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Mobilization of intracellular calcium stores participates in the rise of $[Ca^{2+}]_i$ and the toxic actions of the HIV coat protein GP120

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Abstract

The HIV envelope glycoprotein, GP120, increases intracellular Ca^{2+} concentration and induces degeneration of human and animal neurons in culture. Using patch-clamp recordings and Ca^{2+} imaging techniques, we have now examined the contribution of intracellular stores of calcium in the effects of GP120. We report that in rat hippocampal neuronal cultures, GP120 induces a dramatic and persistent increase in $[Ca^{2+}]_i$ which is prevented by drugs that either deplete (caffeine, carbachol, thapsigargin) or block (dantrolene) Ca^{2+} release from intracellular stores. In contrast, *N*-methyl-D-aspartate (NMDA) receptors or voltage-dependent calcium channels do not participate in these effects, as: (i) the increase in $[Ca^{2+}]_i$ was not affected by NMDA receptor antagonists or calcium channel blockers; and (ii) and GP120 did not generate any current in whole-cell recording. Dantrolene, a ryanodine stores inhibitor, also prevented neuronal death induced by GP120. Our results show that the GP120-induced rise in $[Ca^{2+}]_i$ originates from intracellular calcium stores, and suggest that intracellular stores of calcium may play a determinant role in the pathological actions of GP120.

Introduction

Both native and recombinant envelope glycoprotein GP120 of the human immunodeficiency virus type 1 (HIV-1) induce neurotoxicity in cultures of rodent retinal ganglion cells or hippocampal neurons (Brenneman *et al*., 1988; Dreyer *et al*., 1990; Lo *et al*., 1992; Muller *et al*., 1992; Savio & Levi, 1993; Meuci & Miller, 1996) and human brain neuronal cultures (Lannuzel *et al*., 1995). This protein may therefore participate in the central nervous system (CNS) damage that occurs in patients with acquired immunodeficiency syndrome (AIDS) (Price *et al*., 1988; Ketzler *et al*., 1990). The mechanisms underlying this toxicity have not been determined, but there are several indications that, as for other brain pathologies (Choi, 1988), the damage may be mediated by a toxic accumulation of Ca^{2+}. In cultures of rat neurons, GP120 induced a slow rise in $[Ca^{2+}]_i$, which was blocked by *N*-methyl-D-aspartate (NMDA) receptor antagonists (Lipton *et al*., 1991; Lo *et al*., 1992) or L-type calcium channel blockers (Dreyer *et al*., 1990; Lo *et al*., 1992). However, GP120 did not change $[Ca^{2+}]_i$ in embryonic cultures of rat hippocampal neurons (Meuci & Miller, 1996) or human CNS neurons (Lannuzel *et al*., 1995), but potentiated the NMDA-induced Ca^{2+} rise in both preparations. GP120-induced neurotoxicity is also abrogated by the NMDA receptor-channel blocker MK801 (Lipton *et al*., 1991; Sindou *et al*., 1994; Lannuzel *et al*., 1995; Meuci & Miller, 1996) and by blockers of voltage-gated calcium channels (Dreyer *et al*., 1990), suggesting that external calcium may provide the sources for the toxicity. On the other hand, there are several indications that intracellular stores of calcium may also play a role in various pathologies, including those that depend on the activation of NMDA receptors (review in Mody & MacDonald, 1995).

We have now studied the role of intracellular stores of calcium in the effects of GP120 on hippocampal neurons in culture using whole-cell recordings in parallel with intracellular calcium measurement. We report that in postnatal (Postnatal day 2; P2) rat hippocampal cultures maintained *in vitro* for more than 15 days, GP120 induces a dramatic and persistent rise in $[Ca^{2+}]_i$ in neurons. This rise is entirely due to the release of calcium from intracellular stores, as it was fully blocked by drugs that either deplete stores or prevent Ca^{2+}-induced Ca^{2+}-release, and is not affected by antagonists of NMDA receptors or calcium channels. The toxicity of GP120 was also prevented by dantrolene, a ryanodine stores inhibitor. Therefore, our results suggest that in hippocampal neurons, intracellular calcium stores play a major role both in the Ca^{2+} rise and the GP120 toxicity. A preliminary report of this work has been presented (Medina *et al*., 1996b).

Materials and methods

Primary culture of rat hippocampal neurons

Cultures were prepared from the hippocampi of 2-day-old postnatal (P2) Wistar rats killed by rapid cervical dislocation. The hippocampi were removed and dissected free of meninges in cooled (6 °C) oxygenated phosphate-buffered saline (PBS) with Ca^{2+} and Mg^{2+}. They were then transferred to Ca^{2+}- and Mg^{2+}-free PBS, cut into small pieces (0.2–0.4 mm) and incubated for 30 min at room temperature (20–22 °C) with 0.3% (w/v) protease from *Aspergillus oryzae* (type XXIII) and 0.1% (w/v) DNAse (type I). The reaction

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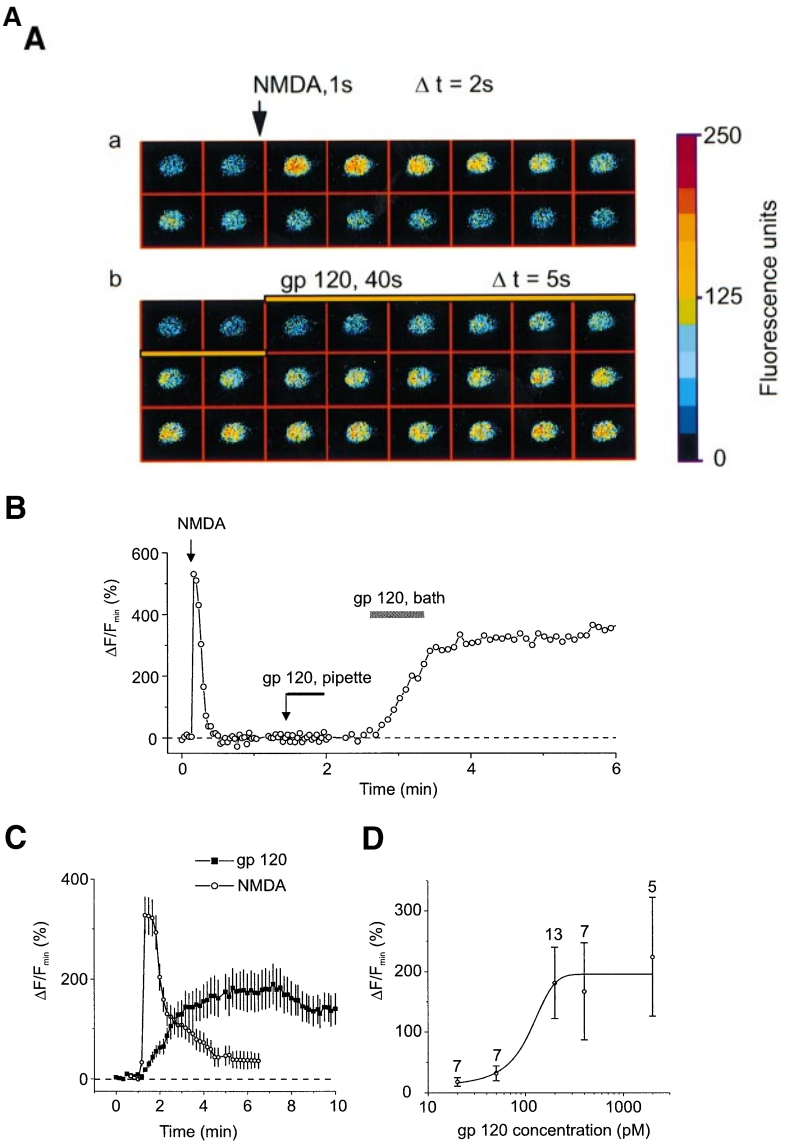

Fig. 1.

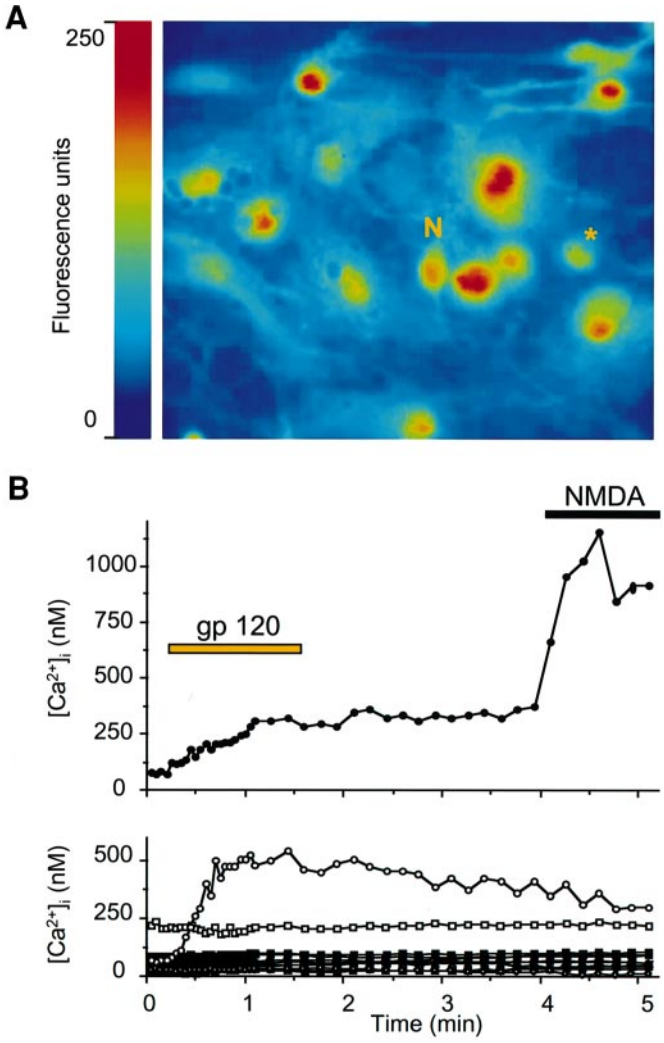

Fig. 2.

