The Cationic Cluster of Group IVA Phospholipase A_2 ($Lys^{488}/Lys^{541}/Lys^{543}/Lys^{544}$) Is Involved in Translocation of the Enzyme to Phagosomes in Human Macrophages

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Abstract

Group IVA cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) plays a role in the microbicidal machinery of immune cells by translocating to phagosomes to initiate the production of antimicrobial eicosanoids. In the present work we have studied the involvement of the cationic cluster of $cPLA_2\alpha$ ($Lys^{488}/Lys^{541}/Lys^{543}/Lys^{544}$) in the translocation of $cPLA_2\alpha$ to the phagosomal cup in human macrophages responding to opsonized zymosan. Phagocytosis was accompanied by an increased mobilization of free arachidonic acid, which was almost completely abrogated by pyrrophenone. In transfected cells a catalytically active EGFP-$cPLA_2\alpha$ translocated to the phagocytic cup, which was corroborated by frustrated phagocytosis experiments utilizing immunoglobulin G-coated plates. However, a $cPLA_2\alpha$ mutant in the polybasic cluster $Lys^{488}/Lys^{541}/Lys^{543}/Lys^{544}$, that cannot bind the anionic phospholipid phosphatidylinositol 4, 5-bisphosphate (PIP_2), did not translocate to the phagocytic cup. Moreover, an EYFP-$cPLA_2\alpha$ and an ECFP-pleckstrin homology (PH) domain of the $PLC\delta_1$ construct that specifically recognizes endogenous PIP_2 in the cells, both localized at the same sites on the phagosome. High cellular expression of the PH domain inhibited EYFP-$cPLA_2\alpha$ translocation. On the other hand, two other members of the phospholipase A_2 superfamily of enzymes were studied as well, namely group V secreted phospholipase A_2 and group VIA calcium-independent phospholipase A_2, but the results indicated that neither of these are involved in zymosan phagocytosis by human macrophages. Collectively, these data indicate that the polybasic cluster $Lys^{488}/Lys^{541}/Lys^{543}/Lys^{544}$ of $cPLA_2\alpha$ regulates the subcellular localization of $cPLA_2\alpha$ in intact immune cells under physiologically relevant conditions.

ABBREVIATIONS: AA, arachidonic acid; PLA_2, phospholipase A_2; $cPLA_2\alpha$, group IVA cytosolic phospholipase A_2; $iPLA_2$, calcium-independent phospholipase A_2; $sPLA_2$, secreted phospholipase A_2; $sPLA_2$-V, group V secreted phospholipase A_2; BEL, bromoenol lactone; bis-BODIPY FL C11-PC, 1,2-bis-(4,4-difluoro-5,7-dimethyl-4-bora-3a, 4a-diaza-*s*indecane-3-undecanoyl)-*sn*-glycero-3-phosphocholine; $PLC\delta_1$-PH, pleckstrin homology domain of $PLC\delta_1$-PH; PIP_2, phosphatidylinositol 4,5-bisphosphate.

Introduction

Phospholipase A_2 (PLA_2) enzymes cleave membrane phospholipids at the *sn-2* position of the glycerol backbone, releasing a free fatty acid and a lysophospholipid [1]. One of the better studied roles of PLA_2 enzymes is their involvement in inflammatory processes, due to their ability to liberate free arachidonic acid (AA) from membrane phospholipids [2-5]. Free AA will in turn be oxygenated by specific enzymes to generate a wide variety of compounds with potent pro- and anti-inflammatory actions, collectively called the eicosanoids [6, 7]. Some of these compounds have also been described as important bactericidal agents [8].

Of the many existing PLA_2 enzymes, only one, the group IVA PLA_2 (also known as cytosolic phospholipase $A_2\alpha$, ($cPLA_2\alpha$) manifests specificity for AA-containing phospholipids [9]. Today it is widely accepted that $cPLA_2\alpha$ is the key enzyme in AA release leading to physiological and/or pathophysiological eicosanoid production [9-11]. Structurally, $cPLA_2\alpha$ possesses a C2 domain containing the binding site for Ca^{2+} --and a binding site for ceramide-1 phosphate as well [12]--, and a catalytic domain, where the residues involved in enzymatic activity are located [9]. The catalytic domain also contains a cluster of cationic amino acids (Lys^{488}, Lys^{541}, Lys^{543}, and Lys^{544}) that may mediate enzyme binding to anionic phospholipids in membranes [13]. In fact, in vitro, anionic phospholipids such as PIP_2, $PI(3,4,5)P_3$ and $PI(3,4)P_2$ strongly increase the $cPLA_2\alpha$ specific activity when incorporated into the vesicle substrate [14, 15]. Mutation of the aforementioned polybasic 4-Lys cluster eliminates the activating effect of PIP_2 on $cPLA_2\alpha$ activity [13]. Also, by plasmon resonance experiments it has been demonstrated that PIP_2 activates $cPLA_2\alpha$ by increasing the catalytic efficiency of the enzyme as a result of increasing membrane penetration [16]. In live cells, exogenous PIP_2 and $PI(3,4)P_2$ induce the translocation of $cPLA_2\alpha$ to the intracellular membranes

[17]. More recently, it has been described that mutation of the Lys^{488}, Lys^{543}, and Lys^{544}, decreases AA release in cells activated by serum, pointing to an important role for that cluster in $cPLA_2$ activation under physiological stimulation [18].

Phagocytosis is a specialized process of cells of the innate immune system to engulf invading microorganisms, foreign particles and apoptotic cells and debris. Phagocytosis is initiated at the site of particle attachment, producing a polarized region within the membrane that enlarges as a pseudopod to engulf the particle and drive it into the cytoplasm, where it will be degraded [19]. It has been previously shown that $cPLA_2\alpha$ translocates to the phagosomal cup during the ingestion of particles, and such a translocation appears to be important for *in situ* eicosanoid generation and efficient elimination of microbes [20-23]. However, the mechanism regulating this translocation remains largely unknown. Utilizing human macrophages responding to opsonized zymosan, we demonstrate in this work that translocation of $cPLA_2\alpha$ to the phagocytic cup depends on an intact cationic cluster $Lys^{488}/Lys^{541}/Lys^{543}/Lys^{544}$) in the catalytic domain of the enzyme. These results reveal a novel role for the cluster and provide new insights into the complex cellular regulation of the $cPLA_2\alpha$.

Materials and Methods

Materials - [5,6,8,9,11,12,14,15-^{3}H]AA 200 Ci/mmol was purchased from Amersham Ibérica (Madrid, Spain). Zymosan labeled with Alexa Fluor 594 and bis-BODIPY FL C11-PC were from Molecular Probes (Carlsbad, CA). Ficoll-Paque™ Plus was from GE Healthcare (Uppsala, Sweden). Gentamicin was purchased from Biowhittaker (Walkersville, MD). Human macrophage Nucleofection solution was from Amaxa (Gaithersburg, MD). Macrophage serum free medium and RPMI 1640 were purchased from GIBCO (Carlsbad, CA). The pEGFP-PLCδ_1-PH domain construct with the pleckstrin domain from the PLCδ_1 was kindly provided by Dr. Tobias Meyer (Stanford University Medical Center, Stanford, CA) [24]. The EGFP-cPLA$_2\alpha$ plasmid, the mutant for the PIP$_2$ binding site EGFP-4KE/A-cPLA$_2\alpha$, the iPLA$_2$-VIA-EYFP plasmid, and the sPLA$_2$-V-EGFP plasmid have been described elsewhere [17, 25, 26]. pDsRed-Monomer-C1 was obtained from Clontech (Mountain View, CA). All other reagents were from Sigma.

