Relationship between Erythrocyte Membrane Phase Properties and Susceptibility to Secretory Phospholipase A$_2$$^\dagger$

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ABSTRACT: Normally, cell membranes resist hydrolysis by secretory phospholipase A$_2$. However, upon elevation of intracellular calcium, the cells become susceptible. Previous investigations demonstrated a possible relationship between changes in lipid order caused by increased calcium and susceptibility to phospholipase A$_2$. To further explore this relationship, we used temperature as an experimental means of manipulating membrane physical properties. We then compared the response of human erythrocytes to calcium ionophore at various temperatures in the range of 20–50 °C using fluorescence spectroscopy and two-photon fluorescence microscopy. The steady state fluorescence emission of the environment-sensitive probe, laurdan, revealed that erythrocyte membrane order decreases systematically with temperature throughout this range, especially between 28 and 45 °C. Furthermore, the ability of calcium ionophore to induce increased membrane order and susceptibility to phospholipase A$_2$ depended similarly on temperature. Both responses to calcium influx were enhanced as membrane fluidity increased. Analysis of the spatial distribution of laurdan fluorescence at several temperatures indicated that the ordering effect of intracellular calcium on fluid membranes generates an increase in the number of fluid—solid boundaries. Hydrolysis of the membrane appeared to progress outward from these boundaries. We conclude that phospholipase A$_2$ prefers to hydrolyze lipids in fluid regions of human erythrocyte membranes, but primarily when those regions coexist with domains of ordered lipids.

Secretnory phospholipase A$_2$ (sPLA$_2$), hydrolyzes phospholipids at the sn-2 position, releasing fatty acids and lysophospholipids. Physiologically, it may participate in a variety of functions including digestion, membrane homeostasis, production of precursors for synthesis of lipid hormones, defense against bacteria, and clearing of dead or damaged cells (1, 2). Furthermore, the enzyme has been very useful in studies of the relationship between bilayer structure and the function of proteins that bind reversibly to the membrane surface.

A large number of studies conducted with sPLA$_2$ and artificial bilayers have indicated that specific membrane properties determine whether that membrane will be resistant or susceptible to hydrolysis. Properties that promote susceptibility include negative charge in the membrane surface, high curvature of the bilayer, and heterogeneity of lipid components (3–10). Furthermore, the activity of sPLA$_2$ is extremely sensitive to the level of ordering of membrane lipids (3, 4, 6, 7, 11).

We are interested in determining whether the principles learned in artificial bilayers apply to biological systems. Human erythrocytes are amenable to such investigations because of their simplicity. In previous studies, it was observed that erythrocytes normally resist the action of sPLA$_2$, but that upon the addition of a calcium ionophore such as ionomycin, they become susceptible (12). This increase in susceptibility appears to be linked to an accompanying increase in the order of membrane lipids (12, 13). Nevertheless, one question that remained unresolved was whether the critical issue for increased susceptibility was the average level of membrane order or the spatial distribution of membrane regions of differential order. We therefore sought independent means of manipulating membrane order to address that question.

Several observations have suggested that erythrocyte membranes display significant temperature dependence of membrane properties in the range of about 20–40 °C indicating that temperature could be an effective means of altering the average level of membrane order (14–16). Accordingly, erythrocytes were treated with and without ionomycin at various temperatures, and the level of lipid order was assessed by fluorescence spectroscopy using laurdan as a probe of membrane structure. We then compared the susceptibility of the membrane to sPLA$_2$ at each condition to determine whether changes in average order alone were sufficient to promote susceptibility. Two-photon scanning
microscopy was used in parallel to identify possible influence of alterations in the spatial distribution of rigid domains.

**EXPERIMENTAL PROCEDURES**

**Materials.** Human erythrocytes were obtained from anonymous residual blood samples following routine physical exams at the student health center at Brigham Young University. These samples were stored overnight at 4 °C in EDTA vacutainers prior to use in experiments. Erythrocytes were isolated by centrifugation and washing, followed by re-suspension to the original hematocrit in MBSS (134 mM NaCl, 6.2 mM KCl, 1.6 mM CaCl₂, 1.2 mM MgCl₂, 18.0 mM HEPES, and 13.6 mM glucose at pH of 7.4) as described (12).

