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# Inhibition of the MEK1/ERK pathway reduces arachidonic acid release independently of cPLA<sub>2</sub> phosphorylation and translocation

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**Keywords:** anisomycin, arachidonic acid, calcium, cytosolic phospholipase A<sub>2</sub>, ERK, fluorescent proteins, phosphorylation, MEK, U0126, translocation

## Abstract

**Background:** The 85-kDa cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) mediates arachidonic acid (AA) release in MDCK cells. Although calcium and mitogen-activated protein kinases regulate cPLA<sub>2</sub>, the correlation of cPLA<sub>2</sub> translocation and phosphorylation with MAPK activation and AA release is unclear.

**Results:** MEK1 inhibition by U0126 inhibited AA release in response to ATP and ionomycin. This directly correlated with inhibition of ERK activation but not with phosphorylation of cPLA<sub>2</sub> on Ser<sup>505</sup>, which was only partially inhibited by ERK inhibition. Inhibition of AA release by U0126 was still observed when stoichiometric phosphorylation of cPLA<sub>2</sub> on Ser<sup>505</sup> was maintained by activating p38 with anisomycin. Translocation kinetics of wild-type cPLA<sub>2</sub> and cPLA<sub>2</sub> containing S505A or S727A mutations to Golgi were similar in response to ATP and ionomycin and were not affected by U0126.

**Conclusions:** These results suggest that the ability of cPLA<sub>2</sub> to hydrolyze membrane phospholipid is reduced by inhibition of the MEK1/ERK pathway and that the reduction in activity is independent of cPLA<sub>2</sub> phosphorylation and translocation to membrane. The results also demonstrate that cPLA<sub>2</sub> mutated at the phosphorylation sites Ser<sup>505</sup> and Ser<sup>727</sup> translocated with similar kinetic as wild-type cPLA<sub>2</sub>.

## Background

Cytosolic PLA<sub>2</sub> specifically hydrolyzes *sn*-2 arachidonate from phospholipid providing the precursors for many different lipid mediators including prostaglandins and leukotrienes [1,2]. These lipid metabolites play a role in acute inflammatory responses and also regulate normal physiological processes. Certain prostaglandins are required for female reproduction and kidney function [3–5]. Because

of its important role in controlling levels of arachidonic acid (AA), much attention has been focused on the regulation of cPLA<sub>2</sub> activation, with particular emphasis on the role of its phosphorylation and Ca<sup>2+</sup>-mediated translocation [6–8].

cPLA<sub>2</sub> is regulated by controlling its cellular localization and access to membrane-phospholipid substrate. An ami-

no terminal, calcium-dependent lipid binding (CaLB or C2) domain regulates  $\text{Ca}^{2+}$ -mediated cPLA<sub>2</sub> translocation to intracellular membranes [9]. In vitro, membrane docking via the C2 domain is necessary and sufficient for catalysis and release of AA [10]. Binding of calcium ions by the cPLA<sub>2</sub> C2 domain is essential for the lipid association in vitro [11,12] and translocation in vivo [13,14]. In response to an increase in  $[\text{Ca}^{2+}]_i$ , cPLA<sub>2</sub> translocates to the Golgi and ER, however translocation to Golgi occurs at a lower  $[\text{Ca}^{2+}]_i$  [15].

Protein kinase pathways play major roles in cPLA<sub>2</sub> activation, and regulation by the mitogen-activated protein kinase kinase (MEK) /extracellular-signal regulated kinase (ERK) signaling pathway has received particular attention. cPLA<sub>2</sub> is phosphorylated by mitogen activated protein (MAP) kinases, including p42/p44 ERKs and p38, on Ser<sup>505</sup> in vitro [16,17] and in response to receptor stimulation [16,18–21]. In addition to phosphorylation by MAP kinase, it has been shown that cPLA<sub>2</sub> is also phosphorylated on Ser<sup>727</sup> by MAPK-interacting kinase I (MNKI) [22] and on Ser<sup>515</sup> by calcium/calmodulin-dependent protein kinase II [23]. Phosphorylation of these sites may also play a role in regulating cPLA<sub>2</sub> function in certain cell models.

Phosphorylation of Ser<sup>505</sup> has been extensively studied because it is readily detected due to a characteristic electrophoretic mobility shift when analyzed by SDS-PAGE [13,16]. The importance of Ser<sup>505</sup> phosphorylation in regulating cPLA<sub>2</sub> has been demonstrated in different cells and in vitro models by using cPLA<sub>2</sub> containing a S505A mutation [16,22]. However, the mechanism whereby Ser<sup>505</sup> phosphorylation regulates cPLA<sub>2</sub> function has been elusive. In vitro studies have demonstrated that dephosphorylated cPLA<sub>2</sub> is catalytically active and that Ser<sup>505</sup> phosphorylation increases activity by only ~30 percent [24]. In contrast, cells expressing the cPLA<sub>2</sub> S505A mutation fail to release AA in response to a low dose of calcium ionophore, but release similar amounts of AA as cells expressing wild-type cPLA<sub>2</sub> in response to high dose ionophore [22]. From these studies, it has been suggested that cPLA<sub>2</sub> Ser<sup>505</sup> phosphorylation may have a role in regulating translocation [22]. A previous study demonstrated translocation of cPLA<sub>2</sub> S505A in response to  $\text{Ca}^{2+}$  ionophore, but did not address the kinetics of translocation, translocation in response to a physiological agonist, or differences in targeting [25].

