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Envelope glycoprotein gp120 of human immunodeficiency virus type 1 alters ion transport in astrocytes: Implications for AIDS dementia complex

(Na$^+$/H$^+$ exchange/glutamate transport/patch clamp/K$^+$ channels/intracellular pH)

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ABSTRACT **Infection by human immunodeficiency virus type 1 (HIV-1) is often complicated by a variety of neurological abnormalities. The most common clinical syndrome, termed acquired immunodeficiency syndrome (AIDS) dementia complex, presents as a subcortical dementia with cognitive, motor, and behavioral disturbances and is unique to HIV-1 infection. The pathogenesis of this syndrome is poorly understood but is believed to involve interactions among virally infected macrophages/microglia, astrocytes, and neurons. In this study, we show that exposure of primary rat and human astrocytes to heat-activated HIV-1 virions, or to eukaryotically expressed HIV-1 and HIV-2 envelope glycoproteins (gp120) stimulates amiloride-sensitive Na$^+$/H$^+$ antiport, potassium conductance, and glutamate efflux. These effects are blocked specifically by amiloride, an inhibitor of Na$^+$/H$^+$ antiport and by the selective removal of gp120 with immobilized monoclonal antibody. As a result of modulation of astrocytic function by gp120, the ensuing neuronal depolarization and glutamate exposure could activate both voltage-gated and *N*-methyl-D-aspartate-regulated Ca^{2+} channels, leading to increases in intraneuronal Ca^{2+} and neuronal death. These findings implicate the astrocyte directly in the pathogenesis of AIDS dementia complex.**

A chronic encephalopathy termed AIDS dementia complex (ADC) afflicts up to 80% of adult patients suffering from human immunodeficiency virus type 1 (HIV-1) infection (1). ADC occurs in the absence of recognized opportunistic pathogens, is associated with direct infection of the central nervous system (CNS) by HIV-1 (2, 3), and is accompanied pathologically by neuronal loss, reactive astrogliosis, and myelin damage (1). Infiltrating monocytes/macrophages, as well as resident microglia, are the principal cell types productively infected with HIV-1 in the CNS (4). Because neither astrocytes, oligodendrocytes, nor neurons are infected with HIV-1 *in vivo*, the causes of neuronal dysfunction in ADC remain enigmatic. There are data concerning the ability of HIV-1 virions, envelope glycoprotein gp120, or products of infected monocytes or microglia to affect directly neuronal function or viability (5–12). Cocultivation of HIV-1-infected macrophages and astrocytes results in the release of soluble factors that exhibit striking toxicity to cultured fetal rat neurons (8, 12). Thus, there is evidence suggesting that a combination of factors including cytokines, arachidonate metabolites, viral proteins, and still-undefined toxic products of infected macrophages/microglia contribute to the neuropathogenesis of HIV disease.

Astrocytes are the most numerous and versatile glial cells within the CNS and are critical to the maintenance of a balanced homeostatic microenvironment for neurons (13), yet their potential involvement in ADC has been largely unexplored. These cells possess a number of transporters, including Na$^+$/H$^+$ exchange, Cl$^-$/HCO$_3^-$ exchange, Na$^+$–K$^+$–Cl$^-$ cotransport, and neurotransmitter uptake systems, and ion channels that may be essential to their function (14). HIV-1 gp120 has been shown to bind specifically to a protein receptor of 180 kDa on human astroglioma cells, and this binding induces activation of tyrosine kinase activity (15). Thus, there is precedent for gp120 modulation of astrocytic intracellular signaling pathways. In this study, we tested the hypothesis that HIV-1 might directly alter normal astrocyte transport functions, including amiloride-sensitive Na$^+$/H$^+$ exchange, K$^+$ conductance, and glutamate efflux. We hypothesize that viral proteins stimulate astrocyte Na$^+$/H$^+$ exchange, thereby increasing intracellular pH (pH$_i$). The resultant cellular alkalinization influences other membrane transport processes, including the opening of K$^+$ channels (14, 16), reduction of Na$^+$-dependent glutamate uptake, and enhancement of glutamate release (14, 17). Increased levels of extracellular K$^+$ and glutamate can cause neuronal depolarization and the opening of voltage-sensitive Ca^{2+} and *N*-methyl-D-aspartate receptor ion channels (18, 19), leading to increases in intracellular calcium, and ultimately, neurotoxicity (20).

