cells

MDPI

Article

Possible Involvement of Intracellular Calcium-Independent Phospholipase A_2 in the Release of Secretory Phospholipases from Mast Cells—Increased Expression in Ileal Mast Cells of Crohn's Disease

Ulrika Christerson [1], Åsa V. Keita [2], Martin E. Winberg [2], Johan D. Söderholm [2,3] and Christina Gustafson-Svärd [1]

1 Department of Chemistry and Biomedical Sciences, Faculty of Health and Life Sciences, Linnaeus University, 391 82 Kalmar, Sweden
2 Department of Clinical and Experimental Medicine, Division of Surgery, Orthopedics & Oncology, Linköping University, 581 85 Linköping, Sweden
3 Department of Surgery, County Council of Östergötland, 581 85 Linköping, Sweden
* Correspondence: asa.keita@liu.se; Tel.: +46101038919

Received: 23 May 2019; Accepted: 1 July 2019; Published: 3 July 2019

Abstract: Increased activity of secretory phospholipases A_2 (sPLA$_2$) type-II was previously observed in ileum of Crohn's disease (CD). Our aims were to explore the involvement of calcium-independent (i)PLA$_2$β in the release of sPLA$_2$s from the human mast cell (MC) line (HMC-1) and investigate expressions of cytosolic (c)PLA$_2$α, iPLA$_2$β, sPLA$_2$-IIA and sPLA$_2$-V in MCs of CD ileum. The release of sPLA$_2$ was investigated in HMC-1 by immunocytochemistry and ELISA. The expression intensities of PLA$_2$s in mucosal MCs, and the proportion of PLA$_2$-positive MCs, were investigated in normal ileum and in ileum from patients with CD by immunohistochemistry. The calcium ionophore-stimulated release of sPLA$_2$-IIA and sPLA$_2$-V from HMC-1 was reduced by the iPLA$_2$-inhibitor bromoenol lactone. All four PLA$_2$s were detectable in mucosal MCs, both in normal ileum and in CD, but the proportion of iPLA$_2$β-containing mucosal MCs and the expression intensity of sPLA$_2$-IIA was increased in CD. Results indicate that iPLA$_2$β is involved in the secretion of sPLA$_2$s from HMC-1, and suggest that iPLA$_2$β-mediated release of sPLA$_2$ from intestinal MCs may contribute to CD pathophysiology. Ex vivo studies on isolated mucosal mast cells are however needed to clarify the precise role of MC PLA$_2$s in the inflammatory processes of CD.

Keywords: phospholipases A_2; mast cells; Crohn's disease; inflammation

1. Introduction

Mediators released from activated intestinal mast cells (MCs) have shown to be of pathophysiological significance in Crohn's disease (CD) [1,2], for instance, by promoting intestinal fibrosis or by decreasing the mucosal barrier against immune-activating antigens [3–5]. However, MC mediators do not necessarily have only detrimental effects in CD, since intestinal MCs also are thought to have a role in host defense against bacterial, viral and parasitic agents [5]. MC mediators of potential relevance for inflammatory conditions include, for instance, eicosanoids [6] and other lipid mediators (i.e., platelet-activating factor and lysophospholipids) generated upon activation of one or several isoforms of the phospholipase A_2 superfamily (PLA$_2$) [7]. The expression of different PLA$_2$ isoenzymes in MCs of the human intestinal mucosa is still unknown, both in the normal intestine and in CD.

The PLA_2s constitute a superfamily of intracellular and secretory isoenzymes that catalyzes hydrolysis of the *sn*-2 ester of glycerophospholipids, thereby producing free fatty acids and lysophospholipids [7]. The high molecular weight intracellular PLA_2s, cytosolic PLA_2 ($cPLA_2$; also named group IV PLA_2) and calcium-independent PLA_2 ($iPLA_2$; also named group VI PLA_2), are ubiquitously expressed in most tissues and cell types [7]. Among the six different $cPLA_2$s known, $cPLA_2\alpha$ (also named group IVA $cPLA_2$) is by far the most studied and evaluated [8]. $cPLA_2\alpha$ shows marked preference for arachidonic acid (AA) over other fatty acids [8] and is activated by an increase in cytosolic free calcium and phosphorylation [8]. Since $cPLA_2\alpha$ is AA-specific, it is generally assumed to be the major contributor to the production of inflammatory eicosanoids [8]. In contrast to $cPLA_2$, $iPLA_2$ shows no strict AA specificity [9], and does not require calcium for its enzymatic activity. $iPLA_2$ has been suggested to have diverse biological functions [9], including release of AA for eicosanoid production [9,10] and participation in various neurodegenerative disorders and inflammatory responses [9]. Until today seven $iPLA_2$s have been identified, $iPLA_2\beta$ (also named group VI-1 and 2 $iPLA_2$) being the most widely evaluated [7,9]. Most interesting, a recent study on mice [11] showed that $iPLA_2\beta$ deficiency increased colitis severity and ileal damage in DSS-induced colitis, suggesting a protective role for $iPLA_2\beta$ in the intestinal mucosa. Indeed, this study [11] points to the importance of further investigations concerning the specific roles of individual PLA_2 isotypes in inflammatory bowel disease (IBD) [12]. To date, no studies on $iPLA_2$ expression or activity in human MCs have been reported.

The mammalian secretory PLA_2s, ($sPLA_2$s) constitute a group of at least eleven different low molecular weight isoforms [13]. They are all Ca^{2+}-dependent and show no apparent fatty acid selectivity [7,13,14]. Individual $sPLA_2$s exhibit unique tissue and cellular localizations and their expression varies among species [14]. $sPLA_2$s have been investigated in several studies on rodent MCs [15–17], whereas only a few studies on $sPLA_2$s in human MCs have been reported so far [18–20]. $sPLA_2$s released to the environment are thought to act in both an autocrine and a paracrine manner [14], and the resulting cellular activities have frequently been associated with various inflammatory conditions [14]. However, the $sPLA_2$s have several diverse functions and in addition to their proposed inflammatory actions they seem to have protective and anti-inflammatory functions as well [14,21,22]. Although $sPLA_2$s release fatty acids from glycerophospholipids, generating lysophospholipids and AA for eicosanoid synthesis [14,23], they may also act by receptor-mediated, non-catalytic, mechanisms [14].

Rodent MCs have shown to express several different $sPLA_2$s, including the two closely related isotypes $sPLA_2$-IIA and $sPLA_2$-V [24], but it is still not known which particular $sPLA_2$s are expressed by human intestinal mucosal MCs. If released from mucosal MCs, however, it seems reasonable to believe that $sPLA_2$s may, in one way or another, participate in modulating the inflammatory process of the intestinal CD mucosa. It is important, therefore, to investigate which particular $sPLA_2$s are present in MCs of the human intestinal mucosa and how the release of these $sPLA_2$s is regulated. Since $iPLA_2$ has shown to participate in processes related to exocytosis and release of enzymes [10,25–28] it is relevant to investigate if this PLA_2 is implicated also in the release of $sPLA_2$s from MCs.

The aims were to explore the possible involvement of $iPLA_2\beta$ in the release of $sPLA_2$s from human MCs using a human MC line (HMC-1) [29] and to investigate the expressions of $cPLA_2\alpha$, $iPLA_2\beta$, $sPLA_2$-IIA and $sPLA_2$-V in mucosal MCs from normal and CD ileum.

2. Materials and Methods

2.1. Cell Culture

The human leukemia MC line-1, HMC-1 [29], was a kind gift from Dr. J.H Butterfield, Mayo Clinic, MN. Cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco BRL, Gaithersburg, MD, USA) supplemented with 100 µg/mL streptomycin (Gibco), 100 U/mL penicillin (Gibco), 10% fetal bovine serum (Gibco), and 1.2 mM α-thioglycerol (Sigma-Aldrich, St. Louis, MO, USA) and kept in a humidified atmosphere with 5% CO_2 at 37 °C. Cell viability was routinely evaluated by the trypan blue

exclusion assay or by a MTT toxicology assay and was not affected during the experimental conditions used in this study.

To investigate if the expressions of iPLA$_2$β and cPLA$_2$α could be further increased upon activation of the MCs, 5 × 10^5 HMC-1 were incubated for 48h in 1 mL culture medium with or without (controls) 25 ng/mL of TNFα. TNFα is of fundamental importance in inflammatory conditions such as CD [30], and may influence PLA$_2$ expression and activity [31,32]. The expressions of cPLA$_2$α, iPLA$_2$β, sPLA$_2$-IIA and sPLA$_2$-V were after incubation analyzed by Reverse Transcriptase-PCR and immunocytochemical staining.

2.2. Reverse Transcriptase-PCR of PLA$_2$s

Total RNA was extracted from HMC-1 using Ultraspec™-II RNA Isolation System (Nordic Biosite, Täby, Sweden). One µg of total RNA was converted into cDNA using Omniscript® Reverse Transcription RT Kit (Qiagen, Solna, Sweden) according to the manufacturer's instructions, and amplified using PuRe Taq RTG PCR beads (GE Healthcare, Buckinghamshire, UK) and primers (Life Technology Ltd., Paisley, UK). Due to a high expression, the cDNA for sPLA$_2$-IIA had to be diluted 10× before subjected to conventional Reverse Transcriptase-PCR. Primers and running schedules used in PCR are summarized in Table 1. The final PCR products were loaded on 1.5% agarose gels, and identified as previously described [33].

