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INVESTIGATING AND MODELING POSSIBLE MECHANISMS BY WHICH HEALTHY CELL MEMBRANES BECOME RESISTANT TO HYDROLYSIS BY SECRETORY PHOSPHOLIPASE A2

By

Jennifer Nelson

A thesis submitted to the faculty of Brigham Young University

In partial fulfillment of the requirements for the degree of

Master of Science

Department of Physiology & Developmental Biology

Brigham Young University

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BRIGHAM YOUNG UNIVERSITY

GRADUATE COMMITTEE APPROVAL

Of a thesis submitted by

Jennifer Nelson

This thesis has been ready by each member of the following graduate committee and by majority vote has been found to be satisfactory.

| Date | John D. Bell, Chair |
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| Date | William S. Bradshaw |

BRIGHAM YOUNG UNIVERSITY

As chair of the candidate's graduate committee, I have read the thesis of Jennifer Nelson in its final form and have found that (1) its format, citations, and bibliographical style are consistent and acceptable and fulfill university and department style requirements; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the graduate committee and is ready for submission to the university library.

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ABSTRACT

INVESTIGATING AND MODELING POSSIBLE MECHANISMS BY WHICH HEALTHY CELL MEMBRANES BECOME RESISTANT TO HYDROLYSIS BY SECRETORY PHOSPHOLIPASE A2

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Department of Physiology and Developmental Biology

Master of Science

Secretory phospholipase A2 (sPLA2) behaves differently toward the membranes of healthy cells compared to those of damaged or dying cells. The enzyme catalyzes rapid and sustained hydrolysis of compromised cells consistent with a simple catalytic mechanism. In contrast, when healthy cells are incubated with sPLA2, they become resistant to hydrolytic attack as manifest by three unusual observations: First, hydrolysis is transient and represents only a small fraction of the total membrane phospholipid content. Second, subsequent

addition of sPLA2 fails to generate additional product. Third, the apparent potency of the enzyme to cause the membrane to be refractory is much greater than the potency for catalyzing hydrolysis. The mechanism responsible for this resistance has not yet been identified. Using Monte Carlo and direct analytical methods, we have developed a model capable of explaining all three of these observations. The model requires two salient elements: only a small pool of phospholipids in the healthy cell membrane is available for catalysis by sPLA2, and hydrolyzed phospholipids are re-acylated and restored very slowly to the accessible pool. The requirement for initial hydrolysis (as opposed to the simple physical presence of the enzyme as previously thought) was confirmed experimentally. Additional evidence has shown that the membrane does not remain permanently in its resistant state. Over time, the membrane resets to its original state. The model also predicts that total substrate, reacylation rate, and the return rate of phospholipids to the membrane should all be constant as enzyme concentration is varied. This prediction was tested by quantitative analysis of hydrolysis time courses at varied enzyme concentrations. Experiments with fluorescent probes, merocyanine 540 and laurdan suggest, that resistance may also involve physical changes to the membrane beyond the kinetic mechanisms hypothesized in the model.

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Introduction

Secretory phospholipase A2 (sPLA₂) belongs to a large family of enzymes that catalyze the hydrolysis of phospholipids. Phospholipases have important physiologic functions, but have also been implicated in many pathophysiologic conditions (Vadas et al., 1993;Murakami et al., 2001). There are many different isoforms that belong to this family of enzymes. Specifically, sPLA₂ hydrolyzes the sn-2 acyl bond in a calcium dependent manner and produces free fatty acid and lysophospholipid as products. sPLA₂ is one of relatively few enzymes capable of catalyzing its reaction at the interface of the cell membrane and the extracellular fluid. Our laboratory has focused on understanding how sPLA2 interacts with the cell membrane. Ample evidence suggests that the intrinsic properties of the membrane govern whether or not sPLA₂ has access to lipid substrate. Catalysis is preceded by two essential steps. First, the enzyme adsorbs to the surface of the cell membrane, and second, substrate migrates from the bilayer into the active site of the enzyme (Figure 1; Gelb et al., 1995; Berg et al., 1997). Regulation of this second step is critical in determining the degree of susceptibly cells exhibit towards sPLA₂.

