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INHIBITION OF PHOSPHOLIPASE A_2 ABROGATES INTRACELLULAR PROCESSING OF NADPH-OXIDASE DERIVED REACTIVE OXYGEN SPECIES IN HUMAN NEUTROPHILS

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Running title: Neutrophil PLA_2 activity in intracellular ROS processing

Abbreviations: AA, arachidonic acid; BEL, bromoenol lactone; CL, chemiluminescence; $cPLA_2$, cytosolic phospholipase A_2; DEDA, 7,7-dimethyleicosadienoic acid; fMLF, formyl-methionyl-leucyl-phenylalanine; HRP, horseradish peroxidase; KRG, krebs-ringer phosphate buffer; MAFP, methyl arachidonyl fluorophosphonate; MPO, myeloperoxidase; OPD, o-phenylenediamine dihydrochloride; $PACOCF_3$, palmityl trifluoromethylketone; PHPA, p-hydroxyphenyl acetic acid; PKC, protein kinase C; PLA_2, phospholipase A_2; PMA, phorbol myristate acetate; PYR, pyrrolidine; ROS, reactive oxygen species; SLD, 12-episcalaradial; SOD, superoxide dismutase; WGA, wheat germ agglutinin

ABSTRACT

Upon activation of human neutrophils, superoxide can be produced at two cellular sites; either in the plasma membrane, giving extracellular release of oxidants, or in intracellular organelles, resulting in oxidants being retained in the cell. The involvement of phospholipase A_2 (PLA_2) in phorbol myristate acetate (PMA)-induced activation of the two pools of NADPH-oxidase was investigated using a variety of PLA_2 inhibitors and the oxidase activity was measured by luminol/isoluminol-amplified chemiluminescence (CL). Two of the seven inhibitors were without effect, two inhibitors inhibited both intra- and extracellular ROS production equally, and three inhibitors inhibited intracellular but not extracellular CL. Using another technique to measure ROS, PHPA oxidation, we found that intracellular ROS production was unaltered with the three last inhibitors, indicating that PLA_2 is not involved in the NADPH-oxidase activity *per se*, but in the intracellular processing of the radicals necessary for the CL reaction to take place. The PLA_2 inhibitors did not abolish the activity of myeloperoxidase (MPO), an enzyme necessary for intracellular CL to occur. Instead, we suggest that these PLA_2 inhibitors block heterotypic granule fusion and prohibit the colocalization of ROS and MPO needed for intracellular CL activity.

Keywords: phagocytes, granules, phospholipases, respiratory burst, membrane fusion

INTRODUCTION

The ability of neutrophils to produce toxic oxygen radicals constitutes a major weapon against microbial intruders. The enzyme system responsible for this respiratory burst, the NADPH-oxidase, functions as a transporter of electrons from NADPH on the cytosolic side of the membrane to oxygen on the other side. The electrons reduce molecular oxygen to superoxide anion [1] which then dismutates to hydrogen peroxide and can be further processed by enzymes such as myeloperoxidase (MPO) to generate yet other reactive oxygen species (ROS) [2]. The NADPH-oxidase is dormant in resting neutrophils and becomes activated when a pro-inflammatory mediator activates the cells and induces a signal transduction cascade resulting in phosphorylation and subsequent translocation of several cytosolic NADPH-oxidase components to the membrane-bound part, the b-cytochrome [3].

A paradigm for activation of the NADPH-oxidase has been that it is assembled in the neutrophil plasma membrane, releasing superoxide anion to the extracellular compartment or into a preformed phagosome. However, in human neutrophils activation of the NADPH-oxidase can occur also without phagosome formation or extracellular release of the produced oxygen metabolites [4-6]. Since 80-85% of the b-cytochrome is localized in the membranes of the peroxidase-negative granules [7], it is reasonable to assume that the NADPH-oxidase can be assembled and activated also in the membrane of these organelles and increasing evidence support this assumption (reviewed in [8]).

Different agonists activate the two pools of NADPH-oxidase (in the plasma membrane or in the granules) differently, suggesting that the signaling as well as the molecular mechanisms for regulation differ depending on the localization of the oxidase [5, 9-11]. Activation of G-protein-

coupled seven-transmembrane spanning cell surface receptors, such as the formyl peptide receptors, generates signals that lead to assembly of the NADPH-oxidase in the plasma membrane, releasing oxidants to the extracellular environment. In contrast, oxygen metabolites formed during activation through CR3 (the receptor for the complement product C3bi) are formed intracellularly [12]. Occupation of receptors for galectin-3 (a human lactose-binding lectin) or wheat germ agglutinin (WGA; a plant lectin specific for sialic acid and/or N-acetyglucosamine) generates signals that activate the oxidase at both sites [11, 13]. Even though detailed knowledge about the exact signals responsible for activation of the oxidase at the two different sites is lacking, we have previously shown that activation of the two pools differ regarding involvement of an intracellular calcium rise, PI 3-kinase, and protein kinase C (PKC) isozymes [14-16].

That not only extracellularly released but also intracellularly produced ROS are of pathophysiological importance in vivo is becoming increasingly evident. Recent findings related to a novel type of chronic granulomatous disease indicate that one of the cytosolic components of the NADPH-oxidase, $p40^{phox}$, is specifically translocated to intracellular phagosomal and granule membranes and could thus be a determining factor for intracellular ROS production [6]. Neutrophils from patients with the inflammatory syndrome SAPHO (synovitis, acne, pustulosis, hyperostosis, osteitis) have also been reported to display severely decreased intracellular ROS production while extracellular production was intact [17].

Arachidonic acid (AA) is a lipid messenger derived through cleavage of membrane phospholipids by phospholipase A_2 (PLA_2). Previous reports have implicated that PLA_2 and AA are important for the translocation, assembly, and/or activation of the NADPH-oxidase in monocytes and

neutrophils [18-21]. Although convincing evidence show that the cytosolic PLA_2 ($cPLA_2$) isozyme is required for superoxide production in monocytes and neutrophils (reviewed in [22]) there are contradicting reports in the literature. E.g., neutrophils from $cPLA_2$ knockout mice have normal ROS producing capacity and the $cPLA_2$ specific inhibitor pyrrolidine is reported not to inhibit ROS production in human neutrophils [23]. In order to contribute to the understanding of PLA_2 participation in neutrophil ROS production, we have investigated the effect of several inhibitors of PLA_2 isozymes on activation of the NADPH-oxidase at different subcellular sites in human neutrophils. We found that one group of PLA_2 inhibitors was altogether without effect, one group blocked NADPH-oxidase activation both at the plasma membrane and intracellularly, while one group appeared to influence the intracellular processing of reactive oxygen species. We suggest that this intracellular processing takes place through intracellular granule fusion.

MATERIALS AND METHODS

Isolation of neutrophils

Human neutrophils were isolated from buffy coats or peripheral whole blood obtained from healthy blood donors. After dextran sedimentation at 1xg and centrifugation in a Ficoll-Paque gradient [24], the remaining erythrocytes were lysed by hypotonic treatment. The neutrophils were washed in Krebs-Ringer phosphate buffer containing glucose (10 mM) and Mg^{2+} (1.5 mM) (KRG, pH 7.3) and finally resuspended ($1x10^7$/ml) in KRG with Ca^{2+} (1 mM). The cells were kept on melting ice until use. For some experiments, neutrophils were primed with TNF-α at 37°C for 20 min prior to use.

Treatment with PLA_2 inhibitors

Seven inhibitors of PLA_2 were employed; 7,7-dimethyleicosadienoic acid (DEDA), palmityl trifluoromethylketone ($PACOCF_3$), pyrrolidine, bromoenol lactone (BEL), 12-episcalaradial (SLD), LY311727, and methyl arachidonyl fluorophosphonate (MAFP)(Table 1). All inhibitors were solubilized to stock solutions in DMSO except MAFP which was solubilized in methyl acetate. Inhibitors were incubated with cells for 15 min at 37°C prior to stimulation. The final concentration of DMSO or methyl acetate in each assay was less than 0.5% for all the inhibitors.

Superoxide production

Superoxide anion produced by the neutrophil NADPH-oxidase was determined using a luminol/isoluminol-enhanced chemiluminescence (CL) system [25]. The CL activity was measured in a six-channel Biolumat LB 9505 (Berthold Co. Wildbad, Germany), using disposable 4-ml polypropylene tubes with a reaction mixture containing 10^6/ml neutrophils. The tubes were equilibrated in the Biolumat at 37°C for 15 min in the presence or absence of inhibitors,

after which the stimulus was added and the light emission recorded continuously. In order to specifically quantify intracellularly and extracellularly generated reactive oxygen species (ROS), two different reaction mixtures were used. Tubes used for measurement of extracellular release of ROS contained neutrophils, horseradish peroxidase (HRP; a cell impermeable peroxidase; 4U) and isoluminol (a cell impermeable CL substrate; 10 μg/ml [26]. Tubes used for measurement of intracellular generation of ROS contained neutrophils, superoxide dismutase (SOD; a cell impermeable scavenger for superoxide; 50 U/ml), catalase (a cell impermeable scavenger for hydrogen peroxide; 2000 U/ml), and luminol (a cell permeable CL substrate; 10 μg/ml). Four different stimuli were used to induce ROS production in neutrophils; PMA ($5x10^{-8}$ M), ionomycin ($5x10^{-7}$ M), galectin-3 (40 μg/ml for the extracellular response, 20 μg/ml for the intracellular response), and formyl-methionyl-leucyl-phenylalanine (fMLF; 10^{-7} M).

Hydrogen peroxide production

Generation of hydrogen peroxide was assayed by measuring the HRP-dependent oxidation of p-hydroxyphenyl acetic acid (PHPA) by fluorometry in a Mithras LB 940 96-well plate reader [27]. Samples (200 μl), containing neutrophils (10^6/ml), PHPA (3.3 mM), and HRP (4U) in KRG, were preincubated in the presence or absence of inhibitors for 15 min at 37°C. PMA (50 nM, 20 μl) was added as stimulus and the increase in fluorescence intensity (excitation wavelength 340 nm, emission wavelength 405 nm) was measured continuously. The PHPA is cell-impermeable, and to measure also intracellular production of hydrogen peroxide, azide (NaN_3; 1 mM) was added to some samples. Azide inhibits the endogenous hydrogen peroxide-consuming enzymes catalase and MPO, allowing for intracellularly produced hydrogen peroxide to leak out of the cell and be determined extracellularly [28, 29]. The intracellular hydrogen peroxide production

was subsequently determined as the difference in fluorescence between samples with azide and without azide.

