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Expression of members of the phospholipase A_2 family of enzymes in human nasal mucosa

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Expression of members of the phospholipase A_2 family of enzymes in human nasal mucosa. J. Lindbom, A.G. Ljungman, M. Lindahl, C. Tagesson. ©ERS Journals Ltd 2001.

ABSTRACT: Phospholipase A_2 (PLA_2) is a family of enzymes thought to play a key role in inflammation by releasing arachidonic acid for the synthesis of eicosanoids and lysophospholipid for the synthesis of platelet-activating factor. However, the precise contribution of different PLA_2 types to the formation of inflammatory lipid mediators in the upper airways is not known and the expression of different PLA_2 genes in the human nasal mucosa has not been examined.

This study therefore investigated the occurrence of messenger ribonucleic acids (mRNAs) for different PLA_2 forms (IB, IIA, IID, IIE, III, IVA, IVB, IVC, V, VI, VII, X, acid calcium-independent (aiPLA$_2$), and calcium-independent membrane bound PLA$_2$, (iPLA$_2$-2)) in the nasal mucosa of five healthy human subjects.

Using reversed transcription-polymerase chain reaction (RT-PCR) techniques it was found that all these PLA_2 types except PLA_2 V were expressed in all subjects, whereas PLA_2 V was detected in only one individual on one single occasion. The relative abundance of the different PLA_2 transcripts were aiPLA$_2$ > X ≈ IVA > IIA ≈ IIE ≈ IVB ≈ VI > IB ≈ IID ≈ III ≈ IVC ≈ VII ≈ iPLA$_2$-2. To further quantify the mRNA-expression of PLA_2 X, IVA and IIA, the samples were reanalysed with a quantitative PCR-technique utilizing competitive deoxyribonucleic acid (DNA) mimics as references. The amounts of PLA_2 X, IVA and IIA mRNA were then estimated to 0.9 ± 0.2, 1.1 ± 0.7, and 0.0025 ± 0.0021 amol (mean ± SE), respectively, confirming the relative abundance of these PLA_2 transcripts and indicating that the recently described PLA_2 X form is relatively strongly expressed.

These findings demonstrate that a large number of PLA_2 types are expressed in the normal human nasal mucosa. Moreover, this investigation demonstrates, for the first time, the presence of the newly discovered phospholipase A_2 forms IID, IIE, III, IVB, IVC, X and calcium-independent membrane bound phospholipase A_2 in the human nasal mucosa and raises the possibility that one or several of these may be involved in inflammatory reactions in the nose.

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Phospholipase A_2 (PLA_2) is the first enzyme in the synthesis of two types of lipid inflammatory mediators with potent effects in the respiratory tract: the eicosanoids and platelet-activating factor (PAF) [1]. Accordingly, PLA_2 hydrolyzes the acyl-ester bond at the *sn-2* position of glycerophospholipids and releases arachidonic acid (AA), which is then metabolized to eicosanoids, and 1-*O*-alkyl-lysophosphatidylcholine (lysoPAF), which is the precursor of PAF. At present, a number of different PLA_2s (IB, IIA, IID, IIE, III, IVA, IVB, IVC, V, VI, VII, X and the cytosolic calcium-independent membrane bound PLA_2 (iPLA$_2$-2)) have been identified in human tissues [2–6]. These PLA_2s belong to different types of enzymes [12]: the secretory type (sPLA$_2$), the cytosolic calcium-dependent type (cPLA$_2$), and iPLA$_2$ [8]. However, the precise contribution of these different PLA_2 types to the formation of inflammatory lipid mediators in the airways is not known, and it may well be that yet other proteins with PLA_2 properties are present. It is important, therefore, to study in more detail the diversity of PLA_2s in the airways and how the different PLA_2s may be activated during inflammation.

Despite the general interest in different PLA_2 types and how they participate in the release of lysoPAF and AA, the role of different PLA_2s in the human nasal mucosa has received rather little attention. Previous investigations have demonstrated increased PLA_2 activity in nasal lavage fluid (NLF) from allergic patients after allergen provocation [10, 11]; although the increased PLA_2 activity was not unequivocally attributed to any specific PLA_2 type, both investigations highlighted an sPLA$_2$ with characteristics similar to PLA_2 IIA. High concentrations of a PLA_2 IIA have also been determined in NLF from healthy subjects after methacholine provocation [12]; however, the PLA_2 levels in NLF were low after obstruction of the flow of tear fluid into the nasal cavity, indicating that the enzyme originated from tear fluid. In addition to PLA_2 IIA, TOUQUI *et al.* [11]

found PAF acetylhydrolase-like activity in NLF, indicating the presence of type VII $sPLA_2$. Despite these original and important findings, it is still unclear how the different PLA_2s participate in the formation and metabolism of lipid mediators in the nasal mucosa. Moreover, a large number of novel PLA_2 types have recently been isolated and cloned [3–6, 13–17], many of which may turn out to be operating in the nasal mucosa or in NLF. These considerations prompted the present effort to examine the occurrence of messenger ribonucleic acids (mRNAs) for different PLA_2 forms in the nasal mucosa of healthy human subjects.

Materials and methods

Subjects

Five subjects volunteered for the investigation. They were all healthy males, 25–45 yrs of age, non-smokers and not taking any drugs. All five subjects were used to obtain nasal brush samples while three of them were used to obtain NLF samples. Brush samples were collected at different occasions from each subject with no less than a week between sampling occasions. All individuals were examined clinically at each sampling occasion, visually checking (by anterior rhinoscopy) that the nasal cavity appeared normal. Thus, the nasal mucosa did not show any signs of inflammation or irritation before nasal lavage or brushing.

