Bovine retinal pigment epithelium contains novel types of phospholipase A₂

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We have recently demonstrated the presence of phospholipase A₂ (PLA₂) activity in cells from bovine retinal pigment epithelium (RPE) [Jacob et al. (1996) J. Biol. Chem. 271, 19209–19218]. We report here our results on the characterization of this RPE-PLA₂ activity. We show that RPE probably contains two types of PLA₂ enzyme, as indicated by the results obtained with different PLA₂-active fractions eluted from cation-exchange columns and treated with Ca²⁺/EGTA, dithiothreitol, *p*-bromophenacyl bromide or heat. These results, in addition to those from PLA₂ assays using different substrates, also suggest that RPE-PLA₂ enzymes are different from the well-known secretory, cytoplasmic and Ca²⁺-independent forms. Sequential extraction of RPE with

(1) isotonic, (2) hypertonic and (3) detergent-containing PBS argues for the presence of weakly membrane-associated enzymes. Control experiments using 'back and forth' TLC allowed us to discriminate between PLA₂ and phospholipase C/diacylglycerol lipase activity and confirmed that, in our assay conditions, the release of fatty acids was indeed due to PLA₂ enzymes. These results, together with those obtained by treating RPE homogenates with H₂SO₄, guanosine 5'-[γ-thio]triphosphate, ATP and different protease inhibitors, permitted us to make the first characterization of these RPE-PLA₂ enzymes. We conclude that RPE contains novel types of PLA₂ that are different from the secretory, cytoplasmic and Ca²⁺-independent forms.

INTRODUCTION

Phospholipases A₂ (PLA₂) are important enzymes involved in the turnover of phospholipids. They catalyse the hydrolysis of *sn*-2 fatty acyl chains and are thus responsible for the production of fatty acids and lysophospholipids, which may then be further metabolized into many types of inflammatory lipid mediators [1–4]. Alternatively, non-esterified fatty acids so generated could act as second messengers in signal transduction [5–8].

There are numerous types of PLA₂. They have been isolated from different sources and classified into three major groups: secretory PLA₂ (sPLA₂), which are subdivided into three subgroups according to their evolutionary relationship [9]; cytosolic PLA₂ (cPLA₂); Ca²⁺-independent PLA₂ (iPLA₂), which are also subdivided into three subgroups according to their biochemical characteristics [10]. These are indeed distinguished on the basis of many criteria, including primary structure, subcellular localization, molecular mass, Ca²⁺ requirement, substrate preference and sensitivity to denaturing treatments [H₂SO₄, dithiothreitol (DTT), *p*-bromophenacyl bromide (pBPB) and heat] [10–13]. This is an operational classification which is widely accepted and used to characterize PLA₂, as well as to define procedures to purify them from new sources. In the future, this classification will certainly be refined, since recent results from Thomson and Clark [14], Buhl et al. [15] and Soubeyrand et al. [16] suggest that other groups or subgroups could be needed to describe newly discovered and cloned PLA₂.

We have recently demonstrated [17] that PLA₂ activity is present in bovine retinal pigment epithelium (RPE), a subretinal fraction that is enriched with RPE cells. We have shown that PLA₂ activity present in RPE homogenate was active at alkaline

pH [17]. In order to characterize, for the first time, the minimum number of PLA₂ enzymes present in RPE, as well as their properties, we tested the effects of H₂SO₄, guanosine 5'-[γ-thio]triphosphate (GTP[S]) and ATP on RPE homogenate. Then we measured the subcellular distribution of PLA₂ activity in RPE by sequentially extracting it with isotonic, hypertonic and detergent-containing PBS. We also tested different substrates for PLA₂ hydrolysis, and control experiments using 'back and forth' TLC (bf-TLC) allowed us to confirm that, in our assay conditions, the hydrolysis of fatty acids was not due to the combined action of phospholipase C (PLC) and diacylglycerol (DAG) lipase but rather to PLA₂. Moreover, we looked at the effects of Ca²⁺/EGTA, DTT, pBPB and heat on different PLA₂-active fractions collected after elution of an RPE high-salt extract on a cation-exchange chromatography column.

We present here the first results on the operational characterization of RPE-PLA₂ activity. From these results, we conclude that RPE probably contains two types of PLA₂ enzyme. Moreover, on comparing the properties of RPE-PLA₂ with those of sPLA₂, cPLA₂ and iPLA₂, RPE-PLA₂ appeared to be quite different, suggesting the presence of novel types of PLA₂ which could be part of either a new group or a new subgroup of PLA₂ enzymes.

