Secreted phospholipases A_2, a new class of HIV inhibitors that block virus entry into host cells

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Mammalian and venom secreted phospholipases A_2 ($sPLA_2s$) have been associated with a variety of biological effects. Here we show that several $sPLA_2s$ protect human primary blood leukocytes from the replication of various macrophage and T cell–tropic HIV-1 strains. Inhibition by $sPLA_2s$ results neither from a virucidal effect nor from a cytotoxic effect on host cells, but it involves a more specific mechanism. $sPLA_2s$ have no effect on virus binding to cells nor on syncytia formation, but they prevent the intracellular release of the viral capsid protein, suggesting that $sPLA_2s$ block viral entry into cells before virion uncoating and independently of the coreceptor usage. Various inhibitors and catalytic products of $sPLA_2$ have no effect on HIV-1 infection, suggesting that $sPLA_2$ catalytic activity is not involved in the antiviral effect. Instead, the antiviral activity appears to involve a specific interaction of $sPLA_2s$ to host cells. Indeed, of 11 $sPLA_2s$ from venom and mammalian tissues assayed, 4 venom $sPLA_2s$ were found to be very potent HIV-1 inhibitors (ID_{50} < 1 nM) and also to bind specifically to host cells with high affinities ($K_{0.5}$ < 1 nM). Although mammalian pancreatic group IB and inflammatory-type group IIA $sPLA_2s$ were inactive against HIV-1 replication, our results could be of physiological interest, as novel $sPLA_2s$ are being characterized in humans.

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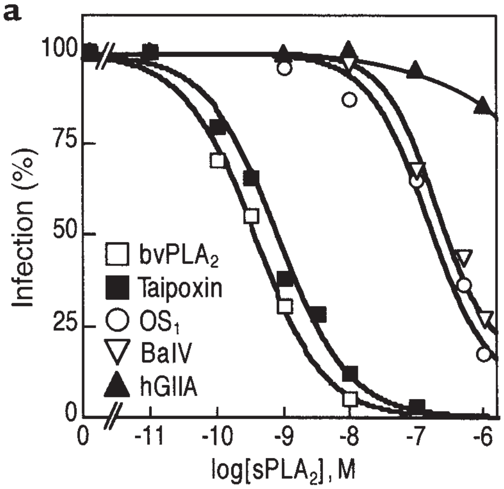
Introduction

HIV-1 infection is initiated by the interaction of the virion envelope complex (gp120/gp41) with at least 2 cellular receptors: the CD4 molecule (1, 2) and a member of the chemokine receptor family (3–6). Subsequent to binding with these cellular receptors, the gp120/gp41 complex undergoes conformational changes that mediate fusion of the viral membrane with the target-cell membrane (7–9). After virus-cell fusion, virion disassembly occurs (uncoating) to release the reverse transcription (RT) complex that dissociates from the plasma membrane and moves toward the cell nucleus (10). This complex contains all the viral functions necessary for the synthesis of the proviral DNA, its transport to the cell nucleus, and its integration into the host cell DNA (11–14). The molecular basis of viral tropism has now been well characterized and resides in the ability of gp120 to interact specifically with a chemokine receptor (3–9). Macrophage-tropic (M-tropic) strains of HIV-1 replicate in macrophages and $CD4^+$ T cells and use the CC chemokine receptor CCR5 (R5 viruses). T-cell–tropic (T-tropic) isolates of HIV-1 replicate in primary $CD4^+$ T cells and established $CD4^+$ T cells and use the CXC chemokine receptor CXCR4 (X4 viruses). Usually, R5 viruses have a non–syncytium-inducing (NSI) phenotype, whereas X4 viruses have a syncytium-inducing (SI) phenotype (10).

Several HIV-1 inhibitors have been described to block HIV entry into cells by antagonizing the interaction between gp120 and the corresponding chemokine receptor. Such inhibitors have been derived from CC or CXC chemokines (3, 5, 15, 16) or are small-molecule inhibitors that bind to the coreceptor (17, 18). In addition, recent advances in AIDS research have focused on the development of new combination therapies that have led to a dramatic and sustained reduction of viral load (19–21). Although these therapies extend the life of patients, such approaches require rigorous compliance with complicated and expensive drug regimens that cause significant side effects. These factors, coupled with the emergence of resistant viruses that escape to treatment with time, argue for the continued development of new compounds capable of protecting cells from HIV replication.

Secreted phospholipases A_2 ($sPLA_2s$; 14 kDa) are found in mammalian tissues and animal venoms and catalyze the hydrolysis of glycerophospholipids to release FFAs and lysophospholipids (22–27). They have been classified into different groups on the basis of the number and position of the cysteine residues present in their sequences (24, 27). These $sPLA_2s$ have a similar overall organization and the same catalytic mechanism but display very distinct pharmacological effects (22, 23, 27). So far, 6 mammalian $sPLA_2s$ referred to as group IB, IIA, IIC, IID, V, and X have been cloned and associated with different physiological and pathological processes (25–29). Aside from their function as enzyme, $sPLA_2s$ have been shown to associate with specific membrane a



b

sPLA$_2$	Origin	ID$_{50}$ (nM)
Venom :		
bvPLA$_2$	*Bee*	0.6
Nmm$_{CMIII}$	*Snake*	0.4
Nigexin	*Snake*	0.4
Taipoxin	*Snake*	0.8
BaIV	*Snake*	400
OS$_1$	*Snake*	300
PA2	*Lizard*	>500
PA5	*Lizard*	>500
Mammalian :		
hGIIA	*Human*	>500
hGIB	*Human*	>500
pGIB	*Hog*	>500

