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GPI-1046 protects dorsal root ganglia from gp120-induced axonal injury by modulating store-operated calcium entry

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Abstract

Human immunodeficiency virus (HIV)-associated sensory neuropathy (HIV-SN) occurs in a large fraction of patients infected with HIV. Viral components, including the coat protein gp120, are thought to exert toxic actions on dorsal root ganglia (DRG) sensory neurons that can be further exacerbated by treatment of HIV infection with some antiretroviral agents. In a tissue culture model of HIV-SN, we found that gp120-induced axonal degeneration in DRG sensory neurons was prevented by treatment with the immunophilin ligand GPI-1046. Gp120 induced a rapid and large release of endoplasmic reticulum (ER) calcium in DRG neurons that was attenuated by treatment with GPI-1046. Further experiments suggested that GPI-1046 reduced the total ER calcium load by attenuating store-operated calcium (SOC) entry. Together, these results suggest that GPI-1046 protects DRG from gp120-induced axonal damage by decreasing the entry of calcium through SOC, thus reducing the total volume of ER calcium that is available to be released by gp120.

Keywords

axon; calcium; gp120; HIV; peripheral neuropathy

Introduction

Human immunodeficiency virus (HIV)-associated sensory neuropathy (HIV-SN) is a common neurological disorder that can occur during the course of HIV infection. HIV-SN is characterized clinically by the gradual onset of a painful burning or tingling sensation in the extremities that is typically more severe on the soles of the feet. Patients often exhibit hyperalgesia, allodynia, and dysesthesia that over the course of a few months can ascend proximally up the lower extremities to involve the fingertips (reviewed in Hoke and Cornblath, 2005; Cornblath and Hoke, 2006). Ankle reflexes are typically reduced or absent. Pathological findings include macrophage and lymphocyte infiltration, increased frequency of Nageotte nodules, distal degeneration of long axons, reduction in the density of small and large myelinated and unmyelinated fibers, and a death of dorsal root ganglia (DRG) sensory neurons (Pardo et al., 2001). HIV-SN can result from toxicities associated with antiretroviral agents (primarily the d-drugs; ddI, ddC, d4T) but may also result from the direct and indirect actions of a number of HIV-proteins (reviewed in Keswani et al., 2002). There is in addition evidence that suggests that there may be a synergistic toxic effect of HIV-1 proteins and antiretroviral agents on DRG neurons (Bodner et al., 2004; Robinson et al., 2007).

A number of molecular mechanisms have been identified that are thought to contribute to the dysfunction and death of DRG in the setting of HIV infection, including complement-dependent mechanisms (Apostolski et al., 1994), activation of the pro-apoptotic caspase pathway (Keswani et al., 2003b; Melli et al., 2006), proinflammatory cytokines (Zhu et al., 2005), upregulation of inducible NO synthase, STAT-1 (Zhu et al., 2005), signaling via the c-Jun N-terminal kinase pathway (Bodner et al., 2004), and dysregulation of cytosolic calcium ($[Ca^{2+}]_C$) handling (Oh et al., 2001). In multiple types of neurons, including DRG, inhibition of inositol triphosphate receptors prevents $[Ca^{2+}]_C$ dysregulation and protects cells from the toxic actions of HIV-1 proteins Tat and gp120 (Zheng et al., 1999), suggesting that drugs that modulate calcium handling may be useful therapeutics in HIV-SN. Recently, we demonstrated that the immunophilin ligand GPI-1046 reduced endoplasmic reticulum (ER) calcium load and protected hippocampal neurons from the toxic effects of gp120 and Tat (Caporello et al., 2006). We now provide evidence that GPI-1046 reduces store-operated calcium (SOC) entry in DRG neurons.

Materials and Methods

Tissue culture

Primary DRG neuronal cells were prepared from 15-day-old embryonic Sprague-Dawley rats as described before (Keswani et al., 2003a). Disassociated DRG neurons were plated onto 15-mm round glass coverslips coated with laminin and a monolayer of Schwann cells in NBI medium containing 1% neuronal growth hormone (Invitrogen).