Table 1. Primers and running schedules used in Reverse Transcriptase-PCR.

Gene	Primers (5′ > 3′)	Product (bp)	Running Scheme [a]
iPLA$_2$β	F: AAGGCCTCATCATCATCCAG R: CGGAACACCTCATCCTTCAT	184	40 cycles: 94 °C, 30 s; 60 °C, 30 s; 72 °C, 30 s
cPLA$_2$α	F: ATGCCCAGACCTACGATTTA R: AGGGGTTTTCTTCATACTTC	737	40 cycles: 94 °C, 30 s; 55 °C, 30 s; 72 °C, 50 s
sPLA$_2$-IIA	F: AAGCCGCACTCAGTTATGG R: GCAGCAGCCTTATCACACT	238	25 cycles: 94 °C, 30s; 55 °C, 30 s; 72 °C, 30 s
sPLA$_2$-V	F: GCTTGGTTCCTGGCTTGTAG R: ACTCGCTGGAGGGTACAGTG	559	30 cycles: 94 °C, 30 s; 55 °C, 30 s; 72 °C, 40 s
18S-rRNA	F: ACGRACCAGAGCGAAAGCAT R: GGACATCTAAGGGCATCACAGAC	531	20 cycles: 94 °C, 20 s; 58 °C, 20 s; 72 °C, 45 s

[a] The first cycle was preceded by an initial denaturation step at 94 °C for 5 min, and the last cycle was followed by an elongation step at 72 °C for 5 or 7 (cPLA$_2$) min.

2.3. Immunocytochemical Staining of PLA$_2$s

HMC-1 were smeared on poly-*L*-lysine coated glass (Sigma) as previously described [33]. The samples were fixed in ice-cold acetone for 5 min at –20 °C and then blocked with 50% of serum in PBS for 1h at room temperature (RT). The samples were incubated with either 1:50 mouse monoclonal FITC-conjugated anti-human sPLA$_2$-V antibody (Santa Cruz, Dallas, Texas, USA) or 1:200 mouse monoclonal anti-human sPLA$_2$-IIA (Cayman Chemical Co, Ann Arbor, MI, USA) for 16h at 4 °C. Biotin-conjugated 1:250 secondary rabbit anti-mouse (DakoCytomation, Glostrup, Denmark) was applied to samples with sPLA$_2$-IIA antibody for 1h at RT and then 1:100 FITC-conjugated streptavidin (DakoCytomation) for 30 min at RT. In addition, samples were incubated with either 1:100 Alexa-488 conjugated mouse monoclonal anti-human cPLA$_2$α (Santa Cruz) or 1:250 rabbit polyclonal anti-human iPLA$_2$β (Cayman) for 16h at 4 °C. FITC-conjugated secondary antibody goat anti-rabbit (Jackson ImmunoResearch Laboratories Inc, West Grove, PA, USA) was applicated at a dilution of 1:400. The slides were mounted with Vectashield® mounting medium with propidium iodide (Vector Laboratories Inc, Burlingame, CA, USA). Negative controls without primary antibodies or with a FITC-conjugated isotype matched irrelevant antibody (Santa Cruz) were included in all experiments.

2.4. Release of Fatty Acids

To further explore the involvement of $cPLA_2$ in AA-mobilization in activated HMC-1, ^{14}C-AA labelled cells were stimulated with the frequently used MC activator calcium ionophore A23187 [10,34–38], in the presence and absence of known enzyme inhibitors. Cells were suspended in 25 mL supplemented medium with 0.1% fatty-acid free bovine serum albumin (Sigma) and labelled for 16 h with 0.1 μCi [1-^{14}C]AA (New England Nuclear, Perkin Elmer, Wellesley, MA, USA) per 5×10^5 cells, before washed two times with PBS supplemented with 0.1% fatty-acid free bovine serum albumin [37]. Labelled cells (5×10^5 cells in a final volume of 2.7 mL) were then treated for 4h with 2 μM of the calcium ionophore A23187 (Sigma) only, or in combination with 200 nM of the protein kinase C activator phorbol myristate acetate (PMA) (Sigma). The combination of A23187 and PMA has previously shown to induce a synergistic release of AA in other cell systems, an effect attributed to an increased activation of $cPLA_2$ [37,39,40].

As an attempt to investigate the relative contribution of $cPLA_2$ and $iPLA_2$ in the A23187-stimulated AA release, cells were pre-incubated with the combined $cPLA_2$ and $iPLA_2$ inhibitor methyl arachidonyl fluoro-phosphonate (MAFP) (Sigma) [41], or the specific $iPLA_2$ inhibitor bromoenol lactone (BEL) (Sigma) [41]. Cells were pre-treated for 30 min with MAFP (0 μM, 10 μM or 25 μM) or BEL (0 μM, 10 μM or 25 μM) prior to incubation with A23187 (2 μM) for an additional 4 h. All treatments with stimulators and inhibitors were performed in the absence of serum but in the presence of 0.1% fatty acid-free bovine serum albumin. The amount of ^{14}C-AA released into the culture medium was analyzed by liquid scintillation counting. The inhibitors were added 30 min prior to adding the stimulators. To evaluate the AA specificity of the involved PLA_2, a comparable stimulation of ^{14}C-oleic acid (OA) (Perkin Elmer) labelled cells was performed.

2.5. Degranulation and Release of $sPLA_2$

Cellular events leading to an increased cytosolic Ca^{2+} concentration may stimulate degranulation of MCs [42]. Therefore, we next investigated if $sPLA_2$-IIA and V were released from A23187-stimulated HMC-1. HMC-1 (5×10^5 cells in a final volume of 150 μL) were treated with A23187 (0 μM, 1 μM, 2 μM, 4 μM) for 4 h.

To investigate if $iPLA_2$ is involved in the ionophore-stimulated $sPLA_2$ secretion in HMC-1, 25 μM of the inhibitor BEL was added 30 min before A23187, when appropriate. All treatments with stimulators and inhibitors were performed in the absence of serum. Cells were centrifuged and the medium was collected. The β-hexosaminidase activity was determined as previously described [43], and the amount of $sPLA_2$-IIA was determined by sandwich-ELISA according to the manufacturer's instructions (Cayman).

The amounts of remaining $sPLA_2$-IIA and $sPLA_2$-V in stimulated cells were investigated by immunocytochemical staining as described above. Due to its low basal expression, $sPLA_2$-V had to be upregulated by 25 ng/mL TNFα (Sigma) for 48h prior to stimulation with A23187 in this set of experiments.

2.6. Patients

Specimens from ileum were achieved during surgery at Linköping University Hospital from 5 patients with ileal CD and 5 patients with colonic cancer, as non-IBD controls. The CD patients constituted of 3 men and 2 women with a median age of 53 years (range 43–65) and disease duration of 15 years (range 9–28). According to the Montreal classification, all patients had an active disease, however, tissue obtained for analyzes were dissected from mild-inflamed ileum. The non-IBD control group constituted of microscopically normal ileal specimens from 3 men and 2 women with a median age of 71 years (range 62–87). None of the patients within the non-IBD control group had received pre-operative chemo- or radiotherapy or had signs of generalized disease. The study was approved

by the Committee of Human Ethics, Linköping (ethical number 02-154, 09/04/2002) and all included subjects gave their informed written consent before the study was initiated.

2.7. Preparation of Ileal Tissues

Surgical ileal specimens from patients with CD and non-IBD controls were immediately after division of the ileocolic artery, put in ice-cold oxygenated Krebs buffer and specimens were stripped of external muscle and myenteric plexus, as previously described [44]. Segments of ileal mucosa were fixed in 4% buffered formaldehyde in PBS for 24h in 4 °C, embedded in paraffin and sectioned to a thickness of 5 µm.

