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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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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 PLA_2 in bovine and human RBCs and purified it to apparent homogeneity with a 14,000-fold purification. The purified enzyme, termed $rPLA_2$, has a molecular mass of 42 kDa and reveals biochemical properties similar to group IV $cPLA_2$, but shows different profiles from $cPLA_2$ in several column chromatographies. Moreover, $rPLA_2$ did not react with any of anti-$cPLA_2$ and anti-$sPLA_2$ 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 $rPLA_2$ and $cPLA_2$, whereas mercurials inhibited $cPLA_2$ but had no effect on $rPLA_2$. Antibody against the 42-kDa protein not only precipitated the $rPLA_2$ 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 $rPLA_2$, 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 diaminobenzidine staining of hemoglobinized fetal liver cells showed that $rPLA_2$ detectable in erythroid cells was down-regulated when differentiated to non-erythroid cells. Together, our results suggest that the 42-kDa $rPLA_2$ identified as a novel form of Ca^{2+}-dependent PLA_2 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)[1] 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 (PLA_2). 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 lipoxygenase 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 PLA_2.

In the last several decades, many types of mammalian PLA_2s 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 PLA_2 activity in RBC membranes

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[1] 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; FPLC, fast-protein liquid chromatography; BSA, bovine serum albumin; EA4, 7-chloro-6-[4-(diethylamino)phenyl]-5,8-quinolinedione; TP1, 2-(3,5-di-*tert*-butyl-4-hydroxyphenyl)-3-chloro-1,4-naphthalene dione; 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 immunochemical studies 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-Stearoyl-2-[1-^{14}C]arachidonyl-*sn*-glycerol-3-phosphocholine (2-[1-^{14}C]AA-GPC, 55.3 mCi/mmol), 1-palmitoyl-2-[1-^{14}C]palmitoyl-*sn*-glycerol-3-phosphocholine (2-[1-^{14}C]PA-GPC, 55.6 mCi/mmol), 1-palmitoyl-2-[1-^{14}C]linoleoyl-*sn*-glycerol-3-phosphocholine (2-[1-^{14}C]LA-GPC, 55.9 mCi/mmol), 1-acyl-2-[1-^{14}C]arachidonyl-*sn*-glycerol-3-phosphoethanolamine (2-[1-^{14}C]AA-GPE, 55.1 mCi/mmol), and [^{3}H]arachidonic acid ([^{3}H]AA, 204 Ci/mmol) were purchased from the radio-chemical center, Amersham Biosciences, Inc. (Buckinghamshire, UK). 1-Stearoyl-2-arachidonyl-*sn*-glycerol-3-phosphocholine (2-AA-GPC), dithiothreitol, A23187, 3,3′-diaminobenzidine (DAB), methylcellulose, erythropoietin, and a Sepharose 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-alkaline phosphatase conjugates were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA. Group IV cytosolic PLA_2 ($cPLA_2$) was purified from porcine spleen, and anti-$cPLA_2$ polyclonal antibody was generated as described previously (21). Group II secretory PLA_2 ($sPLA_2$) was partially purified from bovine platelets as described previously (22). Butyl-Toyopearl 650M gel, preparative Phenyl-5PW, analytical Phenyl-5PW, DEAE-5PW HPLC columns were purchased from Tosoh Co. (Tokyo, Japan). Sephacryl S-300 gel filtration, Superose 12 gel filtration, PD-10 desalting (Sephadex G-25M), and Mono Q FPLC columns, and Protein A-Sepharose CL-4B beads were purchased from Amersham Biosciences, Inc. (Uppsala, Sweden). Arachidonyl trifluoromethyl 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 in heparin (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 (7). The filtered cell suspensions contained the following numbers of blood cells: for human blood, $<3 \times 10^5$ platelets/ml, $<2 \times 10^4$ leukocytes/ml, and $4–5 \times 10^9$ RBCs/ml; for bovine blood, $<4 \times 10^5$ platelets/ml, $<3 \times 10^4$ leukocytes/ml, and $3–5 \times 10^9$ RBCs/ml. Differential cell counts were measured with a Coulter counter (Becton Dickinson UK, Oxford, UK).

Release of [^{3}H]AA by A23187 from Human and Bovine RBCs and in Vitro Assay of PLA_2 Activity—The Sepharose 4B-200 column-purified RBCs suspensions ($\sim 1 \times 10^9$ 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 [^{3}H]AA (1 μCi/μl ethanol)/ml of the same medium. Murine L929 cells ($1–2 \times 10^6$ cells/ml) were labeled for 6 h with 0.1 μCi of [^{3}H]AA (0.1 μCi/μl ethanol)/ml of the same medium. Thereafter, cells were washed three times to remove all unincorporated [^{3}H]AA. The labeled cells were incubated in MEM containing 1 mg/ml BSA as a trap for the released [^{3}H]AA and then stimulated with vehicle (1.0 μl of ethanol/ml medium) or the agonists as indicated. For analysis of [^{3}H]AA release, the RBCs were centrifuged as above, and each aliquot (200 μl) of the supernatants for the RBCs and each aliquot (100 μl) of the conditioned media for the L929 cells was transferred to 2.5 ml of the scintillation solution and counted for radioactivity with a Packard Tri-carb liquid β-scintillation counter (Packard Instrument Co., Meriden, CT). The total incorporated [^{3}H]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 [^{3}H]AA, respectively, and measuring the radioactivity of each aliquot of the supernatants. The total incorporated [^{3}H]AA into L929 cells was measured by counting the radioactivity of an aliquot (50 μl) of the cell lysates obtained after washing the cells three times with 10 ml of phosphate-buffered saline and then adding 1 ml of 0.5 N NaOH solution. On the other hand, PLA_2 activity was measured in an assay system (100 μl) of 75 mM Tris-HCl (pH 7.5) containing 45.0 μM 2-[1-^{14}C]AA-GPC (110,000 cpm/4.5 nmol) mixed with 2-AA-GPC as substrate, 4% glycerol, 5 mM $CaCl_2$, 0.2% BSA as described previously (21).

