Kinetic Evaluation of Cell Membrane Hydrolysis during Apoptosis by Human Isoforms of Secretory Phospholipase A₂ *

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Some isoforms of secretory phospholipase A₂ (sPLA₂) distinguish between healthy and damaged or apoptotic cells. This distinction reflects differences in membrane physical properties. Because various sPLA₂ isoforms respond differently to properties of artificial membranes such as surface charge, they should also behave differently as these properties evolve during a dynamic physiological process such as apoptosis. To test this idea, S49 lymphoma cell death was induced by glucocorticoid (6–48 h) or calcium ionophore. Rates of membrane hydrolysis catalyzed by various concentrations of snake venom and human groups IIa, V, and X sPLA₂ were compared after each treatment condition. The data were analyzed using a model that evaluates the adsorption of enzyme to the membrane surface and subsequent binding of substrate to the active site. Results were compared temporally to changes in membrane biophysics and composition. Under control conditions, membrane hydrolysis was confined to the few unhealthy cells present in each sample. Increased hydrolysis during apoptosis and necrosis appeared to reflect substrate access to adsorbed enzyme for the snake venom and group X isoforms corresponding to weakened lipid-lipid interactions in the membrane. In contrast, apoptosis promoted initial adsorption of human groups V and IIa concurrent with phosphatidylserine exposure on the membrane surface. However, this observation was inadequate to explain the behavior of the groups V and IIa enzymes toward necrotic cells where hydrolysis was reduced or absent. Thus, a combination of changes in the adsorption of enzyme to the membrane surface and subsequent binding of substrate to the active site. Results were compared temporally to changes in membrane biophysics and composition. Under control conditions, membrane hydrolysis was confined to the few unhealthy cells present in each sample. Increased hydrolysis during apoptosis and necrosis appeared to reflect substrate access to adsorbed enzyme for the snake venom and group X isoforms corresponding to weakened lipid-lipid interactions in the membrane. In contrast, apoptosis promoted initial adsorption of human groups V and IIa concurrent with phosphatidylserine exposure on the membrane surface. However, this observation was inadequate to explain the behavior of the groups V and IIa enzymes toward necrotic cells where hydrolysis was reduced or absent. Thus, a combination of changes in the adsorption of enzyme to the membrane surface and subsequent binding of substrate to the active site.

A potential consequence of these events during apoptosis is enzymatic attack of the cell membrane by secretory phospholipase A₂ (sPLA₂). Ordinarily, healthy cells resist hydrolysis, but during apoptosis they become vulnerable to destruction by the enzyme (9–11). Studies with snake venom phospholipase A₂ have identified possible ways by which this phenomenon relates to membrane physical properties (8, 9, 12). Preliminary investigations suggest that human groups IIa (hGIIa) and V (hGV) isoforms may also distinguish healthy and apoptotic cells, although the details of how they do so are uncertain (11, 13, 14). The response of the human group X (hGX) isoyme to apoptosis has not yet been studied.

Hydrolysis of artificial membranes by sPLA₂ involves two precatalytic steps (Scheme 1 (15, 16)). The relationship of each step to membrane behavior varies among the different isoforms (17–20). A prominent example is the degree to which initial adsorption (step 1) depends on the presence of negative charge at the membrane surface (18, 21, 22). For instance, an anionic membrane surface appears required for pancreatic and hGIIa sPLA₂ (18, 23). For hGV, the presence of a tryptophan residue at the interfacial binding surface of the enzyme diminishes this requirement and allows some adsorption to a zwitterionic interface (17, 24). In the case of the hGX enzyme, there appears to be a smaller or perhaps no requirement for an anionic interface (18, 19). For snake venom sPLA₂ from Agkistrodon piscivorus piscivoras, either or both steps may be limiting depending on the physical state of the membrane (15).

This diversity of behaviors among sPLA₂ isoforms suggests that they would respond differently to membrane changes induced by apoptosis. For example, migration of phosphatidyl-
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\[
E + M_S \quad \stackrel{K_d}{\rightleftharpoons} \quad E_B \quad \stackrel{K_\text{cat}}{\rightarrow} \quad E_B^S \quad \stackrel{k_\text{cat}}{\rightarrow} \quad E_B + P
\]

SCHHEME 1. Reaction scheme for interaction between sPLA₂ and cell membranes. \(E\), free sPLA₂; \(E_B\), sPLA₂ adsorbed to membrane surface sites (\(M_S\)); \(S\), available substrate (membrane phospholipid); \(E_B^S\), adsorbed enzyme with substrate bound to the active site; \(K_d\), equilibrium constant for enzyme adsorption to the membrane; \(K_\text{cat}\), equilibrium constant for substrate migration into the enzyme active site; \(k_\text{cat}\), catalytic rate constant for substrate hydrolysis; \(P\), product (fatty acid).