2.8. Immunohistochemical Staining of PLA_2s

Slides with sections were hydrated according to standard procedures followed by incubation for 10 min with background sniper (Histolab, Gothenburg, Sweden). After washed in PBS, slides were incubated for 16h at 4 °C with 1:200 mouse monoclonal-anti-human MC tryptase antibody (Santa Cruz) in combination with either 1:50 rabbit polyclonal-anti-human $sPLA_2$-IIA (Novus Biologicals, Bio-Techne, Abingdon, UK), 1:50 rabbit polyclonal-anti-human $sPLA_2$-V (Bio-Techne), 1:50 goat polyclonal $cPLA_2\beta$ antibody (Santa Cruz), or 1:50 rabbit polyclonal-anti-human $iPLA_2\beta$ (Santa Cruz). Slides were rinsed and incubated with secondary antibodies (MC: 1:4 ready to use Alexa Fluor 594-conjugated-goat-anti-mouse (Invitrogen, Oregon, USA); $cPLA_2\beta$: 1:200 Alexa Fluor 488-conjugated donkey-anti-goat (Life technologies); $iPLA_2\beta$, $sPLA_2$-IIA, $sPLA_2$-V: 1:200 Alexa Fluor 488-conjugated donkey-anti-rabbit (Life technologies) for 1h at RT. After repeated rinsing, slides were mounted with Prolong® Gold Antifade with DAPI (Life Technologies) and evaluated in a Nikon E800 fluorescence microscope connected to software NIS elements (Nikon Instruments Inc. Tokyo, Japan) in a blinded fashion by two independent researchers. Three sections per individual were stained for each double-staining, and negative controls with primary antibodies excluded were included in all experiments. The total number of MCs co-localizing with the different PLA_2s were manually quantified at 600× magnification. The intensities of the different PLA_2-stainings were measured by Image J Fiji software (National Institutes of Health, Bethesda, MD, USA). Approximately 6–8 area-units per section were counted. All area-units were of the same size and only area-units that were fully covered by tissue were used.

2.9. Statistical Analysis

Data were analyzed using the GraphPad Prism Software (GraphPad Software Inc., CA, USA). Parametric data are expressed as mean ± SEM and depending on the experimental layout, statistical analyses were undertaken with one-way ANOVA, repeated measures ANOVA, and Bonferroni post-test. Non-parametric data are given as median (25th–75th interquartile range) and comparisons between groups were done with Kruskal-Wallis and Mann-Whitney U tests.

3. Results

3.1. $iPLA_2$ is the Predominating High-Molecular-Weight PLA_2 Expressed by HMC-1

HMC-1 was found to have a basal expression of both $iPLA_2\beta$ mRNA (Figure 1A) and $iPLA_2\beta$ protein (Figure 1B). In contrast, $cPLA_2\alpha$ revealed no basal mRNA expression (Figure 1A), and the protein expression was very low (Figure 1B). Treatment with 25 ng/mL TNFα for 48 h did neither affect the $iPLA_2\beta$ mRNA expression (Figure 1A) nor the $iPLA_2\beta$ protein expression (Figure 1B). On the contrary, TNFα stimulation had an inconsistent effect on the $cPLA_2\alpha$ expression, increasing the mRNA stimulation had an inconsistent effect on the $cPLA_2\alpha$ expression, increasing the mRNA expression (Figure 1A) without affecting the protein expression (Figure 1B).

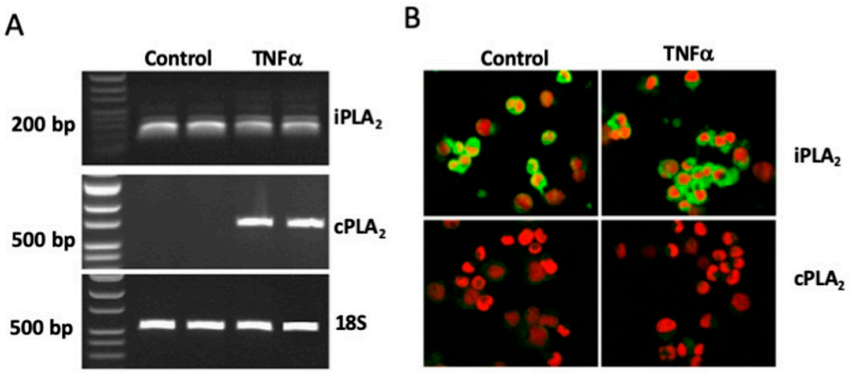

Figure 1. Expression of iPLA$_2$β and cPLA$_2$α in HMC-1. Cells were stimulated with TNFα (25 ng/mL) or culture medium (control) for 48 h. (**A**) Reverse Transcriptase-PCR analysis; PCR products were identified as iPLA$_2$α (184 bp), cPLA$_2$β (737 bp) or 18S rRNA (531 bp). Results are presented as duplicate samples representative of three independent experiments. (**B**) Immunocytochemical analysis; green staining is for either iPLA$_2$β or cPLA$_2$α. Cell nuclei were visualized with propidium iodide staining (red) (magnification × 600). Results are representative for three independent experiments.

3.2. Secretory PLA$_2$-IIA and V are Expressed by HMC-1

Immunostaining revealed a basal expression of sPLA$_2$-IIA mRNA (Figure 2A) and sPLA$_2$-IIA protein (Figure 2B) in HMC-1. Neither the mRNA nor the protein expression was affected in cells stimulated with 25 ng/mL TNFα for 48h (Figure 2A,B). HMC-1 were also found to have a basal expression of sPLA$_2$-V mRNA (Figure 2A) and sPLA$_2$-V protein (Figure 2B), although, less pronounced as compared to corresponding expressions of sPLA$_2$-IIA (Figure 2A,B). However, in contrast to sPLA$_2$-IIA, the expressions of sPLA$_2$-V mRNA and proteins were increased in TNFα-stimulated cells (Figure 2A,B).

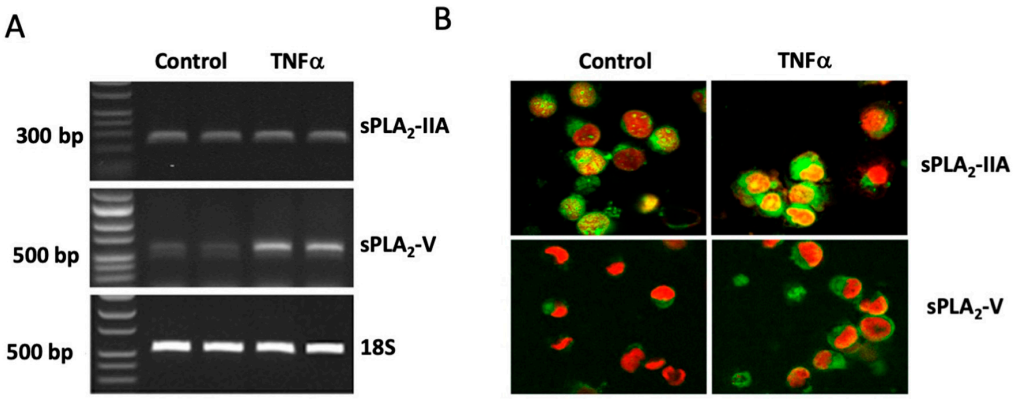

Figure 2. Expression of sPLA$_2$-IIA and sPLA$_2$-V in HMC-1. Cells were either stimulated with TNFα (25 ng/mL) or culture medium (control) for 48 h. (**A**) Reverse Transcriptase-PCR analysis; the PCR products were identified as sPLA$_2$-IIA (238 bp), sPLA$_2$-V (559 bp) or 18S rRNA (531 bp). Note that the cDNA for sPLA$_2$-IIA was diluted ten times compared to the cDNA for sPLA$_2$-V. Samples are two representatives out of seven independent runs. (**B**) Immunocytochemical analysis. Green staining is for sPLA$_2$-IIA or sPLA$_2$-V and red staining is for visualization of cell nuclei (magnification × 600). Results are representative for three independent experiments.

3.3. cPLA$_2$α is not Involved in Calcium Ionophore-Stimulated AA Mobilization in HMC-1

Stimulation with calcium ionophore A23187 caused an obvious time-dependent increase in the release of radioactivity from ^{14}C-AA-labelled cells (Figure 3A). The increase was discernible after 1 h but not significant until 4 h of treatment compared to controls at each time point (Figure 3A). cPLA$_2$α is generally regarded as the main regulator of cellular AA mobilization [8], however, a comparable

release of radioactivity also from A23187-stimulated ^{14}C-OA-labeled HMC-1 clearly demonstrated that the ionophore-stimulated PLA_2 activity was not AA-specific (Figure 3B). Stimulation with the combination of A23187 and the protein kinase C activator PMA showed that PMA had no further impact on the A23187-stimulated AA release, neither at 30 min (data not shown) nor at 4 h (Figure 3C).

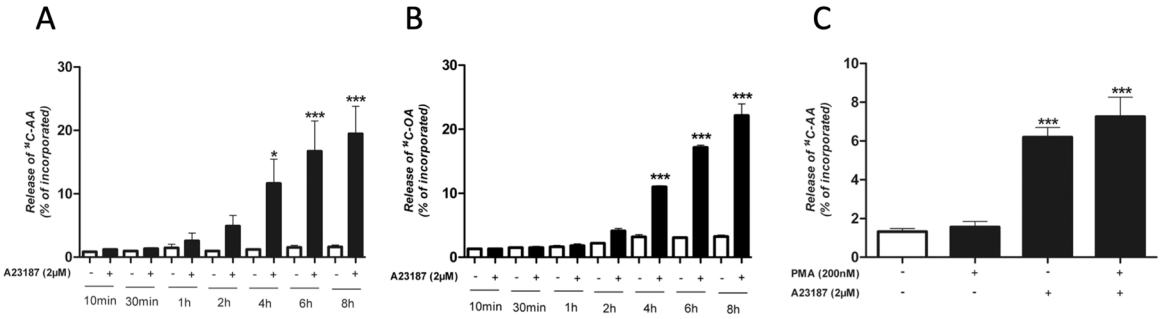

Figure 3. Release of radiolabeled fatty acids from A23187-stimulated HMC-1 cells. Control cells were incubated with culture medium only. (**A**) Time-dependent release of arachidonic acid (AA). (**B**) Time-dependent release of oleic acid (OA). (**C**) Effect of combined stimulation with calcium ionophore A23187 and phorbol myristate acetate (PMA) on the release of AA. PMA and/or A23187 were added for 4 h. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control cells. Data from three independent experiments.