MPO activity assay

To determine the effect of the PLA_2 inhibitors on MPO activity, MPO (12.5 µg/ml; 35 µl/well) was incubated with the peroxidase substrate o-phenylenediamine dihydrochloride (0.4 mg/ml) and hydrogen peroxide (4.9 mM) in the presence or absence of PLA_2 inhibitors (for concentrations see figure legends) or sodium azide (1 mM). All samples were done in triplicates and incubated in the dark for 30 min at RT. The reaction was stopped by adding H_2SO_4 (1 mM; 100 µl/well) and the absorbance was measured at 492 nm.

Degranulation marker analysis by flow cytometry

Isolated neutrophils ($1x10^6$/ml) were incubated with or without DEDA (100 µM), $PACOCF_3$ (100 µM), or pyrrolidine (10 µM) for 15 min at 37°C after which degranulation was stimulated with PMA (5 nM) or TNF-α (10 ng/ml) for 20 min at 37°C. The samples were then put on ice, labeled with anti-CD11b-PE mAb(1/40), one of the component of the complement receptor 3 (CR3), for 30 min at 4-8°C, and then washed. A minimum of 10 000 neutrophils were collected using an Accuri C6 flow cytometer and the results were analyzed using FlowJo software.

Capacitance measurements

For patch-clamp experiments [30], neutrophils isolated from peripheral blood as described above, were allowed to adhere to BSA-coated (0.3%, 10 min) glass cover slips. The cells were washed and kept in a standard extracellular solution (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES, pH 7.2). The pipette solution (112 mM K-Glu, 18 mM NaCl, 1.8 mM $MgCl_2$, 2.5 mM Na_2ATP, 9 mM HEPES, pH 7.2) was supplemented with

suitable concentrations of calcium and calcium chelator to reach the desired concentration of free calcium, as determined by WinMaxChelator 1.78, using the BERS constants [31]. When applied, the PLA_2 inhibitor DEDA (10 μM) was added to the pipette solution. All experiments were performed at room temperature on an inverted microscope (Axiovert 135 TV, Zeiss, Oberkochen, Germany). Patch pipettes were pulled in a PC 10 puller (Narishige, Japan) from borosilicate glass capillaries. The pipettes were coated with Sylgard (Dow Corning Ltd., England) and fire polished (Micro Forge 830, Narishige, Japan), and the pipette resistance was between 4-7 MΩ. The pipettes were slowly moved very close to a neutrophil by a piezodriven micromanipulator (N&F, Bio-Science, Denmark) and a gigaseal formed by suction. The whole-cell configuration was achieved by rupturing of the membrane under the pipette by gentle suction by mouth, and capacitance changes were recorded using an EPC-9 patch-clamp amplifier (HEKA, Lambrecht/Pfalz, Germany). From a holding potential of –10 mV, the cells were exposed to a sine shaped, alternating voltage pulse with a frequency of 1.0 kHz and amplitude of 20 mV. From the resulting current, the capacitance was determined by the Lock-In software based phase-sensitive detector and sampled by the X-Chart extension of Pulse 8.41 (HEKA). The X-chart data were imported into IGOR 3.03 for Macintosh (Wave Metrics Inc., Lake Oswego, OR) for analysis. Rates of exocytosis were determined by fitting the capacitance trace to a straight line, using IGOR curve fitting.

Confocal microscopy

Neutrophils were resuspended in RPMI with 2% FCS and added to coverslips. The cells were treated with PMA (50 nM) for 10 min before fixing the cells with 4% paraformaldehyde for 20 min at room temperature. The cells were washed with PBS and then permeabilized for 5 min in

-20°C acetone and methanol (1:1) and washed again with PBS. Fixed cells were blocked with 10% goat serum in PBS with 2% BSA for 30 min at room temperature and then incubated with primary antibodies for 30 min in a humified dark chamber at room temperature. The primary antibodies used were rabbit anti-human lactoferrin antibody (diluted 1:100) and mouse anti-human CD63 antibody (diluted 1:100). The cells were washed with PBS before incubation with secondary antibodies for 30 min in a humified dark chamber at room temperature. The secondary antibodies used were AlexaFluor goat anti-mouse 647 and AlexaFluor goat anti-rabbit 488. The cells were mounted in mounting medium with DAPI. A confocal microscope (Zeiss LSM700) and Zen software was used to visualize the cells.

Reagents

PHPA, PMA, HEPES, glucose, azide, potassium glutamate, ATP, hydrogen peroxide, formyl-methionyl-leucyl-phenylalanine (fMLF), human TNF-α, isoluminol, luminol, and goat serum were obtained from Sigma-Aldrich (St Louis, MO, USA). Ionomycin, pyrrolidine, and $PACOCF_3$ were purchased from Calbiochem (LaJolla, CA, USA). LY311727 and MAFP were purchased from Tocris Bioscience (Ellisville, MO, USA). Catalase and SOD were from Worthington (Lakewood, NJ, USA). 12-episcalaradial was purchased from Wako Chemicals GmgH (Neuss, Germany). BEL was bought from Enzo Life Science (Lausen, Switzerland). DEDA was bought from Alexis Biochemicals (Farmingdale, NY, USA). HRP was from Roche Diagnostics GmbH (Mannheim, Germany). Bovine serum albumin was purchased from Boehringer Mannheim (Germany). Dextran was from Pharmacosmos (Holbaek, Denmark). Ficoll-Paque was from Fischer Scientific GTF AB (Gothenburg, Sweden). OPD and lactoferrin antibody were purchased from Dako (Glostrup, Denmark). Purified human MPO was a gift from Inge Olsson (Lund, Sweden). Anti-CD11b-PE

mAb (catalogue number, 333142) was from BD (NJ, USA). Alexa Fluor anti-mouse 647 and anti-rabbit 488 secondary antibodies were from Invitrogen (Carlsbad, CA, USA). The CD63 antibody was from Sanquin (Amsterdam, Netherlands).

Statistics

The software GraphPad Prism version 5.0 was used for statistical calculations. One-way ANOVA followed by Dunnett's post-hoc test was used to calculate the difference between control and PLA_2 inhibitor treated samples. A p-value less than 0.05 was considered statistically significant and is indicated in the figures by *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

RESULTS

The neutrophil NADPH-oxidase can be activated at two sites

In human neutrophils, assembly of the NADPH-oxidase can occur not only in the plasma membrane (resulting in a release of ROS) but also in intracellular granule membranes, giving rise to production of retained intracellular oxygen metabolites [8]. An example of a stimulant that induces activation of both of these pools of NADPH-oxidase is PMA that mimics the effects of the natural PKC activator diacylglycerol, and activates the NADPH-oxidase through redistribution of PKC and phosphorylation of several proteins [6, 14] (Fig 1A). Another stimulus, the calcium ionophore ionomycin, induces a rise in cytosolic calcium which gives rise to mainly intracellular ROS production (Fig 1B). The lactose-binding lectin galectin-3 induces ROS production through binding of receptors upregulated on neutrophils after *in vitro* priming of the cells [11], giving rise to both extracellular and intracellular ROS production (Fig 1C). Finally, the bacterium-derived formylated peptide fMLF is a potent inducer of extracellular ROS production with minute intracellular activity (Fig 1D). We decided to start our investigation on the involvement of PLA_2 in neutrophil ROS production using luminol/isoluminol-amplified CL with PMA as a stimulus.

The effect of PLA_2 inhibitors on extracellular and intracellular CL activity

Many authors have reported that PMA induces release of ROS from neutrophils [32, 33] and that this activity is dependent on the production of AA by PLA_2 [18, 34]. There are however, to our knowledge, no reports on the importance of PLA_2 for the intracellular ROS production. To investigate the involvement of PLA_2-derived AA in activation of the two pools of NADPH-oxidase, seven inhibitors of PLA_2, listed in Table 1, were used. Neutrophils were pre-incubated

with or without inhibitor (at a range of concentrations), stimulated with PMA, and ROS production was determined by CL.

DEDA did not affect extracellular ROS release, regardless of the concentration used (1-100 μM; Fig 2A and B). In contrast the intracellular ROS production was dose-dependently inhibited by DEDA (Ic50 ≈10 μM; Fig 2A and B). Two other PLA_2 inhibitors, $PACOCF_3$ and pyrrolidine, showed a similar pattern, inhibiting intracellular CL activity dose-dependently while not affecting the extracellular activity (Fig 2C and D). In contrast, the inhibitors BEL and SLD inhibited both intra- and extracellular CL activity (Fig 2E and F), which is in accordance with previous reports that $cPLA_2$ activity is needed for NADPH-oxidase function (reviewed in [22]). The last two inhibitors, LY311727 and MAFP, affected neither intra- nor extracellular CL activity (Fig 2G and H) at the concentrations used (1-100 μM). The fact that DEDA, $PACOCF_3$, and pyrrolidine had differential effects on intra- and extracellular CL activity inspired us to investigate the effects of these three inhibitors further.

First we studied the effect of DEDA, $PACOCF_3$, and pyrrolidine on intracellular CL responses induced by other stimuli, i.e. ionomycin (that induces primarily intracellular ROS production; Fig 1B) and galectin-3 (that induces both intra- and extracellular ROS production; Fig 1C). DEDA, $PACOCF_3$, and pyrrolidine all inhibited the ionomycin-induced intracellular CL activity (Fig 3A and D). The intracellular galectin-3-induced CL response was also significantly inhibited by all three inhibitors, although the inhibition by $PACOCF_3$ was less prominent than for the PMA- and ionomycin-induced intracellular CL responses (Fig 3B and E). The galectin-3-induced extracellular CL activity was significantly decreased by pyrrolidine pre-treatment while the

response was increased, or unaffected, by pre-treatment with the other inhibitors (Fig 3C and F).

Taken together, the seven PLA_2 inhibitors can be divided into three main groups depending on their action on CL responses; a first group (DEDA, $PACOCF_3$, and pyrrolidine) that inhibited intracellular CL activity while the extracellular activity was mostly unaffected, a second group (BEL and SLD) that inhibited both intracellular and extracellular CL activity, and a third group (LY311727 and MAFP) that had no effect on neither the intracellular nor the extracellular CL activity.

The PLA_2 inhibitors DEDA, $PACOCF_3$, and pyrrolidine do not inhibit the NADPH-oxidase activity per se

We next sought to explain why the inhibition of PLA_2 by DEDA, $PACOCF_3$, and pyrrolidine, resulted in different effects on the intracellular and extracellular CL responses. The CL technique measures the production of superoxide in the presence of a functional peroxidase; for extracellular detection, HRP is added to the system but for intracellular detection a functional intracellular MPO is necessary [35, 36]. To determine the MPO-independent effects of the PLA_2 inhibitors on NADPH-oxidase activation we used the technique of hydrogen peroxide-induced reduction of PHPA.

PHPA is a cell impermeable reagent that only reacts with and measures extracellular hydrogen peroxide. Addition of azide to the system inhibits the endogenous hydrogen peroxide consuming enzymes, allowing intracellularly produced hydrogen peroxide to leak out of the cell, enabling the measurement of total NADPH-oxidase-derived ROS production extracellularly.

