Overexpression of Cytosolic Group IVA Phospholipase A_2 Protects Cells from Ca^{2+}-dependent Death*

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The calcium ionophore ionomycin induces apoptosis-like events in the human embryonic kidney (HEK) cell line at early times. Plasma membrane blebbing, mitochondrial depolarization, externalization of phosphatidylserine and nuclear permeability changes can all be observed within 15 min of treatment. However, there is no activation of caspases or chromatin condensation. Expression of a fusion protein containing the enhanced green fluorescent protein (EGFP) and human cytosolic Group IVA phospholipase $A_2\alpha$ (EGFP-cPLA$_2\alpha$) in these cells prevents the ionomycin-induced phosphatidylserine externalization and death. Cells expressing the cPLA$_2\alpha$ mutant D43N, which does not bind calcium, retain their susceptibility to ionomycin-induced cell death. Both non-expressing and EGFP-D43N-cPLA$_2\alpha$-expressing HEK cells can be spared from ionomycin-induced cell death by pretreating them with exogenous arachidonic acid. Moreover, during calcium overload, mitochondrial depolarization is significantly lower in the EGFP-cPLA$_2\alpha$-expressing cells than in cells expressing normal amounts of cPLA$_2\alpha$. These results suggest that early cell death events promoted by an overload of calcium can be prevented by the presence of high levels of arachidonic acid.

The phospholipase A_2 (PLA_2)[1] superfamily constitutes a heterogeneous group of enzymes whose common feature is to hydrolyze the fatty acid at the *sn*-2 position of glycerophospholipids [1, 2]. Mammalian cells contain structurally diverse forms of PLA_2s which, according to their biochemical characteristics, can be classified into three major families, namely the Ca^{2+}-dependent secreted enzymes, the Ca^{2+}-dependent cytosolic enzymes, and the Ca^{2+}-independent cytosolic enzymes [1, 2]. The presence of PLA_2 enzymes in all cells and tissues underscores their key role in a number of biochemical processes, including the mobilization of free arachidonic acid (AA), and the regulation of membrane phospholipid homeostasis via deacylation/reacylation reactions [1, 2].

Among all the PLA_2 forms, the cytosolic Group IVA phospholipase A_2, also known as cytosolic phospholipase $A_2\alpha$ (cPLA$_2\alpha$), exhibits a unique preference for the hydrolysis of glycerophospholipids that contain AA esterified at the *sn*-2 position. cPLA$_2\alpha$ has emerged as a key enzyme in regulating immunoinflammatory reactions, by catalyzing receptor-coupled AA mobilization and attendant eicosanoid biosynthesis [3, 4]. As a consequence, cPLA$_2\alpha$ has been shown to play pivotal roles in several physiological processes, ranging from parturition to bone resorption, as well as being involved in inflammation and in pathological processes such as arthritis, brain injury, and airway injury [3, 4].

Recently, roles for cPLA$_2\alpha$ have been suggested in mediating different types of cell

death by various agents, particularly oxidant substances [5-7]. However discrepancies exist and, as a matter of fact, cPLA$_2\alpha$ has also been suggested to play a cytoprotective role in some instances [8], and even to be inactivated in apoptotic cells by proteolytic cleavage, thus playing no role in the cell death process itself [9, 10]. In the present work we have studied the role of cPLA$_2\alpha$ during cell death induced by cytoplasmic Ca^{2+} overload by treating cells with the Ca^{2+} ionophore ionomycin. Our results, utilizing cells overexpressing wild-type cPLA$_2\alpha$ and a cPLA$_2\alpha$ mutant that has a greatly impaired ability to bind calcium, suggest that cPLA$_2\alpha$ may protect cells from death via generation of free AA. Moreover, our data suggest that the extent of free AA may regulate the events that are ultimately responsible for the cells surviving or dying.

EXPERIMENTAL PROCEDURES

Materials - [5,6,8,9,11,12,14,15-^{3}H]AA (200 Ci/mmol) was purchased from Amersham Ibérica (Madrid, Spain). MAFP was from Cayman Chemical Co., Inc. (Ann Arbor, MI). Anti-cPLA$_2\alpha$ and anti-caspase 3 antibodies were from Cell Signaling Technology (Beverly, MA). Polyclonal anti-caspase 9 was purchased from BD Biosciences Pharmingen. The general caspase inhibitor Z-VAD-FMK and the calpain inhibitor PD150606 were from Calbiochem. Tetramethyl rhodamine methyl ester (TMRE) was from Molecular Probes. All other reagents were from Sigma.

Cells - HEK cells were cultured in DMEM supplemented with 2 mM glutamine, 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37ºC in a 5% CO_2 humidified incubator. Cells were passaged twice a week by trypsinization. Cells (40-70% confluence) were transfected with 1 μg plasmid/ml using Lipofectamine Plus™ (Invitrogen), following the manufacturer's instructions. For stably transfected cells, 1 mg/ml G418 was used for selection and subsequent passages.

RAW 264.7 cells were cultured in RPMI medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine at 37 ºC in a 5% CO_2 humidified incubator.

Confocal microscopy - Cells were seeded on glass bottom culture dishes (MatTek Corp., Ashland, MA) coated with Poly-L-lysine (Sigma). After 24 hours, the culture medium was replaced by HBSS containing 10 mM HEPES and 1.3 mM $CaCl_2$. Cells were monitored at 37°C by confocal microscopy using a Bio-Rad laser scanning Radiance 2100 system coupled to a Nikon TE-2000U inverted microscope equipped with a DH-35 tissue culture dish heater (Warner Instruments). Most images shown in this paper were obtained with a CFI Plan Apo 60X, oil immersion, 1.40 NA objective, which provided a theoretical confocal layer thickness of approximately 0.4 μm at the wavelengths used. Green fluorescence from the EGFP was monitored at 488 nm argon laser excitation and the combination of a HQ500 long band pass and a HQ560 short band pass blocking filters. Red fluorescence from Cy3-annexin V was monitored at 543 nm HeNe laser excitation using a HQ590/570 long band pass blocking filter. For experiments in witch Cy3-annexin V was used, the cells were incubated in a buffer consisting of 10 mM Hepes, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4, to allow proper binding, and 1 μg/ml Cy3-annexin V was added 15 minutes prior to stimulation with 5 μM ionomycin.

AA release - HEK cells (2.5 x 10^5/well) were labeled with 0.25 μCi [^{3}H]AA overnight. Cells were then extensively washed and overlaid with 0.5 ml serum-free RPMI supplemented with 0.5 mg/ml albumin and treated with 5 μM thimerosal for 15 min to blunt fatty acid reacylation [11]. Cells were then stimulated with 5 μM ionomycin for different time periods. Supernatants were removed and cell monolayers were overlayed with ice-cold phosphate buffer containing 0.05% Triton X-100, and scraped with a cell scraper. Total lipids from supernatants and cells were extracted according to Bligh and Dyer [12]. After extraction, lipids were separated by thin-layer chromatography using the system n-hexane/diethyl ether/acetic acid (70:30:1 by volume). This system clearly separates phospholipid-bound AA from the one in free fatty acid form. Spots corresponding to

AA and phospholipid were scraped and radioactivity was quantified by liquid scintillation counting.

Stimulated [^{3}H]AA release represents a balance between what is being liberated by phospholipases minus what is being reincoporated back by the enzymes of the reacylation pathway. As our assay includes 5 μM thimerosal to blunt fatty acid reacylation, the vast majority of the AA liberated by the ionophore-stimulated PLA_2 will be recovered as free fatty acid. This distinguishes between incorporated and released fatty acid.

Immunoblot - Cells were lysed with lysis buffer, and 20-50 μg of protein was separated by standard 10% SDS-PAGE and transferred to nitrocellulose membranes. Primary (1:1000) and secondary antibodies (1:5000) were diluted in a buffer 25 mM Tris-HCl, 140 mM NaCl, pH 7.4, with 0.5% defatted dry milk and 0.1% Tween 20. Membranes were developed by the chemiluminescent ECL system (Amersham).

Constructs and mutagenesis - The DNA sequence of human $cPLA_2\alpha$ (Group IVA PLA_2) was cloned into the pEGFP vector (Clontech, Palo Alto, CA) using Hind-III and Pst-I cloning sites. This construct codifies for the expression of a fusion protein containing an *N*-terminal EGFP followed by the entire sequence of the human $cPLA_2\alpha$ (EGFP-$cPLA_2$). Wild type $cPLA_2\alpha$ was mutagenized within the calcium-binding site by replacing aspartic acid in position 43 by asparagine (D43N) using the QuikChange XL Site-Directed Mutagenesis kit from Stratagene and the oligonucleotides 5'-CATGCTTGATACTCCAAATCCCTATGTG GAAC-3' (forward) and 5'-GTTCCACATAGGGATTTGGAGTATCAA GCATG-3' (reverse). Mutagenesis was confirmed by sequencing.

Analysis of the mitochondrial membrane potential ($\Delta\Psi_m$) - To study $\Delta\Psi_m$, the cells were stained with either 7.5 μM 5,5′, 6,6′-tetrachloro-1,1′,3,3′-tetraethyl benzimidazol-carbocyanine iodide (JC-1), or 20 nM tetramethyl rhodamine methyl ester (TMRE) for 15-20 min at 37°C. Cells were then detached from the culture flasks by pipetting, washed, and resuspended in PBS containing 1.3 mM $CaCl_2$ and 1 mM $MgCl_2$. The cells were analyzed in a Beckman Coulter flow cytofluorimeter at 37°C. Green fluorescence was monitored at 505-545 nm (FL1), and red fluorescence at 555-600 nm (FL2). For analysis of JC-1 fluorescence under the confocal microscope, the cells were plated in MatTeck dishes, labeled with JC-1, and monitored as mentioned above, using 488 nm argon laser excitation and the combination of a HQ500 long band pass and a HQ560 short band pass-blocking filters for the green fluorescence, and a HQ570 long band pass-blocking filter for the red fluorescence.

Confocal microscopy measurement of TMRE-loaded cells was performed at 530 nm HeNe laser excitation using a 570 long-pass blocking filter. Fluorescence intensity was quantified in a perinuclear ring in which mitochondrial clusters are located.

Cell viability assay. The viability of cells was determined by the measurement of their capacity to exclude the dye Trypan blue. In brief, cells were cultured in 12-well plates and incubated at 37°C in the absence or presence of 5 μM ionomycin for different time periods. After this incubation, the cells were gently scraped, pelleted by centrifugation, resuspended in 500 μl of culture medium containing 0.2% Trypan blue, loaded into a hemocytometer, and examined by light microscopy. Viable and nonviable (blue cells) cells were then counted, and the score obtained expressed dead cells in percent of total cells.

