MOLECULAR ASPECTS OF

PHOSPHOLIPASE A_2 ACTIVATION

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INTRODUCTION

A striking characteristic of soluble phospholipases A_2 is their tendency to become activated at a lipid–water interface.[1] The activity of these enzymes is much greater with aggregated phospholipid substrates than with monomeric substrates. Furthermore, the activity is heavily influenced by the state of the aggregated lipids. Our interests in phospholipase A_2 are focussed on the mechanism of the activation process and the physical basis of the role of lipid structure and/or dynamics in that process.

Early studies is our laboratory involved the various interactions of porcine pancreatic phospholipase A_2 and either small sonicated unilamellar vesicles of dipalmitoylphosphatidylcholine (DPPC) or large fused unilamellar vesicles (LUV) of DPPC.[2,3] It was found that rates of hydrolysis were maximum at initial time with small unilamellar vesicles at temperatures well below the thermotropic transition to the liquid crystalline phase of the phospholipid vesicles (T_m = ~37°C). At temperatures above about 36°C, lag phases prior to rapid hydrolysis are observed, and these lag times become longer at temperatures in the liquid crystalline phase.[2] Somewhat different results were obtained with DPPC LUV. First, lag periods in the hydrolysis time courses were seen at all temperatures. Second, the length of these lag phases is inversely proportional to temperature below T_m, minimal at T_m (~41.5°in DPPC LUV) and increases as a function of temperature in the liquid crystalline phase.[3] Analysis of temperature, calcium, and substrate and enzyme concentration dependence data from those two studies[2,3] led to three conclusions: 1) enzyme activation and substrate hydrolysis depend on the structure of the vesicles; 2) binding of the enzyme to the vesicles is stonger in the gel state and does not require calcium; 3) activation involves aggregation of the enzyme on the surface of the vesicles.

Subsequent investigations focussed on a quantitative analysis of time courses describing pancreatic phospholipase A_2 hydrolysis of DPPC LUV at 38°C. Under such conditions, the initial 10–20% of the time course was directly proportional to At^2 and could be analyzed in terms of a simple activation model.[4] Analysis of the second-order coefficient (A) demonstrated that the rate of activation was proportional to the square of the enzyme concentration and that the rate of activation was an inverse function of the vesicle concentration. Implicit in the analysis was the assumption that activation

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was irreversible within the time frame of the experiment. The conclusion of this work was that the mechanism of activation of phospholipase A_2 involved the formation of enzyme dimers on the surface of the vesicles.[4] Recently, it has been reported that the pancreatic and other soluble phospholipases A_2 become acylated during phospholipid hydrolysis, that the acylated enzyme is a stable dimer and that it is more active than the virgin monomer.[5,6] This acylation reaction, then, could be the basis of the irreversible dimer activation mechanism derived from the kinetic data.

Any simple activation model in which the rate of enzyme activation is constant with time will yield a time course in which the product formed is a second-order function of time. In all other cases, the hydrolysis time course will be a more complex function of time. The complete time courses of phospholipase A_2 hydrolysis of LUV all exhibit such complex behavior, even in cases where simple behavior was observed up to 20% hydrolysis. The deviation from At^2 behavior generally indicates that a second process which increases the rate of activation occurs as hydrolysis proceeds. This second process is most apparent at temperatures above the lipid phase transition temperature for the pancreatic enzyme and at all temperatures for the monomeric aspartate-49 phospholipase A_2 from Agkistrodon piscivorus piscivorus (AppD49).[7]

Unlike the pancreatic enzyme, AppD49 exhibits a large fluorescence change upon interaction with DPPC. Thus, time-dependent changes in the enzymatic activity, the intrinsic enzyme fluorescence and changes in the physical properties of the lipid bilayer can be measured simultaneously in this system.[7] Correlation of these observables has allowed us to begin an examination of the temporal sequence of events in phospholipase A_2 activation and address various models proposed to describe the mechanism of that activation. This paper constitutes a first report of our analysis of experimental hydrolysis data using computer simulations of selected models.

TEMPORAL SEQUENCE

The time course of AppD49-catalyzed hydrolysis of DPPC LUV is complex both as a function of time and temperature.[7] At low temperatures (below 30°C) initial hydrolysis is very slow and is almost undetectable at 25°C. In contrast, hydrolysis is readily detectable at temperatures in the vicinity of the main thermotropic phase transition of the lipid (41.3°C for DPPC LUV). The time courses of DPPC LUV hydrolysis by AppD49 phospholipase A_2 at 39, 41 and 45°C are shown in Fig. 1. Hydrolysis initially proceeds at a relatively slow rate. After a lag time of several hundred seconds, the activity suddenly increases by two to three orders of magnitude within 5 to 10 s. The length of the lag period is dependent on temperature, enzyme concentration and substrate concentration. The lag period is a minimum at approximately the phase transition temperature and becomes dramatically longer as temperature is further increased. The length of this initial phase of the time course increases with increasing substrate concentration and is inversely proportional to enzyme concentration. These observations are consistent with the observations that led to proposal of the dimer activation model,[4] but the complexity of the time courses (Fig. 1) preclude analysis of the data with that model in its simplest form.

Phospholipase A_2 Fluorescence

The interaction of many phospholipases A_2 with lipid substrate can be detected by changes in the intrinsic tryptophan fluorescence of the protein upon mixing with the lipid substrate.[8-10] Generally, the fluorescence intensity increases and the maximum of the emission spectrum shifts some 5 to 10 nm toward lower wavelength. Such spectral changes have been traditionally interpreted as indicating a change in the tryptophan environment to lower polarity.

