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Chapter

Effects of Vesicular Membranes Reordering on the Activity of Lipid Metabolizing Enzymes

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Abstract

The activity of membrane enzymes could be highly determined by the order of the lipid of the membrane and the enzyme distribution. Particularly, the reordering of phospholipid substrates and the local fluctuations of the lipid phases have been included in mathematical models to explain the modulation of the activity of membrane enzymes in extracellular vesicles, liposomes, or microvesicles. The applied principles are different to those derived from the classic considerations such as 3D environment, aqueous, and homogeneous media. Instead, the lateral diffusion of enzyme and substrate and highly nonhomogeneous 2D environment determine fluctuations of enzymatic activity capable to explain metabolic effects, such as in case of peptide-induced membrane components reordering. In this chapter, we review some applications to lipid metabolizing enzymes, due to analytical results of the kinetic theory of membrane enzymes.

Keywords: phospholipid domain, substrate reordering, lipolytic enzyme, phospholipase, enzyme kinetics, lipid phase, micelle, membrane

1. Introduction

The so-called extracellular vesicles (EVs) are either exosomes or microvesicles, which are formed from intracellular multivesicular bodies or plasma membrane, respectively [1, 2]. The lipid content of EVs plays a key role in various pathophysiological processes [3] as well as the native proteins on their surface, many of them having functions in cellular metabolism and signal transductions, such as phospholipases [4]. Interestingly, some membrane protein-related human diseases arise from dysregulation of signal transduction pathways [2]. Moreover, some phospholipases are very important for biogenesis of EVs and there are many phospholipases in EVs [5–7]. About the lipid phase of EVs, lipid exchange between vesicles has been described [8], exosomes can vectorize some lipids acting as transport, and the lipid composition can be modified by in vitro manipulation [7]. On the other hand, microdomains of EVs could be transferred to a target membrane cells by means of membrane fusion, and as a consequence, the lipid substrate redistribution could be able to affect the activity of lipid metabolizing enzymes.

Taking into account all the abovementioned causes and effects of the EVs related to both lipid substrate reordering and their metabolizing enzymes, the understanding of the effect of lipid-substrate reordering over the enzyme activity could be

essential to the development of therapeutic purposes as well as to insight the carcinogenesis and to perform enzyme kinetics experiments. We hope that this purpose of understanding the enzyme kinetics in the lipid phase will be fulfilled at least partially in the remainder of this chapter.

Numerous processes associated with the cell membranes are mediated by the action of lipid metabolizing enzymes. Knowing how the changes of membrane properties affect the activity of these enzymes allows us to explain disease mechanisms and pharmacological activities. Specifically, the knowledge about the mechanisms of the reactions catalyzed by these lipid metabolizing enzymes can contribute to the understanding of several regulation and signaling phenomena in cells. Thus, the enzymes of the phospholipase C family (PLC) [9] are involved in lipid signaling pathways affecting levels of free calcium and protein phosphorylation [10, 11], regulating secretion, transport, metabolism, gene expression, and protein translation. Since phospholipases react in a lipid-water interface, different kinetic experimental systems have been developed using phospholipid vesicles, phospholipid and detergent mixed micelles, or phospholipid monolayers. As a first step, the water-soluble enzyme would bind to the lipid phase, then having many catalytic cycles with the lipid substrate before the enzyme returns to the aqueous solution.

To study the kinetic measurements of phospholipases, the theory known as *surface dilution kinetics* [12] has been applied. This theory allows to estimate the main enzyme kinetic parameters considering the effects of the substrate staying into the lipid phase ("surface dilution") on the enzyme activity. Similar to the most enzyme kinetic models, in this theory, the mass action law and the steady-state assumption for enzyme intermediaries are applied. In the calculations with regard to molecules in water phase, their concentrations are used. Instead, in the case of calculations of molecules dissolved in lipid phase, their mole fractions are used.

Using this theory and its associated experiments, it has been proposed that many lipid metabolizing enzymes follow a mechanism composed by two binding steps of the enzyme on the lipid phase: a first binding step to the lipid phase followed by a second binding step to the substrate. More specifically, depending on the first binding step, there are two possible kinetic models: in the *phospholipid-binding model,* first the enzyme binds specifically to the phospholipid substrate; n the *surface-binding model,* first the enzyme binds to any lipid phase region. In homogeneous substrate distribution conditions, these are the kinetic equations derived for the *phospholipid-binding model* (Eq. 1) and the *surface-binding model* (Eq. 2) [12, 13]:

$$V = \frac{kC_{E_T} f^2}{\frac{k_m k_s}{C_L} + k_m f + f^2} \tag{1}$$

$$V = \frac{kC_{E_T} f}{\frac{k_m k_s}{C_L} + k_m + f} \tag{2}$$

where V is the rate of product formation (mol/[volume·time]), f is the mole fraction of the substrate (dimensionless), C_{E_T} is the total enzyme concentration (mol/volume), C_L is the total lipid concentration (mol/volume), k is the catalytic time constant (time^{-1}), k_s is the dissociation constant (mol/volume), and k_m is the interfacial Michaelis constant (dimensionless).