Intracellular Ca^{2+} measurements. HEK cells were loaded with 3 μM Fluo-5F-AM for 20 min in medium with 10% serum at 37ºC in a 5% CO_2 incubator. Cells were then washed and incubated with HBSS containing 10 mM HEPES and 1.3 mM $CaCl_2$. Fluorescence was monitored under confocal microscope at 488 laser excitation and the combination of a HQ500 long band pass and a HQ560 short band pass blocking filters, having the iris totally open. At the end of each experiment calibration was carried out as previously described by Kao et al. [13], using

$MnSO_4$ at a final concentration of 2 mM, and lysis with 40 μM saponin to obtain the background signal.

RESULTS

Characterization of HEK cells overexpressing cPLA$_2$α and the mutant D43N-cPLA$_2$. Transfection of HEK cells with the construct EGFP-cPLA$_2$α resulted in an elevated AA response to the calcium ionophore ionomycin, as compared with wild type-untransfected cells (Fig. 1A). In contrast, cells transfected with a cPLA$_2$α mutant in which Asp^{43} was replaced with Asn (EGFP-D43N-cPLA$_2$α) [14-16], did not reproduce the increased AA mobilization. This was to be expected, since such a mutation greatly impairs the ability of cPLA$_2$α to interact with the membrane in a Ca^{2+}-dependent manner [14-16]. Fig. 1B shows that overexpression of EGFP-cPLA$_2$α or its D43N mutant in the HEK cells resulted in a robust expression of the fusion proteins, as judged by immunoblot.

Wild type EGFP-cPLA$_2$α was distributed uniformly throughout the cytoplasm of resting HEK cells (Fig. 1C). Shortly after ionomycin stimulation, EGFP-cPLA$_2$α translocated first to perinuclear structures that may represent the Golgi and endoplasmic reticulum (Fig. 1C, 5-8 min), and later to the nuclear envelope (Fig. 1C, 10-30 min). This is in agreement with recent data by others [17]. These localization changes were not observed in cells expressing EGFP only (data not shown). The time course of EGFP-cPLA$_2$α translocation is consistent with the observed time course of AA release in these cells, supporting the notion that binding to intracellular membranes is an essential step in cPLA$_2$α activation. In contrast with the above, the mutant, EGFP-D43N-cPLA$_2$α did not translocate to perinuclear membranes following ionomycin addition.

It is noteworthy that cells transfected with the EGFP-D43N- cPLA$_2$α but not those transfected with EGFP-cPLA$_2$α exhibited notable morphological changes after ionomycin treatment. We consistently observed the appearance of vacuole-like structures (black areas under the microscope), as well as increased nuclear fluorescence, which probably reflects increased nuclear permeability (Fig. 1D). Since both kinds of changes are known to accompany cell death in different cell systems, we sought to characterize the ionomycin-induced HEK cell death, and the intriguing role of cPLA$_2$α in this process.

Analysis of Ca^{2+}-dependent death in HEK cells. Measurement of the intracellular Ca^{2+} level in cells revealed a very rapid rise from ~100 nM in untreated cells to about 4 μM in the ionomycin-treated cells, reflecting cytoplasmic calcium overload (Fig. 2A). No differences in the kinetics or amplitude of calcium overload were found among wild type and cells transfected with plasmids encoding for cPLA$_2$α or the mutant D43N-cPLA$_2$. To assess whether the overload of Ca^{2+} is the cause or a consequence of cell death, viability experiments were conducted in cells exposed to ionomycin in the absence or presence of extracellular Ca^{2+}. Ionomycin-induced death only occurred if the incubation media contained Ca^{2+}, thus providing clear evidence that cell death is caused by cytoplasmic Ca^{2+} overload (Fig. 2B).

The effect of cPLA$_2$α overexpression on cell death was assayed next, and the results are shown in Fig. 2C. Wild type cells underwent massive death when exposed to ionomycin (over 70% of the cells died after 1-2 h exposure to ionomycin). In contrast, cell death in the cPLA$_2$α-transfected cells was kept consistently lower, even after 3 h incubation with the ionophore. These data provide direct evidence that cPLA$_2$α protects cells from Ca^{2+} overload-induced death.

In non transfected HEK cells ionomycin induced a modest release of AA and also morphological changes similar to those observed in the EGFP-D43N-cPLA$_2$α transfected cells. Some of these changes, i.e. cellular shrinking, and nuclear shape alterations, could be easily visualized under bright field microscopy (Fig. 3A).

It is well described that mitochondrial membrane potential ($\Delta\Psi_m$) changes, externalization of anionic phospholipids such as phosphatidylserine, activation of caspases, and chromatin condensation are all events related with programmed cell death [18, 19]. Changes in $\Delta\Psi_m$ were studied by loading HEK cells with the ratiometric indicator JC-1, that selectively accumulates in the mitochondria (monomers, green florescence, low membrane

potential), and subsequently aggregates as a function of $\Delta\Psi_m$ (red fluorescence, high membrane potential). Control untreated HEK cells loaded with JC-1 exhibited a heterogeneous distribution of depolarized (green) and hyperpolarized (red) mitochondria (Fig. 3B), as visualized by confocal microscopy. However, ionomycin-treated cells only showed green fluorescence, presumably due to a diffusion of JC-1 into the cytosol following depolarization of mitochondria. This process was also evaluated by flow cytometry, which is a more quantitative technique (Fig. 3C). Again, cells treated with ionomycin experienced an increase in green fluorescence and a decrease in red fluorescence. The mitochondrial uncoupler p-trifluoromethoxycarbonyl cyanide phenylhydrazone (FCCP), which induces mitochondria depolarization [20], produced fluorescence changes similar to that of ionomycin (data not shown).

Mitochondria depolarization can precede caspase activation, especially that of caspase-9, an initiator caspase that is proteolytically activated by binding to cytochrome c, after the latter is released from the mitochondria. Figure 3D indicates that ionomycin-treated cells showed no appearance of active fragments of caspase-9, except at very long times (i.e. >8 h). No active fragments of the effector caspase-3 could be detected at any time (Fig. 3D).

Chromatin condensation was analyzed by nuclear staining with the DNA-binding fluorophore (DAPI). By this procedure, no signs of chromatin condensation were detected at any time after ionomycin treatment of the cells (data not shown).

Externalization of phosphatidylserine is an event that parallels some types of cell death, and it can be easily detected by analyzing the binding of fluorescent annexin V to the cellular surface [21]. Cells treated with ionomycin for 10 min showed staining of the outer cellular surface with Cy3-annexin V, thus suggesting externalization of phosphatidylserine (Fig. 4A). Increased annexin V binding to the cells was not observed if the cells were treated with the general inhibitor Z-VAD-FMK, suggesting the involvement of caspases and/or calpains [22] (Fig. 4B).

The involvement of caspases in phosphatidylserine externalization can be ruled out by the data of Fig. 3D, where the lack of appearance of active mitochondria-related caspases-3 and -9 was shown. Therefore, experiments were conducted to study the possible involvement of calpains under those conditions. HEK cells preincubated with the specific calpain inhibitor PD150606 blocked annexin-V labeling after 15 min of ionomycin treatment (Fig. 4C), whereas the negative control PD145305 did not (data not shown). These data confirmed the involvement of calpains in phosphatidylserine externalization in this system. Control experiments adding EGTA to the incubation medium confirmed the Ca^{2+}-dependent nature of the ionomycin-induced phosphatidylserine externalization (data not shown).

EGFP-cPLA$_2\alpha$ overexpression prevents phosphatidylserine exposure in response to ionomycin. Initial experiments had shown that ionomycin induced morphological changes in the mutant EGFP-D43N-cPLA$_2\alpha$ overexpressing cells were not present in the wild-type EGFP- cPLA$_2\alpha$-overexpressing cells (Fig. 1), and also that cPLA$_2\alpha$ protected cells for Ca^{2+} overload-induced death (Fig. 2C). Next, we investigated whether these differences correlated with phosphatidylserine externalization. Cells overexpressing EGFP-cPLA$_2\alpha$ did not show increased exposure of phosphatidylserine in response to ionomycin treatment, as judged by annexin V staining (Fig. 5A). Interestingly, in cells overexpressing the calcium-binding domain mutant EGFP-D43N- cPLA$_2\alpha$, labeling of the outer cellular surface with annexin V was detected (Fig. 5B). The images taken from mutant EGFP-D43N-cPLA$_2\alpha$-transfected cells showed that treatment with ionomycin not only increased their intranuclear fluorescence but also induced cellular membrane blebbing (Fig. 5B), which is another striking early feature of some types of cell death [23].

Specific inhibition of cPLA$_2\alpha$ activity abrogates the protective effect of cPLA$_2\alpha$ overexpression. To further demonstrate that the cPLA$_2\alpha$ protective role is dependent upon its enzymatic activity, experiments were conducted in the presence of the active site-directed cPLA$_2\alpha$ inhibitor methyl arachidonyl fluorophosphonate (MAFP) [24]. Control

experiments indicated that treating the cells with 10 μM MAFP for 30 min completely abrogates cellular Ca^{2+}-dependent PLA_2 activity, as judged by an in vitro assay [25]. Figure 6 shows that MAFP did not inhibit the translocation of EGFP-cPLA$_2$α to perinuclear membranes in the ionomycin-treated cells; however, these cells were very strongly labeled with annexin V (Fig. 6). This behavior is similar to the one described above in wild type HEK cells or in cells transfected with the mutant D43N-cPLA$_2$α, and clearly shows that the activity of the cPLA$_2$α is needed for the enzyme to exert an effect.

Effect of exogenous AA. To further characterize cPLA$_2$α overexpression in these cells, we next investigated whether any of the putative products of cPLA$_2$α action on phospholipids, namely free AA and lysophospholipid, could mimic the effect of cPLA$_2$α overexpression on the HEK cells. The results are shown in Fig. 7, and demonstrate that addition of micromolar levels of free AA prior to treatment with ionomycin prevented phosphatidylserine externalization, as judged by Cy3-annexin V plasma membrane labeling. Importantly, the effect of free AA was found to be specific, as neither lysophosphatidylcholine nor other fatty acids like palmitic acid prevented phosphatidylserine externalization after ionomycin treatment (Figure 7).

