Purification, characterization, and cDNA sequencing of cytosolic phospholipase A_2 from equine neutrophils[1]

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Abstract **It has been demonstrated that equine neutrophils, but not eosinophils, require exogenous arachidonic acid for calcium ionophore A23187-induced leukotriene synthesis. Because cytosolic phospholipase A_2 ($cPLA_2$) plays an essential role in leukotriene formation in leukocytes, we investigated the presence of a functional $cPLA_2$ in equine neutrophils. To determine whether $cPLA_2$ from neutrophils was catalytically active, we purified the enzyme >6,500 fold with 3% recovery from equine neutrophils. The full-length cDNA sequence encoded a 749-amino acid protein. The deduced amino acid sequence demonstrated 95% identity with human and mouse $cPLA_2$, as well as 83 and 73% identity with chicken and zebra fish $cPLA_2$ protein, respectively. The equine $cPLA_2$ possessed some properties that distinguished the equine enzyme from the human enzyme. First, the enzyme activity of the equine $cPLA_2$ was differently influenced by cations as compared with the human $cPLA_2$. Second, the equine neutrophil $cPLA_2$ migrated as an approximately 105-kDa protein, in comparison with human $cPLA_2$ which migrated as a 110-kDa protein. A difference between equine neutrophils and eosinophils in the degree of phosphorylation of the $cPLA_2$ protein was observed. Thus, the $cPLA_2$ protein from eosinophils was constitutively phosphorylated, while the $cPLA_2$ protein from neutrophils was unphosphorylated. In summary, these results demonstrate that equine neutrophils indeed express an active $cPLA_2$ protein but that there is a difference in the degree of phosphorylation of the $cPLA_2$ protein between equine neutrophils and eosinophils. This difference might explain the difference between the two cell types in the capacity to produce leukotrienes from endogenous substrate.**—Larsson Forsell, P. K. A., Å. Lindberg, S. Karlsson, J. Å. Lindgren, and H-E. Claesson. **Purification, characterization, and cDNA sequencing of cytosolic phospholipase A_2 from equine neutrophils. *J. Lipid Res.* 2000.** 41: **1222–1230.**

Supplementary key words phospholipase A_2 • arachidonic acid • leukotriene • equine • neutrophils • eosinophils • phosphorylation

The phospholipase A_2 (PLA_2) family of enzymes hydrolyzes the fatty acid ester bond at the *sn*-2 position in phospholipids (1). PLA_2 enzymes play an important role in the release of lysophospholipids and the eicosanoid precursor arachidonic acid. Two major types of intracellular PLA_2 have been recognized, that is, calcium independent and calcium dependent. A group VI calcium-independent PLA_2 ($iPLA_2$) was originally purified from P388D1 cells and was later cloned from several species (2–6). In addition, a group IV cytosolic calcium-dependent PLA_2 ($cPLA_2$) was originally discovered and characterized biochemically from the macrophage cell line RAW 264.7 (7). Subsequently, it was isolated, cloned, and sequenced from the human monocytic cell line U937 (8–10). The cDNA sequence of $cPLA_2$ has also been determined from rat (11), mouse (9), chicken, and zebra fish (12). The $cPLA_2$ enzyme is expressed in a variety of cells including human neutrophils and B lymphoblasts (13, 14). Hormonally induced phosphorylation of $cPLA_2$ occurs in several tissues and cell types and this modification appears to increase the activity of the enzyme in vitro and to decrease the electrophoretic mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15–18). Several investigators have tried to define the role of $cPLA_2$ in leukotriene formation and gene-targeting experiments indicated that $cPLA_2$ is essential for leukotriene synthesis in mice (19, 20).

Arachidonic acid plays a crucial role as a precursor for the synthesis of eicosanoid, such as prostaglandins and leukotrienes. Leukotrienes are mediators of inflammation and are thought to be involved in human diseases such as asthma (21). Leukotrienes have also been suggested to be of importance in several pathophysiological conditions of the horse such as inflammation (22) and chronic obstructive pulmonary disease (23, 24).

Abbreviations: $AACOCF_3$, arachidonoyl trifluoromethyl ketone; DAG, diacylglycerol; DTT, dithiothreitol; LTB_4, leukotriene B_4; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLA_2, phospholipase A_2; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

[1] The sequence reported herein has been submitted to GenBank under the accession number AF092539.

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Equine eosinophils and neutrophils display differential capacity to produce leukotrienes from endogenous substrate after calcium ionophore A23187 stimulation because neutrophils are dependent on exogenous arachidonic acid for leukotriene formation (25). Therefore, we explored the presence of a functional $cPLA_2$ enzyme in equine neutrophils. This article demonstrates the purification, characterization, and cDNA sequence of $cPLA_2$ from these cells. Furthermore, we also present data suggesting a difference in enzyme phosphorylation in equine neutrophils as compared with eosinophils from the same species.

MATERIALS AND METHODS

Materials

High-performance liquid chromatography (HPLC) solvents were purchased from Rathburn Chemicals (Walkerburn, UK) and arachidonic acid from Biomol (Plymouth Meeting, PA). Monoflow™ 2 scintillation liquid was obtained from National Diagnostics (Manville, NJ). 1-palmitoyl 2-[1-^{14}C]arachidonyl phosphatidylcholine (PC), (57 mCi/mmol), 1-palmitoyl 2-[1-^{14}C]palmitoyl PC (55.5 mCi/mmol), 1-palmitoyl 2-[1-^{14}C]arachidonyl phosphatidylethanolamine (PE) (57 mCi/mmol), 1-palmitoyl 2-[1-^{14}C] palmitoyl phosphatidic acid (PA) (144 mCi/mmol), and [1-^{14}C] arachidonic acid (57 mCi/mmol) were obtained from New England Nuclear (Boston, MA); 1-stearoyl 2-[1-^{14}C]arachidonyl PC (56 mCi/mmol) and 1-stearoyl 2-[1-^{14}C]arachidonyl diacylglycerol (DAG) (53 mCi/mmol) were purchased from Amersham International (Amersham, UK). The FPLC System™ and all protein purification columns were from Pharmacia Biotech (Uppsala, Sweden) if not stated otherwise. The rabbit anti-human $cPLA_2$ polyclonal antibody (amino acids 731–749 as immunogen) and the corresponding synthetic peptide were kindly provided by N. Tremblay (Merck Frosst, Pointe-Claire-Dorval, PC, Canada).