Axonal degeneration assay

For toxicity, DRG neurons were plated at 13,000 cells/coverslip in a 24-well plate. After 3 days *in vitro*, neurons were treated with 200 pM gp120_{IIIB} (Advanced Biotechnologies Inc.) with 0–1,000 nM GPI-1046 (Guilford Inc.) for 15 h before fixation. Individual neurites were visualized by immunofluorescence using anti- β III-tubulin antibody (1 : 1,000; Chemicon) conjugated to AlexaFluor 546 (1 : 2000; Molecular Probes Inc). Measurements of the total neurite length were determined using non-biased stereological methods on a Zeiss LSM510 Meta confocal imaging system (Zeiss).

Calcium imaging

DRG neurons loaded with 4 μ M Fura2/AM (containing 0.001% pluronic) for 25 min at 37°C. Cells were washed with Locke's buffer (154 mM NaCl, 3.6 mM NaHCO₃, 5.6 mM KCl, 1 mM MgCl₂, 5 mM HEPES, 2.3 mM CaCl₂, and 10 mM glucose; pH 7.4) to remove extracellular Fura2/AM. Fura2 loaded cells were mounted into a perfusion chamber and maintained at 37°C for the duration of the experiment. Cells were excited at 340 and 380 nm, and emission was recorded at 508 nm with an imaging system (Intracellular Imaging Inc.) equipped with a high performance CCD camera (Cooke Corporation SensiCam QE) and a Bentham (FSM150Xe) shutter system. DRG neurons were perfused with buffer at the rate of 2 ml/min and gp120_{IIIB} or SDF-1 α was applied by pipette. To deplete ER calcium stores, the bathing buffer was switched to calcium-free Lockes (equal molar amount of ethylenedinitrilotetraacetic acid replaced calcium) and thapsigargin was added by pipette. The volume of calcium in ER was determined by measuring the area under the curve that was created when calcium leaked from the ER to the cytosol and out of the cell. The rate of SOC influx was determined during reperfusion with calcium-containing Lockes and was calculated by performing a linear regression from the initial sharp increase in $[Ca^{2+}]_C$ levels to the plateau of calcium entry.

Results

GPI-1046 protects DRG neurons from gp120-induced axonal injury

We first determined if GPI-1046 could protect DRG neurons from gp120-induced neurite degeneration. We used gp120_{IIIB}, a form of this protein that binds CXCR4 and is directly toxic to neurons by mechanisms that involve the mobilization of inositol triphosphate (IP₃)-sensitive calcium pools (Nath et al., 2000; Pandey and Bolsover 2000; Corasaniti et al., 2001; Bachis and Mocchetti 2004). An inhibitory antibody directed against CXCR4 but not CCR5 prevented gp120_{IIIB}-evoked increases of calcium in DRG neurons, confirming that this form of gp120 selectively binds CXCR4 (data not shown). In previous dose- and time-response experiments from our laboratories, we determined that a 200-pM dose of gp120 applied to DRG neurons for 15 h reduced average neurite length, with evidence of apoptosis seen only in a small number of cells (Keswani et al., 2003b). Longer exposures of gp120 to DRG increased the number of cells with apoptotic features, suggesting that neurite degeneration precedes neuronal death (Melli et al., 2006). We therefore used a 200-pM dose of gp120 and measured neurite length after 15 h. gp120 significantly reduced total neurite length in cultured DRG neurons (Fig. 1). Simultaneous treatment of cultures with GPI-1046 dose dependently protected DRG neurons from gp120-induced neurite degeneration and at high dose slightly increased total neurite length, suggesting that this immunophilin ligand is neuroprotective and may promote neurite sprouting in DRG (Fig. 1).