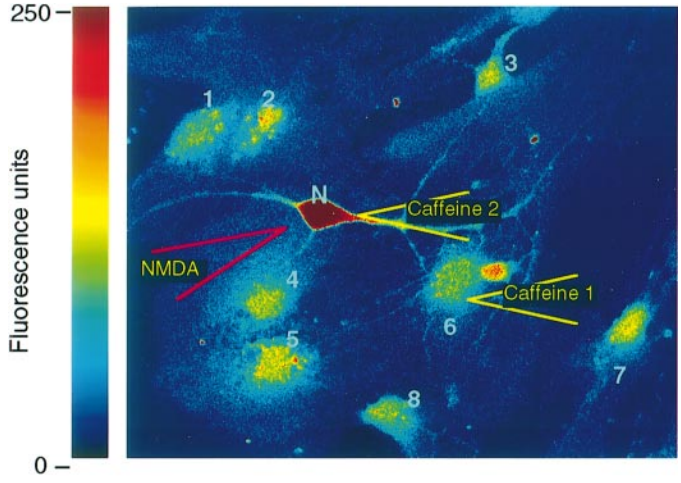

Fig. 3.

was stopped with 10% NU serum (Becton Dickinson, Franklin Lakes, NJ, USA). The tissue was washed and triturated using a fire-polished Pasteur pipette in Ca^{2+}- and Mg^{2+}-free PBS with 0.05% DNAse. After a brief centrifugation (5 min, 3000 r.p.m.), the cell pellet was resuspended in culture medium and plated at a density of 20 000–40 000 per cm^2. Growth medium contained 10% NU serum and 90% Eagle's minimum essential medium (EMEM, Gibco, Rockville, MD, USA) supplemented with penicillin (5 U/mL) and streptomycin (5 μg/mL). Glass coverslips' were coated with 10 μg/mL poly-L-lysine (MW range 30 000–70 000, Sigma, St Louis, MO, USA). Petri dishes were incubated at 37 °C in an atmosphere of carbogen (95% O_2 and 5% CO_2) and high (100%) humidity. Twenty-five per cent of the culture medium was freshly replaced twice a week. Cells were viable for up to 6 weeks.

Measurement of intracellular calcium-dependent fluo-3 fluorescence

To monitor changes in $[Ca^{2+}]_i$, neurons were loaded with 2 μM fluo-3 acetoxymethyl ester (AM). Neurons were washed with Mg^{2+}-free external solution containing (in mM): NaCl, 170; KCl, 1; $CaCl_2$, 2; *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES), 10; glucose, 10; pH 7.4; were incubated with fluo-3 AM (Molecular Probes, Eugene, OR, USA) for 30 min in the dark at room temperature (20–22 °C), the dye washed off and the coverslips reincubated in the dark for a further 30–60 min at room temperature to allow de-esterification of the dye. Petri dishes or coverslips with dye-loaded cells were transferred to the stage of an Axioscope Karl Zeiss microscope (40 × water immersion lens) to which a confocal laser scanning microscope (BioRad, Hercules, CA, USA) equipped with an argon–krypton laser and a photomultiplier is combined. Excitation was delivered at 488 nm and the emission intensity was recorded at $>$ 500 nm. All measurements were made at room temperature, as previously described (Medina *et al*., 1996a). During experiments, neurons were continuously perfused with external solution containing 1 μM tetrodotoxin (TTX) and 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). Drugs (GP120, caffeine, dantrolene, etc.) were applied via five parallel tubes (inner diameter 500 μm) placed at a distance of 2–5 mm from the cell. To prevent leakage of drugs into the bath, the tips of the tubes were filled with external solution. At the beginning or end of each experiment, brief (1-s) test pulses of 50 μM NMDA with 20 μM glycine were applied though a micropipette placed under visual control within 40–60 μm of the soma of a neuron, as previously described (Medina *et al*., 1994, 1996a). This enabled identification of neurons in the mixed culture (functional NMDA receptors having so far been reported only for neurons, McBain & Mayer, 1994). We also used NMDA applications to eliminate neurons whose $[Ca^{2+}]_i$ was initially high; only neurons showing an NMDA-induced increase in fluorescence of at least 200% were analysed. Finally, we used this test to estimate the time of $[Ca^{2+}]_i$ recovery, i.e. the efficiency of the cell's Ca^{2+} extrusion systems; neurons were included in the analysis only when cells recovered to control fluorescence levels (± 10%) within 60 s of NMDA application.

Where mentioned, fluorescence was measured simultaneously with patch-clamp recording. In this case, the patch pipette contained 30 μM fluo-3 to prevent washout during whole-cell recording.

Changes in fluorescence were quantified off-line using the program Fluo (IMSTAR, Paris, France). Part of the image, corresponding to the limits of a cell (mainly the border of the soma) was outlined on one of the images, and fluorescence was quantified within those limits in all successive images, recorded at selected intervals. Changes in fluorescence were calculated as $\Delta F/F_{min}$, where $\Delta F = (F - F_{min})$; F_{min} is the mean fluorescence of four to 10 images before the first application of NMDA or other agents; F is the fluorescence of subsequent images. ΔF, induced by applications of NMDA, caffeine, (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (τACPD) and thapsigargin, was taken as the difference between the peak value of fluorescence changes during drug applications and F_{min}. Changes in fluorescence induced by GP120 were calculated as the difference between F_{min} and the mean fluorescence of five consecutive points, including the maximal one selected during or 60 s after GP120 application. Cells were considered to be non-responding when this mean was not significantly different from F_{min} ($P < 0.05$).

Fura-2 videoimaging

Cells were loaded with 2 μM fura-2 AM using the same procedure as described above for fluo-3. Fura-2 fluorescence was imaged with an Axioscope Karl Zeiss microscope (40 × water immersion lens). The cells were illuminated using the monochromator Polychrome II (Photonics, Planegg, Germany) at 340 and 380 nm. Emitted light passed through a 480 nm barrier filter to an intensified CCD camera (Photonics). Excitation control, image acquisition and outline analyses of images were performed using Axon Imaging Workbench-2 (Axon Instruments, Foster City, CA, USA). Every cell in the image was analysed independently for each time point in the captured sequence. Ratios at saturating and zero Ca^{2+} (R_{max} and R_{min}, respectively) were obtained by perfusing cells with external solution containing 5 mM

FIG. 1. Effects of NMDA (50 μM) and GP120 (200 pM) on calcium-dependent fluorescence in 2-week-old cultured hippocampal neurons. The external solution in all experiments contained 1 μM TTX and 10 μM CNQX (see Materials and methods). (A) Consecutive confocal images obtained every 2 s (a) and 5 s (b) in the same cell. (a) Sixteen consecutive fluorescence changes in response to brief NMDA application to the soma from a distance of about 25 μm (NMDA was applied for 1 s in the pause between the acquisition of the second and third images). (b) Twenty-four consecutive fluorescence changes during GP120 application. The bar above images 3–10 indicates the presence of GP120 in the bath. Note the slow rise in fluorescence induced by GP120. (B) Changes in fluorescence in the neuron illustrated in A during focal application of GP120, which increased the fluorescence. (C) Average changes in fluorescence induced by long (40 s) bath applications of 50 μM NMDA ($n = 18$) and 200 pM GP120 ($n = 19$), as indicated. The plot shows mean ± SEM data. (D) Dose–response curve of GP120 concentration versus fluorescence change; the numbers denote the number of experiments.

FIG. 2. Limited effect of GP120 on $[Ca^{2+}]_i$ in glial cells. (A) Fluorescence of cells loaded with fura-2 AM (2 μM). Excitation wavelength = 380 nm. The microscopic field includes one neuron (marked by 'N') and 15 glial cells. The star indicates a glial cell responding to application of GP120 (B, response in bottom plots). The neuron was identified visually by using morphological criteria and the response evoked by bath application of NMDA (50 μM) with glycine (10 μM, B, top plot). (B) $[Ca^{2+}]_i$ change in the neuron (top plot) and glial cells (bottom plot) during application of GP120 and NMDA. Note that the NMDA application induced an additional $[Ca^{2+}]_i$ increase in the neuron but had no effect on $[Ca^{2+}]_i$ in the glial cells. The only glial cell that responded to GP120 (open circles) is identified in A (star).

FIG. 3. Neurons and glial cells loaded with fluo-3. This image was obtained following focal application of NMDA to the neuron (red arrow). A second micropipette (yellow arrow) containing 20 mM caffeine was positioned close to a glial cell (caffeine 1) or neuron (caffeine 2); the fluorescence changes in the neuron and the eight marked glial cells during focal application of caffeine in position 1 (close to glial cell 4) or position 2 (close to the neuron) are shown below in Fig. 6.

TABLE 1. Summary of fluorescence changes during GP120 application under different experimental conditions

GP120 derivative	$\Delta F/F$ in total			$\Delta F/F$ in responding neurons		Responding neurons	
Experimental condition	(%)	*P* (*t*-test)	*n* (total)	(%)	*P* (*t*-test)	(*n*)	(%)
Bacculovirus							
APV + Cd^{2+}	219 ± 55	0.656	20	312 ± 63	0.954	14	70
APV + D600	160 ± 52	0.794	19	272 ± 73	0.759	11	57
Control (no blockers)	181 ± 59		12	305 ± 67		7	58
MK-801 + D600	163 ± 23	0.762	22	239 ± 11	0.625	14	64
Control (no blockers)	177 ± 35		7	230 ± 11		5	71
MCPG (+ D600 + APV)	102 ± 33	0.797	21	160 ± 46	0.754	13	61
Control (+ D600 + APV)	119 ± 44		7	189 ± 51		4	57
Cho cells							
APV + Cd^{2+}	112 ± 50	0.811	7	195 ± 58	0.93	4	57
Control (no blockers)	129 ± 46		6	188 ± 41		4	67
Drosophila							
APV + Cd^{2+}	202 ± 98	0.714	4	268 ± 102	0.65	3	75
Control (no blockers)	163 ± 56		8	216 ± 59		6	75
Bacculovirus							
+antibodies	8 ± 3		7	0		0	0
+denaturated ab	111 ± 39	0.005	4	143 ± 31	—	3	75
Drosophila							
+antibodies	5 ± 4		6	0		0	0
+denaturated ab	149 ± 68	0.043	5	246 ± 59	—	3	60
+preimmune serum	161 ± 66	0.04	6	242 ± 66	—	4	67

Experiments with different GP120 derivatives and different blockers were performed on different culture batches. *P* shows the significance of the difference in fluorescence, either from no-block control or from antibody condition.