MATERIALS AND METHODS

Materials

1-Palmitoyl-2-[¹⁴C]arachidonoylphosphatidylcholine ([¹⁴C]PAPC), 1-palmitoyl-2-[¹⁴C]arachidonoylphosphatidylethanolamine ([¹⁴C]PAPE) and 1-[¹⁴C]oleoyl-2-[¹⁴C]oleoylphosphatidylcholine

Abbreviations used: pBPB, *p*-bromophenacyl bromide; [¹⁴C]PAPC, 1-palmitoyl-2-[¹⁴C₁]arachidonoylphosphatidylcholine; [¹⁴C]PAPE, 1-palmitoyl-2-[¹⁴C₁]arachidonoylphosphatidylethanolamine; [¹⁴C]DOPC, 1-[¹⁴C₁]oleoyl-2-[¹⁴C₁]oleoylphosphatidylcholine; DAG, 1,2-diacylglycerol; DMPM, dimyristoylphosphatidylmethanol; DTT, dithiothreitol; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; GTP[S], guanosine 5'-[γ-thio]triphosphate; lysoPC, lysophosphatidylcholine; OG, *n*-octyl glucoside; PAPE, 1-palmitoyl-2-arachidonoylphosphatidylethanolamine; PE, phosphatidylethanolamine; PLA₂, phospholipase A₂; cPLA₂, cytosolic PLA₂; iPLA₂, Ca²⁺-independent PLA₂; sPLA₂, secretory PLA₂; PLC, phospholipase C; 10PyPM, 1-O-hexadecyl-2-[pyrene₁₀]decanoyl-*sn*-3-phosphomethanol; 10Py-decanoic acid, [Pyrene₁₀]decanoic acid; RPE, retinal pigment epithelium; bf-TLC, 'back-and-forth' TLC.

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([^{14}C]DOPC) were from Dupont Canada. 1-*O*-Hexadecyl-2-[pyrene $_{10}$]decanoyl-*sn*-3-phosphomethanol (10PyPM) and [pyrene $_{10}$]decanoic and (10Py-decanoic acid) were from Molecular Probes. Dimyristoylphosphatidylmethanol (DMPM), 1-palmitoyl-2-arachidonoylphosphatidylethanolamine (PAPE), egg lysophosphatidylcholine (lysoPC) and egg phosphatidic acid were from Avanti Polar Lipids. 1-Stearoyl-2-arachidonoylphosphatidylcholine, NaN_3 , fatty acid-free BSA and pBPB were from Sigma. Aprotinin, leupeptin, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), DTT, *n*-octyl glucoside (OG), EGTA, EDTA, ATP, GTP[S], 1,2-diolein and arachidonic acid were from Boehringer-Mannheim. Chloroform, methanol, heptane, isopropyl ether, acetic acid and H_2SO_4 were from Fisher Scientific. TLC plates were LK6D silica-gel 60 Å from Whatman.

Preparation of RPE

RPE was prepared from fresh bovine eyes as described previously [17] and was either used immediately or divided into aliquots and stored at -80°C .

Extraction of RPE

After addition of protease inhibitors (2.8 μM E-64, 0.06 μM aprotinin, 1 μM leupeptin and 100 μM EDTA, final concentrations), RPE was homogenized 10 times with 3 vol. of PBS6 $\times$ (50 mM Na_2HPO_4 /600 mM NaCl, pH 7.5) in a tight-fitting glass homogenizer. After centrifugation of the homogenate (140 000 g ; 4°C ; 1 h; Beckman 70.1 Ti rotor), the resulting supernatant was collected and used for all experiments except those presented in Table 1. In this case, RPE was first homogenized with PBS1 $\times$ (50 mM Na_2HPO_4 /100 mM NaCl, pH 7.5) and centrifuged as described above. The resulting supernatant was collected as 'supernatant-PBS1 $\times$ ' and the pellet was washed once with PBS1 $\times$ in the same conditions. 'Wash-PBS1 $\times$ ' supernatant was collected whereas the pellet was treated as described using PBS6 $\times$. After supernatant-PBS6 $\times$ and wash-PBS6 $\times$ had been recovered, the pellet was resuspended in PBS1 $\times$ containing 1 % OG and treated as described above. Both supernatant-PBS1 $\times$ OG and wash-PBS1 $\times$ OG were collected. Before use, supernatant- and wash-PBS6 $\times$, as well as supernatant and wash-PBS1 $\times$ OG were dialysed overnight at 4°C against PBS1 $\times$ with two changes of buffer. The H_2SO_4 extraction was carried out as described by Apitz-Castro et al. [18].

