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Compartmentalisation and Characteristics of a Ca^{2+}-Dependent Phospholipase A_2 in Human Colon Mucosa

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ABSTRACT. The biochemical properties of the phospholipase A_2 (PLA_2) found in the 100,000 × *g* centrifugate cytosol or particulate fractions of human colonic mucosa have been investigated using both deoxycholate-solubilized and *Escherichia coli (E. coli)* phospholipids as substrates. PLA_2 activity was present in both subcellular fractions and the profiles of biochemical activites were similar. Activity in the particulate fraction was approximately twofold greater than the cytosol fraction when expressed on the basis of protein concentration. The PLA_2 is Ca^{2+} dependent and using EGTA-regulated buffers cytosolic or particulate fraction activity was similar at both 10 μm or 10 mm Ca^{2+} concentrations. Using deoxycholate-phospholipid micelles as substrate a small but statistically significant twofold preference for glycero-phosphatidylcholine bearing sn-2-arachidonate compared with sn-2-oleate was seen, but this preference was not noted using arachidonate or oleate labelled *E. coli* membranes. Dithiothreitol (10 mM) reduced colon mucosal cytosol PLA_2 activity significantly by 63.5 ± 1.90% in cytosol and by 30.54 ± 1.27% in microsomes using micelles as substrate or by 84.3 ± 2.30% in cytosol and by 69.33 ± 11.30% in microsomes using oleate-labelled *E. coli* as substrates. Warming at 57°C reduced activity significantly by 35.0 ± 5.80% in microsomes and by 40.0 ± 7.08% in cytosol. Acid treatment increased PLA_2 activity to 148 ± 16.3% in microsomes and 145 ± 18.6% in cytosol. When mucosal preparations were subjected to heparin-Sepharose chromatography, it bound tightly and eluted in the same position on a salt gradient as authentic human group II PLA_2. Further purification by gel-permeation chromatography gave activity in the 14 kDa region of the elution profile. These features have many of the characteristics expected of a 14 kDa isoform of PLA_2 but exhibit activity at concentrations of Ca^{2+} that are relevant in the intracellular environment and may participate in cellular lipid metabolism. BIOCHEM PHARMACOL 53;9:1323–1332, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. phospholipase A_2 human colon mucosa characterisation; calcium dependence

Phospholipase A_2s (PLA_2)¶ are a family of enzymes that catalyze the removal of fatty acids esterified at the sn-2 position of a variety of phosphoglycerides [1, 2]. PLA_2 plays a key role in membrane turnover and is considered to be involved in membrane remodelling [3, 4], exocytosis [5], repair of oxidative damage [6], and the degradation of invading microorganisms and necrotic tissue [7]. The role of the enzyme in cell signalling is linked to the release of fatty acids or lysophospholipids, both of which can be lipid mediators or can be converted to lipid mediators as a result of further metabolism. Such further metabolites include platelet activating factor, prostaglandins, and leukotrienes [8, 9]. Gastrointestinal PLA_2's have a variety of functions including lumenal digestion of dietary and biliary phospholipids, intestinal mucosal barrier functions, membrane signalling [10], and in pathological disorders such as pancreatitis [11, 12], cancer [13], mucosal injury following intestinal ischemia [14, 15], and inflammatory bowel disease [16, 17]. Several subtypes of PLA_2 enzymes, differing in their primary sequence and properties, have been identified in mammalian cells. The calcium-dependent low molecular mass isozymes (14 kDa), including type I and II have generally been reported to be characterized by a requirement for millimolar Ca^{2+} concentrations for optimal activity, lack preference for arachidonate-containing phospholipids, are inactivated by dithiothreitol (DTT), and are acid stable and thermostable at 57°C. On the other hand, the high molecular mass enzymes (85–100 kDa including type IV) are regulated by micromolar calcium concentrations in that

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¶ *Abbreviations:* [^{14}C]PAPC, L-1-palmitoyl-2-[^{14}C]arachidonoyl-sn-glycero-3-phosphocholine; BSA, bovine serum albumin; DTT, dithiothreitol; [^{14}C]POPC, L-1-palmitoyl-2-[1-^{14}C]oleoyl-sn-glycero-3-phosphocholine; [^{3}H]oleic acid, [9,19-^{3}H]oleic acid; PLA, phospholipase A_2; DAUDA, 11-(Dansylamino)undecanoic acid; DOPG, dioleoyl phosphatidylglycerol; EDTA, ethylene (diaminoethylether) N,N,N′, N′tetraacetic acid; EGTA, ethylene glycol-bis(-aminoethylether) N,N,N′, N′tetraacetic acid.

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their catalytic ability and movement from cytosol to membranes are responsive to submicromolar concentrations of calcium [18, 19]. They also show a marked preference for the hydrolysis of arachidonoyl residues [20], are resistant to DTT, and are broken down by acid and treatment at 57°C. Other forms of PLA_2 also exist, most of which have been the subject of limited investigation [2].