Preparation of equine neutrophils

Horse blood from healthy, adult Swedish Warmblood horses was collected by jugular vein puncture into EDTA-containing Vacutainer blood collection tubes (Becton Dickinson, Rutherford, NJ), or into blood bags (Teruflex B-450; Terumo, Tokyo, Japan) containing EDTA (final concentration, 4.1 mM). Equine neutrophils suspensions were prepared as follows: after centrifugation of the equine blood at 140 *g* for 15 min, the platelet-rich plasma was removed and the neutrophils were isolated from the remaining lower phase by dextran sedimentation, hypotonic ammonium chloride lysis, and Lymphoprep™ centrifugation at 400 *g* for 40 min. The isolated cells contained >96% neutrophils, with 1–4% contaminating eosinophils as judged by light microscopy. Highly enriched preparations of equine neutrophils and eosinophils were obtained as described (25). These preparations had less than 1% contaminating cells (data not shown). Cells were resuspended, to a final concentration of 5×10^7 cells/mL, in homogenization buffer (20 mM Tris-HCl [pH 7.5], 2 mM EGTA, 1 mM EDTA, 2 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], soybean trypsin inhibitor [20 μg/mL], bacitracin [0.1 mg/mL], 0.5 mM benzamidine, 0.02 mM leupeptin, and 10% glycerol).

PLA_2 assay

Cells were homogenized twice by nitrogen cavitation at 800 psi for 10 min at 4°C. The supernatant was obtained after centrifugation at 100,000 *g* for 60 min. PLA_2 activity was assayed with 1-palmitoyl 2-[1-^{14}C]arachidonyl phosphatidylcholine (PC) as a standard substrate. Phospholipids were dried under nitrogen and resuspended in assay buffer (final concentration, 80 mM glycine [pH 9.0], 5 mM $CaCl_2$, 5 mM DTT, albumin [1 mg/mL], and 10% glycerol) to yield a final concentration of 2 μM PC. Subsequently this preparation was sonicated in a water bath for 10 min at 4°C. The reaction was initiated by adding either cell homogenate, 100,000 *g* supernatant, or purified PLA_2 and the reaction mixture was incubated for the indicated times at 37°C in a shaking water bath. The reaction was terminated by the addition of 2 volumes of methanol containing 0.5% acetic acid and 40 μM stearic acid, as carrier, followed by vigorous vortexing. Precipitated proteins and cell debris were removed by centrifugation at 800 *g* for 10 min. Thereafter, the supernatants were applied to a octadecyl reversed-phase column (Chromabond, C_{18}, 100 mg; Macherey-Nagel, Duren, Germany). After washing, the fatty acids were eluted with 500 μL of methanol. The samples were analyzed in a reversed-phase (RP) HPLC system equipped with a Radial-Pak cartridge (5 × 100 mm) packed with 4-μm Novapak C_{18} material, guarded by a Novapak C_{18} column (Waters, Milford, MA). The mobile phase was methanol–water–trifluoroacetic acid 85:15:0.007 (v/v/v) and the flow rate was 1.2 mL/min. Analysis was performed by comparing retention times with authentic standards. Radioactivity was detected with a β-RAM HPLC flowthrough monitoring system (Inus System, Tampa, FL) coupled online to a Waters 991 diode array spectrophotometer. Quantitative determination was performed by peak area integration.

Purification of cytosolic PLA_2

Liquid chromatography was performed on a Pharmacia FPLC System™. All buffers were made fresh daily, passed through a 0.22-μm pore size filter, and degassed before use. The 100,000 *g* supernatant was applied to a 10-mL heparin-Sepharose column equilibrated with buffer A (20 mM Tris-HCl [pH 7.5], 1 mM EGTA, 1 mM EDTA, 0.5 mM DTT). Proteins were eluted by a two-step gradient from 0 to 0.5 M NaCl and from 0.5 to 1 M NaCl, respectively. Fractions of 3 mL were collected and 30-μL aliquots of these were assayed for PLA_2 activity. The flowthrough fractions from the heparin-Sepharose step were pooled and applied to a Resource-Q (6-mL) column preequilibrated with buffer A. After washing, a gradient was developed from 0.15 to 0.55 M NaCl in buffer A at a flow rate of 2 mL/min. Fractions of 1.5 mL were collected and 10-μL aliquots of these were then assayed for PLA_2 activity. Sodium chloride (1 M final concentration) was added to pooled, active fractions from the Resource-Q column and in turn applied to a phenyl-Superose HR 5/5 column preequilibrated with 1 M NaCl in buffer A. Proteins were eluted in a stepwise gradient at a flow rate of 0.3 mL/min. Fractions of 0.5 mL were collected and 10-μL aliquots were then assayed for PLA_2 activity. Fractions with enzyme activity were analyzed further on a Resource-Q (1-mL) column equilibrated with buffer A. After washing, proteins were eluted in a linear gradient from 0.15 to 0.5 M NaCl. Aliquots (20 μL) of resulting 0.5-mL fractions were assayed for PLA_2 activity. Active fractions from this purification step were concentrated with a Centricon 10 (Amicon, Danvers, MA) at 4,700 *g* to a final volume of approximately 250 μL and then applied to a Superdex 200 column equilibrated with buffer A supplemented with 0.15 M NaCl. Proteins were eluted at a flow rate of 0.35 mL/min and 0.4-mL fractions were collected. Aliquots (25 μL) of these fractions were analyzed for PLA_2 activity. PLA_2 active fractions were further analyzed by SDS-PAGE. Protein concentrations were measured by Coomassie assay as described by the manufacturer (Bio-Rad, Hercules, CA) against bovine serum albumin as the standard protein. Protein content after the last purification step was estimated from the absorbance at 280 nm.