Secretory PLA₂ (monomeric aspartate 49 isozyme from the venom of *A. piscivorus* *piscivorus*) was isolated according to published procedures (17). Stock solutions were suspended at a concentration of 0.1 mg/mL in 50 mM KCl and 3 mM NaNO₃, and stored at 4 °C. Prior studies have demonstrated that this isoform reports qualitatively the susceptibility of cells to human groups IIa and V sPLA₂ (12, 18).

Acrylodan-labeled fatty acid-binding protein (ADIFAB) and laurdan were both acquired from Molecular Probes (Eugene, OR), while ionomycin was obtained from Calbiochem (La Jolla, CA). Factor Va, factor Xa, prothrombin, and thrombin were all purchased from Hematologic Technologies, Inc. (Essex Junction, VT).

**Fluorescence Spectroscopy.** Washed erythrocytes were suspended in 2 mL MBSS in a fluorometer sample cell to a final density of about (3–4) × 10⁶ cells/mL and equilibrated at the experimental temperature for at least six min prior to addition of fluorescent probes or other agents. Fluorescence measurements were obtained using either a Fluoromax (Jobin Yvon, Edison, NJ) or PC1 (ISS, Urbana, IL) photon-counting spectrophluorometer. Simultaneous assessment of fluorescence intensity at multiple excitation and emission wavelengths was obtained by rapid sluing of monochromator mirrors in the Fluoromax using control software provided with the instrument. Band-pass was set at 4.25 nm for all experiments. Temperature was maintained in each experiment using a circulating water bath, and sample homogeneity was obtained by continual gentle magnetic stirring.

**Membrane Fluidity.** Erythrocyte membrane order was assayed using the fluorescent probe laurdan as explained previously (13). Erythrocyte samples were prepared for fluorescence as described above. After initiating data acquisition, laurdan was added (2.5 μM final) and emission intensity measured (excitation = 350; emission = 435 and 500 nm) as a function of time for 1200 s. Ionomycin (300 nM) or equivalent volume of DMSO was added at the 600 s time point. Laurdan generalized polarization (GP) was then calculated from the time course data (19), and the GP values as a function of time were fit to a series of exponentials by nonlinear regression for both the data before and after the addition of ionomycin or DMSO. The base line established by fits of the time course prior to inclusion of ionomycin or DMSO was subtracted from the data.

Ionomycin treatment caused a small artifactual increase in laurdan GP upon addition to cells that was independent of the presence of calcium. Therefore, control experiments were included in which normal MBSS was replaced with MBSS to which divalent cation was not added (see Figure 3A). The artifactual increase in laurdan GP was then subtracted from all data. Control experiments revealed that similar results were obtained using either MBSS to which calcium had not been added or MBSS containing EDTA to chelate calcium.

**Hydrolysis by sPLA₂.** Release of fatty acids from cells was assayed with ADIFAB (65 nM final, excitation = 390 nm, and emission = 432 and 505) (20). Samples were prepared for fluorescence experiments as described above. After initiating data acquisition, ADIFAB was added followed 100 s later by ionomycin (300 nM) or DMSO, and 700 s later by sPLA₂ (1 μg/mL). The results were quantified by calculation of the generalized polarization (GP) and fit to a double exponential equation by nonlinear regression as described (12). The amount of hydrolysis at 100 s following sPLA₂ addition was then calculated using parameter values from the regression results.

