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HPLC assay with UV spectrometric detection for the evaluation of inhibitors of cytosolic phospholipase A_2

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Abstract

A non-radioactive spectrometric assay for the evaluation of inhibitors of cytosolic phospholipase A_2 ($cPLA_2$) is described. The enzyme was isolated from human platelets applying anion exchange chromatography. Sonicated covesicles consisting of 0.2 mM 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine and 0.1 mM 1,2-dioleoyl-*sn*-glycerol were used as enzyme substrate. The $cPLA_2$ activity was determined by measuring the arachidonic acid released by the enzyme with reversed-phase HPLC and UV detection at 200 nm after cleaning up the samples by solid phase extraction. Two known $cPLA_2$ inhibitors were used to validate the test assay.

Keywords: Cytosolic phospholipase A_2; Inhibitors; HPLC; UV detection

1. Introduction

Phospholipase A_2 (PLA_2) enzymes are a class of esterases that catalyze the hydrolysis of membrane phospholipids at the *sn*-2 position to release fatty acids and lysophospholipids. When the fatty acid is arachidonic acid, the further conversion of this PLA_2 product by cyclooxygenases and lipoxygenases results in the production of eicosanoids, including prostaglandins and leukotrienes. In addition, lysophospholipid metabolism can lead to the formation of platelet-activating factor (PAF). Prostaglandins, leukotrienes, lysophospholipids and the PAF are potent mediators of inflammation [1–3]. Thus, inhibition of PLA_2 is considered as an interesting target for the design of new anti-inflammatory drugs [4–8].

One problem associated with the in vitro search for PLA_2 inhibitors is the selection of the appropriate enzyme, since many different PLA_2s are present in the mammalian organism [9]. They can be divided in PLA_2s utilizing a catalytic histidine and in PLA_2s having a serine in the active site. The small molecular weight (approx. 14 kDa) secretory PLA_2s ($sPLA_2$) are members of the first group. The second group consists of the cytosolic PLA_2s ($cPLA_2$), the calcium-independent PLA_2s ($iPLA_2$) and the lipoprotein-associated PLA_2s (Lp-PLA_2), which have higher molecular weights than the $sPLA_2$s. From all these PLA_2s the α-subtype of $cPLA_2$ seems to play the central role in the arachidonic acid cascade and during the inflammatory response as supported by experiments with cells overexpressing $cPLA_2$

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[10] and with cells from $cPLA_2$ knock-out animals [11–16].

The assays for screening $cPLA_2$ inhibitors in vitro can be divided in two categories, namely in assays with the isolated enzyme, and in assays with intact cells.

In the assays with the isolated enzyme, $cPLA_2$ purified from the human monocytic cell line U937 or human recombinant $cPLA_2$, e.g. expressed in Sf9 insect cells is applied. Most of these procedures are radioactive assays. Vesicles of 1-palmitoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine ([^{14}C]PAPC) [17], covesicles of [^{14}C]PAPC or its 1-stearoyl-analogue 1-stearoyl-2-[1−^{14}C] arachidonoyl-*sn*-glycero-3-phosphocholine ([^{14}C]SAPC) with 1,2-dioleoyl-*sn*-glycerol [18–23] and 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol [24], respectively, or mixed-micelles comprised of [^{14}C]PAPC and Triton X-100 [25–29] serve as substrates. Besides, [^{3}H]arachidonate labelled U937 cell membranes are employed [24,26,30]. After separation from the assay mixture the released radioactive arachidonic acid is quantified by scintillation counting.

Furthermore, several fluorimetric assays have been developed for assessing activity of purified $cPLA_2$. In some of these assays, phosphatidylcholines containing a pyrene fluorophore at the *sn*-1 position and arachidonic acid at the *sn*-2 position are used [31]. Other fluorimetric substrates for assessing activity of isolated $cPLA_2$ are esters formed between a fatty acid, such as γ-linolenic acid, and 7-hydroxycoumarine [22,26,32,33]. Moreover, a spectrophotometric assay has been described for measuring the activity of $cPLA_2$. This utilizes synthetic thiophospholipids as substrates, which possess a thioester linkage to the fatty acid chain instead of an oxyester linkage. Hydrolysis by PLA_2 produces a free fatty acid and a thiolysophospholipid as products. The free thiol-group of the thiolysophospholipid is then available to react with a thiol-sensitive reagent to produce a chromophore [34]. All assays utilizing the isolated enzyme are carried out in the presence of Ca^{2+}, which enables the binding of the $cPLA_2$ to the lipid–water interface via the calcium-lipid binding domain of the enzyme. Since phosphatidylinositol-4,5-diphosphate (PIP_2) and glycerol have been shown to enhance $cPLA_2$ activity [35–37], these substances are added to the test mixtures in several cases. Furthermore, most of the assay mixtures contain bovine serum albumin (BSA). The presence of this plasma protein shall linearize the reaction kinetic by trapping the arachidonic acid released [38].