serine from the inner to outer surface of the cell membrane during apoptosis is an obvious means by which sPLA₂ isoforms sensitive to negative charge might be capacitated to hydrolyze the membrane (25). Recent investigations have revealed additional changes such as increased interlipid spacing that may influence other isoforms (9). This study is designed to explore sPLA₂ species that differ in their membrane requirements and compare their response to bilayer physical properties associated with calcium ionophore and glucocorticoid-initiated apoptosis. Three human isoforms are included (hGIIa, hGV, and hGX). Snake venom (A. p. piscivorus) sPLA₂ (AppD49) is used as a standard for internal comparison to previous studies.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The monomeric aspartic 9 phospholipase A2 from the venom of A. p. piscivorus was isolated according to the procedure of Maraganore et al. (26). The following recombinant human sPLA₂ isoforms were prepared as described previously: hGIIa (27, 28), hGV (29), and hGX (19). The hG enzyme was generously provided by Dr. Wonhwa Cho (University of Illinois, Chicago). Dexamethasone and ionomycin were dissolved in dimethyl sulfoxide (DMSO). Acrylodan-labeled fatty acid-binding protein (ADIFAB), propidium iodide, cell culture medium, and serum were acquired from Invitrogen.

**Cell Culture and Glucocorticoid Treatment**—S49 mouse lymphoma cells were grown as a suspension culture at 37 °C in humidified air containing 10% CO₂ as explained (30). Samples treated with dexamethasone received the drug (100 nM final) 6–48 h before harvesting. Control samples received a corresponding volume of DMSO (0.2% v/v).

**Membrane Hydrolysis**—For hydrolysis assays, cells were harvested by gentle centrifugation, washed, and suspended (0.4–3.5 × 10⁶ cells/ml) in a balanced salt medium (MBSS: NaCl = 134 mm, KCl = 6.2 mm, CaCl₂ = 1.6 mm, MgCl₂ = 1.2 mm, Hepes = 18.0 mm, and glucose = 13.6 mm, pH 7.4, 37 °C). Samples were transferred to quartz fluorometer sample cells and equilibrated for at least 5 min in a spectrofluorometer (Fluoromax 3, Horiba Jobin Yvon, Edison, NJ). Temperature and sample homogeneity were maintained using a water-jacketed sample chamber equipped with magnetic stirring and attached to a circulating water bath. All experiments were performed at 37 °C.

The acrylodan-derivatized fatty acid-binding protein, ADIFAB, was used to assay the release of fatty acids from cell membranes in real time. Data were acquired from cell samples 100 s (excitation = 390 nm, emission = 432 and 505 nm, bandpass = 4 nm) before adding ADIFAB (65 nM final) to assay background intensity. After the addition of ADIFAB and stabilization of the fluorescence intensity (about 300–500 s), one of the four sPLA₂ isoforms was added (0.07–70 nM final), and the time course was continued for an additional 800–2000 s. Fatty acid release was estimated by transforming the raw intensities to generalized polarization values (GP) and then fitting to an arbitrary function by nonlinear regression as described (31).

\[
\text{ADIFAB GP} = \frac{(I_{505} - I_{432})}{(I_{505} + I_{432})} \quad (\text{Eq. 1})
\]

\(I_{505}\) and \(I_{432}\) are the fluorescence emission intensities at 505 and 432 nm. In experiments involving calcium ionophore, ionomycin (300 nM final) or the equivalent diluent (0.25% DMSO) was included in samples with ADIFAB for at least 300 s before adding sPLA₂.

The intensity of propidium iodide fluorescence was used to quantify the fraction of cells susceptible to hydrolysis by sPLA₂ in samples treated with dexamethasone or equivalent DMSO. Cells were harvested, incubated, and mixed with sPLA₂ isoforms as explained above for ADIFAB experiments with differences: propidium iodide (37 μM final) was included instead of ADIFAB, fluorescence intensity was assayed at 617 nm (excitation = 536 nm), and ionomycin was added at the end of the time course to render all the cells hydrolyzable and, thus, provide a maximum signal for internal comparison (9). Data were analyzed by nonlinear regression and by quantifying the various subpopulations as described previously (9). The subpopulation of interest for this study was that in which cells were still alive (i.e. excluded propidium iodide) but susceptible to sPLA₂ (i.e. incorporated the dye upon the addition of sPLA₂). This subpopulation was quantified by calculating the difference in propidium iodide fluorescence intensity before and after the addition of sPLA₂ and normalizing to the maximum fluorescence change observed in samples treated with both AppD49 sPLA₂ and ionomycin. Data were corrected for variations in cell number using direct cell counts (by light microscopy) and light scatter intensity. Because the emission spectra of ADIFAB and propidium iodide do not overlap, the fluorescence of the two probes was assayed simultaneously for some experiments (Fig. 1) as described previously (30).

