Biophysical Chemistry 152 (2010) 109–117

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

A biophysical approach to phospholipase A_2 activity and inhibition by anti-inflammatory drugs

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ARTICLE INFO

Article history:
Received 16 May 2010
Received in revised form 14 August 2010
Accepted 17 August 2010
Available online 25 August 2010

Keywords:
$sPLA_2$
NSAIDs
Enzymatic inhibition
Membrane model systems
Membrane biophysical properties

ABSTRACT

The present study describes the interaction of two nonsteroidal anti-inflammatory drugs (ibuprofen and piroxicam) with PLA_2 from *Naja mossambica mossambica* and seeks to deepen the knowledge about the influence of the biophysical properties of biomembranes, and the inhibitory effect of the drugs on the enzymatic activity. Fluorescent techniques with and without the use of probes, surface pressure/molecular area isotherms, surface pressure/time and molecular area/time measurements combined with circular dichroism spectroscopy and direct techniques of visualization of lipid membranes (Brewster angle microscopy), revealed that both drugs inhibit PLA_2. Additionally, the structure and characteristics of the lipid bilayer, as well as, the direct interaction of drugs with the enzyme seem to play an important role on the hydrolytic activity of PLA_2 towards membrane model systems. These results open a way of finding new and better strategies that can contribute to the development of suitable agents for relieving inflammatory conditions.

1. Introduction

Phospholipase A_2 (PLA_2) is a water-soluble enzyme responsible for the hydrolysis of phospholipids on the sn-2 acyl bond with the consequent release of free fatty acids and lysophospholipids [1]. Arachidonic acid (AA) is among the free fatty acids released representing a possible store of energy [2] and being involved in the biosynthesis of prostaglandins, prostacyclins, thromboxanes and interleukins – eicosanoids, known as mediators of inflammation and signal transduction [2–4]. Plants, mammals, snakes and bee venoms can be the source of PLA_2 and according to their cellular location, these enzymes can be classified as cytosolic ($cPLA_2$), calcium independent ($iPLA_2$) and secretory ($sPLA_2$)[5]. Considering the three types of PLA_2 mentioned, studies revealed that $sPLA_2$ is deeply involved in the inflammatory process of several conditions [3,6] such as rheumatoid arthritis, atherosclerosis, respiratory distress syndrome and septic shock [4,5,7]. Most precisely, $sPLA_2$ leads to the formation of lipid inflammatory mediators through its lipolytic activity and also by activation of $iPLA_2$ [7]. These findings imply that regulation of PLA_2 activity, and in particular $sPLA_2$ activity, is a potential key for controlling inflammatory diseases.

In the presented work, the interaction between two non-steroidal anti-inflammatory drugs (NSAIDs) with $sPLA_2$ from *Naja mossambica mossambica* venom and model membranes was investigated. The choice of this enzyme was based on the large similarity in structure and catalytic functions with mammalian enzymes [8]. Since lipids are the major components of biological membranes and are essential in the regulation of enzymes, lipid model systems were used herein to mimic membranes and to study PLA_2 activity. Indeed, PLA_2 activity is conditioned by the membrane structure [9] and enhanced in presence of phospholipid substrates in the aggregated form [10] such as liposomes and monolayers. In this regard, monolayers used as the half of the membrane bilayers leaflet, provide an adequate system for the investigation of the effects exerted on PLA_2 activity as a consequence of the membrane structure. These model systems also allow controlling the experiments conditions such as the lipid and subphase composition [11]. Besides monolayers, liposomes were also used as representatives of the membrane lipid bilayers. Furthermore, the lipid composition of the model membranes was chosen to promote interfacial activation of PLA_2 activity. In fact it has been observed a substantial increase in the enzymatic activity upon the control of some key properties of the lipid substrate, which include curvature, structural defects, phase separation, surface charge and others [9,12]. In this context a lipid mixture (7:3) of 1, 2-dimyristoyl-sn-glycero-3-phosphocholine (L-DMPC) and 1, 2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (L-DPPE) was used. This lipid mixture was chosen based on the fact that snake venom PLA_2 activity can be enhanced by

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doi:10.1016/j.bpc.2010.08.006

zwitterionic phospholipids [13], and that the presence of lipid domains (de-mixing) results in the adequate environment for PLA_2 hydrolysis [9,14]. Accordingly, the phospholipids DMPC and DPPE are zwitterionic at the pH of the studies (physiological pH 7.4). Furthermore, at the temperature of studies (37 °C), the phase diagram of the aqueous dispersions of the chosen lipid binary system assures the existence of gel-fluid heterogeneity important to activate PLA_2 [15]. The NSAIDs studied, piroxicam and ibuprofen, act as anti-inflammatory drugs by reducing the formation of inflammatory chemical mediators such as prostaglandins and leukotrienes [16]. Another possible target for the anti-inflammatory action is PLA_2, which makes the NSAIDs studied potential inhibitors of this enzyme. In fact, several studies report the inhibition of PLA_2 by NSAIDs. For instance, it has been shown that indomethacin and alminoprofen inhibit rat peritoneal and snake venom PLA_2, respectively [17]. Other molecules, like α-lipoic acid (ALA), inhibit several $sPLA_2$s from human serum and also from snake venoms [3]. Although the mechanism for this inhibition is still unclear, it seems that while some molecules change the membrane biophysical properties, blocking the passage and the contact of PLA_2 with the membrane [3], others have a direct contact with the enzyme and can also form complexes with it, as demonstrated in studies involving ALA [3] and oxyphenbutazone [1]. In view to this, the experiments presented in this work have first the purpose to study the PLA_2 activity and inhibition by the NSAIDs tested, and second, to determine the factor(s) that contribute to that inhibition which might include: (i) the drug induced biophysical changes occurring at the interface where PLA_2 activity takes place; (ii) the direct interaction between the drug and enzyme and/or the drug induced disturbance in the binding of PLA_2 to the substrate. To achieve these purposes, spectrofluorimetric techniques regarding enzymatic activity/inhibition and binding studies were combined with biophysical studies, circular dichroism spectroscopy and Brewster angle microscopy (BAM) as a direct technique of visualization of lipid membranes. Therefore, this work represents a systematic study of evaluation of PLA_2 inhibitors, covering not only the direct inhibitory assays and the induced changes in enzyme's conformation, but also assays that evaluate the role of the membrane biophysical changes induced by drugs on the enzymatic activity. In this context, this study, contributing to identify drugs-induced alterations on membrane lipid physical properties and enzymatic conformation putatively correlated with PLA_2 inhibitory activity, may provide significant insights for predicting or modulating the impact of other related compounds, potentially used for pharmacological purposes, since they may be significant to the development of novel inhibitors with improved anti-inflammatory properties.

