Group V and X secretory phospholipase A₂ prevents adenoviral infection in mammalian cells

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sPLA₂ (secretory phospholipase A₂) enzymes have been implicated in various biological events, yet their precise physiological functions remain largely unresolved. In the present study we show that group V and X sPLA₂s, which are two potent plasma membrane-acting sPLA₂s, are capable of preventing host cells from being infected with an adenovirus. Bronchial epithelial cells and lung fibroblasts pre-expressing group V and X sPLA₂s showed marked resistance to adenovirus-mediated gene delivery in a manner dependent on their catalytic activity. Although adenovirus particles were insensitive to recombinant group V and X sPLA₂s, direct addition of these enzymes to 293A cells suppressed both number and size of adenovirus plaque formation. Group V and X sPLA₂s retarded the entry of adenovirus into endosomes. Moreover, adenoviral infection was suppressed by LPC (lysophosphatidylcholine), a membrane-hydrolytic product of these sPLA₂s. Thus hydrolysis of the plasma membrane by these sPLA₂s may eventually lead to the protection of host cells from adenovirus entry. Given that group V and X sPLA₂s are expressed in human airway epithelium and macrophages and that the expression of endogenous group V sPLA₂ is upregulated by virus-related stimuli in these cells, our present results raise the possibility that group V and X sPLA₂s may play a role in innate immunity against adenoviral infection in the respiratory tract.

Key words: adenovirus, bronchial epithelial cell, infection, lysophosphatidylcholine (LPC), plasma membrane, secretory phospholipase A₂ (sPLA₂).

INTRODUCTION

PLA₂ (phospholipase A₂) catalyses the hydrolysis of the *sn*-2 position of membrane glycerophospholipids to yield free fatty acids, including AA (arachidonic acid) and lysophospholipids. Mammalian PLA₂s have been classified into four major families including sPLA₂ (secretory PLA₂), cPLA₂ (cytosolic PLA₂), iPLA₂ (Ca^{2+}-independent PLA₂) and platelet-activating factor acetylhydrolases, each of which occur as multiple isoforms [1,2]. Of these, the sPLA₂ family represents a group of structurally related, disulphide-rich, Ca^{2+}-dependent, low molecular mass enzymes with a His-Asp catalytic dyad [1,2]. Individual sPLA₂s exhibit unique tissue and cellular localization and enzymatic properties, suggesting distinct roles in various physiological events.

Several sPLA₂s have the ability to release AA from mammalian cell membranes through multiple mechanisms, an event that can be linked to eicosanoid biosynthesis [3–9]. Among these enzymes, group V and X sPLA₂s (sPLA₂-V and -X respectively) are particularly potent in eliciting AA release from intact cellular membranes, most likely because of their high capacity to bind PC (phosphatidylcholine), a phospholipid that is enriched in the outer plasma membrane [4–6]. sPLA₂-IIA and related enzymes often release AA from activated cells in a manner dependent upon their association with HSPGs (heparan sulphate proteoglycans), although the occurrence of this route appears to be cell-type-specific [4,7,8]. It has also been suggested that sPLA₂s can elicit AA release before secretion [9]. sPLA₂s also promote various cellular responses, such as cPLA₂α-dependent AA release, through binding to sPLA₂ receptors in a manner independent of their catalytic activity [10].

In addition to their action on mammalian cells, several sPLA₂s are capable of killing bacteria by degrading bacterial membranes, thereby potentially providing a first line of anti-microbial host defence [11–14]. The bactericidal activity of sPLA₂-IIA, a prototypic anti-bacterial sPLA₂, depends on its catalytic activity, as well as on the overall negative charge of the enzyme [11–14]. The rank order of potency among human sPLA₂s against Gram-positive bacteria has been reported to be IIA > X > V > XII > IIE > IB, IIF [12]. The ability of sPLA₂-X, an acidic sPLA₂ to kill some Gram-positive bacteria, and the observation that another acidic sPLA₂ sPLA₂-XIIA, in which catalytic activity towards phospholipid vesicles is rather low, can also destroy Gram-positive bacteria [12] suggests that the bactericidal activity of sPLA₂s cannot be explained simply by their catalytic activity toward pure phospholipid vesicles and their surface charges but may involve additional unresolved parameter(s).

Although the potential host defence role of sPLA₂s against bacterial infection has thus been emerging, their effect on viral infection has remained largely obscure. The capsid of parvoviruses contains sPLA₂ (group XIII), the activity of which is dispensable for viral attachment to host cell surfaces and subsequent endocytosis but crucial for efficient transfer of the viral genome from endosomes into the nucleus [15,16]. Conversely, exogenous bee venom sPLA₂ (group III) inhibits the entry and thereby replication of HIV in host cells through a mechanism

linked to sPLA$_2$ binding to cells [17,18]. So far, little is known about the effects of mammalian sPLA$_2$s on viral infection into host cells.

Adenoviruses are non-enveloped icosahedral DNA viruses about 90 nm in diameter that cause infections of the respiratory tract [19,20]. The adenovirus particle is composed of an outer capsid consisting mainly of hexon and an inner DNA-associated core with a 36 kbp linear DNA, two terminal proteins and condensing proteins. The vertices are made up of a penton base containing Arg-Gly-Asp motifs and the protruding fibre protein, which binds to CAR (Coxsackie virus B adenovirus receptor) on host cell surfaces and activates integrins locally. Adenoviruses are then internalized via clathrin-mediated endocytosis into the endosomes, a slightly acidic intracellular compartment, and escape into the cytosol after cell signalling. Viruses then move towards the nucleus, dock with the nuclear pore complex and disassemble to release the viral genome into the nucleus.

During our ongoing studies on the functional aspects of mammalian sPLA$_2$s by taking advantage of the adenovirus-mediated gene transfer strategy [21–24], we unexpectedly found that sPLA$_2$-V and -X have the capacity to prevent host cells from being infected with adenoviruses. Unlike the bactericidal action of sPLA$_2$s, the anti-viral effect of these two sPLA$_2$s depends essentially on their ability to hydrolyse phospholipids in host cell membranes. Considering that both sPLA$_2$-V and -X are expressed in the human respiratory tract [23], our present results open new insights into the potential functional role of these enzymes in innate immunity against viral infection.

MATERIALS AND METHODS

Materials

Human sPLA$_2$ cDNAs, pure recombinant human sPLA$_2$ proteins produced in *Escherichia coli* and rabbit antisera specific for individual human sPLA$_2$s were described previously [21–25]. Enzyme immunoassay kit for PGE$_2$ (prostaglandin E$_2$) was purchased from Cayman Chemical. cDNAs for human cPLA$_2\alpha$, cPLA$_2\gamma$, iPLA$_2\beta$, COX (cyclo-oxygenase)-2 and mPGES-1 (microsomal PGE synthase-1) were described previously [3,26–28]. AA, LPC with various *sn*-1 fatty acids (C$_{12:0}$, C$_{14:0}$, C$_{16:0}$ and C$_{18:0}$), 1-palmitoyl-LPE (lysophosphatidylethanolamine), LPS (lysophosphatidylserine), and LPA (lysophosphatidic acid) and human IFN (interferon)-α were purchased from Sigma. Human TNF (tumour necrosis factor)-α was from Genzyme. Poly-I:C was obtained from Amersham Biosciences.

