Artificial membranes may be resistant or susceptible to catalytic attack by secretory phospholipase $A_2$ (sPLA$_2$) depending on the physical properties of the membrane. Living cells are normally resistant but become susceptible during trauma, apoptosis, and/or a significant elevation of intracellular calcium. Intact erythrocytes and ghosts were studied to determine whether the principles learned from artificial systems apply to biological membranes. Membrane properties such as phospholipid and/or protein composition, morphology, and microscopic characteristics (e.g. fluidity) were manipulated by preparing ghosts under different experimental conditions such as in the presence or absence of divalent cations with or without ATP. The properties of each membrane preparation were assessed by biochemical and physical means (fluorescence spectroscopy and electron and two-photon microscopy using the membrane probes bis-pyrene and laurdan) and compared with sPLA$_2$ activity. The properties that appeared most relevant were the degree of phosphatidylserine exposure on the outer face of the membrane and changes to the membrane physical state detected by bis-pyrene and laurdan. Specifically, vulnerability to hydrolysis by sPLA$_2$ was associated with an increase in bilayer order apparently reflective of expansion of membrane regions of diminished fluidity. These results argue that the general principles identified from studies with artificial membranes apply to biological systems.

Secretary phospholipase $A_2$ (sPLA$_2$) is an enzyme that hydrolyzes the acyl bond at the sn-2 position of glycerophospholipids, releasing free fatty acids and lysophospholipids. These hydrolysis by-products are precursors for a variety of chemical messengers involved in metabolism, host defense, and signal transduction (1). Secretary PLA$_2$ is of further interest because its involvement in a number of disease states. A few examples of conditions that correlate with an increase in sPLA$_2$ concentration are inflammation (2–4), sepsis (5), and cancer (6). Secretary PLA$_2$ plays a partially unknown, but potentially important role in these disease states. Healthy cells are resistant to hydrolysis by sPLA$_2$. However, under circumstances involving alterations in membrane structure, cells become susceptible to sPLA$_2$. The mechanisms that promote changes in the physical state of the membrane and eventually lead to hydrolysis by sPLA$_2$ are not well understood.

Much of the present knowledge involving the induction of hydrolysis by sPLA$_2$ has been learned from studies using artificial membranes. However, studies with artificial membranes are potentially limiting because they may lack applicability to biological systems. Nevertheless, experiments using vesicles have contributed a number of insights into the possible physical changes needed for induction of sPLA$_2$ hydrolysis. For example, it has been hypothesized that initiation of hydrolysis by sPLA$_2$ consists of two steps (7–13). The first involves adsorption of sPLA$_2$ to the membrane and the second requires movement of phospholipids from their position in the membrane to the active site of the enzyme. Both of these steps are facilitated by certain alterations in the membrane including changes that increase negative charge on the outer leaflet, increase bilayer curvature, diminish phospholipid/neighbor interactions, and promote microheterogeneities in the organization of membrane lipids (8–10, 13–17). It is reasonable to suppose that when a biological cell becomes susceptible to sPLA$_2$ it is because of changes in these properties.

Experiments with cultured cells have led to some information concerning mechanisms that govern susceptibility. However, there are limitations to the analysis that can be performed on the data because of the complexity of cultured cells. Erythrocytes are an intermediate between artificial membranes and biological systems. They lack the complications arising from multiple intracellular membranes and signaling pathways but retain properties generally common to cell membranes such as a diversity of lipid species, asymmetry between the bilayer leaflets, membrane proteins, and a cytoskeleton.

Intact erythrocytes are naturally resistant to hydrolysis by sPLA$_2$. However, microvesicles released by erythrocytes upon addition of a calcium ionophore are immediately hydrolyzed by sPLA$_2$ (18). Bilayer curvature obviously distinguishes erythrocytes and microvesicles, but it is probable that lipid composition, transbilayer asymmetry, protein distribution, cytoskeleton content, and other physical properties vary as well (19–24). Therefore, one or more of these characteristics probably influence susceptibility. Erythrocytes ghosts represent a model in which several of these parameters can be manipulated by preparing the ghosts in the presence of different ions with or without ATP. The purpose of this study was to use such manipulations to determine whether information concerning induction of susceptibility obtained from studies of artificial membranes pertains to biological membranes.

EXPERIMENTAL PROCEDURES

Materials—Snake venom sPLA$_2$ (monomeric aspartate 49 (AppD49) from the venom of Agkistrodon piscivorus piscivorus) was isolated ac-

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cording to published procedures (25). The protein was stored as a lyophilized powder at −20 °C. Stock solutions were suspended at a concentration of 100 μg/mL in 50 mM KCl with 3 mM Na2SO4 as a preservative and stored at 4 °C.

Acetylated-labeled fatty acid-binding protein (ADIFAB), bis-pyrene, and acetylcholine chloride were purchased from Molecular Probes (Eugene, OR). Factor Xa, Factor X, and prothrombin were purchased from Hematologic Technologies, Inc. (Essex Junction, VT). Thrombin substrate II was obtained from Calbiochem (La Jolla, CA). Gluteraldehyde and osmium tetroxide were procured from Ted Pella, Inc. (Redding, CA). All other reagents were from standard sources.