Cells - Human macrophages were obtained from Buffy coats of healthy volunteer donors obtained from the *Centro de Hemoterapia y Hemodonación de Castilla y León* (Valladolid, Spain). Briefly, blood cells were diluted 1:1 with PBS, layered over a cushion of Ficoll-Paque and centrifuged at 750 x g during 30 min. The mononuclear cellular layer was then recovered and washed three times with PBS, resuspended in RPMI supplemented with 2 mM L-glutamine, 40 μg/ml of gentamicin and allowed to adhere to plastic in sterile dishes for 2 h. Non-adherent cells were then removed by extensively washing with PBS. Macrophage differentiation was achieved by incubating the adhered monocytes in RPMI supplemented with 2 mM L-glutamine, 40 μg/ml of

gentamicin and 5% human serum for two weeks, in the absence of exogenous cytokine mixtures.

Plasmid transfection - Human macrophages were transfected by the Nucleofection technique (Amaxa), following the kit specifications for human macrophages. Briefly, cells were harvested by treatment with trypsin for 90 min and then by gentle scrapping. After washing, the cells were resuspended in 100 μl Human Macrophage Nucleofector solution plus 5 μg of plasmid. Nucleofection was carried out using the program Y-010, and the cells were resuspended in 400 μl of macrophage serum free medium (GIBCO) plus 5% heat inactivated human serum.

Synchronized phagocytosis - These experiments were carried out as described elsewhere with minor modifications [27]. Macrophages were seeded over glass coverslips, allowed to adhere and then washed with RPMI supplemented with 40 μg/ml gentamicin. Cells were then kept at 4ºC for 5 min, and opsonized zymosan was then added. After 15 min incubation, coverslips were transferred to plates with RPMI at 37ºC, and the phagocytosis was allowed to proceed for different periods of time. Reactions were stopped by fixation with 3% paraformaldehyde and 3% sacarose for 15 min if they were to be analyzed by microscopy. After 3 washes with PBS, the coverslips were mounted in glass slides with antifade medium. Samples were than analyzed by epifluorescent or confocal microscopy.

*Confocal microscopy***.** Transfected cells were seeded in MatTek dishes and allowed to adhere for 24 h in RPMI supplemented with 2 mM L-glutamine, 40 μg/ml of gentamicin and 5% human serum. Medium was then changed by HBSS with 10 mM HEPES and

1.3 mM $CaCl_2$ and fluorescence was monitored by confocal a Bio-Rad Radiance 2100 laser-scanning system coupled to a Nikon TE-2000U with a thermostatized chamber (Warner Instruments). The objective was CFI Plan Apo 60X, 1.4 numerical aperture, oil immersion. The fluorescence of ECFP was monitored at 457 nm argon excitation using the combination of a long pass barrier filter HQ470LP and a short pass filter HQ520SP. The fluorescence of EGFP was monitored at 488 nm Argon excitation using the combination of a long pass filter HQ500LP and a short pass filter HQ560SP. The EYFP was monitored at 514 nm Argon excitation and the filters HQ520LP and HQ560 SP. The Alexa Fluor 594 fluorescence was monitored at 543 nm HeNe excitation using a long band pass filter HQ570LP.

AA release. Cells were labeled with 0.5 μCi/ml of [^{3}H]AA overnight. Afterward, the cells were washed three times with PBS supplemented with 0.5 mg/ml fatty acid free-bovine serum albumin and incubated with RPMI supplemented with 0.5 mg/ml fatty acid-free bovine serum albumin and 1% ITS. Cells were then stimulated and supernatants were removed at different time periods. Cells monolayers were overlaid with ice-cold phosphate buffer containing 0.05% Triton X-100 and scraped. Radioactivity was quantified by liquid scintillation counting and AA release was referred to total radioactivity for each condition.

Frustrated phagocytosis – This was carried out as described by Marshall *et al*. [28]. Briefly, human macrophages were dislodged from the culture plates by trypsin treatment, and were resuspended in RPMI containing 10 mM HEPES and 2 mM EDTA. Cells were gently stirred for 2-3 hours to allow for the re-expression of membrane receptors. Cells were then washed, resuspended in RPMI containing 10 mM HEPES and 2 mM $MgCl_2$, and plated over MatTek dishes treated or not with 10 mg/ml pure IgG.

After 30 min at 37°C, the cells were monitored by confocal microscopy. Some pictures were obtained in the XZ axis to have a better view of the cell membranes attached to the glass. In some experiments, the cells were labeled with 5 μM bis-BODIPY FL C_{11}-PC for 30 min, washed twice and processed for frustrated phagocytosis. Fluorescence was monitored by confocal microscopy using 488 argon excitation and the combination of a HQ500 *long band pass* filter and HQ560 *short band pass filter*.

Results

Translocation of $cPLA_2\alpha$ to the phagosome in zymosan-stimulated human macrophages. Human macrophages are capable of recognizing yeast-derived zymosan particles and engulf them. By using transfected cells, we detected the translocation of a chimeric construct EGFP-$cPLA_2\alpha$ from the cytosol to the phagocytic cups (Fig. 1). Translocation of the enzyme was particularly prominent in non-sealed phagocytic cups (Fig 1, 10-15 min). Once the phagosome was sealed and internalized, the EGFP-$cPLA_2\alpha$ separated from it (Fig. 1, 60 min). Experiments were performed next to rule out the possibility that the increased fluorescence arising from EGFP-$cPLA_2\alpha$ in the forming phagosome was due to an increased volume of cytoplasm imaged in the plane. This was addressed by imaging in the same cell the construct EGFP-$cPLA_2\alpha$ and a monomeric form of DsRed —to correct for local variations in cytoplasmic volume— . Fig. 2 shows a clear increase in EGFP-$cPLA_2\alpha$ fluorescence in the phagosomes that does not correspond with an increase in cytoplasmic volume (ratio EGFP-$cPLA_2\alpha$ /DsRed) at any time, thus indicating that enzyme translocation actually occurs.

Phagocytosis of opsonized zymosan activates $cPLA_2\alpha$ in human macrophages - Zymosan induced a significant release of AA (and metabolites) to the extracellular medium, suggesting the activation of a PLA_2 (Fig. 3A). This PLA_2 was identified as $cPLA_2\alpha$ on the basis of complete inhibition of the response by a low concentration of pyrrophenone, a $cPLA_2\alpha$ inhibitor [29] (Fig. 3B).

To confirm that the $cPLA_2\alpha$ that translocates to the phagocytic cup is functionally active, an experiment of frustrated phagocytosis was performed, utilizing glass plates coated with IgG [28]. Macrophages exposed to IgG-coated glass surfaces

responded by translocating the EGFP-cPLA$_2\alpha$ to the membranes more proximal to the glass surface, but not to other cellular membranes (Fig. 4A, B and E). In this experiment, the IgG-coated glass would represent the phagocytosable particle [28]. Next, we loaded the cells with the fluorogenic phospholipase substrate 1,2-bis-(4,4-difluoro-5,7-dimethyl-4-bora-3a, 4a-diaza-*s*indecane-3-undecanoyl)-*sn*-glycero-3-phosphocholine (bis-BODIPY FL C11-PC) [30], and subjected them to the frustrated phagocytosis assay. A dramatic increase in fluorescence was observed, especially in the proximity of the IgG-coated glass (Fig. 4C, D and F), indicating that an A-type phospholipase is acting at that place (where the "phagosome" is being initiated). To confirm that such a phospholipase is actually cPLA$_2\alpha$, we conducted experiments in the presence of pyrrophenone (Fig. 4D and F). As expected, pyrrophenone, at doses as low as 1 μM, strongly blocked the fluorescence increase in the cells exposed to IgG-coated glass, indicating that such a fluorescence increase is mainly due to cPLA$_2\alpha$ activation.