A more complex approach must consider that phospholipids can be reordered in lateral domains [14–16] because of their interactions with either phospholipids, cytoskeleton, or charged soluble molecules, and then more adequate mathematical expressions are necessary involving phospholipid reordering. For example, phosphatidylinositol 4,5-biphosphate (PIP2) and phosphatidylserine (PS) can be reordered in lateral domains because of the direct interactions with Ca^{2+} or basic

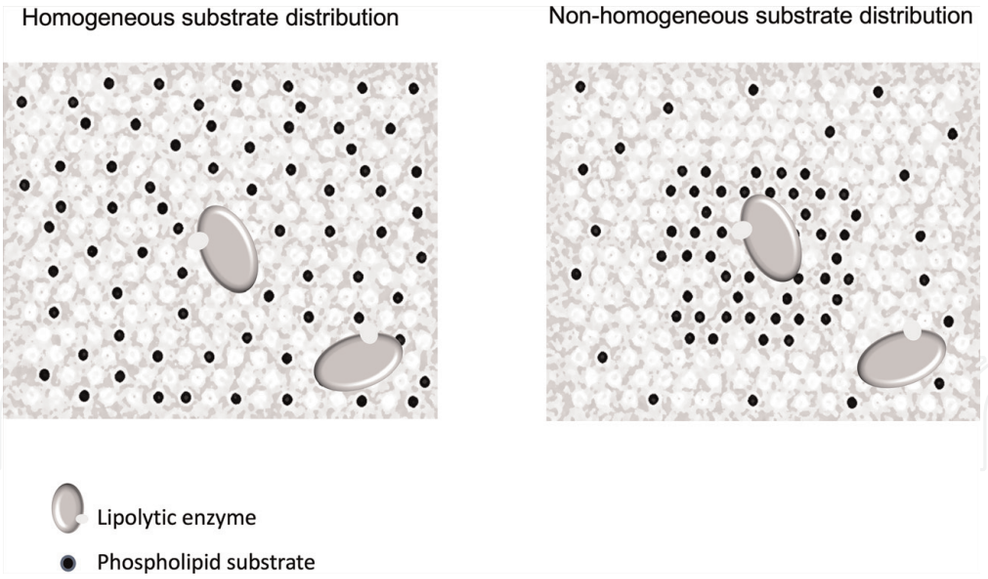

Figure 1.
A generalization of the surface dilution kinetics theory applied to lipolytic enzymes has been necessary for cases of nonhomogeneous substrate distribution. This is because the reordering of the phospholipid substrate could have important effects on the activity of lipolytic enzymes. In the figure, for the nonhomogeneous substrate distribution, two domain phases can be distinguished: enriched substrate domain and nonenriched substrate domain (named elsewhere as nondomain phase) depending on whether the domain phase corresponds to the lipid phase with the largest substrate molar fraction or not, respectively.

molecules such as the protein myristoylated alanine-rich C kinase substrate (MARCKS) or pentalysine (Lys5, one of the first five amino acid residues of the region of bovine MARCKS) [17]. In biological membranes, the microdomain structure and dynamics are widely diverse, considering the scaffolding of cell proteins [18].

In order to find the effects of lipid substrate domain formation on enzyme activities, it is necessary for an extended mathematical formulation starting for similar principles to those of the original surface dilution kinetics theory. That is because the total activity could be integrated by each one of these substrate domains, i.e., whenever there is a phospholipid substrate (e.g., **Figure 1**), and therefore, the formation of domains enriched in a phospholipid substrate could either increase (inside the enriched domain) or decrease (outside the enriched domain; i.e., inside the nonenriched domain) the total enzymatic activity on the membrane. For this reason, it is useful to propose a comprehensive quantitative model that explicitly takes into account the enzyme activity in the different phospholipid phases, which here are frequently called substrate domains or simply "domains," to distinguish them from the eventual thermodynamic phases on membranes. Below, a theoretical frame for lipid binary membrane systems is shown, and then the theoretical frame is extended to a more realistic lipid phases with any number of domains, including continuous gradient of phospholipid substrate (thereby, considering an infinite number of infinitesimal domains).