2. Materials and methods

2.1. Materials

Non steroidal anti-inflammatory drugs, ibuprofen and piroxicam, 1, 2-dimyristoyl-*sn*-glycero-3-phosphocholine (L-DMPC), 1, 2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (L-DPPE) and lyophilized powder of *N. mossambica mossambica* phospholipase A_2 were purchased from Sigma-Aldrich. AcryloDated Intestinal Fatty Acid Binding protein (ADIFAB) was from Molecular Probes. All reagents were used without further purification.

Solutions of ADIFAB and anti-inflammatory drugs were prepared in Tris buffer (Tris 10 mM; NaCl 150 mM; $CaCl_2$ 1 mM; pH 7.4). Solutions were prepared with double-deionised water (conductivity less than 0.1 μS cm^{-1}).

2.2. ADIFAB based fluorescence assay

To monitor the PLA_2 activity, a fluorescence methodology was applied using ADIFAB as a fluorescence probe [18]. ADIFAB is a conjugate of the polarity-sensitive fluorescent probe acrylodan and intestinal fatty acid binding protein (I-FABP), which has a high binding affinity for free fatty acids (FFA) resultant from the lipid hydrolysis catalyzed by PLA_2. Detection of FFA by ADIFAB is based on a change in the position of the acrylodan fluorophore relatively to the nonpolar binding pocket of the protein when it becomes occupied by a fatty acid (FA). The fluorescence intensities at 505 nm and 432 nm corresponding to the bound and unbound probe, as well as single wavelength measurements at 432 nm, allow the determination of FFA released from the liposomes and of the total fatty acid concentration [19].

The experiments were carried out using DMPC + DPPE (7:3) LUVs as PLA_2 substrate. The chosen phospholipids were co-dissolved in chloroform, mixed and dried under nitrogen stream. The resultant lipid film was left under vacuum for complete solvent removal and then dispersed in Tris buffer (Tris 10 mM, NaCl 150 mM, $CaCl_2$ 1 mM, pH 7.4) and vortexed to obtain multilamellar vesicles (MLVs). The MLVs suspension was extruded 10 times in an extruder device (LIPEX™ extruder from Northern Lipids Inc) through polycarbonate filters with a pore diameter of 100 nm to form unilamellar vesicles (LUVs). Both dispersion in Tris buffer and extrusion were performed above the phase transition temperature of the lipid mixture (55 °C).

The reaction mixture of a total volume of 1200 μL was prepared in 1 cm path length quartz cuvette containing Tris buffer (Tris 10 mM, NaCl 150 mM, $CaCl_2$ 1 mM, pH 7.4), 50 μM liposomes of DMPC and DPPE (7:3), ADIFAB 0.108 μM, *N. mossambica mossambica* PLA_2 7.6×10^{-10} M and NSAID 0–20 μM (in the assays attained for studying the PLA_2 enzymatic inhibition by piroxicam and ibuprofen) or buffer (in the control assays). The lipid hydrolysis reaction was monitored in a Perkin-Elmer LS 50B spectrofluorimeter with excitation wavelength set at 386 nm and emission wavelength at 432 nm. Fluorescence values were corrected for light scattering contributions by subtraction of intensities from unlabeled samples at the same conditions. These contributions were always negligible (less than 0.5%).

The assays performed for studying the enzymatic inhibition by piroxicam and ibuprofen required an incubation period of 30 min after which the PLA_2–drug mixture was added to the lipid suspension. The PLA_2 activity obtained without the drug in the control assay was considered to be 100%. All the other activities measured with the PLA_2–drug mixture were compared to the one without drug and considered as relative percentages. The temperature of the measurements was kept constant at 37 °C by an external bath circulator.

2.3. Langmuir isotherms

To study the interaction of the NSAIDs with the lipid monolayers, surface pressure/time (π/t) measurements and surface pressure/molecular area (π/A) isotherms were performed in a Langmuir trough.

Monolayers of the lipid mixture DMPC/DPPE (7:3) were obtained after spreading phospholipid/chloroform solutions (1 mM) on a Tris buffer subphase (Tris 10 mM, NaCl 150 mM, $CaCl_2$ 1 mM, pH 7.4) or on ibuprofen/piroxicam subphases (20 μM) prepared in the same buffer. After waiting 10 min for evaporation of the solvent, isotherms were recorded on a film balance from R&K GmbH (Potsdam, Germany), equipped with a Wilhelmy-type pressure-measuring system using a filter paper plate. Initially, π/t dependencies were measured until a stabilized surface pressure was reached, to assure that the adsorption of NSAIDs to the monolayer reached a steady-state. At this point, π/A isotherms were measured by the compression of the monolayers at a rate of 5 $Å^2$/molecule/min.

Langmuir trough was also used to study the lipid hydrolysis efficiency of PLA_2 and its inhibition by the NSAIDs studied. In this case, monolayers of DMPC/DPPE (7:3) were obtained in a similar way as described above on a Tris buffer subphase (Tris 10 mM, NaCl 150 mM, $CaCl_2$ 1 mM, pH 7.4). Initially, the monolayer was compressed until a

desired surface pressure was reached. At this point, the compression was stopped, and the monolayer was left for stabilization until the molecular area has reached a constant value. After this, A/t dependencies were plotted using two different types of incubation procedures. In the first procedure, PLA_2 (4 μM) was injected into the subphase and the variation of the molecular area over time was recorded. The final concentration of PLA_2 in the subphase was 0.4 nM. In the second procedure, PLA_2 (4 μM) and drug (50 nM) were incubated for 30 min after which the mixture was injected into the subphase and the variation of the molecular area over time was recorded. More than 120 min after this first injection, 1×10^{-3} M of PLA_2 was injected into the subphase, and the variation of the molecular area was once again registered.

