HIV-1 Tat Inhibits Human Natural Killer Cell Function by Blocking L-Type Calcium Channels

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Herein we show that functional phenylalkylamine-sensitive L-type calcium channels are expressed by human NK cells and are involved in the killing of tumor targets. Blocking of these channels by phenylalkylamine drugs does not affect effector/target cell binding but inhibits the release of serine esterases responsible for cytotoxicity. Interestingly, treatment of NK cells with HIV-1 Tat, which is known to affect several calcium-mediated events in immune cells, impairs their cytotoxic activity. In addition, Tat inhibits the rise in intracellular free calcium concentration upon cross-linking of the adhesion molecule CD11a, engaged during effector/target cell interaction, and the activation molecule CD16. Exogenous Tat does not influence NK-target cell binding but prevents NK cell degranulation. We propose that the molecular structure(s) on NK cells mediating the inhibitory effects HIV-1 Tat belong to L-type calcium channels, based on three lines of evidence: 1) binding of phenylalkylamine derivatives to these channels is cross-inhibited by Tat; 2) L-type calcium channels from NK cell lysates bind to Tat linked to Sepharose columns; 3) the inhibitory effect of HIV-1 Tat on NK cell function is prevented by the agonist of L-type calcium channels, Bay K 8644. Altogether, these results suggest that exogenous Tat is deeply involved in the impairment of NK cell function during HIV-1 infection. The Journal of Immunology, 1998, 161: 2938–2943.

Impairment of NK cell function and loss of natural immunity in AIDS patients contribute to progressive immunosuppression during HIV-1 infection (1–4). However, the molecular bases of NK cell dysfunction are debated. Cytotoxicity mediated by NK cells is essentially of two types: FcγRIII (CD16)-initiated Ab-dependent cellular cytotoxicity (ADCC), i.e., killing of opsonized targets, and natural killing, i.e., the ability to lyse tumor or virus-infected targets in the absence of Ab (5–9). Both mechanisms are highly dependent on intracellular calcium rise. Indeed, one of the earliest detectable events following CD16 ligation is an increase in intracellular free calcium concentration ([Ca2+]i) (5, 7, 9). In addition, natural cytotoxicity is initiated by adhesion receptors that drive NK cells and their targets to close contact and trigger NK cell activation (5–9); these receptors include the αβ integrin (CD11a/CD18), which is known to elicit a [Ca2+]i elevation, mainly due to extracellular calcium influx (9–11). The rise in [Ca2+]i is needed for the granule release involved in the delivery of a lethal hit (7, 12).

The molecular structures mediating extracellular calcium influxes in NK cells are still poorly characterized. Recently, the presence of functional L-type calcium channels, whereby extracellular calcium can enter the cell, has been observed in B lymphocytes (13) and dendritic cells (14), raising the possibility that similar structures are present also in NK cells. L-type calcium channels are composed of three transmembrane subunits (αC, γ, and the α2δ complex) and one cytoplasmic chain (the β1 chain) (15, 16). These structures can be identified using compounds such as the phenylalkylamine (PAA) derivatives, which specifically bind with high affinity to the pore-forming αC chains, regulating their functional state from blocking to opening (15, 16).

Interestingly, we have recently observed that exogenous HIV-1 Tat inhibits some dendritic cell functions by competing with L-type calcium channels expressed by these cells (14). Several lines of evidence indicate that extracellular Tat plays important roles in the development of immunodeficiency. Indeed, the HIV-1 transactivating factor Tat can be released by infected cells and exert many biologic effects on bystander cells (17–20), including inhibition of several calcium-dependent immune cell functions (14, 21–25).

In this paper, we demonstrate that L-type calcium channels are expressed by NK cells and regulate the killing of tumor cells. Furthermore, we show that L-type calcium channels are the molecular target of HIV-1 Tat on NK cells: blocking of these channels by either Tat of PAA drugs results in lack of degranulation, which eventually leads to impairment of NK cell-mediated cytotoxicity.

