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Auxins action on *Glycine max* secretory phospholipase A_2 is mediated by the interfacial properties imposed by the phytohormones

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ABSTRACT

Secretory phospholipase A_2 ($sPLA_2$) are soluble enzymes that catalyze the conversion of phospholipids to lysophospholipids and free fatty acids at membrane interfaces. The effect of IAA and IPA auxins over the activity of recombinant $sPLA_2$ isoforms from *Glycine max* was studied using membrane model systems including mixed micelles and Langmuir lipid monolayers. Both phytohormones stimulate the activity of both plant $sPLA_2$ using DLPC/Triton mixed micelles as substrate.

To elucidate the mechanism of action of the phytohormones, we showed that both auxins are able to self-penetrate lipid monolayers and cause an increment in surface pressure and an expansion of lipid/phytohormone mixed interfaces. The stimulating effect of auxins over phospholipase A_2 activity was still present when using Langmuir mixed monolayers as organized substrate regardless of $sPLA_2$ source (plant or animal). All the data suggest that the stimulating effect of auxins over $sPLA_2$ is due to a more favorable interfacial environment rather to a direct effect over the enzyme.

1. Introduction

Secretory phospholipases A_2s ($sPLA_2$s) hydrolyze glycerophospholipids at the *sn*-2 position to produced free fatty acids and lysophospholipids. Rate of hydrolysis depends on the physicochemical properties of the membrane. Animals $sPLA_2$s are rather well studied and it has been reported that they play pivotal roles in diverse cellular responses, as host defense, signal transduction, phospholipid digestion, metabolism (Liscovitch and Cantley, 1994) and generation of precursors (Murakami et al., 1997). In plants, $sPLA_2$s activity has been reported for partially purified enzymes (Stahl et al., 1998, 1999). Several reported cDNA showed to encode putative $sPLA_2$s (Dhondt et al., 2000; Kim et al., 1999; Lee et al., 2005; Mansfeld and Ulbrich-Hofmann, 2007; Ryu et al., 2005). In a previous work, five *Glycine max* $sPLA_2$ isoforms were found to encode putative functional $sPLA_2$ enzymes (Mariani et al., 2012). Both of them, called $GmsPLA_2$-XIA-1 and $GmsPLA_2$-XIB-2, showed an optimum pH of 7, micromolar calcium requirement and a preference of zwitterionic over anionic phospholipids (Mariani et al., 2015).

Studies in plants demonstrated that $sPLA_2$s play important roles in signal transduction regulating cellular processes and probably they are implicated in phospholipid signaling (Ryu et al., 2005; Scherer et al., 2010; Wang, 2001). Till today, poor is known about the molecular mechanism and the effect of the auxins over $sPLA_2$s activation and further investigation is needed. A rapid increase in PLA_2 activity was first verified by treating isolated microsomes and cell cultures with auxins (André and Scherer, 1991; Scherer, 1990; Scherer and André, 1993, 1989; Scherer, 1992, 1996) and microsomes isolated from hypocotyls segments (Blanchet et al., 2008b).

At the cellular level, auxins control cell division, growth, extension, and differentiation (Davies, 1995). Over whole-plant, auxins play an essential role in processes such as apical dominance, lateral/adventitious root formation, tropisms, fruit set and development, vascular differentiation, and embryogenesis (Friml, 2003).

Indole-3-acetic (IAA) is the most abundant and potent naturally occurring auxin active in higher plants (Simon and Petrasek, 2011). It exerts control over many important developmental processes in plants (Eckardt, 2001). Earlier studies indicated that applications of auxins to plants cause profound changes in plant growth and development (Bonner, 1952). IAA and indole-3-propionic acid (IPA) are comprised of a hydrophobic indole ring linked to the hydrocarbon chain of an acetic acid or propionic acid, respectively (Fig. 1).

Abbreviations: DLPC, 2-dilauroyl-*sn*-glycero-3-phosphatidylcholine; IAA, indole-3-acetic acid; IPA, indol-3-propionic acid; $sPLA_2$, secretory phospholipase A_2.