PLA_2 enzymes of human intestinal origin have been only partially characterized. Using immunohistochemical analysis a PLA_2-type II was identified in human esophagus, duodenum, small intestine, ascending colon, pancreas, hepatocytes, and Kupffer cells of liver and macrophages of spleen [21]. However, using similar techniques another investigation found immunoreactive PLA_2 type II only in Paneth cells and not in any other cell types in the digestive tract [22]. In a series of articles from one laboratory [23–25], immunohistochemical, radioimmunoassay, and micellar assay techniques were used to identify the presence of a type II PLA_2 in the serum and ileal and colonic mucosae in humans, and levels were elevated in patients with active inflammatory bowel disease. The PLA_2 appeared to be of the synovial fluid type II PLA_2 and not the pancreatic type. Using PCR and Northern blot analysis, mRNA for PLA_2 type II and also for the type I and IV PLA_2 was identified in human ileal mucosa [26]. Only two studies have assessed PLA_2 activity in human gastrointestinal mucosa using radiolabelled *E. coli* as substrate [16, 17]. They reported increased levels of PLA_2 activity in ileal and colonic mucosa of patients with inflammatory bowel diseases but did not characterise the subtype present. There are virtually no detailed data on distribution, biochemical characteristics, substrate preferences, and properties of human intestinal PLA_2s.

In the present study, further information is provided about the properties and the subcellular distribution of human colonic mucosal PLA_2. Some of these data have been presented in abstract form [27].

MATERIALS AND METHODS

Materials

[^{3}H]oleic acid (sp. act. 9.2 Ci/mmol) and [^{14}C]PAPC (sp. act. 57 mCi/mmol) were obtained from New England Nuclear Du Pont, [^{14}C]POPC (sp. act. 55 mCi/mmol) and [1–^{14}C]arachidonic acid (sp. act. 55.9 mCi/mmol) were from Amersham International; L-1 palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, L-1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine were from Sigma; fatty acid poor Fraction V bovine serum albumin (BSA) and porcine pancreatic PLA_2 suspension in $(NH_4)_2SO_4$ were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Five hundred milligram silica bonded phase columns (Bond Elut) were from Analytichem International Varian. All other chemicals were of reagent grade. *Escherichia coli* K 12 C600 strain was kindly donated by Dr. Y. Li, Department of Biochemistry, University of Southampton.

A human nonpancreatic secreted PLA_2 (hnps PLA_2) and rat liver Fatty Acid Binding Protein (FABP) were purified from synthetic genes expressed in *E. coli* [28, 29]. Dioleoyl phosphatidylglycerol and oleic acid were obtained from Sigma. DAUDA was obtained from Molecular Probes, Junction City, USA.

METHODS

Preparation of Crude Microsomal and Cytosol PLA_2 From Human Colon Mucosa

Macroscopically normal full thickness specimens of colon were obtained from patients undergoing resection of the ascending or sigmoid colon for carcinoma. The age of the patients ranged from 35 to 70 years. The specimens were taken at least 10–15 cm from the tumour or close to the resection margins and immediately transferred to ice-cold oxygenated (95% O_2–5% CO_2) Krebs solution. The mucosa was dissected away from the muscle by means of scissors and stored at −70°C. Particulate and cytosol fractions were prepared [30]. The mucosal specimens were thawed, weighed, finely chopped with scissors, and 10 vol of 50 mM Tris/HCl buffer pH 7.5, containing 20 μg/mL trypsin inhibitor, 20 μg/mL aprotinin, 20 μM leupeptin, 1 mM phenylmethylsulphonylfluoride were added. These were homogenized with a motor driven homogeniser for 90 sec at 4°C, the homogenate was centrifuged at 1000 × *g* for 10 min to sediment the nuclear fraction, and the supernatant centrifuged at 10,000 × *g* for 20 min to sediment the mitochondrial fraction. The supernatant was further centrifuged for 60 min at 100,000 × *g* at 4°C to obtain microsomes and cytosol fractions. The microsomal fraction was finally resuspended in 1 mL of homogenizing buffer. Particulate and cytosol fractions were stored at −70°C.

Preparation of Radiolabelled E. Coli *Membranes*

A single colony of *E. coli* bacteria obtained from a freshly inoculated agar plate [31] was grown overnight in 5 mL Todd Hewitt nutrient broth at 37°C. One hundred microliters were then diluted with 10 mL fresh medium and subcultured for 3 hr at 37°C, during which 50 μCi of radioactively labelled [^{3}H]oleate or 5 μCi of [^{14}C]arachidonate were incorporated into phospholipids as previously described [32]. *E. coli* with incorporated labelled fatty acid or unlabelled bacteria prepared in the same way were sedimented by centrifugation at 6,000 × *g* for 10 min, resuspended in 10 mL fresh-growth medium, and reincubated for 30 min at 37°C to aid in the incorporation of the fatty acids. Finally bacteria were washed twice with 5 mL 1% (w/v) bovine serum albumin to remove unincorporated fatty acids and then harvested by centrifugation at 6000 × *g* for 5 min. The bacteria were then resuspended in sterile saline containing 0.2% (w/v) sodium azide. Suspensions were then autoclaved for 20 min at 120°C and 65 p.s.i. and stored at −20°C. Of the incorporated label about 90% (for [^{3}H]oleate) and 80% (for [^{14}C]arachidonate) was found in phosphatidylethanolamine, as shown by thin layer chromatography (data not shown). The phosphorus content of

labelled and unlabelled *E. coli* membranes was estimated [33].

Typically 10 nmol of *E. coli* phosphorus/phospholipid contained 180,000 dpm [^{3}H]oleate or 14,000 dpm [^{14}C]arachidonate.

Preparation of Deoxycholate Solubilised Micelles

Chloroform solutions of [^{14}C]POPC or [^{14}C]PAPC and unlabelled phosphoglycerides were mixed and evaporated under nitrogen. The phosphoglycerides were then resuspended in 2% (w/v) sodium deoxycholate (0.1%) and Tris/HCl buffer, pH = 8.0 to provide a suspension of detergent-phospholipids in a micellar form with a final concentration of 48 nmol phospholipid/mL and 0.3 μCi/mL.

