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Cholera toxin inhibits HIV-1 replication in human colorectal epithelial HT-29 cells through adenylate cyclase activation

Sonia Gauthier, Michel J. Tremblay*

Centre de Recherche en Infectiologie, Centre Hospitalier Universitaire de Québec-CHUL, and Département de Microbiologie-Infectiologie et Immunologie, Faculté de Médecine, Université Laval, Québec, Canada

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ABSTRACT

Mixed feeding, combining breast milk and nonhuman milk and/or solid food, is a common practice in developing countries that increases the risk of vertical HIV-1 transmission. It also enhances the risk of infection by waterborne microorganisms such as *Vibrio cholerae*, a diarrhoea-causing pathogen that frequently infects children below 18 months of age. Although both HIV-1 and *V. cholerae* affect young children and target intestinal epithelial cells, no information is currently available on possible interactions between these two pathogens. In this study, we show for the first time that cholera toxin (CTx), at a concentration as low as 100 pg/ml, inhibits HIV-1 infection of HT-29, a human colorectal epithelial cell line. The CTx-mediated inhibitory effect does not result from a down-regulation of receptor/co-receptor expression or a modulation of viral transcription. Nevertheless, additional experiments indicate that a yet to be identified early step in the virus life cycle is targeted by CTx since the enterotoxin similarly reduces infection of HT-29 cells with AMLV-I, HTLV-I and HIV-1 pseudotyped viruses while exerting no effect on infection with VSV-G pseudotypes. Furthermore, our results indicate that the CTx-dependent suppression is not due to the cholera toxin subunit B but linked instead to the action of cholera toxin subunit A (CTA). Altogether our data indicate that the CTA subunit of CTx is negatively affecting an early event in HIV-1 replication in human colon cancer HT-29 cells.

1. Introduction

Mother-to-child transmission, which can occur *in utero*, *intrapartum*, or during breastfeeding (Lehman and Farquhar, 2007), is responsible for the majority of pediatric HIV-1 infection, which is considered as a major health issue in developing countries (Van de Perre, 1999). In the absence of antiretroviral therapy, HIV-1 vertical transmission rate is estimated to be between 30% and 45% in breastfed children and 15% and 30% in formula-fed infants (Lehman and Farquhar, 2007). Although formula feeding carries no risk of HIV-1 transmission, it augments sharply, in developing countries, the mortality risk due to increased infection by pathogens present in water (Coovadia and Kindra, 2008). Consequently, many women living in developing countries choose to breastfeed their child despite their HIV-1 positive status. *Post-partum* transmission of HIV-1 following ingestion of breast milk contaminated with cell-free or cell-associated HIV-1 involves the baby's gastrointestinal system, especially the intestine (Janoff and Smith, 2001; Smith et al., 2003; Van de Perre, 1999).

After reaching the intestinal lumen, HIV-1 may gain access to the gut-associated lymphoid tissue (GALT) by being transported by M cells, being captured by dendritic cells extending their dendrites into the intestinal lumen and/or by infecting intestinal epithelial cells (Janoff and Smith, 2001; Smith et al., 2003). Intestinal epithelial cells form a polarized monolayer separating the GALT from the intestinal lumen. Their apical membranes are facing the intestinal lumen and are in continual contact with microorganisms, which can be both commensal and/or pathogenic. Their basolateral membranes are facing the GALT and are in close contact with immune cells such as $CD4^+$ T lymphocytes, the main target of HIV-1 in peripheral blood. *In vitro* infection of intestinal epithelial cells by HIV-1 is well documented and occurs primarily through the major receptor galactosylceramide (GalCer) and the coreceptor CXCR4 (Delezay et al., 1997). *In vivo* infection of those cells by HIV-1 has also been reported (Flemstrom and Sjoblom, 2005; Nelson et al., 1988) and may occur through the CCR5 coreceptor since they express GalCer and CCR5 (Meng et al., 2002). Some studies have also reported CXCR4 expression on rectal and colonic epithelial cells in addition to CCR5 (Jordan et al., 1999).

* Corresponding author at: Centre de Recherche en Infectiologie, RC709, Centre Hospitalier Universitaire de Québec-CHUL, 2705 boul. Laurier, Québec, QC, Canada, G1V 4G2. Tel.: +1 418 656 4141/48274; fax: +1 418 654 2715.
E-mail address: michel.j.tremblay@crchul.ulaval.ca (M.J. Tremblay).

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Many bacteria, both commensal and pathogenic, can be present in the intestinal system. For instance, *Vibrio cholerae* (*V. cholerae*) is a gram-negative pathogenic bacterium mainly found in developing countries where HIV-1 transmission via breast milk is frequent (Sack et al., 2004; Vanden Broeck et al., 2007). Cholera affects 1.8 million people yearly, causing over 27,000 deaths annually (Girard et al., 2006). Transmission to humans occurs through ingestion of contaminated food or water (Girard et al., 2006; Steinberg et al., 2001; Vanden Broeck et al., 2007). *V. cholerae* produces an enterotoxin, the cholera toxin (CTx), which acts on intestinal epithelial cells and causes diarrhoea (Lencer et al., 1999).

CTx is a multimeric protein composed of an active subunit A (CTA) surrounded by five subunits B (CTB) (Vanden Broeck et al., 2007). CTA is causing the toxic effect of the enterotoxin, whereas CTB is responsible for attachment to the cell membrane (Lavelle et al., 2004; Sanchez and Holmgren, 2008). Subunit A is composed of two polypeptide chains, CTA1 and CTA2, linked by a disulfide bound. The CTA1 subunit exerts an ADP-ribosylating activity, whereas the CTA2 subunit links CTA1 to the CTB pentamer (Zhang et al., 1995). Upon binding of CTB to the cell surface ganglioside GM_1, CTx is internalized by lipid raft/caveolae (Orlandi and Fishman, 1998), clathrin (Broeck et al., 2007), or a non-caveolar/non-clathrin-mediated endocytic pathway (Kirkham et al., 2005; Massol et al., 2004). Thereafter, CTx traffics initially to the trans-Golgi network, followed by a retrograde transport to the endoplasmic reticulum (ER) (Lencer et al., 1995). In the ER, CTA1 is dissociated from the CTA2/CTB complex, unfolded and transported to the cytoplasm by a process termed retro-translocation (Hazes and Read, 1997). CTA1 then ADP-ribosylates Gsα, a protein regulating adenylate cyclase function (Vanden Broeck et al., 2007). This modification constitutively activates Gsα, which will constantly activate adenylate cyclase and cause an increase in cyclic AMP (cAMP), an important cellular second messenger (Serezani et al., 2008) regulating multiple biological processes (De Haan and Hirst, 2004). The biological effect of cAMP is mediated by two ubiquitary receptors, protein kinase A (PKA) and the exchange proteins activated directly by cyclic AMP (Epac) system (Serezani et al., 2008). Once activated, PKA phosphorylates its substrates, which can be both cytoplasmic and/or nuclear proteins, and affects many cellular events. For the Epac system, its activation leads to interaction with different effectors which influence many biological functions such as actin dynamics (Kooistra et al., 2007; Rangarajan et al., 2003) and gene expression (Lotfi et al., 2006).