Intracellular hydrogen peroxide production can thus be determined by calculating the difference between total (+azide) and extracellular (-azide) production (Fig 4A) [37].

In contrast to the CL results, the intracellular hydrogen peroxide production was unaltered in the presence of DEDA (Fig 4B and D), $PACOCF_3$, as well as pyrrolidine (Fig 4D). Thus, the intracellular NADPH-oxidase activity *per se* was not influenced by these PLA_2 inhibitors, giving that the decreased CL must be explained by negative effects on some other part of the intracellular CL reaction. Contrarily, the two inhibitors BEL and SLD, which inhibited both intracellular and extracellular CL activity (Fig 2E and F), clearly inhibited the NADPH-oxidase derived hydrogen peroxide production detected by PHPA (Fig 4C and D).

Effect of PLA_2 inhibitors on MPO

The intracellular CL response in neutrophils is dependent on a functional MPO, shown by that neutrophils from MPO-deficient individuals exhibit no intracellular CL responses [35, 36]. The PLA_2 inhibitors (DEDA, $PACOCF_3$, and pyrrolidine) had no effect on MPO activity *per se* when measured in an assay with purified MPO and OPD as a substrate (Fig 5A). The effects of the PLA_2 inhibitors were also tested in a modified extracellular CL system where the peroxidase HRP was exchanged for MPO, with the rationale that if the inhibitors affect MPO activity, extracellular CL activity would decrease in this set-up. However, neither DEDA nor $PACOCF_3$ inhibited MPO-dependent extracellular CL activity. A slight inhibitory effect was seen for pyrrolidine (Fig 5B). In conclusion, the inhibitory effect of DEDA and $PACOCF_3$ on the intracellular neutrophil CL response was not due to interference with MPO activity while part of the pyrrolidine effect on intracellular CL activity might be due to interference with this endogenous neutrophil peroxidase.

Effects of PLA_2 inhibition on granule – plasma membrane fusion

The MPO is stored in the peroxidase-positive azurophil granules in neutrophils, a granule type devoid of the b-cytochrome component of the NADPH-oxidase [38]. We have previously suggested that MPO reaches the ROS substrates (i.e., superoxide anion and/or hydrogen peroxide) produced in peroxidase-negative granules through an intracellular granule-granule fusion process involving different granule subsets [8, 16]. The effect of DEDA, $PACOCF_3$, and pyrrolidine, i.e., inhibiting intracellular CL without affecting MPO activity or NADPH-oxidase activity, could possibly be explained by inhibition of such fusion events. Unfortunately, no technique exists to date with resolution high enough to enable direct quantification of such intracellular fusion events in neutrophils.

Instead, we studied the effect of PLA_2 inhibition on granule fusion with the plasma membrane, which is possible to measure. We first investigated whether granule fusion induced by intracellular calcium elevations was affected by DEDA, by measuring membrane capacitance increase by patch-clamp technique. With this technique, the capacitance recorded correlates to the area of the plasma membrane [39] and during exocytosis of intracellular granules this area increases. The plasma membrane increase in capacitance, due to granule fusion induced by calcium, was not affected by DEDA (Fig 6A), indicating that DEDA-sensitive PLA_2 does not play a crucial part in granule fusion with the plasma membrane.

Granule mobilization induced by PMA or TNF-α in the presence and absence of DEDA, $PACOCF_3$, and pyrrolidine was also investigated by measuring the exposure of complement receptor 3 (CR3) on the cell surface. A large proportion of CR3 is stored in granule membranes of resting

cells and the receptor is upregulated, through degranulation, upon cell activation. Activation of cells with PMA or TNF-α for 20 min resulted in approximately four-fold increase in CR3 as compared to unstimulated cells. No difference in CR3 increase could be detected between the inhibitor-treated and untreated cell populations stimulated with PMA or TNF-α (Fig 6B, C). These data indicate that the PLA_2 inhibitors DEDA, $PACOCF_3$, and pyrrolidine do not interfere with granule fusion with the plasma membrane. It should be stressed that neither of the techniques used here are able to measure intracellular fusion events, and thus the inhibition of agonist-induced intracellular CL could still be explained by abrogation of such processes, although no techniques are available to prove it.

DISCUSSION

This work has been focused on studying the effects of different PLA_2 inhibitors on the regulation of the neutrophil NADPH-oxidase. PMA induces release, as well as intracellular production, of ROS in neutrophils and is thus a good tool to investigate the signal transduction pathways leading to activation of the NADPH-oxidase in the plasma membrane and intracellular (granule) membranes, respectively. The metabolic response induced in neutrophils upon interaction with PMA was followed by luminol/isoluminol-amplified CL, a technique that is peroxidase-dependent and utilizes the fact that isoluminol and luminol differ with respect to ability to pass neutrophil membranes (for a review see [25]). Using this technique, three PLA_2 inhibitors, DEDA, $PACOCF_3$, and pyrrolidine, exhibited inhibitory effects on the intracellular CL response only, indicating a difference in regulation between the two pools of oxidase with regards to the involvement of PLA_2. Also, intracellular ROS production induced by the calcium ionophore ionomycin as well as galectin-3, could be inhibited by DEDA, $PACOCF_3$, and pyrrolidine, arguing that the inhibitory effect is not restricted to a certain stimulus but is a more general phenomenon. The fact that galectin-3 is a receptor-dependent stimulator of the NADPH-oxidase might explain why the inhibition on intracellular CL was not as strong as with PMA or ionomycin. The same argument could also explain why the extracellular CL response to galectin-3 was slightly inhibited by pyrrolidine. More complex signaling mechanisms are likely triggered by receptor ligation than by direct PKC activation by PMA, and these may be only partially blocked by PLA_2 inhibition.

Our data show diversity among the PLA_2 inhibitors in their effects on the CL responses. As stated above, three inhibitors, DEDA, $PACOCF_3$, and pyrrolidine, exhibited inhibitory effects on the

intracellular CL response only, while two PLA_2 inhibitors, BEL and SLD, inhibited both intra- and extracellular CL responses and the last two inhibitors, LY311727 and MAFP, inhibited neither. The grouping of the inhibitors with regard to effect on ROS-production does not correlate with the presumed isozyme specificity of the different inhibitors (Table 1). However, several of the inhibitors have effects on more than one isozyme, especially at high concentrations [40-43] making it hard to pinpoint the specific isozyme accountable for the responses we see. Further, the isozyme specificities given in the Table 1 have been decided by studies of cell types different from neutrophils, or even in cell free systems, and the relevance of these results for neutrophils may be limited. In fact, the isozyme profile of each cell type with regard to both composition and abundance may influence the response to different inhibitors. For one inhibitor, LY311727, membrane impermeability may explain the lack of effects in neutrophils [40].

The relative non-specificity of pharmacological inhibitors is a well-known and accepted drawback to these kinds of studies, but the importance of individual PLA_2 isozymes in intracellular ROS production in neutrophils is difficult to examine by other techniques. The option of using mouse neutrophils in which specific PLA_2 isozymes have been genetically removed is not possible, since murine neutrophils apparently lack the ability to form intracellular ROS [44], and specific knockdown of enzymes in primary cells cannot be performed since the cells do not divide and therefore cannot be cultured.

To account for the effect of the inhibitors on intracellular CL, several aspects of the CL technique must be considered. This reaction is dependent not only on the NADPH-oxidase activity, but also on a functional MPO [35, 36]; MPO-deficient cells do not display intracellular CL while their production of intracellular oxidants is normal or even enhanced [45]. Our results show that the

inhibitory effect of DEDA, $PACOCF_3$, and pyrrolidine on the intracellular CL activity was not due to inhibition of the intracellular production of ROS, since PHPA oxidation by intracellular hydrogen peroxide was unaffected. Secondly, the enzymatic activity of MPO was largely unabridged by DEDA, $PACOCF_3$, and pyrrolidine. Hence, the inhibitors affected neither of the molecules (ROS and MPO) necessary for the CL activity.

As previously mentioned, MPO resides in the matrix of the azurophil granules [46] whereas the intracellular superoxide-producing NADPH-oxidase is located in the peroxidase-negative granules [7]. A prerequisite for the CL reaction to take place is the colocalization of MPO and newly produced superoxide anion in the same intracellular compartment [8]. Thus, the most plausible explanation for the effect on intracellular CL seen with DEDA, $PACOCF_3$, and pyrrolidine is that they inhibit intracellular granule fusion which prevents the radicals from interacting with MPO. A schematic drawing of this hypothesis is shown in Fig 7A. We found no effect of the inhibitors on neutrophil degranulation or exocytosis in general, and closer investigation of intracellular heterotypic granule fusion is unattainable, as there are at this time and to our knowledge no quantitative assays to study intracellular granule fusion *per se.* However, we have made some attempts to use immunostaining of distinct granule markers and confocal microscopy to visualize heterotypic granule fusion. Ten minutes after PMA stimulation of neutrophils, specific granules (lactoferrin, green) and azurophil granules (CD63, red) appear to fuse in parts of the cells (yellow) (Fig 7B). This is in agreement with a paper by Kobayashi et al, who have shown with electron microscopy that the oxygen radical forming granules fuse with other types of granules after PMA stimulation in human neutrophils [47].

The process of membrane fusion, the energy-consuming joining of two separate cellular compartments, has been extensively studied in several cell types, including neutrophils. Primarily the granule to plasma membrane fusion has been studied, resulting in identification of an abundance of regulatory molecules for this process [48-50]. The involvement of AA in fusion events is well established, e.g., PLA_2 may regulate as diverse fusion processes as sperm-egg fusion [51], synaptic vesicle fusion with presynaptic membranes [52] and endosomal fusion [53, 54]. In fact, PLA_2-derived AA has been shown to promote the fusion of neutrophil granules, both specific and azurophil granules, with synthetic liposomes [55], which is very interesting in light of the results presented here.

Although there are no functional assays to study intracellular granule fusion *per se*, compound exocytosis, i.e., intracellular granule-to-granule fusion followed by extracellular release of the newly formed vacuole, has been demonstrated by various means in several different secretory cells, including eosinophils [56-58], mast cells [59] and pancreatic β-cells [60, 61]. Compound exocytosis has been demonstrated also in PMA-stimulated neutrophils [49], indicating occurrence of granule-to-granule fusion following activation with this stimulus, supporting the data presented here. The fact that compound exocytosis has been suggested to be regulated differently from granule-to-plasma membrane fusion [58, 62] may explain the absence of effect of inhibitors in our membrane fusion experiments, since our assays only measure the granule-to-plasma membrane fusion and not granule-to-granule fusion that take place within the cells. The biological reasons for intracellular granule fusion to occur could be several; localized secretion at the plasma membrane of several consecutive granules, compound fusion of granules with the phagosome or an intracellular processing of pro-enzymes prior to release. For

example, hCAP18 (residing in neutrophil specific granules) has been shown to be processed by proteinase 3 (residing in azurophil granules) extracellularly [63]. Intracellular granule fusion has been suggested as a possible way to further enhance this process, preceding the extracellular interaction [49]. Furthermore, pro-gelatinase has been shown to be processed and activated by ROS [64], and fusion of azurophil and gelatinase granules would accommodate such a process. Fusion of several intracellular granules has also been shown to occur through stimulation of the CD44 surface protein, which is then followed by programmed necrosis of neutrophils [65]. Taken together, this study shows that concomitant to NADPH-oxidase activation, the PKC agonist PMA, as well as ionomycin and galectin-3, activates additional effects that influence the oxygen radical metabolism in neutrophils. One such effect appears to be granule-to-granule fusion, and we suggest that this event is dependent on PLA_2-derived AA.