$CaCl_2$ and 4 μM ionomycin, and subsequently with a Ca^{2+}-free solution containing 5 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA). The $[Ca^{2+}]_i$ was determined according to the equation of Grynkiewicz *et al*. (1985).

Electrophysiological recording

Electrophysiological recordings were performed using the patch-clamp technique in whole-cell configuration. Currents were recorded using EPC-9 amplifier (HEKA Electronics, Liambrecht) and stored on digital tape using a DTR 1201 recorder (Bio-Logic, Claix, France). Unless otherwise mentioned in the text, the internal solution contained (in mM): CsCl, 100; Cs-gluconate, 80; EGTA, 1.1; $CaCl_2$, 0.2; HEPES-Cs, 10; Mg-ATP, 4.0; guanosine 5′-triphosphate (GTP), 0.6; creatine phosphate, 6.0; pH 7.2. The resistance of recording borosilicate glass pipettes was 3–6 MΩ and series resistance was in the range of 10–30 MΩ. The external solution contained (in mM): NaCl, 170; KCl, 1; $CaCl_2$, 2; $MgCl_2$, 1; HEPES, 10; glucose, 10; pH 7.4. TTX (1 μM) and CNQX (10 μM) were routinely added to block synaptic activity.

To record current–voltage relationships, ramps of potential from –100 mV to +30 mV (duration 1 s) were applied using pCLAMP 6 software (Axon Instruments, USA), a Labmaster DMA board (DIPSI, France) and an IBM AT computer (Intersys, USA). For analysis, data were filtered at 1–3 kHz and digitized at 5–10 kHz using pCLAMP-6 software.

Cytotoxicity assay

To study the toxic effect of GP120, 14-day-old cultures of neurons were incubated for 24 h with 400 pM GP120 and/or other drugs as mentioned. Then, fluorescein diacetate (10 μM) or propidium iodide (7.5 μM) were applied for 2 h to stain viable and dead cells, respectively. Cells were washed with external solution and visualized using a Nikon fluorescence microscope with Nomarski optics or a confocal scanning microscope. At least 100 neurons (five to 10 microscopic fields) were counted per coverslip, with two to three coverslips for each experiment. Cell survival was calculated as the percentage of neurons incorporating fluorescein diacetate (or not incorporating propidium iodide). Quantitative measurements after GP120 treatment gave similar results with either dye. However, the results obtained with propidium iodide are presented as they allowed clear discrimination between viable and damaged cells, being impermeable to the intact membrane. Fluorescein diacetate-treated coverslips always had ≈ 25% weakly fluorescent neurons, which were difficult to classify as 'living' or 'dead'. All quantifications were made on living cultures because both the fluorescein diacetate and propidium iodide was washed out from part of cells after fixation with 4% paraformaldehyde. Neurons were identified visually as cells with characteristic shape (dendritic tree). After some experiments ($n = 5$), cells were fixed and neurons were identified by using monoclonal β-tubulin III antibodies (Sigma Chemical, St Louis, MO, USA). No difference in the amount of neurons identified visually and immunocytochemically was found (100–300 neurons per experiment).

Materials

Three types of recombinant HIV-1 GP120 were used in the present study: GP120 from a baculovirus expression system (GP120-bac) (Intracel Corporation, Washington D.C., USA); GP120 from the mammalian (cho cells) expression system (GP120-cho) (Intracel Corporation); and GP120 derivative JR-FL produced from Drosophila Schneider 2 cells (GP120-JR-FL) was obtained as described (Wu *et al*., 1996), and was provided by R. Wyatt (Dana-Faber Cancer institute, Boston, MA, USA). GP120 was kept as 200 nM stock solution at –20 °C. This stock solution was diluted to 200 pM with the external solution 2–10 min before application. Rabbit polyclonal antibody to GP120 (IgG purified, Intracel Corporation) was reconstituted with water at a concentration of 1 mg/mL, stored at –20 °C and diluted to 4 μg/mL with a 200 pM GP120 solution extempora before an experiment.

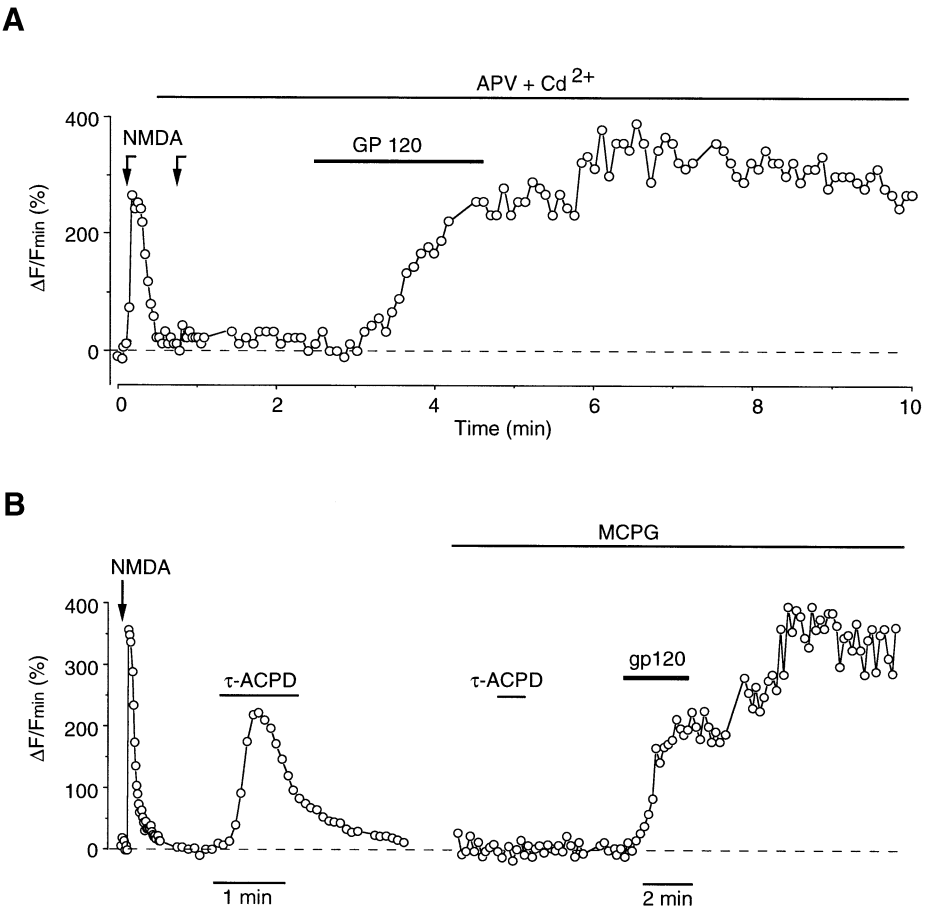

FIG. 4. Antagonists of voltage-gated calcium channels, NMDA receptor channels and metabotropic glutamate receptors do not significantly reduce the neuronal calcium response to bath application of GP120 (200 pM). All experiments were performed in the presence of 1 μM TTX and 10 μM CNQX. (A) Changes in fluorescence induced by GP120 in the presence of 100 μM D-APV and 100 μM Cd^{2+}. Bars indicate the duration of bath application of D-APV + Cd^{2+} and GP120. In this set of experiments, the pipette used for NMDA application also possessed 100 μM D-APV. (B) Bath application of τ-ACPD (10 μM, 70 s) induced a transient increase in fluorescence. A similar application of τ-ACPD to neuron from a new (τ-ACPD untreated) coverslip in the presence of MCPG (10 μM) failed to induce a change in fluorescence, whereas bath application of 200 pM GP120 had a strong effect. This set of experiments was performed in the presence of D-600 (100 μM) and D-APV (100 μM) in the external solution.

Dantrolene was from Sigma Chemical; a 20 mM stock solution was prepared in dimethyl sulphoxide (DMSO) (20 mM), taking steps to avoid exposure to strong light at any stage and stored at –20 °C. Thapsigargin was from Alomone Laboratories, Jerusalem, Israel. Propidium iodide and fluorescein diacetate were from Molecular Probes, Eugene, OR, USA. α-methyl-4-carboxyphenylglycine (MCPG), CNQX, D-2-amino-5-phosphonopentanoic acid (D-APV), NMDA were from Cookson Tocris, Bristol, UK. All other reagents, unless otherwise specified, were from Sigma Chemical Co.

All results are presented as mean values ± SEM, and Student's *t*-test was used to compare two groups of data with significant difference taken to be at the 5% level.

Results

GP120 induces a persistent increase in $[Ca^{2+}]_i$

Figure 1A and B illustrates the increase in $[Ca^{2+}]_i$ induced by application of NMDA and GP120-bac to hippocampal neurons from P2 rats maintained in culture for more than 15 days. NMDA was applied at the beginning and/or the end of each experiment to confirm that cells were neurons and not glia, and to estimate the amplitude of fluorescence change during the increase in $[Ca^{2+}]_i$ (see Materials and methods). A brief 1-s pulse of NMDA (50 μM) with glycine (20 μM) through a micropipette (1–2 μm in diameter) induced a fast rise in Ca^{2+}-dependent fluo-3 fluorescence (by 533 ± 58%; $n = 12$) that recovered to control levels within 10–30 s. A similar focal application of GP120 (400–600 pM) through a micropipette (10–30 μm from the soma) did not have any significant effect on $[Ca^{2+}]_i$ ($\Delta F/F_{min} = 19 \pm 13\%$, $n = 12$, Fig. 1B). In contrast, bath application of GP120 (200 pM) induced a slow (30–90 s) increase in fluorescence in 12 out of 19 experiments. The average fluorescence change was 179 ± 39%, $n = 19$ (unless otherwise mentioned, the mean ± SE level of fluorescence change was calculated for all the experiments). Because GP120 increased Ca^{2+} in only 60% of neurons, we mention the level of fluorescence in these responding neurons as well as their number (for details see Table 1). The apparent half-maximal activation concentration (EC50) of GP120 was 110 pM (Fig. 1D). This value is in the same range as that reported previously (≈ 60 pM) by Dreyer *et al*. (1990). The high level of fluorescence persisted in all neurons for at least 3–7 min after washout ($\Delta F/F_{min} = 180 \pm 40\%$, $n = 19$, 4 min after washout, Fig. 1C). The slow time course of GP120 action and the persistence of Ca^{2+} rise were not due to the perfusion system, as application of NMDA (50 μM with 20 μM glycine) produced a fast (< 5 s) and reversible rise in Ca^{2+}-dependent fluorescence that recovered immediately after washout in 11 out of 18 neurons. Four minutes after NMDA application, $\Delta F/F_{min}$ was 37 ± 17% ($n = 18$) and was significantly different from fluorescence observed 4 min after GP120 application (Fig. 1C). Interestingly, the effects of GP120 were observed only during the first application. A second application, even 3 h later, had no effect on $[Ca^{2+}]_i$ ($\Delta F/F = 2 \pm 3\%$, $n = 13$). Therefore, all experiments were performed on neurons from separate cover slips or culture dishes.