Although HIV-1 and *V. cholerae* can be found simultaneously in the digestive tract where they both interact with intestinal epithelial cells, data on their putative relationships are very scarce. Few reports have been published on the effects of cAMP on HIV-1 infection, mostly concerning $CD4^+$ T lymphocytes. These studies revealed that cAMP increases CXCR4 expression resulting in an increased susceptibility to infection with X4-using virions (Cole et al., 1999; Cristillo et al., 2002a). Since CTx activation of adenylate cyclase eventually results in increased intracellular cAMP levels (Vanden Broeck et al., 2007), we investigated if the presence of CTx modulates HIV-1 infection of intestinal epithelial cells, more precisely, by monitoring the pre- and post-integration events. We provide evidence here that CTx reduces HIV-1 infection in the human colorectal cell line HT-29.

2. Materials and methods

2.1. Reagents

CTx and CTB were purchased from List Biological Laboratories (Campbell, CA) while recombinant TNF-α was obtained from PeproTech (Rocky Hill, NJ). Cytochalasin D and forskolin were purchased from Calbiochem (Gibbstown, NJ). Tetramethylrhodamine- and Alexa 633-conjugated transferrin were obtained from Molecular Probes (Invitrogen Life Technologies, Burlington, ON).

2.2. Cells

The human colorectal epithelial cell line HT-29 (ATCC HTB-38; American Type Culture Collection, Manassas, VA) was grown in DMEM/F12 supplemented with 10% foetal bovine serum (FBS) (Invitrogen Life Technologies) and antibiotics. The human embryonic kidney cell line 293T was grown in DMEM (Invitrogen Life Technologies) supplemented with 10% FBS and antibiotics. Peripheral blood mononuclear cells (PBMCs) from healthy donors were obtained by Ficoll gradient centrifugation. Next, $CD4^+$ T cells were purified from freshly isolated PBMCs by immunomagnetic negative selection (Stem Cell Technologies Inc., Vancouver, BC, Canada). Purified $CD4^+$ T cells were activated by culturing them for 3 days in RPMI-1640 culture medium (Wisent, St-Bruno, QC, Canada) supplemented with 10% fetal bovine serum in the presence of phytohemagglutinin (1 μg/ml) and recombinant human interleukin-2 (30 U/ml).

2.3. Plasmids

The full-length infectious molecular clone NL4-3Bal*env* has been constructed by replacing the *env* gene of the X4-tropic NL4-3 strain with that of the R5-using Bal strain. The pNL4-3Luc$^+$Env$^-$Vpr$^+$ expression vector encodes the complete HIV-1 genome, in which the envelope gene has been inactivated, and the luciferase gene inserted in the region coding for Nef. The pcDNA-1-AMLV-Env vector expresses the amphotropic murine leukemia virus (MLV) envelope (Env) gene under the control of the MLV long terminal repeat (LTR). Both vectors were kindly provided by Dr. N.R. Landau (NYU School of Medicine, New York, NY). The pSV-HTLV-I-Env vector carries the HTLV-I Env under the control of the SV40 promoter (kindly supplied by Dr. R. Sutton, Baylor College of Medicine, Houston, TX). The pJD19 vector expresses the wild type HIV–1 NDK Env (X4-tropic) under the control of the NDK LTR (obtained from Dr. U. Hazan, Institut Cochin de Génétique Moléculaire, Paris, France). The pHCMV-G vector expresses the Env glycoprotein G of the broad host-range vesicular stomatitis virus (VSV-G), under the control of the human cytomegalovirus (CMV) promoter (obtained from Dr. J.C. Burns, University of California, San Diego, CA). The pLTRX-Luc vector contains the HIV-1 LTR from the HIV-1 LAI strain positioned upstream of the luciferase reporter gene (kindly provided by Dr. O. Schwartz, Unité d'Oncologie Virale, Institut Pasteur, Paris, France). The following reagent was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pCEP4-Tat from Dr. Lung-Ji Chang. The pCEP4-Tat vector contains the HIV-1 SF2 *tat* gene from pSP72-tat ligated to pCEP4 (Chang et al., 1999).

2.4. Virus production

Virus stocks were prepared as previously described (Cantin et al., 1997). Briefly, 293T cells (2×10^6) were seeded in 75-cm^2 flasks and cotransfected with the AMLV-, HTLV-I- or NDK-Env expression vector (20 μg) and the Env-deficient HIV-1 backbone plasmid pNL4-3Luc$^+$Env$^-$Vpr$^+$ (10 μg) by the calcium–phosphate method. For VSV-G pseudotyped viruses, pHCMV-G (2 μg) was cotransfected with pNL4-3Luc$^+$Env$^-$Vpr$^+$ (28 μg). Viruses were harvested 40 h after cotransfection, filtered through a 0.22 μm-pore-membrane and stored at −80 °C until used. The virus content was measured using a sensitive in-house double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) specific for the viral p24 protein (Bounou et al., 2002).

2.5. Infection assays

HT-29 cells (2×10^5) were either left untreated or treated for 24 h with CTx (0.01–10,000 pg/ml), CTB (0.01–10,000 pg/ml), or forskolin (50 μM), as indicated, and washed. The cells were then infected for 24 h with a standardized amount of NL4-3Luc$^+$Env$^-$Vpr$^+$ virus pseudotyped with AMLV-Env, HTLV-I-Env, NDK-Env, or VSV-G (20 ng of p24 each). In the case of forskolin, the drug was left during the infection assay. Thereafter, the cells were washed and either left untreated or treated with TNF-α (10 ng/ml), as indicated, for 24 h and lysed in 100 μl of lysis buffer (125 mM Tris–Base, 10 mM DTT, 5% Triton X-100 and 50% glycerol). Luciferase activity was measured using an MLX microtiter luminometer (Dynex Technology, Chantilly, VA) and expressed as Relative Luciferase Units (RLU). For CD4$^+$ T cells, 2×10^5 cells were either left untreated or treated for 24 h with CTx (0.01–10,000 pg/ml) and washed extensively. The cells were then infected for 24 h with a standardized amount of NL4-3Bal*env* virus (20 ng of p24). Thereafter, the cells were washed and extracellular p24 was measured on day 3, 6 and 9 post-infection using the p24 test.

2.6. Virus entry test

HT-29 cells (2×10^5) were either left untreated or treated with CTx (0.01–10,000 pg/ml) for 24 h and washed. Cells were then incubated for 4 h with NL4-3Luc$^+$Env$^-$Vpr$^+$ virus pseudotyped with NDK-Env (20 ng of p24). Cells were trypsinized, washed four times with PBS and resuspended in 100 μl of lysis buffer (20 mM HEPES, 150 mM NaCl, 0.05% Tween-20 and 0.5% Triton X-100). Virus entry was estimated by quantifying the p24 content.