The assays for screening inhibitors in a cellular situation use intact U937 cells, macrophages, human fibroblasts, platelets, rat basophilic leukemia (RBL) cells or human monocytic leukemia (THP-1) cells [8]. Depending on the cell type, the $cPLA_2$ is activated by stimuli such as calcium ionophore A23187, TPA, zymosan, *N*-formyl-methionyl-leucylphenylalanine, IL-1, dinitrophenyl(DNP)-albumin or collagen. Usually, the enzyme activity is measured by determination of the release of radiolabeled arachidonic acid and its metabolites, respectively, from pre-labeled cells by scintillation spectrometry. Besides for assessment of enzyme activity the liberation of the natural arachidonic acid is measured by HPLC or GC after derivatization of the arachidonic acid. In some cases metabolites of arachidonic acid such as prostaglandin D_2 or E_2 are used to determine the inhibition of $cPLA_2$ activity. We have developed a cellular test system, in which the calcium ionophore A23187-induced arachidonic acid release from human platelets is evaluated directly with HPLC and UV detection at 200 nm [39]. In this assay, an amount of arachidonic acid sufficient for quantification by UV-absorption is generated by the blockade of the platelet arachidonic acid metabolism enzymes. Recently, a method was described in which the fluorescent substrate 1,2-bis-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indecene-3-undecanoyl)-*sn*-glycero-3-phosphocholine is incorporated into the cellular membranes of U937 cells. The increase of the fluorescence of the hydrolysis product of this compound generated after activation of the $cPLA_2$ by A23187 can be measured by fluorimetry [40].

In comparison with test systems which use the isolated enzyme, cellular assays have the advantage that they better correlate with the conditions prevailing in vivo. In cells the $cPLA_2$, once activated, interacts with the substrate in its physiological form by cleaving phospholipids of intact cell membranes. Furthermore, whole cell assays provide information on the ability of the test compounds to penetrate into the cells. On the other hand, a weakness of screening systems with intact cell is the evidence that in the cells other isoforms of the enzyme may be present such as $sPLA_2$s or $iPLA_2$s. These could be involved in substrate cleavage

[41]. Furthermore, since cells are complex systems, a reduction of arachidonic acid release does not necessarily prove a direct interaction of a test compound with the enzyme. Finally, cytotoxic compounds, which cause cell lysis or loss of viability, may misleadingly indicate enzyme inhibition.

Therefore, in search for true $cPLA_2$ inhibitors with a potential in vivo activity in our opinion it is recommendable to apply a test system with the isolated enzyme and a test system with intact cells in parallel.

As mentioned above, we have already established an assay for $cPLA_2$ inhibitors using intact human platelets. In completion of this screening system, we have now developed an analogous HPLC/UV assay for measuring the effect of agents on the activity of the $cPLA_2$ enzyme isolated from human platelets. This assay for the first time provides the opportunity to measure the inhibition of the release of arachidonic acid from naturally occurring, non-radioactive phospholipids by the isolated $cPLA_2$ with HPLC and UV detection directly without derivatization.