2. Changes in the lipolytic enzyme activity due to substrate reordering

In the calculations of the lipase activities in membranes, it is assumed that whatever the structure of the lipid phase (micelle, liposome, or monolayer), all the lipids in the lipid-water interface expose the same area to the aqueous phase. As a consequence, the area of the lipid phase surface is proportional to the amount of lipid molecules. Then, at the beginning of the reaction, the lipid area will be

constant regardless of any substrate reordering. However, depending on the enzymatic model, the substrate reordering effectively could change the enzyme activity, having many differences between homogenous or nonhomogeneous substrate distributions.

To understand how the substrate lateral reordering might affect the enzyme kinetics, a mathematical approach has been developed for the models having two steps binding between the enzyme and the lipid phase. First, the simple nonhomogeneous case of a binary condition is considered where the substrate can be distributed in two coexisting lateral phases: an enriched domain and a nonenriched domain (usually named nondomain). Finally, a more general expression corresponding to any gradients of substrate molar fraction will be shown.

As in the case of homogenous distribution of substrate on lipid phase [12], in the simple nonhomogeneous distribution given by a binary substrate distribution (i.e., two mixed lipid molecules, one of them being the substrate), the kinetics surface dilution theory is applied to the *surface-binding model* and to the *phospholipid-binding model* [19]:

1. For the *phospholipid-binding model* (**Figure 2A**), the enzyme activity (V) depends on the substrate reordering in according to

$$V = \frac{kC_{E_T}\left(a_1 f_{S^1}^2 + a_2 f_{S^2}^2\right)}{\frac{k_m k_s}{C_L} + k_m f + a_1 f_{S^1}^2 + a_2 f_{S^2}^2} \tag{3}$$

