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# Off-target effect of the cPLA$_2\alpha$ inhibitor pyrrophenone: Inhibition of calcium release from the endoplasmic reticulum

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## Abstract

Cytosolic phospholipase A$_2\alpha$ (cPLA$_2\alpha$) mediates agonist-induced release of arachidonic acid from membrane phospholipid for production of eicosanoids. The activation of cPLA$_2\alpha$ involves increases in intracellular calcium, which binds to the C2 domain and promotes cPLA$_2\alpha$ translocation from the cytosol to membrane to access substrate. The cell permeable pyrrolidine-containing cPLA$_2\alpha$ inhibitors including pyrrophenone have been useful to understand cPLA$_2\alpha$ function. Although this serine hydrolase inhibitor does not inhibit other PLA$_2$s or downstream enzymes that metabolize arachidonic acid, we reported that it blocks increases in mitochondrial calcium and cell death in lung fibroblasts. In this study we used the calcium indicators G-CEPIA1*er* and CEPIA2*mt* to compare the effect of pyrrophenone in regulating calcium levels in the endoplasmic reticulum (ER) and mitochondria in response to A23187 and receptor stimulation. Pyrrophenone blocked calcium release from the ER and concomitant increases in mitochondrial calcium in response to stimulation by ATP, serum and A23187. In contrast, ER calcium release induced by the sarco/endoplasmic reticulum $Ca^{2+}$-ATPase inhibitor thapsigargin was not blocked by pyrrophenone suggesting specificity for the calcium release pathway. As a consequence of blocking calcium mobilization, pyrrophenone inhibited serum-stimulated translocation of the cPLA$_2\alpha$ C2 domain to Golgi. The ability of pyrrophenone to block ER calcium release is an off-target effect since it occurs in fibroblasts lacking cPLA$_2\alpha$. The results implicate a serine hydrolase in regulating ER calcium release and highlight the importance of careful dose-response studies with pyrrophenone to study cPLA$_2\alpha$ function.

### Keywords

cPLA$_2\alpha$; calcium; pyrrophenone; endoplasmic reticulum; mitochondria

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## 1. Introduction

The Group IVA cytosolic phospholipase $A_2$ (cPLA$_2\alpha$) is a widely expressed enzyme that releases arachidonic acid for production of eicosanoids [1]. Arachidonic acid is metabolized by cyclooxygenases to prostaglandins and thromboxane $A_2$, and by 5-lipoxygenase to generate leukotrienes [2, 3]. cPLA$_2\alpha$ is composed of two domains, an N-terminal calcium and phospholipid binding C2 domain, and a globular catalytic domain that contains the active site Ser/Asp catalytic dyad and phosphorylation sites [4]. cPLA$_2\alpha$ is activated in cells by diverse stimuli that trigger common signaling pathways including calcium mobilization and activation of mitogen-activated protein kinases [1]. When calcium binds to the cPLA$_2\alpha$ C2 domain, the affinity of cPLA$_2\alpha$ for membrane increases, and it translocates from the cytosol to the Golgi apparatus and endoplasmic reticulum/nuclear envelope for accessing phospholipid substrate [5]. The phosphorylation of cPLA$_2\alpha$ on Ser505 by mitogen-activated protein kinases enhances its catalytic activity [6, 7].

cPLA$_2\alpha$ is the only mammalian $PLA_2$ that exhibits preference for *sn-2* arachidonic acid and its role in initiating eicosanoid production is well established [8, 9]. However, mammalian cells contain a number of $PLA_2$ enzymes that can potentially release arachidonic acid for lipid mediator production [10]. A common approach to study $PLA_2$ enzymes involves the use small molecule cell permeable inhibitors [11]. Potent cPLA$_2\alpha$ inhibitors containing 1,2,4-trisubstituted pyrrolidine have been generated and are used widely to study the role of cPLA$_2\alpha$ in cells [12, 13]. The pyrrolidine inhibitors, such as pyrrophenone, are more potent than other commonly used cPLA$_2\alpha$ inhibitors such as arachidonyl trifluoromethyl ketone and methyl arachidonyl fluorophosphonate [12–14]. They are also more selective and do not inhibit Group VI $PLA_2$s or downstream enzymes that metabolize arachidonic acid [12–15]. Small molecule inhibitors are important for probing the cellular function of $PLA_2$ enzymes, however, there is the potential for concentration-dependent off-target effects.