PLA_2 Assay Using E. coli *Membranes*

PLA_2 activity was assayed by incubating 12.5 μg to 200 μg of cytosolic or microsomal protein with *E. coli* membranes containing 10 nmol phospholipid/phosphorus (10–15 μL) containing approximately 180,000 dpm [^{3}H]oleate or 14,000 dpm [^{14}C]arachidonate, 25 μL of 100 mM $CaCl_2$, 12.5 μL of 1 M Tris/HCl buffer with a final incubation volume of 250 μL made up with physiologic saline [34]. Incubations were carried out for 15 min with shaking at 37°C. The reaction was stopped by addition of 250 μL of ice-cold 0.5% BSA and 20 min incubation on ice, followed by centrifugation for 4 min at 8730 × *g* to separate the albumin-complexed products of hydrolysis from the pelleted bacteria membranes containing the undegraded phospholipids. An aliquot of the supernatant (400 μL) containing the fatty acid released was removed, mixed with 4 mL liquid scintillation fluid, and analyzed for radioactivity by liquid scintillation counting.

PLA_2 Assay Using Phospholipid Micelles

Deoxycholate-solubilised micelles (2.4 nmol in approximately 50 μL of 50 mM Tris buffer, pH 8.0), 5 mM $CaCl_2$, and 50 or 200 μg of cytosolic or microsomal proteins in a total volume of 150 μL Tris-buffer were incubated for 30 min [35]. The reaction was terminated by addition of 8 M aqueous acetic acid. Lipids were then extracted according to the modified method of Folch [36]. Released fatty acids were separated on BondElut silica minicolumns [37]. Lipids were loaded in 2 mL chloroform:acetic acid (100:1 v/v). Free fatty acids were eluted with 6 mL chloroform:acetic acid (100:1 v/v) followed by 5 mL methanol:chloroform:water (20:10:8 v/v) to elute the undegraded phospholipids. Preliminary experiments, carried out to determine the column separation characteristics by applying mixtures of standard fatty acids or phospholipids, have shown 92.1 ± 3.85% of fatty acid was recovered in the first fraction and 98.8 ± 0.91% of the phospholipid in the second fraction (n = 3).

Results are expressed by the dpm fatty acid released as a percentage of the total dpm phospholipid added or as nmol of fatty acid hydrolyzed per mg protein per minute. In each assay nonspecific release of radiolabel was monitored by performing control incubations in the absence of enzyme, and this was subtracted in each sample. Each assay was performed in duplicate and the mean value calculated.

Displacement Fluorescence Assay for PLA_2

The assay is based upon the displacement of DAUDA from the highly fluorescent DAUDA/FABP complex when fatty acid is released by PLA_2. The displacement leads to a fall in fluorescence with time [38] that can be calibrated to determine specific activity. Each cuvette (1 mL) contained 0.1 M Tris/HCl, pH 8.0, 0.1 M NaCl, 2.5 mM $CaCl_2$, 1 μM DAUDA, 1 μM FABP, and 50 μg of DOPG. DAUDA and phospholipid were added as 1.0 mM and 10 mg/mL solutions in methanol, respectively. All assays were performed with an Hitachi F2000 fluorescent spectrometer at 37°C in plastic cuvettes, with an excitation wavelength of 350 nm and an emission wavelength of 500 nm. Calibration was achieved by adding sequential 1 μL aliquots of 0.2 mM oleic acid in methanol.

Purification and Molecular Mass Estimation of PLA_2

Partial purification of PLA_2 was achieved by heparin-sepharose affinity chromatography [39–41]. The crude human colon subcellular fractions were exposed to 1 M KCl and gently homogenised. The suspension was centrifuged for 15 min at 400,000 × *g* using a Beckman TLX benchtop ultracentrifuge. The supernatant was decanted and diluted fivefold with 100 mM Tris/HCl (pH 7.4) containing 1 mM EDTA. Three millilitres were loaded onto a Heparin Sepharose HITRAP column (1 mL; Pharmacia, LKB Technology) equilibrated with 100 mM Tris/HCl (pH 7.4) and 1 mM EDTA. The column was washed extensively with the starting buffer and then eluted with the same buffer containing an increasing linear gradient of 0 M to 1 M KCl at a flow rate of 0.5 mL/min as shown in Fig. 5. One millilitre fractions were collected. Fifty microlitres of each fraction were monitored for PLA_2 activity by the fluorescence displacement assay. One hundred microlitres of the most active fractions were applied to a Superdex 75 gel filtration column (10 mm × 30 cm; Pharmacia LKB Technology) equilibrated with 0.1 M Tris/HCl (pH 7.4), 1 mM EDTA, 0.5 M KCl. Samples were eluted in this buffer at a flow rate of 0.5 mL/min. The column was calibrated with recombinant human nonpancreatic $sPLA_2$ of 14 kDa molecular weight.

Preparation of Buffers Containing Defined Calcium Ion Concentrations

Ca^{2+} concentrations were controlled by using Ca^{2+}/EGTA buffers as calculated by Eqcal system for Windows (Biosoft). Micromolar and millimolar concentrations of $CaCl_2$ were

added to 50 mM Tris buffer (pH 7.5) plus 1 mM EGTA to achieve the required final free Ca^{2+} concentrations.

