HIV-1 gp120 glycoprotein affects the astrocyte control of extracellular glutamate by both inhibiting the uptake and stimulating the release of the amino acid

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Abstract The mechanisms of HIV-1 neurotoxicity remain still undefined although the induction of signalling events and a modest inhibition of glutamate uptake induced by the envelope glycoprotein, gp120, have called attention to astrocytes. Here we demonstrate that the levels at which the viral glycoprotein affects glutamate homeostasis of astrocyte cultures are at least two: not only the inhibition of uptake, due to an effect at site(s) away from the transporters of the amino acid but also a slow stimulation of release. The combination of these two events accounts for a considerable steady increase of the extracellular concentration of the excitatory amino acid which could play an important role in the neurotoxicity often observed in AIDS patients.

Key words: Astrocyte; gp120; Glutamate uptake; Glutamate release; HIV neurotoxicity

1. Introduction

Neurological disorders characterized by impairment of cognition and motor performance have often been reported in patients infected by the human immunodeficiency virus type 1, HIV-1 [1]. In many such cases the brain infection, although confined to macrophages and microglia [1,2], was accompanied by striking losses of neurons [3,4]. Death of the latter cells appears therefore to develop indirectly, most likely via shedding of injurious proteins from infected cells. In previous studies, the HIV-1 coat protein gp120 was shown to be neurotoxic in vivo, both when injected intracerebrally [5] and when expressed by astrocytes in transgenic mice [6]. Moreover, in mixed cultures of neurons and glia the gp120-induced neurotoxicity was prevented by NMDA receptor antagonists [7,8], suggesting the sensitization of these receptors to the activation by endogeneous glutamate. Whether these effects are due to a direct action of gp120 on neurons [9,10] or require the involvement of other cells, not only the infected microglia and monocytes [11] but possibly also astrocytes [7,12], remains debated (for reviews see [2] and [13]). A role of astrocytes is suggested by a multifarious experimental evidence. Exposure to gp120 induces in these cells a number of signalling events such as increases of $[Ca^{2+}]_i$ [14,15] and of protein tyrosine phosphorylation [15]; depolarization [16]; activation and redistribution of protein kinase C [17] as well as inhibition of glutamate uptake [16] mediated via microglia-released arachidonic acid [18]. Whether the above effects constitute the entire spectrum induced by gp120 in astrocytes has not been established yet.

By using primary astrocyte cultures we demonstrate here that the action of subnanomolar concentrations of gp120 on glutamate control by astrocytes is more complex than previously envisaged since it includes not only a blockade of uptake but also a stimulation of release. The overall consequence of these processes, the rise of extracellular glutamate, $[Glu]_o$, could have an important role in the chain of events leading to widespread damage of brain cells, as observed in the course of HIV-1 infection.

2. Materials and methods

2.1. Cell cultures

Confluent monolayers of rat cortical astroglial cells were obtained as previously described [19] and plated in 35 mm Petri dishes. They were enriched (>97%) in flat type-1 astrocytes. For fluorometric determinations in continuous, cultures were plated on coverslips sized to fit into the instrument cuvettes.

2.2. [^{3}H]Glutamate uptake

In order to assay uptake into astrocytes [20] cell monolayers were incubated for 60 min in the presence of gp120 or other agents in a buffer containing (mM): 25 glucose, 1.8 $CaCl_2$, 1 $MgCl_2$, 4 KCl, 116 NaCl, 10 HEPES, pH 7.4, and supplemented with 10 μM [^{3}H]glutamate (53 Ci/mmol, isotopic dilution 1/4000). 5 min later the cells were rapidly washed with ice-cold buffer containing excess glutamate, scraped from the culture dishes with NaOH (0.2 N) and counted by liquid scintillography for incorporated radioactivity.

To assay transport into liposomes, glutamate transporters from rat forebrain were partially purified and reconstituted as described [21]. This preparation contains immunoreactivity to the GLT1, GLAST and EAAC1 transporter subtypes [22]. Proteoliposomes with KPi as their internal medium were pre-incubated in a phosphate buffer pH 7.4 (containing 130 mM NaCl, 20 mM NaP_i and 1% glycerol) ± gp120 for 15 min, after which uptake was started by the addition of 1.4 μCi of [^{3}H]glutamate+2.8 μM valinomycin. 70 s later liposomes were collected on filters and counted for radioactivity.

