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Activation of Group III Metabotropic Glutamate Receptors Inhibits the Production of RANTES in Glial Cell Cultures

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The chemokine RANTES is critically involved in neuroinflammation and has been implicated in the pathophysiology of multiple sclerosis. We examined the possibility that activation of G-protein-coupled metabotropic glutamate (mGlu) receptors regulates the formation of RANTES in glial cells. A 15 hr exposure of cultured astrocytes to tumor necrosis factor-α and interferon-γ induced a substantial increase in both RANTES mRNA and extracellular RANTES levels. These increases were markedly reduced when astrocytes were coincubated with L-2-amino-4-phosphonobutanoate (L-AP-4), 4-phosphonophenylglycine, or L-serine-*O*-phosphate, which selectively activate group III mGlu receptor subtypes (i.e., mGlu4, -6, -7, and -8 receptors). Agonists of mGlu1/5 or mGlu2/3 receptors were virtually inactive. Inhibition of RANTES release produced by L-AP-4 was attenuated by the selective group III mGlu receptor antagonist (*R,S*)-α-methylserine-*O*-phosphate or by pretreatment of the cultures with pertussis toxin. Cultured astrocytes expressed mGlu4 receptors, and the ability of L-AP-4 to inhibit RANTES release was markedly reduced in cultures prepared from mGlu4 knock-out mice. This suggests that activation of mGlu4 receptors negatively modulates the production of RANTES in glial cells. We also examined the effect of L-AP-4 on the development of experimental allergic encephalomyelitis (EAE) in Lewis rats. L-AP-4 was subcutaneously infused for 28 d by an osmotic minipump that released 250 nl/hr of a solution of 250 mM of the drug. Detectable levels of L-AP-4 (~100 nM) were found in the brain dialysate of EAE rats. Infusion of L-AP-4 did not affect the time at onset and the severity of neurological symptoms but significantly increased the rate of recovery from EAE. In addition, lower levels of RANTES mRNA were found in the cerebellum and spinal cord of EAE rats infused with L-AP-4. These results suggest that pharmacological activation of group III mGlu receptors may be useful in the experimental treatment of neuroinflammatory CNS disorders.

Key words: chemokines; glial cultures; experimental allergic encephalomyelitis; multiple sclerosis; mGlu4 receptor; leukocytes

Chemokines constitute a growing family of low molecular weight cytokines (8–14 kDa) that play a pivotal role in many biological processes, from routine immunosurveillance to the control of inflammation and cell infection by human immunodeficiency virus. They are divided into four distinct subfamilies (designated as α or CXC, β or CC, γ or CX3C, and δ or C chemokines) and activate G-protein-coupled receptors, which are named according to the classification of their specific ligands (Bazan et al., 1997; Pan et al., 1997; Wells et al., 1998). Chemokines have been implicated in the modulation of numerous biological functions in both the developing and mature CNS, including neuropoiesis, oligodendrocyte proliferation, regulation of synaptic plasticity, and, particularly, leukocyte recruitment in response to traumatic injury, stroke, autoimmunity, and inflammation (Mennicken et al., 1999). Chemokines are the only group of inflammatory mediators endowed with cell type-selective chemotactic activity, and hence, they play a vital role in defining the cellular composition of inflammatory infiltrates at the sites of tissue damage. Because of this, chemokines are becoming potential targets for therapeutic intervention in inflammatory disorders of the CNS, including multiple sclerosis (MS) (Godiska et al., 1995; Miyagishi et al., 1997; Ransohoff and Bacon, 2000) (see Discussion and references therein). Chemokines are constitutively expressed at low-to-negligible levels in neurons, astrocytes, and microglia, but are markedly upregulated in response to proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (Barnes et al., 1996; Mennicken et al., 1999). The identification of membrane receptors that control the induction of chemokines is an obligatory step in the search for drugs acting on leukocyte recruitment in neuroinflammation. We now report that pharmacological activation of group III mGlu receptors reduces the synthesis and release of the β-chemokine RANTES (from regulated upon activation of normal T cell expressed and secreted) induced by TNF-α and IFN-γ in cultured astrocytes and increases the rate of functional recovery in an *in vivo* model of neuroinflammation.

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MATERIALS AND METHODS

Materials. (*S*)-4-carboxy-3-hydroxyphenylglicine (4C3HPG), L-2-amino-4-phosphonobutanoate (L-AP-4), (*R,S*)-α-methylserine-*O*-phosphate (MSOP), L-serine-*O*-phosphate (L-SOP), (*RS*)-3,5-dihydroxyphenylglycine (DHPG), and (2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylate (2*R*,4*R*-APDC) were purchased from Tocris Cookson Ltd. (Bristol, UK). 4-phosphonophenylglycine (PPG) was a generous gift from Dr. Peter J. Flor and Dr. Fabrizio Gasparini (Novartis Pharma, Basel, Switzerland). All other drugs or chemicals were purchased from Sigma (Milan, Italy).

Preparation of primary cultures of mouse or rats cortical astrocytes. Primary cultures of cortical astrocytes were prepared from neonate CD1 mice (Charles River, Calco, Italy), mGlu4 knock-out mice (Jackson Laboratories, Bar Harbor, MN), or Lewis rats (Charles River), as described by Rose et al. (1992). Dissociated cortical cells were grown in MEM–Eagle's salts, supplemented with 10% horse serum, 10% fetal calf serum, 2 mM glutamine, 25 mM sodium bicarbonate, and 21 mM glucose. Cultures were kept at 37°C in a humidified 5% CO_2 atmosphere until they reached confluence (7–14 d *in vitro*). The number of microglial cells contaminating the cultures was assessed in cultures fixed with 2% paraformaldehyde by using the lectin, isolectin B_4 *Banderiera simplicifolia I,* coupled to biotin. After 30 min of incubation at room temperature with lectin at 1:50 dilution, lectin binding was identified by using the ABC Vectastain kit (Vector Laboratories, Burlingame, CA). The efficacy of the method for the detection of microglial cells was proven by staining spinal cord sections of Lewis rats 11 d after immunization with myelin basic protein (MBP) (data not shown).

Measurement of extracellular RANTES in the glial medium. Glial cultures were washed with serum-free medium and, 1 hr later, were incubated for 15 hr with IFN-γ (10 U/ml) and TNF-α (0.1 ng/ml), in the absence or presence of specific mGlu receptor ligands. At the end of the incubation, the medium was removed and used for the determination of RANTES using the mouse RANTES QUANTIKINE M immunoassay ELISA kit (R & D Systems, Minneapolis, MN).