Erythrocyte Ghost Preparation—Blood samples were collected and stored in EDTA Vacutainers from the Brigham Young University Student Health Center. These samples were anonymous surpluses from healthy patients undergoing routine physical exams. Samples were stored overnight at 4 °C and used the next day. Erythrocytes were washed three times in phosphate-buffered saline solution (PBS: 137 mM NaCl, 2.7 mM KCl, 10.6 mM Na2HPO4, 8.5 mM KH2PO4, pH 7.4), suspended to their original hematocrit (0.75 mL) 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, pH 7.4, 37 °C) and used forghost preparation or stored at 4 °C overnight for experiments.

Washed erythrocytes (1.5 mL) were suspended in 10 mL of one of the following buffers: PBS diluted to 1/25 in water (PBS ghosts), 1/25 PBS containing (Mg2+ ghosts), 1/25 PBS containing water, 1 mL CaCl2 (Ca2+ ghosts). After 30 min at 0 °C, 5-fold concentrated PBS alone or containing 1 mM MgCl2, 1 mM MgATP, or 1 mM CaCl2 was added to the corresponding ghosts to restore isotonicity. Next, suspensions were incubated for 45 min at 37 °C to reseal the membrane, and sealed ghosts were collected by centrifugation at 2,500 × g for 10 min. The ghosts were washed in PBS until the supernatant appeared free from hemoglobin. The PBS ghosts, Mg2+ ghosts, and Mg2+-ATP ghosts were then resuspended in MBSS, and the Ca2+ ghosts were resuspended in MBSS without Mg2+ (26) and stored at 4 °C overnight. To determine membrane protein content, samples were lysed by freezing in liquid nitrogen, and membranes were isolated by rapid centrifugation in a microcentrifuge. Membranes were then diluted 1:1 in 0.4 M NaOH and warmed for 30 min at 37 °C. Protein concentration was determined by the method of Bradford (27).

Acetylcholinesterase Activity—Membrane sidedness was determined by acetylcholinesterase activity using an adaptation of the method of Steck and Kant (28). Red blood cell or ghost samples (10 μg of membrane protein) were suspended in 600 μL of 100 mM sodium phosphate (pH 8.0) containing 5 mM sodium phosphate (pH 8.0) or 0.2% Triton X-100 to adjust the volume to 700 μL. Acetylcholine chloride (0.78 mM) and 5,5′-dithiobis-(2-nitrobenzioic acid) (0.63 mM) were added to the mixture, and the reaction optical density was monitored at 412 nm for 90 s after addition of acetylcholinesterase using an adaptation of the method of Ferguson and Ott (29). Following subsequent addition of β-nicotinamide adenine dinucleotide (1 μM), the optical density was measured at 412 nm for 3 min. Membrane sealing was verified by comparing glyceraldehyde-3-phosphate dehydrogenase activity in the absence of Triton X-100 to that observed in its presence.

Phosphatidylserine (PS) Exposure Assayed by Prothrombinase Activity—Exposure of PS was measured using a variation of the method of de Jong and Ott (29). Membrane preparations or erythrocytes were diluted to 3 × 107 cells or ghosts/mL with either MBSS or hypotonic MBSS (10% MBBS diluted in water). The preparations suspended in hypotonic MBSS were then frozen in liquid nitrogen and thawed immediately to lyse the samples before addition to the reaction mixture. These lysed samples were added to the reaction mixture that contained as an effective substrate 10% PS exposure (12 exposure). To quantify the amount of PS exposed in the various samples, factor V (6 ng final), factor X (3 ng final), and 3 × 105 cells/mL were added to 25 μL of buffer containing 10 mM Tris-HCl, 136 mM NaCl, 2.7 mM KCl, 4 mM CaCl2, and 0.5 mg/mL bovine serum albumin at pH 7.9. This reaction mixture was allowed to incubate for 2 min at 37 °C, after which prothrombin (4 μL final) suspended in a solution containing 5.6 mM CaCl2 and 0.5 mg/mL bovine serum albumin was added (final volume = 30 μL). The mixture was incubated 5 min at 37 °C, and the reaction was then stopped by adding the entire mixture to 920 μL of pre-warmed buffer (50 mM Tris-HCl, 120 mM NaCl, 2 mM EDTA, pH 7.5) in a spectrophotometer cuvette. The spectrophotometer was zeroed and 50 μL of thrombin (2000 units/mL) was added. The mixture was then incubated at 0 °C for 4 min. The amount of PS exposure was measured using Fluoromax (Spex Industries) photon-counting spectrophotometer. Band pass was set at 4.25 nm for both monochromators in these experiments. Simultaneous assessment of fluorescence intensity at multiple excitation and emission wavelengths was obtained by rapid slicing of monochromator mirrors using control software provided with the instrument. Laudan anisotropy measurements were obtained in the L-format using a PCI fluorometer from ISS (Urbana, IL) equipped with Glan-Thompson polarizers and 16-nm band pass on both monochromators. Temperature was maintained at 37 °C in all experiments using circulating water baths. Continuous gentle magnetic stirring preserved sample homogeneity in both instruments.

Light Scattering—The amount of light scattering was assayed as a function of wavelength for each preparation by synchronous scanning of excitation and emission wavelengths from 250 to 700 nm with a 0-nm offset and a 0.4-nm band pass. This procedure allowed us to distinguish between variations in size, shape, and concentration. Control experiments with microscopical beads of uniform size indicated that the light scattering intensity below 280 nm normalized to that at 390–350 nm was most useful for determining ghost size. Relative hemoglobin concentration of each preparation was determined by comparing the average normalized light scattering between 408 and 416 nm.

