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Relationship between membrane permeability and specificity of human secretory phospholipase A_2 isoforms during cell death

Jennifer Nelson, Elizabeth Gibbons, Katalyn R. Pickett, Michael Streeter, Ashley O. Warcup, Celestine H.-Y. Yeung, Allan M. Judd, John D. Bell *

Department of Physiology and Developmental Biology, Brigham Young University, Provo, UT 84602, USA

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ABSTRACT

During apoptosis, a number of physical changes occur in the cell membrane including a gradual increase in permeability to vital stains such as propidium iodide. This study explored the possibility that one consequence of membrane changes concurrent with early modest permeability is vulnerability to degradation by secretory phospholipase A_2. The activity of this hydrolytic enzyme toward mammalian cells depends on the health of the cell; healthy cells are resistant, but they become susceptible early during programmed death. Populations of S49 lymphoma cells during programmed death were classified by flow cytometry based on permeability to propidium iodide and susceptibility to secretory phospholipase A_2. The apoptotic inducers thapsigargin and dexamethasone caused modest permeability to propidium iodide and increased staining by merocyanine 540, a dye sensitive to membrane perturbations. Various secretory phospholipase A_2 isozymes (human groups IIa, V, X, and snake venom) preferentially hydrolyzed the membranes of cells that displayed enhanced permeability. In contrast, cells exposed briefly to a calcium ionophore showed the increase in cell staining intensity by merocyanine 540 without accompanying uptake of propidium iodide. Under that condition, only the snake venom and human group X enzymes hydrolyzed cells that were dying. These results suggested that cells showing modest permeability to propidium iodide during the early phase of apoptosis are substrates for secretory phospholipase A_2 and that specificity among isoforms of the enzyme depends on the degree to which the membrane has been perturbed during the death process. This susceptibility to hydrolysis may be important as part of the signal to attract macrophages toward apoptotic cells.

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1. Introduction

Early attempts at distinguishing apoptotic and necrotic cells often focused on permeability of the cells to vital stains such as propidium iodide (reviewed in [1]). The original paradigm was that necrotic cells are immediately permeable to the dye while apoptotic cells display a significant temporal delay before they become stained. It was soon discovered that the latent permeability to propidium iodide during apoptosis is not an "all or none" phenomenon. Instead, there is a gradual acceleration of probe uptake that initially produces faint cellular fluorescence quantifiable only by flow cytometry but eventually culminating in complete staining of the cells [2,3]. Presumably, this gradual acceleration represents alterations to the structure and dynamics of the cell membrane that progressively become more pronounced. Although these observations have been substantiated by several investigators, the focus has been confined to development of assay methods; determinations of mechanisms and physiological/pathological consequences have lagged.

Another membrane event that occurs during early apoptosis is an increase in the ability of secretory phospholipase A_2 ($sPLA_2$) to hydrolyze phospholipids and release fatty acids and lysophospholipids from the outer face of the plasma membrane [4–8]. This interesting relationship between $sPLA_2$ and apoptosis is an extension of a broader paradigm that healthy cells resist hydrolysis by the enzyme whereas membranes of damaged or dying cells are vulnerable [4–10]. At least some of this enhanced vulnerability to hydrolytic attack is observed in apoptotic cells that have not yet become fully stained by propidium iodide [5–8]. Apparently, the increased susceptibility to hydrolysis also represents alterations to the structure and dynamics of the cell membrane [6,7,9,11–15]. Biophysical studies of this phenomenon have yielded some clues as to what these alterations might involve. Possible candidates include increased lipid spacing, decreased lipid order, and increased exposure of phosphatidylserine on the outer face of the cell membrane [6–8]. Nevertheless, a complete understanding of the nature of relevant membrane changes during early apoptosis has not yet been achieved. These observations raise the question of whether the alterations that permit hydrolysis by $sPLA_2$ might correspond to those that allow modest permeability to propidium iodide. Answering that question could clarify mechanisms involved in controlling $sPLA_2$ activity as well as

* Corresponding author. Tel.: +1 801 422 2353; fax: +1 801 422 0263.
E-mail address: john_bell@byu.edu (J.D. Bell).

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identify possible novel biological significance for subtle changes in membrane permeability to vital stains during apoptosis.

To address this question, we examined responses to various death stimuli using flow cytometry to classify populations of cells based on their intensity of staining with fluorescent probes that detect specific membrane properties. The objective was to identify which populations were most susceptible to enzymatic attack and determine how those populations related to permeability to propidium iodide and other physical properties. We included agents that initiated death through endoplasmic reticulum stress, glucocorticoid receptor stimulation, and calcium loading. Although we compared results using each of these stimuli, we focused most of our attention on endoplasmic reticulum stress caused by the calcium ATPase inhibitor thapsigargin [5,16–18]. Thapsigargin was emphasized because it induces apoptosis rapidly in S49 cells thereby augmenting the size of relevant populations by maintaining high synchrony of cells as they proceed through the death process. In addition to propidium iodide, merocyanine 540 was used as a marker of increases in interlipid spacing and membrane lipid disorder that have been reported to enable hydrolysis by $sPLA_2$[6–8,19–21]. Exposure of phosphatidylserine was eliminated as a variable by including death stimuli and incubation times known to result in optimal exposure of the anionic phospholipid on the extracellular membrane surface [5,22–25].