RNA extraction and Northern blot analysis of RANTES mRNA. Total RNA was prepared from cultured astrocytes and from cerebellum and spinal cord of Lewis rats (Chomczynski and Sacchi, 1987). Thirty micrograms of total RNA were denatured, subjected to electrophoresis on 1% formaldehyde agarose gel, and transferred to a nylon membrane Hybond-N (Amersham Pharmacia Biotech, Milan, Italy). Membranes were fixed by UV irradiation using an XL-1500 UV cross-linker (Spectrolinker, Spectronics Corporation, Westbury, NY) and stained with 0.04% methylene blue and 0.5 M sodium acetate. Membranes were hybridized with a random primed [α-^{32}P]-dCTP-labeled probe consisting of RANTES 0.7 kb cDNA insert in a pCR II vector (Invitrogen, Groningen, The Netherlands) cloned at the *Bst*XI/*Not*I site. Hybridizations were performed overnight at 42°C. Blots were washed twice using 2× SSC–0.1% SDS for 15 min at 42°C and then twice with 0.1× SSC–0.1% SDS for 15 min at 42°C. The filters were then exposed to Hyperfilm-MP (Amersham Pharmacia Biotech) and exposed at −80°C for 18 hr. Filters were reprobed with β-actin cDNA, and autoradiograms were quantified by densitometry using a computerized image-processing system (NIH Imaging, Bethesda, MD).

RT-PCR analysis of mGlu4 receptors. Two micrograms of total RNA extracted from cultured astrocytes and 100 ng of random hexamers dissolved in 10 μl of RNase-free water were heated to 65°C for 10 min and then cooled on ice. Reverse transcriptase (RT) buffer (10 mM DTT, 500 μM dNTP, and 200 U of Moloney murine leukemia virus RT enzyme) was added to a final volume of 25 μl. The incubation was continued at 42°C for 1 hr, and the reaction was terminated by a 10 min incubation at 99°C. PCR was performed for 35 cycles in final volume of 50 μl with appropriate quantity of buffer and $MgCl_2$, 200 μM of dNTP, 50 pmol of either forward or reverse primers, and 2.5 U of AmpliTaq Gold (Perkin-Elmer Cetus Corp., Norwalk, CT). RT-PCR negative control was performed loading dH_2O instead of cDNA. Primers and PCR conditions were as follows: mGlu4 receptor: GenBank accession #M90518; 1.5 mM $MgCl_2$; annealing at 60°C; amplimer 567 bp; forward: 5′-TGAGCTACGTGCTGCTGGCG-3′; reverse: 5′-TGTCGGCTGA-CTGTGAGGTG-3′.

Western blot analysis of RANTES and mGlu4 receptors. Western blot analysis in protein extracts from lysates of cultured astrocytes prepared from mice or rats or from mouse cerebral cortex (used as a positive control) was performed as described by Ciccarelli et al. (2000). RANTES expression was detected with 4 μg/ml of a monoclonal antibody (R & D Systems). mGlu4a receptors were detected with 0.5 μg/ml of a polyclonal antibody raised against synthetic peptides corresponding to the following amino acid sequences CGGLETPALATKQTYVTNHAI corresponding to the putative intracellular C-terminal domain of rat mGlu4a receptor (Upstate Biotechnology, Inc. Lake Placid, NY; residues 893–912) (Bradley et al., 1996, 1999).

Induction of experimental allergic encephalomyelitis in Lewis rats. For experimental allergic encephalomyelitis (EAE) induction, Lewis rats (225–250 gm, body weight) were immunized in the proximal portion of the tail with 50 μg of guinea pig MBP, 2 mg *Mycobacterium tuberculosis* in 100 μl saline, and 100 μl Freund's incomplete adjuvant. Animals were implanted with subcutaneous osmotic minipumps (Alzet; Alza, Palo Alto, CA) containing 200 μl of 250 mM of L-AP-4 dissolved in PBS, which release 250 nl/d for 28 d. Control animals were implanted with minipumps containing PBS alone. Immunization was performed 48 hr after implanting the osmotic minipumps. In standard experiments, immunized animals developed clinical signs of EAE 10 d after immunization. Symptoms of EAE were scored using the disability scale described by Godiska et al. (1995) in which 0 = absence of clinical signs; 1 = loss of motor control in the tail; 2 = hindquarter weakness or the inability to turn over when placed on the back; 3 = total hindquarter paralysis; 4 = total hindquarter paralysis with incontinence and/or forearm involvement; and 5 = death caused by EAE. In addition, body weight was monitored every day during the development of EAE.

Some animals were killed 12 d after immunization (i.e., at the time of the peak of clinical symptoms) for the detection of RANTES in the cerebellum and spinal cord. RANTES protein and mRNA levels were assessed by Western and Northern analysis, as described above.

Immunohistochemical analysis of spinal cords. Two established parameters of neuroinflammation, i.e., the expression of major histocompatibility complex (MHC) class II antigens and the presence of $CD4^+$ cells were examined by immunohistochemistry in Lewis rats immunized with MBP and treated with L-AP-4, as described above. Animals were killed by CO_2 inhalation at 11 or 27 d after immunization, and spinal cords were collected and snap-frozen in liquid nitrogen. For immunohistochemical analysis, spinal cord cryostat sections (10 μm) were exposed to appropriate dilutions of the mouse FITC-conjugated monoclonal antibodies (Seralab, Crawley-Down, UK) directed against the rat homologs of human CD4, (W3/25), and MHC class II antigens (OX6). Coded slides were examined by fluorescence microscopy at 40× magnification. Inflammation grade was assessed blindly and scored from grade 1–4 in relation to the intensity of immunostaining, density of immunopositive cells, and distribution of clusters of immunopositive cells (total score: 0–12) (Di Marco et al., 2001).

Measurements of L-AP-4 levels by in vivo *microdialysis.* To assess whether subcutaneously infused L-AP-4 can penetrate the brain, we measured the amount of the drug present in the striatal dialysate of freely moving animals. Lewis rats, 250–300 gm, were implanted with microdialysis intracerebral guides in the striatum using the following coordinates: 2.0 mm anterior to bregma, 2.6 mm lateral to the midline, and 4–6 mm ventral, according to the atlas of Pellegrino et al. (1992), under pentobarbital anesthesia (50 mg/kg, i.p.). After surgery, rats were housed in separate cages in a temperature-controlled environment on a 12 hr light/dark cycle, with *ad libitum* access to water and food, and allowed to recover. Forty-eight hours later rats were implanted with subcutaneous osmotic minipumps (Alzet), containing L-AP-4 (200 μl, 250 mM) that release 250 nl/d for 28 d of L-AP-4, and were immunized 48 hr later as described above. Microdialysis was performed 9 d after immunization. Twelve hours before the experiment, a concentric vertical probe (2-mm-long and 0.5 mm in outer diameter having a polycarbonate membrane; molecular cutoff: 20,000 Da; CMA/12; CMA Microdialysis, Stockholm, Sweden) was inserted into the intracerebral guide cannula, and rats were transferred to a plastic bowl cage with a moving arm. The animals had *ad libitum* access to water and food. The probe was perfused continuously with artificial CSF (ACSF), at a flow rate of 0.1 μl/min, using a microinjection pump. ACSF contained in mM: 150 NaCl, 3 KCl, 1.7 $CaCl_2$, and 0.9 $MgCl_2$. On the next day, perfusate sample fractions were continuously collected by a fraction collector. Analysis of L-AP-4 present in the dialysate was performed by HPLC with fluorescence detection.