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REFERENCES

[1] B.M. Babior, NADPH oxidase: An update, BLOOD 93 (1999) 1464-1476.

[2] S.J. Klebanoff, Myeloperoxidase, Proc Assoc Am Physicians 111 (1999) 383-389.

[3] F.R. DeLeo, M.T. Quinn, Assembly of the phagocyte NADPH oxidase: molecular interaction of oxidase proteins, J Leukoc Biol 60 (1996) 677-691.

[4] C. Dahlgren, A. Johansson, H. Lundqvist, O.W. Bjerrum, N. Borregaard, Activation of the oxygen-radical-generating system in granules of intact human neutrophils by a calcium ionophore (ionomycin), Biochem Biophys Acta 1137 (1992) 182-188.

[5] H. Lundqvist, P. Follin, L. Khalfan, C. Dahlgren, Phorbol myristate acetate induced NADPH-oxidase activity in human neutrophils: Only half the story has been told, J Leuk Biol 59 (1996) 270-279.

[6] J.D. Matute, A.A. Arias, N.A. Wright, I. Wrobel, C.C. Waterhouse, X.J. Li, C.C. Marchal, N.D. Stull, D.B. Lewis, M. Steele, J.D. Kellner, W. Yu, S.O. Meroueh, W.M. Nauseef, M.C. Dinauer, A new genetic subgroup of chronic granulomatous disease with autosomal recessive mutations in p40 phox and selective defects in neutrophil NADPH oxidase activity, BLOOD 114 (2009) 3309-3315.

[7] N. Borregaard, J.M. Heiple, E.R. Simons, R.A. Clark, Subcellular localization of the b-cytochrome component of the human neutrophil microbicidal oxidase: translocation during activation, J Cell Biol 97 (1983) 52-61.

[8] J. Bylund, K.L. Brown, C. Movitz, C. Dahlgren, A. Karlsson, Intracellular generation of superoxide by the phagocyte NADPH oxidase: how, where, and what for?, Free Radic Biol Med 49 (2010) 1834-1845.

[9] C. Dahlgren, Difference in extracellular radical release after chemotactic factor and calcium ionophore activation of the oxygen radical-generating system in human neutrophils, Biochim Biophys Acta 930 (1987) 33-38.

[10] F. Watson, J. Robinson, S.W. Edwards, Protein kinase C-dependent and -independent activation of the NADPH oxidase of human neutrophils, J Biol Chem 266 (1991) 7432-7439.

[11] A. Karlsson, P. Follin, H. Leffler, C. Dahlgren, Galectin-3 activates the NADPH-oxidase in exudated but not peripheral blood neutrophils, Blood 91 (1998) 3430-3438.

[12] L. Serrander, J. Larsson, H. Lundqvist, M. Lindmark, M. Fallman, C. Dahlgren, O. Stendahl, Particles binding beta(2)-integrins mediate intracellular production of oxidative metabolites in human neutrophils independently of phagocytosis, Biochim Biophys Acta 1452 (1999) 133-144.

[13] A. Karlsson, Wheat germ agglutinin induces NADPH-oxidase activity in human neutrophils by interaction with mobilizable receptors, Infect Immun 67 (1999) 3461-3468.

[14] A. Karlsson, J.B. Nixon, L.C. McPhail, Phorbol myristate acetate induces neutrophil NADPH-oxidase activity by two separate signal transduction pathways: dependent or independent of phosphatidylinositol 3-kinase, J Leukoc Biol 67 (2000) 396-404.

[15] D. Granfeldt, M. Samuelsson, A. Karlsson, Capacitative Ca2+ influx and activation of the neutrophil respiratory burst. Different regulation of plasma membrane- and granule-localized NADPH-oxidase, J Leukoc Biol 71 (2002) 611-617.

[16] A. Karlsson, C. Dahlgren, Assembly and activation of the neutrophil NADPH oxidase in granule membranes, Antioxid Redox Signal 4 (2002) 49-60.

[17] P.J. Ferguson, M.A. Lokuta, H.I. El-Shanti, L. Muhle, X. Bing, A. Huttenlocher, Neutrophil dysfunction in a family with a SAPHO syndrome-like phenotype, Arthritis Rheum 58 (2008) 3264-3269.
[18] R. Dana, H.L. Malech, R. Levy, The requirement for phospholipase A2 for activation of the assembled NADPH oxidase in human neutrophils, Biochem J 297 (Pt 1) (1994) 217-223.
[19] R. Dana, T.L. Leto, H.L. Malech, R. Levy, Essential requirement of cytosolic phospholipase A2 for activation of the phagocyte NADPH oxidase, J Biol Chem 273 (1998) 441-445.
[20] P.K. Tithof, M. Peters-Golden, P.E. Ganey, Distinct phospholipases A2 regulate the release of arachidonic acid for eicosanoid production and superoxide anion generation in neutrophils, J Immunol 160 (1998) 953-960.
[21] X. Zhao, E.A. Bey, F.B. Wientjes, M.K. Cathcart, Cytosolic phospholipase A2 (cPLA2) regulation of human monocyte NADPH oxidase activity. cPLA2 affects translocation but not phosphorylation of p67(phox) and p47(phox), J Biol Chem 277 (2002) 25385-25392.
[22] R. Levy, The role of cytosolic phospholipase A2-alfa in regulation of phagocytic functions, Biochim Biophys Acta 1761 (2006) 1323-1334.
[23] B.B. Rubin, G.P. Downey, A. Koh, N. Degousee, F. Ghomashchi, L. Nallan, E. Stefanski, D.W. Harkin, C. Sun, B.P. Smart, T.F. Lindsay, V. Cherepanov, E. Vachon, D. Kelvin, M. Sadilek, G.E. Brown, M.B. Yaffe, J. Plumb, S. Grinstein, M. Glogauer, M.H. Gelb, Cytosolic phospholipase A2-alpha is necessary for platelet-activating factor biosynthesis, efficient neutrophil-mediated bacterial killing, and the innate immune response to pulmonary infection: cPLA2-alpha does not regulate neutrophil NADPH oxidase activity, J Biol Chem 280 (2005) 7519-7529.
[24] A. Böyum, Isolation of mononuclear cells and granulocytes from human blood., Scand J Lab Invest 21 (1968) 77-89.
[25] C. Dahlgren, A. Karlsson, Respiratory burst in human neutrophils, J Immunol Methods 232 (1999) 3-14.
[26] H. Lundqvist, C. Dahlgren, Isoluminol-enhanced chemiluminescence: A sensitive method to study the release of superoxide anion from human neutrophils, Free Radic Biol Med 20 (1996) 785-792.
[27] P.A. Hyslop, L.A. Sklar, A quantitative fluorimetric assay for the determination of oxidant production by polymorphonuclear leukocytes: its use in the simultaneous fluorimetric assay of cellular activation processes, Anal Biochem 141 (1984) 280-286.
[28] R.K. Root, J. Metcalf, N. Oshino, B. Chance, H2O2 release from human granulocytes during phagocytosis. I. Documentation, quantitation, and some regulating factors, J Clin Invest 55 (1975) 945-955.
[29] R.K. Root, J.A. Metcalf, H2O2 release from human granulocytes during phagocytosis. Relationship to superoxide anion formation and cellular catabolism of H2O2: studies with normal and cytochalasin B-treated cells, J Clin Invest 60 (1977) 1266-1279.
[30] O.P. Hamill, A. Marty, E. Neher, B. Sakmann, F.J. Sigworth, Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches, Pflugers Arch 391 (1981) 85-100.
[31] D. Bers, C. Patton, R. Nuccitelli, A practical guide to the preparation of Ca buffers, A practival guide to the study of Ca2+ in living cells, Academic Press, San Diego, 1994, Vol. 40.

[32] C. Laudanna, F. Rossi, G. Berton, Effect of inhibitors of distinct signalling pathways on neutrophil Q2- generation in response to tumor necrosis factor-alpha, and antibodies against CD18 and CD11a: evidence for a common and unique pattern of sensitivity to wortmannin and protein tyrosine kinase inhibitors, Biochem Biophys Res Commun 190 (1993) 935-940.
[33] C.J. Vlahos, W.F. Matter, R.F. Brown, A.E. Traynor-Kaplan, P.G. Heyworth, E.R. Prossnitz, R.D. Ye, P. Marder, J.A. Schelm, K.J. Rothfuss, Investigation of neutrophil signal transduction using a specific inhibitor of phosphatidylinositol 3-kinase, J Immunol 154 (1995) 2413-2422.
[34] M.D. Rosenthal, R.C. Franson, Separation of agonist-stimulated arachidonate mobilization from subsequent leukotriene B4 synthesis in human neutrophils: different effects of oleoylacetylglycerol and phorbol myristate acetate as priming agents, J Cell Physiol 160 (1994) 522-530.
[35] P. Stevens, D.J. Winston, K. Van Dyke, In vitro evaluation of opsonic and cellular granulocyte function by luminol-dependent chemiluminescence: utility in patients with severe neutropenia and cellular deficiency states, Infect Immun 22 (1978) 41-51.
[36] C. Dahlgren, O. Stendahl, Role of myeloperoxidase in luminol-dependent chemiluminescence of polymorphonuclear leukocytes, Infect Immun 39 (1983) 736-741.
[37] C. Dahlgren, A. Karlsson, J. Bylund, Measurement of respiratory burst products generated by professional phagocytes, Methods Mol Biol 412 (2007) 349-363.
[38] M. Faurschou, N. Borregaard, Neutrophil granules and secretory vesicles in inflammation, Microbes Infect 5 (2003) 1317-1327.
[39] J.M. Fernandez, E. Neher, B.D. Gomperts, Capacitance measurements reveal stepwise fusion events in degranulating mast cells, Nature 312 (1984) 453-455.
[40] W.C. Hope, T. Chen, D.W. Morgan, Secretory phospholipase A2 inhibitors and calmodulin antagonists as inhibitors of cytosolic phospholipase A2, Agents Actions 39 Spec No (1993) C39-42.
[41] K. Conde-Frieboes, L.J. Reynolds, Y.C. Lio, M.R. Hale, H.H. Wasserman, E.A. Dennis, Activated Ketones as Inhibitors of Intracellular Ca2+-Dependent and Ca2+-Independent Phospholipase A2, J Am Chem Soc 118 (1996) 5519-5525.
[42] K. Seno, T. Okuno, K. Nishi, Y. Murakami, F. Watanabe, T. Matsuura, M. Wada, Y. Fujii, M. Yamada, T. Ogawa, T. Okada, H. Hashizume, M. Kii, S. Hara, S. Hagishita, S. Nakamoto, K. Yamada, Y. Chikazawa, M. Ueno, I. Teshirogi, T. Ono, M. Ohtani, Pyrrolidine inhibitors of human cytosolic phospholipase A(2), J Med Chem 43 (2000) 1041-1044.
[43] F. Ghomashchi, A. Stewart, Y. Hefner, S. Ramanadham, J. Turk, C.C. Leslie, M.H. Gelb, A pyrrolidine-based specific inhibitor of cytosolic phospholipase A(2)alpha blocks arachidonic acid release in a variety of mammalian cells, Biochim Biophys Acta 1513 (2001) 160-166.
[44] J. Bylund, M. Samuelsson, L.V. Collins, A. Karlsson, NADPH-oxidase activation in murine neutrophils via formyl peptide receptors, Exp Cell Res 282 (2003) 70-77.
[45] W.M. Nauseef, J.A. Metcalf, R.K. Root, Role of myeloperoxidase in the respiratory burst of human neutrophils, BLOOD 61 (1983) 483-492.
[46] N. Borregaard, J.B. Cowland, Granules of the human neutrophilic polymorphonuclear leukocyte, Blood 89 (1997) 3503-3521.