The PLA_2-inhibitors MAFP (general) and BEL ($iPLA_2$-specific) were found to reduce the A23187-stimulated AA release in a dose-dependent manner and at a comparable extent (Figure 4A,B).

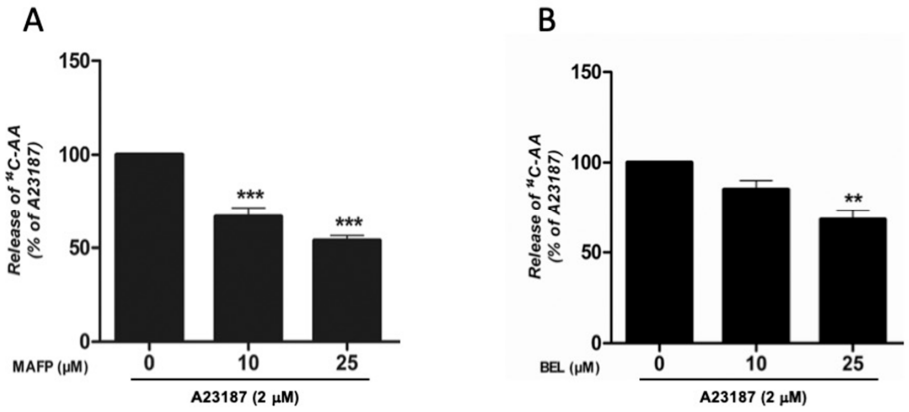

Figure 4. Effect of PLA_2 inhibitors on the calcium ionophore A23187-stimulated release of radiolabeled arachidonic acid (AA) from HMC-1. Cells were pre-treated for 30 min with various concentrations of PLA_2 inhibitors, prior to incubation with A23187 (2 μM) for an additional 4 h. (**A**) Effect of the combined $cPLA_2$ and $iPLA_2$ inhibitor methyl arachidonyl fluoro-phosphonate (MAFP). (**B**) Effect of the specific $iPLA_2$ inhibitor bromoenol lactone (BEL). *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. A23187-stimulated cells. Data from three independent experiments.

3.4. $iPLA_2$ is Involved in the A23187-Stimulated Release of $sPLA_2$-IIA and $sPLA_2$-V from HMC-1

Stimulation with A23187 induced degranulation of the HMC-1 cells in a dose-dependent manner, demonstrated as an increased β-hexosaminidase release (Figure 5A). Simultaneously, A23187 caused a dose-dependent release of $sPLA_2$-IIA, as detected by ELISA (Figure 5B) and further confirmed by immunocytochemical visualization (Figure 5C). In addition, A23187 caused a dose-dependent release of $sPLA_2$-V, as visualized by immunocytochemistry (Figure 5C). Due to the low basal expression of $sPLA_2$-V, the immunocytochemistry was performed after up-regulation of $sPLA_2$-V with TNFα, as illustrated in Figure 2B.

Pre-incubation with the $iPLA_2$-specific inhibitor BEL prior to A23187 stimulation, diminished both the degranulation of HMC-1 (Figure 6A) and the release of $sPLA_2$-IIA and $sPLA_2$-V (Figure 6B,C).

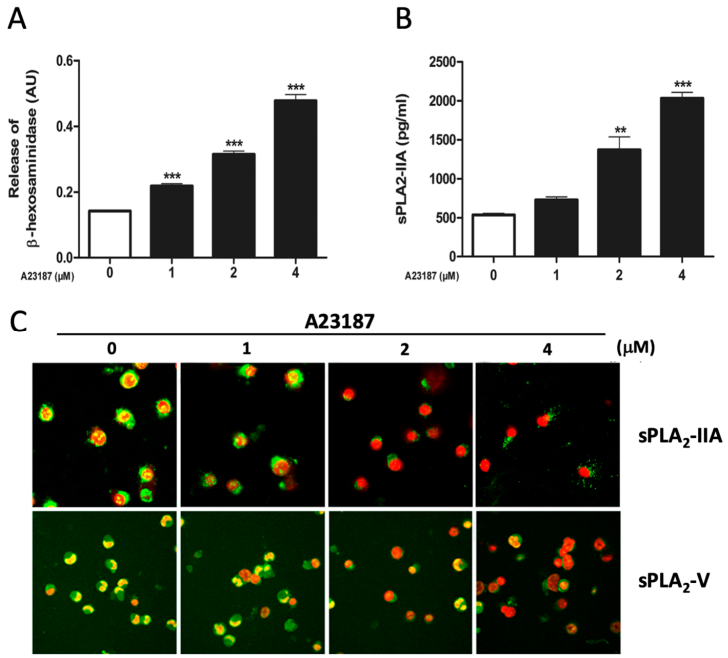

Figure 5. Degranulation and release of $sPLA_2$-IIA and $sPLA_2$-V in A23187-stimulated HMC-1. Cells were stimulated for 4 h with various concentrations of calcium ionophore A23187. Control cells were incubated with culture medium only. (**A**) Release of β-hexosaminidase. (**B**) ELISA analysis. Release of $sPLA_2$-IIA. (**C**) Immunocytochemical analysis, visualizing the effect of A23187 on the release of $sPLA_2$-IIA and $sPLA_2$-V. Green staining is for $sPLA_2$-IIA or $sPLA_2$-V and red staining is for visualization of cell nuclei (magnification × 600). Note that the expression of $sPLA_2$-V had to be upregulated by TNFα, as described in Figure 2A and B. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. controls. Data from three independent experiments.

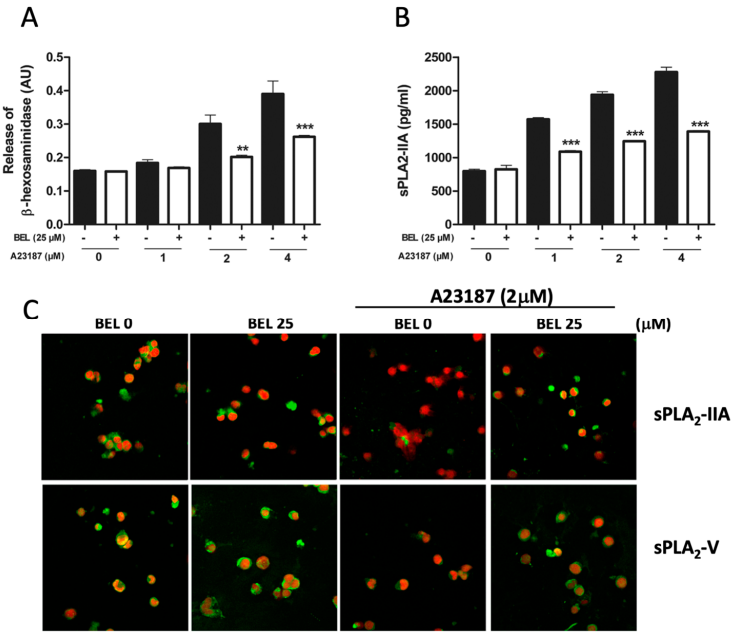

Figure 6. Effect of $iPLA_2$ inhibition on A23187-induced degranulation and release of $sPLA_2$-IIA and $sPLA_2$-V in HMC-1. (**A**) Effect of the specific $iPLA_2$ inhibitor bromoenol lactone (BEL) on the release of β-hexosaminidase. (**B**) Effect of the specific $iPLA_2$ inhibitor BEL on the release of $sPLA_2$-IIA measured by ELISA. (**C**) Immunocytochemical analysis, visualizing the effect on the release of $sPLA_2$-IIA and $sPLA_2$-V. Green staining is for $sPLA_2$-IIA or $sPLA_2$-V and red staining is for visualization of cell nuclei (magnification × 600). Note that the expression of $sPLA_2$-V had to be upregulated by TNFα, as described in Figure 2A and B, to be illustrated. ** $p < 0.01$, *** $p < 0.001$ vs. controls. Data from three independent experiments.

3.5. Mucosal MCs Express all four PLA_2 Isoforms Investigated

Cells positively stained with the MC tryptase antibody were found in both control and CD ileal mucosa. MCs from controls and CD patients were found to express all four PLA_2 isoforms investigated, i.e., the two intracellular high molecular isoforms, cPLAα and iPLA$_2\beta$, and the two secretory isoforms, sPLA$_2$-IIA and sPLA$_2$-V (Figure 7A–D). Both intracellular and secretory PLA$_2$s were also found on cells not positive for MC tryptase, and in addition, there were MCs present not expressing any PLA_2.