Hydrolysis by sPLA2—Release of fatty acids from cells was assayed with ADIFAB (65 mM final, excitation = 390 nm, emission = 432 and 505 nm; Ref. 30 and 31). The results were quantified by calculation of the generalized polarization (GP) as described (31, 32) and fit to a double exponential equation by nonlinear regression. The amount of hydrolysis at 100 s after addition of sPLA2 (1 μg/mL) was calculated using parameter values from the nonlinear regression results. This value was chosen as a parameter for comparison with the various physical parameters because it provided information related to both the extent and rate of membrane hydrolysis.

Membrane Fluidity—The fluidity of the membrane was determined with the use of laurdan and bis-pyrene (32, 33). Background contribution by light scattering of the individual preparations was subtracted prior to analysis. Laurdan fluorescence (2.5 μM final, excitation = 350 nm, emission = 435 and 505 nm) was used to monitor the overall movement and/or amount of water in the membrane. Laurdan fluorescence was monitored as a function of time after addition of the probe and analyzed by calculating its components of generalized polarization (32). Kinetic parameters for describing laurdan equilibration with various environments in the membrane were estimated by fitting the GP values to a single exponential (see Equation 1 under “Results”) by nonlinear regression. Laurdan anisotropy at 435 nm was used to infer the relative ability of laurdan to rotate in the membrane as described (8). Bis-pyrene monomer emission intensity (1.25 μM final, excitation = 344 nm, emission = 377 nm) was divided by the intensity of bis-pyrene in water to quantify membrane viscosity.

Two-photon Excitation Scanning Microscopy—The distribution of laurdan GP values on ghost membranes was visualized using two-photon microscopy (34). The two-photon excitation images were collected on an Axiovert 35 inverted microscope (Zeiss, Thornwood, NY), with a Zeiss 20X LD-Achroplan (0.4 N.A., air) using a titanium-sapphire laser excitation source (Coherent, Palo Alto, CA) tuned to 770 nm and pumped by a frequency doubled Nd:Vanadate laser (Coherent, Palo Alto, CA). The laser was guided by a galvanometer-driven x-y scanner (Cambridge Technology Watertown, MA) to achieve beam scanning in both x and y directions. A frequency synthesizer (Hewlett-Packard, Santa Clara, CA) controlled the scanning rate of a to acquire a 256 × 256 image. A rapid-scan confocal image was recorded in 0.4 of the field. Dual images were collected simultaneously using a beam-splitter, two emission short-pass filters (centered at about 450 and 500 nm), and two detectors for calculation of GP (32). Laurdan was added to a suspension of ghost preparations as described above. After equilibration, a two-photon image was obtained. Secretary PLA2 was then added, and two-photon micrographs of the same field were acquired at several time intervals thereafter.
**Susceptibility of Erythrocyte Ghosts to sPLA$_2$**

**FIG. 1.** Variability of PS exposure and susceptibility of erythrocytes and ghosts to hydrolysis by sPLA$_2$. Panel A, the proportion of PS exposed on the outer bilayer leaflet for multiple samples of erythrocytes ($n = 10$), Mg$^{2+}$ ghosts ($n = 5$), and Mg$^{2+}$-ATP ghosts ($n = 5$). Data were obtained and calculated as explained under “Experimental Procedures.” Data are expressed as the mean ± S.E. The groups were significantly different by analysis of variance ($p < 0.0001$). Individual differences were identified by a Bonferroni post-test comparing all three permutations of groups ($p < 0.01$ in each case). Panel B, erythrocytes (solid squares), Mg$^{2+}$ ghosts (solid triangles), and PBS ghosts (open circles) were mixed with sPLA$_2$, the time courses of fatty acid release were monitored using ADIFAB, and the data fit by nonlinear regression as described under “Experimental Procedures.” Panel C, the amount of fatty acid produced at 100 s (calculated as described under “Experimental Procedures”) for multiple samples of each membrane preparation ($n = 10$ for erythrocytes, $n = 5$ for each of the ghost preparations).

**Scanning Electron Microscopy**—Samples were prepared for scanning electron microscopy by a modification of Schneider’s method (35). Briefly, the preparations were washed in PBS at pH 7.4. Ten ml of 4 × 10$^6$ cells/ml were incubated in a jar having a 5.5-cm diameter and allowed to settle onto poly-l-lysine-coated cover glasses at 4 °C overnight. Samples were then fixed in 2% glutaraldehyde for 2.5 h. Following fixation, the cells were washed six times in PBS, fixed in 2% osmium tetroxide for 2 h at 23 °C, and washed six more times in PBS. Samples were dehydrated through a graded series of ethanol solutions (10, 30, 50, 70, 95, and 100%) for 10 min each then washed three times in acetone. The slides were then subjected to critical point drying, using liquid carbon dioxide. Finally, samples were sputter coated with gold for 2 min. Images were obtained on a JEOL JSM 840A scanning electron microscope.

The electron micrographs were analyzed using three parameters. These parameters were the membrane size, surface texture, and shape distortion from the original biconcave disc of erythrocytes. Images were arranged randomly and labels removed so that scoring of these parameters could be done without bias. Four raters then visually scored each image for these parameters. The size of the membrane was measured in millimeters along the largest diameter and normalized by the magnification of the picture. The surface texture and the shape distortion were rated on a scale of 1–5 (5 high). The values obtained from each of the raters were averaged. Correlations in scores among raters ranged from 0.72 to 0.99.