2.4. Brewster angle microscopy (BAM)

Morphological images of the monolayers were obtained with a Brewster angle microscope; model BAM1, NFT – Nanofilm Technologie GmbH, Goettingen, Germany, settled up onto the computer-interfaced Langmuir trough. The microscope was equipped with a He–Ne laser (632.8 nm, 10 mW), a polarizer, an analyzer and a CCD camera. BAM images were recorded on a video tape and then transferred to a personal computer [20,21].

2.5. Fluorescence binding measurements

The intrinsic fluorescence intensity of PLA_2 (0.36 μM) was measured in the presence of several concentrations of piroxicam and ibuprofen (0–150 μM). All measurements were carried out in a Perkin-Elmer LS 50B spectrofluorimeter. Excitation and emission wavelength were set at 220 nm and 345 nm, respectively. Fluorescence emission spectra were obtained between 275 and 410 nm. All fluorescence intensity data were corrected from reabsorption and inner filter effects [22]. The temperature of all measurements was kept constant at 37 °C with an external bath circulator.

2.6. Circular dichroism (CD) spectroscopy

CD spectra were obtained on a JASCO J-715 Spectropolarimeter (Jasco Co., Tokyo, Japan) using a cuvette with 1 mm path length. Data were collected at 0.2 nm intervals from 195 to 260 nm for wavelength scans, with the average of ten scans recorded at a speed of 50 nm/min. To evaluate the enzyme conformational changes due to membrane binding, CD spectra of PLA_2 (8.9 μM) were measured in Tris buffer (Tris 10 mM, NaCl 150 mM, $CaCl_2$ 1 mM, pH 7.4) and after incubation with LUVs of DMPC + DPPE (7:3) (430 μM). Similar measurements were performed for the drugs tested, piroxicam and ibuprofen, in buffer or in lipid media. To study the effect of drugs on PLA_2 structure in presence of DMPC + DPPE liposomes, the samples were incubated by two different procedures. In one case, the enzyme was first mixed with piroxicam or ibuprofen (77.5 μM) and then added to the liposome suspension. In a different approach, drugs were first incubated with liposomes and the enzyme solution was added afterwards. The protein spectra were corrected by subtracting the appropriate blank runs (buffer, drug and/or liposomes in buffer) and converted to mean residue molar ellipticity, represented by $[\theta]$. Spectra were smoothed using the noise reduction routines provided with the J-715 spectropolarimeter.

3. Results and discussion

Biophysical studies concerning PLA_2 activity led to the assumption that the reaction taking place at the interface can be visualized as two distinct steps, interfacial recognition and catalytic cleavage [10,23] and that the enzymatic activity is enhanced in the presence of aggregated phospholipids, e.g., monolayers at the air/water interface [23]. The explanation regarding this behavior is covered by two theories that should be considered simultaneously: the enzyme theory, where PLA_2 undergoes a conformational change favorable to the reaction, and the substrate theory, where the characteristics of the substrate (like molecular orientation and fluidity) have an important role in the course of the reaction [10]. To understand the mechanism underlying the inhibition of $sPLA_2$ by piroxicam and ibuprofen, it is necessary to determine whether the drugs are responsible for changes in the lipid structure and lipid packing leading to the inability of PLA_2 to access the substrate, or if the drugs show a direct interaction with the enzyme inhibiting its activity. Regarding these aims, different studies were made to understand if the inhibitory effect of the NSAIDs studied is related with their action on the membrane structure and/or their direct binding to PLA_2.

3.1. Studies of membrane biophysical changes induced by NSAIDs

The adsorption behaviour of piroxicam and ibuprofen to a monolayer of DMPC/DPPE (7:3) was the first step for assessing the effect of the drugs on membrane structure. During the adsorption process of the drugs to the monolayer it was possible to see a time-dependent increase of the pressure (π) as a result of the insertion of the drug molecules into the lipid monolayer. The adsorption was complete only after 4–6 h, which was the time needed to achieve the steady-state pressure. The steady-state pressure reached for the same NSAID concentration was 10 mN/m and 4.5 mN/m for piroxicam and ibuprofen, respectively. The observed surface pressure increase indicates that both NSAIDs are able to adsorb/penetrate to the monolayer and that this effect is more pronounced in the case of piroxicam. Similar effect of ibuprofen on monolayers of DPPC has been shown recently [24]. Indeed, Jablonowska et al. demonstrated that when ibuprofen is present in the subphase the organization of molecules into a liquid monolayer starts immediately at larger areas per molecule corresponding to an earlier onset of the increase of surface pressure. However, in the current study, the NSAIDs' time dependent interaction with lipid component of membranes was also assessed, since this aspect is most times overlooked. Therefore, it was possible to conclude that there is a time-dependent increase of the surface pressure until a steady-state was reached where the drug adsorption/penetration into the monolayers was considered to reach its maximum. It is thus interesting to notice that similar effects obtained by other authors for higher concentrations of ibuprofen [24], where attained here for smaller concentrations of the same drug but with larger times of interaction with the lipid component.The NSAIDs' adsorption process to the lipid monolayers was also followed visually by BAM. This technique takes advantage of the Brewster angle, 53.1°, where no reflection arises from the interface after it has been illuminated with p-polarized light. Consequently, when a monolayer is present at the interface it causes a change in the Brewster angle conditions by changing the refractive index, thus reflecting p-polarized light [23]. This permits the visualization of domains arising during first-order phase transitions between fluid and condensed phases or due to de-mixing into fluid and condensed regions. In the case of the interface studied, BAM images show a darker area representing the fluid phase (DMPC) and bright spots correspondent to condensed domains (phase-separated DPPE). A comparison of BAM images obtained at different points of the adsorption process of the drugs (as can be seen in Fig. 1 for piroxicam) shows that the penetration of the drug molecules into the phospholipid layer results in changes in the membrane domains with different contrast effects. At the steady-state pressure where the drug shows the highest adsorption to the monolayer, there is a well defined contrast between a fluid dark area correspondent to the membrane fluid phase and brighter areas correspondent to spherical condensed domains (Fig. 1 – number 3). The size of the condensed phase domains is not changed, but the density is drastically increased.