To verify whether the observed effects were specific for GP120, we have used recombinant glycoprotein from three different sources: (i) from bacculovirus; (ii) mammalian; and (iii) drosofila expression systems (see Materials and methods). All three GP120 derivatives had similar effects on neuronal Ca^{2+} (Table 1). Another test for specificity of the effect of GP120 was performed by using GP120

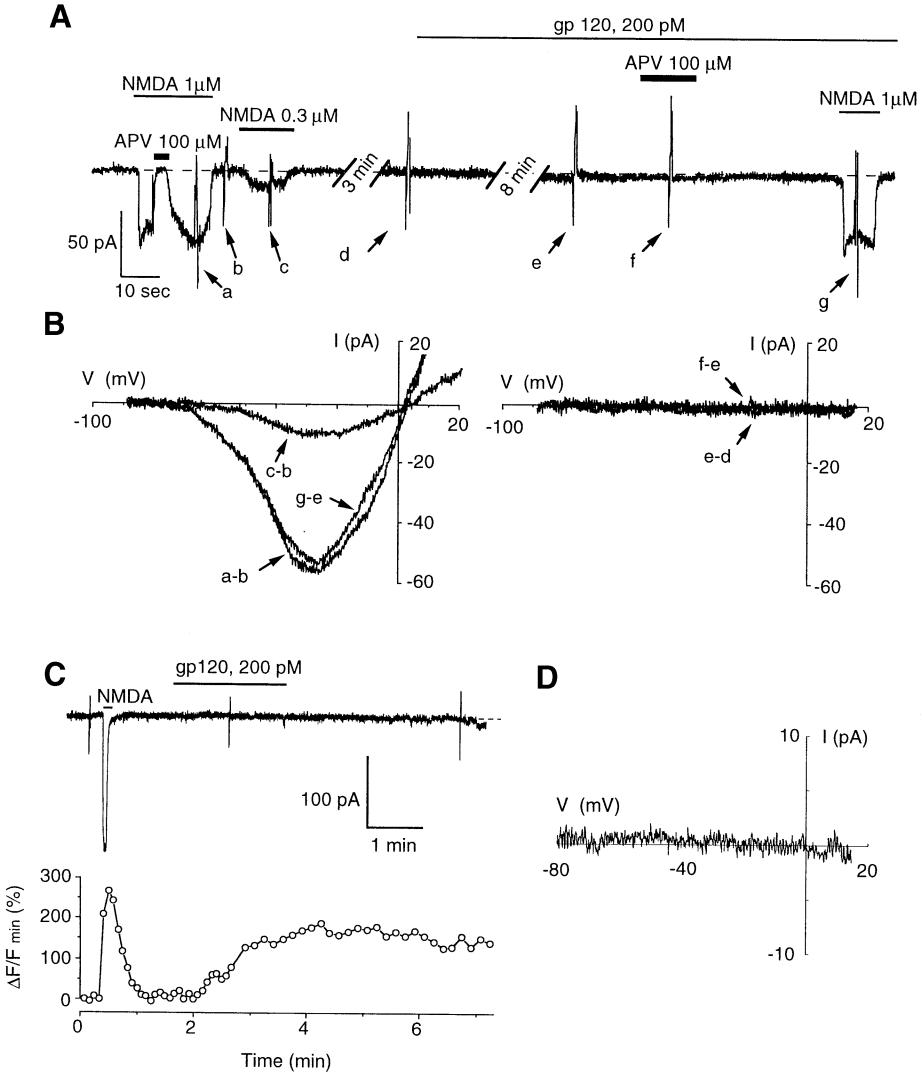

FIG. 5. GP120 does not modulate NMDA or any other current resolvable in whole-cell recording. (A) The application of NMDA (1 μM) using a fast perfusion system induces a current which is completely blocked by D-APV (100 μM). A 10 min application of GP120 (200 pM) does not result in the appearance of an APV-sensitive current or in an increase in NMDA current amplitude. This set of experiments was performed in the presence of 0.5 mM Mg^{2+}, 100 μM Cd^{2+} and 10 μM glycine. (B) Current–voltage relationships of NMDA (left) and GP120 (right) induced currents. I–V relations were obtained by subtraction of responses marked by the corresponding letters in (A). Note that the application of even a low concentration of NMDA (0.3 μM) induces a current with the characteristic I–V curve, whereas GP120 does not induce any current. (C) Simultaneous recording of ionic current at holding potential –60 mV and fluo-3 fluorescence during application of NMDA and GP120, as shown by bars. Note the GP120-induced increase in calcium-dependent fluorescence in the absence of any change in ionic current. (D) Mean current–voltage relationship obtained by subtraction of currents before and during GP120 application in seven cells showing an increase in fluorescence during GP120 application. Experiments were performed in the presence of 100 μM Cd^{2+}.

immunodepletion with 4 μg/mL purified polyclonal anti-GP120 antibodies. Solution of the immunodepleted GP120 from bacculovirus and the drosofila expression system failed to change the neuronal calcium in 10 and 12 experiments, respectively (Table 1). In contrast, the application of GP120 (200 pM) incubated with denatured antibodies (15 min at 80 °C) or rabbit preimmune serum significantly increased neuronal Ca^{2+} (Table 1).

Ratiometric calcium measurement using fura-2AM confirmed these observations: GP120-bac (200 pM) increased neuronal $[Ca^{2+}]_i$ by 328 ± 89 nM (n = 11), whereas the immunodepleted solution had no effect on $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$ was 15 ± 6 nM, n = 9, SD, $P < 0.05$).

Limited effects of GP120 on glia

Because glial cells have been suggested to be involved in signal transduction from GP120 to neurons (Dreyer & Lipton, 1995; Meuci & Miller, 1996; see also below and Discussion), we simultaneously measured GP120-induced calcium changes in both neurons and glia (e.g. see Fig. 2). Using the above approach we could visualize one to three neurons and five to 20 glial cells in a single image (see Fig. 3). Bath application of GP120-bac increased $[Ca^{2+}]_i$ in eight out of 91 glial cells studied in 14 experiments (on average $\Delta F/F$ was 13 ± 4%, n = 91 and mean $\Delta F/F$ in the eight responding cells was 109 ± 22 nM). Similar results were observed after application of GP120-cho (five out of 73 responding glia) and GP120-JR-FL (two out of 64 responding glia). Ratiometric calcium measurement showed GP120-bac-induced increase in $[Ca^{2+}]_i$ in five out of eight neurons (by 317 ± 121 nM, n = 8) but in only 15 out of 236 glial cells (263 ± 62 nM, n = 15 and 20 ± 6 nM, n = 236, respectively; Fig. 2).

The rise in $[Ca^{2+}]_i$ is not mediated by glutamate receptors or voltage-gated calcium channels

Early studies suggested that GP120 may act via the release of glutamate, and an indirect activation of both NMDA receptors and voltage-gated channels (Dreyer *et al*., 1990; Lipton *et al*., 1991). We therefore studied the effect of GP120 on $[Ca^{2+}]_i$ in the presence of blockers of NMDA and voltage-gated calcium channels. The Ca^{2+} influx through voltage-gated calcium channels, activated by voltage steps from –80 to –20 mV, during simultaneous fluorescence and whole-cell recording, was effectively blocked by 100 μM Cd^{2+} as shown previously (Medina *et al*., 1994) or by 100 μM methoxyverapamil (D600) (not shown). In contrast, the L-type calcium channel blocker nimodipine (10 μM) did not block the increase in $[Ca^{2+}]_i$ induced by depolarization (not shown). NMDA receptors were effectively blocked by the competitive NMDA channel antagonist D-APV (100 μM, Fig. 4A). In the presence of D-APV (100 μM) and Cd^{2+} (100 μM) or D600 (100 μM), GP120 induced changes of Ca^{2+}-