2. Materials and methods

2.1. Reagents

Secretory phospholipase A_2 isoforms were isolated and prepared as described: monomeric aspartate-49 $sPLA_2$ from the venom of *Agkistrodon piscivorus piscivorus* (AppD49) [26], hGIIa [27,28], hGV [29], and hGX [30]. Thapsigargin was obtained from Enzo (Plymouth Meeting, PA). Dexamethasone was purchased from Sigma-Aldrich (St. Louis, MO). Ionomycin, acrylodan-labeled intestinal fatty acid-binding protein (ADIFAB), propidium iodide and merocyanine 540 were acquired from Invitrogen (Carlsbad, CA). Dipalmitoylphosphatidylglycerol (DPPG) was obtained from Avanti Polar Lipids (Birmingham, AL). Multilamellar liposomes were prepared by hydration and vortex agitation of dried lipid samples at 50 °C as described [31].

2.2. Cell culture and treatment with agents

S49 mouse lymphoma cells were grown in suspension in Dulbecco's Modified Eagle Medium at 37 °C (10% CO_2) as explained [32]. Cells were treated in culture with thapsigargin (5 μM, 3–4 h), dexamethasone (100 nM, 24 h), or equivalent volumes of the drug vehicle dimethylsulfoxide (DMSO, parallel incubation times, ≤0.1% v/v). The treatment times were established through preliminary experiments as the moment corresponding to optimal hydrolysis of the dying cells by $sPLA_2$. For experiments, cells were collected by centrifugation, washed, and suspended ($0.4–3.5\times10^6$ cells/ml) in a balanced salt medium (NaCl = 134 mM, KCl = 6.2 mM, $CaCl_2$ = 1.6 mM, $MgCl_2$ = 1.2 mM, Hepes = 18.0 mM, and glucose = 13.6 mM, pH 7.4, 37 °C). Treatments with ionomycin (300 nM) were for 10 min in the balanced salt medium. All experiments, treatments, and incubations were conducted at 37 °C.

2.3. Flow cytometry

Washed samples of cells in 200 μl aliquots were incubated for 15 min (or as otherwise indicated in figure legends) with 10 μM propidium iodide or 250 nM merocyanine 540. Where indicated, 70 nM $sPLA_2$ was also included. Separate aliquots were incubated with Alexafluor-labeled annexin (Invitrogen, Carlsbad, CA) as positive controls to verify exposure of phosphatidylserine, an effect expected and assumed for all of the treatments used in this study [5,22–25]. Samples were then immediately processed (without fixation) by flow cytometry using a BD FACSCanto flow cytometer (BD Biosciences, San Jose, CA). An argon laser was used for excitation (488 nm) with emission assessed using a bandpass filter at 564–606 nm. The number of cells containing hypodiploid-staining DNA due to fragmentation was quantified by flow cytometry after treatment with Triton X-100 as described [33].

2.4. Hydrolysis

Cell membrane hydrolysis catalyzed by various $sPLA_2$ isozymes (35–70 nM) was measured by assaying fatty acid release using ADIFAB (65 nM final) as described [8]. Most experiments were conducted using the AppD49 isozyme as a standard for comparison to previous studies and because it mimics many general behaviors of the human enzymes [8]. Key results were then repeated with hGIIA, hGV, and hGX. These assays were conducted on bulk samples in photon-counting spectrofluorometers (Fluoromax 3, Horiba Jobin Yvon, Edison, NJ) with magnetic stirring and thermostated at 37 °C by circulating water baths. Data were gathered in real time during incubation of the cells with ADIFAB and the indicated $sPLA_2$ isozyme. Fluorescence emission was acquired by rapid sluing of monochromator mirrors between two emission wavelengths (excitation = 390 nm, emission = 432 and 505 nm, 4 nm bandpass). The amount of fatty acid released was estimated using the following equation:

$$GP = \frac{(I_{505} - I_{432})}{(I_{505} + I_{432})} \quad (1)$$

GP stands for generalized polarization, and I_{505} and I_{432} are the fluorescence emission intensities at 505 and 432 nm.

The total amount of hydrolysis possible under the various experimental conditions was estimated using AppD49 $sPLA_2$. Cells treated with DMSO, thapsigargin, or dexamethasone were incubated for 600 s with the enzyme. The maximum displacement in ADIFAB GP during this incubation period was then divided by the equivalent value obtained from parallel experiments with cells treated 10 min with 300 nM ionomycin. Ionomycin renders 100% of S49 cells vulnerable to hydrolysis by AppD49 $sPLA_2$[6] and was therefore used as a standard to establish the maximum for normalization.