Mutation of a four-lysine cluster in cPLA$_2\alpha$ that is involved in binding of anionic phospholipids suppresses EGFP-cPLA$_2\alpha$ translocation to the phagocytic cup - We have previously described that mutations on Lys488, Lys541, Lys543 and Lys544 of cPLA$_2\alpha$ result in a defective translocation of cPLA$_2\alpha$ to intracellular membranes in response to exogenous PIP$_2$ [17]. Because it is well described that PIP$_2$ increases in the phagosome [31], we studied next the behavior of the 4-Lys mutant (EGFP-4KE/A-cPLA$_2\alpha$) in macrophages exposed to opsonized zymosan. The results, as shown in Fig. 5A, clearly indicated that this mutant does not translocate to the phagocytic cup in activated cells at any time tested, suggesting that a functional binding site in cPLA$_2\alpha$ for anionic phospholipids is necessary for such a translocation to be observed. To further substantiate this observation, we performed experiments of frustrated phagocytosis, similar to those shown in Fig. 4, utilizing cells transfected with the EGFP-4KE/A-

$cPLA_2\alpha$ mutant. Fig. 5B shows that, unlike the wild type enzyme, the mutant did not translocate to the membranes that are closer to the IgG-coated plate. These data highlight the importance of the 4-Lys cluster for proper binding of $cPLA_2\alpha$ to the phagosomal membranes.

Experiments were conducted next to study a possible role for anionic phospholipids in $cPLA_2\alpha$ translocation to the phagocytic cup. To this end, we took advantage of the PIP_2-binding properties of a fluorescent chimeric protein of EGFP with the pleckstrin homology domain of the $PLC\delta_1$ (EGF-$PLC\delta_1$-PH) [28]. Figure 6A shows that, when transfected into human macrophages, the chimera labels the main cellular reservoir of PIP_2, i.e. the plasma membrane [24]. Immediately after promoting PIP_2 hydrolysis by activating the cells with a calcium ionophore, the fluorescence disappears from the plasma membrane, and accumulates in the cytoplasm, as would be expected from a functional PH domain (Fig. 6A). By using this chimera, we confirmed in human macrophages previous observations by Botelho *et al.* [31], indicating that PIP_2 levels increase at the phagocytic cup while the phagosome is being formed, and decrease when it seals (Fig. 6B).

Subsequently, the cells were co-transfected with both constructs, namely ECFP-$PLC\delta_1$-PH and EYFP-$cPLA_2\alpha$. In resting cells, ECFP-$PLC\delta_1$-PH was present only in the plasma membrane, whereas EYFP-$cPLA_2\alpha$ was found primarily in the cytoplasm (Fig. 7). However, after exposure of the cells to opsonized zymosan both chimeric proteins localized at the forming phagocytic cups (Fig. 7B). There was no co-localization in the cytoplasm of resting or stimulated cells. We noticed also that in those cells where ECFP-$PLC\delta_1$-PH construct was expressed at higher levels the translocation of the $cPLA_2\alpha$ to the phagosomes was inhibited (Fig. 7 C).

The behavior of EGFP-$PLC\delta_1$-PH and EGFP-$cPLA_2\alpha$ were analyzed in more

detail by confocal analysis of z-stack series from cells that were engulfing particles at similar stages of phagocytosis (Figs. 8 and 9). We observed that both constructs were enriched in the same sites of the phagosomes, especially along the particles and in the base. It is worth noting that translocation of the EGFP-cPLA$_2\alpha$ to the base of some of the phagosome was more prominent than that of EGFP-PLCδ_1-PH. It is possible that in the base of the phagosome other factors, in addition to or independently of PIP$_2$, contribute to the translocation of cPLA$_2\alpha$.

Secreted group V and cytosolic calcium-independent group VIA PLA$_2$s do not translocate to the phagocytic cup - To address whether other PLA$_2$s in addition to cPLA$_2\alpha$ could also translocate to the phagocytic cup during phagocytosis of opsonized zymosan, we utilized human macrophages transfected with either sPLA$_2$-V-EGFP or iPLA$_2$-VIA-EYFP. In agreement with our previous studies in murine macrophages [25, 32], sPLA$_2$-V-EGFP was found in resting cells associated with secretory granules and Golgi-like structures (Fig 10A). iPLA$_2$-VIA-EYFP had a mitochondrial localization in unstimulated cells (Fig. 10B), which is in accordance with previous estimates [33]. After the cells where challenged with opsonized zymosan, localization was studied between the chimeric proteins and the fluorescent particles. We failed to detect association of the fluorescence arising from either sPLA$_2$-V-EGFP or iPLA$_2$-VIA-EYFP to the phagocytic cups under any condition (Fig. 10). Thus, of the three major PLA$_2$ classes potentially capable of generating lipid mediators during inflammation [2-5, 34], only the cPLA$_2\alpha$ translocates to the phagocytic cup in human macrophages.

Discussion

A major regulatory mechanism of cPLA$_2\alpha$ activity in cells is the Ca^{2+}-dependent control of the physical state of the enzyme. In resting cells, the enzyme resides in the cytosol and hence, having no access to its substrate in the membrane, has no activity. In stimulated cells cPLA$_2\alpha$ translocates to the membrane in a Ca^{2+}-dependent process, this resulting in phospholipid hydrolysis and free AA release [9]. Most of the research carried out to date has thoroughly documented the translocation of cPLA$_2\alpha$ to intracellular membranes such as those of the nuclear envelope, Golgi complex or endoplasmic reticulum [9]. A few instances have been reported of translocation of cPLA$_2\alpha$ to cellular membranes different to those indicated above [20, 35, 36]. This study adds to these studies by showing that the phagosomal membrane of human macrophages is also a site for cPLA$_2\alpha$ translocation during activation conditions. Particularly relevant to our report is the work by Girotti et. al. showing translocation of cPLA$_2\alpha$ to forming phagosomes in murine macrophages [20]. Although the molecular mechanism was not investigated, the authors noted that chelation of extracellular calcium decreased the total number of phagocytic events, but cPLA$_2\alpha$ still remained associated to the lasting phagosomes at intracellular calcium levels equaling those of resting cells [20]. In previous work from our laboratory, we showed that introducing exogenous short-chain PIP_2 into cells promotes the translocation of cPLA$_2\alpha$ from cytosol to perinuclear membranes at basal levels of intracellular Ca^{2+} [17]. Moreover, other studies have demonstrated that PIP_2 transiently increases in the forming phagosome and disappears after phagosome sealing, being undetectable in the sealed phagosome [31]. Intrigued by these observations, we speculated that translocation of cPLA$_2\alpha$ to the forming phagosome could require the cationic cluster of four Lys

(Lys488/Lys541/Lys543/Lys544) that is present in $cPLA_2\alpha$ and has been shown to bind anionic phospholipids such as PIP_2 [13]. Using confocal microscopy techniques, we document the localization of $cPLA_2\alpha$ and a PIP_2-binding construct, ECFP-PLCδ_1-PH, in forming phagosomes. Also, high level expression of the ECFP-PLCδ_1-PH inhibits the translocation of the $cPLA_2\alpha$ to phagosomes. Moreover, mutation of the cationic cluster of Lys488, Lys541, Lys543 and Lys544 that serves as a PIP_2-binding site, eliminates the ability of the enzyme to translocate to the phagosomal membrane. Collectively, these results provide the first example of a physiologically-relevant condition where the cationic cluster of $cPLA_2\alpha$ participates in regulating enzyme association to membranes and hence, its activity in intact cells.

The presence of $cPLA_2\alpha$ at the membrane of the nascent phagosome may serve important pathophysiological roles, since during phagocytosis large quantities of eicosanoids are produced which could be involved in the killing of the ingested microorganism at the phagosome [8, 21-23]. In the mouse model, other PLA_2s in addition of $cPLA_2\alpha$ have been suggested to be related to phagocytic events. Particularly relevant to this work, it has been shown that group V secreted PLA_2 also translocates to the phagosome in zymosan-stimulated murine peritoneal macrophages [23]. Intrigued by this report, we also studied the possible movement of $sPLA_2$-V to phagosomes in our human macrophage cell system. We failed to detect translocation of a chimeric $sPLA_2$-V-EGFP protein to phagosomes in our systems. We had previously demonstrated that this chimeric $sPLA_2$-V behaves the same as the native $sPLA_2$-V protein in terms of biochemical properties, enzymatic activity and subcellular localization [25, 32]. There are many differences between the murine and human macrophage models of zymosan phagocytosis that may explain these different results. For instance, in murine macrophages, zymosan induces abundant AA release [37-43] and may be internalized

primarily via dectin-1 receptors [44]. However, human macrophages do not respond readily to zymosan by releasing AA, and opsonization of the particle appears to be required for full responses, which may occur primarily via Fc receptors. Thus, it appears likely that the different mechanisms of internalization of phagocytosable particles in mouse *versus* human may account, at least in part, for the remarkable differences in the translocation ability of $sPLA_2$–V to the phagosomes.