Materials and Methods

Isolation and culture of CD3+ CD16+ NK cells

Highly purified CD3+ CD16+ NK cells were obtained from PBL after depletion of monocytes by plastic adherence and of CD3+, CD4+, and CD8+ cells upon incubation with anti-CD3 (Leu4a), anti-CD4 (Leu3a), and anti-CD8 (Leu2a) mAbs (Becton Dickinson, Mountain View, CA) followed by immunomagnetic beads (Unipath, Milan, Italy), as described (10). NK cells were stimulated with 10 μg/ml PHA (Sigma Chemicals, St. Louis, MO) and then cultured in 96-well U-bottom microplates (Greiner Labortechnik, Nurtingen, Germany) with RPMI 1640 medium supplemented with 10% FCS, 1 mM glutamine, 100 U/ml penicillin-streptomycin (all from Biochrom, Berlin, Germany) and 100 U/ml of rIL-2 (Cetus, Emeryville, CA) in a final volume of 200 μl/well.
**Indirect immunofluorescence and cytofluorometric analysis**

Immunofluorescence staining was performed as described (10). Briefly, aliquots of 10^5 cells were stained with the anti-CD11a 70H2a mAb (10) or the anti-CD16 KDI mAb (IgG1 (26)) followed by phycoerythrin (PE), or isotype-specific goat anti-mouse serum (GAM; Southern Biotechnology, Birmingham, AL). Control aliquots were stained with the fluorescent reagent alone. Samples were analyzed on a flow cytometer (FACSort, Becton Dickinson) equipped with an argon ion laser exciting FITC and PE at 488 nm, gated to exclude nonviable cells and debris. Data were analyzed using the Lysis II computer program (version 1.1, Becton Dickinson).

**Analysis of calcium fluxes by spectrofluorometry**

NK cells were loaded with the acetoxymethyl-ester of fura 2 (fura 2-AM, 1 μM Sigma) for 1 h at 37°C, placed in a quartz 2-nl cuvette and maintained at 37°C by a thermostatically controlled water bath. Fura 2-AM was excited at 334 and 380 nm; emitted light was filtered at 510 nm; and fluorescence was monitored with an LS-50B spectrofluorometer (Perkin-Elmer, Pomona, CA) (10). [Ca^{2+}]_i, was calculated according to Grynkiewicz et al. (27). [Ca^{2+}]_i increases were measured upon cross-linking of CD11a or CD16 molecules, obtained by incubating NK cells with the Fab'2 (5 µg/ml of the specific anti-CD11a 70H2a mAb (10) or anti-CD16 KDI mAb (20 min at 4°C) followed by adding 10 µg/ml of Fab)'2. GAM-Ig (H+L) (Zymed, San Francisco, CA) during the test at 37°C as described (10). In some experiments, NK cells were pretreated with the L-type Ca^{2+} channel antagonist verapamil (VPM; Calbiochem-Intalco, Milan, Italy; 10 or 1 µM) or chemically synthesized, >96% pure, HIV-1 Tat (Tecnogen, Piana di Monteverna, Italy, 10 or 100 µM) for 20 min (14), then washed before challenge with the various abs. Alternatively, [Ca^{2+}]_i increases were elicited with 10 µM (2) Bay K 8644 (Calbiochem; the net functional effect of the racemic mixture is that of the negative enhancer, which is an L-type Ca^{2+} channel agonist), as described (14, 15). When indicated, experiments were performed in the presence of 2 nM EGTA (Sigma).

