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Novel translocation responses of cytosolic phospholipase $A_2\alpha$ fluorescent proteins

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ABSTRACT

Cytosolic phospholipase A_2 ($cPLA_2$)α responds to the rise in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) attending cell stimulation by moving to intracellular membranes, releasing arachidonic acid (AA) from these membranes, and thereby initiating the synthesis of various lipid mediators. Under some conditions, however, $cPLA_2\alpha$ translocation occurs without any corresponding changes in $[Ca^{2+}]_i$. The signal for such responses has not been identified. Using confocal microscopy to track fluorescent proteins fused to $cPLA_2\alpha$ or $cPLA_2\alpha$'s C2 domain, we find that AA mimics Ca^{2+} ionophores in stimulating $cPLA_2\alpha$ translocations to the perinuclear ER and to a novel site, the lipid body. Unlike the ionophores, AA acted independently of $[Ca^{2+}]_i$ rises and did not translocate the proteins to the Golgi. AA's action did not involve its metabolism to eicosanoids or acylation into cellular lipids. Receptor agonists also stimulated translocations targeting lipid bodies. We propose that AA is a signal for Ca^{2+}-independent $cPLA_2\alpha$ translocation and that lipid bodies are common targets of $cPLA_2\alpha$ and contributors to stimulus-induced lipid mediator synthesis.

1. Introduction

Cytosolic phospholipase A_2 ($cPLA_2\alpha$) is critical for the production of various active lipids [1]. The rise in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) attending cell stimulation causes $cPLA_2\alpha$ to move from the cytosol to the perinuclear ER and Golgi, deacylate resident phospholipids (PLs), and thereby generate arachidonic acid (AA) and lyso-PLs; these products are then metabolized to eicosanoids, platelet-activating factor, and other mediators [2–6]. $cPLA_2\alpha$ thereby converts $[Ca^{2+}]_i$ signals into the synthesis of lipids controlling various biological reactions. However, some stimuli cause $cPLA_2\alpha$ to translocate and lipid mediators to form without altering $[Ca^{2+}]_i$ [7–11]. Moreover, many cell stimulating conditions lead to $[Ca^{2+}]_i$ rises and $cPLA_2\alpha$ translocations that rapidly reverse. Such fleeting changes do not lead to lipid mediator synthesis. Other stimulating conditions, contrastingly, cause $cPLA_2\alpha$ translocations that persist long after $[Ca^{2+}]_i$ normalizes [9,12,13] and evoke the synthesis of lipid mediators. The persistent translocation of $cPLA_2\alpha$ appears due to an irreversible change in its structure that may or may not result from the stimulus-induced formation of phosphatidylinositol polyphosphates [7,14,15] or from subtle differences in the duration or subcellular location of Ca^{2+} rises [3,9,12,13,16]. Using $cPLA_2\alpha$ fluorescent fusion proteins to track the movements of $cPLA_2\alpha$, we show here that a product of $cPLA_2\alpha$'s action, AA, causes Ca^{2+}-independent and sustained $cPLA_2\alpha$ translocation responses. Thus, AA may serve as a signal for the Ca^{2+}-independent and irreversible translocation of $cPLA_2\alpha$. In the course of these studies, we also found that AA, $[Ca^{2+}]_i$ rises, and receptor agonists stimulate $cPLA_2\alpha$ fluorescent proteins to translocate to a novel site, the lipid body. Lipid bodies, we suggest, are targets for translocating $cPLA_2\alpha$ and provide substrates for lipid mediator synthesis under diverse cell stimulating conditions.

2. Materials and methods

2.1. Materials

Stock solutions of AA (NuChek Prep, Elysian, MN) and α-linolenic and 11Z,14Z-eicosadienoic acids (Matreya LLC, Pleasant Gap, PA) (>99% purity) were stored in ethanol at −70 °C under argon. Triacsin C (Biomol International, Plymouth Meeting, PA) was stored in DMSO at −70 °C under argon. Sphingosine-1-phosphate (S1P) was taken up in saline-BSA just before use as instructed by Avanti Polar Lipids, Alabaster, AL; ionomycin (Io, Squibb & Sons, Inc., Cranbury, NJ), A23187 (Calbio-chem, San Diego, CA) and GTPγS (Sigma-Aldrich, St. Louis, MO) were dissolved in ethanol and water, respectively; nordihydroguairetic acid and indomethacin (Sigma-Aldrich) were dissolved in ethanol and incubated with cells for 30 min; pertussis toxin (List Biological Laboratories, Inc., Campbell, CA) was dissolved in water and incubated with cells for 18 h before stimulation. At the final concentrations used, ethanol,

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Abbreviations: $cPLA_2$, cytosolic phospholipase A_2; PL, phospholipids; AA, arachidonic acid; $[Ca^{2+}]_i$, cytosolic Ca^{2+}; EGFP, EYFP, or ECFP enhanced green, yellow, or cyanine fluorescent protein; Io, ionomycin; S1P, D-*erythro*-sphingosine-2-phosphate; DIC, differential interference contrast; 293, HEK 293 cells

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DMSO, and saline-BSA (0.1%), nordihydroguairetic acid and indomethacin (10 μM), and pertussis toxin (1 μg/ml) did not alter the location of the fluorescent proteins examined here.