Purification of $rPLA_2$ from Bovine RBCs—The packed RBCs were prepared from bovine blood (4 liters) described as above and re-suspended in buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM 2-mercaptoethanol) containing 1 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride and used as the enzyme source for purification of PLA_2. First, to obtain cytosolic and membrane fractions from bovine RBCs, the resuspended packed cells were homogenized by sonicating in an ice bath at 40-watt output and 40% duty cycle for 20 s with a sonicator (Sonics & Materials Inc., Danbury, CT). The debris and unlysed cells were removed by centrifuging the homogenates at 3000 × *g* at 4 °C for 30 min. After the supernatants were again centrifuged at 100,000 × *g* at 4 °C for 2 h, the resulting supernatants and pellets were obtained as the cytosolic and membrane fractions, respectively. For the first step, the cytosolic fractions were adjusted to 0.5 M $(NH_4)_2SO_4$, stirred at 4 °C for 5 min, and loaded onto a Butyl-Toyopearl hydrophobic column (15.0 cm × 5.0 cm) pre-equilibrated with buffer A containing 0.5 M $(NH_4)_2SO_4$ at a flow rate of 20 ml/min. After washing with buffer A until no protein was eluted, the column-binding proteins were eluted at a flow rate of 20 ml/min with a stepwise gradient of distilled water. Next, a pool of the active fractions was adjusted to 0.5 M $(NH_4)_2SO_4$ and then loaded onto a preparative Phenyl-5PW hydrophobic HPLC column (21.3 mm × 15 cm) pre-equilibrated with buffer A containing 0.5 M $(NH_4)_2SO_4$ at a flow rate of 5.0 ml/min. The column-binding proteins were eluted at a flow rate of a 100-ml linear gradient of 0.5–0.0 M $(NH_4)_2SO_4$, and 5-ml fractions were collected. The active fractions were pooled and loaded onto a DEAE-5PW HPLC column (7.5 mm × 7.5 cm) pre-equilibrated with buffer A at a flow rate of 1.0 ml/min. Proteins bound to the column were eluted with a 20-ml linear gradient of 0.0–1.0 M NaCl, and 1-ml fractions were collected. The active fractions from the DEAE-5PW column were pooled and injected onto a Sephacryl S-300 gel filtration column (30 mm × 60 cm) pre-equilibrated with buffer A containing 0.1 M NaCl. The column was eluted with the same buffer at a flow rate of 1 ml/min. The active pool was continuously adjusted to 0.5 M $(NH_4)_2SO_4$ and then loaded onto an analytical Phenyl-5PW hydrophobic HPLC column (7.5 mm × 7.5 cm) pre-equilibrated with buffer A containing 0.5 M $(NH_4)_2SO_4$ at a flow rate of 1.0 ml/min. The column-binding proteins were eluted at a flow rate of 1 ml with a 20-ml linear gradient of 0.5–0.0 M $(NH_4)_2SO_4$. The fractions of the major peak activity eluted were pooled and used for further purification. The active pool was concentrated into ~250 μl using a Centricon 10 (Amicon Co., Beverly, MA) and injected onto a Superose 12 gel filtration FPLC column (10 mm × 30 cm) pre-equilibrated with buffer A containing 0.1 M NaCl. The column was eluted with the same buffer at a flow rate of 0.5 ml/min. 0.5-ml fractions were collected. Finally, this active fractions were loaded onto a Mono Q FPLC column (5.0 mm × 5.0 cm) pre-equilibrated with buffer A adjusted to pH 8.0 at a flow rate of 1.0 ml/min. Proteins bound to the column were eluted with a 20-ml linear gradient of 0.0–1.0 M NaCl, and 1-ml fractions were collected. To monitor the amount of protein during purification of $rPLA_2$, the A_{280} was measured by a UV detector. Protein concentration of each sample was measured with Bradford reagents (Bio-Rad, Hercules, CA) using BSA as a standard.

SDS-PAGE—One-dimensional denaturing SDS-PAGE was performed on 10% polyacrylamide gels according to Laemmli's procedure (23) in a Bio-Rad Protean II electrophoresis system. Two-dimensional gel electrophoresis was performed according to O'Farrell (24) using the IPG-phor (Amersham Biosciences, Inc., Uppsala, Sweden) system according to the instructions of the manufacturer. The separated proteins

were stained with a PlusOne silver staining kit (Amersham Biosciences, Inc., Piscataway, NJ).

Immunochemical Study of rPLA$_2$—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 antiserum (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, 2.0% (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 PLA_2 activity. The pellets were washed six times with buffer B containing 0.1% Tween 20 and 0.5 M 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 (Amersham Biosciences, Inc. UK Ltd., Buckinghamshire, UK), and visualized as described previously (21). The membranes were exposed to the antisera against $rPLA_2$ (1:5000), $cPLA_2$ (1:2000), and $sPLA_2$ (1:2000), respectively, and incubated with a 1:2500 dilution of goat anti-rabbit or anti-mouse-alkaline phosphatase conjugate in Tris-buffered saline (25 mM Tris-HCl, pH 8.0, 143 mM NaCl, 3 mM KCl) containing 0.1% Tween 20 and 5% skim milk 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 α-cyano-4-hydroxycinnamic acid as the matrix. Trypsin autolysis products were used for internal calibration. Delayed ion extraction resulted in peptide masses with better than 50 ppm mass accuracy on average. Comparison of the mass value against the Swiss-Prot data base was performed using 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 *N*,*N*-diethylaniline (Aldrich). Briefly, a solution of 5,8-quinolinedione (6.28 mmol) and $Cu(CH_3COO)_2 \cdot H_2O$ (6.28 mmol) in 80 ml of acetic acid was added to a solution of *N*,*N*-diethylaniline (6.28 mmol) 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-*tert*-butyl-4-hydroxyphenyl)-3-chloro-1,4-naphthalene dione (TP1) was synthesized and characterized as described previously (29).