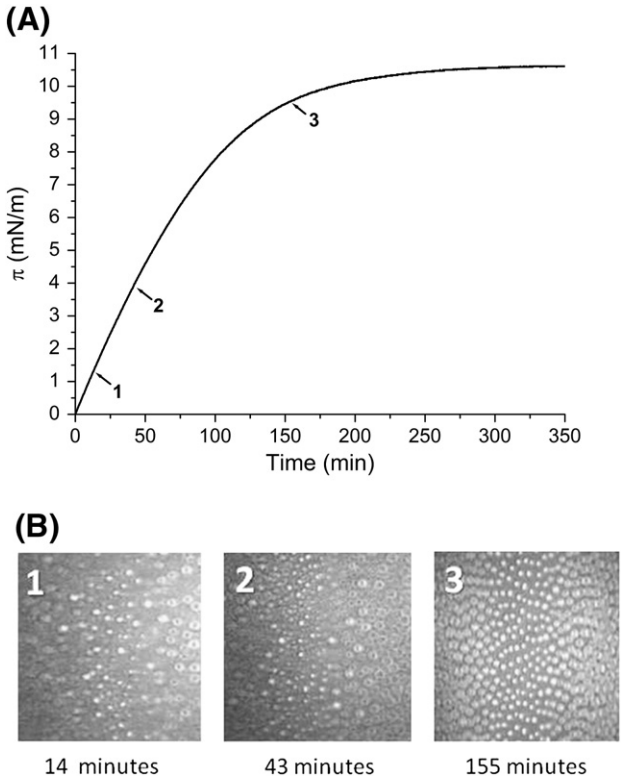

Fig. 1. (A) Surface pressure as a function of time of DMPC/DPPE (7:3) monolayer on a buffered subphase containing piroxicam (20 μM) and (B) BAM images showing the morphology of the mixed monolayer at different time periods. The images size is $280 \times 300\ \mu m^2$.

After the adsorption process has reached the maximum pressure, the monolayer was compressed and π/A isotherms were plotted. Two types of π/A isotherms were measured from the monolayer compression on a buffer subphase with or without the NSAIDs tested. The resultant isotherms are presented in Fig. 2.

As a reference, the π/A isotherm of the lipid mixture in the absence of drugs (Fig. 2A-line 1) showed an expanded state, with no evidence of any phase transition. The condensed phase domains are observable already at large molecular areas indicating that a phase-separation into a liquid phase, mainly composed of DMPC, and a condensed phase, mainly composed of DPPE, occurs. From the extrapolation of the steeper portion of the isotherm slope to zero surface pressure, it is possible to obtain the limiting molecular area A_0 ($\approx 6.5\ nm^2$/molecule) of the mixture which is in good agreement with values reported for DMPC monolayers [25]. Indeed, it has also been reported that the effective cross-sectional area of DMPC in a mixed monolayer is similar to the area occupied by a DMPC molecule in a tightly packed monolayer [26], and thus the small amount of condensed DPPE does not seem to have great influence on the limiting molecular area of the studied monolayer.

The addition of both NSAIDs to the subphase changes the π/A isotherms revealing the penetration of the drugs which now occupy parts of the surface area of the trough. At large molecular areas of the lipid mixture, the drug penetration leads to the same pressure as observed for the π/t measurements. On compression, the drugs are progressively squeezed out from the lipid monolayer and at higher lateral pressures one observes the same molecular area as for the pure lipid monolayer. Although these common effects were found for both NSAIDs, isotherms with different profiles were obtained for each one of these drugs. At larger surface areas, piroxicam revealed higher penetration since the isotherm starting pressure value was greater than the observed for ibuprofen (Fig. 2A – lines 3 and 2). However, with increasing compression ibuprofen revealed a shift of the isotherm to higher surface pressures indicating that this NSAID is better stabilized in the lipid layer compared to piroxicam. This isotherm shift caused by ibuprofen decreases with the onset of a plateau at 18 mN/m (73 Å^2/molecule) indicating a partial expulsion of the drug. At the end of this plateau, the isotherms from the lipid mixture on the pure buffer solution and on the buffer containing ibuprofen almost superimpose (Fig. 2A – line 2).

The described observations support the assumption that piroxicam is more surface active whereas ibuprofen is better stabilized in a lipid monolayer.

The process of compression was followed with BAM which showed once more the co-existence of fluid and condensed domains (Fig. 2B). The BAM micrographs indicate that, in the presence of NSAIDs in the subphase, the domains characteristic for the coexistence of liquid expanded (LE) and liquid condensed (LC) states coalesce at slightly higher surface pressures compared to those of the lipid mixture in the absence of the drug, which is in accordance with the shift of the compression isotherms to higher surface pressures indicating the penetration of the drugs. It seems that the drugs are preferentially localized in the disordered phase since the condensed domains did not change.

Furthermore, comparing with the lipid mixture alone, BAM images (Fig. 2B) reflect the existence of less condensed domains in the case of ibuprofen (Fig. 2B – number 2b shows less brighter spots than number 1b), whereas in the case of piroxicam the condensed domains are more evident than in the non-influenced monolayer (Fig. 2B – number 3a shows bigger brighter spots than number 1b).

Moreover, it is also evident that the drugs are expelled from the monolayer at smaller molecular areas, and at 53 Å^2/molecule the BAM images (Fig. 2B) look almost the same whether we are observing images of the lipid mixture alone (Fig. 2B – number 1d), or lipid mixture with the drug (Fig. 2B – numbers 2d and 3c). This is also visible on the isotherms (Fig. 2A – numbers 1d, 2d and 3c) where the π/A plots from the lipid mixture and NSAIDs almost superimpose.