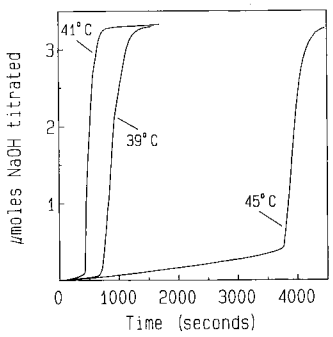


Fig. 1. Time courses of hydrolysis of DPPC LUV by AppD49 phospholipase A_2. Reactions were initiated by the addition of enzyme (final concentration = 310 nM) to 2.5-ml samples containing 35 mM KCl, 10 mM $CaCl_2$ and 1.5 mM DPPC LUV at 39, 41 or 45°C and monitored at pH 8.0 with a pH stat mounted in the sample chamber of an SLM 8000C spectrofluorometer. Used by permission from Ref. 7.

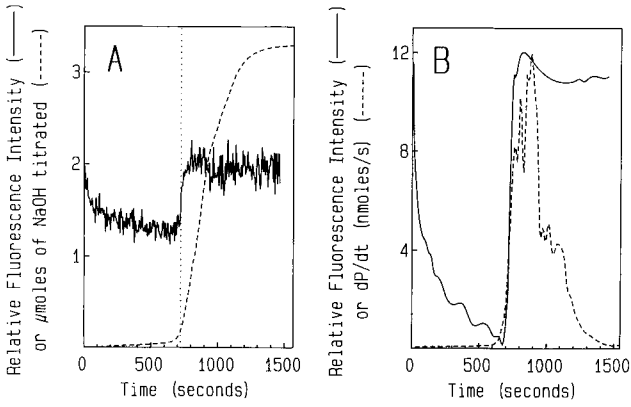


Fig. 2. Correlation of AppD49 fluorescence with the time courses of vesicle hydrolysis (dashed line) and enzyme fluorescence (excitation = 280 nm, emission = 340 nm, solid line) from the 39°C experiment described in Fig. 1. Panel B, the derivative of the hydrolysis time course (dP/dt) was calculated from the data in A. The enzyme fluorescence was rescaled and smoothed to demonstrate more clearly the temporal correlation of the fluorescence change with dP/dt. The decrease in enzyme fluorescence during the first 500 s is independent of the presence of vesicles and is due to binding of some of the enzyme to the walls of the cuvette. Used by permission from Ref. 7.

Upon mixing with DPPC small unilamellar vesicles, the AppD49 phospholipase A_2 fluorescence increases 70%, and the emission spectrum shifts 6 nm to lower wavelength. This fluorescence change does not require calcium but is sensitive to temperature. In the absence of vesicle hydrolysis, it only occurs in the gel state of the lipid.[10] These results substantiate the conclusion derived from studies with the pancreatic phospholipase that binding does not require calcium but is stronger to the gel state of the lipid.[3]

The addition of AppD49 phospholipase A_2 to DPPC LUV results in no measurable fluorescence change at time zero. However, after an initial latency, the fluorescence intensity suddenly increases some 70%.[7] This increase in intensity is accompanied by a shift in the maximum of the emission spectrum from 348 nm to about 340 nm. By combining the pH stat instrument used to measure the hydrolysis reaction with the fluorometer cell,[7] we are able to temporally correlate the fluorescence and hydrolysis time courses. As shown in Fig. 2, the abrupt increase in enzyme fluorescence intensity superimposes on the derivative of the hydrolysis time course (Fig. 2B). This result has been found to be reproducible at all temperatures and concentrations of enzyme or substrate and strongly suggests that enhanced fluorescence intensity is directly correlated to formation of the active state of the enzyme–lipid complex.

Membrane Structure

Two observations are of substantial importance in an effort to understand the mechanisms of these unusual time courses. First, the onset of rapid hydrolysis can be an extremely abrupt phenomenon. Second, the mole fraction of hydrolysis products formed at the time of the abrupt increase in activity is constant at a given temperature regardless of enzyme or substrate concentration (.071 $\pm$ standard deviation of .016 at 39°C).[7] These observations suggest that a cooperative change in membrane structure occurs within a specific concentration range of the reaction products and that this change promotes formation of the active enzyme–lipid complex. We have employed two spectroscopic observations that can be monitored simultaneously with the hydrolysis or fluorescence time courses looking for evidence of such a phenomenon. The first is the intensity of the light scattered by the vesicles and the second is the fluorescence of the membrane probe trimethylammonium diphenylhexatriene (TMA–DPH).

Changes in light scattering are indicative of changes in vesicle size which could be related to the abrupt increase in activity. As shown in curve b of Fig. 3, a sudden change in the intensity of scattered light at 290 nm occurs near the onset of the enzyme fluorescence increase (curve c) and the onset of rapid hydrolysis of the substrate (dotted line in Fig. 3). The light scattering first decreases and then increases as a function of time. The ultimate increase in both the intensity and noise of the light scattering is due to the formation of a precipitate of the calcium and palmitic acid released in the late phase of vesicle hydrolysis. At low calcium concentrations ($\leq$100 μM), no visible precipitate is formed, and the increases in intensity and noise of the light scattering are not observed. The magnitude of the initial decrease in light scattering is less than or equal to that produced by sonication of the DPPC LUV to form small unilamellar vesicle (corresponding to a reduction in vesicle diameter from 900 to 200 Å). The most important aspect of the light scattering change is that it consistently occurs after the onset of the fluorescence change and rapid hydrolysis. In over 50 experiments, the onset of increased protein fluorescence has occurred from 0–60 s prior to the onset of the light scattering change depending on the experimental conditions. We have never found any evidence that the light scattering change occurs prior to the enzyme fluorescence increase. Therefore, changes in the vesicles during the hydrolysis time course which are reflected by the change in light scattering are not responsible for the sudden increase in AppD49 phospholipase A_2 fluorescence or hydrolytic activity.