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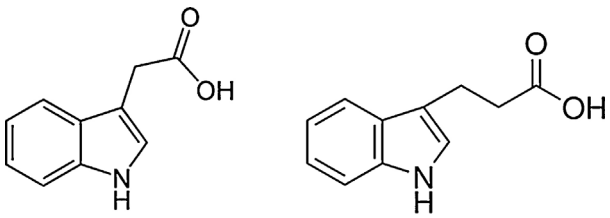

Fig. 1. Chemical structure of the auxins used.

It is known that in the biological medium, enzymes frequently act in association to membranes (Gelb et al., 2000). A common concept in plant growth regulatory theory, is the vision of a direct auxin–protein interaction and, in this sense, it has been proposed that the auxin mechanism of action would involve an association to proteins (Sakai, 1992). Nevertheless, the mechanism by which auxins produce $sPLA_2$ activation has not been firmly established yet.

The aim of the present work was to elucidate the effect of phytohormones, specifically IPA and IAA, on the activity of $sPLA_2$s from *G. max* comparatively with the behavior elicit on pancreatic $sPLA_2$ (from animal source). The studies were performed by using organized substrate in either mixed micelles or Langmuir lipid monolayers. Studies on Langmuir monolayers are ideal for understanding auxin–membrane interactions, since lipid monolayers constitute simple models to study intermolecular interactions (Albrecht et al., 1981; Demel, 1974; Möhwald, 1993) and the interface can be easily modulated by changing interfacial composition or lateral packing (Imbenotte and Verger, 1973; Maggio et al., 2014). This technique allowed us to monitor not only the thermodynamic trend of self insertion of auxins into biomembrane models, but also we analyze the effect of auxin adsorption on the activity of two *G. max* $sPLA_2$s isoforms assayed.

2. Methods

2.1. Chemicals and materials

Pancreatic $sPLA_2$ and Triton X-100 were from Sigma Chem. Co. (St. Louis, MO, USA). 1,2-dilauroyl-*sn*-glycero-3-phosphatidylcholine (DLPC) was from AVANTI polar Lipids Inc. (Birmingham, AL, USA). IAA was from Research Organics, Inc. (Cleveland, Ohio, USA) and IPA from BDH laboratory chemical division (London, England). All other reagents were of the highest analytical grade. Solutions were prepared with double-deionized water.

2.2. Expression, purification and folding of the recombinant $GmsPLA_2$s proteins

Expression of the recombinant proteins (Poly-His-Ub-$GmsPLA_2$-XIA-1 and Poly-His-Ub-$GmsPLA_2$-XIB-2) in *E. coli* BL21 strain (DE3), isolation and folding were carried out following identical procedures as mentioned in (Mariani et al., 2012).

2.3. Effect of auxins on $sPLA_2$ activity using mixed micelles

$sPLA_2$ activities were determined using DLPC/Triton X-100 mixed micelles (1:4, mole ratio) following the liberated fatty acids during the reaction by using the adapted NEFA-HR (2) kit from WAKO (WAKO Pure Chemical Industries, Japan) according to (Hoffmann et al., 1986) and expressed as $\mu mol\,min^{-1}\,mg^{-1}$. Substrate mixture composed of 5 mM DLPC, 20 mM Triton X-100, 10 mM $CaCl_2$, and 10 mM Tris/HCl pH 8 in a total reaction volume of 30 μl. Assays were performed at 37 °C. Auxins stock solutions (10 mM) were dissolved in absolute ethanol.

Enzymes were pre-incubated with auxin during 1 min to provide the necessary time for equilibrium. Free-phytohormone samples were used as controls. After enzyme addition samples were agitated with an appropriate mixer at 60 rpm. The reaction was stopped by addition of 6 μl of 0.5 M EDTA after 5 min of incubation. The reported data are the averages of, at least, two independent experiments performed in duplicate. Bars represent ± S.D.

2.4. Surface pressure (π)–molecular area (A) isotherms

Compression isotherms (π/A isotherms) were measured by the compression of monolayers containing DLPC, DLPC/IAA and DLPC/IPA, using a KSV Microtrough (KSV NIMA_Biolin Scientific AB, Västa Frölunda, Sweden). Langmuir monolayers were prepared by spreading pure DLPC or DLPC/auxin (8:2) mixture dissolved in chloroform/methanol (2:1) on the aqueous surface of a Teflon™ through filled with 10 mM Tris–HCl buffer pH 8, containing 100 mM NaCl and 20 mM $CaCl_2$ as subphase. After solvent evaporation (5–10 min), the film was compressed isometrically at constant rate of $5 \pm 1\,Å^2\,mol^{-1}\,min^{-1}$ until reaching the target pressure. π was determined with a platinum plate using the Wilhelmy method (Verger and De Haas, 1973). Isotherms were averaged at least from duplicates. All assays were performed at 25 °C. For mean molecular area (MMA^2) determinations, auxin proportion was taken into account in the mixture.