In a recent study we investigated the role of cPLA$_2\alpha$ in regulating cell death in lung fibroblasts by using the pyrrolidine derivative pyrrophenone, and by comparing fibroblasts from cPLA$_2\alpha$ wild type and knockout mice [16]. Cell death was induced in lung fibroblasts with the calcium ionophore A23187, a known inducer of necrotic cell death due to mitochondrial calcium overload and cyclophilin D-dependent opening of the mitochondrial permeability transition pore (MPTP) [16–18]. Cell death was induced to a similar extent in A23187 treated cPLA$_2\alpha^{+/+}$ and cPLA$_2\alpha^{-/-}$ lung fibroblasts indicating no role for cPLA$_2\alpha$ [16]. However, cell death in response to A23187 was blocked by pyrrophenone in both cPLA$_2\alpha^{+/+}$ and cPLA$_2\alpha^{-/-}$ lung fibroblasts by inhibiting mitochondrial calcium uptake and MPTP [16]. The ability of pyrrophenone to block cell death in cells lacking cPLA$_2\alpha$ represents an off-target effect suggesting that it may target a novel pathway involving a serine hydrolase that regulates mitochondrial calcium uptake. Calcium is transferred from the ER to mitochondria through specialized contact sites, a process that is important for regulating mitochondrial function but that also promotes cell death when not properly controlled [19]. In this study we specifically addressed whether pyrrophenone inhibits the release of calcium from the ER thereby preventing calcium transfer to mitochondria. We monitored the effect of pyrrophenone on regulating agonist-stimulated ER and mitochondrial calcium levels by using the recently developed calcium-measuring organelle-

entrapped protein indicators (CEPIA) that can be targeted to specific organelles for evaluating intra-organelle calcium levels [20].

## 2. Material and methods

### 2.1 Cells

Mouse lung fibroblasts were isolated from cPLA$_2\alpha^{+/+}$ and cPLA$_2\alpha^{-/-}$ mice and immortalized with SV40 to generate immortalized mouse lung fibroblasts (IMLF$\alpha^{+/+}$ and IMLF$\alpha^{-/-}$) as previously described [21, 22]. Unless otherwise noted all experiments were carried out with IMLF$\alpha^{-/-}$.

### 2.2 Transfection protocol

IMLF$\alpha$ ($5 \times 10^3$ cells) were plated on the glass insert of MatTek dishes (MatTek Corp.) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (growth media) for 24 hr as previously described [6]. The cells were transfected using Lipofectamine 3000 (ThermoFisher Scientific) according to the manufacturer's protocol with pCMV G-CEPIA*er* and pCMV CEPIA2*mt* (gifts from Masamitsu Iino) (Addgene plasmids #58215 and #58218, respectively) and mCherry-ER-3 (a gift from Michael Davidson) (Addgene plasmid # 55041) [20]. After 24 hr the medium was replaced with serum-free DMEM containing 0.1% bovine serum albumin and the cells incubated for an additional 24 hr.

The monomeric (A206K) enhanced yellow fluorescent protein (EYFP)-C2 domain of cPLA$_2\alpha$ was cloned into pVQAd5CMVK-NpA shuttle plasmid (ViraQuest, Inc) and recombinant adenoviruses generated by ViraQuest. IMLF$\alpha$ plated on the glass insert of MatTek dishes were cultured in growth media as described above for 18 hr. The medium was replaced with serum- and antibiotic-free DMEM containing 0.1% bovine serum albumin and cells incubated with recombinant adenovirus for 26 hr as previously described [6].

### 2.2 Live-cell imaging

For imaging cytosolic $Ca^{2+}$, IMLF$\alpha$ were loaded with Fura Red-AM for 15 min as previously described [16]. Imaging of ER and mitochondrial calcium was carried out in IMLF$\alpha$ expressing G-CEPIA*er* or CEPIA2*mt*, respectively. Cells were pre-incubated with the indicted concentration of pyrrophenone (Cayman Chemical) or DMSO vehicle for 30 min at 37°C in serum-free phenol red-free DMEM containing 25 mM Hepes, pH 7.4 and 0.1% BSA. Calcium transients were induced by stimulation with A23187 (1 μg/ml), mouse serum (5%), ATP (200 μM) or thapsigargin (3 μM) and cells imaged on an inverted Zeiss 200M microscope using Intelligent Imaging Innovations Inc. (3I) software (Slidebook 6). In some experiments the cells were incubated in media containing 3 mM EGTA. Fluorescence intensities of Fura Red excited at 403/490 nm for bound/unbound calcium were used for cytosolic calcium analysis as described [16]. The ratio of fluorescence at each time point ($R_t$) after cell stimulation was determined after correcting for background fluorescence. Fluorescence data ($R_t/R_0$) represents the fold-increase at $R_t$ relative to the ratio of fluorescence at time 0 ($R_0$) set at 1. For imaging ER calcium, fluorescence (488nm/525nm excitation/emission) of G-CEPIA1*er* was recorded every 5 sec for ATP and A23187, and

every 15 sec after thapsigargin stimulation. For imaging mitochondrial calcium, fluorescence (488nm/525nm excitation/emission) of CEPIA2*mt* was recorded every 5 sec after ATP, A23187, and thapsigargin stimulation.