MATERIALS AND METHODS

Cell Culture. Primary glial cell cultures were established from neonatal rat cerebra (21). Rat astrocyte cultures were routinely >97% positive for glial fibrillary acidic protein (GFAP, an antigen specific to astrocytes), and <2% of the cells were microglia.

Human brain material resected from a patient who underwent surgery for intractable epilepsy was used to culture human astrocytes, as described (22). The enriched astrocyte cultures were 87–93% GFAP-positive.

Measurement of ^{22}Na Uptake and [^{3}H]Aspartate Efflux. ^{22}Na influx into rat astrocytes was measured as described (21). [^{3}H]Aspartate efflux was measured from astrocytes previously loaded for 60 min at 37°C with D-[^{3}H]aspartate (1 μCi/ml; 1 μCi = 37 kBq) a nonmetabolized substrate that can substitute for glutamate on the Na$^+$-dependent glutamate transporter (23). Efflux curves were constructed by plotting the percent tracer remaining in the cells versus time. Rate

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Abbreviations: ADC, AIDS dementia complex; BCECF, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; CNS, central nervous system; DSP, *Drosophila* secreted protein(s); GFAP, glial fibrillary acidic protein; HBsAg, hepatitis B surface antigen; HIV, human immunodeficiency virus; [K$^+$]$_o$, external K$^+$ concentration; pH$_i$, intracellular pH.

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constants were calculated from exponential fits to the curves (24).

pH_i Measurements. Human astrocytes were plated on glass coverslips and loaded with the pH-sensitive fluorescent dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF, 3 μM) as described (25). The nigericin technique (26) was used to clamp pH_i to predetermined values to obtain intracellular calibration of the excitation ratio. A coverslip with cells was placed in a quartz cuvette that was inserted into a thermostatically controlled holder in a Photon Technology International (South Brunswick, NJ) ratio spectrophotometer. Cells on coverslips were continuously superfused at 6.8 ml/min at 37°C with phosphate-buffered saline solution, removed from the chamber, incubated with either gp120 or indifferent proteins (*Drosophila* secreted proteins, DSP) for 10 min at 37°C, and then returned to the holder for fluorescence measurements.

Whole-Cell Patch-Clamp Studies. Astrocytes were plated onto glass coverslips and placed into a 1-ml chamber mounted on an inverted microscope. The cells were continuously superfused with 150 mM NaCl/5 mM Hepes, pH 7.2/1 mM $CaCl_2$ (flow rate, 7 ml/min). The pipette solution was filled with 150 mM KCl/1 mM EGTA and was usually unbuffered (pH 7.0). Cells were held at −60 mV; 50-ms test pulses, generated by the CLAMPEX component of the PCLAMP program with the aid of a computer, ranging from −160 to +40 mV in 20-mV increments and returning to the holding potential for 2 s between each test pulse, were used to elicit inward and outward currents. Data were analyzed with a Labmaster TL-1 analog-to-digital converter and the Axon PCLAMP 5.5 software.