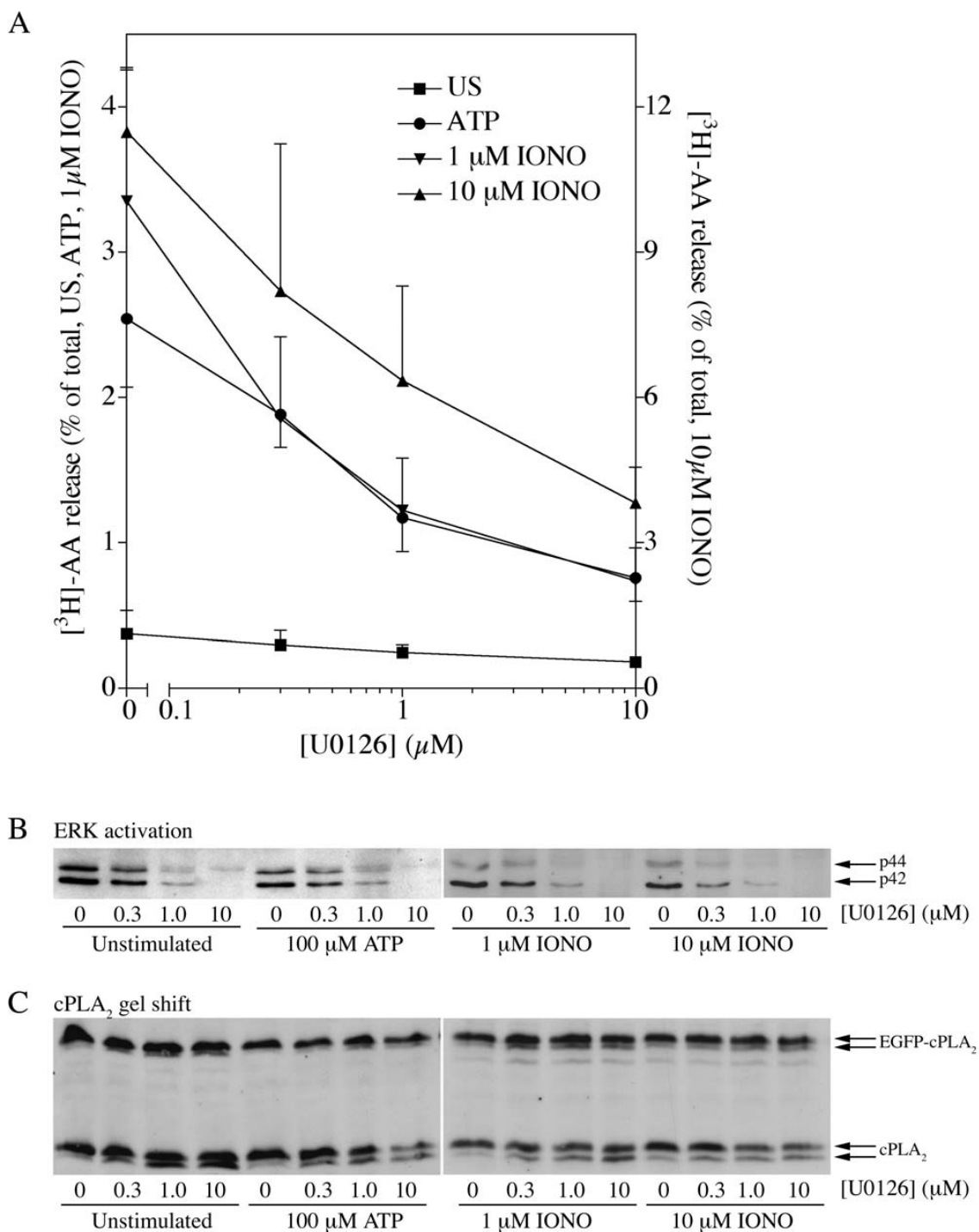
To better understand the regulation of cPLA<sub>2</sub> by the MEK1/ERK pathway and  $\text{Ca}^{2+}$ , we investigated the effect of MEK inhibitors on AA release, cPLA<sub>2</sub> phosphorylation of Ser<sup>505</sup>, cPLA<sub>2</sub> translocation kinetics, and  $[\text{Ca}^{2+}]_i$  increase in Madin-Darby canine kidney (MDCK) cells. We found that inhibition of MEK1 by U0126 significantly in-

hibited AA release and this was correlated with inhibition of ERK activation. However, MEK inhibition only partially affected cPLA<sub>2</sub> phosphorylation and had no effect on the kinetics of  $\text{Ca}^{2+}$ -mediated cPLA<sub>2</sub> translocation to membrane. In addition, using cells expressing wild-type cPLA<sub>2</sub> and cPLA<sub>2</sub> with S505A or S727A mutations, it was found that translocation kinetics and membrane targeting in response to ATP or ionomycin was similar to wild-type cPLA<sub>2</sub>. These data suggest that MEK1 inhibition reduces cPLA<sub>2</sub> catalytic activity and AA release independently of phosphorylation and translocation.

## Results

### **Effect of MEK inhibition on AA release, ERK activation, and cPLA<sub>2</sub> Ser<sup>505</sup> phosphorylation**

To study the role of the MEK1/ERK pathway in cPLA<sub>2</sub> activation, quiesced MDCK cells were treated with the MEK1 inhibitor U0126, and the effect on AA release, ERK activation, and cPLA<sub>2</sub> gel shift determined (Fig. 1). For equivalence with the imaging studies, cells expressing EGFP-cPLA<sub>2</sub> were used in all experiments. EGFP-cPLA<sub>2</sub> was expressed to similar levels as endogenous enzyme but did not contribute significantly to AA release in stably transfected cells. However, EGFP-cPLA<sub>2</sub> is functional since it dose-dependently catalyzes release of AA when expressed in cells that lack endogenous cPLA<sub>2</sub>, such as Sf9 cells [13] and immortalized mouse lung fibroblasts from cPLA<sub>2</sub>α knock-out mice [26]. In order to enhance AA release in cells containing endogenous cPLA<sub>2</sub>, it is necessary to overexpress the enzyme several fold as previously reported [16]. Cytosolic PLA<sub>2</sub> has been shown to mediate  $\text{Ca}^{2+}$ -induced AA release in MDCK cells treated with ATP and IONO in experiments using the group IV cPLA<sub>2</sub>α-specific inhibitor pyrrolidine-1 [27]. To measure cPLA<sub>2</sub> mediated AA release, EGFP-cPLA<sub>2</sub>-transfected MDCK cells labeled with [<sup>3</sup>H]-AA were incubated with 0.3, 1 or 10 μM U0126 for 15 min prior to stimulation with 100 μM ATP, 1 μM IONO, or 10 μM IONO. AA release was measured at 3 min because we have shown that ATP- and IONO-stimulated AA release peaks between 3 to 5 min post-stimulation [15]. Agonist-induced AA release was inhibited dose-dependently by U0126 (Fig. 1A) with the highest U0126 concentration used (10 μM) reducing AA release by 72–80% with all agonists (Table 1). This inhibition was independent of the total amount of AA released, since AA release stimulated by 10 μM IONO was 3-fold greater than release stimulated with 1 μM IONO or 100 μM ATP, but the percent inhibition by U0126 was similar. Treatment of MDCK cells with 30 μM PD098059, a less potent inhibitor of MEK [28,29], resulted in a ~50% reduction in AA release in response to 100 μM ATP, 1 μM IONO, and 10 μM IONO (data not shown). Thus, in MDCK cells, MEK1 inhibition significantly reduces the ability of cPLA<sub>2</sub> to hydrolyze AA from membrane phospholipids.



**Figure 1**  
**Effect of MEK inhibition on AA release, ERK inhibition, and cPLA<sub>2</sub> Ser<sup>505</sup> phosphorylation** (A) EGFP-cPLA<sub>2</sub>-transfected cells were incubated with the indicated concentrations of U0126 for 15 min prior to stimulation with 100 μM ATP, 1 μM IONO, or 10 μM IONO. AA release was assayed at 3 min. Results show the average ± SD of 3 experiments (error bars are down for ATP for clarity). Protein extracts from EGFP-cPLA<sub>2</sub>-transfected cells incubated with the indicated concentrations of U0126 for 15 min prior to stimulation with 100 μM ATP, 1 μM IONO, or 10 μM IONO for 3 min were subjected to immunoblotting using (B) anti-phospho-ERK and (C) anti-cPLA<sub>2</sub> polyclonal antibodies. Results are from 3 to 5 independent experiments.

**Table 1: Inhibition of AA release by 10  $\mu$ M U0126** See "Experimental Procedures" for experimental conditions and methods to determine AA release. Percent inhibition of AA release after 3 min of agonist treatment are average  $\pm$  S.D. for N experiments performed in duplicate.