### 2.3 EYFP-C2 domain translocation

IMLFα$^{-/-}$ expressing EYFP-C2 domain were pre-incubated with the indicted concentration of pyrrophenone or DMSO vehicle for 30 min and then stimulated with 5% mouse serum to induce EYFP-C2 domain translocation to the Golgi as previously described [6]. Images were collected every 5 sec using a YFP filter with the microscopy system described above. Translocation data were calculated based on average fluorescence intensity of EYFP-C2 domain on the Golgi in each cell. Values were corrected for background fluorescence and differential bleaching at each wavelength through the duration of the imaging and expressed relative to time 0 ($F_t/F_0$).

## 3. Results

### 3.1 Pyrrophenone blocks receptor-mediated release of calcium from intracellular stores

We previously reported that pyrrophenone blocked A23187-induced increase in mitochondial calcium $[Ca^{2+}]_{mt}$ and partially suppressed cytosolic calcium $[Ca^{2+}]_c$ increases [16]. To determine if pyrrophenone inhibited receptor-mediated increases in $[Ca^{2+}]_c$, fibroblasts were treated with serum or ATP. For these experiments, IMLFα$^{-/-}$ were used to avoid any potential effects of cPLA$_2$α activation and arachidonic acid release in influencing calcium mobilization. We previously reported that serum stimulates a rapid increase in $[Ca^{2+}]_c$ in IMLFα with an initial peak at 15 sec followed by lower amplitude oscillations, a pattern typical of release from intracellular stores followed by capacitative influx of extracellular calcium [6]. As shown in Fig. 1A, serum-stimulated calcium mobilization was dose-dependently inhibited with pyrrophenone that was almost completely blocked at a concentration of 2 μM. Pyrrophenone inhibited the peak of $[Ca^{2+}]_c$ increase, which occurred 15 sec after serum addition, with an IC$_{50}$ of ~1 μM (Fig. 1B). The increase in $[Ca^{2+}]_c$ induced by ATP was also inhibited by pyrrophenone. Serum and ATP stimulated the release of calcium from intracellular stores since they trigger $[Ca^{2+}]_c$ increase in fibroblasts incubated in media containing EGTA to chelate extracellular calcium (Fig. 1C and Fig. 1D). Pyrrophenone inhibited serum and ATP-stimulated release of calcium from intracellular stores to basal levels. The results are not unique to fibroblasts lacking cPLA$_2$α since similar effects of pyrrophenone were observed using IMLF$^{+/+}$ (data not shown).

### 3.2 Use of the CEPIA calcium indicators to monitor changes in $[Ca^{2+}]_{er}$ in IMLF

Since the ER is the main intracellular store of $Ca^{2+}$ that is released in response to cell stimulation, we tested the ability of pyrrophenone to block calcium release from the ER by imaging ER calcium in IMLFα$^{-/-}$ expressing G-CEPIA1*er*. This calcium indicator contains an enhanced green fluorescent protein (EGFP) tag, and an ER localization and retention sequence [20]. It was designed to have low $Ca^{2+}$ affinity (672 μM) and high dynamic range ($F_{max}/F_{min}$=4.7) in order to measure calcium changes in the ER lumen, which contains high levels of calcium. We first confirmed that G-CEPIA1$_{er}$ localized to the ER in IMLF. G-CEPIA1$_{er}$ co-localized with the mCherry-tagged ER marker (calreticulin + KDEL) when co-

expressed in IMLF as previously reported in HeLa cells (Fig. 2A) [20]. To evaluate the ability of G-CEPIA1$_{er}$ to monitor changes in $[Ca^{2+}]_{er}$ IMLF were treated with the sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) inhibitor thapsigargin, which promotes calcium release from the ER [23]. Thapsigargin induced a decrease in fluorescence of G-CEPIA1$_{er}$ consistent with release of calcium from the ER (Fig. 2B). Our protocol for testing the effect of pyrrophenone involved pre-treating cells for 30 min with the inhibitor or DMSO prior to cell stimulation. It was important to determine if pyrrophenone affected the steady state concentration of $[Ca^{2+}]_{er}$ during the pre-incubation time. There was no difference in the fluorescence intensity of G-CEPIA1$_{er}$ in IMLF 30 min after incubation with either pyrrophenone (2 μM) or DMSO suggesting that the inhibitor does not influence steady state $[Ca^{2+}]_{er}$ (Fig. 2C). In additional control experiments we also found that pyrrophenone had no effect on $[Ca^{2+}]_{c}$ when monitored for 5 min after its addition to IMLF (data not shown).