IP₃ receptor-mediated calcium release is reduced by GPI-1046

Forms of gp120 that bind the chemokine receptor CXCR4 are directly toxic to neurons by mechanisms that involve the release of IP₃-sensitive ER calcium stores (Nath et al., 2000; Pandey and Bolsover 2000; Corasaniti et al., 2001; Bachis and Mocchetti 2004). We therefore determined if GPI-1046 modified gp120-evoked release of ER calcium in DRG neurons. gp120 (200 pM) directly applied to cultured DRG neurons rapidly increased [Ca²⁺]_C levels in DRG neurons. Inhibition of ryanodine-sensitive ER calcium stores with the plant alkaloid ryanodine (1 μM) did not reduce gp120-evoked increases of [Ca²⁺]_C, suggesting that these calcium-induced calcium release channels are not activated by gp120. Inhibition of IP₃-sensitive ER stores with the marine alkaloid xestospongine (10 μM), reduced gp120-evoked increases of [Ca²⁺]_C by twofold, suggesting that the primary source of calcium was from IP₃-gated stores (Fig. 2A). We next determined if GPI-1046 modified gp120-evoked release of ER calcium stores. A 20-min pretreatment of DRG neurons with GPI-1046 (100 nM) reduced basal levels of [Ca²⁺]_C and reduced the amplitude of gp120-evoked increases of [Ca²⁺]_C (Figs. 2B and 2C). To determine if GPI-1046 interfered with gp120-binding to CXCR4 or IP₃-sensitive ER calcium release, we used SDF-1α (a natural ligand for CXCR4) to induce the generation of IP₃ and trigger ER calcium release. Direct applications of SDF-1α (100 nM) rapidly increased [Ca²⁺]_C in DRG neurons, and these responses were dose dependently inhibited by pretreatment with GPI-1046 (Fig. 2D). SDF-evoked increases of [Ca²⁺]_C were not inhibited by ryanodine but were blocked by preincubation of DRG neurons with xestospongine and reduced twofold by GPI-1046, suggesting that GPI-1046 reduced the amount of calcium released from IP₃-sensitive ER (Fig. 2E).

GPI-1046 reduces ER calcium

We next sought to determine if GPI-1046 reduced IP₃-sensitive calcium release by interfering with IP₃-channel activation or by a direct modification of ER calcium load. To accomplish this, we removed calcium from the bathing solution, depleted ER calcium stores with the sarco-endoplasmic reticulum calcium (SERCA) pump inhibitor thapsigargin (1 μM), and measured area under the curve created as calcium flows down its concentration gradient from the ER to the cytosol and out of the cell (Figs. 3A and 3B). Pretreatment of DRG neurons with GPI-1046 (100 nM) reduced area under the [Ca²⁺]_C curve, suggesting that this immunophilin ligand

reduced the amount of thapsigargin releasable calcium in the ER (Figs. 3B and 3C). The prototypic immunophilin ligand FK506 (100 nM) also lowered the thapsigargin releasable pool of ER calcium, but the inactive immunophilin ligand GPI1334 did not modify the amount of calcium stored in ER (Fig. 3D).

GPI-1046 regulates SOC channels

A reduction in the calcium concentration within ER activates signaling pathways that open plasma membrane located SOC to initiate the refilling of calcium stores. To determine if this mechanism was perturbed by GPI-1046, we bathed cells in calcium-deficient media, depleted ER calcium stores with thapsigargin, and then reintroduced calcium into the bathing buffer. The slope and maximal amount of calcium entry into the cytosol are measures of SOC activity (Fig. 4A). There were significant reductions in the slope and rate of SOC entry in DRG neurons pretreated with GPI-1046, suggesting that this immunophilin ligand interfered with function of SOC (Figs. 4B–4D).