2. Experimental

2.1. Materials

1-Stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine, 1,2-dioleoyl-*sn*-glycerol, arachidonic acid, $CaCl_2$, dithiothreitol (DTT), phosphate buffered saline tablets, nordihydroguaiaretic acid (NDGA), protease-inhibitor cocktail (104 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 1.5 mM pepstatin A, 1.4 mM *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane, 3.6 mM bestatin, 2.1 mM leupeptin, and 0.08 mM aprotinin in DMSO), Tris, Hepes (Sigma); methanol HPLC grade, EDTA-Na_2, KCl, NaCl (Merck); dimethyl sulfoxide (DMSO), EGTA (Fluka); 4-undecyloxy benzoic acid (Aldrich); acetonitrile HPLC grade (Biosolve LTD); octadecyl endcapped reversed-phase extraction columns 200 mg, 3 ml (Baker); bromoenol lactone (Cayman chemical company); buffy coats of human blood (German Red Cross); HiTrap Q Sepharose Fast Flow anionic exchange columns 5 ml (Amersham Biosciences); Centriplus 15 ml centrifugal filter devices YM 50000 MWCO (Millipore); Vivaspin 2 ml concentrator 50000 MWCO PES (Vivascience); *N*-{(2*S*,4*R*)-4-[biphenyl-2-ylmethyl(isobutyl)amino]-1-[2-(2,4-difluorobenzoyl)benzoyl]pyrrolidin-2-ylmethyl}-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)phenyl]acrylamide [18] (Merckle, Blaubeuren); AR-C67047MI (dicyclohexylamine salt of *(S)*-5-(4-benzylphenylsulfanyl)-4-(7-phenylheptanoylamino)pentanoic acid) [42] (Astra Zeneca, Leicestershire); AR-C70484XX (4-[3-(4-decyloxyphenoxy)-2-oxopropoxy]benzoic acid) and its alcohol analogue were sythesized according to published procedures [22].

2.2. Isolation of the $cPLA_2$ from human platelets

About 50 ml buffy coat was centrifuged in three polypropylene tubes at 2000 × *g* for 2 min at 4 °C and the platelet-rich supernatants were carefully separated by aspiration. The obtained platelet-rich fractions were combined and centrifuged at 1000 × *g* for 15 min at 4 °C. The pellet was resuspended in a mixture of cold phosphate buffered saline and 3.7% aqueous EDTA-Na_2 (97:3, v/v). The volume of this mixture was adequate to the volume of the platelet-rich supernatant obtained before. The suspension was centrifuged at 1000 × *g* for 15 min at 4 °C and the platelets were resuspended in 5 ml cold lysis-buffer (Hepes 10 mM, DTT 1 mM, EGTA 2 mM, NaCl 140 mM, KCl 27 mM, pH 7.4) containing freshly added protease-inhibitor cocktail solution (10 μl/ml). The cells were disrupted by sonication (Branson sonifier B15, level 4, 4 × 15 s) at 0 °C and then stored at −20 °C.

After thawing the suspension was subjected to ultracentrifugation at 100,000 × *g* for 1h at 4 °C and the supernatant dialyzed for 20 h against 500 ml Tris-buffer (Tris 10 mM, DTT 1 mM, EGTA 2 mM, pH 7.4) at 4 °C with one chance of dialysis buffer after 4 h. The precipitate formed during dialysis was separated by ultracentrifugation at 100,000 × *g* for 1 h at 4 °C. The clear supernatant was diluted with 6 ml buffer A (25 mM Tris, 1mM EGTA, 2 mM DTT, pH 8) and loaded onto a HiTrap Q Sepharose FF anionic exchange column (5 ml) that had been conditioned previously with 25 ml of buffer A, 25 ml of buffer B (25 mM Tris, 1 M NaCl, 1 mM EGTA, 2 mM DTT, pH 8) and again 35 ml of buffer A at a flow rate of 5 ml/min. The column was washed with 25 ml of buffer A and then eluted at a flow rate of 5 ml/min in four steps with 15 ml aliquots of mixtures of buffer A and B containing 0.15, 0.30, 0.45 and 0.60 M NaCl, respectively, and the four

fractions were collected. The fraction containing the enzyme (fraction 3) was first concentrated with Centriplus 15 ml central filter devices with a cut-off of 50 kDa (75 min at 3000 × *g* at 4 °C) to a volume of about 2 ml and then with Vivaspin 2 ml concentrators with a cut-off of 50 kDa (15 min at 4000 × *g* at 4 °C) to a final volume of about 1 ml. The concentrate was incubated with 5 μl of a bromoenol lactone solution (1 mM in DMSO) for 15 min at 25 °C to inhibit any $iPLA_2$ present in the sample. In the enzyme assay 20 μl of this enzyme solution liberated about 3 nmol arachidonic acid/100 μl in 60 min. The enzyme preparation was stored at −20 °C until used.

