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Selective Effects of Human Immunodeficiency Virus (HIV) gp120 on Invertebrate Neurons

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SUMMARY

1. HIV gp120 selectively reduces the glutamate-induced inward current and the acetylcholine-induced outward current in specific and identified *Aplysia* neurons without affecting dopamine (DA)- and serotonin (5-HT)-induced responses.

2. gp120 specifically decreases DA levels without significantly altering norepinephrine and 5-HT levels in *Mytilus* pedal ganglia.

3. The gp120-associated decrease in DA levels in *Mytilus* is dose dependent and exhibits a threshold level.

4. The alteration of *in vitro* DA levels is specific for gp120 since anti-gp120 blocks the effect.

5. gp120 and its effects appear to be stable due to the duration of treatment and the failure of secondary effects to materialize following antibody treatment.

INTRODUCTION

Among the major pathologies observed in the acquired immunodeficiency syndrome (AIDS) are the development of various neurological and neuropsychiatric deficits (Kent *et al.*, 1993; Rao *et al.*, 1993; Stefano *et al.*, 1993a). The

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agent believed to cause AIDS, human immunodeficiency virus (HIV), may invoke these deficits by gaining entrance into the central nervous system and brain via infected macrophages (Kent *et al.*, 1993; Rao *et al.*, 1993; Stefano *et al.*, 1993*a*). It has been proposed that the major envelope glycoprotein of HIV, gp120, initiates the development of these pathologies by directly binding to neurons by a CD4-dependent mechanism (Rao *et al.*, 1993; Stefano *et al.*, 1993a). However, the presence of CD4 on neurons is highly questionable, as is evidence for an HIV productive infection in neurons (Kent *et al.*, 1993; Rao *et al.*, 1993). Interestingly, alternate mechanisms for HIV penetration into cells have been described (Harouse *et al.*, 1991). Thus, in the absence of productive HIV infection of neurons, gp120, which by itself has been shown to be neurotoxic (Harouse *et al.*, 1991; see da Cunha and Eiden, 1993; Merrill, 1993; Stefano *et al.*, 1993a), could possibly produce neuropathology through mechanisms that are distinct from those involving CD4/gp120 interactions.

The observations showing that HIV can remain latent in the body for periods of up to several years without causing overt disease suggests that mechanisms exist for it to escape immune surveillance. One of these mechanisms may be our recent description of gp120's ability to inhibit chemotaxis of cells treated with neuropeptides or cytokines (Stefano *et al.*, 1993b). Specifically, we found that D-Ala^2-Met-enkephalinamide (DAMA) and interleukin-1 (IL-1) increased the velocity of chemotaxis in human macrophages and granulocytes as well as in invertebrate immunocytes obtained from the bivalve *Mytilus edulis*. The addition of gp120 to these cells resulted in a marked decrease in chemotaxis speed and directionality. We found that the gp120 effect was likely due to a calcium-dependent mechanism based on the effects of the selective calcium channel antagonist nimodipine and the finding that gp120's effects were blocked by anti-gp120 but not soluble CD4. Since the gp120 effects occurred in such highly divergent organisms, these results suggest a universal inhibitory mechanism.

In the present study, we have extended our observations on gp120 inhibitory effects into invertebrate nervous tissue. Here we demonstrate for the first time that gp120 can influence highly specific invertebrate neuronal activities.

MATERIALS AND METHODS

Subtidal *Mytilus edulis* were collected from the shore area of Wading River and Montauk Point, Long Island Sound, New York, and maintained in the laboratory for 1 day prior to testing. They received frequent changes of fresh seawater filtered through gauze. Pedal ganglia were prepared for monoamine analysis as extensively documented elsewhere (Stefano, 1990). Incubations were carried out at a 1:1 ratio of artificial seawater and *Mytilus* hemolymph devoid of hemocytes. The pedal ganglia, 5 pairs /1 ml of solution, were maintained at room temperature with constant aeration for up 48 hr in the presence of recombinant gp120, gp120 + gp120 antibody, or vehicle alone (bovine serum albumin; BSA). In all assay systems polymyxin B was used at a concentration of 15 μg/ml to

minimize any effects due to contaminating endotoxin, which exerts potent stimulatory activities on invertebrate immune cells (Hughes *et al.*, 1991a,b).