Treatment With Dithiothreitol

An aliquot of cytosol or microsomal fraction containing 200 μg of protein or porcine pancreatic PLA_2 (100 ηg protein) was incubated with 10 mM DTT or water for 30 min at 37°C. The samples were then assayed for PLA_2 activity using the micellar or *E. coli* assay.

Treatment With Acid at pH 1.8 and Stability at Elevated Temperatures

Aliquots of microsomes or cytosol or porcine pancreatic PLA_2 were incubated at 4°C in 100 μL volume of 0.36 N H_2SO_4 (pH 1.8) or water for 60 min.

The pH was adjusted to 7.5 by addition of 2 M Tris/HCl buffer, pH 9. Samples were then centrifuged at 10,000 × *g* for 5 min and the supernatant was assayed for PLA_2 activity using *E. coli* membranes.

Aliquots of microsomal or cytosol preparations were incubated at 57°C for 5 min before comparing the PLA_2 activity with suitable controls.

Statistical Analysis

All values are presented as the mean ± SEM. Statistical significance was evaluated using the Student's paired t-test with $P < 0.05$ regarded as significant.

RESULTS

PLA_2 Activity in Mucosal Microsomes and Cytosol

Using [^{3}H]oleate-labelled *E. coli* phospholipids as substrate PLA_2 activities in microsomal and cytosolic fractions were quantitatively related to the amount of protein added between 12.5 μg and 50 μg protein added per assay tube but with further enzyme added (up to 200 μg) there was little further increase in [^{3}H]oleate release (Fig. 1). Hydrolysis with 50 μg protein was 24.7 ± 2.30% ($n = 3$) in the cytosol and 62.7 ± 3.60% ($n = 3$) in microsomes. Hydrolysis was significantly greater in the microsomes compared with the cytosol at each concentration of protein ($P < 0.05$). Using *E. coli* phospholipids as substrate but purified porcine pancreatic type I PLA_2 (100 ηg) or *Naja naja* snake venom type II PLA_2 (100 ηg) as the enzyme source it was possible under our assay conditions to achieve 90–95% PLA_2 hydrolysis indicating that the majority of the fatty acid was probably incorporated in the sn-2 position of *E. coli* phospholipids.

Time Course of PLA_2 Mediated Hydrolysis by Cytosol or Microsomal PLA_2

Hydrolysis of *E. coli* [^{3}H oleate] or micellar [^{14}C]PAPC by cytosol or microsomal PLA_2 activity was measured in incubates at time points from 5 to 60 min (Fig. 2). With *E. coli* membranes as substrate hydrolysis by microsomal PLA_2

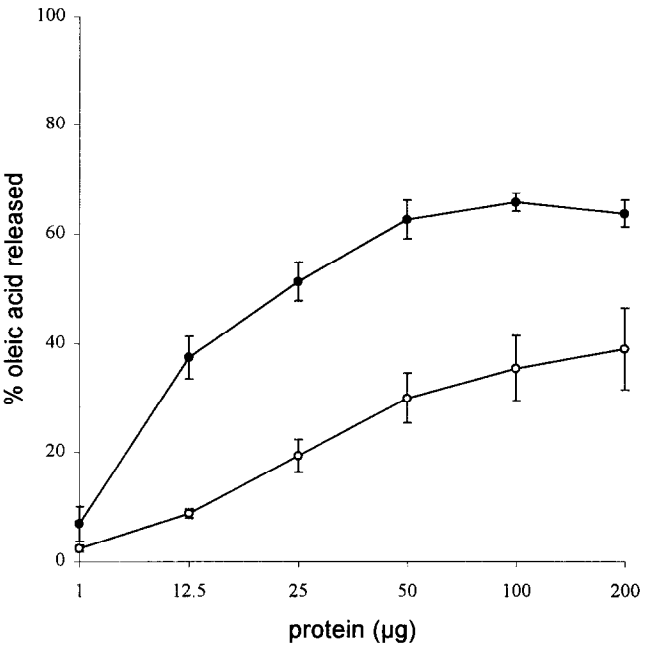

FIG. 1. Phospholipase A_2 activity in 100,000 × *g* colonic mucosa cytosol (○) or microsomal (●) preparations. PLA_2 activity was measured using [^{3}H]oleate *E. coli* membranes. Data points represent fatty acid released in a 30-min incubation as percentage of total radioactivity added in 10 nmol substrate. Values are expressed as mean ± SEM ($n = 3$).

(50 μg protein) was 37.2 ± 0.90% at 15 min and 49.3 ± 1.13% at 60 min (difference $P < 0.01$). For cytosol at these time points hydrolysis was 19.6 ± 3.53% and 52.4 ± 7.71%, respectively (difference $P < 0.05$). Using micellar deoxycholate [^{14}C POPC] and 200 μg microsomal protein, hydrolysis was 5.07 ± 0.78% and 12.5 ± 1.33% at 30 min and 60 min, respectively. Therefore, 15 min and 30 min were used respectively for all subsequent *E. coli* and micellar assays.