Polyacrylamide gel electrophoresis

SDS-PAGE was performed as described previously (26). Samples were mixed with loading buffer (50 mM Tris [pH 6.8], 10% glycerol, 0.1% SDS, 3 mM 2-mercaptoethanol, and 0.005% bromophenol blue), heated for 3 min at 95°C, and loaded onto a 5–16% SDS-PAGE (16 × 18 or 8 × 10 cm). After the run, proteins were visualized by silver staining (Bio-Rad silver stain kit), as described by the manufacturer, or subjected to Western blot. Molecular weight markers, SeeBlue and Mark 12, were obtained from Novex (San Diego, CA).

Immunoblotting

Western blots were performed according to Towbin et al. (27). Briefly, after SDS-PAGE the samples were transferred to Hybond™-C nitrocellulose sheets (Amersham, Buckinghamshire, UK). Afterward, membranes were blocked with 1% milk powder and 0.5% bovine albumin in Tris-buffered saline with 0.1% Tween (TBS-T) for 60 min. After blocking, the membranes were incubated overnight at 4°C with a sequence-specific rabbit anti-human $cPLA_2$ polyclonal antibody (1:1,000 dilution). In one set of control experiments the corresponding synthetic peptide was added to the polyclonal antibody before immunodetection. The peptide/antibody ratio exceeded a 100:1 molar ratio in these experiments. The second antibody was anti-rabbit (1:10,000) coupled to horseradish peroxidase. Enhanced chemiluminescence (ECL™) was used for detection as described by the manufacturer (Amersham).

Isolation of total cellular RNA, reverse transcription, and PCR

Total cellular RNA was isolated with Trizol reagent (Life Technologies, Gaithersburg, MD) according to the instructions of the manufacturer. The amount and purity of isolated total RNA were determined spectrophotometrically by analyzing the absorbance at 260 nm. Isolated total RNA (2 μg) was subjected to reverse transcription in order to obtain cDNA. The reverse transcription mixture contained 2 μg of total RNA, 1× reverse transcriptase buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 2 mM $MgCl_2$, and 0.01% gelatin), 5 mM DTT, 0.5 mM dNTPs (SDS-Promega, Falkenberg, Sweden), 200 ng of oligo(dT)$_{12–18}$ (Pharmacia, Uppsala, Sweden), 39 U of RNasin (SDS-Promega), and 500 U of Moloney murine leukemia virus reverse transcriptase (Mo-MuLV RT; United States Biochemical, Cleveland, OH) in a total volume of 40 μL. The reverse transcription reaction was carried out at 37°C for 60 min and then terminated by heating for 5 min at 95°C. To determine whether a successful cDNA was obtained, polymerase chain reaction (PCR) amplification of a 784-bp β-actin fragment was performed (data not shown). PCR was performed with the following cycle parameters. First cycle: denaturation for 4.0 min at 94°C, annealing for 1.0 min at 65°C, and extension for 1.5 min at 72°C. Subsequent cycles: denaturation for 1.0 min at 94°C, annealing for 1.0 min at 65°C, and extension for 1.5 min at 72°C. Last cycle: denaturation for 1.0 min at 94°C, annealing for 1.0 min at 65°C, and a final extension of 10.0 min at 72°C. Altogether, 33 cycles were carried out for each fragment to be amplified. The following $cPLA_2$ primer pairs were used:

1. 5′-CTCGCTCGCCCACTCTAGTCCTCCGTTCAAGGAAC-3′
   5′-CTGGTTCGGCCCATTGCAAACTGCCTCAGCATCAG-3′
2. 5′-CTCGCTCGCCCAGGCTCCACAATGGAGGAAGAAT-3′
   5′-CTGGTTCGGCCCAGTTCCTTGAACGGAGGACTAGA-3′
3. 5′-AGCCACAATCCCCTTTTACTT-3′
   5′-GCTATCATTACTCACAATATGCT-3′
4. 5′-AGTGCCAAAGAAGACTTTGAAGT-3′
   5′-TCTCTTAACTTTCTGTGGCGTGA-3′

The primer pairs used were designed on the basis of the human $cPLA_2$ cDNA sequence. After the PCR, aliquots of the amplified product was analyzed on a 1% low melting point agarose gel. The PCR-derived fragment was excised and extracted with a Qiaex II agarose gel extraction kit (Qiagen, Chatsworth, CA).

Cloning and sequencing of the PCR products

The PCR products were cloned with the PCR-DIRECT cloning system (Clontech, Palo Alto, CA) or with the pCR-Script Amp SK(+) cloning kit (Stratagene, La Jolla, CA). Plasmid DNA was purified and the insert was sequenced with M13 forward and reverse primers as well as internal sequencing primers, using the T7 sequencing kit (Pharmacia). An aliquot (2 μL) of the sequencing reaction was subjected to electrophoresis with a Macrophore sequencing system (Pharmacia LKB Biotechnology, Uppsala, Sweden). After electrophoresis the gels were fixed, dried, and subjected to autoradiography.

RESULTS

Purification of $cPLA_2$ from equine neutrophils

Because equine neutrophils are unable to produce leukotrienes from endogenous arachidonic acid after calcium ionophore A23187 stimulation (25), we investigated whether these cells contained active $cPLA_2$ enzyme. When 100,000 *g* supernatants obtained from neutrophil homogenates were incubated with PC and PE containing ^{14}C-

TABLE 1. Scheme for purification of cytosolic phospholipase A_2 from equine neutrophils

Step	Protein	Total Activity	Specific Activity	Yield	Purification
	mg	*nmol/min*	*nmol/min × mg*	*%*	*fold*
I. Homogenate	76.3	0.865	0.011	—	1
II. 100,000 *g* sup	31.8	1.25	0.039	—	3.5
III. Heparin-Sepharose	27.8	6.16	0.22	100	20 (1)
IV. Resource-Q (6 ml)	1.65	2.55	1.54	41.4	140 (7)
V. Phenyl HR 5/5	0.086	1.46	16.9	23.7	1,536 (77)
VI. Resource-Q (1 ml)	0.011	0.46	41.8	7.5	3,800 (190)
VII. Superdex 200	<0.0025	0.18	>72	2.9	>6,545(>327)

PLA_2 activity was determined with a mixture (1:1) of 2 μM 1-palmitoyl 2-[^{14}C]arachidonyl PC and 1-palmitoyl 2-[^{14}C]arachidonyl PE as substrate. The incubation period was 30 min. The protein content in the last purification step was estimated from the absorbance at 280 nm. The calculations of fold purification (in parentheses) are based on step III as the enzyme source. The data are from one of three representative experiments.

labeled arachidonic acid at the *sn*-2 position, a phospholipase activity corresponding to 39 pmol mg^{-1} min^{-1} was observed. For comparison, 100,000 *g* supernatants from a corresponding number of U937 cells, possessing a phospholipase activity of 46 pmol mg^{-1} min^{-1}, were used.