Aplysia kurodai (100–300 g) used in these experiments were collected along the shore of Hinomisaki Bay in Japan. Isolated, desheathed abdominal ganglia of *Aplysia kurodai* were pinned in a plexiglass chamber. The ganglia were superfused with buffered saline [normal seawater (NSW) (m*M*): 587 Na^+, 12 K^+, 671 Cl^-, 14 Ca^{2+} and 52 Mg^{2+}] at room temperature (19–20°C). The pH was adjusted to 7.6 with Tris and HCl. Two glass microelectrodes filled with 4 *M* K-acetate were inserted into *Aplysia* neuron R12 for conventional two-electrode voltage clamp (Sawada *et al.*, 1991).

For analysis of the content of biogenic amines from *Mytilus* pedal ganglia, five ganglia were combined and assayed for dopamine (DA) and norepinephrine (NE). The tissues were homogenized by a polytron (setting 4 for 10 sec). In tests for DA, the ganglia were homogenized by a polytron before undergoing a radioenzymatic assay as modified by Stefano and Catapane (1980). After homogenization and centrifugation a 10-μl aliquot of the supernatant was incubated for 1 hr at 37°C in medium containing 50 μg of dithiothreitol, 0.05 μmol of $MgCl^2$, 14 μmol of Tris–HCl buffer, pH 9.6, 1 μl of partially purified catechol-o-methyl transferase, and 1 μl of *S*-[*methyl*-^{3}H]adenosyl-L-methionine (SAM; 14.1 Ci/mmol). After incubation the reaction vials were placed in an ice-water bath and the pH adjusted by the addition of 0.5 μl of 0.5 *M* borate buffer, pH 10. The samples were then processed by thin-layer chromatographic (TLC) separation (Stefano and Catapane, 1980). internal standards consisted of 40-μl aliquots of homogenate plus 10 μl of epinephrine. Blanks consisted of 40 μl of 0.2 *N* perchloric acid or tissue homogenate added to the incubation medium. The method described is able to detect 35 pg of NE/50 μl of sample and 25 pg of DA/50 μl of sample.

Recombinant gp120 and anti-gp120 were obtained from the Repligen Corporation, Cambridge, MA.

RESULTS

Based on our findings that gp120 inhibited chemotaxis of immunocytes stimulated with IL-1 or DAMA and that these materials could modulate voltage-activated ion currents on identified invertebrate neurons, we determined whether gp120 could influence highly specific neuronal activities. Thus, the effects of focal, pressure application of gp120 (P) for 2 min (Fig. 1A) and 4 min (Fig. 1B) on the glutamate-induced inward current recorded from neuron R12 in *Aplysia kurodai* were determined (Sawada *et al.*, 1991). This was performed by micropressure ejection of a 100 m*M* Na-glutamate solution from an electrode onto the neuron, which was voltage clamped at −60 mV. gp120, 1 μg/ml, was then applied in pressure pulses at intervals of 1.5 sec, for a duration of 200 msec, at an intensity of 2 kg/cm. As shown in Figs. 2 and 3, gp120 reduced the glutamate-inward current and the acetylcholine (ACH) outward current without affecting the DA and serotonin (5-HT) responses.