2.3. Fluorometric assay of $[Glu]_o$

Astrocyte cultures were incubated for 60 min in HEPES buffer (see above) ± different agents. At the end, incubation media were collected and their $[Glu]_o$ measured by means of a specific fluorescence assay (excitation and emission at 335 and 430 nm, respectively) performed at 37°C in a Perkin-Elmer LS50B computerized spectrofluorometer [23]. Samples (1 ml) were transferred to a cuvette containing glutamate dehydrogenase (GDH, 40 U/ml, Sigma G2626, batch 64H7130) and 1 mM $NADP^+$. Glutamate oxidation by GDH to α-ketoglutarate led to formation of NADPH with fluorescence emission. $[Glu]_o$ was calculated by referring to standard curves constructed with exogenous glutamate (in the linear range 0.1–1 μM) and by normalizing for the protein content of each sample.

In order to monitor continuously the endogenous glutamate release, glass coverslips with cultured astrocytes were lodged in a 1×1 cm cu-

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vette (2 ml volume) inside the spectrofluorometer in HEPES buffer and incubated at 37°C under stirring. gp120 and other agents were added directly to the cuvette via a microsyringe. Unidirectional glutamate efflux was monitored in continuous by the enzymatic assay described above which ensures immediate reaction of cell-released glutamate with GDH, thereby preventing the reuptake process. Efflux, here expressed as nmol glutamate released/mg prot, was calculated as above.

2.4. Materials

Recombinant gp120 was purchased from Intracel (London, UK); [^{3}H]glutamate from Amersham International; MEM, FCS, penicillin/streptomycin and glutamine for cell cultures from ICN Flow (Costa Mesa, CA); dihydrokainate (DHK) and all other chemicals from Sigma.

3. Results and discussion

In agreement with previous reports [18,24] native (but not heat-inactivated) gp120 was found to induce a slight, but significant reduction ($-15 \pm 3\%$, $n = 5$) of [^{3}H]glutamate uptake into cultured cortical astrocytes (Fig. 1). In contrast, the viral protein had no appreciable effect on the purified brain glutamate transporters functionally reconstituted in liposomes ($n = 3$, not shown). As this preparation is enriched in GLT1 and also contains GLAST [22], the two glial transporters, we conclude that inhibition by gp120 is not due to a direct interaction with these specific targets (see also [18]).

To study whether the uptake inhibition by gp120 is paralleled by the elevation of $[Glu]_o$ of astrocyte cultures, medium assays were carried out at the end of the 60 min incubation with 200 pM of the viral protein. To our surprise, the same treatment that reduced uptake only slightly was able to almost double the $[Glu]_o$ of the cultures ($n = 8$). In contrast, equimolar boiled gp120 had almost no effect (Fig. 2).

To clarify whether uptake inhibition was indeed responsible for the marked $[Glu]_o$ enhancement by gp120, parallel experiments were performed using a specific glutamate uptake blocker drug, DHK [25]. In contrast to most other blockers including the potent *t*-pyrrolidine-2,4-dicarboxylate, DHK is known to block uptake without being transported and therefore cannot be exchanged with cytosolic glutamate during the transport process [19]. Because of this property (see also Fig. 3, insert), DHK appears well suited to identify the contribution of the uptake inhibition process to $[Glu]_o$ increase. 1 h incubation with 2 mM DHK, on the one hand induced uptake inhibition stronger than gp120 ($n = 3$, Fig. 1); on the other hand, enhanced $[Glu]_o$ only slightly ($n = 6$, Fig. 2). Thus, the larger $[Glu]_o$ elevation of gp120 necessarily requires additional mechanisms.

