca$^{2+}$-dependent PLA$_2$, termed rPLA$_2$, from bovine RBCs and characterized it as a novel form of Ca$^{2+}$-dependent PLA$_2$ through biochemical and immunochromatography and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometric analysis. We showed that rPLA$_2$ is responsible for the Ca$^{2+}$-dependent release of AA from human and bovine RBCs by using a quinone derivative newly developed for rPLA$_2$ inhibitor.

EXPERIMENTAL PROCEDURES

Materials—1-Stearyl-2-[1-14C]arachidonoyl-sn-glycero-3-phosphocholine (2-[1-14C]AA-GPC, 55.3 Ci/mmol), 1-palmitoyl-2-[1-14C]palmitoyl-sn-glycero-3-phosphocholine (2-[1-14C]PPA-GPC, 24.9 Ci/mmol), and 1-[1-14C]cholesterol, and 1-acyl-2-[1-14C]arachidonyl-sn-glycero-3-phosphoethanolamine (2-[1-14C]AA-GPE, 55.1 Ci/mmol), and [1-14C]arachidonic acid ([1-14C]AA, 204 Ci/mmol) were purchased from the radiochemical center, Amersham Biosciences, Inc. (Buckinghamshire, UK). 1-Stearyl-2-arachidonoyl-sn-glycero-3-phosphocholine (2-AA-GPC), dithiothreitol, 21S187, 3.5-diaminobenzidine (DAB), methylcellulose, erythropoietin, and a Sephrose 4B-200 gel filtration column were purchased from Sigma Chemical Co. (St. Louis, MO). Anti-human secretory 14-kDa sPLA$_2$ antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Goat anti-rabbit- and anti-mouse-immunoglobulin alkaline phosphatase conjugates were purchased from Santa Cruz Biotechnology, Inc. (Lake Placid, NY). Group IV cytosolic PLA$_2$ (cPLA$_2$) was purified from bovine platelets as described previously (21). Butyl-Toyopearl 650M gel, preparative Phenyl-5PW, analytical Phenyl-5PW, DEAE-5PW columns were purchased from Tosoh Co. (Tokyo, Japan). Sephacryl S-300 gel filtration, Superose 12 gel filtration, PD-10 desalting (Phadex G-25M), and Mono Q FPLC columns, and Protein A-Sepharose CL-4B beads were purchased from Amersham Biosciences, Inc. (Uppsala, Sweden). Arachidonyl triethylammonium ketone was obtained from BIOMOL (Plymouth Meeting, PA). Complete Freund’s adjuvant and minimal essential medium (MEM) were obtained from Invitrogen (Grand Island, NY). All other chemicals were of the highest purity or molecular biology grade available from commercial sources.

Isolation of Human and Bovine RBCs—Human venous blood was collected by venepuncture into 40-unit/ml from some healthy volunteers among the Korean graduate students in our laboratory and bovine blood freshly collected in heparin (40 unit/ml) in a local slaughterhouse. After blood was centrifuged at 500 × g for 20 min, the resulting supernatants of the platelet-rich plasma, the buffy coat, and the leading edge of the packed RBCs were completely removed by aspiration. Sedimented RBCs, leukocytes, and platelets were re-suspended in a sterile buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.12 M NaCl). This centrifugation and aspiration cycle was repeated six times, taking care to removing leukocytes and platelets and the top 10% of the RBC suspensions. Washed cell suspensions (10 ml) were subsequently depleted of residual leukocytes and platelets by filtration through a Sepharose 4B-200 column (20 × 2.5 cm) pre-equilibrated with sterile saline (0.9% w/v NaCl) as described previously (21). The filtered cell suspensions contained the following numbers of blood cells: for human blood, <3 × 10$^8$ platelets/ml, <2 × 10$^8$ leukocytes/ml, and <5 × 10$^8$ RBCs/ml; for bovine blood, <4 × 10$^8$ platelets/ml, <3 × 10$^8$ leukocytes/ml, and 3–5 × 10$^8$ RBCs/ml. Differential cell counts were measured with a Coulter counter (Becton Dickinson UK, Oxford, UK).