Murine Erythroid Progenitor Cell Assay—To obtain murine fetal liver (MFL) cells, adult male and female CD-1 mice (Dae Han Biolink Co., Ltd., Eumsung-Gun, Chungbuk, Korea) underwent timed matings. At days 12–13 after mating, the female mice were killed while 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 minimum essential medium with glutamine (α-MEM, Invitrogen, Grand Island, NY), and resuspended in 5 ml of α-MEM. Isolated murine fetal liver cells (1×10^5/ml) were plated in a mixture (DAB mixture) containing α-MEM, 0.8% methylcellulose, 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% CO_2. After 3 or 7 days, the dishes were stained for pseudoperoxidase with DAB and hydrogen peroxide as described previously (31).

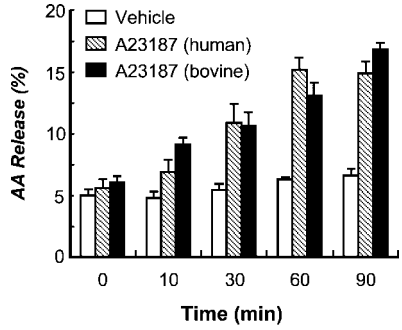

FIG. 1. **Release of [^{3}H]AA by a calcium ionophore from human and bovine RBCs.** Human and bovine RBCs (2 ml/sample, 1.0 ~ 1.2 × 10^9 cells/ml in MEM containing 1 mg/ml BSA) were labeled with 1.5 μCi of [^{3}H]AA/ml for 1 h, washed with MEM, and incubated with A23187 (2 μM) in MEM containing 1 mg/ml BSA for the indicated times at 37 °C, respectively. Each sample (250 μl) of the incubation media was obtained for analysis of [^{3}H]AA release as described under "Materials and Methods." The data presented are from a representative experiment that was repeated five times with similar results.

RESULTS

Detection of a Cytosolic Ca^{2+}-dependent PLA_2 in Human and Bovine RBCs—A calcium ionophore A23187 released [^{3}H]AA from the purified human and bovine RBCs in a time-dependent manner (Fig. 1). The releases of [^{3}H]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, PLA_2 activity was assayed by analyzing the reaction products with thin layer chromatography using various phospholipids as described previously (21). A Ca^{2+}-dependent PLA_2 activity, which preferred 2-[1-^{14}C]AA-GPC to 2-[1-^{14}C]LA-GPC and 2-[1-^{14}C]PA-GPC by 8.5- and 25.2-fold, respectively, was detected in the cytosolic fractions and hydrolyzed preferentially 2-[1-^{14}C]AA-GPE to 2-[1-^{14}C]AA-GPC by 1.7-fold. On the other hand, the membrane-bound PLA_2 activity from the 100,000 × *g* pellets was Ca^{2+}-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 PLA_2 from the cytosolic fractions suggests that this enzyme may be similar to group IV $cPLA_2$. To further examine this, elution profiles between the RBC PLA_2 and $cPLA_2$ from porcine spleen were compared in hydrophobic, anionic exchange, and gel filtration column chromatographies, respectively. As shown in Fig. 2, each of these two PLA_2 enzymes were eluted at different fractions in all of the columns utilized, and in particular, the RBC PLA_2 migrated as a molecular mass of ~40 kDa in a Superose 12 gel filtration FPLC column (Fig. 2*D*).

Purification of a Cytosolic PLA_2 from Bovine RBCs—As shown in Table I, the purification of the cytosolic PLA_2 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 PLA_2. 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 (2000 kDa), 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

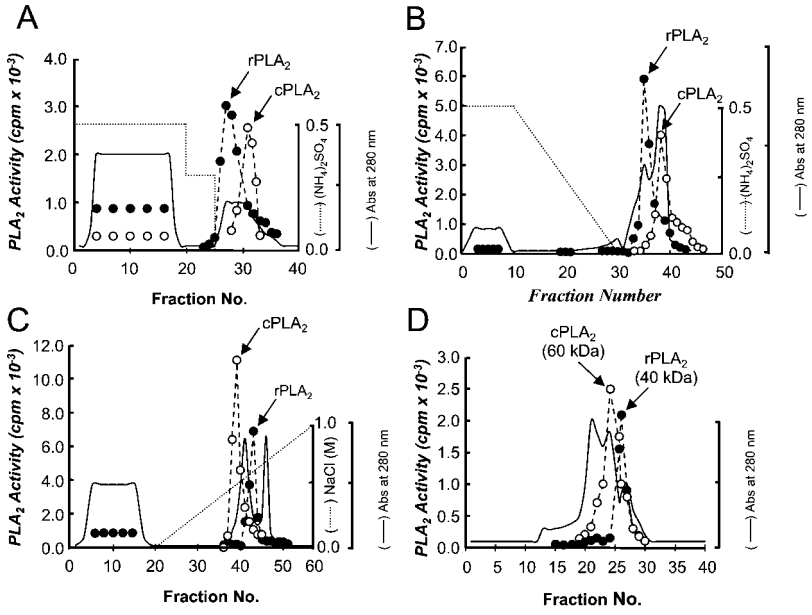

FIG. 2. **Comparison of elution profiles between PLA_2 enzymes from bovine RBCs and porcine spleen.** The PLA_2 enzymes from RBCs and spleen were prepared by homogenizing the purified packed bovine RBCs and porcine spleen tissue slices, respectively, as described under "Materials and Methods." Elution 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 I
Summary of purification of rPLA$_2$ from bovine RBCs

Purification step	Total protein	Total activity	Yield	Specific activity	Change
	mg	*pmol/min*	*%*	*pmol/min/mg protein*	*-fold*
S100	46,000	18,400	100.0	0.4	1
Butyl-Toyopearl	700	7,280	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
Superose 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. 3*A*, *inset*), and a single spot was observed in a two-dimensional SDS-PAGE (Fig. 3*B*), 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, antiserum against the 42-kDa protein precipitated the PLA_2 activity in a time-dependent manner (Fig. 3*C*). In addition, when each of the immunoprecipitates of pre-immune serum and antiserum was washed and subjected to SDS-PAGE and silver staining, only the antiserum precipitated the 42-kDa protein (Fig. 3*D*).