2.2. Cells and transfections

HEK-293, MDA-MB-231, and PC3 cells (ATCC, Manassas, VA) were cultured (37 °C, 5% CO_2) in DMEM containing penicillin, streptomycin, L-glutamine, and 10% FBS (Invitrogen, Carlsbad, CA). Cells were transfected stably with pEGFP-cPLA$_2$ or transiently with pEYFP-cPLA$_2$,±pECFP-golgi subcellular localization vector (Clontech, Palo Alto, CA), using Fugene 6 Transfection Reagent as directed by the manufacturer (Roche Diagnostics, Indianapolis, IN). Primers for the C2 domain of cPLA$_2$α (IDT, Greensboro, NC) were used to obtain cPLA$_2$α's nucleotide sequence (49–444) encoding amino acids 17–148. The sequence was inserted into the HindIII and SmaI restriction sites of pEYFP-C2 (Clontech); the resulting vector (pC2-EYFP) was verified to have the same sequence as pC2-EGFP between the appropriate restriction sites by the Wake Forest University Health Sciences DNA Sequencing Laboratory. Transfected cells were plated for 18 h in single chamber cover glasses (Fisher Scientific, Pittsburgh, PA) and incubated in serum-free DMEM for 30–60 min before challenge. Where specified, the cells were treated with 10 μg/ml of BODIPY 493/503 for 10 min to stain lipid bodies as recommended by the supplier (Molecular Probes), washed twice, incubated in serum-free DMEM for 120 min, and challenged.

2.3. $[Ca^{2+}]_i$ measurements

Cells were loaded with 1 μM of X-rhod-1 Ca^{2+} indicator (Invitrogen) in 0.01% Pluronic F127 (Invitrogen) for 20 min, washed twice, and incubated in DMEM for 30 min before challenge. The intensity of X-rhod-1 fluorescence was monitored in a predetermined cytosolic region of cells each minute for 0–120 min after challenge. Results are reported as the fractional changes in fluorescent emissions or maximal fractional rises in emission relative to $t=0$ values. For some studies, cells were incubated in Ca^{2+}-free medium and 10 μM of BAPTA-AM (Invitrogen) for 30 min.

2.4. Fluorescent microscopy

Fluorescence was monitored with a Zeiss LSM 510 laser scanning microscope (Carl Zeiss, International, Oberkochen, Germany) equipped with a 63× water corrected lens and external heating source (stage temperature, 37 °C). ECFP, EGFP, and EYFP were detected by argon (respective emissions 458, 488, and 514), X-rhod-1 by helium neon (emission 563 nm), and BODIPY by argon (emission 458 nm) lasers. We used filters to distinguish the fluorescent emission of EYFP from that of BODIPY and eliminate bleed-through: a longpass filter selecting excitation at ≥560 nm captured EYFP, a dichroic filter for excitation at 480–520 nm captured BODIPY emissions. Since the BODIPY emission closely overlapped that of EGFP, experiments on BODIPY tracked EYFP but not EGFP fusion proteins. DIC and fluorescent images were taken concurrently. Pinhole size was 1 Airy unit, zoom was at 2×. Cells were scanned at 1 min intervals from 0–120 min after challenge. For 3-dimensional images, scans of cells were taken in successive increasing slices at 0, 30, 60, 90, or 120 min after challenge. Photo-bleaching of fluorescent probes and movement or detachment of cells limited experiments to 2 h. In studies using ≥10 μM of a Ca^{2+} ionophore, cells often detached after 30 min of observation. These studies were ended after cell detachment.

2.5. Phase-contrast microscopy

Cells were visualized at 37 °C by phase-contrast microscopy to identify refractive bodies and, within 1 min thereafter, fluorescence microscopy (Zeiss Axioplan 2 fluorescent microscope) to determine the fluorescence of these bodies.

3. Results

293 cells expressing EGFP-cPLA$_2$ emitted fluorescence homogeneously throughout the cytoplasm. On exposure to Io, the cells shifted this fluorescence to the perinuclear area and an irregularly-shaped cytosolic site, as found by others, but also to sites not previously described as targets for this protein viz., small, spherical, cytoplasmic sites (Fig. 1, panels A–F). Responses began within 5, 10, 15, 20, and 40 min of exposure to 33, 10, 1, 0.1, and 0.01 μM of Io, respectively, and did not reverse during 2 h of observation. Ca^{2+} ionophore A23187 had similar actions (not shown). AA also caused the translocation of EGFP-cPLA$_2$. However, these responses began only after ~20, 40, 60, 90, and 100 min of exposure to 33, 10, 1, 0.1, and 0.01 μM of AA, respectively, and targeted the protein to the perinuclear and spherical but not irregular sites (Fig. 1, panels G–L). AA and Io exhibited the same differential targeting action in cells expressing EYFP-cPLA$_2$ and a Golgi marker: Io caused EYFP-cPLA$_2$ to translocate to the perinuclear, spherical, and irregular sites while AA caused it to move to the perinuclear and spherical but not irregular sites; the irregular but not other sites overlap our Golgi marker (Fig. 2). Finally, the vehicle for AA and the ionophores, ethanol, did not alter EGFP-cPLA$_2$'s location (Fig. 1, panels M–O) and neither Io [17] nor AA (Fig. 1,

