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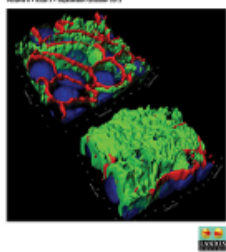
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# HIV-1 Tat protein perturbs diacylglycerol production at the plasma membrane of neurosecretory cells during exocytosis

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**Keywords:** HIV-1 Tat, neurosecretion, PI(4,5)P<sub>2</sub>

Human immunodeficiency virus (HIV)-infected cells actively release the transcriptional activator (Tat) viral protein that is required for efficient HIV gene transcription. We recently reported that extracellular Tat is able to enter uninfected neurosecretory cells. Internalized Tat escapes endosomes to reach the cytosol and is then recruited to the plasma membrane by phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P<sub>2</sub>). Tat strongly impairs exocytosis from chromaffin and PC12 cells and perturbs synaptic vesicle exo-endocytosis cycle through its ability to interact with PtdIns(4,5)P<sub>2</sub>. Among PtdIns(4,5)P<sub>2</sub>-dependent processes required for neurosecretion, we found that Tat impairs annexin A2 recruitment involved in the organization of exocytotic sites at the plasma membrane. Moreover Tat perturbs the actin cytoskeleton reorganization necessary for the movement of secretory vesicles toward their plasma membrane fusion sites during the exocytotic process.

Here, we investigated whether extracellular Tat affects PtdIns(4,5)P<sub>2</sub> metabolism in PC12 cells. Using a diacylglycerol (DAG) sensor, we found that ATP stimulation of exocytosis triggers the production of DAG at the plasma membrane as seen by the relocation of the DAG probe from the cytosol to the plasma membrane. Exposure to Tat strongly delayed the recruitment of the DAG sensor, suggesting a reduced level of DAG production at the early phase of ATP stimulation. These observations indicate that Tat reduces the hydrolysis rate of PtdIns(4,5)P<sub>2</sub> by phospholipase C during exocytosis. Thus, the neuronal disorders often associated with HIV-1 infection may be linked to the capacity of Tat to interact with PtdIns(4,5)P<sub>2</sub> and alter both its metabolism and functions in neurosecretion.

The human immunodeficiency virus type I (HIV-1) encodes three structural (*gag*, *pol* and *env*), four accessory (*vif*, *vpr*, *vpu* and *nef*) and two regulatory (*tat* and *rev*) genes, the products of which are responsible for establishing sophisticated interactions between the virus and human host. HIV-1 transcriptional activator (Tat) is a small (~11 kDa) basic protein that binds to the trans-activation-response element (TAR) on the nascent viral RNA and recruits molecular components for efficient transcriptional elongation.<sup>1</sup> Thus, Tat displays an array of functions that are essential for viral gene expression and replication, but also affects host gene expression.<sup>2</sup> Tat is an early regulatory protein that has a variable length of 86–103 aa (depending on viral isolates) and is encoded by two exons. The first exon encodes the first 72 amino acids and the second exon encodes for the remaining residues. Tat is actively released by infected T-cells into the extracellular medium by an unconventional mechanism<sup>3,4</sup> and the concentration of Tat in the serum of HIV-1 infected patients reaches nanomolar levels.<sup>5</sup> Tat can cross the blood brain barrier<sup>6,7</sup> and be produced in situ by HIV-1 infected cells such as perivascular macrophages, astrocytes and microglia. Tat is thus present within

the central nervous system and could potentially be involved in HIV-1 neuropathogenesis. HIV-1-associated neuronal symptoms include motor or cognitive dysfunctions and behavioral changes affecting nearly one third of patients undergoing intensive tri-therapy. Moreover, one patient out of ten develops HIV-1 associated dementia at the late stage of AIDS.<sup>8</sup> Although some signs of neuronal death have been reported in these patients, they most likely do not explain the extent of cognitive alterations, suggesting alternative unknown mechanism altering neuronal functions.

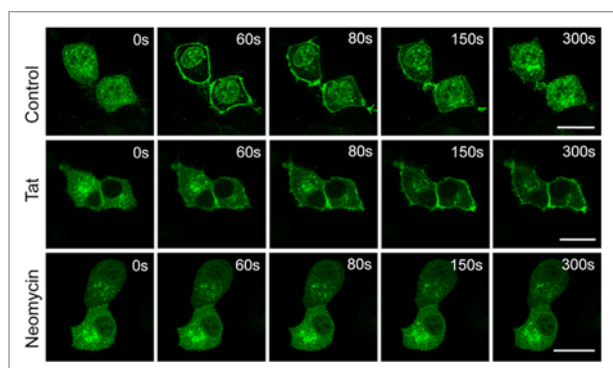
Extracellular Tat has been shown to penetrate several cell types through different pathways<sup>9</sup> and the capacity of Tat to reach the cytosol of uninfected cells likely enables circulating Tat to interfere with key cellular processes in these cells. Moreover, the recent breakthrough identifying the membrane inner leaflet phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P<sub>2</sub>] as a high affinity ligand for Tat,<sup>10</sup> suggests that key cellular activities relying on PtdIns(4,5)P<sub>2</sub> such as exocytosis might be affected in neurosecreting cells.