Figure 1
Inhibitory effects of sPLA$_2$s on HIV-1$_{BRU}$ replication. P4 cells were infected with HIV-1$_{BRU}$ in the presence of various concentrations of sPLA$_2$s for 8 hours. Cells were then washed, and AZT (50 μM) was added to prevent a second round of viral replication. Two days after infection, the level of viral replication was determined by β-gal assay. (**a**) Representative dose-response curves obtained with the venom sPLA$_2$s taipoxin, bvPLA$_2$, OS$_1$, BaIV, and hGIIA. (**b**) Concentration of sPLA$_2$s (ID$_{50}$) that inhibit 50% of HIV-1$_{BRU}$ replication. Values are representative of results obtained with at least 2 or 3 independent experiments with SEM less than 10%.

receptors that participate to their biological activities (27). To date, 2 main types of sPLA$_2$ receptors have been identified. N-type receptors are expressed at high levels in brain, but they are also present in other tissues (30–32). These receptors bind with high affinities different venom sPLA$_2$s, such as bee venom sPLA$_2$ (bvPLA$_2$) (31). The 180-kDa M-type receptor is expressed in various tissues including lung, kidney, and liver and belongs to the C-type lectin superfamily (27). The M-type receptor has been proposed to be involved in a variety of biological effects of sPLA$_2$s including cell migration, eicosanoid release, and septic shock (33, 34), and recent data have indicated that this receptor is the physiological target for the mammalian endogenous group IB and group IIA sPLA$_2$s (35).

Mammalian sPLA$_2$s are likely to play important roles in host defense (25, 27, 36); sPLA$_2$ products have been shown to interfere with viral infection (37, 38); and venom sPLA$_2$s display a wide and intriguing array of biological effects. We therefore analyzed whether sPLA$_2$s have antiviral properties against HIV-1. Our results indicate that several, but not all, assayed sPLA$_2$s can protect efficiently various host cells from the replication of HIV-1 isolates and are likely to define a new class of HIV-1 inhibitors.

Methods

Plasmids. Plasmids encoding HIV-1 virus (pNL.AD8, pYU2, pBru-2), HIV-2 virus (pROD10), and pCMVTat were kindly provided by P. Charneau, K. Peden, and N. Israel, respectively (Pasteur Institute, Paris, France). The human CCR5 chemokine receptor construct (pCCR5) was obtained after insertion of the cloned receptor cDNA in the mammalian expression vector pCEP4 (Invitrogen BV, Groningen, the Netherlands).

Antibodies. Polyclonal antibodies to bvPLA$_2$ (rbv$_{Ab}$) were obtained after immunization of rabbits by 4 successive injections of 350 μg of bvPLA$_2$. These antibodies specifically recognize bvPLA$_2$ in Western blot (working dilution 1:1000) and prevent bvPLA$_2$ antiviral effect (working dilution 1:40). Anti-human CD4 mAb Leu3A was from Becton Dickinson (Pont de Claix, France).

Reagents. Purified HIV-1$_{Lai}$ gp120 and SDF-1α were from Neosystem Laboratories (Strasbourg, France) and PeproTech (London, United Kingdom), respectively. OS$_1$, bvPLA$_2$, taipoxin, basic sPLA$_2$ from *Naja mossambica mossambica* snake venom (Nmm$_{CMIII}$), porcine group IB (pGIB), and recombinant human group IIA sPLA$_2$ (hGIIA) were prepared as described (31, 35). Nigexine from *Naja nigricollis* (39), PA2 and PA5 sPLA$_2$s from the venom of the lizard *Heloderma suspectum* (40), human group IB sPLA$_2$ (hGIB), and catalytically inactive BaIV sPLA$_2$-like protein from *Bothrops asper* (41) were kindly provided by André Ménez (C.E.A. Saclay, Gif/Yvette, France), Jean Christophe (Université libre de Bruxelles, Belgium), the late Hubertus Verheij (University of Utrecht, the Netherlands), and José Maria Gutiérrez and Sergio Lizano (University of Costa Rica, San José, Costa Rica), respectively. Oleoyloxyethylphosphocholine was obtained from Calbiochem-Novabiochem (Meudon, France). Other reagents were from Sigma-Aldrich (St. Quentin Fallavier, France).

Cell culture. Activated PBMCs (10^6 cells/mL) were obtained after treatment of cells with 3 μg/mL phytohemagglutinin for 48 hours in RPMI-1640 medium (GIBCO BRL, Cergy Pontoise, France) supplemented with 20% FBS (BioWhittaker Europe, Verviers, Belgium) followed by a 24-hour incubation with 20 UI/mL of recombinant human IL-2 (Roche, Meylan, France). MT4s and CEMs are CD4$^+$ T-cell lines obtained from