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. 4*A*), 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. 4*B* 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-^{14}C]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 μM and the V_{max} value was 7.4 nmol/min/mg of protein with 2-[1-^{14}C]AA-GPC (Fig. 5*A*) and revealed the high selectivity for phospholipids containing AA at the *sn*-2 position (Fig. 5*B*). The pI of rPLA$_2$ has ranged from about 3.9 to 4.1 (Fig. 3*B*). Although profiles of Ca^{2+} requirements (Fig. 5*C*), pH dependence (Fig. 5*D*), effects on some enzymatic inhibitors (Fig. 5, *E*, *F*, and *G*), and divalent metals such as Zn^{2+}, Fe^{2+}, 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. 5*H*), mercuric chloride (data not shown), and quinone derivative TP1 (Fig. 6*B*) 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. 6*A*). Although EA4 inhibited both rPLA$_2$ and cPLA$_2$, TP1 inhibited cPLA$_2$ but not rPLA$_2$ (Fig. 6*B*). A Dixon plot was constructed to show that the inhibition of rPLA$_2$ by EA4 is competitive, but not uncompetitive, with an inhibition constant

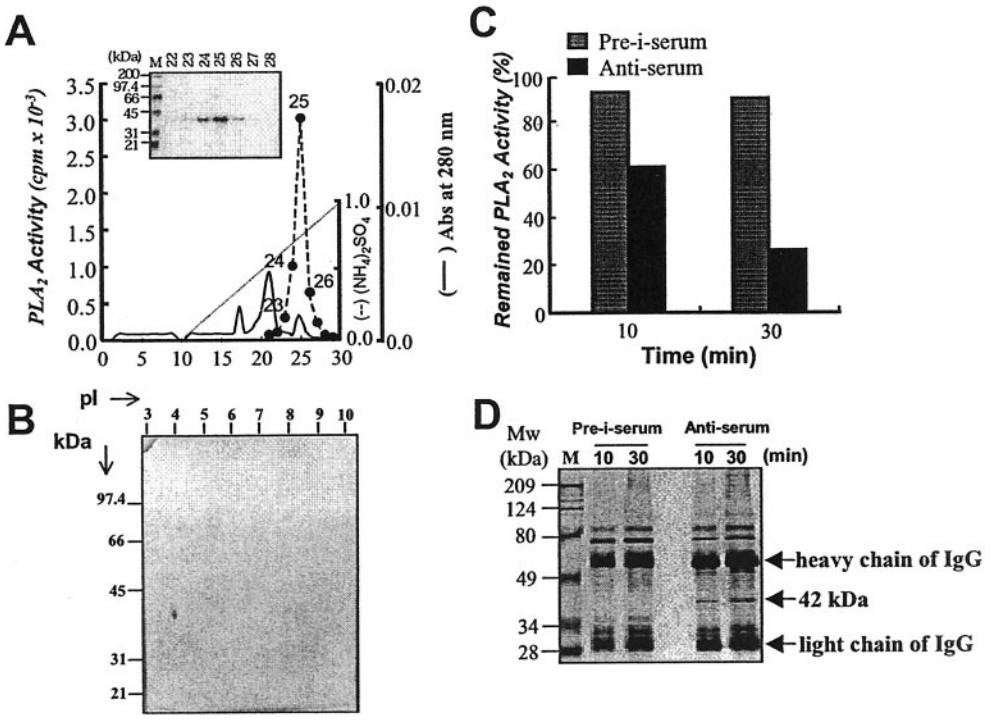

FIG. 3. **Purification and immunoprecipitation of $rPLA_2$.** *A*, the active pool from the Superose 12 column was applied to the Mono Q column as described under "Materials and Methods." 20 μl of each fraction (1 ml) was assayed for the PLA_2 activity using 2-[1-^{14}C]AA-GPC as substrate. Each aliquot (20 μl) of the active fractions from the Mono Q column was subjected to 10% SDS-PAGE followed by silver staining, and the *numbers of the lanes* correspond to those of the active fractions from the column (*inset*). *B*, two-dimensional electrophoresis of $rPLA_2$ was performed. In the first dimension an aliquot (20 μl) of fraction 25 from the Mono Q column was separated on a 13-cm IPG-Strip (pH 3–10). A 12% Tris-HCl gel was used for the second dimension. Proteins were visualized by silver staining. *C*, the PLA_2 activity partially purified from the Superose 12 column was immunoprecipitated and (*D*) the 42-kDa protein in the immunoprecipitates was analyzed by immunoblot as described under "Materials and Methods." Total activity of the control was 11.5 pmol/min at 0 min after incubating with the beads.