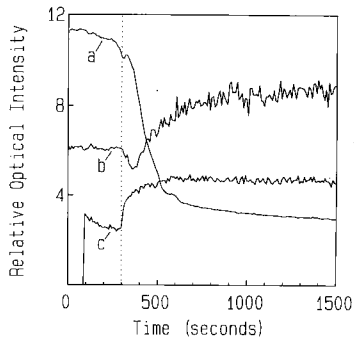


Fig. 3. Correlation of time courses of AppD49 fluorescence, vesicle light scattering and TMA-DPH fluorescence during the hydrolysis of DPPC LUV. LUV (0.4 mM DPPC) were equilibrated with 0.4 µM TMA-DPH in 35 mM KCl, 10 mM $CaCl_2$ and 10 mM sodium borate at pH 8. TMA-DPH fluorescence (excitation = 360 nm, emission = 430 nm, curve a), light scattering at 290 nm (curve b) and enzyme fluorescence (curve c) were simultaneously recorded as described in Figs. 1 and 2. The enzyme (140 nM final) was added to the sample at 90 s into the time course. For explanation of the initial time dependence of the enzyme fluorescence, see legend to Fig. 2. Used by permission from Ref. 7.

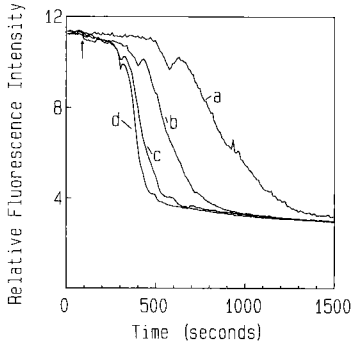


Fig. 4. Enzyme concentration dependence of TMA-DPH fluorescence during hydrolysis of DPPC LUV by AppD49 phospholipase A_2. LUV (0.4 mM DPPC) equilibrated with TMA-DPH were mixed with 36 nM (curve a), 71 nM (curve b), 140 nM (curve c) or 570 nM (curve d) phospholipase A_2 at 39°C, and the TMA-DPH fluorescence was monitored as described in Fig. 3. The enzyme was added to the sample at 90 s as indicated by the arrow. Used by permission from Ref. 7.

The fluorescence of TMA-DPH is sensitive to changes in membrane structure such as those that occur at the thermotropic phase transition.[11] Fig. 3 (curve a) shows a typical time course of TMA-DPH fluorescence during hydrolysis of DPPC LUV. During the initial slow phase of hydrolysis, the TMA-DPH fluorescence gradually decreases as a function of time. Just prior to the increase in AppD49 phospholipase A_2 fluorescence, the TMA-DPH fluorescence increases slightly and then rapidly decreases. The small temporary increase in TMA-DPH fluorescence is a highly reproducible phenomenon and is most apparent at low enzyme concentrations when the hydrolysis time course is expanded in time (Fig. 4). In all experiments in which TMA-DPH and phospholipase A_2 fluorescence have been monitored concurrently, the time of onset of the sudden increase in intensity for both fluorophors has been identical. The possibility that the coincidence is due to energy transfer from tryptophan to TMA-DPH or other optical artifacts has been ruled out. We thus conclude that the TMA-DPH data indicates a change in the internal bilayer structure that is presumably coupled to the concentration of reaction products in the bilayer and promotes the rapid activation of the phospholipase A_2. Jain et al. have recently reported that the autoquenching of the fluorescent probe NK-529 increases during hydrolysis of vesicles of dimyristoyl-phosphatidylcholine.[12] Their study also suggested a temporal coincidence between the fluorescence changes of this probe and a rapid increase in hydrolytic activity of the porcine pancreatic phospholipase A_2.

The putative lipid structural change induced by reaction products is probably not an isolated phenomenon produced only by palmitic acid and lysophospholipid. Rather, we think that membrane changes that promote enzyme activation can be induced by other perturbations. Several observations are consistent with such a notion. First, it has been found with vesicles of egg phosphatidylcholine mixed with cholate that the rate of activation of porcine pancreatic phospholipase A_2 is directly proportional to the mole fraction of cholate in the vesicle.[13] This dependence is smooth until about 0.22 mole fraction where a further increase of .02 to .04 results in a 15 to 30-fold increase in the rate of enzyme activation. Second, osmotic shock of DPPC LUV causes apparently instantaneous activation of pancreatic phospholipase A_2 under conditions where lag periods would normally be observed.[3,4] Third, Cunningham and co-workers have reported that critical concentrations of diacylglycerol increase pancreatic phospholipase A_2 activity several-fold.[14] An additional interesting observation that may also be related is that a threshold electric field can induce large increases in phopholipase A_2 activity toward monolayer substrate.[15]

MODELS FOR REACTION PRODUCT COUPLING

Important questions are how might a cooperative lipid structural change be induced by products and how might such a process be coupled to activation of the enzyme. Two-component systems in which each molecule occupies a site of a two dimensional lattice are topologically described in terms of the distribution of the two different molecules among the sites. If the two components demonstrate no preferential association (i.e. nearest neighbors), then the distribution will be random. These distributions will include small clusters of connected molecules of the minor component until a critical value of the mole fraction, called the percolation point, is reached. At that point, all molecules become essentially connected in a single, very large cluster, analogous to phase-separation. The percolation point for a random system is precisely defined by the coordination geometry (i.e. the number of nearest neighbors) of the lattice. For a hexagonal lattice this is when the mole fraction of the minor component equals 0.5.[16]

The details of percolation become more complex when preferential interaction exists between molecules. If the interactions between the components are very strong, complexes may be formed and percolation can be

acheived at concentrations well below the "random" percolation point. Such appears to be the case for cholesterol-DPPC vesicles where 2:1 lipid-cholesterol complexes form and the percolation point is deduced to be about 0.2 from Monte Carlo calculations and calorimetric data.[17,18] In the case of two immisible lipids, the components will always be "phase-separated" and the minor component will always exist in a single distinct cluster. Thus, in an hexagonal lattice, the sudden change from a large number of small clusters to a small number of large clusters can occur at any mole fraction of the minor component, dependent only on the magnitude of its nearest neighbor interactions.