3.2. NSAIDs binding studies to PLA_2

Binding studies of NSAIDs to PLA_2 were performed to evaluate the contribution of the direct interaction between drugs and enzyme on the inhibitory effect of the enzymatic activity. The fluorescence of the aromatic region of PLA_2, due to the existence of tryptophans, gives the possibility to evaluate the binding of the enzyme to a drug molecule [27]. The emission spectra of PLA_2 were examined in the absence and presence of piroxicam and ibuprofen after 2, 5, 8 and 10 min reaction in a concentration range of 0–150 μM. For both drugs studied, a concentration dependent decrease of the fluorescence intensity was obtained and this quenching effect was time independent. The PLA_2–drug binding constants were calculated by a graphical method where Langmuir equation was used to fit the fluorescence quenching intensities (expressed as % of quenching) according to the concentration of NSAID [27]:

$$Quenching(\%) = \frac{y_{max}}{1 + \frac{K_d}{Log[NSAID]}} \quad (1)$$

The values of y_{max} (maximum quenching value obtained) and K_d (dissociation constant which is inversely proportional to the binding constant, K_b) are determined from fitting a plot of quenching (% of fluorescence deactivation correspondent to the % of bound enzyme molecules) as a function of Log[NSAID] (logarithm of NSAID concentration). A Langmuir plot illustrating the binding behaviour for piroxicam is presented in Fig. 3. A similar plot was obtained for ibuprofen. For both drugs, the plot is an "S-shaped"

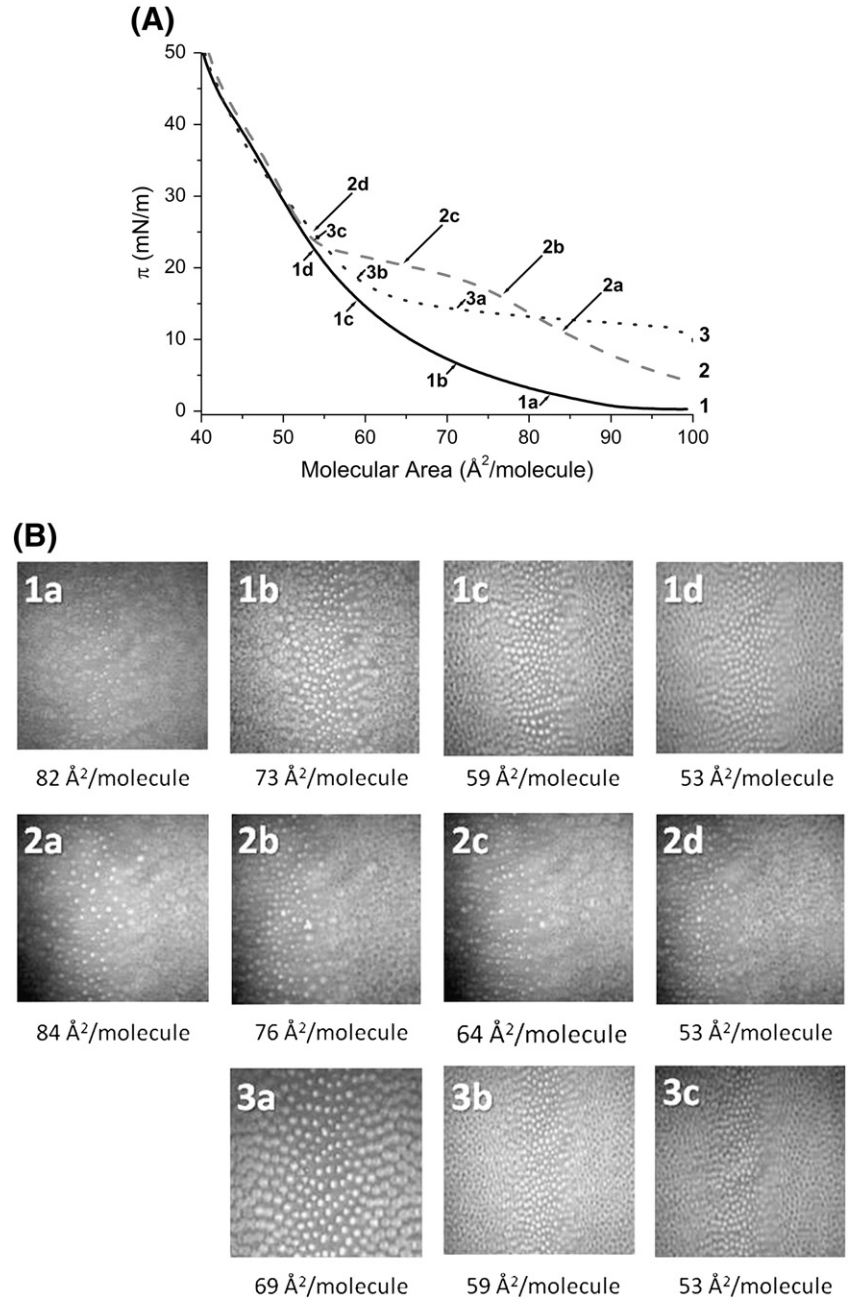

Fig. 2. (A) Surface pressure/area isotherms of DMPC/DPPE (7:3) monolayers on a buffered subphase (Tris 10 mM, NaCl 150 mM, Ca^{2+} 1 mM, pH 7.4) (Line 1) and on a buffered subphase containing 20 μM of ibuprofen (Line 2) or of piroxicam (Line 3) and respective (B) BAM images (first row on buffer, second row on ibuprofen and third row on piroxicam solutions). The images' size is $280 \times 300\ \mu m^2$.

curve and K_d corresponds to the midpoint of the quasi-linear portion of the curve.

The binding constants to PLA_2 obtained for piroxicam and ibuprofen were 6.0×10^{-2} μM and 1.5×10^{-2} μM, respectively, which indicates that piroxicam has a more extensive binding to the enzyme.

3.3. Evaluation of PLA_2 activity and its inhibition by the NSAIDs

The inhibition of PLA_2 enzyme can be evaluated by several methods. One of them is the determination of the amount of free fatty acid (FFA) that is released over time as a consequence of the enzyme hydrolytic activity against a lipidic substrate [28]. In this case the lipidic substrates were LUVs of DMPC/DPPE (7:3) and the chosen method for the quantification of the phospholipid hydrolysis was based on the use of a fluorescent probe (ADIFAB) with high binding affinity for FFA. According to this method, the concentration of FFA ([FFA]) was quantified at 432 nm using the following expression:

$$[FFA] = K_D \frac{F - F_0}{F_{max} - F} \quad (2)$$

Where F are the values of relative fluorescence, F_0 is the fluorescence obtained in the absence of the enzyme; $F_{max} = F_o/19.5$ and $K_D = 0.34$ [18].