Measurement of extracellular RANTES in leukocytes. Peripheral blood mononuclear cells (PBMCs) were prepared using Ficoll HyPaque according to the manufacturer's recommended protocol. Enriched monocytes were obtained from whole PBMCs by incubation with RPMI 1640 + 10% FCS at 4°C, for 30 min at constant rotation. Nonaggregated cells were removed, and monocyte-enriched pellet was resuspended in RPMI and plated 2×10^{-6} cells per well in 6-well plates. After a 2 hr incubation at 37°C, nonadherent cells were removed, and adherent cells were

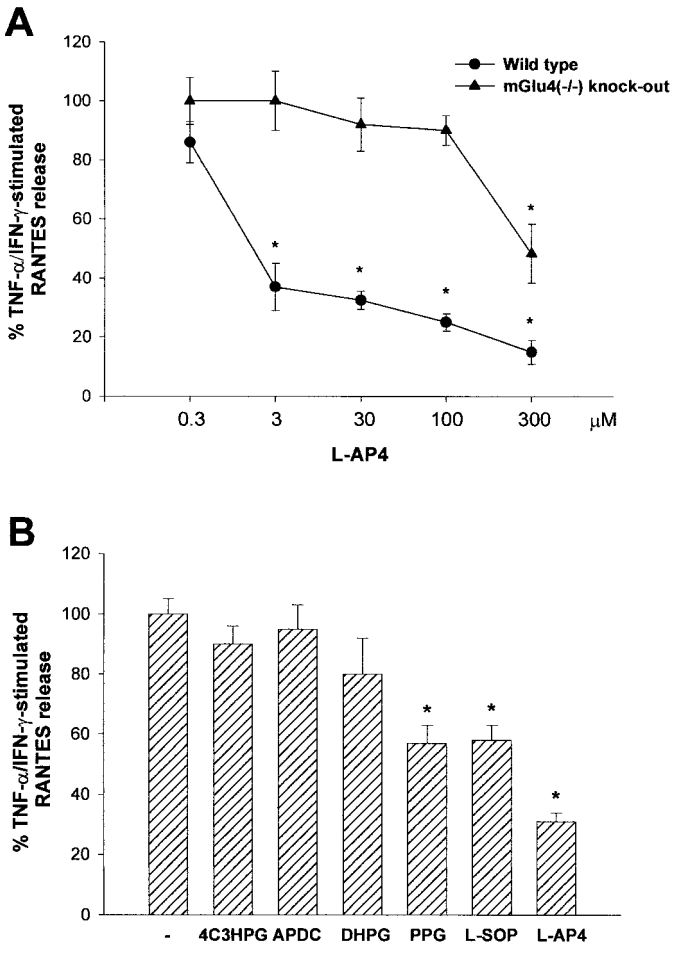

Figure 1. Extracellular RANTES levels in mouse cultured astrocytes exposed to TNF-α and IFN-γ in the absence or presence of mGlu receptor ligands. *A*, Concentration-dependent effect of L-AP-4 on the increase in extracellular RANTES induced by TNF-α and IFN-γ in cultured cortical astrocytes prepared from wild-type or knock-out mouse cortical astrocytes. Addition of TNF-α and IFN-γ increased extracellular RANTES levels from 0.15 ± 0.02 to 6.7 ± 0.81 ng/mg protein in wild-type cultures and from 0.08 ± 0.04 to 5.9 ± 0.63 ng/mg protein in mGlu4(−/−) cultures. Values were calculated as a percentage of cytokine-stimulated RANTES release and represent the means ± SEM of six determinations. $^*p < 0.05$ (one-way ANOVA + Fisher's PLSD) as compared with TNF-α and IFN-γ alone. *B*, Effect of different mGlu receptor ligands on the increase in extracellular RANTES induced by TNF-α and IFN-γ in mouse cultured astrocytes. Values were calculated from 6–12 determinations from at least three individual multiplates. All mGlu receptor ligands were added at concentrations of 100 μM with the exception of L-SOP (300 μM). $^*p < 0.05$ (one-way ANOVA and Fisher's PLSD) as compared with values obtained in the absence of mGlu receptor ligands (−).

washed. Adherent cells were then removed from the plate using trypsin. This enriched monocyte fraction was 85–90% $CD4^+$ by flow cytometric analysis. $CD8^+$ cells were obtained from whole PBMCs by negative selection. Briefly, cells were incubated with an antibody cocktail containing microbeads against CD4, 11b, 14, 16, 19, 36, 56, and IgE (Miltenyi Biotec) for 30 min at 4°C. Cells were washed and passed over a permanent magnet. The negative fraction was >95% $CD8^+$ as determined by flow cytometric analysis. Cells were preincubated with appropriate concentration of L-AP-4 for 30 min at 37°C and seeded at 2×10^5 cells per well in 96-well plates. Phorbol-12-myristate-13-acetate (PMA) was added, and cultures were incubated for 48 hr at 37°C. Supernatants were collected and stored at −80°C. For the determination of extracellular RANTES, human anti-RANTES antibody (Genzyme Techne) was coated on a 96-well plate (10 μg/ml) at 4°C for 18 hr. Plate was washed three times with PBS. Nonspecific sites were blocked by an incubation with PBS supplemented with 5% BSA. After washing plate with PBS + 0.05% Tween 20, 50 μl/well supernatant was added and incubated at room temperature for 2 hr. A Europium-labeled anti-RANTES antibody was added (a kind gift from H. Inbe, Department of Biological Chemistry, Yamaguchi University, Yamaguchi, Japan) and incubated for a further 2 hr at room temperature. Plate was washed using PBS + 0.05% Tween 20 at 300 μl/well. Enhancement solution (LKB-Wallac, Gaithersburg, MD) was added at 100 μl/well and incubated at room temperature for 10 min. The plate was then measured for Europium counts using a Wallac Arvo SX Multi-Label counter (Wallac).

Table 1. MSOP antagonizes the reduction in extracellular RANTES levels induced by L-AP4

	ng/mg prot.
TNF-α + IFN-γ	7.14 ± 0.66
+ L-AP4, 30 μM	3.56 ± 0.12*
+ MSOP, 100 μM	8.53 ± 0.38
+ L-AP4 + MSOP	9.17 ± 1.42

Values are means ± SEM of four individual determinations.
$^*p < 0.05$ (one-way ANOVA + Fisher's PLSD) compared with all other groups.