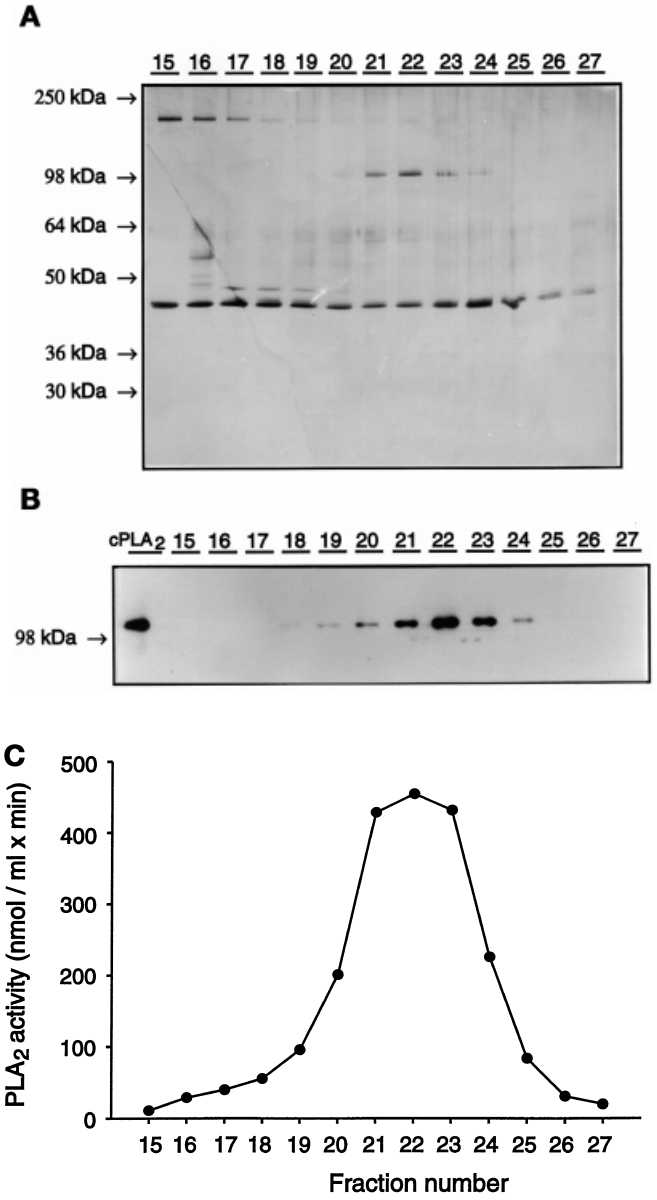

Fig. 1. Analysis of PLA_2 active fractions after gel filtration. (A) Silver staining of PLA_2 active fractions after gel electrophoresis. An aliquot of each fraction from Superdex 200 chromatography was subjected to SDS-PAGE on a 12% gel. The fraction numbers are indicated above the lanes. Molecular mass markers are indicated on the left. (B) Western blot analysis of $cPLA_2$. An aliquot of each fraction from the Superdex 200 column was subjected to SDS-PAGE followed by immunoblotting. Recombinant $cPLA_2$ (15 ng) was run in parallel as indicated. Immunoblot analysis was performed with polyclonal antibodies raised against a C-terminal peptide of $cPLA_2$. Arrow on the left indicates the position of the 98-kDa marker. (C) PLA_2 activity profile of fractions from the Superdex 200 column. PLA_2 activity was determined with 1 μM 1-palmitoyl 2-[^{14}C]arachidonyl PC as substrate. Aliquots from each fraction were incubated for 30 min. The activity represents the activity found in each analyzed aliquot.

To investigate whether the phospholipase activity observed in the 100,000 *g* supernatants from equine neutrophils could be ascribed to $cPLA_2$, the PLA_2 enzyme was isolated and purified by sequential heparin-Sepharose chromatography, anion-exchange chromatography, hydrophobic interaction chromatography, and a second anion-exchange chromatography followed by size-exclusion chromatography (Superdex 200) (**Table 1**). After the last purification step, PLA_2 activity declined rapidly, either because of inactivation or aggregation. Aliquots of PLA_2 active fractions eluting from the Superdex 200 column were subjected to gel electrophoresis. One major band with the apparent molecular mass of 105 kDa was detected in the PLA_2 active fractions by silver staining (**Fig. 1A**). The intensity of the 105-kDa protein, eluting in fractions 21–23, correlated with PLA_2 activity (Fig. 1C). Furthermore, Western blot analysis of the PLA_2 active fractions demonstrated correlation between immunodetected proteins, PLA_2 activity, and the silver-stained 105-kDa protein (Fig. 1B). One contaminating protein was present in all examined fractions. The contaminating protein, with a molecular mass of approximately 45 kDa, is likely to be the equine form of a protein disulfide isomerase, as previously reported by Gronich et al. (28). The identity of this contaminating protein was not further analyzed. In summary, these results demonstrate that active $cPLA_2$ from equine neutrophils has been purified to near homogeneity. This purification scheme resulted in >6,500-fold purification above levels found in total cellular homogenate, with a recovery of 2.9% (Table 1). The apparent increase in total activity after the first chromatographic steps might be due to the removal of either endogenous substrate or inhibiting factors.