**Calcium channel detection by fluorescence or Western blot**

NK cells (10^5/sample), untreated or preincubated with 10 µM VPM or 0.5 µM HIV-1 Tat or 1 µM recombinant fibronectin type III repeat (Fn-III, from amino acid 1086-1172, kindly provided by Dr. L. Zardi, IST-CBA, Genoa, Italy) as a control, were stained with 3 nM fluorescent DMBODIPY PAA or fluorescent ura-conotoxin GVIA (Molecular Probes Europe, Leiden, The Netherlands) and run on a FACSort (14, 28). Alternatively, NK cells, untreated or pretreated for 20 min with either 0.5 µM HIV-1 Tat or 10 µM VPM or 1 µM Fn-III, were stained with 100 nM biotinylated HIV-1 Tat (Tecnogen) (14) followed by PE-streptavidin and analyzed by FACSort. Western blot was performed as described (14). Lyases from NK or A431 cells (positive control, Transduction Laboratories, Lexington, KY) were separated by SDS-PAGE (12% gel) under reducing conditions and electrotransferred onto nitrocellulose filters (Hybond ECL; Amersham Italia, Milan, Italy). In other experiments, biotinylated Tat or biotinyl transferrin (Sigma) were cross-linked to avidin-Sepharose (Boehringer Mannheim Italia, Monza, Italy) and incubated with NK cell lysates at 4°C overnight. After elution, samples were run and transferred as above. Membranes were then incubated for 1 h with the anti-calcium channel p_b subunit mAb (clone 44, Transduction Laboratories) at 1:250 dilution, followed by horsedarized peroxidase-conjugated GAM Ig (Dako, Milan, Italy; 1:10,000 dilution). The immunoreactive bands were revealed by luminol reaction (ECL; Amersham).

**Cytolytic assay and serine esterase measurement.** Cytolytic activity of CD3 CD16+ or activated NK cells against the human erythroleukemia K562 cell line, the murine mastocytoma cell line P815, or the human lung adenocarcinoma cell line P71 was tested in a 4-h 51Cr release assay as described (29). NK cells were used as effectors at different E:T ratios, from 40:1 to 1:1, in a final volume of 200 µL of RPMI 1640. NK cells were also tested in a redirected killing assay using FC-yR+ P815 target cells, by adding saturating amounts (1 µg/10^6 cells) of the anti-CD16 mAb at the onset of the cytolytic assay. In some experiments, NK cells were pretreated for 20 min with 10 µM VPM, 100 nM Tat alone, or 100 nM Tat followed by 10 µM Bay K 8644 or 10 µM Bay K 8644 followed by 100 nM Tat. Drug concentrations were chosen on the basis of titration experiments (14). Results are expressed as percentage of cytotoxicity calculated as described (29). Measurement of serine-esterases in the supernatants or in cell lysates of NK cells, challenged as described above, was conducted according to Shiver et al. (30). Briefly, 100 µL of supernatants (SN) or cell lysates (CL, 0.5% Triton X-100) obtained from 10^5 NK cells, activated upon CD16 or CD11a cross-linking before or after treatment with 10 µM VPM or 100 nM Tat, were added to 50 µL of 1 mM dithionitrobenzoic acid (DTNB, Sigma) and 50 µL of 1 mM N-benzoylcarboxyl-l-lysine thiobenzylester (BLT; Calbiochem, La Jolla, CA), and the reaction was performed at room temperature for 30 min. ODs were measured at 414 nm, and results expressed as percentage of esterase release were calculated as follows: (OD_{414} SN/OD_{414} CL + OD_{414} SN) x 100.

**Binding assay**

Highly purified fresh or cultured NK cells were labeled with 3Cr (NEN, Boston, MA), washed, and added (10^5/well) to confluent monolayers of either the ICAM-1+ P71 tumor target cells or ICAM-1-transfected NIH/3T3 murine fibroblasts (31) and incubated at 37°C for 1 h. In some experiments, NK cells were preincubated for 20 min at 4°C with saturating amounts (5 µg/ml) of the anti-CD11a (70H2a) Fab'2, with 10 µM VPM, or with 100 nM Tat and washed twice before the onset of the adhesion assay. Nonadherent cells were removed by washing, and adherent cells were lysed with 1% Triton X-100 in 100 mM Tris buffer. The radioactivity...
of the samples was measured in a gamma counter (Packard, Sterling, VA). Results are expressed as percentage of adherent cells, calculated as previously described (31).