Release of [3H]AA by 21S187 from Human and Bovine RBCs and in Vitro Activity of PLA$_2$ Activity—The Sepharose 4B-200 column-purified RBCS suspensions (~1 × 10$^8$ cells/ml) were twice washed with serum-free MEM containing 1 mg/ml fatty acid-free bovine serum albumin (BSA) and labeled for 1 h with 1.5 µCi of [3H]AA (1 µCi/µl ethanol/ml) of the same medium. Murine L929 cells (1–2 × 10$^6$ cells/ml) were labeled for 6 h with 0.1 µCi of [3H]AA (0.1 µCi/µl ethanol/ml) of the same medium. Afterward, cells were washed three times to remove all unincorporated [3H]AA. The labeled cells were incubated in MEM containing 1 mg/ml BSA as a trap for the released [3H]AA and then stimulated with vehicle (1.0 µl of ethanol/ml medium) or the agonists as indicated. For analysis of [3H]AA release, the RBCs were centrifuged as above, and each aliquot (200 µl) of the supernatants for human and bovine RBCs and for each reservoir (100 µl) of the supernatants for the L929 cells was transferred to 2.5 ml of the scintillation solution and counted for radioactivity with a Packard tri-carboxylate β-scintillation counter (Packard Instrument Co., Meriden, CT). The total incorporated [3H]AA into the RBCs was determined by centrifuging the RBC suspensions at 10,000 × g for 1 min immediately and 1 h after addition of [3H]AA, respectively, followed by counting the radioactivity of each aliquot of the supernatants.

In the present study we purified a cytosolic 42-kDa Ca$^{2+}$-dependent PLA$_2$, termed rPLA$_2$, from bovine RBCs and characterized it as a novel form of Ca$^{2+}$-dependent PLA$_2$ through biochemical and immunochromatography and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometric analysis. We showed that rPLA$_2$ is responsible for the Ca$^{2+}$-dependent release of AA from human and bovine RBCs by using a quinone derivative newly developed for rPLA$_2$ inhibitor.
were stained with a PlusOne silver staining kit (Amersham Biosciences, Inc., Piscataway, NJ).

**Immunochemical Study of rPLA2—**To prepare mouse anti-42-kDa protein polyclonal antibody, the active pool obtained from the Mono Q column was concentrated using a Centri-Prep (Amicon Co., Beverly, MA) by ~5-fold, and an aliquot (~25 µg of protein in 0.25 ml) was mixed with the same volume of complete Freund’s adjuvant and injected into a BALB/c mouse via an intraperitoneal route. After boosting four times at a 3-week interval, the immunized mouse was sacrificed and the serum was obtained. First, for immunoprecipitation study, pre-immune serum (50 µl) and anti-42-kDa protein antisera (50 µl) were mixed with packed Protein A-Sepharose CL-4B beads (bed volume, 25 µl), respectively, and incubated overnight at 4 °C as described previously (25). The beads were then washed six times with 1.0 ml of buffer B (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2% (w/v) BSA) and incubated with an active pool (protein 8.2 µg) from the Superose 12 column for the indicated times at 4 °C with constant shaking. Then, the beads were pelleted by centrifuging at 1300 × g at 4 °C for 1 min, and each aliquot of the resulting supernatants was assayed for PLA2 activity. The pellets were washed six times with buffer B containing 0.1% Tween 20 and 0.5 mM NaCl, separated on 10% SDS-PAGE, and visualized by a silver staining kit. Second, for immunoblotting analysis, samples were separated by 10% SDS-PAGE, transferred to a Hybond ECL nitrocellulose membrane (Amer sham Biosciences, Inc. UK Ltd., Buckinghamshire, UK), and visualized as described previously (21). The membranes were exposed to the antisera against rPLA2 (1:5000), CPLA2 (1:2000), and sPLA2 (1:2000), respectively, and incubated with a 1:2500 dilution of goat anti-rabbit or anti-mouse-alkaline phosphatase conjugate in Tris-phosphate, 30 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM MgCl2, and 0.1% Tween 20 as a blocking buffer for 2 h, respectively. The membranes were developed with a preformulated substrate kit (1-Step nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate, Pierce Co., Rockford, IL).