Nasal mucosal samples

Cells from the nasal mucosa were obtained either by nasal lavage or by a brush technique as previously described [18]. To obtain NLF, 15 mL of saline were inserted by means of a syringe connected to a commercial Foley catheter (Bard Ltd, Crawley, West Sussex, UK) with the cuff adapted in the vestibulum nasi. The saline was recovered from the nasal cavity after 10 min and the cell content was collected by centrifugation. To obtain nasal brush samples, small plastic-coated, steel-wire brushes with nylon bristles were employed. The brush was placed between the septum and inferior turbinate and rotated while being removed. Cells obtained by brushing were immediately collected by twisting the brush in 1 mL of phosphate buffered saline at pH 7.3 followed by centrifugation. The total number of cells in the nasal brush and NLF samples were $2.7 \times 10^5 \pm 2.1 \times 10^5$ and $2.3 \times 10^4 \pm 1.5 \times 10^4$, respectively. The viability of the cells exceeded 97%, in both preparations, as determined by Tryptan blue exclusion. Differential countings stained with Wright stain (Accustain, Sigma-Aldrich, St Louis, MO, USA) showed that the mucosal samples obtained by either method consisted mainly of epithelial cells. An average of $5.6 \pm 1.8\%$ of the total number of cells were leukocytes and among these only occasional eosinophils were detected.

Detection of messenger ribonucleic acid by reverse transcriptase-polymerase chain reaction

The cell pellets were immediately lysed and total cellular ribonucleic acid (RNA) isolated by the method of acid guanidium thiocyanate/phenol/chloroform extraction as previously described [19]. RNA was reverse-transcribed into complementary DNA (cDNA) with Moloney Murine Leukaemia Virus (MMLV) Reverse Transcriptase (RT) (SuperScript™ II, Life Technologies Ltd, Paisley, UK) according to the manufacturers instructions. Briefly, total RNA was mixed with a buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 0.6 mM of each deoxyribonucleotide triphosphate (dNTP), and 0.5 μg oligonucleotide (deoxythumidylate)$_{18}$ (oligo $(dT)_{18}$), and heated for 5 min at 65°C. After the incubation, the samples were chilled on ice and RT SuperScript™ II enzyme (200 units), RNAsine (20 units) and dithiothreitol (DTT) (100 mM) was added in a final volume of 20 μL. The samples were incubated for 60 min at 40°C followed by 5 min at 95°C. The cDNAs were stored at -20°C until used in PCR amplifications.

For polymerase chain reaction (PCR), cDNA from all subjects were pooled and 1 μL of the pooled cDNA was used per PLA_2 form amplified. All PCR reactions contained 2 μL PCR buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.6), 2 mM $MgCl_2$, 0.2 mM dNTP mix, 0.1 mM oligonucleotide primers, and 0.5 units *Taq* DNA polymerase in a total volume of 20 μL. For the amplification of PLA_2 IIA and IV, Platinum *Taq* DNA polymerase (Life Technologies) was used. All primers (table 1) used were constructed from published sequences and then synthesized by Life Technologies. The PCR reactions were carried out in a PTC-100 (MJ Research Inc., Watertown, MA, USA) programmable thermal controller with an initial 2 min denaturation at 94°C followed by the cycled program 1 min at 94°C, 1 min at 55–65°C (varying between primer sets) and 1 min at 72°C. All PCR reactions were carried out for 40 cycles (figs. 1–5) except the competitive analyses of PLA_2 IVA and X which were carried out for 28 cycles (figs. 6–7). A final extension of 7 min at 72°C ended the reaction. Ten microlitres from each PCR reaction was separated on a 1.5% agarose gel and ethidium bromide-stained. The stained gels were digitized into gray-scale images with a DC120 Zoom Digital Camera (Kodak Digital Science, Rochester, NY, USA) and analysed with the Kodak Digital Science lD Image Analysis Software. The PCR products obtained corresponded to the expected product sizes as listed in table 1. The PCR products were confirmed by Southern blot using oligonucleotide probes complementary to sequences within the different PLA_2 PCR products (the oligonucleotide probes used are listed in table 1) or by restriction enzyme digestion using PVU II (isolated from *Proteus vularis*) for the IID, IVB and $aiPLA_2$ forms, Hha I (isolated from *Haemophillus haemidyticus*) for the IIE form, Sty I (isolated from *Salmonella Typhi*) for the III form and Hind III (isolated from *Haemophillus influence* R_d) for the $iPLA_2$-2 form (Life Technologies).

To allow a comparison of the relative abundance of

Table 1. – Description of primers used in polymerase chain reactions and oligonucleotides used in Southern blots