Biochemical characterization of equine neutrophil $cPLA_2$

The substrate specificity of partially purified $cPLA_2$ (Table 1, step V) was investigated with various radiolabeled substrates as outlined in **Table 2**. The enzyme selectively hydrolyzed phospholipids containing arachidonic acid, as compared with palmitic acid, at the *sn*-2 position. As shown in Table 2, the PLA_2 activity toward PC or PA, containing palmitic acid at the *sn*-2 position, and 1-stearoyl 2-[1-^{14}C]arachidonoyl DAG was below detection limits in a vesicle-based assay. As shown in **Fig. 2**, the equine neutro-

TABLE 2. Acyl-chain specificity of partially purified equine $cPLA_2$

Substrate	Specific Activity
	nmol/mg × min
1-Palmitoyl 2-[1-^{14}C]arachidonyl PC	10.6 ± 0.46
1-Stearoyl 2-[1-^{14}C]arachidonyl PC	15.6 ± 2.4
1-Palmitoyl 2-[1-^{14}C]arachidonyl PE	22.5 ± 1.2
1-Palmitoyl 2-[1-^{14}C]palmitoyl PC	n.d.
1-Stearoyl 2-[1-^{14}C]arachidonyl DAG	n.d.
1-Palmitoyl 2-[1-^{14}C]palmitoyl PA	n.d.

The acyl-chain specificity of equine PLA_2 was determined with partially purified $cPLA_2$, isolated from step V. The enzyme was incubated with various phospholipids (2 μM) as described in Materials and Methods. The incubation time was 30 min and the results are the mean values of triplicate samples ± range from one representative experiment out of two; n.d., not detectable.

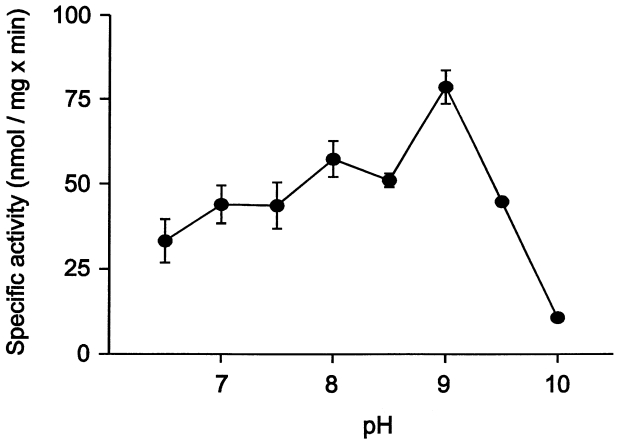

Fig. 2. pH dependency of the release of arachidonic acid. Partially purified PLA_2 (step V) was added to the assay mixture, containing 80 mM HEPES (pH 6.5–8.0) or 80 mM glycine (pH 8.5–10), with pH as indicated, and incubated for 30 min with a mixture (1:1) of 2 μM 1-palmitoyl 2-[^{14}C]arachidonyl PC and 1-palmitoyl 2-[^{14}C]arachidonyl PE. The results represent the means ± range of duplicate samples from one of two representative experiments.

phil $cPLA_2$ had an alkaline pH optimum, with maximal enzyme activity at pH 9.0. Furthermore, the $cPLA_2$ active site inhibitor, arachidonyl trifluoromethyl ketone ($AACOCF_3$), dose dependently inhibited equine neutrophil $cPLA_2$ activity (**Fig. 3**). The inhibition displayed an apparent median inhibitory concentration (IC_{50}) of less than 0.1 μM on partially purified equine $cPLA_2$. The enzyme was calcium dependent because no release of arachidonic acid from PC:PE was observed in the presence of EGTA (**Table 3**). No other divalent metal cations such as Sr^{2+}, Ba^{2+}, Mn^{2+}, Mg^{2+}, Cd^{2+}, and Zn^{2+} could replace Ca^{2+} and restore activity completely, but Sr^{2+}, Ba^{2+}, and Mn^{2+} were capable

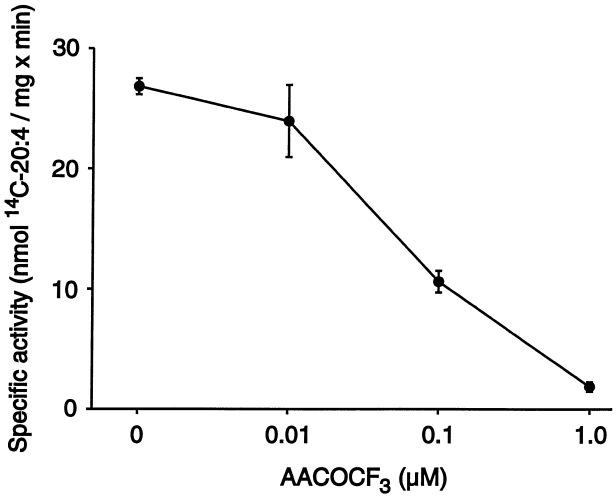

Fig. 3. Inhibition of equine $cPLA_2$ activity by $AACOCF_3$. Partially purified PLA_2 (step V) was added to the assay mixture, containing the indicated concentrations of arachidonyl trifluoromethyl ketone ($AACOCF_3$), and incubated for 30 min with a mixture (1:1) of 2 μM 1-palmitoyl 2-[^{14}C]arachidonyl PC and 1-palmitoyl 2-[^{14}C]arachidonyl PE. The results represent the means ± range of triplicate samples from one of two representative experiments.

TABLE 3. Effects of divalent cations on the activities of equine and human $cPLA_2$

	Equine	Human
$CaCl_2$	100 ± 9.2	100 ± 10.2
$BaCl_2$	92.9 ± 9.3	23.4 ± 1.8
$SrCl_2$	78.5 ± 7.2	69.4 ± 9.9
$MnSO_4$	41.1 ± 3.5	117 ± 13.2
$MgCl_2$	8.5 ± 3.4	14.0 ± 4.5
EGTA/EDTA	n.d.	n.d.