2.3. Incubation procedure for measuring $cPLA_2$ activity

The phospholipid substrate 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine was dissolved in $CHCl_3$ (10 mg/ml). An aliquot of this solution was thoroughly dried under a stream of nitrogen together with an aliquot of a solution of the 1,2-dioleoyl-*sn*-glycerol (10 mg/ml $CHCl_3$). The residue was resuspended in Tris-buffer (Tris 50 mM, DTT 1 mM, NaCl 150 mM, $CaCl_2$ 1 mM, pH 8.0) by sonication to obtain vesicles. 78 μl of this suspension were added to 2 μl of a DMSO solution of the test compound or, in case of the controls, to 2 μl of DMSO. The mixture was incubated for 5 min at 37 °C. Then the enzyme reaction was started by adding 20 μl of the enzyme solution. The final reaction mixture (100 μl) contained 0.2 mM of SAPC phospholipid and 0.1 mM 1,2-dioleoyl-*sn*-glycerol. The incubation was performed at 37 °C for 60 min. In case of the kinetic tests the incubation time was variable (1–75 min). The enzyme reaction was terminated by the addition of 400 μl of a mixture of acetonitrile, methanol and 0.1 mM aqueous EDTA-Na_2 solution (16:15:1, v/v/v), which contained 4-undecyloxybenzoic acid as internal standard (0.62 μg/400 μl) and NDGA as oxygen scavanger (1.2 μg/400 μl). The obtained samples were cooled in an ice bath, diluted with 2 ml 0.005 M NaOH and then applied to an octadecyl reversed-phase extraction column, which has previously been washed with methanol (6 ml) and water (6 ml). After washing the column with water (1 ml) the adsorbed arachidonic acid was eluted with methanol (2 × 200 μl). The eluate was diluted with water (500 μl) and then subjected to HPLC. Control incubations in the absence of the enzyme and in the absence of substrate, respectively, were carried out in parallel and used to calculate the specific hydrolysis.

2.4. HPLC analysis

The HPLC system consisted of a Waters HPLC-pump model 515, a Waters autosampler model 717 plus and a Waters UV-Vis-detector model 2487. Separation was achieved on a Nucleosil 100 C18 analytical column (3 mm inside diameter × 125 mm, particle size 3 μm) protected with a Nucleosil 100 C18 guard column (3 mm inside diameter × 20 mm, particle seize 5 μm) (Macherey and Nagel). 100 μl of each sample were injected onto the HPLC system. The mobile phase consisted of acetonitrile-water-phosphoric acid (85%) (77:23:0.1, v/v/v). The flow rate was 0.4 ml/min. The effluents were monitored at 200 nm.

For the evaluation of enzyme inhibition the mean level of arachidonic acid concentration obtained in presence of a test compound ($n = 3$) was compared with the mean level of arachidonic acid obtained in absence of test compounds (control tests, $n = 3$). All data were analyzed by Student's *t*-test. A value of $P < 0.05$ was considered significant. The IC_{50} values were calculated with the aid of Probit transformation. For the calculation of the absolute amount of the arachidonic acid released by the $cPLA_2$ a calibration curve was used. The linearity of the calibration curve was assessed in the range of 0.25–1.50 μg/ml. The correlation coefficient for peak area versus concentration was 0.998.

3. Results and discussion

3.1. Isolation of the $cPLA_2$

The 85 kDa $cPLA_2$ has been purified from a variety of mammalian sources such as neutrophils, macrophages, platelets, fibroblasts, spleen and kidney [43]. Since we have already established a cellular $cPLA_2$ assay with intact human platelets [39], we decided to purify the enzyme from the same cell type. Human platelets previously isolated from buffy coats by centrifugation were lysed by sonication in the presence of a cocktail of protease inhibitors, dithiothreitol

and EGTA according to a published procedure [44]. After ultracentifugation the supernatant was dialyzed against Tris-buffer. The dialysate was ultracentrifuged and the supernatant applied on an anion exchange column that was developed with a gradient of NaCl. The fraction containing the enzyme was concentrated by ultrafiltration using filtration tubes with a cut-off of 50 kDa. The concentrate was incubated with bromoenol lactone to inactivate the $iPLA_2$ probably present in the enzyme preparation. To inhibit the activity of platelet secretory PLA_2, DTT was added to all buffers. In the enzyme assay 20 μl of the enzyme solution obtained liberated about 3 nmol/100 μl arachidonic acid from the phospholipid substrate in 60 min.