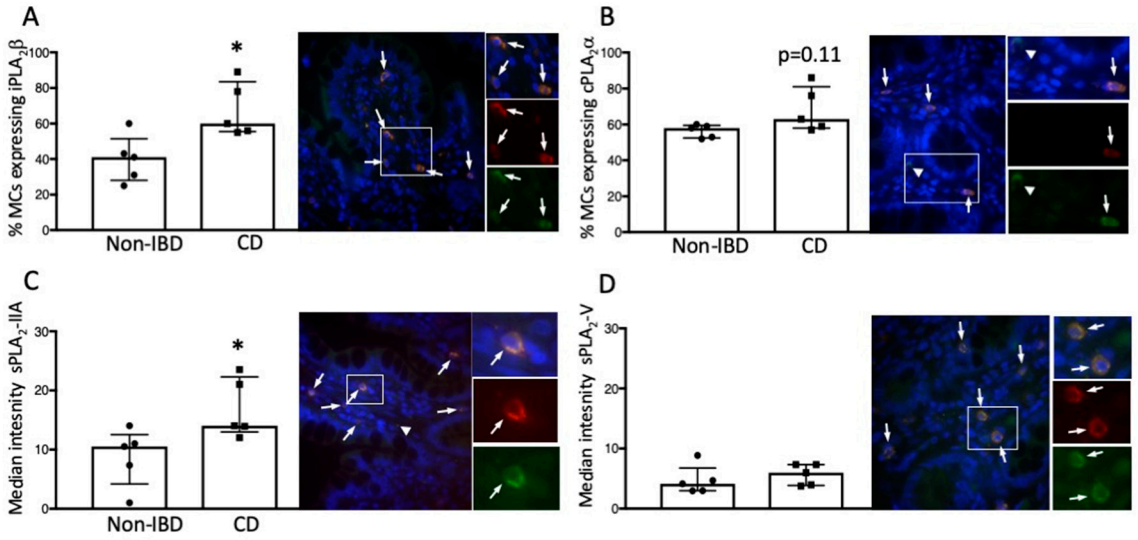

Figure 7. Expression of iPLA$_2\beta$, cPLA$_2\alpha$, sPLA$_2$-IIA and sPLA$_2$-V on mast cells (MCs) in the intestinal mucosa of 5 patients with Crohn's disease (CD) and 5 controls. (**A**) Percentage of MCs expressing iPLA$_2\beta$. Arrows indicate MCs co-localizing with iPLA$_2\beta$ in a patient with CD. (**B**) Percentage of MCs expressing cPLA$_2\alpha$. Arrows indicate MCs co-localizing with cPLA$_2\alpha$ in a control patient. Arrow-head indicates cPLA$_2\alpha$ expression in a cell not positive for MC tryptase. (**C**) Expression intensity of sPLA$_2$-IIA on MCs. Arrows indicate MCs co-localizing with sPLA$_2$-IIA in a patient with CD. Arrow-head indicates sPLA$_2$-IIA expression in a cell not positive for MC tryptase. (**D**) Expression intensity of sPLA$_2$-V on MCs. Arrows indicate MCs co-localizing with sPLA$_2$-V in a control patient.

MC and PLA_2 expressions were quantified manually at 600× magnification and results are given as median (25th–75th percentile). Red = MCs, Green = PLA_2, Blue = DAPI, nuclei staining. *$p < 0.05$ vs. controls.

3.6. Increased Proportion of iPLA$_2\beta$-Containing Mucosal MCs of CD Ileum

For the intracellular forms there was a higher percentage of MCs expressing iPLA$_2\beta$ in CD compared to controls, $p < 0.05$ (Figure 7A), but no significant difference in expressions of cPLA$_2\alpha$, $p = 0.11$ (Figure 7B). Measurements of intensity (Median (25th–75th percentile)) showed no difference between the groups (iPLA$_2\beta$: CD 13.1 (12.1–16.3); non-IBD 12.9 (11.2–14.5), $p = 0.69$, and cPLAα; CD 21.8 (18.1–32.3); non-IBD 17.4 (14.2–20.9), $p = 0.22$).

3.7. Increased Expression Intensity of sPLA$_2$-IIA in Mucosal MCs of CD Ileum

For the secretory PLA$_2$s, there was no difference in the percentage of MCs expressing either sPLA$_2$-IIA (Non-IBD 71.0% (52.3–74.1); CD 69.0 (48.5–73.5)) or sPLA$_2$-V (Non-IBD 37.0 (25.5–57.5); CD 50.0 (30.0–50.1)). In contrast, intensity measurements showed a significantly higher expression intensity of sPLA$_2$-IIA in MCs of CD patients compared to controls, $p < 0.05$ (Figure 7C), but no difference between groups in the expression of sPLA$_2$-V (Figure 7D).

4. Discussion

The present study demonstrates, for the first time, that human ileal MCs of normal and CD mucosa contain the sPLA$_2$ isoforms sPLA$_2$-IIA and sPLA$_2$-V, as well as the intracellular high molecular isoforms cPLA$_2\alpha$ and iPLA$_2\beta$. In addition, studies on the human MC cell line HMC-1 demonstrated that iPLA$_2\beta$ might have a role in the release of sPLA$_2$-IIA and sPLA$_2$-V. Thus, our results point to a possible role of iPLA$_2\beta$ in the release of sPLA$_2$s from MCs of the human ileal mucosa.

sPLA$_2$-IIA and V are frequently associated with inflammatory conditions [14,23]. Even though sPLA$_2$-II is known to be present in the CD intestine [45,46], including submucosal MCs [18], no studies on sPLA$_2$-V expressions in CD intestine, or sPLA$_2$-II expressions in intestinal mucosal MCs, have been reported. We previously demonstrated [46] that the distal ileal mucosa is rich in PLA$_2$-II mRNA and that the expression of this mRNA and the corresponding enzyme activity accompanies recurrent new ileal inflammation after ileocolonic resection for CD. However, the cells responsible for this increased expression and activity have previously not been identified. In the present study we demonstrated that the expression of sPLA$_2$-IIA was higher in MCs from ileal CD mucosa compared to MCs from control patients. Further, we found that the proportion of iPLA$_2\beta$-expressing mucosal MCs was increased in CD ileum compared with controls; i.e., among all MCs present, more MCs expressed iPLA$_2\beta$ in ileum from CD patients. These findings suggest that MCs may contribute to the increased sPLA$_2$-II expression and activity in CD ileum [46].

Although iPLA$_2\beta$ is generally thought to be involved in various cellular and pathological conditions [9], its expression and role in the human intestine has never been investigated. However, our results on HMC-1 support previous findings demonstrating a possible role for iPLA$_2\beta$ in MC exocytosis [10], and one might speculate that the increased proportion of iPLA$_2\beta$-expressing MCs found in CD may reflect a greater release of various MC mediators in the CD intestine. Intestinal barrier dysfunction, leading to increased transfer of luminal bacteria to the lamina propria is thought to be a factor of importance in the pathogenesis of CD [47]. Considering the proposed protective role of iPLA$_2\beta$ in the intestine [11,12], it is tempting to speculate that iPLA$_2\beta$ might have a role in releasing bactericidal sPLA$_2$s from MCs in the intestinal mucosa. Indeed, several sPLA$_2$s, in particular sPLA$_2$IIA, are known to have antibacterial activities [7,13,21].

Considering the proposed species differences with regard to both MC characteristics [48] and PLA$_2$ expression [14] a human experimental MC cell model was used for the studies on sPLA$_2$ release. Although various aspects of PLA$_2$s have been extensively studied in rodent MCs [10,15–17,38,49–51], not much is known about the expression and regulation of these enzymes in MCs of human origin. We chose to work with the human MC cell line HMC-1 [29] because it has been frequently used for studies on various aspects of MC biology, and this cell line has been reported to produce several different eicosanoids upon stimulation with calcium ionophore [35,36]. However, the PLA$_2$s responsible for generating the required free AA is not known, and studies concerning the expression and activity of PLA$_2$-enzymes of HMC-1 are still lacking. It was necessary thus to confirm the presence of intracellular and secretory PLA$_2$s in this cell line before using it for studies on sPLA$_2$ release. Interestingly the HMC-1 was found to have a basal expression of iPLA$_2\beta$ protein, whereas the expression of cPLA$_2\alpha$ was very low. Neither the protein nor the mRNA expression of iPLA$_2\beta$ was apparently affected by TNFα. This lack of effect of TNFα suggests that iPLA$_2\beta$ is not regulated by inflammatory agents in HMC-1, a finding well in line with the proposed role of iPLA$_2\beta$ as a homeostatic enzyme in cellular phospholipid metabolism [9]. In contrast, TNFα increased the mRNA but not the protein expression of cPLA$_2\alpha$. Thus, increasing the level of cPLA$_2\alpha$ mRNA in HMC-1 seems not to per se induce translation into cPLA$_2\alpha$ protein, but additional stimulators of translation seem to be needed. These findings are in line with a previous study [52], showing that transforming growth factor β-1 stimulates cPLA$_2$ gene expression in human intestinal MCs without affecting the level of cPLA$_2$ protein. The translation of gene expressions to protein levels is a multistep process and Schwanhausser et al. [53] has concluded that translational rate constants were the dominant factors in controlling protein levels, and that half-life of the proteins are highly involved in the translation as well. In addition to the findings of

increased iPLA$_2$β, the HMC-1 were found to have a basal expression of both sPLA$_2$-V and sPLA$_2$-IIA, which is in line with previous reports on rodent MCs [16] and human lung MCs [20]. However, when HMC-1 were stimulated with TNFα, both mRNA and protein expressions of sPLA$_2$-V were increased, whereas the mRNA and protein levels of sPLA$_2$-IIA was unaltered. Our results on HMC-1 are in line with previous studies showing that despite close similarities between group IIA and V [14], their expression and regulation may differ [54].

