The Human Immunodeficiency Virus-1 Protein Transactivator of Transcription Up-Regulates N-Methyl-D-aspartate Receptor Function by Acting at Metabotropic Glutamate Receptor 1 Receptors Coexisting on Human and Rat Brain Noradrenergic Neurones

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ABSTRACT

We investigated the effects of the human immunodeficiency virus-1 transactivator of transcription (Tat) on the release of norepinephrine (NE) from human and rat brain synaptosomes. Tat could not evoke directly release of [3H]NE. In the presence of Tat (1 nM), N-methyl-D-aspartate (NMDA) concentrations unable to release (human synaptosomes) or slightly releasing (rat synaptosomes) [3H]NE became very effective. The NMDA/Tat-evoked release depends on NMDA receptors (NMDARs) since it was abolished by MK-801 (dizocilpine). Tat binding at NMDARs was excluded. The NMDA-induced release of [3H]NE in the presence of glycine was further potentiated by Tat. The release evoked by NMDA/glycine/Tat depends on metabotropic glutamate receptor 1 (mGluR1) activation, since it was halved by mGluR1 antagonists. Tat seems to act at the glutamate recognition site of mGluR1. Recently, Tat was shown to release [3H]acetylcholine from human cholinergic terminals; here, we demonstrate that this effect is also mediated by pre-synaptic mGluR1. The peptide sequence Tat41–60, but not Tat61–80, mimicked Tat. Phospholipase C, protein kinase C, and cytosolic tyrosine kinase are involved in the NMDA/glycine/Tat-evoked [3H]NE release. To conclude, Tat can represent a potent pathological agonist at mGlu1 receptors able to release acetylcholine from human cholinergic terminals and up-regulate NMDARs mediating NE release from human and rat noradrenergic terminals.

AIDS is often accompanied by neuropsychiatric symptoms known as the human immunodeficiency virus (HIV)-1-associated dementia (HAD; Navia et al., 1986; Diesing et al., 2002; Lawrence and Major, 2002), the origins of which are poorly understood. Neuroinflammation and neuronal death (collectively termed HIV-1 encephalopathy) are common in AIDS patients and have been implicated in the pathogenesis of HAD (Masliah et al., 1992; Everall et al., 1999). Reducing the viral load by antiretroviral therapy does not impede HAD development and neuronal damage (Lawrence and Major, 2002). Since HIV-1 encephalopathy is a gradual process, dramatic neurodegenerative events may not be necessary to explain neuropsychiatric deficits that, in some patients, rep-
recent early manifestations of AIDS (Lawrence and Major 2002). In fact, subtle changes in neurotransmission induced by viral components, including proteins gp120 and Tat, could underlie various functional impairments.

Evidence has been provided that HIV-1 proteins can affect neuronal functions through glutamate receptor activation (Savio and Levi, 1993; Pittaluga and Raiteri, 1994; Magnuson et al., 1995; Pittaluga et al., 1996, 2001; Kaul and Lipton, 1999; Feligioni et al., 2003; Self et al., 2003; Behnisch et al., 2004; Gelman et al., 2004), although the exact targets for gp120 and Tat remain undetermined.

If subtle changes in neurotransmission, independent of or preceding neurotoxicity, underlie the development of HAD, it is worthwhile to investigate whether and how HIV-1 proteins alter neurotransmitter systems potentially involved in functions that can be impaired during HIV-1 infection. We already found that gp120 acts as a potent glycine site agonist at glutamate N-methyl-d-aspartate (NMDA) receptors (NMDARs) mediating norepinephrine (NE) release from human and rat central nervous system (CNS) nerve endings (Pittaluga and Raiteri, 1994; Pittaluga et al., 1996, 2001; Pattarini et al., 1998). More recently, we reported that Tat releases acetylcholine (ACH) from human neocortical cholinergic terminals by targeting group I metabotropic glutamate receptors (mGluRs; Feligioni et al., 2003).

Tat has been reported to positively modulate NMDARs in neuronal and organotypic slices (Haugaey et al., 2001; Pendergast et al., 2002; Self et al., 2003; Song et al., 2003), but the site of action, whether on NMDARs or on receptive sites that can cross-talk with NMDARs or on both, is not well understood. To shed light on this question, here we investigated whether and how Tat can affect the function of NMDARs known to exist on noradrenergic terminals of human and rat brain.

Our results show that Tat is a potent agonist at mGluR1 localized on human and rat noradrenergic terminals. Tat is ineffective on its own but acts in cooperation with coexisting NMDARs. The mGluR1-NMDAR interaction, triggered by concomitant exposure to Tat and NMDA, leads to enhancement of NE release. In addition, we report that the release-enhancing group I mGluRs, previously found to exist on human neocortical cholinergic terminals and to be targeted by Tat (Feligioni et al., 2003), also belong to the mGluR1 subtype.