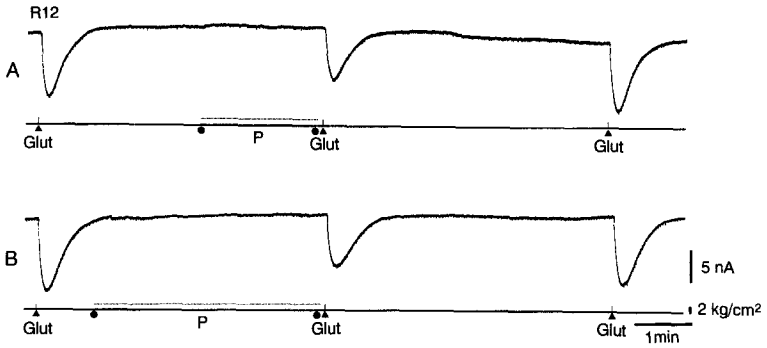

Fig. 1. Effects of focal, pressure application of gp120 (P) for 2 min (A) and 4 min (B) on the glutamate (Glut)-induced inward current recorded from neuron R12 voltage clamped at −60 mV. Glut was applied by micropressure ejection from a electrode filled with 100 m*M* Na-glutamate. gp120 was applied to the soma of the neuron by pressure pulses at intervals of 1.5 sec with a duration of 200 msec (between filled circles). The concentration of gp120 in the pipette was 1 μg/ml. The Glut-induced inward current is due to an increase in nonspecific cation conductance and reversed at a holding potential of −27 mV, although we could not identify the type of the glutamate receptor, *N*-methyl-D-aspartate, K, and Q. Glut was applied with a constant pressure pulse (200 msec in duration, 2 kg/cm^2 in intensity).

On the other hand, *Mytilus* pedal ganglia incubated in gp120 (10 n*M*) for 48 hr exhibited a decrease in DA levels compared to vehicle-treated controls (−33 and −8%, respectively; $P < 0.01$) (Fig. 4). gp120 at 1 n*M* had no effect, while 100 n*M* treatment gave the same results (−37%) as 10 n*M*, indicating a threshold effect of gp120 in this system. The effect appeared to be due specifically to gp120 since anti-gp120 reversed the decrease in DA levels to vehicle-treated control values (−10%) (Fig. 4).

There was a slight reduction in NE levels (−18%) but this was not statistically significant compared to controls. Interestingly, prior addition of

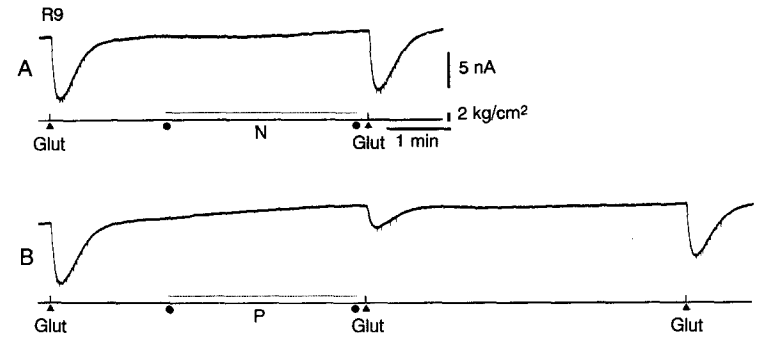

Fig. 2. Effects of focal, pressure application of (A) NSW (N) and (B) gp120 (P) on the Glut-induced inward current recorded from neuron R9 voltage clamped at −50 mV. Glut was ejected with a constant pressurre pulse (500 msec, 2 kg/cm^2). NSW and gp120 (1 μg/ml) were applied for 3 min by pressure pulses at intervals of 1.5 sec with a duration of 400 msec (between filled circles).