The initial hydrolysis rate was quantified for each isozyme by measuring the displacement in ADIFAB GP during the initial 5 s (AppD49), 20 s (hGX), or 50 s (hGV or hGIIa) of the hydrolysis time courses and then dividing that value by the length of the incubation. The specific activity of each enzyme preparation was assayed by likewise assessing the initial hydrolysis rate using DPPG liposomes (25 μM lipid) as substrate.

3. Results

3.1. Susceptibility to $sPLA_2$ of moderately-permeable cells

S49 lymphoma cells were treated for 3 h with thapsigargin or control vehicle (DMSO), stained with propidium iodide, and classified based on staining intensity by flow cytometry. Fig. 1 illustrates contour plots of the flow cytometry results. As shown in Fig. 1A, three distinct populations were identified in control samples based on their level of propidium iodide fluorescence intensity (ordinate axis). The designation "P" refers to those cells staining positive for propidium iodide. This population was indistinguishable from cells that had been permeabilized by the detergent Triton-X 100 (not shown). Therefore, it is assumed to represent cells with permeable membranes. The negative population ("N") did not stain with propidium iodide since the apparent fluorescence intensity was comparable to the background observed with cells that had never been exposed to the dye. A third population was labeled "I" for intermediate. The average staining intensity of this population was about 10 times that of the

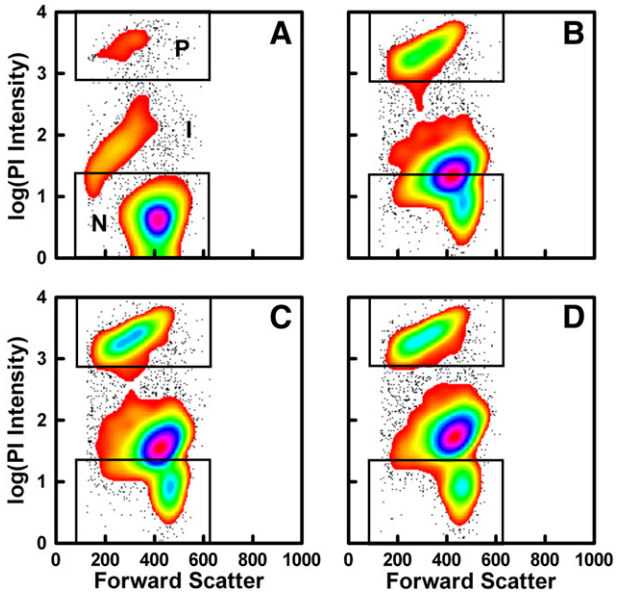

Fig. 1. Contour plots of propidium iodide fluorescence and forward light scatter. Cells were treated with DMSO (panel A) or thapsigargin (panels B–D). Samples were stained for 10 min with propidium iodide in panel A and fluorescence intensity ("PI Intensity") and forward light scatter were assessed for individual cells by flow cytometry. In panels B–D, cells were stained for 1 min (B), 5 min (C), or 10 min (D) with propidium iodide prior to immediate flow cytometry. Labels: "N," negative (same as background); "I," intermediate propidium iodide staining; "P," positive or complete propidium iodide staining. This figure is a representative example from three independent experiments.

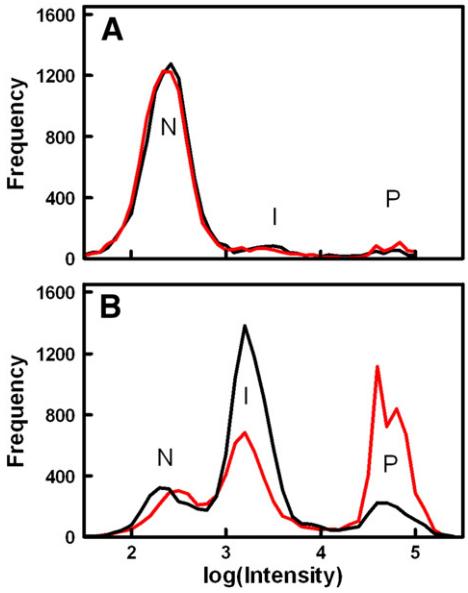

Fig. 2. Histograms of propidium iodide fluorescence intensity before and after hydrolysis by $sPLA_2$. Cells were treated with DMSO (panel A) or thapsigargin (panel B). Samples were stained for at least 10 min with propidium iodide without (black curves) or with (red curves) AppD49 $sPLA_2$. Fluorescence intensity per cell was assessed by flow cytometry. For key to labels, see Fig. 1. This figure is a representative example from three independent experiments.

negative population but only1/30 that of the positive population. Cells in the negative and positive populations exhibited stable fluorescent intensities over time. However, for the intermediate population, the degree of staining increased incrementally over time. The cells shown in Fig. 1A were representative of typical control samples (84.3% negative, 11.3% intermediate, 4.4% positive).