**Two-Photon Microscopy.** The two-photon excitation images were collected on an Axiovert 35 inverted microscope (Zeiss, Thornwood, NY) at the Laboratory for Fluorescence Dynamics (Urbana, IL) as described previously (12, 13, 21). The excitation source was a titanium-sapphire laser (Coherent, Palo Alto, CA) tuned to 780 nm and pumped by a...
frequency-doubled Nd:Vanadate laser (Coherent, Palo Alto, CA). To obtain laurdan GP values, dual images were collected simultaneously using two detectors, a dichroic beam-splitter, and emission interference filters (Ealing 440 and 490) \((\text{21})\). In preparation for the images, washed cells (about 2 \(\times 10^6\) cells) and 2 mL MBSS were combined in a dish and incubated 5 min at the desired temperature. Laurdan (250 nM final) was then added and the incubation continued for an additional 15 min. After equilibration, multiple two-photon images were obtained. Ionomycin or DMSO was then added (300 nM), and images acquired every two min for 10 min. Finally, sPLA\(_2\) (1 \(\mu\)g/mL) was mixed with the samples and images again acquired repeatedly for an additional 10 min.

**RESULTS**

The steady-state emission spectrum of laurdan in human erythrocytes is broadened toward longer wavelengths at 44 °C compared to 20 °C (Figure 1A). This difference in emission spectrum is reminiscent of results obtained in artificial bilayers above and below the gel-liquid crystalline phase transition \((\text{5})\). To further explore the temperature dependence of erythrocyte membrane properties, we repeated the experiment of Figure 1A at a variety of temperatures between 20 and 50 °C. The data were quantified by calculating GP, a quantitative means of evaluating shifts in laurdan spectra associated with the solvent relaxation effect \((\text{19})\). Increased values of GP correspond to reduced solvent relaxation consistent with a reduction in membrane water content. Generally, this reduction in water results from an increase in the order of lipids in the membrane. Likewise, a reduction in GP suggests that the membrane lipids have become more disordered. As shown in Figure 1B, the value of GP was relatively constant from 20 °C to about 28 °C. The value declined steadily from that point to about 46 °C. Above 46 °C, GP values changed little.

Figure 2 displays time courses of laurdan GP upon addition of ionomycin at 20 and 40 °C (parts A and B). At both temperatures, the GP value increased immediately upon addition of ionomycin. The GP value then declined steadily until about 46 °C, at which point it stabilized. The addition of sPLA\(_2\) caused a decrease in GP, indicating an increase in membrane disorder. The decrease in GP upon addition of sPLA\(_2\) was more pronounced at 40 °C than at 20 °C, reflecting the increased membrane disorder at higher temperatures. This result is consistent with the observed decline in GP above 46 °C, indicating a further increase in membrane disorder at this temperature.
addition of the ionophore. In the case of 40 °C, the rise in GP was stable. At 20 °C, the rise was transient, and the steady state value of GP was displaced little from the original value. The unusual kinetics of laurdan changes shown in part A were frequently observed at temperatures below 25 °C. Apparently, either equilibration of ionomycin-induced changes in membrane properties or reequilibration of laurdan following these changes is slow at low temperature. To avoid complications due to these kinetics, all measurements with laurdan as well as hydrolysis experiments involving ionomycin incubation were executed after equilibration was complete (10 min). Hydrolysis of erythrocytes by sPLA2 also appeared temperature-dependent in that hydrolysis of ionomycin-treated cells was minimal at 20 °C compared to 40 °C (Figure 2C,D).

A summary of the complete temperature dependence of laurdan GP (at steady state) and susceptibility to sPLA2 is shown in Figure 3. At low temperatures, the change in laurdan GP upon addition of ionomycin was indistinguishable from the small artifactual change observed with ionomycin in the absence of calcium (Figure 3A; also see legend to the figure). As temperature was raised, the change in GP caused by ionomycin also increased. The amount of phospholipids hydrolyzed by sPLA2 after incubation of the cells with ionomycin also increased. The amount of phospholipids also increased. The amount of phospholipids also increased.