It is this clustering phenomenon that may be coupled to activation of the phospholipase A_2. Three current pieces of evidence substantiate this hypothesis: 1) Calorimetric and fluorescence polarization experiments indicate that the critical mole fraction for the compositional transition from mixtures of phosphatidylcholine and diacylglycerol to formation of "specific complexes or preferred packing assays" occurs at 0.25 mole fraction diacylglycerol.[14] The activity of pancreatic phospholipase A_2 increases several-fold abruptly as the mole fraction of diacylglycerol is raised from 0.20 to 0.25 mole fraction. 2) The apparent rate of activation of porcine pancreatic phospholipase A_2 increases up to 30-fold between 0.22 and 0.26 mole fraction cholate in egg phosphatidylcholine vesicles.[13] The structural similarity between cholate and cholesterol suggests that the two may have similar percolation points in mixtures with phosphatidylcholine. As stated above, the percolation point for cholesterol is estimated to be 0.20.[17,18] 3) The fluorescent probe NK-529 has been found to detect apparent sudden clustering of reaction products at the time of the burst in activity of pancreatic phospholipase A_2 during the time course of phosphatidylcholine vesicle hydrolysis.[12]

The structural change induced by reaction products during vesicle hydrolysis can hypothetically be described by a two-state model. At very low concentrations of reaction products, the bilayer exists in state A. At high concentrations, it exists in state B. The critical mole fraction of reaction products is defined as the concentration at which the ratio of A to B equals 1.0. The observed activation of the phospholipase A_2 could be coupled to this compositional transition in at least two ways. The first is that either the equilibrium amount of active enzyme or the rate of activation is directly proportional to the amount of lipid in state B. One can mathematically describe the proportion of lipid in state B with the following relationship:

$$f_B = \frac{1}{e^{n(X_c - X(t))} + 1} \qquad (1)$$

where f_B is the fraction of lipid in state B and the exponential term is the statistical weight of lipid in state A. X_c is the critical mole fraction of reaction products and $X_{(t)}$ is the mole fraction existing in the bilayer as a function of time. The coefficient n is the cooperative unit size or the number of molecules participating as a cluster in the transition. At small values for n, the transition from state A to B will occur gradually as the concentration of reaction products increases. Large values produce a sharp transition. To simulate a given model for the mechanism of phospholipase A_2 activation and its coupling to the compositional transition, one would simply set the appropriate model parameter (i.e. rate constant or equilibrium constant) proportional to f_B.

A second possibility for the coupling of enzyme activation to the compositional transition is that activation depends not on the presence of state A or B of the lipid; but rather, it depends on dynamic changes or fluctuations in structure that occur during the transition from state A to B. If this possibility were true, activation would be a maximum at the

critical mole fraction of reaction products and would decrease at higher or lower concentrations of reaction products. This suggestion is analogous to a previous proposal that the effect of the thermotropic phase transition to enhance phospholipase A_2 activation is coupled to fluctuations of clusters of gel or liquid crystalline lipid at that temperature.[3] This type of model for the effect of reaction product accumulation on phospholipase A_2 activation assumes that it is the rate of enzyme activation that is coupled to the compositional transition and that the activation is essentially irreversible for the duration of the time course. Formulation of this coupling to the dynamics of the compositional transition is analogous to the formulation described above for coupling to the fraction of lipid in state B except that the rate of activation is proportional to $f_T = f_B(1-f_B)$ where f_T is the magnitude of the structural fluctuations as a function of the mole fraction of reaction product.

MODELS FOR PHOSPHOLIPASE A_2 ACTIVATION

As stated in the introduction, the simple time dependence of the model for activation that led to the conclusion that activation of the pancreatic phospholipase A_2 involves dimerization is not adequate to describe the complex time courses shown in Fig. 1. Furthermore, the possible effect of reaction products on the activation process were not explicitly addressed by the model.[4] However, the inclusion of a product-dependent activation step could be readily incorporated as will be described. Other models which will be considered are based on a variety of experimental evidence and suggestions in the literature including activation being the result of enhanced binding of the enzyme to the bilayer surface,[19] penetration of the enzyme into the bilayer,[1] increased catalytic efficiency due to reorientation of the phospholipid molecules[20] and conformational changes of the enzyme.[21,22] In simple terms, three general models will be used to describe these various hypotheses: 1) binding activation, 2) equilibrium activation, and 3) kinetic activation.

Theory

For the purpose of this discussion, we will defer several of the proposed details of specific activation mechanisms such as whether the activation step is an enzyme conformation change, a substrate conformation change or enzyme dimerization. Some of these details can be incorporated into the various models but are beyond the scope of this manuscript.