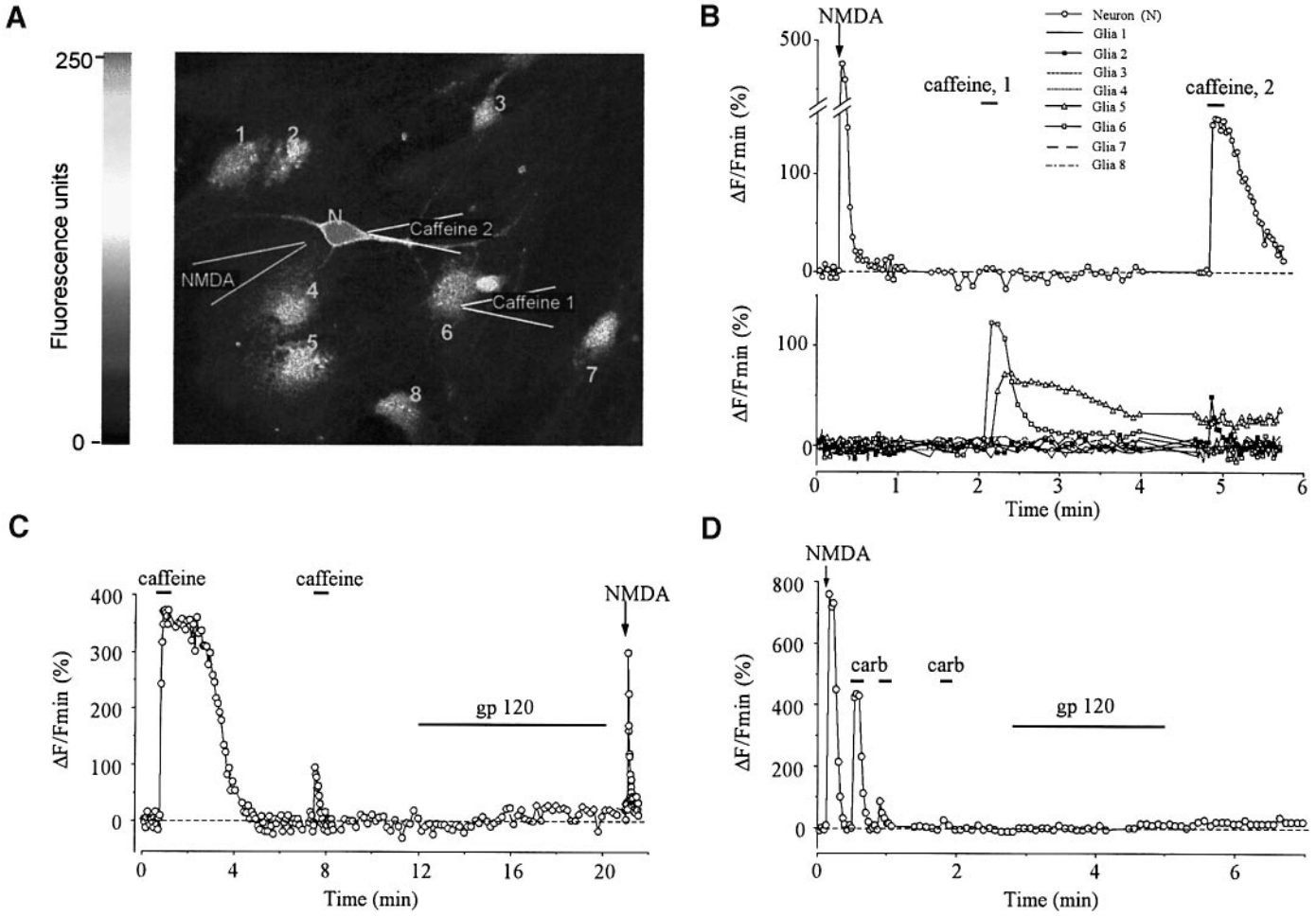

FIG. 6. (A) Neurons and glial cells loaded with fluo-3. This image was obtained following focal application of NMDA to the neuron. A second micropipette containing 20 mM caffeine was positioned close to a glial cell (caffeine 1) or neuron (caffeine 2); the fluorescence changes in the neuron and the eight marked glial cells during focal application of caffeine in position 1 (close to glial cell 4) or position 2 (close to the neuron); see Fig. 3 for full colour version of this panel. (B) Application of caffeine at position 1 induced a calcium rise in glial cells 6 and 5, but not in the neuron. Application of caffeine at position 2 induced a calcium rise in neuron and only one glial cell 2. (C,D) Effects of focal applications of 20 mM caffeine (C) or 30 μM carbachol (D) on the response induced by a subsequent bath application of 200 pM GP120. Note that a second application of caffeine (C) or carbachol (D) induced a much smaller increase in fluorescence than the first one, suggesting that the stores are empty.

dependent fluorescence similar to those observed in control conditions (Fig. 4A, Table 1). MK801, an open NMDA channel blocker, also did not significantly affect changes in $[Ca^{2+}]_i$ induced by GP120 (Table 1).

We then studied a possible involvement of metabotropic glutamate receptor (mGluR)-dependent calcium release from intracellular stores. Application of 10 μM τACPD, a mGluR agonist, induced a transient increase in fluorescence by 251 ± 72% ($n = 12$, Fig. 4B, Table 1). MCPG (10 μM), which antagonizes mGluRs in certain populations of hippocampal neurons, effectively prevented this rise ($\Delta F/F = 4 \pm 1\%$, $n = 8$, SD, $P = 0.001$), but failed to block the effects of GP120 (Fig. 4B and Table 1).

We also tested a possible action of GP120 on NMDA receptor currents. Application of the protein did not modify the amplitude of currents induced by 50 μM NMDA or the voltage-dependent Mg^{2+} block of these channels. Besides, no activation of membrane current was observed in the range of potentials from –90 to +15 mV ($n = 43$), [Fig. 5A and B, see also Lipton *et al*., 1991]. This set of experiments cannot, however, exclude the possibility that the patch-clamp recordings were performed on neurons insensitive to GP120. Thus, we made simultaneous measurements of fluo-3 fluorescence and whole-cell current during GP120 application (Fig. 5C and D). In the presence of D-APV, TTX, CNQX and Cd^{2+} (to block all ongoing synaptic activity), GP120 increased intracellular calcium in seven out of 13 experiments [$\Delta F/F = 174 \pm 41\%$ in responding cells ($n = 7$) and 94 ± 33% in all cells ($n = 13$)]. The analysis of voltage–current relationships in responding cells before, during and after GP120 application did not reveal any transmembrane ionic current (Fig. 5D).

Therefore, the rise in $[Ca^{2+}]_i$ induced by GP120 in 2-week-old cultured hippocampal neurons is not due to the activation of ionotropic or metabotropic glutamate receptors. It is also not mediated by voltage-gated calcium channels or other channels that can be measured by whole-cell patch-clamp.

To prevent a possible contamination of the calcium signal, subsequent experiments were performed in the presence of 100 μM Cd^{2+} or 100 μM D600 and 100 μM APV.

Depletion of intracellular stores by caffeine or carbachol prevents the effect of GP120

Two types of intracellular Ca^{2+} stores have been characterized, i.e. the inositol trisphosphate (IP_3)-sensitive and ryanodine-sensitive stores (for reviews see Henzi & MacDermott, 1992; Berridge, 1993, 1997; Simpson *et al*., 1995). These stores can be activated and depleted by

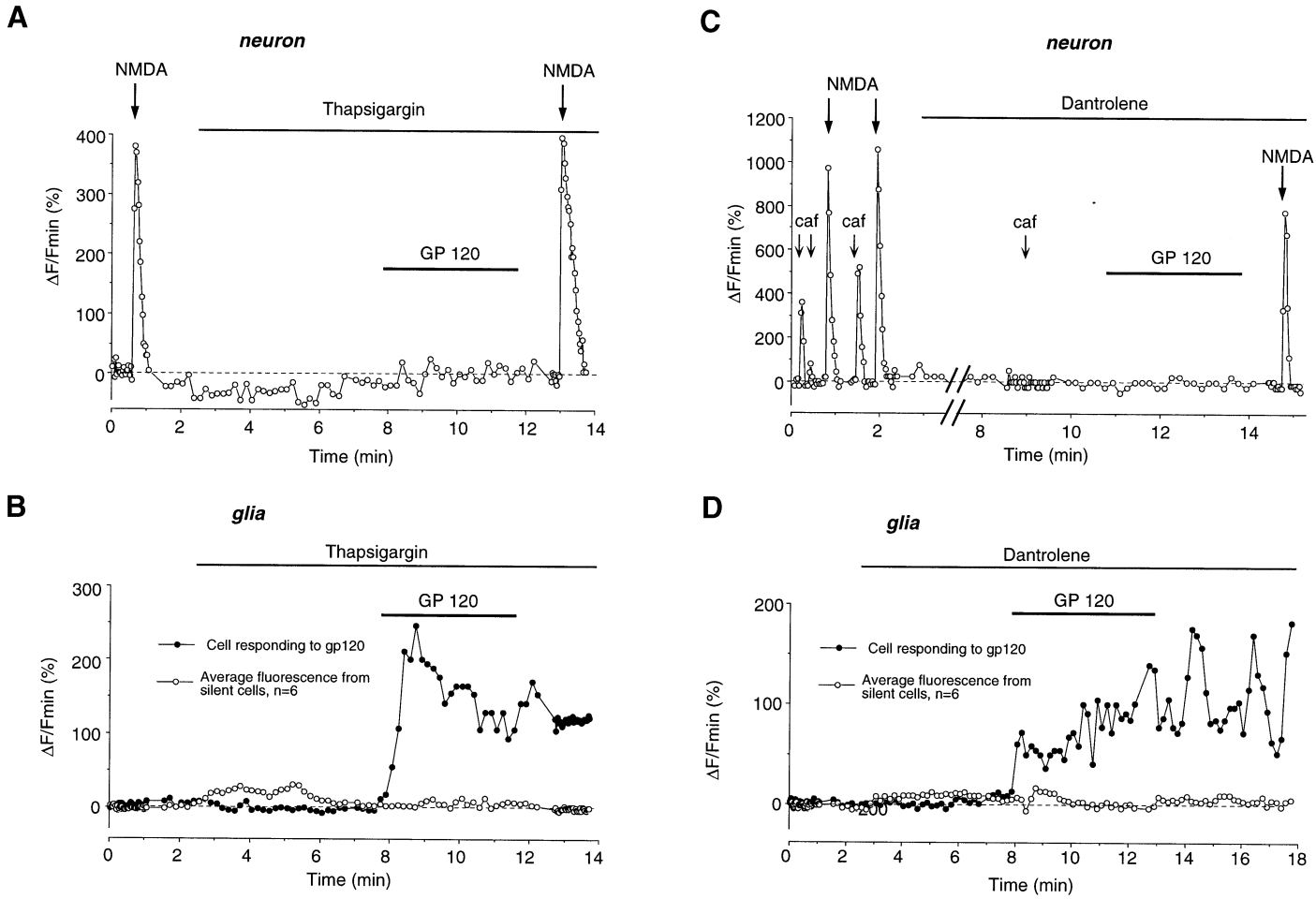

FIG. 7. Inhibitors of intracellular stores prevent the GP120-induced calcium response in neurons, but not in adjacent glial cells. (A,B) The fluorescence change in neuron (A) and glial cells (B) during bath application of 10 μM thapsigargin and 200 pM GP120, as shown by bars. One neuron (A) and seven glial cells (B) were visualized in one frame. (B) Fluorescence changes in the single responding glial cell and the mean fluorescence of non-responding cells ($n = 6$). (C) Dantrolene (10 μM) blocks the effects of GP120 (120 pM) on neurons. Note that dantrolene also abolished the calcium rise induced by caffeine and decreased the NMDA-induced calcium rise by 30%. Caffeine (20 mM, 10 s) was applied focally as in Fig. 6A. Dantrolene and GP120 were bath applied. (D) Out of seven glial cells observed, in only one did GP120 change the fluorescence and the effect was not blocked by dantrolene (10 μM).