RESULTS

Activation of group III mGlu receptors reduces the production of RANTES in primary cultures of glial cells

We used confluent cultures of glial cells, which were primarily constituted of flat and polygonal type-1 astrocytes. Cultures were virtually devoid of microglial cells (only 6–10 cells per well stained with the lectin, *Banderiera simplicifolia I*). A 15 hr exposure of mouse cultured glial cells to 0.1 ng/ml of TNF-α plus 10 U/ml of IFN-γ led to a substantial increase in the amount of RANTES released into the medium. L-AP-4 applied in combination with TNF-α and IFN-γ reduced extracellular RANTES levels in a concentration-dependent manner, with an apparent EC_{50} value of 6 μM (Fig. 1*A*). Among other mGlu receptor ligands, L-SOP (300 μM) and PPG (100 μM) were also able to reduce extracellular RANTES. 4C3HPG, 2*R*,4*R*-APDC, and DHPG (all at 100 μM) had negligible, if any, effect on the cytokine-stimulated increase in extracellular RANTES (Fig. 1*B*). The group III mGlu receptor antagonist MSOP (100 μM) produced a slight increase in extracellular RANTES on it own, but markedly reduced the inhibitory action of L-AP-4 (30 μM) (Table 1). None of the mGlu receptor ligands induced changes in extracellular RANTES in the absence of TNF-α and IFN-γ (data not shown). We also assessed intracellular RANTES mRNA and protein levels in mouse cultured glial cells. Northern blot analysis showed that RANTES mRNA levels were nearly undetectable in untreated cultures but increased substantially after a 15 hr treatment with TNF-α and IFN-γ. This increase was markedly reduced by L-AP-4 (100 μM) or PPG (100 μM), but was minimally affected by 2*R*,4*R*-APDC (100 μM) or DHPG (100 μM) (Fig. 2*A,B*). Similarly, L-AP-4 reduced the cytokine-induced increase in RANTES protein levels, and its action was attenuated in cultures pretreated with pertussis toxin (PTX) (Fig. 3).

Finally, we measured the ability of L-AP-4 to reduce cytokine-stimulated RANTES production in cultured glial cells prepared from knock-out mice lacking mGlu4 receptors. In mGlu4(−/−) cultures a 15-hr treatment with TNF-α + IFN-γ increases the extracellular levels of RANTES to the same extent as in cultures from wild-type mice. L-AP-4 could still reduce cytokine-stimulated RANTES release in mGlu4(−/−) cultures, but only at concentrations of 300 μM (Fig. 1*A*).

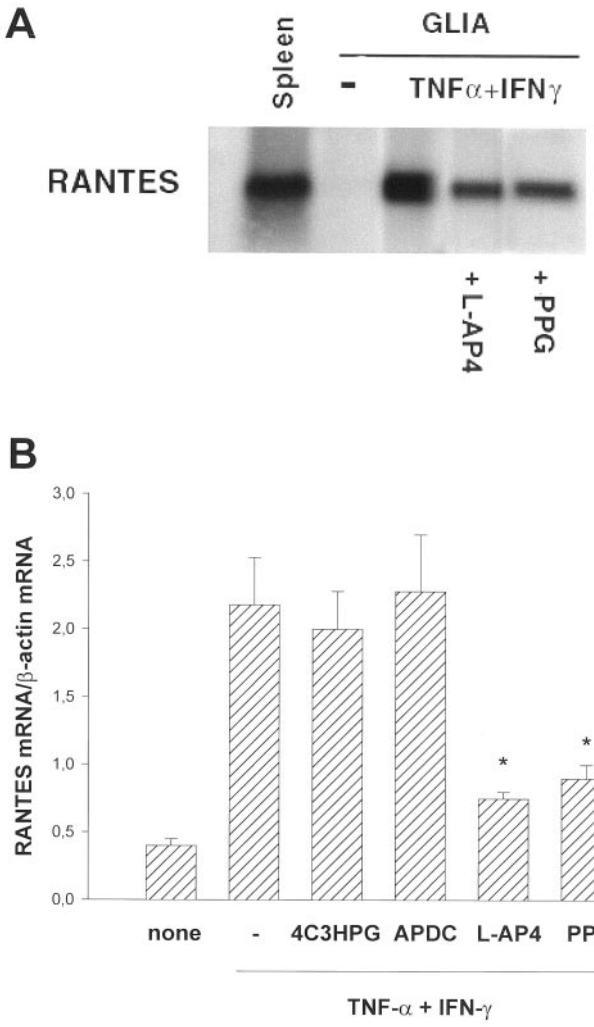

Figure 2. RANTES mRNA levels in mouse cultured astrocytes incubated for 15 hr with TNF-α and IFN-γ in the absence or presence of different mGlu receptor agonists. *A*, Representative Northern Blot of RANTES mRNA in mouse cultures of astrocytes. In the first lane, the spleen is shown as a positive control. Note that RANTES mRNA levels were nearly undetectable in untreated cultures (−), but increased substantially after a 15 hr treatment with TNF-α and IFN-γ. This increase was markedly reduced by L-AP-4 or PPG (both at 100 μM). *B,* Densitometric analysis of RANTES mRNA levels in cultured astrocytes incubated for 15 hr with TNF-α and IFN-γ in the absence or presence of 4C3HPG, 2*R*,4*R*-APDC (*APDC*), L-AP-4, or PPG (all at 100 μM). Values were normalized by the amount of β-actin mRNA and represent the means ± SEM of three determinations. $*p < 0.05$ (one-way ANOVA plus Fisher's PLSD) versus cultures treated with TNF-α and IFN-γ alone (−).

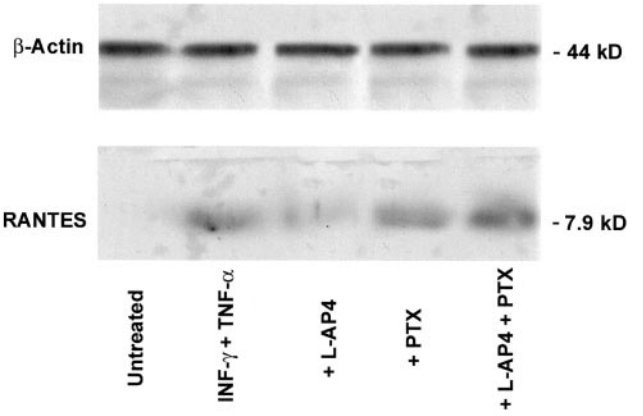

Figure 3. Western blot analysis of RANTES in mouse cultured astrocytes incubated for 15 hr with TNF-α and IFN-γ in the absence or presence of L-AP-4 (100 μM) and/or PTX (0.5 μg/ml, preincubated for 16 hr before the incubation with the cytokines). Note that PTX abolished the reduction in RANTES levels induced by L-AP-4.

Detection of mGlu4 receptors in cultured glial cells

In immunoblots, mGlu4 antibodies labeled a major band at ~100 kDa, which corresponds to the receptor monomer. This band was detected in cultured glial cells from wild-type mice or Lewis rats, but not in cultures from mGlu4(−/−) mice (Fig. 4*A*). The presence of mGlu4 receptors in cultured glial cells was confirmed by RT-PCR analysis (Fig. 4*B*).