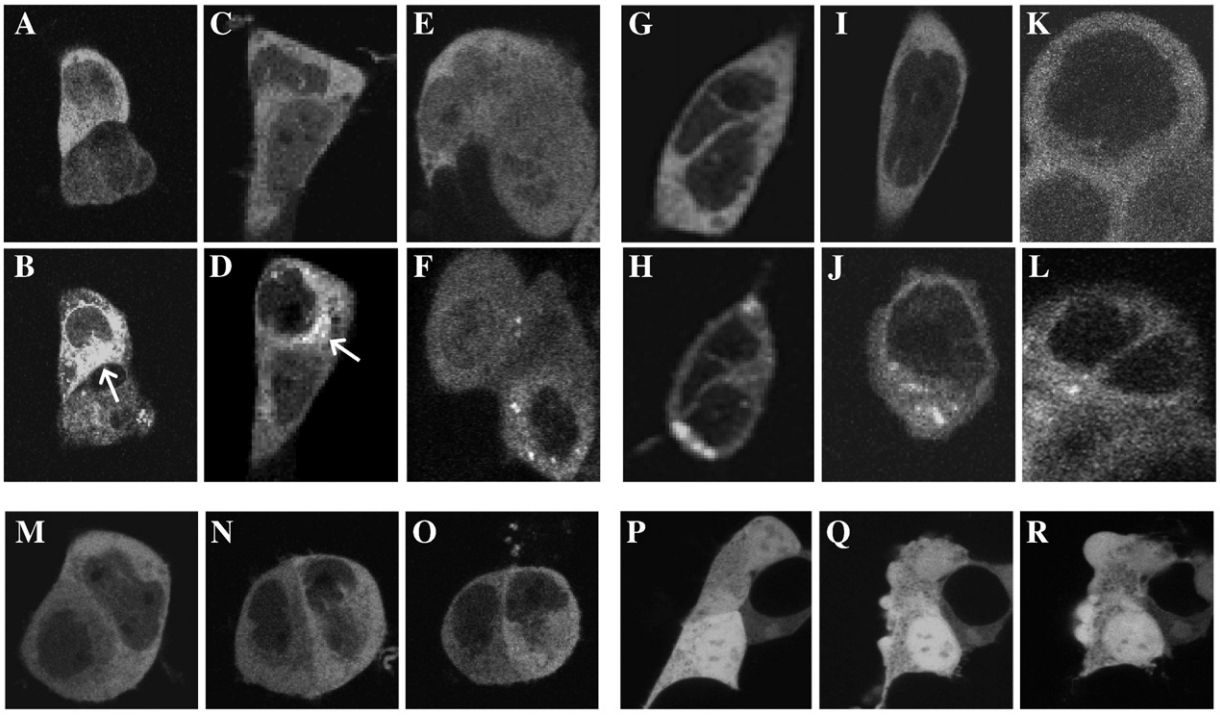

Fig. 1. EGFP-cPLA$_2$ translocation responses. Panels A–O: 293 cells expressing EGFP-cPLA$_2$ were scanned by confocal microscopy for EGFP just before (A, C, E, G, I, and K) or after challenge with 1 μM of Io for 4 min (B); 100 nM of Io for 15 min (D); 10 nM of Io for 120 min (F); 1 μM of AA for 45 min (H); 100 nM of AA for 90 min (I); 10 nM of AA for 120 min (L); or the solvent for AA and Io (ethanol) for 0 (M), 60 (N) or 120 (O) min. Panels P–R: Cells expressing EGFP not fused to cPLA$_2$ were challenged with 33 μM of AA for 0 (P), 60 (Q), or 120 (R) min. Arrows point to the irregular site referred to in the text. Results are representative of ≥3 studies.