Partially purified equine neutrophil $cPLA_2$ or U937 $cPLA_2$, both from step IV, was added to the assay mixture, containing a 2 mM concentration of each salt, and incubated for 30 min with a mixture (1:1) of 2 μM 1-palmitoyl 2-[^{14}C]arachidonyl PC and 1-palmitoyl 2-[^{14}C] arachidonyl PE. The PLA_2 activity found with $CaCl_2$ set to 100% and was 1.54 nmol mg^{-1} min^{-1} and 2.46 nmol mg^{-1} min^{-1} for equine $cPLA_2$ and U937 $cPLA_2$, respectively. The results are the mean values of duplicate samples ± range from one representative experiment out of two; n.d., not detectable.

of partial restoration (Table 3). For comparison, $cPLA_2$ from U937 cells, purified 380-fold in a similar manner as described for equine neutrophil $cPLA_2$ (steps I–IV), was analyzed for cation dependency under identical conditions (Table 3). In contrast to equine neutrophil $cPLA_2$, Mn^{2+} could replace Ca^{2+} to restore full activity of U937 $cPLA_2$, as reported by others (29, 30). On the other hand, Ba^{2+} restored more activity in equine neutrophil $cPLA_2$ than in U937 $cPLA_2$ preparations. The other divalent cations were equipotent in restoring the activity of equine neutrophil $cPLA_2$ as compared with U937 $cPLA_2$. Thus, these results might demonstrate a species difference in cationic ability to restore PLA_2 activity.

Preliminary Western blot results demonstrated that the equine $cPLA_2$ had higher electrophoretic mobility than human recombinant $cPLA_2$ protein when analyzed by SDS-PAGE (data not shown). To explore further the observed size differences, $cPLA_2$ partially purified from equine as well as human neutrophils and from unstimulated U937 cells, and recombinant $cPLA_2$, were loaded on a 5–10% polyacryl-

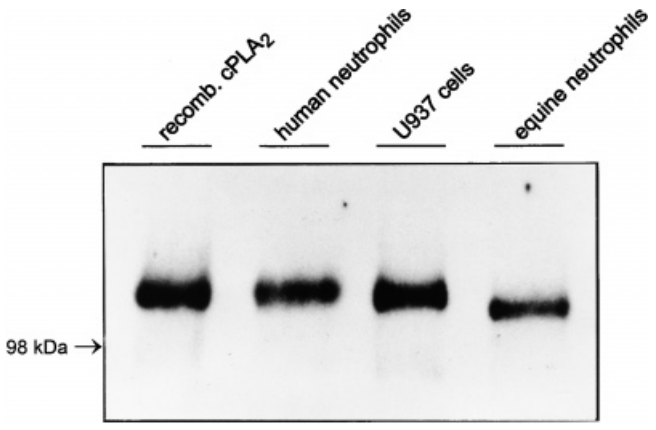

Fig. 4. Western blot analysis of $cPLA_2$ purified from different sources. Recombinant human $cPLA_2$ protein (15 ng) and partially purified $cPLA_2$ (0.5–1 μg from step V) from equine neutrophils, human neutrophils, and U937 cells were subjected to SDS-PAGE, on a 5–10% gel, followed by immunoblotting. Immunoblot analysis was performed with a rabbit anti-human $cPLA_2$ polyclonal antibody. Arrow on the left indicates the position of the 98-kDa marker.

amide gel (**Fig. 4**). The gel was run for an additional 30 min after the front had eluted from the gel in order to obtain sufficient separation. Subsequently, Western blotting was performed and immunoreactive proteins were detected with a polyclonal anti-cPLA$_2$ antibody. Figure 4 demonstrates that equine neutrophil cPLA$_2$ had a higher electrophoretic mobility than either cPLA$_2$ derived from human neutrophils or U937 cells, or recombinant cPLA$_2$, and that equine neutrophil cPLA$_2$ migrated as a 105-kDa protein. This observed apparent size difference is unlikely to be due to phosphorylation differences between the equine and human cPLA$_2$ proteins because both partially purified and recombinant human cPLA$_2$ had a lower electrophoretic mobility than the equine cPLA$_2$ protein (Fig. 4).

cDNA sequencing of equine neutrophil cPLA$_2$

In an attempt to explain the observed differences in migration on SDS-polyacrylamide gels between human and equine cPLA$_2$, the equine cPLA$_2$ cDNA sequence was determined. **Figure 5** depicts the deduced amino acid sequence of the equine neutrophil cPLA$_2$ aligned with the human sequence. The equine protein demonstrated 95% identity with the human protein. As indicated in Fig. 5, two charged amino acids were different in the vicinity of Ser-505, which is the target for phosphorylation by mitogen-activated protein (MAP) kinase. This phosphorylation, and hence the introduction of a negatively charged phosphate group, is suggested to result in a slower migrating protein cPLA$_2$ (31). Ser-508 and Asp-509 in the human sequence were found to be Arg-508 and Asn-509 in the equine sequence, and thus a net charge corresponding to −1 in the human sequence is replaced by a net charge of +1 in the equine sequence, in this specified region of the protein. We suggest that this charge difference is the cause for the different mobility observed between the two species (Fig. 4). Furthermore, the equine protein demon-

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Equine SSTPDSRKRTRHFNNNINPVWNETFEFILDPNQENVLEITLMDANYVMDE 100
       |.|||||||||||||.|||||||||||||||||||||||||||||||||||
Human  STTPDSRKRTRHFNNDINPVWNETFEFILDPNQENVLEITLMDANYVMDE 100

Equine TLGTATFTLSSMKVGEKKEVPFIFNQVTEMILEMSLEVCSCPDLRFSMAL 150
       ||||||||.|||||||||||||||||||:||||||||||||||||||||||
Human  TLGTATFTVSSMKVGEKKEVPFIFNQVTEMVLEMSLEVCSCPDLRFSMAL 150

Equine CDQEKTFRQQRKENIKENMKKLLGPKKSEGLYSTRDVPVVAILGSGGGFR 200
       |||||||||||||.|:|.|||||||| ||||:| ||||||||||||||||
Human  CDQEKTFRQQRKEHIRESMKKLLGPKNSEGLHSARDVPVVAILGSGGGFR 200