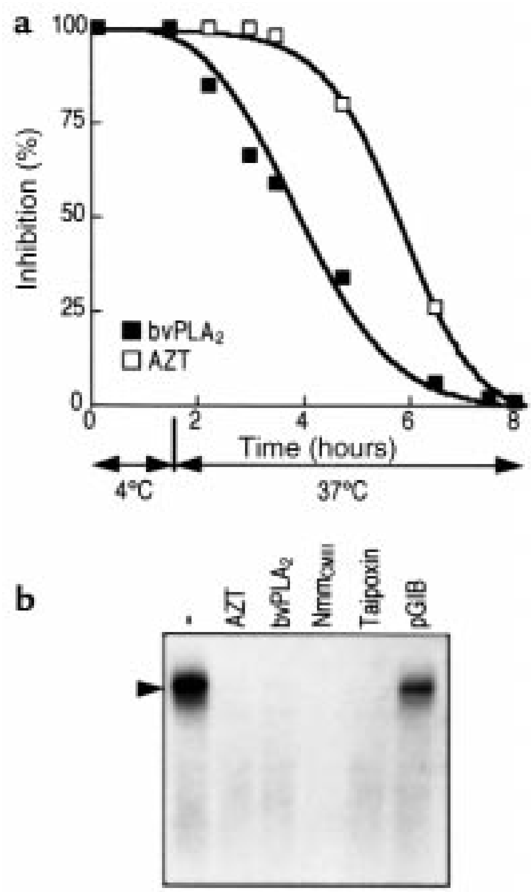


Figure 2
$sPLA_2$s block HIV-1 viral cycle before RT. (**a**) Time-of-addition experiments with $bvPLA_2$ and AZT on the replication of HIV-1_{BRU}. P4 cells were incubated with virus at 4°C for 90 minutes to allow virus binding. Unbound viruses were then removed by several washes and cells were shifted to 37°C to allow virus entry and infection. $bvPLA_2$ (100 nM) or AZT (50 μM) were added at different times after virus addition until 8 hours, after which the cell culture medium was replaced by fresh medium containing AZT. Two days later, the level of viral replication was measured by β-gal assay. (**b**) $sPLA_2$ effect on proviral DNA synthesis. MT4 cells (10^7 cells) were infected with high doses of infectious HIV-1_{BRU} supernatant in the presence of AZT or 100 nM $bvPLA_2$, Nmm_{CMIII}, taipoxin, or pGIB. Early after infection (8 hours), HIV-1 proviral DNA was extracted and analyzed by Southern blot. Each lane was loaded with 10 μg of soluble DNA, and the blot was hybridized with a specific ^{32}P-labeled HIV-1 riboprobe. The arrow indicates the position of the unintegrated linear proviral HIV-1 DNA (9.2 kb).

the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program (Rockville, Maryland, USA). P4 cells are $CD4^+$ HeLa cells in which transactivation by the HIV-1 tat protein induces expression of the *Escherichia coli* LacZ gene from the HIV-1 long-terminal repeat (42). P4-CCR5 cells were obtained after transfection of P4 cells with pCCR5 and selection of clones that are permissive for the replication of both X4 and R5 viruses. For P4 and P4-CCR5 cells, geneticin (300 μg/mL; GIBCO BRL) or geneticin plus hygromycin (150 μg/mL; GIBCO BRL) was added to the cell culture media, respectively. Cellular viability of PBMCs and MT4, CEM, and P4 cells was monitored using the XTT assay according to the manufacturer's instructions (Roche).

HIV-1 viral stocks and infection assays. The primary HIV-1 isolates CMU02, 91US054, 91US056, and 92US657 were obtained from the NIH AIDS Research and Reference Reagent Program. These primary HIV-1 isolates were propagated on activated PBMCs and harvested from the medium at the peak times of Gag p24 production. HIV virus stocks were produced by transient transfection of pBru-2, pROD10, pNL.AD8, or pYU2 plasmids in the human embryonic kidney 293 cells (CRL-1573; American Type Culture Collection, Manassas, Virginia, USA) using a calcium phosphate mammalian transfection kit (Stratagene, Montigny le Bretonneux, France). Three days after transfection, supernatants were collected and centrifuged (1,500 *g* for 15 minutes). High-titer viral stocks were obtained by mixing 10^7 HIV-1_{BRU}–infected MT4 cells with 4×10^8 MT4 cells in 40 mL of culture medium containing 20 μg/mL polybrene (Sigma-Aldrich). The next day, cells were diluted to 2.5×10^6 cells/mL and further incubated for 1–2 days until cell lysis was about 50%. Infectious supernatants were clarified by centrifugation (1,500 *g* for 30 minutes) and filtration through a 0.8-μm filter (Sartorius, Göttingen, Germany). Viral stocks were evaluated for HIV-1 viral capsid protein (Gag p24) content using an ELISA kit (Organon Teknika, Fresnes, France). Single rounds of viral replication in P4 cells were performed as follows: P4 cells, seeded in 24-well plates (8×10^4 cells per well), were infected the next day with 100 μL of HIV viral supernatant (100 ng of Gag p24). Virus and cells were left in contact for 8 hours at 37°C in the presence or absence of the different effectors, and the culture medium was then replaced with fresh medium containing 50 μM 3′-azido-3′-deoxythymidine (AZT; Sigma-Aldrich). Two days after infection, P4 cells were lysed and β-galactosidase (β-gal) activity was used as an index of HIV replication (42). Activated PBMCs (10^6 cells) were infected with different HIV-1 isolates (100 ng of Gag p24) for 2 hours at 37°C with or without $sPLA_2$s (100 nM). Infected PBMCs were then cultured in the presence or absence of $sPLA_2$s (100 nM) in medium containing IL-2 (20 UI/mL) with fluid renewal each other day. Three and 6 days after infection, Gag p24 content in cell supernatants was determined as already indicated earlier here. The different inhibitors or catalytic products of PLA_2 activity were used in single-round infection assays and were preincubated with P4 cells in the presence or absence of $sPLA_2$s for 15 minutes before the addition of virus.