HIV-1, Recombinant HIV-1 Proteins, and Monoclonal Antibodies. Purified virions of HIV-1 strain YU-2 (27) were prepared as follows. COS-1 monkey cells (10^6) were transfected with 20 μg of plasmid pYU-2, which contains a complete HIV-1 provirus cloned directly from uncultured brain tissue of a patient who died with ADC. Phytohemagglutinin-stimulated peripheral blood mononuclear cells (10^7) were added directly to the transfected COS-1 cells and cocultivated for 3 days. The peripheral blood cells were then removed and grown independently for an additional 18 days. At the peak of virus production as judged by reverse transcriptase activity, culture supernatants were centrifuged through a 30% sucrose cushion (100,000 × *g*; 45 min). The resultant viral pellet was washed and suspended in 1 ml of RPMI 1640 medium. Viral content was quantitated by measuring p24 antigen levels. A "mock viral preparation" was prepared in an identical fashion, except that the plasmid was omitted. The virus was heat-inactivated by incubation at 60°C for 45 min. Three different preparations of recombinant gp120 were used in this study. They were HIV-1_{IIIB} gp120 (gp120-DSP) and HIV-2_{ST} gp120 (gp120-2-DSP) produced in *Drosophila* S2 cells (28) and HIV-1_{IIIB} gp120 expressed in Chinese hamster ovary (CHO) cells (gp120-CHO; ref. 29). Monoclonal antibodies against gp120-CHO, gp41-CHO, or hepatitis B surface antigen (HBsAg), coupled to agarose beads (30), were used.

RESULTS

HIV-1 Virions and Recombinant gp120 Activate an Amiloride-Sensitive Na^+/H^+ Antiport System in Astrocytes. Previous work has demonstrated that primary rat astrocytes possess an amiloride-sensitive Na^+/H^+ antiport system that can be functionally activated by the cytokine interferon γ (21). Interferon γ is found in cerebrospinal fluid and the CNS of ADC patients, presumably arising from infiltrating activated T cells (31). We next determined the effect of heat-inactivated HIV-1 virions on $^{22}Na^+$ uptake in rat astrocytes (Fig. 1*A*). In these experiments, the macrophage-tropic YU-2 strain (27) was used. Intracellular acidification, produced by exposing astrocytes to 15 mM NH_4Cl for 15 min and then washing it away (the NH_4 pulse protocol; ref. 32), maximally activated amiloride-sensitive Na^+/H^+ antiport (115 ± 17 nmol/mg of protein per min, $n = 12$) and served as a positive control. Heat-inactivated HIV-1 virus, at 50 ng of p24 per ml, stimulated amiloride-sensitive $^{22}Na^+$ uptake, whereas mock-infected supernatants had no effect. To evaluate whether stimulation of $^{22}Na^+$ influx was due to a specific effect of the envelope glycoprotein gp120 and to determine whether the effect was restricted to certain strains of HIV-1 or HIV-2, we tested three different sources of recombinant gp120. HIV-1_{IIIB} gp120 (28) expressed in *Drosophila* (gp120-DSP) or CHO cells (gp120-CHO; ref. 29) and HIV-2_{ST} gp120 (28) expressed in Drosophila (gp120-2-DSP) all stimulated amiloride-sensitive $^{22}Na^+$ influx (Fig. 1*B*). Dose–response experiments