Cell culture

Human bronchial epithelial BEAS-2B cells were cultured in RPMI 1640 medium (Nissui Pharmaceutical) containing 10% (v/v) foetal calf serum as described previously [28]. BEAS-2B cells stably expressing various PLA$_2$s or COX-2 were established as described previously [23,28]. NHPF (normal human pulmonary fibroblasts) and culture media with supplements for these cells were purchased from BioWhittaker [23].

Preparation and activation of peritoneal macrophages

C57BL/6 mice (Saitama Animal Centre) were housed in microisolator cages in a pathogen-free barrier facility and all experiments were performed under approved institutional guidance. Peritoneal cells were recovered from mice that had received thioglycollate medium (Difco) (1 ml per 20 g of body weight) 4 days previously [29]. The peritoneal cells were seeded into 12 well plates (Iwaki Glass) at a cell density of 2×10^6 cells/ml

in 1 ml of RPMI medium supplemented with 10% (v/v) foetal calf serum. After incubation for 2 h in a CO$_2$ incubator, the supernatants and non-adherent cells were removed. More than 90% of adherent cells were macrophages.

Northern-blotting

Equal amounts (approx. 10 μg) of total RNA obtained from cells by use of TRIzol® reagent (Invitrogen) were applied to separate lanes of 1.2% (w/v) formaldehyde-agarose gels, electrophoresed and transferred to Immobilon-N membranes (Millipore). The resulting blots were then probed with appropriate cDNA probes that had been labelled with [^{32}P]dCTP (Amersham Bioscience) by random priming (Takara Biomedicals). Hybridization and subsequent membrane washing were carried out as described previously [3].

Immunoblotting

Lysates from 2×10^5 cells were subjected to SDS/PAGE on 15% gels under reducing conditions with 2-mercaptoethanol. The separated proteins were electroblotted on to nitrocellulose membranes (Schleicher and Schuell) with a semi-dry blotter (MilliBlot-SDE system, Millipore). After blocking with 5% (w/v) skimmed milk in TBS containing 0.05% (v/v) Tween-20 (TBS-T), the membranes were probed with rabbit antibody against human sPLA$_2$-V or -X at 1:5000 dilution in TBS-T for 2 h, followed by incubation with horseradish peroxidase-conjugated anti-(rabbit IgG) (Zymed) at 1:5000 dilution in TBS-T for 2 h and were visualized using the ECL (enhanced chemiluminesence) Western blot system (PerkinElmer) as described previously [22,23].

RT (reverse transcription)-PCR

Synthesis of cDNA was performed using 0.5 μg of total RNA from cells and tissues, and AMV (avian myeloblastosis virus) reverse transcriptase, according to the manufacturer's instructions supplied with the RNA PCR kit (Takara Biomedicals). Subsequent amplification of the cDNA fragments was performed using 0.5 μl of the reverse-transcribed mixture as a template with specific primers for each sPLA$_2$ (Greiner, Japan). For amplification of human and mouse sPLA$_2$-V and -X, a set of 23 bp oligonucleotide primers corresponding to 5′- and 3′-nt sequences of their open reading frames were used as primers, as described previously [22,23]. The PCR conditions were 94 °C for 30 s and then 30 cycles of amplification at 94 °C for 5 s and 68 °C for 4 min, using the Advantage cDNA polymerase mix (Clontech). The PCR conditions for G3PDH (glyceraldehyde-3-phosphate dehydrogenase) was described previously. The PCR products were analysed by 1% agarose gel electrophoresis with ethidium bromide.

Adenovirus expression system

Adenoviruses bearing cDNAs for human sPLA$_2$s and their mutants; cPLA$_2$s, iPLA$_2\beta$, COX-2 and mPGES-1 were prepared using the ViraPower Adenovirus Expression System (Invitrogen) as described previously [21–24]. Briefly, the full-length cDNAs were subcloned into the pENTER/D-TOPO vector using a pENTER Directional TOPO Cloning kit (Invitrogen). After purification of the plasmids from the transformed Top10 competent cells (Invitrogen) the cDNA inserts were transferred to the pAd (adenovirus)/CMV/V5-DEST vector (Invitrogen) by means of the Gateway® system using LR Clonase™ (Invitrogen). The plasmids were purified and digested with Pac I (New England BioLabs). The linearized plasmids (1–2 μg) were then mixed with

4 μl of Lipofectamine™ 2000 (Invitrogen) in 200 μl of Opti-MEM® medium (Invirogen) and transfected into subconfluent 293A cells (Invitrogen) in 1 ml of Opti-MEM® in 6 well plates (Iwaki Glass). Then 293A cells were cultured for 1–2 weeks in RPMI 1640 medium containing 10 % foetal calf serum, with replacement of the medium every 2 days. When most cells became detached from the plates the cells and culture medium were harvested together, freeze-thawed twice and centrifuged to obtain the adenovirus-enriched supernatants. Then aliquots of the supernatants were added to fresh 293A cells and cultured for 2–3 days to amplify adenoviruses. After 2–4 amplifications, the resulting adenovirus-containing media were used as virus stock. Viral titres were determined by the plaque-forming assay using 293A cells. As a control, PacI-digested pAd/CMV/V5-GW/*lac*Z vector (Invitrogen) was transfected into 293A cells to produce a *lac*Z-bearing adenovirus. Aliquots of the adenovirus-containing medium were added to cells and cultured for appropriate periods to allow the expression of adenovirus-delivered genes.

Construction of sPLA₂-V and -X mutants

cDNAs for the catalytic centre mutants of human sPLA₂-V and -X (H48Q; numbering in alignment with sPLA₂-IB) were prepared by the mismatched PCR method as described previously [4]. The primers used were as follows: V-HQ-S primer, 5′-TGTT-GGGCGCAGGACCACTGCTATG-3′; V-HQ-AS primer, 5′-CA-TAGCAGTGGTCCTGCGCCCAACA-3′; X-HQ-S primer, 5′-TG-CCATGGCCAGGACTGTTGTTAC-3′; X-HQ-AS primer, 5′-GTAACAACAGTCCTGGCCATGGCA-3′; V-5′ primer, 5′-AT-GAAAGGCCTCCTCCCACTGGC-3′; V-3′ primer, 5′-GGCC-TAGGAGCAGAGGATGTTGG-3′; X-5′ primer, 5′-ATGGGG-CCGCTACCTGTGTGCC-3′ and X-3′ primer, 5′-TCAGTCAC-ACTTGGGCGAGTCCG-3′. To construct the mutants, PCR was conducted with a set of V- or X-5′ primers and V- or X-HQ-AS primers (for the forward strand), and with a set of V- or X-HQ-S primers and V- or X-3′ primers (for the reverse strand) using Pyrobest polymerase (Takara Biomedicals) and sPLA₂-V or -X cDNA as a template with 25 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30s. The resulting forward and reverse strands were mixed, heated, annealed and subjected to a second PCR with V- or X-5′ primers and V- or X-3′ primers under the same thermal conditions. The PCR products were subcloned into the pENTER/D-TOPO vector for adenoviral expression as described above. The plasmids were sequenced with a Taq cycle sequencing kit (Takara Biomedicals) and an autofluorometric DNA sequencer 310 Genetic Analyser (Applied Biosystems) to confirm the sequences.