Agonist	% Inhibition	N
100 $\mu$ M ATP	76.1 $\pm$ 6.8	7
1 $\mu$ M IONO	80.2 $\pm$ 5.9	8
10 $\mu$ M IONO	72.2 $\pm$ 7.3	6

The effect of MEK1 inhibition on activation of p42/p44 ERK measured by immunoblot analysis using phospho-specific antibodies in cells treated with U0126 and stimulated as above was determined (Fig. 1B). Work in our laboratory has shown that recognition of ERK by anti-phospho-ERK antibodies correlates with an increase in ERK activity [21,30,31]. Interestingly, the anti-phospho-ERK immunoblots revealed that ERKs were constitutively activated in untreated, quiesced MDCK cells and activation was not enhanced further by ATP or IONO (Fig. 1B, left panel). ERK activation was diminished by increasing concentrations of U0126 and was quantitatively inhibited after 15 min incubation in 10  $\mu$ M U0126. U0126 decreased ERK activation following ATP or IONO stimulation in the same fashion as in unstimulated cells. Consequently, there was a direct correlation between the reduction of AA release (Fig. 1A) and inhibition of ERK activation (Fig. 1B) in MDCK cells treated with U0126.

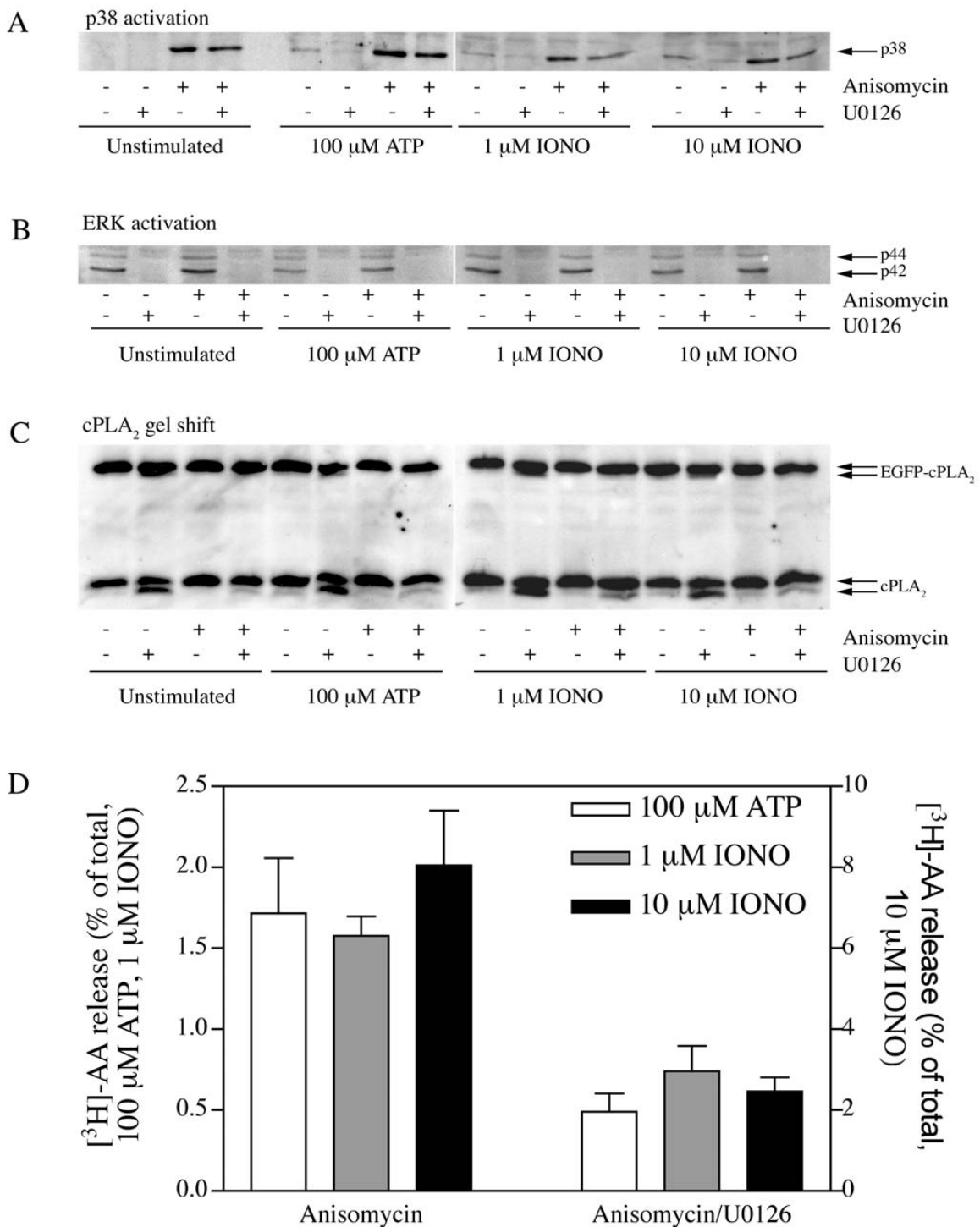
Because cPLA<sub>2</sub> is a target of the MEK1/ERK signaling cascade, we assayed the effect of MEK1 inhibition by U0126 on cPLA<sub>2</sub> phosphorylation by analyzing gel shift of cPLA<sub>2</sub>. Phosphorylation of Ser<sup>505</sup> results in a retardation of its electrophoretic mobility (gel shift) [13,16]. In unstimulated cells, EGFP-tagged and endogenous cPLA<sub>2</sub> were nearly completely gel shifted, indicating that most cPLA<sub>2</sub> was phosphorylated on Ser<sup>505</sup> (Fig. 1C), which is consistent with the observation that ERKs are constitutively activated. Incubation with U0126 resulted in a partial reversal of the gel shift although, at 10  $\mu$ M U0126, approximately half of cPLA<sub>2</sub> remained phosphorylated on Ser<sup>505</sup>. Thus, unlike the quantitative effect of U0126 on ERK activation, inhibition of MEK1 with U0126 only partially reversed the gel shift of cPLA<sub>2</sub>. The reversal of the gel shift was similar in cells treated with ATP and 1 and 10  $\mu$ M IONO. Due to the increased molecular weight of the EGFP-tagged cPLA<sub>2</sub>, the two forms of cPLA<sub>2</sub> did not separate as well, making the gel shift more difficult to visualize, but generally mirrored the gel shift characteristics of the endogenous cPLA<sub>2</sub>. These results suggest that, in response to MEK1 inhibition, there is a quantitative, dose-dependent

decrease in AA release that correlates well with the loss of ERK activation, but not with the extent of cPLA<sub>2</sub> Ser<sup>505</sup> phosphorylation.