A likely interpretation of the intermediate population was suggested in a study by Vitale [2]. The researchers proposed that it represents a trait inherent to apoptotic cells—slightly compromised membranes that have therefore become modestly permeable to propidium iodide. To examine the applicability of this idea to apoptotic S49 cells, we monitored the distribution of propidium iodide staining at different times after introduction of the dye to a thapsigargin-treated sample. We assumed that if the intermediate population represented cells with modest permeability, the population would gradually migrate along the fluorescence intensity axis of the contour plot while the negative and positive populations would remain stable. As shown in panels B–D, the positive population was clearly defined, contained a greater number of cells than in the control sample (12% compared to 4%), and remained stable in intensity over time. Initially, cells staining with low intensity were distributed across both the negative and intermediate regions (panel B). As time progressed, a population with higher intensity staining emerged and migrated along the ordinate indicating that the amount of staining per cell was increasing. By 10 min, the two populations had resolved into the negative and intermediate regions.

Fig. 2 shows histograms of fluorescence intensity from an experiment like that of Fig. 1 for cells incubated without (black curves) or with (red curves) $sPLA_2$. In control samples (panel A), 10-min treatment with $sPLA_2$ produced only minor alterations. With thapsigargin-treated cells (panel B), the effects of hydrolysis by $sPLA_2$ were obvious. The proportion of cells found in the positive peak tripled. These were accounted for quantitatively by a comparable reduction in the number occupying the intermediate peak. The proportion of cells found in the negative population stayed about the same with a small increase in staining intensity, suggesting a minor perturbation to those cells, perhaps from modest hydrolysis. This result suggested that cells in the intermediate peak are a prominent target of $sPLA_2$.

Fig. 3A represents the evolution of the various populations of apoptotic cells over time after addition of propidium iodide. Consistent with the results in Fig. 1, the main change was a time-dependent migration of cells from the negative region (black lines and symbols) to the intermediate region (red). A moderate rise in the number of cells staining positive was also observed (blue). Panels B and C demonstrate the effect of $sPLA_2$ on the distribution of populations over time. The main effect was conversion of the intermediate population to positive cells, presumably due to membrane damage during hydrolysis. As expected, very little hydrolysis was detected in untreated control samples (open symbols).

As indicated by the relative areas under the black and red curves in Fig. 3D, only about 40% of the cells in the intermediate population was hydrolyzed by $sPLA_2$ (i.e. 22–29% of the total sample). There were at least two reasons that cells remained in the intermediate population after addition of $sPLA_2$. First, a subpopulation of the intermediate group was already fully permeable to propidium iodide but stained with low intensity because of DNA fragmentation. The existence of this subpopulation was verified by addition of Triton X-100 [33]. Obviously, hydrolysis would have had no impact on the intensity of propidium iodide staining of this subpopulation. We performed parallel control experiments to quantify the contribution of these hypodiploid cells to the result. We found that about 10% of the sample contained fragmented DNA under the conditions of Fig. 3, which would account for about a fourth of the intermediate cells that did not change staining intensity after $sPLA_2$ treatment (not shown).

The second reason why the remaining cells in this population did not appear to be hydrolyzed is not clear; however, one clue may come from close examination of the histograms shown in Fig. 2. The average staining intensity for the intermediate population was reduced by an average of 0.15 log units after hydrolysis by $sPLA_2$. This effect is shown in Fig. 3D comparing the residual intermediate subpopulation (red) with that apparently removed by hydrolysis (blue). This difference between