**Flow Cytometry**—These experiments were done in parallel with spectrofluorometric measurements of hydrolysis and propidium iodide uptake. Cells were washed and suspended in MBSS. After washing, aliquots of the control and treatment samples were transferred to flow cytometry sample tubes and incubated for 5 min with propidium iodide (10 μM final). Cell subpopulations were then identified based on the level of fluorescence intensity using a BD FACSCanto flow cytometer (BD Biosciences) with excitation at 488 nm, and emission was detected in the range of 564–606 nm.

**RESULTS AND DISCUSSION**

Are Subpopulations of Cells Hydrolyzed Differentially?—Fig. 1A displays a time course of hydrolysis of a sample of S49 cells by extracellular AppD49 sPLA₂. As is typical for healthy untreated samples, a small amount of fatty acid (and lysophospholipid) was produced upon the addition of sPLA₂ followed by a gradual return to base line as the reaction ended, and the products were salvaged by reacylation (13). This transient burst
of hydrolysis was accompanied by a small increase in the permeability of the cells to the fluorescent dye propidium iodide (Fig. 1B). Normally, healthy cells exclude the dye, but when their membranes are damaged by hydrolysis, the dye enters the cells and binds to DNA and emits with higher intensity (10). This small rise in propidium iodide fluorescence, thus, represents cells that are initially alive (impermeable) but are then killed by the action of the phospholipase (labeled Alive & Susceptible in the Fig. 1 inset). Although the increased intensity in Fig. 1B was very small, it was consistent with previous studies comparing healthy and apoptotic cells (10). In contrast, when ionomycin was subsequently introduced into the sample, more extensive hydrolysis was observed, as indicated by a large elevation of free fatty acid (Fig. 1A) and propidium iodide emission (Fig. 1B). The enhanced enzymatic activity and resulting membrane damage upon the addition of ionomycin represented a hydrolytic attack of 100% of the cells as the level of propidium iodide fluorescence could not be further increased by subsequent incubation of the sample with a detergent (Triton X-100, 0.25% v/v).

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Interpretation of the experiments in this study requires that we determine whether the data in Fig. 1A represent uniform levels of hydrolysis among all the cells in the sample or extensive sensitivity of a small subpopulation of vulnerable cells. This question is of particular concern for experiments involving sPLA₂ because the products of hydrolysis from one cell may be able to diffuse to another cell and induce hydrolysis as has been observed with artificial membranes (32). The matter was addressed by analysis of hydrolysis time courses in the context of flow cytometry data gathered on the same samples as explained below (Figs. 1, C and D).

Previously, the transient burst of activity in control samples (Fig. 1A) was assumed to represent minor hydrolysis of all cells. To account for the complete cessation of hydrolysis even though substrate was restored by reacylation, a model was created in which cells become refractory after initial exposure to sPLA₂ (13). However, an alternative model in which a minority of the cells is susceptible to the enzyme and the remainder is resistant can also account quantitatively for the result if two assumptions are made. First, hydrolysis ceases because the membranes of vulnerable cells have been consumed. Second, uptake and reacylation of reaction products is accomplished by the remaining healthy cells in the sample.

To distinguish these possibilities, we used flow cytometry to separate healthy cell samples into subpopulations based on propidium iodide permeability (Fig. 1C). Data were then compared with and without sPLA₂ treatment to see whether all the cells were uniformly affected by the enzyme or whether certain subpopulations were preferentially altered. As shown in Fig. 1C, three subpopulations were identified as peaks in the flow cytometry histogram. Peak 1 represented cells that did not stain with propidium iodide. Peak 2 represented cells that displayed very low permeability to propidium iodide, an observation described for thymocytes early during apoptosis and necrosis (33, 34). Although the fluorescence intensity of these cells was 10 times that of background (Peak 1), it represented only 1% that observed for cells that were fully permeable to the dye (Peak 3). The complete permeability of the subpopulation shown in Peak 3 was confirmed by comparison to samples treated with the detergent Triton X-100 (0.1% v/v). Traditionally, cells arriving spontaneously in Peak 3 would be considered necrotic (34).