Effect of L-AP-4 on the development of EAE in Lewis rats

To assess whether the modulation of RANTES formation in glial cells has any functional relevance, we examined the effect of systemically injected L-AP-4 in the EAE model of neuroinflam-

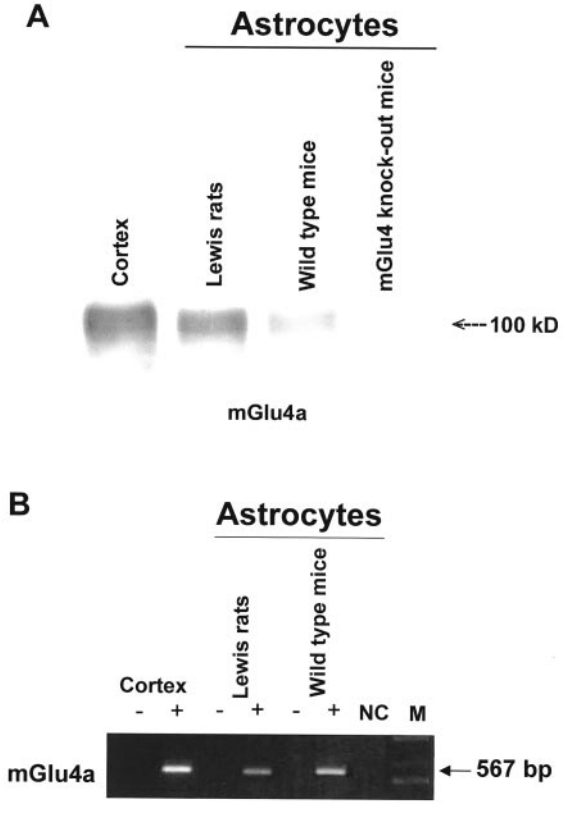

Figure 4. Expression of mGlu4a receptor in cultured astrocytes. *A*, Western blot analysis of mGlu4a receptor in cultured astrocytes from wild-type mice, mGlu4(−/−) mice, or Lewis rats. Expression in the cerebral cortex of wild-type mice is shown as a positive control. The antibody recognized a specific band corresponding to the monomeric form of receptor at 100 kDa (*arrow*). *B,* RT-PCR analysis of mGlu4a receptor mRNA in cultured astrocytes from wild-type mice or Lewis rats. (−) and (+) refer to the absence or presence of reverse transcriptase. Mouse cerebral cortex is shown as reference tissue. NC represents a negative control in which dH_2O has been loaded instead of cDNA. Size markers (*M*) are on the last lane on the *right*.

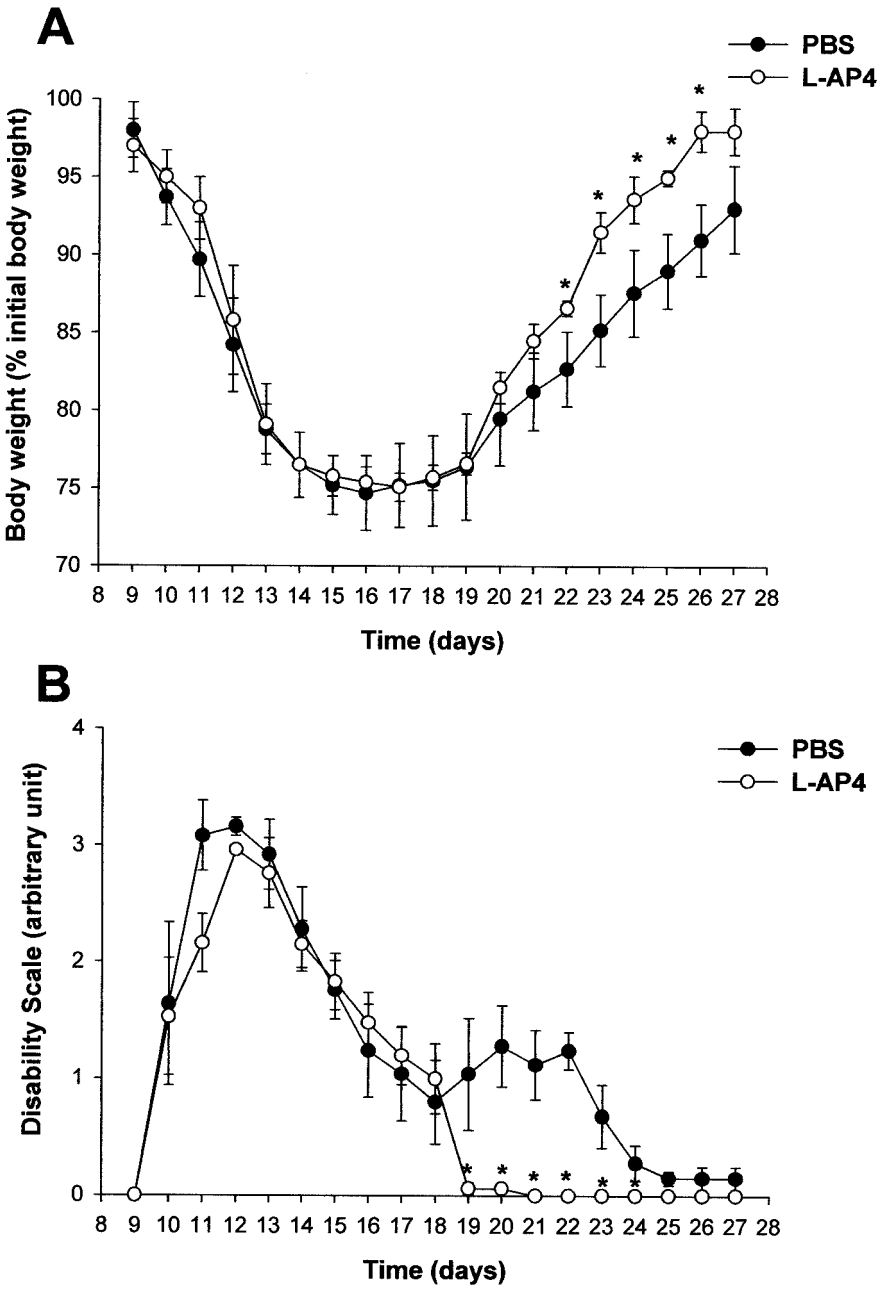

Figure 5. Effect of L-AP-4 on the development of EAE in Lewis rats. *A*, Temporal profile of body weight in animals immunized with MBP implanted with an osmotic minipump, which released 250 nl/d of PBS or a PBS solution containing 250 mM of L-AP-4. *B*, Temporal profile of the disability score of EAE. Note that rats treated with L-AP-4 showed a faster recovery rate from EAE than control rats. Values express the means ± SEM of 10 animals per group. $^{*}p < 0.05$ (one-way ANOVA and Fisher's PLSD) versus PBS-treated animals.