Discussion

gp120 induced a ‘dying back’ of axons in DRG neurons that was dose dependently inhibited by the immunophilin ligand GPI-1046. A complete protection of DRG axons was achieved with concentrations of GPI-1046 ≥ 100 nM. This concentration of GPI-1046 is achievable in plasma with once daily dosing of the drug, in amounts that do not result in any adverse effects (J. Steiner, Department of Neurology, Johns Hopkins University School of Medicine, personal communication). Currently, there is no treatment available for HIV-infected patients who suffer from distal sensory polyneuropathy. Although the macrolide antibiotic FK506 showed promising neuroprotective potential in tissue culture and rodent models of neuronal injury, including antiretroviral-induced toxic neuropathy (Steiner et al., 1997; Bavetta et al., 1999; Guo et al., 2001b; Tanaka et al., 2002; Keswani et al., 2003a; Furuichi et al., 2004), potent immunosuppressant properties and side effects linked to its immunosuppressive actions made this drug unsuitable for use in humans. The immunophilin ligand, GPI-1046, was synthesized as a non-immunosuppressive analog of FK506. The immunosuppressant actions of FK506 result from the FK506/FKBP12 complex binding to and inactivating protein phosphatase 2B (calcineurin). Similar to FK506, GPI-1046 also interacts with FKBP12-binding proteins, however, GPI-1046, does not inhibit calcineurin and therefore does not have immunosuppressive effects.

GPI-1046 has been shown to be neuroprotective in models of HIV-1 Tat- and gp120-induced death of hippocampal neurons (Caporello et al., 2006), and our current findings suggest that GPI-1046 can also protect DRG sensory axons from gp120-induced damage. There are additional reports that suggest GPI-1046 also has neurotrophic effects that can stimulate the outgrowth of damaged peripheral and central neurons (Guo et al., 2001b; Tanaka et al., 2002; Burnett and Becker, 2004; Valentine et al., 2007). These neuroprotective and neurotrophic actions of GPI-1046 are independent of FKBP12 (Guo et al., 2001b), suggesting that the mechanism of neuroprotection by GPI-1046 is independent of calcineurin. Although the exact mechanism of neuroprotection is still unknown, roles for modulating redox balance and ER calcium have been suggested (Tanaka et al., 2001; Caporello et al., 2006). Potential roles for GPI-1046 in modulating ER calcium are especially intriguing because dysregulated IP₃ receptor function has been implicated as a key mechanism of neural dysfunction in several neurodegenerative diseases, including Alzheimer's, stroke, and HIV dementia (Huang et al., 1991; Kirino et al., 1992; Haughey et al., 1999; 2001; Leissring et al., 1999). It has been previously reported that T-cell trophic forms of gp120 can directly induce the death of neurons by activation of CXCR4 (Hesselgesser et al., 1998; Catani et al., 2000; Corasaniti et al., 2001; Bachis and Mocchiatti, 2004). Activation of the α -chemokine receptor CXCR4 promotes

the hydrolysis of phosphatidylinositol bisphosphate to diacylglycerol and a soluble messenger IP_3 that triggers the release of Ca^{2+} from IP_3 -sensitive ER Ca^{2+} stores. Inhibition of this pathway either by blocking the interaction of gp120 with CXCR4 or preventing IP_3 -triggered ER calcium release is neuroprotective (Meucci et al., 1998; Catani et al., 2000; Pandey and Bolsover, 2000; Bachis and Mocchetti, 2004). In experimental models of HIV-associated neurological disease, it has been demonstrated that gp120-induced calcium release via IP_3 receptors is related to a deregulation of glutamate transport, cytokine and chemokine expression in glia, and sensitizes neurons to excitotoxic insult by modifying the kinetics of NMDA and voltage-operated calcium channels (Dreyer et al., 1990; Lipton, 1992; Lo et al., 1992; Lannuzel et al., 1995; Holden et al., 1999; Medina et al., 1999; Hegg et al., 2000; Liu et al., 2000). In DRG neurons, gp120 interacts with chemokine receptors on axons and activates a mitochondrial caspase pathway that triggers axonal degeneration and ultimately neuronal death (Melli et al., 2006).