The expression of cPLA$_2$α protein appears to be very low in HMC-1. Therefore, to clarify if cPLA$_2$α activity is present in HMC1, the release of AA and OA was compared in A23187-stimulated cells. The calcium ionophore A23187 caused a marked elevation of fatty acid release from the HMC-1. This fatty acid release was not restricted to AA, and about equally reduced by the specific iPLA$_2$ inhibitor BEL [41] and the combined iPLA$_2$ and cPLA$_2$ inhibitor MAFP [41]. Also, the A23187-stimulated AA release was not augmented by the attempt to increase the cPLA$_2$α activity by combined stimulation with PMA [37,39,40]. Taken together, these findings strongly suggest that one or several PLA$_2$s, different from the AA specific cPLA$_2$α, is accountable for the A23187-stimulated AA release in HMC-1. One possible candidate is iPLA$_2$β, since the AA release was reduced by BEL and iPLA$_2$ is known to release AA in other cell systems [9,10]. However, BEL and MAFP reduced about 50% of the AA release induced by A23187, indicating contribution of one or several MAFP/BEL-insensitive PLA$_2$s, for instance sPLA$_2$s [14]. It was out of the scope of the present study to investigate in detail which particular PLA$_2$s are involved in the AA release from HMC-1. However, our results may suggest a role for iPLA$_2$ and clearly indicate that the cPLA$_2$α activity of HMC1 is very low and in line with the low cPLA$_2$α protein levels found.

Whereas several studies have implicated a role for cPLA$_2$ and sPLA$_2$ in the release of AA from rodent MCs [6,15,17,50,51,55], only one study, so far, has reported involvement of iPLA$_2$ [10]. Indeed, A23187 was found to release radiolabeled AA from mouse bone marrow-derived MCs (BMMCs) and rat basophilic leukemia MCs (RBL 2H3) by an iPLA$_2$-dependent mechanism [10], a finding in line with our results in HMC-1.

The mechanism of MC degranulation involves cellular events leading to an increased cytosolic Ca^{2+}- concentration [42]. Evidently, we found that A23187 stimulates degranulation (i.e., stimulated the release of β-hexosaminidase) of HMC-1 and release of sPLA$_2$. This is in line with a previous study on ionophore-stimulated BMMCs [34]. The A23187-stimulated release of sPLA$_2$-IIA and sPLA$_2$-V was reduced by the iPLA$_2$ inhibitor BEL, suggesting a role for iPLA$_2$ in the A23187-stimulated sPLA$_2$ release from HMC-1. Although BEL is known to inhibit degranulation of BMMCs and RBL 2H3 cells [10], and also to inhibit exocytosis in other cell types [25,28], this is, as far as we know, the first study suggesting a role for iPLA$_2$ in the regulation of sPLA$_2$ release. Indeed, our finding that BEL inhibited not only the A23187-stimulated release of sPLA$_2$, but also the release of β-hexosaminidase, may indicate a role of iPLA$_2$ in MC degranulation and release of MC mediators in general.

Although the results of the present study suggest that iPLA$_2$β is involved in the release of sPLA$_2$s from A23187-stimulated cells, the precise mechanism by which iPLA$_2$β is activated by A23187 has to be evaluated. However, one possible mechanism might be that depletion of calcium stores by A23187 results in displacement of inhibitory calmodulin from iPLA$_2$ [49].

Both iPLA$_2$β [10,25–28], and cPLA$_2$α [8,56,57] have been implicated in vesicle trafficking and exocytosis. However, due to the low (perhaps absent) cPLA$_2$α activity of the HMC-1, it is not likely that cPLA$_2$α is involved in the release of sPLA$_2$s. Our finding that cPLA$_2$α is expressed in human intestinal MCs may suggest, however, that also this intracellular PLA$_2$ might be involved in MC exocytosis in the human intestine. Clearly, further studies on MCs isolated directly from the human intestine are needed to evaluate the precise roles of iPLA$_2$β and cPLA$_2$α in the release of sPLA$_2$s from MCs in the normal and inflamed human intestine.

Although our results suggest that iPLA$_2$β is involved in the degranulation and release of sPLA$_2$ in HMC-1, this is not necessarily true for other experimental MC models or during other experimental settings. For example, a study on BMMCs [38] demonstrated, in contrast with a previous report [10],

that iPLA$_2$β is not involved in the release of β-hexosaminidase from these MCs. It is also worth mentioning that species differences among MCs may influences their behavior [48], and that it is unknown to what extent the role and regulation of a particular PLA$_2$ in rodent MCs correspond to its role and regulation in human MCs.

BEL is a widely used inhibitor of iPLA$_2$, with limited effect on cPLA$_2$ and sPLA$_2$ [9,41]. Indeed, BEL is to date the only irreversible specific inhibitor of iPLA$_2$ available, however, BEL may have other unspecific side effects as well, resulting in cytotoxic effects [58]. In the present study, the viability of HMC-1 was routinely evaluated and no detrimental effect of BEL was found. Thus, it seems likely that iPLA$_2$ was the target of BEL in HMC-1. However, to verify this, further studies using gene silencing techniques are needed.

5. Conclusions

In conclusion, this study suggests that iPLA$_2$β might be involved in the secretion of sPLA$_2$s from HMC-1, suggesting that an iPLA$_2$β-mediated release of sPLA$_2$ from intestinal MCs may contribute to increased sPLA$_2$-II activity. Further, cPLA$_2$α, iPLA$_2$β, sPLA$_2$- IIA and sPLA$_2$-V are all present in mucosal MCs of both normal ileum and in the mild-inflamed ileum of CD. However, CD ileum possessed an increased proportion of iPLA$_2$β-containing MCs. Taken together, results may suggest that iPLA$_2$β may have a previously unrecognized role in human MCs, i.e., regulation of sPLA$_2$ secretion. However, further ex vivo studies are needed to confirm this and to evaluate the precise role of iPLA$_2$β in the release of sPLA$_2$s from isolated ileal MCs and its importance in the pathophysiology of CD.

Author Contributions: Conceptualization, U.C., Å.V.K., J.D.S., C.G.-S.; methodology, U.C., Å.V.K., M.E.W., C.G.-S.; validation, U.C., Å.V.K., M.E.W., C.G.-S.; formal analysis, U.C., Å.V.K., M.E.W., C.G.-S.; investigation, U.C., M.E.W.; data curation, U.C., M.E.W.; writing—original draft preparation, C.G.-S.; writing—review & editing, U.C., Å.V.K., M.E.W., J.D.S., C.G.-S.; supervision, Å.V.K., J.D.S., C.G.-S.; project administration, Å.V.K., C.G.-S.; funding acquisition, Å.V.K., J.D.S., C.G.-S.

Funding: This study was supported by grants from the Medical Research Council of Southeast Sweden (C.G.-S), the Faculty of Health and Life Sciences, Linneaus University, Sweden (C.G.-S), the Swedish Research Council VR-Medicine and Health, 2014-02537, 2017-02475 (JDS) and LIONS international Foundation (Å.V.K.).

Acknowledgments: We thank Master´s student Hanna Carlsson, Kalmar, for valuable laboratory work during the initial phase of this study, and lab technician Lena Svensson, Linköping, for assistance with immunohistochemical stainings.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

A23187, calcium ionophore; AA, arachidonic acid; BEL, bromoenol lactone; CD, Crohn's disease; cPLA$_2$, cytosolic phospholipase A$_2$; IMDM, Iscove's Modified Dulbecco's Medium; IBD, inflammatory bowel disease; iPLA$_2$, calcium-independent phospholipase A$_2$; MAFP, methyl arachidonyl fluoro-phosphonate; MC, mast cell; OA, oleic acid; PLA$_2$, phospholipase A$_2$; PMA, phorbol myristate acetate; RT, room temperature; sPLA$_2$, secretory phospholipase A$_2$; sPLA$_2$-IIA, secretory phospholipase A$_2$ group IIA; sPLA$_2$-V, secretory phospholipase A$_2$ group V.