2.7. Viral transcription assay

HT-29 cells (7.5×10^6 in a 75-cm^2 flask) were transfected by Lipofectamine™ with the pLTRX-Luc and pCEP4-Tat vectors (ratio 2:1) according to the manufacturer's instructions (Invitrogen Life Technologies) and, 5 h after adding the DNA/lipofectamine mixture, cells (2×10^5) were seeded in a 24-well plate. The next day, transfected HT-29 cells were either left untreated or stimulated with CTx (100 pg/ml) for 24, 48, or 72 h, and then lysed in 100 μl of lysis buffer. Luciferase activity was measured as specified above.

2.8. Flow cytometry analysis

HT-29 cells (1×10^6) were either left untreated or treated with CTx (0.01–10,000 pg/ml) for 24 h. Cells were washed with phosphate-buffered saline (PBS), incubated with Versene and detached from the culture plate by scraping with a rubber policeman. Non-specific sites were blocked by incubating cells with 1% bovine serum albumin (MP Biomedicals, Solon, OH), 10% human serum and 20% goat serum (Jackson ImmunoResearch, West Grove, PA) in PBS. Cells were then incubated for 30 min at room temperature with a phycoerythrin (PE)-conjugated monoclonal antibody against CXCR4 or an isotype-matched irrelevant control antibody (both from BD Pharmingen). In some experiments, cells were incubated for 30 min at room temperature with an antibody directed against GalCer (Millipore, Billerica, MA). Cells were then washed twice with PBS, fixed with 2% paraformaldehyde for 30 min at room temperature and then analyzed by flow cytometry (Epics ELITE ESP; Coulter Electronics, Burlington, ON).

For transferrin entry analysis, HT-29 cells (1×10^6) were either left untreated or treated with CTx (0.01–10,000 pg/ml) or cytochalasin D (5 μM) for 24 h, washed and incubated for 1 h with Alexa 633-conjugated transferrin (50 μg/ml) (Molecular Probes). Cells were then detached with trypsin, washed twice with PBS, fixed with 2% paraformaldehyde for 30 min at room temperature and finally analyzed by flow cytometry.

2.9. Confocal microscopy analysis

HT-29 cells (1×10^5) were either left untreated or treated with CTx (100 pg/ml) or cytochalasin D (1 μM) for 24 h, washed and incubated for 1 h with transferrin-tetramethylrhodamine (50 μg/ml) (Molecular Probes). Cells were then fixed with 2% paraformaldehyde, permeabilized and non-specific sites blocked by using PBS 1× containing 1% BSA and 0.1% Triton X-100. Finally, cells were stained with phalloidin-488 (1/100) (Molecular Probes, Invitrogen) and examined using a FluoView FV300 confocal laser scanning confocal microscope (Olympus America, Melville, NY).

2.10. Statistics

Statistical analyses were carried out according to the methods outlined in Zar (1984). Means were compared using either the Student's test or single-factor ANOVA followed by Bonferroni's multiple comparison when more than two means were compared. Because values vary between different experiments, a repeated-measures ANOVA test had to be used. *P* values of less than 0.05 were deemed statistically significant, whereas *P* values lower than 0.01 were considered highly significant. Computations were carried out using GraphPad PRISM statistical software, version 3.03.

3. Results

To study the effect of CTx on the susceptibility of human intestinal cells to HIV-1 infection, we used the colorectal epithelial cell line HT-29. As for the viral strain used to infect them, we used an NL4-3-based vector coding for the luciferase reporter gene. As luciferase is only produced following productive virus infection, using such a reporter virus to assess the infection level presents the advantage of not measuring the initial virus input, which is normally the case when assessing the major viral core protein p24. Virus preparations were made in 293T cells using reporter virions pseudotyped with the NDK Env because the NDK strain is commonly used to infect HT-29 cells.

3.1. CTx impedes HIV-1 infection of HT-29 cells

In order to determine whether CTx can modulate HIV-1 infection of epithelial intestinal cells, HT-29 cells were treated for 24 h with increasing concentrations of CTx and next infected with reporter viruses. As illustrated in Fig. 1A, CTx represses HIV-1 infection in a dose-dependent manner reaching a 60% inhibition at a final dose of 10,000 pg/ml. A time-course experiment also shows that 16 h of treatment with CTx were necessary before resistance was conferred (data not shown). To eliminate the possibility that a modulation of cell proliferation is responsible for the observed CTx-mediated inhibitory effect, we performed a cell proliferation assay. No statistical difference was seen between HT-29 cells treated with CTx and the untreated control (data not shown). Additional experiments indicate that the CTx-mediated diminution in HIV-1 infection is not a generalized phenomenon since pre-treatment of primary human CD4$^+$ T cells with increasing concentrations of CTx (i.e. ranging from 0.01 to 10,000 pg/ml) has no effect on virus replication (Fig. 1B).

3.2. Virus transcription is not affected by CTx

To further investigate the effect of CTx on virus gene expression, HT-29 cells were transiently cotransfected with HIV-1 LTR-driven luciferase and Tat-encoding vectors, and then stimulated with CTx

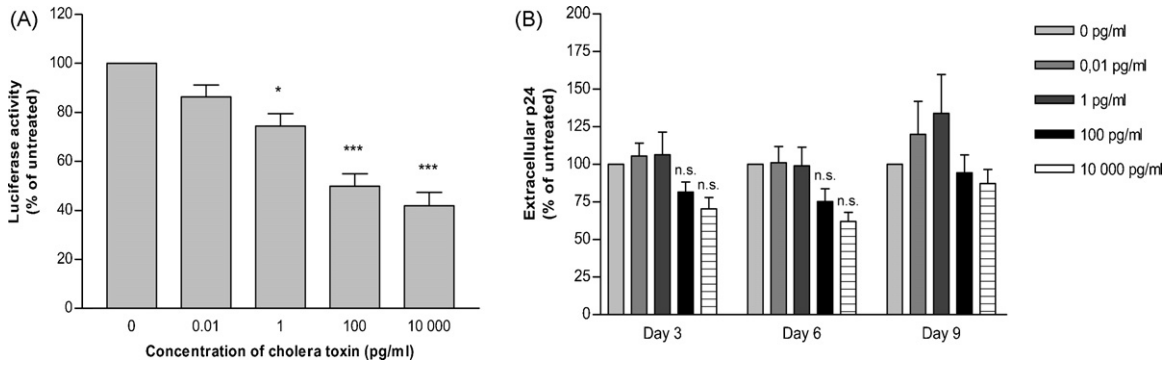

Fig. 1. CTx inhibits HIV-1 replication in HT-29 cells. (A) HT-29 cells were either left untreated or treated with CTx (i.e. 0.01–10,000 pg/ml) for 24 h and then washed extensively. Cells were next infected for 24 h with NDK-Env pseudotypes and washed again. Virus infection was estimated by measuring virus-encoded luciferase activity at 24 h post-infection. The results shown represent the means ± standard deviations calculated from three independent experiments with quadruplicate samples and are expressed as percentages of untreated cells (*, $P < 0.05$; ***, $P < 0.001$). (B) Purified $CD4^+$ T cells were either left untreated or treated with CTx (i.e. 0.01–10,000 pg/ml) for 24 h and then washed extensively. Cells were next infected for 24 h with NL4-3Bal*env* and washed again. Virus infection was estimated by measuring the extracellular p24 content at 3, 6 and 9 days post-infection. The results shown represent the means ± standard deviations calculated from six independent experiments with quadruplicate samples and are expressed as percentages of untreated cells (n.s., non significant).

for 24–72 h. Data shown in Fig. 2A indicate that CTx has no significant effect on viral transactivation. In addition to the viral transactivating Tat protein, HIV-1 transcription requires cellular transcription factors such as NF-κB and Sp1. To determine if CTx can exert its effect through NF-κB, HT-29 cells were pretreated with CTx for 24 h, washed and infected with NDK pseudotypes. Cells were then treated with the cytokine TNF-α, which is a potent inducer of NF-κB. Our results suggest that a CTx treatment does not affect the TNF-α-mediated induction of HIV-1 expression (Fig. 2B). Altogether, our results suggest that HIV-1 inhibition by CTx occurs prior to viral transcription.