The one other PLA_2 that we investigated is $iPLA_2$-VIA, a calcium-independent enzyme. Unlike $cPLA_2\alpha$ and $sPLA_2$ enzymes, the involvement of $iPLA_2$-VIA in receptor-mediated AA mobilization appears not to be a general one, but to depend on cell type and stimulation conditions [2, 45-48]. Using a chimeric construct, $iPLA_2$-VIA-EYFP, we have detected no appreciable change in the subcellular localization of this enzyme during opsonized zymosan challenge; the enzyme always remained associated to mitochondria, which is consistent with previous data [33]. Thus, from the three major PLA_2 families potentially capable of effecting AA release for eicosanoid production, only one, $cPLA_2\alpha$, translocates to the phagosome in human cells.

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Figure Legends

Figure 1. *Translocation of $cPLA_2\alpha$ to the phagosome in zymosan-treated human macrophages.* Human macrophages were transfected with the construct EGFP-$cPLA_2\alpha$, treated with 1 mg/ml opsonized-zymosan for the indicated times, fixed and analyzed by confocal microscopy. In panels A and E Alexa Fluor 594-labeled zymosan was used to better visualize the particles. Fluorescence arising from the EGFP-$cPLA_2\alpha$ (green) or zymosan (red) is shown on the left column. Middle-column panels show a pseudocolored fluorescence intensity from the EGFP-$cPLA_2\alpha$. Panels on the right column show a detailed amplification of the phagosomes framed in the middle panels. Scale bar = 10 μM.

Figure 2. *Analysis of the translocation of $cPLA_2\alpha$ and the monomeric DsRed to phagosomal membranes.* Human macrophages were co-transfected with the construct EGFP-$cPLA_2\alpha$ or the soluble fluorescent protein DsRed (monomeric form). Cells were stimulated with opsonized zymosan, fixed, and fluorescence was analyzed by confocal microscopy at different times. A) The figure represents the fluorescence ratio EGFP-$cPLA_2\alpha$ / DsRed in phagosomes versus cytosol at each time point. At least 4 phagosomes per cell were analyzed from many cells. B) Pictures of the cellular fluorescence of the EGFP-$cPLA_2\alpha$ and the DsRed at different times of phagocytosis are shown.

Figure 3. *AA release in human macrophages challenged with opsonized zymosan.* (A) Human macrophages labeled with [^{3}H]AA were treated with 1 mg/ml opsonized

zymosan (black triangles) or vehicle (black squares). AA release was assessed at different times as described under Materials and Methods. (B) Cells labeled with [^{3}H]AA were pretreated with 1 μM pyrrophenone (pyrr) or vehicle (control) for 30 min and then treated with 1 mg/ml opsonized zymosan (black bars) or vehicle (grey bars) for 1 h.

Figure 4. *cPLA$_2\alpha$ is functionally active in the phagosomal cup in human macrophages.* (A) Cells transfected with EGFP-cPLA$_2$ were subjected to a frustrated phagocytosis assay on non-coated glass (Ctrl) or IgG-coated glass (IgG), as indicated, and analyzed by confocal microscopy. Pictures of the XZ axis of the cells were taken. (B) The intensity of the fluorescence obtained in A was analyzed in pseudocolor. (C) Cells, labeled with bis-BODIPY FL C$_{11}$-PC, were plated on non-coated glass (Ctrl) or on IgG-coated glass (IgG) for 30 min, and fluorescence was analyzed by confocal microscopy. The mean of fluorescence intensity in Ctrl was 66 and in IgG was 128. (D) The cells were labeled with bis-BODIPY FL C$_{11}$-PC and subjected to a frustrated phagocytosis assay. Pictures of the XZ axis of the cells were taken, and the intensity of the fluorescence was analyzed in pseudocolor. In the picture on the bottom, the cells were treated with 1 μM pyrrophenone (Pyrr). White bar = 10 μm. E) Statistical analysis of the fluorescence intensity in cells assayed for frustrated phagocytosis as in D. Data are represented as relative fluorescence intensities (mean fluorescence intensity in membranes closer to the glass/mean fluorescence intensity in the cytosol). At least 15 different cells were analyzed.

Figure 5. *The mutant EGFP-4KE/A-cPLA$_2\alpha$ does not translocate to the phagosome in human macrophages.* A) Human macrophages transfected with the mutant EGFP-

4KE/A-cPLA$_2\alpha$ were subjected to synchronized phagocytosis with Alexa Fluor 594-labeled opsonized zymosan for the indicted times, fixed and analyzed by confocal microscopy. Framed phagosomes have been enlarged and the intensity of the fluorescence analyzed by pseudocolor (left upper inserts at 15 and 30 min). B) Cells transfected with the construct EGFP-cPLA$_2\alpha$ or EGFP-4KE/A-cPLA$_2\alpha$ were subjected to a frustrated phagocytosis assay, and plated over non-coated glasses (Ctrl) or IgG-coated glasses (IgG) as indicated, and analyzed by confocal microscopy. Pictures of the XZ axis of the cells were taken and the intensity of the fluorescence obtained was analyzed in pseudocolor. The figure is representative of more than 40 cells that were analyzed per experiment, and the experiment was repeated four times. White bar = 10 μm.

Figure 6. *PIP_2 accumulation in the forming phagosomes during zymosan phagocytosis in human macrophages.* (A) Cells were transfected with the construct EGFP-PLCδ_1-PH and analyzed in vivo by confocal microscopy. Pictures were taken before (control) and after 5 min of treatment with 5 μM ionomycin (Iono 5 μM). (B) Cells were transfected with the construct EGFP-PLCδ_1-PH, treated with opsonized zymosan, an analyzed in vivo by confocal microscopy. Pictures were taken at different time points after exposure to zymosan, as indicated. The pictures on the bottom right correspond to the phagosomes selected with a dotted white box at each time point, and the fluorescence intensity has been analyzed in pseudocolor.

Figure 7. *Localization of ECFP-PLCδ_1-PH and EYFP-cPLA$_2\alpha$ during phagocytosis in human macrophages.* Human macrophages, co-transfected with the ECFP-PLCδ_1-PH and the EYFP-cPLA$_2\alpha$ constructs, were subjected to synchronized phagocytosis using

Alexa Fluor 594-labeled opsonized zymosan, fixed at 0 (Control) and 15 min and analyzed by confocal microscopy (A). B) A cell with high expression of the construct ECFP-PLCδ_1-PH is shown. In A and B, middle panels, fluorescence intensities are shown in pseudocolor, and detailed fluorescence in forming phagosomes is shown in panels to the right.

Figure 8. Analysis *of human macrophages transfected with EGFP-cPLA$_2\alpha$ during phagocytosis of zymosan.* Human macrophages transfected with the construction EGFP-cPLA$_2\alpha$ were treated with opsonized zymosan for 20 min, fixed, and fluorescence analyzed by confocal microscopy. A) Ten different z-stacks of the same cell are shown (from top to bottom). B) Pseudocolor analysis of the intensity of fluorescence of the cell in A is shown. C and D) Detailed phagosomes from B.

Figure 9. Analysis *of a human macrophage transfected with EGFP-PLCδ_1-PH during phagocytosis of zymosan.* Human macrophages transfected with the construction EGFP-PLCδ_1-PH were treated with zymosan for 20 min, fixed, and fluorescence analyzed by confocal microscopy. A) Ten different z-stacks of the same cell are shown (from top to bottom). B) Pseudocolor analysis of the intensity of fluorescence from some phagosomes is shown (white square in A).