Results

**Human NK cells express functional L-type calcium channels**

Peripheral blood resting or rIL-2-cultured CD3−CD16−CD8+ cells were stained with DM-BODIPY R PAA, a fluorescent PAA derivative that binds to the α1C chain of L-type calcium channels (28), and FACS analysis was performed. As shown in Figure 1 (one representative experiment of six) freshly purified CD3−CD16−CD8+ NK cell populations (>96% CD16+) express surface structures recognized by this fluorescent compound (Fig. 1C). Conversely, the fluorescent α-conotoxin GVIA failed to stain both cultured (Fig. 1D) and resting (not shown) NK cells, thus ruling out the surface expression of N-type calcium channels. The presence of L-type calcium channels on NK cells was confirmed by the finding of a specific 58-kDa band corresponding to the β1 calcium channel subunit in NK cell lysates (Fig. 1E). Moreover, treatment of cultured NK cells with Bay K 8644, a drug known to bind the open state of L-type calcium channels and prolong their open time (15), elicits a sustained [Ca2+]i rise (Fig. 1F). Unlike in excitable tissues, these channels are voltage independent, since exposure of NK cells to 50 mM of KCl failed to induce a calcium influx (not shown).

To investigate the possible involvement of calcium channels in NK cell function, we studied the effect of the PAA derivative VPM, a blocker of L-type calcium channels (15, 16), on NK cell-mediated lytic activity. Figure 2 shows that VPM inhibits, in a dose dependent manner, the lysis of K562 cell line by fresh NK cells (Fig. 2A) and of the human lung adenocarcinoma cell line P71 (Fig. 2B) by cultured NK cells. A similar impairment of NK cell-mediated cytotoxicity by VPM was also observed using the murine mastocytoma P815 (Fig. 2C). Since CD16 represents an important pathway of NK cell activation, which can trigger the cytolytic activity (5), we asked whether VPM could also block CD16-induced cytotoxicity in a redirected killing assay with P815 target cells. Interestingly, VPM significantly decreases the CD16-mediated lysis of P815 targets by cultured (Fig. 2D) and resting (not shown) NK cells.

Adhesion of NK cells to tumor targets is largely mediated by the interaction between CD11a/CD18 molecules on effector cells and ICAM-1 ligands on target cells (9, 10, 32, 33). Engagement of CD11a/CD18 adhesion system induces an extracellular calcium influx in NK cells (10); likewise, ligation of CD16 elicits a [Ca2+]i rise in NK cells (5, 9, 10). We thus investigated the effects of L-type calcium channel blockers on CD11a/CD18- and CD16-induced calcium mobilization. As shown in Figure 3, exposure of cultured NK cells to VPM completely prevented the [Ca2+]i rise detected upon CD11a (Fig. 3A) and partially prevented calcium mobilization induced by CD16 cross-linking (Fig. 3B). When CD16 triggering was performed in the presence of 2 mM EGTA to chelate extracellular calcium, a slight and quick [Ca2+]i increase was observed, conceivably due to mobilization from internal stores (Fig. 3C). This calcium mobilization was not inhibited by VPM, supporting the conjecture that this drug blocks the entry of extracellular calcium (Fig. 3C).

**HIV-1 Tat inhibits NK cell-mediated cytotoxicity and prevents the opening of L-type calcium channels**

As HIV-1 Tat can inhibit several calcium-dependent immune cell functions (21–25), we addressed the question of whether Tat can block NK cell-mediated cytotoxicity. Exogenous Tat proved to
inhibit both the lysis of the K562 cell line by fresh NK cells (from 44 ± 12% to 15 ± 4% of specific lysis) and the killing of P815 targets by cultured NK cells (Fig. 4A); a similar inhibitory effect was observed on CD16-triggered cytotoxicity (Fig. 4B). In both cases, inhibition was prevented by exposing NK cells to the calcium channel agonist Bay K 8644 before adding Tat (Fig. 4, A and B). However, Bay K 8644, when added after the binding of this protein to NK cells, was not able to revert the effect of Tat (Fig. 4 A and B).