Assays for AA release and PGE₂ generation

Cells grown to near confluency in 24-well plates (Iwaki Glass) were incubated with [³H]AA (Amersham Bioscience) (0.1 μCi/ml) overnight. After 3 washes with fresh medium, 250 μl of culture medium with or without 100 units/ml TNFα was added to each well and the radioactivities, released into the supernatants after incubation for appropriate periods, were measured. The percentage release was calculated using the formula $[S/(S + P)] \times 100$, where S and P are the radioactivities measured in the supernatant and cell pellet respectively, as described previously [3,4]. Aliquots of the supernatants from replicate positive cells (without preincubation with [³H]AA) were subjected to PGE₂ enzyme immunoassay.

Plaque-forming assay

Adenovirus solution in serial dilutions was added to subconfluent 293A cells cultured in 1 ml of culture medium in 24 well plates.

After 5 days of culture, the number of plaques formed on the 293A cell monolayer was counted. Photographs were taken using an ECLIPSE TE300 microscope (Nikon).

Measurement of sPLA₂ activity

sPLA₂ activities in cell lysates and culture supernatants were assayed by measuring the amount of radiolabelled linoleic acid released from the substrate 1-palmitoyl-2-[¹⁴C]linoleoyl-phosphatidylethanolamine (Amersham Bioscience). The substrate in ethanol was dried under N_2 stream and was dispersed in water by sonication. Each reaction mixture (total volume 250 μl) consisted of appropriate amounts of the required samples, 100 mM Tris/HCl (pH 7.4), 4 mM CaCl₂ and 2 μM substrate. After incubation for 30 min at 37 °C, [¹⁴C]linoleic acid was extracted and the radioactivity was quantified by liquid-scintillation counter as described previously [3,4].

Confocal laser microscopy

Cells grown to subconfluency on glass-bottom dishes (Matsunami) pre-coated with 5 μg/ml fibronectin (Sigma) were incubated with adenovirus at 4 °C or 37 °C for adequate periods. Then the cells were washed twice with PBS and fixed with 3 % paraformaldehyde in PBS for 1 h. After 3 washes with TBS, the fixed cells were treated with 1 % (w/v) BSA and 0.5 % (w/v) saponin in TBS (blocking solution) for 1 h, with rabbit anti-adenovirus hexon protein (Chemicon) or goat anti-EEA1 (early endosomal antigen 1) antibody (B.D. Biosciences) at 1:200 dilution in blocking solution for 2 h and then with species-matched FITC-conjugated or carbocyanine 3-conjugated second antibody at 1:200 dilution in blocking solution for 2 h, with 3 washes after each interval. After 6 washes with TBS, specific immunofluorescent signals were visualized using a laser scanning confocal microscope (IX70, Olympus).

ESI (electrospray ionization) MS

MS spectra were obtained using a Quattro Micro tandem quadrupole mass spectrometer (Micromass) equipped with an ESI as described previously [24,27]. Lipid extracts obtained from BEAS-2B cells with or without sPLA₂ transfection [100 μg protein equivalents; protein concentrations were determined by BCA (bicinchoninic acid) protein assay kit (Pierce)] were reconstituted in chloroform/methanol (2:1) (100–300 μmol phosphorus/l), and 2 μl of the sample was injected per run. As internal standards, 100 pmol of PC (C₁₄:₀–C₁₄:₁, diacyl), LPC (C₁₂:₀) and PE (C₁₄:₀–C₁₄:₀, diacyl) were added to the samples. The samples were introduced by means of a flow injector into the ESI chamber at a flow rate of 4 μl/min in a solvent system of acetonitrile/methanol/water (2:3:1, by vol.) containing 0.1 % (v/v) ammonium formate (pH 6.4). The mass spectrometer was operated in the positive and negative scan modes. The flow rate of the nitrogen drying gas was 12 l/min at 80 °C. The capillary and cone voltages were set at 3.7 kV and 30 V respectively, argon at $3–4 \times 10^4$ Torr was used as the collision gas and a collision energy of 30–40 V was used for obtaining fragment ions for precursor ions.

In situ hybridization

In situ hybridization experiments were performed on the paraffin embedded tissue serial sections with the Ventana HX system (Ventana Medical System) as described previously [30]. Briefly, sPLA₂ cDNAs were each subcloned into the pCR-II vector (Invitrogen) and the sense and antisense cRNA probes for sPLA₂s were synthesized from the T7 and SP6 promoters respectively, on the Not I-digested, linearized plasmid. Sections were pre-treated and hybridized with a Ventana Ribo Map kit (Ventana Medical

Systems) on the automated Ventana HX system. Detection of hybrid was performed using a digoxigenin nucleic kit (Boehringer Mannheim GmbH), according to the manufacturer's instructions. Signal detection with Nitro Blue tetrazolium salt was performed at 20 °C for 30 h in the dark. Sections were then dehydrated through an increasing concentration of ethanol (30, 50, 70, 90 and 100 % ethanol, 2 min each) and washed twice for 5 min in xylene before mounting in Malinol mounting medium (Muto Pure Chemicals).

RESULTS

Overexpression of sPLA$_2$-V and -X prevents adenoviral infection in host cells

In an initial effort to address the eicosanoid-biosynthetic capacity of various PLA$_2$s in combination with downstream COX and PGES enzymes, human bronchial epithelial cells BEAS-2B, which had been stably transfected with various PLA$_2$ and COX enzymes, were infected with adenovirus expressing mPGES-1 (Ad-mPGES-1), the most downstream enzyme in the PGE$_2$-synthetic pathway. During the course of this study, we unexpectedly found that the expression level of adenovirally delivered mPGES-1 was significantly lower in cells pre-expressing sPLA$_2$-V or -X than that in cells pre-expressing other sPLA$_2$s (sPLA$_2$-IIA, -IID and -IF), cPLA$_2\alpha$, and COX-2 (Figure 1A). It is unlikely that the event observed in cells pre-expressing sPLA$_2$-V or -X was due to overexpression of mPGES-1, since decreased expression of other adenovirus-delivered genes (e.g. iPLA$_2\beta$) was also evident in cells pre-expressing sPLA$_2$-V or -X, but not in cells pre-expressing other PLA$_2$s and COX-2 (Figure 1A).

To verify if the above result is particular to BEAS-2B cells or also occurs in other cell types, normal human fibroblasts NHPF were first transfected adenovirally with various sPLA$_2$s and cPLA$_2$s and then subjected to second transfection with Ad-mPGES-1 or Ad-iPLA$_2\beta$. As shown in Figure 1(B), expression of the genes delivered by Ad-mPGES-1 and Ad-iPLA$_2\beta$ was significantly lower in cells pre-expressing sPLA$_2$-V or -X than in cells pre-expressing other PLA$_2$s or control cells. Moreover, adenoviral expression of sPLA$_2$-IIA-G30S, a mutant that lacks catalytic activity and thus is devoid of the cellular AA-releasing function [3], was significantly decreased in cells pre-expressing sPLA$_2$-V or -X relative to that in control cells (results not shown), indicating that the adenoviral delivery of the gene irrelevant to eicosanoid biosynthesis is also suppressed by sPLA$_2$-V or -X.