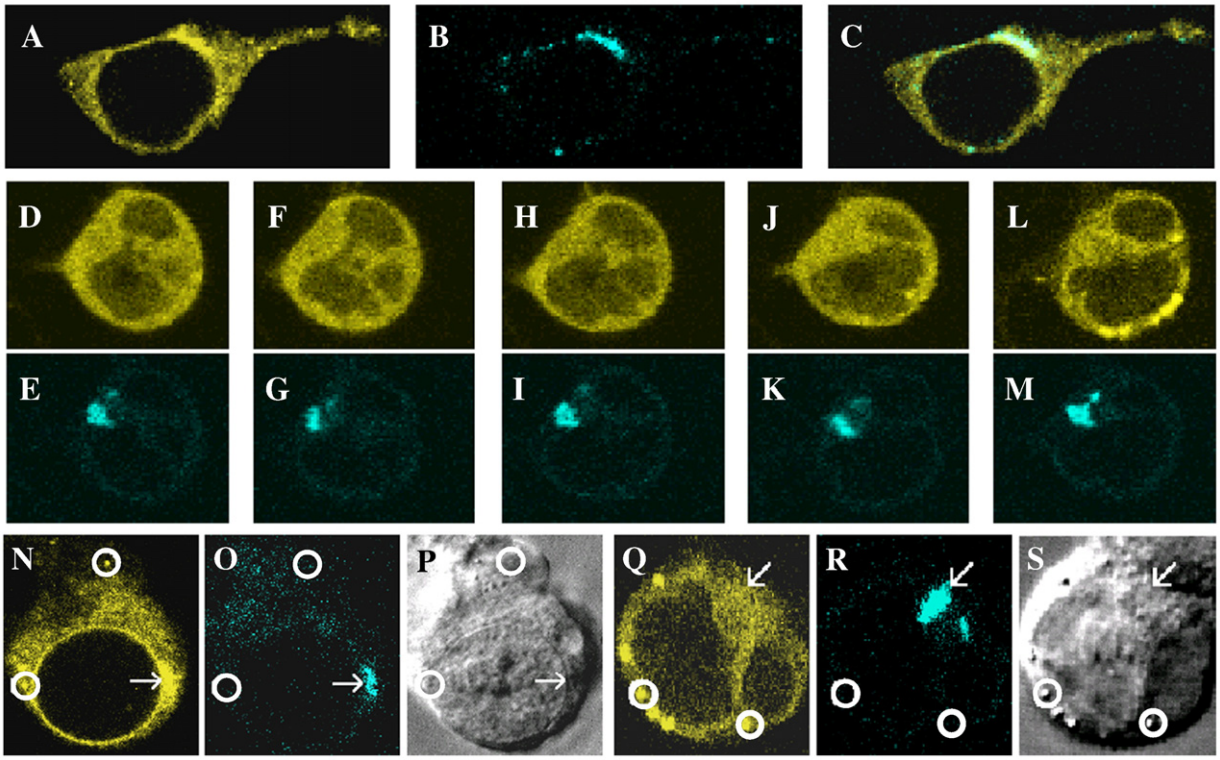

Fig. 2. Translocations to the Golgi. Panels A–S: 293 cells expressing EYFP-cPLA$_2$ and ECFP-golgi marker were scanned for: EYFP (A) and ECFP (B) after an 8 min challenge with 1 μM of Io (panel C superimposes A and B); EYFP (D, F, H, J, L) and ECFP (E, G, I, K, M) after challenge with 1 μM of AA for 0 (D, E), 30 (F, G), 60 (H, I), 90 (J, K), or 120 (L, M) min; or EYFP (N, Q), ECFP (O, R), and DIC (P, S) images after challenge with 10 μM of Io for 30 min (N–P) or 33 μM of AA for 60 min (Q–S). Arrows indicate the Golgi marker; circles enclose the spherical sites. Results are typical of ≥3 studies.

panels P–R) altered the distribution of EGFP not fused to cPLA$_2\alpha$. These results agree with reports [2–6] that $[Ca^{2+}]_i$ rises like those caused by ionophores induce cPLA$_2\alpha$ fluorescent proteins to translocate to the perinuclear ER and Golgi. They also show that AA stimulates irreversible translocation responses that target the perinuclear ER although not Golgi and that AA and the ionophores stimulate the proteins to move to

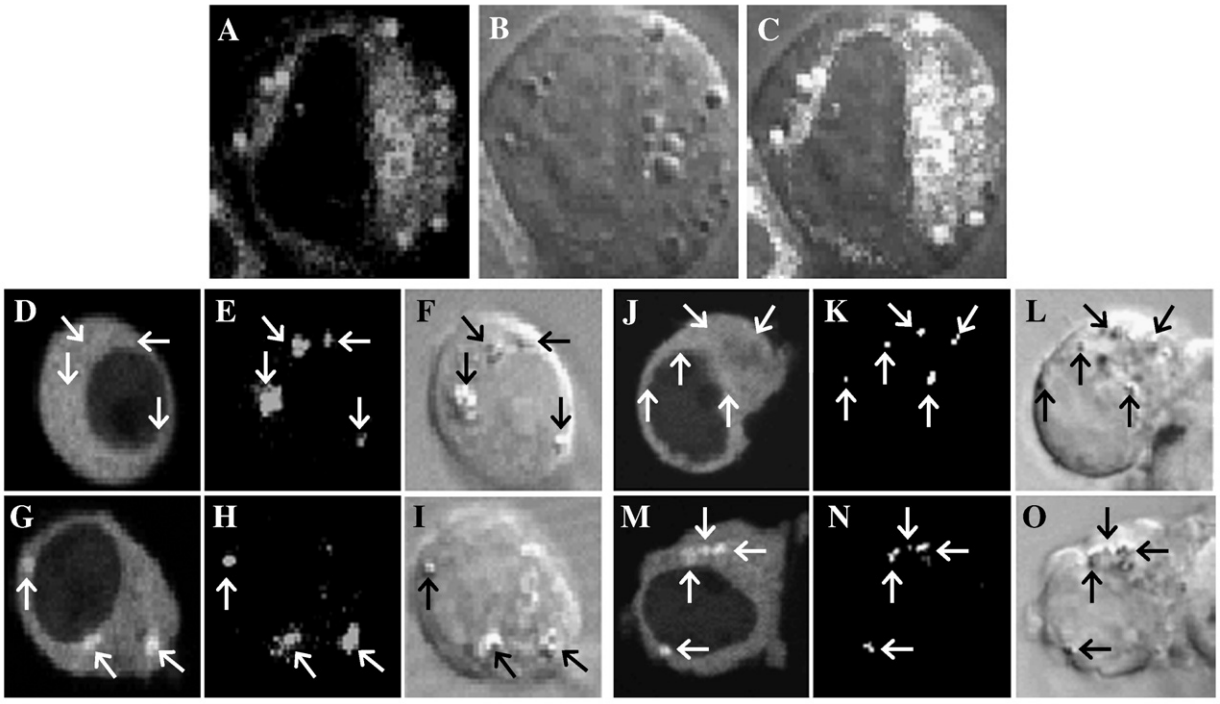

Fig. 3. Translocations to lipid bodies. Panels A–C: 293 cells expressing EYFP-cPLA$_2$ were scanned for EYFP (A) and DIC (B) images after challenge with 10 μM of AA for 80 min (panel C superimposes A and B). Panels D–O: Cells expressing EYFP-cPLA$_2$ and stained with BODIPY were scanned for EYFP (D, G, J, M), BODIPY (E, H, K, N), and DIC (F, I, L, O) images before (D–F, J–L) or after challenge with 1 μM of Io for 4 min (G–I) or 10 μM of AA for 60 min (M–O). Arrows point to BODIPY-stained bodies. Results are typical of ≥5 studies. Studies with 0.1 and 1 μM of AA or Io gave results similar to these (not shown).