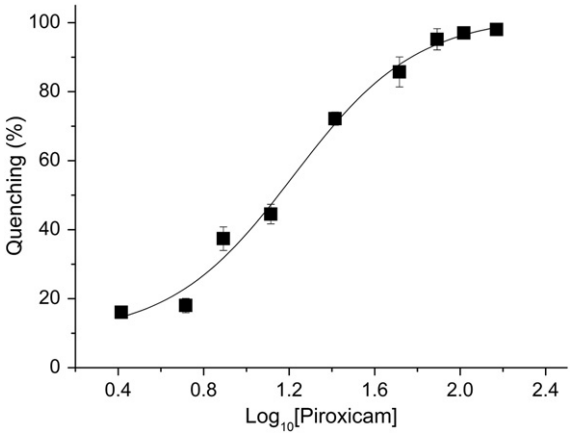

Fig. 3. Langmuir plot for PLA_2-piroxicam binding obtained from the fitting of % quenching of PLA_2 fluorescence as a function of piroxicam concentrations. Values represent the mean of three experiments.

After determining the FFA concentrations it was possible to calculate total fatty acid concentrations ($[FA]_{TOTAL}$) according to the expression:

$$[FA]_{TOTAL} = K_P \frac{V_m}{V_a} \times [FFA] + [FFA] \quad (3)$$

Where K_P is the partition coefficient of FFA, V_m is the volume of the membrane phase and V_a is the volume of the aqueous phase [18].

The inhibition capacity of the NSAIDs tested was quantified according to the reduction of $[FA]_{TOTAL}$ considering that $[FA]_{TOTAL}$ is 100% in the absence of the inhibitors. Ibuprofen and piroxicam were tested for different concentrations (0–20 μM) using two types of incubation. When the NSAIDs were incubated in LUVs before the addition of PLA_2, there was no visible alteration in the hydrolysis. On the other hand, when PLA_2 was incubated with NSAIDs before the addition to the liposomes, it was found that the enzyme inhibition occurred and was concentration dependent. Fig. 4 shows PLA_2 inhibition with increasing concentrations of piroxicam and ibuprofen plotted as a function of the total fatty acid concentration ($[FA]_{TOTAL}$ %) produced after 5 min reaction time.

From Fig. 4 it is possible to conclude that the total fatty acid concentration reduced significantly with increasing concentrations of NSAIDs and a small concentration of 6 μM was enough to provoke an extensive inhibition of PLA_2 (correspondent to 60% and 50% of total fatty acid concentration obtained with piroxicam and ibuprofen, respectively). According to the extent of reduction of fatty acid produced, ibuprofen revealed to be a stronger inhibitor of PLA_2.

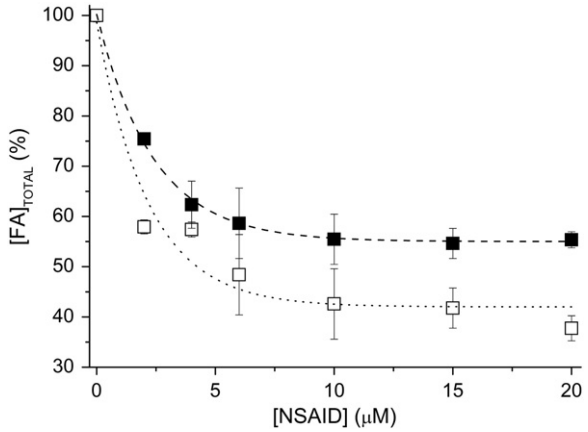

Fig. 4. Fatty acid concentration (%) obtained after 5 min hydrolysis reaction catalyzed by PLA_2 in DMPC/DPPE (7:3) LUVs after incubation of the enzyme with piroxicam (■) or ibuprofen (□). Values represent the mean of three experiments.

Since $[FA]_{TOTAL}$ reduction occurred only when the drugs were previously incubated with the enzyme this indicates that possibly these NSAIDs require to be in direct contact with the enzyme to be able to act as inhibitors.

The PLA_2 activity and its inhibition by the NSAIDs studied was evaluated by another method that makes use of the molecular area/time dependency (A/t) for testing whether a direct interference with the enzyme is playing a role in the inhibition process. Fig. 5 shows a typical example of an (A/t) plot obtained in the absence and in the presence of NSAID (piroxicam).

The lipid substrate was a monolayer of DMPC/DPPE (7:3) spread on the aqueous phase (Tris 10 mM; NaCl 150 mM, $CaCl_2$ 1 mM, pH 7.4). The initial molecular area (Fig. 5 – point A) corresponds to the area occupied by the monolayer with fully expanded barriers. During compression, the area decreases steeply until a fixed pressure of 25 mN is reached (Fig. 5 – point B). After waiting some time for stabilization of the monolayer, the enzyme was injected (alone or after incubation with NSAID) into the subphase (Fig. 5 – point C). When the enzyme is active, and in the absence of drug (Fig. 5 – Line 1), the hydrolysis of the monolayer occurs at a constant pressure, and as a result, the produced lysophospholipids are dissolved in the aqueous subphase and thus the molecular area of the monolayer decreases over time. In contrast, when the enzyme and drug are injected into the subphase (Fig. 5 – Line 2) the hydrolytic activity is inhibited and the phospholipid molecular area remains constant. More than 2 h after the NSAIDs' inhibitory studies have been started another injection of 1×10^{-3} M PLA_2 (not incubated with the drug) was carried out (Fig. 5 – point D). This injection reinforces the enzyme concentration and as a consequence, the phospholipid hydrolysis starts, as observed by the decrease of the molecular area (Fig. 5 – from point D to the end).