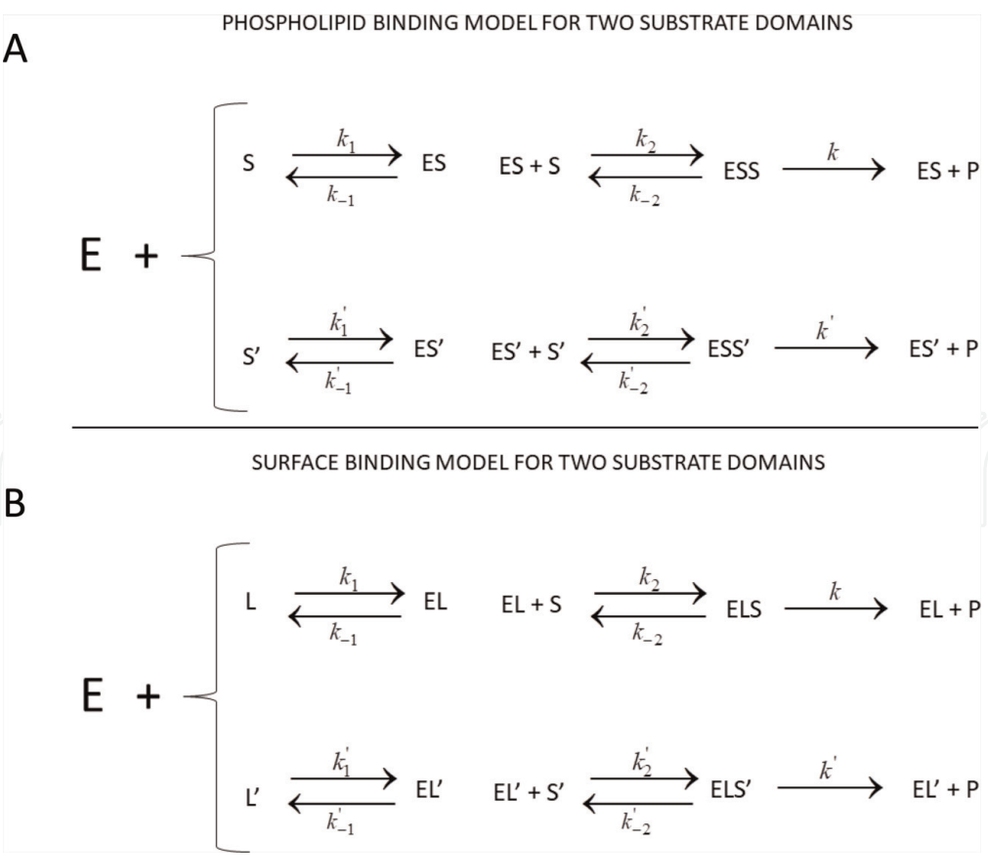

Figure 2.
A and B represent two different Lipolytic enzyme kinetic models for a lipid phase with two substrate domains: enriched substrate domain and nonenriched substrate domain. L, lipid molecule; S, phospholipid substrate; E, lipolytic enzyme; ES and ESS, enzyme-substrate complexes; and P, product. Individual kinetic constants are shown (k, $k^{'}$, k_1, $k_1^{'}$, k_{-1}, $k_{-1}^{'}$, k_2, $k_2^{'}$, k_{-2}, $k_{-2}^{'}$). In the text: $k_m \equiv \frac{k_{-2}+k}{k_2}$ and $k_s \equiv \frac{k_{-1}}{k_1}$. The association of symbols and parameters to a particular domain is indicated by mean of primed or nonprimated signs in each case.

where f_{S^i} and a_i are the substrate molar fraction and the fraction of the total lipid area in the ith phase (i = 1 or 2), respectively, and such that

$$a_1 f_{S^1} + a_2 f_{S^2} = f \tag{4}$$

and

$$a_1 + a_2 = 1 \tag{5}$$

According to Eq. (4), f is the average of the substrate molar fraction weighed by the domain areas.

In Eq. 3, we can see that V depends hyperbolically on

$$\left\langle f_{S^i}^2 \right\rangle \equiv a_1 f_{S^1}^2 + a_2 f_{S^2}^2 \tag{6}$$

the average of the square of the substrate mole fraction weighted by the phase area. These mean values have minimal and maximal values equal to f^2 and f, respectively. Therefore, there are for V minimal and maximal values ($V_{\min}$ and $V_{\max}$, respectively):

$$V_{\min} = \frac{E_T k f^2}{\frac{k_m k_s}{L} + k_m f + f^2} \tag{7}$$

We can see that $V_{\min}$ corresponds to V for a lipid homogeneous phase (Eq. 1) [12, 13]

$$V_{\max} = \frac{E_T k f}{\frac{k_m k_s}{L} + k_m f + f} \tag{8}$$

Curiously, in spite of Eq. 8 deduced for the phospholipid-binding model with any substrate distribution, this equation is equal to Eq. 2, which corresponds to the case of surface-binding model with homogeneous substrate distribution on the membrane.

According to Eq. 3, if the homogeneous distribution of the substrate on the membrane is broken (i.e., substrate reordering such that $\langle f_{S^i}^2 \rangle > f^2$), then the enzyme activity will increase. In particular, the total enzyme activity increases when the recruitment of substrate to the enriched-substrate domain (e.g., phase 1) increases, due to an increase of either the domain mol fraction (f_{S^1}) or the extension of the domain (a_1).

2. On the other hand, for the surface-binding model (**Figure 2B**), the enzymatic activity in a two-phase membrane equals to the enzyme activity in a homogeneous lipid phase, following the equation:

$$V = \frac{C_{E_T} k f}{\frac{k_m k_s}{C_L} + k_m + f} \tag{9}$$

and applying the same restrictions given by Eqs. 4 and 5.

The differences between the behaviors of both enzymatic models are much more than the differences between the corresponding equations for the enzymatic activities (Eqs. 3 and 9). Following this theory, important differences exist in both the ratio of the substrate regarding the two substrate domains and the total enzyme binding to membrane.

The ratio between the enzymes binding into the two domains (indicated as primed and nonprimed) is described as follows:

for the *phospholipid-binding model*:

$$\frac{E_S + E_{SS}}{E_S^{'} + E_{SS}^{'}} = \frac{f_d\left(k_m + f_d\right)}{f_n\left(k_m + f_n\right)} \tag{10}$$

for the *surface-binding model*:

$$\frac{E_S + E_{SS}}{E_S^{'} + E_{SS}^{'}} = \frac{k_m + f_d}{k_m + f_n} \tag{11}$$

Furthermore, there are different expressions for the molar concentration of total enzyme binding to the lipid phase, $[E_B]$:

Naming the molar concentration of total enzyme binding to the lipid phase $[E_B]$, we have:

$$\begin{aligned}[E_B] &\equiv La(E_S + E_{SS}) + L(1-a)\left(E_S^{'} + E_{SS}^{'}\right) \\ &= E_T\left(\frac{k_m f + \left\langle f_i^2\right\rangle}{\frac{k_m k_s}{L} + k_m f + \left\langle f_i^2\right\rangle}\right),\end{aligned} \tag{12}$$

for the *phospholipid-binding model*,

and

$$\begin{aligned}[E_B] &\equiv La(E_S + E_{SS}) + L(1-a)\left(E_S^{'} + E_{SS}^{'}\right) \\ &= E_T\left(\frac{k_m + f}{\frac{k_m k_s}{L} + k_m + f}\right),\end{aligned} \tag{13}$$

for the *surface-binding model*.