**Electrophoresis**—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using 10% pre-cast gels in a mini electrophoresis unit (Mini-PROTEAN 3, Bio-Rad). Buffers and protein samples from ghost preparations and intact erythrocytes were accomplished by one-step analysis of the picture. The surface texture and the shape distortion from the original biconcave disc of erythrocytes. Images were arranged randomly and labels removed so that scoring of these parameters could be done without bias. Four raters then visually scored each image for these parameters. The size of the membrane was measured in millimeters along the largest diameter and normalized by the magnification of the picture. The surface texture and the shape distortion were rated on a scale of 1–5 (5 high). The values obtained from each of the raters were averaged. Correlations in scores among raters ranged from 0.72 to 0.99.

**Statistical Analysis**—The relationships between susceptibility to sPLA$_2$ and the various physical properties were quantified by linear regression. When relationships were significant (i.e. $p \leq 0.05$), the analysis was repeated with removal of data points at the extremes to ensure that overall trends represented real covariance rather than the influence of possible outliers. Also, the analysis was repeated again with only the data from ghosts (no erythrocytes) included. This manipulation ensured that the relationship observed represented true covariance between the property in question and susceptibility rather than a simple difference between erythrocytes and ghosts in general. Only those relationships that remained significant after these manipulations are reported as such under “Results.” Quantitative comparisons among ghost preparations and intact erythrocytes were accomplished by one-way analysis of variance or Student’s $t$ test as appropriate. The combined contribution of more than one physical property to susceptibility was assessed by multiple regression.

**RESULTS**

Erythrocyte ghosts were prepared in which membrane properties hypothesized to be responsible for the level of susceptibility to sPLA$_2$ were varied. For example, we manufactured ghosts by including either Mg$^{2+}$ or Mg$^{2+}$-ATP in the lysing and resealing media in order to control the degree to which the normal phospholipid asymmetry of the bilayer was maintained. The inclusion of ATP would be expected to support the activity of aminodiacylphospholipid translocase and thus maintain the asymmetry (19). As shown in Fig. 1A, these protocols generated ghosts that differed in the amount of PS exposed on the outer leaflet of the membrane. In an attempt to obtain additional variation in membrane properties, we experimented with other preparation conditions such as the absence of divalent cations or with Ca$^{2+}$ instead of Mg$^{2+}$. Fig. 2 demonstrates that these conditions produced different morphological characteristics among the ghost preparations. Importantly, they also generated a series of membrane preparations for which the variation in susceptibility to sPLA$_2$ was high. Fig. 1B displays the time course of hydrolysis of intact erythrocytes and two example ghost preparations by sPLA$_2$. Fig. 1C summarizes the degree of variation in susceptibility among all of the ghost preparations. Not only did the different types of ghosts differ from intact erythrocytes in their susceptibility to sPLA$_2$ ($p = 0.0005$ by analysis of variance), the individual preparations within each category varied substantially from each other. Rather than discard preparations that behaved differently from the norm for a given type of ghost, we elected instead to make use of the variability by comparing membrane properties with susceptibility for each preparation independently. In essence, this choice provided a broad continuum allowing a more complete analysis in which 30 sets of properties could be compared instead of multiple replicates of only five sets.

A series of control experiments were conducted to determine whether the exposure to PS and/or susceptibility to sPLA$_2$ might be attributed to inside-out or leaky ghosts. Acetylcholinesterase activity was used to judge whether the ghosts were oriented outside out. Fig. 3A displays the results of acetylcholinesterase assays on each of the ghost and erythrocyte preparations considered in Fig. 1C. As shown by the small standard errors, the ghost preparations differed little in the amount of activity present. Furthermore, they were identical to native erythrocytes in the level of acetylcholinesterase exposed to the extracellular medium. This result sug-
The average levels of susceptibility in these experiments were shown on the same scale. Panel A, intact erythrocyte; panel B, Ca\(^2+\) ghosts; panel C, Mg\(^2+\) ghosts; panel D, PBS ghosts; and panel E, Mg\(^2+\)-ATP ghosts. The images are shown on the same scale.

FIG. 2. Scanning electron micrograph images representative of the various preparations. Panel A, intact erythrocyte; panel B, Ca\(^2+\) ghosts; panel C, Mg\(^2+\) ghosts; panel D, PBS ghosts; and panel E, Mg\(^2+\)-ATP ghosts. The images are shown on the same scale.

FIG. 3. Tests for sidedness (A) and integrity (B) of ghost membranes. Acetylcholinesterase (A) or glyceraldehyde-3-phosphate dehydrogenase activity (B) was assayed for the various ghost preparations shown in Fig. 1C before and after addition of detergent as described under “Experimental Procedures.” Panel A, data are expressed as the amount of activity prior to membrane lysis divided by the total activity and normalized to the average observed in native erythrocytes. Panel B, the data are expressed as the increase in activity upon lysis divided by the total activity and normalized to the average observed in native erythrocytes. Activity prior to lysis was frequently undetectable.