Radiometric measurement of PLA $_2$ activity

PLA $_2$ assays with [^{14}C]PAPE + DMPM, [^{14}C]PAPC + DMPM, [^{14}C]DOPC + DMPM or [^{14}C]PAPC were performed as described previously [17]. Briefly, the assay buffer contained 50 mM Tris-HCl, pH 9.0 (or 50 mM glycine), 3 mM CaCl_2 , 30 mM MgCl_2 and 100 mM NaCl. [^{14}C]PAPE + DMPM, [^{14}C]PAPC + DMPM and [^{14}C]DOPC + DMPM were used at 1:1 molar ratios. Where indicated, GTP[S] (200 μM) or ATP (10 mM) was included. Incubation was at 37°C with 1.85 nmol of phospholipid and 50 μg of protein per tube. Total volume was 200 μl . The enzymically released [^{14}C]oleic or [^{14}C]arachidonic acids were extracted and quantified by liquid-scintillation counting. PLA $_2$ activity was expressed as pmol of [^{14}C]oleic or [^{14}C]arachidonic acid released/mg of protein and was corrected for the presence of unlabelled DMPM, if present. When homogenate was assayed, the activity was corrected for the presence of endogenous unlabelled phospholipids as described [17]. Zero-time values were subtracted.

Fluorimetric measurement of PLA $_2$ activity

PLA $_2$ assays with 10PyPM were performed as described by Bayburt et al. [19]. Enzyme reactions were started by the addition of 1.85 nmol of phospholipid/well. The assay buffer contained 50 mM Tris-HCl, pH 9.0, 100 mM NaCl, 2 mM CaCl_2 , 1 mg/ml BSA. Incubation was at 22°C with 20 μl of protein samples in a total volume of 200 μl . Fluorescence (excitation wavelength 345 nm; emission wavelength 377 nm) was read with a Perkin-Elmer LS50B spectrofluorimeter. PLA $_2$ activity was expressed as pmol of 10Py-decanoic acid released/mg of protein. Zero-time values were subtracted. For Table 3, protein samples were first incubated for 15 min at 37°C (as described by Hara et al. [20]) with 2 mM Ca^{2+} (control), 3 mM DTT, 1 mM pBPB or 3 mM EDTA and then cooled to 4°C , before the addition of the substrate and transfer to 22°C . Heat-treated samples (20 min at 60°C [21]) were also cooled to 4°C before addition of 10PyPM and transfer to 22°C for the activity measurements.

Discrimination between PLA $_2$ and PLC activity using bf-TLC

To determine whether ^{14}C -labelled fatty acids were released as a consequence of PLA $_2$ activity or PLC coupled to DAG lipase activity, we used the radiometric assay of RPE, using [^{14}C]DOPC + DMPM as described. However, total lipids were extracted with chloroform/methanol by the procedure of Miljanich [22]. The extract was then evaporated to dryness by heating under an argon stream, resuspended in 100 μl of chloroform/methanol (2:1, v/v), applied to TLC plates and resolved according to a new method (bf-TLC). The first solvent system consisted of chloroform/methanol/water (14:6:1, by vol.). Migration was stopped 2 cm away from the top of the plate. This first step allowed the separation of [^{14}C]lysoPC, [^{14}C]DOPC and DMPM, whereas [^{14}C]oleic acids and [^{14}C]DAGs both migrated with the solvent front [23]. Solvent was evaporated from the plate which was then put upside-down in a second TLC chamber containing just enough of the second solvent system (heptane/isopropyl ether/acetic acid, 60:40:4, by vol.) to cover 1 cm of the plate height. Solvent was allowed to migrate over only half of the plate to separate [^{14}C]oleic acids and [^{14}C]DAGs [23] without overlapping the compounds separated in the first migration. Solvent was evaporated and separation was visualized with I_2 vapours. Spots corresponding to fatty acid, DAG, lysoPC and 1-stearoyl-2-arachidonoylphosphatidylcholine standards were identified, individually scraped and collected in scintillation vials. I_2 was allowed to evaporate and liquid-scintillation cocktail was added for quantification. PLA $_2$ activity was expressed as pmol of ^{14}C -labelled hydrolysis products/mg of protein and was corrected for the presence of unlabelled DMPM.

Cation-exchange chromatography

RPE homogenate was extracted directly with PBS6 $\times$ and centrifuged (140 000 g ; 4°C ; 1 h; Beckman 70.1 Ti rotor). The supernatant was collected, adjusted to pH 6.0, agitated for 1 h at 4°C and centrifuged as described above. The resulting supernatant was collected, mixed with Sephadex G10 (1.5 ml of swollen gel/50 ml), gently agitated for 1 h at 4°C to adsorb suspended lipids and centrifuged again as described above. Cleared supernatant was filtered (1 μm Whatman filters) and then injected on to a Fractogel EMD SO_3^- -650 column (150 mm $\times$ 10 mm) equilibrated with PBS1 $\times$, pH 6. Unbound proteins were eluted with PBS1 $\times$, pH 6, and a single-step gradient (50 mM Na_2HPO_4 /2 M NaCl, pH 6.0) was then applied to elute bound proteins.