We discovered an intriguing phenomenon: sPLA₂ behaves differently toward healthy cell membranes than toward those of damaged or dying cells. The enzyme catalyzes rapid and sustained hydrolysis of compromised cells

consistent with a simple catalytic mechanism (Figure 2, Panel A, dashed curve; Wilson et al., 1999; Gelb et al., 1995; Wilson et al., 1999). In contrast, when healthy cells are incubated with sPLA₂, they become resistant to hydrolytic attack as manifest by three unusual observations. First, hydrolysis is transient and represents only a small fraction of the total membrane phospholipid content (Figure 2, Panel A, solid curve; (Wilson et al., 1999). The amount of product decreases due to reacylation of species generated in the reaction. Second, subsequent addition of sPLA₂ fails to generate additional product (Figure 2, Panel B; Wilson et al., 1999). Third, the enzyme's apparent potency to cause the membrane to be refractory is much greater than its potency for catalyzing hydrolysis (Figure 2, Panel C). The mechanism responsible for this resistance has not yet been elucidated. The first part of this project focuses on developing a theoretical model capable of explaining resistance, and the second part tests the model experimentally and characterizes the biophysical changes that take place as a cell membrane becomes resistant to hydrolysis.

Materials and Methods

Reagents

Acrylodan-labeled fatty acid-binding protein (ADIFAB), laurdan, and merocyanine 540 (MC540) were obtained from Invitrogen (Carlsbad, CA). Laurdan and MC540 were suspended in dimethylsulfoxide (DMSO). ADIFAB was suspended in 50 mM KCl and 3 nM NaN₃. Aspartate-49 phospholipase (APPD49 sPLA₂) was purified from the venom of *A. p. piscivorus* using the protocol outlined in (Maraganore et al., 1984).

Cell Culture

The cells used in all experiments were S49 mouse lymphoma cells. Cells were maintained in a carefully controlled environment: 37° C, humidified air, and 10% CO₂ (Wilson et al., 1997).

*Ca*²⁺ *and EDTA experiments*

Cells were suspended in MBSS that did not contain Ca²⁺. The reaction progress was monitored using ADIFAB (excitation, 390 nm; emission 432 nm and 505 nm; 65 nM final concentration; (Harris et al., 2002). EDTA, a Ca²⁺ chelator was added at 150 seconds, 20 μ L of 0.1 mg/mL of sPLA₂ were added at 500 seconds, and 20 μ L of 3 nM Ca²⁺ were added at 750 seconds. In the control experiment, the addition order of sPLA₂ and Ca²⁺ was reversed to ascertain that the amount added of Ca²⁺ was sufficient to activate sPLA₂.

Two Hit Experiments

An aliquot (0.5-4 x 10⁶) of cells suspended in MBSS were transferred to a cuvette and placed in the fluorometer (Fluoromax 3, Horiba Jobin-Yvon, Edison, NJ) and maintained at 37° C. At 100 seconds, ADIFAB was added to monitor fatty acid production (Wilson et al., 1997). At 400 seconds, a dose of APPD49 sPLA₂ (0.02-70 nM final concentration) was added. At 900 seconds, an additional dose of 20 μ L of 0.1 mg/mL APPD49 sPLA₂ (70 nM final concentration) was added.

Long Reversal Experiments

An aliquot (0.5-4 x 10⁶) of cells suspended in MBSS were transferred to a cuvette and placed in the fluorometer and maintained at 37° C. At 100 seconds, ADIFAB was added to monitor the reaction progress. At 400 seconds, a dose of APPD49 sPLA₂ (70 nM final concentration) was added. At 900 seconds, an additional dose of 20 μ L of 0.1 mg/mL APPD49 sPLA₂ (70 nM final concentration) was added. At one concentration) was added. Cells were then transferred to a centrifuge tube, washed, and resuspended in medium with or without serum and incubated for 0 to 24 hrs. After incubation, cells were again washed, resuspended in MBSS, and transferred to a cuvette, and the same experimental time course was repeated.

Quantification of Experiments Using ADIFAB

Data acquired from the ADIFAB experiments were quantified by calculation of the generalized polarization (GP): $(I_{505} - I_{432})/(I_{505} + I_{432})$. The GP was then fit using a derived equation designed to model the system under study (see section below).

To determine how much hydrolysis and resistance occurred for a particular dose of sPLA₂, the following formulas were used (Figure 3):

Hydrolysis equaled the change in ADIFAB GP from the first dose of sPLA₂ (Δ Hy) divided by the change in GP from a control dose of 70 nM, a concentration which causes maximum hydrolysis (Δ To).

$$Hydrolysis = \frac{\Delta Hy}{\Delta To}$$

Resistance equaled the value one subtracted by the change in ADIFAB GP from the second dose of sPLA₂ (Δ Re) divided by the change in GP from a control dose of 70 nM (Δ To).