Substrate Specificity of Colon Mucosal PLA_2 Activity

The ability of the colon mucosal particulate and cytosol PLA_2s to catalyse the hydrolysis of [^{14}C]arachidonate or [^{4}C]oleate from the sn-2 position of micellar deoxycholate [^{14}C]POPC or [^{14}C]PAPC and [^{14}C]oleate or [^{14}C]arachidonate from radiolabelled *E. coli* membranes is shown in Table 1. No significant difference was observed in the rates of hydrolysis from [^{14}C]arachidonate or [^{3}H]oleate-labelled *E. coli* membranes. Using deoxycholate-solubilised phospholipid micelles as substrates a small but statistically significant preference for the hydrolysis of [^{14}C]arachidonate from [^{14}C]PAPC compared with [^{14}C]oleate from [^{14}C]POPC was seen in 30-min incubates.

PLA_2 Activity at μM or mM Ca^{2+} Concentrations

Figures 3 and 4 show the effect of calcium ion concentrations upon microsomes and cytosol PLA_2 activity. Micro-

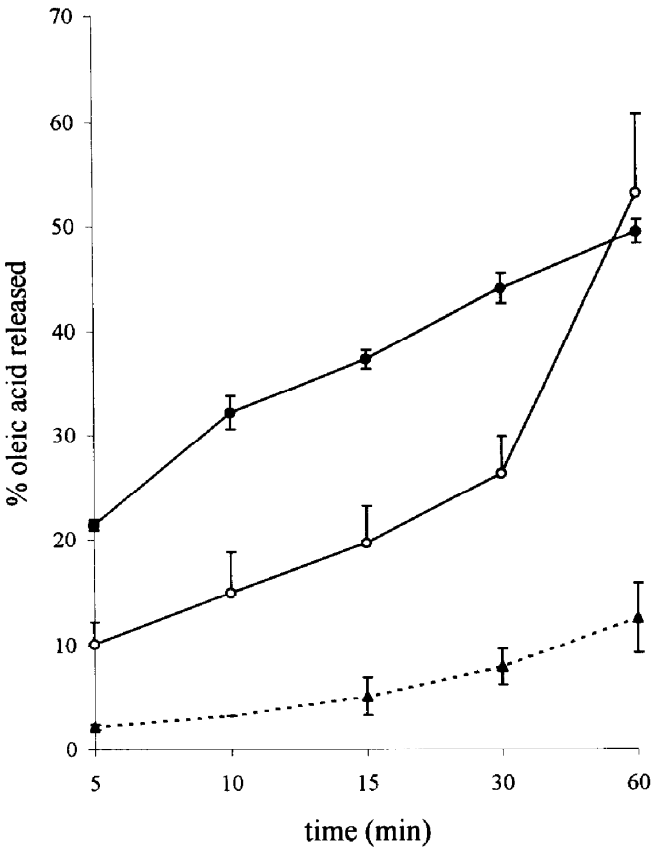

FIG. 2. Time course of the hydrolysis of [^{3}H]oleate labelled *E. coli* (10 nmol) by cytosolic (○) and microsomal (●) preparations of colonic mucosa (50 μg protein) compared with the time course of the hydrolysis of deoxycholate-solubilised sn-2-oleate phosphatidylcholine (2.4 nmol) by colonic microsomal fraction (200 μg protein) (▲). The data points represent mean ± SEM (*n* = 3–4).

somal and cytosol PLA_2 enzymes (50 μg protein) behaved similarly, and no hydrolysis was detected when 1 mM EGTA was added in the absence of added Ca^{2+}. Hydrolysis was close to optimum with Ca^{2+} concentrations as low as 10 μM. Using [^{3}H]oleate-labelled *E. coli* membranes as substrate and 10 mM Ca^{2+}, hydrolysis was 47.5 ± 7.44% (*n* = 4) in microsome and 28.0 ± 5.75% (*n* = 4) in cytosol and

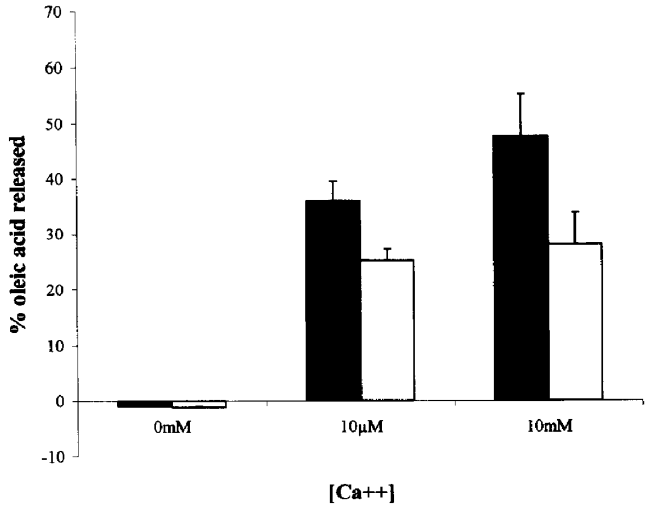

FIG. 3. Effect of calcium ion concentrations on PLA_2-mediated hydrolysis of [^{3}H]oleate labelled *E. coli* (10 nmol) by cytosolic (□) and microsomal (■) fractions of colonic mucosa in EGTA-regulated buffers as described in Materials and Methods. The data points represent mean ± SEM (*n* = 4).

with 10 μM free Ca^{2+} was 35.8 ± 3.73% (*n* = 8) in microsomes and 25.1 ± 2.02% (*n* = 8) in cytosol (Fig. 3). A similar pattern was also observed using the deoxycholate-solubilised micellar synthetic phospholipids as substrate (Fig. 4). With 10 mM Ca^{2+} hydrolysis was 8.28 ± 2.41% (*n* = 3) in microsomes and 7.26 ± 2.54 (*n* = 3) in cytosol and with 10 μM Ca^{2+} was 7.78 ± 1.66% (*n* = 6) in microsomes and 6.7 ± 1.94% (*n* = 6) in cytosol.