Other methods

Protein concentration was determined using the Lowry protein assay kit from Sigma.

RESULTS AND DISCUSSION

We have recently [17] published data on the presence of PLA₂ activity in bovine RPE, a subretinal fraction enriched in RPE cells. There had been two previous reports on uncharacterized PLA activity in RPE [24,25], but these papers focused on lysosomal PLA activity which was optimal at pH 4.5. This is thus very different from the PLA₂ activities that we measured and which were shown to be optimally active at alkaline pH [17]. To identify better and classify these RPE-PLA₂ enzymes more efficiently, we have tested the effects of Ca²⁺/EGTA, DTT, pBBP, H₂SO₄, GTP[S], ATP and different substrates on PLA₂-active fractions obtained using different methods. We report here the first operational characterization of the minimum number of PLA₂ enzymes detected in RPE.

RPE-PLA₂ enzymes are inactivated by H₂SO₄ like cPLA₂ but inhibited by GTP[S] and ATP unlike sPLA₂, cPLA₂ and iPLA₂

Given that sPLA₂ but not cPLA₂ may be acid-extracted without inactivation [18], we measured PLA₂ activity in H₂SO₄ extracts from RPE. Since we detected no more than 0.5 ± 0.1 % (mean ± S.D. of triplicates from two separate experiments) of PLA₂ activity in neutralized RPE-H₂SO₄ extracts, as compared with untreated controls, it is clear that RPE-PLA₂ was inactivated by H₂SO₄. This suggests that RPE-PLA₂ enzymes are not of the sPLA₂ type. We also tested the effects of GTP[S] and ATP on RPE-PLA₂ activity because of reports on the regulation of cPLA₂ and iPLA₂ by these nucleotides [26–35]. GTP[S] and ATP caused inhibition to 54.7 ± 4.6 and 59.6 ± 6.2 % (mean ± S.D. of triplicate determinations from two separate experiments) of untreated control RPE-PLA₂ activity respectively. The ineffectiveness of ATP in stimulating RPE-PLA₂ activity suggests that RPE does not contain iPLA₂ of the myocardial, platelet, pancreatic or macrophage type (as described by Hazen et al. [32], Margalit et al. [33], Ramanadham et al. [34] and Ackermann et al. [35]), which were all shown to be stimulated two- to six-fold by ATP. Similarly, the ineffectiveness of GTP[S] in stimulating

RPE-PLA₂ activity suggests that it is different from cPLA₂ since none was shown to be inhibited by GTP[S] [26–31]. Thus these results suggest that RPE-PLA₂ represent novel types of PLA₂ which may be down-regulated by ATP and GTP[S] through kinases and/or G-proteins.

Identification of weakly membrane-associated PLA₂ in RPE; evidence for the presence of membrane-bound regulatory components in RPE

To determine the subcellular localization of RPE-PLA₂, we measured PLA₂ activity in supernatants sequentially collected after extraction of the homogenate with PBS1 ×, PBS6 × and PBS1 × OG. Interestingly, Table 1 shows that RPE apparently contains an endogenous PLA₂ inhibitor since the total of all supernatant and wash fractions yielded more than twice (238.4 ± 20.1 %) the activity originally measured in the homogenate-PBS1 × (100.0 ± 12.2 %). Given that PBS1 × and PBS6 × extracted 220.4 ± 19.9 % of PLA₂ activity as compared with the homogenate (100.0 ± 12.2 %), this suggests that PBS1 × and PBS6 × did not co-extract the PLA₂ inhibitor and thus that it is not a soluble or weakly membrane-associated one. This is consistent with our results on the effect of GTP[S] and ATP, which suggest that RPE-PLA₂ activity could be down-regulated by a more tightly membrane-bound protein, namely a G-protein [36–40]. Moreover, it seems very improbable to us that this increase in activity reflects the removal of endogenous unlabelled phospholipids (which would dilute the radiolabelled substrate), since, as described under 'Radiometric measurement of PLA₂ activity', PLA₂ activity of the homogenate was corrected for the presence of these endogenous phospholipids.

After normalization of the PLA₂ activity recovered in each fraction to 100 %, the resulting 'relative percentage of PLA₂ activity extracted' indicates that most, if not all, of RPE-PLA₂ is weakly membrane-associated (see Table 1). PBS1 ×, PBS6 × and PBS1 × OG extracted 32.1 ± 0.5, 61.7 ± 7.8 and 6.3 ± 0.1 % of PLA₂ activity respectively. PBS1 × and PBS6 × were thus able to extract more than 90 % of total RPE-PLA₂ activity, suggesting that RPE mainly possesses PLA₂ enzymes that are soluble and/or weakly membrane-associated.