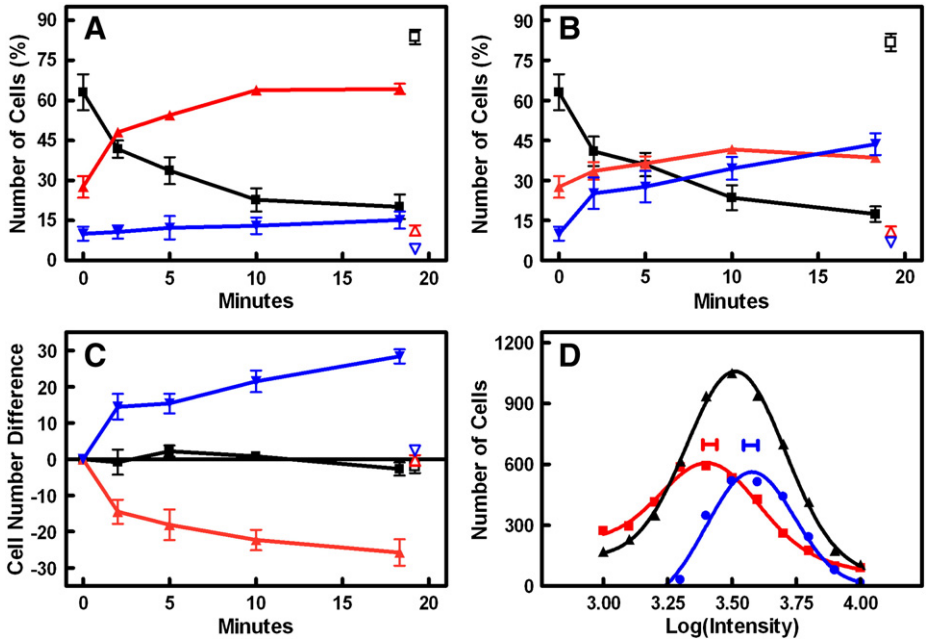

Fig. 3. Time course of distribution of thapsigargin-treated cells among populations defined by propidium iodide fluorescence intensity before and after hydrolysis by $sPLA_2$. Panel A: data from three independent experiments such as that shown in Fig. 1 were quantified at the indicated time points following mixing with propidium iodide—black, percentage of the cell sample staining negative for propidium iodide intensity (see field marked "N" on Fig. 1); red, intermediate for propidium iodide ("I" on Fig. 1); blue, propidium iodide positive ("P" on Fig. 1). Panel B: The experiments and analysis of panel A were repeated for samples incubated with $sPLA_2$ simultaneously during the propidium iodide staining. Panel C: The data represent the differences between panels A and B. Open symbols in panels A–C represent data from three control (treated with equivalent DMSO) samples. Panel D: Histograms of the intermediate cohort corresponding to the last time point from one of the samples in panels A–C for cells without $sPLA_2$ (black), with $sPLA_2$ (red), and the arithmetic difference between the two (blue). Data were fit by nonlinear regression to a Gaussian function. The brackets represent the 95% confidence intervals for the fitting parameter corresponding to the mean values. Error bars represent SEM (smaller than the size of the symbols for some of the data).

the vulnerable and resistant subpopulations in the intermediate cohort was reproducible; the lack of overlap between the 95% confidence intervals for the respective histogram means shown in Fig. 3D was true for all of the replicate samples in Fig. 3. This result indicates that the vulnerable subpopulation has a slightly greater permeability to propidium iodide (about 41% greater) compared to those that remain resistant. If the intermediate population represents cells with membranes perturbed in a way that makes them susceptible to hydrolysis, then the data presented here would suggest that the enzyme is quite sensitive to the magnitude of that perturbation, and only those cells with sufficient permeability above a narrow threshold are attacked. This result is reasonable for $sPLA_2$ given the extreme sensitivity of the enzyme to subtle changes in artificial membranes [34–36].

3.2. Quantification of hydrolysis

Experiments parallel to those in Fig. 3 were conducted spectroscopically on bulk samples using the fluorescent fatty acid binding protein ADIFAB to assess the total amount of hydrolysis occurring during incubation with $sPLA_2$ (Fig. 4A). Consistent with previous reports, the control cells displayed transient hydrolysis detected by ADIFAB indicative of a small subset of cells (10–15% of the sample based on Figs. 2 and 3) that were compromised in culture or during sample preparation and therefore became vulnerable to the enzyme [8]. After treatment with thapsigargin, the amount of lipid hydrolyzed was 52% of the total achieved when 100% of the cells are susceptible to the enzyme (Fig. 4C, green bar).

Previous reports have indicated that the total number of cells vulnerable to $sPLA_2$ can also be quantified by counting the number stained by merocyanine 540 [6,7]. Accordingly, we also assessed merocyanine 540 staining by flow cytometry (Fig. 4B). Quantitatively, the number of cells staining brightly for merocyanine 540 in control and thapsigargin samples (violet bars, Fig. 4C) matched the percentage hydrolyzed (green; see statistical details in the legend to Fig. 4).

3.3. Comparison between hydrolysis and propidium iodide uptake for various inducers of cell death

The purpose for obtaining these two estimates of the number of cells vulnerable to enzymatic attack was to compare to the propidium iodide flow cytometry results and identify those populations of cells likely to be targets for $sPLA_2$. For control samples and those treated with thapsigargin, the positive (blue) and intermediate (red) populations were adequate to account for the amount of hydrolysis and the apparent numbers of susceptible cells (Fig. 4C). As predicted by the data in Fig. 3D (red curve), the amount of hydrolysis was less than the sum of the intermediate and positive populations ($p<0.001$). In contrast, cells treated with dexamethasone exhibited a greater amount of hydrolysis than could be explained by flow cytometry assessments of merocyanine 540 fluorescence or PI permeability ($p<0.01$). This result implies that either there is a greater amount of lipid hydrolyzed per cell, or some of the negative propidium iodide population (gray) is hydrolyzed at a rate low enough that it does not produce enhanced permeability to propidium iodide. One way in which the first of these possibilities could be realized is that cells treated with dexamethasone, but not thapsigargin or ionomycin, may break into fragments during hydrolysis that exposes additional intracellular membranes to $sPLA_2$. Further experiments will be required to resolve this difference and to identify the extent to which it is a common feature among various apoptotic stimuli.