Resistance =
$$1 - \frac{\Delta \text{Re}}{\Delta To}$$

Cell Growth Curve Experiments

An aliquot (5 mL) of cells which had been suspended to come to full density in 48 hours were treated with 50 μ L sPLA₂. Control cells received 50 mM KCl and 3 mM NaN₃ (solution in which sPLA₂ is suspended in). Every 6 to 12 hours a 100 μ L sample was taken from both the control and treatment flasks and gently mixed with 100 μ L trypan blue. The number of both viable cells and dead cells was recorded. Data were normalized by dividing all subsequent time points by the initial cell count. Data were plotted and fit using nonlinear regression.

Assessment of Physical Changes that Correspond to Membrane Resistance

Two probes were used to quantify and characterize membrane properties of resistant cells: MC540 (excitation 540 nm, emission wavelengths recorded: 570, 585 nm), which measures interlipid spacing and laurdan (excitation 350 nm, emission wavelengths recorded: 435, 500 nm), which measures invasion of the bilayer surface by water molecules. Experiments were performed using a fluorometer, which permits assessment of global changes.

Cells were harvested, washed, resuspended in 2 mL of MBSS, and incubated with MC540, 170 nM Final, (or laurdan, 50 nM Final) at 37° C in the fluorometer. After equilibrium was reached, a dose of sPLA₂ (0.7 nM to 70 nM) was added, and changes in the fluorescence were monitored during a time course of 1800 seconds. The time courses generated were fitted using nonlinear regression. For MC540, these fits allowed for determination of the maximum change in the ratio of intensities observed at 570 and 585 nm as well as the maximum change in the total intensity at 570 and 585 nm. For laurdan, the maximum change in total intensities as well as maximum change in GP were measured.

Theory

Monte Carlo Simulations

The Monte Carlo program consists of a continuous two-dimensional hexagonal array capable of simulating the cell surface. Enzymes can bind to any of the 2000 available sites provided that another enzyme is not bound to an adjacent site (to accommodate steric hindrance). Each binding site consists of 7 phospholipids. The program is capable of varying a large number of parameters (see Table 1), including those in the reaction scheme shown in Figure 4, and can accommodate a second addition of enzyme. The properties of the membrane are adjustable. A proportion of the membrane can be nonhydrolyzable (the enzyme can bind to the site but no catalysis can occur). The affinity of the enzyme can be different for hydrolyzable and nonhydrolyzable sites. The affinity of the enzyme can also be orientation-dependent; i.e. the binding depends on which part of the enzyme surface faces the membrane. There are several ways in which an enzyme can move to a new site on the membrane. In addition to the typical mode of disassociation and readsorption to a new site on the membrane, the enzyme can roll (new orientation) or scoot (same orientation) directly across the surface to a new site. Once the properties of the membrane and enzyme have been set, the program iterates a decision-making routine (Figure 5), and data are generated in the form of a time course. There are two outcomes that are reported at the end of

each cycle: the number of enzymes molecules adsorbed to the membrane and the amount of product.

Analytical Derivation

The distribution of phospholipids among structural states (substrate (S), reaction product (P) and reacylated product (P*)) was modeled by the following system of differential equations:

$$\frac{dP}{dt} = E_B k_{cat} \left(S_T - P - P^* \right) - k_{acyl} P \tag{1}$$

$$\frac{dP^*}{dt} = k_{acyl}P - k_rP^*$$
⁽²⁾

ST is the total amount of substrate. k_{cat} is the catalysis rate. k_{acyl} is the rate of reacylation. k_r is the rate of return of reacylated lipids to the hydrolyzable pool.

The system assumes rapid equilibrium between the bound and free states of the enzyme and was derived in terms of E_B (the bound state).

$$E_B = \frac{E_T K_{app}}{1 + E_T K_{app}} M_T \tag{3}$$

 E_T is the total amount of enzyme. K_{app} is the equilibrium constant for enzyme adsorption and substrate migration. M_T is the total amount of membrane.

Integration of Equation 1 yields Equation 4 with the substitutions listed in Equations 5 and 6.

$$P = \frac{A}{a-b} \left(e^{-at} - e^{-bt} \right) + \frac{Ak_r}{ab} \left(1 - \frac{b}{b-a} e^{-at} + \frac{a}{b-a} e^{-bt} \right)$$
(4)

$$a = \frac{(k_r + A + k_{acyl}) + \sqrt{(k_r + A + k_{acyl})^2 - 4[k_r(A + k_{acyl}) + k_{acyl}A]}}{2}$$
(5)

$$b = \frac{(k_r + A + k_{acyl}) - \sqrt{(k_r + A + k_{acyl})^2 - 4[k_r(A + k_{acyl}) + k_{acyl}A]}}{2}$$
(6)

To facilitate fitting with nonlinear regression, the data were fit with Equation 7, a simplified version of Equation 4.