2.5. Penetration and adsorption isotherms. Lipid interface partition

In order to study the penetration of the IAA and IPA into DLPC lipid monolayers, we performed surface pressure increment *vs.* time (and concentration) plots in a Langmuir trough at constant total surface area ($\Delta\pi$ *vs.* time/isotherms). A phospholipid monolayer was spread previous to auxin injection into the subphase until achieving the target π of $13\,mN\,m^{-1}$. Controls experiments were done to assure surface stability of spread monolayers (see Fig. S3). After waiting 10 min, $\Delta\pi$ were recorded on a home-made circular Langmuir trough balance controlled by an electronic unit (Monofilmetter, Mayer, Gottingen) at 25 °C. The surface activity of both auxins was checked by testing the self-adsorption into lipid free air/aqueous interface. The aqueous subphase was 10 mM Tris–HCl buffer pH 8 containing 100 mM NaCl and 20 mM $CaCl_2$.

Penetration (into lipid covered interface) or adsorption (into lipid free interface) experiments were performed by injections of auxins from 10 mM stock solution in absolute ethanol (< 20 μl) into the subphase under continuous stirring (trough volume of 52 ml, auxin final subphase concentration was 5 μM). The same amount of absolute ethanol was assay as negative control. The changes in π at constant area were register as a function of time. Initially, π/t isotherms were measured until an equilibrium surface pressure was reached (changes in pressure less than $1\,mN\,m^{-1}$ per hour).

The partition coefficients of auxins into biomembranes were theoretically estimated by the octanol–water partition coefficient ($\log P$, K_{ow}) using ChemSketch, version 14.01, from Advanced Chemistry Development (www.acdlabs.com).

2.6. Determination of $sPLA_2$s activity on Langmuir monolayers

A zero order Langmuir trough was used to study the lipid hydrolysis of $sPLA_2$s and the stimulation exerted by auxins. Medium chain DLPC was employed as substrate because the activity can be measured at zero order kinetics following the

reduction of substrate monolayer area from the reservoir compartment at constant surface pressure (surface barostat), since a rapid desorption of the medium-chain products into the aqueous subphase in the reaction compartment occur after lipid hydrolysis (Panaiotov and Verger, 2000; Verger and De Haas, 1973). Monolayers of DLPC and DLPC/auxin (8:2) were obtained in similar way as described above. Initially, the monolayer was compressed until a constant surface pressure of 13 mN m^{-1} was reached. We have choose this value because it is near to the optimum surface pressure of *G. max* $sPLA_2$ activity (Mariani et al., 2015) and that observed for pig pancreatic $sPLA_2$ (Pattus et al., 1979). The monolayer was left for stabilization without any appreciable leakage (see Fig. S3). Changes in the film area over time at a constant surface pressure was recorded after the enzyme injection (0.085 μg/ml of $GmsPLA_2$-XIA-1, 0.148 μg/ml of $GmsPLA_2$-XIB-2 and 0.027 μg/ml of pancreatic $sPLA_2$) into the subphase of the reaction compartment (Fig. S2). Linear least square fitting was performed over the area *vs.* time data to determine the activity expressed as molecule cm^{-2} min^{-1} mg^{-1}.

3. Results

3.1. Stimulating effect of auxins on $sPLA_2$ activity using mixed micelles

The stimulation on $sPLA_2$ activity is observed with both auxins, 3-IPA and 3-IAA, after 5 min of incubation using mixed micelle as substrate and in presence of different concentrations of either $GmsPLA_2$s or pancreatic PLA_2 (Fig. 2). Mixed Triton X-100/phospholipids micelles have been extensively used for $sPLA_2$ studies (Reynolds et al., 1991). An excess of detergent in the mixture avoids the aggregate structure from being greatly affected by the introduction of phospholipids or by inhibitors/activators (Ribeiro and Dennis, 1975).