PLA_2-type	Primer and oligonucleotide sequence	Expected product size (bp)	Access code
IB	[s], 5′-AAA TGATCAAGTGCGTGATCC-3′	243	HuMPLA2RA
	[a], 5′-TTGCTGCTACAGGTGATTGC-3′		E02268
	[oligo] 5′-GACACATGACAACTGCTATGACCAG GCCAA-3′		
IIA	[s], 5′-ATCCCCCAAGGATGCAAC-3′	195	HuMRASFAB
	[a], 5′-AAAACAGGTGGCAGCAGC-3′		
	[oligo] 5′-ACAAGTTTAGCAACTCGGGGAGCAG AATCA-3′		
IID	[s], 5′-ATCCTGAACCTGAACAAGATG-3′	328	AF112982
	[a], 5′-AGTCGCTTCTGGTAGGTGTC-3′		
IIE	[s], 5′-GATGATCGAGAAGATGACAG -3′	324	AF189279
	[a], 5′-AGCTTGTTGGGATAATGG -3′		
III	[s], 5′-ACAACTCTTCTATGCCTGG-3′	256	AF220490
	[a], 5′-TGTGACATCCCTAACTTCC-3′		
IVA	[s], 5′-GTTGCTGGTCTTTCTGGCTC-3′	313	HuMPLA2A
	[a], 5′-GGTAAAGGGCATTGTGCAGT-3′		
	[a], 5′-AGGTTTGACATGAAGACAGGTG-3′	341	
	[oligo] 5′-TCCCCTTTTACTTCTCACACCACAGA AAGT-3′		
IVB	[s], 5′-GAGCTTCGTGCTGGATGTG-3′	273	AF065215
	[a], 5′-CGGATGGAACAGGAAATGC-3′		NM_005090
IVC	[s], 5′-CGATTTACCCGAGGAGTGG-3′	329	AF058921
	[a], 5′-GCTTCCGAAGTGGGTTATGG-3′		AF065214
	[oligo] 5′-GAAGAAGCCCGTGGAAGAAGGGACACTACC-3′		
V	[s], 5′-GGGCTGCAACATTCGCACAC-3′	278	HSU03090
	[a], 5′-CCTCTCTCAGGAACCAGGCAG-3′		
	[oligo] 5′-CCATGTGAACCTCTGTGCCTGTGAC CGGAA-3′		
VI	[s], 5′-ACCTGGCTGTGGAGCTAGG-3′	293	AF064594
	[a], 5′-GCCCTTGGTTATTCACCTGG-3′		
	[oligo] 5′-GTGGAGCTGGTGCAGTACTGCCACACTCAG-3′		
VII	[s], 5′-ATTCTGCTATTGGCATTGAC-3′	470	HSU24577
	[a], 5′-AAAAGA GGGGCTGAGGAAT-3′		HSU20157
	[oligo] 5′-AGCAAAAGAATGTTCCCAAGCTCT CAGTCT-3′		
X	[s], 5′-CCATCGCCTATATGA AATATGG-3′	295	HSU95301
	[a], 5′-TAGGAACTGGGGGTAGAAGAG-3′		
	[oligo] 5′-CCTGTGCGGACCGGCAGAGAACAAAT GCCA-3′		
$aiPLA_2$	[s], 5′-GCTTGCTTCACTCCATCAG-3′	454	KIAA0106
	[a], 5′-TGTGTGAAAGGCACATGG-3′		
$iPLA_2$-2	[s], 5′-CATTAGAGCCTCATCTGC-3′	303	AB041261
	[a], 5′-CATTAGAGCCTCATCTGC-3′		

s: sense; a: antisense; oligo: oligonucleotide used in Southern blot; $aiPLA_2$: acid calcium-independent phospholipase A_2; $iPLA_2$-2: calcium independent phospholipase A_2.

the PLA_2 forms, the amplification efficiency (E) was determined for each primer set. Aliquots of cDNA were subjected to varying numbers of amplification cycles and the PCR product was treated as described earlier. The cDNA amounts (expressed as net intensities) from respective number of cycles were then plotted as the log (amount PLA_2 form) *versus* number of cycles. After linear regression, the E value was deduced from the slope of the semi-log plot. The E values were: PLA_2 IB (E = 0.68), IIA (E = 0.77), IID (E = 1.19), IIE (E = 0.68), III (E = 0.56), IVA (E = 0.84), IVB (E = 0.53), IVC (E = 1.13), V (E = not

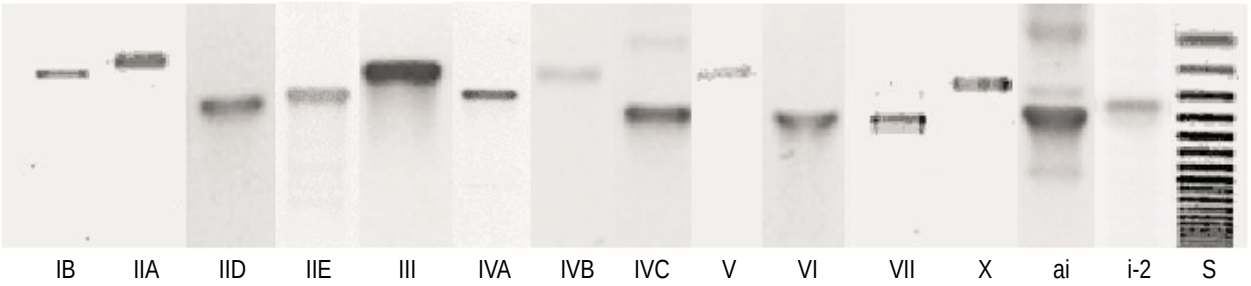

Fig. 1. – Gene expression of different phospholipase A_2 (PLA_2) types in nasal mucosal cells obtained through lavage. Total ribonucleic acid isolated from the cells was converted to complementary deoxyribonucleic acid through reverse transcription, and analysed by polymerase chain reaction for the IB, IIA, IID, IIE, III, IVA, IVB, IVC, V, VI, VII, X, and calcium-independent membrane bound PLA_2 ($aiPLA_2$; ai) and calcium-independent membrane bound PLA_2 ($iPLA_2$-2; i-2) types. S: 100 base pair size marker.