[47] T. Kobayashi, J.M. Robinson, H. Seguchi, Identification of intracellular sites of superoxide production in stimulated neutrophils, J Cell Sci 111 (Pt 1) (1998) 81-91.
[48] B. Martin-Martin, S.M. Nabokina, J. Blasi, P.A. Lazo, F. Mollinedo, Involvement of SNAP-23 and syntaxin 6 in human neutrophil exocytosis, BLOOD 96 (2000) 2574-2583.
[49] K. Lollike, M. Lindau, J. Calafat, N. Borregaard, Compound exocytosis of granules in human neutrophils, J Leukoc Biol 71 (2002) 973-980.
[50] F. Mollinedo, B. Martin-Martin, J. Calafat, S.M. Nabokina, P.A. Lazo, Role of vesicle-associated membrane protein-2, through Q-soluble N-ethylmaleimide-sensitive factor attachment protein receptor/R-soluble N-ethylmaleimide-sensitive factor attachment protein receptor interaction, in the exocytosis of specific and tertiary granules of human neutrophils, J Immunol 170 (2003) 1034-1042.
[51] M.S. Riffo, M. Parraga, Role of phospholipase A2 in mammalian sperm-egg fusion: development of hamster oolemma fusibility by lysophophatidylcholine, J Exp Zool 279 (1997) 81-88.
[52] H. Nishio, T. Takeuchi, F. Hata, O. Yagasaki, Ca(2+)-independent fusion of synaptic vesicles with phopholipase A2-treated presynaptic membranes in vitro, Biochem J 318 (1996) 981-987.
[53] L.S. Mayorga, M.I. Colombo, M. Lennartz, E.J. Brown, K.H. Rahman, R. Weiss, P.J. Lennon, P.D. Stahl, Inhibition of endosome fusion by phospholipase A2 (PLA2) inhibitors points to a role for PLA2 in endocytosis, Proc Natl Acad Sci USA 90 (1993) 10225-10229.
[54] L.S. Mayorga, W. Beron, M.N. Sarrouf, M.I. Colombo, C. Creutz, P.D. Stahl, Calcium-dependent fusion among endosomes, J Biol Chem 269 (1994) 30927-30934.
[55] R.A. Blackwood, A.T. Transue, D.M. Harsh, R.C. Brower, S.J. Zacharek, J.E. Smolen, R.J. Hessler, PLA2 promotes fusion between PMN-specific granules and complex liposomes, J Leukoc Biol 59 (1996) 663-670.
[56] D.J. McLaren, C.D. Mackenzie, F.J. Ramalho-Pinto, Ultrastructural observations on the in vitro interaction between rat eosinophils and some parasitic helminths (Schistosoma mansoni, Trichinella spiralis and Nippostrongylus brasiliensis), Clin Exp Immunol 30 (1977) 105-118.
[57] S. Scepek, M. Lindau, Focal exocytosis by eosinophils--compound exocytosis and cumulative fusion, EMBO J 12 (1993) 1811-1817.
[58] J. Hartmann, S. Scepek, I. Hafez, M. Lindau, Differential regulation of exocytotic fusion and granule-granule fusion in eosinophils by Ca2+ and GTP analogs, J Biol Chem 278 (2003) 44929-44934.
[59] Z. Guo, C. Turner, D. Castle, Relocation of the t-SNARE SNAP-23 from lamellipodia-like cell surface projections regulates compound exocytosis in mast cells, Cell 94 (1998) 537-548.
[60] L. Orci, W. Malaisse, Hypothesis: single and chain release of insulin secretory granules is related to anionic transport at exocytotic sites, Diabetes 29 (1980) 943-944.
[61] K. Bokvist, M. Holmqvist, J. Gromada, P. Rorsman, Compound exocytosis in voltage-clamped mouse pancreatic beta-cells revealed by carbon fibre amperometry, Pflugers Arch 439 (2000) 634-645.

[62] A.J. Cochilla, J.K. Angleson, W.J. Betz, Differential regulation of granule-to-granule and granule-to-plasma membrane fusion during secretion from rat pituitary lactotrophs, J Cell Biol 150 (2000) 839-848.
[63] O.E. Sorensen, P. Follin, A.H. Johnsen, J. Calafat, G.S. Tjabringa, P.S. Hiemstra, N. Borregaard, Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3, BLOOD 97 (2001) 3951-3959.
[64] G.J. Peppin, S.J. Weiss, Activation of the endogenous metalloproteinase, gelatinase, by triggered human neutrophils, Proc Natl Acad Sci U S A 83 (1986) 4322-4326.
[65] C.C. Mihalache, S. Yousefi, S. Conus, P.M. Villiger, E.M. Schneider, H.U. Simon, Inflammation-associated autophagy-related programmed necrotic death of human neutrophils characterized by organelle fusion events, J Immunol 186 (2011) 6532-6542.
[66] M.D. Lister, K.B. Glaser, R.J. Ulevitch, E.A. Dennis, Inhibition studies on the membrane-associated phospholipase A2 in vitro and prostaglandin E2 production in vivo of the macrophage-like P388D1 cell. Effects of manoalide, 7,7-dimethyl-5,8-eicosadienoic acid, and p-bromophenacyl bromide, J Biol Chem 264 (1989) 8520-8528.
[67] N. Cohen, G. Weber, B.L. Banner, A.F. Welton, W.C. Hope, H. Crowley, W.A. Anderson, B.A. Simko, M. O'Donnell, J.W. Coffey, et al., Analogs of arachidonic acid methylated at C-7 and C-10 as inhibitors of leukotriene biosynthesis, Prostaglandins 27 (1984) 553-562.
[68] R.E. Duncan, E. Sarkadi-Nagy, K. Jaworski, M. Ahmadian, H.S. Sul, Identification and functional characterization of adipose-specific phospholipase A2 (AdPLA), J Biol Chem 283 (2008) 25428-25436.
[69] E.J. Ackermann, K. Conde-Frieboes, E.A. Dennis, Inhibition of macrophage Ca(2+)-independent phospholipase A2 by bromoenol lactone and trifluoromethyl ketones, J Biol Chem 270 (1995) 445-450.
[70] C.R. Jan, K.J. Chou, K.C. Lee, J.L. Wang, L.L. Tseng, J.S. Cheng, W.C. Chen, Dual action of palmitoyl trifluoromethyl ketone (PACOCF3) on Ca2+ signaling: activation of extracellular Ca2+ influx and alteration of ATP- and bradykinin-induced Ca2+ responses in Madin Darby canine kidney cells, Arch Toxicol 74 (2000) 447-451.
[71] J. Balsinde, E.A. Dennis, Distinct roles in signal transduction for each of the phospholipase A2 enzymes present in P388D1 macrophages, J Biol Chem 271 (1996) 6758-6765.
[72] M.S. Barnette, J. Rush, L.A. Marshall, J.J. Foley, D.B. Schmidt, H.M. Sarau, Effects of scalaradial, a novel inhibitor of 14 kDa phospholipase A2, on human neutrophil function, Biochem Pharmacol 47 (1994) 1661-1667.
[73] Y. Chen, E.A. Dennis, Expression and characterization of human group V phospholipase A2, Biochim Biophys Acta 1394 (1998) 57-64.
[74] J. Marshall, E. Krump, T. Lindsay, G. Downey, D.A. Ford, P. Zhu, P. Walker, B. Rubin, Involvement of cytosolic phospholipase A2 and secretory phospholipase A2 in arachidonic acid release from human neutrophils, J Immunol 164 (2000) 2084-2091.
[75] Y.C. Lio, L.J. Reynolds, J. Balsinde, E.A. Dennis, Irreversible inhibition of Ca(2+)-independent phospholipase A2 by methyl arachidonyl fluorophosphonate, Biochim Biophys Acta 1302 (1996) 55-60.
[76] Z. Huang, S. Liu, I. Street, F. Laliberte, K. Abdullah, S. Desmarais, Z. Wang, B. Kennedy, P. Payette, D. Riendeau, P. Weech, M. Gresser, Methyl arachidonyl

fluorophosphonate, a potent irreversible cPLA inhibitor, blocks the mobilization of arachidonic acid in human platelets and neutrophils, IAIS Symposium on Novel Molecular Approaches to Anti-inflammatory Therapy, Mediators Inflamm, Toronto, Canada, 1994, Vol. 3, pp. 303-311.

FIGURE LEGENDS

Figure 1

PMA induces extracellular and intracellular ROS production in neutrophils

ROS production was measured by luminol/isoluminol-amplified chemiluminescence (CL). Neutrophils were preincubated for 5 min at 37°C, prior addition of PMA (**A**; $5x10^{-8}$ M), ionomycin (**B**; $5x10^{-7}$ M), galectin-3 (**C**; extracellular, 40 μg/ml; intracellular, 20 μg/ml), or fMLF (**D**; 10^{-7} M). The extracellular ROS production was measured in the presence of isoluminol and HRP (extracellular response; black), while the intracellular activity of the NADPH-oxidase was measured in the presence of luminol, SOD, and catalase (intracellular response; gray). The time-course of the responses were followed and the magnitude of the responses is given in Mcpm (10^6 counts per minute). Representative experiments are shown.