Materials and Methods

Human Brain Tissue Samples. Samples of human cerebral cortex were obtained from informed and consenting HIV-1-negative patients undergoing neurosurgery, each on a different day, to reach deeply seated tumors. The samples represented parts of frontal (n = 20) and temporal (n = 24) lobes obtained from 16 women and 28 men (age 30–70 years). Immediately after removal, the tissue was placed in a physiological salt solution at 2–4°C, and crude synaptosomes were prepared within 90 min. The experimental procedures were approved by the Ethical Committee of the Pharmacology and Toxicology Section, Department of Experimental Medicine, in accordance with the European legislation (European Communities Council Directive of November 24, 1986, 86/609/EEC).

Preparation of Synaptosomes. Crude synaptosomes were prepared according to Raiteri and Raiteri (2000). In brief, the tissue was homogenized in 40 volumes of 0.32 M sucrose and buffered at pH 7.4 with phosphate (final concentration 0.01 M). The homogenate was first centrifuged at 1000g for 5 min to remove nuclei and cellular debris, and synaptosomes were then isolated by centrifugation at 13,000g for 20 min. The synaptosomal pellet was resuspended in a physiological medium having the following composition: 125 mM NaCl, 3 mM KCl, 1.2 mM MgSO4, 1.2 mM CaCl2, 1 mM NaH2PO4, 22 mM NaHCO3, and 10 mM glucose (aerated with 95% O2 and 5% CO2). pH 7.2 to 7.4. When indicated, brain tissue was homogenized in buffered sucrose containing 5 mM pertussis toxin or 20 µg of antibody anti-phosphotyrosine to trap these agents into subsequently isolated synaptosomes (Raiteri et al., 2000).

Release Experiments. Human or rat synaptosomes were labeled with [3H]NE (final concentration, 30–50 nM) in the presence of the transporter blockers 6-nitroquipazine (0.1 µM) and GBR 12909 (0.1 µM) to avoid false labeling with [3H]NE of serotoninergic and dopaminergic nerve terminals, respectively. In a set of experiments, human cortical synaptosomes were preloaded with [3H]choline (final concentration, 60 nM). Incubation was performed at 37°C for 15 min in a rotary water bath and in an atmosphere of 95% O2 and 5% CO2. After the labeling period, identical portions of the synaptosomal suspensions were layered on microporous filters at the bottom of parallel superfusion chambers thermostated at 37°C (Raiteri and Raiteri, 2000). Synaptosomes were superfused at 0.5 ml/min with standard physiological solution aerated with 95% O2 and 5% CO2 at 37°C. When indicated, the medium was replaced, at t = 20 min, with a medium from which Mg2+ ions were omitted, to permit NMDAR activation. The system was first equilibrated during 38 min of superfusion; subsequently, eight consecutive 1-min fractions were collected. Synaptosomes were exposed to agonists at the end of the first fraction collected (t = 39 min) until the end of the superfusion, whereas antagonists were added 8 min before agonists. Fractions collected and superfused synaptosomes were counted for radioactivity.

Calculations and Statistics. The amount of radioactivity released into each superfusate fraction was expressed as a percentage of the total synaptosomal tritium content at the start of the fraction collected (fractional efflux). Drug effects were expressed as percent increase over basal release and were evaluated as the ratio between the percentage of tritium released into the fraction where the maximal releasing effect was observed and that in the first fraction collected; this ratio was compared with the corresponding ratio obtained under control conditions (no drug added). Analysis of variance was performed by ANOVA followed by Dunnett’s test multiple-comparison test or Student’s t test as appropriate. Data were considered significant for p < 0.05 at least. Appropriate controls with antagonists and inhibitors were always run in parallel.

Materials. 1-[7,8-3H]NE (specific activity, 39 Ci/mmol) and [methyl-3H]choline (specific activity, 83 Ci/mmol) were from Amer sham Radiochemical Centre (Buckinghamshire, UK). NMDA, 3-(4-chlorophenyl)-1-(1,1-dimethylethyl)-1H-pyrrolizolo[3,4-d]pyrimidin-4-amine (PP2), 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP), 7-hydroxyimino)cyclopenta[b]chromen-1a-carboxylic ethyl ester (CPCCOEt), LY 367385, 7-Chr-KYNA, and GF 109203X were obtained from Tocris Cookson (Bristol, UK). Glycine, aracine, U 73122, and H89 were purchased from Sigma Chemical Co. (St. Louis, MO). Ac-RQIKIWFQNRRMKWKKKKKLRRQEAFDAL-OH (autocamtide-2 related inhibitor peptide II; Ant-AIP-II) and pertussis toxin (PTx) were from Calbiochem (La Jolla, CA). Mouse antiphosphotyrosine (clone 4G10) was from Upstate Biotechnology (Lake Placid, NY). The recombinant HIV-1 protein gp120 (strain MN) was from AMS Raggio Italgene (Milan, Italy). The following compounds were gifts: 6-nitroquipazine maleate (Duphar, Amsterdam, The
Netherlands), GB 12909 (Gist-Brocades, Delft, The Netherlands), and MK-801 (Merck-Sharp & Dohme, Whitehouse Station, NJ). Recombinant Tat (HIV-1 clade B HAN2), Tat peptide 41 to 60 (HIV-1 strain LAI), and Tat peptide 61 to 80 (HIV-1 strain LAI) were kindly donated by European program EVA (779.5-779.1-7) (National Institute for Biological Standards and Control, Hertfordshire, UK). The amount of contaminant glycine or glutamate in the protein solution (at the final concentration of 1 nM) amounted to 65 ± 7.8 glycine nM and 53 ± 4.3 nM for glutamate.