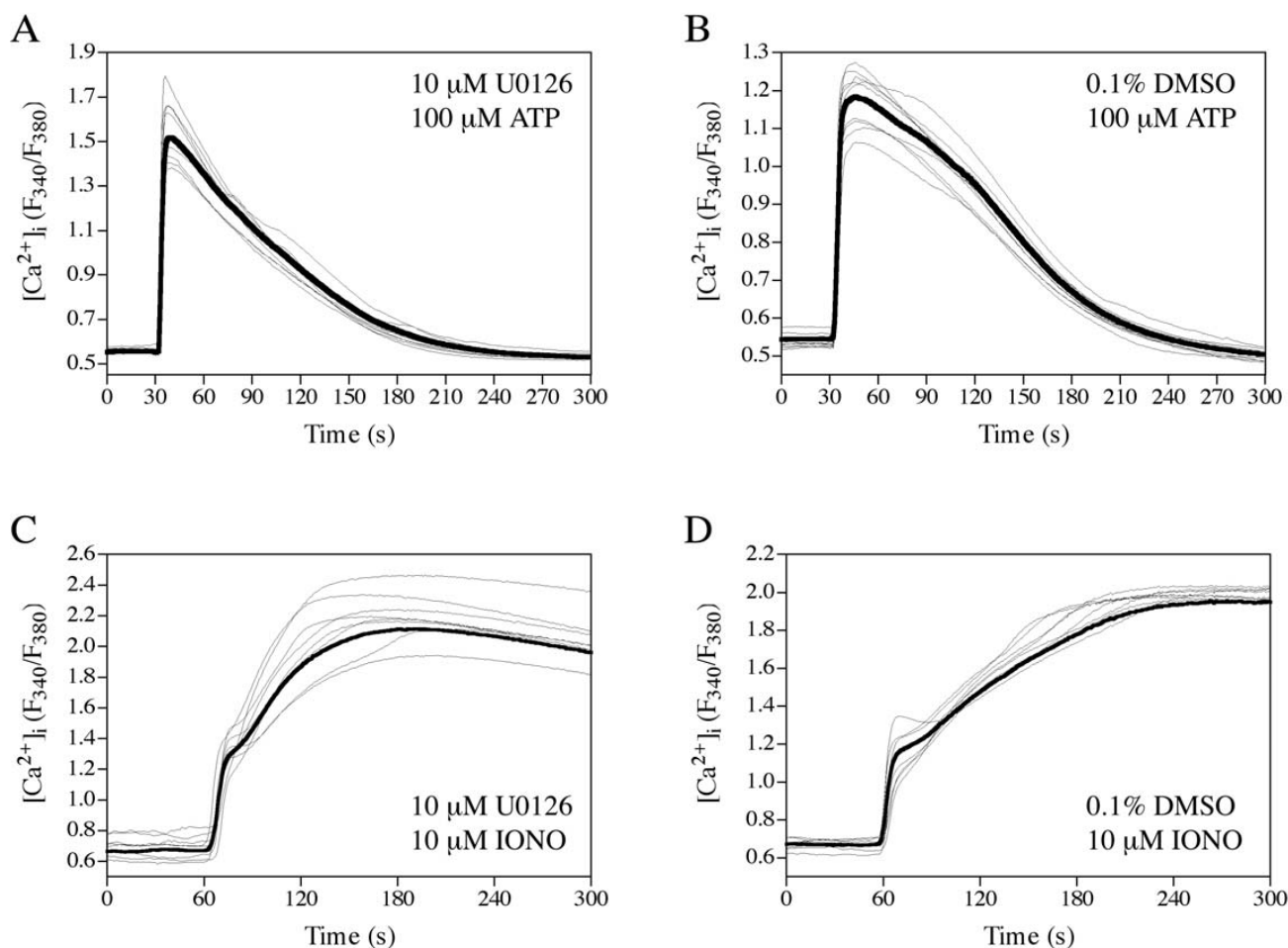
To further investigate whether the MEK1/ERK pathway played a role in regulating AA release independent of Ser<sup>505</sup> phosphorylation, we treated cells with anisomycin, which activates the MAPK homolog p38, but not the MEK1/ERK pathway [32]. Activation of p38 in response to anisomycin treatment was analyzed by immunoblotting using an anti-phospho-p38 antibody (Fig. 2A). The immunoblots demonstrate that 30 min treatment in 25 ng/ml anisomycin resulted in phosphorylation of p38 in unstimulated MDCK cells and in cells treated with ATP or IONO. ATP and ionomycin treatment in the absence of anisomycin only weakly increased p38 phosphorylation. As expected, the MEK inhibitor U0126 did not significantly affect anisomycin-stimulated p38 phosphorylation. In control experiments, anisomycin treatment did not induce ERK activation nor interfere with inhibition of ERK activation by 10  $\mu$ M U0126 treatment (Fig. 2B). We have previously reported that p38 is also selectively activated in anisomycin-treated macrophages [21]. Importantly, pretreatment of cells for 30 min with anisomycin resulted in maintenance of the cPLA<sub>2</sub> gel shift in the presence of U0126 in unstimulated cells and in cells stimulated with ATP and 1 and 10  $\mu$ M IONO (Fig. 2C). AA release assays show that, in MDCK cells treated with anisomycin, U0126 resulted in an AA release reduction of ~67–76% (Fig. 2D) similar to the inhibition observed without anisomycin. These results demonstrate that activation of the MEK1/ERK pathway is required for AA release even under conditions where cPLA<sub>2</sub> Ser<sup>505</sup> phosphorylation is maintained, suggesting an alternative role for the MEK1/ERK pathway in regulating cPLA<sub>2</sub>.

#### [Ca<sup>2+</sup>]<sub>i</sub> increase is independent of MEK1/ERK pathway

One explanation for the decrease in AA is that U0126 inhibits [Ca<sup>2+</sup>]<sub>i</sub> mobilization in response to ATP or IONO, thereby preventing translocation of cPLA<sub>2</sub>. In chick ventricular myocytes, inhibition of MEK1/ERK by PD98059 inhibits zinterol-mediated AA release, but also inhibits zinterol-induced stimulation of [Ca<sup>2+</sup>]<sub>i</sub> cycling in electrically stimulated cells [33]. In MDCK cells, extracellular ATP acts via P<sub>2Y2</sub> receptors to elicit an IP<sub>3</sub>-mediated [Ca<sup>2+</sup>]<sub>i</sub> increase [34] and IONO acts to increase [Ca<sup>2+</sup>]<sub>i</sub> by permeabilizing cell membranes to Ca<sup>2+</sup>. To determine the effect of U0126 on intracellular Ca<sup>2+</sup> mobilization by 100  $\mu$ M ATP or 10  $\mu$ M IONO, we utilized single-cell fluorescence microscopy on cells loaded with the calcium indicator Fura2. Analysis of the [Ca<sup>2+</sup>]<sub>i</sub> increase in individual cells reveals the heterogeneity in the response to ATP, although most cells exhibited [Ca<sup>2+</sup>]<sub>i</sub> spikes of similar magnitude and duration (Fig. 3A and 3B, thin lines). Analysis of the [Ca<sup>2+</sup>]<sub>i</sub> increase in several cells (Fig. 3A and 3B,



**Figure 2**  
**Effect of anisomycin and U0126 on AA release, ERK inhibition, and cPLA<sub>2</sub> Ser<sup>505</sup> phosphorylation**  
 EGFP-cPLA<sub>2</sub>-transfected cells were incubated with 25 ng/ml anisomycin for 30 min, 10 μM U0126 for 15 min, or with both drugs prior to stimulation with 100 μM ATP, 1 μM IONO, or 10 μM IONO. Protein extracts were subjected to immunoblotting using (A) anti-phospho-p38, (B) anti-phospho-ERK, and (C) anti-cPLA<sub>2</sub> antibodies. (D) AA release in response to 100 μM ATP, 1 μM IONO, or 10 μM IONO in the presence and absence of the inhibitors. Anisomycin did not stimulate AA release in unstimulated cells. The results show average ± SEM for 3 to 5 independent experiments.