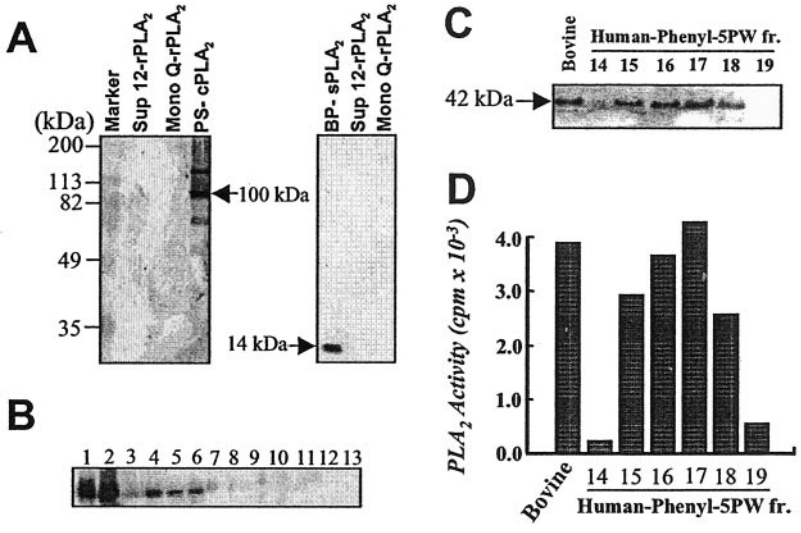

FIG. 4. **Detection of $rPLA_2$ in various tissues and cells.** *A*, the Superose 12 column-purified bovine RBC PLA_2, Mono Q column-purified bovine RBC PLA_2, porcine spleen $cPLA_2$, and bovine platelet $sPLA_2$ were immunoblotted with anti-$cPLA_2$ or anti-$sPLA_2$ antiserum as described under "Materials and Methods." *B*, Phenyl 5PW-purified $rPLA_2$ (*lane 1*), Mono Q-purified $rPLA_2$ (*lane 2*), MDCK cells (*lane 3*), MFL cells were obtained as described under "Materials and Methods" and treated for 2 h with 1% (v/v) water as vehicle (*lane 4*), 0.2 units (*lane 5*), 0.5 units (*lane 6*) of EPO, L929 cells (*lane 7*), and U937 cells (*lane 8*) were maintained in MEM at 37 °C under 5% CO_2 in air at a density of 2–3 $\times$ 10^6/ml. These cultured cells were passaged once or twice each week to maintain exponential phase growth. Rat tissues, brain (*lane 9*), kidney (*lane 10*), lung (*lane 11*), liver (*lane 12*), and spleen (*lane 13*), were dissected from Sprague-Dawley male rat under ether anesthesia. The cells and tissues were resuspended in buffer A containing 0.12 M NaCl and sonicated for 20 s in an ice bath at 40-W output and 40% duty cycle with a sonicator. The lysates were centrifuged at 100,000 $\times$ *g* at 4 °C for 1 h. Each sample (50 μg of protein) was immunoblotted using anti-$rPLA_2$ antiserum as described under "Materials and Methods." According to the purification procedure identical to that for bovine RBCs, (*C*) human RBC PLA_2 was partially purified from the 100,000 $\times$ *g* supernatants. The Phenyl-5PW(I) column fractions were subjected to immunoblotting analysis and (*D*) assayed for PLA_2 activity. For comparison, the Phenyl-5PW(I)-purified bovine RBC PLA_2 was also loaded on the first lane.

of K_i = 130 μM (Fig. 6*C*). 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 [^{3}H]AA from human (Fig. 7*A*) and bovine (Fig. 7*B*) 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. 7*C*) and human U937 cells (data not shown). These results strongly suggest a potential involvement of $rPLA_2$ in the AA release.

High Expression of $rPLA_2$ in Hemoglobinized Cells—It has been reported that activation of phospholipase A_2 (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 $rPLA_2$ and pseudoperoxidase activity of hemoglobinized cells. The PLA_2 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. 8*A*, 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 $rPLA_2$ as shown in immunoblotting analysis (Fig. 8*B*). Interestingly, despite disappearance of $rPLA_2$ at 7 days of culture, the PLA_2 activity of the cell lysate was not significantly reduced and eventually identified as the activity preferentially hydrolyzing 2-[1-^{14}C]AA-GPC to 2-[1-^{14}C]LA-GPC and inhibited by mercurial compounds (data not shown), suggesting the induction of $cPLA_2$ at this time. When the protein level of $cPLA_2$ was measured by immunoblotting analysis using anti-$cPLA_2$ antibody, it was found that $cPLA_2$ was detected at 7 days of culture but not at 3 days (Fig. 8*C*). It was also shown that the levels of $rPLA_2$ 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. 8*A*, *panel b versus c*). These results strongly suggest the correlation between activation of $rPLA_2$ 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 PLA_2 as a major pathway leading to the production of the bioactive molecules from mammalian RBCs. In the present study we identified a novel Ca^{2+}-dependent form of 42-kDa cytosolic PLA_2, termed $rPLA_2$, from bovine RBCs and demonstrated that $rPLA_2$ can play an important role in the Ca^{2+}-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 Ca^{2+}-dependently release AA in a time-dependent manner (Fig. 1), assuming the presence of a Ca^{2+}-dependent PLA_2. 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 $cPLA_2$ as majority (13) but lack $rPLA_2$ as shown in Fig. 4*B*. 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 PLA_2 activity of RBCs, we compared the amounts of [^{3}H]AA released between bovine RBCs and U937 cells for the same cell counts and found that the incorporation rate and release of [^{3}H]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