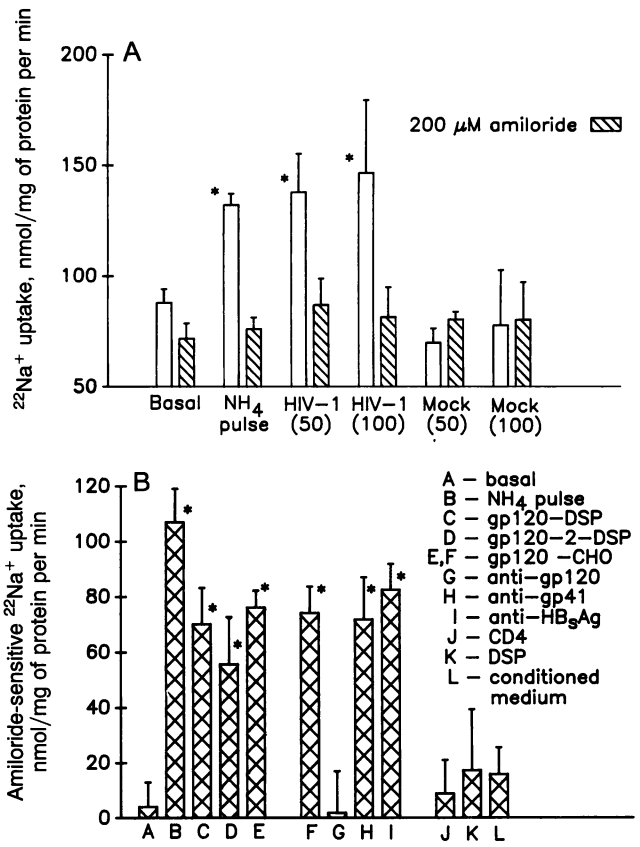

FIG. 1. (*A*) Effect of heat-inactivated HIV-1 virions on $^{22}Na^+$ influx into primary rat astrocytes. Rat astrocytes were preincubated for 5 min in the absence or presence of amiloride (200 μM) and then incubated in the absence or presence of various agents for 10 min. At this time, 0.6 μCi of $^{22}Na^+$ was added to each well (1-ml total volume), the plates were incubated at 37°C for 3 min, and then the fluxes were terminated by aspirating the radioactive medium and washing the cells four times with ice-cold 150 mM NaCl/5 mM Hepes, pH 7.4. At least six replicates were done for each experimental condition. Each experiment was repeated at least three times, and the data are expressed as the mean ± SEM. The $^{22}Na^+$ influxes measured for NH_4 pulse (positive control) and HIV-1 (both 50 and 100 ng of p24 per ml) in the absence of amiloride (open bars) were significantly enhanced with respect to basal levels (*, $P < 0.01$). Fluxes measured in the presence of amiloride are shown by the hatched bars. (*B*) Effect of recombinant gp120 on amiloride-sensitive $^{22}Na^+$ influx into rat astrocytes. Three different preparations of recombinant gp120 were used at 25 nM. Each experiment was repeated 6–29 times, and the data are expressed as the difference of the means determined in the absence and presence of 200 μM amiloride. Conditioned medium from mock-transfected cells was used as a negative control as were recombinant soluble CD4 and DSP (all at 5 μg/ml). *, $P < 0.01$.

indicated that stimulation of amiloride-sensitive $^{22}Na^+$ influx was observed with 1 nM gp120, but stimulation was maximal at 25 nM (data not shown). Medium from *Drosophila* cultures not expressing gp120, soluble CD4 expressed in *Drosophila*, and other irrelevant proteins expressed in *Drosophila* (DSP) failed to stimulate $^{22}Na^+$ influx. To ensure the specificity of this response, gp120-CHO was preincubated for 30 min at 37°C with a 10-fold molar excess of anti-HIV-1 gp120 monoclonal antibodies, anti-HIV-1 gp41, or anti-HBsAg coupled to agarose beads. After removal of the beads by centrifugation (16,000 × *g* for 10 min), the clarified supernatant was added to astrocytes for the standard 10-min incubation. There was total abrogation of gp120-CHO-stimulated Na^+/H^+ exchange after incubation of gp120-CHO with anti-gp120 antibodies, but not with anti-gp41 or anti-HBsAg antibodies (Fig. 1*B*).