3.2. Choice of substrate

The analysis of inhibitors of lipolytic enzymes such as $cPLA_2$ is more difficult than with enzymes that operate in the aqueous phase because the enzymatic hydrolysis of naturally occurring, long-chain lipids and phospholipids, respectively, takes place at a lipid–water interface. For assessment of $cPLA_2$ inhibitors usually substrates are applied, which form vesicles, or micelle forming detergents such as Triton X-100 are added, in which the substrate is intercalated. One problem of some of these assays is the fact that they are carried out with a concentration of inhibitors comparable to the concentration of the substrate used in the assay. Under such conditions the amount of the inhibitor in the interface is quite high, and it is possible that the presence of the inhibitor causes desorption of the enzyme from the interface into the aqueous phase by altering the physical nature of the substrate thus falsely indicating enzyme inhibition. Therefore, for the development of true PLA_2 inhibitors, assays should be used in which the mole fraction of inhibitor in the interface is kept low so that desorption of the enzyme into the aqueous phase does not occur [26]. Another approach to discriminate between "true" enzyme inhibitors and compounds, which act via perturbation of the enzyme–lipid interaction, is the use of a dual screening strategy. In this, the inhibitors are tested in presence of substrate vesicles, and also against the enzyme working against a soluble monomeric substrate, where complexities associated with lipid aggregation are minimized [22].

We have chosen the first approach testing inhibitors at low mole fractions in the substrate interface, since for an assay with a monomeric substrate high amounts of enzyme are necessary as a result of the very slow hydrolysis of non-aggregated substrates by $cPLA_2$. Sonicated vesicles consisting of 0.1 mM of 1,2-dioleoyl-*sn*-glycerol and 0.2 mM of the substrate 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (SAPC) were used. Unlike the non-physiological fluorescence probes used in fluorimetric assays, SAPC is a natural substrate of the $cPLA_2$. The arachidonic acid released from SAPC by the $cPLA_2$ can be measured by HPLC with UV detection. The maximal inhibitor concentration in the assay was 10 μM. As already mentioned above, in most of the $cPLA_2$ assays described BSA is added to the incubation mixtures. Since it was imaginable that the addition of BSA may decrease inhibition values of such inhibitors, which are highly bound to this plasma protein, we omitted the addition of this substance to the assay buffer.

3.3. Incubation procedure and HPLC analysis

The reaction mixtures were incubated in Tris-buffer in the presence of Ca^{2+}. The enzyme reaction was terminated by adding a mixture of acetonitrile, methanol and aqueous EDTA containing 4-undecyloxybenzoic acid as internal standard and nordihydroguaiaretic acid as oxygen scavanger. After addition of dilute NaOH, the samples were cleaned up by solid phase extraction employing octadecyl reversed-phase extraction columns. The recovery of the extraction procedure was determined applying an analogous matrix spiked with arachidonic acid (1.0 μg/ml) and the internal standard (0.62 μg/ml). Absolute recoveries were calculated from the peak areas of the compounds in extracted and non-extracted samples. The mean absolute recovery for arachidonic acid was $75 \pm 2.0\%$ (mean ± S.D., $n = 5$) and for the internal standard $74 \pm 2.6\%$ (mean ± S.D., $n = 5$). The relative recovery calculated by comparing the ratios of peak areas of arachidonic acid and internal standard in extracted and non-extracted samples was $100 \pm 1.0\%$ ($n = 5$).