Then, due to the difference between Eqs. 12 and 13, the two enzymatic models could be easily distinguishable by means of the observed change in the total lipid metabolizing enzyme binding to the lipid phase under substrate reordering: *phospholipid-binding model* predicts changes in total membrane enzyme upon domain formation, unlike the *surface-binding model*, in which there are no changes in total membrane enzyme, whatever the distribution of the substrate is.

3. Redistribution effect versus competitive effect of a lipid-inducing domain peptide

Similar to the enzyme, again we will not consider the substrate dilution due to the protein insertion in the bilayer. That is because the number of molecules binding to the lipid phase is much smaller than the number of the phospholipid substrate molecules, and besides, the domain-inducing peptide either would not penetrate the lipid phase or its interface concentration can be considered negligible.

As seen before, depending on the enzymatic model, substrate redistribution such as the transitions from homogeneous distribution to nonhomogeneous

distribution could change the enzyme activity. However, another effect must be considered when there are domains that have been induced by soluble peptides (e.g., basic peptides such as pentalysine), which interact directly with the phospholipid substrate of the membrane (i.e., acidic phospholipid such as PIP2). In this case, such interaction could be enough to consider a competitive effect over the enzyme activity; i.e., in the lipid-water interface, the domain-inducing peptide would compete with the enzyme for the substrate, because there would be less free substrate to bind to the enzyme. Then, we have proposed that the superposition of both redistribution and competitive effects may explain some results in the literature that appear as contradictory. [19]

3.1 The kinetic effects of peptide induction of phospholipid domains

In order to calculate the effects on the PLC-β activity (a lipolytic enzyme) on PIP_2 (lipid substrate) due to pentalysine-induced domain formation, it has been assumed that the stoichiometry of binding is one lipid substrate per one domain-inducing peptide [19]. In case of larger stoichiometry for the phospholipid binding to the peptide (as Kim et al. describes [20]), this would imply that the competitive effect from peptides tends to decrease the enzyme activity more dramatically at low substrate molar fractions in the 1:1 stoichiometric case. Then, to estimate the amount of substrate bound to all the domain-inducing peptides in any lipid phase, it was assumed that the domain-inducing peptide near the surface of the lipid phase is in equilibrium with the phospholipid substrate, obeying a Langmuir isotherm, and this peptide concentration was determined by the electrochemical equilibrium in according with a Boltzmann-like relationship, which included the membrane potential in the lipid phase and the peptide concentration in the bulk solution [21]. Moreover, knowing the substrate binding to the domain-inducing peptide, free substrate can be calculated, and then the molar fraction of free substrate can be taken into account into the deduced previous kinetic models (Eqs. 3 and 9).

As a result, if there is competition effect due to peptide binding substrate, in case of the *surface-binding model*, the enzymatic activity will always diminish because the substrate reordering has no effect in the enzyme activity. Instead, in case of the *phospholipid-binding model* with peptide-induced breakage of substrate homogeneity the enzyme activity may either increase or decrease depending on the difference between the competitive effect (diminishing the enzyme activity) and the substrate distribution effect (increasing the enzyme activity) [19]. A theoretical estimation of PLCβ, acting on PIP2 as substrate, and having enriched substrate domain induced by pentalysine, has been shown in Figure 4 of Salinas et al. 2005 [19]. A maximum for an enriched domain, with acute declination for others, are shown.

4. Lypolitic enzyme activity in lipid phases with multiple substrate domains

The above kinetics expressions can be generalized to any amount of substrate domains, even to infinite number of domains, and this latter is very useful for modeling any kind of substrate distribution in the total lipid phase [22].

In this extended theoretical frame, we have the following:

In homogenous condition, *f* is the molar fraction of phospholipid substrate.

S reorders into n homogeneous domains, with the ith domain (i = 1, 2,..., n) with normalized area ai and molar fraction f_i of S.