AA induces $\Delta\Psi_m$ changes in HEK cells. Free fatty acids have been shown to directly act on the mitochondria by increasing proton permeability, opening the mitochondrial permeability transition pore [26], and/or influencing the activity of mitochondrial uncoupling proteins [27]. Since either of these actions would have an effect on $\Delta\Psi_m$, we studied whether AA changes the mitochondrial potential of HEK cells. JC-1 labeled HEK cells were analyzed by flow cytometry. As shown in figure 8, exogenous free AA produced a rapid and robust increase in JC-1 green fluorescence (within the first 5 min of treatment), while palmitic acid, a fatty acid that does not induce phosphatidylserine externalization (Fig. 7), had no effect, either if added alone, before or after AA. The mitochondrial uncoupler FCCP, produced a fluorescence increase similar to that of AA (not shown).

Overexpression of EGFP-cPLA$_2$α changes mitochondrial polarization after ionomycin treatment of HEK cells. It has been described that $\Delta\Psi_m$ is the driving force for mitochondrial Ca^{2+} overload [28]. Thus, the above results would suggest that, after ionomycin treatment, the high levels of AA produced in the EGFP-cPLA$_2$α transfected HEK cells may act to change $\Delta\Psi_m$ and, hence, inhibit mitochondrial calcium uptake and overload. The ultimate consequence would be that further mitochondrial damage would not take place.

To experimentally validate the above sequence of events, we proceeded to analyze $\Delta\Psi_m$ changes in the EGFP-cPLA$_2$α transfected HEK cells. Since EGFP-cPLA$_2$α expressing HEK cells display green fluorescence, it was not possible to use JC-1. We used instead a red reversible potentiometric fluorescence dye, TMRE that preferentially accumulates in the mitochondria [29]. During the course of these experiments we noticed that the EGFP-cPLA$_2$α transfected HEK cells were composed of two populations with varying degrees of EGFP-cPLA$_2$α expression, as judged by flow cytometry (Fig. 9). To compare wild type and EGFP-cPLA$_2$α overexpressing cells within the same experiment, we took advantage of the fact that in flow cytometry it is possible to separately analyze populations that exhibit a distinct fluorescent pattern. We mixed wild type cells –exhibiting green autofluorescence only--, with EGFP-cPLA$_2$α overexpressing cells, --exhibiting low and high green fluorescence--, and labeled them all with TMRE. Analysis of red fluorescence (TMRE fluorescence) in each single population was then performed by flow cytometry. The results are shown in Figure 9, and indicate that, after ionomycin challenge, there is a rapid decrease in red fluorescence. However, wild type cells had a deeper and quicker decrease in fluorescence than the EGFP-cPLA$_2$α-transfected cells. Maximum decrease was observed at about 2 min after ionomycin treatment for wild type HEK cells, but EGFP-cPLA$_2$α transfected cells demonstrated a slower kinetics, reaching the plateau at about 5 min after ionomycin treatment (Fig. 9B). Moreover, the high-expressing EGFP-cPLA$_2$α population experienced the lowest changes in TMRE fluorescence, as compared with the low-expressing EGFP-cPLA$_2$α, and wild type

cells. Those decrements of mitochondrial fluorescence primarily reflected mitochondrial depolarization, as further confirmed by the use of FCCP. The latter compound decreased TMRE fluorescence to an extent comparable to that induced by ionomycin (data not shown). Collectively, these data highlight the correlation between the amount of EGFP-cPLA$_2\alpha$ expressed by the cells and the degree of mitochondrial depolarization after ionomycin treatment.

In agreement with the above observations, pretreating the cells with the cPLA$_2\alpha$ inhibitor MAFP potentiated the TMRE fluorescence decrease provoked by ionomycin in the three cell populations analyzed (Fig. 10).

Confocal microscopy experiments were also performed in the TMRE-loaded cells. Fig. 11A shows that mitochondria did exhibit significant, bright staining with TMRE. No other cellular compartment incorporated the probe at least at detectable levels. By measuring fluorescence intensity only from the perinuclear ring where mitochondrial clusters are located, a time-dependent decrease in fluorescence in response to ionomycin was detected Collectively, these results from TMRE confocal microscopy (Figs. 11B & 11C), are fully consistent with the TMRE flow cytometry measurements described above (Figs. 9 and 10).

Studies with RAW 264.7 macrophages. To study whether the protective effect of AA on Ca^{2+} overload-induced cell death is a general one, we conducted studies with the murine macrophage-like cell line RAW264.7. These cells showed increased surface appearance of phosphatidylserine in response to inomycin, as judged by Cy3-annexin V staining (Fig. 12). Importantly, pretreating the cells with exogenous AA prevented the surface staining with Cy3-annexin V (Fig. 12).

To assess whether cPLA$_2\alpha$ has similar cytoprotective effects under more physiological conditions, we studied the effect of cPLA$_2\alpha$ overexpression on the cell death response of RAW 264.7 macrophage-like cells to peroxynitrite. Peroxynitrite, the coupling product of nitric oxide and superoxide, is thought to play a pivotal role in eliciting tissue damage during inflammation [30]. Exposure of RAW264.7 to 500 μM peroxynitrite for 1 h resulted in 40 ± 5% of the cells being positive for Trypan blue staining. Interestingly, in cells overexpressing cPLA$_2\alpha$, the percentage of cells positive for Trypan blue under exact experimental conditions was lowered down to 12 ±3.

DISCUSSION

Previous studies have demonstrated that cPLA$_2\alpha$ is the key effector involved in stimulated AA mobilization in response to Ca^{2+} ionophores [31, 32], and the results reported in this study are consistent with these previous observations. The response of EGFP-cPLA$_2\alpha$ transfected HEK cells to the Ca^{2+} ionophore ionomycin is very rapid, linearly increasing up to 15 min of exposure of the drug to the cells, and reaching a plateau thereafter. Under these conditions, cell death became apparent as early as 15 min after addition of ionomycin to the cells, as judged by increased blebbing, nuclear protrusion and permeability, as well as exposure of anionic aminophospholipids on the cell surface. These data are generally consistent with previous reports on the effects of cytoplasmic Ca^{2+} overload on early cell death, but they also provide important insights on to the role of cPLA$_2\alpha$-derived free AA levels as regulators of the balance between cell survival and death.

We have found that overexpression of cPLA$_2\alpha$ in HEK cells, results in the cells being protected from ionomycin-induced early signals of cell death. Importantly, this protective effect is not observed in cells overexpressing a cPLA$_2\alpha$ mutant in which the Asp^{43} has been replaced with Asn. Such a mutation causes the enzyme not to translocate to intracellular membranes in response to Ca^{2+} elevations, making it unable to reach its substrate and thus catalyze phospholipid hydrolysis [14-16].

The above data strongly suggested that the pro-survival effect of cPLA$_2\alpha$ documented in this work is caused by the elevated formation of a metabolite derived from the

action of cPLA$_2\alpha$ on phospholipids, namely free AA or a lysophospholipid. This prompted us to analyze the effect of treating the cells with low micromolar doses of free AA or lysophosphatidylcholine during ionomycin-induced cell death. The results indicate that only free AA exerts an effect. Interestingly, palmitic acid, at the same concentrations used for AA, fails to protect the cells, a finding that is consistent with the well known fact that cPLA$_2\alpha$ exhibits striking specificity for AA-containing phospholipids [1].

Our results appear to be in line with those studies that have suggested that, when cells commit to die in response to death ligands such as tumor necrosis factor α or Fas ligand, an early cellular event is the proteolytic cleavage of cPLA$_2\alpha$ by caspases to prevent liberation of AA and its subsequent metabolism to eicosanoids [9, 10]. It is inferred from these results that cPLA$_2\alpha$ activation may help promote cell survival under certain conditions. Similarly, it has also been suggested that PLA$_2$s may function to remove phospholipids damaged by or during the death process, thus allowing membrane repair and helping promote cell survival [33]. Our observation that not only cPLA$_2\alpha$ *per se* but also micromolar levels of exogenous AA are cytoprotective for ionomycin-treated HEK cells suggests that the fatty acid itself may be involved in a pathway leading to cell survival.

In keeping with the above we characterized further the protective mechanism mediated by AA. We observed that micromolar concentrations of free AA induce mitochondrial depolarization. It is unlikely that such elevated free AA levels are reached during ionomycin stimulation of the cells, even those overexpressing cPLA$_2\alpha$. Free AA being released upon ionophore stimulation would therefore induce smaller changes in $\Delta\Psi_m$ than those induced by exogenous, micromolar levels of AA. Such an effect could be produced by at least three different routes, namely (i) AA acting as a mitochondrial protonophore, (ii) AA opening the mitochondrial permeability transition pore, or (iii) AA acting on uncoupling proteins. The latter possibility appears unlikely in view of the lack of an effect of palmitic acid on HEK cell depolarization, for palmitic acid is known to be an effective activator of uncoupling mitochondrial proteins [33].

Given that accumulation of Ca^{2+} into the mitochondria is driven by the $\Delta\Psi_m$, [28] it can be concluded from our data that, in the overexpressing-cPLA$_2\alpha$ HEK cells, ionomycin will induce both Ca^{2+} entry into the mitochondria and the release of high amounts of AA via cPLA$_2\alpha$ activation. Intracellular AA would then produce small changes in $\Delta\Psi_m$, and blunt the capture of Ca^{2+} by this organelle. Then, complete dissipation of $\Delta\Psi_m$ that usually takes place after mitochondrial Ca^{2+} overload would not take place. As an ultimate consequence, mitochondrial damage would cease, and the subsequent events that parallel cell death, i.e. phosphatidylserine externalization, are stopped or delayed.

Collectively, our work leads to the important conclusion that high levels of free AA derived from cPLA$_2\alpha$ activation may play a cytoprotective role in cells exposed to a cytoplasmic Ca^{2+} overload. It is noteworthy that the mode of cell death induced by Ca^{2+} ionophores has been related to both their chemical nature as well as the concentrations utilized, lower concentrations causing apoptosis and higher concentrations causing necrosis in some cell types [35, 36]. In between these two extremes, it has also been reported that in certain cells, Ca^{2+} overload may produce cell death with characteristics of both necrosis and apoptosis [37]. Consistent with the latter, in the cell death system described here there is a very rapid mitochondrial depolarization, externalization of phosphatidylserine, blebbing, nuclear shape changes and increased nuclear permeability. All of these events occur during the first 15-30 min after ionomycin treatment, and most of them are typical of cells committed to apoptotic death. At longer times, there is neither caspase activation nor nuclear chromatin condensation, and these are processes more consistent with a necrotic type of death.