Figure 10. sPLA$_2$-V and iPLA$_2$-VIA do not translocate to the phagocytic cup in human macrophages. Cells transfected with the fluorescent constructs sPLA$_2$-V-EGFP (A) or iPLA$_2$-VIA-EYFP (B) were subjected to synchronized phagocytosis using Alexa Fluor 594-labeled opsonized zymosan (op-zym) or vehicle (control) for 15 min, fixed and analyzed by confocal microscopy. Images are the projection to the Z axis of more than

13 stacks (0.25 μm each). Transmission images are shown in the right panel. White bar = 10 μm.

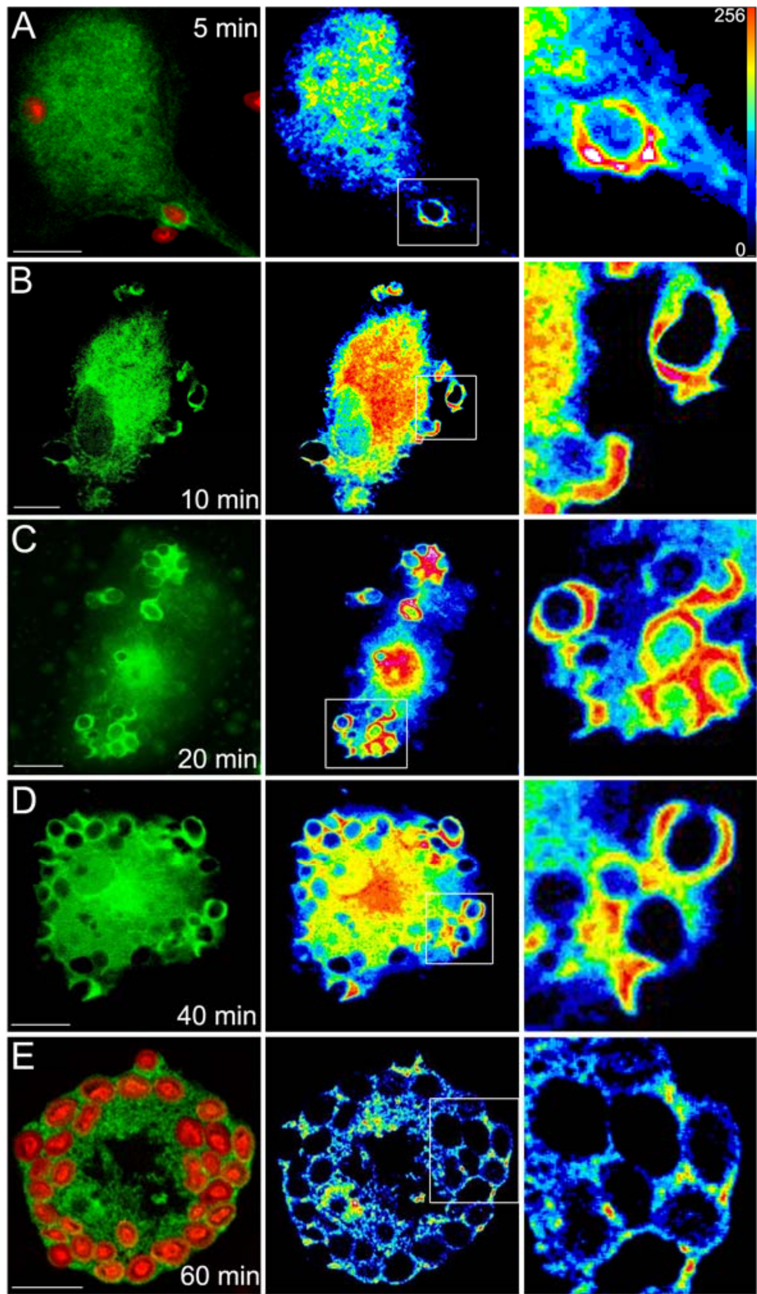


Figure 1

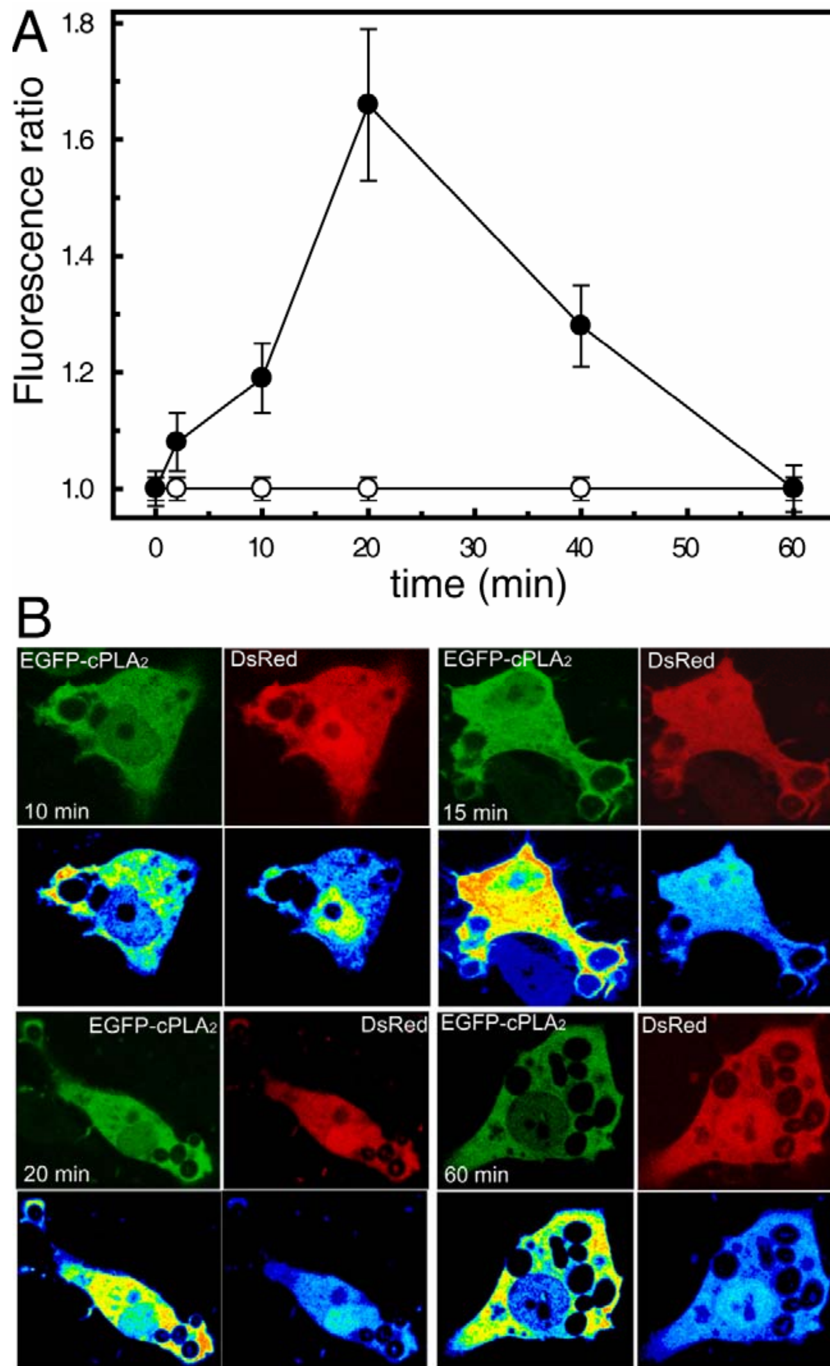
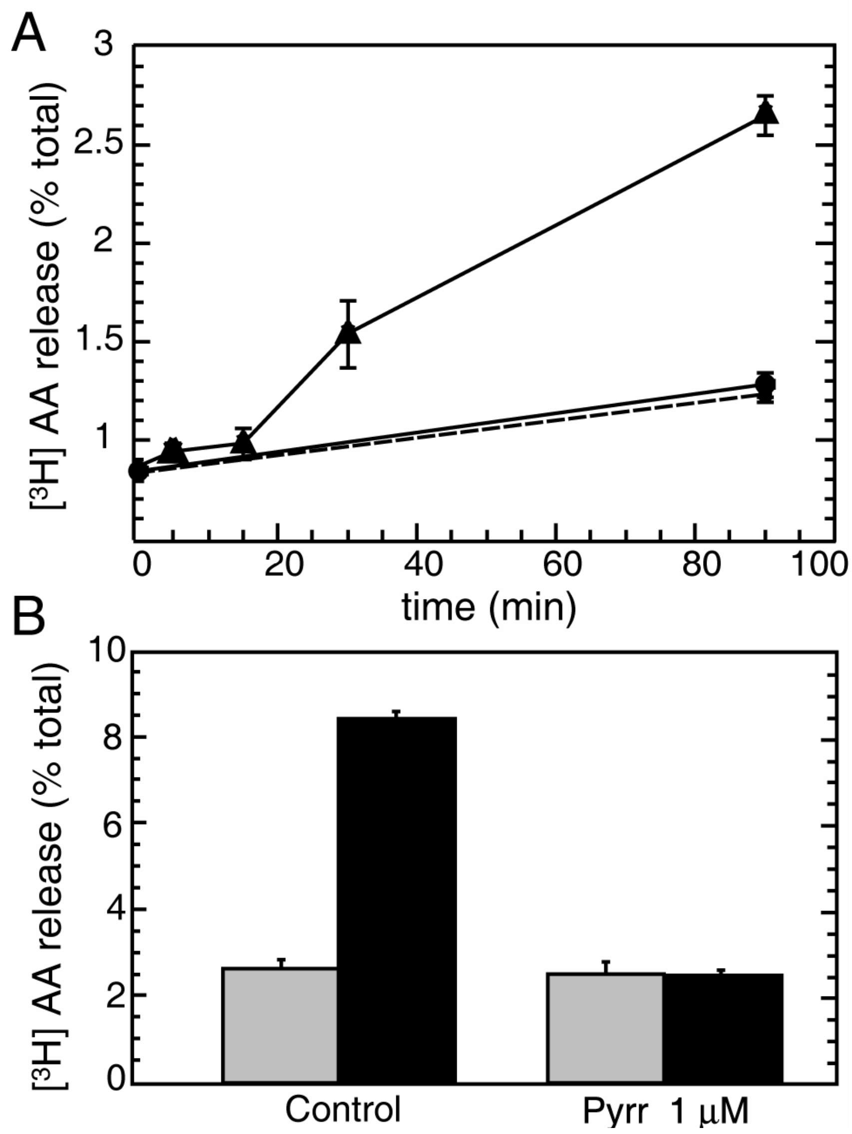