The total conservation equation is given as:

$$1 = \sum_{i=1}^{n} a_i \tag{14}$$

The cross-sectional areas of any lipid molecules in any phases are equal and conserved, and then the total phospholipid S normalized area conservation equation is given as:

$$f = \sum_{i=1}^{n} a_i f_{S^i} \tag{15}$$

Thus, again, it can be demonstrated that the enzyme activity for the *surface kinetic model* does not depend on the substrate ordering, and it is equal to the enzyme activity for the completely homogenous substrate distribution case (Eq. 2). For the *phospholipid-binding model*, the enzyme activity even depends on the reordering of the substrate on the lipid phase, such that some terms in Eq. 1 must be replaced by more general ones, even more than in Eq. 3. Thus, in case of multiple domains in the *phospholipid-binding model*, it has been demonstrated theoretically that the enzymatic activity on n substrate domains is given as:

$$V = \frac{kC_{E_T}\left\langle f_{S^i}^2 \right\rangle}{\frac{k_m k_s}{C_L} + k_m f + \left\langle f_{S^i}^2 \right\rangle} \tag{16}$$

where f is defined in according to Eq. 15 and $\langle f_{S^i}^2 \rangle$ is the average of the square of the substrate molar fraction weighted by the domain areas:

$$\left\langle f_{S^i}^2 \right\rangle \equiv \sum_{i=1}^{n} a_i f_{S^i}^2 \tag{17}$$

Two abovementioned results can be represented by Eqs. 16 and 17: First, when the lipid substrate distribution is completely homogeneous (n = 1), Eq. 1 is obtained. Secondly, when there are only two domains of lipid substrate (enriched substrate domain and nonenriched substrate domain; n = 2), Eq. 3 is obtained.

To calculate any V-value, $\langle f_{S^i}^2 \rangle$ must be calculated as a summation over the whole surface of the lipid phase. Thus, minimum and maximum V-values are calculated from minimum and maximum $\langle f_{S^i}^2 \rangle$ values, respectively:

$$\left\langle f_{S^i}^2 \right\rangle_{\min} = \sum_{i=1}^{n} a_i f^2 = f^2 \sum_{i=1}^{n} a_i = f^2 \tag{18}$$

(using Eq. 14, i.e., the conservation of the total lipid area)
and

$$\left\langle f_{S^i}^2 \right\rangle_{\max} = f \tag{19}$$

Thus, the minimum V-value as a function of substrate distribution was obtained for a homogeneous distribution ($f_{S^i} = f$). The maximum V-value was obtained for one domain composed only by molecules of phospholipid substrate, and the other one without substrate (e.g., f_1 = 1 and f_2 = 0). Then, the enzymatic activities are within the following limiting values when there are multiple membrane domains:

$$\frac{kC_{E_T} f^2}{\frac{k_m k_s}{C_L} + k_m f + f^2} \le V \le \frac{kC_{E_T} f}{\frac{k_m k_s}{C_L} + k_m f + f} \tag{20}$$

5. Effects of Poisson distribution of substrate on enzyme activity following the *phospholipid binding model*

Since the extended theory shown above does not consider boundaries, the same can be applied to a population of lipid particles (like vesicles or micelles), each one as a substrate domain represented in a summation term in Eq. 17.

Frequently, data from *in vitro* kinetic studies of lipid metabolizing enzymes have been interpreted as indicating cooperative phenomena [23–25]. Alternatively, there is an explanation based on the idea that phospholipid substrate molecules are not homogeneously distributed within a population of lipid particles, although simultaneously we can suppose the substrate having homogeneous distribution within each particle. Then, modeling the nonhomogeneous substrate distribution on the population of lipid particles, it is assumed that the probability of finding a substrate molecule on a lipid particle does not depend on the number of previous substrate molecules in the same lipid particle. A consequence of this assumption is a Poisson distribution of the substrate on the mixture of the lipid particles.

Defining α as the average number of lipid molecules per lipid particle (in micellar case, this parameter is known as aggregation number), and according to Eq. 17 and Poisson-distributed substrate, it can be demonstrated that [22]

$$\left\langle f_{S^i}^2 \right\rangle = f^2\left(1+\frac{1}{f\alpha}\right) \tag{21}$$

We consider a lipolytic enzyme following the phospholipid-binding model in a system of multiple domains of substrate, and such that the lipid phase is composed by a mixture of lipid particles, each one with homogeneously distributed substrate, but Poisson distributed over the same population of particles. Then, the $\left\langle f_{S^i}^2 \right\rangle$ value in Eq. 21 is replaced into Eq. 16 obtaining

$$V = \frac{kC_{E_T}f^2\left(1+\frac{1}{f\alpha}\right)}{\frac{k_m k_s}{C_L}+k_m f+f^2\left(1+\frac{1}{f\alpha}\right)} \tag{22}$$

That is, Eq. 22 is an expression of the rates of enzyme activity on Poisson-distributed phospholipid substrates. Applying this equation to published kinetic parameters for PLC acting on PIP2 in Triton X-100 micelles (C_L = 200 μM, k_m = 0.13, and k_s = 170 μM) [23], the ratio between "the enzyme activity on micelles with Poisson-distributed substrate" (Eq. 22) and "the enzyme activity on micelles with homogeneously distributed substrate" (Eq. 1) was calculated. Assuming a range of f from 10^{-1} to 10^{-3} (as in published work [12, 13]), activity ratios between 1.0 and 6.0 ($\alpha = 200$) and between 1.1 and 11.0 ($\alpha = 100$) were obtained. We can see that without considering cooperative effects, a simple explanation for a very high departure from the homogeneous standard model may be that the increases in enzyme activity are due to Poisson distribution of the substrate.