Effect of Dithiothreitol on PLA_2 Activity

Table 2 shows the effect of DTT upon the activity of the mucosal microsomal or cytosol PLA_2 or porcine pancreatic (14 kDa type I) PLA_2. DTT-treated and control samples were analyzed for PLA_2 activity using both *E. coli* membranes or synthetic phospholipid micelles labelled with [^{14}C]arachidonate. Microsomal and cytosol PLA_2 and porcine pancreatic PLA_2 were significantly reduced by DTT treatment using the micellar or *E. coli* assay.

TABLE 1. Substrate selectivity of PLA_2 activity in subcellular fractions of human colonic mucosa

PLA_2 source	PLA_2 activity (pmol hydrolyzed/min/mg protein)			
	Micellar assay		*E. coli* assay	
	[^{14}C]PAPC	[^{14}C]POPC	[^{14}C]Arachidonate	[^{14}C]Oleate
Cytosol	18.8 ± 2.22	10.0 ± 1.66*	1571 ± 297	1408 ± 225
Microsomes	20.0 ± 1.60	14.6 ± 1.73*	2108 ± 21.5	2305 ± 70.3

Substrate selectivity was assessed using the deoxycholate-solubilized synthetic phospholipids sn-2 [^{14}C]arachidonate or [^{14}C]oleate phosphatidylcholine, or [^{14}C]arachidonate, or [^{3}H]oleate labeled *E. coli* membranes as substrates with 200 μg microsomal or cytosolic protein. Other conditions as described in methods. Values are means ± SEM (n = 3).

* Paired *t*-test compared with PAPC. $P < 0.05$.

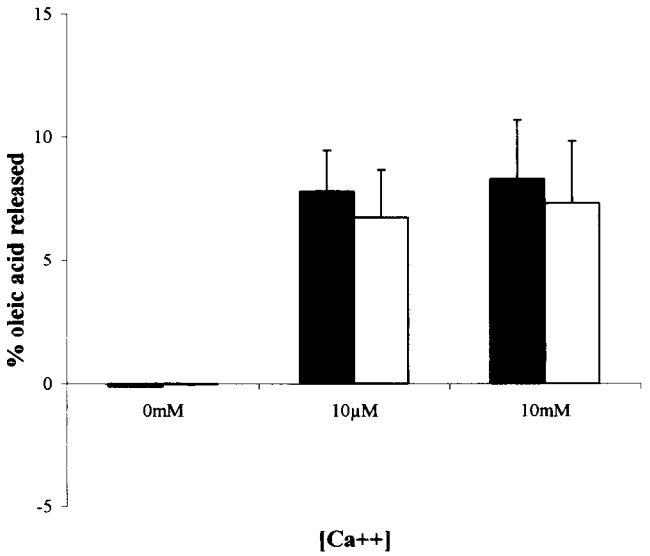

FIG. 4. Effect of calcium ion concentrations on PLA_2-mediated hydrolysis of deoxycholate-solubilized sn-2-oleate phosphatidylcholine (2.4 nmol) by cytosolic (□) and microsomal (■) fractions of colonic mucosa in EGTA-regulated buffers as described in Materials and Methods. The data points represent mean ± SEM (n = 4).

Stability of PLA_2 in Acid Conditions at pH 1.8 and PLA_2 Thermostability

Type II (14 kDa) PLA_2 enzymes but not the high molecular mass form are resistant to acid denaturation because of their rigid tertiary structure [42]. Table 3 shows the effect of treatment with acid at pH 1.8 on microsome, cytosol and porcine pancreatic PLA_2. PLA_2 was not diminished in acid conditions; in fact, an elevation of PLA_2 activities was seen that reached significance in microsomes (n = 3). Porcine pancreatic PLA_2 activity was not significantly altered by acid conditions (n = 4).

Table 4 shows the effect of preincubation of microsome and cytosol and porcine pancreatic PLA_2 activity after warming for 5 min at 57°C prior to assay using *E. coli* membranes. Microsome and cytosol PLA_2 were significantly reduced (n = 4–5) while porcine pancreatic PLA_2 was not affected (n = 3).

Purification and Molecular Mass Estimation of Human Colon Mucosa PLA_2 Activity

PLA_2 enzyme from cytosol, microsomal, and mitochondrial fractions behaved similarly on heparin-Sepharose column chromatography. Activity was estimated by the fluorescence displacement assay in fractions eluted by an increasing gradient of KCl. Figure 5 illustrates the chromatographic profile of the mucosal mitochondrial fraction. It showed marked affinity for heparin-Sepharose with a single peak of activity eluting at a concentration of about 0.6 M KCl. Recombinant hnps PLA_2 elutes in the same position, while cytosolic PLA_2 does not normally bind to heparin-Sepharose.

Aliquots of the active fractions from the heparin-Sepharose columns were further examined by gel filtration chromatography using a Superdex 75 column. The PLA_2 derived from human colon mucosa eluted with the same volume as recombinant hnps PLA_2 having a molecular mass of 14 kDa.

Characteristics of Semipurified PLA_2

The purified PLA_2 enzyme from microsomal and cytosolic fractions efficiently hydrolysed DOPG as measured by the fluorescence displacement assay. No detectable hydrolysis of SAPC or DOPC was obtained under otherwise identical conditions. These results are in accordance with observations made for the human group II enzyme [38] and confirm the original observations with PLA_2 derived from pig ileum [39] that intestinal PLA_2 has a marked preference for PG substrates in single component assays.