Of special relevance to the results presented in this work is a recent study by Tommasini *et. al.* [8] in peroxynitrite-treated U937 cells. These authors found that cPLA$_2\alpha$ activation promoted survival in cells exposed

to peroxynitrite and the effect was mediated by free AA. By utilizing different maneuvers to decrease the activation state of the cPLA$_2\alpha$ and the capacity of the cells to generate AA, (i.e. chemical inhibitors, antisense oligonucleotides) Tommasini *et. al*. [8] made the key observation that the extent of AA formation regulates the balance between cell survival and death. By means of a different experimental approach (i.e. increasing the cellular levels of cPLA$_2\alpha$ activity by overexpressing the enzyme), we have obtained results that are in agreement with the work by Tommasini *et. al*. [8]. It appears therefore that the cytoprotective effect of elevated levels of free AA is not restricted to the context of Ca^{2+}-dependent cell death, but may operate in other settings as well, such as triggering of cell death after an oxidative insult. As a matter of fact, we have obtained evidence that overexpression of cPLA$_2\alpha$ in RAW264.7 macrophages also prevents peroxynitrite-induced cell death.

On the other hand, there are a number of reports suggesting that free AA and cPLA$_2\alpha$ activation may act to favor cell death under a variety of settings. For example, tumor necrosis factor-α mediated apoptosis is impaired in cells deficient in cPLA$_2\alpha$ [38] or when the enzyme is inhibited by pharmacological means [39]. Inhibition of AA metabolizing enzymes results in elevated AA levels which has been related to apoptosis [40,41], and the proapoptotic effects have also been reproduced by direct addition of exogenous fatty acid in some instances [42]. Moreover, overexpression of either long chain fatty acyl-CoA synthetase or cyclooxygenase-2, --which scavenge free AA by converting it into the CoA thioester derivative or prostaglandin, respectively--, protects cells from apoptosis [43]. These results are consistent with the proposed proapoptotic effects on nonsteroidal anti-inflammatory drugs under certain conditions [44]. In rat hepatoma MH1C1 cells, it was shown that free AA increases upon stimulation of cPLA$_2$a by the ionophore A23187 and causes cell death through the mitochondrial pathway [6]. Clearly, the role of cellular free AA acid on apoptosis is a debatable issue, and appears to be strikingly dependent on experimental conditions and agonists under study.

From a pathophysiological point of view, the overexpression of cPLA$_2\alpha$ resulting in enhanced cell survival may bear important consequences with regard to mammalian cell defense against virus infection. In a recent report, Allal *et. al.* [45] have found that cPLA$_2\alpha$ is one of the several host-derived proteins carried by human cytomegalovirus. Although the function of these host-cell derived proteins is mostly unknown, cPLA$_2\alpha$has been suggested to be required for proper infectivity [46, 47]. Based on our data demonstrating that overexpression of cPLA$_2\alpha$ protects from cell death, it is tempting to speculate that a critical function of the cPLA$_2\alpha$ borne by the human cytomegalovirus and injected into the host cell may be that of ensuring survival of the infected cell by maintaining free AA at high levels. In this manner, the execution of the host cell's death program would be blocked and the virus would buy the time necessary to fully replicate inside the host cell.

In conclusion, our data demonstrate that overexpression of cPLA$_2\alpha$ in HEK cells protects them from death induced by cytoplasmic Ca^{2+} overload, and that the mechanism involves the generation of elevated levels of free AA, and changes in $\Delta\Psi m$. Since the cPLA$_2\alpha$ ordinarily present in wild type cells appears not to produce AA at high enough levels to counteract the early death induced by ionomycin, it appears that under conditions of Ca^{2+} overload, it is the extent of free AA availability that determines whether the cell will survive or die.

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FOOTNOTES

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[1]The abbreviations used are: PLA_2, phospholipase A_2; AA, arachidonic acid; $cPLA_2\alpha$, Group IVA cytosolic phospholipase $A_2\alpha$; EGFP, enhanced green fluorescent protein; HEK, human embryonic kidney; MAFP, methyl arachidonyl fluorophosphonate; $\Delta\Psi_m$, mitochondrial membrane potential.

[2]J. Casas and M. A. Balboa, unpublished observation.

FIGURE CAPTIONS

Figure 1. *[^{3}H]AA release and $cPLA_2$ translocation in HEK cells.* A) Wild type HEK cells, cells transfected with EGFP-$cPLA_2\alpha$, and cells transfected with the mutant EGFP-D43N-$cPLA_2\alpha$ were labeled overnight with [^{3}H]AA as described under Experimental Procedures. The cells were then stimulated with 5 μM ionomycin for the indicated periods of time, and [^{3}H]AA release was analyzed by thin-layer chromatography. The data are expressed as percent of total label incorporated in phospholipids. B) Cell lysates from wild type HEK cells, EGFP-$cPLA_2\alpha$–transfected HEK cells, EGFP-D43N-$cPLA_2\alpha$ transfected HEK cells or RAW 264.7 cells were analyzed by immunoblot using specific antibodies against $cPLA_2\alpha$. C, D) HEK cells, transfected with EGFP-$cPLA_2\alpha$ (C) or the mutant EGFP-D43N- $cPLA_2\alpha$ (D), were stimulated with 5 μM ionomycin and images were recorded under confocal microscopy at the indicated times. [^{3}H]AA release measurements were carried out in triplicate and a representative experiment out of three is shown. Confocal experiments were done at least four times and many cells were analyzed each time.

Figure 2. *Calcium dependence of cell death*. A) Wild type (closed circles), $cPLA_2\alpha$-transfected (open circles), or D43N-$cPLA_2\alpha$-transfected HEK cells (close triangles) were loaded with fluo-5F-AM, and intracellular calcium measurements were performed. The arrow indicates the time at which 5 μM ionomycin was added to the cells. For these experiments cells expressing $cPLA_2\alpha$s withou EGFP were used so as to avoid interference with the fluo-5F fluorescence. B) Wild type (circles) or EGFP-D43N-$cPLA_2\alpha$ transfected HEK cells (triangles) were treated with 5 μM ionomycin in the absence (open simbols) or presence (closed simbols) of 1.3 mM $CaCl_2$ in the incubation medium. After the indicated times, cell viability was assessed by Trypan Blue exclusion. C) Wild type

(open bars) or cPLA$_2\alpha$-overexpressing (closed bars) HEK cells were treated with 5 μM ionomycin for the indicated periods of time. Afterward, cell viability was assessed by Trypan Blue exclusion.

Figure 3. *Cellular changes induced by ionomycin.* Wild type HEK cells were treated with 5 μM ionomycin or vehicle for the indicated periods of time, and analyzed for A) changes in cell shape under bright field microscopy; B) JC-1 staining observed by confocal microscopy; C) JC-1 staining studied by flow cytometry (left panel, green fluorescence; right panel, red fluorescence); D) the presence of active fragments of caspase-9 and caspase-3 by immunoblot. As a control to visualize active caspases Jurkat cells treated with Camptothecin were used (Camp, panel D, first lane from the right). Experiments were carried at least three times, and representative experiments are shown.

Figure 4. *Ionomycin induces Cy3-annexin V binding to HEK cells.* HEK cells were preincubated with Cy3-annexin V (1 μg/ml) for 15 min in the absence (Control) or presence of the general caspase/calpain inhibitor Z-VAD-FMK (A) or the specific calpain inhibitor PD156060 (20 μM) (B). Cells were then stimulated with 5 μM ionomycin for 10 min. Confocal microscopy images were taken before and after ionomycin treatment. Experiments were done at least thrice, and many cells were analyzed in each experiment.

Figure 5. *cPLA$_2\alpha$ overexpression prevents ionomycin-induced Cy3-annexin V binding.* HEK cells transfected with EGFP-cPLA$_2\alpha$ (A) or the EGFP-D43N- cPLA$_2\alpha$ mutant (B) were preincubated with 1 μg/ml Cy3-annexin V for 15 min and then stimulated with 5 μM ionomycin for 10 min. Cells were monitored by confocal microscopy and pictures were taken before and after ionomycin treatment. Experiments were done at least three times, and many cells were analyzed in each experiment.

Figure 6. *Annexin V labeling of cPLA$_2$a-overexpressing cells treated with MAFP*. HEK cells transfected with EGFP-cPLA$_2$ were preincubated with 1 μg/ml Cy3-annexin V for 15 min in the absence (A) or presence of the cPLA$_2$ inhibitor MAFP (10 μM) (B). Cells were then stimulated with 5 μM ionomycin for 10 min, observed by confocal microscopy and pictures were taken at the indicated time points. Experiments were done at least three times, and many cells were analyzed.

Figure 7. *Exogenous AA prevents ionomycin-induced Cy3-annexin V in HEK cells*. HEK cells were preincubated with 1 mg/ml Cy3-annexin V for 15 min in the absence (A) or presence of 10 μ M AA (B), lysophosphatidylcholine (10 μM) (C), or palmitic acid (10 μM) (D). Cells were then stimulated with 5 μM ionomycin. Fluorescence was monitored by confocal microscopy and images were taken before and after 10 min ionomycin stimulation. Experiments were done at least three times, and many cells were analyzed.

Figure 8. *Time-course of JC-1 fluorescence changes in HEK cells*. Wild type HEK cells were labeled with 7.5 μM JC-1 for 15 min, and analyzed for changes in JC-1 fluorescence by flow cytometry as described in Experimental Procedures. At the indicated time points 10 μM palmitic acid or 10 μM AA was added to the cells. Experiments were done at least three times and fluorescence from 50-100 x 10^3 cells was analyzed each time.

Figure 9. *Time-course of TMRE fluorescence changes in wild type and two populations of EGFP-cPLA$_2\alpha$-expressing HEK cells.* (A) Green fluorescence was analyzed by flow cytometry in wild type (light grey) and EGFP-cPLA$_2\alpha$–transfected HEK cells (dark grey). (B) HEK cells, and the two populations of EGFP- cPLA$_2\alpha$–expressing cells were mixed, labeled with 20 nM TMRE and red fluorescence was monitored by flow cytometry. 5 μM ionomycin was added to the medium at the indicated time points. After data collection red fluorescence was analyzed for each population separately. Experiments were done at least three times and fluorescence from 50-100 x 10^3 cells was analyzed each time.