**Results**

Tat Up-Regulates Presynaptic NMDARs Mediating NE Release without Binding at the NMDAR Complex. Human cortical noradrenergic nerve endings are endowed with release-enhancing NMDARs (Fink et al., 1992; Pittaluga et al., 1996). In the absence of extracellular Mg2+ and of exogenously added glycine, neither NMMA (1 mM) nor Tat (1 nM) elicited significant release of [3H]NE from superfused human neocortical synaptosomes prelabeled with the [3H]catecholamine (Fig. 1A). Concomitant addition of glycine (3 or 10 μM) permitted NMMA to evoke significant release of [3H]NE [Fig. 1A, F(7,30) = 22.1; p < 0.05 and p < 0.001, respectively]. On the contrary, Tat remained ineffective in the presence of 10 μM glycine (Fig. 1A).

Release-enhancing NMDARs also exist on rat hippocampal noradrenergic nerve endings (Fink et al., 1990; Pittaluga and Raiteri, 1990). Since NMDARs mediating NE release in the hippocampus has been the object of a number of our investigations (Pittaluga and Raiteri, 1990, 1992, 1994; Pittaluga et al., 2001, 2005), the present work was carried out with rat hippocampal synaptosomes with some confirmatory experiments performed with rat cortical preparations (see below). In the absence of extracellular Mg2+ and of exogenously added glycine, NMMA (100 μM), but not Tat (1 nM), elicited small but significant [3H]NE release (~25% over basal release) from rat hippocampal synaptosomes (Fig. 1B). It had been reported that this effect of NMMA alone is due to unavoidable small glycine contamination (40 ± 10 nM; see also Kew et al., 1998) in the solutions and could only be observed in experiments with rodent synaptosomes (cf. Pittaluga and Raiteri, 1990; Pittaluga et al., 1996), probably due to their better viability. Figure 1B also reports that Tat (1 nM) was ineffective when added together with 1 μM glycine.

When human synaptosomes were exposed to NMMA plus Tat (1 nM), without adding glycine, a dramatic release of [3H]NE was observed [Fig. 1A; F(7,30) = 22.1, p < 0.001]. This effect was entirely dependent on NMDAR activation since it was almost eliminated by the NMDA receptor antagonist MK-801 (Fig. 1A; F(7,30) = 22.1, p < 0.001).

In rat synaptosomes, Tat increased in a concentration-dependent manner the release of tritium caused by 100 μM NMMA in the absence of exogenously added glycine; the maximal effect was reached when Tat was present at 1 nM [Fig. 1C; F(4,24) = 14.75, p < 0.01]. As in human synaptosomes, the effect of NMMA/Tat depended on NMDAR activation since it was abrogated by MK-801 [Fig. 1B; F(6,36) = 23.78, p < 0.01].

Based on these results, the effects of Tat would appear compatible with a potent action at the glycine site of the NMDARs. It should be reminded that gp120 was previously found to behave as an extremely potent glycine-like agonist, capable (similar to glycine) to competitively reverse and surmount the antagonism by 7-Cl-KYNA (a selective blocker of the glycine site) of the NMDA-evoked [3H]NE release (Pittaluga and Raiteri, 1994; Pattarini et al., 1998). In contrast, Table 1 shows that Tat failed to revert and surmount the 7-Cl-KYNA antagonism of the NMDA/Tat effects both in human [F(5,22) = 15.76, p < 0.001] and rat [F(5,23) = 79.93, p < 0.001] synaptosomes, a result inconsistent with an action of Tat at the NMDA glycine site.

Importantly, the potentiation by 3 μM (human) or by 1 μM (rat) glycine (Fig. 1, A and B) as well as by gp120 (Fig. 2, A and B) of the NMDA-evoked [3H]NE release (Pittaluga and Raiteri, 1994; Pittaluga et al., 1996) could be further increased by 1 nM Tat (human synaptosomes, F(2,12) = 70.15, p < 0.01; rat synaptosomes, F(2,10) = 52.02, p < 0.05), results consistent with Tat and glycine (or gp120) acting at different sites on noradrenergic terminals.