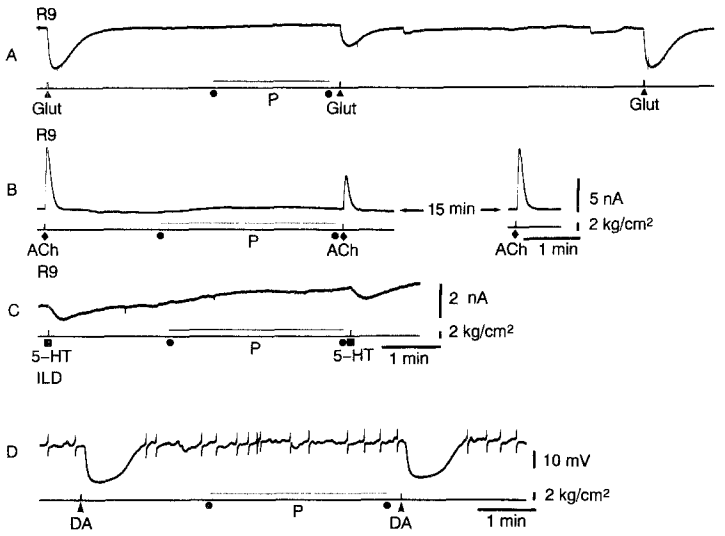

Fig. 3. Effects of focal, pressure application of gp120 on the Glut-induced inward current (A), the ACH-induced outward current (B), and 5-HT-induced inward current (C) recorded from the same R9 neuron voltage clamped at −50 mV. (D) Effects of focal application of gp120 on the DA-induced hyperpolarization recorded from neuron ILD (Sawada *et al.*, 1980). gp120 (P) was applied to some of the neuron for 2 min (A) or 3 min (B–D) by pressure pulses at intervals of 1.5 sec with a duration of 300 msec. Glut was ejected by a constant pressure pulse (700 msec, 2 kg/cm^2). ACH was ejected by a constant pressure pulse (500 msec, 2 kg/cm^2). 5-HT was ejected by a constant pressure pulse (900 msec, 3 kg/cm^2). DA was ejected by a constant pressure pulse (500 msec, 2 kg/cm^2). The resting potential of neuron ILD was −45 mV. The DA-induced outward current is due to an increase in K^+ conductance. The 5-HT-induced inward current is due to a decrease in K^+ conductance.

anti-gp120 prevented the nonsignificant drop in NE levels. There were no effects on the 5-HT levels. It is likely that when a greater number of ganglia are used to determine the effects of gp120 on catecholamine levels, statistically significant effects will be observed for NE. Finally, in light of the known presence of high concentrations of proteolytic enzymes in these preparations (Leung *et al.*, 1992; Stefano, 1992), it appears that gp120 is stable since (1) its effect occurred maximally after 48 hr and (2) secondary effects did not materialize over the 48-hr treatment period in the groups initially treated with gp120 plus anti-gp120.

DISCUSSION

Taken together, our results demonstrate that (1) gp120 can selectively reduce the glutamate-induced inward current and the ACH-induced outward current in specific and identified *Aplysia* neurons without affecting DA- and 5-HT-induced responses; (2) gp120 specifically decreases DA levels without significantly altering NE and 5-HT levels in *Mytilus* pedal ganglia; (3) the decrease in DA levels in