Because of the limited numbers of astrocytes generally attainable from human clinical biopsy specimens, $^{22}Na^+$ influx experiments on human astrocytes were impractical. However, Na^+/H^+ antiport can also be assessed by measuring changes in pH_i with the fluorescent dye BCECF (25). Human astrocytes bathed in a bicarbonate-free, phosphate-buffered saline solution at pH 7.4 at 37°C had a steady-state pH_i value of 6.99 ± 0.04. When exposed to 25 nM gp120-CHO for 10 min, pH_i increased to 7.26, and this increase was blocked by amiloride (Fig. 2). On average, gp120-CHO stimulated a 0.26 ± 0.06 ($n = 3$, $P < 0.005$) increase in pH_i in human astrocytes and a 0.14 ± 0.03 ($n = 3$, $P < 0.005$) increase in pH_i in rat astrocytes. These observations are consistent with the interpretation that HIV-1 gp120 increases pH_i via activation of amiloride-sensitive Na^+/H^+ exchange in rat and human astrocytes.

gp120 Activation of Outward K^+ Currents in Astrocytes. In other systems (33–35), increases in pH_i have been shown to activate K^+ currents. To examine whether an activation of Na^+/H^+ exchange with subsequent cytoplasmic alkalinization increases K^+ conductance in astrocytes, we performed whole-cell patch-clamp experiments using gp120-CHO as a stimulus. Fig. 3 shows an example of such measurements in an astrocyte bathed in medium with a high Na^+ concentration (an unbuffered K^+ solution was inside the patch pipette) under basal conditions (Fig. 3*A*) and 5 min after treatment with gp120-CHO (Fig. 3*B*). In the untreated cell, most of the resting membrane current was due to outward K^+ movement; the magnitudes of these outward K^+ currents were stable for up to 1 hr (or as long as the seal was intact), even when the external solution was continuously perfused. After gp120-CHO treatment, outward K^+ current increased by 160% and 280% after 3 and 5 min, respectively, and remained constant thereafter. Furthermore, the resting membrane potential decreased by 30–40 mV after gp120 exposure. Identical findings were obtained when either sulfate or gluconate was substituted for chloride. Again, the specificity of this HIV-1 gp120 mediated effect was shown by its elimination by preincubation with anti-gp120 antibodies (data not shown). To test whether the activation of outward K^+ currents was mediated by cellular alkalinization following increased Na^+/H^+ exchange, we performed three additional experi-