Cells induced to die by brief treatment (10 min) with a calcium ionophore, ionomycin, also behaved differently. In this case, all the cells were vulnerable to the enzyme and stained brightly for merocyanine 540 as reported previously [6]. However, only about 30% of the cells sorted into the positive or intermediate propidium

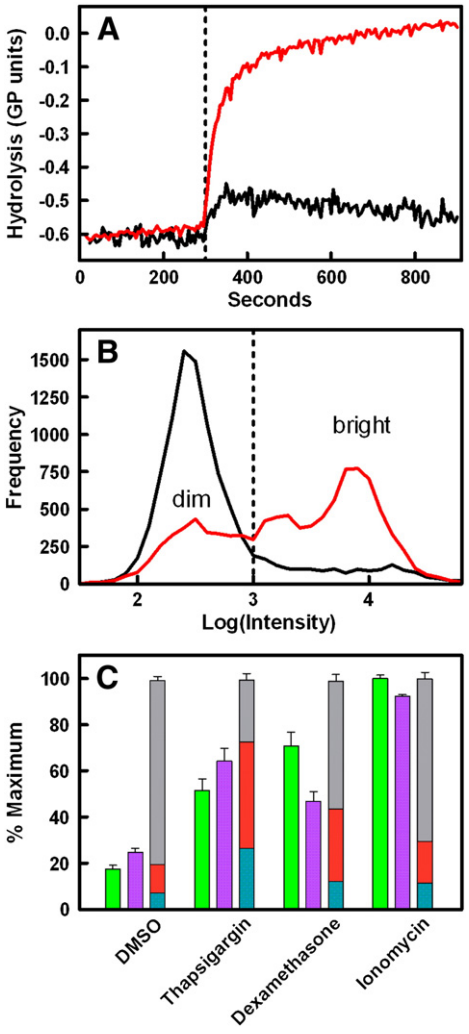

Fig. 4. Comparison of compartment sizes assessed by various methods for cells treated with thapsigargin, dexamethasone or ionomycin. Panel A: Time profile of cell membrane hydrolysis by AppD49 $sPLA_2$ (added at dotted line) for control (DMSO) cells (black) and cells treated with thapsigargin (red). Hydrolysis was assayed by ADIFAB fluorescence as explained in Materials and methods. Panel B: Histograms of merocyanine 540 staining resolved by flow cytometry for cells treated with DMSO (black) or thapsigargin (red). Panel C: Cell compartment sizes were estimated by fluorescence spectroscopy or flow cytometry and normalized to maxima as explained in Materials and methods. Green bars: total amount of lipid hydrolyzed by AppD49 $sPLA_2$ in 600 s (spectroscopy of ADIFAB, as in panel A). Violet bars: number of cells staining brightly for merocyanine 540 (flow cytometry, as in panel B). Blue, red, and gray bars: number of cells staining positive, intermediate, and negative for propidium iodide (flow cytometry as in Figs. 1–3). Error bars represent SEM ($n=3$–42). The three sets of observations representing hypothesized $sPLA_2$-susceptible cohorts (green, violet, and blue + red) were analyzed by two-way analysis of variance followed by a Bonferroni post test for DMSO, thapsigargin, and dexamethasone treatments. The cohorts for ionomycin were not analyzed in this manner because the values were used as normalization standards for the other measurements, which would bias the ionomycin means for statistical comparisons. The overall effect of treatment was significant ($p<0.0001$, 49% of the total variation) but not the overall effect of measurement type (0.06% of the total variation). A significant interaction was detected ($p<0.0001$, 12% of the total variation) suggesting that differences among measurements exist for one or more of the treatments. The post test revealed three sources of the interaction: For thapsigargin, the flow cytometry (blue + red) cohort was statistically different from hydrolysis detected by ADIFAB ($p<0.001$). For dexamethasone, both merocyanine 540 and propidium iodide flow cytometry results were statistically significant compared to hydrolysis ($p<0.01$).

iodide populations by flow cytometry, indicating that most of the hydrolysis observed with ionomycin-treated samples originates from cells that exclude propidium iodide completely.

3.4. Comparisons among $sPLA_2$ isoforms

To test the potential physiological relevance of these findings, we compared the relative abilities of three human isoforms of $sPLA_2$ to hydrolyze samples using data obtained with the AppD49 enzyme as a standard. We compared initial rates rather than total hydrolysis amounts because they more readily resolve quantitative differences related to enzyme activity. Fig. 5 summarizes the initial rate of hydrolysis for all four isozymes under various experimental conditions. As suggested by Fig. 4A, the initial rate catalyzed by AppD49 toward thapsigargin-treated cells was similar to that observed for unhealthy cells in the control sample ($p>0.05$) even though the total available substrate was very different (Fig. 5A). Dexamethasone induced susceptibility to $sPLA_2$ at a slightly higher initial rate that was distinguishable from that observed toward damaged cells in the control sample ($p<0.05$). As shown in prior studies, the rate toward ionomycin-treated samples was much greater than with any of the other conditions ($p<0.01$ compared to DMSO) [6,8]. A similar pattern was obtained with the hGX isoform toward the weakened population in control samples and toward cells treated with the various inducers of cell death (Fig. 5B). In this case, only the ionomycin group was statistically different from the control group ($p<0.01$). In contrast to the results with the AppD49 and hGX isozymes, the hGIIa and hGV isozymes generally showed a different pattern with more obvious differences in the rate of hydrolysis between control and apoptotic samples (thapsigargin and dexamethasone) but diminution of the rate toward samples incubated briefly with ionomycin (Fig. 5C and D; see legend for statistical details). As explained for the AppD49 isoform, the amount of lipid hydrolyzed in the various treatment groups was always much greater than observed in the control sample (reflecting the larger number of cells participating in biochemically-programmed death) even though the initial rate was sometimes comparable [6].