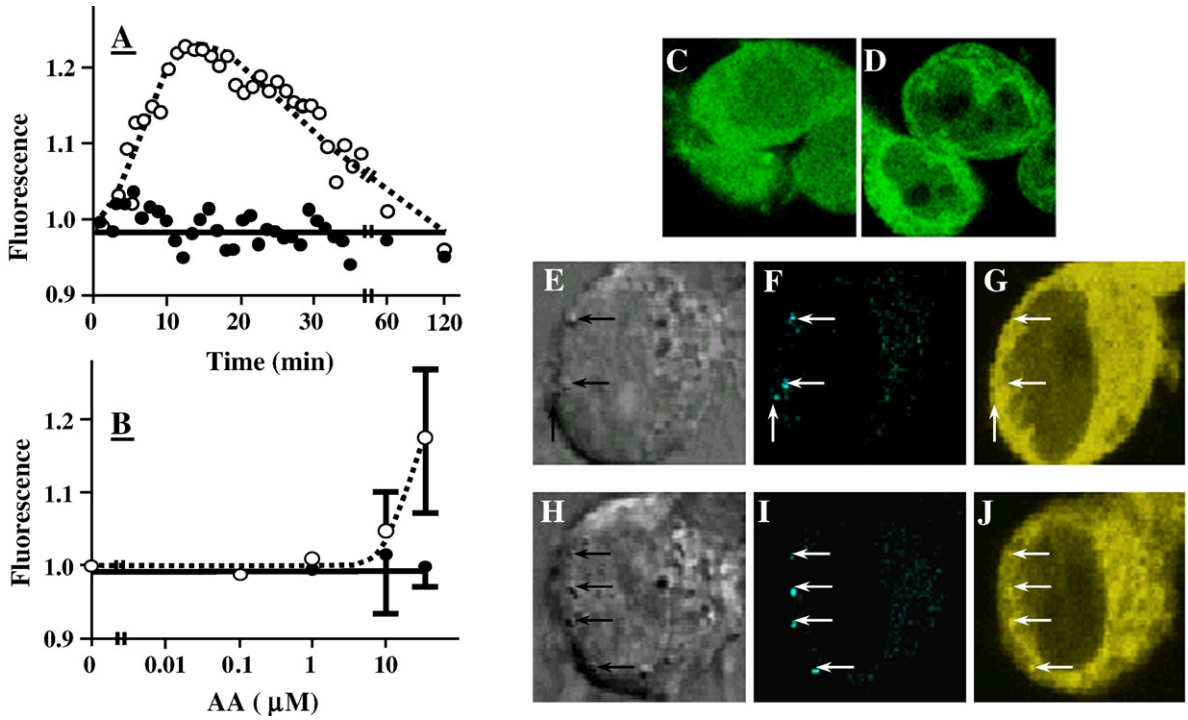

Fig. 4. Effect of $[Ca^{2+}]_i$ on translocation. Panels A–B: X-rhod-1-loaded 293 cells were $[Ca^{2+}]_i$-fixed (closed circles) or not (open circles), challenged, and assayed for X-rhod-1 fluorescent emissions. Data are the fractional rise in emissions relative to pre-challenge levels in cells stimulated with 33 μM of AA for 0–120 min (A) or the mean (±SEM, N ≥ 3) of the highest fractional rise (B) in emissions in cells following challenge with 0.1–33 μM of AA. Panels C–D: Ca^{2+}-fixed cells expressing EGFP-cPLA$_2$ were scanned for EGFP after challenge with 10 μM of AA for 0 (C) or 60 min (D). Panels E–J: Ca^{2+}-fixed cells expressing EYFP-cPLA$_2$ and stained with BODIPY were scanned for DCI (E, H), BODIPY (F, I), and EYFP (G, J) images after challenge for 0 (E–G) or 55 min (H–J) with 10 μM of AA. Arrows indicate lipid bodies. Results for panels C–J are typical of ≥ 3 studies.

sites 0.2–2 μm in diameter and more or less evenly distributed throughout the cytoplasm.

DIC microscopy identified the spherical cytoplasmic sites to which EGFP-cPLA$_2$α translocated as discrete spherical organelles distinct from the Golgi (Fig. 2, panels N–S). After challenge with AA or Io, EYFP-cPLA$_2$ layered onto the surface of these organelles (Fig. 3, panels A–C). This surface-layering effect is most clearly seen in Supplementary Figs. 2S, panels C, E, I, and M and 3S, panel S. The organelles were highly refractive on phase-contrast microscopy (not shown) and, in cells pre-stained with BODIPY, emitted the fluorescent signature of this lipid body marker (Fig. 3, panels D–O). 3-Dimensional analysis of such responses revealed that EYFP-cPLA$_2$ and BODIPY fluorescent emissions exactly overlapped