**Protein Identification by Peptide Mass Fingerprinting Analysis—** Protein peptide fingerprinting analysis was performed as described previously (26). Briefly, the 42-kDa spot was stained with Coomassie Brilliant Blue and excised from a two-dimensional electrophoresis gel and digested with trypsin. A 1-µl aliquot of the total digest (total volume 30 µl) was used for peptide mass fingerprinting. The masses of the tryptic peptides were measured with a Bruker Reflex III mass spectrometer. MALDI-TOF analysis was performed with a Bruker Reflex III mass spectrometer. The tryptic peptides were measured with a Bruker Reflex III mass spectrometer. The masses with better than 50 ppm mass accuracy on average. Comparison of the mass value against the Swiss-Prot data base was performed using the Peptide Search (27).

**Preparation of Quinone Derivatives, EA4 and TP1—**First, 7-chloro-6-[4-(diethylamino)phenyl]-5,8-quinolinedione (EA4) was prepared by substitution of 5,8-quinolinedione (28) with 6-[4-(diethylamino)phenyl]-5,8-quinolinedione (EA4) was prepared by substitution of 5,8-quinolinedione (28) with Cu(CH3COO)2.H2O (6.28 mmol) in 80 ml of acetic acid was added to a solution of N,N-diethylaminocarbonylhydrazine in 20 ml of acetic acid with stirring at room temperature for 2 h. After the reaction mixture was kept overnight, the precipitate was collected by filtration. Second, 2-(3,5-di-tet-butyl-4-hydroxyphenyl)-3-chloro-1,4-naphthalene dione (TP1) was synthesized and characterized as described previously (28).

**Murine Erthyroid Progenitor Cell Assay—**To obtain murine fetal liver cells, adult male and female CD-1 mice (Duess Han Biolink Co., Ltd., Eumsung-Gun, Chungbuk, Korea) underwent timed matings. At days 12–13 after mating, the female mice were killed under ether anesthesia. According to the method of Mason-Garcia et al. (30), the fetal livers were removed from the fetuses and gently teased free of the abdominal cavity. MFL cells were gently disaggregated by sequential passage through 18-, 21-, and 23-gauge hypodermic needles, washed twice in α-modified Eagle’s medium essential medium with glutamine (α-MEM, Invitrogen, Grand Island, NY), and resuspended in 5 ml of α-MEM. Isolated murine fetal liver cells (1 × 10^7/ml) were plated in a mixture (DAB mixture) containing α-MEM, 0.8% methycellulose, 20% fetal bovine serum, 10^{-4} M mercaptoethanol, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.2 unit/ml highly purified human recombinant EPO (specific activity >160,000 units/mg of protein). For DAB staining, 1 ml of the DAB mixture was plated in each 10 × 35-mm Petri dishes and incubated under a humidified atmosphere of 95% air and 5% CO2. After 3 or 7 days, the dishes were stained for pseudoperoxidase with DAB and hydrogen peroxide as described previously (31).

**RESULTS**

**Detection of a Cytosolic Ca2+-dependent PLA2 in Human and Bovine RBCs—**A calcium ionophore A23187 released [3H]AA from the purified human and bovine RBCs in a time-dependent manner (Fig. 1). The releases of [3H]AA in these cells were relatively rapid as significantly observed at 10 min and gradually increased up to 60 min. Furthermore, after the 100,000 × g supernatants and pellets were prepared from bovine RBCs, PLA2 activity was assayed by analyzing the reaction products with thin layer chromatography using various phospholipids as described previously (21). A Ca2+-dependent PLA2 activity, which preferred 2-[1-14C]AA-GPC to 2-[1-14C]LA-GPC and 2-[1-14C]PA-GPC by 8.5- and 25.2-fold, respectively, was detected in the cytosolic fractions and hydrolyzed preferentially 2-[1-14C]AA-GPE to 2-[1-14C]AA-GPC by 1.7-fold. On the other hand, the membrane-bound PLA2 activity from the 100,000 × g pellets was Ca2+-dependent and markedly increased by 2 mM sodium deoxycholate in a total activity nearly similar to that of the cytosolic fraction. This substrate specificity for the RBC form of PLA2 from the cytosolic fractions suggests that this enzyme may be similar to group IV cPLA2. To further examine this, elution profiles between the RBC PLA2 and cPLA2 from porcine spleen were compared in hydrophobic, anionic exchange, and gel filtration column chromatographies, respectively. As shown in Fig. 2, each of these two PLA2 enzymes were eluted at different fractions in all of the columns utilized, and in particular, the RBC PLA2 migrated as a molecular mass of ~40 kDa in a Superose 12 gel filtration FPLC column (Fig. 2D).