A possible alternative interpretation of the data in Fig. 3A is that the ghosts were permeable to the assay reagents used. To test this possibility and assess any relationship between membrane leakiness and susceptibility to sPLA\(_2\), the integrity of the ghost membranes was assessed by several means. First, examples of each type of ghost preparation were tested for their permeability to hemoglobin (by absorbance of samples and supernatant at 412 nm). The amount of hemoglobin released from the ghosts following >24 h incubation was 3.5 ± 0.4% (mean ± S.E., n = 8). Experiments were completed within a couple of days of preparation of the ghosts. Nonetheless, ghosts retained their integrity much longer in that hemoglobin retention was stable for more than 1 month. Rarely we did observe that ghosts become leaky after extended storage (i.e. >1 month); however, the tendency to eventually lose membrane integrity was not predictable based on the original level susceptibility to sPLA\(_2\). Second, ghosts were loaded with propidium iodide during preparation, washed, and incubated overnight. The amount of propidium iodide retained by the ghosts was then assessed by incubation with extracellular DNA before and after membrane lysis by freeze-thaw. For each type of ghost preparation, the transmembrane propidium iodide gradient was maintained during overnight incubation. Third, ghosts prepared in Mg\(^2+\) were stored overnight in buffer with or without Ca\(^2+\) to identify whether possible permeability to Ca\(^2+\) might influence the behavior of the ghosts toward sPLA\(_2\). The average levels of susceptibility in these experiments were 0.10 ± 0.02 ADIFAB GP units for ghosts incubated without Ca\(^2+\) added to the storage buffer and 0.11 ± 0.02 ADIFAB GP units for ghosts stored in 1.6 mM Ca\(^2+\) (mean ± S.E., p = 0.77, n = 3 and 7). Therefore, storage with or without Ca\(^2+\) had no influence on the susceptibility of the ghosts. Fourth, the integrity of all of the ghost preparations used in Fig. 1C and the other experiments reported in this paper was assessed by measuring the activity of an intracellular enzyme, glyceraldehyde-3-phosphate dehydrogenase, before and after membrane lysis. The results of these experiments are shown in Fig. 3B. As with the acetylcholinesterase assay, the data for the various ghost preparations were indistinguishable from those obtained for native intact erythrocytes. Nevertheless, since some variation was observed among the samples, we tested whether the observed values for these assays could account for differences in susceptibility to sPLA\(_2\) or exposure of PS among the preparations. No correlation was observed for either susceptibility or the fraction of PS exposed (p > 0.15 in each case, n = 30). Therefore, we were confident that observed variations in susceptibility among the ghost preparations were due to factors other than membrane sidedness or leakiness.

Membrane morphology was considered since bilayer curvature has been shown to be a critical factor in determining whether artificial membranes are resistant or susceptible to catalysis by sPLA\(_2\). For example, small vesicles composed of phosphatidylcholine are immediately hydrolyzed upon addition of sPLA\(_2\), while larger vesicles are only vulnerable after perturbation of the membrane with contaminants (8, 9, 14, 15, 37). Both gross (size and distortion of shape) and fine (surface texture) morphology were scored from micrographs such as those shown in Fig. 2 and compared with the level of susceptibility to sPLA\(_2\) for the various preparations. None of these
properties predicted the level of hydrolytic attack upon addition of sPLA_2 (p = 0.81, 0.84, and 0.52 for size, surface texture, and shape distortion). Size was additionally assessed by light scattering. Comparison of those values with the level of susceptibility verified the result from electron microscopy (p = 0.1).

One obvious distinction between the artificial membranes used previously to study sPLA_2 and biological systems is the presence of membrane proteins and cytoskeleton. It is therefore possible that differences in the vulnerability of the various ghost preparations to sPLA_2 are attributable to variations in protein content. In order to explore this possibility, membrane and cytoskeleton proteins were isolated from each ghost preparation and separated by SDS-polyacrylamide gel electrophoresis. In preliminary experiments, Coomassie Blue staining of the gels reveals the usual pattern of erythrocyte membrane proteins (spectrin, ankyrin, bands 3–7). No obvious differences in the content of these proteins were observed among the preparations. Nevertheless, many proteins do not stain well with Coomassie Blue due to either sensitivity or heavy glycosylation. Since it seemed important to consider all membrane proteins, we chose to focus our analysis on gels that had been developed by silver staining. Densitometric profiles of sample gels from ghost preparations (curves a–d) and erythrocyte membranes (curve e) are displayed in Fig. 4. Due to the large number of proteins visible with silver staining, the individual bands apparent with Coomassie Blue staining are more difficult to discern. The approximate locations of some of those proteins along the gels (based on Coomassie Blue staining) are labeled on the figure.

Each preparation displayed a somewhat different pattern of protein bands on the gels (Fig. 4). To identify which, if any, of these differences could account for the differences in susceptibility, the staining intensity of each of 250 equal segments of the gel was compared with the susceptibility of the corresponding preparation to sPLA_2. In general, no consistent relationships among the 20 samples of ghosts were identified. In a few cases, correlations that appeared significant statistically were observed, but for each of these, the significance was abolished by removal of a single point from the data set. Thus, all apparent relationships observed were excluded based on the criteria explained under "Experimental Procedures." To avoid possible bias based on the choice of the segment of the gels used for normalization, the procedure was repeated four more times using the intensity of a different segment in each case as the denominator for normalization. This repetition validated the interpretation that protein content of the various preparations was not a determinant of susceptibility. A similar result was found when comparing the hemoglobin content (assessed from light scattering spectra) to susceptibility. A significant correlation was identified, but it was abolished when erythrocyte samples were excluded from the analysis (see “Experimental Procedures”).

It is clear from studies with artificial membranes that microscopic physical properties of the membrane are the major factors responsible for the level of susceptibility to hydrolytic attack (8–10, 13–17, 37). Prominent among these properties is membrane surface charge (15, 16). Specifically, membranes that contain excess negative charge are more susceptible to attack because of important structural relationships between the membrane surface and the interfacial recognition region of the enzyme (38–40). Additional properties shown to be relevant include the membrane phase state and dynamics, the strength of phospholipid/neighbor interactions, and the presence of compositional microheterogeneities (8–10, 14, 16, 17, 37). Fluorescent probes such as laurdan and pyrene probes have been useful in delineating some of these relationships (8, 9, 15).