Studies with neuronal and organotypic slice cultures of rat hippocampus, aimed at understanding the mechanisms of NMDAR up-regulation by Tat, reported that the neurotoxicity of the protein may require action at an NMDAR polyamine-sensitive site (Prendergast et al., 2002; Self et al., 2003). These authors found that both the neurotoxicity produced by Tat in the CA1 and CA3 pyramidal cell layers (Prendergast et al., 2002) and the elevations in intracellular Ca2+ caused by the protein in the same regions (Self et al., 2003) could be significantly attenuated by the NMDAR polyamine site antagonist arcaine. As shown in Table 2, the potentiation by Tat of the NMMA/glycine-evoked release of [3H]NE in human and rat synaptosomes was insensitive to...
Tat does not revert the antagonism by 7-Cl-KYNA of the NMDA-evoked \([^{3}H\)NE release from human and rat nerve endings. Human and rat synaptosomes were superfused with Mg\(^{2+}\)-free medium and exposed to 1 \(\mu\)M NMDA (human synaptosomes) or to 100 \(\mu\)M NMDA (rat synaptosomes), respectively. When indicated, Tat was added concomitantly with NMDA at the end of the first fraction collected. 7-Cl-KYNA (1 \(\mu\)M) was added 8 min before agonists and maintained until the end of superfusion. Results are expressed as percentage increase over basal release; data are means \(\pm\) S.E.M. of three to six (human) and four to six (rat) experiments run in triplicate (three superfusion chambers for each experimental condition).

<table>
<thead>
<tr>
<th></th>
<th>Human NMDA</th>
<th>Human NMDA + 7-Cl-KYNA</th>
<th>Rat NMDA</th>
<th>Rat NMDA + 7-Cl-KYNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.99 (\pm) 3.03</td>
<td>11.69 (\pm) 2.76</td>
<td>32.30 (\pm) 3.05</td>
<td>11.21 (\pm) 2.30</td>
</tr>
<tr>
<td>1 nM Tat</td>
<td>91.46 (\pm) 13.18(^b)</td>
<td>18.26 (\pm) 15.87(^b)</td>
<td>79.32 (\pm) 6.05(^e)</td>
<td>18.44 (\pm) 6.02(^e)</td>
</tr>
<tr>
<td>10 nM Tat</td>
<td>86.32 (\pm) 8.93(^c)</td>
<td>15.54 (\pm) 12.78(^b)</td>
<td>74.34 (\pm) 5.99(^e)</td>
<td>19.31 (\pm) 7.55(^f)</td>
</tr>
</tbody>
</table>

\(^a\) \(p < 0.001\) vs. NMDA.
\(^b\) \(p < 0.001\) vs. NMDA + Tat.

30 \(\mu\)M arcaine, suggesting that the Tat effects on \([^{3}H\)NE release do not involve the polyamine sites of the NMDARs located on noradrenergic axon terminals.

The Effects of Tat on NMDAR Functions Are Mediated by Activation of a Pertussis Toxin-Sensitive Mechanism. Because Tat failed to mimic NMDA, glycine, or polyamines, the possibility existed that it could bind to a "receptor" coexisting with NMDARs on noradrenergic terminals. Receptor-receptor interactions are diffuse mechanisms generally occurring postsynaptically (for review, see Agnati et al., 2003). However, interactions between presynaptic receptors, both ionotropic and metabotropic, have also been described previously (Dese et al., 1992; Raiteri et al., 1992; Diaz-Hernandez et al., 2004; Risso et al., 2004; Pittaluga et al., 2005). One interesting aspect of receptor-receptor interactions is that receptors sometimes unable to mediate responses when exposed to selective agonists become functional when other receptors coexisting on the same membrane are activated.

We first investigated whether Tat activates G protein-coupled receptors and started by verifying the possible involvement of PTx-sensitive G proteins in the control by Tat of NMDAR function. To avoid excessively long incubations with PTx, we prepared synaptosomes by homogenizing brain tissues in the presence of PTx, a technique previously shown to successfully entrap the toxin into subsequently isolated synaptosomes (Raiteri et al., 2000). Figure 3B shows that entrapped PTx did not modify the release of \([^{3}H]NE elicited from rat synaptosomes either by 100 \(\mu\)M NMDA alone or in the presence of 1 \(\mu\)M glycine. On the contrary, PTx totally prevented the Tat-mediated component of the NMDA-evoked release either in the absence or presence of glycine \(F(7,28) = 12.11, p < 0.05\) and \(p < 0.001\), respectively.