### 3.3 Pyrrophenone blocks ER calcium release and mitochondrial calcium uptake in response to ATP and A23187 but not to thapsigargin

The effect of pyrrophenone on the release of calcium from the ER was investigated in IMLF stimulated with a variety of calcium mobilizing agonists. Stimulation of IMLF with ATP, which triggers receptor-mediated release of calcium from the ER, resulted in a decrease in G-CEPIA1*er* fluorescence that was inhibited by pyrrophenone (Fig. 3A). A23187, which is known to induce release of calcium from intracellular ER stores, also triggered a decrease in fluorescence of G-CEPIA1*er* that was inhibited by pyrrophenone (Fig. 3B) [24]. However, the release of calcium from the ER induced by thapsigargin was not inhibited by pyrrophenone suggesting that the serine hydrolase inhibitor targets specific pathways for calcium release (Fig. 3C).

Since calcium is transferred from the ER to mitochondria, we investigated the effect of pyrrophenone on agonist-induced increases in $[Ca^{2+}]_{mt}$ in IMLF$^{-/-}$ expressing CEPIA2*mt*. This indicator contains mitochondrial localization sequences in tandem, a GFP fluorescent tag and has a relatively high affinity for calcium ($K_d$ 160 nM) [20]. The ability of ATP and A23187 to stimulate ER calcium release was accompanied by an increase in $[Ca^{2+}]_{mt}$, which was inhibited by pyrrophenone (Fig. 1E and 1F, respectively). Although thapsigargin treatment promoted release of $Ca^{2+}$ from the ER, this resulted in only a small increase in $[Ca^{2+}]_{mt}$ that was not inhibited by pyrophenone (Fig. 3G). We previously reported that another serine hydrolase inhibitor, the triazole urea compound KT195, also inhibited A23187-stimulated cell death by blocking mitochondrial calcium uptake [16]. Similar to the effect of pyrophenone, KT195 inhibited ER calcium release (Fig. 3D) and mitochondrial calcium uptake (Fig. 3H) in cells stimulated with ATP.

### 3.4 Pyrrophenone inhibits serum-stimulated translocation of the cPLA$_2$α C2 domain to Golgi

The ability of pyrrophenone to block ER calcium release suggested that it would inhibit the translocation of cPLA$_2$α to Golgi mediated by the C2 domain, which requires an increase in $[Ca^{2+}]_{c}$ [25]. We have previously reported that the cPLA$_2$α C2 domain translocates to Golgi in proportion to the increase in $[Ca^{2+}]_{c}$ indicating that it functions as a calcium sensor [26]. To investigate the effect of pyrrophenone on translocation, the EYFP-C2 domain was

expressed in IMLF$^{-/-}$ and translocation induced by serum stimulation monitored by live-cell imaging (Fig. 4). Serum stimulated the rapid translocation of ECFP-C2 domain to Golgi seen as intense juxta-nuclear fluorescence that was completely blocked by pyrrophenone (2 μM). The results suggest that pyrrophenone blocks serum-induced increase in $[Ca^{2+}]_c$ to levels below the threshold required for membrane binding of the C2 domain.

## 4. Discussion

The serine hydrolase inhibitor pyrrophenone has been used extensively to understand the role of cPLA$_2$α in mediating lipid mediator production and cell function. Pyrrophenone inhibits cPLA$_2$α-mediated arachidonic acid release from serum-stimulated IMLF$^{+/+}$ with an $IC_{50}$ of ~0.05 μM with 80–90% inhibition occurring at 0.3–1 μM [16]. We previously reported that pyrrophenone inhibits mitochondrial calcium uptake and necrotic cell death due to MPTP formation [16]. This is an off-target effect since it occurs in IMLF lacking cPLA$_2$α. We now show that pyrrophenone blocks calcium release from the ER triggered by receptor stimulation and to A23187, and the concomitant transfer of calcium to the mitochondria. The off-target effects of pyrrophenone occur at an $IC_{50}$ of ~0.5–1 μM [16]. These results highlight the importance of careful dose response studies with pyrrophenone to study the role of cPLA$_2$α in specific cell types. Pyrrophenone is a potent cPLA$_2$α inhibitor that can be used at concentrations sufficient to inhibit cPLA$_2$α catalytic activity in cells (<0.2 μM) that will limit off-target effects we described previously and in this study [16]. The results demonstrate that with concentrations exceeding ~0.5 μM, pyrrophenone can affect cPLA$_2$α function by blocking calcium-dependent translocation as well as catalytic activity. In addition pyrrophenone has the potential of blocking cellular processes that require calcium mobilization.

The catalytic activity of cPLA$_2$α is likely inhibited by pyrrophenone through formation of a hemiketal between its ketone carbonyl and the active site serine of the enzyme based on the ability of polarized ketones to form stable hemiketals with serine proteases and esterases [27–29]. In support of this mechanism, when the ketone of pyrrophenone is reduced to the secondary alcohol, it poorly inhibits serum-stimulated arachidonic acid release from IMLF$^{+/+}$ [16]. The reduced form of pyrrophenone also does not effectively block cell death due to calcium overload in A23187-stimulated IMLF supporting involvement of a serine hydrolase that is inhibited through interaction with the ketone carbonyl group of pyrrophenone [16]. This off-target effect of pyrrophenone has uncovered a novel pathway implicating a serine hydrolase in regulating the release of calcium from the ER.