To investigate the possibility that changes in membrane surface charge are involved in determining the susceptibility of red blood cell membranes to sPLA_2, we assayed the degree of exposure of the anionic phospholipid, PS on the membrane surface for erythrocytes and the various ghost preparations using an assay for PS-dependent conversion of prothrombin to thrombin. Figs. 5, A and B, demonstrate that the assay successfully distinguished PS exposed on the outer leaflet from total PS exposed after lysis of the cells by freeze-thaw in hypotonic medium. The degree of exposure was a significant predictor of the level of susceptibility of each corresponding preparation to sPLA_2 (Fig. 5C).

Additional microscopic membrane properties were investigated using the fluorescent probes laurdan and bis-pyrene. Laurdan emission at two wavelengths (435 and 500 nm) was observed as a function of time after addition of the probe. The data were quantified by calculating the generalized polarization value (32). The GP measurement is a function of the degree

![Figure 4. Densitometry spectra of electrophoretic gels containing membrane proteins obtained from the various preparations. Curve a, Ca\(^{2+}\) ghosts; curve b, Mg\(^{2+}\) ghosts; curve c, PBS ghosts; curve d, Mg\(^{2+}\)-ATP ghosts; and curve e, erythrocytes. The positions of some of the major cytoskeleton and membrane proteins identified in Coomassie Blue-stained gels are indicated for orientation. The curves are offset along the ordinate for clarity of presentation.](image)
of solvent relaxation experienced by the excited fluorophore. In general, an increase in the value of GP represents a diminution in the degree and/or rate of solvent relaxation suggesting a decrement in membrane water content and/or membrane fluidity (32). These measurements also allowed us to assess the dynamics of interaction of these probes with the membranes. Membrane order was further assessed by laurdan anisotropy and by the intensity of bis-pyrene monomer emission (33). Possible formation of phospholipid microheterogeneities in the membrane was examined by two-photon excitation scanning microscopy with laurdan as the probe.

The value of laurdan GP displayed time-dependent changes reflecting the interaction of the probe with erythrocyte or ghost membranes (Fig. 6). This slow equilibration of the probe with the membranes appeared to follow first-order kinetics in that it was well fit to the following equation.

\[
GP = a(1 - e^{-\beta t}) + \gamma t + \delta \quad \text{(Eq. 1)}
\]

In order to interpret the four coefficients \((\alpha, \beta, \gamma, \delta)\), we conducted two control experiments. In the first, the value of laurdan GP was measured over time in the absence of cells or ghosts but in the presence of various concentrations of hemoglobin. This experiment provided three important insights. First, laurdan formed micelles upon introduction to aqueous solution under the conditions used in Fig. 6. The value of GP for laurdan in these micelles was \(-0.061\). Second, the presence of hemoglobin interfered slightly with laurdan fluorescence. This interference altered the value of GP causing a small increase at low hemoglobin concentrations and a small decrease at higher concentrations. These effects corresponded well with variations in the initial value of laurdan GP \((\delta)\) observed upon addition of laurdan to suspensions of erythrocytes or ghosts when those variations were compared with a quantitative assessment of the hemoglobin content of each preparation. Therefore, we assumed that \(\delta\) represented the sum of micellar laurdan GP and the effect of hemoglobin. Accordingly, we were able to calculate a correction factor for hemoglobin optical effects by taking the arithmetic difference between \(\delta\) and \(-0.061\). Third, the coefficient \(\gamma\) was a baseline value independent of the presence of membranes and therefore unimportant in the interpretation of the data. In the second control experiment, time courses such as those shown in Fig. 6 were repeated with a subsequent separation of residual laurdan micelles from laurdan partitioned into erythrocyte or ghost membranes by rapid centrifugation in a microcentrifuge. This experiment demonstrated that about 80% of the value of GP at steady state was due to laurdan bound to membranes, and the remainder represented micelles consistent with the expected tight binding of laurdan to lipid membranes (41).

The coefficient \(\alpha\) represents the magnitude of change in GP during binding. Using the value of \(\alpha\) and the information described above, we calculated the value of GP for laurdan bound to the membrane \((GP_m)\) with Equation 2.

\[
GP_m = \frac{(\alpha t + \beta) - (\frac{\alpha}{0.8}) - (\delta - \frac{\alpha}{0.8}) - (0.2t - 0.061)}{0.8}
\]

Fig. 7 reveals that a significant relationship between susceptibility to sPLA2 and \(GP_m\) was observed. The kinetics of laurdan binding given by the coefficient \(\beta\) also displayed a weak relationship. However, this relationship was no longer statistically significant when only the data obtained with ghosts were considered (see "Experimental Procedures").

The positive relationship between laurdan \(GP_m\) and susceptibility suggested that an increase in membrane molecular viscosity could be important for rendering the membrane receptive to attack by sPLA2. To explore this possibility further, we also compared the value of two other parameters sensitive to membrane order: laurdan anisotropy and normalized bis-pyrene monomer fluorescence. As shown in Fig. 8, both measurements also suggested a positive relationship between membrane order and susceptibility to sPLA2. Interestingly, it appeared that bis-pyrene and laurdan fluorescence reported distinguishable properties of the membrane since the values obtained with each probe did not correlate with each other.