In our efforts to unravel the potential effect of Tat on neurosecretion, we first tested the ability of extracellular Tat to enter

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**Figure 1.** HIV-1 Tat perturbs the recruitment to the plasma membrane of a DAG biosensor in PC12 cells stimulated for exocytosis. Cells expressing a fluorescent DAG probe were incubated 2 h with neomycin (4 mM) or treated with Tat (50 nM, 30 min 0°C, then chased for 4 h) at 37°C before exocytosis stimulation using 300  $\mu$ M ATP. Pictures extracted from a representative video of each condition show the distribution of the pKDC1ab-GFP in control (i.e., vehicle), Tat or neomycin treated cells at the indicated time points after cell stimulation. Bars: 10  $\mu$ m.

conventional neurosecretory cell models i.e., chromaffin and PC12 cells.<sup>11</sup> Consistent with the Tat uptake pathway observed in other cell types,<sup>12,13</sup> our results indicate that internalized Tat is first found in early endosomal structures, as revealed by co-localization with EEA1 and then in late endo-lysosomal Lamp-1 positive structures.<sup>14</sup> After 4 h, Tat-containing vesicular structures were barely detectable and, concomitantly, Tat signal became visible on the inner leaflet of the plasma membrane.<sup>14</sup> In addition, when overexpressed in PC12 cells, Tat localized essentially at the plasma membrane.<sup>14</sup> The binding of Tat to  $\text{PtdIns}(4,5)\text{P}_2$  is largely responsible for this recruitment of Tat to the plasma membrane because both neomycin, a  $\text{PtdIns}(4,5)\text{P}_2$  masking drug, and  $\text{PtdIns}(4,5)\text{P}_2$  5-phosphatases, which reduce cellular  $\text{PtdIns}(4,5)\text{P}_2$  levels when overexpressed, efficiently displaced Tat from the plasma membrane to the cytosol.<sup>14</sup>

Most strikingly, once internalized by chromaffin and PC12 cells, Tat strongly inhibited the number of exocytotic events as revealed by a reduction in amperometric spike frequency and growth hormone release, respectively.<sup>14</sup> Inhibition of secretion was observed at nanomolar concentrations of Tat, comparable with the concentrations reported for circulating Tat,<sup>5</sup> thereby arguing for the physiological significance of our findings. Expression of Tat mutants unable to bind  $\text{PtdIns}(4,5)\text{P}_2$  did not inhibit secretion, demonstrating that the effect of Tat on secretion is directly linked to its interaction with  $\text{PtdIns}(4,5)\text{P}_2$  at the plasma membrane and does not result from a transcriptional effect of Tat.<sup>14</sup> Overexpression of phosphatidylinositol 4 phosphate 5-kinase was able to partially rescue secretion from cells exposed to Tat, in line with the idea that Tat blocks secretion through its capacity to specifically bind  $\text{PtdIns}(4,5)\text{P}_2$ . Interaction of Tat with  $\text{PtdIns}(4,5)\text{P}_2$  occurs with such a high affinity that Tat sequesters this phosphoinositide and thereby strongly impairs the translocation of the  $\text{PtdIns}(4,5)\text{P}_2$ -binding protein annexin A2 to the plasma membrane following cell stimulation. Annexin

A2 plays an essential role in calcium-regulated exocytosis by promoting the formation of exocytotic platforms in neuroendocrine cells.<sup>15,16</sup> Inhibition of annexin A2 translocation in cells exposed to Tat led to a reduction in the number of functional exocytotic sites and to a concomitant decrease in the number of exocytotic events.<sup>14</sup> Furthermore, we found that Tat hampers the actin cytoskeleton reorganization during neurosecretion, an effect that may also be a direct consequence of Tat binding to  $\text{PtdIns}(4,5)\text{P}_2$ . Indeed, available  $\text{PtdIns}(4,5)\text{P}_2$  can be a limiting factor for exocytosis efficacy since it orchestrates the actin-mediated movements of secretory granules to exocytotic sites.<sup>17</sup> In chromaffin cells, the cortical F-actin network constitutes a negative clamp preventing the movement of secretory vesicles to release sites, and it must be locally disassembled and reorganized in a  $\text{PtdIns}(4,5)\text{P}_2$ -dependent manner to allow recruitment of secretory vesicles in preparation for exocytosis.<sup>18</sup> Thus, the inhibitory effect of Tat on subplasmalemmal actin may also explain its capacity to impair the exocytotic response.