mation in Lewis rats. Immunization of Lewis rats with guinea pig MBP induced the classical symptoms of EAE after a latency of 10 d, as assessed by the disability scale and by the loss of body weight. In standard experiments, the course of EAE was biphasic, showing a peak at 11–13 d after immunization, followed by a drop in the score during days 14–18 and by a plateau phase with scores of 1–1.5 lasting until day 23. No relapses were observed after full recovery of EAE. Figure 5 shows the temporal profile of EAE in control rats and in rats implanted with a subcutaneous osmotic minipump releasing 250 nl/d of a PBS solution containing 250 mM of L-AP-4. The pump was implanted 2 d before immunization. Detectable amounts of L-AP-4 (105 ± 37 nM; $n = 3$) were detected in the striatal dialysate of freely moving rats, suggesting that some amounts of subcutaneously infused L-AP-4 can penetrate the brain. No gross abnormalities in motor behavior were observed in nonimmunized rats treated with L-AP-4 or in immunized rats infused with L-AP-4 before the clinical onset of EAE, as detected by measuring locomotor activity in an open field apparatus (data not shown). In rats treated with L-AP-4 there was no difference in the time at onset and in the peak of the disability score of EAE, as compared with control rats implanted with a minipump containing PBS alone, although L-AP-4-treated rats reached the peak of the disability score 1 d later than control rats (i.e., 12 instead of 11 d after immunization). However, rats treated with L-AP-4 showed a faster recovery rate from EAE, as indicated by the absence of the plateau phase, and showed a full recovery after 19 d (vs 24 or 25 d in control rats) (Fig. 5*A,B*). Independent groups of animals were used for histological examination. Neuroinflammation was assessed by immunohistochemical analysis of MHC-II^{+} and CD4^{+} cells in the spinal cord (MHC-II^{+} cells in the spinal cord of immunized animals are shown in the lower panel of Fig. 6*A*). We adopted a semiquanti-

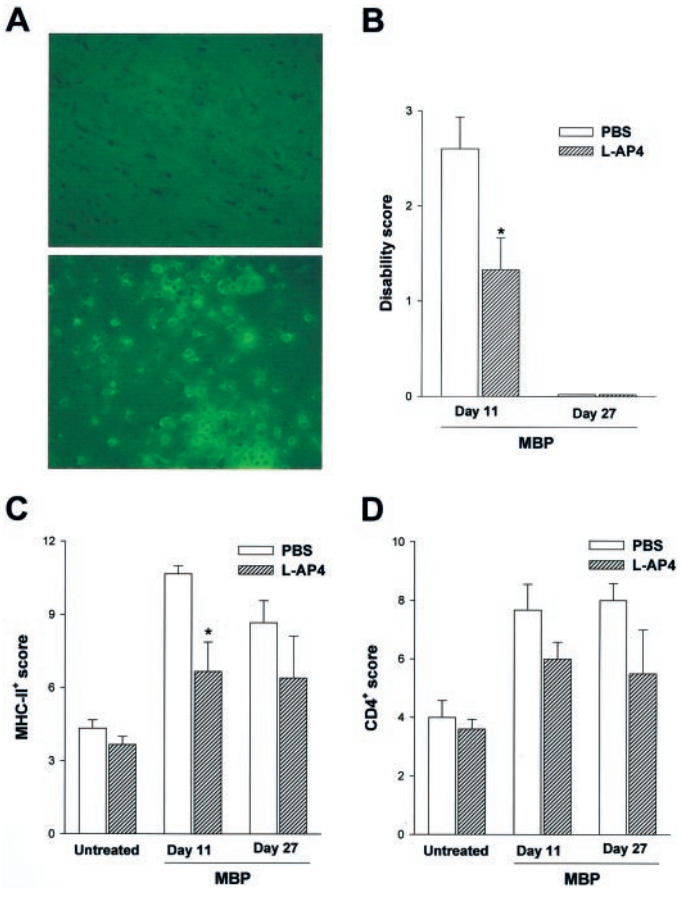

Figure 6. Immunohistochemistry of MHC-II$^+$ and CD4$^+$ cells in the spinal cord of nonimmunized or immunized Lewis rats subcutaneously infused with L-AP-4. An example of MHC-II immunostaining in the spinal cord of nonimmunized (*top panel*) and immunized (*bottom panel*) rats is shown in *A*. The disability score of immunized animals killed at 11 or 27 d after MBP injection and used for immunohistochemical analysis is shown in *B* (mean + SEM of three to five determinations; $^*p < 0.05$; Student's *t* test vs the respective group of rats subcutaneously infused with saline). Quantification of MHC-II and CD4 immunostaining in nonimmunized (*untreated*) or immunized (*MBP*) rats subcutaneously infused with PBS or L-AP-4 is shown in *C* and *D*, respectively. Immunized rats were examined at day 11 or 27 after injection of MBP. Untreated rats were examined 13 d after implantation of osmotic minipumps releasing either saline or L-AP-4. Values are means + SEM of three to five determinations; $^*p < 0.05$ (one-way ANOVA and Fisher's PLSD, as compared with the respective group implanted with minipumps releasing saline).

tative scale with scores from 0 to 4 referred to the intensity of staining, number of immunopositive cells within identified clusters of cells, and distribution of clusters of immunopositive cells (maximal score = 12; modified from Di Marco et al., 2001). Animals were examined at day 11 or 27 after immunization. A substantial increase in the neuroinflammation score for both MHC-II and CD4 was observed in the spinal cord of immunized animals after 11 d, i.e., at the time of the peak of motor symptoms. The score was only slightly reduced at 27 d, when animals were apparently asymptomatic (Fig. 6*B–D*). Immunized rats that received L-AP-4 subcutaneously had a lower disability score at day 11 (Fig. 6*B*) and a lower number of MCH-II$^+$ cells in the spinal cord (Fig. 6*C*). The MCH-II score showed a trend to a reduction also at 27 d in immunized animals treated with L-AP-4 (Fig. 6*C*). This trend with L-AP-4 was also observed by scoring CD4 immunoreactivity at 11 and 27 d after immunization (Fig. 6*D*). In other animals we measured RANTES mRNA levels in the cerebellum and spinal cord at the time of the peak of the disability score. Northern blot analysis showed that treatment with L-AP-4 reduced RANTES mRNA levels in the cerebellum and spinal cord of EAE rats (Fig. 7). Because systemically injected L-AP-4 gave only a partial protection against EAE, we decided to examine whether the drug had any effect on the production of RANTES by other cells that contribute to the pathophysiology of EAE, such as leukocytes. Experiments were performed on human purified protein derivative-derived Th1 clones, CD8$^+$ leukocytes, and monocytes, in which the production and release of RANTES was stimulated by PMA (0.1–10 ng/ml). Addition of L-AP-4 (0.1–1000 μM) had no effect on extracellular RANTES in Th1 clones and showed a trend to reduction of extracellular RANTES in CD8$^+$ cells and monocytes. However, this trend was observed only with high concentrations of L-AP-4 (100 or 1000 μM) and was not statistically significant (Fig. 8*A–C*).