5.1 Substrate-distribution dependence of PLA2 activity in mixed micelles

The mentioned kinetic theory applied to Poisson-distributed substrate on lipid particles has been verified with experimental results, and their obtained parameters have been compared with those of the canonical phospholipid-binding model originally developed for homogeneously distributed substrate on mixed micelles

		Phospholipid-binding model with homogeneous substrate distribution	Phospholipid-binding model with Poisson-distributed substrate
Kinetic parameters for PLA2 activity on Triton X-100/thio-PC mixed micelles	k_m =	0.0532	0.0216
	k_S =	1.9168 mM	6.2170 mM
Kinetic parameters for PLA2 activity on Triton X-100/thio-PE mixed micelles	k_m =	0.1379	0.0942
	k_S =	0.1132 mM	0.4107 mM

Parameter values are taken from Table II in Salinas et al. 2011 [14].

Table 1.
Values for fitting kinetic parameters for PLA2 activity on Triton X-100 mixed micelles regarding either homogeneous substrate distribution or Poisson substrate distribution, with adjustable ε_{T_t} parameter (the molar concentration of detergent that is not kinetically active, a proposed parameter that enhance the fitting).

[12, 13]. Both models ("nonhomogeneous model" and "homogeneous model," respectively) can be represented simultaneously by the following general equation:

$$V = \frac{kC_{E_T} f^2}{\frac{\left(k_m/F_{f,\ \alpha}\right)k_s}{C_L} + \left(k_m/F_{f,\alpha}\right)f + f^2} \tag{23}$$

where

$$F_{f,\alpha} = 1, \tag{24}$$

in a homogeneous model (Eq. 1), and

$$F_{f,\alpha} = 1 + \frac{1}{\alpha f}, \tag{25}$$

in a nonhomogeneous model (Eq. 22).

In micelles, it has been found that α, the average number of lipid molecules per mixed micelle (i.e., the aggregation number), depends on the molar fraction of phospholipid (but not on total detergent concentration [26]) within the concentration range of Triton X-100 and phospholipid used in Hendrickson et al.'s study [13]. Therefore, in order to find the parameters for modeling, the functional dependence of α from f must be taken into account in micellar experiments [22].

In **Table 1**, all the values of parameters k_m and k_S obtained for homogeneous and nonhomogeneous substrate distributions are compared. The differences indicate that the values of these kinetic parameters can depend critically on the distribution of the substrate.

6. Discussion

To understand the effect of lipid substrate reordering on their metabolizing enzymes, theoretical results are shown. A simple kinetic model considers a nonhomogeneous membrane with the lipid substrate reordered in two domains with different molar fractions. The results are included in a more general extended theory considering substrate multidomains on either lipid surface of vesicles. Because the calculations do not regard any domain boundaries, the same models obtained from this theory (Eqs. 2 and 16) can be applied on a mixture of lipid particles (vesicles or micelles).

Here, only two kinetic models have been considered, but similar theoretical framework could be applied on other ones. The general mathematical expression for the surface-binding model does not depend on whether the substrate distribution is homogeneous or nonhomogeneous (Eq. 2). Thus, in this kinetic model, any substrate distribution changing has no kinetic effect.

On the contrary, considering the phospholipid-binding model, the calculations predict how the substrate distribution may affect the activity of the lipid metabolizing enzyme. In particular, the enzyme activity is increased by the transition from the homogeneous substrate distribution to any nonhomogeneous one (Eqs. 16 and 17).