RPE-PLA₂ enzymes have a substrate selectivity that is different from that of sPLA₂, cPLA₂ and iPLA₂

Since sPLA₂ forms are known to preferentially hydrolyse phosphatidylethanolamine over phosphatidylcholine without being selective for the *sn*-2 fatty acid [13], whereas cPLA₂ and soluble iPLA₂ are selective for *sn*-2 arachidonic acid but not for polar headgroups [10,11], we measured the PLA₂ activity of a high-salt extract of RPE towards different substrates. We compared the hydrolytic rates of [¹⁴C]PAPC, [¹⁴C]PAPC + DMPM, [¹⁴C]PAPE + DMPM and 10PyPM and found that RPE-PLA₂ has a strong preference for the anionic phosphomethanol group of 10PyPM over the zwitterionic phosphocholine group of [¹⁴C]PAPC. Indeed, even at 22 °C, we observed more than a 100-fold increase in the hydrolysis of 10PyPM (2224.1 ± 569.3 pmol/h per mg) over [¹⁴C]PAPC (21.4 ± 3.9 pmol/h per mg) at 37 °C. This preference is also evidenced, although less markedly, by the threefold increase obtained by simply adding DMPM (50 % molar ratio) to [¹⁴C]PAPC (63.7 ± 3.4 pmol/h per mg). Results with [¹⁴C]PAPC and [¹⁴C]PAPC + DMPM are means ± S.D. of triplicate determinations from two separate experiments whereas results with 10PyPM are means ± S.D. of triplicate determinations from five separate experiments.

Although we cannot rule out the possibility that this increase

Table 1 Subcellular distribution of PLA₂ activity in RPE

The different supernatant and wash fractions were sequentially extracted from RPE homogenate with isotonic (PBS1 ×), hypertonic (PBS6 ×) and OG-containing (PBS1 × OG) buffers. Aliquots of each fraction were then incubated for 30 min with [¹⁴C]PAPC + DMPM as the substrate. The results are means ± S.D. of triplicate determinations from two experiments. The percentage of PLA₂ activity extracted was calculated on the basis that the homogenate contained 100 % of PLA₂ activity, whereas the relative percentage of PLA₂ activity extracted was obtained by taking the sum of all cytosol and wash fractions to be equal to 100 %.

Fractions	PLA ₂ activity extracted (%)	Relative percentage of PLA ₂ activity extracted
RPE		
Homogenate-PBS1 ×	100.0 ± 12.2	
Supernatant-PBS1 ×	15.6 ± 0.2	6.6 ± 0.1
Wash-PBS1 ×	60.8 ± 1.1	25.5 ± 0.4
Supernatant-PBS6 ×	94.3 ± 5.5	39.6 ± 2.3
Wash-PBS6 ×	52.7 ± 13.1	22.1 ± 5.5
Supernatant-PBS1 × OG	8.3 ± 0.1	3.5 ± 0.1
Wash-PBS1 × OG	6.7 ± 0.1	2.8 ± 0.1
Total	238.4 ± 20.1	100.0 ± 8.4

reflects acyl chain selectivity, we do not favour this possibility because no PLA_2 has yet been shown to be selective for fatty acids other than polyunsaturated fatty acids (11–13, 41–42). Nevertheless, if RPE- PLA_2 were found to be selective for saturated fatty acids, this would also constitute a novel property. Moreover, this increase would not be due to the presence of BSA (which is known to bind free fatty acids) in the 10PyPM assay buffer, since addition of BSA to the [^{14}C]PAPC + DMPM assay buffer did not allow us to recover as much activity with [^{14}C]PAPC + DMPM (81.5 ± 1.8 pmol/m per mg; mean $\pm$ S.D. of triplicate determinations from two separate experiments) as with 10PyPM. We thus interpret these results as indicating that RPE- PLA_2 binds more tightly to substrate vesicles with a negatively charged surface (phosphomethanol) instead of having a preference for saturated myristoyl-containing phospholipids over polyunsaturated arachidonoyl-containing phospholipids.