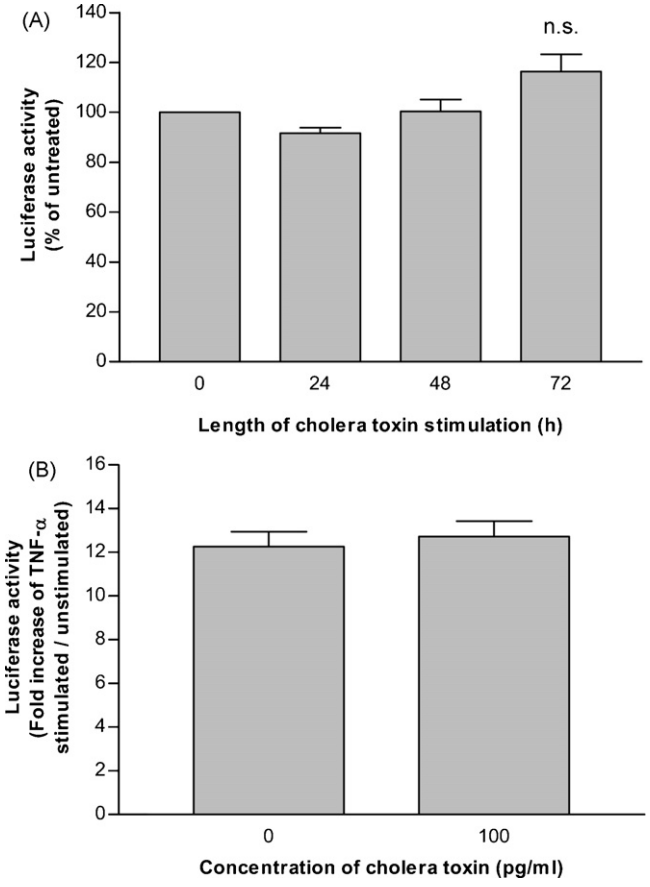

Fig. 2. CTx does not modulate HIV-1 transcription in HT-29 cells. (A) HT-29 cells were first cotransfected with pLTRX-Luc and pCEP4-Tat and next either left untreated or stimulated with CTx (100 pg/ml). Luciferase activity was measured 24, 48, or 72 h later. (B) HT-29 cells were either left untreated or treated with CTx (100 pg/ml) for 24 h and then washed extensively. Cells were next infected with NDK-Env pseudotypes (20 ng of p24) for 24 h, washed and treated or not with TNF-α (10 ng/ml). Luciferase activity was measured at 24 h post-infection. The results shown represent the means ± standard deviations calculated from three independent experiments with quadruplicate samples and are expressed as percentages of untreated cells.

3.3. CTx modulates an early step in the virus life cycle

Having eliminated the possibility that CTx is exerting an effect on viral transcription, we focused our attention to earlier events in the virus replication cycle. It has been shown that HIV-1 uses GalCer and CXCR4 as receptors before fusion at the surface of HT-29 cells. The use of pseudotyped viruses is helpful as it allows dissecting the contribution of virus attachment and fusion. In an attempt to determine if CTx is modulating virus entry, HT-29 cells were treated with CTx and infected with viruses pseudotyped with AMLV-Env and HTLV-I-Env because such pseudotypes employ respectively Pit2 and a yet to be identified receptor to infect target cells. In addition, cells were infected also with VSV-G pseudotypes, which can achieve infection through endocytosis. As illustrated in Fig. 3A and B, CTx inhibits infection of HT-29 cells with AMLV-Env and HTLV-I-Env pseudotyped viruses. Interestingly, the process of infection with VSV-G pseudotypes was unaffected (Fig. 3C), therefore suggesting that CTx influences attachment and/or fusion events.

However, it is known that HT-29 cells are 2–3 orders of magnitude more susceptible to infection by VSV-G pseudotypes compared to viruses pseudotyped with NDK Env. To determine whether the superior infectivity of VSV-G pseudotypes might mask the CTx-mediated suppressive effect, we conducted infection studies but using lower virus inputs (i.e. 10- and 100-fold less). Infection of HT-29 cells was still unaffected by CTx when using 100-fold less VSV-G pseudotyped viruses although the final output of luciferase activity was similar to the one obtained with AMLV, HTLV and NDK pseudotyped viruses (data not shown).

As stated above, GalCer and CXCR4 behave as attachment factors for HIV-1 on HT-29 cells. CTx is known to increase production of cAMP, and previous studies have shown that cAMP increases CXCR4 expression on peripheral blood lymphocytes. We thus examined by flow cytometry if surface expression of both GalCer and CXCR4 was possibly affected by CTx. Our results indicate that although CTx increases CXCR4 expression (Fig. 4A), it has no modulatory effect on GalCer (Fig. 4B). Therefore, it can be concluded that CTx is not down-regulating expression of cellular receptor and coreceptor.