The binding activation model assumes that increased activity is solely the result of the products increasing the amount of enzyme bound to the substrate surface and is described by the following equilibrium:

$$E + S \underset{}{\overset{K_B}{\rightleftharpoons}} \overset{S_m}{+} E_B$$
$$\Big\downarrow\Big\uparrow K_m$$
$$E_B \cdot S_m$$
$$\Big\downarrow\ k_{cat}$$
$$E_B + P_n$$

where E and S are the enzyme and lipid, S_m is the lipid mole fraction in the bilayer and P_n are the hydrolysis products (H^+, fatty acid, lysophospholipid). K_B is the apparent association constant of the enzyme to the surface of the membranes, K_m is the Michaelis constant for the bound enzyme interacting with phospholipid monomers in the bilayer and k_{cat} is the catalytic rate constant or turnover number. K_m is a thermodynamic quantity relating to the

conditional probability that a substrate molecule is bound to the enzyme's active site given that the enzyme is bound to the vesicle surface. The increase in activity occurs because the enzyme binds better to state B of the lipid. This is reflected in the value of K_B which is related to f_B in the following way

$$K_B = K_{B0} + f_B(K_{B1} - K_{B0}) \tag{2}$$

where K_{B0} is the initial value of K_B and K_{B1} is the maximum value. The rate of hydrolysis is

$$\frac{dP}{dt} = \frac{k_{cat}E_B S_m}{S_m + K_m} \tag{3}$$

where

$$E_B = \frac{E_T K_B S}{1 + K_B S} \tag{4}$$

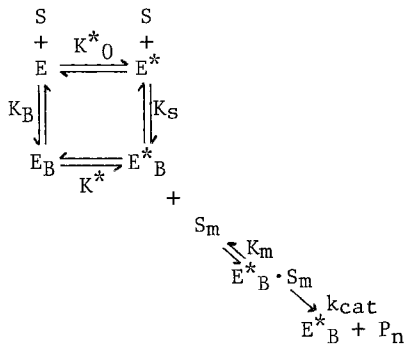
with

$$S = \frac{\sqrt{b^2 + 4K_B S_T} - b}{2K_B} \tag{5a}$$

$$b = 1 + K_B(NE_T - S_T) \tag{5b}$$

E_T and S_T are the total concentrations of enzyme and phospholipid and N is the number of phospholipid molecules that define the surface binding site of one enzyme molecule. Thus, it is assumed that the first enzyme to bind to the vesicles has an equal probability of binding to a number of sites equal to the total phospholipid concentration. Each enzyme that binds reduces the probability of the next enzyme binding by removing N binding sites. Note that S_m and K_m are both expressed in mole fraction units since they represent phospholipid concentrations within the bilayer. The depletion of substrate by hydrolysis is included by setting S_m equal to $1-X_{(t)}$ where $X_{(t)} = P_{(t)}/S_T$. The possibility of product inhibition[23] has not been incorporated into these models.

The equilibrium activation model is one step more complex than the binding activation. It states that the enzyme exists as an equilibrium between active and inactive species both in solution and on the vesicle surface and that the equilibrium on the membrane surface is altered by the presence of products as described by the following scheme.



The alteration of the position of the equilibrium is described explicitly by assuming that the apparent equilibrium constant K^* equals K^*_0 when the lipid is in state A and K^*_1 when the lipid is in state B.

$$K^* = K^*_0 + f_B(K^*_1 - K^*_0) \tag{6}$$

The equations describing the hydrolysis reaction for this equilibrium model, then, are:

$$\frac{dP}{dt} = \frac{k_{cat}E^*_B S_m}{S_m + K_m} \tag{7}$$

$$E^*_B = \frac{E_T K^* K_B S}{1 + K^*_0 + K_B S + K^* K_B S} \tag{8}$$

where

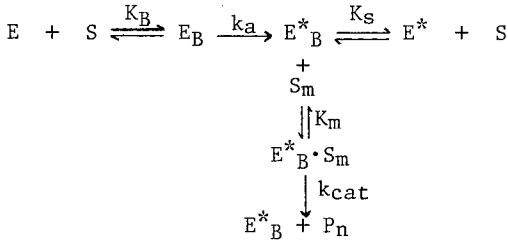
$$S = \frac{\sqrt{b^2 + 4ac} - b}{2a} \tag{9a}$$

$$a = K_b(1 + K^*) \tag{9b}$$

$$b = 1 + K^*_0 + K_B(1 + K^*)(NE_T - S_T) \tag{9c}$$

$$c = S_T(1 + K^*_0) \tag{9d}$$

The third model to be discussed is a kinetic activation model.



In this model, it is the rate of activation, k_a, that is enhanced by reaction product accumulation. The activation step on the membrane surface has been written as irreversible for simplicity, but it could also be written as a slowly reversible reaction. The most important feature of this model is that $E \rightarrow E^*$ is a reaction that does not occur in the absence of phospholipid. Conformation changes of the protein–lipid complex such as penetration into the bilayer[1] or covalent modification of the enzyme[5,6] are examples of such a phenomenon. This model requires two differential equations:

$$\frac{dP}{dt} = \frac{k_{cat}E^*_B S_m}{S_m + K_m} \tag{7}$$

where

$$E^*_B = \frac{E^*_T K_S S}{1 + K_S S} \tag{10}$$

and

$$\frac{dE^*_T}{dt} = k_a E_B \tag{11}$$

We assign two rates of activation k_{a0} and k_{a1} depending on whether the lipid exists in state A or B as defined by equation 1. Thus,

$$k_a = k_{a0} + f_B(k_{a1} - k_{a0}) \tag{12}$$

where k_{a0} and k_{a1} are the initial and maximum values of k_a. The other relationships necessary to integrate equations 7 and 11 for this model are:

$$E_B = \frac{(E_T - E^*_T)K_B S}{1 + K_B S} \tag{13}$$

and equations 5a and 5b from the binding activation model using a constant value for K_B. The calculation of S using equations 5a and 5b for this model does ignore the fact that E^*_B will become very large after the latency period and the value of S will therefore be overestimated by equations 5a and 5b. However, this only produces inaccuracies in the calculations at low substrate concentrations ($S < K_B^{-1}$) after the lag phase in the time course. Since most of our analysis of these time courses involves the early phase (see below), this is not a problem.