Figure 1: Quantification of the cellular basis for sample hydrolysis under control conditions. Panel A, hydrolysis of S49 cell membranes was assayed using ADIFAB as described under “Experimental Procedures.” The amount of hydrolysis was estimated by calculating the ADIFAB GP (Equation 1) and expressing the results as a percentage of the maximum ADIFAB GP change observed in the presence of ionomycin and the AppD49 sPLA₂ (plateau after the second dotted line). Dotted lines represent the addition of AppD49 sPLA₂ (70 nM) then ionomycin (300 nM). Panel B, propidium iodide uptake (fluorescence intensity) was assayed simultaneously with the ADIFAB fluorescence from Panel A. The inset narrows the intensity range for the first 1000 s. Panel C, cell subpopulations in a companion sample were identified by flow cytometry. Peak areas were quantified by nonlinear regression using sums of multiple Gaussian distributions. Solid black curve, control sample; dashed red curve, parallel sample incubated 15 min with 70 nM AppD49 sPLA₂. The inset shows the details of Peak 2. Panel D, the experiments of panels A and B were repeated for a total of six samples. Fatty Acid represents the height of the initial hydrolysis burst (before the addition of ionomycin) in panel A. Stressed Cells represents the sum of the percentage of cells incorporating propidium iodide at the first dotted line in Panel B (Alive & Susceptible) plus the areas of Peaks 2 and 3 in Panel C. The means of the two bars were compared by paired Student’s t test (p = 0.74).
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Previous studies have suggested that necrotic cells are fully susceptible to hydrolysis by sPLA$_2$ (8, 13, 30). This interpretation was confirmed indirectly using samples that contained varied proportions of necrotic cells (6–81%). Aliquots of these samples were analyzed by flow cytometry in parallel with measurements of hydrolysis using ADIFAB. A strong very correlation was observed with respect to the amount of hydrolysis and the area of Peak 3 in the flow cytometry histogram ($r^2 = 0.93$, $p < 0.0001$, $n = 9$). Moreover, the slope of the regression line was $0.91 \pm 0.09$, suggesting that the majority of the hydrolysis observed could be accounted for by the fully-permeable, necrotic cells of Peak 3.

We were able to assess the hydrolytic susceptibility of the cells in Peak 2 more directly because these cells were only modestly permeable to propidium iodide, and hydrolysis would, therefore, have a distinct measurable effect by making the subpopulation more permeable. Accordingly, the area defined by this part of the histogram was compared before and after treatment with sPLA$_2$. As shown by the dashed curve in Fig. 1C, Peak 2 was reduced dramatically after exposure to sPLA$_2$ ($p = 0.002$, $n = 19$), presumably because the cells had become permeabilized by the enzyme and, thus, shifted into Peak 3.

It is likely that the shallow slope of the time course in Fig. 1B represents the low permeability of the cells designated by Peak 2. If this is the case, the slope should be reduced after treatment with sPLA$_2$; depletes the subpopulation. In fact, the slope of the time profile was reduced by 35% ($p = 0.02$, $n = 6$) upon the addition of sPLA$_2$, again suggesting that the small burst of propidium iodide fluorescence seen in the inset of Fig. 1B represents hydrolytic attack of this tiny subpopulation.

The large population of healthy impermeable cells in Peak 1 was also analyzed as described for Peak 2. In this case, the subpopulation was mostly unaffected by sPLA$_2$; the area of Peak 1 was identical regardless of the presence of the phospholipase (Fig. 1C, $p = 0.21$, $n = 19$). Nevertheless, the mode of the peak was shifted slightly (7% increase in propidium iodide intensity compared with the 1000-fold increase when cells become fully permeable) but reproducibly ($p = 0.006$, $n = 19$). This result suggests that the enzyme probably does hydrolyze lipids of healthy cells but that the level is extremely small compared with the attack of the Peak 2 and Peak 3 subpopulations.

To determine whether hydrolysis of these two apparently vulnerable subpopulations could account quantitatively for the transient fatty acid release observed from ADIFAB data, we added the area of Peaks 2 and 3 and compared that sum to the size of the hydrolysis data. As shown in Fig. 1D, the size of these two subpopulations and the amount of hydrolysis observed in aliquots from the same samples were indistinguishable ($p = 0.74$, $n = 6$). Even though these results are based on correlation, they demonstrated that a simple model can account quantitatively for the observation of transient hydrolysis rather than having to invoke the complex ideas proposed previously (13). Therefore, we concluded that the hydrolysis of healthy cell samples by sPLA$_2$ as observed with ADIFAB fluorescence, reflects the action of the enzyme on small vulnerable subpopulations without adverse impact on the remaining cells.