The arachidonic acid released by the enzyme was measured by reversed-phase HPLC and UV detection at 200 nm. Fig. 1 shows a typical HPLC chromatogram

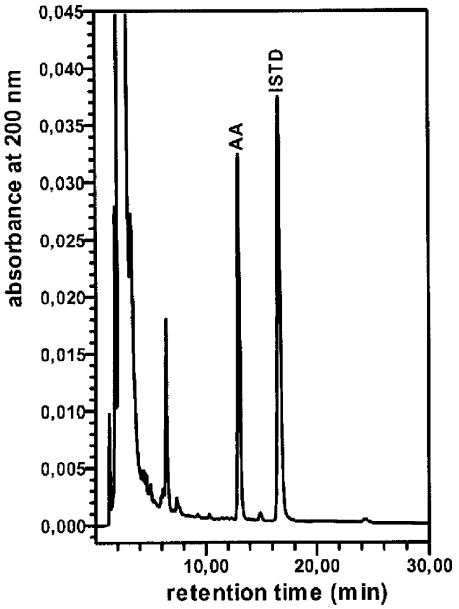

Fig. 1. HPLC analysis of the arachidonic acid released from 1-stearoyl-2-arachido noyl-*sn*-glycero-3-phosphocholine (SAPC) by human platelet $cPLA_2$ in the absence of an enzyme inhibitor. Sonicated vesicles consisting of 0.1 mM of 1,2-dioleoyl-*sn*-glycerol and 0.2 mM of SAPC were incubated with the enzyme in Tris-buffer (Tris, DTT, NaCl, $CaCl_2$, pH 8) for 60 min at 37 °C. The enzyme reaction was terminated by the addition of a mixture of acetonitrile, methanol and 3 mM aqueous EDTA-Na_2, which contained 4-un decyloxybenzoic acid as internal standard and NDGA as oxygen scavenger. After cleaning up by solid phase extraction the samples were subjected to reversed-phase HPLC with UV detection at 200 nm. *Peaks:* ISTD, internal standard (4-undecyloxybenzoic acid); AA, arachidonic acid.

of the arachidonic acid released by the $cPLA_2$ in absence of an inhibitor.

3.4. Kinetic of the arachidonic acid formation

The reaction progress curve for the hydrolysis of the phospholipid vesicles by the $cPLA_2$ shows a biphasic course (Fig. 2). From 0 min to about 30 min it has a sigmoid character showing a significant lag phase in the first 5 min. In the time interval from 30 to 75 min, the increase of arachidonic acid concentration seems to be linear. Similar reaction progress curves for substrate hydrolysis by $cPLA_2$ have been described before [32,45]. Regarding this kinetic, for the evaluation of enzyme inhibitors an incubation time of 60 min was chosen.

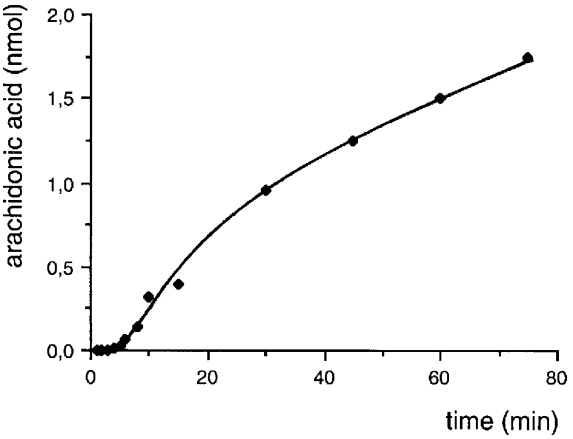

Fig. 2. Reaction progress curve of the action of $cPLA_2$ isolated from human platelets on vesicles of 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (0.2 mM) and 1,2-dioleoyl-*sn*-glycerol (0.1 mM). The reaction was conducted in a final volume of 0.1 ml of Tris-buffer in the presence of calcium at pH 8.0.

3.5. Testing of inhibitors

A known pyrrolidine $cPLA_2$-inhibitor from Shionogi (1) [18] and the AstraZeneca $cPLA_2$-inhibitor AR-C70484XX (2) [22] (Fig. 3) were evaluated under the standard assay conditions. Both compounds inhibited the $cPLA_2$ with a high potency. The IC_{50} values obtained were 31 and 11 nM, respectively (Table 1). In the literature IC_{50} values of 1.8 and 8 nM, respectively, have been reported for these compounds applying vesicle assays with differing substrate compositions. A total inhibition of the enzyme could be achieved in our assay e.g. with a concentration of 0.33 μM of 2. Contrary, the $sPLA_2$ inhibitor AR-C67047MI (4) [42] did not effect enzyme activity at 10 μM, indicating that $sPLA_2$ isoforms are not present or active, respectively, in the enzyme preparation. As already mentioned, for inactivation of $iPLA_2$, which is a putative constituent of platelet cytosol [41,46], the irreversible $iPLA_2$ inhibitor bromoenol lactone was added to the $cPLA_2$ preparation.