A maximum of auxin stimulation was observed at around 100 μM of 3-IAA auxin concentration for both recombinant enzymes obtained from *G. max* (Fig. 2A). The stimulating effect clearly decreased at higher concentrations of 100 μM and a plateau level of activity is attained, indicating that a further stimulating effect cannot be achieved beyond a saturating effect. The same tendency was observed for 3-IPA, but a higher stimulation level of $sPLA_2$ activity is obtained at 100 μM (Fig. 2B). Clearly this indicates that a maximum stimulating effect on $sPLA_2$ activity is achieved at around 100 μM of auxin, regardless of the source of the $sPLA_2$ (animal or plant), since the same effect was observed for porcine pancreatic PLA_2. The effect observed coincides with the concentration found in biological medium where the concentrations of auxins are in the micromolar order (Paul et al., 1998; Scherer, 2002; Scherer et al., 2007). At millimolar auxin level, an attenuation of the stimulation effect was observed (see Table 1).

3.2. Interaction of auxins with lipid interfaces. Stability of phytohormones in a membrane-like environment

As we also studied the influence of phytohormones on PLA_2 activity by using lipid monolayers as membrane model systems, firstly it was necessary to evaluate the interaction and penetration capability of IAA and IPA into lipid monolayers. IAA and IPA phytohormones are amphiphilic molecules (Fig. 1) with affinity for lipid interfaces with an estimated partition coefficient $\log K_{wo}$ of 1.43 ± 0.23 and 1.78 ± 0.22, respectively. The DLPC–phytohormones interaction was studied by the ability of the auxins to self-penetrate lipid monolayers as function of time. Fig. 3A shows an increase in surface pressure upon addition of auxins into the subphase. The change in surface pressure was rapidly obtained and practically a 90% of the extent of the interaction is achieved at 2–3 min. The interaction of IPA is greater than that obtained with IAA in keeping with the higher hydrophobicity expected for the former. An increment in the lateral pressure ($\Delta\pi$) is interpreted in terms of penetration of at least part of the amphiphilic molecule into the monomolecular film causing its increase in surface pressure (Law and Davies, 1990; Serville et al., 1973). Both phytohormones did not self-adsorb into a clean air/water interface (interface without lipid, data not shown). This indicates that IPA and IAA behave strongly stabilized in a membrane like environment only when the interface is covered with phospholipids.

The capability of the phytohormones to self-penetrate DLPC monolayers was studied at different auxin concentrations (Fig. 3B). For the more hydrophobic IPA, a saturating effect is achieved at 10 μM and posses a higher capacity than IAA to interact wiht DLPC monolayers. For IAA, the saturating effect is reached at 2 μM but the extent of interaction is lower (Fig. 3B). Penetration experiments undoubtedly indicated that the phytohormones were stabilized at the interface in presence of phospholipids. Previous to consider their influence on PLA_2 activity, we also studied the stability of mixed phospholipid/auxins interfaces by direct spreading into air/water interface of pre-mixed solution made of lipid with each phytohormone. Compression π-area isotherms of mixed lipid/phytohormone