**Purification of a Cytosolic PLA2 from Bovine RBCs—**As shown in Table I, the purification of the cytosolic PLA2 from bovine RBCs was summarized. Two hydrophobic columns as initial steps typically resulted in a 273-fold purification and 22.3% yield of bovine RBC cytosolic PLA2. The activities from these columns were stable for several weeks at ~75 °C. A Superose 12 gel filtration FPLC column resulted in a 1.3-fold purification with an efficient yield of 62% and was calibrated as a molecular mass of ~43 kDa by the molecular standards: myosin (200,000 Da), phosphorylase b (97.4 kDa), bovine serum albumin (66.7 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14.4 kDa). Finally, the active pool from the Superose 12 column was further purified by a Mono Q anion-exchange FPLC column. This final step resulted in a 3.4-fold purification with a high yield of 81%. To assess the purity, a portion of each fraction was analyzed on one-dimensional and two-dimensional...
**A 42-kDa Ca$$^{2+}$$-dependent PLA$_2$ in Cytosol of Mammalian RBCs**

**Fig. 2.** Comparison of elution profiles between PLA$_2$ enzymes from bovine RBCs and porcine spleen. The PLA$_2$ enzymes from RBCs and spleen were prepared by homogenizing the purified packed bovine RBCs and porcine spleen tissue slices, respectively, as described under "Materials and Methods." Elution profiles between these PLA$_2$ enzymes were compared in sequential chromatographies of Butyl-Toyopearl hydrophobic (A), Phenyl-5PW hydrophobic (B), DEAE-5PW anion exchange (C), and Superose 12 gel filtration (D) columns. The data presented are from a representative experiment that was repeated three times with similar results.

### Table 1

**Summary of purification of rPLA$_2$ from bovine RBCs**

| Purification step | Total protein | Total activity | Yield | Specific activity | Change |
|------------------|---------------|---------------|-------|------------------|--------|
| S100             | 46,000        | 18,400        | 100.0 | 0.4              | 1      |
| Butyl-Toyopearl  | 700           | 7,200         | 39.6  | 10.4             | 26     |
| Phenyl-5PW(I)    | 37.50         | 4,095         | 22.3  | 109.2            | 273    |
| DEAE-5PW         | 7.00          | 1,873         | 10.2  | 267.6            | 669    |
| Sephacryl S-300  | 1.70          | 1,804         | 9.8   | 1,061.2          | 2,653  |
| Phenyl-5PW(II)   | 0.52          | 656           | 3.6   | 1,261.6          | 3,154  |
| Superase 12      | 0.25          | 412           | 2.2   | 1,648.0          | 4,120  |
| Mono Q           | 0.06          | 336           | 1.8   | 5,600.0          | 14,000 |

SDS-PAGE gels, respectively. The relative PLA$_2$ activity from the final step paralleled the intensity of the 42-kDa band as a single protein band (Fig. 3A, inset), and a single spot was observed in a two-dimensional SDS-PAGE (Fig. 3B), indicating that this 42-kDa band represents the RBC PLA$_2$, termed rPLA$_2$. MALDI-TOF mass spectrometric analysis of the single spot showed no apparent homology to any known protein (data not shown).

To verify that the 42-kDa protein was responsible for rPLA$_2$, mouse polyclonal antibodies against the 42-kDa protein were raised. Although incubation of the Superose 12 column-active fractions with pre-immune serum did not result in any time-dependent loss of PLA$_2$ activity, antisera against the 42-kDa protein precipitated the PLA$_2$ activity in a time-dependent manner (Fig. 3C). In addition, when each of the immunoprecipitates of pre-immune serum and antisera was washed and subjected to SDS-PAGE and silver staining, only the antisera precipitated the 42-kDa protein (Fig. 3D).