Calcium release from the ER in response to receptor stimulation with agonists such as ATP and serum is mediated by the production of $IP_3$ that acts through receptors in the ER [30]. Calcium is transferred from the ER to mitochondria through sites where the membranes are in close contact, the mitochondrial-associated membranes [31, 32]. Mitochondrial calcium uptake is important for regulating mitochondrial function and cell survival, but when there is calcium overload in mitochondria that occurs with A23187 there is opening of MPTP and cell death [18, 33]. In addition to ATP, pyrrophenone also inhibited calcium release from the ER in response to A23187. Studies have shown that the calcium ionophore increases $IP_3$ levels in cells that may play a role in inducing the release of calcium from the ER [34–36].

In contrast, the release of calcium from the ER in response to the SERCA inhibitor thapsigargin, which has been reported not to promote $IP_3$ production, was not inhibited by pyrrophenone [23, 37]. Thapsigargin does not generate the formation of high-concentration $Ca^{2+}$ micro-domains that are required for mitochondrial calcium uptake consistent with studies showing that it is not as effective in increasing mitochondrial calcium as the $IP_3$-mediated pathway [31, 38, 39]. Steady state ER calcium levels are maintained by the continuous cycling of calcium in and out of the ER through SERCA-mediated uptake and passive leakage through basal ER calcium-leak channels [40]. Our results suggest that pyrrophenone targets a serine hydrolase in the $IP_3$-mediated ER calcium release pathway that couples to mitochondrial calcium uptake and not pathways that control basal calcium release.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Abbreviations

| | |
|---|---|
| **cPLA$_2\alpha$** | cytosolic phospholipase A$_2\alpha$ |
| **ER** | endoplasmic reticulum |
| **DMEM** | Dulbecco's Modified Eagle's Medium |
| **MPTP** | mitochondrial permeability transition pore |
| **IMLF** | immortalized mouse lung fibroblasts |
| **EYFP** | enhanced yellow fluorescent protein |
| **CEPIA** | calcium-measuring organelle-entrapped protein indicators |
| **SERCA** | sarco/endoplasmic reticulum $Ca^{2+}$-ATPase. |

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## Highlights

1. cPLA$_2$α inhibitor pyrrophenone blocks calcium increase and C2 domain translocation.
2. Pyrrophenone inhibits ATP stimulated transfer of calcium from ER to mitochondria.
3. Effects of pyrrophenone are off-target occurring in cPLA$_2$α-deficient cells.
4. Pyrrophenone targets a novel serine hydrolase that regulates calcium mobilization.