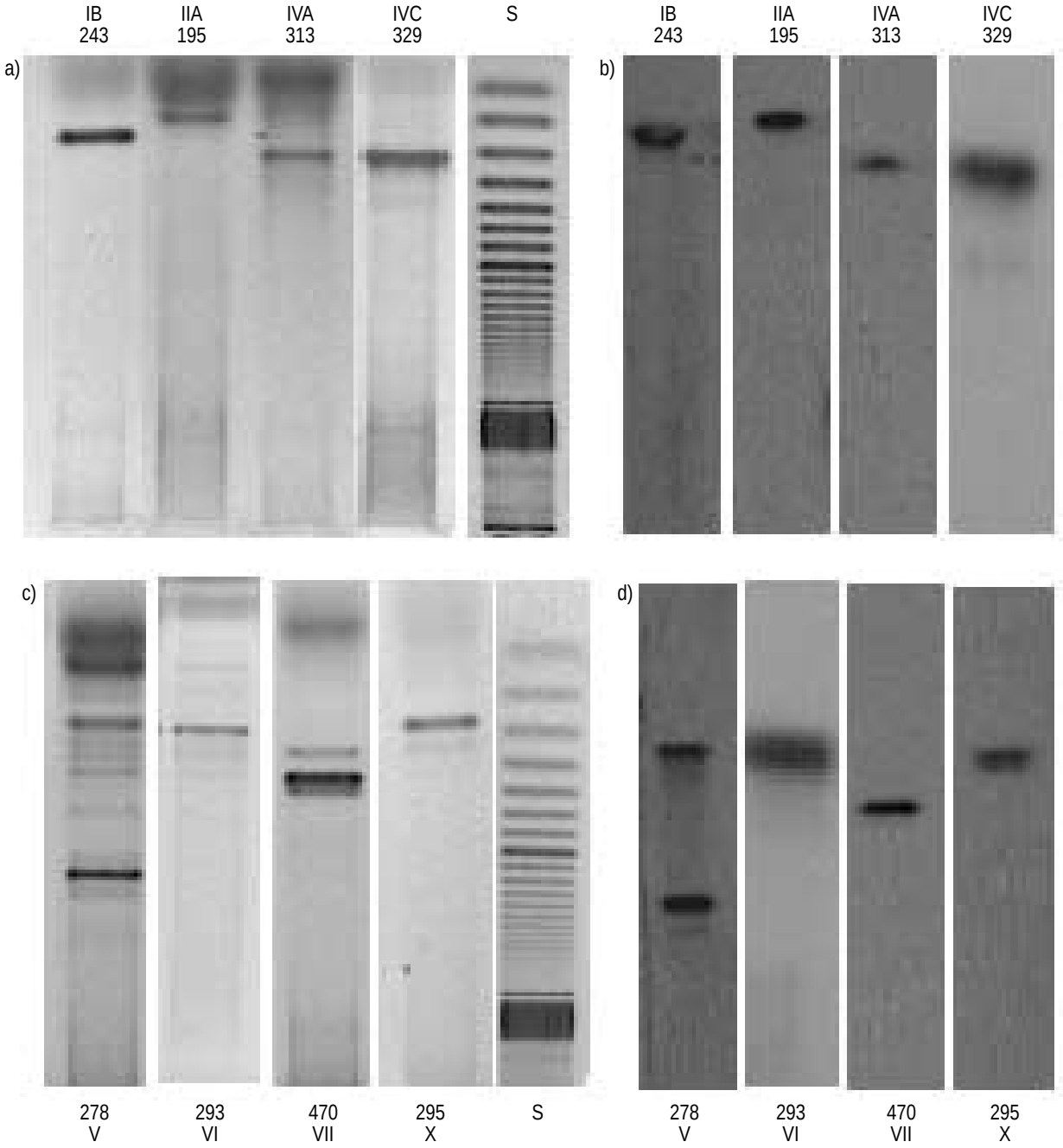

Fig. 2. – Gene expression of different phospholipase A_2 (PLA_2) types in nasal mucosal cells obtained by brushing. Total ribonucleic acid (RNA) isolated from the cells was converted to complementary deoxyribonucleic acid through reverse transcription, and analysed by polymerase chain reaction (PCR) for the IB, IIA, IVA, IVC, V, VI, VII, and X types (left panels a, c). The 1,000 base pair band visible in the Southern blot for the V form (d) is probably due to genomic contamination. The expected PCR-product size for each PLA_2 type is indicated. S: 100 base pair size marker. The right panels (b, d) show Southern blot confirmations of the PLA_2 PCR fragments utilizing γ-^{32}P-labelled oligonucleotide probes.

determined), VI (E = 0.90), VII (E = 0.67), X (E = 0.64), $iPLA_2$-2 (E = 0.88), and $aiPLA_2$ (E = 0.53).

Quantitative polymerase chain reaction

In order to further quantify the amounts of mRNA, competitive DNA fragments (PCR mimics) were constructed for the PLA_2 forms IIA, IVA and X. The PCR mimics were generated by two successive PCR amplifications of heterologous DNA (pGEM® plasmide control DNA, Promega Corp., Madison, WI, USA). For the first PCR, composite primers were used. One contained the upstream target primer (PLA_2 IIA, IVA or X; see table 1) linked to a primer that anneals to the heterologous DNA. The other contains the downstream PLA_2 target primer and the oligonucleotide sequence for the PLA_2 target (table 1) linked