$$P = \frac{A}{a-b} \left(e^{-at} - e^{-bt} \right) + \frac{B}{ab} \left(1 - \frac{b}{b-a} e^{-at} \right) + I$$
(7)

Theoretical Results

Monte Carlo Simulations

let $A = E_B k_{cat} S_T$

In simulation trials, each parameter was systematically varied to assess its effect on the product time course. If no reacylation occurred, a typical time course looked like Panel A Figure 6. If reacylation occurred but reacylated lipids were available for rehydrolysis, a typical time course looked like Panel B Figure 6. In contrast, if the reacylated substrate was not available for hydrolysis, the shape of the time course changed dramatically, and the shape depended greatly on the reacylation rate (Panel C Figure 6). Simulated data matched experimental time courses best when the reacylation rate was very small (0.005). When parameters were set to the values indicated in Table 2, the simulated product time course exhibited a form very similar to experimental results (Figure 7).

Fitting Routine

A, *a*, and *b* were constrained based on enzyme concentration and independent assessment of K_{app} .

The value of k_r was allowed to float.

Since the amount of substrate remaining at the time of the second addition of enzyme is unknown, the value of A for the second enzyme addition (A2) was allowed to float.

The model predicted that k_r should vary randomly around a constant value while A2 should decrease with increasing initial dose of enzyme. When applied to actual experimental data, the parameters from the fits behaved as expected (Figure 8). Fits of experimental data for three different enzyme concentrations are shown in Figure 9.

Experimental Results

Resistance is Hydrolysis-Dependent

Incubation of cells while temporarily inhibiting sPLA₂ catalysis showed that hydrolysis is required for resistance to occur. In the presence of EDTA, no catalysis occurred because no free Ca²⁺, a necessary cofactor, was available. If adsorption of sPLA₂ alone were capable of inducing resistance by some biophysical or biochemical means, then subsequent addition of Ca²⁺ would have yielded no hydrolysis. However, when Ca²⁺ was added, hydrolysis occurred (Panel B Figure 10). Positive controls were also included to ascertain that the amount of Ca²⁺ added was sufficient to restore the enzyme to full catalytic activity (Panel A Figure 10). These experiments provided evidence that adsorption of enzyme to the surface of the cell is not enough to cause resistance.

Reversibility of Resistance

After cells had become resistant to the enzyme (exposure to 70 nM final concentration sPLA₂), they were washed and resuspended in various solutions (MBSS, medium, and medium plus serum) and incubated at 37° C for different periods of time (0, 1, 6, 12, and 24 hours). After the prescribed incubation time, cells were washed and resuspended in MBSS. Another dose of sPLA₂ was added and the amount of hydrolysis was measured. If the cell membrane was still resistant, then minimal or no hydrolysis was expected. If the cell membrane had begun to revert to its state prior to sPLA₂ exposure, then an appreciable amount of hydrolysis was expected. Interestingly, the rate at which the membrane reverted to its normal state (condition prior to exposure to sPLA₂) depended on what the cells were suspended in. Cells suspended in MBSS showed little or no

reversal (Panel A Figure 11). Cells in culture medium (Panel B Figure 11) with no serum (or with serum, not shown) showed rapid reversal.

Biophysical Changes in the Cellular Membrane

Previous experiments conducted with S49 mouse lymphoma cells revealed pronounced changes in membrane structure that corresponded to increased susceptibility to sPLA₂ (Bailey et al., 2007). The order of lipids in the membrane decreased and interlipid spacing increased. Similar experiments were conducted to assess what changes, if any, occurred as the membrane became resistant to hydrolytic attack.

Lipid Order in the Resistant Cell Membrane

Addition of sPLA₂ to the cell suspension caused a rapid decrease in apparent lipid order, as assessed by laurdan GP (Figure 12). Repetition of the experiment at various sPLA₂ concentrations demonstrated that the effect was saturable and reproducible. The maximum change was 0.02 ± 0.01 GP units with an EC₅₀ value of 5.79 ± 3.48 nM sPLA₂ (blue curve, Figure 12). The total intensity of laurdan also decreased with increasing concentration of sPLA₂ (maximum change of 5% decrease, pink curve, Figure 12) with an EC₅₀ value of 0.23 ± 0.005 .

Lipid Spacing in the Resistant Cell Membrane

Interlipid spacing also changed as the membrane became resistant to hydrolysis. The dose dependent decrease in total MC540 intensity with increasing concentration of sPLA₂ (maximum decrease of 12%, EC₅₀ 0.71 ± 0.43) indicated that the lipids in the cell membrane became more tightly packed (Figure 13).