Two-photon microscopy allowed direct confirmation of the steady state fluorescence results obtained with laurdan (i.e., Fig. 7). Figs. 9, A and B, display two-photon images of erythrocytes and Mg\(^{2+}\) ghosts as examples of preparations with low and high susceptibility. Histograms representing the frequency distribution of GP values for multiple images such as those shown in Fig. 9, A and B, are exhibited in Fig. 9C. The absolute values of GP were not the same when obtained with the two-photon technique compared with the results shown in Figs. 6 and 7. This is due to differences in the optics of the two detection systems. However, the increase in average GP between erythrocytes and susceptible ghosts was quantitatively similar for both methods.

The two-photon technique also allows one to identify changes in the microheterogeneity of the membrane. Such changes appear as alterations in the number and/or size of domains with distinct physical properties illustrated by the spatial distribution of GP values (34). Inspection of images such as those shown in Fig. 9 reveals that the value of GP was not homogeneous across the surface of the cells and ghosts. This microheterogeneity was verified quantitatively by the breadth of the frequency distribution of GP values (Fig. 9C). Comparison of the frequency distribution did not indicate the presence of gross differences in the degree of membrane heterogeneity between resistant and susceptible membranes. However, repeated imaging of the same cells or ghosts before and after mixing with sPLA2 suggested that membrane heterogeneity may be an important factor in determining loci of membrane attack by the...
enzyme. Fig. 10 displays such a series of images of the same ghost. As illustrated by the arrows, regions of the membrane with higher GP values expanded systematically and became more ordered following sPLA$_2$ addition. Inspection of multiple images such as these confirmed this observation.

Based on the data obtained, three distinguishable factors appeared relevant as determinants of membrane susceptibility to sPLA$_2$: the amount of PS exposed on the outer leaflet, the order of the membrane assessed by bis-pyrene, and the value of laurdan GP$_m$. We used multiple regression analysis to determine whether these factors were sufficient to account for the variation in the degree of membrane hydrolysis catalyzed by sPLA$_2$ among the different preparations and to identify the relative contribution of each. These three parameters were considered together as well as the all the permutations of pairs. Incorporating all three parameters provided the best fit, as one would expect (Table I). The fact that all three parameters contributed significantly to the fit suggested that all three were relevant. The values of the F-statistic and correlation coefficient indicate that these three variables accounted well for the observed data. After adjusting for the number of independent variables, the value of the square of the correlation coefficient was 0.79, suggesting that only 21% of the variation among samples was due to experimental error or unexplained factors. Hence, the $p$ value for the regression was extremely low ($1 \times 10^{-9}$). Since the range of measured susceptibility was 0.2 units (see Fig. 5, for example), the 21% unexplained in our analysis would represent about 0.04 units. The best estimate of the range of random population variation was about 0.02 units based on data from native erythrocyte. Thus, at least half of the 0.04 units for which our model could not account was probably a result of individual and unidentified differences among donors and/or experimental error. In terms of relative contributions to the regression slope, GP$_m$ was the major determinant in that it accounted for almost twice as much of the variation as either of the other two factors.

**DISCUSSION**

Much of the understanding of relationships between membrane properties and susceptibility to sPLA$_2$ has been obtained using artificial membranes of defined composition as models. These numerous studies have identified a broad range of phenomenology about which a few key generalizations can be made. First, for the enzyme to be active on the surface of a membrane, the enzyme must first adsorb to the surface, and phospholipids within the bilayer must then migrate up into the active site of the bound enzyme (7–9, 12–15, 42). Second, changes in membrane physical properties that promote one or both of these events render the membrane more susceptible to catalysis (8–10, 14–17). The properties found to be important for inducing susceptibility can be summarized in two categories: those that promote negative charge at the bilayer surface and those that perturb the interactions among phospholipid molecules (9, 10, 13–17).

The purpose of this study was to use erythrocyte ghosts as simple models to determine whether these same principles apply to biological membranes. Properties that distinguish biological membranes from artificial bilayers were manipulated and compared systematically to the susceptibility of the corresponding membranes to sPLA$_2$. Among the properties evaluated, two appeared related to the level of susceptibility. They were membrane order and exposure of the anionic phospholipid, PS, on the cell exterior. As discussed below, these characteristics correspond to some of those previously found to promote hydrolysis of artificial membranes by sPLA$_2$. Other major properties that distinguish artificial from biological membranes appeared not to be important, i.e. membrane mor-
The increased value of laurdan GP and bis-pyrene monomer fluorescence was normalized to the intensity of emission in water prior to the addition of ghosts. Panel A, \( r = 0.65, p = 0.0001 \). Panel B, \( r = 0.64, p = 0.0001 \).

**Fig. 8.** Relationship between susceptibility to sPLA\(_2\) and membrane order. Panel A, laurdan anisotropy (A) or bis-pyrene monomer fluorescence (B) was measured as described under “Experimental Procedures” for each of the samples in Fig. 1C and compared with the corresponding amount of hydrolysis by sPLA\(_2\). Bis-pyrene fluorescence was normalized to the intensity of emission in water prior to the addition of ghosts.

**Fig. 9.** Distribution of laurdan GP in erythrocytes and Mg\(^{2+}\) ghosts. Panels A and B, two-photon micrographs of erythrocytes (A) and Mg\(^{2+}\) ghosts (B). The colors represent the relative fluidity of each area of the membrane. Blue proceeding through red indicates an increase in membrane order. Panel C, histograms representing the laurdan GP distribution obtained from the two-photon images of the erythrocytes (solid squares; GP = -0.21 ± 0.20, mean ± S.D. for the image) and Mg\(^{2+}\)-ATP ghosts (open circles, GP = 0.018 ± 0.18). The curves represent nonlinear regression of the data using the Gaussian distribution.

