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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.

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

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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trifluoromethyl ketone and methyl arachidonyl fluorophosphonate [12–14]. They are also more selective and do not inhibit Group VI PLA_2s 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

IMLFα (5×10^3 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 h 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 h the medium was replaced with serum-free DMEM containing 0.1% bovine serum albumin and the cells incubated for an additional 24 h.

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. IMLFα plated on the glass insert of MatTek dishes were cultured in growth media as described above for 18 h. The medium was replaced with serum- and antibiotic-free DMEM containing 0.1% bovine serum albumin and cells incubated with recombinant adenovirus for 26 h as previously described [6].

2.3. Live-cell imaging

For imaging cytosolic Ca^{2+}, IMLFα were loaded with Fura Red-AM for 15 min as previously described [16]. Imaging of ER and mitochondrial calcium was carried out in IMLFα 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/525 nm excitation/emission) of G-CEPIA1*er* was recorded every 5 s for ATP and A23187, and every 15 s after thapsigargin stimulation. For imaging mitochondrial calcium, fluorescence (488nm/525 nm excitation/emission) of CEPIA2*mt* was recorded every 5 s after ATP, A23187, and thapsigargin stimulation.

2.4. EYFP-C2 domain translocation

$IMLF\alpha^{-/-}$ 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 s 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 mitochondrial 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, $IMLF\alpha^{-/-}$ were used to avoid any potential effects of $cPLA_2\alpha$ 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 s 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 s 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 D).