**Figure 3**

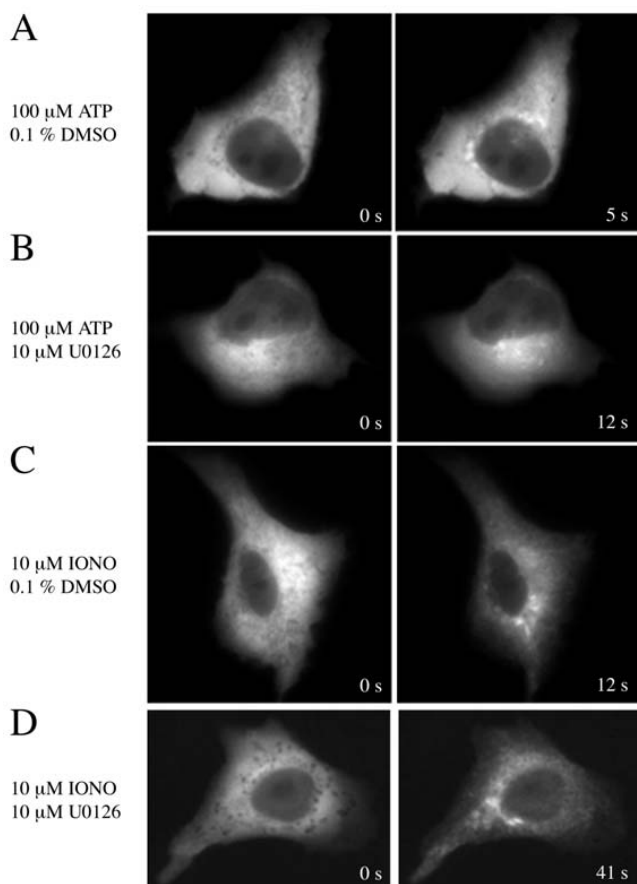
**Effect of U0126 on ATP- and IONO-induced  $[Ca^{2+}]_i$  change** MDCK cells were loaded with the fluorescent  $Ca^{2+}$  indicator Fura2, incubated with 10  $\mu$ M U0126 or vehicle for 15 min, and stimulated with 100  $\mu$ M ATP or 10  $\mu$ M IONO. Graphs show changes in  $[Ca^{2+}]_i$  (expressed as the 340/380 ratio of Fura2 fluorescence) with respect to time from 8 individual cells (thin traces) and from the average of 16 cells (thick trace) treated with (A) U0126 and ATP, (B) vehicle and ATP, (C) U0126 and IONO, and (D) vehicle and IONO. Results are representative of 4 independent experiments in each condition and analysis of several cells per experiment.

thick line) revealed that although the duration of the  $[Ca^{2+}]_i$  increase elicited by ATP in control cells was the same as in the U0126-treated cells, approximately 3–4 min, the amplitude of the  $[Ca^{2+}]_i$  increase was slightly higher ( $\sim$ 20%) in the U0126-treated cells. IONO elicited a sustained, supraphysiological  $[Ca^{2+}]_i$  increase in cells that was also slightly enhanced by U0126 (Fig. 3C and 3D). These experiments demonstrate that U0126 does not decrease  $[Ca^{2+}]_i$  mobilization, and the inhibition of AA release by U0126 cannot be ascribed to a failure in  $[Ca^{2+}]_i$  mobilization.

#### **cPLA<sub>2</sub> translocation is independent of MEK1/ERK pathway**

The effect of inhibition of the MEK1/ERK pathway by U0126 on translocation of cPLA<sub>2</sub> was investigated. Al-

though there is no inhibition of  $[Ca^{2+}]_i$  release by U0126 and little effect on Ser<sup>505</sup> phosphorylation, it is possible that MEK1 inhibition by U0126 prevents cPLA<sub>2</sub> translocation by another mechanism. To investigate this possibility, cells were transfected with a wild-type cPLA<sub>2</sub> fused to EYFP (EYFP-cPLA<sub>2</sub>) and the distribution of EYFP-cPLA<sub>2</sub> was imaged in response to  $[Ca^{2+}]_i$  transients elicited by ATP and sustained  $[Ca^{2+}]_i$  elevations elicited by IONO, in the presence and absence of U0126. Following stimulation with 100  $\mu$ M ATP, there was a rapid translocation of EYFP-cPLA<sub>2</sub> to Golgi that was unaffected by U0126 (Fig. 4A,4B). In response to physiological agonists that elicit transient  $[Ca^{2+}]_i$  changes, only a small fraction of the cPLA<sub>2</sub> translocates. This observation is consistent with our previous results [15] and has been demonstrated by Hira-



**Figure 4**  
**Effect of U0126 on translocation of EYFP-cPLA<sub>2</sub> following ATP or IONO stimulation** Cells expressing EYFP-cPLA<sub>2</sub> were incubated with 10 μM U0126 (B, D) or vehicle (A, C) for 15 min prior to stimulation with 100 μM ATP (A, B) or 10 μM IONO (C, D). Representative frames from time-lapse images show the distribution of EYFP-cPLA<sub>2</sub> fluorescence before and after stimulation. Results are representative of 4 independent experiments and analysis of several cells per experiment.

bayashi et al. [35]. Most studies of cPLA<sub>2</sub> translocation have utilized ionophore, which elicits a large, supraphysiological sustained increase in [Ca<sup>2+</sup>]<sub>i</sub> [14,15,25,35–38], or agonists that produce a sustained [Ca<sup>2+</sup>]<sub>i</sub> increase [35]. Under these conditions, a large proportion of cPLA<sub>2</sub> binds to membrane. These studies show extensive translocation to the endoplasmic reticulum (ER), nuclear envelope and Golgi [14,15,35,36,38]. We found that U0126 also failed to alter extensive EYFP-cPLA<sub>2</sub> translocation to Golgi and ER in response to 10 μM IONO (Fig. 4C,4D). These results demonstrate that MEK inhibition has no effect on cPLA<sub>2</sub> translocation.

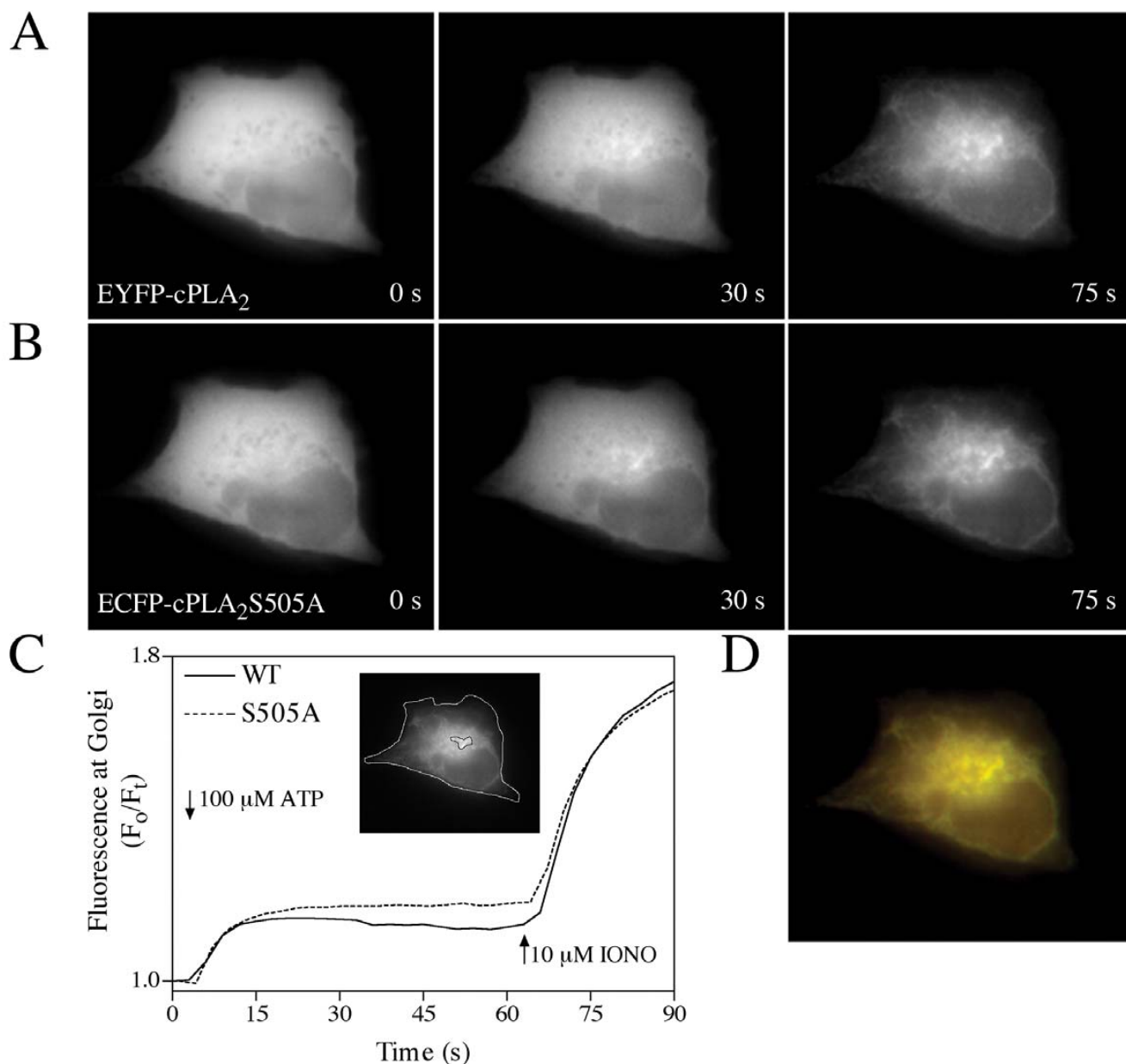
#### **Translocation of phosphorylation site mutants S505A or S727A is similar as wild-type cPLA<sub>2</sub>**