References

1. Hamilton, M.J.; Frei, S.M.; Stevens, R.L. The multifaceted mast cell in inflammatory bowel disease. *Inflamm. Bowel Dis.* **2014**, *20*, 2364–2378. [CrossRef] [PubMed]
2. Bischoff, S.; Gebhardt, T. Role of mast cells and eosinophils in neuroimmune interactions regulating mucosal inflammation in inflammatory bowel disease. *Adv. Exp. Med. Biol.* **2006**, *579*, 177–208. [PubMed]
3. Gelbmann, C.; Mestermann, S.; Gross, V.; Köllinger, M.; Schölmerich, J.; Falk, W. Strictures in Crohn´s disease are characterised by an accumulation of mast cells colocalised with laminin but not with fibronectin or vitronectin. *Gut* **1999**, *45*, 210–217. [CrossRef] [PubMed]
4. Boeckxstaens, G. Mast cells and inflammatory bowel disease. *Curr. Opin. Pharmacol.* **2015**, *25*, 45–49. [CrossRef] [PubMed]

5. Bischoff, S.C. Mast cells in gastrointestinal disorders. *Eur. J. Pharmacol.* **2016**, *778*, 139–145. [CrossRef] [PubMed]
6. Boyce, J. Mast cells and eicosanoid mediators: A system of reciprocal paracrine and autocrine regulation. *Immunol. Rev.* **2007**, *217*, 168–185. [CrossRef] [PubMed]
7. Dennis, E.A.; Cao, J.; Hsu, Y.H.; Magrioti, V.; Kokotos, G. Phospholipase A2 enzymes: Physical structure, biological function, disease implication, chemical inhibition, and therapeutic intervention. *Chem. Rev.* **2011**, *111*, 6130–6185. [CrossRef] [PubMed]
8. Leslie, C.C. Cytosolic phospholipase A(2): Physiological function and role in disease. *J. Lipid Res.* **2015**, *56*, 1386–1402. [CrossRef] [PubMed]
9. Ramanadham, S.; Ali, T.; Ashley, J.W.; Bone, R.N.; Hancock, W.D.; Lei, X. Calcium-independent phospholipases A2 and their roles in biological processes and diseases. *J. Lipid Res.* **2015**, *56*, 1643–1668. [CrossRef] [PubMed]
10. Fensome-Green, A.; Stannard, N.; Li, M.; Bolsover, S.; Cockcroft, S. Bromoenol lactone, an inhibitor of group VIA calcium-independent phospholipase A_2 inhibits antigen-stimulated mast cell exocytosis without blocking Ca^{2+} influx. *Cell Calcium* **2007**, *41*, 145–153. [CrossRef]
11. Jiao, L.; Inhoffen, J.; Gan-Schreier, H.; Tuma-Kellner, S.; Stremmel, W.; Sun, Z.; Chamulitrat, W. Deficiency of Group VIA Phospholipase A2 (iPLA2beta) Renders Susceptibility for Chemical-Induced Colitis. *Dig. Dis. Sci.* **2015**, *60*, 3590–3602. [CrossRef] [PubMed]
12. Petan, T.; Krizaj, I. Is iPLA2beta a Novel Target for the Development of New Strategies to Alleviate Inflammatory Bowel Disease? *Dig. Dis. Sci.* **2015**, *60*, 3504–3506. [CrossRef] [PubMed]
13. Murakami, M.; Sato, H.; Miki, Y.; Yamamoto, K.; Taketomi, Y. A new era of secreted phospholipase A(2). *J. Lipid Res.* **2015**, *56*, 1248–1261. [CrossRef] [PubMed]
14. Murakami, M.; Taketomi, Y.; Girard, C.; Yamamoto, K.; Lambeau, G. Emerging roles of secreted phospholipase A_2 enzymes: Lessons from transgenic and knockout mice. *Biochimie* **2010**, *92*, 561–582. [CrossRef] [PubMed]
15. Fonteh, A.; Atsumi, G.-I.; Laporte, T.; Chilton, F. Secretory phospholipase A_2 receptor-mediated activation of cytosolic phospholipase A_2 in murine bone marrow-derived mast cells. *J. Immunol.* **2000**, *165*, 2773–2782. [CrossRef] [PubMed]
16. Bingham, C.; Fijneman, R.; Friend, D.; Goddeau, R.; Rogers, R.; Austen, K.; Arm, J. Low molecular weight group IIA and group V phospholipase A_2 enzymes have different intracellular locations in mouse bone marrow-derived mast cells. *J. Biol. Chem.* **1999**, *274*, 31476–31484. [CrossRef]
17. Diaz, B.; Satake, Y.; Kikawada, E.; Balestrieri, B.; Arm, J. Group V secretory phospholipase A_2 amplifies the induction of cyclooxygenase 2 and delayed prostaglandin D_2 generation in mouse bone marrow culture-derived mast cells. *Biochim. Biophys. Acta* **2006**, *1761*, 1489–1497. [CrossRef]
18. Lilja, I.; Gustafson-Svärd, C.; Franzén, L.; Sjödahl, R.; Andersen, S.; Johansen, B. Presence of group IIa secretory phospholipase A_2 in mast cells and macrophages in normal human ileal submucosa and in Crohn´s disease. *Clin. Chem. Lab. Med.* **2000**, *38*, 1231–1236. [CrossRef]
19. Jamal, O.; Conaghan, P.; Cunningham, A.; Brooks, P.; Munro, V.; Scott, K. Increased expression of human type IIa secretory phospholipase A_2 antigen in arthritic synovium. *Ann. Rheum. Dis.* **1998**, *57*, 550–558. [CrossRef]
20. Triggiani, M.; Giannattasio, G.; Calabrese, C.; Loffredo, S.; Granata, F.; Fiorello, A.; Santini, M.; Gelb, M.H.; Marone, G. Lung mast cells are a source of secreted phospholipases A_2. *J. Allergy Clin. Immunol.* **2009**, *124*, 558–565. [CrossRef]
21. Wu, Y.; Raymond, B.; Goossens, P.L.; Njamkepo, E.; Guiso, N.; Paya, M.; Touqui, L. Type-IIA secreted phospholipase A_2 is an endogenous antibiotic-like protein of the host. *Biochimie* **2010**, *92*, 583–587. [CrossRef] [PubMed]
22. Boilard, E.; Lai, Y.; Larabee, K.; Balestrieri, B.; Ghomashchi, F.; Fujioka, D.; Gobezie, R.; Coblyn, J.S.; Weinblatt, M.E.; Massarotti, E.M.; et al. A novel anti-inflammatory role for secretory phospholipase A_2 in immune complex-mediated arthritis. *EMBO Mol. Med.* **2010**, *2*, 172–187. [CrossRef] [PubMed]
23. Granata, F.; Balestrieri, B.; Petraroli, A.; Giannattasio, G.; Marone, G.; Triggiani, M. Secretory phospholipases A_2 as multivalent mediators of inflammatory and allergic disorders. *Int. Arch. Allergy Immunol.* **2003**, *131*, 153–163. [CrossRef] [PubMed]
24. Murakami, M.; Taketomi, Y. Secreted phospholipase A2 and mast cells. *Allergol. Int.* **2015**, *64*, 4–10. [CrossRef] [PubMed]