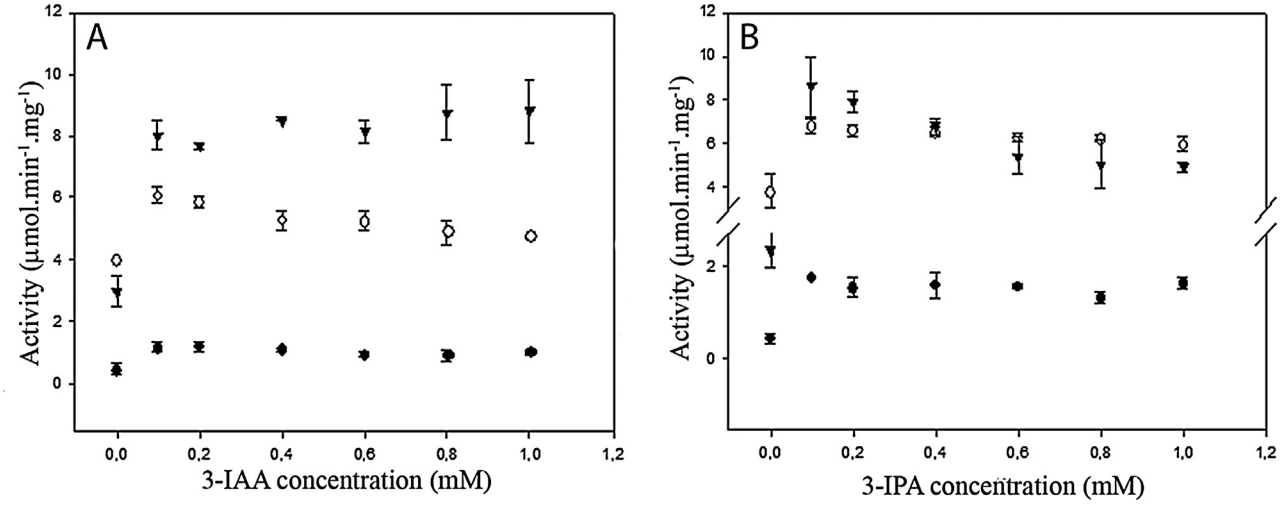

Fig. 2. Effect of auxins over $sPLA_2$ activity in mixed micelles. Interaction of $GmsPLA_2$–XIA-1 (○), $GmsPLA_2$-XIB-2 (▼) and Pancreatic $sPLA_2$ (●) with A) 3-IAA and B) 3-IPA. Substrate: DLPC/Triton X-100 (1:4 ratio).

Table 1
Percentage of stimulation on PLA2 activity induced by the addition of auxins in mixed Triton X-100/DLPC mixed micelles.

% Stimulation						
Auxin (mM)	$GmsPLA_2$-XIA-1/IPA	$GmsPLA_2$-XIA-1/IAA	Pancreatic PLA_2/IPA	Pancreatic PLA_2/IAA	$GmsPLA_2$-XIB-2/IPA	$GmsPLA_2$-XIB-2/IAA
0	0	0	0	0	0	0
0.1	78.7	53.0	286.7	155.3	271.1	170.7
0.2	73.7	47.5	240.0	151.1	240.1	157.9
0.4	71.8	32.9	255.6	131.9	192.7	186.2
0.6	66.5	32.2	246.7	102.1	132.8	174.4
0.8	64.5	24.2	188.9	100.0	118.1	194.9
1.0	56.5	19.8	257.8	119.1	112.9	196.3