Effect of sPLA2 on Cell Growth

Over an incubation period of 48 hours with 70 nM dose of sPLA₂, cell growth appeared to be unaffected by the presence of enzyme. The doubling time for the control cells was 10.32 ± 0.10 hours and 13.27 ± 0.37 hours for the cells incubated with sPLA₂ (Figure 14).

Discussion

It is simple to establish that cell membranes become resistant to sPLA₂ (Wilson et al., 1999); however, the mechanism governing resistance needs to be better understood. Two plausible hypotheses are easily excluded. The first centers on the idea of rapid endocytosis of sPLA₂. Sequestration of the enzyme inside the cell would prevent hydrolysis of the outer-leaflet; however, three pieces of evidence refute this idea. First, a membrane that has become resistant to hydrolysis can rapidly be rendered susceptible by the addition of ionomycin, a calcium ionophore (Bailey et al., 2007). Thus, if the enzyme were sequestered, it is unlikely that it would be regurgitated on the time scale of the experiment. Second, flow cytometry data reveal that only a small percentage of the sPLA₂ is endocytosed; a large proportion is still present extracellularly and presumably capable of carrying out catalysis (Bailey et al., 2007). Third, a second maximum dose of enzyme (70 nM final concentration) elicits no hydrolysis. It is difficult to imagine a situation in which the cell could completely sequester the second dose of enzyme before any hydrolysis occurred since it was unable to do so with the first.

The second possible explanation for resistance centers on a receptormediated response to the presence of sPLA₂. However, two pieces of evidence suggest that this is not the case. First, if resistance depended on sPLA₂ binding to a receptor, then enzymes which do not bind to the receptor would be incapable of causing resistance. However, experiments done by Wilson et al. (1999) showed that bee venom sPLA₂, which does not bind to sPLA₂ receptor (M-type, Ancian et al., 1995), can cause resistance. Second, our experiments indicate that hydrolysis is required for resistance to occur. When cells were incubated in Ca²⁺-free MBSS with sPLA₂, the presence of the enzyme on the cell surface did not trigger resistance. It was only after Ca²⁺ had been added back into solution and sPLA₂ became active that resistance occurred. These two observations argue that the resistance is not a receptor-mediated response.

The next general hypothesis is whether resistance can be explained entirely by the interactions of sPLA₂ with the membrane. When we simulated the interactions between sPLA₂ and the cell membrane using a Monte Carlo

algorithm, we identified what could potentially be the most important variables. From the many simulations performed, two critical conclusions emerged. First, the hydrolysis reaction must slow significantly or come to a complete stop. The easiest way to satisfy this criterion is to limit the amount of substrate available to the enzyme. Exhaustion of this pool would explain why the cell membrane has become resistant. Second, the rate at which reacylated lipids are returned to the available substrate pool must be much smaller than the rate of reincorporation of fatty acid into phospholipids. When these two conclusions are in force, the results of simulated time courses closely resemble experimental time courses (Figure 15). To restate, the minimum model for resistance centers on a small, rapidly hydrolyzable pool of lipids that upon exhaustion is slowly repopulated over time.

A system of differential equations based on this minimum model (limited substrate hypothesis) is capable of fitting experimental data even when parameters are highly constrained by values determined from independent experimental results. Parameters that were allowed to float behaved as predicted, providing further evidence that the model could correctly provide a mechanism for resistance.

Previous experiments published in Wilson et. al. (1999) showed that resistance occurs even when little or no initial hydrolysis is observed

(experiments used weakly active human group IIA and venom Lys-49). However, due to the high concentrations used in those experiments, the resistance observed could be the result of competition for binding. If the catalytically weak enzymes saturate the surface of the cells, then highly active Asp-49 venom sPLA₂ would have diminished access to the membrane. Additionally, these isoforms are incapable of inducing complete resistance (Wilson et al., 1999).

Two lines of evidence suggest that resistance could be more complex than previously thought. The first refers to the rate at which the initial naïve state of the membrane can be restored following exposure to sPLA₂. If the limited substrate model applied, one would expect that the reversal of resistance would involve restoration of the original pool of membrane phospholipids and that the timing of the restoration would be fixed based on cellular metabolic processes. Instead, the reversal rate of resistance depended on the solution in which cells were suspended. Since the reversal rate was dependent on the type of cell suspension, a different mechanism could be responsible for the reestablishment of original membrane conditions.