To confirm these observations further, we used several stable clones of sPLA$_2$-V- or -X-transfected BEAS-2B cells, in which the expression levels of these enzymes differed among individual clones, as assessed by RNA blotting (Figure 1C, top panel) and by enzymatic activity released into the culture supernatants (Figure 1C, bottom panel). When individual clones were infected with Ad-mPGES-1, the expression of mPGES-1 showed a reciprocal correlation with that of sPLA$_2$-V or -X (Figure 1C). Thus the expression of mPGES-1 was lower in cells expressing higher levels of sPLA$_2$-V or -X. Time-course experiments demonstrated that the adenoviral delivery of mPGES-1 into BEAS-2B (Figure 1D) and NHPF (results not shown) was markedly delayed in sPLA$_2$-V- or -X-expressing cells compared with that in control cells. On the basis of these observations, we hypothesized that sPLA$_2$-V and -X have the unique ability to prevent adenoviral infection into mammalian cells.

BEAS-2B cells expressing sPLA$_2$-V or -X, but not sPLA$_2$-IIA and -IID and cPLA$_2\gamma$, exhibited increased AA release (Figure 1E) and PGE$_2$ production (when cotransfected with COX-2) (Figure 1F) compared with replicate control cells, indicating that sPLA$_2$-V and -X can hydrolyse BEAS-2B cell membranes

to supply AA for eicosanoid production. However, greater AA release (Figure 1E) and PGE$_2$ production (Figure 1F) were evident in cells expressing cPLA$_2\alpha$, which displayed no inhibitory effect on adenoviral infection (Figure 1A) than in cells expressing sPLA$_2$-V or -X. Moreover, cells transfected with COX-2 alone, in which no inhibition of adenoviral infection occurred (Figure 1A) produced more PGE$_2$ than did cells transfected with sPLA$_2$-V or -X alone (Figure 1F). These observations thus argue against a contribution of AA or its metabolites to sPLA$_2$-V- or -X-mediated suppression of adenoviral infection.

To assess whether adenovirus entry into the cells was indeed perturbed in cells expressing sPLA$_2$-V or -X, we performed immunocytostaining of adenovirus-infected BEAS-2B cells with an antibody against adenoviral hexon protein. When control BEAS-2B cells were incubated with adenovirus, adenovirus signals exhibited a punctate pattern in the cytoplasm, which was largely colocalized with the endosomal marker EEA1 (Figure 2A). As shown in Figure 2(B), endosomal localization of adenovirus particles was markedly decreased in sPLA$_2$-V- or -X-expressing cells compared with that in control cells, implying that adenoviral entry into cells is prevented by sPLA$_2$-V or -X. By contrast, such a prevention of adenoviral entry was not appreciably observed in sPLA$_2$-IIA-expressing cells (Figure 2B).

Exogenous sPLA$_2$-V and -X prevent adenoviral infection into host cells

To further explore the inhibitory effect of sPLA$_2$-V and -X on adenoviral infection, we conducted a plaque formation assay using 293A cells to determine whether or not purified recombinant sPLA$_2$-V and -X added exogenously are capable of preventing adenoviral infection. First, in order to assess if these enzymes would inactivate adenovirus directly, the virus was preincubated with recombinant sPLA$_2$-V or -X for various periods of time. Then the virus stock was diluted appropriately and added to 293A cells to observe the effect on adenoviral plaque formation. After this treatment (even for up to 1 week), however, we observed no appreciable inhibition of adenoviral plaque formation by these enzymes (results not shown), suggesting that adenovirus particles themselves are refractory to these enzymes. By contrast, when recombinant sPLA$_2$-V or -X was added directly to 293A cells in the presence of adenovirus, there was an sPLA$_2$ dose-dependent decrease in the number of adenoviral plaques formed (Figure 3A). A marked decrease in plaque number was observed using as little as 0.1 μg/ml of enzyme (Figure 3A), a concentration almost equivalent to that released from BEAS-2B or NHPF cells stably transfected with these enzymes (see Figure 1). In addition, each plaque in cells treated with recombinant sPLA$_2$-V or -X appeared to be smaller than that observed in cells without addition of each enzyme (Figure 1B). By comparison, recombinant sPLA$_2$-IIA failed to decrease adenoviral plaque formation in terms of number (Figure 3A) and size (Figure 3B) even at 1 μg/ml. Thus sPLA$_2$-V and -X suppress adenoviral infection by acting on host cells rather than on adenoviral particles.

Requirement for catalytic activity

To assess whether the enzymatic activities of sPLA$_2$-V and -X are required for their ability to block adenoviral infection, we prepared BEAS-2B transfectants stably expressing catalytically inactive sPLA$_2$-V and -X mutants in which the catalytic centre histidine was replaced with asparagine (H48Q). We then examined the infection efficiency of Ad-mPGES-1 in these cells by monitoring mPGES-1 expression. Neither the sPLA$_2$-V H48Q mutant (Figure 4A) or the sPLA$_2$-X H48Q mutant (Figure 4B) decreased adenoviral expression of mPGES-1 in BEAS-2B cells under the
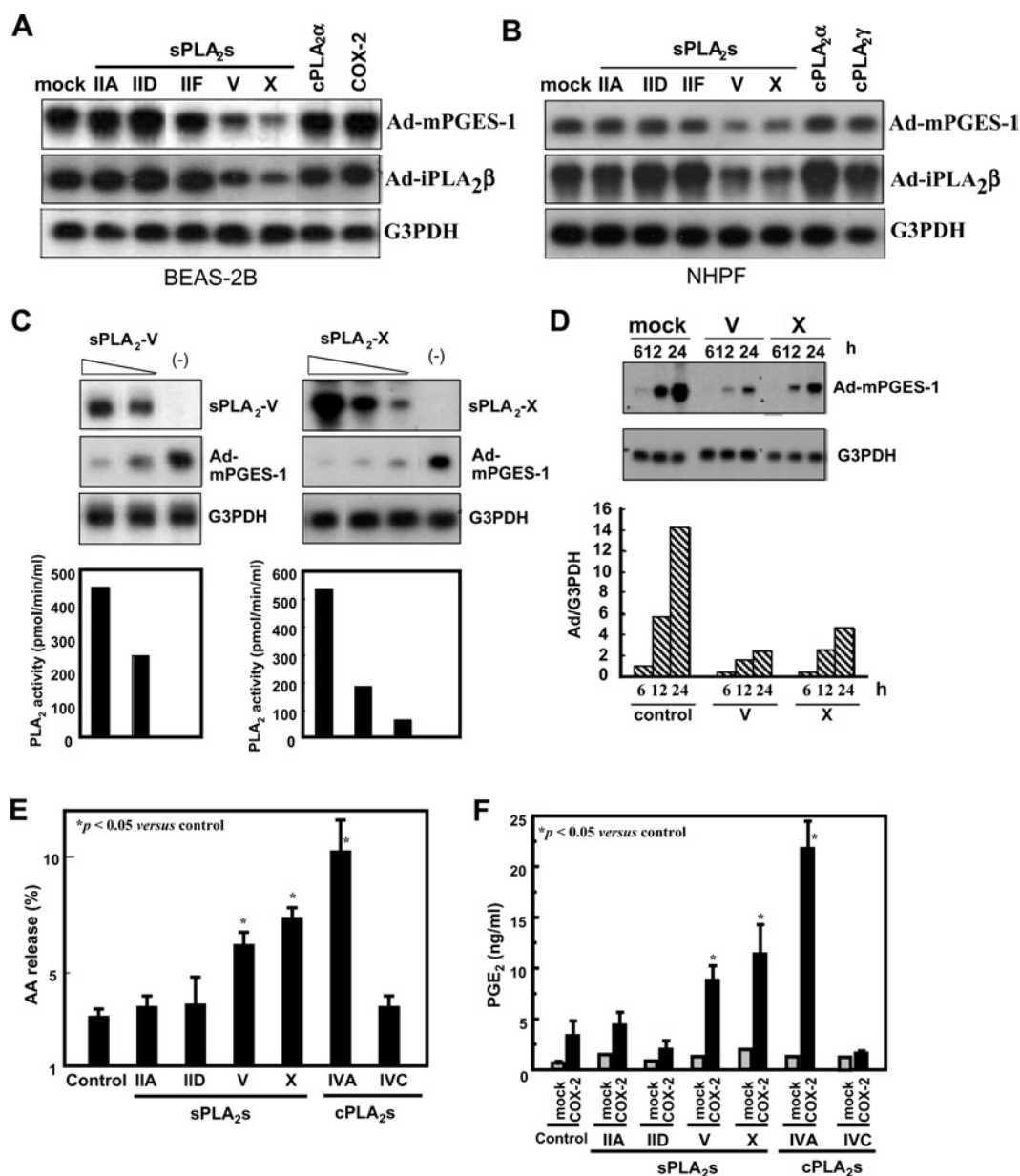
**Figure 1   sPLA₂-V and -X suppress adenovirus-mediated gene delivery into human lung-derived cells**