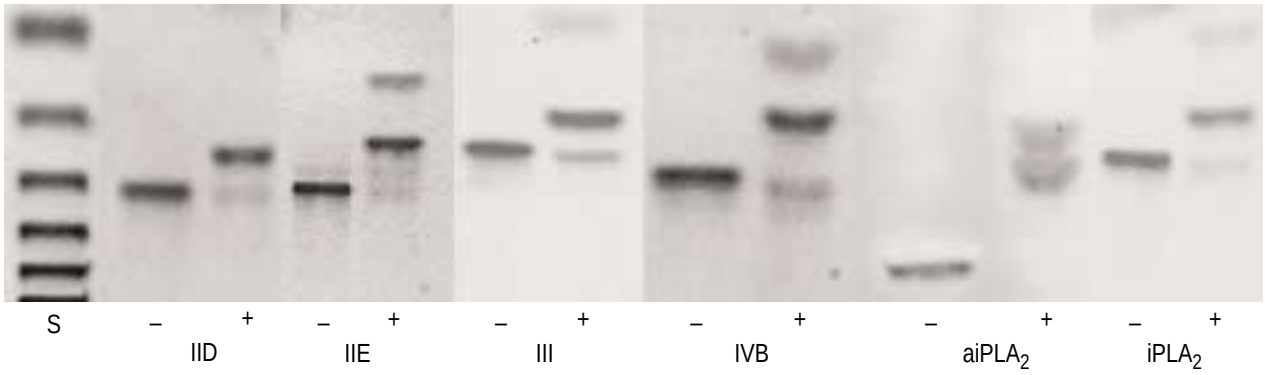

Fig. 3. – Gene expression of phospholipase A_2 (PLA_2) IID, IIE, IVB and acid calcium-independent membrane bound PLA_2 in nasal mucosal cells obtained by brushing. Total ribonucleic acid isolated from the cells was analysed by reverse transcription-polymerase chain reaction (RT-PCR) and the PCR products were treated with PVU II (PLA_2 IID, IVB and $aiPLA_2$), Hha I (PLA_2 IIE) or Sty I (PLA_2 III). This way, the 328 base pair PLA_2 IID product was cut into 259 and 69 bp fragments, the 324 bp PLA_2 IIE product into 207 and 117 bp fragments, the 266 bp PLA_2 III product into 191 and 65 bp fragments, the 272 bp PLA_2 IVB product into 174 and 98 bp fragments, the 451 bp $aiPLA_2$ product into 250 and 201 bp fragments, and the 303 calcium-independent membrane bound PLA_2 ($iPLA_2$-2) product into 211 and 92 bp fragments, as predicted by the restriction enzyme map. –: uncut fragment; +: cut fragment; S: 100 base pair size marker.

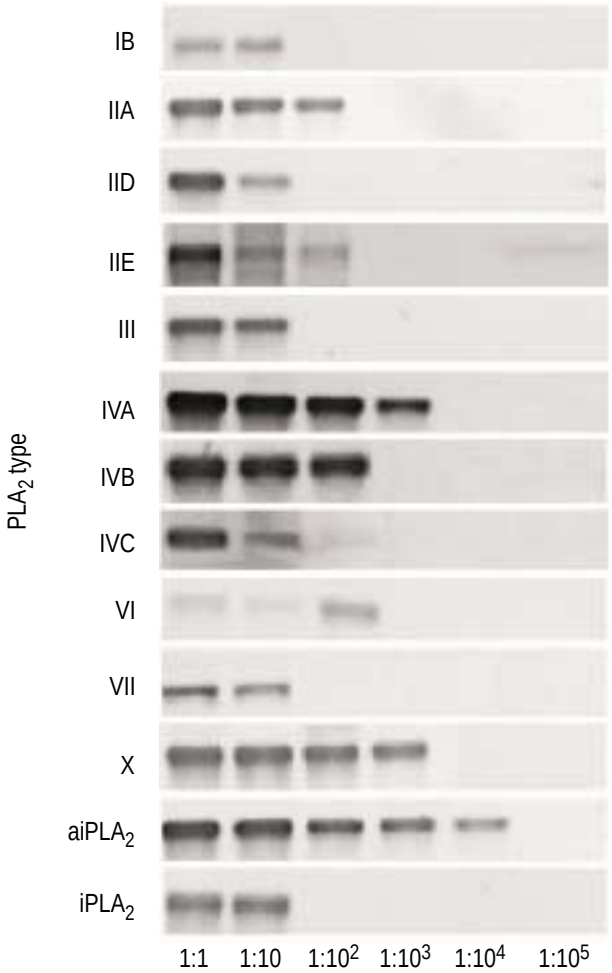

Fig. 4. – Expression of different phospholipase A_2 (PLA_2) types in nasal mucosal cells obtained by brushing. A 10-fold dilution series of pooled complimentary deoxyribonucleic acid was analysed by polymerase chain reaction.

to the opposite strand of the heterologous DNA. The second PCR is performed with the PLA_2-specific primer. The PLA_2 mimics thus generated contain the target sequences for the PLA_2-specific primers and the target sequence for the internal oligonucleotide probe, allowing confirmation of the specific PLA_2 product as well as the mimic product by Southern blot. The internal control fragments (mimics) and cDNA fragments from the PLA_2 forms were simultaneously

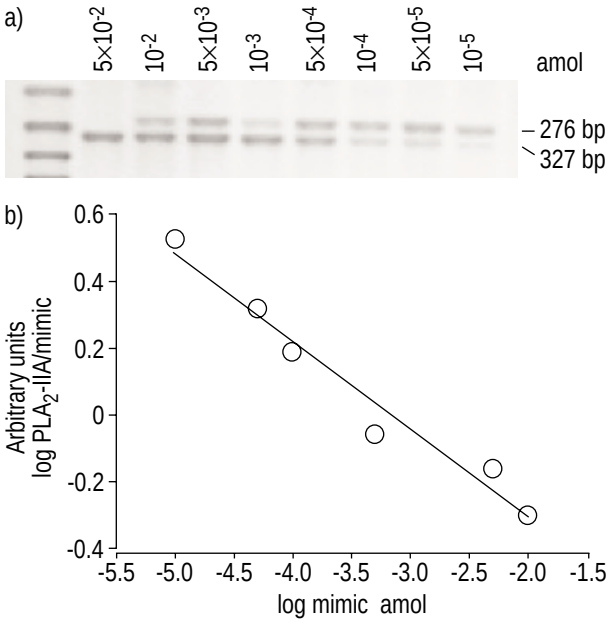

Fig. 5. – a) Competitive polymerase chain reaction (PCR) analysis of the phospholipase A_2 (PLA_2) IIA(276 base pairs (bp)) messenger ribonucleic acid (mRNA) level in nasal mucosal cells obtained by brushing. Total RNA was used as template for complementary deoxyribonucleic acid (cDNA) synthesis and a portion of the cDNA was amplified in the presence of different amounts of a PLA_2 IIA mimic (327 bp). Lanes 2–9 contain decreasing concentrations of PLA_2 IIA mimic, and lane 1 contains a 100 bp size marker. b) The quantitative analysis of the competitive PCR shown in a): $y = -0.26x - 0.83$; $R^2 = 0.97$.