The low levels of activity observed towards [^{14}C]PAPE + DMPM (16.6 ± 6.3 pmol/h per mg) suggest that, in contrast with what is known for s PLA_2 , it is not a good substrate for RPE- PLA_2 . At first glance, one might think that this low level of [^{14}C]arachidonic acid release from [^{14}C]PAPE is due to a property of phosphatidylethanolamine (PE) bearing unsaturated fatty acid(s) at the *sn*-1 and/or *sn*-2 position(s). Indeed, 'unsaturated PE' has a natural tendency to form hexagonal_{II} (H_{II}) phases at low temperatures in an aqueous environment, instead of lamellar phases [43]. Given that the lamellar- H_{II} transition temperature of PAPE has not yet been determined [44], and that saturated fatty acids are known to hinder this H_{II} phase-forming tendency of unsaturated PE [43,44], DMPM (50% molar ratio) was added to [^{14}C]PAPE in the PLA_2 assay. Moreover, ^{31}P NMR of this mixture (PAPE/DMPM; 1:1 molar ratio) allowed us to determine the distribution of PAPE between lamellar and H_{II} phases. We found that, in our assay conditions, PAPE was essentially organized into lamellar structures. This means that, even in the presence of DMPM, which ensures the formation of mixed vesicles, [^{14}C]PAPE is still not a good substrate for RPE- PLA_2 . Taken together, these results support our hypothesis that RPE- PLA_2 enzymes are different from the well-known s PLA_2 , c PLA_2 and i PLA_2 forms.

Release of fatty acids resulting from PLA_2 activation in RPE

Given that PLCs are also ubiquitous enzymes [45,46] acting on phospholipids, and that arachidonic acid may be generated through the sequential action of PLC and DAG lipases, we measured both PLC and PLA_2 hydrolysis products in our assay conditions. Since RPE- PLA_2 was not strictly selective for arachidonoyl-containing phospholipids (see above), [^{14}C]DOPC with both chains radiolabelled was used as the substrate (in mixed vesicles with DMPM).

In order to separate all possible PLA_2 and PLC/DAG lipase hydrolysis products, we developed a rapid and simple modified version of the TLC technique which we called bf-TLC. As described in the Materials and methods section, the first solvent system allowed the separation of lysoPC (R_{F1} 0.15), phosphatidylcholine (R_{F1} 0.33), phosphatidic acid (R_{F1} 0.43) and phosphatidylmethanol (R_{F1} 0.52) in the 'forth' dimension, whereas the second solvent system allowed the separation of DAG (R_{F2} 0.25) and free fatty acids (R_{F2} 0.5) in the 'back' dimension. Thus parallel formation of [^{14}C]lysoPC and [^{14}C]oleic acid would indicate PLA_2 activity, whereas the formation of [^{14}C]DAG or only [^{14}C]oleic acid would indicate PLC or PLC plus DAG lipase activity. Moreover, as suggested by the migration pattern of the phosphatidic acid standard, this technique would also allow us to detect phospholipase D activity.

The production of [^{14}C]oleic acid and [^{14}C]lysoPC with RPE

Figure 1 Discrimination between PLA_2 and PLC activity

Aliquots of RPE-cytosol were incubated for different periods of time with [^{14}C]DOPC + DMPM as the substrate. Hydrolysis products were extracted, separated by bf-TLC, identified, scraped and quantified. Production of [^{14}C]oleic acid and [^{14}C]lysoPC reflected PLA_2 activity whereas production of [^{14}C]DAG and/or [^{14}C]oleic acid reflected PLC activity. These results are means $\pm$ S.D. of duplicate determinations from two separate experiments.

increased similarly with time (Figure 1), thus confirming the presence of PLA_2 activity. There was also a small increase in [^{14}C]DAG with time, which is likely to be due to PLC activity. If there had been a concerted action of a PLC and a DAG lipase, then the amount of [^{14}C]DAG should have decreased as the amount of [^{14}C]oleic acid increased, which did not happen. Figure 1 shows that the contribution of PLC/DAG lipase activities to the accumulation of [^{14}C]oleic acid is negligible, since the level of [^{14}C]DAG remained almost constant over time. Since there was no production of phosphatidic acid, we conclude that there is no phospholipase D activity in RPE in our assay conditions (not shown). Figure 1 thus shows that, in our assay conditions, the release of ^{14}C -labelled fatty acids was a direct consequence of PLA_2 activity.

RPE contains different types of PLA_2 -active fractions

Elution of a high-salt extract of RPE on a cation-exchange chromatography column revealed the presence of more than one PLA_2 -active fraction. Figure 2(A) shows a typical elution profile of proteins, and Figure 2(B) shows the distribution of PLA_2 activity among the diverse fractions tested. There were two types of PLA_2 -active fractions in RPE: RPE-1, which was eluted with unbound proteins by a low-salt buffer, and RPE-10, which bound to the column and was eluted by a high-salt buffer. Although RPE-1 and RPE-10 originate from different elution peaks and should thus contain different types of protein (Figure 2A), they had similar levels (26 and 36%) of PLA_2 activity (Table 2). Thus, the elution time, the low protein content and the high activity content of both RPE-1 and RPE-10, as well as the decrease in PLA_2 activity from fractions 1 to 8, suggest that PLA_2 activity in RPE-10 is not due to overloading of the column with PLA_2 activity from RPE-1 (see Figure 2 and Table 2). Together, these results suggest that RPE-1 and RPE-10 contain different types of PLA_2 , and both were thus selected for further characterization.