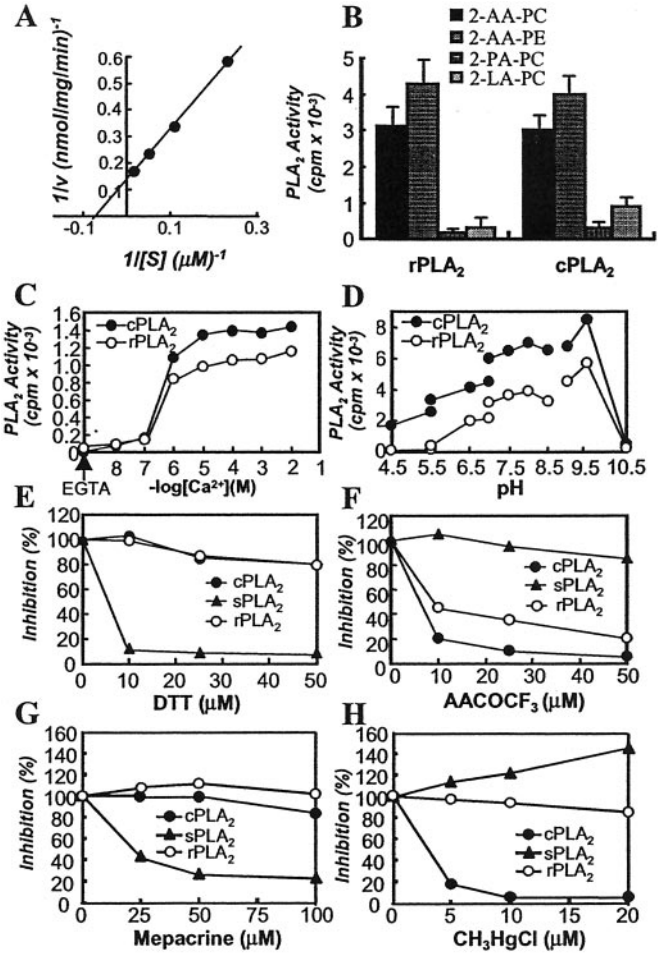

FIG. 5. **Lineweaver-Burk analysis and characterization of $rPLA_2$.** The active pool from the Mono Q column was desalted using a PD-10 desalting column pre-equilibrated with a buffer (10 mM Tris-HCl, pH 7.5). In some experiments, the enzyme sources and appropriate amounts of inhibitors or metals were mixed and preincubated for 5 min at 37 °C, followed by addition of the substrate. *A*, an aliquot of the active pool from the Mono Q column was incubated with 2-[1-^{14}C]AA-GPC of the indicated concentrations. Results are means of duplicate determinations. *B*, an aliquot of the active pool from the Mono Q column was assayed for the activity using 0.9 μM 2-[1-^{14}C]AA-GPC, 2-[1-^{14}C]AA-GPE, 2-[1-^{14}C]LA-GPC, and 2-[1-^{14}C]PA-GPC as substrates, respectively. *C*, Ca^{2+} dependence and *D*, pH dependence as described previously (21, 25), and *E–H*, for dose-dependent inhibition of various PLA_2 inhibitors, $rPLA_2$ was obtained from the active pool from the Mono Q column, $cPLA_2$ was purified as described previously (21), and $sPLA_2$ was partially purified from bovine platelets as described previously (22). Each aliquot of these PLA_2 enzymes, equivalent to 0.18–0.21 nmol/10 min for 45.0 μM 2-[1-^{14}C]AA-GPC, was preincubated with the indicated concentrations of inhibitors at 37 °C for 5 min followed by addition of the substrate. Each assay was further incubated at 37 °C for 10 min for the PLA_2 activity. Data presented are from a representative of four experiments with similar results.

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 $rPLA_2$. However, they proposed that the attack by PLA_2 is the most likely mechanism for deacylation of radiolabeled AA and showed that, concomitant with this deacylation, was the appearance of the lipoxygenase products (7), suggesting that this low level of HETEs may be caused by the low PLA_2 activity. The possibility cannot be excluded that the low total activity results from a small count of contaminating platelets or neu-

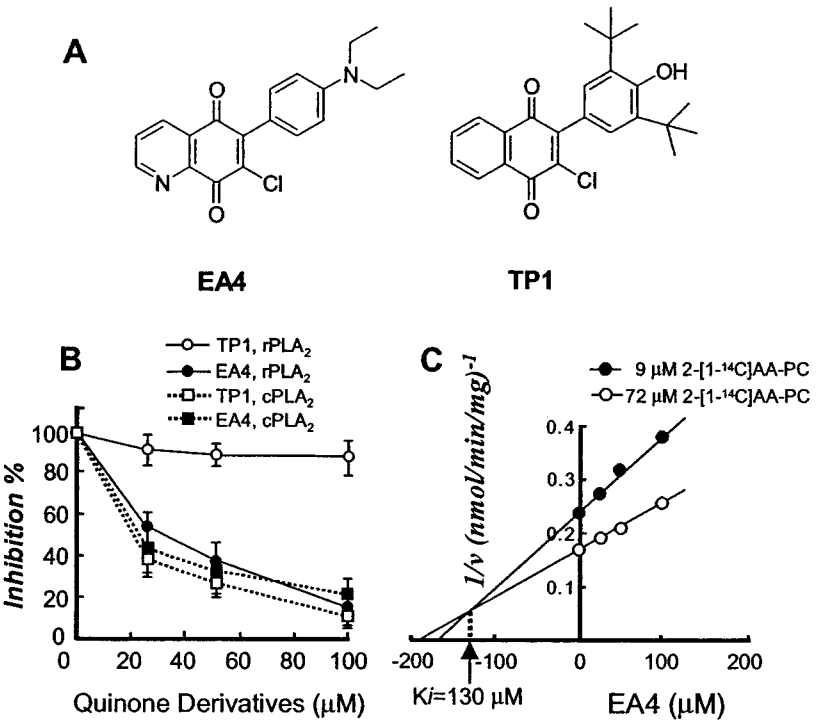

FIG. 6. **Inhibition of $rPLA_2$ by quinone derivatives, EA4 and TP1.** *A*, structure of quinone derivatives, EA4 and TP1. *B*, quinone derivatives were chemically synthesized as described under "Materials and Methods," and two derivatives, EA4 and TP1, were obtained by determining the inhibitory activity for $rPLA_2$ and $cPLA_2$. The purified $rPLA_2$ and $cPLA_2$ were incubated at 37 °C with the inhibitors of the indicated concentrations dissolved in 5 μl of Me_2SO for 10 min followed by the addition of the substrate 2-[1-^{14}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-^{14}C]AA-GPC as described under "Materials and Methods." Shown are values from one experiment representative of three independent experiments producing similar results.