The second reason for believing that resistance is more complex than the limited-substrate model is that fluorescent probes detected changes in the membrane when cells were incubated sPLA₂. If these changes were reflective of

resistance, they would be small, logical with respect to susceptibility, and dependent on sPLA² dose in the appropriate range. As predicted, the observed changes in membrane order (0.02 GP units and 5% maximum drop in normalized total laurdan intensity) and interlipid spacing (12% maximum drop in normalized MC540 intensity) were small (Figures 12 and 13). Moreover, these changes were logical. As cell membranes become susceptible, lipid order decreases and interlipid spacing increases which improves the ability of phospholipids to migrate into the enzyme's active site (Bailey et al., 2007). In contrast, the opposite occurred as resistance emerged; cell membranes became more ordered and interlipid spacing decreased (Figures 12 and 13).

An intriguing observation was the difference in sPLA₂ potency for inducing resistance (EC₅₀ = 0.49) and catalyzing hydrolysis (EC₅₀ = 3.39, Panel C Figure 2). Although the limited-substrate model can reconcile at least a small difference in potency (Figure 16), a more compelling explanation relates to the fluorescence observations. The potencies of the change in total laurdan intensity (EC₅₀ = 0.23) and total MC540 intensity (EC₅₀ = 0.79) were very similar to the EC₅₀ of resistance (0.49). Thus, since the fluorescence changes occurred at the same range of sPLA₂ dose as resistance, it is reasonable to conclude that the observed changes in interlipid spacing correspond to resistance. Interestingly, the EC₅₀ of laurdan GP (5.79) was very similar to the EC₅₀ of hydrolysis (3.39). This result suggests that the changes in lipid order are more closely linked to hydrolysis. Taken together, the results encourage a hypothesis that products of hydrolysis either directly or indirectly alter membrane structure in a way that makes it resistant to the action of sPLA₂.

How might the dose of sPLA₂ be different for inducing resistance than for hydrolysis since resistance appears to depend on the enzyme's catalytic activity? When a relatively small amount of available substrate has been hydrolyzed, the reaction products generated in the membrane could be sufficient to alter the domains where productive catalysis can occur, making subsequent hydrolysis less favorable. For this explanation to work, the rate of domain alteration must be slower than the rate of hydrolysis. If the rate of hydrolysis and domain alteration equaled each other, there would be no transient burst of hydrolysis.

Conclusion

The cell membrane is a dynamic environment. The results of this study show the delicate balance between two competing needs: first, the need to generate product that will be used in synthesis of inflammatory molecules in a physiological setting, and second, the need to preserve membrane integrity. Studying how membranes become resistant to sPLA₂ reveals the importance of lipid organization in the bilayer. In this case, the properties of the membrane, not the enzyme, regulate the reaction. Based on the experiments performed in this study, we conclude the following:

Monte Carlo simulations can adequately simulate the interaction of sPLA₂ with the cell membrane and indicate the minimum requirements for the resistance phenomenon.

Hydrolysis is required experimentally for resistance to occur.

Tests of the Limited Substrate Model have shown that it is capable of explaining resistance.

Resistance is a reversible phenomenon.

Changes in total MC540 and laurdan fluorescence intensity suggest resistance may involve physical changes in the membrane that interfere with hydrolysis and limit the available substrate pool.

The reversibility of resistance and the physical changes in the membrane that occur during the onset of resistance suggest that resistance may be more complex than previously thought.

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Tables

Table 1: Monte Carlo Parameters

| K ₁ = rate of enzyme binding |
|---|
| K ₂ = enzyme disassociation rate |
| \mathbf{K}_{e} = probability that the lipid makes it into the |
| enzyme active site |
| K _i = rate of inhibition on hydrolysis of |
| occupied, non-hydrolysable sites. |
| \mathbf{K}_{acyl} = rate of reacylation |
| Orientation effect = binding strength is |
| altered by some factor if the enzyme active- |
| site is down. |
| # non hydrolysable sites = establishes x/2000 |
| sites are unavailable for hydrolysis |
| Ratio of affinity for non-hydrolysable sites = |
| enzyme affinity for non-hydrolysable sites can |
| multiplied by some factor |
| T = length of experiment |
| Initial enzyme concentration |
| Determines how much additional enzyme |
| will be added at a time point |
| When will the additional enzyme be added? |
| Reacylation dependent on binding = the |
| reacylation rate can increase every time an |
| enzyme binds to a lipid |
| Reacylation dependent on hydrolysis = |
| reacylation rate can increase every time a lipid |
| is hydrolyzed. |
| Reacylated lipids may become unavailable for |
| hydrolysis once they have been reacylated, |
| they become labeled as non-hydrolysable |
| sites. |

Table 2: Parameters Values Used to Generate Figure 19A

| Parameter | Value |
|------------------------|-----------|
| K1 | 0.5 |
| K ₂ | 0.5 |
| ke | 0.5 |
| kacyl | 0.005 |
| Experiment length | 2500 |
| Initial dose of enzyme | 50 to 600 |
| Second dose of | |
| enzyme | 600 |
| Time of second dose | |
| addition | 1750 |
| Lipids available for | |
| rehydrolysis | No |

Figure Legends

Figure 1: Reaction Scheme of sPLA2 with a Membrane E: sPLA₂, EB: sPLA₂ adsorbed to membrane surface, S: substrate, E_B^S: sPLA₂ absorbed to membrane and with substrate bound in the active site, P: hydrolysis product (lysophospholipid and fatty acid), K₁: equilibrium constant for adsorption, K₂: equilibrium constant for substrate migration, k_{cat}: rate of catalysis.