Along this line, the calcium influx elicited in cultured NK cells by cross-linking CD11a (Fig. 5A) or CD16 (Fig. 5B) molecules is strongly decreased by pretreatment of NK cells with HIV-1 Tat. In contrast, Tat does not affect the calcium channel-independent calcium rise that follows NK cell exposure to the ionophore ionomycin (not shown). Interestingly, Tat was not able to inhibit the calcium rise elicited upon CD16 cross-linking in the presence of 2 mM EGTA (Fig. 5C), supporting the concept that Tat, similarly to VPM (Fig. 3B), blocks the entry of extracellular calcium. Moreover, Tat did not compete with the anti-CD11a or anti-CD16 mAbs for binding to NK cells (Fig. 6), thus ruling out the possibility that the inhibition of calcium mobilization elicited via CD11a or CD16 was due to the inaccessibility of these molecules.

Altogether, these results suggest that HIV-1 Tat interacts with L-type calcium channels on NK cells. Support for this hypothesis comes from the observation that the binding of biotinylated Tat to NK cells is cross-inhibited by pretreatment of these cells with VPM (Fig. 7A); in turn, Tat antagonizes the binding of DM-BODIPY® PAA to NK cells (Fig. 7B), whereas the unrelated peptide Fn-III, displaying the same length of HIV-1 Tat, has no effect (not shown). Furthermore, we found that the β1 subunit of calcium channels is eluted, after chromatography of NK cell lysates, from an avidin column immobilizing biotinylated Tat (Fig. 7C). These data indicate that the inhibitory effect of HIV-1 Tat on NK cell function is mediated by its interaction with surface structures related to L-type calcium channels.

**HIV-1 Tat inhibits the secretion of serine esterases by NK cells**

In NK cell-mediated cytotoxicity, three steps can be identified: binding to target cells, degranulation, and killing (6, 7, 9). In our experiments, adhesion of NK cells to the ICAM-1 + P71 tumor cell line or to ICAM-1 transfected cells was unaffected by HIV-1 Tat (Fig. 8A), indicating that this protein does not interfere with the first step of the cytotoxic process. Similarly, VPM does not impair NK-target cell binding, ruling out the involvement of L-type calcium channel in this step. To understand whether secretion of cytolytic enzymes is affected by HIV-1 Tat, we evaluated the percent of release of serine esterases by cultured NK cells pretreated with 1 μM fura 2-AM, and fluorescence was monitored the same way as described in the legend to Figure 1. [Ca^{2+}], increases were measured upon cross-linking of CD11a (A) or CD16 (B) molecules, in the absence (closed squares) or presence (triangles) of 100 nM HIV-1 Tat. Open squares, baseline (GAM alone). C, Calcium mobilization evaluated in the presence of 2 mM EGTA. Results are expressed as [Ca^{2+}], nM and are representative of six independent experiments.

**FIGURE 4.** HIV-1 Tat blocks NK cytolytic activity. A, Cytolytic activity of rIL-2-cultured NK cell populations against P815 targets was tested in a 4-h 51Cr release assay. E:T ratios, from 10:1 to 2.5:1. B, Redirected killing assay using NK cells stimulated with the anti-CD16 mAb (1 μg/106 cells) and P815 targets. NK cells were untreated (open squares) or pre-treated for 20 min with 100 nM Tat alone (closed squares); with 100 nM Tat before the addition of 10 μM Bay K 8644 (open circles); with 10 μM Bay K 8644 before the addition of 100 nM Tat (closed triangles); or with 10 μM Bay K 8644 alone (open triangles). Results are expressed as percentage of cytotoxicity and are the mean ± SD from six independent experiments.