Simulations

Numerical integration of the differential equations describing the three models yields theoretical time courses of phospholipid hydrolysis (Fig. 5) reminiscent of the experimental data shown in Fig. 1. These simulated results suggest that the models are distinguishable by the shapes of the time course. However, this requires extremely precise and reproducible data which are difficult to obtain. For the time being, we have adopted the strategy of finding as many measurable parameters of the time courses as possible and then comparing relative changes in the values of these parameters as a function of experimental perturbations explicitly contained within the models.

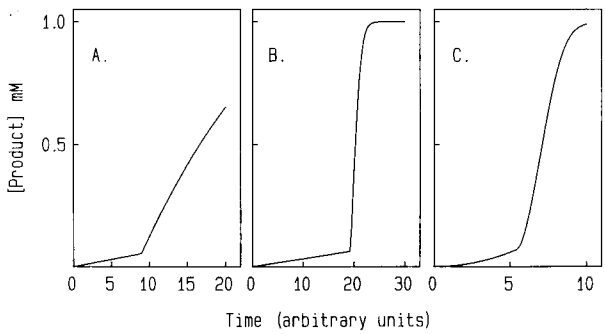


Fig. 5. Time courses of DPPC LUV hydrolysis simulated by the A, binding activation; B, equilibrium activation; or C, kinetic activation model. The time courses were calculated by numerical integration of the differential equations described in the text pertaining to each model using the Runge-Kutta fourth order algorithm. The integration interval was 0.01 arbitrary time units. Parameter values: $E_T = 10^{-7}$ M, $S_T = 10^{-3}$ M, $K_m = 0.5$, $n = 10^3$, $X_c = 0.07$, $N = 50$; Panel A: $k_{cat} = 10^3$ units time^{-1}, $K_{B0} = 100$ M^{-1}, $K_{B1} = 10^9$ M^{-1}; Panel B: $k_{cat} = 10^4$ units time^{-1}, $K_B = 10^3$ M^{-1}, $K^*_0 = 0.01$, $K^*_1 = 10$; Panel C: $k_{cat} = 10^4$ units time^{-1}, $K_B = 3 \times 10^3$ M^{-1}, $K_S = 3 \times 10^4$ M^{-1}, $k_{a0} = 0.01$, $k_{a1} = 1.0$.

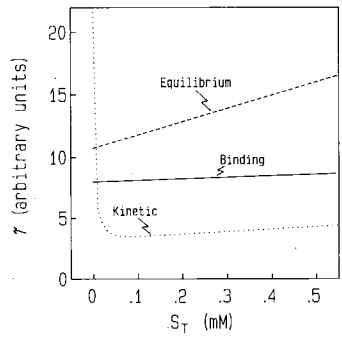


Fig. 6. Simulated dependence of τ on S_T for each of the three models. Time courses were simulated as in Fig. 5 using the same parameters at the values of S_T indicated. τ was calculated as the time at which $P/S_T = X_C$. Solid curve: binding activation model, dashed curve: equilibrium activation model, dotted curve: kinetic activation model.

We have initiated such a protocol using time course data of DPPC LUV hydrolysis by the AppD49 phospholipase A_2. One promising experimental observable is to measure the time at which the activity of the enzyme rapidly increases (τ) as a function of substrate concentration. The fact that the enzyme fluorescence change correlates well with enzyme activation (Fig. 2), allows us to measure τ at concentrations of substrate lower than we use in the pH stat enzyme activity assay. Fig. 6 demonstrates the τ versus substrate concentration dependence predicted by each of the three models. Both the binding (solid curve) and equilibrium (dashed curve) activation models predict that τ will be independent of substrate concentration at low concentrations and will be an increasing function at high concentrations. This behavior is always true of these two models, and in the case of the equilibrium model, does not matter whether the equilibrium is slow or fast. In contradistinction, the kinetic model (dotted curve) predicts a completely different dependence of τ on substrate concentration. The value of τ is first a decreasing function of substrate concentration, reaches a minimum and then increases with further increases in substrate concentration. The concentration at which the minimum occurs depends on the values of K_B and K_s.

The experimentally determined behavior of τ as a function of substrate concentration is shown in Fig. 7. The value τ is a decreasing function at low substrate concentration, an increasing function at high substrate concentrations and reaches a minimum at ~0.1 mM DPPC. This kind of behavior is highly reproducible and has been found without failure in 10 experiments, although the exact position of the minimum varies between 0.03 mM and 0.2 mM DPPC. Similar behavior has been seen in preliminary experiments with the phospholipase A_2 from Crotalus atrox venom. Obviously, these results are not consistent with either the binding or equilibrium models as defined here. They are completely consistent with the kinetic model.