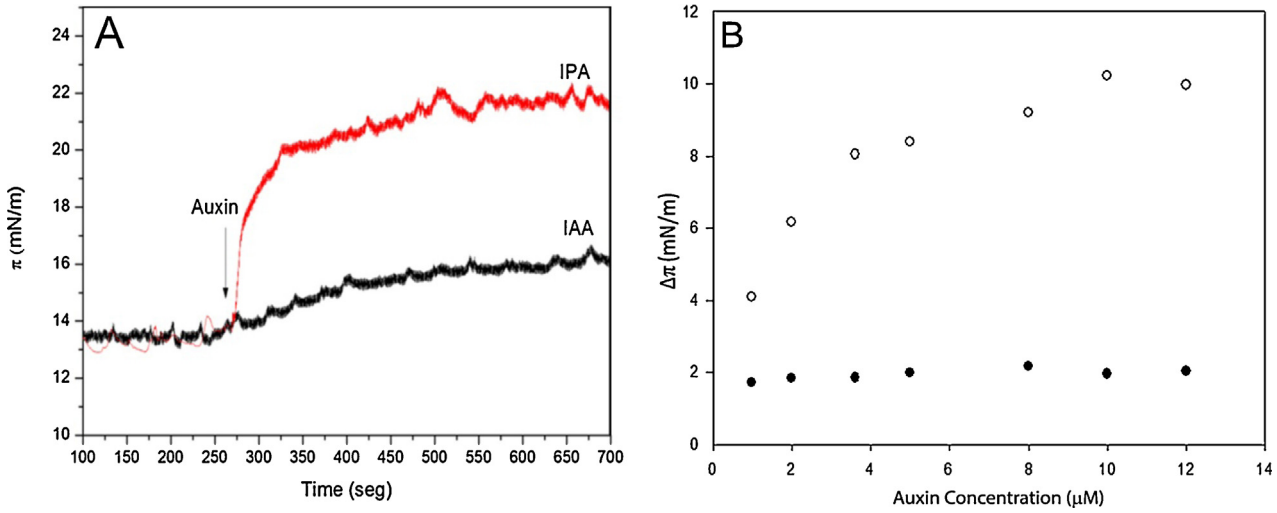

Fig. 3. Interaction of auxins into lipid monolayers. A) Time course of auxins penetration into DLPC monolayers set at 13.5 mN m^{-1}. B) Adsorption isotherm of auxins into DLPC monolayers set a 13.5 mN m^{-1} at different phytohormone concentration; (●) IAA and (○) IPA. The points represented in B are the equilibrium penetrating values achieved at 5–10 min when changes in surface pressure is lower than 0.01 mN m^{-1} per minute.

monolayers indicated that they can form coherent and reproducible films that can be compressed up to a lateral pressure up to 20 mN m^{-1} without appreciable desorption. Both auxins (IAA and IPA) mixed with DLPC have the capacity to remain at the lipid/phytohormone mixed monolayers and, in turn, they modified the interfacial characteristics of DLPC, causing the expansion of the monolayer when compared with the pure lipid (see Figs. S1 and S3). Expansion effect of IAA was recently observed on DPPC monolayers spread over a subphase containing 1 μM of this phytohormone (Gzyl-Malcher et al., 2011).

3.3. $sPLA_2$ activity on auxin/DLPC mixed monolayers

Activity of $sPLA_2$s from different sources was studied using Langmuir monolayers of DLPC in presence and absence of auxins, in order to determine the direct effect of phytohormones on $sPLA_2$ activity when they are directly spread at the interface. In a zero order kinetics regime, the amount of area displaced in the trough from the reservoir at constant surface pressure (surface barostat) is equivalent to the amount of substrate degraded (Point et al., 2013; Verger et al., 1978). Fig. S2 shows the profile for $sPLA_2$ activity (area *vs.* time) obtained for monolayers of DLPC and DLPC/auxin mixtures. After the enzyme injection into the reaction compartment velocity of degradation remains almost linear after an initial lag time of few minutes. Specific activities obtained for each condition and enzyme assayed is shown in Table 2. The presence of auxin in mixed monolayers follows the same tendency of that found for mixed micelles. Phytohormones showed an increase in $sPLA_2$ activity when disposed directly onto the surface mixed with the DLPC substrate regardless of enzyme source and type of auxin.

Table 2
Action of auxins on $sPLA_2$ activity[a] against phospholipd monolayers.

Enzyme	DLPC	DLPC/IPA	DLPC/IAA
Pancreatic PLA_2	157.8 ± 0.1	368.4 ± 0.1	926.3 ± 0.4
$GmsPLA_2$-XIA-1	71.4 ± 0.2	140.3 ± 0.1	159.7 ± 0.1
$GmsPLA_2$-XIB-2	38.6 ± 0.1	184.1 ± 0.1	138.6 ± 0.1

[a] Activity was expressed as (molecules cm^{-2} min^{-1}) × 10^{13}/mg enzyme. DLPC/auxin (mole ratio 8:2).

4. Discussion

$sPLA_2$s, like other lipolytic enzymes, are interfacial enzymes, since they access the water insoluble substrate organized into the interface to carry out the catalytic turnover. For this systems, the activity of the enzyme is directly modulated at the interface by the supramolecular organization of the substrate summarized in the concept of "interfacial quality" (e.g., the physical state of the lipids, proper lateral packing, modulation by non-substrate lipids, "membrane lateral defects"; surface charge density; etc. (Blanchet et al., 2008a; Campagnoli et al., 2008; Daniele et al., 1996; De Tullio et al., 2013; Fico et al., 2008; Jain and Berg, 2006; Verger et al., 1978).