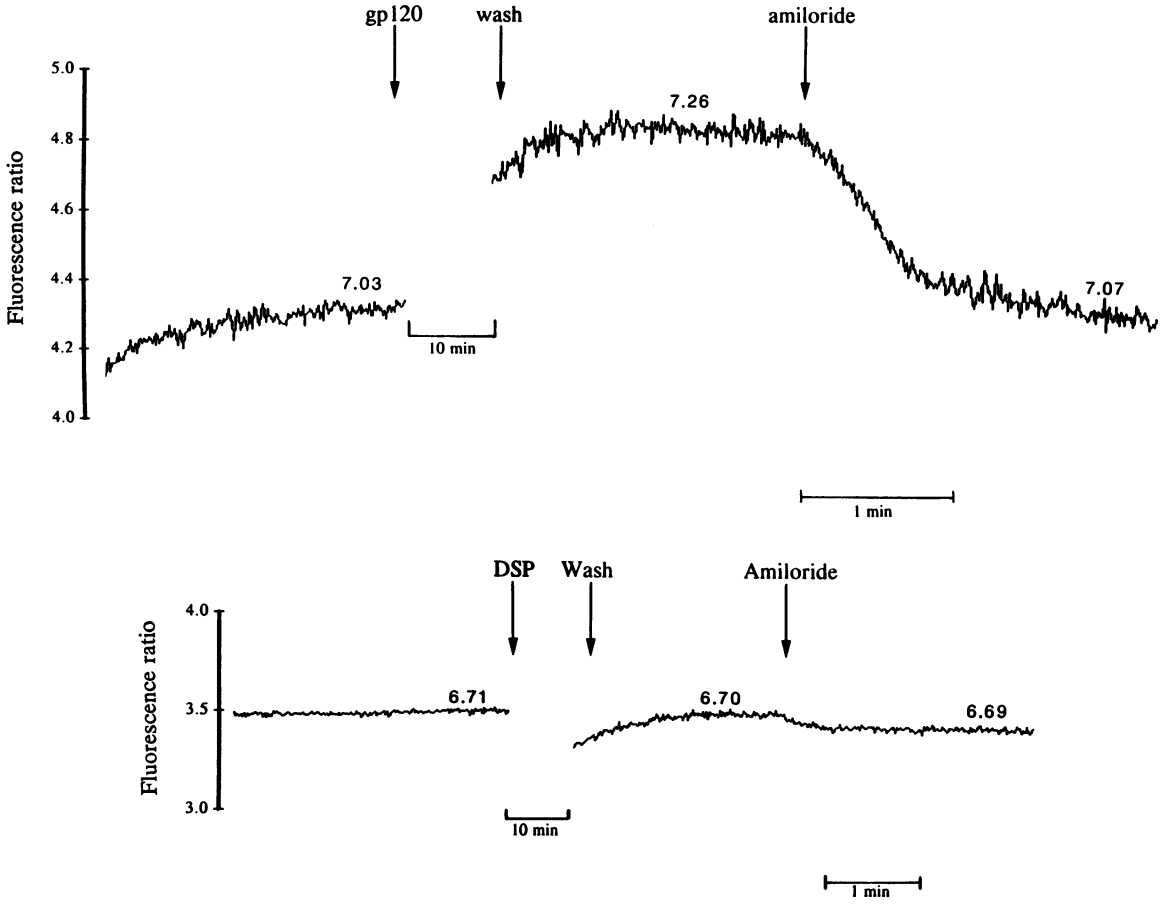

FIG. 2. Effect of gp120 on pH_i in primary human astrocytes. Human astrocytes were cultured as described. Cells were continuously superfused with phosphate-buffered saline at 6.8 ml/min at 37°C. At the indicated times, the coverslips were removed from the chamber, incubated with either 25 nM gp120 or an equivalent concentration of DSP for 10 min at 37°C, and then returned to the chamber for fluorescence measurements (ratio of emission at 520 nm with excitation at 500 nm to that with excitation at 440 nm). pH_i values are shown above the traces. Amiloride was used at 200 μM.

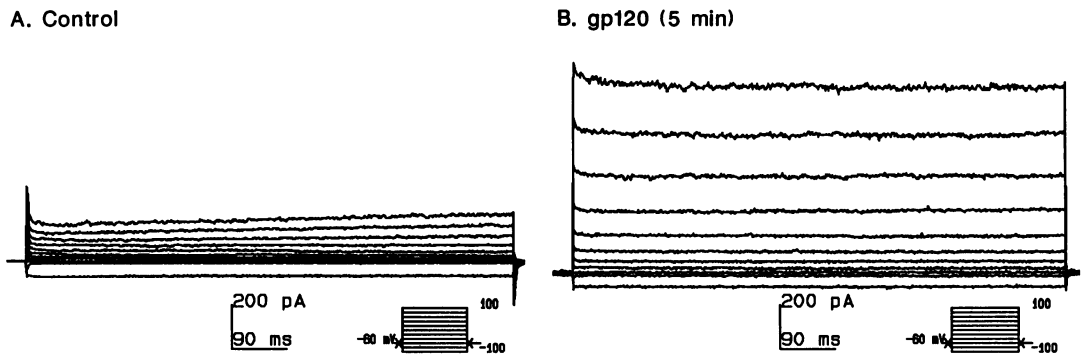

FIG. 3. Effect of gp120 on whole-cell currents recorded from primary cultured rat astrocytes. Cells were cultured in serum-free medium for 24 hr prior to experimentation. The bath solution contained 150 mM NaCl, 6 mM Hepes (pH 7.25), 1 mM EGTA, and 2 mM $CaCl_2$. The pipette contained 150 mM KCl and 1 mM EGTA. Control currents were stable for at least 1 hr, or as long as the seal was intact. (*A*) Control. (*B*) Five minutes after exposure to 25 nM gp120-CHO.