Figure 10. *Effect of MAFP on TMRE fluorescence in ionomycin-treated HEK cells*. Wild type and EGFP- cPLA$_2\alpha$ transfected HEK cells were labeled with 20 nM TMRE, and treated with vehicle or 10 μM MAFP for 10 min. Red fluorescence was monitored by flow cytometry. 5 μM Ionomycin was added at the indicated time points. After data acquisition, analysis of red fluorescence was done separately for each population of cells: A) wild type, B) low EGFP-cPLA$_2\alpha$–expressing HEK cells, and C) high EGFP-cPLA$_2\alpha$–expressing cells. Experiments were done at least three times and fluorescence from 50-100 x 10^3 cells was analyzed each time.

Figure 11. *Confocal microscopy analysis of TMRE-loaded cells*. HEK cells were loaded with 20 μM TMRE, and analyzed under the confocal microscope (A). The cells were treated with vehicle (open circles) or 5 μM ionomycin (closed circles) and fluorescence was analyzed every 15 s (B). The transmission images and TMRE fluorescence of the analyzed cells at 0 and 15 min after ionomycin treatment is shown in panel C.

Figure 12. *Binding of Cy3-annexin V to RAW 264.7 cells treated with ionomycin*. RAW 264.7 cells were preincubated with 1 mg/ml Cy3-annexin V for 15 min in the absence (A) or presence of 10 μM AA (B). Cells were then stimulated with 5 μM ionomycin. Fluorescence was monitored by confocal microscopy and images were taken before and after 10 min ionomycin stimulation. Experiments were done at least twice and many cells were analyzed each time.

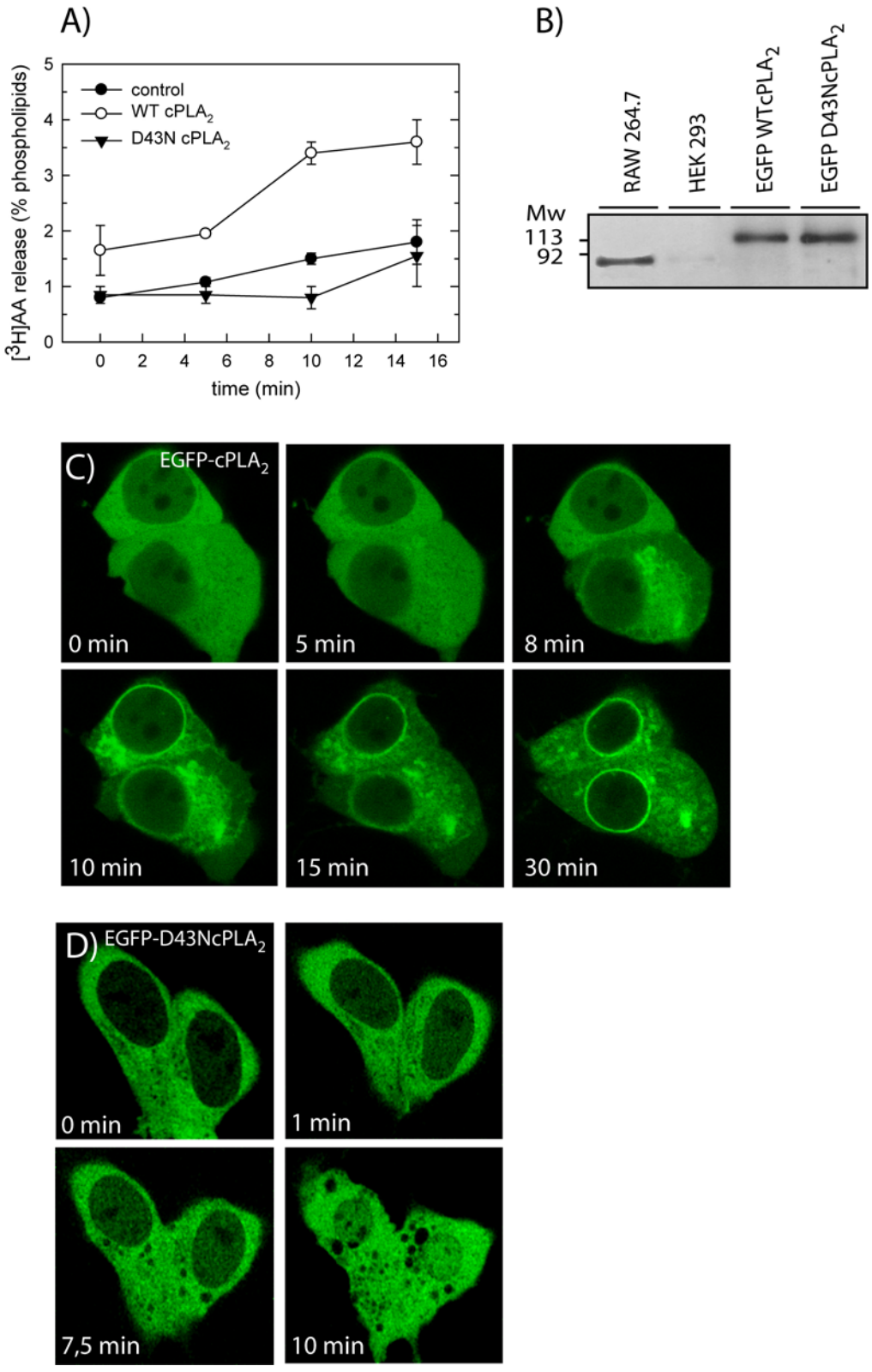

A)
control
WT cPLA2
D43N cPLA2
[3H]AA release (% phospholipids)
time (min)
B)
RAW 264.7
HEK 293
EGFP WTcPLA2
EGFP D43NcPLA2
Mw
113
92
C)
EGFP-cPLA2
0 min
5 min
8 min
10 min
15 min
30 min
D)
EGFP-D43NcPLA2
0 min
1 min
7,5 min
10 min

Figure 1

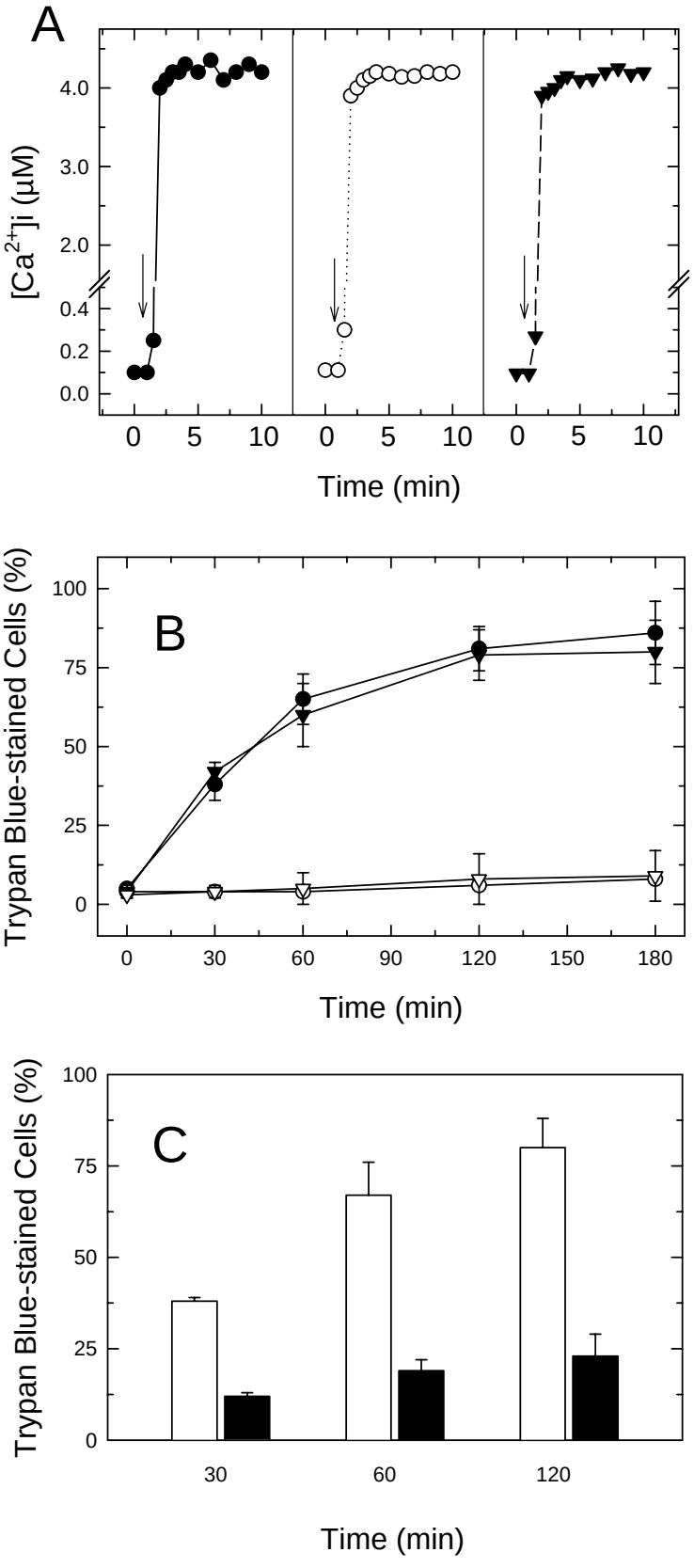

A
[Ca2+]i (μM)
4.0
3.0
2.0
0.4
0.2
0.0
0
5
10
Time (min)
B
Trypan Blue-stained Cells (%)
100
75
50
25
0
0
30
60
90
120
150
180
Time (min)
C
Trypan Blue-stained Cells (%)
100
75
50
25
0
30
60
120
Time (min)