The state of our interpretations at this point regarding the mechanism of activation of the pancreatic and AppD49 phospholipase A_2 is as follows. 1) Both enzymes can bind to DPPC vesicles in the absence of calcium and this binding is stronger when the lipid is in the gel phase. 2) Both enzymes are activated on the surface of DPPC LUV. 3) This activation is, at least in part, coupled to a membrane structural change which occurs as reaction products accumulate in the bilayer. 4) The kinetics of hydrolysis for both enzymes are consistent with the occurrence of a quasi-irreversible step in the activation mechanism. 5) The enzyme and substrate concentration dependencies

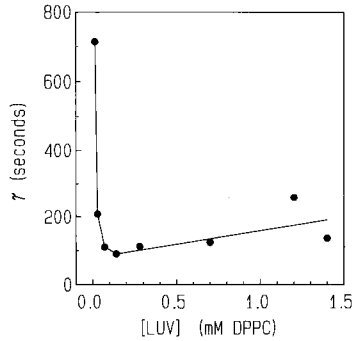


Fig. 7. Experimental dependence of τ on the concentration of DPPC LUV. The intrinsic AppD49 enzyme fluorescence (290 nM) was monitored during hydrolysis of LUV at the indicated DPPC concentrations at 39°C using the protocol described in Fig. 3 (no TMA-DPH). τ was measured as the time of the fluorescence increase.

of the rate of activation of the pancreatic enzyme at 38°C indicate that the activation mechanism involves dimerization on the membrane surface. Both a dimer and monomer kinetic activation model predict that τ versus S_t will exhibit a minimum as shown in Fig. 7 and distinction between those two models for the AppD49 enzyme will require a more sophisticated analysis which is currently underway. It is worth noting, however, that both the pancreatic and AppD49 enzymes have been reported to become acylated and stabilized as a dimer during the activation process.[5,6]

While the binding activation model cannot account for the results shown in Fig. 7, the binding of the enzyme to the vesicles must indeed improve upon activation. Thermodynamically, K_s must be greater than K_B if enzyme activation is to occur on the surface of the vesicles. We therefore do not dispute previous data reporting that the enzyme binds better in the presence of the appropriate concentration of reaction products;[19] rather, we argue that improved binding is not the activation step per se but is a thermodynamic consequence of the fact that activation occurs on the vesicle surface.

CALCIUM

The calcium requirement for the activity of most phospholipases A_2 has generally been reported to be in the millimolar concentration range.[1] However, the role of calcium in the temporal sequence of events in phospholipase A_2 activation is not clear. Specifically, is calcium required for enzyme binding to the bilayer, for activation and/or for catalysis? It has frequently been reported that calcium is required for "catalytically effective" phospholipase A_2 binding to lipid substrate.[19] However, calorimetric[3] and fluorescence,[10] studies have demonstrated that calcium is not required for enzyme-lipid interaction. We have also obtained similar results with the Crotalus atrox enzyme.

Fig. 8 shows time courses of DPPC LUV hydrolysis by AppD49 phospholipase A_2 at three concentrations of calcium. These preliminary results show that the lag time is sensitive to calcium in the low millimolar to high micromolar ranges; but the maximum rate of hydrolysis is not. The increase in the intrinsic enzyme fluorescence at time τ, simultaneously measured for each of these time courses, was found to be identical for all three calcium concentrations. Therefore, we conclude that the maximum degree of enzyme activation can be acheived at very low calcium.

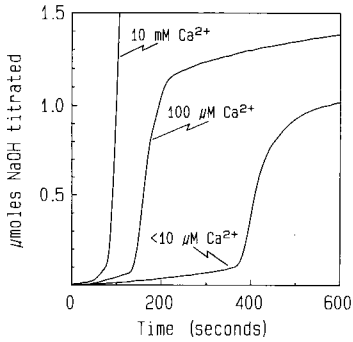


Fig. 8. Time courses of DPPC LUV hydrolysis by AppD49 phospholipase A_2 as a function of $CaCl_2$ concentration. Hydrolysis reactions were monitored at 41.5°C with 380 nM enzyme and 1.5 mM DPPC as described in Fig. 1. $CaCl_2$ concentrations were 10 mM, 0.1 mM and <0.01 mM (50 mM KCl instead of $CaCl_2$).

Preliminary studies on the effect of calcium on the hydrolysis of small unilamellar vesicles sheds some additional light on the problem. As mentioned, the maximal rate of hydrolysis of small unilamellar vesicles is observed at time zero. At 10 mM calcium, the hydrolysis time-course is essentially a true hyperbolic function of time. However, at lower calcium concenrtions (in 50 mM KCl) the time course is non-hyperbolic and of such a form as to suggest severe product inhibition as the reaction progresses. Such inhibition has previously been reported during the hydrolysis of vesicular substrate by Crotalus atrox PLA_2.[23] Furthermore, the concentration of calcium required to acheive half maximal initial activity is on the order of 0.5 to 1 mM. This can be seen in Fig. 9 where the estimated initial velocity versus [calcium] is displayed (circles). The apparent inhibition by product can be relieved by 10 mM magnesium which, in the absence of calcium, is incapable of supporting catalysis.[1] This relief of apparent product inhibition is reflected by an increase in the estimated initial velocity. In Fig. 9, the initial velocity as a function of calcium in the presence of 10 mM magnesium is displayed and the estimated calcium dissociation constant under these conditions is approximately 2×10^{-5} M (triangles). These results clearly show that the maximal catalysis can be achieved at calcium concentrations much lower than previously reported and at calcium concentrations much lower than the dissociation constant for the isolated enzyme.

These results suggest the following. Since calcium does not appear to affect the binding of phospholipase A_2 to zwitterionic vesicles and since the apparent calcium binding constant for catalysis is much stronger than that of the free enzyme, the structure of the active enzymes on the membrane surface must be different than the inactive solution form or the form which is initially bound to the lipid. This conclusion, if correct, means that increased activity of the protein cannot be solely due to increased binding, as has been deduced from comparison of computer simulations of such a model and the experimental data from r versus S_T (Figs. 6 and 7). These calcium results reported here do not directly address the role of calcium in the activation process per se, but are consistent with calcium being required as previously suggested.[2,3] A detailed reevaluation of the role of calcium in the overall process of phospholipase activation is currently underway.