ments. We predicted that gp120-activated outward K^+ current would be prevented if amiloride was present in the bathing medium (thus inhibiting Na^+/H^+ exchange activity), if external Na^+ was replaced by the impermeant cation *N*-methyl-D-glucamine (also eliminating Na^+/H^+ exchange), or if the pipette solution was highly buffered (thus resisting changes in pH_i). All three predictions were borne out by experiment (data not shown). Thus, activation of Na^+/H^+ exchange by HIV-1 gp120 leads to intracellular alkalinization with a subsequent shift of resident K^+ channels to a more open configuration. The whole-cell patch-clamp experiments, because they were performed on single cells that were continuously perfused, ruled out the possibility that low-level contamination of astrocyte cultures by microglia (0–3%) influenced the gp120-mediated effects on Na^+/H^+ antiport stimulated K^+ conductance.

gp120 Stimulation of D-Aspartate Efflux. Increases in pH_i and external K^+ concentration ($[K^+]_o$) (via membrane depolarization) have previously been shown to enhance glutamate release and inhibit its uptake from neurons and astrocytes (14, 17). We verified these observations in the rat astrocytes used in these studies (data not shown) and further demonstrated that after a 10-min exposure of rat astrocytes to 25 nM gp120-CHO, the D-[^{3}H]aspartate efflux rate constant increased nearly 2.5-fold, from an average of 0.204 min^{-1} to 0.504 min^{-1} (n = 4) (Fig. 4). Preincubation of gp120 with anti-gp120 antibodies prevented the stimulation of D-aspartate efflux. Importantly, when D-[^{3}H]aspartate efflux was measured from gp120-treated astrocytes in the presence of 200 μM amiloride, efflux rates did not increase (0.219 min^{-1}, n = 4). Amiloride or DSP alone had no effect on the efflux of D-aspartate, indicating that neither substance directly affects the glutamate transporter. Amiloride did not inhibit the K^+-induced increase in D-aspartate efflux. These findings thus suggest that increases in pH_i resulting from gp120-induced activation of Na^+/H^+ exchange enhance glutamate release from the astrocyte.

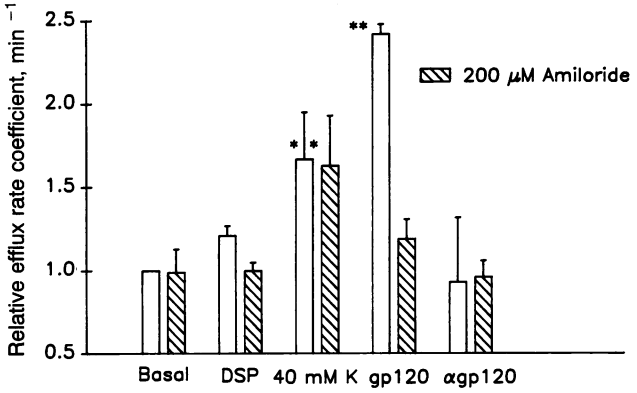

FIG. 4. Effect of gp120 on efflux of D-[^{3}H]aspartate from rat astrocytes. Effluxes were normalized to that observed under basal conditions in the absence of amiloride (0.204 ± 0.013 min^{-1}, n = 5) and are expressed as the mean ± SEM. αgp120, gp120 preincubated with anti-gp120. *, $P < 0.01$; **, $P < 0.001$.