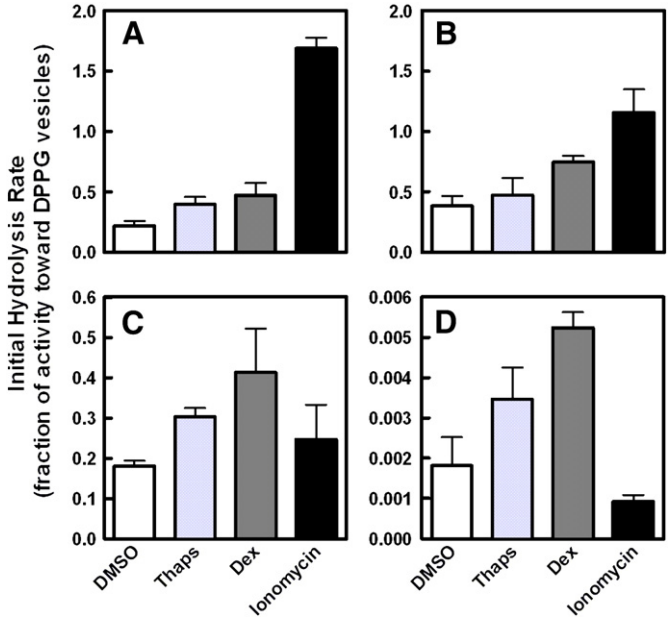

Fig. 5. Comparison of initial hydrolysis rates catalyzed by snake venom and human $sPLA_2$ isoforms among cells treated with DMSO, thapsigargin, dexamethasone, or ionomycin. Hydrolysis was measured by ADIFAB fluorescence as in Fig. 4A for cells treated as indicated. The initial rate was quantified as described in Materials and methods. Panel A: AppD49; panel B: hGX; panel C: hGV; panel D: hGIIa. For the AppD49, hGX, and hGIIa isozymes, the results were significant by one-way analysis of variance ($p<0.008$). As indicated by Dunnett's post-test, the following treatments were statistically different from the corresponding DMSO controls ($p<0.05$): AppD49—dexamethasone and ionomycin, hGX—ionomycin, hGIIa—dexamethasone.

The lower activity of hGIIa toward mammalian cell membranes has been reported previously [12,37–39] and probably relates to the absence of tryptophan at its interfacial binding surface [12,27,40]. This difference was not due to low activity of the enzyme preparation since hGIIa was the most active of the four toward the DPPG liposomes used as a positive control (AppD49 = 0.05 GP units·s^{-1}, hGIIa = 0.08 GP units·s^{-1}, hGV = 0.005 GP units·s^{-1}, hGX = 0.01 GP units·s^{-1}). This dichotomy between hGIIa activities toward liposomes *versus* apoptotic cells probably reflects the much higher density of negative charge on the liposome compared to that achievable in S49 cells [10].

4. Discussion

The data in this study argue strongly that one consequence of the modest increase in membrane permeability detected by propidium iodide as an intermediate-staining population is enhanced susceptibility to hydrolysis by sPLA$_2$. Moreover, this enhanced susceptibility is likely to have physiological significance because several sPLA$_2$ isoforms present in human plasma responded to these early apoptotic cells (Fig. 5). In contrast, cells dying by ionophore treatment showed two important deviations from this pattern. First, the cells did not display a sizeable intermediate-staining propidium iodide population even though they stained brightly with merocyanine 540 and were aggressively attacked by AppD49 and hGX sPLA$_2$. Second, the ionophore-treated cells were hydrolyzed at a much lower rate by hGV and hGIIa sPLA$_2$. This second observation was reported previously and raised the question of whether there might be differences in physical properties of the membranes of apoptotic and ionophore-treated cells that explain this apparent specificity of the isozymes [8]. The presence of anionic lipids such as phosphatidylserine on the membrane surface and enhanced spacing of bilayer lipids, two properties known to be relevant to the activity of these enzymes, were excluded as candidates since both are expressed more completely in ionophore-treated cells than in those undergoing apoptosis (Fig. 4 and Ref. [8]). However, the relationship between hydrolysis and the intermediate-staining propidium iodide population discovered here (Figs. 4 and 5) is a match with the prior observations and may explain the specificity of hGV and hGIIa sPLA$_2$ isoforms toward apoptotic cells.

