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Ionomycin causes susceptibility to phospholipase A_2 while temperature-induced increases in membrane fluidity fail: Possible involvement of actin fragmentation

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ABSTRACT

A diminution in the order of membrane lipids, which occurs during apoptosis, has been shown to correlate with increased membrane susceptibility to hydrolysis by secretory phospholipase A_2. Studies with artificial membranes, however, have demonstrated that the relationship between membrane order and hydrolysis is more complex than suggested thus far by cell studies. To better resolve this relationship, this study focused on comparisons between increasing temperature and calcium ionophore as means of decreasing membrane order in S49 cells. Although these two treatments caused comparable changes in apparent membrane order as detected by steady-state fluorescence measurements, only ionophore treatment enhanced phospholipase activity. Experiments with exogenously-added phosphatidylserine indicated that the difference was not due to the presence of that anionic phospholipid in the outer membrane leaflet. Instead, analysis of the equilibration kinetics of various cationic membrane probes revealed that the difference could relate to the spacing of membrane lipids. Specifically, ionophore treatment increased that spacing while temperature only affected overall membrane order and fluidity. To consider the possibility that the distinction with ionophore might relate to the actin cytoskeleton, cells were stained with phalloidin and imaged via confocal microscopy. Ionophore caused disruption of actin fibers while increased temperature did not. This apparent connection between membrane hydrolysis and the cytoskeleton was further corroborated by examining the relationship among these events during apoptosis stimulated by thapsigargin.

1. Introduction

Prior studies have investigated the role of biophysical alterations to the plasma membrane that govern its susceptibility to hydrolysis by secretory phospholipase A_2 ($sPLA_2$). Environment-sensitive fluorescent membrane probes such as merocyanine 540, Laurdan, diphenylhexatriene derivatives, and Patman have been used to detect changes in lipid order that correlate temporally and quantitatively with susceptibility [1–6]. In lymphocytes, reductions in lipid order consistently correlate with conditions that render the cells vulnerable to $sPLA_2$[1,2,6,7]. This apparent reduction in lipid order has been detected only by probes sensitive to the head-group region of the membrane and have been quantified from steady-state measurements of generalized polarization (GP) of the naphthalene derivatives (Laurdan and Patman) or probe anisotropy [1,2,6,7]. However, experiments with artificial bilayers and human erythrocytes have demonstrated that the relationship between membrane order and $sPLA_2$ activity is more complex than implied by the results from nucleated cells. For example, under some conditions, raising the experimental temperature reduces the order of liposome membranes and yields the same changes in probe steady-state fluorescence observed in cells, yet the rate of hydrolysis by $sPLA_2$ is not affected [8–10]. In erythrocytes, conditions that promote hydrolysis actually increase the average lipid order, but in a way that enhances the structural diversity in the membrane. Hydrolysis by $sPLA_2$ appears to localize at boundaries along regions of that diversity [3,4,11].

Hence, if membrane lipid order is relevant to the activity of $sPLA_2$, there must be additional information about those properties that cannot be discerned from the anisotropy or GP of these probes. Recent studies suggested that monitoring the pre-steady-state equilibration of Patman may provide that additional information. Patman is an analog of Laurdan that contains a trimethylammonium group attached to the naphthalene moiety to minimize transbilayer migration and a two-carbon extension of the aliphatic tail to retain the probe in the membrane [12]. Specifically, analysis of Patman kinetics distinguished subtle

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differences between reductions in bilayer order in cells when $sPLA_2$ activity is enhanced [6] compared to those in artificial membranes that do not affect hydrolysis [9]. The results of this comparison supported the hypothesis that $sPLA_2$ activity is promoted by changes in membrane order only when those changes facilitate migration of phospholipid molecules perpendicular to the plane of the bilayer [6,13,14]. Consequently, it was proposed that quantifying the kinetics of Patman equilibration in addition to measuring its end-point GP can be an effective tool for detecting these subtleties of membrane order.

This study was designed to test this hypothesis and proposal in cells without relying on artificial bilayers. This was accomplished by comparing two experimental conditions that produce the same changes in membrane properties detected by the end-point fluorescence of environment-sensitive membrane probes yet differ in their ability to stimulate hydrolysis by $sPLA_2$. A known paradigm for producing changes in membrane properties that induce hydrolysis by $sPLA_2$ is cellular apoptosis [1,2,6,15]. In this study, the calcium ionophore ionomycin was chosen to induce cell death because its effects are rapid, uniform, and synchronous [1]. In order to produce changes in steady-state probe fluorescence without stimulating $sPLA_2$ activity, sample temperature was manipulated. Comparison of these two treatments was then used to clarify the nature of the relationship between membrane physical properties and $sPLA_2$ activity in cells.