3.4. Evaluation of PLA_2 conformational changes

CD spectroscopy allowed the study of possible PLA_2 conformational changes. PLA_2 spectrum shows two negative bands near 209 nm and 223 nm and a positive band at 195 nm, typical of α-helical conformation (Fig. 6A – black curve). The $[\theta]_{223}/[\theta]_{209}$ ratio is

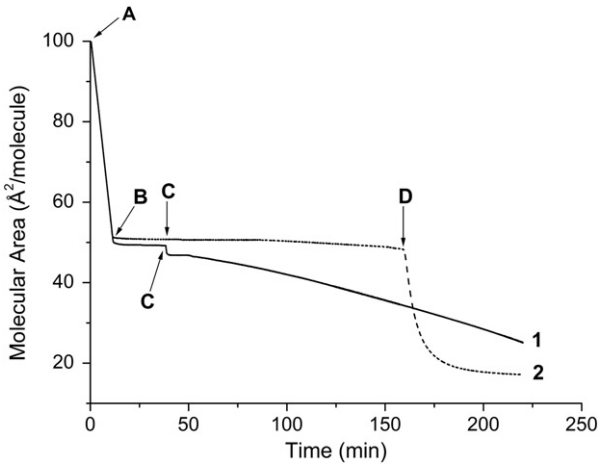

Fig. 5. Area/time plot of a DMPC/DPPE (7:3) monolayer showing the hydrolysis reaction catalyzed by PLA_2 in the absence (line 1) and presence (line 2) of 50 nM piroxicam (Tris 10 mM; NaCl 150 mM; Ca^{2+} 1 mM; pH 7.4). The initial molecular area (point A) corresponds to the area occupied by the monolayer with fully expanded barriers. During compression, the area decreases until a fixed pressure of 25 mN/m is reached (point B). After waiting some time for stabilization of the monolayer, the enzyme was injected (4 μM) (alone or after incubation with NSAID) into the subphase (point C). Point D indicates the moment of another PLA_2 injection (1×10^{-3} M).

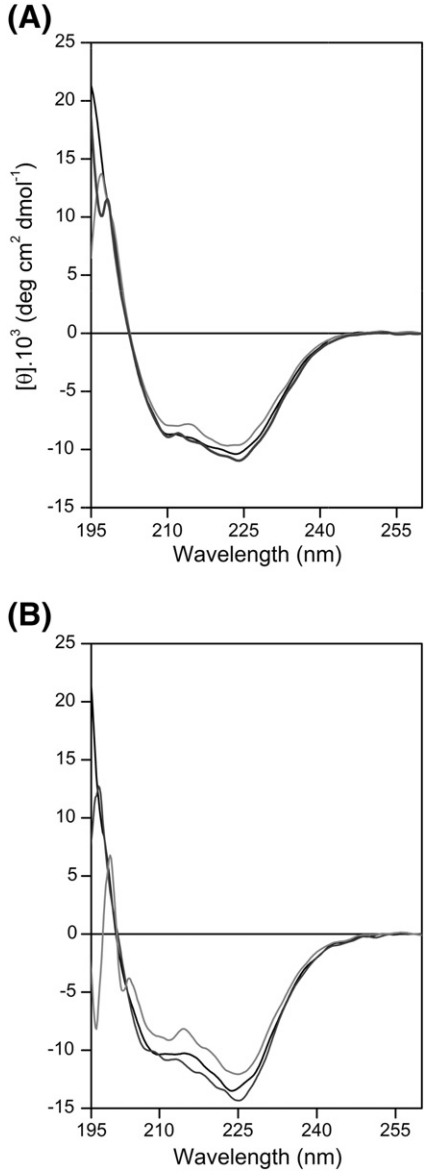

Fig. 6. (A) CD spectra of PLA_2 (8.9 μM) alone (black curve); with piroxicam (dark gray curve); or with ibuprofen (light gray curve). (B) CD spectra of PLA_2 (8.9 μM) after addition of LUVs of DMPC/DPPE (7:3) (black curve) and CD spectra of PLA_2 (8.9 μM) that was first mixed with NSAIDs (piroxicam (dark gray curve) or ibuprofen (light gray curve)) and then added to the liposomes. The noise below 200 nm is due to the NaCl in the solution.

greater than 1 (1.18, Table 1) indicating superhelical twist associated with coiled-coils or other assemblies of helices [29]. The n–π^* transition (~220 nm CD band) is responsive to the α-helical content, whereas the π–π^* excitation band at ~208 nm polarizes parallel to the helix axis and is sensitive to whether the α-helix is single-stranded or is an interacting helix as in the case of the two-stranded coiled-coils [30].This is supported by the crystal structure of the enzyme [31]. Each NSAID was studied individually regarding its ability to modify the enzyme's secondary structure. The binding of ligands can affect the structure of enzymes and it has been previously reported the ability of inhibitors to change PLA_2 conformation through the increase or decrease in the α-helical content [3,32,33]. It is visible that PLA_2 CD spectra after addition of the NSAIDs show minor differences comparing with that from the enzyme alone, indicating lack of major changes in the secondary structure (Fig. 6A). The ellipticity ratio $[\theta]_{223}/[\theta]_{209}$ increases in the presence of NSAIDs particularly piroxicam, indicating a stronger interaction between the enzyme and this drug, which is supported by the higher binding constant found for this case (Table 1).

Table 1
Ellipticities and $[\theta]_{223}/[\theta]_{209}$ ratio of PLA_2.

	$[\theta]_{223}$ ($\times 10^3$ °cm^{2}dmol^{-1})	$[\theta]_{223}/[\theta]_{209}$
PLA_2	−11.0	1.18
PLA_2 + piroxicam	−10.6	1.30
PLA_2 + ibuprofen	−9.8	1.25
PLA_2 + LUV	−12.6	1.29
PLA_2 + piroxicam + LUV	−13.7	1.35
PLA_2 + ibuprofen + LUV	−11.8	1.36

In buffer solution, the enzyme presents mainly an α-helix conformation where as in the presence of liposomes, PLA_2 exhibits as well a spectrum characteristic of α-helical, but with the band at 223 nm more pronounced, indicating higher helical content and $[\theta]_{223}/[\theta]_{209}$ ratio (Fig. 6B and Table 1) [34,35]. The increase of the ellipticity ratio $[\theta]_{223}/[\theta]_{209}$ indicates helices with increased flexibility [34–36]. Therefore, CD data suggest an increased level of more flexible α-helices in PLA_2 when the enzyme is bound to its natural substrate, phospholipid membranes.