In our attempt to decipher the mechanism(s) by which Tat alters  $\text{PtdIns}(4,5)\text{P}_2$ -dependent membrane trafficking processes in neurosecretory cells, we explored the ability of Tat to directly affect  $\text{PtdIns}(4,5)\text{P}_2$  metabolism. Indeed, the quantity of available  $\text{PtdIns}(4,5)\text{P}_2$  can influence the efficacy of exocytotic process.  $\text{PtdIns}(4,5)\text{P}_2$  synthesis and hydrolysis is regulated by a complex set of inositol kinases, lipases and phosphatases, including  $\text{PtdIns}(4,5)\text{P}_2$ -specific phospholipase C (PLC).  $\text{PtdIns}(4,5)\text{P}_2$  hydrolysis by PLC generates two second messengers, namely diacylglycerol (DAG) and inositol 1,4,5-trisphosphate, which both play a major role in neuronal plasticity and exocytosis.<sup>19</sup>

To examine whether Tat interferes with the production of second messengers through hydrolysis of  $\text{PtdIns}(4,5)\text{P}_2$  by PLC during exocytosis, we monitored DAG production in PC12 cells in situ upon neurosecretion stimulation. To this end, cells were transfected with a high affinity DAG sensor using the tandem C1 domains (C1ab) of protein kinase D tagged with GFP at the C terminus (pKDC1ab-GFP).<sup>20</sup> This probe enables to track the in situ production of DAG from  $\text{PtdIns}(4,5)\text{P}_2$  upon ATP stimulation, used here as a secretagogue to trigger exocytosis.

In resting PC12 cells, we found that pKDC1ab-GFP mostly localized in the cytoplasm and in perinuclear Golgi-like structures, but also weakly in the nucleus of some cells. Indeed, pKDC1ab-GFP possesses a nuclear export signal at the N terminus of the probe that may influence its nuclear accumulation.<sup>20</sup> Cell stimulation induced a dramatic and transient recruitment of pKDC1ab-GFP to the cell periphery (Fig. 1, upper panel), revealing the rapid and robust production of DAG at the plasma membrane of stimulated PC12 cells. Image quantification indicated that recruitment of the DAG probe to the plasma membrane peaked between 20 sec and 40 sec after stimulation and thereafter decreased to reach basal level 3 min after stimulation (Fig. 2). In cells exposed to exogenous Tat, the translocation of the DAG sensor to the plasma membrane in response to secretagogue stimulation was significantly delayed reaching a plateau only 200 sec after stimulation (Fig. 1, middle panel and Fig. 2). Interestingly, the highest fluorescence intensity reached at the plasma membrane in Tat-treated cells was similar to that

in control cells. Thus, the total level of DAG production was similar in Tat-treated and control cells, but with strikingly different kinetics. DAG generation was rapid and transient in control cells whereas it increased slowly and progressively in cells exposed to Tat (Fig. 2). In Tat-treated cells the DAG probe remained recruited at the plasma membrane for up to 8 min and subsequently slowly translocated back to the cytoplasm (not shown).