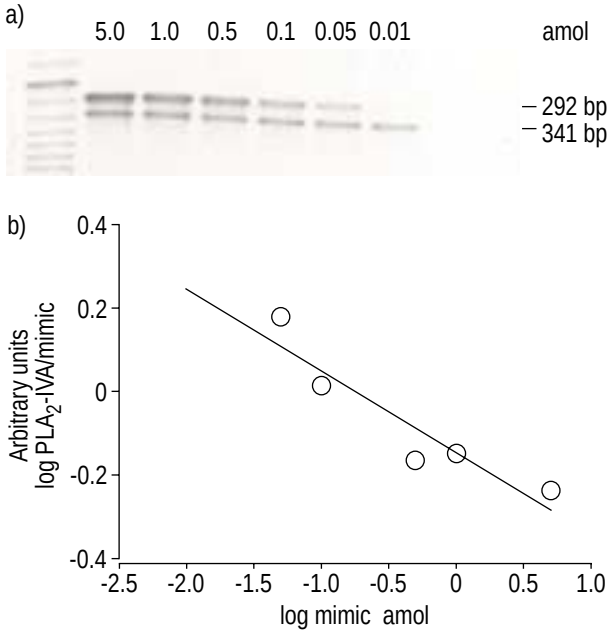

Fig. 6. – Competitive polymerase chain reaction (PCR) analysis of the A_2 (PLA_2) IVA (341 base pairs (bp)) messenger ribonucleic acid (mRNA) level in nasal mucosal cells obtained by brushing. Total RNA was used as template for complementary deoxyribonucleic acid (cDNA) synthesis and a portion of the cDNA was amplified in the presence of different amounts of a PLA_2 IVA mimic (292 bp). Lanes 2 – 7 contain decreasing concentrations of PLA_2 IVA mimic, and lane 1 contains a 50 bp size marker with the 250 bp band enhanced. b) The quantitative analysis of the competitive PCR shown in (a): $y=-0.2x-0.15$; $R^2=0.87$.

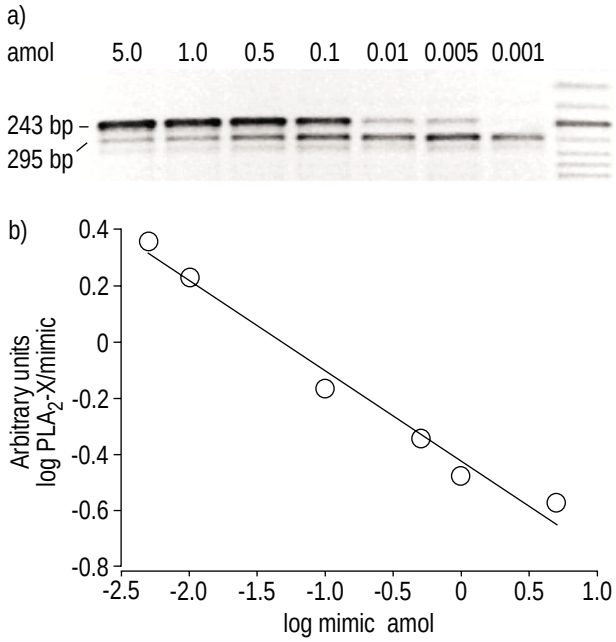

Fig. 7. – a) Competitive polymerase chain reaction (PCR) analysis of the phospholipase A_2 (PLA_2) X (295 base pairs (bp)) messenger ribonucleic acid (mRNA) level in nasal mucosal cells obtained by brushing. Total RNA was used as template for complementary deoxyribonucleic acid (cDNA) synthesis and a portion of the cDNA was amplified in the presence of different amounts of a PLA_2 X mimic (243 bp). Lanes 1 – 7 contain decreasing concentrations of PLA_2 X mimic and lane 8 contains a 50 bp size marker with the 250 bp band enhanced. b) The quantitative analysis of the competitive PCR shown in a): $y=-0.32x-0.44$; $R^2=0.98$.

amplified to ensure a parallel accumulation over the whole cycle range and a similar E-value for the accumulation for the two fragments. Due to large E-value differences between the cDNA PLA_2 IVA and its mimic, a new antisense primer was constructed giving a 341 bp PCR product (fig. 6). The E-values were: mimic PLA_2 IIA and cDNA PLA_2 IIA 0.81 and 0.78, mimic PLA_2 IVA and cDNA PLA_2 IVA 0.71 and 0.72, and mimic PLA_2 X and cDNA PLA_2 X 0.59 and 0.53, respectively. The amounts of PCR product generated in the coamplification of cDNA from a sample with different amounts of mimic was used to determine the level of specific PLA_2 mRNA present in the sample. The amounts of the two products separated on an agarose gel (Life Technologies) ethidium bromide stained, digitized into gray-scale images and expressed as net intensity were plotted as the log (PLA_2-form/mimic) *versus* log (mimic concentration). After linear regression and expression in the format $y=kx+l$, the amount of PLA_2 in the sample was calculated from $y=0$ (figs. 5 – 7). The intra-assay and interassay variation of the determinations were tested by amplifying a dilution series of the PLA_2 mimic with 0.1 amol, respectively, 1 amol of PLA_2 cDNA added to each dilution. The PCR reactions were performed in triplicate at three different occasions. The mean $\pm$ SD values for the detected amounts of PLA_2 cDNA in the nine determinations were for the 0.1 amol samples: PLA_2 IIA 0.07 ± 0.02 amol, PLA_2 IVA 0.29 ± 0.04 amol, PLA_2 X 0.10 ± 0.02 amol, and for the 1 amol samples: PLA_2 IIA 1.08 ± 0.36 amol, PLA_2 IVA 2.99 ± 0.92 amol, and PLA_2 X 1.20 ± 0.25 amol.