Treatment of S49 cells for 18 h with dexamethasone produced a level of hydrolysis by AppD49 sPLA$_2$ intermediate between that observed in control versus ionomycin-treated samples (Fig. 2). As in Fig. 1A, subsequent addition of ionomycin induced hydrolysis of the remaining sample. Hence, as in the control samples, hydrolysis in apoptotic cells appeared confined to a sensitive subpopulation. Treatments, then, that enhance hydrolysis do so by increasing the size of the pool of susceptible cells.

Action of Human sPLA$_2$ Isoforms toward Apoptotic S49 Cells—Fig. 3 displays time courses of hydrolysis of S49 cell phospholipids by extracellular AppD49 (panel A), hGX (panel B), hGV (panel C), or hGIIa sPLA$_2$ (panel D) under control conditions (black curves). The data are expressed as a percentage of the maximum potential amount of fatty acid released as explained for Fig. 1. The hGV, hGX, and AppD49 isoforms each catalyzed a modest release of fatty acid. In contrast, no change hydrolysis was detected upon the addition of hGIIa (panel D).

Exposure of the cells to ionomycin produced an increase in both the rate and amount of hydrolysis by the hGV and hGX isoforms but not hGIIA (Fig. 3, red curves). The extent of these increases depended upon the isoform and was always less than that observed with the AppD49 enzyme (AppD49 > hGX > hGV >> hGIIA = 0). As shown in Fig. 4, incubation of the cells with the glucocorticoid dexamethasone for 24 h also rendered the cells susceptible to hydrolytic attack compared with cells treated with control vehicle only. In contrast to ionomycin treatment, dexamethasone was effective at causing hydrolysis of the cells by the hGIIA isoform (Fig. 4D).

Fig. 5 illustrates the effect of variation in enzyme concentration on the initial hydrolysis rate from experiments analogous to those of Figs. 3 and 4. The purpose of these experiments was to address the question of which steps from Scheme 1 account for the increased activity observed during apoptosis. Based on Figs. 1 and 2, the various experimental conditions used for Figs. 3–5 can be described in terms of the proportions of two subpopulations of cells: the resistant healthy subpopulation and the susceptible subpopulation. The observed initial hydrolysis rate ($dP/dt$), then, is the sum of the rates pertaining to each subpopulation (Res and Sus) weighted by the fraction susceptible ($f_{sus}$).

$$\frac{dP}{dt_{observed}} = (1 - f_{sus}) \frac{dP}{dt_{res}} + f_{sus} \frac{dP}{dt_{sus}}$$

(Eq. 2)
Explicit generic descriptions of $dP/dt$ that can be applied to each subpopulation are derived as described previously (8, 12),

$$\frac{dP}{dt} = k_{cat}E^s_g$$  \hspace{1cm} (Eq. 3)

Adsorbed enzyme with substrate occupying the active site ($E^s_g$) can be defined in terms of total added enzyme ($E_g$) and adsorption site ($M_{ST}$) concentrations by considering the equilibrium constants and law of conservation of mass.

$$K_A = \frac{E_g}{(E_g)(M_{ST})}$$  \hspace{1cm} (Eq. 4)

$$K_{E^s} = K_{E^s}/M_{ST} = \frac{E^s_g}{E_g}$$  \hspace{1cm} (Eq. 5)

$$M_{ST} = M_s + E_g + E^s_g$$  \hspace{1cm} (Eq. 6)

Substitution of Equations 4–6 into Equation 3 yields the complete generic relationship

$$\frac{dP}{dt} = \frac{k_{cat}M_{ST}K AE}{1 + K_{E^s}/(1 + K_{E^s})^2}$$  \hspace{1cm} (Eq. 7)

Equations 4–7 assume that the experimental conditions are designed so that most of the enzyme added remains free in solution (i.e. $E \approx E_g$). This assumption was verified by analysis of binding studies conducted previously with AppD49, the isoform that adsorbs most tightly to the membrane among those tested here (8). In those experiments, the amount of enzyme adsorbed to the cell surface was negligible compared with the amount that was free.

$K_A$ and $K_{E^s}$ are the only constants in the equation that would be expected to vary depending on membrane structure as $k_{cat}$ is a property of the enzyme relevant only after substrate has bound to the active site and as the total number of cells ($M_{ST}$) and enzyme concentration relate to the entire sample ensemble. Hence,

$$\frac{dP}{dt} = k_{cat}M_{ST}(1 - f_{Sus})\frac{K_A E^s_g}{1 + K_{E^s}/(1 + K_{E^s})^2}$$  \hspace{1cm} (Eq. 8)

where $K_{A^*}$ and $K_{E^s}$ are the values of $K_A$ and $K_{E^s}$ for the cells that have become susceptible to sPLA$_2$.