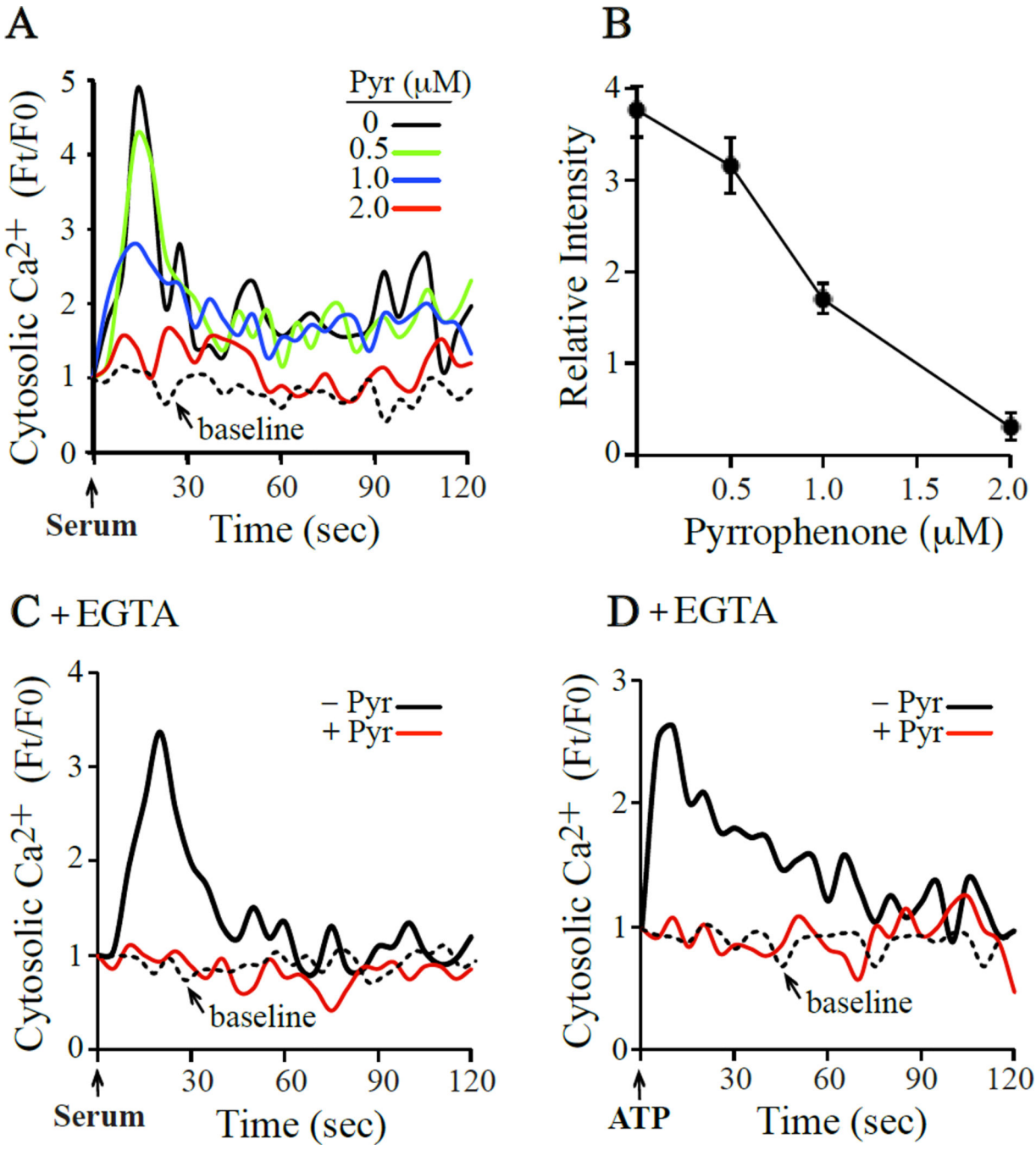


**Fig. 1. Pyrrophenone blocks calcium mobilization induced by serum, ATP and A23187**

IMLFα$^{-/-}$ loaded with FuraRed-AM were pre-incubated with (A,B) the indicated concentrations of pyrrophenone followed by stimulation with serum, or with 2 μM pyrrophenone (red line) or DMSO (black line) followed by stimulation with (C) serum or (D) ATP in media containing EGTA. Line traces show the fold changes in cytosolic calcium over time. The hatched lines show baseline calcium over-time in untreated, un-stimulated cells. The data represents the average of three independent experiments from analysis of at

least 5 cells/experiment. From the data in (A), the effect of pyrrophenone dose on cytosolic calcium levels 15 sec after adding serum is shown (B).