(**A**) Human bronchial epithelial BEAS-2B cells, which had been stably transfected with various PLA₂s and COX-2 were subjected to infection with Ad-mPGES-1 or Ad-iPLA₂β at MOI (multiplicity of infection) = 10. After 24 h the cells were harvested and taken for Northern-blotting with probes against mPGES-1, iPLA₂β and G3PDH. (**B**) NHPF were infected with adenoviruses for various PLA₂s at MOI = 10 for 24 h and were then infected with Ad-mPGES-1 or Ad-iPLA₂β at MOI = 2 for an additional 24 h. The cells were harvested and taken for Northern-blotting with probes against mPGES-1, iPLA₂β and G3PDH. (**C**) BEAS-2B cells stably expressing different amounts of sPLA₂-V (left panel) or -X (right panel) or mock-transfected cells (−) were infected with Ad-mPGES-1 at MOI = 10 for 24 h. Then the cells were harvested and taken for Northern-blotting with probes for sPLA₂-V or -X, mPGES-1 and G3PDH. sPLA₂ activity released into the supernatants from individual clones is shown in the bottom panel. (**D**) Control BEAS-2B cells and those stably transfected with sPLA₂-V or -X were infected for the indicated periods with Ad-mPGES-1 at MOI = 10. Then the cells were subjected to Northern-blotting with probes against mPGES-1 and G3PDH. The bottom panel shows the ratio of signal intensities between mPGES-1 and G3PDH. (**E**) AA release from BEAS-2B cells transfected with various PLA₂s. Cells prelabelled with [³H]AA for 24 h were stimulated for 4 h with 100 units/ml TNFα to assess [³H]AA release. (**F**) BEAS-2B cells stably expressing various PLA₂s with or without cotransfection of COX-2 were incubated with TNFα for 4 h to assess generation of PGE₂. (**A**) to (**D**) representative results of 3–5 experiments and values are mean ± S.E.M. for three independent experiments in (**E**) and (**F**).

conditions where wild-type enzymes significantly prevented it. This result implies that the catalytic activity of sPLA₂-V and -X is a prerequisite for their anti-viral effect.

sPLA₂-V and -X increase LPC levels in cells

It has been reported that LPC, a PLA₂ reaction product of PC, disturbs virus-mediated membrane fusion and thereby viral infection [31–35]. In the light of the proposal that sPLA₂-V and -X are much more potent than other sPLA₂s in acting on the PC-rich external leaflet of the plasma membrane [4–6], we hypothesized that the hydrolysis of membrane PC, and thereby increased production of LPC, by sPLA₂-V or -X might be functionally linked to their inhibitory effects on adenoviral infection. To corroborate this possibility, we investigated whether LPC or other lysophospholipids added exogenously could prevent adenoviral
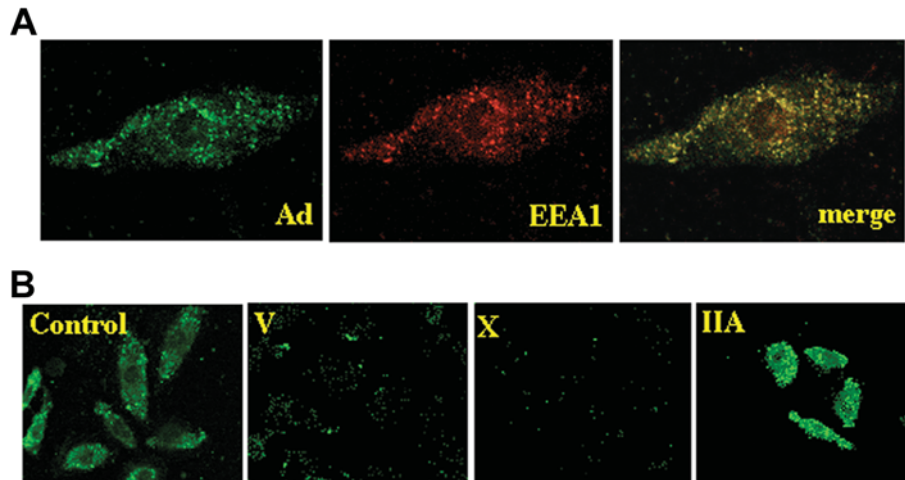
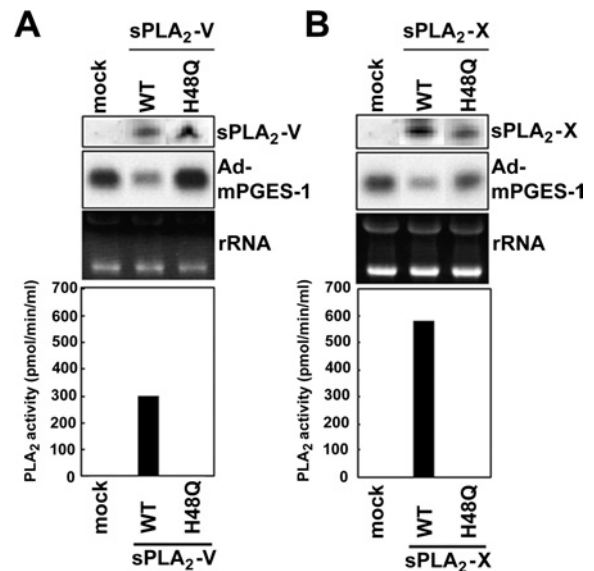**Figure 2   Immunostaining of adenoviral particles in BEAS-2B cells**