The effect of the protein on the release of glutamate from astrocytes was next investigated. For this purpose, the enzymatic assay of Nicholls and Sihra [23] was modified to continuously monitor the unidirectional efflux of glutamate from our cultures. Fig. 3 shows that addition of 200 pM gp120 after a 40 min recording of baseline enhanced, slowly but consistently, the release of glutamate for about 70 min. When employed at 1 nM, the total release induced by gp120 was similar but the rate was faster as the process lasted about 40 min. At the two concentrations release took place in fact at 0.017 and 0.031 nmol/min/mg protein, respectively. No significant enhancement from basal release was observed after addition of either equimolar boiled gp120 ($n = 3$, not shown) or 2 mM DHK (Fig. 3, insert). This last observation is important, as it confirms that in our assay glutamate release is specifically revealed, without contribution of uptake.

In conclusion, our data indicate that gp120 markedly elevates $[Glu]_o$ of astrocyte cultures by two parallel mechanisms: (a) activation of a slow release process; (b) inhibition of uptake. The mechanism of release remains to be understood and

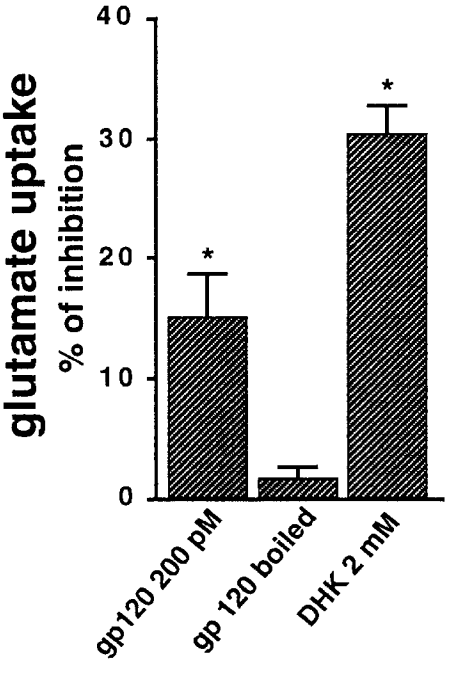

Fig. 1. Inhibition of glutamate uptake into cultures of cortical astrocytes by gp120 and DHK. 100% uptake of [^{3}H]glutamate was $55\,800 \pm 1000$ cpm. Results shown are averages ± S.E.M. of 3–5 experiments. *, $P < 0.01$, one-way ANOVA followed by the Tukey method for multiple comparisons.

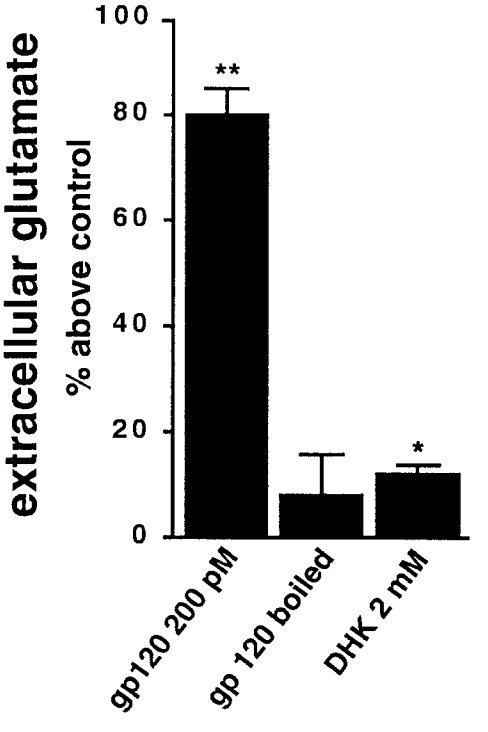

Fig. 2. $[Glu]_o$ increases induced by gp120 and DHK in cultures of cortical astrocytes. Basal $[Glu]_o$ was 451 ± 32 nM. Results shown are averages ± S.E.M. of 6–8 experiments. **, $P < 0.001$; *, $P < 0.01$, one-way ANOVA followed by the Tukey method for multiple comparisons.