RPE probably contains two different PLA_2 enzymes

Since the different types of PLA_2 so far characterized differ in their sensitivity to Ca^{2+} /EGTA, DTT, pBPB and heat [10–13], we have tested the effects of these agents on the selected PLA_2

Figure 2 Cation-exchange chromatography of RPE-cytosol

RPE-Cytosol was applied to a Fractogel EMD SO₃⁻-650S column. After elution of unbound proteins with PBS1 × , pH 6.0, bound proteins were eluted with 2 M NaCl, as indicated by the arrow. Flow rate was 1 ml/min. Aliquots of eluted fractions were assayed for PLA₂ activity using 10PyPM. Incubation time was 30 min. PLA₂ activity was expressed as pmol of 10Py-decanoic acid hydrolysed/20 µl of sample. (A) Protein elution profile. (B) PLA₂ activity elution profile. The width of each bar in (B) corresponds to the total volume collected in that fraction.

Table 2 Characteristics of RPE-PLA₂-active fractions eluted from a cation-exchange chromatography column

RPE-1 and RPE-10 were collected from the cation-exchange column, and tested for PLA₂ activity compared with RPE-cytosol. Aliquots of each fraction were incubated for 60 min with 10PyPM. Protein content and PLA₂ activity of each fraction were calculated on the basis that the cytosol contained 100% of the protein and PLA₂ activity respectively. The results are mean ± S.D. of triplicate determinations from four separate experiments.

Fractions	Protein concentration (mg/ml)	Specific activity (pmol/h per mg)	Purification factor	Percentage of total protein	Percentage of total PLA ₂ activity
RPE-cytosol	2.28	1 878 ± 486		(100)	(100)
RPE-1	0.53	6 701 ± 745		8	26
RPE-10	0.02	882 160 ± 34 919	470	0.08	36

active fractions, namely RPE-1 and RPE-10 (Figure 2, Table 2). As can be seen in Table 3, RPE-PLA₂ activity is essentially Ca²⁺-dependent since EGTA caused 99.3 ± 1.1 and 94.8 ± 4.6 % inhibition in RPE-1 and RPE-10 respectively. This is consistent with the 89.0 ± 0.0 % inhibition observed for the whole cytosol. Moreover, it indicates that there are probably two types of PLA₂

Table 3 Effects of EGTA, DTT, pBPB and heat on RPE-PLA₂ activity

Aliquots of RPE-cytosol, RPE-1 and RPE-10 were either heated or pretreated with EGTA, DTT or pBPB before PLA₂ assay with 10PyPM as the substrate. PLA₂ activity is expressed as a percentage of control (presence of 2 mM Ca²⁺) PLA₂ activity. The results are means ± S.D. of triplicate determinations from three separate experiments.

Fractions	PLA ₂ activity (% of control)			
	EGTA	DTT	pBPB	Heat
RPE-cytosol	11.0 ± 0.0	38.0 ± 11.3	0.0 ± 0.0	57.5 ± 10.6
RPE-1	0.7 ± 1.1	85.5 ± 7.8	0.0 ± 0.0	89.7 ± 5.3
RPE-10	5.2 ± 4.6	24.6 ± 12.2	0.0 ± 0.0	92.7 ± 7.4

enzyme in RPE, since PLA₂ activity found in RPE-1 was DTT-resistant whereas that in RPE-10 was DTT-sensitive (14.5 ± 7.8 and 75.4 ± 12.2 % inhibition respectively). Again, this is consistent with the intermediate response (62.0 ± 11.3 % inhibition) of the whole cytosol (Table 3). Concerning the effect of pBPB, PLA₂ activity in RPE-cytosol, RPE-1 and RPE-10 was completely abolished (100.0 ± 0.0 % inhibition in all cases, determined in comparison with a vehicle control) by this histidine-modifying reagent well-known as an inhibitor of type-I and -II sPLA₂.