caffeine (an agonist of ryanodine-sensitive stores) and carbachol, which increases the turnover of inositol phosphates by muscarinic receptor activation (Simpson *et al.*, 1995). The interpretation of experiments based on bath applications of caffeine or carbachol is, however, complicated, as both agonists increase calcium in glial cells, which subsequently modulate $[Ca^{2+}]_i$ in neighbouring neurons (Nedergaard, 1994). Furthermore, glial cells may be involved in signal transduction from GP120 to neurons (see Dreyer & Lipton, 1995; Meuci & Miller, 1996). We therefore developed a procedure that enables selective stimulation and depletion of calcium stores in neurons or glial cells under visual control (Fig. 6A and B). Caffeine (20 mM) or carbachol (30 μM) were applied focally (to neurons or glial cells) by micropipettes (tip diameters: 0.5–1 μm). As shown in Fig. 6, a application of caffeine to a neuron induced Ca^{2+} rise in neuron but not glial cells ($n = 18$ out of 21 experiments), or in neuron and one to two adjacent glia (three experiments). The caffeine application to a single glial cell increased calcium in the target cell and in one to five adjacent glial cells. In most experiments (12 out of 14), the activation of glial cells had no effect on calcium in adjacent neurons ($\Delta F/F_{min} = 8 \pm 3$, $n = 12$). Only in two experiments did the activation of glial cell by caffeine induce a neuronal calcium rise ($\Delta F/F = 360$ and 520%). Therefore, this approach is suitable to selectively stimulate Ca^{2+} stores in neurons without affecting the Ca^{2+} stores in glial cells. Repeated tests showed that the effects of bath applications of GP120 were fully prevented by focal applications of caffeine ($\Delta F/F = 39 \pm 12\%$, $n = 16$) or carbachol ($\Delta F/F = 18 \pm 13\%$, $n = 6$) to neurons, suggesting an involvement of neuronal Ca^{2+} stores in the actions of GP120 (Fig. 6C and D).

Because these results suggest that GP120 induces release of Ca^{2+} from intracellular stores, we wanted to know whether the failure of GP120 to induce the $[Ca^{2+}]_i$ rise in 30% of neurons during the first application and in all neurons during the second application is related to the refilling of stores by calcium. We found that all neurons ($n = 5$) which did not respond to GP120 in this set of experiments showed an increased $[Ca^{2+}]_i$ in response to focal applications of caffeine ($\Delta F/F = 201 \pm 56$) and carbachol ($\Delta F/F = 171 \pm 40$, carbachol was applied 2 min after caffeine). GP120 (200 pM) was routinely present in the bath during caffeine and carbachol application. Moreover, four out of four neurons which responded to the first application of GP120 ($\Delta F/F = 208 \pm 38\%$) and showed no change in $[Ca^{2+}]_i$ during the second application 45 min later ($\Delta F/F = 11 \pm 5$), responded to caffeine ($\Delta F/F = 222 \pm 34$) and carbachol ($\Delta F/F = 232 \pm 29$).

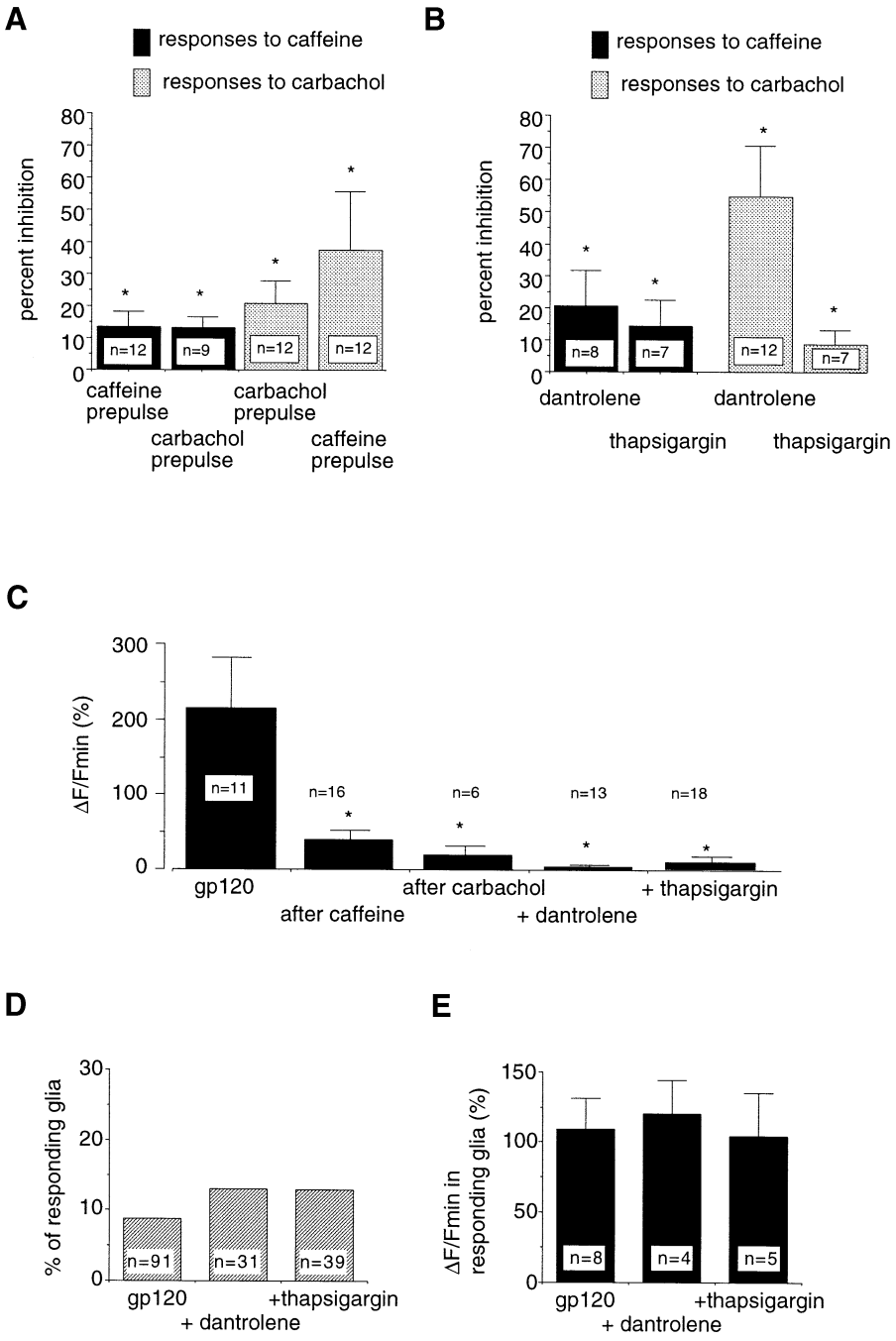

FIG. 8. Summary data for fluorescence changes induced in neurons and glia. (A) Effects of caffeine and carbachol on intracellular stores. Cells were focally treated by two successive applications, 60 s apart, of either drug, and subsequent fluorescence changes were compared to control values obtained at the beginning of each experiment. Asterisks show values significantly different from controls (paired *t*-test). In experiments with caffeine application after a carbachol prepulse, caffeine was first applied to estimate the control value, then the intracellular calcium was increased by a 1–5 -s application of NMDA to recharge stores, as shown in Fig. 8C, and a carbachol pulse was finally applied. A similar approach was used to restore the calcium pool mobilized by carbachol. *n* denotes the number of experiments. (B) Inhibition of neuronal responses evoked by caffeine (20 mM) and carbachol (30 μM). As in Fig. 8C, the intracellular store agonist (caffeine or carbachol) was first applied, the stores were then recharged by NMDA, and then 10 μM dantrolene or thapsigargin was bath applied for at least 60 s. Ca^{2+} was mobilized by a focal application of caffeine or carbachol, and the peak amplitude of responses was compared to that obtained at the beginning of the experiment. Asterisks denote values significantly different from controls (paired *t*-test). (C) The effects of GP120 are prevented by caffeine, carbachol, dantrolene or thapsigargin. Similar experiments are shown in Figs 6C and D and 8A and C. (D) Glial cell responses to GP120 in control conditions and in the presence of 10 μM dantrolene or thapsigargin. *n* denotes the number of glial cells tested in 14 control experiments, seven experiments with dantrolene and eight experiments with thapsigargin. (E) Mean ± SEM changes in fluorescence of glial cells responding to GP120 alone, and in the presence of dantrolene or thapsigargin. Glia were considered to be non-responding when this mean was not significantly different from F_{min} ($P < 0.05$).