Notwithstanding the apparent complexity of Equation 8, the outcomes are straightforward depending on whether it is step one ($K_A^* > K_A, K_{E^s}^* = K_{E^s}$) or step two ($K_A^* = K_A, K_{E^s}^* > K_{E^s}$) that is rate-limiting and, therefore, responsible for low activity in healthy cells. As shown in Fig. 6, the distinction can be made by comparing different treatments in which the proportion of cells susceptible to sPLA$_2$ varies. Panel A displays the expected result when susceptibility induced by apoptosis relieves low adsorption affinity by increasing $K_A$ 10-fold. The effect can be approximated by fitting the data to a traditional rectangular hyperbola function analogous to a binding isotherm or a Michaelis-Menten equation.

As suggested by the appearance of the curves, $EC_{50}$ varies by a factor of 5, whereas $v_{max}$ remains nearly constant (varies by only 16%) in this case. Conversely, if step 2 is rate-limiting and apoptosis relieves that limitation by increasing $K_{E^s}$ (without a change in $K_A$), $v_{max}$ increases proportional to the fraction of cells that have become susceptible (panel B), whereas $EC_{50}$ stays essentially identical (varies by only 4%).

Accordingly, the data of Fig. 5 were fit by nonlinear regression to Equation 9. In each case, the regression was conducted as a global analysis for the three treatment groups (blue, DMSO control; red, dexamethasone; yellow, ionomycin). The regression performed three different ways. First, the value of $EC_{50}$ (solid colored curves in Fig. 5) was shared across the entire data set to see whether the data were accommodated when only $v_{max}$ was allowed to vary. Second, the value of $v_{max}$ (dashed colored curves in Fig. 5) was shared across the entire data set, allowing $EC_{50}$ to float. Third, the values of both $EC_{50}$ and $v_{max}$ were left unconstrained to obtain an optimal fit as a standard for the other two.

The data with the AppD49 enzyme were fit better by varying $v_{max}$ instead of $EC_{50}$ (Fig. 5A). Quantitative comparison to the standard optimal fit (black curves in Fig. 5A) revealed that the correlation coefficient for the fit with $v_{max}$ unconstrained (solid-colored curves) was 48-fold closer to the standard fit than was the fit with $EC_{50}$ unconstrained (dashed colored curves). When $v_{max}$ was allowed to float, its value varied significantly (95% confidence intervals: control, 0.014–0.018; dexamethasone, 0.028–0.032; ionomycin, 0.080–0.11 GP units$^{-1}$) and was greatest for ionomycin-treated samples. Thus, induction of apoptosis appeared not to affect the ability of the AppD49 enzyme to adsorb (since saturation was reached at the same concentration for each treatment). Instead, the increased $v_{max}$ in proportion to the number of cells engaged in cell death under these conditions (control < dexamethasone < ionomycin = 100%) suggested that the second step in Scheme 1, substrate migration into the enzyme active site, was the one most facilitated by apoptosis. This conclusion substantiates interpretations reached previously for the AppD49 enzyme in model systems, human erythrocytes, and S49 cells treated with ionomycin (8, 12, 14, 15, 31).

Human group X sPLA$_2$ gave results qualitatively identical to the AppD49 enzyme (Fig. 5B). The correlation coefficient was 8-fold closer to the optimal standard fit when $v_{max}$ was unconstrained instead of $EC_{50}$ and $v_{max}$ values differed significantly (95% confidence intervals: control, 0.004–0.007; dexamethasone, 0.009–0.010; ionomycin, 0.012–0.019 GP units$^{-1}$). Therefore, hGX apparently also adsorbed well to the membrane surface regardless of the experimental treatment. Apoptosis presumably improved hydrolysis by enhancing substrate access to the enzyme active site, as for AppD49.

We note that a result similar to that seen for AppD49 and hGX would also be obtained if apoptosis promoted the adsorption step in the extreme; that is, negligible adsorption to healthy cells but saturation of treated cells. However, several lines of evidence argue against this alternative. Binding experiments...
In contrast to AppD49 and hGX, the data for the hGV isoform were better fit by Equation 9 when $E_{C50}$ was varied rather than $V_{max}$ (dashed curves Fig. 5C). In this case, the correlation coefficient was 18-fold closer to the optimal standard fit when $E_{C50}$ was unconstrained. The value of $E_{C50}$ varied from 154 nM (95% confidence interval = 150–158 nM) in control samples to 87 nM (76–98 nM) in ionomycin-treated samples and reached 24 nM (10–38 nM) for cells treated with dexamethasone. Thus, the ability of the hGV isoform to adsorb to the membrane was increased during apoptosis.