FIGURE 3: Temperature dependence of the change in laurdan fluorescence (A) or hydrolysis (B) in erythrocyte membranes change upon addition of ionomycin. Part A: data were obtained from time courses such as those shown in Figure 2A,B by calculating the value of laurdan GP at the 600 s time point after addition of ionomycin from the nonlinear regression described in Experimental Procedures. Data were obtained in the presence (solid squares) or absence (open triangles) of extracellular calcium. The background change in GP obtained in the absence of calcium was averaged by linear regression and subtracted from all data (background intercept = 0.065 GP units; background slope = −0.0013 GP units °C−1). Part B: data were obtained from time courses such as those shown in Figure 2C,D by calculating the value of ADIFAB GP at the 100 s time point after addition of sPLA2 from the nonlinear regression described in Experimental Procedures. The curve shown in each part was obtained by a fit of the data points to an arbitrary function and has no theoretical basis.

The response chosen for examination was transmembrane migration of phosphatidylserine (23). The average amount of phosphatidylserine exposed in the presence of ionomycin was 92 ± 20% of that exposed upon lysis in liquid nitrogen (mean ± S. E. M., n = 7). Importantly, no trend with temperature was observed (p = 0.999 by linear regression). Hence, the results shown in Figure 3 appeared not to be the consequence of a differential ability of ionomycin to act on erythrocytes as a function of temperature.

Using two-photon excitation microscopy facilitated interpretation of these temperature effects. Figure 4 compares typical images of erythrocytes at 25 and 42 °C before and after ionomycin treatment. The value of GP was higher in untreated cells at 25 °C than at 42 °C (by 0.15 units in the images shown in Figure 4A,C) concurring with Figure 1B. In both cases, regions of higher GP values (orange) were located on the periphery of the cells, consistent with previous accounts (12, 24). Addition of ionomycin elevated the GP of cells modestly at 25 °C (part B compared to part A, change = 0.061 units) and more substantially at 42 °C (part D compared to part C, change = 0.118 units) as expected. Spatially, the increase in GP values included an invasion of the central regions of lower GP (blue and green) by dendritic structures of higher GP. This invasion was most apparent at the higher temperature. This observation was made more apparent at the higher temperature. This invasion was most apparent at the higher temperature.
obvious by considering the spatial frequency of abrupt changes in GP values as shown in Figure 5A, B. This increase in membrane diversity was a consistent observation under conditions that resulted in elevated susceptibility to sPLA2 (Figure 5C). Examination and quantification of additional images such as those in Figure 4 at a variety of temperatures confirmed the trends and interpretations of the data in Figures 1 and 3.

**DISCUSSION**

The level of membrane order in artificial phosphatidylcholine bilayers has profound influence on the susceptibility of that membrane to sPLA2 (3, 4, 6, 7, 11). For example, the adsorption of sPLA2 to the surface of small unilamellar vesicles in the absence of calcium requires the membrane to be in an ordered phase (25). Alternatively, the rate of turnover of substrate by bound enzyme is higher at or above the melting temperature of pure phosphatidylcholine bilayers (6). The tendency of the thermotropic phase transition to promote hydrolysis by sPLA2 in artificial membranes probably relates to dynamic heterogeneity among domains of ordered and disordered lipids present under that condition (11, 26). This assertion is supported by studies in which the ability of contaminating neutral lipids such as lysophospholipid, protonated fatty acid, diacylglycerol, and triacylglycerol to promote hydrolysis of phospholipid bilayers appears linked to the existence of lipid domains in the membrane (5, 6, 8, 9).
Several reports have suggested that human erythrocyte membranes display temperature-dependent changes in membrane properties between 20 and 40 °C (16). For example, the membrane capacitance of intact erythrocytes increases sharply between 30 and 40 °C (14). Likewise, amphotericin B derivatives to permeabilize erythrocyte membranes changes substantially between 20 and 30 °C (15). The data of Figure 1B demonstrate changes in membrane order in the range of 28–46 °C, consistent with these reports. It appeared, then, that human erythrocytes could serve as a useful model to examine whether the sensitivity of sPLA2 to membrane order would apply to biological systems.