Similar results were obtained in experiments with human synaptosomes. As shown in Fig. 3A, entrapped PTx prevented the Tat-mediated component of the release elicited by NMDA/glycine/Tat \(F(2,17) = 18.3, p < 0.01\). The inhibition produced by PTx was higher in human than in rat synaptosomes, in line with the larger contribution by Tat observed in human neocortex. Entrapping PTx failed to modify the basal release of tritium (data not shown).

**The Tat-Mediated Effects Involve the mGlu1 Receptor Subtype.** In a recent work (Feligni et al., 2003), Tat was shown to directly activate release-enhancing group I mGluRs presynaptically located on human neocortex cholinergic nerve endings. This finding prompted us to verify whether the indirect Tat-mediated effect on the function of NMDARs sited on noradrenergic terminals also could involve mGluRs.

Human synaptosomes were exposed to NMDA/glycine/Tat in the presence of CPCCoEt or MPEP, two noncompetitive antagonists at mGluR1 or mGluR5, respectively. As shown in Fig. 4A, CPCCoEt (5 \(\mu\)M) totally prevented the Tat-mediated component of the NMDA/glycine/Tat-evoked release \(F(4,23) = 10.39, p < 0.001\), whereas MPEP (1 \(\mu\)M) only exerted a slight nonsignificant inhibition. To confirm the involvement of a mGlu1 receptor subtype, we tested LY 367385, a selective competitive mGluR1 antagonist. As reported in Fig. 4A, LY 367385 (1 \(\mu\)M) abrogated the Tat-mediated component of the NMDA/glycine/Tat-evoked \([^{3}H]NE release from human nerve terminals \(F(4,23) = 10.39, p < 0.001\).

When rat synaptosomes were exposed to NMDA/glycine/Tat in the presence of mGluR antagonists, CPCCoEt (5 \(\mu\)M) totally prevented the Tat-mediated component of the evoked release \(F(4,38) = 13.9, p < 0.001\). Moreover, LY 367385 concentration-dependently (10 nM-1 \(\mu\)M) inhibited the Tat-mediated release, causing complete inhibition when added at 0.1 \(\mu\)M \(F(6,38) = 13.9, p < 0.001\). At the maximal concentration applied, CPCCoEt, MPEP, and LY 367385 failed to affect the basal release of tritium or that evoked by NMDA/glycine from human and rat synaptosomes (data not shown).
TABLE 2
Arcaine does not affect the release of \(^{3}H\)NE evoked by NMDA/glycine in absence or in presence of Tat from human and rat nerve endings. Human and rat synaptosomes were superfused with Mg\(^{2+}\)-free medium and exposed, respectively, to 1 mM NMDA + 3 \(\mu M\) glycine (human synaptosomes) or to 100 \(\mu M\) NMDA + 1 \(\mu M\) glycine (rat synaptosomes). When indicated, 1 nM Tat was added together with agonists, whereas arcaine was present from 8 min before agonists. Results are expressed as percentage increase over basal release; data are means ± S.E.M. of five (human) and three to six (rat) experiments run in triplicate. Arcaine did not affect on its own the spontaneous release of tritium (not shown).

<table>
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<tr>
<th></th>
<th>Human</th>
<th>Rat</th>
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<tbody>
<tr>
<td></td>
<td>NMDA + Glycine</td>
<td>NMDA + Glycine + Tat</td>
</tr>
<tr>
<td>Control</td>
<td>21.22 ± 3.05</td>
<td>72.02 ± 11.78*</td>
</tr>
<tr>
<td>+ Arcaine (30 (\mu M))</td>
<td>18.46 ± 3.46</td>
<td>75.83 ± 12.41*</td>
</tr>
</tbody>
</table>

* \(p < 0.05\) vs. respective control [human, \(F(3,16) = 8.29\); rat, \(F(3,23) = 9.71\)].

Because LY 367385 is a competitive antagonist of mGluR1, we investigated whether Tat could indeed compete with the drug. As shown in Table 3, 0.1 \(\mu M\) LY 367385 inhibited the Tat-mediated component of the NMDA/glycine/Tat-evoked \(^{3}H\)NE release \([F(5,14) = 31.21, p < 0.001]\); increasing Tat concentration up to 10 nM did not further potentiate the NMDA/glycine-evoked release, but it reversed completely the antagonism by LY 367385 \([F(5,14) = 31.21, p < 0.001]\).

**Tat Activates mGlu1 Receptors Also in Human Cortical Cholinergic Nerve Endings.** As mentioned above, group I mGluRs were found to exist on human (but not rat) neocortex cholinergic axon terminals whose activation by Tat (in the absence of NMDA) brings about ACh release (Feligioni et al., 2003). Therefore, it was important to better characterize the pharmacology of these presynaptic mGluRs. Synaptosomes from human neocortex were labeled with \(^{3}H\)choline and exposed during superfusion (with Mg\(^{2+}\)-containing medium) to Tat (1 nM), in the absence or presence of the selective mGlu1 receptor antagonist LY 367385. Figure 5 shows that the Tat-induced release of \(^{3}H\)ACh was completely abolished by 1 \(\mu M\) LY 367385 (df = 5, \(t = 5.99\), \(p < 0.05\)).