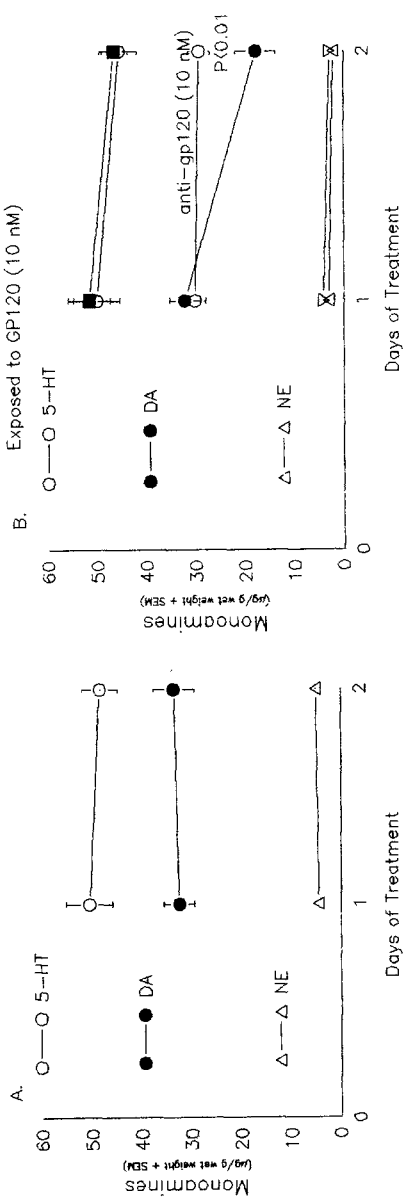

Fig. 4. HIV gp120 *in vitro* incubation and subsequent alteration of DA levels in the pedal ganlia of *M. edulis*. For analysis of the content of biogenic amines from *Mytilus* pedal ganglia, five ganglia were combined and assayed per treatment per dose and replicated five times. Additionally, five ganglia were incubated with gp120 and anti-gp120. The group incubated for 48 hr with anti-gp120 alone exhibited DA levels similar to those of the group incubated alone (data not shown). After 48 hr ganglia incubated alone (A) exhibited significantly ($P < 0.01$, Student's t test) higher DA levels than those incubated with gp120 (B).

Mytilus is dose dependent and exhibits a threshold level; (4) this decrease is specific for gp120 since anti-gp120 blocks the effect; and (5) gp120 and its effects appear to be stable due to the duration of treatment and the failure of secondary effects to materialize following antibody treatment.

In mammals, gp120 has been shown to be highly toxic to nervous tissue. It has been shown to kill neurons (Brenneman *et al.*, 1988), presumably following its release and metabolism from $CD4^+$-infected cells (Hart *et al.*, 1991). The neurotoxic effect can be blocked in mammalian nervous tissue by anti-gp120 (Kaiser *et al.*, 1990), suggesting that the neurotoxic effect may be due specifically to gp120 in the absence of infection (Wiley *et al.*, 1986; Ketzler *et al.*, 1990; Masliah *et al.*, 1992). In mammalian neurons gp120 may exert its effects by way of a glutaminergic mechanism involving calcium, enhancing the intracellular calcium level to the point of causing toxicity (Dreyer *et al.*, 1990; Lipton *et al.*, 1990, 1991; da Cunha-Eiden, 1993). Induction of quinolinic acid would also be a calcium-dependent, indirect neurotoxic mechanism (da Cunha and Eiden, 1993). It is important to point out that these neurotoxic products [IL-1, tumor necrosis factor, (TNF), nitric oxide, and quinolinic acid] may emerge from macrophages (Stefano *et al.*, 1994) whose behavior has been altered from exposure to HIV, gp120, or a combination of the two (Stefano *et al.* 1993a,b). Indeed, the calcium involvement noted above may stimulate nonspecific signal molecule release from macrophage/microglial cells. The fact that invertebrate immunocytes act and function as macrophage-like cells (Hughes *et al.*, 1990) may help to explain the results of the present study, since these cells can be found in invertebrate ganglia and peripheral nerves (Paemen *et al.*, 1992; Stefano, 1992; Stefano *et al.*, 1989, 1993c).

The present study, demonstrating the alteration in the glutaminergic responses in *Aplysia* identified neurons, offers an interesting similarity since it, too, may be operating through a calcium-dependent mechanism. In support of this contention, we have observed that exposure of *Mytilus* pedal ganglia to sodium glutamate (10^{-6} *M*) results in decreased DA levels (unpublished observations). Thus, the present results obtained with *Mytilus* suggest that a common glutaminergic mechanism may be occurring.

Of equal interest concerning the potential of gp120 to cause neuronal damage is the alternate hypothesis that gp120 may be damaging nervous tissue indirectly through its induction of cytotoxic levels of cytokines such as TNF and IL-1 (Merrill *et al.*, 1992). Supporting this, we have shown that *Mytilus* contains immunoreactive TNF and IL-1 in both immune and nervous tissue (Hughes *et al.*, 1992; Paemen *et al.*, 1992; Stefano, 1992). Our finding that the calcium channel blocker nimodipine blocks gp120 activity in invertebrate immunocytes (Stefano *et al.*, 1993b) further demonstrates the similarity of gp120 in vertebrates and invertebrates. The presence of these cytokines in invertebrate neural tissue, as well as macrophage/glial cells, and their ability to initiate specific neurophysiological activity (Sawada *et al.*, 1991; Szûcs *et al.*, 1992; Stefano *et al.*, 1994) further support the hypothesis that gp120 may be exerting its effects through a highly conserved mechanism. The information also suggests that this type of viral activity may not be unique to man.

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