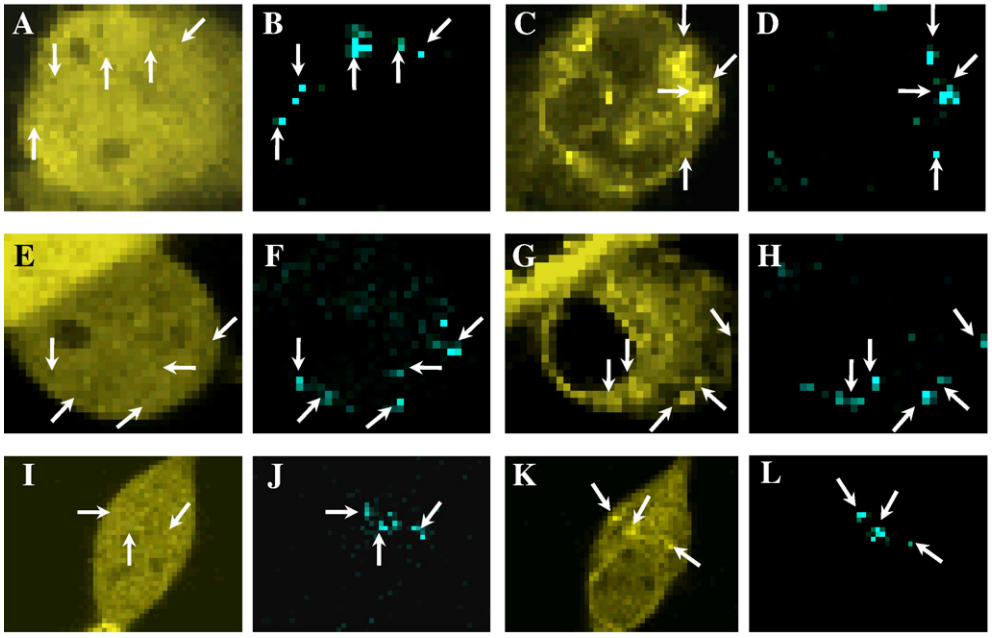

Fig. 5. C2-EYFP translocation responses. Panels A–H: 293 cells expressing C2-EYFP and stained with BODIPY were scanned for EYFP (A, C, E, G, I, K) and BODIPY (B, D, F, H, J, L) after challenge with 10 μM of A23187 for 0 (A, B) or 5 (C, D) min or with 10 μM of AA for 0 (E, F) or 90 (G, H) min. Panels I–L: Ca^{2+}-fixed cells were challenged with 10 μM of AA for 0 (I, J) or 95 (K, L) min. Arrows indicate lipid bodies. Results are typical of ≥ 3 studies; ethanol did not alter C2-EYFP's location (not shown).

and that virtually all EYFP-tagged spherical organelles had incorporated BODIPY (not shown). AA and the ionophores thus stimulate $PLA_2\alpha$ fluorescent proteins to translocate to the surface of lipid bodies.

AA's failure to target $cPLA_2\alpha$ fluorescent proteins to the Golgi implies that it acts by a different mechanism than Ca^{2+} ionophores. Other studies supported this. Io and AA stimulated 293 cells to raise $[Ca^{2+}]_i$, as defined by the emissions of $[Ca^{2+}]_i$ reporter X-rhod-1. All translocating concentrations (≥10 nM) of Io raised X-rhod emissions (Fig. 1S, panel A, interrupted line in Appendix A, Supplementary data). In contrast, at 10–30 μM, AA caused ≤1.2-fold rises and at <10 μM no rise in these emissions (Fig. 4, panels A and B, interrupted lines). Since AA's translocating action occurs at ≥10 nM, these results tend to dissociate AA's $[Ca^{2+}]_i$-raising from its translocating action. This was confirmed in cells loaded with Ca^{2+} chelator BAPTA and maintained in Ca^{2+}-free medium. These cells held $[Ca^{2+}]_i$ at pre-stimulatory levels after challenge with 33 μM of AA (Fig. 4, panels A and B, solid lines) or 10 μM of Io (Fig. 1S, panel A, solid line in Appendix A, Supplementary data) and, as expected, failed to translocate EGFP-cPLA2 in response to Io (Fig. 1S, panels B–J in Appendix A, Supplementary data). The $[Ca^{2+}]_i$-fixed cells nonetheless mounted full translocation responses to the perinuclear ER (Fig. 4, panels C and D) and lipid bodies (Fig. 4, panels E–J) in response to AA.

Studies on $cPLA_2\alpha$'s C2 domain fused to EYFP (C2-EYFP) indicated that AA did have at least one key mechanistic similarity with the ionophores: A23187 (Fig. 5, panels A–D) and Io (not shown) translocated C2-EYFP to the perinuclear ER, lipid bodies, and Golgi; AA translocated it to perinuclear ER and lipid bodies (Fig. 5, panels E–H); and AA (Fig. 5, panels I–L) but not A23187 (Fig. 1S, panels K–S in Appendix A, Supplementary data) or Io (not shown) was active in Ca^{2+}-fixed cells. The C2 domain thus houses the triggering and membrane-recognizing motifs responsible for initiating and targeting the translocation of $cPLA_2\alpha$ fluorescent proteins whether elicited by $[Ca^{2+}]_i$, as found previously [2,14,15,18–22], or by AA.