**The Tat Peptide Fragment 41 to 60 Mimics the Intact Protein.** In our previous work (Feligioni et al., 2003), the amino acid sequence 41 to 60 of Tat could mimic the intact protein at the mGluRs mediating ACh release from human cortical nerve endings. In contrast, the effect of Tat on ACh release from rat cortical synaptosomes (mediated by an unidentified receptor) was reproduced by the sequence 61 to 80, indicating species specificity.
Experiments were performed to test the effects of Tat$_{41–60}$ and Tat$_{91–80}$ on the NMDA/glycine-evoked release of [³H]NE from human and rat noradrenergic axon terminals. As reported in Table 4, Tat$_{41–60}$ potentiated the evoked release from both human [F(2,9) = 8.77, p < 0.01] and rat [F(2,9) = 21.83, p < 0.001] synaptosomes, whereas the sequence 61 to 80 was ineffective.

**Tat Up-Regulation of NMDARs Occurs through a Phospholipase C/Protein Kinase C/Cytosolic Tyrosine Kinase (Src) Pathway.** In rat hippocampal noradrenergic terminals, the [³H]NE release evoked by NMDA/glycine/Tat was significantly diminished by U 73122, a selective phospholipase C (PLC) inhibitor, by GF 109203X, a selective protein kinase C (PKC) blocker, and by PP2, a selective inhibitor of Src [F(7,23) = 5.05, p < 0.05]. On the contrary, H89, a protein kinase A inhibitor and Ant-AIP-II, a selective inhibitor of Ca$^{2+}$/calmodulin-dependent protein kinase II, left unaffected the evoked release (Fig. 6B). The effective compounds appear to block the Tat-mediated component of the NMDA/glycine/Tat-evoked release since U 73122 or PP2 did not inhibit significantly the release evoked by NMDA plus glycine (data not shown).

The involvement of Src implies phosphorylation of tyrosine residues. Accordingly, entrapping of antibodies against phosphotyrosinases into rat synaptosomes inhibited the release of [³H]NE elicited by NMDA/glycine/Tat to the same extent as PP2 [Fig. 6B; F(7,23) = 5.05, p < 0.05].

**Table 4**

<table>
<thead>
<tr>
<th>Effects of Tat fragments 41 to 60 and 61 to 80 on the [³H]NE release evoked by NMDA/glycine from human and rat noradrenergic nerve endings</th>
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<tbody>
<tr>
<td>Synaptosomes were superfused with Mg$^{2+}$-free medium; human synaptosomes were exposed to 1 mM NMDA + 3 μM glycine, whereas rat synaptosomes were exposed to 100 μM NMDA + 1 μM glycine. Tat fragments (final concentration, 1 nM) were added concomitantly with NMDA/glycine. Results are expressed as percentage increase over basal release; data are means ± S.E.M. of 4 (human) and 4 (rat) experiments run in triplicate.</td>
</tr>
<tr>
<td>Human: NMDA</td>
</tr>
<tr>
<td>Glycine</td>
</tr>
<tr>
<td>Control</td>
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<tr>
<td>Fragment 41 to 60</td>
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<td>Fragment 61 to 80</td>
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</table>

$^*$p < 0.01 vs. respective control.

Finally, U 73122 and PP2 produced significant inhibition of the [³H]NE release provoked by NMDA/glycine/Tat in human synaptosomes [F(3,12) = 23.61, p < 0.001], suggesting participation of a PLC/Src pathway also in human neocortex noradrenergic nerve endings (Fig. 6A). At the concentrations applied, antagonists or enzyme inhibitors did not affect, on their own, the release of tritium from human and rat synaptosomes (data not shown).

**Tat Activates Presynaptic mGlu1 Receptors Colocalized with NMDA Receptors on Rat Cortical Cholinergic Nerve Endings.** In rat central nervous system, cortical noradrenergic nerve endings are endowed with NMDA receptors, whose activation elicits the release of preloaded [³H]NE; previous results suggested that NMDARs located on cortical axon terminals display a pharmacological profile similar to that of NMDARs situated on hippocampal nerve endings (Pittaluga and Raiteri, 1994). However, these similarities do not allow the assumption that the Tat-NMDAR interaction characterized in the hippocampus also occurs in the neocortex. Therefore, a set of experiments was carried out with rat cortical synaptosomes. Table 5 shows that enhancement of [³H]NE release provoked by 100 μM NMDA + 1 μM glycine in rat cortical synaptosomes is significantly increased by 1 nM Tat [F(3,8) = 10.31; p < 0.01, data from three experiments run in triplicate]. Moreover, LY 367385 (1 μM) but not MPEP (1 μM) significantly prevented the Tat-mediated potentiation of the NMDA/glycine-induced [³H]NE release.
TABLE 5
Effects of Tat on the [\( ^{3}H \)]NE release evoked by NMDA/glycine from rat cortical noradrenergic nerve endings: effects of I group mGluR antagonists