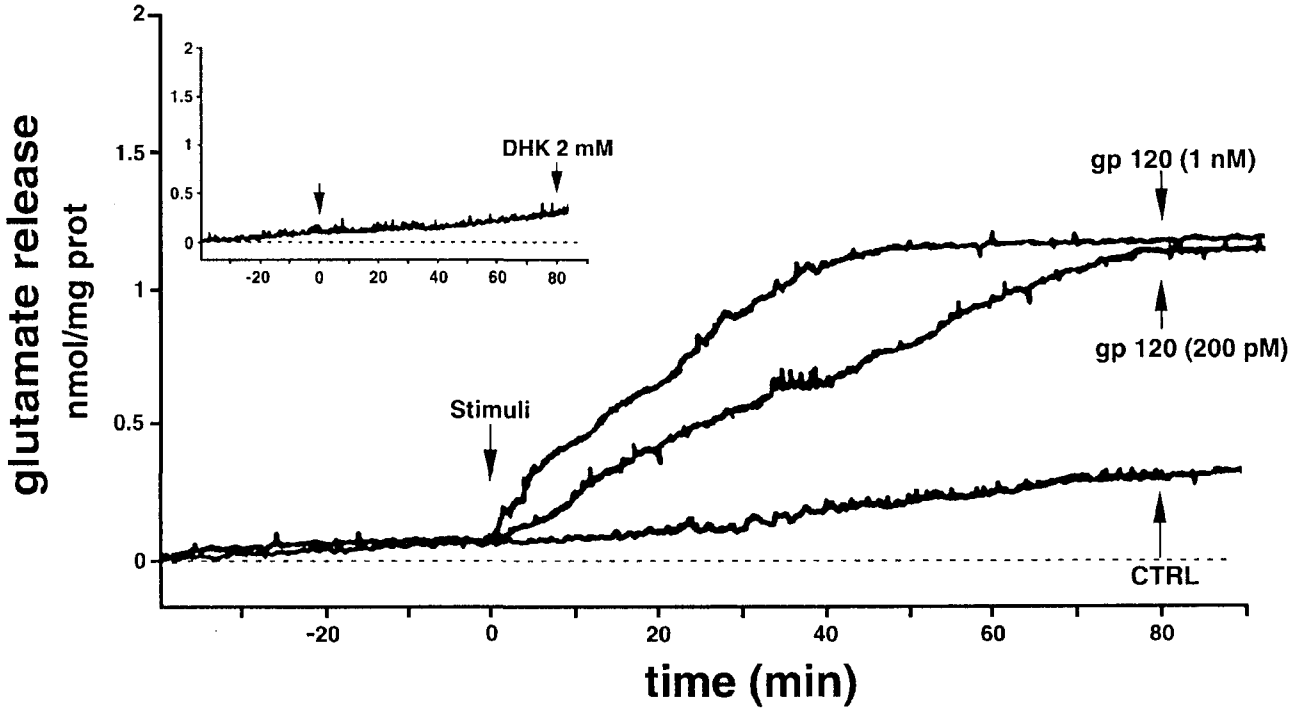

Fig. 3. Glutamate release from cultures of cortical astrocytes as revealed in continuum by the GDH fluorescence procedure. Administration of DHK and gp120 at time 0 are indicated by an arrow in the small and large panel, respectively. The arrows with indications of DHK, gp120 and CTRL identify the treatments (with doses) and the controls. Overall glutamate release values were (in nmol/mg protein): 0.337 ± 0.017 for control ($n = 4$); 0.34 ± 0.023 for DHK ($n = 3$)-, 1.167 ± 0.07 gp120 (200 pM, $n = 7$)- and 1.22 ± 0.2 for gp120 (1 nM, $n = 3$)-treated samples.

could be peculiar. So far, in fact, at least two processes, one Ca^{2+}-independent, the other Ca^{2+}-dependent, have been described, activated in response to swelling or neuroligands [26,27]. Both of them, however, occur at rates much faster than those observed here. With gp120, on the other hand, $[Glu]_o$ remains elevated at the end of the incubation, most likely because the enhanced release is not compensated by adequate uptake. The duality of the gp120 action might therefore ultimately induce synergistic effects on glutamate homeostasis. Since in the brain astrocytes are intimately connected to neurons and synapses, the effects of gp120 here observed on astrocyte cultures could have important toxic consequences. The raised ambient glutamate levels, even if of moderate extent, might in fact induce in the long run injurious overactivation of neuronal excitatory amino acid receptors, a process already suggested to play a key role in the development of gp120-mediated neurotoxicity [7,8].

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