It has been proposed that the tendency of ionomycin to cause erythrocyte membranes to become more susceptible to sPLA2 relates to increased membrane order reflected by changes in laurdan GP as calcium enters the cell (12). The observations in Figure 3 support this idea and, in fact, the values for susceptibility and the change in laurdan GP correlated well (p < 0.0008; n = 40). However, if the average value of laurdan GP for the cell was the only issue, then one would expect that simply lowering the temperature to raise GP would render the cells susceptible without requiring ionomycin treatment. As shown in Figure 3B, this did not happen. In fact, cells at lower temperature were less vulnerable to the action of sPLA2 even in the presence of ionomycin. Hence, it appears that it is the change in GP imposed by ionomycin instead of the absolute value of GP that predicts susceptibility to sPLA2. Previous proposals that external exposure of phospholipids from the interior of the cell membrane might be the relevant factor controlling sPLA2 activity (1, 27) are dismissed by the observation that ionomycin-induced phosphatidylserine migration displayed no temperature dependence in the range investigated in Figure 3.

How could the change in membrane order caused by increased intracellular calcium be different from that caused by lowering temperature? A possible explanation is that the effect of ionomycin on membrane order is not uniform, and remnants of the initial state are preserved. The resulting coexistence of these remnants and new regions of increased membrane order could create heterogeneity of structure not present in the untreated cells. If so, then the issue relevant to sPLA2 susceptibility might be enhanced heterogeneity of membrane properties. To address this possibility, we used two-photon fluorescence microscopy to examine the spatial distribution of membrane properties as reported by laurdan GP. Figures 4 and 5 verified that ionophore treatment produced an increase in membrane heterogeneity, especially at elevated temperature. In fact, the argument that increased heterogeneity is the relevant issue for susceptibility to sPLA2 seems to be the most logical explanation for the combined effects of temperature and ionomycin. If the presence of fluid lipids were the only necessity for hydrolysis of erythrocyte membranes, then raising the temperature would have been sufficient to render them susceptible without requirement for ionomycin. Likewise, if an increase in order were the only factor, lowering the temperature would have been sufficient without ionomycin, and the effect of ionomycin treatment would probably not have been temperature-dependent.

The interpretation that these heterogeneities are relevant to increased susceptibility to sPLA2 was supported by two additional observations. First, the progression of hydrolysis upon introduction of sPLA2 appeared to radiate from domains of high GP (Figure 6). This result corroborates earlier observations at 37 °C showing that elevation of GP values during erythrocyte hydrolysis was not homogeneous (12). Second, if these heterogeneities are relevant to the increased susceptibility to sPLA2, they should be present in cells treated with ionomycin at high temperature but absent from untreated cells that have the same average GP at lower temperature. Figure 7 displays such a comparison. The average GP of the cells treated with ionomycin at 40 °C was −0.280. A comparable value was obtained for untreated erythrocytes at 29 °C (−0.295). As shown in the figure, ionomycin-treated cells contained greater GP heterogeneity in the central region of the cell than did untreated cells even though the average GP was similar.

Why might increased heterogeneity of membrane properties promote susceptibility to sPLA2? The answer may lie in considering how sPLA2 interacts with the membrane surface to hydrolyze phospholipids. Several lines of evidence suggest that the action of sPLA2 involves two initial steps (9, 10, 28). The first is adsorption of the enzyme to the membrane surface. In the second, phospholipids migrate from their normal position in the bilayer up into the enzyme active site. The higher rate of substrate turnover observed with artificial membranes in a disordered phase compared to the ordered phase probably reflects the reduction in interactions among phospholipids characteristic of a disordered state. At the interface between domains of the two phases, interactions would be the weakest, and phospholipids would therefore be more likely to leave their normal position in the bilayer to enter the active site of the enzyme. Thus, one would expect the membrane to be most susceptible under conditions that promote the greatest heterogeneity of structure.

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Figure 7: Two-photon excitation microscopy images comparing untreated cells at 29 °C (part A) with those at 40 °C treated with ionomycin (part B). Cells were treated and images acquired as described in Experimental Procedures. The color scheme is the same as for Figure 4.
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