Figure 2

The effect of PLA_2 inhibitors on ROS production as measured with CL

A. Neutrophils were preincubated in the presence or absence of indicated concentrations of DEDA at 37°C for 15 min prior to stimulation with PMA ($5x10^{-8}$ M). The figure shows the time-course of the extra- and intracellular CL responses, measured as described for Figure 1.

B-H. Neutrophils were pretreated with different concentrations of the indicated PLA_2 inhibitors at 37°C for 15 min prior to stimulation with PMA ($5x10^{-8}$ M). The extracellular (blue) and intracellular (red) responses were measured for 20 min and the peak values (maximal CL readings) are expressed as percent of control (in the absence of inhibitor), is given as mean ± SD; n=3 for each inhibitor concentration.

Figure 3

The effects of DEDA, $PACOCF_3$, and pyrrolidine on ionomycin- and galectin-3-induced CL responses

Neutrophils were preincubated in the presence (dashed lines; open bars) or absence (solid line; closed bar) of DEDA (100 µM), $PACOCF_3$ (100 µM), or pyrrolidine (10 µM) at 37°C for 15 min prior to stimulation with ionomycin (**A**; $5x10^{-7}$ M) or galectin-3 (**B**; intracellular, 20 µg/ml; **C**; extracellular, 40 µg/ml). The figures show the time-course of representative CL responses, measured as described for Figure 1. Also shown is the peak ROS production as a percent of control (**D-F**), given as mean ± SD, n=3 independent experiments. *P<0.05, **P<0.01, ***P<0.001; One-way ANOVA, Dunnett's multiple comparison test.

Figure 4

The effect of PLA_2 inhibitors on PMA-induced hydrogen peroxide production as measured by PHPA oxidation

A. Neutrophils were preincubated for 5 min at 37°C in the presence of HRP (4 U/ml) and PHPA (0.5 mg/ml). After addition of PMA ($5x10^{-8}$ M) the fluorescence emitted by oxidized PHPA was recorded continuously and given as arbitrary fluorescence units. This system measures the extracellular hydrogen peroxide production (light blue), whereas the total hydrogen peroxide production (extra- plus intracellular) was measured in the presence of azide (1 mM; dark blue). Neutrophils preincubated (10 min) with the NADPH-oxidase inhibitor DPI (10 µM; dotted lines) failed to generate detectable levels of hydrogen peroxide, indicating that the ROS detected in this system stem from the NADPH-oxidase. A representative experiment is shown. Neutrophils were preincubated for 15 min at 37°C with HRP and PHPA with or without DEDA (**B**; 100 µM) or BEL (**C**; 100 µM) prior to stimulation with PMA ($5x10^{-8}$ M). Azide was present in some samples for total hydrogen peroxide

detection. Representative experiments are shown. Intracellular hydrogen peroxide production (**D**) was measured by PHPA-oxidation in the absence or presence of PLA_2 inhibitor (DEDA, 100 µM; $PACOCF_3$, 100µM; pyrrolidine, 10 µM; BEL, 100 µM; SLD, 1 µM) for 10 min at 37°C prior to stimulation with PMA ($5x10^{-8}$ M). The intracellular ROS production was determined as the difference between total (+azide) and extracellular (-azide) hydrogen peroxide production at a time-point 18 min after stimulation and is given as percent of control (absence of PLA_2 inhibitor), mean ±SD, n=3 independent experiments.

Figure 5

The effects of PLA_2 inhibitors on MPO activity

A. The peroxidase substrate (OPD; 0.4 mg/ml) and hydrogen peroxide (4.9 mM) were incubated with PLA_2 inhibitors and purified MPO (12.5 µg/ml) for 30 min. The reaction was stopped with H_2SO_4 (1 mM) and the absorbance measured. The figure shows a representative experiment in triplicate as percent of control. **B**. The effects of PLA_2 inhibitors on MPO function was measured by extracellular CL in the presence of exogenously added purified MPO instead of HRP. The MPO (1 µg/ml) was mixed with neutrophils and isoluminol and incubated for 15 min at 37°C with or without PLA_2 inhibitors (DEDA, 100 µM; $PACOCF_3$, 100 µM; pyrrolidine (PYR), 10 µM). Cells were stimulated with PMA ($5x10^{-8}$ M) and shown are peak responses as percent of control (no PLA_2 inhibitor), given as mean ±SD, n=4 independent experiments.

Figure 6

The effect of DEDA, $PACOCF_3$, and pyrrolidine on granule fusion with the plasma membrane

A. Capacitance recordings from neutrophils stimulated with pipette solutions containing 10 µM of free calcium, with (dotted line, closed bar, n=5) or without DEDA (solid line, open bar, n=11). The capacitance values obtained directly after break through (initial or resting

capacitance, typically around 2.5 pF) have been subtracted from the starting values for clarity. Shown is the rate of exocytosis measured in femtofarads/s, determined by fitting a straight line to the capacitance traces. The inset data are mean ± SD of all experiments. **B and C.** The effect of PLA_2 on upregulation of CR3 to the plasma membrane was measured using flow cytometry. The PLA_2 inhibitors, DEDA (100 μM), $PACOCF_3$ (100 μM), and pyrrolidine (PYR; 10 μM), were incubated with neutrophils for 15 min at 37°C after which the cells were stimulated with PMA (**B**; 5nM) or TNF-α (**C**; 10 ng/ml) for 20 min at 37°C and stained with CD11b-PE mAb. The figure shows the geometrical mean from three independent experiments expressed as percent of control (unstimulated), given as mean ±SD, n=3 independent experiments.

Figure 7

Proposed mechanism of heterotypic granule fusion in PMA-stimulated neutrophils and result of PLA_2 inhibition

A. PMA stimulates NADPH-oxidase activation both at the plasma membrane and in intracellular granules, through direct activation of PKC. In order to detect intracellularly generated ROS with the chemiluminescence technique, the ROS produced by the NADPH-oxidase need to come in contact with MPO, and we hypothesize that this takes place through heterotypic fusion of the specific and azurophil granules. The PLA_2 inhibitors DEDA, PACOCF3 and pyrrolidine are proposed to inhibit this type of fusion, since they neither inhibit NADPH-oxidase activity nor MPO function. **B.** A confocal micrograph of a PMA-stimulated neutrophil labeled with DAPI (nucleus; blue), lactoferrin (specific granules; green), and CD63 (azurophil granules; red). Colocalization of lactoferrin and CD63 is indicated by the arrows.

Table 1. Selected PLA_2 inhibitors.

Name	Full name	Specificity	IC_{50}	Assay	Comments
DEDA	7,7-Dimethyleicosadienoic acid	$sPLA_2$ [40]	16 μM[66] 14 μM [67] 5.8 μM [40]	Inhibits P388 cell PLA_2 activity [66]. Activity on soluble Naja snake venom PLA_2[67]. Inhibition of human placental $sPLA_2$ activity [40].	Inhibits also 5-lipoxygenase [67], $AdPLA_2$ [68].
$PACOCF_3$	Palmitoyl trifluoro-methyl ketone	$iPLA_2 > cPLA_2$	$iPLA_2$: 3.3-3.8 μM [41, 69] $cPLA_2$: 47 μM [41]	Inhibition of P388 $iPLA_2$ [69].	Induces Ca^{2+} influx [70].
Pyrrolidine		$cPLA_2 > iPLA_2$	13-50 nM[42, 43]	Inhibition of AA, PGE_2, and LTC_4 release in THP-1 cells [42] and AA release by CHO cells [43].	Non-specific inhibition of $iPLA_2$ at higher concentrations, micromolar range [43].
BEL	Bromoenol lactone	$iPLA_2 > cPLA_2$	60-180 nM [69]	Inhibition of $iPLA_2$ activity in P388D cells [69].	Inhibits also phosphatidic acid phosphohydrolase (PAP) [71].
Scalaradial (SLD)	12-Episcala-radial	$sPLA_2 > cPLA_2$	$sPLA_2$: 5.4 μM [40] $cPLA_2$: 28 μM [40]	Inhibition of recombinant $sPLA_2$ and $cPLA_2$ [40].	Inhibits degranulation in human neutrophils [72].
LY311727		$sPLA_2$	36 nM [73]	Inhibition of human $sPLA_2$ [73].	Probably not cell permeable [74].
MAFP	Methyl arachidonyl fluorophos-phonate	$cPLA_2$ & $iPLA_2$	$iPLA_2$: 0.5 μM [75] $cPLA_2$: 0.6 μM [76]	Inhibition of P388D $iPLA_2$ activity [75]. Inhibition of $cPLA_2$ in human platelets [76].	