DISCUSSION

RANTES is a β chemokine that is gaining more and more interest for its role in neuroinflammation. Similar to monocyte chemoattractant protein-1 (MCP-1) and interferon-γ-inducible protein-10 (IP-10), RANTES is chemoattractant for monocytes, and contributes to the pathophysiology of immune disorders, including MS (Ransohoff, 1999). RANTES can also attract memory T and NK cells, which are involved in MS, and act as an antigen-independent activator of T cells *in vitro* (Schall et al., 1990; Bacon et al., 1995; Taub et al., 1995). In EAE, RANTES amplifies the inflammatory process, and its expression correlates with the intensity of neuroinflammation (Glabinski et al., 1998; Ransohoff, 1999). In addition, RANTES has been associated with the early formation of plaques (Simpson et al., 1998), and the RANTES receptor CCR5 has been found in lymphocytes and phagocytes of actively demyelinating lesions in MS (Bacon and Oppenheim, 1998; Sorensen et al., 1999). Interestingly, the production of RANTES by peripheral mononuclear cells is reduced after treating MS patients with IFN-β-1b (Iarlori et al., 2000; Zang et al., 2001), suggesting that the modulation of RANTES production may be a valuable target in the pharmacological treatment of MS patients. We examined the possibility that the production of RANTES could be modulated by the activation of glial membrane receptors. In particular, we focused on mGlu receptors, which are G-protein-coupled receptors activated by glutamate and other excitatory amino acids. mGlu receptors form a family of eight subtypes, which are subdivided into three groups on the basis of their sequence homology, pharmacological profile, and transduction pathways. Group I includes mGlu1 and -5 receptors, which are coupled to inositol phospholipid hydrolysis and activated by DHPG. Group II includes mGlu2 and -3 receptors, which are coupled to G_i-proteins and activated by 2*R*,4*R*-APDC. 4C3HPG behaves as a mixed mGlu2/3 agonist/mGlu1 antagonist. Group III includes mGlu4, -6, -7, and -8 receptors, which are also coupled to G_i-proteins and selectively activated by L-AP-4, PPG, and L-SOP (Pin and Duvoisin, 1995; Schoepp et al., 1999). Whereas mGlu3 and -5 receptors are expressed by astrocytes (Miller et al., 1995; Petralia et al., 1996; Ciccarelli et al., 1999), evidence for the expression of group III mGlu receptors in glial cells is still lacking. Hence, we used primary cultures of glial cells, expecting that mGlu3 or -5 receptor agonists could modulate the production of RANTES. Unexpectedly, however, only group III

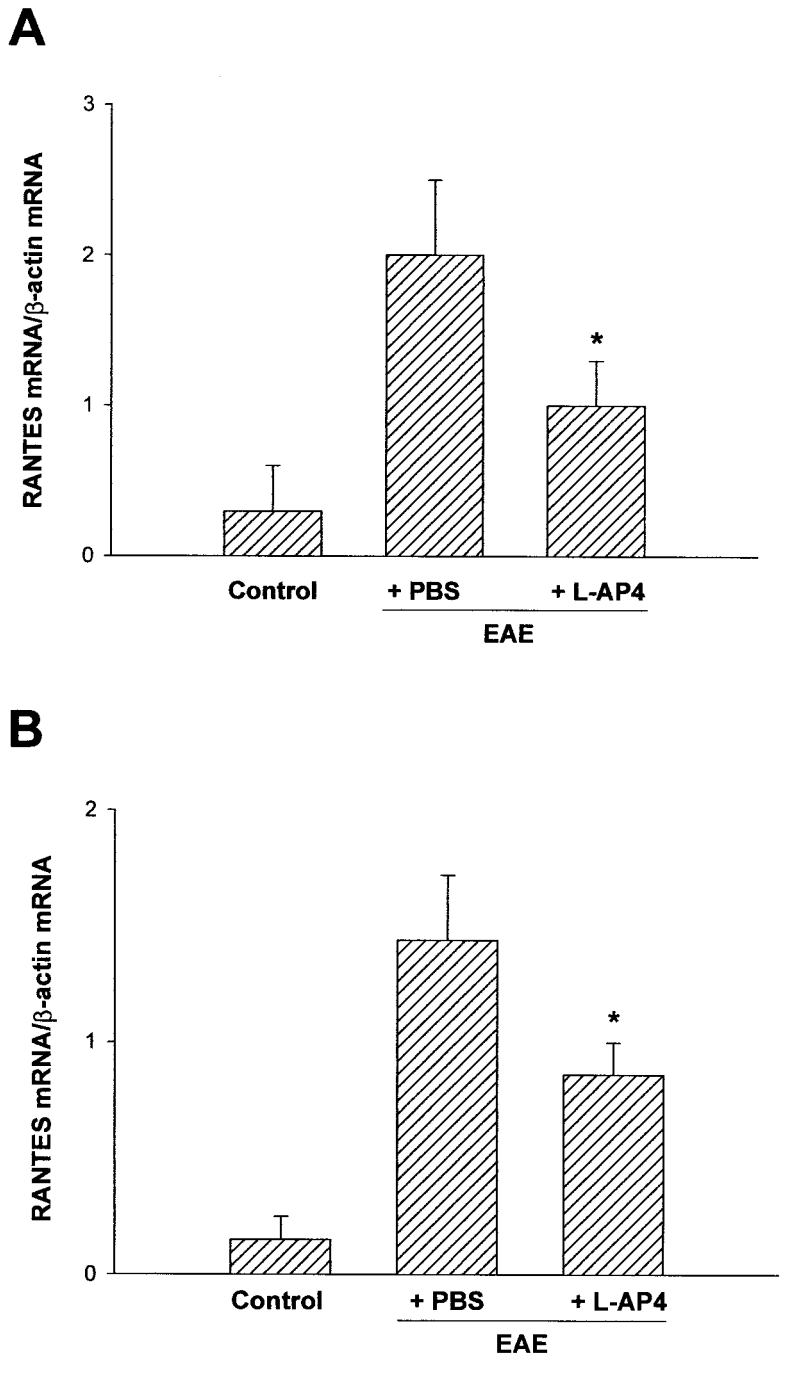

Figure 7. Expression of RANTES mRNA levels in the cerebellum and spinal cord of EAE rats subcutaneously infused with PBS or L-AP-4. *A*, Densitometric analysis of RANTES mRNA levels in the cerebellum and spinal cord is shown in *A*, and *B*, respectively. Values were normalized by the amount of β-actin mRNA and express the means ± SEM of three determinations. $^*p < 0.05$ (one-way ANOVA and Fisher's PLSD) versus treated animals. Subcutaneous infusion of L-AP-4 in nonimmunized rats did not induce changes in RANTES mRNA levels in the cerebellum and spinal cord (93 + 7.8% of controls in the cerebellum and 137 + 28% of controls in the spinal cord, 13 d after implantations of osmotic minipumps; $n = 4$).