The alcohol derivative 3 (Fig. 3) obtained by reduction of the ketone moiety of 2 was reported to be active against the enzyme in a vesicle assay at an IC_{50} level of about one micromolar [22]. Since this substance was essentially inactive in a monomeric substrate assay applied in parallel, it was concluded that the level of activity seen in the vesicle assay was likely

Table 1
Inhibition of the $cPLA_2$ isolated from human platelets by a pyrrolidine $cPLA_2$-inhibitor (**1**), the $cPLA_2$-inhibitor AR-C70484XX (**2**), its alcohol analogue (**3**), and the $sPLA_2$ inhibitor AR-C67047MI (**4**)

Compound	Inhibition of $cPLA_2$ isolated from human platelets IC_{50} (μM)
1 (pyrrolidine-derivative) ($cPLA_2$-inhibitor)	0.031 ± 0.0049
2 (AR-C70484XX) ($cPLA_2$-inhibitor)	0.011 ± 0.0010[a]
3 (Alcohol analogue of AR-C70484XX) (non-specific inhibitor of $cPLA_2$-activity)	Not active at 10 μM[b]
4 (AR-C67047MI) ($sPLA_2$-inhibitor)	Not active at 10 μM[b]

[a] Value is the mean ± S.D. of independent experiments ($n = 3$) performed at different days.
[b] $n = 3$.

to be due to non-specific inhibition. In our assay the alcohol 3 was inactive at 10 μM. This means that a non-specific inhibition, which can be seen for a large number of lipophilic acids, does not occur under the conditions used here. The reason for this difference in behavior is probably the fact that in our vesicle assay the concentration of the components forming the substrate vesicles is much higher than in the vesicle assay reported for the evaluation of 3 [22] (300 μM versus 9.3 μM). This result again shows that for the discrimination between apparent and true $cPLA_2$ inhibitors the use of a vesicle assay is sufficient as far as the mole fraction of the inhibitor in the interface is kept low.

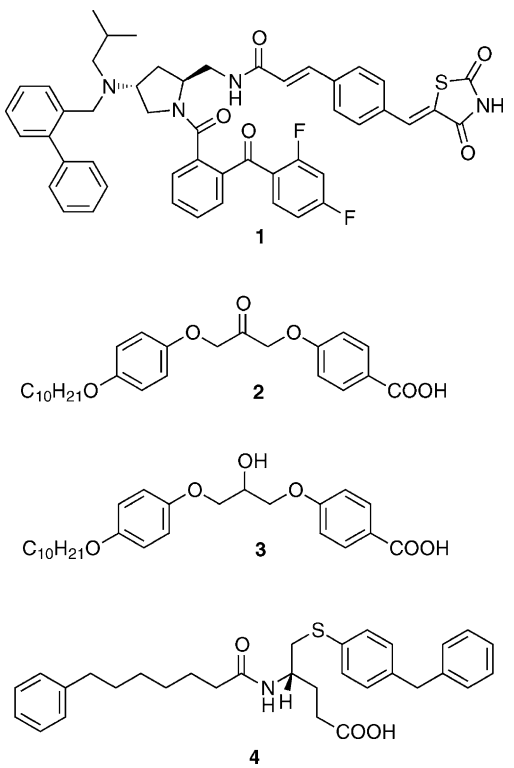

Fig. 3. Structure of a pyrrolidine $cPLA_2$-inhibitor from Shionogi (**1**), the AstraZeneca $cPLA_2$-inhibitor AR-C70484XX (**2**), its alcohol analogue (**3**), and the $sPLA_2$-inhibitor AR-C67047MI (**4**).

In conclusion, we have developed a non-radioactive assay for the evaluation of $cPLA_2$ inhibitors, in which vesicles of the substrate 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine with 1,2-dioleoyl-*sn*-glycerol are applied. The enzyme product arachidonic acid is quantified after solid phase extraction without derivatization by HPLC and UV detection at 200 nm. The method allows $cPLA_2$-inhibitor screening even in laboratories, which do not have a sophisticated instrumentation.

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