We previously reported that GPI-1046 protected hippocampal neurons from the lethal effects of gp120 and Tat by reducing ER calcium load (Caporello et al., 2006). In the current study, we found that GPI-1046 has a similar mechanism of protection in DRG neurons exposed to gp120 and extended these initial observations with evidence that suggests that GPI-1046 reduces ER calcium load by down modulating SOC. Several macrocyclic lactone compounds in addition to GPI-1046 are known to modulate ER calcium flux including ivermectin, FK506, ascomycin, and rapamycin. FK506 has been shown to enhance IP_3 and ryanodine receptor-mediated calcium release and can inhibit SERCA pumps (Bultynck et al., 2000; Bilmen et al., 2002). FK506 modulates ER calcium by binding FKBP12 and preventing association of the FKBP12/calcineurin complex with IP_3 receptors. Calcineurin is typically anchored to IP_3 receptors by FKBP12, where it participates in channel gating by regulating the phosphorylation of IP_3 and ryanodine receptor subunits (Cameron et al., 1995a; 1995b; Snyder et al., 1998). Several lines of evidence suggest that GPI-1046 may lower ER calcium load by a mechanism distinct from the actions of FK506 on ER calcium. GPI-1046 does not interfere with calcineurin and the neuroprotective actions of GPI-1046 are similar in FKBP12 knockout mice (Guo et al., 2001a), suggesting that GPI-1046 does not modulate ER calcium load by interfering with the actions of the FKBP12/calcineurin complex on ER channel gating. Our findings suggest that a mechanism by which GPI-1046 reduces ER calcium load may involve a down modulation of the rate of calcium influx through SOC.

Release of ER Ca^{2+} is typically followed by Ca^{2+} entry into cells via SOC channels. To date, a number of SOC have been identified including calcium release-activated calcium channels (Hoth and Penner, 1992) and several members of the transient receptor potential superfamily (see Smyth et al., 2006, for a review). Although the properties of these channels have been studied for nearly two decades, the mechanisms that regulate their function remain largely obscure (see Vig and Kinet, 2007; Putney and Bird, 2008, for recent reviews). Based on evidence that the neuroprotective actions of GPI-1046 are independent of calcineurin, a known modulator of SOC and FK506 (Albert et al., 2006), it is likely that the modulation of SOC by GPI-1046 is independent of these proteins. Work is underway in our labs to identify the SOC(s) modulated by GPI-1046 and the mechanism of the drugs action on SOC.

Despite promising experimental results that suggest modulating ER calcium may be neuroprotective, the translation of these findings to therapeutics has been difficult because of the inability of many IP_3 receptor antagonists to enter into cells (such as heparin, which has limited cell permeability) and because of toxicity that can occur when IP_3 signaling is blocked. Because GPI-1046 reduces total ER calcium load, residual IP_3 and ryanodine receptor-mediated calcium flux still occur. Thus, normal cellular function may be preserved. Indeed, a second generation non-immunosuppressive immunophilin ligand, GPI-1485, is under investigation in a human phase II clinical trial for Parkinson's disease with no reported adverse

effects. The efficacy of this drug to slow or halt the progression of HIV-SN remains to be determined.

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Figure 1.

GPI-1046 rescued dorsal root ganglia (DRG) neurons from gp120-induced axonal injury. DRG neuronal cultures were exposed to the indicated compounds for 15 h and total neurite length was measured. Representative micrographs of DRG neurons with (A) no treatment (control), (B) gp120 (200 pM), and (C) GPI-1046 (100 nM) + gp120 (200 pM). (D) gp120 significantly reduced the total neurite length of DRG neurons compared with untreated cultures. GPI-1046 (1–1000 nM) dose dependently protected neurites from the toxic effects of gp120. ANOVA with Tukey post-hoc comparisons. Each condition was performed in triplicate and experiments were repeated three to six times. * $p < 0.05$ compared with gp120; ** $p < 0.001$ compared with control.

Figure 2.