trophils in the purified RBCs suspensions rather than RBCs. However, this is not the case, because the Sepharose 4B-200 gel filtration of a RBC suspension that already had been washed six times reduced the cell counts of RBCs, white blood cells, and platelets to an additional 9%, 85%, and 92%, respectively, without affecting the total activity. Together, this low release of AA or its metabolites is likely to be due to the low PLA_2 activity in the cytosolic fractions of RBCs, which would have made it difficult for previous researchers to detect a cytosolic form of PLA_2 activity in the cells. On the other hand, like the previous observations, the membrane-bound PLA_2 activity was Ca^{2+}-dependent and too low to be detectable but markedly enhanced by deoxycholate. Moreover, we found that this membrane-bound enzyme is different from $rPLA_2$, $cPLA_2$, or $sPLA_2$ as evidenced by some biochemical and immunochemical data.[2] Thus, bovine and human RBCs appears to consist of at least two novel forms of PLA_2 in cytosolic and membrane fractions, respectively.

In this report we purified for the first time a cytosolic form of PLA_2 to near homogeneity from bovine RBCs and characterized it as a novel RBC form of PLA_2 through immunochemical experiments (Figs. 3*C*, 3*D*, 4) and inhibitor studies (Figs. 5*H*, 6*B*). $rPLA_2$ exhibited biochemical properties similar to $cPLA_2$ but was different in many features: chromatographic profiles (Fig. 2), immunoreactivity with anti-$cPLA_2$ and anti-$sPLA_2$ polyclonal antibodies (Fig. 4*A*), and sensitivity to several chemicals (Figs. 5*H*, 6*B*). If not unexpected, the most prominent biochemical property of $rPLA_2$ is a very low specific activity of 5.6 nmol/min/mg of protein compared with 3800~8630 nmol/min/mg of protein for $cPLA_2$ (37, 38) or 40~1500 μmol/min/mg of protein for $sPLA_2$ (22, 39). Although at present the reason for this remains unknown, it is not likely that this is a result of less contribution of $rPLA_2$ to the AA release from RBCs in circulation, because RBCs constitute ~99% of the blood cell mass that may compensate for this low specific activity.

Several possibilities for this low specific activity can be raised as follows: 1) this 42-kDa protein is not a full-length protein but a proteolytic fragment. However, this may be not the case, because the addition of the various protease inhibitors into the homogenizing buffer could not be of any noticeable help to the increase in the activity of the resulting cytosolic fraction compared with their absence. In an independent experiment, when the active fractions of the Butyl-Toyopearl column obtained by the use of the inhibitors in the whole process were concentrated and applied to a gel filtration column, the activity was eluted at the same fractions of a molecular mass of ~40 kDa compared with those without the inhibitors (data not shown). Moreover, Western blotting analysis showed that the anti-42-kDa protein antibody reacted with ~55-kDa protein as well as the 42-kDa protein in the active fractions of the early steps of the purification. However, the intensity of this cross-reacted band was not paralleled with the PLA_2 activity in the active fractions of the Superose 12 gel filtration HPLC column, and this band appeared in the residual fractions showing no PLA_2 activity, but not in the active fractions, in the Mono Q column of the final step in the purification process (data not shown); 2) the $rPLA_2$ activity may require a cofactor for the full activity, but we could find neither significant decrease of <40% in the total activity in any step of the purification as shown in Table I nor fraction increasing the activity from the residual fractions of each column; 3) the present assay condition including 2-[1-^{14}C]AA-GPC as the substrate can not be fully optimized for the $rPLA_2$ activity. However, so far we have not found any better substrate and assay condition. 4% glycerol and 0.2%

[2] H. S. Shin, M.-R. Chin, J. S. Kim, S. Y. Jung, and D. K. Kim, unpublished data.

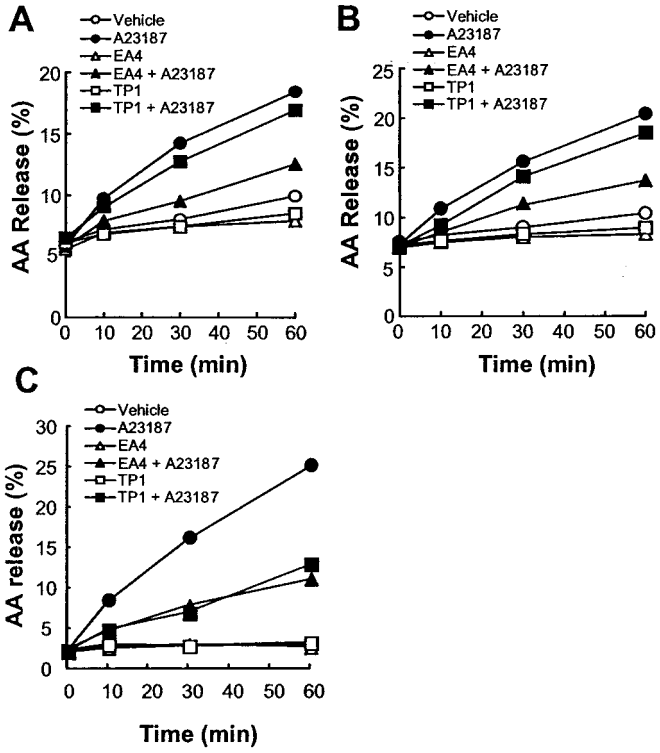

FIG. 7. **Inhibition of Ca^{2+} ionophore-induced AA release from mammalian RBCs by EA4.** *A*, human (2 ml/sample, 1.0–1.2 × 10^9 cells/ml in MEM containing 1 mg/ml BSA); *B*, bovine RBCs (2 ml/sample, 1.0–1.2 × 10^9 cells/ml in MEM containing 1 mg/ml BSA); and *C*, L929 cells (2 ml/sample, 1.2 × 10^6 cells/ml in MEM containing 1 mg/ml BSA) were labeled and washed three times with MEM, respectively, as described under "Materials and Methods." Human and bovine RBCs and L929 cells were pretreated with 50 μM EA4 or 50 μM TP1 (vehicle, 2 μl of Me_2SO) for 20 min and incubated with 2 μM A23187 (vehicle, 2 μl of Me_2SO) at 37 °C for the indicated times. The released [^{3}H]AA was measured as described in Fig. 1. Shown are values from one experiment representative of five times independent experiments producing similar results.