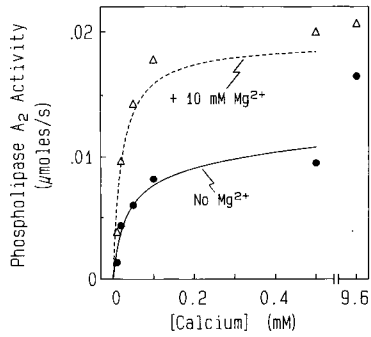


Fig. 9. $CaCl_2$ dependence of AppD49 phospholipase A_2 activity without (solid line) or with (dashed line) $MgCl_2$. Enzyme (90 nM final) was added to a 2-ml reaction cocktail containing small unilamellar vesicles (1 mM DPPC), the indicated concentrations of $CaCl_2$, ± 10 mM $MgCl_2$ and 50 mM KCl at 25°C. The reaction was monitored at pH 8.0 with a pH stat. The activity was determined from the maximum initial slope of the time courses. The curves have no theoretical significance.

SIGNAL TRANSDUCTION

What might be learned from these soluble phospholipases A_2 in terms of the regulation of the enzyme in a living cell? A cell must accomplish four tasks in regulating phospholipase A_2 for signal transduction. 1) It must protect itself from extensive hydrolysis which could lead to solubilization of membrane components and lysis. 2) It must be capable of reversibly activating the enzyme. 3) It must be capable of limiting the number of phospholipids that will be hydrolyzed by the active enzyme. 4) The activation process must be specific.

A plethora of phospholipase A_2 have now been purified from a variety of mammalian cells and tissues. One interesting consistency is that the enzymes are generally active upon purification requiring only calcium and the appropriate pH and substrate. One could argue that the enzymes responsible for signal transduction have not yet been isolated and would behave differently. Nevertheless, the cell must have some way of protecting itself from all these phospholipases. An obvious answer is that the enzyme requires calcium which is normally very low intracellularly. Calcium may indeed be one level of regulation of phospholipase A_2 activity, but it probably does not suffice. Certainly, it would lack specificity in the sense that all the phospholipase A_2 in the cell would be activated every time there was a calcium flux. Such could be disastrous in muscle cells and neurons. In addition, some studies have suggested that hormonal-stimulated calcium flux is insufficient in itself to induce activation of phospholipase A_2.[24,25]

It would seem, then, that cellular regulation of phospholipase A_2 must involve more than modulation of intracellular calcium concentration. A variety of other mechanisms have also been suggested in the literature which could presumably act as additional levels of regulation. One suggestion is that hormonally-regulated phospholipase is activated by phosphorylation via protein kinase C.[26] Other reports suggest a role of intracellular pH[27] and the cytoskeleton.[28] Substrate specificity is probably also important.[29-32] Finally, a variety of reports have proposed that phospholipase A_2 is hormonally regulated by one or more GTP-binding proteins analogous to those that regulate adenylate cyclase.[33]

Notwithstanding these several possible mechanisms, one might ask whether membrane structure could not play a significant role in the regulation of cellular phospholipase A_2 as it apparently does for the soluble enzymes from snake venom or pancreas. Initially, the cellular membrane would exist in a state which is either not susceptible to hydrolysis or incapable of activating the phospholipase. This would confer protection to the membrane. The activation of the enzyme could then be initiated by applying a reversible perturbation to the membrane structure which renders a small number of lipids susceptible to hydrolysis or which activates a small number of enzymes. Upon removal of the perturbation, the enzyme could presumably relax to the inactive state. The specificity could be achieved in the following ways. First, the extreme sensitivity of phospholipase A_2 to membrane perturbation could mean that a perturbation of small magnitude that would not affect the majority of cellular membrane-bound proteins could have a larger effect on the phospholipase A_2. Second, a typical theme in cellular homeostasis is to regulate an important process at several levels to make the system fail-safe and finely controllable. One can imagine that phospholipase A_2 activation might require a calcium influx plus interaction with a transducer protein plus an appropriate membrane perturbation. Such a multi-level mechanism would be especially important if the GTP-binding transducer protein component responsible for phospholipase A_2 regulation turns out to be the beta-gamma subunit as has been proposed.[33] The same or a similar beta-gamma subunit exists in all GTP-binding transducer proteins which couple to a large number of hormone receptors.[34] Thus, since a number of receptors on a given cell would cause release of the beta-gamma subunit upon stimulation, additional levels of phospholipase A_2 regulation would be absolutely required to maintain specificity.

A variety of experimental evidence supports the possibility that the membrane structure and perturbations of the same play a role in cellular phospholipase A_2 regulation. Like the soluble enzymes, the activity of many cellular phospholipases A_2 depends on the structure of the bilayer upon which they act.[35-39] Perturbations by molecules such as glycerol[37] or diacylglycerol[38-40] have also been reported to activate cellular phospholipase A_2. A phospholipase A_2 activating protein structurally similar to mellitin which probably activates phospholipase A_2 by effects on the membrane[20] has also been described.[41] In fact, it has even been speculated that the conformational change of hormone receptors or transducer proteins upon binding of ligands could induce the necessary membrane perturbations.[20] Clearly, increased physical characterization of the role of membrane structure on the temporal sequence of events in the activation of phospholipase A_2 will aid in the understanding of the regulation of this and other membrane-bound cellular proteins.

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