GPI-1046 reduced gp120-evoked increases in cytosolic calcium. Cytosolic calcium ($[Ca^{2+}]_c$) concentrations in dorsal root ganglia (DRG) neurons were determined using Fura2-based imaging techniques. (A) gp120-evoked (200 pM) increases of $[Ca^{2+}]_c$ were not altered by pretreatment of DRG neurons with the ryanodine receptor blocker ryanodine (1 μ M) but were significantly reduced by pretreatment with the IP₃ receptor antagonist xestospongine (10 μ M). (B) Representative traces of calcium responses to gp120 (200 pM) and gp120 following preincubation with GPI-1046 (100 nM); arrowhead indicates the point of gp120 addition. (C) Quantification of peak $[Ca^{2+}]_c$ responses to gp120 showing a significant reduction after pretreatment of DRG neurons with GPI-1046. ANOVA with Tukey post-hoc comparisons. $n = 3-6$ cells per experiment with three experiments per condition. * $p < 0.05$; *** $p < 0.001$.

Figure 3.

GPI-1046 reduced endoplasmic reticulum calcium load. Dorsal root ganglia (DRG) neurons were bathed with Ca^{2+} -free buffer (a molar amount of ethylenedinitrilotetraacetic acid equal to the missing Ca^{2+} was added to chelate any trace amounts of calcium and normalize osmolarity), and ER calcium stores were released by inhibiting sarco-endoplasmic reticulum calcium pumps with thapsigargin (1 μ M). (A) Representative images (approximately every 10th image of an experiment is shown) of DRG neurons pretreated with vehicle or GPI-1046 (100 nM) showing gp120-evoked increases of $[Ca^{2+}]_c$. Original grayscale images were converted to pseudocolor in which increasing calcium concentration is depicted by warmer colors. (B) Summary traces showing $[Ca^{2+}]_c$ levels after thapsigargin (1 μ M) in DRG neurons pretreated with vehicle or GPI-1046 (100 nM). (C) Summary traces showing $[Ca^{2+}]_c$ levels after thapsigargin (1 μ M) in DRG neurons pretreated with vehicle, FK506 (5 μ M), or/and inactive GPI-1046 analog GPI1334 (100 nM). (D) Quantitative analysis showing that pretreatment with GPI-1046 significantly reduced the thapsigargin releasable pool of ER Ca^{2+} in DRG neurons. Data are the summary of 44–61 neurons from three independent experiments per condition. ANOVA with Tukey post-hoc comparisons. ***p < 0.001.

Figure 4.

GPI-1046 reduced the rate of calcium influx through store-operated calcium channels. Dorsal root ganglia (DRG) neurons were bathed with Ca^{2+} -free buffer (a molar amount of ethylenedinitrilotetraacetic acid equal to the missing Ca^{2+} was added to chelate any trace amounts of calcium and normalize osmolality), and ER calcium stores were released by inhibiting sarco-endoplasmic reticulum calcium pumps with thapsigargin ($1\ \mu\text{M}$). After $[\text{Ca}^{2+}]_i$ levels returned to a stable baseline, DRG neurons were bathed in calcium-containing buffer to allow capacitive calcium entry. (A) Representative images of capacitive calcium entry in DRG neurons directly following the reintroduction of calcium-containing buffer. Original grayscale images were converted to pseudocolor in which increasing calcium concentration is depicted by warmer colors. (B) Summary traces showing $[\text{Ca}^{2+}]_i$ levels during (1) baseline in Ca^{2+} -containing buffer; (2) the introduction of calcium-free buffer; (3) thapsigargin ($1\ \mu\text{M}$)-induced release of ER calcium stores; and (4) the reintroduction of calcium-containing buffer in vehicle and GPI-1046 pretreated DRG neurons. (C) The slope of calcium entry during the reintroduction of Ca^{2+} was less in GPI-1046 pretreated DRG neurons. (D) The rate of Ca^{2+} influx was significantly slower in DRG neurons pretreated with GPI-1046; $n = 44\text{--}61$ cells

derived from three separate experiments per condition. ANOVA with Tukey post-hoc comparisons. *** $p < 0.0001$.