DISCUSSION

The results show that HIV virions, and specifically gp120, can substantially alter Na^+/H^+ exchange, K^+ conductance, and glutamate fluxes in primary human and rat astrocytes. The concentration of purified virus used in these model studies (50 ng of p24 antigen per ml, or 5×10^7 to 5×10^8 virions per ml) is within the range attainable clinically in tissues of patients with acute [Centers for Disease Control and Prevention (CDC) stage I] or far advanced (CDC stage IVC1) infection (36). The concentration of envelope glycoproteins employed, although much higher overall than that present in circulating tissues such as plasma or cerebrospinal fluid, could nonetheless approximate that present in the microenvironment of astrocytes and macrophages/microglia within the tightly compacted neuropil, analogous to other biologically active substances such as neurotransmitters (37). We have shown that cell-free virus, as well as purified gp120, directly alter normal astrocyte Na^+/H^+ exchange function resulting in intracellular alkalinization and K^+ leakage from the cell. The increase in $[K^+]_o$ in the restricted extracellular space surrounding the astrocytes and the neuron can depolarize both cells because large (>35 mM) changes in $[K^+]_o$ can result from small (<1%) losses of K^+ from the astrocytes (38), thereby producing significant depolarizations (30–40 mV) of neuronal membrane potential (39). Neuronal membrane depolarization in turn has been shown to open voltage-dependent Ca^{2+} channels, thereby altering neuronal Ca^{2+} metabolism and eventually producing a rise in neuronal intracellular Ca^{2+} activity. In parallel, we have shown that both elevated $[K^+]_o$ and cytoplasmic alkalinization lead to a diminution in glutamate uptake as well as enhancement of glutamate release by the astrocyte (Fig. 4 and refs. 14 and 17). Glutamate in turn can then activate receptors both on the neuronal and astrocyte membrane, further increasing Ca^{2+} entry (18). Importantly, separate studies have shown that glutamate activation of these receptors on the astrocyte membrane depolarizes the membrane potential, thus further enhancing K^+ loss from the cells into the external compartment (40, 41). Thus, our finding that gp120-induced activation of Na^+/H^+ exchange in astrocytes leads to activation of outward K^+ currents and enhanced glutamate release sug-

gests that direct alteration of astrocyte function by gp120 may contribute to neurotoxicity.

In this regard, there is increasing evidence that gp120 alone cannot directly induce neurotoxicity; rather, glial cells such as the astrocyte and other excitotoxins such as glutamate are required for HIV-1 mediated neurotoxicity. Dreyer *et al.* (6) have shown that gp120 treatment (in picomolar concentrations) of cultured rat retinal ganglion cells and hippocampal neurons increases intracellular free Ca^{2+} with subsequent neurotoxicity. More recent work has suggested that gp120 alone does not induce neurotoxicity, but both gp120 and glutamate-related molecules are necessary for neuronal cell death (7). Both voltage-dependent Ca^{2+} channels and *N*-methyl-D-aspartate receptor-operated channels are required for this form of HIV-related neurotoxicity (7, 18–20).

Recent work from Gendelman and coworkers (8, 12) suggests that HIV-induced CNS disorders are mediated in part through cytokines (tumor necrosis factor α, interleukin 1) and arachidonate metabolites produced by macrophages and that gp120 itself is not neurotoxic. Importantly, they showed that a requirement of HIV-induced neurotoxicity was cell–cell interactions between HIV-infected macrophages and astrocytes. Astrocytes themselves are a direct target for gp120 as evidenced by the data shown in this paper and other studies demonstrating (*i*) gp120 activation of tyrosine kinase in human astroglioma cells (15), (*ii*) gp120 inhibition of β-adrenergic regulation of astroglial function (42), and (*iii*) gp120 inhibition of GFAP expression in human astrocytes (43). These studies indicate that gp120 directly modulates astrocyte function. Thus, in the CNS of ADC patients, neuronal injury is likely mediated by a complex interaction of cytokines, HIV-1 proteins, and neural toxic factors, interacting with macrophages/microglia, neurons, and astrocytes. The ability of astrocytes to respond directly to gp120 by activation of Na^+/H^+ antiport activity, which then leads to subsequent enhanced glutamate efflux, implicates the astrocyte as an important CNS cell type involved in the pathogenesis of HIV encephalopathy.

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