**Fig. 10.** Change in laurdan GP distribution during hydrolysis by sPLA\(_2\). Panel A, two-photon micrographs of a Mg\(^{2+}\) ghost before sPLA\(_2\) addition (GP = 0.037 ± 0.20, mean ± S.D. for the image); panel B, 2 min after sPLA\(_2\) addition (GP = 0.035 ± 0.20); panel C, 4 min after sPLA\(_2\) addition (GP = 0.059 ± 0.20); panel D, 10 min after sPLA\(_2\) addition (D; GP = 0.067 ± 0.20).

The conclusion that changes in membrane order play an important role in the susceptibility of the ghosts was based on three types of fluorescence measurements: laurdan GP\(_m\), laurdan anisotropy, and normalized bis-pyrene monomer fluorescence. The increased value of laurdan GP\(_m\) observed with increased susceptibility suggests a decrease in the mobility of membrane water molecules and/or a decrement in the amount of water in the membrane, either of which may be caused by an increase in bilayer viscosity. This interpretation seemed to be validated by anisotropy measurements assessing rotational motion of laurdan in the membrane and by bis-pyrene monomer fluorescence which is sensitive to the translational movement of molecules at the level of the phospholipid acyl chains (Figs. 7 and 8, Ref. 33).

Closer scrutiny of the results with laurdan and bis-pyrene argued that the two probes were not reporting identical phenomena. The correlation in values between the two was very poor \( (r = 0.1, p = 0.6) \) suggesting that the two observations were independent. In agreement with that assessment, both laurdan GP\(_m\) and bis-pyrene monomer fluorescence contributed significantly in the multiple regression analysis, which would not be expected if they were redundant measurements (Table I). Independence of results with these two probes is not unprecedented (8). The basis for the independence of these measurements probably lies in one or both of two distinctions between the biophysics of the probes. First, they assay the ability of molecules to diffuse on two different scales; bis-pyrene assesses diffusion of molecules the size of phospholipids, whereas laurdan assays water movement. Second, the vertical depth reported by the two probes is different with bis-pyrene probably located deep in the region of the phospholipid acyl chains (33) and laurdan positioned more superficially (49). Laurdan anisotropy correlated better with bis-pyrene fluores-
Heterogeneity of structure either through compositional disorder, but rather it is the organization of lipids within the membrane that determines susceptibility, not the fluidity of the membrane per se. This is evidenced by the fact that ordered lipids are more readily hydrolyzed by sPLA2 than disordered lipids. For example, bilayers composed of pure phosphatidylcholine are more vulnerable to attack when the membrane is in a state that involves reorganization and/or altered physical properties of the molecular environment. From this perspective, the similarity in behavior to that observed with artificial systems is striking (8–17, 37, 50).

In summary, we have provided evidence supporting the conclusion that the general biophysical principles that govern the susceptibility of artificial membranes to sPLA2 pertain to biological membranes. This important observation emphasizes the need for future studies. Nevertheless, the results support the interpretation that the capacity of the membrane for enzymatic attack by sPLA2 depends on changes to the membrane structure that involve reorganization and/or altered physical properties of the molecular environment. From this perspective, the similarity in behavior to that observed with artificial systems is striking (8–17, 37, 50).

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Table I

Results of multiple regression analysis comparing susceptibility to sPLA2 and the percentage of PS exposed on the outer membrane face, bis-pyrene monomer fluorescence and the GPm for each of the erythrocyte and ghost preparations.

| R² | N | p | p² | % Contribution |
|----|---|---|----|--------------|
| 0.9 | 30 | | | |
| Intercept | -0.046 | 0.012 | 0.0006 | 27.0 |
| % PS exposed | 0.00089 | 0.00025 | 0.002 | 27.8 |
| Bis-pyrene monomer | 0.018 | 0.0044 | 0.0004 | 45.2 |
| GPm | 0.45 | 0.10 | 0.0001 | 20.2 |

Correlation coefficient for the fit. R² adjusted for the number of variables was 0.79.
Statistical significance of the overall regression by distinguishing total variation, that accommodated by the regression fit, and residual error by analysis of variance.
Regression coefficients in the same units as Figs. 5C, 7, and 8. Statistical significance of each regression coefficient.
Percent contribution to the regression slope when the coefficient is multiplied by the average value of the relevant observed parameter.

"Coefficients" is used to denote the coefficients of regression. The four values listed for each coefficient are: Coefficient, Statistical significance (p), Coefficient square (p²), and percent contribution to the regression slope when the coefficient is multiplied by the average value of the relevant observed parameter.

The two-photon micrographs in Fig. 10 suggest that hydrolysis of monolayers may be sensitive to boundaries around ordered membrane domains as it is in artificial membranes (50). The data do not distinguish whether sPLA2 attacked regions of lower GP causing them to expand or regions of higher GP causing them to contract. Resolution of this issue will require future studies. Nevertheless, the results support the interpretation that the capacity of the membrane for enzymatic attack by sPLA2 depends on changes to the membrane structure that involve reorganization and/or altered physical properties of the molecular environment. From this perspective, the similarity in behavior to that observed with artificial systems is striking (8–17, 37, 50).
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