Here, we report for the first time that the stimulation effect of auxins over recombinant $sPLA_2$s from soybean (*G. max*) is rather an interfacial effect. Porcine pancreas $sPLA_2$ presents low identity with the known reported $sPLA_2$s from plants source (Schaloske and Dennis, 2006). But they show a significant similarity in the active site and calcium binding loop regions (Mansfeld and Ulbrich-Hofmann, 2007), thus, it serves as an acceptable model for

comparison in the present study. Auxins are phytohormones that are found solely in plants. In bulk assays, using micelles, the effect of auxins on $sPLA_2$ stimulation dependents on the concentration of the phytohormone employed with an optimum around 100 μM (Fig. 2 and Table 1). This behavior may be interpreted as either a direct action over $sPLA_2$ enzyme molecule or a synergic effect on the micelle surface doing more favorable the interface for lipolysis. The fact that the effect of phytohormones were not only specific for $sPLA_2$ coming from plants but rather it was also effective on pancreatic $sPLA_2$ and, together with all the data about the concept of "surface quality" acting on lipolytic enzymes, makes more likely the second hypothesis. The tendency of both phytohormones assayed was quite similar toward both plant $GmsPLA_2$s and also for the pancreatic PLA_2, suggesting that no direct specific enzyme–phytohormone interaction is involved.

To ascertain to the interfacial hypothesis we firstly tested the self partition of the phytohormones into lipid interfaces. Both phytohormones assayed, IAA and IPA (Fig. 1), did not show surface activity *per se* toward aqueous lipid–free interfaces (self-adsorption). But they certainly interact with DLPC measured by their capability of lipid penetration into lipid monolayers (Fig. 3). Besides, they behave surface stable in mixed monolayers when an adequate mixed lipid/phytohormone solution is directly spread into air/water interface (Figs. S1 and S3). Results showed that the more hydrophobic auxin, IPA, can self penetrate more effectively into the lipid monolayer than IAA (higher $\Delta\pi$,Fig. 3A) and, it corresponds with a greater expansion of monolayer observed in π-area isotherms in mixed spread monolayers (see Fig. S1). To emphasize the lateral stability observed of π-area isotherms of mixed lipid/phytohormones is important for non specialist readers in surface chemistry, since in the experimental set up of measuring $sPLA_2$ activity in lipid mixed monolayers the amount of phytohormone spread constrained at the interface are few nanomoles forming an insoluble lipid/hormone interface.

Once it was clearly ascertained the surface stability of mixed lipid/phytohormone interface, experiments using mixed monolayers were performed to study the influence of auxins on PLA_2 activity. The amount of phytohormone relative to substrate necessary for an optimal stimulation was about 0.2 of auxin mole fraction in the mixed monolayers suggesting that the mechanism is not catalytically driven over the enzyme but it rather occurs via the interface quality (Table 2). These results are consistent with those obtained on mixed micelles where, at auxin concentrations above 100 μM (Table 1), no further significant stimulation can be observed. This was the maximum perturbing effect and consequently, the maximum increase on PLA_2 stimulation that can be achieved. These results, added to the unspecific nature of the stimulation demonstrate that the effect was clearly over the interfacial quality of the organized substrate than over the enzyme. A direct interpretation of a probably more "disordered" state that could be imposed by the auxins when they interact with phospholipids is not also as direct as it could be thought in a first glance, since the stimulating effect of auxins is present either over mixed Triton/DLPC micelles or the more organized lipid monolayers. The molecular details by which the particular mixed interfaces formed by auxins/phospholipids is more subtle to be degraded by the $sPLA_2$, regardless of the enzyme origin, remain to be elucidated.

5. Conclusion

We demonstrate that the more abundant natural occurring auxins indole-3-acetic acid (IAA) and indole-3-propionic acid (IPA) are amphiphilic compounds that can self- penetrate lipid monolayers and become incorporated into membranes in an organized and stable mixed interface. These auxins exerted a stimulating effect on soybean (*G. max)* recombinant phospholipases A_2 by favorable changes induced at the membrane interface level, discarding a direct action in bulk over the enzyme. The studies were done in comparison with a $sPLA_2$ from animal source. The stimulation exerted by auxins over lipolytic activity was effective regardless the source of $sPLA_2$ (plant or animal) and the membrane model assayed, indicating a rather general effect imposed by auxins at the interface level.

Author contributions

R.R.M. and G.D.F. have designed the general research project. M. E.M has conducted the experiments. M.E.M and G.D.F. have written the paper. This paper is part of the experimental work from M.E.M required to get her PhD degree in the National University of Cordoba, Argentina, and supervised by R.R.M. All authors read, corrected and approved the paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemphyslip.2015.05.003.

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