Equine AMVGFSGVMKALYESGILDCATYLAGLSGSSWYMSTLYSHPDFPEKGPEE 250
       ||||||||||||||||||||||||. |||||.||||||||||||||||||
Human  AMVGFSGVMKALYESGILDCATYVAGLSGSTWYMSTLYSHPDFPEKGPEE 250

Equine INKELMKNVSYDPLLLLTPQKIKRYVESLWKKKSSGQPVTFTDIFGMLIG 300
       ||.|||||||:.|||||||||:||||||||||||||||||||||||||||
Human  INEELMKNVSHNPLLLLTPQKVKRYVESLWKKKSSGQPVTFTDIFGMLIG 300

Equine ETLIHNRMNTTLSSLKEKVNTAQCPLPLFTCLHVKPDVSELMFADWVEFS 350
       ||||||||||||||||||||||||||||||||||||||||||||||||||
Human  ETLIHNRMNTTLSSLKEKVNTAQCPLPLFTCLHVKPDVSELMFADWVEFS 350

Equine PYEIGMAKYGTFMAPDLFGSKFFMGTVVKKYEENPLHFLMGVWGSAFSIL 400
       ||||||||||||||||||||||||||||||||||||||||||||||||||
Human  PYEIGMAKYGTFMAPDLFGSKFFMGTVVKKYEENPLHFLMGVWGSAFSIL 400

Equine FNRVLGVSGSQNKGSTMEEELENITAKHIVSNDSSDSDDESQEPKGTENE 450
       ||||||||||.:|||||||||||| |||||||||||||| ||||||||
Human  FNRVLGVSGSQSRGSTMEEELENITTKHIVSNDSSDSDDESHEPKGTENE 450

Equine DAERDYQNDNQASWVHRMLMALVSDSALFNTREGRAGKVHNFMLGLNLNT 500
       ||  |||.|||||||:|||:||||||||||||||||||||||||||||||
Human  DAGSDYQSDNQASWIHRMIMALVSDSALFNTREGRAGKVHNFMLGLNLNT 500

Equine SYPLSPLRNFTTQESLDDDELDAAVADPDEFERIYEPLDVKSKKIHVVDS 550
       ||||||| .| ||:| ||||||||||||||||||||||||||||||||||
Human  SYPLSPLSDFATQDSFDDDELDAAVADPDEFERIYEPLDVKSKKIHVVDS 550
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Fig. 5. Partial alignment of equine and human cPLA$_2$ proteins. The deduced amino acid sequences of equine and human cPLA$_2$ proteins were aligned by the Genetics Computer Group (GCG) program GAP. Bars indicate identical amino acid residues, dots indicate similar amino acid residues. The consensus sequence, GXSXG, is boxed and in boldface. Charged amino acids adjacent to Ser-505 that are not identical between the two species are indicated in boldface and underlined. Ser-505 is indicated in boldface.

strated 95% identity with human and mouse $cPLA_2$ as well as 83 and 73% identity with chicken and zebra fish $cPLA_2$ protein, determined by using the Genetics Computer Group (GCG, Madison, WI) program GAP.

Expression of $cPLA_2$ protein in equine eosinophils and neutrophils

Having established that equine neutrophils indeed express an active $cPLA_2$ enzyme, we continued to explore the discrepancy between equine neutrophils and eosinophils in their capacity to produce leukotrienes from endogenous substrate by analyzing phosphorylation of $cPLA_2$ in highly enriched preparations of these cells. It is well documented that the phosphorylated $cPLA_2$ enzyme migrates slower than the unphosphorylated enzyme (15, 32). Interestingly, $cPLA_2$ from both resting and calcium ionophore A23187-stimulated eosinophils had slower electrophoretic mobility than $cPLA_2$ from neutrophils (**Fig. 6**), suggesting that eosinophilic $cPLA_2$ was phosphorylated to a major extent because no slower migrating band could be observed in these samples. On the other hand, $cPLA_2$ protein from resting neutrophils seemed to be unphosphorylated and did not change its electrophoretic mobility after calcium ionophore A23187 stimulation (Fig. 6). Thus, the phosphorylation pattern of $cPLA_2$ in equine neutrophils and eosinophils correlates with the capacity of these cells to produce leukotrienes after calcium ionophore stimulation (Fig. 6 and ref. 25). However, incubation of total homogenate from eosinophils with alkaline phosphatase did not affect the mobility of $cPLA_2$ from equine eosinophils (data not shown).

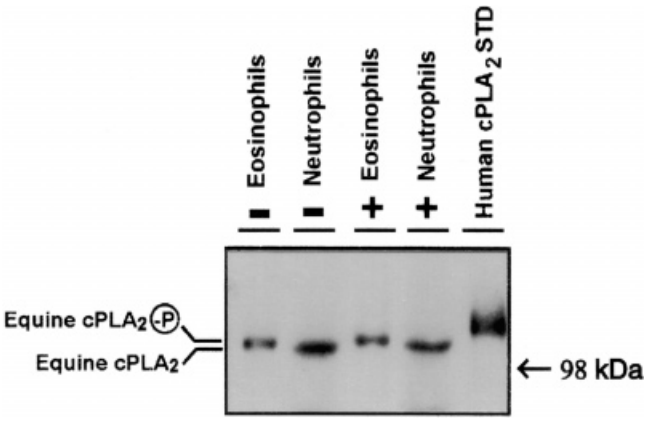

Fig. 6. Western blot analysis of $cPLA_2$ content in equine eosinophils and neutrophils Equine eosinophils or neutrophils were incubated with (+) or without (−) calcium ionophore A23187 for 10 min. The resulting homogenates of these cells were subjected to SDS-PAGE, on a 5–10% gel, followed by immunoblotting. Immunoblot analysis was performed with a rabbit anti-human $cPLA_2$ polyclonal antibody. Recombinant $cPLA_2$ protein (15 ng) was loaded as control. Markers on the left indicate the position of phosphorylated and unphosphorylated $cPLA_2$ protein. Arrow on the right indicates the position of the 98-kDa marker.