Effects of $sPLA_2$s on proviral DNA synthesis, syncytium formation, and cytosolic Gag p24 release. MT4 cells (10^7 cells) were infected with HIV-1_{BRU} (2.5 μg of Gag p24) in the absence or presence of effectors. Eight hours after virus addition, low–molecular-weight DNA was extracted from cells (43) and analyzed by Southern blot with an HIV-1_{BRU} gag riboprobe (nucleotides 914–1920). For syncytium formation assay, P4 cells were mixed in a 3:1 ratio with 293 cells cotransfected 1 day before with pBru-2 and pCMVTat. Cells were cocultured in the presence of AZT (50 μM) and the different effectors for 48 hours before β-gal determination. Intracellular levels of cytosolic Gag p24 were determined as described previously (44). Briefly, 10^7 CEM cells were incubated at 4°C for 45 minutes with HIV-1_{BRU} (1 μg of Gag p24); unbound viruses were then removed by 2 cold washes, and cells were incubated for 1 hour at 37°C in culture medium in the presence or absence of effectors. Infected cells were then treated with 50 μg/mL of pronase (Roche) for 5 minutes at 4°C to remove adherent viral particles. After extensive washes, cells were lysed by Dounce homogenization, and cytosolic fractions were prepared by ultracentrifugation at 150,000 *g* for 10 minutes at 4°C and used to determine the content of Gag p24.

$sPLA_2$ and gp120 binding assays. $sPLA_2$ binding experiments and cell-membrane preparations were performed as described using OS_2 as iodinated ligand (31). Briefly, membranes from P4 cells, ^{125}I-OS_2, and unlabeled competitors were incubated at 20°C in 1 mL of binding buffer (140 mM NaCl, 0.1 mM $CaCl_2$, 20 mM Tris [pH 7.4], and 0.1% BSA) for 90 minutes and then filtered through GF/C glass fiber filters (Whatman, Maidstone, United Kingdom) presoaked in 0.5% polyethylenimine. Filters were washed twice with binding buffer, and bound radioactivity was counted. gp120 (0.17 nmol) was labeled with ^{125}I-Na (0.5 nmol) to a specific activity of approximately 2,500 Ci/mmol using lactoperoxidase as described (45). ^{125}I-gp120 binding assays were performed on P4 cells that had been dissociated with PBS containing 2 mM EDTA, washed, and resuspended at 10^7 cells/mL in gp120 binding buffer (50 mM HEPES [pH 7.2], 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.5% BSA). Binding assays were performed in 150 μL of gp120 binding buffer containing P4 cells (10^6 cells), ^{125}I-gp120 (3×10^5 cpm, 0.5 nM), and various unlabeled competitors. After 30 minutes at 37°C, incubations were layered on 500 μL of FBS, centrifuged for 10 minutes at 20,000 *g*, and analyzed for radioactivity associated with cell pellets. Nonspecific ^{125}I-gp120 binding was determined in the presence of 300 nM unlabeled gp120.

Results

$sPLA_2$s have antiviral properties against T-tropic (X4 viruses) and M-tropic (R5 viruses) HIV-1 isolates. The effect of various $sPLA_2$s on HIV-1_{BRU} infectivity was first analyzed with a single-round infection assay using $CD4^+$ HeLa cells (P4 cells). As shown in Figure 1, 4 venom $sPLA_2$s ($bvPLA_2$, Nmm_{CMIII}, taipoxin, and nigexine) appear as very potent inhibitors of HIV-1 infection. These 4 $sPLA_2$s reduce HIV-1_{BRU} replication with ID_{50} values lower than 1 nM and completely prevent infection above 10 nM (Figure 1). In contrast, other venom $sPLA_2$s have weaker (OS_1, BaIV) or no (PA2, PA5) antiviral activity (Figure 1). The inhibitory effect observed with BaIV appears interesting, as BaIV is a naturally occurring catalytically inactive $sPLA_2$ (41). We also analyzed the effect of human inflammatory-type group IIA $sPLA_2$ (hGIIA) and human and porcine pancreatic-type group IB $sPLA_2$s (hGIB and pGIB, respectively) (25). As shown in Figure 1, hGIIA, hGIB, and pGIB are very weak inhibitors of HIV-1_{BRU} replication. Finally, we analyzed whether these $sPLA_2$s can interfere with the antiviral activity of $bvPLA_2$. In these experiments, P4 cells were infected with HIV-1_{BRU} in the simultaneous presence of $bvPLA_2$ (10 nM) and either hGIB or hGIIA (up to 1 μM). In all cases, $bvPLA_2$ was found to retain full antiviral activity (data not shown), indicating that the inactive mammalian $sPLA_2$s do not interfere with the inhibitory activity of $bvPLA_2$.