Phosphorylation of cPLA<sub>2</sub> on Ser<sup>505</sup> has been hypothesized to play a role in Ca<sup>2+</sup>-mediated translocation since Ser<sup>505</sup> phosphorylation is required for cPLA<sub>2</sub>-mediated AA release in response to low-dose, but not high-dose, ionophore [22]. Translocation of cPLA<sub>2</sub> S505A in CHO cells has been reported in response to ionophore stimulation [25], but the effect of Ser<sup>505</sup> phosphorylation on the kinetics of translocation, targeting, and in response to a physiological agonist was not investigated. MDCK cells were co-transfected with EYFP-cPLA<sub>2</sub> and a cPLA<sub>2</sub> with a S505A mutation fused to ECFP (ECFP-cPLA<sub>2</sub>S505A). Using dual EYFP/ECFP imaging, we were able to directly compare translocation of both constructs in the same cell. The resting distribution of EYFP-cPLA<sub>2</sub> was similar to that of ECFP-cPLA<sub>2</sub>S505A and, in response to ATP followed by IONO, the pattern of translocation of EYFP-cPLA<sub>2</sub> was similar to ECFP-cPLA<sub>2</sub>S505A (Fig. 5 panels A and B, D). Analysis of the increase in fluorescence at the Golgi with respect to time demonstrates that the rates of translocation of cPLA<sub>2</sub> and cPLA<sub>2</sub>S505A elicited by ATP followed by IONO are very similar (Fig. 5C). As previously reported [22], the cPLA<sub>2</sub> S727A mutation has a similar phenotype on AA release as the S505A mutation and Ser<sup>727</sup> was found to be phosphorylated in tandem with Ser<sup>505</sup>. Imaging experiments were performed using EYFP-cPLA<sub>2</sub> and ECFP-cPLA<sub>2</sub>S727A and we found that the distribution of EYFP-cPLA<sub>2</sub> was identical to that of ECFP-cPLA<sub>2</sub>S727A before and after stimulation with 10 μM IONO (Fig. 7 panels A and B, D). Analysis of the increase in fluorescence at the Golgi with respect to time demonstrates that the rates of translocation of cPLA<sub>2</sub> and the cPLA<sub>2</sub>S727A elicited by IONO are very similar (Fig. 6C).

#### **Discussion**

The MEK1/ERK pathway regulates cPLA<sub>2</sub> and ERKs phosphorylate cPLA<sub>2</sub> on Ser<sup>505</sup>. The results of this study demonstrate that this pathway is required for cPLA<sub>2</sub>-mediated AA release independent of Ser<sup>505</sup> phosphorylation and extend our previous work in macrophages [13] by demonstrating that this alternative role of the MEK1/ERK pathway is not involved in regulating [Ca<sup>2+</sup>]<sub>i</sub> change or cPLA<sub>2</sub> translocation kinetics or targeting, but is required for optimal hydrolytic activity and AA release.

The results shown here demonstrate that inhibition of MEK with U0126 quantitatively inhibits both ERK phosphorylation and AA release in MDCK cells in response to [Ca<sup>2+</sup>]<sub>i</sub> mobilization. The MEK inhibitor PD098059, a less potent inhibitor [28,29], also inhibited ATP- and IONO-induced AA release (data not shown). ERK was found to be constitutively phosphorylated in our study using MDCK cells from ATCC, in contrast to what has been reported previously for MDCK-D<sub>1</sub>, a subclone of