**Characterization of rPLA$_2$**—rPLA$_2$ revealed different profiles from spleen cPLA$_2$ in hydrophobic and ion exchange column chromatographies and a gel filtration FPLC (Fig. 2). Moreover, rPLA$_2$ did not react with anti-spleen cPLA$_2$ and anti-sPLA$_2$ antisera in immunoblotting analysis (Fig. 4A), strongly suggesting that rPLA$_2$ could be a novel form of Ca$$^{2+}$$-dependent enzyme. Interestingly, rPLA$_2$ was specifically detected in murine fetal liver (MFL) cells, a rich source of erythroid precursors, among various tissues and cells tested. Furthermore, EPO is known to induce the proliferation and differentiation of MFL cells (32), where a PLA$_2$ may be involved (33), prompting us to examine whether EPO can induce rPLA$_2$ in the cells. Fig. 4B showed that rPLA$_2$ could not be up-regulated by treatment of the progenitor cells with EPO. A cytosolic PLA$_2$ activity of human RBCs was partially purified with similar column profiles by identical procedure (data not shown) and detected as the 42-kDa protein with correlation to the relative activity in immunoblot (Fig. 4, C and D).

To determine whether the 2-[1-14C]AA-GPC-hydrolyzing activity results from PLA$_2$ activity, the reaction products were separated by thin layer chromatography as described previously (21). No radioactive diacylglycerol or lyso-phosphatidylcholine was detected, suggesting that there may be little phospholipase C or phospholipase A$_1$ activity present. The apparent $K_m$ value was $13.9$ $\mu$M and the $V_{max}$ value was $7.4$ nmol/min/mg of protein with 2-[1-14C]AA-GPC (Fig. 5A) and revealed the high selectivity for phospholipids containing AA at the sn-2 position (Fig. 5B). The pl of rPLA$_2$ has ranged from about 3.9 to 4.1 (Fig. 5B). Although profiles of Ca$$^{2+}$$ requirements (Fig. 5C), pH dependence (Fig. 5D), effects on some enzymatic inhibitors (Fig. 5E, F, and G), and divalent metals such as Zn$$^{2+}$$, Fe$$^{3+}$$, Cu$$^{2+}$$, Sr$$^{2+}$$, Ba$$^{2+}$$, Mn$$^{2+}$$, and Mg$$^{2+}$$(data not shown) between rPLA$_2$ and cPLA$_2$ were similar, methyl mercury (Fig. 5H), mercuric chloride (data not shown), and quinone derivative TP1 (Fig. 6B) potently inhibited cPLA$_2$, but not rPLA$_2$.

**Inhibition of Ca$$^{2+}$$-dependent AA Release by Quinone Derivatives**—To assess a role of rPLA$_2$ in the Ca$$^{2+}$$-dependent release of AA in RBCs, two quinone derivatives were developed (Fig. 6A). Although EA4 inhibited both rPLA$_2$ and cPLA$_2$, TP1 inhibited cPLA$_2$ but not rPLA$_2$ (Fig. 6B). A Dixon plot was constructed to show that the inhibition of rPLA$_2$ by EA4 is competitive, but not competitive, with an inhibition constant...
of $K_i = 130 \ \mu M$ (Fig. 6C). Accordingly, EA4 and TP1 are likely to be useful agents for examining whether rPLA$_2$ is involved in the Ca$^{2+}$-dependent AA release from RBCs. The A23187-stimulated release of $[3H]$AA from human (Fig. 7A) and bovine (Fig. 7B) RBCs was significantly inhibited by EA4, but not TP1, whereas both EA4 and TP1 significantly inhibited the Ca$^{2+}$-
dependent release of AA from L929 cells (Fig. 7C) and human U937 cells (data not shown). These results strongly suggest a potential involvement of rPLA2 in the AA release.

High Expression of rPLA2 in Hemoglobinized Cells—It has been reported that activation of phospholipase A2 (33, 34) and AA metabolites, especially lipoxygenase products (10, 35), play an important role in erythropoiesis. This prompted us to examine the correlation between the level of rPLA2 and pseudoperoxidase activity of hemoglobinized cells. The PLAA activity was measured, and DAB staining for pseudoperoxidase was performed in MFL cells cultured in the presence and absence of EPO. As shown in Fig. 8A, when isolated fetal liver cells at 12 days of gestation were cultured for 3 days, single cells were largely reduced and instead DAB-positive colonies were found with the majority being colony-forming unit erythroid (CFU-E), which consist of 10–20 cells with morphological appearance of basophilic erythroblasts and numerous mitotic figures. In contrast, by 7 days of culture, few erythroid colonies could be seen and the benzidine-positive colonies disappeared with concomitant loss of rPLA2, as shown in immunoblotting analysis (Fig. 8B). Interestingly, despite disappearance of rPLA2 at 7 days of culture, the PLAA activity of the cell lysate was not significantly reduced and eventually identified as the activity preferentially hydrolyzing 2-[1-14C]AA-GPC to 2-[1-14C]LA-GPC and inhibited by mercurial compounds (data not shown), suggesting the induction of cPLA2 at this time. When the protein level of cPLA2 was measured by immunoblotting analysis using anti-cPLA2 antibody, it was found that cPLA2 was detected at 7 days of culture but not at 3 days (Fig. 8C). It was also shown that the levels of rPLA2 and hemoglobin were not significantly changed by EPO during differentiation, but a significant increase in the number of CFU-E was observed in EPO-treated cells (see Fig. 8A, panel b versus c). These results strongly suggest the correlation between activation of rPLA2 and definitive erythropoiesis of MFL cells.