Taipoxin, $bvPLA_2$, and Nmm_{CMIII} were then assayed for their ability to inhibit the replication of primary HIV-1 isolates and cell line–adapted HIV-1 strains that use either CXCR4 or CCR5 as a coreceptor. The replication of these HIV-1 isolates was measured in activated PBMCs maintained in the continuous presence or absence of $sPLA_2$s for 6 days (Table 1). HIV-1 replication, as monitored by the production of the HIV-1 Gag p24 protein, was dramatically reduced by $sPLA_2$s when PBMCs were infected either with a X4 virus (HIV-1_{BRU}), a R5 virus (HIV-1_{ADA}), or primary HIV-1 isolates of SI (91US054, CMU02) and NSI phenotype (91US056, 92US657). Similar antiviral activity of $sPLA_2$s was also observed when P4-CCR5 cells were infected with the HIV-1 R5 viruses HIV-1_{ADA} or HIV-1_{YU2} (data not shown). Taken together, these data indicate that several, but not all, $sPLA_2$s protect efficiently different cellular models from the replication of HIV-1 isolates independently of the coreceptor usage.

$sPLA_2$s do not exert virucidal or cytotoxic effects and block virus entry. The lipid composition of the HIV viral membrane is similar to that of cell membranes and mainly contains glycerophospholipids, which are $sPLA_2$ substrates (46). A first possible mechanism for the inhibition of virus infection would then be that $sPLA_2$s inactivate viral particles by hydrolyzing the lipids of the viral membrane. To address this possibility, HIV-1_{BRU} viral particles were treat-

Table 1
$sPLA_2$ inhibition of HIV-1 replication in human PBMCs[A,B]

			Production of Gag p24 (ng/mL)			
HIV-1	SI/NSI capacity[C]	Days after infection	–	$bvPLA_2$	Taipoxin	NMM_{CMIII}
91US054	+	3	210	21	30	10
		6	1,800	310	380	380
CMU02	+	3	7	6	8	8
		6	460	30	17	4
BRU	+	3	340	26	15	11
		6	2,600	270	160	160
91US056	–	3	370	9	7	8
		6	2,300	170	260	260
92US657	–	3	260	6	8	8
		6	2,500	70	290	100
ADA	–	3	270	9	11	4
		6	1,100	270	160	160

[A]Activated human PBMCs were infected with the indicated primary HIV-1 isolates or laboratory adapted HIV-1 strains (100 ng of Gag p24). The amount of Gag p24 released into culture supernatant was measured after 3 and 6 days of culture in the continuous presence or absence of $sPLA_2$ (100 nM). [B]Cell viability was always greater than 80% in all conditions, as measured with the XTT assay. [C]Syncytium induction capacity in PBMCs.

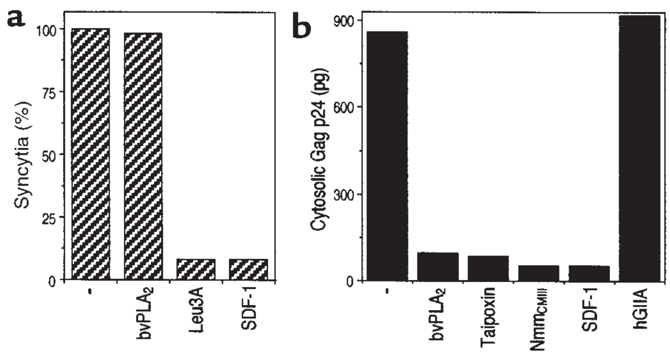


Figure 3
Effect of sPLA$_2$s on HIV-1 entry into host cells. (**a**) Effect of bvPLA$_2$ on the cell-cell fusion process. P4 cells and 293 cells expressing HIV-1 proteins, including gp120/gp41, were mixed and treated with or without bvPLA$_2$ (10 nM), SDF-1 (250 nM), or anti-CD4 mAb (Leu3A, 1/40). Levels of cell-cell fusion were estimated after 48 hours by β-gal assay. (**b**) Effect of sPLA$_2$s and SDF-1 on the intracellular release of Gag p24, used as an index of virus entry. CEM cells (10^7 cells) were incubated at 4°C for 45 minutes with HIV-1$_{BRU}$, washed extensively, and then incubated for 1 hour at 37°C in the presence or absence of sPLA$_2$s (10 nM) or SDF-1 (250 nM). After pronase treatment, cells were lysed, ultracentrifuged, and analyzed for Gag p24 content in the cytosolic fraction. Results are representative of at least 2 (**a**) or 4 (**b**) independent experiments with SEM less than 10%.

ed with bvPLA$_2$ and then used to infect P4 cells (Table 2). Before infection, viral particles were centrifuged and the viral pellet was resuspended in the presence of specific bvPLA$_2$ antibodies (rbv$_{Ab}$) added to neutralize the residual bvPLA$_2$ that contaminates the viral pellet. As shown in Table 2, HIV-1$_{BRU}$ viral particles treated with bvPLA$_2$ were still able to infect P4 cells, clearly indicating that the antiviral activity of bvPLA$_2$ does not result from a direct action on the viral particles. Furthermore, pretreatment of target cells with bvPLA$_2$ followed by extensive washes and infection with HIV-1 does not lead to inhibition of HIV-1 replication (Table 2). Another possibility would be that sPLA$_2$ exerts a cytotoxic action on the HIV-1 host cells. However, using the XTT cytotoxic assay, we found that the growth of the various HIV-1 host cells, including PBMCs (Table 1), was not affected by sPLA$_2$ treatment, indicating that the antiviral activity of sPLA$_2$s does not result from a cytotoxic effect (data not shown).