Figure 2

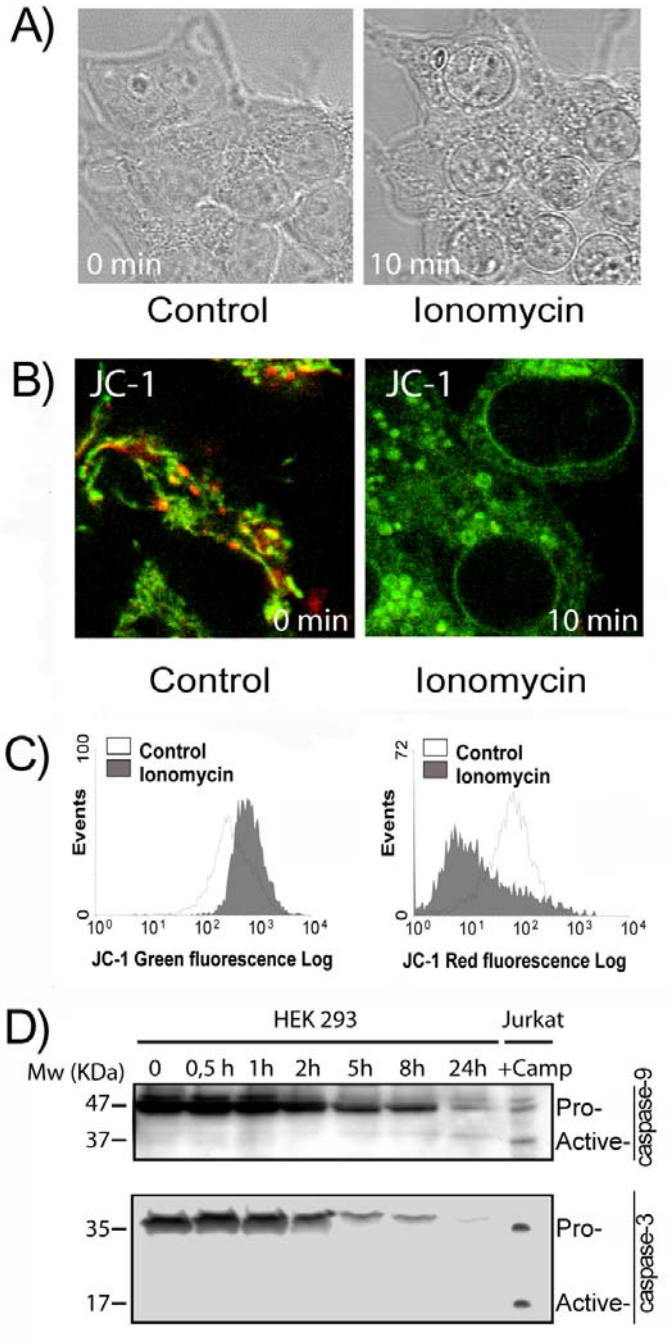

A)
0 min
10 min
Control
Ionomycin
B)
JC-1
JC-1
0 min
10 min
Control
Ionomycin
C)
Control
Ionomycin
Events
100
0
10^0 10^1 10^2 10^3 10^4
JC-1 Green fluorescence Log
Control
Ionomycin
Events
72
0
10^0 10^1 10^2 10^3 10^4
JC-1 Red fluorescence Log
D)
HEK 293
Jurkat
Mw (KDa)
0 0,5 h 1h 2h 5h 8h 24h +Camp
47–
37–
Pro-
Active-
caspase-9
35–
17–
Pro-
Active-
caspase-3

Figure 3

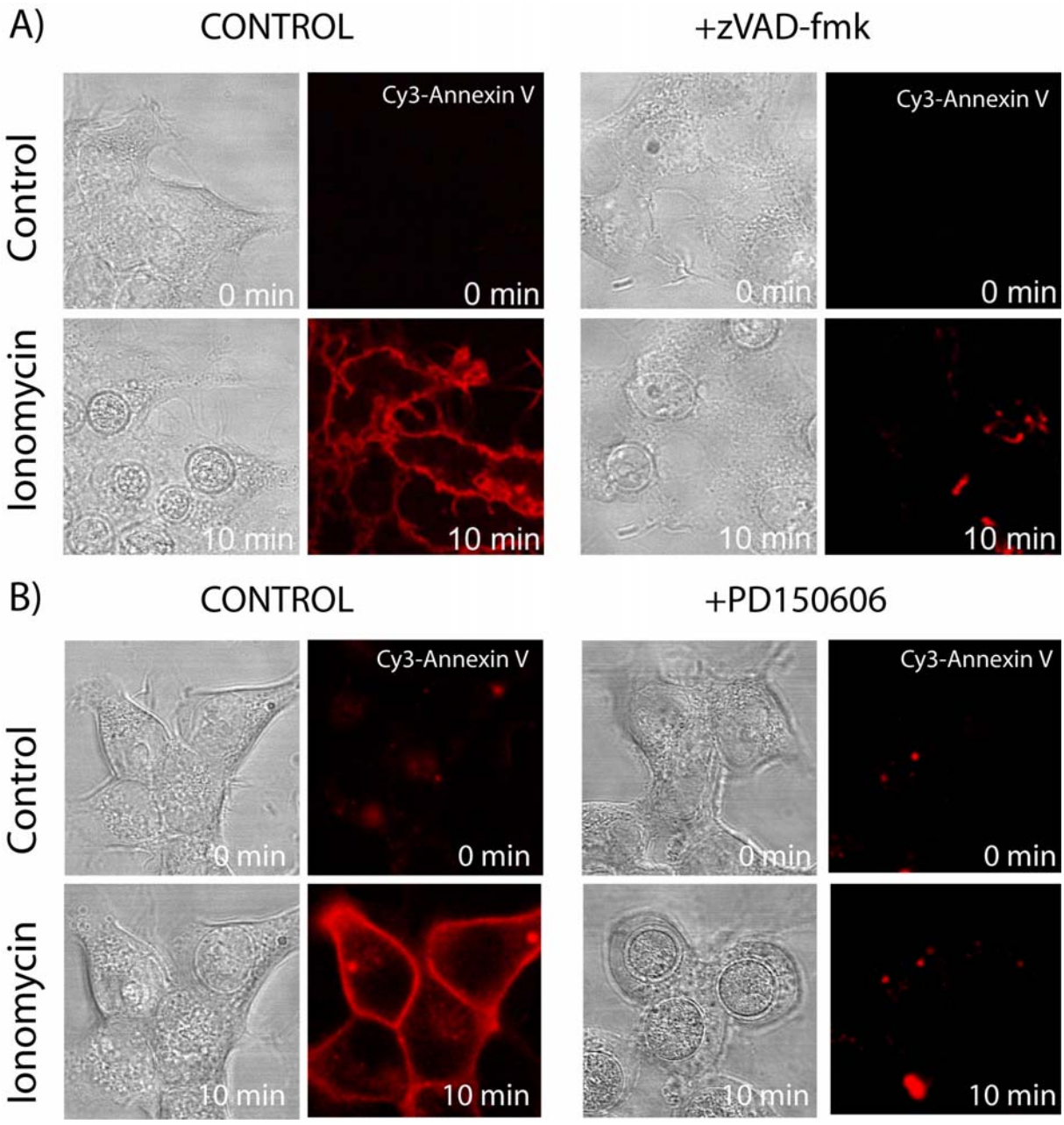

A)
CONTROL
+zVAD-fmk
Control
Ionomycin
Cy3-Annexin V
0 min
10 min
B)
CONTROL
+PD150606
Control
Ionomycin
Cy3-Annexin V
0 min
10 min

Figure 4

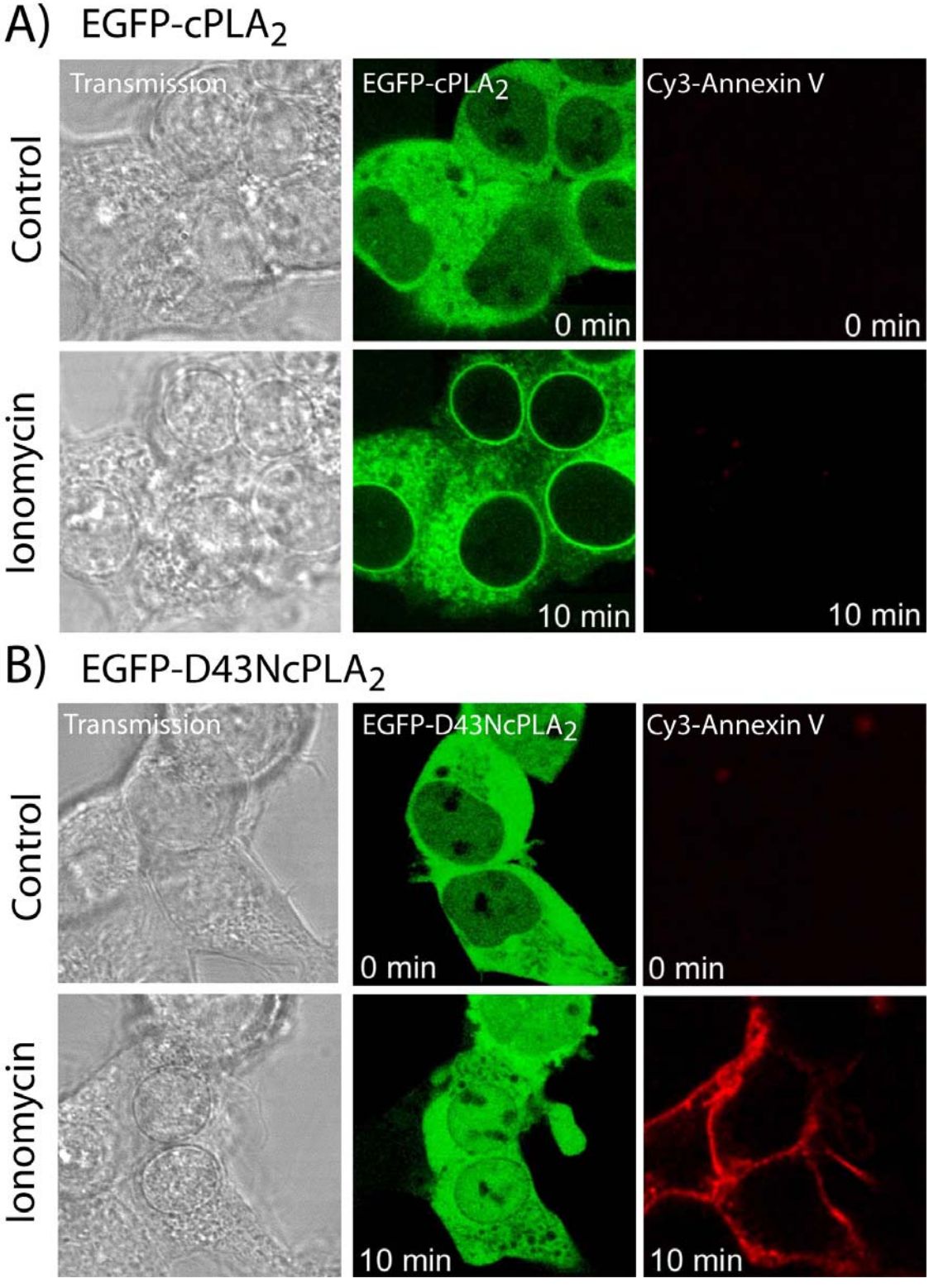

A) EGFP-cPLA2
Transmission
EGFP-cPLA2
Cy3-Annexin V
Control
0 min
0 min
Ionomycin
10 min
10 min
B) EGFP-D43NcPLA2
Transmission
EGFP-D43NcPLA2
Cy3-Annexin V
Control
0 min
0 min
Ionomycin
10 min
10 min