Figure 1

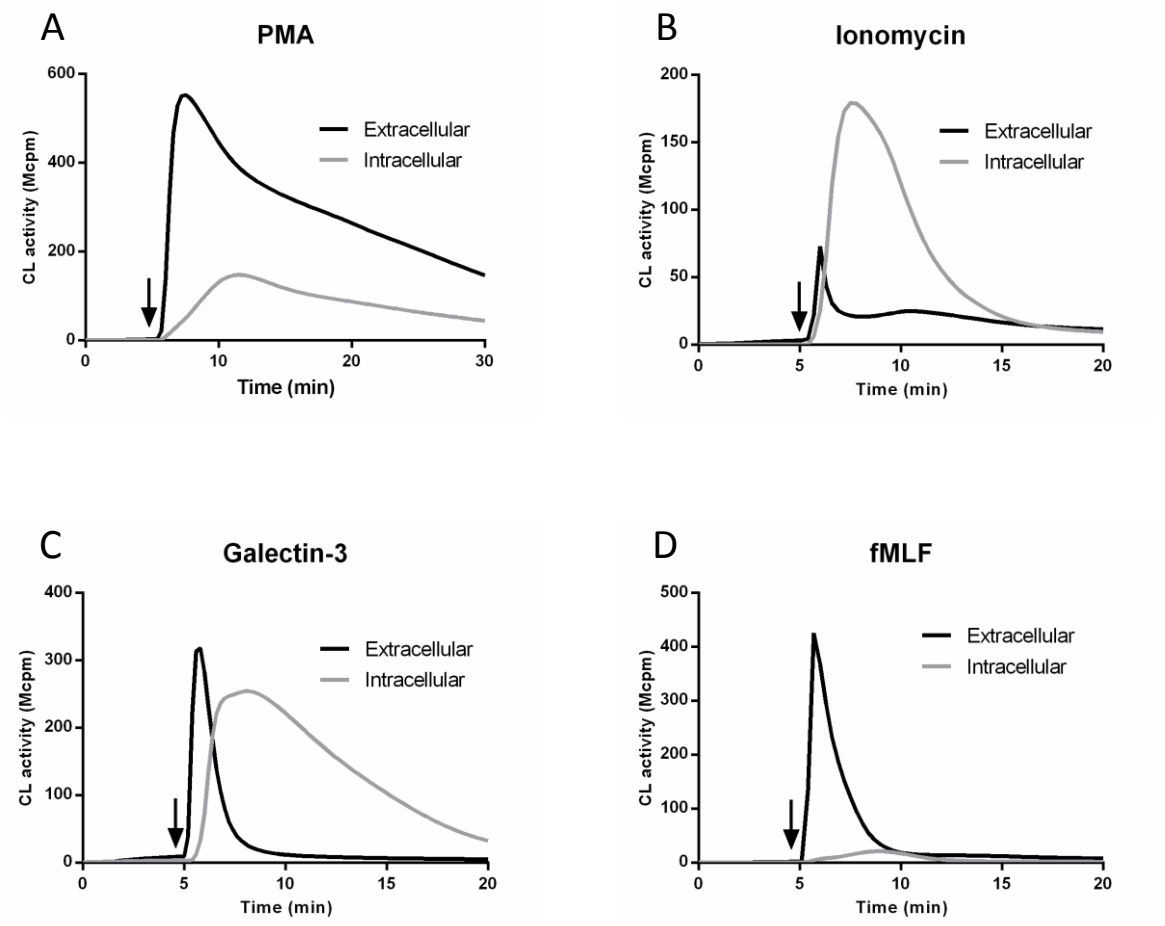

A
PMA
CL activity (Mcpm)
600
400
200
0
0
10
20
30
Time (min)
Extracellular
Intracellular
B
Ionomycin
CL activity (Mcpm)
200
150
100
50
0
0
5
10
15
20
Time (min)
Extracellular
Intracellular
C
Galectin-3
CL activity (Mcpm)
400
300
200
100
0
0
5
10
15
20
Time (min)
Extracellular
Intracellular
D
fMLF
CL activity (Mcpm)
500
400
300
200
100
0
0
5
10
15
20
Time (min)
Extracellular
Intracellular

Figure 2

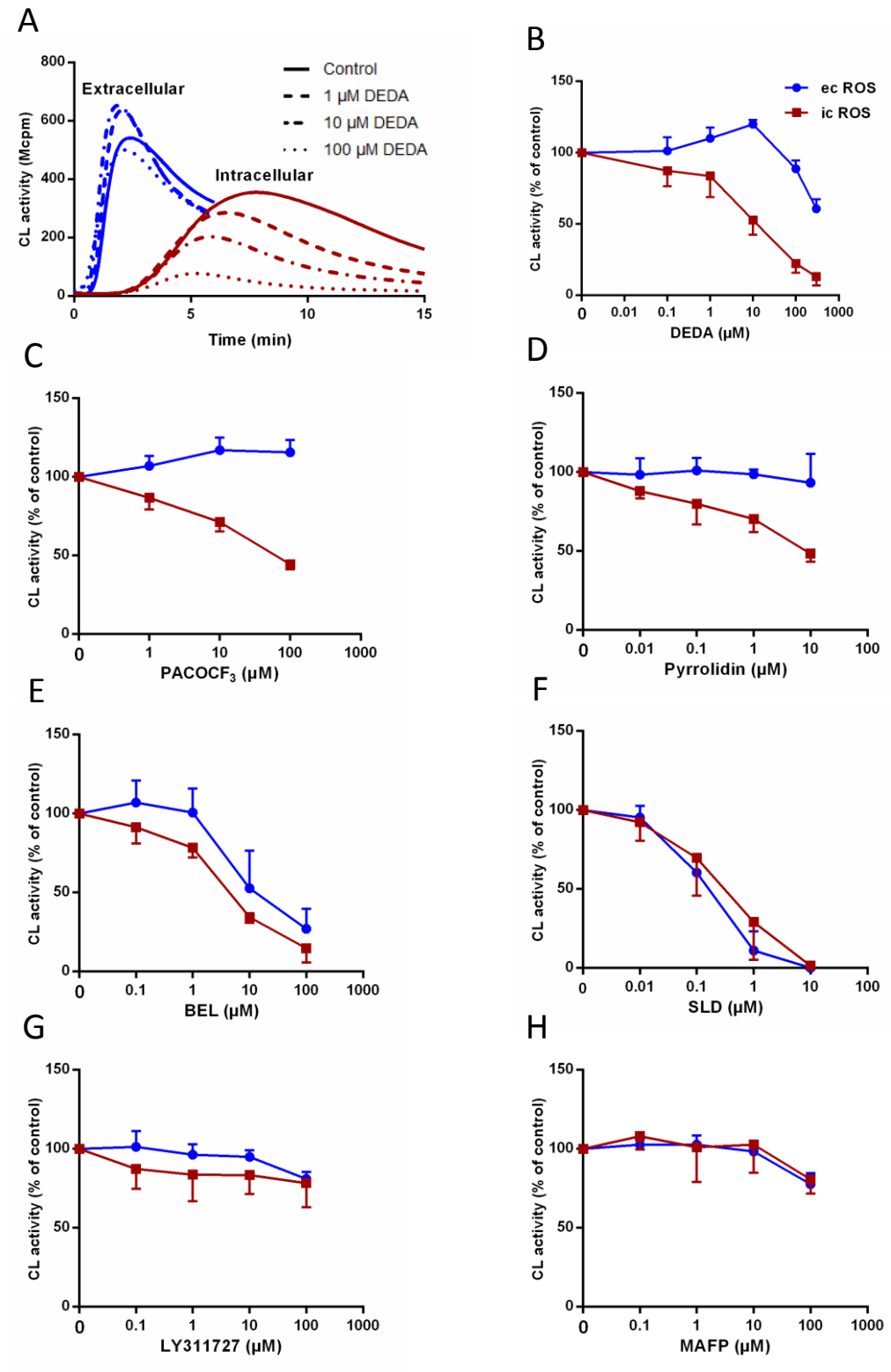

A
Extracellular
Intracellular
Control
1 μM DEDA
10 μM DEDA
100 μM DEDA
CL activity (Mcpm)
Time (min)
B
ec ROS
ic ROS
CL activity (% of control)
DEDA (μM)
C
PACOCF3 (μM)
D
Pyrrolidin (μM)
E
BEL (μM)
F
SLD (μM)
G
LY311727 (μM)
H
MAFP (μM)

Figure 3

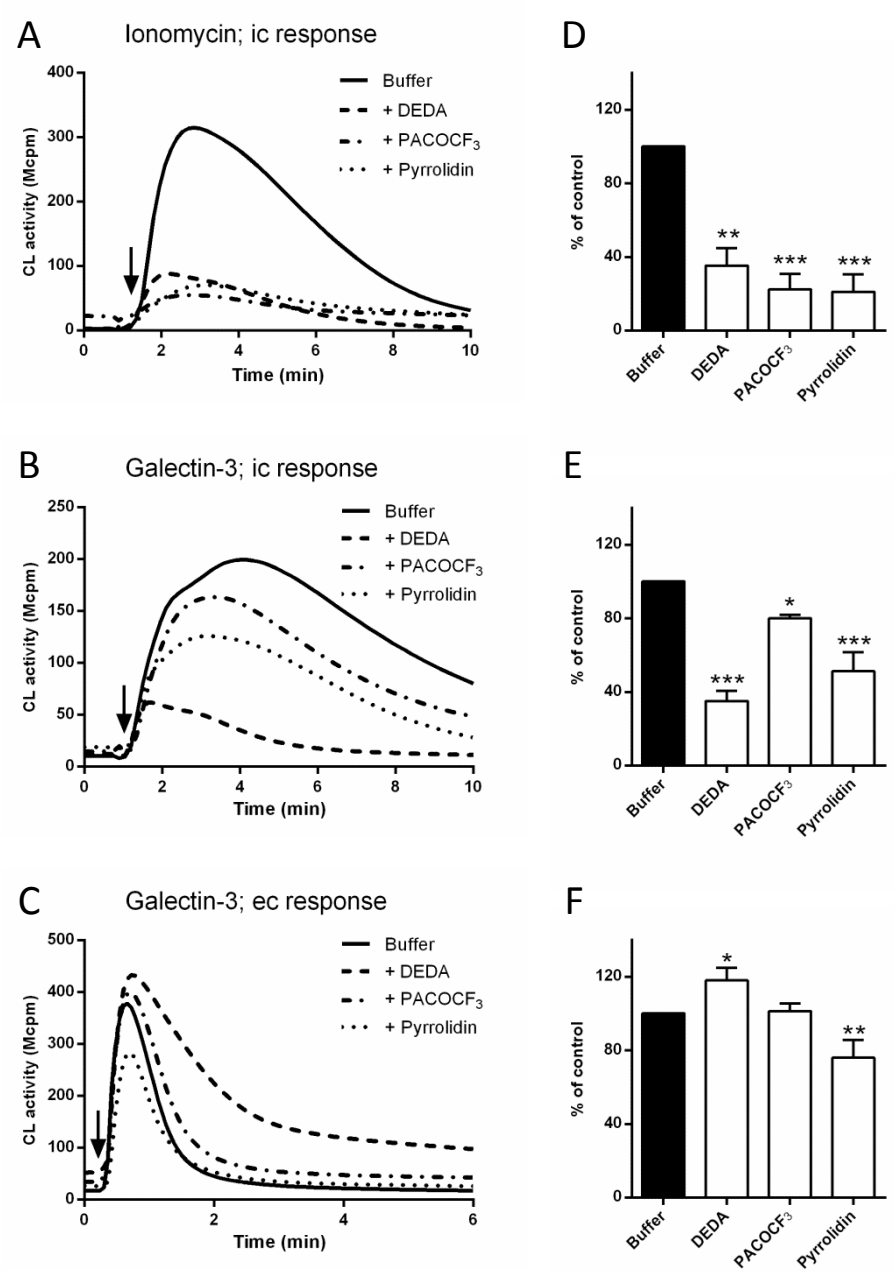

A
Ionomycin; ic response
Buffer
+ DEDA
+ PACOCF3
+ Pyrrolidin
CL activity (Mcpm)
Time (min)
B
Galectin-3; ic response
C
Galectin-3; ec response
D
E
F
% of control
Buffer
DEDA
PACOCF3
Pyrrolidin