HIV-1 infection is initiated by the binding of gp120 to the CD4 receptor. This step was not blocked by sPLA$_2$s, as the binding of ^{125}I-gp120 to CD4 was not inhibited by sPLA$_2$s including bvPLA$_2$ (data not shown). This conclusion was confirmed by time-of-addition experiments showing that sPLA$_2$s are still able to inhibit HIV-1 replication when added to cells after virus binding (Figure 2a). In these experiments, P4 cells were incubated with HIV-1 at 4°C to allow virus binding, but not virus entry. Unbound viruses were then washed away, and cells were shifted to 37°C to trigger the virus-cell fusion process that initiates virus entry into host cells. At different times after virus addition to cells, bvPLA$_2$ was added until virus entry was fully achieved (8 hours). As shown in Figure 2a, a complete inhibition of HIV-1 infection was observed when bvPLA$_2$ was added before or after virus binding to cells. After the temperature shift, the sensitivity to bvPLA$_2$ was gradually lost with an inhibitory half-time effect of about 2 hours. The inhibitory effect of bvPLA$_2$ on virus entry occurs before the RT step, as the half-time effect of AZT, which is known to inhibit this step, was about 4 hours (Figure 2a). The half-time effects of taipoxin and Nmm$_{CMIII}$ (data not shown) were found to be similar to those of bvPLA$_2$, indicating that all of these sPLA$_2$s are acting after virus binding to cells, but before the RT step. These results were confirmed by Southern blot analysis of the proviral DNA synthesis in HIV-1–infected cells (Figure 2b). For these experiments, MT4 cells were preferred because these cells are highly susceptible to HIV-1 infection and are also sensitive to sPLA$_2$ action (data not shown). Early after virus addition (8 hours), no unintegrated proviral DNA was detected in cells treated with bvPLA$_2$, Nmm$_{CMIII}$, or taipoxin (Figure 2b), whereas it was abundantly accumulated in untreated cells and in cells treated with the mammalian sPLA$_2$s pGIB (Figure 2b) and hGIIA (data not shown).

The fusion between viral and cellular membranes is usually addressed indirectly by syncytium formation assays between CD4$^+$ cells and cells expressing gp120. The possible effect of sPLA$_2$s on the formation of syncytia was thus analyzed by cocultivation of P4 cells with 293 cells expressing HIV-1$_{BRU}$ proteins, including gp120. As shown in Figure 3a, bvPLA$_2$ was unable to prevent the formation of syncytia, whereas both the chemokine SDF-1 (known to block virus-cell fusion by interacting with CXCR4; ref. 16) and an mAb specific for CD4 (Leu3A) led to a dramatic decrease in the formation of syncytia. These results indicate that the formation of syncytia between cells is not inhibited by sPLA$_2$s and suggest that the virus-cell fusion process is probably not affected by sPLA$_2$s.

Recently, Maréchal et al. have shown that the detection of Gag p24 in the cytosol of host cells can be used as a good index to monitor productive entry of HIV-1 into host cells (44). We therefore analyzed the effect of sPLA$_2$s on the level of cytosolic Gag p24 to determine whether sPLA$_2$s are inhibiting HIV-1 entry before or after virion dissociation from the cell membrane (Figure 3b). These experiments were carried out with different cell systems (P4, CEM, and MT4 cells), and suitable conditions were obtained with CEM cells. As shown in Figure 3b, CEM cells infected with HIV-1$_{BRU}$ in the presence of SDF-1, bvPLA$_2$, taipoxin, or Nmm$_{CMIII}$ contain much lower amounts of cytosolic Gag p24 compared with untreated cells or cells treated with hGIIA. Interestingly, sPLA$_2$s were as efficient as SDF-1 in preventing cytosolic Gag p24 accumulation (Figure 3b), indicating that these enzymes are potent inhibitors of virus entry into host cells.

Antiviral activity of $sPLA_2$s is linked to $sPLA_2$ binding to host cells, rather than $sPLA_2$ catalytic activity. The role of $sPLA_2$ catalytic activity in the HIV-1 antiviral action of $sPLA_2$s was addressed by using various $sPLA_2$ inhibitors that are known to inhibit $sPLA_2$ activity in vitro (23). Phenacyl bromide (0.1 mM), aristolochic acid (0.1 mM), and oleoyloxyethylphosphocholine (10 μM), which do not show antiviral activity by themselves, were unable to prevent the HIV-1 inhibitory effects of $bvPLA_2$ (data not shown). The cyclooxygenase blocker indomethacin (0.1 mM), which has no effect on HIV-1 infection, had also no effect on the antiviral activity of $bvPLA_2$ (data not shown). The effect of mepacrine (0.1 mM), a general PLA_2 inhibitor, and nordihydroguaiaretic acid (0.1 mM), a lipoxygenase inhibitor, was not addressed because these inhibitors were toxic to P4 cells. On the other hand, several catalytic products of $sPLA_2$s, such as arachidonic acid, lysophosphatidylethanolamine, lysophosphatidic acid, and oleoyl- and palmitoyl-lysophosphatidylcholine (up to 10 μM), were also found to be unable to block HIV-1 replication (data not shown). We also addressed the possible role of leukotriene B4 (LTB4), a downstream metabolite of PLA_2 activity whose receptor has recently been shown to act as a coreceptor for HIV-1 entry (47). Addition of LTB4 (up to 1 μM) to P4 cells did not block HIV-1_{BRU} replication (data not shown), making it unlikely that $sPLA_2$s mediate their inhibitory effect through production of LTB4. Finally, the relatively weak but significant ability of the catalytically inactive $sPLA_2$ BaIV (41) to block HIV-1 replication (Figure 1) suggests that the $sPLA_2$ catalytic activity is not involved in HIV-1 inhibition. Together, these results indicate that the catalytic activity of $sPLA_2$s would not play a crucial role in the $sPLA_2$ antiviral effect.