Concordantly, in erythrocytes, the Ca^{2+}-induced domains increase the activity of PLA2 [27], an enzyme that follows the phospholipid-binding model [12, 13]. The increased activity agrees with an observed enzyme reordering, which may be due to formed enriched-substrate domains. Then, there will be more enzyme molecules binding to areas of higher substrate molar fraction, causing a larger local enzyme activity. On the other hand, PLCβ kinetic data from micellar experiments have fitted to the *phospholipid-binding model* using Hill coefficients [28–30], but the usage of this type of coefficients was not useful in monolayers having with large increases in enzyme activity after small increases in the PIP2 fraction [31]. The analysis of pressure versus area isotherm of the monolayers suggested a nonhomogeneous distribution of the lipids and was proposed that the PIP2 molecules get together into enriched lateral domains, favoring the PLCβ activity, an enzyme following the *phospholipid-binding model*. This agrees with what is expected from the theory presented here.

In other cases, since basic molecules can induce formation of acidic phospholipid domains in membranes, the increased activity of PLC δ1 and PLC δ3 by addition of polyamines or basic proteins such as spermine, protamine, histone, and melittin [32] also can be explained by substrate redistribution. Differently, PLCβ activity decreases in experiments with vesicles containing acidic phospholipid domains induced by the basic molecules, pentalysine, spermine, and MARCKS (151–175) [17]. However, assuming that PLCβ is a *phospholipid-binding* enzyme, the decrease in enzyme activity may be due to a high competitive effect of the substrate-domain-inducing peptide. Such competitive effect overcomes the rise in activity that substrate redistribution would produce. Finally, the importance of each effect is dependent on the used amount of domain-inducing molecule and this could explain the apparent contradictory results of the activities of lipid-metabolizing enzymes, such as PLC (an enzyme following the phospholipid-binding model [24, 29, 30] upon addition of domain-inducing molecules [17, 23, 32, 33]).

Substrate distribution also must be considered in *in vitro* kinetic experiments of enzymes following the phospholipid-binding model. In this sense, the application of the theory developed here is useful for kinetic experiments with mixed lipid particles (i.e., liposomes or micelles, instead of lipid domains). We assumed that each one of the particles will have a homogeneous molar fraction, which follows a Poisson distribution on the lipid particles [34, 35].

If the average of substrate molecules per lipid particle in suspension ($f\alpha$) is very large ($f\alpha \gg \mathbf{1}$), then Eq. 22 predicts that the enzymatic activity tends to the value obtained for a homogeneously distributed substrate (Eq. 1, or its equivalent, Eqs. 23 and 24). However, in case of decreased average of substrate molecules per lipid particle in suspension ($f\alpha \ll 1$), Eq. 22 predicts that the enzyme activity will be larger than in the homogeneous case at equal f value. Concordantly, some PLC isoenzymes [24, 29, 30] and PLA2 [13] have an increased activity in cases of small substrate molar fractions, similar to cooperative phenomena. However, if these kinetic data could be fitted to Eq. 22, they will contribute to a more simple

explanation based on the substrate distribution. In other case, in experiments with PLA2, increase in enzyme activity has been associated with decrease of the size of lipid vesicles, suggesting that PLA2 activity happens in areas with structural defects [36]. Again, here our approach based on substrate distribution provides a simple alternative explanation: if the substrate molar fraction in the mixture of lipid particles is Poisson distributed, then Eq. 22 could be applied. Considering that substrate is fixed (f fixed), the decreasing vesicle sizes (α decrease) produces decreasing average of substrate molecules per vesicle ($f\alpha$). Therefore, in according to Eq. 22, at low values of $f\alpha$ and for Poisson-distributed substrate, the relative enzyme activity must increase more notoriously, regarding the case of homogeneous distribution of substrate as reference.

The application of the theoretical model (Eq. 22) in published results of the PLA2 activity on Triton X-100 mixed micelles of phospholipids [13], considering Poisson-distribution substrate, allows a very good fit to the data. Interestingly, the estimated values of the kinetic parameters strongly depend on whether the substrate distribution used in the fitting is distributed either homogeneously or according to Poisson.

On the other hand, detergent-based micelles are not capable to mimic the lipid environment of membranes. In such case, the activities of most membrane protein could be affected. As a solution, liposomes or high-density apolipoprotein particles have been proposed [2]. However, compared with those experimental models, the EVs offer a number of potential benefits, such as providing a more adequate membrane environment for membrane proteins, in terms of both dynamics and stability.

In summary, depending on the enzyme model, the lipid substrate reordering can regulate the enzyme activity, giving to the membrane organization a topological role in the control of cell process. In order to a good estimation of kinetic parameters in phospholipase enzymology, *in vitro* kinetic experiments must consider the substrate distribution effects. Also, many complex metabolic effects of substrate-domain-inducing molecules can be explained by a result of the balance between the competitive effects of the substrate domain inducers and substrate redistribution. All these considerations should be taken into account even in case of EVs, in relation to their formation, functionality, and action on membrane targets.

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