2. Materials and methods

2.1. Reagents

Monomeric aspartate-49 $sPLA_2$ from the venom of *Agkistrodon piscivorus piscivorus* (AppD49) was isolated and prepared as described [16]. Ionomycin, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), acrylodan-labeled fatty acid-binding protein (ADIFAB), 6-palmitoyl-2-[[2-(trimethylammonio) ethyl]-methylamino]naphthalene chloride (Patman), merocyanine 540 (MC540), and phalloidin and annexin V conjugates with Alexa Fluor® 488 were purchased from Life Technologies (Grand Island, NY). Thapsigargin (TG) was acquired from Life Technologies or Enzo Life Sciences (Plymouth Meeting, PA). Formaldehyde (16%, no methanol) was obtained from Thermo Fisher Scientific (Rockford, IL). Porcine brain L-α-phosphatidylserine (PS) and chicken egg L-α-phosphatidylcholine (PC) were purchased from Avanti Polar Lipids (Birmingham, AL). All other reagents were obtained from standard suppliers.

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 [17]. Thapsigargin treatments (5 μM) or equivalent volumes of DMSO (0.1% v/v) were done in culture medium. For experiments, cells were collected by centrifugation, washed, and suspended ($0.25–3.5 \times 10^6$ 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 done in the balanced salt medium after cell harvesting. All experiments, treatments, and incubations were conducted at 37 °C unless otherwise specified. Samples were equilibrated at the indicated temperature for at least 10 min prior to addition of probes or treatment agents. Sample viability was assessed by trypan blue exclusion.

2.3. Addition of exogenous PS

S49 cell plasma membranes were enriched with exogenous PS using liposomes containing 50% PS and 50% PC. Lipids were dried under nitrogen and resuspended in PBS (1 mM final). In order to make unilamellar liposomes, samples were sonicated in a Misonex Sonicator 3000 (Farmingdale, NY) at 2 W for 3 min. Cells were harvested as described above and resuspended to a density of $0.5–1.5 \times 10^6$ cells/ml. Cells were then incubated with liposomes (50 μM final lipid concentration) for 30 min at 37 °C, collected by centrifugation, and resuspended in balanced salt medium for experimentation.

2.4. Fluorescence spectroscopy

Time-based fluorescence intensities were assayed with a Fluoromax (Horiba Jobin Yvon, Edison, NJ) photon-counting spectrofluorometer. Anisotropy measurements were collected with a PC-1 (ISS, Champaign, IL) photon-counting spectrofluorometer equipped with Glan–Thompson polarizers. Continuous gentle stirring with a magnetic stir bar ensured sample homogeneity, and temperature was maintained by a jacketed sample chamber fed by a circulating water bath. Bandpass was set at 16 nm for anisotropy measurements and 4 nm for all other experiments. When necessary, fluorescence emission at multiple wavelengths was acquired by rapid sluing of monochromator mirrors. Cell samples were treated and prepared as described above and equilibrated in the fluorometer sample chamber for at least 5 min before initiating data acquisition.

Cell membrane hydrolysis catalyzed by $sPLA_2$ was measured by assaying fatty acid release using ADIFAB (65 nM final) as described [18]. The amount of fatty acid released was quantified by comparing ADIFAB fluorescence emission (excitation = 390 nm) at 432 and 505 nm (I_{432} and I_{505}) [19] and calculating the generalized polarization (GP_{ADIFAB}) as described [17]:

$$GP_{ADIFAB} = \frac{I_{505} - I_{432}}{I_{505} + I_{432}}. \quad (1)$$

Measurements of TMA-DPH (250 nM final) fluorescence were collected with different configurations of excitation (350 nm) and emission (452 nm) polarizers in the vertical and horizontal positions after 10 min equilibration of cells with the probe. The anisotropy (r) was then calculated from the fluorescence intensity when both polarizers were vertical ($I_{//}$) and when the excitation was vertical and the emission horizontal ($I_{\perp}$). Correction for differential transmission and detection at the two polarizer positions was included (G) according to convention:

$$r = \frac{I_{//} - GI_{\perp}}{I_{//} + 2GI_{\perp}}. \quad (2)$$

Merocyanine 540 (170 nM) fluorescence intensity was acquired as a function of time with excitation at 540 nm and emission at 585 nm. Patman (250 nM final) data were collected in a similar manner with excitation at 350 nm and emission at both 435 and 500 nm (I_{435} and I_{500}). Patman spectral changes were quantified by calculating the generalized polarization (GP) as follows [20]:

$$GP = \frac{I_{435} - I_{500}}{I_{435} + I_{500}}. \quad (3)$$

For Patman equilibration analyses of temperature studies, time courses of probe intensity were smoothed by nonlinear regression to an arbitrary function (analogous to Eq. (4)) and normalized to the intensity at 435 nm at 400 s to aggregate data from multiple replicate samples. These pooled data were then fit by nonlinear regression to the following:

$$I = A\left(1 - e^{-bt}\right) + C\left(1 - e^{-dt}\right) + F \quad (4)$$

where A and C are arbitrary scalars, b and d are rate constants, and F is the intercept intensity. Intensities at both wavelengths were fit together with b and d constrained as shared parameters. These fitting parameters were then used to calculate model parameter values (fraction of probe