Because $sPLA_2$s have previously been shown to bind specifically to membrane receptors (27), we analyzed the presence of $sPLA_2$ binding sites in the different cell systems used in this study. A specific $sPLA_2$ binding was observed in P4 cells (Figure 4, inset), as well as in PBMCs and CEM and MT4 cells (data not shown). Competition binding experiments (Figure 4) indicate that $K_{0.5}$ values for $bvPLA_2$, taipoxin, nigexine, and Nmm_{CMIII}, which all block HIV-1 replication efficiently (Figure 1), are 0.08, 0.15, 0.06, and 0.17 nM, respectively (Figure 4 and data not shown). Conversely, $K_{0.5}$ values for $sPLA_2$s that have weak or no antiviral activity (Figure 1) are in the micromolar range (OS_1, BaIV) or higher (PA2, PA5, pGIB, hGIB, and hGIIA) (Figure 4 and data not shown). Taken together, the observed binding profile shows that the $sPLA_2$s that efficiently inhibit HIV-1 replication also display high affinities to P4 cell membranes, whereas the $sPLA_2$s that have no or weak inhibitory effect have much lower affinities.

Table 2

Pretreatment of cells or viral particles with $bvPLA_2$ is not sufficient to inhibit HIV-1 replication

Pretreatment of viral particles[A]	Pretreatment of cells[B]	Condition of infection[C]	Viral replication[D,E] (%)
–	–	–	100
–	$bvPLA_2$	–	94
–	–	$bvPLA_2$	7
$bvPLA_2$	–	–	52
$bvPLA_2$	–	rbv_{Ab}	87
–	–	rbv_{Ab}	103

[A]Viral particles (HIV-1_{BRU}) were incubated for 60 minutes at 37°C in the presence or absence of $bvPLA_2$ (10 nM), ultracentrifuged, and then used to infect P4 cells. [B]P4 cells were incubated for 2 hours in medium containing or not containing $bvPLA_2$ (10 nM), washed, and infected with HIV-1_{BRU}. [C]P4 cells were infected with HIV-1 viral particles in medium containing $bvPLA_2$ (10 nM) or a neutralizing rabbit polyclonal serum raised against $bvPLA_2$ (rbv_{Ab}, 1:40), as indicated. [D]The level of viral replication was determined 48 hours after infection by measuring β-gal activity. [E]Each value represents the mean of at least 3 independent experiments with an SEM less than 10%.

Discussion

Mammalian and venom $sPLA_2$s have been implicated in a variety of physiological and pathological effects including cell proliferation, cell contraction, hormone release, inflammation, cancer, and antibacterial defense and exert various types of toxicities (22, 25, 30, 36, 48). We show here that several, but not all, $sPLA_2$s are potent inhibitors of HIV-1 replication. These $sPLA_2$s were found to protect different types of host cells efficiently from the replication of various HIV-1 isolates independently of the viral phenotype. Furthermore, $bvPLA_2$ was also able to inhibit the replication of HIV-2_{ROD10} in P4-CCR5 cells with ID_{50} values close to 1 nM (data not shown), indicating that $sPLA_2$s can protect cells from infection with both HIV-1 and HIV-2 viruses.

Our results show that HIV inhibition by $sPLA_2$s takes place early during the viral life cycle. HIV enters the cell by fusion at the plasma membrane, a process that is triggered by the binding of gp120 to CD4 and chemokine receptors. Our data indicate that the binding of gp120 to these cellular receptors is not inhibited by $sPLA_2$s, because (a) binding of gp120 to CD4 is not inhibited by $sPLA_2$s; (b) $sPLA_2$s can block the entry of viruses that have been first bound to cells; (c) both X4 and R5 viruses that use different chemokine receptors are sensitive to $sPLA_2$s; and (d) syncytia formation between gp120 and CD4 expressing cells is not affected by the presence of $sPLA_2$s. The absence of $sPLA_2$ effect on syncytia formation also suggests that $sPLA_2$s do not act by blocking the virus-cell fusion process. However, this assumption must be tempered, as cell to cell fusion experiments do not always reflect the virus-cell fusion process (49).