PLA₂ activity of both RPE-1 and RPE-10 is essentially thermo-resistant, as shown by the 89.7 ± 5.3 and 92.7 ± 7.4 % activity still remaining after the heat-treatment. This is slightly different from what we observed for RPE-cytosol which showed only 57.5 ± 10.6 % activity after such a treatment (Table 3). Although we have no definite explanation for this decrease, it might be due to non-specific aggregation or trapping of PLA₂ enzymes by thermo-sensitive proteins. Since over 90 % of the proteins were removed by cation-exchange chromatography (Figure 2 and Table 2), aggregating proteins would probably be absent from the RPE-1 and RPE-10 fractions, leaving the thermo-resistant RPE-1- and RPE-10-PLA₂ activity. Moreover, these results were very reproducible, and purified cPLA₂ and sPLA₂ were included in the assay as well-characterised positive (heat-sensitive) and negative (heat-resistant) controls respectively. These two points strongly argue against the possibility of spurious results. Table 3 thus strengthens our conclusion from Figure 2 and Table 2 that RPE possesses more than one type of PLA₂ enzyme, probably two.

Summary of evidence for RPE-PLA₂ being different from sPLA₂, cPLA₂ and iPLA₂

Table 4 summarizes our results from Table 3 and shows that: (1) RPE-1 PLA₂ is Ca²⁺-dependent, pBPB-sensitive, and heat- and DTT-resistant, (2) RPE-10 PLA₂ is Ca²⁺-dependent, pBPB-sensitive and heat-resistant, but is also DTT-sensitive. These results strongly argue for the presence of two different PLA₂ enzymes in RPE and suggest that they are different from the already known forms of the enzyme. Indeed, there are actually three groups of PLA₂ that can be distinguished according to their sensitivity to Ca²⁺/EGTA, DTT, pBPB, heat, H₂SO₄, nucleotides and different substrates [9–13,18]: sPLA₂ (subgroups I, II and III), cPLA₂ and iPLA₂ (subgroups I, II and III). In the future, this operational classification will have to be refined as more PLA₂ enzymes are cloned and characterized and other groups (or subgroups) delineated [14–16]. When we compare the characteristics of RPE-PLA₂ with those of sPLA₂, cPLA₂ and iPLA₂ (Table 4), we conclude that the RPE-PLA₂ enzymes are

Table 4 Comparison of the characteristics of sPLA₂, cPLA₂, iPLA₂ and RPE-PLA₂

n.a., Not available.

	H ₂ SO ₄	Ca ²⁺	DTT	pBPB	Heat
sPLA ₂ [*]	—	+	+	+	—
cPLA ₂ [*]	+	+	—	—	+
iPLA ₂ [†]	n.a.	—	—	—	+
RPE-1	+	+	—	+	—
RPE-10	+	+	+	+	—

^{*} See references [10–13] for reviews on the characteristics of sPLA₂, cPLA₂ and iPLA₂.[†] See references [30–35] for additional information on the characteristics of iPLA₂.

different. They appear to share some of the characteristics of sPLA₂ and cPLA₂ at the same time. The most prominent point is that the RPE-PLA₂ enzymes are all pBPB-sensitive, like sPLA₂, whereas they are all inactivated by H₂SO₄ extraction, like cPLA₂. This suggests that RPE-PLA₂ may have to be included in either a new group or subgroup of PLA₂. Further experiments are certainly needed to confirm our results, but it could be significant that the eye is a closed organ because of the blood/ocular barrier, and retinal cells may have evolved expressing PLA₂ of a new group (or subgroup). Two interesting recently published papers [47,48] support this idea by demonstrating that transducin, a heterotrimeric G-protein present in rod cells, also shares some characteristics with the small monomeric 'Ras-like' G-proteins.

In summary, RPE-PLA₂ enzymes have been shown to be H₂SO₄-sensitive, like cPLA₂, but pBPB-sensitive, like sPLA₂. Moreover, RPE-PLA₂ activity is partly inhibited by GTP[S] and ATP, unlike cPLA₂ and iPLA₂, which are known to be stimulated by these nucleotides. RPE-PLA₂ has also been shown to be regulated by an endogenous PLA₂ inhibitor which could be removed by PBS extraction and to exhibit substrate selectivity different from that of sPLA₂, cPLA₂ and iPLA₂. This means that the RPE-PLA₂ enzymes probably represent novel types of PLA₂, and may form either a new group of a new subgroup. Of course, it will be of prime importance to purify and sequence these enzymes in order to classify them. Considering that this is the first report on RPE-PLA₂, we expect that the purification of one or another of these enzymes to now be possible. In this regard, one should note that, after five freeze–thaw cycles, RPE homogenate still exhibited 81 ± 19% of untreated control PLA₂ activity. Moreover, of the different protease inhibitors tested, namely E-64, pepstatin, aprotinin, PMSF, Pefabloc, EDTA, leupeptin and α-macroglobulin, only Pefabloc was found to strongly inhibit PLA₂ activity of RPE homogenate, leaving only 29 ± 2% of control PLA₂ activity.

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