Figure 2



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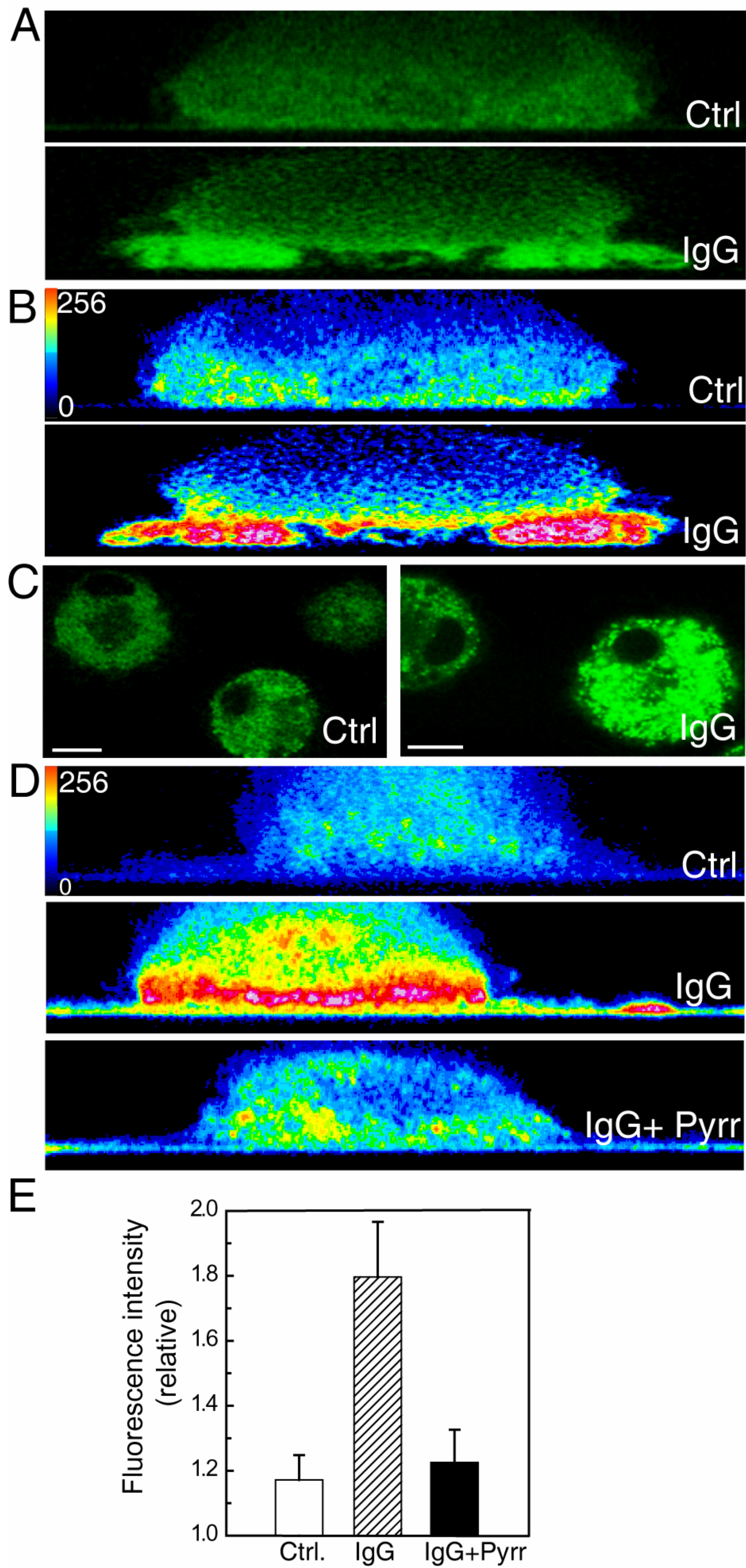

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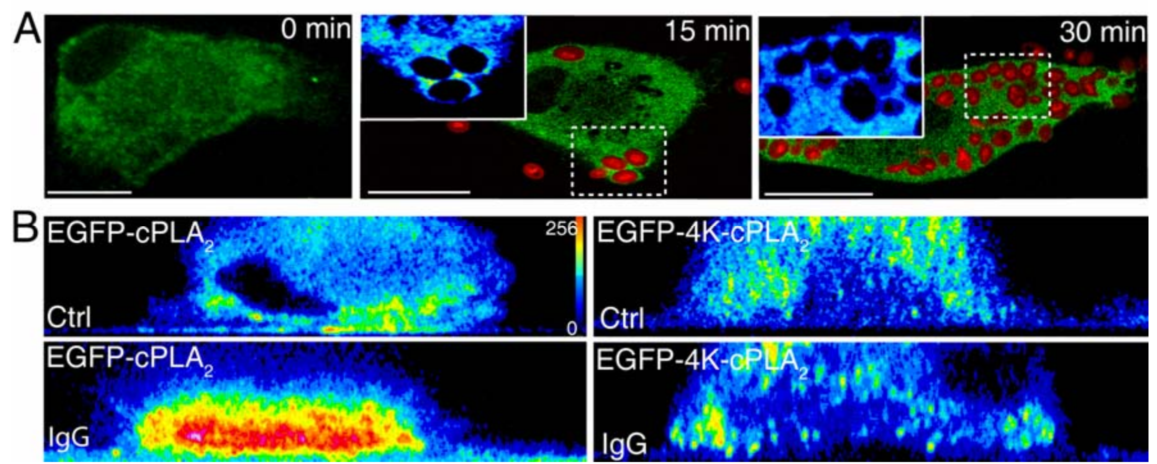