Figure 2: Panel A S49 cells were incubated with 5 uL of ionomycin or DMSO, 20 μ L of sPLA2 was added, and the amount of reaction product generated was measured over time using ADIFAB. **Panel B** This figure shows the result of a typical "two hit" experiment. At the gray arrow 20 μ L of sPLA2 was added. After a new baseline had been established, a second 20 μ L dose of sPLA2 was added as indicated by the black arrow. **Panel C** Analysis of a series of "two hit" experiments. The results of the analysis were fit using non-linear regression. The dashed curve is the resistant curve and the solid curve is the hydrolysis curve.

Figure 3: Method Used to Calculate Resistance and Total Hydrolysis

Hydrolysis equaled the change in ADIFAB GP from the first dose of sPLA2 (Δ Hy) divided by the change in GP from a control dose of 70 nM, a concentration which causes maximum hydrolysis (Δ To). Resistance equaled the value one subtracted by the change in ADIFAB GP from the second dose of sPLA2 (Δ Re) divided by the change in GP from a control dose of 70 nM (Δ To).

Figure 4: Expanded Reaction Scheme of sPLA2 with a Membrane E: sPLA2, EB: sPLA2 adsorbed to membrane surface, S: substrate, E_BS: sPLA2 absorbed to membrane and with substrate bound in active the site, P: hydrolysis product (lysophospholipid and fatty acid), K₁: equilibrium constant for adsorption, K₂: equilibrium constant for substrate migration, k_{cat}: rate of catalysis, k_{acyl}: rate of reacylation, k_r: rate of return of reacylated lipids to the membrane.

Figure 5: Flow Chart of the Algorithm Executed by the Monte Carlo Simulation

Figure 6: Simulated Time Courses: Panel A Example of a typical time course when the reacylation rate (k_{acyl}) is set to zero. **Panel B** Example of a typical time course when the reacylation is a small number (0.005) and lipids are available for

rehydrolysis. **Panel C** Example of a typical time course when reacylation is a small number (0.005) and lipids are not available for rehydrolysis.

Figure 7: Simulated Time Course: Variation of Initial Enzyme Dose Initial enzyme concentration varied between 50 and 600, kacyl equaled 0.005, and lipids were not available for rehydrolysis.

Figure 8: Results of Fitting Double Dose Experiments with Equation 7 Green curve: parameters A2 (reflects amount of substrate left at time of second addition of enzyme) and purple curve: k_r (reflects reacylation rate).

Figure 9: Examples of Fitting "Two Hit" Experiments with Equation 7 *A*, *a*, and *b* were constrained based on enzyme concentration and independent assessment of K_{app}. The value of k_r was allowed to float. Since the amount of substrate remaining at the time of the second addition of enzyme is unknown, the value of A for the second enzyme addition (A2) was allowed to float. Fits for three different experiments are shown (70.0 nM, 0.2 nM, 0.7 nM).

Figure 10: Results of Ca²⁺ and EDTA experiments Cells were suspended in MBSS that did not contain Ca²⁺. **Panel A** EDTA was added at 150 seconds, 20 µL of 3 nM Ca²⁺ was added at 500 seconds, and 20 µL of 0.1 mg/mL of sPLA₂ was added 750 seconds. **Panel B** EDTA was added at 150 seconds, 20 µL of 0.1 mg/mL of sPLA₂ was added at 500 seconds, and 20 µL of 3 nM Ca²⁺ were added at 750 seconds.

Figure 11: Reversal of Resistance: Cells Incubated in MBSS and Medium Panel A Amount of initial hydrolysis (black curve), amount of hydrolysis after incubation 15 minute incubation in MBSS (purple curve). **Panel B** Amount of initial hydrolysis (as measured by change in ADIFAB GP, black curve), amount of hydrolysis after incubation in medium (as measured by change in ADIFAB GP, purple curve).