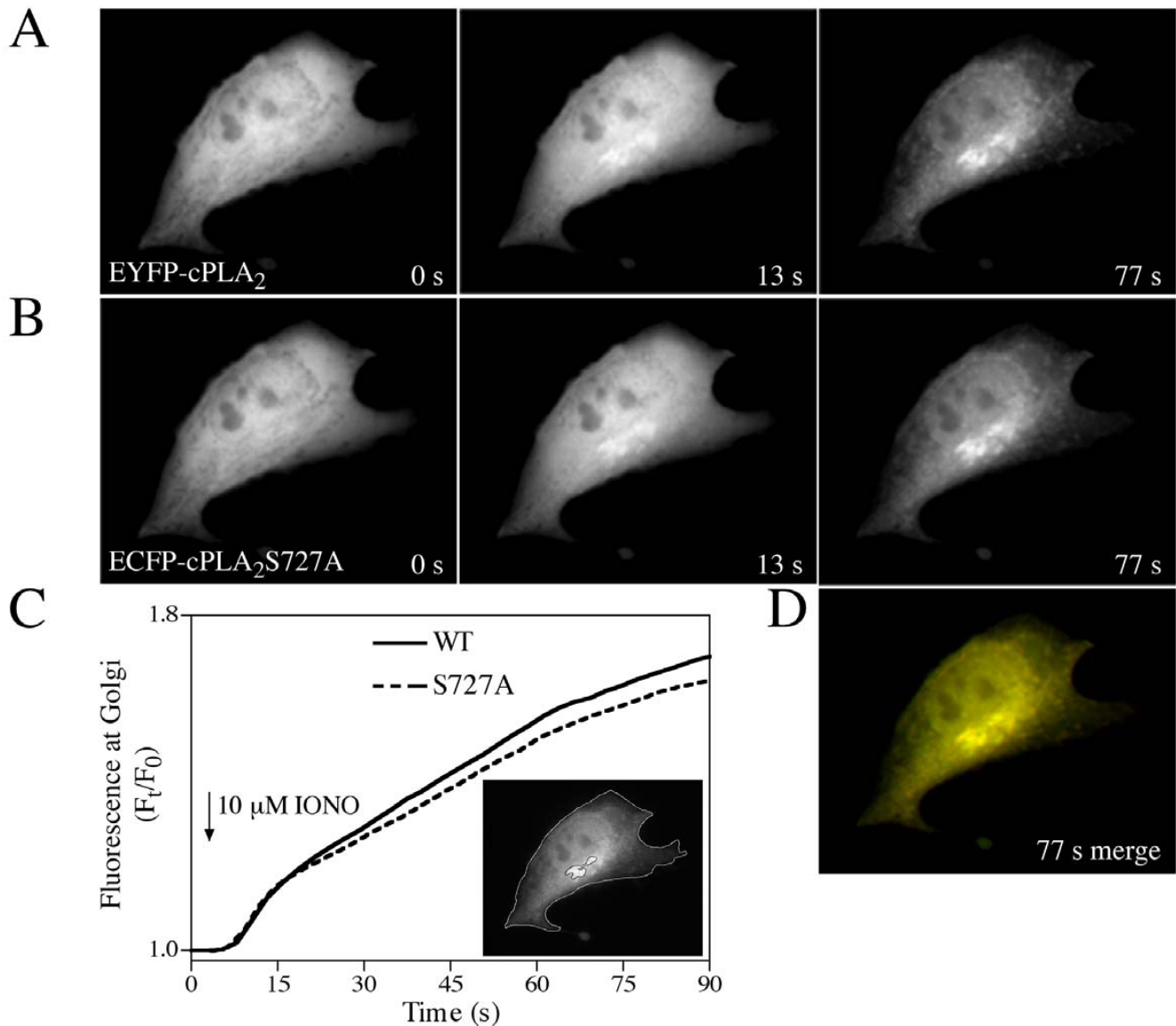
**Figure 5**  
**Translocation of ECFP-cPLA<sub>2</sub>S505A following ATP and IONO stimulation** Cells expressing ECFP-cPLA<sub>2</sub>S505A and EYFP-cPLA<sub>2</sub> were stimulated with ATP and IONO. ECFP and EYFP fluorescence images were taken at 3 s intervals. Representative frames from time-lapse images show the distribution of EYFP-cPLA<sub>2</sub> and ECFP-cPLA<sub>2</sub>S505A fluorescence before and 30 s after addition of 100 μM ATP, followed by addition of 10 μM IONO at 1 min (panels A and B). (C) Fluorescent protein (FP) fluorescence from the area of the Golgi (inset) was analyzed with respect to time before and after ATP and IONO addition (arrows). (D) Overlay of the 75 s images from panels A and B. Results are representative of 5 independent experiments and analysis of several cells per experiment.

MDCK selected for adrenergic receptor expression [39,40]. However, a side-by-side comparison of MDCK cells from ATCC and the MDCK-D<sub>1</sub> subclone (kindly provided by Dr. Paul Insel, UCSD) demonstrated constitutive activation of ERKs when both are grown at low density

(not shown). However, at high density ERKs are less active and can be further activated by phorbol ester (not shown).

In MDCK-D<sub>1</sub> cells, without constitutively active ERKs, AA release is delayed after [Ca<sup>2+</sup>]<sub>i</sub> mobilization, and is tem-





**Figure 6**  
**Translocation of ECFP-cPLA<sub>2</sub>S727A following IONO stimulation** Cells expressing ECFP-cPLA<sub>2</sub>S727A and EYFP-cPLA<sub>2</sub> were stimulated with IONO. Representative frames from time-lapse images show the distribution of EYFP-cPLA<sub>2</sub> and ECFP-cPLA<sub>2</sub>S727A fluorescence before and 13 s after addition of 10 μM IONO (panels A and B). (C) Fluorescent protein (FP) fluorescence from the area of the Golgi (inset) was analyzed with respect to time before and after IONO addition (arrow). (D) Overlay of the 77 s images from panels A and B. Results are representative of 4 independent experiments and analysis of several cells per experiment.

porally correlated with ERK activation [40], whereas in MDCK cells with constitutively active ERKs, AA release is rapid, with significant AA release measured 30 s after  $[Ca^{2+}]_i$  increase [15]. This temporal correlation between AA release and ERK activation has also been reported in CHO cells in response to PAF stimulation [35,41]. These results support the observations made here that ERK activation

is required for phospholipid hydrolysis independently of cPLA<sub>2</sub> translocation.

cPLA<sub>2</sub>-mediated AA release must be preceded by translocation of the enzyme to its membrane substrate which is a  $Ca^{2+}$ -dependent process and is a function of the calcium-dependent lipid-binding (C2) domain. cPLA<sub>2</sub> translocates primarily to Golgi in response to a transient  $[Ca^{2+}]_i$

changes and to Golgi and ER in response to a sustained  $[Ca^{2+}]_i$  increase [15]. The reduction in AA release by MEK inhibition did not involve a failure in  $[Ca^{2+}]_i$  release or translocation. These results show that translocation is necessary but not sufficient for optimum hydrolytic activity.

Measuring cPLA<sub>2</sub> translocation is not a trivial matter when investigating mechanisms of AA release. For example, cPLA<sub>2</sub> constructs with a S505A mutation have long been recognized as unable to support AA release in response to physiological agonists or low-dose ionophore when transfected in cells. In contrast, cPLA<sub>2</sub> S505A is active in vitro and phosphorylation only modestly increases the activity of the enzyme [16,17,24]. Interestingly, the inhibitory effect of the S505A mutation on AA release is obviated by a high  $[Ca^{2+}]_i$  increase. In light of these observations, it is possible that Ser<sup>505</sup> phosphorylation may alter the  $[Ca^{2+}]_i$  sensitivity of the enzyme, its rate of translocation, its intracellular targeting or, as has been previously suggested [22], the ability of cPLA<sub>2</sub> to release from a non-membrane sequestering agent. Although one report has shown that cPLA<sub>2</sub> S505A translocates in CHO cells in response to ionophore, we were able to directly compare rates of translocation between cPLA<sub>2</sub> and cPLA<sub>2</sub> S505A to a physiological agonist and found no difference between translocation rates or intracellular targeting. We also demonstrated that there was no difference in translocation rates or intracellular targeting between wild-type cPLA<sub>2</sub> and cPLA<sub>2</sub> S727A, which has the same phenotype as S505A with regard to AA release when transfected into cells. Thus, the role of cPLA<sub>2</sub> phosphorylation in mediating AA release remains unclear.

Although the alternative mechanism whereby the MEK1/ERK pathway regulates cPLA<sub>2</sub> is not known, it is possible that it affects membrane properties and/or cPLA<sub>2</sub> conformation that promotes optimal hydrolytic activity. It is also possible that the alternative mechanism is due to phosphorylation of cPLA<sub>2</sub> on a novel site by a kinase that is downstream of the MEK1/ERK pathway or phosphorylation of a regulatory protein.

### Conclusions

Translocation to membrane is a critical regulatory step for the action of cPLA<sub>2</sub> because it is necessary for access to substrate. In this study we demonstrate, however, that association of cPLA<sub>2</sub> with membrane when phosphorylated on Ser<sup>505</sup> is not sufficient for its full activity in vivo. This is demonstrated by the results showing that inhibition of the MEK1/ERK pathway significantly blocks AA release but has no effect on  $[Ca^{2+}]_i$  mobilization or cPLA<sub>2</sub> translocation and targeting. Diminution of AA release by MEK1/ERK is also independent of cPLA<sub>2</sub> phosphorylation on Ser<sup>505</sup>. Consequently, our results demonstrate in living cells that the translocation process and subsequent cata-

lytic activity on the membrane are two independently regulated steps.