Results

Expression of phospholipase A_2 in human nasal mucosal cells

The expression of different PLA_2 forms in NLF and nasal brush samples are illustrated in figures 1 and 2. It thus appeared that the IB, IIA, IID, IIE, III, IVA, IVB, IVC, V, VI, VII, X, $iPLA_2$-2 and $aiPLA_2$ forms were all expressed in the mucosal cell samples, regardless of sampling technique. The different PCR products were confirmed using Southern blot and γ-^{32}P-oligonucleotides (fig. 2), or by use of the restriction enzymes PVU II, Hha I or Sty I (fig. 3). Repeated experiments showed essentially the same pattern of PLA_2 expression, except that PLA_2 V mRNA was not detected; this particular PLA_2 form was thus found only in pooled samples from the first sampling round. Analysis of mRNA from the five different subjects revealed that the PLA_2 V expression originated from one single individual. Repeated samples were taken from this individual and analysed; however, no PLA_2 V mRNA was found on these later occasions. By contrast, all the other PLA_2 forms were found to be expressed in all subjects, including the individual that expressed the PLA_2 V form on one single occasion.

To investigate the relative abundance of the different PLA_2 mRNA forms, a ten-fold dilution series of pooled cDNA was analysed (fig. 4). This revealed that $aiPLA_2$ was detected in the $1{:}10^4$ dilution, followed by PLA_2 IVA and X in the $1{:}10^3$ dilution, IIA, IIE, IVB, and VI in the $1{:}10^2$ dilution, and IB, IID, III, IVC, VII, and $iPLA_2$-2 in the 1:10 dilution. The large amounts of $aiPLA_2$ mRNA detected were not due to a more efficient amplification reaction; in fact, the PCR for this PLA_2 type had the lowest E-value of all.

Quantitative messenger ribonucleic acid analysis of phospholipase A_2 IIA, IVA and X

In order to quantitatively assess the expression of PLA_2 IIA, IVA and X mRNA, competitive PCR analyses were performed using mimics for these forms. The application of these mimics is illustrated in figures 5–7, which show the measurements of PLA_2 IIA, IVA and X mRNA in a nasal mucosal sample. After linear regression analysis, the amounts of mRNA in this specific sample were calculated to be 6.8×10^{-3}, 0.18 and 0.05 amol, respectively.

The mimics were then used to determine the amounts of PLA_2 IIA, IVA and X mRNA in nasal mucosal samples from the five subjects. After normalization to their reduced glyceraldehyde-phosphate-dehydrogenase (GAPDH) level the amounts of PLA_2 IIA, IVA and X mRNA were estimated to 0.0025 ± 0.0021, 1.1 ± 0.7, and 0.9 ± 0.2 amol (mean $\pm$ SE), respectively (table 2). The relative abundance of these PLA_2 transcripts (as demonstrated in fig. 4) was thus confirmed.

Discussion

The present investigation showed that PLA_2 IB, IIA, IID, IIE, III, IVA, IVB, IVC, VI, VII, X, $iPLA_2$-2 and $aiPLA_2$ mRNA were expressed in the human nasal mucosa in all subjects investigated. This study thus not only confirmed the presence of PLA_2 IIA [12] and VII [11] but also demonstrated the presence of a large number of other members of the PLA_2 superfamily, including the newly described PLA_2 IID [14], IIE [4], III [6], IVB [16], IVC [17], X [20], and $iPLA_2$-2 [3, 5]. It should be emphasized, however, that the presence of PLA_2 gene transcripts does not necessarily mean that the mRNA is translated into proteins or that these proteins, if present, are activated PLA_2 enzymes. Further studies must be undertaken in order to reveal which PLA_2 enzyme proteins are operating in the nasal mucosa.

Table 2. – Competitive polymerase chain reaction (PCR) analysis of the phospholipase A_2 (PLA_2) IIA, IVA and X messenger ribonucleic acid (RNA) levels in nasal mucosal cells from five healthy subjects

Subject	PLA_2 mRNA (amol/GAPDH)		
	IIA	IVA	X
A	<0.0001	1.6	0.9
B	0.0003	0.1	0.3
C	0.01	3.7	0.9
D	<0.0001	<0.1	1.1
E	0.0008	0.1	1.4

Total RNA was used as templates for complementary deoxyribonucleic acid (cDNA) synthesis and the amount of cDNA for the different PLA_2 types determined by competitive PCR, utilizing cDNA mimics corresponding to respective PLA_2 type. Each sample was then normalized to its reduced glyceraldehyde-phosphate dehydrogenase (GAPDH) level, giving a quantitative estimate of the amount of the different PLA_2 mRNA types.

Previous studies have identified some of the different PLA_2 types in other parts of the human respiratory tract, such as PLA_2 IB [21], IIA [12], IID [14], IIE [4], VII [22, 23], and X [20]. PLA_2 III was cloned from the foetal lung, but Northern blot did not reveal any transcript in the lung [6]. Cytosolic PLA_2 IVA has been identified in human alveolar macrophages and respiratory cell lines [24, 25], and the recently discovered paralogues, PLA_2 IVB and IVC, in human lung tissue [16, 17]. By contrast, cytosolic PLA_2 VI ($iPLA_2$) has, to the present authors' knowledge, not been demonstrated in human airways until now. PLA_2 VI mRNA corresponds to an 85 kDa murine PLA_2 that has been identified in P388D1 cells [26]. Although this enzyme shares no homology with the calcium-dependent PLA_2 IVA and possesses no clear preference for AA-containing phospholipids, it is thought to play a role in the remodelling of membrane phospholipid and has been shown to be of importance in regulating the AA incorporation into membranes [2]. It is possible, therefore, that this enzyme plays an important part in the overall AA metabolism.