**DISCUSSION**

Despite accumulating evidence that lipid-derived bioactive mediators such as AA and its metabolites play a potential role in pathophysiology of RBCs, little is known about PLAA as a major pathway leading to the production of the bioactive molecules from mammalian RBCs. In the present study we identified a novel Ca2+-dependent form of 42-kDa cytosolic PLAA, termed rPLA2, from bovine RBCs and demonstrated that rPLA2 can play an important role in the Ca2+-dependent release of AA from bovine and human RBCs.

Consistent with the previous study (7), we also found that the purified human and bovine RBCs could Ca2+-dependently release AA in a time-dependent manner (Fig. 1), assuming the presence of a Ca2+-dependent PLAA. The specific activity of the 100,000 × g supernatants from bovine and human RBCs was 0.4 and 0.3 pmol/min/mg of protein, respectively, whose values were much lower by ~500- to ~1000-fold under our assay system compared with other non-erythroid cells. Human monocytic U937 cells are known to contain cPLA2 as majority (13) but lack rPLA2, as shown in Fig. 4B. Moreover, the total activity of the cytosolic fractions per 10^6 cells was very low by 0.060 pmol/min in bovine RBCs and 0.045 pmol/min in human RBCs compared with 30 pmol/min in U937 cells. To further explain this low PLAA activity of RBCs, we compared the amounts of [3H]AA released between bovine RBCs and U937 cells for the same cell counts and found that the incorporation rate and release of [3H]AA in the RBCs were less by ~2- and ~1000-fold than those in U937 cells, respectively. Although the reason for this low activity remains unknown, in this context, it will be noteworthy that Kobayashi and Levine (7) pointed out that the level of HETEs produced by lipoxygenase was very low in A23187-stimulated RBCs (0.01–0.2 ng of 12-HETE/10^6 cells) compared with that produced by A23187-stimulated rat basophil leukemia cells (160 ng/10^6 cells (36)) or even A23187-stimulated mouse lymphoma cells (32 ng/10^6 cells (36)). It may be, of course, possible that this extremely low level of HETEs in RBCs is due to a low level of lipoxygenase rather than rPLA2. However, they proposed that the mechanism by PLAA is the most likely mechanism for deacylation of radiolabeled AA and showed that, concomitant with this decylation, was the appearance of the lipoxygenase products (7), suggesting that this low level of HETEs may be caused by the low PLAA activity. The possibility cannot be excluded that the low total activity results from a small count of contaminating platelets or neu-
A 42-kDa Ca^{2+}-dependent PLA_2 in Cytosol of Mammalian RBCs

Fig. 6. Inhibition of rPLA_2 by quinone derivatives, EA4 and TP1. A, structure of quinone derivatives, EA4 and TP1. B, quinone derivatives were chemically synthesized as described under “Materials and Methods,” and two derivatives, EA4 and TP1, were obtained by determining the inhibitory activity for rPLA_2 and cPLA_2. The purified PLA_2 and cPLA_2 were incubated at 37 °C with the inhibitors of the indicated concentrations dissolved in 5 μl of MeSO for 10 min followed by the addition of the substrate 2-[1-C]AA-GPC. Then the assay system was further incubated for 30 min, and the residual PLA_2 activity was measured as described under “Materials and Methods.” C, determination of the inhibitory pattern on rPLA_2 by EA4. The rPLA_2 activity was assayed for 15 min at 37 °C in the presence of the indicated concentrations of EA4 and 9 μM (■) or 72 μM (○) of 2-[1-C]AA-GPC as described under “Materials and Methods.” Shown are values from one experiment representative of three independent experiments producing similar results.