The purified PLA_2 enzyme absolutely required the presence of calcium. The addition of 1 mM EGTA to the fluorescence assay cocktail suppressed the PLA_2 activity completely. Full activity was detected at both 10 μM and 2.5 mM Ca^{2+} concentrations in EGTA-regulated buffers.

TABLE 2. The effect of dithiothreitol on PLA_2 activity in subcellular fractions of human colonic mucosa

PLA_2 source	PLA_2 activity (pmol hydrolysed/min/mg protein)			
	Micellar assay		*E. coli* assay	
	Control	DTT	Control	DTT
Cytosol	16.0 ± 3.0	5.80 ± 0.99*	1755 ± 289	264 ± 11.6*
Microsomes	29.1 ± 5.04	20.20 ± 3.09*	1878 ± 64.6	576 ± 131*
Porcine† pancreatic PLA_2	225 ± 5.22	84.72 ± 4.12*		

The effect of 10 mM dithiothreitol on colon microsomal or cytosol PLA_2 activity (200 μg protein) or porcine pancreatic PLA_2 activity (100 ηg) using micellar phospholipids [^{14}C]PAPC or *E. coli* membranes as substrate (50 μg protein). Values are mean ± SEM (n = 3).

* Paired t-test compared with respective controls. $P < 0.05$.

† Data for porcine pancreatic PLA_2 are in nmol.

TABLE 3. The effect of acid pH on PLA_2 activity in subcellular fractions of human colonic mucosa

PLA_2 source	Percent fatty acid released		Percent of control
	Control	Incubation at pH 1.8	
Cytosol	12.3 ± 2.56	17.1 ± 3.40	145 ± 18.6
Microsomes	23.1 ± 4.88	32.8 ± 3.47*	148 ± 16.3
Porcine pancreatic PLA_2	67.4 ± 0.40	54.5 ± 4.16	81.2 ± 5.14

The effect of incubating at pH 1.8 for 1 hr on colon microsomal or cytosol PLA_2 activity (50 μg protein) or porcine pancreatic PLA_2 activity (100 ηg) using *E. coli* membranes as substrate. Values are mean ± SEM (n = 3–4).

* Paired t-test compared with control. $P < 0.05$.

DISCUSSION

The subtype of PLA_2 found in a variety of human intestinal tissues using mainly immunochemical and radioimmunoassay techniques was identified as the type II 14 kDa PLA_2 [21–25]. Subsequently, however, one report using PCR and Northern blot analysis has suggested the additional presence of type I and type IV PLA_2 in human ileal mucosa [26]. The coexistence of low and high molecular mass like PLA_2 isoforms is not uncommon, both having been described in human platelets, neutrophils, and monocytes [43]. Coexistence of both calcium dependent low molecular weight and calcium independent high molecular weight isoforms have also been found in rat stomach and small intestine [44–46].

Evidence for an elevation in PLA_2 in serum [23] and in intestinal mucosa [16, 17, 25, 26] in bowel disorders such as inflammatory bowel disease has also been described.

In this study we have focused our attention on the subcellular localization and the biochemical features of the PLA_2 activities present in human colonic mucosa. Using biochemical techniques we found that PLA_2 activity was found in greater amounts associated with the 100,000 × g particulate fraction than the cytosol while enzyme activity could be released from membranes by treatment with a high salt concentration indicating the peripheral nature of the enzyme. The hydrolysis of *E. coli* membrane lipids was not complete, reaching a maximum of about 25% with cytosol PLA_2 and 60% with microsomal PLA_2. This observation was somewhat predictable as crude fractions that were used contain many enzymes and proteins, other than PLA_2, that possibly interfere with the PLA_2 activity. There are, in fact, reports of PLA_2 inhibition by various factors including product inhibition [47], inhibitory proteins such as annexins, gravidin [48, 49] and vasoactive intestinal peptide [50]. To what extent and the way each factor may operate to regulate PLA_2 in these preparations is not yet defined. The validity of this is born out by a comparison with the enzymatic activities of two purified commercially supplied PLA_2s, porcine pancreatic PLA_2, and venom PLA_2 of *Naja naja*, where 90% hydrolysis of *E. coli* membrane lipids was seen (data not shown).

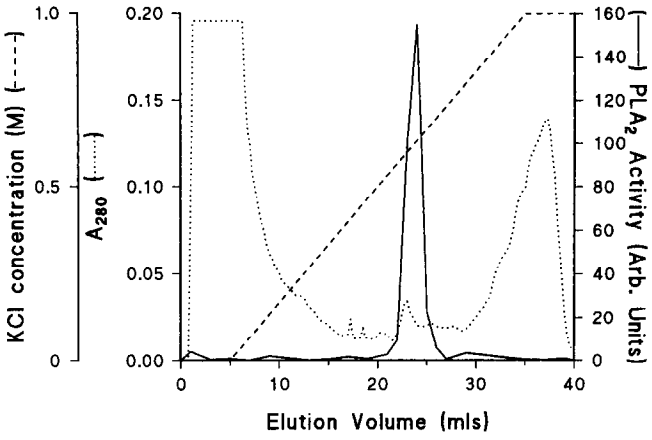

FIG. 5. Elution profile of phospholipase A_2 activity (—) and protein (- - -) from heparin-sepharose binding columns. This illustrates a typical elution pattern using a mitochondrial extract. The soluble fraction was applied and eluted with a gradient of KCl (— —) from zero to one molar. PLA_2 activity was measured in aliquots using the fluorescence displacement assay. The column was calibrated with hnps PLA_2, which eluted in the same volume as the colon mucosal extract.