The properties of intracellular calcium stores in the CNS have not been fully characterized (for review see Simpson *et al*., 1995). In cerebella granule cells, IP_3-sensitive and ryanodine-sensitive stores are coupled (Irving *et al*., 1992). We therefore studied the interactions between caffeine and carbachol responses in hippocampal neurons in order to evaluate whether GP120 responses are mediated by one and/or the other Ca^{2+} store. Both types of intracellular stores were effectively mobilized by brief 10-s applications of agonists (caffeine, 20 mM or carbachol, 30 μM), and consecutive applications of the same agonist had a much weaker effect (Fig. 6C and D, for mean data see also Fig. 8A,). Pretreatment with carbachol significantly reduced the response evoked by caffeine (caffeine-induced rise of fluorescence by 228 ± 61% in control and 30 ± 7% after carbachol pretreatment, $n = 9$, $P = 0.01$, Fig. 8A). Similarly, caffeine also had a significant, albeit smaller, effect on carbachol-induced calcium release ($\Delta F/F = 212 \pm 61\%$ in control and 79 ± 39% after caffeine application, $n = 12$, $P = 0.016$, Fig. 8A). Caffeine and carbachol responses were significantly decreased both by thapsigargin (10 μM), a blocker of the endoplasmic reticulum Ca^{2+}-ATPase (Fig. 8B) and dantrolene (10 μM), a ryanodine stores inhibitor. However, dantrolene was more effective on the caffeine response ($\Delta F/F = 270 \pm 72\%$ in control and 55 ± 29% in the presence of dantrolene, $n = 8$, $P = 0.01$) than on the carbachol one ($\Delta F/F = 272 \pm 80\%$ in control and 149 ± 42% in the presence of dantrolene, $n = 12$, $P = 0.017$). Therefore, as in the cerebellum, IP_3-sensitive and ryanodine-sensitive stores are coupled in hippocampal neurons.

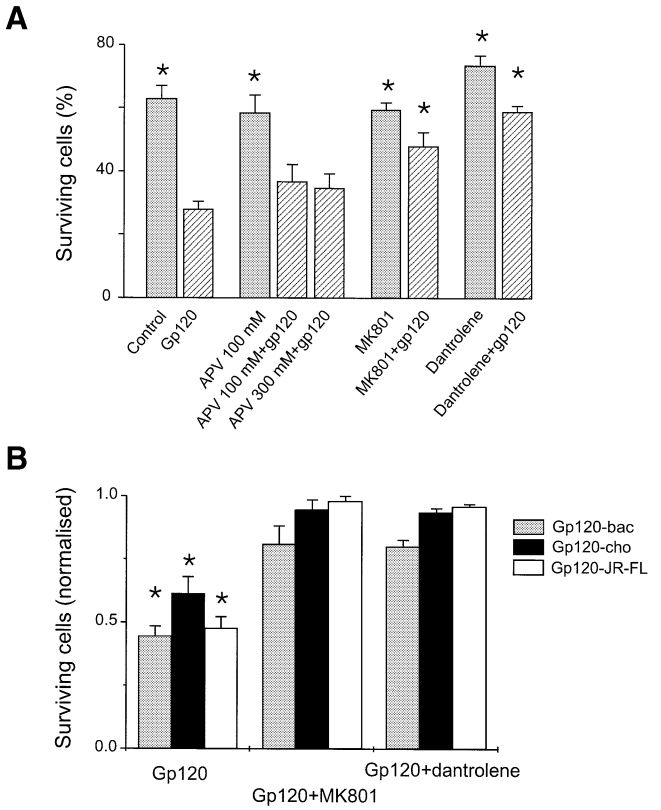

FIG. 9. Dantrolene abrogates neuronal injury mediated by GP120. (A) Neurotoxicity induced by GP120-bac. Cultured neurons were incubated with GP120 (400 pM) for 24 h with or without various antagonists as indicated. Note that GP120-induced toxicity was prevented both by dantrolene (10 μM) and MK801 (10 μM) but not by APV, even at a relatively high concentration (300 μM). Asterisks indicate values significantly different from those observed with GP120 alone ($P < 0.05$). Each column represents the average result from six to 22 experiments. (B) Similar neurotoxic effects of the different GP120 derivatives. Results obtained during neurons incubation with GP120 (400 pM), GP120 + MK801 and GP120 + dantrolene are normalized to corresponding control levels [no GP120 added, MK801 (10 μM) and dantrolene (10 μM), respectively]. Asterisks indicate values significantly different from those observed at control (no GP120 added).

Inhibitors of intracellular stores prevent GP120-induced rise in $[Ca^{2+}]_i$

We tested the effect of thapsigargin and dantrolene on the GP120-induced increase in $[Ca^{2+}]_i$. *Thapsigargin (10* μM) strongly prevented the effects of GP120-bac (mean $\Delta F/F = 9 \pm 6\%$, $n = 18$, Figs 7A and 8C); only in one experiment did GP120 induce an increase in fluorescence in the presence of thapsigargin (110%, not shown). Dantrolene (10 μM) also completely prevented the increase in fluorescence induced by a subsequent application of GP120-bac ($\Delta F/F = 3 \pm 1\%$, $n = 13$, Figs 7C and 8C). Similarly, dantrolene prevented the effect of GP120-JR-FL ($\Delta F/F = 15 \pm 6\%$, $n = 7$). Parallel experiments were performed in order to determine a possible role of glial cells in mediating the actions of dantrolene and thapsigargin on neurons. As shown in Figs 7B and D, and 8D and E, neither dantrolene nor thapsigargin affected the $[Ca^{2+}]_i$ changes produced in glial cells by GP120. Therefore, the effects of the envelope protein are prevented when neuronal stores are blocked or depleted.

Dantrolene prevents GP120-induced neurotoxicity

To study GP120-induced neurotoxicity, we have used several approaches (see Materials and methods). In 14-day-old cultures from P2 rat hippocampi, GP120 (400 pM) did not induce significant changes in neuron density even 4 days after application (not shown). However, the number of neurons incorporating fluorescein diacetate (10 μM, viable cell marker) or propidium iodide (7.5 μM, dead cell marker) changed significantly. Figure 9 shows the changes in the number of living neurons (neurons not incorporating propidium iodide) induced by GP120 in different experimental conditions. A 24 h GP120 (400 pM) treatment of cultures with either GP120 derivative significantly decreased the population of viable neurons. The application of dantrolene (10 μM) protected neurons from GP120 toxicity. Interestingly, the neurotoxicity of GP120 was effectively reduced by MK801, but not APV, even at relatively high concentrations (Fig. 9).

Discussion

The principal observation of our study is that the increase in $[Ca^{2+}]_i$ generated by GP120 in hippocampal neurons is due to Ca^{2+} release from intracellular stores. The effects of GP120 on neuronal $[Ca^{2+}]_i$ do not involve NMDA receptors or other measurable channels.

Earlier studies suggested that the rise in $[Ca^{2+}]_i$ induced by GP120 is due to activation of the NMDA receptors or voltage-gated calcium channels (Dreyer *et al*., 1990; Lipton *et al*., 1991; Lo *et al*., 1992). However, in whole-cell recordings, GP120 did not generate or modulate ionic currents, nor did it affect NMDA-receptor mediated currents (Lipton *et al*., 1991). As a possible explanation for the contradiction between Ca^{2+}-measurements and electrophysiological recordings, the authors proposed a local rise in endogenous glutamate which could be generated by the glycoprotein but was not detectable in whole-cell experiments. Our whole-cell patch-clamp recordings confirmed that GP120 does not generate ionic currents, and provided direct evidence that in hippocampal neurons, the rise in $[Ca^{2+}]_i$ is mediated neither by NMDA receptors nor by voltage-gated calcium channels, as: (i) a large Ca^{2+} rise was observed in the presence of blockers of NMDA and voltage-gated calcium channels at concentrations that fully block currents generated by their activation; and (ii) in simultaneous whole-cell recording and fluorescence monitoring, the rise in $[Ca^{2+}]_i$ was not associated with ionic currents.

Our results strongly suggest that GP120-induced increase in neuronal $[Ca^{2+}]_i$ is due to release from intracellular stores. Both IP_3-sensitive and ryanodine-sensitive stores are involved, as GP120 did not evoke a rise in $[Ca^{2+}]_i$ when they were either depleted (by caffeine, carbachol or thapsigargin) or blocked by dantrolene. Because these stores interact and are coupled in hippocampal neurons, as shown in the present study, we cannot exclude the possibility that actions of GP120 primarily involve one of them.

To determine the mechanisms underlying the effects of the protein, great efforts were made to directly induce the rise in $[Ca^{2+}]_i$ by focal applications of GP120 on visually identified neurons or glia. These were, however, unsuccessful, and even high concentrations of GP120 (400–600 pM) did not increase $[Ca^{2+}]_i$. We cannot offer a simple explanation for this failure. One possible explanation is that the target of GP120 is found in dendritic sites that were not affected by focal application of the glycoprotein. An alternative explanation is that the protein does not act directly on neurons but on other populations of cells, and in particular glial cells including macrophages that were present as in earlier studies. Earlier studies have suggested that GP120 acts on glial cells to release a neurotoxic substance that potentiates the activation of NMDA receptors (Dawson *et al*., 1993; Hewett *et al*., 1994; Ushijima *et al*., 1995; also see below). Our results, however, indicate that the rise in neuronal $[Ca^{2+}]_i$ induced by GP120 does not involve glutamate receptors, either directly or indirectly, as it was observed even when ionotropic (AMPA and NMDA) and

metabotropic glutamatergic receptors were fully blocked. Furthermore, the simultaneous measurement of $[Ca^{2+}]_i$ in neurons and glia showed that the envelope protein increased $[Ca^{2+}]_i$ in less than 10% of glia (23 out of a total of 337 glia studied, see also Codazzi *et al*., 1995). The glial response was not affected by the application of dantrolene at a concentration that fully blocked the neuronal rise in $[Ca^{2+}]_i$. Therefore, if a messenger is released by glia to increase neuronal $[Ca^{2+}]_i$: (i) it is not glutamate or any agent that generates a voltage-gated or receptor-coupled ionic current in hippocampal neurons in culture; (ii) its action is not mediated by glutamatergic metabotropic receptors; (iii) its release does not require the activation of sodium or calcium channels and it is not associated with a rise of $[Ca^{2+}]_i$ in glia; (iv) it initiates a $[Ca^{2+}]_i$ rise in neurons by a pharmacologically selective interaction with neuronal IP_3 and/or ryanodine-sensitive $[Ca^{2+}]_i$ stores. Failure to induce the calcium rise after the second application of GP120 to neurons with refilled intracellular stores suggests that some pathways involved in GP120 action need a long time for recovery (e.g. accumulation or synthesis of the diffusible messengers between target cells and neurons). Alternatively, it may suggest a high affinity of GP120 association with the putative receptor.