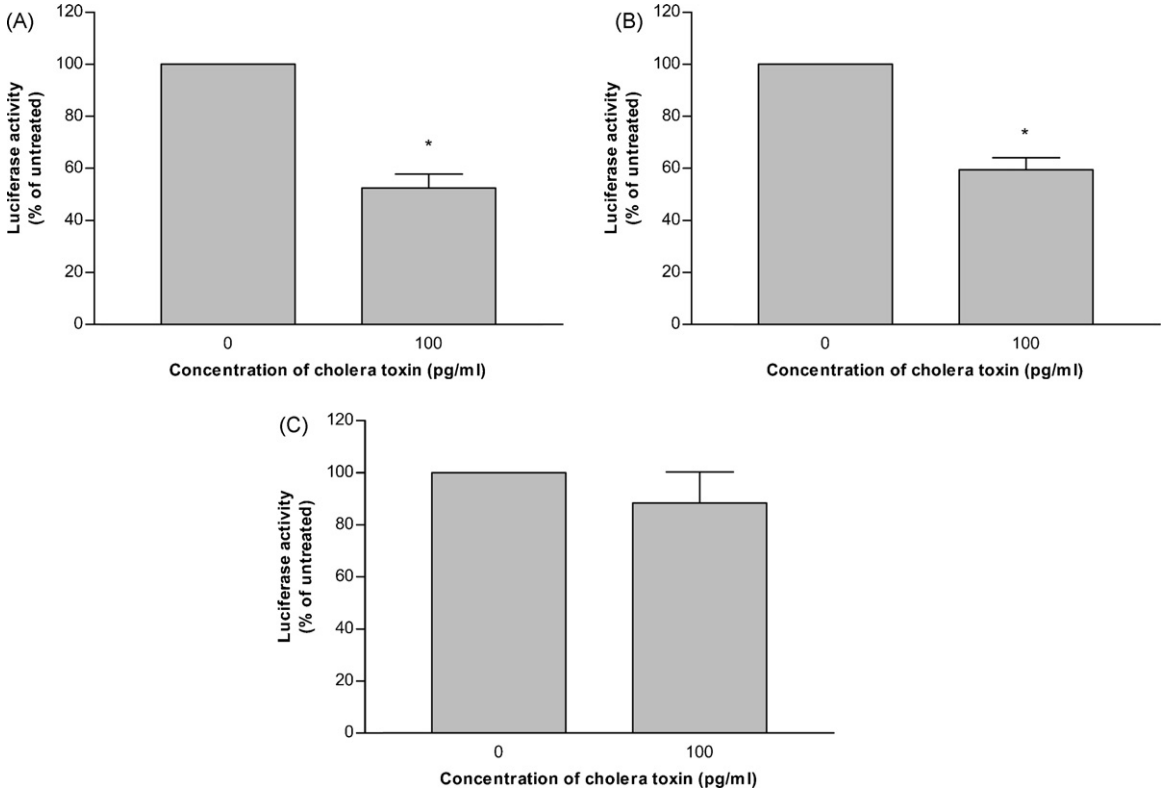

Fig. 3. CTx modulation of HIV-1 infection in HT-29 cells is Env dependent. HT-29 cells were either left untreated or treated with CTx (100 pg/ml) for 24 h and then washed. Next, cells were infected either with AMLV-Env (A), HTLV-I-Env (B), or VSV-G (C) pseudotypes (20 ng of p24) for 24 h and washed. Virus infection was estimated by measuring virus-encoded luciferase activity 24 h post-infection. The results shown represent the means ± standard deviations calculated from three independent experiments with quadruplicate samples and are expressed as the percentage of untreated cells (*, $P < 0.05$).

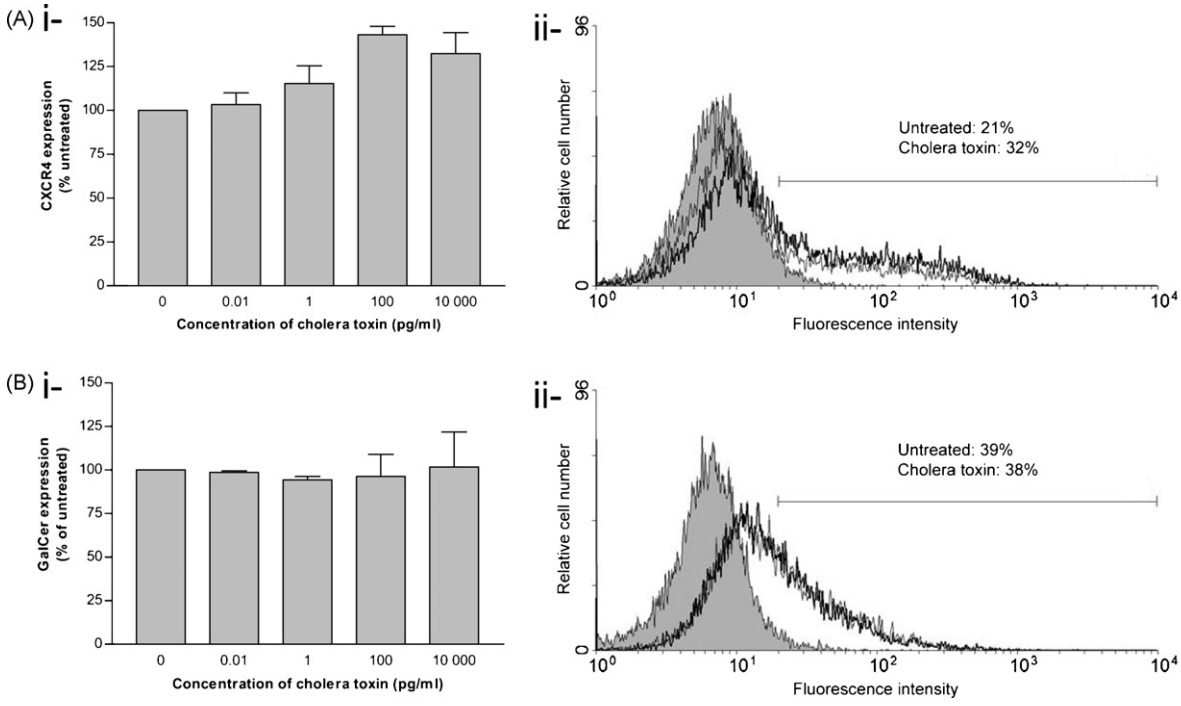

Fig. 4. CTx does not down-regulate HIV-1 receptor and coreceptor expression in HT-29 cells. HT-29 cells were either left untreated or treated with CTx (i.e. 0.01–10,000 pg/ml) for 24 h. Next, surface expression of CXCR4 (A) and GalCer (B) was analyzed by flow cytometry using specific antibodies. The results shown in the left panels represent the means ± standard deviations calculated from three independent experiments and are expressed as percentages of untreated cells (*, $P < 0.05$). The results shown in the right panels are histograms representative of three independent experiments. Isotype-matched controls antibodies (thin lines/shaded) are compared with CXCR4- or GalCer-specific antibodies from untreated (thin lines/unshaded) and cholera toxin-treated (100 pg/ml) (bold lines/unshaded) cells.

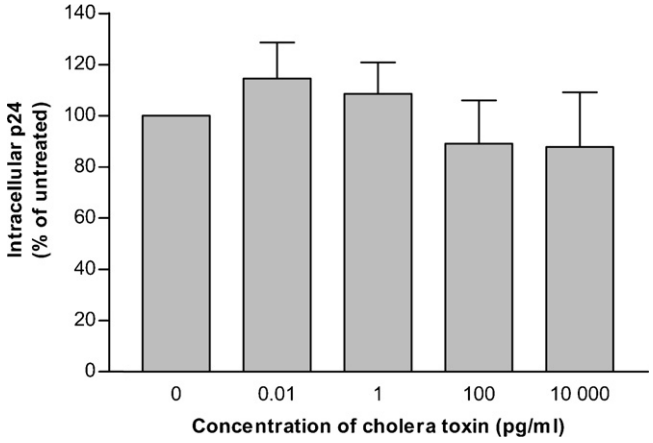

Fig. 5. CTx does not modulate HIV-1 entry in HT-29 cells. HT-29 cells were either left untreated or treated with CTx (i.e. 0.01–10,000 pg/ml) for 24 h. Cells were then incubated with NDK pseudotyped viruses for 4 h, detached with trypsin, washed and resuspended in lysis buffer before assessing the p24 content. The results shown represent the means ± standard deviations of three independent experiments and are expressed as the percentage of untreated cells.