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further study, these inhibitors rendered us to obtain similar results for shear stress-induced AA release from the RBCs. It seems to be especially important that rPLA₂ requires Ca²⁺ for its activation. It is known that shear stress upon the RBCs induces the elevation of intracellular Ca²⁺ concentration (40), and RBC pathophysiology such as an alteration of deformability and aging is provoked by this increased Ca²⁺ (41, 42). More importantly, it may be that lysophosphatidic acid (43), which is a lipid-derived second messenger generated possibly by activation of PLA₂ and prostaglandin E₉ (43) is required for catalytic activity of PLA₂ in RBCs. These observations suggest that PLA₂ may not only play an important role in pathophysiology of RBCs through the production of AA and eicosanoids but also amplify this process through its further activation by the increased Ca²⁺. To investigate the role of rPLA₂ in the Ca²⁺-dependent AA release, we developed an inhibitor for rPLA₂ by chemically synthesizing quinone derivatives. EA4, an inhibitor for both rPLA₂ and cPLA₂, significantly inhibited A23187-induced AA release from both human and bovine RBCs in a time-dependent manner, whereas TP1, which inhibited cPLA₂, not rPLA₂, failed to reduce the AA release in these RBCs (Fig. 7). However, TP1 markedly reduced A23187-induced AA release from murine L929 cell line (Fig. 7C) and human U937 leukemia cells (data not shown), where cPLA₂ exists as major protein for rPLA₂ and cPLA₂, respectively) was obtained from cultured MFL cells as described in Fig. 4 and immunoblotted using anti-rPLA₂ (a), anti-cPLA₂ (c) antisera as described under "Materials and Methods." Lanes 1–5 of each of the immunoblotting gels indicate samples prepared from MFL cells a–e of A, respectively. Data presented are from a representative of three experiments with similar results. Finally, it is known that, during normal murine embryogenesis, beginning on days 7–8 of gestation, the yolk sac blood islands serve as the site for primitive erythropoiesis and, by day 11, the fetal liver becomes the major site of RBCs production as erythroid progenitor cells of >90% (44, 45). During such latter process termed definitive erythropoiesis, CFU-E can be found as an enriched population in the fetal liver; at days 12–13 of gestation, it is estimated that 70–80% of fetal liver cells are CFU-E (46). In the present study, DAB staining for pseudoperoxidase of hemoglobin as a marker for erythroid cells suggests that rPLA₂ plays an important role in the erythropoiesis of fetal liver cells. As shown in Fig. 8, the protein level of rPLA₂ paralleled the pseudoperoxidase activity of hemoglobinized
cells. Despite disappearance of rPLA₂ at 7 days of culture, the PLA₂ activity of the cell lysate at this point was not significantly reduced and inhibited by mercurial compounds, suggesting cPLA₂ activity (data not shown). When the protein level of rPLA₂ was measured by immunoblot analysis using anti-cPLA₂ antibody, we found that cPLA₂ was detected in the cell lysate at 7 days of culture, suggesting a possible role of rPLA₂ and cPLA₂ in erythroid and non-erythroid cells, respectively. On the other hand, it has been generally accepted that EPO and EPO receptor are crucial and irreplaceable for definitive erythropoiesis in vivo, whereas none of these are required for erythroid lineage commitment or for the proliferation and differentiation of MFL cells (47). Consistent with this, our results showed that the levels of rPLA₂ and hemoglobin were not significantly changed by EPO during differentiation (Fig. 8). Despite many lines of evidence that PLA₂ and AA metabolites are involved in erythropoiesis (10, 30, 33), at the present time, whether rPLA₂ affects such definitive erythropoiesis of MFL cells remains to be studied.

In summary, we present here for the first time that bovine and human RBCs are equipped with a novel form of 42-kDa Ca²⁺-dependent PLA₂ in cytosol, which is likely to be involved in the Ca²⁺-dependent release of AA from the RBCs. Our results could be of importance to better understand a phenomenon that may be related to the known clinical participation of RBCs in hemostasis, thrombosis, and/or erythropoiesis. Further studies are currently underway to elucidate molecular mechanisms leading to its activation by pathophysiological stimuli on RBCs, to clone cDNA encoding rPLA₂, and to link AA generated by rPLA₂ to the production of eicosanoids in RBCs or platelets.

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