**FIGURE 5.** HIV-1 Tat blocks extracellular calcium entry in NK cells. NK cells were loaded with 1 μM fura 2-AM, and fluorescence was monitored as described in the legend to Figure 1. [Ca^{2+}], increases were measured upon cross-linking of CD11a (A) or CD16 (B) molecules, in the absence (closed squares) or presence (triangles) of 100 nM HIV-1 Tat. Open squares, baseline (GAM alone). C, Calcium mobilization evaluated in the presence of 2 mM EGTA. Results are expressed as [Ca^{2+}], nM and are representative of six independent experiments.
Ca2+CD16 cross-linking and cytolytic activity is inhibited by L-type voltage sensitive (34), PAA-sensitive Ca2+ channels in NK cells. The presence of functional L-type Ca2+ channels in NK cells is supported by three lines of evidence: 1) Ca2+ entry upon CD11a or CD16 ligation and Ca2+-dependent NK cell cytotoxicity. The effect of Tat on NK-mediated killing is not due to a defective binding between effector and target cells, in agreement with the fact that adhesion is dependent on the presence of calcium ions in the extracellular milieu, rather than on [Ca2+]i rise (9, 32). Conversely, [Ca2+]i increases are essential for NK cell degranulation and secretion of the lytic enzymes that contribute to target cell damage (7, 12); it is noteworthy that Tat inhibits the release of serine esterases induced in NK cells by CD16 or CD11a ligation. Thus, Tat interferes with the earliest signaling events following effector/target interaction and impairs NK cell-mediated cytotoxicity by blocking the delivery of a lethal hit.

We show that rises in [Ca2+]i, levels occurring upon CD11a or CD16 engagement in NK cells, mainly due to extracellular calcium entry (5, 10, 11), are mediated by L-type calcium channels. The presence of functional L-type Ca2+ channels in NK cells is supported by three lines of evidences: 1) Ca2+ entry upon CD11a or CD16 cross-linking and cytolytic activity is inhibited by L-type Ca2+ channel blockers; 2) fluorescent PAA drugs, specific for the α1C chain of L-type channels, bind to NK cell surface; and 3) the calcium channel β1 chain is detectable in NK cell lysates. At variance with L-type calcium channels in excitable tissues, which are voltage sensitive (34), PAA-sensitive Ca2+ channels in NK cells are voltage independent, as described in B lymphocytes and dendritic cells (13, 14). This points to a common mechanism responsible for Ca2+ entry in immune cells, based on specialized transmembrane structures.

L-type calcium channels appear to be the molecular target of HIV-1 Tat on NK cells: indeed, the inhibitory effect of Tat on NK cell activity is prevented by the activator Bay K 8644, which selectively binds to L-type calcium channels, prolonging their open time (15). Along this line, the β1 calcium channel subunit is eluted by avidin-Sepharose columns linked to biotinylated Tat, and the binding of PAA derivatives to these channels is cross-inhibited by Tat. In turn, PAA drugs are able to strongly decrease the binding of biotinylated Tat to NK cells, indicating that PAA-sensitive calcium channels represent one major ligand for Tat on NK cells. The finding that Bay K 8644 is unable to restore cytotoxicity when NK cells are pretreated with Tat is in agreement with the observations that calcium channel antagonists can be displaced from purified receptors only by very high (1000-fold) doses of agonists (15, 16); we could not use these concentrations (>10 μM), as they lead to NK cell dysfunction in our system (not shown).

The physiologic relevance of our findings is supported by two previous observations: 1) nanomolar concentrations of HIV-1 Tat are detectable in the sera of AIDS patients (24), and 2) a serum factor contributes to the suppression of NK cell function (35). It is tempting to speculate that this factor is HIV-1 Tat: the local amount of Tat in the mucosal and lymphoid tissues should be conceivably higher, due to the concentrating effect of heparan sulfates (36), thus contributing to decreased NK cell function at the site of infection.

We cannot rule out that other mechanisms, operating synergistically, may contribute to NK cell deficiency in AIDS (3). Nevertheless, our data provide evidence for a molecular mechanism, based on the blocking of L-type calcium channels, possibly leading to NK cell impairment during HIV-1 infection. As L-type calcium channels are expressed by other immune cells (13, 14), they could represent a common target for HIV-1 Tat. Interaction with Tat...
would impair several immune functions that are dependent on extracellular calcium entry, thus contributing to a generalized immune dysfunction.

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