DISCUSSION

Human neutrophils produce leukotriene B_4 (LTB_4) after stimulation with calcium ionophore A23187. A species difference, between human and horse, in leukotriene production after A23187 stimulation has been demonstrated (25). Thus, equine neutrophils did not produce leukotrienes after calcium ionophore A23187 stimulation, unless low concentrations of exogenous arachidonic acid were added. However, calcium ionophore A23187 was a sufficient stimulus to induce leukotriene synthesis in equine eosinophils (25). To elucidate why equine neutrophils failed to produce leukotrienes after calcium ionophore A23187 stimulation, we investigated whether the $cPLA_2$ enzyme from neutrophils was catalytically active. The equine form of the $cPLA_2$ protein was purified >6,500 fold with a recovery of 3% from equine neutrophils. The equine cytosolic PLA_2 activity could be ascribed to a protein migrating as a 105-kDa protein on SDS-PAGE (Fig. 1). This protein was also recognized by $cPLA_2$ polyclonal antibody, thus establishing that the purified protein was the equine $cPLA_2$. The partially purified $cPLA_2$ was insensitive to reducing conditions and displayed an alkaline pH optimum. The enzyme was dependent on Ca^{2+} for its activity and showed a marked preference for phospholipids containing arachidonic acid at the *sn*-2 position, as compared with palmitic acid, when sonicated phospholipid vesicles were used as substrates (Table 2).

The equine neutrophil $cPLA_2$ possessed some biochemical features that distinguished it from the human $cPLA_2$ and hence could represent species-specific differences. First, the dependence on divalent metal cations was not similar for the equine neutrophil $cPLA_2$ as compared with U937 $cPLA_2$ (Table 3), THP-1 $cPLA_2$ (33), or recombinant $cPLA_2$ (30). The observed differences in cation dependence for the equine neutrophil $cPLA_2$, as compared with previous studies, could simply be due to different assay conditions. However, $cPLA_2$ from U937 cells, isolated in a similar manner as $cPLA_2$ from equine neutrophils, showed a dependency on divalent cations different from that of equine neutrophil $cPLA_2$ (Table 3). A possible explanation for this finding might be differences in the calcium-binding domain between the two species. The crystal structure of the C2 domain of $cPLA_2$ was resolved and it was demonstrated that two calcium ions were ligated to this domain (34). The amino acid residue Asn-65 participates in ligation of one of the calcium ions via its side-chain oxygen. Interestingly, one neighboring amino acid residue to Asn-65 was found to be different in the equine $cPLA_2$ protein as compared with the human protein. The exchange of Asp-66 in the human protein to Asn-66 in the equine protein might affect the binding of divalent cations and thereby explain the observed differences in the dependency on cations for activity (Table 3). Interestingly, mutation of Asn-65 to alanine had a larger effect on $cPLA_2$ activity than on membrane binding, suggesting that Asn-65 is important for activity (35). Thus, comparative studies using $cPLA_2$ from human and horse might be useful for further exploration of cation-mediated mechanisms.

Second, a significant difference in migration on SDS-PAGE could be observed between equine neutrophil $cPLA_2$ as compared with $cPLA_2$ derived from human neutrophils or U937 cells or with recombinant $cPLA_2$, with the equine neutrophil $cPLA_2$ having a higher electrophoretic mobility (Fig. 4). Phosphorylation of $cPLA_2$ results in a gel mobility shift, with the phosphorylated protein migrating slower than the unphosphorylated protein (15, 17, 18). However, in this study both the phosphorylated form of equine $cPLA_2$ from equine eosinophils, and the unphosphorylated form of $cPLA_2$, from equine neutrophils, had higher electrophoretic mobility than the human forms (Figs. 4 and 6). It is therefore highly unlikely that the observed difference in electrophoretic mobility of $cPLA_2$ between human and equine cells could be due to phosphorylation differences. A more likely explanation for the differences in electrophoretic mobility might involve differences in the primary structure of the enzymes. The equine neutrophil $cPLA_2$ protein demonstrated two changes in charged amino acids in the vicinity of Ser-505 (Fig. 5), which when phosphorylated is known to cause a slower migrating protein on SDS-PAGE (15, 17, 18). Furthermore, mutation of Ser-505 to glutamate resulted in a protein with gel mobility properties similar to those of phosphorylated $cPLA_2$ (31). This demonstrates that reduced gel mobility is likely to be dependent on the presence of a negatively charged residue in this region. Taken together, the changes in charged amino acid residues between equine and human $cPLA_2$ might explain the observed gel mobility differences (Fig. 4).

Having established that equine neutrophils indeed express an active $cPLA_2$ enzyme, we tried to elucidate the role of phosphorylation of $cPLA_2$ in leukotriene synthesis in the horse. Our results indicate that $cPLA_2$ in equine eosinophils is constitutively phosphorylated, whereas the $cPLA_2$ in neutrophils is unphosphorylated, even after calcium ionophore A23187 stimulation (Fig. 6). There was a clear difference in electrophoretic mobility between the $cPLA_2$ from eosinophils as compared with neutrophils and it is well known that the phosphorylated enzyme migrates slower than the unphosphorylated enzyme. No other posttranslational modification of the $cPLA_2$ protein is known to cause a gel shift similar to that caused by a phosphorylation event (36).

Phosphorylation of Ser-505 by MAP kinase has been demonstrated to increase the specific activity of the enzyme in vitro. However, the increase in activity is only modest and it has been suggested that phosphorylation of $cPLA_2$ facilitates the calcium-dependent association between enzyme and membrane, thereby leading to increased release of fatty acid (18, 37). In this study, a majority of $cPLA_2$ from equine eosinophils seemed to be in the phosphorylated state, because no faster migrating form could be observed, whereas a majority of $cPLA_2$ from equine neutrophils seemed to be nonphosphorylated. This coincided with the greater capacity of the eosinophils to produce leukotrienes from endogenous arachidonic acid, as compared with equine neutrophils (25). Phosphorylation of $cPLA_2$ might be one of several mechanisms necessary for the formation of leukotrienes from endogenous substrate in horse neutrophils. However, the precise mechanism governing leukotriene formation in equine neutrophils needs further elucidation. In conclusion, comparative studies of $cPLA_2$ from different species could contribute to a better mechanistic understanding of the enzyme.

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