The data for hydrolysis by hGIIa did not match the functional form of Equation 9, as illustrated by the upward concavity of the curves (Fig. 5D). The lack of a plateau in the data indicated that adsorption of the enzyme was weak and that saturation of the cell surface was not achieved at any of the concentrations tested. Nevertheless, the enzyme responded with the highest potency to cells treated with dexamethasone, analogous to the behavior of hGV. This result also suggests that there was virtually no adsorption of hGIIa to untreated cells as has been suggested previously (35) and that ionomycin treatment did little or nothing to alter that condition.

**Relationship to Physical Changes in the Membrane**—The underlying assumption in these investigations is that changes occur in the composition and/or physical structure of the cell membrane during apoptosis that facilitate whatever step(s) in Scheme 1 is limiting in healthy untreated cells. Because different steps appear critical for different isoforms, it is also likely that the various enzymes are also sensitive to different physical membrane properties. Therefore, the timing of onset of hydrolysis during apoptosis should vary among the isoforms and correspond temporally with the relevant physical changes. Accordingly, we compared the rate of hydrolysis (Fig. 7) and fraction of cells still alive but susceptible to sPLA$_2$ (Fig. 8) as a function of treatment time with dexamethasone for each of the isoforms. These time profiles were then compared with the timing of previously mea-

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**FIGURE 3.** Hydrolysis of S49 cell membranes by different sPLA$_2$ isoforms in the presence and absence of ionomycin. S49 cells were incubated with ADIFAB and treated for at least 300 s with DMSO (black curves, 0.25% v/v, control solvent for drugs) or ionomycin (red curves, 300 nM) before the addition of sPLA$_2$ (dotted lines) as in Fig. 1. A, 35 nm AppD49; B, 35 nm hGX; C, 35 nm hGV; D, 70 nm hGIIa.

**FIGURE 4.** Effects of dexamethasone treatment on the time course of membrane hydrolysis by sPLA$_2$. The experiments of Fig. 3 were repeated with cells that had previously been treated for 24 h with dexamethasone (100 nM) instead of DMSO or ionomycin. The dotted lines indicate the addition of sPLA$_2$. A, 35 nm AppD49; B, 35 nm hGX; C, 35 nm hGV; D, 70 nm hGIIa.

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have demonstrated that the AppD49 enzyme adsorbs equally well to treated and untreated cell membranes (8, 12). Likewise, previous studies have observed high affinity adsorption of hGX sPLA$_2$ to zwitterionic artificial membranes and to non-apoptotic cells (18, 21, 35). Thus, the interpretation that step two is limiting for these isoforms seems to be the most plausible explanation for the data.
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FIGURE 5. Dependence of the initial rate of hydrolysis on sPLA₂ concentration. The experiments of Figs. 3 and 4 were repeated at the indicated concentrations of sPLA₂ for cells treated with DMSO (blue symbols and curves), ionomycin (yellow), or dexamethasone (red). The initial rates were calculated from the amount of product generated during the first 5 s (AppD49 (A)), 20 s (hGX (B)), or 50 s (hGV (C) and hGIIa (D)) after sPLA₂ addition (n = 1–14). Error bars represent the S.E. (when n > 2) or range (when n = 2). Data in Panels A–C were fit by nonlinear regression to Equation 9 with the constraint that either the parameter value for EC₅₀ (solid colored curves) or vₘₐₓ (dashed colored curves) was shared and, therefore, constant among the three data sets. The black curves in panels A–C depict fits in which neither EC₅₀ nor vₘₐₓ was constrained.

FIGURE 6. Theoretical description of kinetic behavior of sPLA₂ depending on whether apoptosis promotes step 1 (panel A) or step 2 (panel B). Data were calculated using Equation 8 for values of fₜₐₐₜ corresponding to susceptible subpopulations representing 90, 50, or 10% of the cell sample. In panel A, kₐ = 0.01 nM⁻¹ s⁻¹, kₕ = 0.1; in panel B, kₐ = 0.1 nM⁻¹ s⁻¹, kₕ = 0.01. The other parameter values were kₜₐₜ = 1 units⁻¹, Mₛₜ = 1000 arbitrary units, Kₐ* = 0.1 nM⁻¹, and Kₕ* = 0.1.