A CTx-mediated increase in CXCR4 expression on HT-29 cells does not, however, exclude a negative modulation of the internalization process by the toxin. To determine if CTx can regulate virus entry, HT-29 cells were treated with CTx for 24 h followed by a 4 h incubation with NDK-Env pseudotyped viruses. After a trypsin treatment and three extensive washes to eliminate uninternalized virus, the intracellular p24 content was measured. Data illustrated in Fig. 5 demonstrate that entry of NDK-Env pseudotyped viruses was not reduced upon a treatment of HT-29 cells with CTx. However, this classical HIV-1 entry test does not allow to discriminate between viral material localized in the cytoplasm (thought to lead to productive infection) or vesicular machinery (thought to lead to virus degradation). Therefore, we next performed a fusion assay to specifically estimate viral delivery into the cytoplasm. Briefly, HT-29 cells were pretreated with CTx and next inoculated with AMLV-Env, HTLV-I-Env, NDK-Env, or VSV-G pseudotyped viruses containing chimeric protein made of Gag and Renilla luciferase (called $Pr55^{Gag}$/RLuc). The fusion was then analyzed by measuring luciferase activity as described recently (Gauthier and Tremblay, 2010). Unfortunately, fusion naturally occurs at a very low rate in HT-29 cells, and thus the assay was not sensitive enough to determine if CTx affects fusion of AMLV-Env, HTLV-I-Env, or NDK-Env pseudotyped viruses. However, our results demonstrate that CTx has no effect on fusion of VSV-G pseudotyped viruses (data not shown).

3.4. CTx-mediated suppressive effect on HIV-1 replication relies on adenylate cyclase activation

Although we were unable to define the precise mechanism of action of CTx and knowing that the molecule exerts its effects through activation of cAMP, we next attempted to identify the contribution of the two different subunits of CTx in the observed phenomenon. CTx is a multimeric protein composed of an active subunit A (CTA) surrounded by five subunits B (CTB) (Vanden Broeck et al., 2007) responsible for attachment to the cell membrane (Lavelle et al., 2004; Sanchez and Holmgren, 2008). CTA mediates most of the effects of CTx by activating adenylate cyclase, but some studies have reported that CTB can also induce signalling events and is partly responsible for some of the functional effects of the toxin (Buckley et al., 1995; Fantini et al., 2000; Massi et al., 1993; Uhal et al., 1998). As illustrated in Fig. 6A, CTB does not modulate HIV-1 infection of HT-29 cells suggesting a classical mechanism of action involving the enzymatic subunit CTA.

Considering that CTA needs to be linked in a complex with CTB in order to enter into the cell, purified CTA cannot be used to determine if adenylate cyclase activation is involved in the inhibition observed. To overcome this problem, forskolin, a cell-permeable diterpene activating adenylate cyclase, was used. Results depicted in Fig. 6B demonstrate that activation of adenylate cyclase via forskolin induces a similar level of inhibition of HIV-1 infection as CTx, thus indicating that activation of adenylate cyclase by CTA is responsible for the CTx-mediated reduction in HIV-1 infection. To discard the possibility that cell toxicity is responsible for the effect of forskolin, a cell proliferation assay was performed. No statistical difference was seen in cell proliferation of treated as compared to untreated cells (data not shown).

3.5. Actin organisation and functionality are not affected by CTx

Actin reorganisation is important for HIV-1 fusion with susceptible target cells (Eitzen, 2003) and adenylate cyclase activation by CTx has been shown to modify actin polymerisation (Hirshman et al., 2005). To determine if the observed reduction in HIV-1 infection results from actin reorganisation by CTx, HT-29 cells were either

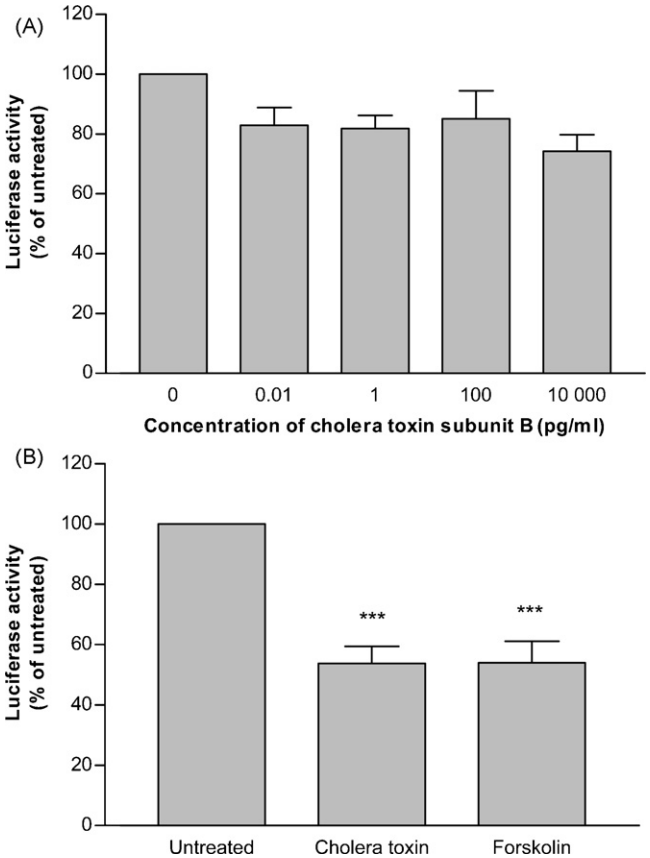

Fig. 6. CTx modulates HIV-1 infection through adenylate cyclase activation. (A) HT-29 cells were either left untreated or treated with CTB (0.01–10,000 pg/ml) for 24 h and then washed extensively. Cells were next infected with NDK-Env pseudotypes (20 ng of p24) for 24 h and washed again. (B) HT-29 cells were either left untreated or treated for 24 h with CTx (100 pg/ml) or forskolin (50 μM) and then washed extensively. Cells were next infected with NDK-Env pseudotypes (20 ng of p24) for 24 h. Virus infection was estimated by measuring virus-encoded luciferase activity 24 h post-infection in both cases. The results shown represent the means ± standard deviations calculated from three independent experiments with quadruplicate samples and are expressed as percentages of untreated cells (***, $P < 0.001$).