Cells pre-treated with nordihydroguairetic acid at 10 μM, a concentration inhibiting the cyclooxygenase, lipoxygenase, and cytochrome P450 enzymes which metabolize AA into eicosanoids, translocated EGFP-$cPLA_2$ normally in response to AA (Fig. 6, panels A–C). Cells treated with 10 μM of indomethacin for 30 min likewise did not translocate $cPLA_2$-EYFP when challenged with ethanol but mounted normal translocation responses to 1 and 10 μM of AA (not shown), In addition, 11Z,14Z-eicosadienoic acid, α-linolenic acid, and triacsin C (a straight-chain undecene with *cis* double bonds at C2–3 and C5–6 and hydroxytriazine double-bonded to C1) also translocated EGFP-$cPLA_2$ to the perinuclear ER and spherical sites but not Golgi (Fig. 6, panels D–I, and Fig. 2S, panels A–F in Appendix A, Supplementary data). These unsaturated compounds were active at ≥0.1–1 μM while the saturated analog of AA, eicosanoic acid, was inactive at 100 μM (Fig. 6, panels J–L). Neither 11Z,14Z-eicosadienoic acid nor triacsin C are known to be metabolized to active products by mammalian AA oxygenases. Finally, cells treated with pertussis toxin demonstrated full translocation responses to AA (Fig. 6, panels M–O). Pertussis toxin would block these responses if AA acted after conversion to leukotriene B_4, 5-hydroxyicosatetraenoate, or other metabolite acting through receptors linked to pertussis toxin-sensitive G proteins [23]. Taken together, these results render it unlikely that AA's translocating activity requires it to be oxygenated by the cited enzymes.

Our final studies probed the generality of lipid body targeting by $cPLA_{2\alpha}$. Two agonists that act via different surface membrane receptors, S1P and GTPγS, stimulated 293 cells to translocate EYFP-$cPLA_2$ to among other sites lipid bodies (Fig. 2S, panels G–N in Appendix A, Supplementary data). In addition, Io and AA stimulated MBA-MB-231 breast cancer cells to translocate EYFP-$cPLA_2$ to lipid

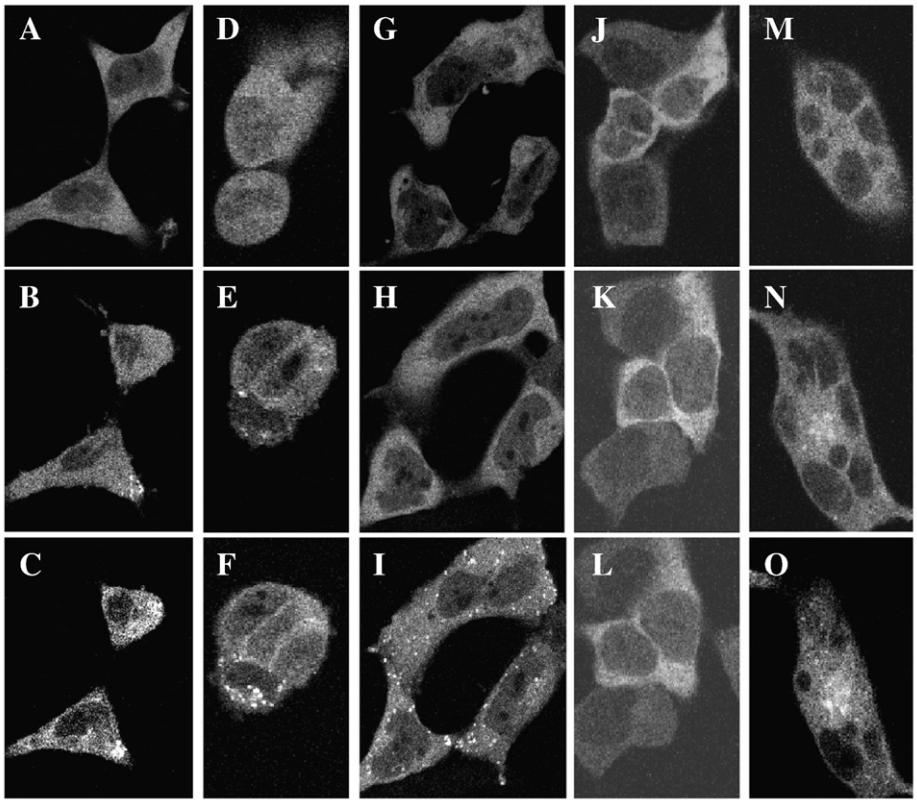

Fig. 6. Translocating effects of selected agents. 293 cells expressing EGFP-$cPLA_2$ were scanned for EGFP. Panels A–C: Cells incubated with 10 μM of nordihydroguairetic acid for 30 min were challenged with 10 μM of AA for 0 (A), 30 (B), or 45 (C) min. Panels D–L: Cells were challenged with 10 μM of α-linolenate for 0 (D), 60 (E), or 90 (F) min; 33 μM of eicosadienoate for 0 (G), 90 (H), or 115 min (I); or 100 μM of eicosanoate for 0 (J), 90 (K), or 120 (L) min. Panels M–O: Cells were incubated with 1 μg of pertussis toxin for 18 h and challenged with 1 μM of AA for 0 (M), 45 (N), or 90 (O) min. Nordihydroguairetic acid, pertussis toxin, and the vehicles for these drugs did not alter EGFP-$cPLA_2$'s location (not shown). Results are representative of ≥3 studies.

bodies although modest levels of EYFP-cPLA$_2$ were on lipid bodies prior to stimulation in some of these cells (Fig. 3S, panels A–P in Appendix A, Supplementary data). The latter type of localization was far more evident in resting PC3 prostate cancer cells, virtually all of which presented with EYFP-cPLA$_2$ almost exclusively on their lipid bodies (Fig. 3S, panels Q–T in Appendix A, Supplementary data). In any event, AA's translocating action is not limited to 293 cells and the translocation of cPLA$_2\alpha$ fluorescent proteins to lipid bodies occurs in response to diverse stimuli.