TABLE 4. The effect of heating on PLA_2 activity in subcellular fractions of human colonic mucosa

PLA_2 source	Percent fatty acid released		Percent of control
	Control	Heated at 57°C	
Cytosol	18.6 ± 3.24	10.6 ± 0.99*	60.5 ± 7.10
Microsomes	36.8 ± 2.40	23.7 ± 2.10*	65.1 ± 5.90
Porcine pancreatic PLA_2	60.2 ± 2.90	59.4 ± 1.90	98.8 ± 1.60

The effect of heating for 5 min on colon microsomal or cytosol PLA_2 activity (50 μg protein) or porcine pancreatic PLA_2 using *E. coli* membranes as substrate. Values are mean ± SEM (n = 3–4).

* Paired t-test compared with control. $P < 0.05$.

The resistance to acid treatment (pH 1.8 for 60 min) is typical of the 14 kDa isozyme, whereas the 85 kDa isozyme is inactivated by this treatment [42, 51]. The significant enhancement of activity that was seen following acid treatment has also been reported in human polymorphonuclear leukocytes [52] and has been attributed, in part, to the removal of pH-sensitive endogenous inhibitors such as acid-labile free fatty acids. The purified pancreatic PLA_2 tested did not show increased activity following acid treatment.

DTT treatment substantially reduced the PLA_2 activity in both human colonic mucosa and porcine pancreatic PLA_2 (14 kDa type I). This is in accord with the accepted properties of the 14 kDa isozyme having seven disulfide bridges [42] and, consequently, a rigid tertiary structure, which are susceptible to sulphydryl reducing agents.

Treatment at 57°C for 5 min has been reported to reduce activity of the 85 kDa PLA_2 isoform [2], whereas the 14

kDa isoform is heat-stable [41, 50, 58]. We found a small but significant reduction in the activity of crude colon mucosal PLA_2 by 35–40% but no reduction in the activity of purified porcine pancreatic PLA_2.

In contrast to the 85 kDa PLA_2 isozyme, the 14 kDa form is generally considered as an enzyme with no preference for hydrolysis of arachidonate in the sn-2 position of phospholipids [2, 35, 56]. The arachidonate-preference of high molecular weight isoforms has in the main been demonstrated using detergent-solubilized phospholipids [53–55], with an extent of preference ranging from about 4- to 100-fold greater hydrolysis of arachidonate in the *sn-2* position compared with other fatty acids. Quite recently, preference for arachidonate hydrolysis among secretory type II PLA_2 has been reported in mast cells and human bronchoalveolar lavage fluid [59, 60]. We found a small but statistically significant twofold greater hydrolysis of arachidonate compared with oleate using micellar preparations of pure phosphatidylcholine. However *E. coli* membranes labelled with arachidonate or oleate showed similar levels of hydrolysis by human cytosol or microsomal preparations. This difference may be attributable to differences in substrate composition and presentation to which PLA_2s are known to be susceptible [56]. We know, for example, that the majority of incorporated labelled fatty acid in *E. coli* membranes was in the phosphatidylethanolamine. There could also be variabilities in measuring substrate selectivity using labelled *E. coli* membranes as has been reported in studies using crude subcellular fractions of human platelets, monocytes, and neutrophils [42].

Using heparin-Sepharose and Superdex sizing columns and a fluorescence displacement assay for PLA_2 the predominant PLA_2 activity in colonic mucosal fractions coeluted with the 14 kDa type PLA_2. However, a feature of the properties of this intestinal PLA_2 is its ability to be fully activated by micromolar amounts of Ca^{2+}. No activity was detected in the absence of Ca^{2+}, but using both *E. coli* or deoxycholate solubilised phosphatidylcholine as substrates to ensure that the results were not an artifact in our system, the PLA_2 activity in the presence of 10 μM Ca^{2+} did not differ from that seen in the presence of 10 mM Ca^{2+} using EGTA-containing buffers. In preliminary work the semipurified enzyme from heparin-Sepharose columns and using the fluorescence-displacement assay for detection of PLA_2 displayed similar activity whether 10 μM or 2.5 mM Ca^{2+} was used in EGTA-containing assay buffers, and there was an absence of activity in Ca^{2+} free/EGTA buffers. A calcium requirement of low molecular mass PLA_2s in the micromolar range has been reported for human neutrophils and platelets [57], and led the authors to conclude that the micromolar or millimolar requirement of Ca^{2+} to support enzyme activity could not necessarily be used to distinguish between high and low molecular mass PLA_2s. However, contrary to this study, Marshall and McCarte-Roshak [57] found, using human synovial fluid PLA_2 or rat recombinant low molecular mass PLA_2, that activity in the presence of micromolar Ca^{2+} was still significantly less than when 10 mM Ca^{2+} was included indicating that micromolar amounts of Ca^{2+} did not fully activate the low molecular mass PLA_2. We suggest that the human colon mucosal cytosol and particulate compartments has a PLA_2 that has properties suggestive of a 14 kDa isoenzyme and is activated by intracellular concentrations of Ca^{2+} and could participate in the release of arachidonate for the synthesis of eicosanoids.

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