Figure 5

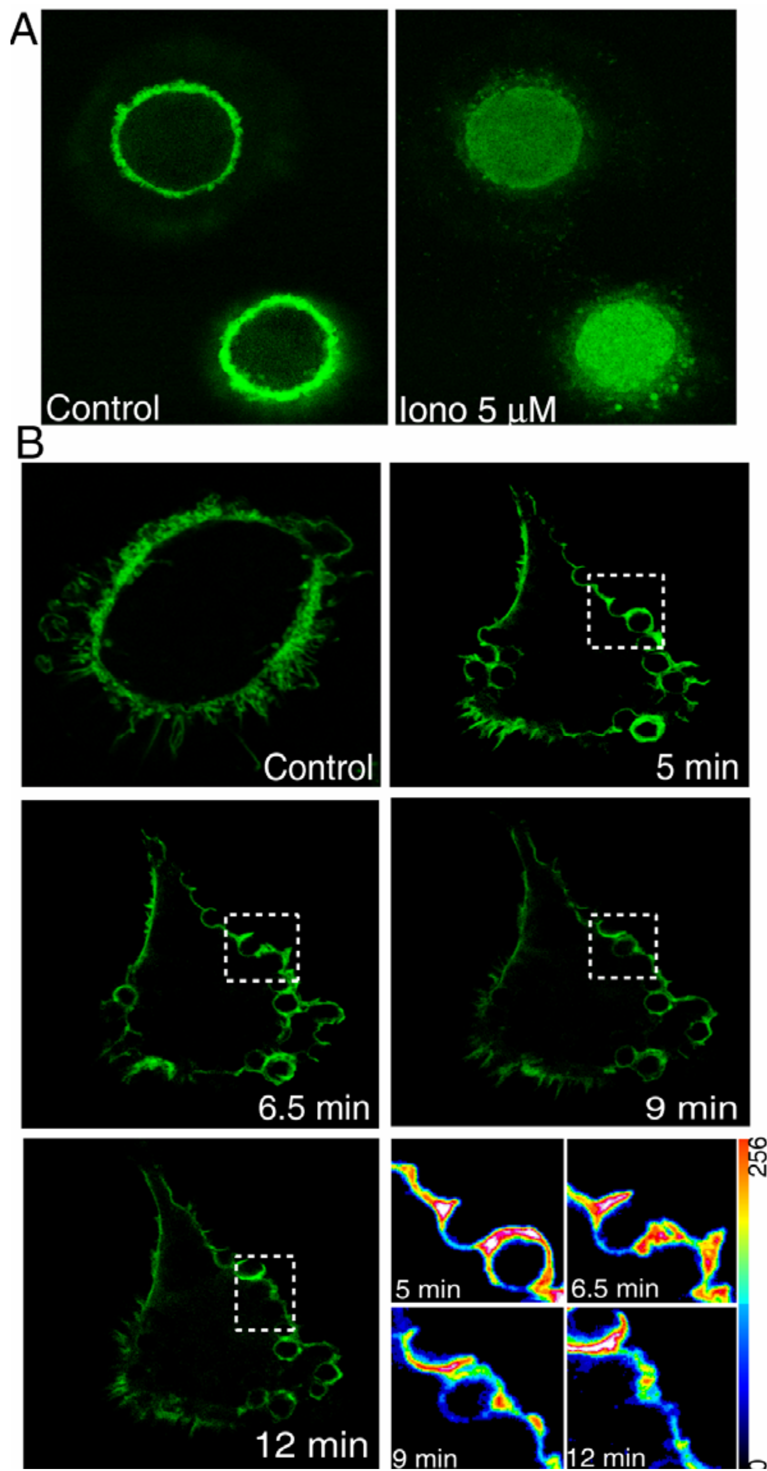

Figure 6

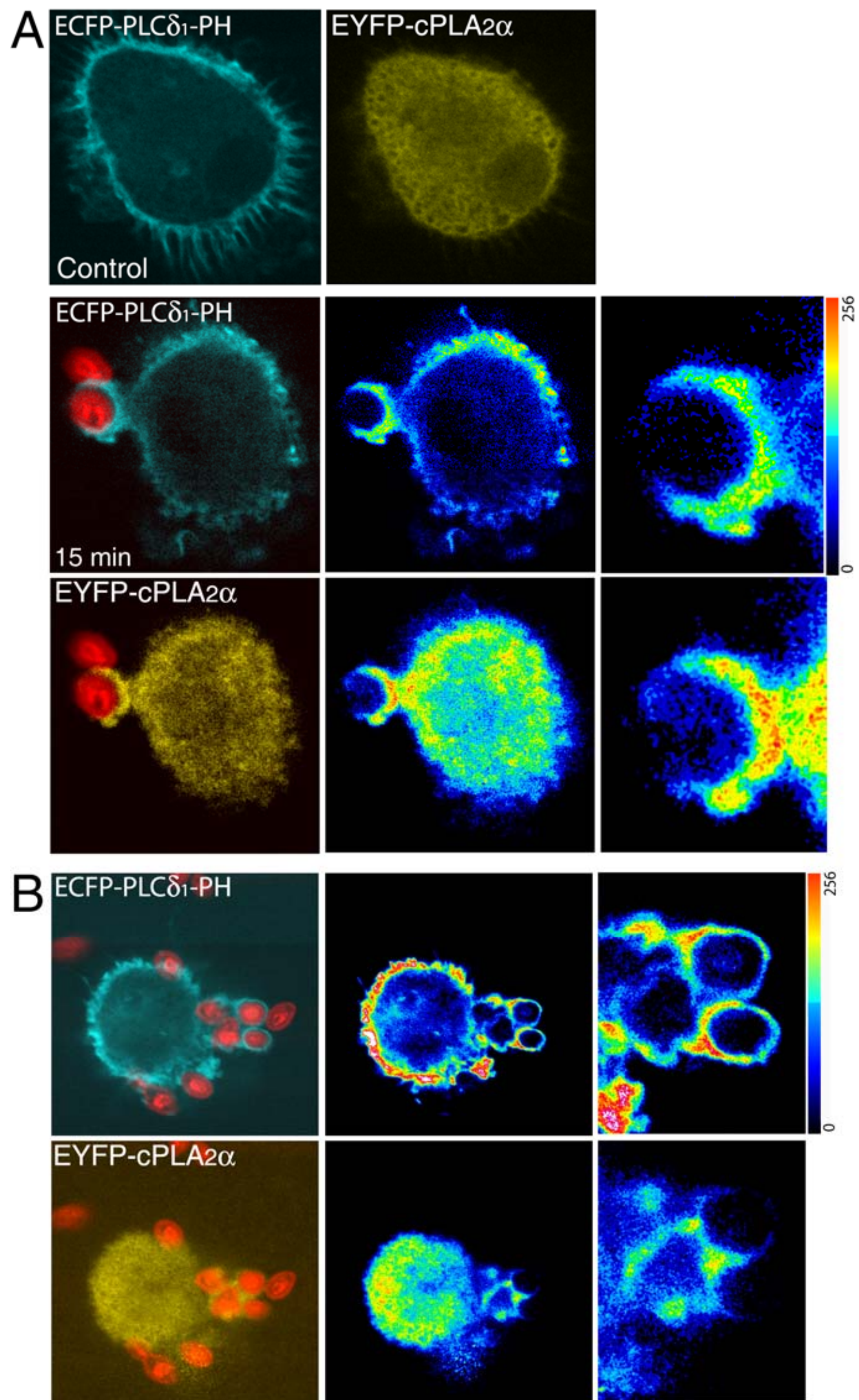

Figure 7