mGlu receptor agonists (i.e., L-AP-4, PPG, or L-SOP) reduced RANTES levels in cultures stimulated with TNF-α and IFN-γ. L-AP-4, the prototypic agonist of group III mGlu receptors, produced the more substantial and reproducible effect on RANTES levels, and its action was sensitive to the group III mGlu receptor antagonist MSOP (Schoepp et al., 1999) and to PTX, which inhibits the activity of G_i-proteins. The reduction of RANTES mRNA levels by L-AP-4 or PPG suggested that these drugs act at transcriptional level or decrease the stability of RANTES mRNA. The calculated EC_{50} value for L-AP-4 is consistent with the activation of mGlu4 or -8 rather than mGlu7 receptors, which can be recruited only by concentrations of L-AP-4 >100 μM (Schoepp et al., 1999). At least mGlu4 receptors appeared to be expressed by cultured glial cells. It is unlikely that expression of mGlu4 receptors derived from contaminating microglia, because only 6–10 microglial cells per well were detected with a method that was highly efficacious in detecting microglia in the inflammatory infiltrate of EAE rats (data not shown). In cultures from mGlu4 knock-out mice, only concentrations of L-AP-4 > 100 μM were still able to reduce the production of RANTES. This indicates that glial mGlu4 receptors primarily contribute to the regulation of RANTES formation in astrocytes. Whether the effect produced by 300 μM L-AP-4 reflects the recruitment of mGlu7 receptors or rather represents a nonspecific effect of the drug remains to be determined. Moving from the *in vitro* data, we decided to examine the effect of L-AP-4 on EAE in Lewis rats. Because EAE develops gradually after a latency period of ~10 d, we continuously infused L-AP-4 by means of a subcutaneous

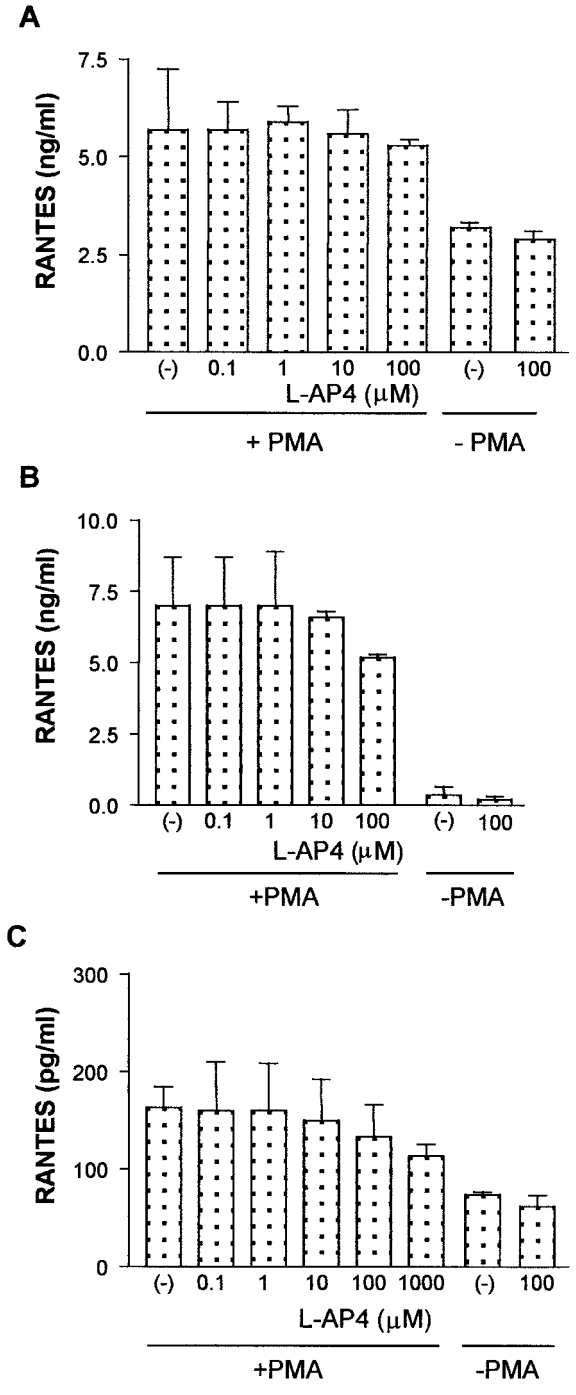

Figure 8. Effect of L-AP-4 on RANTES release in Th1 clones (*A*), $CD8^+$ leukocytes (*B*), or monocytes (*C*), stimulated for 48 hr with 0.1 ng/ml of PMA. Values are means ± SEM.

osmotic minipump. We used this strategy because the increased permeability of the blood–brain barrier during the development of EAE (Perry et al., 1997) could allow a sufficient penetration of L-AP-4 into the brain. To assess whether this assumption was correct, we measured extracellular brain levels of L-AP-4 by *in vivo* microdialysis. The amount of L-AP-4 found in the dialysate (~100 nM) suggests that concentrations of L-AP-4 in the range of 0.1–1 μM should be present in the extracellular space. These concentrations may be sufficient to activate mGlu4 receptors (Schoepp et al., 1999). Infusion of L-AP-4 neither delayed the time at onset nor reduced the severity of EAE symptoms, although treated animals reached the peak of the disability score 1 d later. Interestingly, however, treatment with L-AP-4 increased the rate of recovery from EAE. The second phase of motor disability, which was present in animals infused with PBS from day 18 to day 23 after immunization, was not observed in animals that received L-AP-4. Because EAE in Lewis rats represents a model of neuroinflammation, but not of demyelination, we suggest that L-AP-4 infusion reduces the inflammatory infiltrate in the brain parenchyma to an extent that does not prevent the development of EAE but allows a faster recovery from the disease (Miyagishi et al., 1997). Accordingly, immunized animals treated with L-AP-4 showed a reduced immunostaining for MHC class II in the spinal cord and a trend to a reduction in CD4 immunostaining in the spinal cord at day 11 after immunization. The partial reduction of RANTES mRNA levels produced by L-AP-4 infusion in the cerebellum and spinal cord of EAE rats might account for the 1 d delay in reaching the peak of the disability score, the reduced extent of neuroinflammation, and the lack of the delayed plateau phase of the disability score. The possibility that the delayed plateau phase is sustained by the release of RANTES from glial cells is interesting and deserves further investigation. The lack of a more robust effect of L-AP-4 on EAE may reflect the inability of the drug to reduce the production of RANTES from other cells that directly contribute to the inflammatory infiltrate, such as leukocytes. Accordingly, L-AP-4 did not reduce the release of RANTES stimulated by phorbol esters in lymphocytes or monocytes, although a trend to a reduction with high concentrations of L-AP-4 was seen in $CD8^+$ lymphocytes and in monocytes.

In conclusion, present results offer the first demonstration that group III mGlu receptors are expressed and functional in glial cells. The unexpected finding that activation of these receptors reduces the production of RANTES in astrocytes suggests that brain-permeable agonists may be useful in the experimental treatment of neuroinflammatory disorders of the CNS. The potential usefulness of group III mGlu receptor agonists in human pathology awaits the demonstration that these drugs show good safety and tolerability when systemically injected.

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