Figure 5

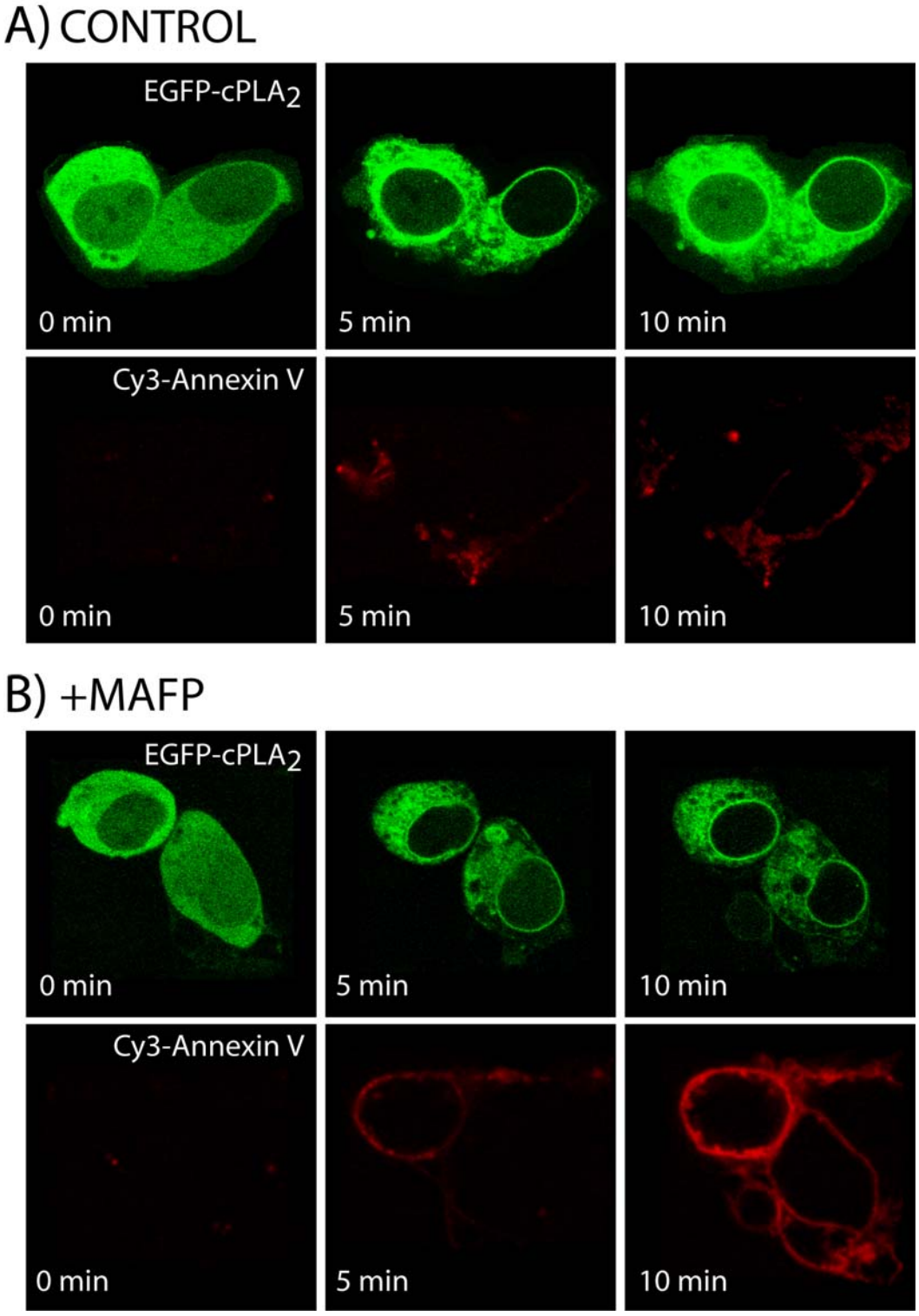

A) CONTROL
EGFP-cPLA2
0 min
5 min
10 min
Cy3-Annexin V
0 min
5 min
10 min
B) +MAFP
EGFP-cPLA2
0 min
5 min
10 min
Cy3-Annexin V
0 min
5 min
10 min

Figure 6

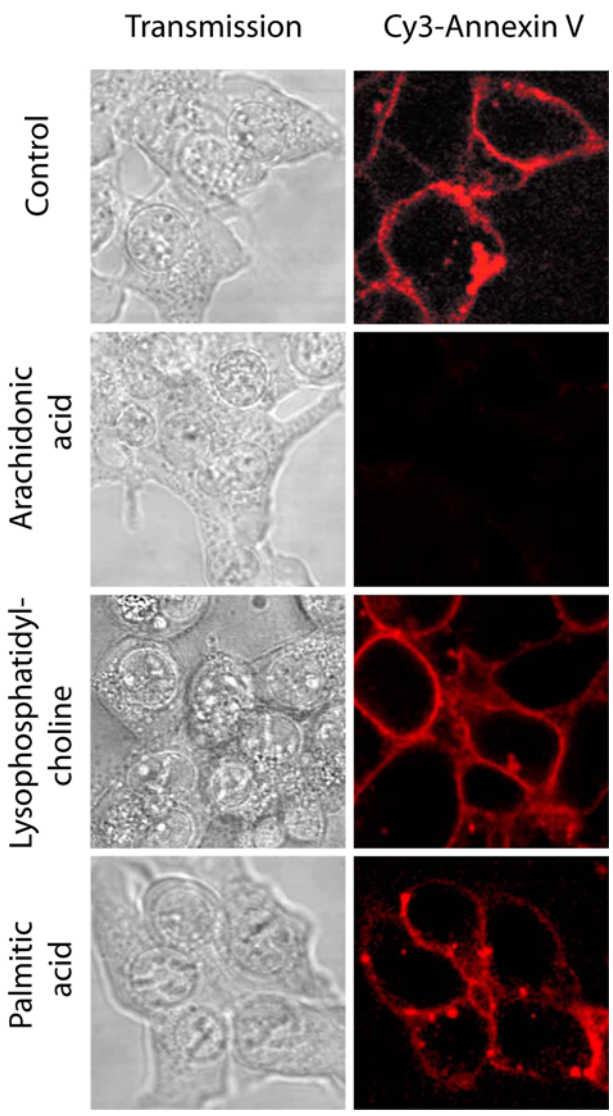

Transmission
Cy3-Annexin V
Control
Arachidonic acid
Lysophosphatidyl-choline
Palmitic acid

Figure 7

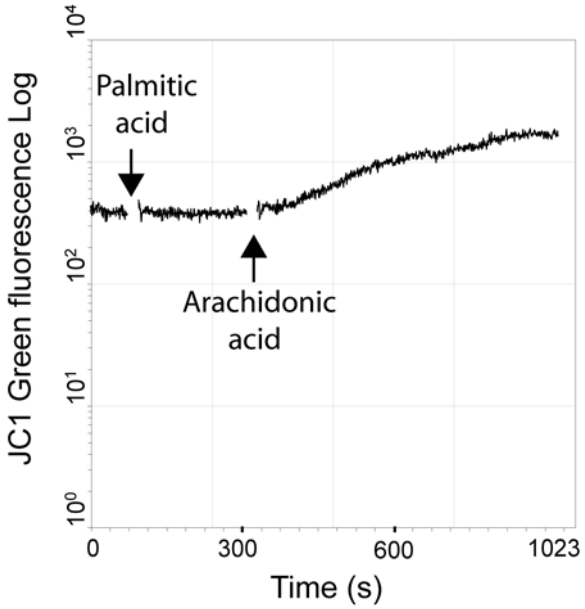

JC1 Green fluorescence Log
10^4
10^3
10^2
10^1
10^0
Palmitic acid
Arachidonic acid
0
300
600
1023
Time (s)

Figure 8

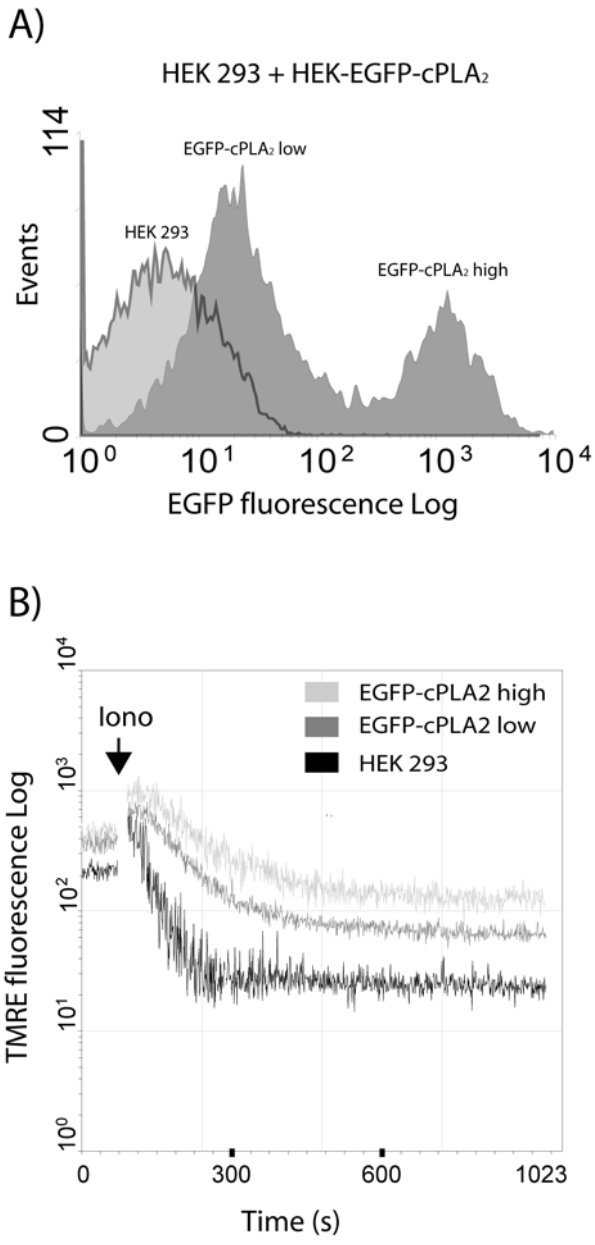

A)
HEK 293 + HEK-EGFP-cPLA2
114
Events
0
EGFP-cPLA2 low
HEK 293
EGFP-cPLA2 high
10^0
10^1
10^2
10^3
10^4
EGFP fluorescence Log
B)
TMRE fluorescence Log
10^4
10^3
10^2
10^1
10^0
Iono
EGFP-cPLA2 high
EGFP-cPLA2 low
HEK 293
0
300
600
1023
Time (s)