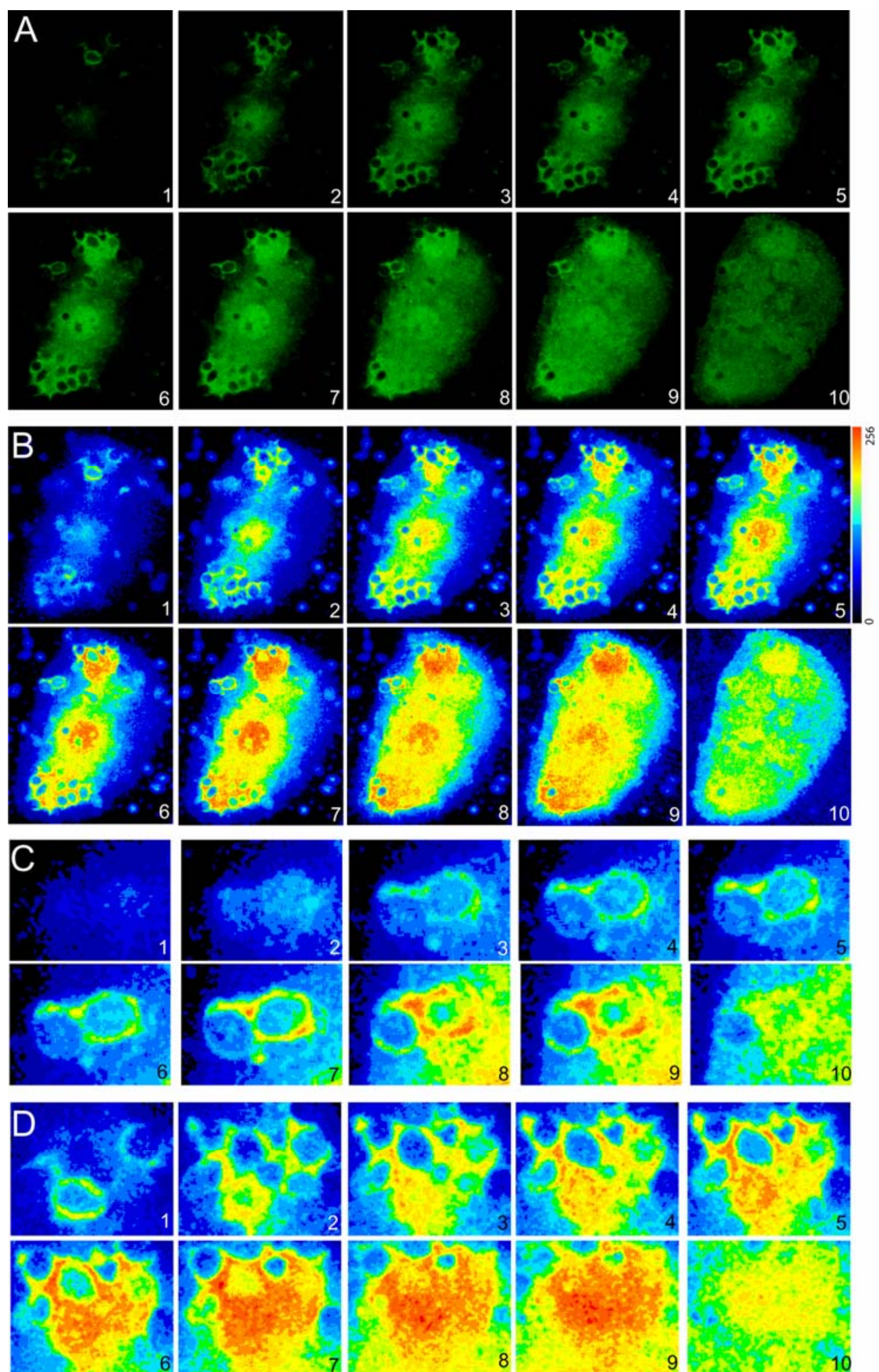

Figure 8

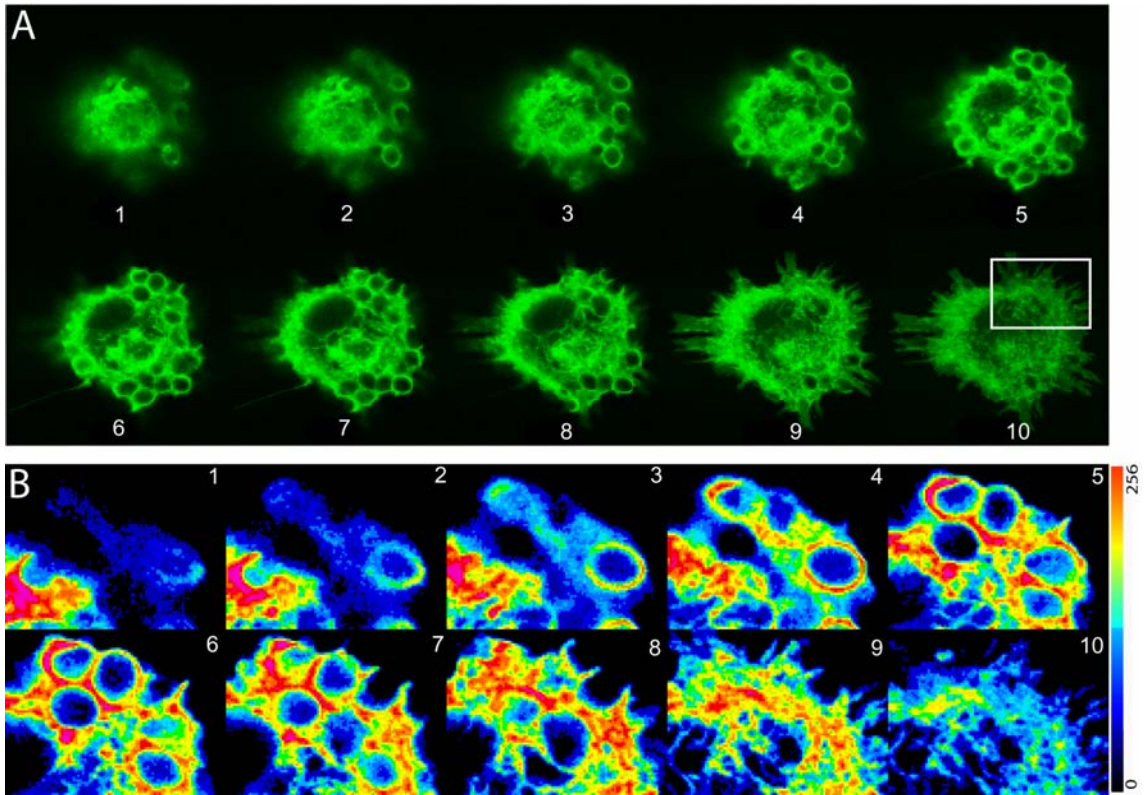

Figure 9

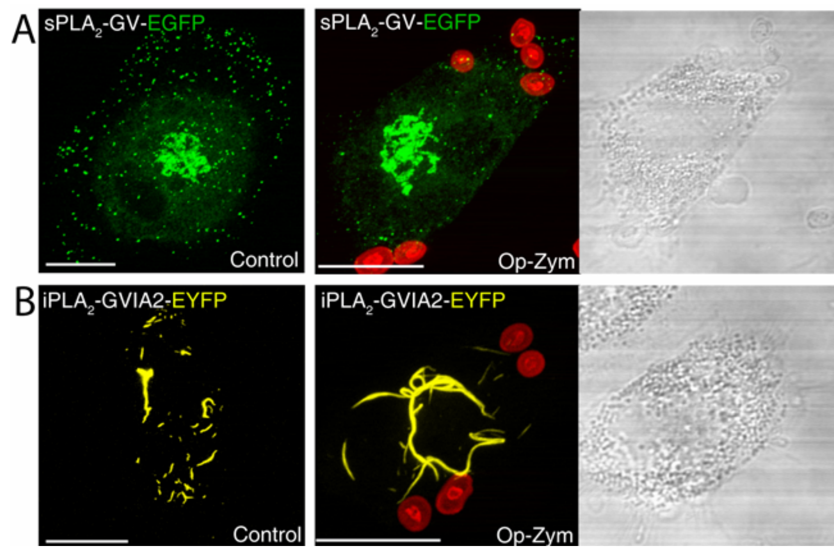

Figure 10