In agreement to these results, destabilization of enzymatic helices upon membrane binding have been shown previously for several proteins or their fragments, such as cytochrome *c*, α-lactalbumin, and the pore-forming domain of colicin resulting in structural changes in these proteins to more flexible conformations [12]. A possible mechanism for the formation of more flexible helices in PLA_2 induced by binding to lipid bilayers may be disruption of a few intrahelical hydrogen bonds and formation of such bonds between the enzyme and the phosphate, carbonyl, and hydroxyl groups of membrane lipids. These results imply that during interfacial activation via membrane binding, the PLA_2 molecules acquire more dynamic properties, which presumably confer plasticity to the enzyme that is required for the multistep catalytic process. This might render the enzyme more flexible and stabilize the transition-state complex which may contribute to enzyme activation and facilitate catalysis [12]. The incubation of liposomes with the drugs followed by the addition of PLA_2 provided spectra resembling the one of PLA_2 only in presence of LUVs (data not shown). The incubation of PLA_2 with piroxicam followed by the addition of liposomes showed similar tendency with slight increase of the absolute value of $[\theta]_{223}$ (Fig. 6B – dark gray curve). In the case of PLA_2 incubated first with ibuprofen and then with LUVs (Fig. 6B– light gray curve), the overall CD signal decreases and the noise increases, suggesting the occurrence of aggregation. The presence of aggregates is known to reduce the signal intensity due to light scattering and a shadowing effect [37]. The formation of aggregates upon interaction of PLA_2 with ibuprofen in the presence of liposomes can be correlated with the better stabilization of this NSAID in the lipid layer which might have implications on the interactions of the enzyme with the substrate.

The increase of the ratio $[\theta]_{223}/[\theta]_{209}$ is large in samples containing both liposomes and NSAIDs when compared to the samples with enzyme and drug or only liposomes indicating a pronounced alteration in the distorted structure of the enzyme (Table 1).

A $[\theta]_{223}/[\theta]_{209}$ amplitude ratio near 1.5 rather than near 1 has been attributed to tryptophan contributions [38]. In general, pairwise or multiple coupling between any of the aromatic or other side-chain chromophores could lead to substantial effects in the 200–250 nm region and has been proposed to explain the unusual CD of fd Bacteriophage [39]. Considering that the intact tryptophan residues and certain level of helix distortion are essential for the enzymatic activity of PLA_2 [39,40] the changes observed in the $[\theta]_{223}/[\theta]_{209}$ ratio induced by the drugs might be associated with the rearrangement of functional residues toward the interaction with phospholipids/substrate, and might be associated with these NSAIDs' capacity to

inhibit the interaction of the enzyme with the lipid substrate. In fact, enzymes like PLA_2 from snake venom, that are known to bind well to zwitterionic surfaces, tend to have one or more tryptophans located on their interfacial surfaces taking active part in the interaction of the enzyme with the lipid membrane where these residues are inserted into the glycerol region of the bilayer [41].

4. Conclusions

Piroxicam and ibuprofen, the two NSAIDs chosen for this study, were able to inhibit $sPLA_2$ from *N. mossambica mossambica* as proved by fluorescent measurements using ADIFAB. The interactions between drug molecules and biomembranes were studied since they could induce alterations in the lipid structure and modulate the enzymatic performance to different levels of activity. Results have shown that the drugs studied were able to adsorb or to penetrate into lipid monolayers due to their intrinsic surface activity. However, ibuprofen has been shown to be better stabilized in the lipid layer, which might be related with its capacity to affect the performance of lipid-modifying enzymes like $sPLA_2$.

The possibility of a direct effect of the anti-inflammatory agents on PLA_2 was also assessed. In the molecular area/time dependency it was visible that the enzyme ability to hydrolyze the monolayer was clearly decreased, resulting from the direct interaction PLA_2 – anti-inflammatory agents, latter regained by the excess of enzyme added to the subphase medium.

The enzyme secondary structure was also evaluated by CD spectroscopy. The increase observed in $[\theta]_{223}/[\theta]_{209}$ ratio caused by both anti-inflammatory agents and particularly in the presence of liposomes indicates a change in the helical structure related to PLA_2 flexibility when interacting with the model membrane. This might be in turn related to an alteration of the distorted structural elements that determine the interfacial activation competence of the enzyme. The ratio closer to 1.5 also indicates an alteration associated to tryptophans. Spectrofluorometric analysis showed the tryptophan quenching by both drugs and further confirmed that piroxicam and ibuprofen prevent the enzyme from binding to its lipid substrate, either by shielding the tryptophan residue(s) and limiting the access to the active site, or causing a conformational change of PLA_2 structure reducing its ability for catalysis. In this regard, it is also possible to observe that piroxicam has demonstrated a higher binding constant to PLA_2 through the tryptophan residues, which has been further confirmed by CD measurements where the ellipticity $[\theta]_{223}/[\theta]_{209}$ ratio increases particularly in the presence of piroxicam, indicating a stronger interaction between this NSAID and the enzyme. Despite the smaller binding effect to the enzyme tryptophan residues, ibuprofen has proven to be a stronger inhibitor of PLA_2 activity. In this case, the biophysical changes induced by this drug in the lipid interface seem to have their own contribution to the whole inhibition process. Indeed, upon interaction of PLA_2 with ibuprofen in the presence of liposomes, the CD signal decreases and the noise increases, suggesting the occurrence of aggregation. This can be correlated with the better stabilization of ibuprofen in the lipid layer which, added to its binding capacity to the enzymatic tryptophan residues, might promote desorption of PLA_2 from the substrate. In agreement to this, it has been reported that the surface properties of the membrane are a prerequisite for binding of PLA_2 in a proper way, required for the induction of the conformational changes that ultimately activate the enzyme [42].

In conclusion, the results in the present study clearly show that the NSAIDs studied inhibit PLA_2 and that the inhibition of interfacial enzymes should be studied by a combination of methods that are able to cover the enzyme conformational changes upon binding and their correlation with membrane biophysical effects, since these aspects can constitute synergistic determinants of the overall inhibitory effect.

Acknowledgements

Partial financial support for this work was provided by FCT (Lisbon), through the contract PTDC/SAU-FCF/67718/2006. Diana Gaspar thanks FCT (Lisbon) for the fellowship (SFRH/BD/29308/2006).

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