This result begs the interesting question: what is the molecular source of the intermediate-staining population, and why does it appear to relate to enhanced enzymatic activity of hGIIa and hGV sPLA$_2$ isoforms? An attractive possibility is that both events are produced by lipid oxidation. This idea stems from three published observations. First, lipid peroxidation appears to occur during apoptosis, perhaps catalyzed by cytochrome c oxidase released from mitochondria [41–44]. Second, studies with artificial bilayers suggest that lipid oxidation can perturb the physical properties of membranes in ways that enhance their permeability [42,45–47]. Third, membranes containing oxidized lipids are more vulnerable to hydrolysis by sPLA$_2$, especially the hGIIa isozyme [48–52].

A second possibility relates to studies with artificial membranes demonstrating that fluctuations caused by changes in membrane order and fluidity can permit charged molecules like propidium iodide to temporarily traverse the bilayer [53–57]. The increased staining of the membrane by merocyanine 540 (Fig. 4 and Ref. [6,7]) probably also represents changes of that nature [19–21,31,58]. Thus, it may be that the intermediate propidium iodide population does not represent emergence of an additional membrane physical trait but instead, greater magnitude of the same perturbations detected by merocyanine 540. In other words, perhaps merocyanine 540 is sensitive to low levels of perturbation as perhaps occurs with ionomycin. When the perturbation escalates to the point that it becomes detectable by propidium iodide, such as during apoptosis, there is no additional staining by merocyanine 540 because the intensity was already maximal at a weaker perturbation. If this is true, then the differences among isoforms with respect to membrane physical properties would be quantitative rather than qualitative and could be summarized as follows.

1. Cells that are negative for both merocyanine 540 and propidium iodide were not attacked by any of the sPLA$_2$ isoforms studied here.
2. Cells that are weakly perturbed, as during ionomycin treatment, stain brightly by merocyanine 540 but are still negative for propidium iodide. These cells were good substrates only for the AppD49 and hGX isoforms.
3. Cells that are more strongly perturbed stain brightly by merocyanine 540 and at an intermediate level with propidium iodide. These cells were observed during apoptosis and were susceptible to all four isoforms.
4. The cells that are most heavily perturbed are positive for both propidium iodide and merocyanine 540. A previous study demonstrates these cells are also hydrolyzed by the various isozymes, at least during mid-apoptosis [8].

Finally, there is evidence that membrane permeability is enhanced in a more specific fashion early during apoptosis via pannexin 1 channels [59]. This rise in permeability allows release of nucleotides such as ATP and UTP from apoptotic cells. These nucleotides function as signals to attract macrophages in preparation for clearance of apoptotic remnants [59,60]. Whether activation of these channels accounts for the modest rise in permeability to propidium iodide is unknown. Other vital stains such as YO-PRO-1 and TO-PRO-3 appear to be conducted by pannexin channels, but propidium iodide was reported not to stain cells at a stage of apoptosis when pannexin channels are open [59]. Unfortunately, the experimental data were not reported in sufficient detail to allow one to ascertain whether the more moderate propidium iodide diffusion characteristic of early apoptotic cells could be explained by pannexin channel activation. Moreover, it is unclear how pannexin channel activation might relate directly to increased vulnerability of the membrane to hydrolysis by sPLA$_2$.

The data in this study suggest the possibility that the temporary moderate increase in membrane permeability during apoptosis could have significant consequences because these cells can be hydrolyzed by pro-inflammatory enzymes present in the plasma. In general, it is thought that this kind of problem would be avoided through elimination of apoptotic cells by macrophages [61–63]. The issue, then, becomes one of timing. Recent studies have suggested that macrophage participation involves two signals: a recognition or "find-me" signal followed by an "eat-me" signal [59]. The first appears to involve increases in membrane permeability via the pannexin 1 channel mentioned above [59]. The second is the exposure of phosphatidylserine on the external face of the cell membrane [64–67]. Since the hGV and hGIIa isoforms require the presence of phosphatidylserine in addition to enhanced membrane permeability, they would not be capable of generating inflammatory precursors until the "eat-me" signal and the resulting protection by phagocytosis. Therefore, these isoforms are unlikely to produce an untoward inflammatory response. However, as recently demonstrated, the hGX isozyme actively hydrolyzes the cell membrane during apoptosis *prior* to the exposure of phosphatidylserine [8]. Accordingly, the potential exists for production and release of inflammatory fatty acids and lysophospholipids during apoptosis before the dying cells can be cleared by macrophages. This scenario may not be a bad thing, however. Although pannexin channel activation has been proposed as one mechanism for releasing certain "find-me" signals, additional molecules that fulfill the same function have also been reported to be released during early apoptosis through undetermined means [61–63]. Among those other signals is lysophosphatidylcholine [68–70]. It is thus tempting to speculate that hGX sPLA$_2$ may play a major role in releasing lysophosphatidylcholine as a "find-me" signal through mechanisms described in this study.

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