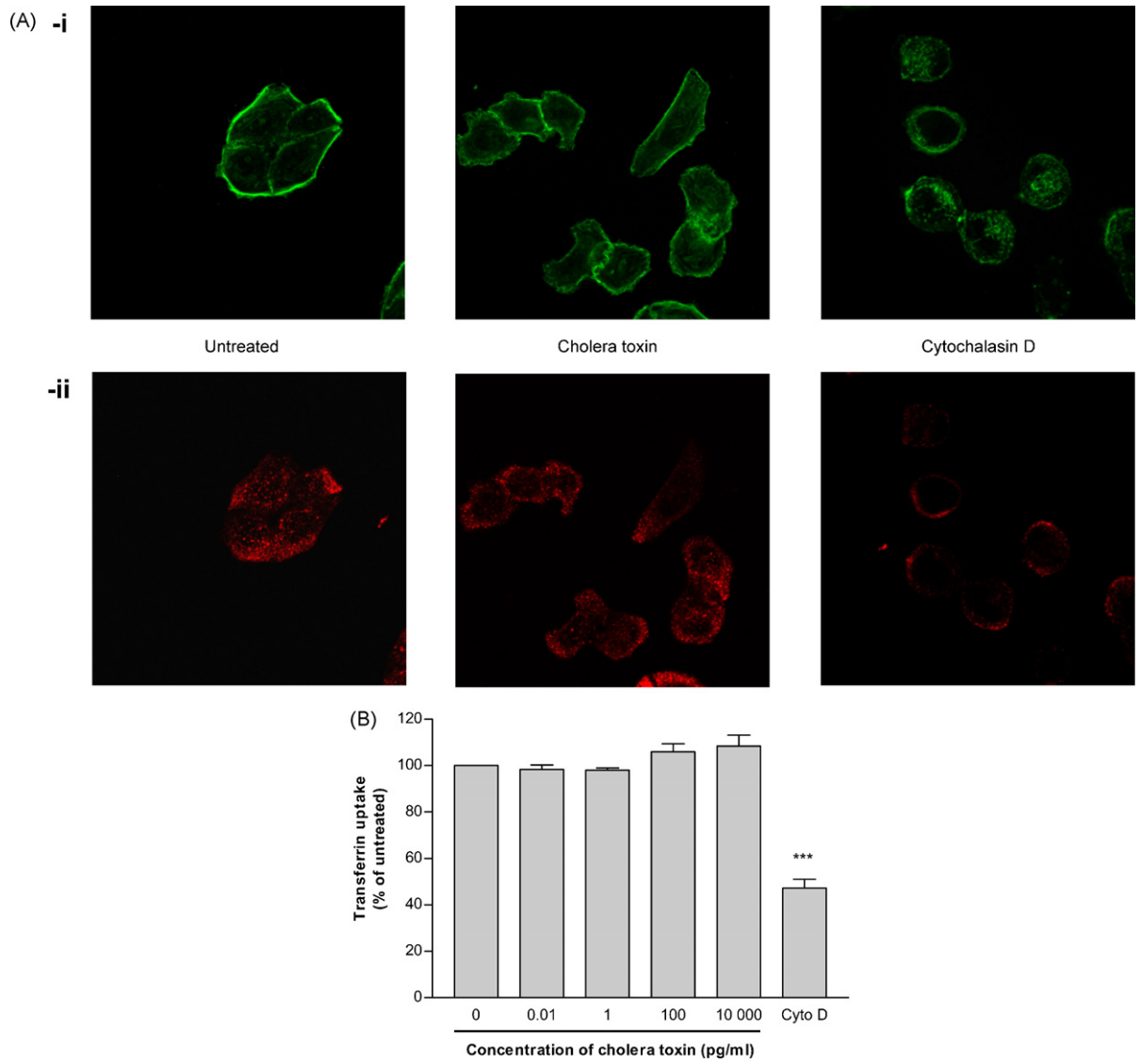

Fig. 7. CTx does not modulate actin organisation or functionality. (A) HT-29 cells were first either left untreated or treated with CTx (100 pg/ml) or cytochalasin D (1 μM) for 24 h and next incubated for 1 h with tetramethylrhodamine-conjugated transferrin (50 μg/ml). Cells were then fixed, stained for actin and analyzed by confocal microscopy. The results shown represent actin staining (i) and transferrin uptake (ii) and are representative of three independent experiments. (B) HT-29 cells were first either left untreated or treated with CTx (0.01–10,000 pg/ml) or cytochalasin D (5 μM) for 24 h, washed and next incubated for 1 h with Alexa 633-conjugated transferrin (50 μg/ml). Finally, transferrin uptake was analyzed by flow cytometry. The results shown represent the means ± standard deviations calculated from three independent experiments and are expressed as percentages of untreated cells (***, $P < 0.001$).

left untreated or treated for 24 h with CTx or cytochalasin D, a drug preventing actin assembly by capping actin filaments. The actin cytoskeleton was finally analyzed by confocal microscopy. Our observations suggest that CTx, in contrast to cytochalasin D, does not disrupt actin organisation (Fig. 7A).

Although cytoskeleton organisation does not seem to be affected by CTx, its functionality may be compromised. To define whether CTx can modify the functionality of cortical actin filaments, we investigated its effect on transferrin uptake. As actin plays a variable role in transferrin endocytosis depending on the cell type (Fujimoto et al., 2000), we first analyzed the effect of cytochalasin D on transferrin endocytosis in HT-29 cells. Data depicted in Fig. 7B show that transferrin entry is inhibited by 50% following cytochalasin D treatment, which confirms the validity of this combination of reagents to demonstrate actin functionality. However, our results show that both untreated and CTx-treated HT-29 cells display similar transferrin uptake, therefore suggesting that the functionality of cortical actin filaments is not affected by CTx.

4. Discussion

A few reports have been published on the effects of cAMP on HIV-1 infection, mostly concerning $CD4^+$ T lymphocytes. These studies reveal that cAMP increases CXCR4 expression resulting in an increased susceptibility to infection with X4-using viruses (Cole et al., 1999; Cristillo et al., 2002a). Since CTx activation of adenylate cyclase eventually results in an increased intracellular cAMP level (Vanden Broeck et al., 2007), we investigated if the presence of CTx modulates HIV-1 infection of intestinal epithelial cells. Our results unexpectedly showed that CTx inhibits HIV-1 replication in HT-29 cells, in a dose-dependent fashion. We first postulated that this reduction in HIV-1 infection could be explained by a decrease of virus gene expression. CTx is a potent activator of the cAMP/PKA pathway and although a majority of the studies revealed that cAMP increases HIV-1 transcription (Chowdhury et al., 1993; Kagnoff and Roebuck, 1999; Rabbi et al., 1997, 1998), it had been previously published that cAMP can inhibit transcription from the

HIV-1 regulatory elements (LTR) in a CD4-expressing T cell line. This was attributable to an increased phosphorylation of CREB, which then competes with NF-κB for the binding of two important co-activators connecting transcription factors to the transcriptional machinery (i.e. CBP and p300) (Banas et al., 2001). Our results, however, do not support this hypothesis since, in HT-29 cells, a CTx treatment fails to inhibit transactivation from the HIV-1 LTR and does not affect transactivation of viral DNA by NF-κB. Those data suggest that CTx is exerting its inhibitory effect at an early step in the virus replicative cycle.