As illustrated in figure 4, there were striking differences in the relative abundance of the different PLA_2 types. The strongest expression was shown by $aiPLA_2$, which has been proposed to play a major part in the recycling of lung surfactant, as it has preference for dipalmitoyl phosphatidylcholine [9, 27]. However, it is questionable whether this enzyme is a true PLA_2 and if it has PLA_2 properties at physiological pH. The $aiPLA_2$ also has glutathione peroxidase activity, and has instead been proposed as an antioxidative protein [28]. Interestingly, PLA_2 X was also relatively strongly expressed (fig. 4), indicating that this PLA_2 type is abundantly present in the nasal mucosa. The results obtained with the mimic technique confirmed the order of appearance of PLA_2 X, IVA and IIA shown by the dilution series, with PLA_2 X and IVA being about 400-fold more strongly expressed than PLA_2 IIA (table 2). There is therefore reason to believe that PLA_2 X is strongly expressed in the human nasal mucosa.

PLA_2 X is a low molecular weight (13.6 kDa), calcium-dependent, secretory PLA_2 that contains 16 cysteine residues [8, 29]. Because it binds to a PLA_2 lung membrane receptor [30], it is thought to play a role in cell signaling. PLA_2 X has been detected in human adult lung [20] and type II alveolar epithelial cells [31] but not in the human nasal mucosa until now. Interestingly, NLF from individuals exposed to cold air or methacholine has high PLA_2 activity. A previous study using a fluoroimmunoassay has suggested that this activity is due to PLA_2 IIA derived from tear fluid [12]. In the same study, however,

immunostaining of nasal biopsies showed only few PLA_2 IIA-positive cells, which is in accordance with the present findings that the mRNA expression of PLA_2 IIA is weak. Altogether, these findings suggest that PLA_2 X, rather than PLA_2 IIA, should be considered as the major $sPLA_2$ in the nasal tract of healthy individuals. The function of PLA_2 X is not known. Notably, it has been demonstrated that PLA_2 X releases significantly larger amounts of AA than do other $sPLA_2$ forms (IB, IIA and V) when added exogenously to adherent cells [8, 29, 31], suggesting that PLA_2 X may play an important role in AA liberation.

As to which PLA_2 type(s) are involved in airway inflammation and disease, much attention has been given to the secretory (14 kDa)-type ($sPLA_2$). The PLA_2 IIA has been connected to inflammatory diseases such as Crohn's disease and endotoxic shock [32]. This form is inducible by endotoxin in rats [14, 33] and in guinea pigs [34] and has also been proposed to take part in the defence against bacterial infection [35]. The recently discovered $sPLA_2$ II forms, IID and IIE, might also take part in the bacterial defence, as both are able to hydrolyze phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), the major components of bacterial phospholipids.

Aside from the $sPLA_2$ group, $cPLA_2$ has also been considered important for inflammatory signaling [36]. The present results showed that the expression of PLA_2 IVA mRNA was greater than that of PLA_2 IIA in the nasal mucosa of healthy subjects. This $cPLA_2$ is known to be of importance for AA liberation with the most convincing results obtained through experiments with PLA_2 IVA deficient mice. Such studies have revealed that the IVA form is of importance in the allergic response and for macrophage production of inflammatory mediators [36, 37]. This suggests a role for PLA_2 IVA in airway inflammation. In addition to PLA_2 IVA, it was also found that mRNA of the two novel IVB and IVC forms in the nasal mucosa. Biochemical characterization by Song *et al.* [38] has shown that IVA has a preference for the sn-2 position and the IVB for the sn-1 position, while IVC enzymes can cleave efficiently well at both positions. The IVA and IVB are both calcium-dependent enzymes while IVC is not. This indicates different biological roles or regulations of these $cPLA_2$ forms.

During recent years another $sPLA_2$ (type V) has been implicated in inflammatory signalling and it has been suggested that some of the results concerning PLA2 IIA should be reconsidered in the light of the presence of PLA_2 V [7]. In the present study the V form was only detected in one out of five healthy individuals. Repeated efforts to demonstrate PLA_2 V in mucosal samples from this one individual were unsuccessful, indicating that the expression was occasional and that this form was induced by some stimuli at that specific time point. Further studies are needed to investigate if this PLA_2 form perhaps is more highly expressed in patients with airway inflammation.

Taken together, the issue of PLA_2 activity and regulation is growing more complex. New PLA_2 forms with unknown functions, cross-talking between different PLA_2s [39], splice variants of certain PLA_2s [15], and a large number of proteins that may affect PLA_2 activity [13, 40, 41] all add up to a picture of great complexity. It is important, however, to gain a deeper knowledge about the role and regulation of each PLA_2 and so to be able to develop new and more specific anti-PLA_2 drugs. In light of the present study the nasal mucosa offers a location for further studies, in which most, if not all the PLA_2 forms are expressed.

In conclusion, it has been found that a large number of phospholipase A_2 types are expressed in the normal human nasal mucosa. Moreover, the investigation demonstrates for the first time, the presence of the newly discovered phospholipase A_2 forms IID, IIE, III, IVB, IVC, X and the cytosolic calcium-independent membranebound phospholipase A_2 ($iPLA_2$-2). The precise function of each of these phospholipase A_2 forms is not clear. The nasal mucosa is constantly exposed to environmental factors such as particles, chemicals, allergens and microorganisms and the presence of messenger ribonucleic acid of several phospholipase A_2 forms may therefore reflect a need for a fast response and a first line of defence towards all these stimuli. It is likely, however, that one or several of these PLA_2 enzymes are important to the arachidonic acid and/1-*O*-alkyl-lysophosphatidylcholine release in the nasal mucosa. Future studies aiming at identifying the molecular mechanisms underlying inflammatory reactions or diseases of the nasal mucosa will therefore have to take these findings into consideration.

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