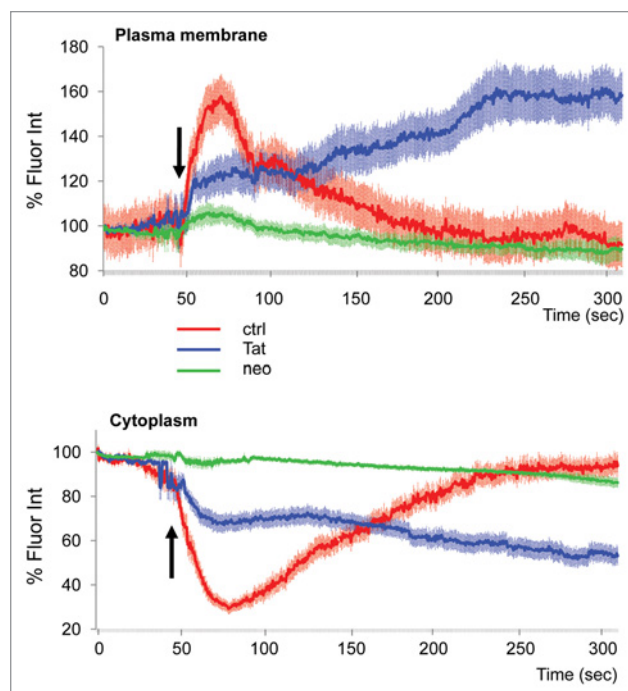
To test the idea that Tat may affect PLC activity by reducing substrate [PtdIns(4,5) $P_2$ ] availability, we tested the effect of neomycin, a non-specific PLC inhibitor known to prevent DAG production by sequestering PtdIns(4,5) $P_2$ . As illustrated in Figure 1 (lower panel), neomycin treatment (4 mM for 2 h) completely blocked the recruitment of the DAG probe to the plasma membrane in response to exocytosis stimulation. This result suggests that neomycin and Tat affect DAG production through distinct mechanisms in ATP stimulated PC12 cells that undergo exocytosis. Hence, Tat may be progressively displaced from PtdIns(4,5) $P_2$  allowing its slow hydrolysis by PLC or Tat might be able to directly affect the enzymatic activity of PLC. The latest possibility is however unlikely because we recently reported that Tat has no impact on the fast and transient calcium increase in PC12 cells undergoing exocytosis,<sup>14</sup> which reflects normal PLC activity in these cells under these conditions. An alternative possibility is that in the presence of Tat, the cell differently handles DAG. It is noteworthy to mention that DAG can be transformed into phosphatidic acid (PA) by DAG-kinase. On the other hand PA itself, which is produced at exocytotic sites in neurosecreting cells,<sup>21</sup> can also be converted into DAG by PA phosphohydrolase. This PA phosphohydrolase activity, as an alternative pathway in these cells, might thus be responsible for some of the lately produced DAG explaining the delay in the pKDC1ab-GFP probe recruitment to the plasma membrane. It is likely that precise lipidomic analyzes will be required to fully understand the profound effect of Tat on lipid turnover in neurosecretory cells.

In conclusion, we found that Tat dramatically inhibited neurosecretion by a mechanism that most likely reflects a general alteration of the availability and/or the metabolism of PtdIns(4,5) $P_2$ . These effects of Tat might contribute to the neuronal disorders associated with HIV-1 infection.

## Materials and Methods

**Cell culture, transfection and imaging.** PC12 cells were cultured as described previously<sup>22</sup> and transfected with pKDC1ab-GFP expression vector (0.8 to 1  $\mu$ g total DNA,  $15 \times 10^4$  cells, 24-well plate) using Lipofectamine 2000 as previously reported.<sup>23</sup> Under these conditions, transfection efficiency was around 50%.

Twenty-four h after transfection, PC12 cells were treated 2 h with 4 mM neomycin or incubated for 30 min on ice with 50 nM Tat or citrate buffer (vehicle, 100 mM NaCl, 50 mM Na-citrate, pH 7) and chased for 4 h at 37°C as described.<sup>14</sup> Cells were then washed three times with Locke's buffer (140 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, 11 mM glucose and 15 mM HEPES, pH 7.4) and stimulated with 300  $\mu$ M ATP in Locke's buffer<sup>24</sup> to trigger exocytosis. GFP-tagged



**Figure 2.** Tat modifies the DAG biosensor translocation kinetics to the plasma membrane in PC12 cells stimulated for exocytosis. Intensities of fluorescence of the DAG probe were quantified in each video frame. The plots show the time-dependent increase of fluorescence at the plasma membrane and the concomitant decrease of signal in the cytoplasm in control (ctrl), Tat-treated (Tat) and neomycin-treated (neo) cells. Arrow indicates exocytosis stimulation (at 45 sec of the recordings). Mean values  $\pm$  s.e.m. obtained from 8 (Tat-treated or control) or 5 cells (neomycin-treated) are shown.

DAG biosensor (pKDC1ab-GFP) recruitment to the plasma membrane of PC12 cells was recorded using a SP5II Leica confocal microscope in xyt mode using a 63X oil-immersion objective and a heated stage (37°C). Recordings were started 45 sec before cell stimulation, and a total of 601 frames were taken at every 500 ms during 5 min.

**Data and image analysis.** The recruitment of GFP tagged DAG biosensor was analyzed using the SP5II Leica quantification software (LAS AF Lite). Three regions of interest (ROI) were determined to delimitate cell periphery including plasma membrane, cytoplasm and nucleus of each cell. Average fluorescence intensities of different ROIs were analyzed through the 601 frames of recorded videos for each condition. Time dependent variation of fluorescence intensities at plasma membrane level and cytoplasm is expressed as mean  $\pm$  SEM. Values are normalized to starting point fluorescence intensities that are fixed as 100%. Data were generated from 8 control, 8 Tat-treated and 5 neomycin-treated cells respectively issued from two independent cell cultures. Statistical significance was estimated using Student's t-test and data were considered significantly different when  $p < 0.01$ . Gaussian distribution of the data was verified.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.



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