The use of pseudotyped viruses is helpful to analyze early events in virus replication since attachment and fusion are the only steps that will differ from those of wild-type viruses. In the current study, we used three envelopes that use different receptors and entry pathways to pseudotype HIV-1, i.e. AMLV-Env, HTLV-I-Env and VSV-G. The first two are retrovirus envelopes of murine and human origin respectively. Fusion of these two pseudotypes normally occurs at the cell surface and is pH-independent (Daenke and Booth, 2000; McClure et al., 1990; Rodrigues and Heard, 1999). VSV-G is the envelope of the vesicular stomatitis virus (VSV), a member of the rhabdoviridae family (Superti et al., 1987). VSV enters into the cell by endocytosis and its fusion occurs in the endosomes through a pH-dependent process (Superti et al., 1987). A similar infection inhibition of all three pseudotyped viruses by CTx would have suggested a post-fusion step to be affected. However, our results show an inhibition of virus infection only with AMLV-ENV and HTLV-I-Env pseudotyped viruses, indicating that the binding and/or fusion step may be the viral step affected by CTx. Previous observations demonstrate that GalCer and CXCR4 are the receptor and coreceptor used by HIV-1 to infect HT-29 cells (Delezay et al., 1997). We thus examined whether a decrease in receptor and/or coreceptor expression could explain the inhibition observed following CTx treatment. Our results, however, show that CTx has no effect on GalCer expression and slightly increases CXCR4 expression on HT-29 cells. A positive modulation of CXCR4 expression was expected since adenylate cyclase activation by CTx ultimately leads to an increased intracellular cAMP level, which was shown to increase CXCR4 expression in T lymphocytes (Cole et al., 1999; Cristillo et al., 2002a; Goichberg et al., 2006). The presence of a cAMP-responsive element (CRE) site within the CXCR4 promoter allows the binding of the transcription factor cAMP-responsive element binding protein-1 (CREB-1), and is responsible for the cAMP-dependent up-regulation of CXCR4 expression.

An increased CXCR4 expression on HT-29 cells by CTx does not, however, exclude that the toxin can negatively modulate the entry and/or fusion process(es). For example, a study performed by Cristillo and colleagues has demonstrated a 60% decrease in HIV-1 fusion in CEM cells treated with dibutyryl cAMP without any modulation in CXCR4 expression at the protein level (Cristillo et al., 2002b). Interestingly, activation of PKA by 8-Br-cAMP has also been shown to inhibit envelope-induced cell-to-cell fusion of AMLV (Wang et al., 2005) without affecting the cellular level of PiT2, the AMLV cellular receptor. It was proposed that PKA phosphorylation of PiT2, or a cofactor associated with PiT2, may be responsible for the decrease of AMLV-induced cell-to-cell fusion. In contrast to those two studies where cells expressing the viral envelope were used, our study is using a cell-free virus system. Data from a virus entry test demonstrate that CTx is not affecting HIV-1 internalization in HT-29 cells. However, it is important to specify that this assay measures both the cytoplasmic (resulting from fusion at the cell membrane and leading to productive infection) and endosomal viral material (resulting from a vesicular uptake and leading to degradation by lysosomal enzymes). Unfortunately, viral fusion in HT-29 cells is a very inefficient process and the test used was not sensitive enough to determine if CTx affects viral fusion of NDK-Env pseudotyped viruses.

CTx is a multimeric protein composed of a CTA subunit surrounded by five CTB subunits (Vanden Broeck et al., 2007). CTB is responsible for attachment to the cell membrane, whereas CTA is responsible for the toxic effect (Lavelle et al., 2004; Sanchez and Holmgren, 2008). In the cytoplasm, CTA ADP-ribosylate Gsα, a protein regulating adenylate cyclase function (Vanden Broeck et al., 2007). This modification constitutively activates Gsα, which will continually activate adenylate cyclase and cause an increase in cAMP. Although CTA is responsible for the majority of CTx effects, it has been reported that some effects are independent of cAMP production (Buckley et al., 1995; Uhal et al., 1998). In our study, we show that the CTB subunit cannot induce the diminished virus gene expression seen with the whole toxin. Furthermore, treatment of HT-29 cells with forskolin, an adenylate cyclase activator, mimics the effect of CTx, thus supporting the hypothesis that CTA mediates its regulatory effect through cAMP production. The fact that a 16 h pre-treatment period with CTx is needed to reduce virus infection suggests that a late phase in the adenylate cyclase → cAMP → PKA signaling cascade is involved. cAMP is an important cellular second messenger (Serezani et al., 2008) regulating many biological processes (De Haan and Hirst, 2004). Interestingly, cAMP is able to modify the actin cytoskeleton through PKA (Howe, 2004) and activation of the Epac system (Bos et al., 2003; Hochbaum et al., 2003; Huang et al., 2003; Maillet et al., 2003; Rangarajan et al., 2003).

From an HIV-1 point of view, actin remodelling has been suggested to be important for HIV-1 receptors clustering (Iyengar et al., 1998; Jimenez-Baranda et al., 2007) and to allow fusion between viral and cellular membranes (Eitzen, 2003). Several recent papers have shown that gp120 binding to CD4 and/or CXCR4 activates proteins involved in actin remodelling such as RhoA (Jimenez-Baranda et al., 2007), cofilin (Yoder et al., 2008) and ezrin–radixin–moesin (ERM) (Barrero-Villar et al., 2009) which induce actin polymerisation or depolymerisation. In addition, it has been proposed that the viral Nef protein, following fusion, induces local actin remodelling to facilitate HIV-1 infection (Campbell et al., 2004). Interestingly, increased infection following actin remodelling seems to be specific to virus pseudotyped with a pH-independent envelope, such as AMLV (Howe, 2004) and HIV-1 (Barrero-Villar et al., 2009; Howe, 2004; Iyengar et al., 1998; Jimenez-Baranda et al., 2007; Yoder et al., 2008), while infection with pH-dependent pseudotyped virus, such as VSV-G, remains unaffected (Barrero-Villar et al., 2009; Campbell et al., 2004; Jimenez-Baranda et al., 2007). Considering that CTx is only inhibiting the process of infection with pH-independent pseudotyped viruses (i.e. AMLV, HTMV and HIV-1) and that the CTA subunit is responsible for the modulatory effect of CTx, it was reasonable to suspect that actin remodelling may alter viral fusion and affect virus replication. However, our results show that actin polymerisation is not affected by CTx. In addition, our observations indicate also that the functionality of the actin network does not seem to be modulated by CTx since transferrin endocytosis, which is affected by drugs impairing F-actin polymerisation, remains untouched following CTx treatment. Those results make unlikely an involvement of actin remodelling in the observed CTx-mediated effect on HIV-1 life cycle.

Although numerous pathogens have been shown to promote HIV-1 replication, our study shows that low concentrations of CTx limit virus production in HT-29 cells through adenylate cyclase activation. CTx is already used in clinical research as a vaccine adjuvant. Our results suggest that CTx may also be useful for controlling intestinal transmission of HIV-1. A better understanding of the mechanism by which CTx inhibits HIV-1 infection of intestinal epithelial cells may lead to new strategies for reducing virus propagation, notably to young children in developing countries.

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