(**A**) Endocytosis of adenoviral particles into endosomes. BEAS-2B cells were incubated with adenovirus (MOI = 20) for 6 h and were immunostained with anti-(adenoviral hexon) (left panel) and anti-EEA1 (middle panel) antibodies. A merged image is shown in the right panel. (**B**) Control BEAS-2B cells and cells expressing sPLA$_2$-V, -X or -IIA were incubated with adenovirus (MOI = 20) for 6 h and were immunostained with anti-adenoviral hexon antibody.
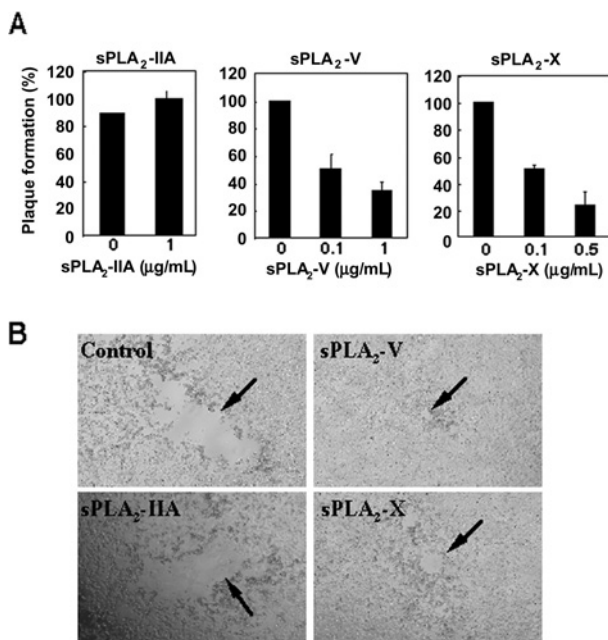
Figure 3   Exogenous sPLA$_2$-V and -X block adenoviral plaque formation in 293A cells

(**A**) Effects of sPLA$_2$s on plaque numbers. 293A cells were incubated for five days with adenovirus in the presence or absence of the indicated amounts of sPLA$_2$s, and plaque numbers formed on 293A cell monolayers were counted. Values are expressed as percentages of the number of plaques compared with that of the control as 100 % (mean ± S.E.M., $n = 3$). (**B**) Photographs of the plaques formed on a 293A cell monolayer after culture for five days in the presence or absence (control) of 1 μg/ml sPLA$_2$-IIA, 1 μg/ml sPLA$_2$-V or 0.5 μg/ml sPLA$_2$-X.


Figure 4   Requirement for sPLA$_2$ catalytic activity

Control BEAS-2B cells and those stably expressing wild-type enzymes (WT) or the catalytically inactive H48Q mutants of sPLA$_2$-V (**A**) or sPLA$_2$-X (**B**) were infected with Ad-mPGES-1 for 24 h. Then the cells were subjected to Northern-blotting to assess the expression of mPGES-1 and sPLA$_2$s. Equal loading of RNA in each lane was verified by ribosomal RNA (rRNA) stained with ethidium bromide. Bottom graphs indicate PLA$_2$ activity released into the supernatants, which confirmed that the H48Q mutants were catalytically inactive.

infection in BEAS-2B cells. As shown in Figure 5(A), infection of Ad-mPGES-1 in BEAS-2B cells, as revealed by mPGES-1 expression, was markedly decreased when the cells were incubated with exogenous LPC containing relatively long *sn*-1 fatty acyl chains (C$_{14:0}$, C$_{16:0}$ and C$_{18:0}$, but not C$_{12:0}$) (Figure 5A). By contrast, none of the other lysophospholipids (LPE, LPS and LPA with C$_{16:0}$ at *sn*-1) (Figure 5A) or AA (Figure 5B)

affected the adenoviral delivery of mPGES-1, thus revealing LPC specificity. The inhibitory effect of LPC on the adenoviral delivery of mPGES-1 was dependent on the concentration ($> 10 \mu$M) of this lysophospholipid added (Figure 5C).

We then investigated whether sPLA$_2$-V and -X are indeed capable of increasing levels of endogenous LPC in cells. ESI/MS analysis of phospholipids extracted from BEAS-2B cells, with or without sPLA$_2$ transfection, revealed that there were marked increases in the amount of endogenous LPC molecular species with C$_{16:0}$, C$_{18:0}$ and C$_{18:1}$ in sPLA$_2$-V-expressing cells (19-, 39- and 10-fold increases respectively, as evaluated by peak areas)
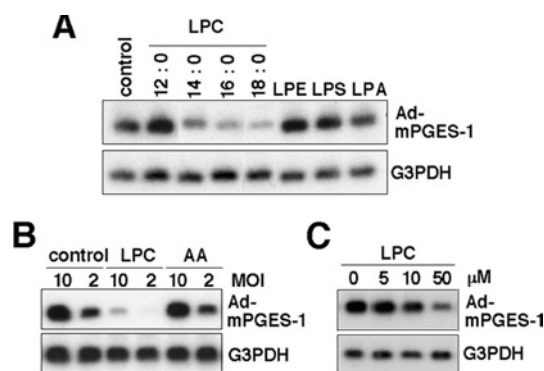
Figure 5   LPC blocks adenoviral infection into BEAS-2B cells

(**A**) and (**B**) BEAS-2B cells were incubated with Ad-mPGES-1 at MOI = 10 (**A**) and at MOI = 2 or 10 (**B**) in the presence or absence (control) of various lysophospholipids or AA (each at 50 μM) for 24 h. In the case of LPC, molecular species having $C_{12:0}$, $C_{14:0}$, $C_{16:0}$ and $C_{18:0}$ at sn-1 were used. (**C**) The cells were incubated with Ad-mPGES-1 at MOI = 10 in the presence or absence of the indicated concentrations of LPC with $C_{18:0}$ at sn-1. The cells were then harvested and subjected to Northern-blotting with probes against mPGES-1 and G3PDH.

and in sPLA₂-X-expressing cells (4-, 12- and 3-fold increases respectively) compared with those in control cells (Figure 6A). Concomitantly, there were approx. 32 % and 24 % decreases in the amount of PC species with $C_{16:0}$–$C_{16:1}$ and $C_{16:0}$–$C_{18:1}$ respectively, in sPLA₂-V-expressing cells and an approx. 40 % decrease in the amount of PC containing $C_{16:0}$–$C_{16:1}$ in sPLA₂-X-expressing cells as compared with those in control cells (Figure 6B). Thus the increased amount of LPC containing $C_{16:0}$ in sPLA₂-V- or -X-expressing cells may be derived from these decreased PC species with $C_{16:0}$ at sn-1. Irrespective of marked increases in the amount of LPC with $C_{18:0}$ and $C_{18:1}$, however, no appreciable changes in the number of PC species with $C_{18:0}$ or $C_{18:1}$ at sn-1 were found in sPLA₂-V- or -X-expressing cells. Although the reason for this discrepancy is unknown, it may reflect accelerated membrane remodelling (hydrolysis by sPLA₂s followed by rapid reacylation) of PC species with $C_{18:0}$ or $C_{18:1}$ at sn-1 during culture. Supporting this idea, PC containing $C_{18:0}$–$C_{20:4}$ was significantly increased in sPLA₂-V- and -X-expressing cells (2- and 1.6-fold increases respectively) compared with that in control cells (Figure 6B). No alterations in LPC (Figure 6A) or PC (Figure 6B) species were observed in cells transfected with sPLA₂-IIA or sPLA₂-X H48Q, which failed to suppress adenoviral infection (see above), compared with those in control cells.