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[( ^{3}H )]NE Release Percentage of Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>33.50 ± 3.20</td>
</tr>
<tr>
<td>+Tat</td>
<td>55.00 ± 3.24(^a)</td>
</tr>
<tr>
<td>+Tat + LY367385</td>
<td>28.86 ± 3.22(^a)</td>
</tr>
<tr>
<td>+Tat + MPEP</td>
<td>47.83 ± 2.33(^a)</td>
</tr>
</tbody>
</table>

\(^a\) p < 0.001 vs. respective control.

Discussion

The main result of this work is that the HIV-1 protein Tat can potentiate NMDAR-mediated responses, also in conditions of glycine deficiency, by acting at mGlur1 receptors coexisting with NMDARs on the same neuronal membrane. The technique used is particularly suitable to identify receptor coexistence and to study receptor-receptor interactions. Monolayers of synaptosomes, plated on microporous filters in parallel chambers, are up-down superfused, and transmitter releases are measured in the superfusate fractions collected. In these conditions, the compounds released are immediately removed by the superfusing solution before they can activate targets on the releasing nerve endings or on adjacent particles, so that indirect effects are prevented (see Raiteri and Raiteri, 2000). In the present work, synaptosomes are selectively prelabeled with [\( ^{3}H \)]NE, which therefore can only be released from noradrenergic terminals; moreover, release modifications by added agents reflect direct action on noradrenergic terminals. If NMDA or Tat alone is unable to elicit NE release, whereas NMDA and Tat added together provoke release sensitive to different receptor antagonists, it can be concluded that the two corresponding receptors coexist and cross-talk on the same noradrenergic terminal.

It is well known that activation of NMDARs requires glutamate (or NMDA) and glycine/D-serine. Concomitant addition of NMDA (without glycine) and Tat, inactive when added alone, produced dramatic release of NE from human noradrenergic terminals, suggesting that Tat could mimic glycine but three to four orders of magnitude more potent than the natural cotransmitter of glutamate at NMDARs.

If Tat mimics glycine, it would behave like gp120, previously found to be a potent agonist at the NMDAR glycine site on human and rat noradrenergic terminals (Pittaluga and Raiteri, 1994; Pittaluga et al., 1996; Pattarini et al., 1998). However, gp120 was able to reverse and surmount the antagonism of the NMDA-evoked NE release provoked by 7-CI-KYNA (Pittaluga et al., 1996; Pattarini et al., 1998), whereas Tat failed to compete with 7-CI-KYNA for the glycine site, indicating that, in human and rat brain, gp120/glycine and Tat act at different sites on noradrenergic nerve terminals, a view strengthened by the ability of Tat to further enhance the maximal effects of gp120 (see Pittaluga and Raiteri, 1994; Pittaluga et al., 1996; Fig. 2).

The release evoked by NMDA/glycine/Tat was PTX-sensitive, whereas that of NMDA/glycine was insensitive to the toxin, suggesting that Tat acts through G protein-coupled receptors. We previously found that Tat could directly activate group I mGlur located on human cortical cholinergic nerve terminals to elicit ACh release (Feligioni et al., 2003). Since group I mGlur have been reported to up-regulate NMDAR currents (Nicoletti et al., 1996; Skeberdis et al., 2001), it was of interest to investigate whether Tat could activate mGlur and interact with NMDARs possibly coexisting on noradrenergic terminals. This seems to be the case, as shown in Fig. 4; more precisely, our pharmacological characterization with selective group I mGlur antagonists suggests that Tat activates mGlur of subtype 1. Interestingly, here we find that the mGlur present on human cholinergic terminals also belong to the mGlur1 subtype (Fig. 5). Moreover, results with the competitive mGlur antagonist LY 367385 are consistent with the idea that Tat is a mGlur1 agonist able to bind the glutamate recognition site on the outer side of mGlur1.