Shortly after fusion, the disengagement of the RT complex from the cell membrane (often referred as uncoating) leads to the cytosolic accumulation of virion components. This step is believed to be a prerequisite for the synthesis of the proviral DNA that then moves toward the cell nucleus (10, 13, 14, 50). Our data show that $sPLA_2$s do not act directly on viral particles (Table 2), do not act on cells before infection (Table 2), do not block CD4-gp120 interaction, and have no antiviral activity when added a few hours after virus addition (Fig-

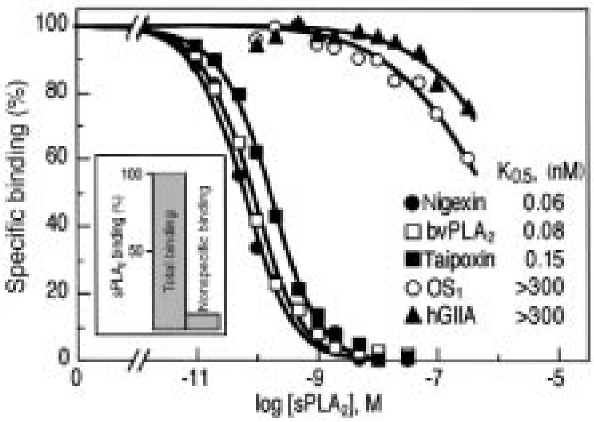


Figure 4
Binding properties of $sPLA_2s$ to P4 cell membranes. P4 cell membranes (20 μg/mL) were incubated with iodinated $sPLA_2$ (25 pM) and various concentrations of unlabeled $sPLA_2s$. Results are expressed as percentage of the maximal specific binding measured in the absence of $sPLA_2$ competitor. A total of 100% corresponded to 1.8 pM of specifically bound labeled $sPLA_2$. Nonspecific binding was measured with 100 nM unlabeled ligand and accounted for 10% of total binding.

ure 2). We thus conclude that $sPLA_2s$ display antiviral activity only when they are present during the first hours of virus entry that correspond to the critical period of $sPLA_2$ action. This is in agreement with our results showing that $sPLA_2s$ prevent the release of Gag p24 and the synthesis of proviral DNA in the cytosol of cells infected with HIV-1 (Figures 2 and 3), and suggests that $sPLA_2s$ block virus entry by preventing virion uncoating and dissociation of the RT complex from host cell membranes. This blockade of virion disassembly is likely to explain the lack of proviral DNA synthesis in the presence of $sPLA_2s$, as these enzymes were unable to inhibit the RT activity (data not shown), as measured in both exogenous and endogenous in vitro assays (51). Several previous reports have shown that postfusion steps can be modulated by different cellular and viral factors. The phosphorylation of RT complex components by a virion-associated kinase facilitates the disengagement of this complex from the cell membrane and its subsequent nuclear targeting (12, 14, 52, 53). Cyclophilin A, a host protein isomerase, is required for an early step in the HIV-1 life cycle, probably to favor virion uncoating by destabilizing the capsid structure (54, 55). Nef, a regulatory viral protein found associated to the virion (56), increases the efficiency of RT (51, 57) and may increase viral nuclear import (58). It thus appears that postfusion steps are tightly regulated, making it possible that $sPLA_2s$ act on one of these steps through direct or indirect interaction with viral or cellular factors.

The $sPLA_2$ catalytic activity does not appear to be involved in HIV-1 antiviral effect. First, different $sPLA_2$ inhibitors were unable to prevent $sPLA_2$ antiviral activity. Second, $sPLA_2$ products, such as arachidonic acid and various lysophospholipids, do not prevent HIV-1 replication. Third, several $sPLA_2s$ like PA2, PA5, pGIB, hGIB, and hGIIA are catalytically active enzymes (25, 27), but do not have antiviral activities (Figure 1). Finally, BaIV, a catalytically inactive $sPLA_2$-like protein (41), was found to have antiviral properties, although with a relatively low ID_{50} value of 400 nM (Figure 1). On the other hand, the $sPLA_2$ antiviral activity appears to be associated to the ability of $sPLA_2s$ to bind to host cells. Indeed, the $K_{0.5}$ values measured for the binding of the various $sPLA_2s$ including BaIV to P4 cells are in good agreement with their ID_{50} values to inhibit HIV-1 infection (Figures 1 and 4). So far, 2 main types of $sPLA_2$ receptors have been identified (27). N-type receptors display high affinities for $bvPLA_2$, taipoxin, and Nmm_{CMIII} $sPLA_2$, and low affinities for OS_1 and pGIB. Conversely, M-type receptors have high affinity for OS_1 and pGIB, and do not bind $bvPLA_2$ and Nmm_{CMIII}. Based on these binding profiles, the $sPLA_2$ binding sites detected in P4 cells appear most similar to N-type receptors. Furthermore, no M-type receptors were detected in P4 cells (data not shown). The molecular nature of the $sPLA_2$ binding sites found in P4 cells remains to be determined, but are likely to be related to the N-type like receptors that have been identified in different tissues and cells including immune cells (27, 31, 32).

In conclusion, our data indicate that several venom $sPLA_2s$ can protect various types of host cells including PBMCs from the replication of primary HIV-1 isolates. Conversely, human group IB and human group IIA $sPLA_2s$ were unable to block HIV-1 replication. However, because several other $sPLA_2s$ are being identified in mammals (26, 28, 29), it is tempting to speculate that some of these novel human $sPLA_2s$ may have clinical relevance in HIV infection. In individuals repeatedly exposed to HIV but who remain uninfected, several possible reasons for protection have been proposed but not clearly elucidated (59). In this respect, the putative antiviral activity of the novel group IID, V, and X $sPLA_2s$, which are expressed in immune tissues and cells such as macrophages and PBMCs (28, 29), will be particularly interesting to analyze in the future, when these enzymes will be available in high enough amounts to assay them, as we have assayed venom $sPLA_2s$.

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