25. Takuma, T.; Ichida, T. Role of Ca^{2+}-independent phospholipase A_2 in exocytosis of amylase from parotid acinar cells. *J. Biochem.* **1997**, *121*, 1018–1024. [CrossRef] [PubMed]
26. Balboa, M.A.; Saez, Y.; Balsinde, J. Calcium-independent phospholipase A2 is required for lysozyme secretion in U937 promonocytes. *J. Immunol.* **2003**, *170*, 5276–5280. [CrossRef] [PubMed]
27. Mikami, S.; Aiboshi, J.; Kobayashi, T.; Kojima, M.; Morishita, K.; Otomo, Y. Discrete roles of intracellular phospholipases A2 in human neutrophil cytotoxicity. *J. Trauma Acute Care Surg.* **2015**, *79*, 238–246. [CrossRef]
28. Abi Nahed, R.; Martinez, G.; Escoffier, J.; Yassine, S.; Karaouzene, T.; Hograindleur, J.P.; Turk, J.; Kokotos, G.; Ray, P.F.; Bottari, S.; et al. Progesterone-induced Acrosome Exocytosis Requires Sequential Involvement of Calcium-independent Phospholipase A2beta (iPLA2beta) and Group X Secreted Phospholipase A2 (sPLA2). *J. Biol. Chem.* **2016**, *291*, 3076–3089. [CrossRef]
29. Butterfield, J.; Weiler, D.; Dewald, G.; Gleich, G. Establishment of an immature mast cell line from a patient with mast cell leukemia. *Leuk. Res.* **1988**, *12*, 345–355. [CrossRef]
30. Van Assche, G.; Vermeire, S.; Rutgeerts, P. Infliximab therapy for patients with inflammatory bowel disease: 10 years on. *Eur. J. Pharmacol.* **2009**, *623*, S17–S25. [CrossRef]
31. Seeds, M.; Jones, D.; Chilton, F.; Bass, D. Secretory and cytosolic phospholipases A_2 are activated during TNF priming of human neutrophils. *Biochim. Biophys. Acta* **1998**, *1389*, 273–284. [CrossRef]
32. Wu, T.; Ikezono, T.; Angus, W.; Shelhamer, J. Tumor necrosis factor-a induces the 85-kDa cytosolic phospholipase A_2 gene expression in human bronchial epithelial cells. *Biochim. Biophys. Acta* **1996**, *1310*, 175–184. [CrossRef]
33. Christerson, U.; Keita, Å.; Söderholm, J.; Gustafson-Svärd, C. Increased expression of protease-activated receptor-2 in mucosal mast cells in Crohn´s ileitis. *J. Crohns Colitis* **2009**, *3*, 100–108. [CrossRef] [PubMed]
34. Murakami, M.; Kudo, I.; Suwa, Y.; Inoue, K. Release of 14-kDa group-II phospholipase A_2 from activated mast cells and its possible involvement in the regulation of the degranulation process. *Eur. J. Biochem.* **1992**, *209*, 257–265. [CrossRef]
35. Macchia, L.; Hamberg, M.; Kumlin, M.; Butterfield, J.; Haeggström, J. Arachidonic acid metabolism in the human mast cell line HMC-1: 5-lipoxygenase gene expression and biosynthesis of thromboxane. *Biochim. Biophys. Acta* **1995**, *1257*, 58–74. [CrossRef]
36. Meyer, G.K.; Neetz, A.; Brandes, G.; Tsikas, D.; Butterfield, J.H.; Just, I.; Gerhard, R. Clostridium difficile toxins A and B directly stimulate human mast cells. *Infect. Immun.* **2007**, *75*, 3868–3876. [CrossRef]
37. Christerson, U.; Keita, Å.; Söderholm, J.; Gustafson-Svärd, C. Potential role of protease-activated receptor-2-stimulated activation of cytosolic phospholipase A_2 in intestinal myofibroblast proliferation: Implications for stricture formation in Crohn´s disease. *J. Crohns Colitis* **2009**, *3*, 15–24. [CrossRef]
38. Ueno, N.; Taketomi, Y.; Yamamoto, K.; Hirabayashi, T.; Kamei, D.; Kita, Y.; Shimizu, T.; Shinzawa, K.; Tsujimoto, Y.; Ikeda, K.; et al. Analysis of two major intracellular phospholipases A(2) (PLA(2)) in mast cells reveals crucial contribution of cytosolic PLA(2)alpha, not Ca^{2+}-independent PLA(2)beta, to lipid mobilization in proximal mast cells and distal fibroblasts. *J. Biol. Chem.* **2011**, *286*, 37249–37263. [CrossRef]
39. Shimizu, M.; Azuma, C.; Taniguchi, T.; Murayama, T. Expression of cytosolic phospholipase A_2a in murine C12 cells, a variant of L929 cells, induces arachidonic acid release in response to phosbol myristate acetate and Ca^{2+} ionophores, but not to tumor necrosis factor-a. *J. Pharm. Sci.* **2004**, *96*, 324–332. [CrossRef]
40. Lin, M.T.; Wang, Y.-H.; Chen, Y.-L.; Chang, W.-C. The effect of copper ion on arachidonic acid metabolism in the porcine corneal epithelium. *Biochem. Biophys. Res. Commun.* **1993**, *190*, 1122–1129. [CrossRef]
41. Balsinde, J.; Balboa, M.; Insel, P.; Dennis, E. Regulation and inhibition of phospholipase A_2. *Annu. Rev. Pharmacol.* **1999**, *39*, 175–189. [CrossRef] [PubMed]
42. Kalesnikoff, J.; Galli, S. New developments in mast cell biology. *Nat. Immunol.* **2008**, *9*, 1215–1222. [CrossRef] [PubMed]
43. Baram, D.; Vaday, G.G.; Salamon, P.; Drucker, I.; Hershkoviz, R.; Mekori, Y.A. Human mast cells release metalloproteinase-9 on contact with activated T cells: Juxtacrine regulation by TNF-α. *J. Immunol.* **2001**, *167*, 4008–4016. [CrossRef] [PubMed]
44. Keita, Å.; Gullberg, E.; Ericson, A.; Salim, S.; Wallon, C.; Kald, A.; Artursson, P.; Söderholm, J. Characterization of antigen and bacterial transport in the follicle-associated epithelium of human ileum. *Lab. Invest.* **2006**, *86*, 504–516. [CrossRef] [PubMed]
45. Haapamäki, M.; Grönroos, J.; Nurmi, H.; Alanen, K.; Nevalainen, T. Gene expression of group II phospholipase A_2 in intestine in Crohn´s disease. *Am. J. Gastroenterol.* **1999**, *94*, 713–720. [PubMed]

46. Lilja, I.; Smedh, K.; Olaison, G.; Sjödahl, R.; Tagesson, C.; Gustafson-Svärd, C. Phospholipase A_2 gene expression and activity in histologically normal ileal mucosa and in Crohn´s ileitis. *Gut* **1995**, *37*, 380–385. [CrossRef] [PubMed]
47. Keita, A.V.; Söderholm, J.D. Barrier dysfunction and bacterial uptake in the follicle-associated epithelium of ileal Crohn´s disease. *Ann. N. Y. Acad. Sci.* **2012**, *1258*, 125–134. [CrossRef] [PubMed]
48. Bischoff, S. Role of mast cells in allergic and non-allergic immune responses: Comparison of human and murine data. *Nat. Rev. Immunol.* **2007**, *7*, 93–104. [CrossRef] [PubMed]
49. Csutora, P.; Zarayskiy, V.; Peter, K.; Monje, F.; Smani, T.; Zakharov, S.; Litvinov, D.; Bolotina, V. Activation mechanism for CRAC current and store-operated Ca^{2+} entry. Calcium influx factor and Ca^{2+}-independent phospholipase A_2ß-mediated pathway. *J. Biol. Chem.* **2006**, *281*, 34926–34935. [CrossRef] [PubMed]
50. Fujishima, H.; Sanchez Mejia, R.; Bingham, C.; Lam, B.; Sapirstein, A.; Bonventre, J.; Austen, K.; Arm, J. Cytosolic phospholipase A_2 is essential for both the immediate and the delayed phases of eicosanoid generation in mouse bone marrow-derived mast cells. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 4803–4807. [CrossRef]
51. Cho, S.-H.; You, H.-J.; Woo, C.-H.; Yoo, Y.-J.; Kim, J.-H. Rac and protein kinase C-delta regulate ERKs and cytosolic phospholipase A_2 in FcERI signaling to cysteinyl leukotriene synthesis in mast cells. *J. Immunol.* **2004**, *173*, 624–631. [CrossRef] [PubMed]
52. Gebhardt, T.; Lorentz, A.; Detmer, C.; Trautwein, C.; Bektas, H.; Manns, M.; Bischoff, S. Growth, phenotype, and function of human intestinal mast cells are tightly regulated by transforming growth factor β1. *Gut* **2005**, *54*, 928–934. [CrossRef] [PubMed]
53. Schwanhausser, B.; Busse, D.; Li, N.; Dittmar, G.; Schuchhardt, J.; Wolf, J.; Chen, W.; Selbach, M. Global quantification of mammalian gene expression control. *Nature* **2011**, *473*, 337–342. [CrossRef] [PubMed]
54. van der Helm, H.A.; Buijtenhuijs, P.; van den Bosch, H. Group IIA and group V secretory phospholipase A_2: Quantitative analysis of expression and secretion and determination of the localization and routing in rat mesangial cells. *Biochim. Biophys. Acta* **2001**, *1530*, 86–96. [CrossRef]
55. Ashraf, M.; Murakami, M.; Shimbara, S.; Amakasu, Y.; Atsumi, G.-I.; Kudo, I. Type II phospholipase A_2 is linked to cyclooxygenase-2-mediated delayed prostaglandin D_2 generation by cultured mouse mast cells following FcERI- and cytokine-dependent activvation. *Biochem. Biophys. Res. Commun.* **1996**, *229*, 726–732. [CrossRef] [PubMed]
56. Regan-Klapisz, E.; Krouwer, V.; Langelaar-Makkinje, M.; Nallan, L.; Gelb, M.; Gerritsen, H.; Verkleij, A.J.; Post, J.A. Golgi-associated cPLA2alpha regulates endothelial cell-cell junction integrity by controlling the trafficking of transmembrane junction proteins. *Mol. Biol. Cell* **2009**, *20*, 4225–4234. [CrossRef]
57. Schmidt, J.A.; Kalkofen, D.N.; Donovan, K.W.; Brown, W.J. A role for phospholipase A2 activity in membrane tubule formation and TGN trafficking. *Traffic* **2010**, *11*, 1530–1536. [CrossRef]
58. Fuentes, L.; Perez, R.; Nieto, M.; Balsinde, J.; Balboa, M. Bromoenol lactone promotes cell death by a mechanism involving phosphatidate phosphohydrolase-1 rather than calcium-independent phospholipase A2. *J. Biol. Chem.* **2003**, *278*, 44683–44690. [CrossRef]