The effect of Tat as a mGlur1 agonist on both human and rat noradrenergic terminals is retained by the peptide fragment Tat41–60 but not by Tat61–80. Of note, Tat41–60 contains the arginine-rich basic region (Tat49–57) that is required to induce dephosphorylation and internal Ca\(^{2+}\) mobilization in human fetal neurons (Nath et al., 1996). Accordingly, Tat41–60 had been found to imitate the intact protein and to directly release ACh from human (but not rat) neocortex cholinergic terminals (Feligioni et al., 2003). Altogether, the results with mGlur antagonists and with Tat peptide fragments lead to the following conclusions. 1) Tat is an extremely potent mGlur1 agonist; 2) mGlur1 receptors exist on human cholinergic terminals and on human and rat noradrenergic terminals; 3) mGlur1 on human cholinergic terminals directly mediates ACh release through internal Ca\(^{2+}\) mobilization from IP\(_3\)-sensitive stores (Feligioni et al., 2003), whereas mGlur1 on noradrenergic terminals indirectly up-regulates NMDAR-evoked NE release; 4) the rodent model of NMDAR-mediated NE release appears appropriate to study effects of Tat and fragments on glutamate receptors; and 5) Tat41–60 or shorter fragments might be of help in drug design projects aimed at developing selective potent mGlur1 ligands.

The Tat effect on NE release thus requires a mGlur1-NMDAR cross-talk through intraterminal pathways. The release of NE elicited by NMDA/glycine/Tat was significantly reduced when PLC, PKC, or Src activity was inhibited by selective blockers. The release also was reduced in synaptosomes entrapped with antiphosphotyrosine antibodies. All of these agents inhibited to a similar extent the NE release elicited by NMDA/glycine/Tat, the sensitive portion of the release probably representing the Tat-mediated component of the evoked NE efflux. Thus, PLC activation by Tat acting at mGlur1 linked to PTX-sensitive G proteins plays an important role in the mGlur1-NMDAR interaction. The downstream signaling pathway includes PKC- and Src-mediated tyrosine phosphorylation.

The involvement of Src is supported by the inhibition of the evoked NE release observed in the presence of the Src blocker PP2, as well as by the finding that anti-phosphotyrosine antibodies prevented the effect of NMDA/glycine/Tat. Src is associated with NMDARs and phosphorylation by Src was reported to up-regulate NMDAR currents (Yu et al., 1997). Notably, activation of Src family kinase can be triggered by PKC (Salter and Kalia 2004). Thus, the PLC/PKC/Src path-
way seems to couple mGluR1 activation and NMDAR function in brain noradrenergic terminals. This cascade has been proposed to mediate up-regulation of NMDA currents by various G protein-coupled receptors, including group I mGluRs (Salter and Kalia, 2004).

Experiments concerning NMDAR function are usually performed with Mg^{2+}-free solutions. However, NMDA was reported to elicit NE release in the presence of physiological Mg^{2+} concentrations during concomitant activation of AMPA receptors, which was found to coexist with NMDARs on noradrenergic terminals (Raiteri et al., 1992). The following scenario could be envisaged: glutamate reaching noradrenergic terminals activates depolarizing AMPA receptors, which in turn permit Mg^{2+} removal and NMDAR activation; if Tat mimics glutamate and binds at mGluR1, the NMDAR-mediated response could be strongly enhanced. In addition, Tat might also contribute by releasing ACh (Feligioni et al., 2003) onto α7 nicotinic receptors located on glutamatergic terminals and that mediate glutamate release (Risso et al., 2004).

The lack of releasing effect of Tat added alone suggests that the mere activation of mGluR1 stimulates the above enzymes insufficiently to trigger NE release. Nonetheless, mGluR1 activation can up-regulate the functions of NMDARs, an event particularly impressive when these receptors, almost silent in human terminals when glycine is not added, become fully responsive in the presence of 1 nM Tat (see Fig. 1A) as in human terminals, is almost silent in the absence of added glycine (Fig. 1A). If glycine is an obligatory coagonist, a reasonable explanation for the Tat effect could be that the protein causes, through mGluR1 activation and the above phosphorylative pathway, an increase in the affinity of the glycine site for glycine. Unfortunately, this hypothesis cannot be verified by glycine binding experiments or by morphological techniques, due to the very low density of noradrenergic terminals in the CNS.

The concentrations of Tat used here are lower than those causing overt neurotoxicity. It is therefore possible that mGluR1 represents a major target through which Tat produces early impairments in neurotransmission independent of cell death. Circulating levels of Tat have been measured to be ~2.5 nM in AIDS patients (Westendorp et al., 1995), but, according to Nath and Geiger (1998), concentrations are likely to be higher in microenvironments around brain cells. Since Tat or its fragments cannot be removed as easily as glutamate in the CNS, mGluR1 could be “pathologically” activated. The persistent effects of Tat may provoke release of ACh and NE outside of the physiological range and with abnormal kinetic characteristics, potentially contributing to the cognitive impairments occurring in HAD. The situation could become particularly serious if gp120 is also present, considering that this HIV-1 coat protein is an agonist at the glycine site of NMDARs and that the effects of Tat and gp120 can be additive (Fig. 2A; see also Nath et al., 2000). Assuming that Tat and its fragments are pathological mGluR1 agonists, HAD symptoms might be controlled, in part, by mGluR1 antagonists.

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