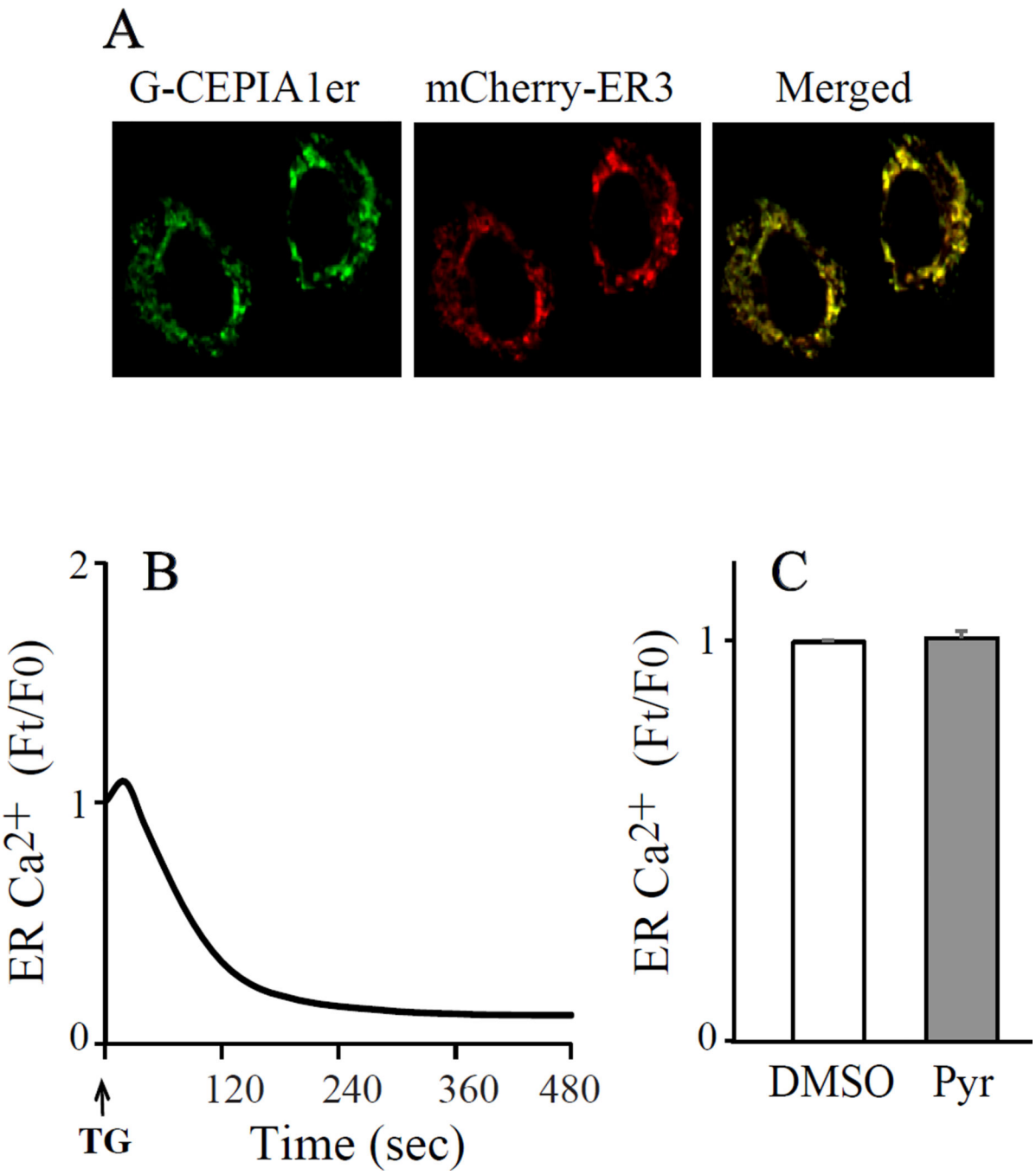


**Fig. 2. Use of G-CEPIA1*er* for imaging ER calcium in IMLF**

**(A)** Live-cell images of G-CEPIA1*er* and the ER marker mCherry-ER3 co-expressed in IMLFα$^{-/-}$ are shown along with the merged image. (B) Live-cell imaging of G-CEPIA1*er* fluorescence in IMLFα$^{-/-}$ stimulated with thapsigargin is shown. (C) Live-cell imaging of G-CEPIA1*er* fluorescence in IMLFα$^{-/-}$ treated with 2 μM pyrrophenone or vehicle (DMSO) for 30 min. The data (B,C) show the relative levels of ER calcium in three independent experiments from analysis of at least 5 cells/experiment.