### Materials and Methods

#### Fluorescent protein-cPLA<sub>2</sub> fusion constructs

DNA encoding the full-length human cPLA<sub>2</sub> was cloned into the vector pEGFP-C3 (Clontech) to create pEGFP-cPLA<sub>2</sub>, as previously described [15]. The XbaI/PstI fragment from a cPLA<sub>2</sub>α clone containing S505A or S727A mutations [13] was inserted into an XbaI/PstI site in pEGFP-cPLA<sub>2</sub> to generate pEGFP-cPLA<sub>2</sub>S505A and pEGFP-cPLA<sub>2</sub>S727A. Different fluorescent-protein tagged constructs were produced by exchanging the NheI/BsrGI fragment containing the fluorescent protein coding sequence between EGFP, EYFP, and ECFP. All constructs were confirmed by sequencing.

#### Cell culture

MDCK cells obtained from ATCC were cultured in DMEM containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.292 mg/ml glutamine (growth medium) in 5% CO<sub>2</sub> at 37°C. Subconfluent cells ( $5 \times 10^3$  cells/cm<sup>2</sup>) were transfected with 2 µg of the relevant plasmid using Fugene-6 (Boehringer Mannheim) in DMEM containing 0.2% BSA, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.292 mg/ml glutamine (serum-free medium) following the manufacturer's protocol. Stable lines expressing EGFP-cPLA<sub>2</sub> were generated by growing transfected cells in growth medium for 3 d, supplementing the growth medium with 5 mg/ml Geneticin (antibiotic G418-sulfate), and culturing for an additional 2 wk in Geneticin. Cells expressing EGFP fluorescence were selected using a fluorescence-activated cell sorter. The EGFP-positive cells were maintained in growth medium supplemented with 5 mg/ml Geneticin. For imaging studies, MDCK cells were plated on glass-bottomed 35 mm culture dishes (MatTek) at  $5 \times 10^3$  cells/cm<sup>2</sup> in growth medium and incubated overnight, transfected with the relevant plasmid(s), changed into serum-free medium to quiesce the cells, incubated overnight, and used the next day.

#### Immunoblotting

Stable EGFP-cPLA<sub>2</sub> transfectants were grown on 100 mm dishes at  $5 \times 10^3$  cells/cm<sup>2</sup> in growth medium for one day, then quiesced in serum-free medium overnight. Cells were scraped into ice-cold lysis buffer: 50 mM HEPES, pH 7.4, 150 mM sodium chloride, 1.5 mM magnesium chloride, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 200 µM sodium vanadate, 10 mM tetrasodium pyrophosphate, 100 mM sodium fluoride, 10 µg/ml leupeptin, and 10 µg/ml aprotinin. Lysates were centrifuged at  $15,000 \times g$  for 15 min, and protein concentration of the supernatant was determined by the bicinchoninic acid method. Laemmli electrophoresis sample buffer (5×) was added to the lysates, and SDS-polyacrylamide gel electrophoresis and

immunoblotting were performed using 35  $\mu$ g lysate protein, phospho-specific antibodies for ERK and p38, and rabbit polyclonal antibody for cPLA<sub>2</sub>[21].

#### Dual imaging microscopy of fluorescent protein translocation

In order to compare the characteristics of full-length cPLA<sub>2</sub> and cPLA<sub>2</sub>S505A or cPLA<sub>2</sub>S727A translocation, while controlling for cell-to-cell heterogeneity, we used a dual CFP/YFP imaging approach. EYFP-cPLA<sub>2</sub>/ECFP-cPLA<sub>2</sub>S505A- or pECFP-cPLA<sub>2</sub>S727A-transfected MDCK cells grown on MatTek plates were quiesced overnight in serum-free medium, washed with and incubated in Hank's balanced salt solution (HBSS) additionally buffered with 25 mM HEPES pH 7.4 (HHBSS). Cells were imaged using an Olympus inverted microscope equipped with a 60 $\times$ , 1.25 NA oil immersion objective, CFP and YFP emission filters (Chroma) in a Sutter filter wheel, a dual CFP/YFP dichroic mirror, and a TILL Imago CCD camera (TILL Photonics). Excitation light of 430 and 510 nm for CFP and YFP, respectively, was provided using a Polychrome IV monochromator (TILL Photonics). TILLvisION software was used for acquisition and analysis. Bleach values for ECFP and EYFP were calculated by determining the background-corrected fluorescence for the entire cell with respect to time and normalizing each value to the initial value. ECFP/EYFP fluorescence changes with respect to time for regions of interest corresponding to an area of Golgi membrane were determined by calculating the  $F_t/F_0$ , where  $F_t$  is the background- and bleach-corrected ECFP or EYFP fluorescence at time = t and  $F_0$  is the background-corrected ECFP or EYFP fluorescence at time = 0 s. Fluorescence was normalized to the  $F_0$  value, which resulted in  $F_t/F_0$  representing the fraction of total cell fluorescence at Golgi. Final images were produced using Adobe Photoshop.

#### Calcium imaging

MDCK cells grown on MatTek plates were quiesced overnight in serum-free medium, washed with HHBSS containing 1 mM probenecid and incubated with 5  $\mu$ M Fura2-AM (Calbiochem) in HHBSS, 1 mM probenecid, and 1% DMSO for 45 min at 37°C. Cells were then washed with HHBSS containing 1 mM probenecid and imaged after a 30 min incubation for de-esterification of the Fura2-AM. Single-cell imaging was performed on the Olympus system described above, but using a 40 $\times$ , 1.35 NA oil immersion objective and a Fura2 dichroic mirror and emission filter (Chroma). Fura2 image pairs illuminated at 340 and 380 nm were taken at 1 Hz. The  $[Ca^{2+}]_i$  increase is expressed as the ratio of the background-corrected Fura2 fluorescence at 340 and 380 nm [42].

#### Measurement of AA release

The protocol for determining AA release is essentially as described [15,21]. MDCK cells stably expressing EGFP-cPLA<sub>2</sub> were plated in 12-well plates at  $5 \times 10^3$  cells/cm<sup>2</sup> and incubated in growth medium overnight. Cells were then washed twice with serum-free medium and incubated with 0.25  $\mu$ Ci [<sup>3</sup>H]-AA/well in serum-free medium overnight. U0126 or vehicle was added to each well and the cells were then incubated for 15 min at 37°C in 5% CO<sub>2</sub>. Cells were washed to remove unincorporated [<sup>3</sup>H]-AA and then incubated in HHBSS supplemented with 0.05% BSA with either U0126 or vehicle. Cells were stimulated with the agonist of choice and the medium was collected at appropriate time points. The medium was centrifuged at 500 g for 5 min, and the amount of radioactivity in the supernatant was determined by scintillation counting. Cells were scraped in 0.5 ml 0.1% Triton X-100 for determining the total cellular radioactivity.

#### Authors' contributions

JHE carried out the Ca and FP imaging studies, participated in the design and coordination of the study, and drafted the manuscript. DJF performed the AA release and Western blot studies and participated in the design and coordination of the study. CCL conceived of the study, participated in its design and coordination, and participated in writing the draft. All authors read and approved the final manuscript.

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