Expression of sPLA₂-V is induced by virus-related stimuli

We next examined whether the expression levels of endogenous sPLA₂-V and -X are altered in cells treated with certain stimuli related to viral infection. Treatment of BEAS-2B cells and mouse peritoneal macrophages with IFN-α, an anti-viral cytokine [36], resulted in a significant increase in the expression of sPLA₂-V at both protein (Figure 7A) and mRNA (Figure 7B) levels, as assessed by immunoblotting and RT-PCR respectively. Increased expression of sPLA₂-V was also observed in macrophages after stimulation with poly(I:C), a synthetic ligand for a Toll-like receptor (TLR3) that binds to double-stranded viral nucleic acid and activates the type I IFN response [37], and even after infection with adenovirus (Figure 7C). In contrast with sPLA₂-V, sPLA₂-X was expressed in macrophages at an almost constant level following treatment with IFN-α (Figure 7A), poly-I:C or adenovirus (results not shown). Expression of sPLA₂-X protein was very faint and hard to detect in BEAS-2B cells under all conditions tested (results not shown) even though mRNA was

weakly and constantly detectable as assessed by high sensitivity RT-PCR [23].

### sPLA₂-V and -X mRNAs are expressed in human respiratory tract

In situ hybridization of the bronchial epithelium from human lungs affected by pneumonia with sPLA₂-specific cRNA anti-sense, but not sense, probes revealed the existence of sPLA₂-V and -X mRNAs in epithelial cells (see Supplementary Figure 1S at http://www.BiochemJ.org/bj/393/bj3930097add.htm). sPLA₂-IID mRNA was also detected, whereas signals for sPLA₂-IIA and -IIF mRNAs were very weak in epithelium (see Supplementary Figure 1S). These results are in agreement with our recent immunohistochemical study in which immunoreactive sPLA₂-V, -X and -IID proteins were detected in the epithelium of human respiratory tract [23].

DISCUSSION

The role of sPLA₂s (sPLA₂-IIA in particular) in host defence against bacterial infection has been well documented by recent studies [11–14]. By contrast, the contribution of sPLA₂s to innate immunity against viral infection is poorly understood. In the present study, we have provided evidence that sPLA₂-V and -X, two sPLA₂s that possess potent ability among sPLA₂s to hydrolyse mammalian cell membranes [4–6], prevent host cells from being infected with adenoviruses, which often causes infectious respiratory diseases. Unlike the anti-bacterial function of sPLA₂s, which depends on their hydrolytic action on bacterial membranes [11–14], the anti-viral action of sPLA₂-V and -X depends on their ability to act on host cell membranes. Thus these two sPLA₂s hydrolyse phospholipids in the plasma membrane, eventually leading to a decrease in adenoviral entry into cells. Since sPLA₂-V and -X are expressed in the human respiratory tract at both protein [23] and mRNA (Supplementary Figure 1) levels, it can be speculated that these two sPLA₂s may function as part of the anti-viral arsenal to protect the respiratory (and possibly other) organs against adenoviral invasion in particular pathological circumstances.

Several lines of evidence support the idea that sPLA₂-V and -X inhibit adenoviral infection by hydrolysing the plasma membrane of host cells. Among sPLA₂s tested so far, this anti-viral effect was observed only with sPLA₂-V and -X (Figure 1), the two most potent plasma membrane-acting sPLA₂s [4–6]. Immunofluorescence microscopy revealed that these sPLA₂s prevent adenoviral entry into the endosomes of host cells (Figure 2). By comparison, parvovirus-associated sPLA₂ (as opposed to host sPLA₂-V and -X), which is essential for effective infection of the virus acts on a later step; transfer of the viral genome from the endosomes into the nucleus [15,16]. Although cPLA₂α released more AA and PGE₂ than did sPLA₂-V and -X, it was unable to suppress adenovirus-mediated gene delivery (Figure 1), indicating that no arachidonate metabolite participates in the anti-viral effect of sPLA₂-V and -X. Furthermore, considering that cPLA₂α-mediated AA release occurs in the perinuclear region [38,39], it is unlikely that hydrolysis of the perinuclear membrane would affect viral entry.

More direct evidence for the anti-viral effect of sPLA₂-V and -X was obtained from experiments using pure recombinant enzymes in the plaque-forming assay (Figure 3). Adenovirus amplification occurring in 293A cells was markedly suppressed by adding exogenous sPLA₂-V and -X to the culture, whereas preincubation of adenovirus with these recombinant enzymes did not decrease the capacity of the virus to infect cells. As little as 100 ng/ml recombinant sPLA₂-V and -X was able to decrease
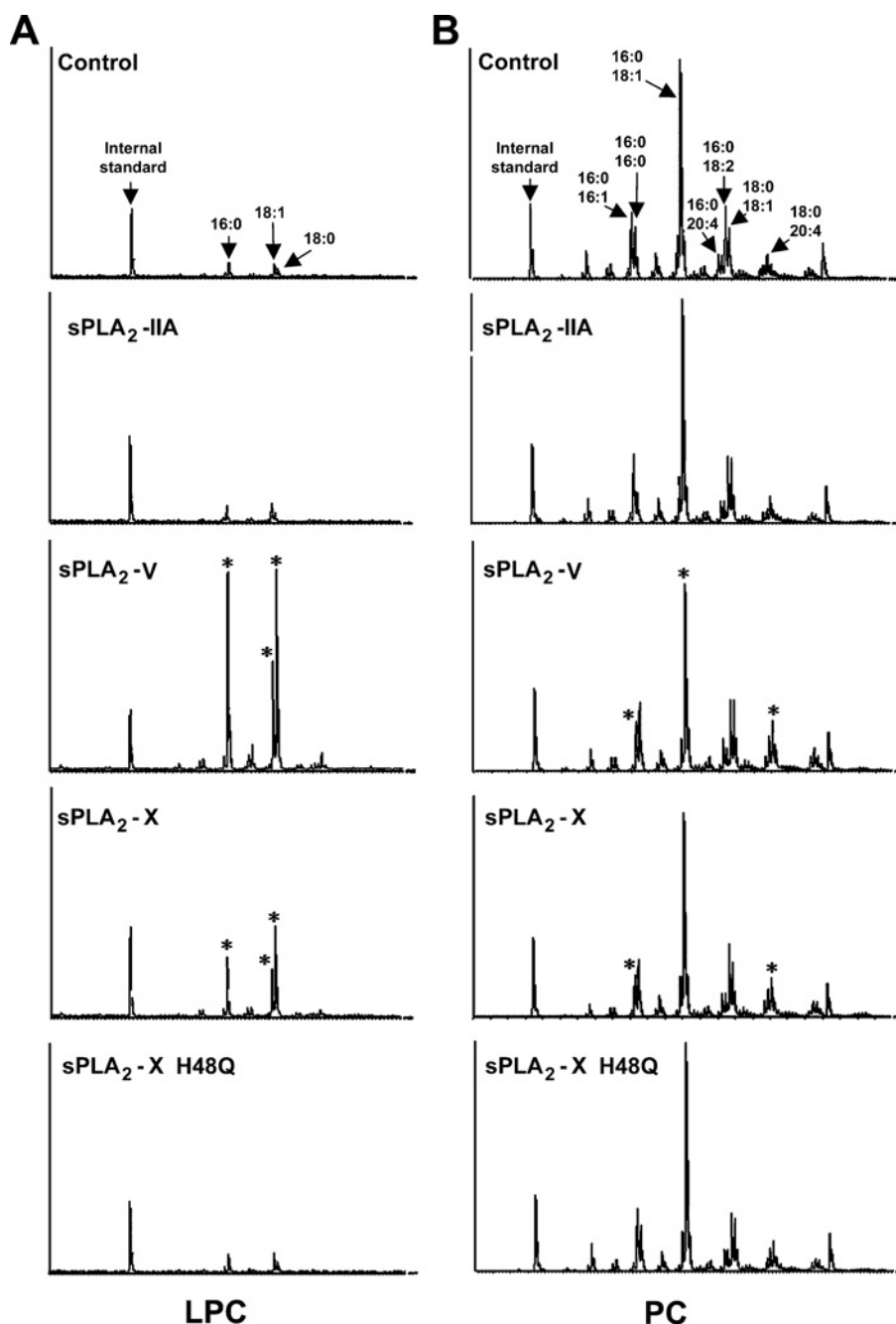
**Figure 6   ESI-MS analyses of LPC and PC species**