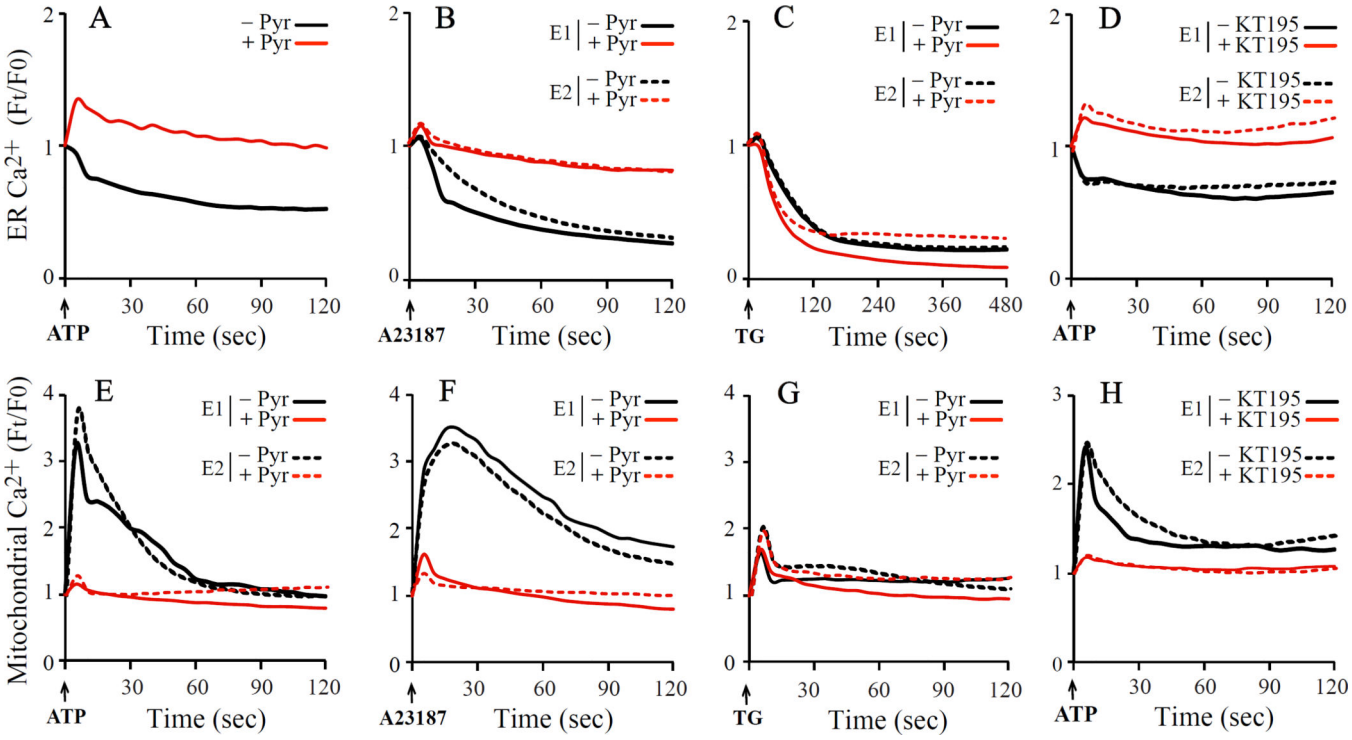


**Fig 3. Pyrrophenone inhibits calcium release from ER and calcium increase in mitochondria triggered by ATP and A23187 but not by thapsigargin**

IMLFα$^{-/-}$ expressing G-CEPIA1*er* or CEPIA2*mt* were pre-treated with 2 μM pyrrophenone (Pyr), 2 μM KT195 or vehicle (DMSO) for 30 min and then stimulated with ATP, A23187 or thapsigargin (TG). The relative changes in ER (upper panels) or mitochondrial (lower panels) calcium were determined by live-cell fluorescent imaging. The data are the average of three independent experiments (A), or from two independent experiments (E1, E2) from analysis of at least 5 cells/experiment.

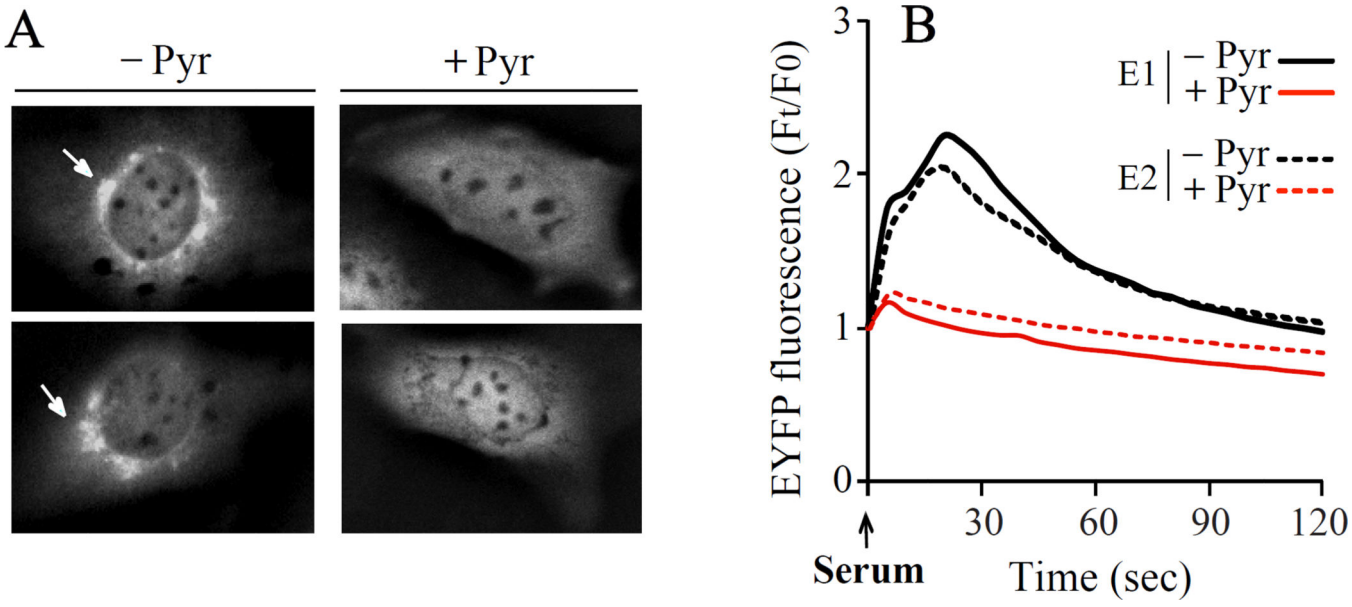


**Fig. 4. Pyrrophenone inhibits translocation of the cPLA$_2\alpha$ C2 domain in serum-stimulated IMLF$\alpha$**

IMLF$\alpha^{-/-}$ expressing the cPLA$_2\alpha$ C2 domain were pre-treated with 2 μM pyrrophenone or vehicle (DMSO) for 30 min and then stimulated with serum. (A) Representative images show (left panel) translocation of EYFP-C2 domain to Golgi (arrow) 30 sec after stimulation with serum. (B) Graphical representation showing EYFP-C2 domain translocation to Golgi over time after stimulation with serum in two independent experiments (E1, E2) from analysis of at least 5 cells/experiment.