Neurotoxicity of GP120

Our observations suggest that the initial action of GP120 that leads to a rise in $[Ca^{2+}]_i$ does not involve glutamate receptors and is due to release from intracellular stores. The protective effect of dantrolene suggests an important role of this release in subsequent neurotoxicity. Neuroprotective effects of dantrolene have been reported in several pathologies (for review see Mody & MacDonald, 1995; Simpson *et al*., 1995). The contribution of NMDA receptors in GP120 toxicity in the present, as in several earlier studies (Lipton *et al*., 1991; Giulian *et al*., 1993; Sindou *et al*., 1994; Dreyer & Lipton, 1995; Lannuzel *et al*., 1995; Meuci & Miller, 1996), is based on the highly reproducible neuroprotective actions of MK801. However, there are also indications that MK801 may not be a fully selective NMDA channel blocker. Thus, several actions of MK801 have been described that are clearly not due to NMDA receptors (Olney *et al*., 1991; Sharp *et al*., 1992; Charriaut-Marlangue *et al*., 1994). Furthermore, in our cultures, APV did not protect neurons from GP120 toxicity, even at a relatively high concentration (300 μM). It is therefore possible that NMDA receptors are not involved at least in the initial stages of the toxic effects of the protein. Future studies will have to determine whether these receptors are activated in the steps that following calcium release from intracellular stores lead to cell death. It will also be important to establish whether the multiple pleiotropic effects of GP120 exposure represent distinct arms of a complex signalling pathway or whether they are the consequences of Ca^{2+} release.

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Abbreviations

AIDS, acquired immunodeficiency syndrome; AM, acetoxymethyl ester; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CNS, central nervous system; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; EMEM, Eagle's minimum essential medium; *F*, fluorescence; GP120, HIV envelope glycoprotein; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; HIV-1, human immunodeficiency virus type 1; IP_3, inositol triphosphate; MCPG, α-methyl-4-carboxyphenylglycine; mGluR, metabotropic glutamate receptor; NMDA, *N*-methyl-D-aspartate; P2, postnatal day 2; PBS, phosphate-buffered saline; τACPD, (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid; TTX, tetrodotoxin.

References

Berridge, M.J. (1993) Inositol trisphosphate and calcium signaling. *Nature*, **361**, 315–325.

Berridge, M.J. (1997) Elementary and global aspects of calcium signaling. *J. Physiol.*, **499**, 291–306.

Brenneman, D.E., Westbrook, G.L., Fitzgerald, S.P., Ennist, D.L., Elkins, K.L., Ruff, M.R. & Pert, C.B. (1988) Neuronal cell killing by the envelope protein of HIV and its prevention by vasoactive intestinal peptide. *Nature*, **335**, 639–642.

Charriaut-Marlangue, C., Dessi, F. & Ben-Ari, Y. (1994) Inhibition of protein synthesis by the NMDA channel blocker MK-801. *Neuroreport*, **5**, 1110–1112.

Choi, D.W. (1988) Glutamate neurotoxicity and diseases of the nervous system. *Neuron*, **1**, 623–634.

Codazzi, F., Menegon, A., Zacchetti, D., Ciardo, A., Grohovas, F. & Meldolesi, J. (1995) HIV-1 gp120 glycoprotein induces $[Ca^{2+}]_i$ responses not only in type-2 but also type-1 astrocytes and oligodendrocytes of the rat cerebellum. *Eur. J. Neurosci.*, **7**, 1333–1341.

Dawson, V.L., Dawson, T.M., Uhl, G.R. & Snyder, S.H. (1993) Human immunodeficiency virus type 1 coat protein neurotoxicity mediated by nitric oxide in primary cultures. *Proc. Natl Acad. Sci. USA*, **90**, 3256–3259.

Dreyer, E.B., Kaiser, P.K., Offerman, J.T. & Lipton, S.A. (1990) HIV-1 coat protein neurotoxicity prevented by calcium channel antagonists. *Science*, **248**, 364–367.

Dreyer, E.B. & Lipton, S.A. (1995) The coat protein gp120 of HIV-1 inhibits astrocyte uptake of excitatory amino acids via macrophage arachidonic acid. *Eur. J. Neurosci.*, **7**, 2502–2507.

Giulian, D., Wendt, E., Vaca, K. & Noonan, C.A. (1993) The envelope glycoprotein of human immunodeficiency virus type 1 stimulates release of neurotoxins from monocytes. *Proc. Natl Acad. Sci. USA*, **90**, 2769–2773.

Grynkiewicz, G., Poenie, M. & Tsien, R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem*, **260**, 3440–3450.

Henzi, V. & MacDermott, A.B. (1992) Characteristics and function of $[Ca^{2+}]$- and inositol 1,4,5-trisphosphate-releasable stores of Ca^{2+} in neurones. *Neuroscience*, **46**, 251–273.

Hewett, S.J., Csernansky, C.A. & Choi, D.W. (1994) Selective potentiation of NMDA-induced neuronal injury following induction of astrocytic iNOS. *Neuron*, **13**, 487–494.

Irving, A.J., Collingridge, G.L. & Schofield, J.G. (1992) Interactions between Ca^{2+} mobilizing mechanisms in cultured rat cerebellar granule cells. *J. Physiol.*, **456**, 667–680.

Ketzler, S., Weis, S., Haug, H. & Budka, H. (1990) Loss of neurones in the frontal cortex in AIDS brains. *Acta Neuropathol.*, **80**, 92–94.

Lannuzel, A., Lledo, P.-M., Lamghitnia, H.O., Vincent, J.-D. & Tardieu, M. (1995) HIV-1 envelope proteins gp120 and gp160 potentiate NMDA-induced $(Ca^{2+})_i$ increase, alter $(Ca^{2+})_i$ homeostasis and induce neurotoxicity in human embryonic neurones. *Eur. J. Neurosci.*, **7**, 2285–2293.

Lipton, S.A., Sucher, N.J., Kaiser, P.K. & Dreyer, E.B. (1991) Synergistic effects of HIV coat protein and NMDA receptor-mediated neurotoxicity. *Neuron*, **7**, 111–118.

Lo, T.M., Fallert, C.J., Piser, T.M. & Thayer, S.A. (1992) HIV-1 envelope protein evokes intracellular calcium oscillations in rat hippocampal neurones. *Brain Res.*, **594**, 189–196.

McBain, C.J. & Mayer, M.L. (1994) *N*-methyl-D-aspartic acid receptor structure and function. *Physiol. Rev.*, **74**, 723–760.

Medina, I., Filippova, N., Bakhramov, A. & Bregestovski, P. (1996a) Calcium-induced inactivation of NMDA receptor-channels evolves independently of run-down in cultured rat brain neurones. *J. Physiol.*, **495**, 411–427.

Medina, I., Filippova, N., Barbin, G., Ben-Ari, Y. & Bregestovski, P. (1994) Kainate-induced inactivation of NMDA currents via an elevation of intracellular Ca^{2+} in hippocampal neurones. *J. Neurophysiol.*, **72**, 456–465.

Medina, I., Ghose, S., Esplin, B.A. & Ben-Ari, Y. (1996b) The gp120-induced rise in $[Ca^{2+}]_i$ in rat hippocampal neurones in culture is not mediated by NMDA receptors or voltage-gated calcium channels. *Soc. Neurosci.*, **1**, 11.6, p. 9.

Meuci, O. & Miller, R.J. (1996) Gp120-induced neurotoxicity in hippocampal pyramidal neuron cultures: protective action of TGF-b1. *J. Neurosci.*, **16**, 4080–4088.

Mody, I. & MacDonald, J.F. (1995) NMDA receptor-dependent excitotoxicity: The role of intracellular Ca^{2+} release. *Trends Pharmacol. Sci.*, **16**, 356–359.
Muller, W.E.G., Schroder, H.C., Ushijima, H., Dapper, J. & Bormann, J. (1992) Gp120 of HIV-1 induces apoptosis in rat cortical cell cultures: prevention by memantine. *Eur. J. Pharmacol.*, **226**, 209–214.
Nedergaard, M. (1994) Direct signaling from astrocytes to neurones in cultures of mammalian brain cells. *Science*, **263**, 1768–1771.
Olney, J.W., Labruyere, J., Wang, G., Wozniak, D.F., Price, M.T. & Sesma, M.A. (1991) NMDA antagonist neurotoxicity: mechanism and prevention. *Science*, **254**, 1515–1518.
Price, R.W., Brew, B., Sidtis, J., Rosenblum, M., Scheck, A.C. & Cleary, P. (1988) The brain in AIDS: central nervous system HIV-1 infection and AIDS dementia complex. *Science*, **239**, 586–592.
Savio, T. & Levi, G. (1993) Neurotoxicity of HIV coat protein gp120, NMDA receptors, and protein kinase C: a study with rat cerebellar granule cell cultures. *J. Neurosci. Res.*, **34**, 265–272.
Sharp, F.R., Butman, M., Wang, S., Koistinaho, J., Graham, S.H., Sagar, S.M., Noble, L., Berger, P. & Longo, F.M. (1992) Haloperidol prevents induction of the hsp70 heat shock gene in neurones injured by phencyclidine (PCP), MK801, and ketamine. *J. Neurosci. Res.*, **33**, 605–616.
Simpson, P.B., Challiss, R.A.J. & Nahorski, S.R. (1995) Neuronal Ca^{2+} stores: activation and function. *Trends Neurosci.*, **18**, 299–306.
Sindou, P., Couratier, P., Esclaire, F., Yardin, C., Bousseau, A. & Hugon, J. (1994) Prevention of HIV coat protein (gp120) toxicity in cortical cell cultures by riluzole. *J. Neurol. Sci.*, **126**, 133–137.
Ushijima, H., Nishio, O., Klocking, R., Perovic, S. & Muller, W.E.G. (1995) Exposure to gp120 of HIV-1 induces an increased release of arachidonic acid in rat primary neuronal cell culture followed by NMDA receptor-mediated neurotoxicity. *Eur. J. Neurosci.*, **7**, 1353–1359.
Wu, L., Gerard, N.P., Wyatt, R., Choe, H., Parolin, C., Ruffing, N., Borsetti, A., Cardoso, A.A., Desjardin, E., Newman, W., Gerard, C. & Sodroski, J. (1996) CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5. *Nature*, **384**, 179–183.