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enzyme (8, 12, 14, 15, 31) and suggest that the behavior of hGX is the same as that proposed for the venom as follows. 1) Catalytic activity toward cell membranes is limited by step 2 in Scheme 1. 2) This limitation is relieved by diminished interactions among neighboring phospholipids, which facilitate upward migration of phospholipids into the active site of adsorbed enzyme. 3) Anionic phospholipids are not required for enhanced hydrolytic activity during apoptosis, but they help. 4) A highly ordered membrane impairs activity.

In contrast, reduced interlipid interactions (blue curve in Fig. 9B) were either irrelevant or insufficient to explain enhanced activity of the hGIIa and hGV isozymes. Exposure of phosphatidylserine (green curve), however, corresponded exactly with the enhanced activity until membrane order began to change (red curve). This result matched well with the observation that the activity of these isozymes seemed limited by their affinity for the membrane surface (step 1 in Scheme 1) and because in vitro studies indicate that these enzymes have a strong (hGV) or absolute (hGIIa) requirement for negative charge on the membrane surface (17, 18, 21, 25, 35–37). As with the other isozymes, the highly ordered cell membranes present during the late phase of apoptosis appeared to inhibit hydrolytic activity and override the beneficial effect of phosphatidylserine exposure.

In contradistinction to dexamethasone, ionomycin treatment immediately (within 10 min) induces 100% of the cells to experience both the reduction in lipid-neighbor interactions and maximal exposure of phosphatidylserine (10). This result seems adequate to explain the high level of hydrolysis observed with the AppD49 and hGX enzymes toward ionomycin-treated cells (i.e. as in Figs. 1, 3, and 5). However, the observation that ionomycin treatment had essentially no effect on hydrolysis by hGIIa and a weak effect on hGV was a complete surprise. As argued in the previous paragraph, the observations with dexamethasone treatment strongly supported the hypothesis that hydrolysis by these enzymes is capacitated by exposure of phosphatidylserine on the cell surface. Thus, one would have expected ionomycin treatment to stimulate both isozymes strongly as it produces maximum exposure of phosphatidylserine (i.e. twice that observed at 24 h with dexamethasone). Clearly, some unidentified membrane event associated with ionomycin treatment impairs the binding and activity of hGIIa and hGV, or some additional event during glucocorticoid treatment is required beyond phosphatidylserine exposure.

The nature of this implied difference between the membrane behavior provoked by glucocorticoid and calcium ionophore
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Surface charge is fundamental determinants of the ability of this enzyme to function at the bilayer surface (8, 12, 14, 15, 17, 18, 21, 23, 25, 31, 35–37, 47). Such studies are commonly criticized with respect to their relevance to complex biological systems. This study addresses that criticism by applying the biophysical information gleaned from model systems and with model enzymes (such as snake venom sPLA₂) to the process of programmed cell death and human isozymes. Although, these results relate specifically to S49 lymphoma cells, observations of sPLA₂-catalyzed hydrolysis of various other cell lines during apoptosis or necrosis suggest that they apply broadly (10, 11, 13, 35, 48, 49).

In summary, there are five novel conclusions resulting from the data of this project. First, the properties of healthy cell membranes naturally resist hydrolysis by all three human sPLA₂ isoforms tested. However, all three isoforms actively hydrolyze the membranes of cells rendered apoptotic by glucocorticoid. Second, the hGX isoform appears to benefit from a reduction in the strength of interactions among neighboring lipids resulting in easier migration of phospholipids into the enzyme active site. Third, although this reduction in packing density may be sufficient for hGX sPLA₂, the hGV and hGIIa isozymes appear to require an additional alteration that improves the ability of these enzymes to adsorb to the membrane surface. This additional alteration is probably the exposure of phosphatidylserine on the membrane surface. Fourth, in addition to glucocorticoid-stimulated apoptosis, cell damage from calcium influx also renders the membranes susceptible to hGX and (to a lesser extent) hGV but not hGIIa. The relevant distinguishing element between death caused by calcium influx and glucocorticoid is not yet known. Fifth, for hGV and hGIIa isoforms, the ability to hydrolyze cells during apoptosis is transient. The highly ordered cell corpses appear to be substrates (albeit weaker) only for hGX.

These conclusions imply possible physiological roles for the three human isoforms. The fact that all three ignore or display reduced activity toward healthy cells is beneficial because they are capable of killing cells by inflicting membrane damage and because the hydrolysis products are proinflammatory. All three are potentially bactericidal and, hence, may play a role in the innate immune system (50–56). In addition, the hGX isoform appears capable of performing a general role for clearing damaged cells and cellular debris after traumatic injury. The hGV and especially the hGIIa isoforms, in contrast, may only generate significant hydrolysis of apoptotic cells.

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