4. Discussion

Ca^{2+} coordinates with acidic residues on the membrane-binding face of cPLA$_2\alpha$'s C2 domain; this lowers the face's electronegativity thereby enabling it to bind electrically neutral membranes, insert hydrophobic residues into these membranes, and form a complex persisting as long as Ca^{2+} remains available. Since Ca^{2+}-coordinated cPLA$_2\alpha$ retains its repulsion for negatively charged membranes [2,4,14,18–22], rises in $[Ca^{2+}]_i$ cause cPLA$_2\alpha$ to translocate to the perinuclear ER and Golgi, organelles with cytoplasmic leaflets rich in electrically neutral PL, but not to the plasma membrane whose cytoplasmic leaflet is rich in electronegative PLs [4,15,20,22]. The single-leafleted lipid body membrane may originate from the ER [24,25], has a PL content level similar to the ER [26], and may be constitutively associated with numerous lipid mediator-forming enzymes including cPLA$_2\alpha$ in some cell types [27–29]. These considerations suggest that the lipid body may be a common target for translocating cPLA$_2\alpha$. Indeed, we found that AA, Io, A23187, GTPγS, and/or S1P did in fact cause 293 and MDA-MB-231 cells to move cPLA$_2\alpha$ fluorescent proteins to cytoplasmic organelles identified as lipid bodies in phase-contrast and BODIPY studies (Figs. 1–3, Fig. 3S, Appendix A, Supplementary data). We also found that PC3 and to a lesser extent MBA-MD-231 cells data) localize EYFP-cPLA$_2$ on their lipid bodies in the absence of stimulation (Fig. 2S, Appendix A, Supplementary data). Resting A549 lung [30] and Caco-2 colon [31] cancer cells likewise localize cPLA$_2\alpha$ to internal membranes. Since PC3, MDA-MB-231, A549, and Caco-2 cells overproduce eicosanoids or platelet-activating factor and use these products to promote their own growth [27,32–35], constitutive localization of cPLA$_{2\alpha}$ to membranes may underlie the over-production of PL-derived mediators and thereby certain aspects of the malignant phenotype.

AA and the ionophores also caused C2-EYFP to translocate. Like their effects on cPLA$_2\alpha$ fluorescent proteins, AA induced slowly evolving responses targeted to the perinuclear ER and lipid bodies but not Golgi (Fig. 5); ionophores induced more rapid responses targeting all three sites (Fig. 5), and neither AA (Fig. 1, panels P–R) nor Io [17] induced EGFP not fused to cPLA$_2\alpha$ to translocate. Thus, AA and the ionophores act via the C2 domain to trigger and direct cPLA$_2\alpha$'s movements. In spite of this similarity, however, AA's ability to translocate cPLA$_2\alpha$ and C2 domain fluorescent proteins, unlike those of the ionophores, did not require Ca^{2+} (Figs. 4 and 5, and 1S in Appendix A, Supplementary data). We evaluated three other mechanisms that might explain its action. AA did not operate after being metabolized by AA oxygenases to receptor-interacting products, as found for the C2 domain-dependent translocations of certain protein kinases C [27,36,37]: nordihydroguairetic acid and indomethacin, which block these enzymes, did not inhibit AA; eicosadienoic acid and triacsin C, while not known to be metabolized by the oxygenases, had translocating activity; and pertussis toxin failed to alter AA's translocating action under conditions where it blocks responses to various AA metabolites (Fig. 6). AA also did not act through GPR40 or GPR120 long-chain fatty acid receptors: these receptors have similar affinities for α-linolenate and AA [27,38–41] whereas AA is 100-fold more potent than α-linolenic acid in translocating EYFP-cPLA$_2$ (Fig. 6). Moreover, 293 cells do not express these receptors [27,38,39,42–44]. Finally, AA did not act by being acylated into membrane PLs to form a cPLA$_2\alpha$ docking site: triacsin C lacks the carboxy residue needed for this acylation yet has potent translocating activity (Fig. 2S in Appendix A, Supplementary data). These compounds thus appear to raise the affinity of cPLA$_2\alpha$'s C2 domain for electrically neutral membranes without requiring a metabolic processing step.

The results of our studies lead us to propose that cPLA$_2\alpha$ translocates to lipid bodies in cells responding to a wide range of stimuli and that these translocations contribute to the stimulus-induced synthesis of PL-derived mediators. This agrees with prior studies implicating lipid bodies in the production and priming for the production of eicosanoids in stimulated and/or primed leukocytes [45–47]. We further propose that AA is a Ca^{2+}-independent signal for translocating cPLA$_2\alpha$, at least when this occurs in the absence of $[Ca^{2+}]_i$ rises. The AA performing this role may arise from Ca^{2+}-independent cPLA$_2$'s, extracellular PLA$_2$'s, other cells by *trans*-cellular passage, or cPLA$_2\alpha$ itself. Relevant to the last point, suboptimal cell stimulation causes fleeting cPLA$_2\alpha$ translocations that are not accompanied by AA release or lipid mediator synthesis whereas optimal stimulation causes irreversible cPLA$_2\alpha$ translocation, AA release, and eicosanoids synthesis [9,12]. While proposed as resulting from the more prolonged $[Ca^{2+}]_i$ rise that occurs under the latter conditions [9], these effects ultimately may result from AA released by translocated cPLA$_2\alpha$. In this scenario, an initial product of cPLA$_2\alpha$'s action sustains the translocation response to convert a non-productive event into one productive of lipid mediators.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2008.03.008.

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