Figure 4

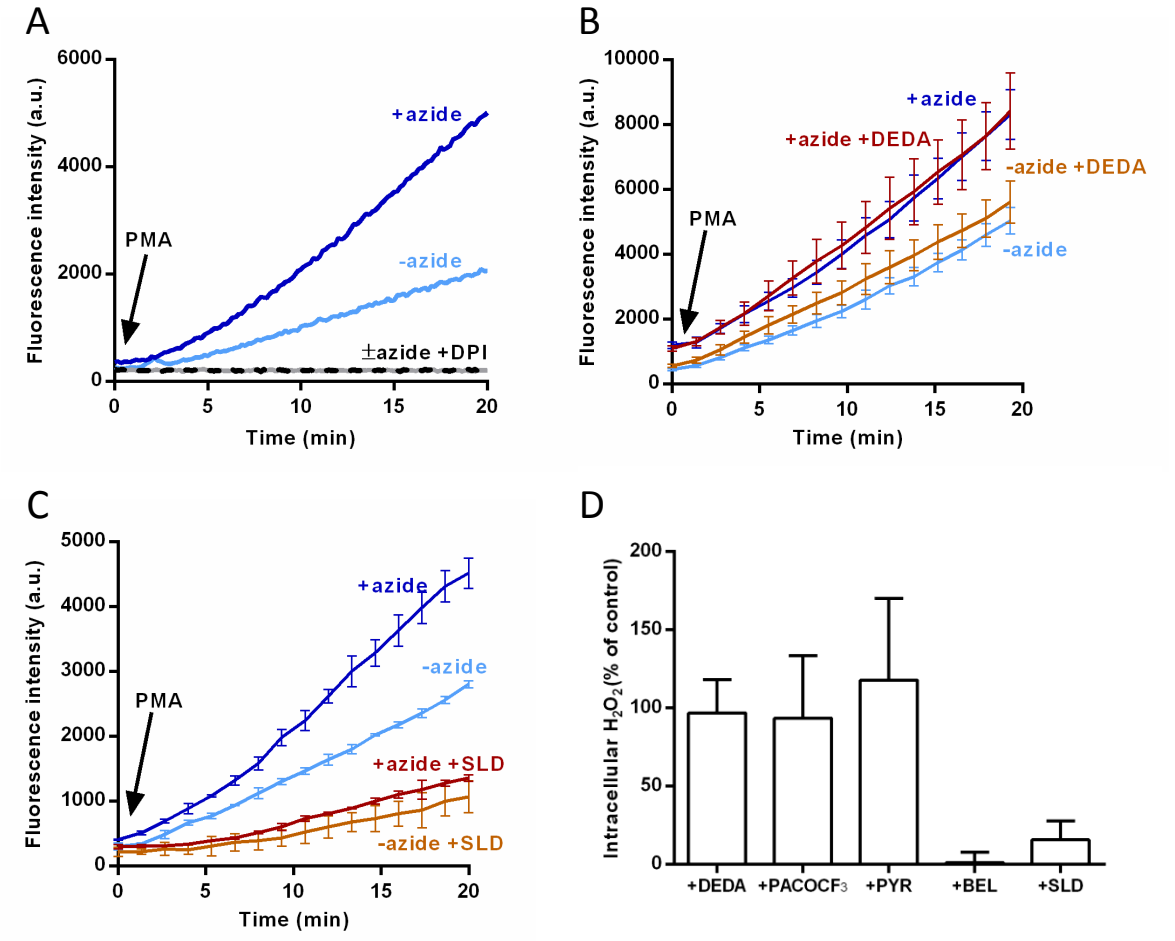

A
Fluorescence intensity (a.u.)
6000
4000
2000
0
PMA
+azide
-azide
±azide +DPI
0
5
10
15
20
Time (min)
B
Fluorescence intensity (a.u.)
10000
8000
6000
4000
2000
0
PMA
+azide
+azide +DEDA
-azide +DEDA
-azide
0
5
10
15
20
Time (min)
C
Fluorescence intensity (a.u.)
5000
4000
3000
2000
1000
0
PMA
+azide
-azide
+azide +SLD
-azide +SLD
0
5
10
15
20
Time (min)
D
Intracellular H_2O_2 (% of control)
200
150
100
50
0
+DEDA
+PACOCF3
+PYR
+BEL
+SLD

Figure 5

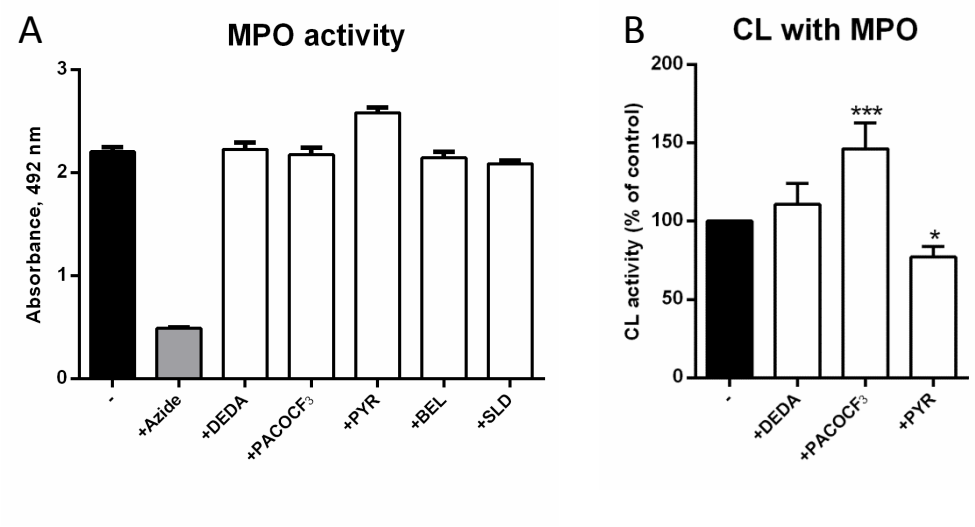
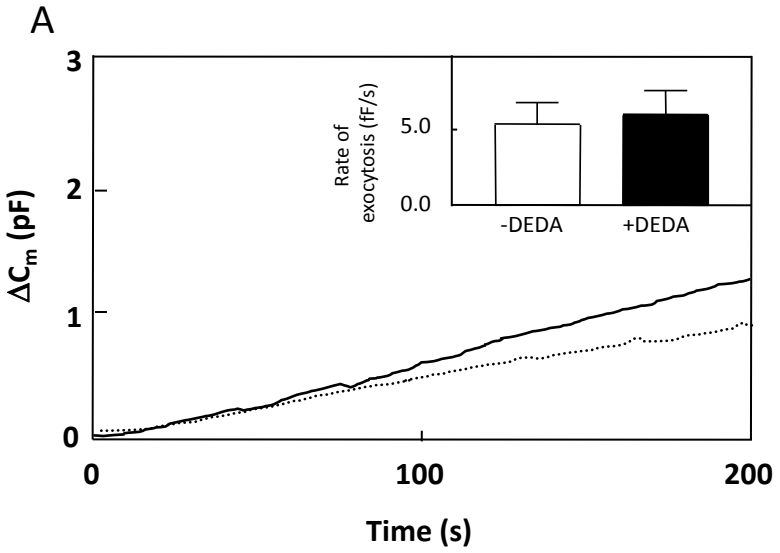
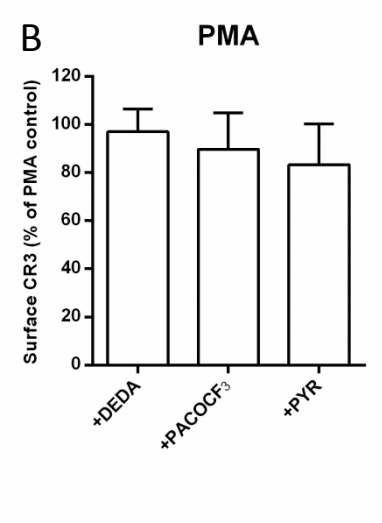

A
MPO activity
Absorbance, 492 nm
3
2
1
0
-
+Azide
+DEDA
+PACOCF3
+PYR
+BEL
+SLD
B
CL with MPO
CL activity (% of control)
200
150
100
50
0
***
*
-
+DEDA
+PACOCF3
+PYR

Figure 6


A
3
2
1
0
ΔC_m (pF)
0
100
200
Time (s)
Rate of exocytosis (fF/s)
5.0
0.0
-DEDA
+DEDA


B
PMA
Surface CR3 (% of PMA control)
120
100
80
60
40
20
0
+DEDA
+PACOCF3
+PYR

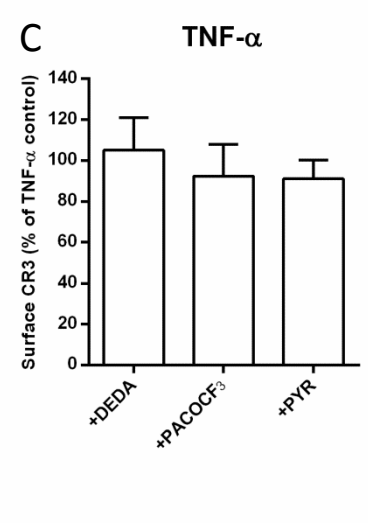
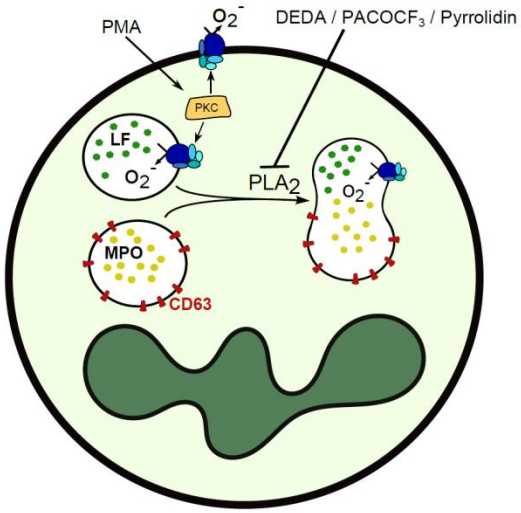
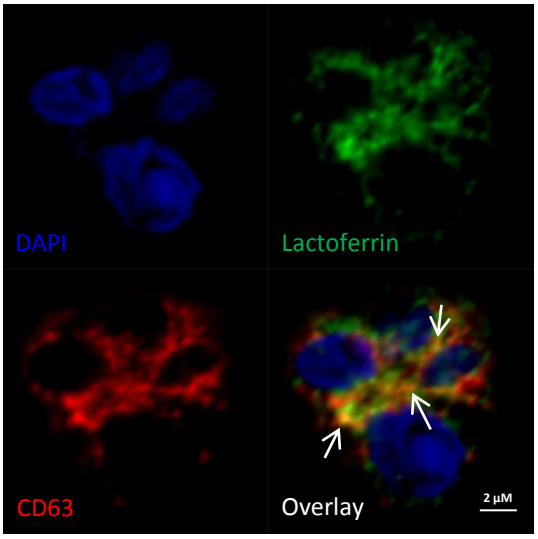

C
TNF-α
Surface CR3 (% of TNF-α control)
140
120
100
80
60
40
20
0
+DEDA
+PACOCF3
+PYR

Figure 7

A


PMA
O_2^-
DEDA / PACOCF$_3$ / Pyrrolidin
PKC
LF
O_2^-
PLA$_2$
O_2^-
MPO
CD63

B


DAPI
Lactoferrin
CD63
Overlay
2 µM