Lipids extracted from control and sPLA$_2$-expressing BEAS-2B cells (100 μg protein equivalents) were subjected to ESI-MS to assess the composition of LPC (**A**) and PC (**B**) species. LPC with C$_{12:0}$ at sn-1 and PC with C$_{14:0}$–C$_{14:1}$ were added to samples as internal standards before lipid extraction. Asterisks indicate molecular species for which amounts were markedly changed in comparison with those in control cells. A representative result of two reproducible experiments is shown.

adenoviral plaque formation by $\geqslant 50\,\%$. In the case of sPLA$_2$-V, this concentration is almost comparable with that reported to be sufficient for this enzyme to exert its bactericidal activity [12,14,40]. By contrast, exogenous sPLA$_2$-IIA failed to suppress plaque formation even at $1\,\mu$g/ml, probably because it hardly acts on the plasma membrane of host cells [5]. Although the *in vivo* concentrations of sPLA$_2$-V or -X under physiological and pathological conditions are unclear, it is known that the levels of sPLA$_2$-IIA in severely inflamed sites often reach the order of μg/ml [41]. This fact, together with the intense immunoreactivity

of sPLA$_2$-V and -X in the respiratory epithelium of human lungs affected by pneumonia [23], suggests that local concentrations reached in the respiratory tract could be high enough to elicit their anti-viral effect, a possibility that should be confirmed by future studies.

Mutagenesis studies have revealed that the ability of sPLA$_2$-V and -X to suppress adenoviral infection depends on their catalytic activity (Figure 4). This is in contrast with the suppressive action of bee venom sPLA$_2$ on HIV entry into host cells; this function depends on the binding of the enzyme to cells, and a peptide of
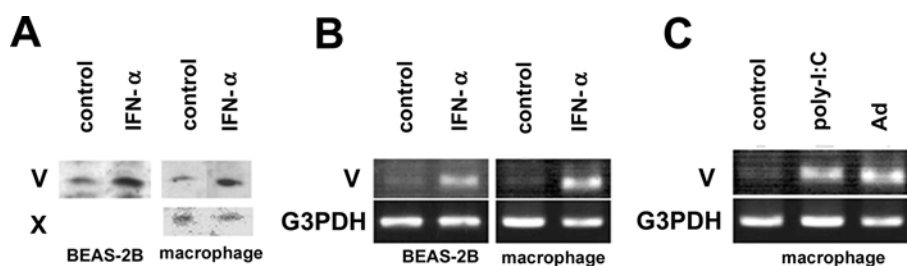
Figure 7   Effects of virus-related stimuli on the expression of endogenous sPLA$_2$-V and -X

(**A** and **B**) Human BEAS-2B cells and mouse peritoneal macrophages were incubated with or without 0.2 μg/ml IFN-α for 24 h. Then the cells were subjected to immunoblotting with anti-sPLA$_2$-V and anti-X antibodies (**A**) or to RT-PCR for sPLA$_2$-V and G3PDH (**B**). (**C**) Macrophages were incubated with or without adenovirus (MOI = 20) or poly-I:C (100 μg/ml) for 24 h and then subjected to RT-PCR for sPLA$_2$-V or G3PDH. Representative results of three independent experiments are shown.

15 amino acids that corresponds to a surface-exposed loop of the enzyme displays anti-HIV activity through interacting with the HIV coreceptor CXCR4 [17,18]. Nonetheless, the requirement of catalytic activity for the anti-viral effect of sPLA$_2$-V and -X was further supported by the findings that LPC, a PLA$_2$-hydrolytic product of PC that is richly present in the outer leaflet of the plasma membrane, suppressed adenoviral infection (Figure 5A), and that substantial amounts of LPC species were indeed produced in cells transfected with these enzymes (Figure 5B). To our knowledge, this is the first quantitative demonstration by ESI/MS that sPLA$_2$-V and -X produce LPC from the cellular membrane. Since LPC binds to serum albumin, serum in medium already contains substantial amounts of albumin-bound LPC [42,43], and LPC undergoes further metabolizing such as reacylation, it was difficult to precisely evaluate the actual working concentration of LPC and the amounts of LPC produced by these sPLA$_2$s in cells. We speculate that the local spatiotemporal concentration of LPC produced by these sPLA$_2$s in the plasma membrane may be sufficient to facilitate their suppressive effect on adenovirus entry.

LPC has been shown to inhibit membrane fusion elicited by a variety of viruses, such as influenza, simian immunodeficiency, Sendai and rabies [31–35]. This 'inverted-cone' shape lyso-phospholipid, when present in the outer leaflet of the plasma membrane, prevents the formation of stalks, a common step in viral entry into host cells [34,35]. Alternatively, LPC could interact directly with viral fusion proteins [31–33]. For instance, inhibition of influenza virus-induced membrane fusion by LPC is increased with increasing acyl chain length and is caused by the binding of LPC to fusion peptides [32]. It is thus speculated that LPC-mediated inhibition of adenoviral infection might involve similar machinery, although the precise mechanisms for LPC action need further investigation. As another possibility, LPC, by acting as a ligand for putative LPC receptor(s) [42], might generate particular signals that lead to prevention of adenovirus internalization.

In the context of this study, increased expression of endogenous sPLA$_2$-V in response to stimuli related to viral infection (Figure 7) may represent a host response directed towards anti-viral defence. On the contrary, the expression of sPLA$_2$-X was relatively constant after viral-related stimuli, in agreement with previous observations that the expression of this enzyme is poorly affected by proinflammatory stimuli [22,23,44]. Conceivably, sPLA$_2$-X may be regulated by cleavage of the N-terminal pro-peptide by certain proteases that are induced or activated during pathological processes, a possibility that is now under investigation. If the anti-viral effect of these two sPLA$_2$s is mainly if not solely mediated by the production of LPC, which suppresses infection by various viruses [31–35], the innate immune action of these sPLA$_2$s may be directed not only at adenovirus but also at other infectious viruses. In support of this, we have recently shown that the expression and localization of sPLA$_2$-V in the liver of patients with viral hepatitis are closely correlated with those of hepatitis C antigen [45]. Thus although it has been proposed that the control of particular sPLA$_2$s may have advantages over the inhibition of selective lipid mediator pathways and of other biological events in the treatment of pathological states, one should consider that pharmacological inhibition of sPLA$_2$s would hamper their anti-microbial roles, possibly causing disadvantageous side-effects.

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