Figure 9

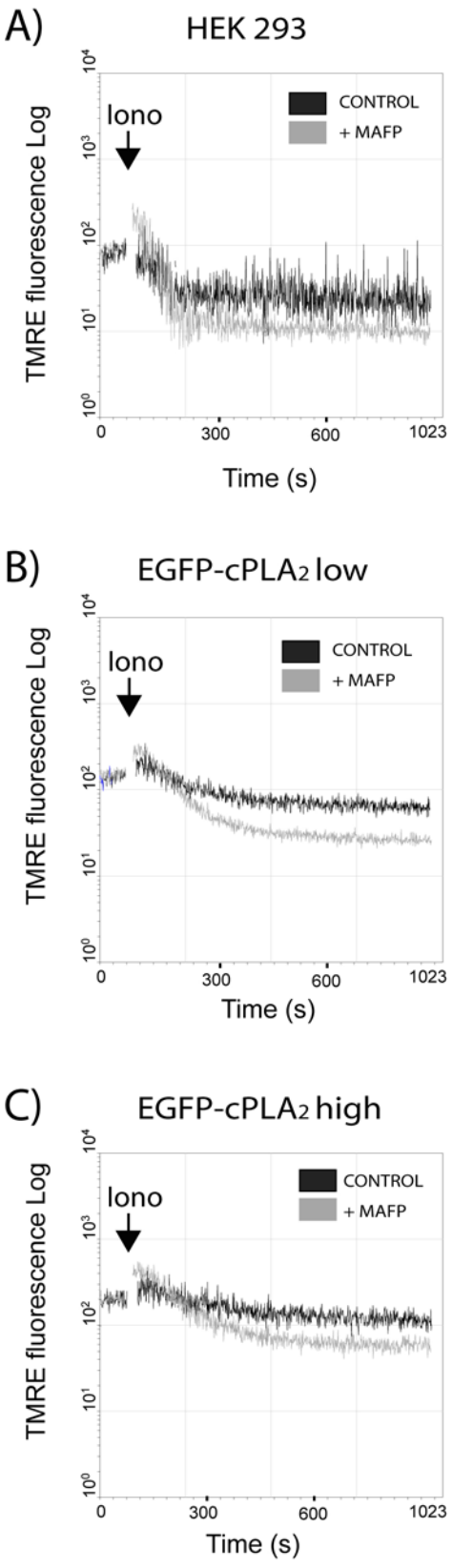

A)
HEK 293
TMRE fluorescence Log
Iono
CONTROL
+ MAFP
Time (s)
B)
EGFP-cPLA2 low
TMRE fluorescence Log
Iono
CONTROL
+ MAFP
Time (s)
C)
EGFP-cPLA2 high
TMRE fluorescence Log
Iono
CONTROL
+ MAFP
Time (s)

Figure 10

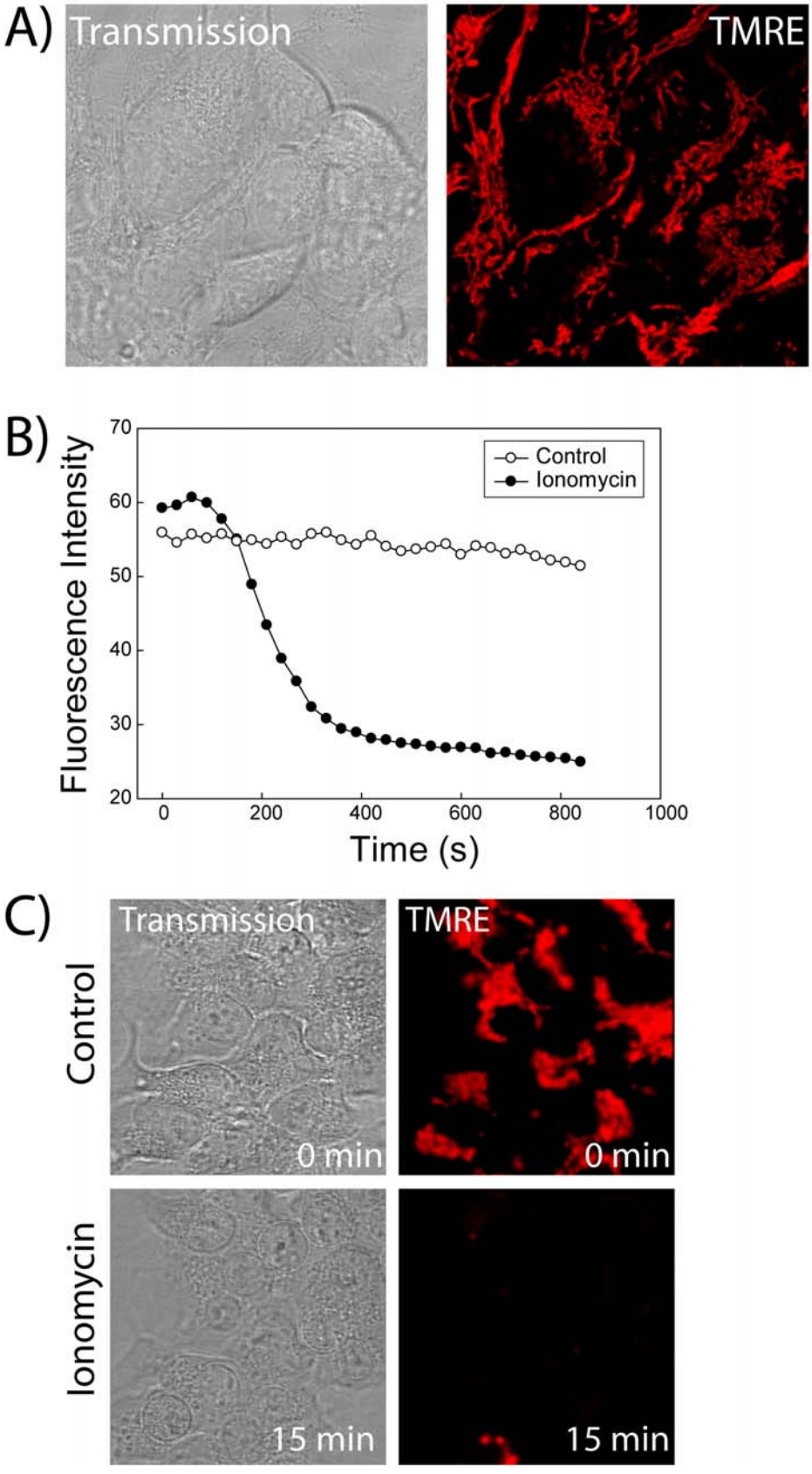
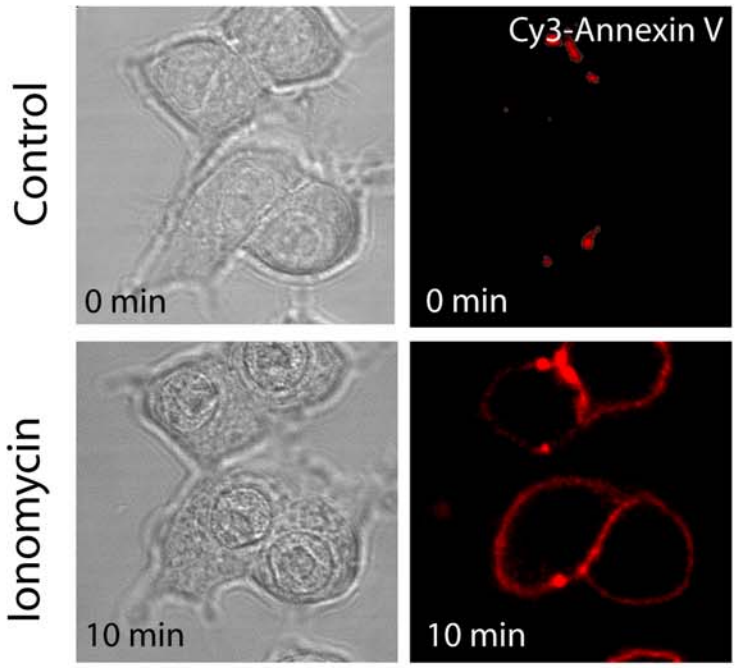

A)
Transmission
TMRE
B)
Fluorescence Intensity
70
60
50
40
30
20
0
200
400
600
800
1000
Time (s)
Control
Ionomycin
C)
Control
Transmission
TMRE
0 min
0 min
Ionomycin
15 min
15 min

Figure 11

A) CONTROL



B) +Arachidonic acid

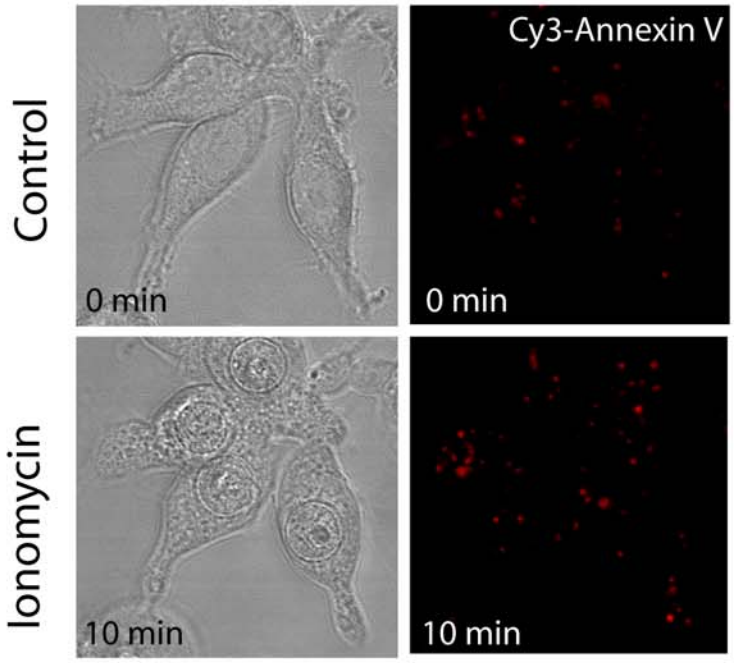


Figure 12