Figure 12: Change of Laurdan Fluorescent Intensity and GP as a Function of SPLA2 Concentration Cells were suspended in MBSS. After equilibrium was reached, a dose of sPLA2 (0.7 nm to 70 nM) was added and changes in the laurdan fluorescence were monitored during a time course of 1800 seconds. The maximum change in the intensities (blue curve) and GP (pink curve) were calculated.

Figure 13: Change of MC540 Fluorescent Intensity Cells were suspended in MBSS. After equilibrium of the probe was reached, a dose of sPLA2 (0.7 nm to 70 nM) was added and changes in the fluorescence were monitored during a time

course of 1800 seconds. The maximum change in MC540 total intensity (blue curve) was calculated.

Figure 14: Cell Growth is Not Affected by sPLA² The growth of cells was with and without sPLA₂. The growth of control cells is reported in the black curve and the growth of cells treated with 70nM (final concentration) sPLA₂ is reported in the green curve.

Figure 15: Comparison of Simulated Double Dose Experiments to Actual Double Dose Experiments Panel A Results of Monte Carlo simulations in which the initial dose of enzyme was varied (50 to 600). The second dose of enzyme (600) was added at 1750. **Panel B** Results of actual double dose experiments in which the initial dose of enzyme was varied (0.02 to 70 nM). The second dose of enzyme (70 nM) was added at 600 seconds.

Figure 16: Comparison of Potencies Generated from Simulated "Two Hit" Experiments The amount of resistance and hydrolysis caused by a particular dose of sPLA2 in the simulated experiments from Panel B Figure 15 was calculated using the method described in Figure 3. The results of the analysis were fit using non-linear regression. The green curve is the resistant curve and the black curve is the hydrolysis curve.

$$E + M_T \stackrel{\kappa_1}{\longrightarrow} E_B \stackrel{s}{\longleftarrow} E_B^{S} \stackrel{\kappa_{cat}}{\longrightarrow} E_B + P$$



 $Hydrolysis = \frac{\Delta Hy}{\Delta To}$

Resistance = $1 - \frac{\Delta \text{Re}}{\Delta To}$ -0.405-Hydrolysis Product -0.430 (ADIFAB GP) -0.455 -0.480 ΔTo -0.505 70 n M -0.530 0 250 500 750 1000 Time -0.405-Hydrolysis Product -0.430 (ADIFAB GP) -0.455 ∆Re -0.480 -0.505 ΔHy 0.7 nM -0.530 250 500 * 1000 0 750

Time * 2nd Addition of Enzyme 70 nM

Figure 4







Figure 7



Figure 8





Figure 10









Figure 13



Figure 14



Figure 15





В



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| Grants & | Grant: Office of F | Research and Creative Activities | | | |
|---|---|--|----------------------|--|--|
| Scholarships | 2005-2006 | Brigham Young University | Provo, UT | | |
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| Oral Presentations & Research Abstracts | Biophysical Soci Congress February 2008 | ety 52 nd Annual Meeting & International Bio | ophysics | | |
| | <u>Nelson-Keller J</u> , Yeung CH, LaFrance D, Bell JD, Judd AM Abstract Title: Investigating and Modeling Possible Mechanisms by which Healthy Cell Membranes Become Resistant to Secretory Phospholipase A2 | | | | |
| | Bailey RW, Gibbons Biophysical Changes Promote Hydrolysis b | E, Robertson L, Nguyen T, <u>Nelson J</u> , Judd Am, Bell J s in the Plasma Membrane during Glucocorticoid-stimu by Secretory Phospholipase A2 | D Jated Apoptosis | | |
| | Biophysical Soci | ety 52 nd Annual Meeting & International Bio | ophysics | | |
| | February 2008 | | | | |
| | <u>Nelson-Keller J</u> , Yeu Abstract Title: Investi Membranes Become | ng CH, LaFrance D, Bell JD, Judd AM igating and Modeling Possible Mechanisms by which Resistant to Secretory Phospholipase A2 | Healthy Cell | | |
| | American Societ | ty for Cell Biology Annual Meeting | | | |
| | Bradshaw W, <u>Keller .</u> Abstract Title: Persor Tasks in Cell Biology | <u>J</u> , Boren K, Bell JD nal and Attitudinal Predictors of Performance on Analy r: Helping Students Improve Their Study Methods | tical Reasoning | | |
| | American Societ | ty for Cell Biology Annual Meeting | | | |
| | Bell JD, Bradshaw W Abstract Title: The St Efforts to Improve Te | /, <u>Nelson J</u> tudent Mobility Profile: An Instrument for Measuring th eaching and Learning | e Effectiveness | | |
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| | 20th Annual Spring Research Conference | | | |
|----------------------------|---|--------------------------|-----------|--|
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