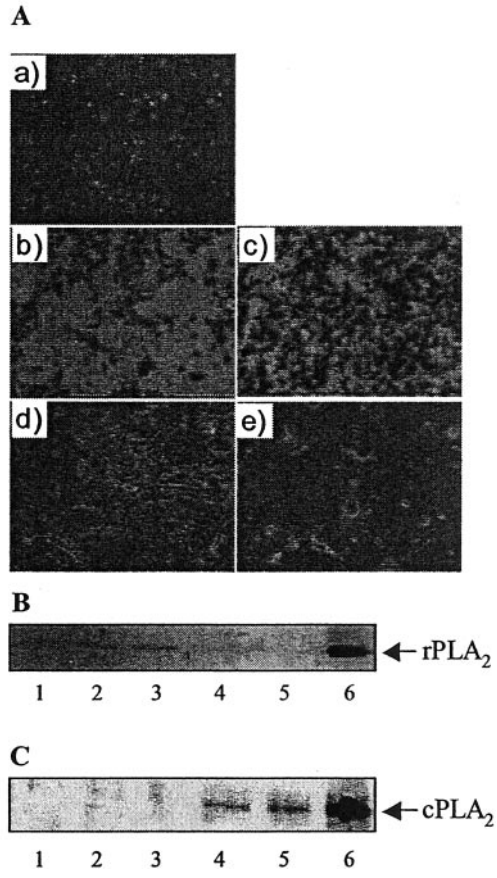

FIG. 8. **Induction of $rPLA_2$ during definitive erythropoiesis in murine fetal liver.** *A*, DAB staining of hemoglobinized MFL cells. The MFL cells were isolated from fetal liver at 12 days of gestation and plated as described under "Materials and Methods." The cells were cultured for 0 days (*a*), for 3 days in the absence of EPO (*b*), for 3 days in the presence of EPO (*c*), for 7 days in the absence of EPO (*d*), and for 7 days in the presence of EPO (*e*). Each sample (50 μg and 150 μg of protein for $rPLA_2$ and $cPLA_2$, respectively) was obtained from cultured MFL cells as described in Fig. 4 and immunoblotted using anti-$rPLA_2$ (*B*) and anti-$cPLA_2$ (*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.

BSA increased the activity by ~2-fold, respectively, whereas various detergents, including deoxycholate and Triton X-100, largely inhibited the activity even in the concentration of <0.1%.

It seems to be especially important that $rPLA_2$ requires Ca^{2+} for its activation. It is known that shear stress upon the RBCs induces the elevation of intracellular Ca^{2+} concentration (40), and RBC pathophysiology such as an alteration of deformability and aging is provoked by this increased Ca^{2+} (41, 42). More importantly, it may be that lysophosphatidic acid (43), which is a lipid-derived second messenger generated possibly by activation of PLA_2, and prostaglandin E_2 (11) are known to enhance intracellular Ca^{2+} in RBCs. These observations suggest that PLA_2 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 Ca^{2+}. To investigate the role of $rPLA_2$ in the Ca^{2+}-dependent AA release, we developed an inhibitor for $rPLA_2$ by chemically synthesizing quinone derivatives. EA4, an inhibitor for both $rPLA_2$ and $cPLA_2$, significantly inhibited A23187-induced AA release from both human and bovine RBCs in a time-dependent manner, whereas TP1, which inhibited $cPLA_2$, not $rPLA_2$, 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. 7*C*) and human U937 leukemia cells (data not shown), where $cPLA_2$ exists as majority, but lacks the 42-kDa $rPLA_2$ as shown in Fig. 4*A*. In our further study, these inhibitors rendered us to obtain similar results for shear stress-induced AA release from the RBCs.[2] These results demonstrate that $rPLA_2$ can play an important role in the Ca^{2+}-dependent AA release from bovine and human RBCs. It is also suggested that the membrane-bound PLA_2, as a different enzyme from $rPLA_2$, may be involved in the residual AA release, because the A23187-stimulated release of AA from both RBCs was not completely blocked by the inhibitor (Fig. 7). Whether Ca^{2+} for $rPLA_2$ activity is required for catalytic activity like $sPLA_2$ or triggering its translocation to the substrate membrane like $cPLA_2$ remains to be studied.

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 later 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 $rPLA_2$ plays an important role in the erythropoiesis of fetal liver cells. As shown in Fig. 8, the protein level of $rPLA_2$ paralleled the pseudoperoxidase activity of hemoglobinized

cells. Despite disappearance of $rPLA_2$ at 7 days of culture, the PLA_2 activity of the cell lysate at this point was not significantly reduced and inhibited by mercurial compounds, suggesting $cPLA_2$ activity (data not shown). When the protein level of $cPLA_2$ was measured by immunoblot analysis using anti-$cPLA_2$ antibody, we found that $cPLA_2$ was detected in the cell lysate at 7 days of culture, not at 3 days (Fig. 8*B*), suggesting a possible role of $rPLA_2$ and $cPLA_2$ 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 $rPLA_2$ and hemoglobin were not significantly changed by EPO during differentiation (Fig. 8). Despite many lines of evidence that PLA_2 and AA metabolites are involved in erythropoiesis (10, 30, 33–35), at the present time, whether $rPLA_2$ 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 Ca^{2+}-dependent PLA_2 in cytosol, which is likely to be involved in the Ca^{2+}-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 $rPLA_2$, and to link AA generated by $rPLA_2$ to the production of eicosanoids in RBCs or platelets.

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