The growth-related gene product β (GROβ) is a small chemoattractant cytokine that belongs to the CXC chemokine family, and GROβ receptors are expressed in the brain, including the cerebellum. We demonstrate that rat cerebellar granule neurones express the GROβ receptor CXCR2. We also show that, in addition to the known stimulation of a phosphoinositide-specific phospholipase C, GROβ activates both neutral (N-) and acidic (A-) sphingomyelinases (SMase) and the stress-activated JNK1, c-Jun N-terminal kinase 1 (JNK1). Although both exogenous ceramide and bacterial SMase stimulate JNK1, GROβ-induced JNK1 activation is an event probably independent of ceramide generated by A-SMase, since it is maintained in the presence of compounds that block A-SMase activity. This is the first report on the activation of the SMase pathway by chemokines.

Moreover, chemokines and their receptors are expressed in different regions of the central nervous system: human cerebellar neurones stain positively for CXCR2 and Duffy antigen/chemokine receptor, which are expressed mainly in Purkinje cells and in neurite processes projecting to Purkinje cells (10) and we have recently shown that agonists of the CXCR2 have neuromodulatory roles in mouse and rat cerebellum, increasing the frequency of postsynaptic currents and impairing the induction of long-term depression (11, 12). Chemokine levels in the central nervous system increase in several neurological pathologies (reviewed in Ref. 13), and deletion of chemokine receptor CXCR4 has severe consequence on the correct cerebellar development (14). For all these reasons, primary cultures of cerebellar granules have been chosen to study the biochemical pathways activated by GROβ treatment of homogeneous populations of cerebellar neurones. While previous reports demonstrated that, in addition to PLC, chemokine signaling can occur via phospholipase D and phospholipase A2 activation (15, 16), in this paper we describe for the first time that a chemokine induces the activation of sphingomyelin hydrolysis. We also report that GROβ mediates the activation of the stress-activated protein kinase c-Jun N-terminal kinase 1 (JNK1) and that this effect is not influenced by A-SMase inhibitors.

**EXPERIMENTAL PROCEDURES**

Materials—Lipid standards, mosenins, imipamine, and desipramine were from Sigma Italy; [γ-32P]ATP (6000 Ci/mmol) was from NEN Life Science Products Inc.; N-[methyl-3H]sphingomyelin, [1-14C]arachidonic acid, InsP3 assay system, [3H]serine, and nitrocellulose membrane were from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom); U73122 was from Alexis Corp. (San Diego, CA); affinity purified polyclonal antibodies for JNK1, ERK2, p38, IL8RB, secondary antibodies, and IL8RB peptide were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-phospho-JNKs was from Promega (Madison, WI); anti-phospho-p38 and anti-phospho-ERKs were from New England BioLabs; Cs-ceramide was from BIOMOL Res. Lab. Inc. (Plymouth Meeting, PA); Ca2+-Green-1 AM was from Molecular Probes Inc. (Eugene, OR); rat GROβ was from Peprotech Inc. (Rocky Hill, NJ); chemiluminescent substrate and BCA protein assay kit were from Pierce; non-fat dry milk was from Bio-Rad; GST-c-Jun was kindly provided by Dr. C. J. Der (University of North Carolina, Chapel Hill, NC).

**Granule Neurone Cultures**—Cerebellar granule cells were obtained from 8-day-old Wistar rats (17), and cultured in basal Eagle's medium containing 25 mM KCl and 10% heat-inactivated fetal calf serum. Cells were used after 8 days of culture when they had fully differentiated. Most of the experiments were comparably carried out in Locke’s solution (154 mM NaCl, 5.6 mM KCl, 5.6 mM NaHCO3, 3.8 mM NaH2PO4, 2.5 mM CaCl2, 5.6 mM glucose, buffered with 5 mM Hepes, pH 7.4) or in phosphate-buffered saline (PBS) containing 5 mM glucose.

**Immunofluorescence**—Rat granule cells were plated on coverslips coated with poly-L-lysine (Sigma). Cells were rinsed in PBS, fixed in 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100 in PBS.
for 5 min. After extensive washing, primary antibody was applied as indicated by the supplier and incubated at 4–8 °C in PBS. Cells were washed and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody (Sigma). The samples were routinely examined with a microscope (Dialux, Leica, Northvale, NJ, equipped with a Leica TCS 4D system, equipped with a 100 × 1.3–0.6 oil immersion lens.

Western Blot Analysis of CXCR2 Expression—Cerebellar granule cells were scraped off the plate in a buffer containing 10 mM Tris-HCl, pH 7.5, 10 mM NaN3, 0.5 mM EDTA, 1% Nonidet P-40, 10 μM leupeptin, 10 mM aprotinin, and 1 mM PMFS, sonicated with a probe sonicator (Bandelin, Germany), and centrifuged for 15 min at 15,000 rpm in a microcentrifuge. Proteins were analyzed on 10% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose paper at 4 °C for 2 h. Blots were incubated for 1 h with 5% non-fat dry milk to block nonspecific binding sites and then incubated with affinity purified rabbit antibody to CXCR2 (K-19). The immunoreactivity was detected with a chemiluminescent substrate (ECL).

Confocal Microscopy—Fluorescence determinations were made using a real time confocal laser microscope (Odyssey, Noran Instruments, Redwood City, CA) equipped with an argon laser and interfaced to an upright microscope (Axioscope, Zeiss, Germany). The unit was driven by Image-1 software (Universal Imaging Corp., West Chester, PA). An excitation wavelength (λ) of 488 nm and emission was monitored at A > 515 nm, with a confocal slit of 100 μm. The laser beam was set at 30% intensity, with minimum power. Real time acquisition was performed at an effective resolution of 20 Hz. Granule cells were incubated at 37 °C for 30–60 min with the dye Ca2+-Green-1 AM (4–5 μM), extensively washed with medium and transferred to the stage of the confocal microscope for fluorescence recording. Images were captured in a video recorder (Sony VO 9600P, Japan) for further analysis. The cytosolic Ca2+ concentration induced by GROβ was expressed as the ratio of fluorescence increase after treatment/basal fluorescence (ΔF/ΔF0).

Cells were continuously superfused with external medium. For this and the following assays the concentration of GROβ was 60 nM, which gave the most reproducible results. agonists were applied by a gravity-driven perfusion system using independent external pipettes connected to a fast exchanger system (RSC-100, BioLogic, France).

Determination of Cellular Inositol 1,4,5-Trisphosphate (InsP3)—Primary cultures of rat granule cells (8-day-old) were incubated for 2–3 h in PBS and stimulated with GROβ. To investigate the effect of the phospholipase inhibitor U73122, this compound was given 10–30 min before GROβ poly(3-keto)polyanal 10 min on ice with a probe sonicator. The reaction was expressed as the ratio over basal levels, and greatly reduced InsP3 formation over basal levels, and greatly reduced InsP3 formation.

RESULTS

Rat Granule Cells Express CXCR2—We investigated the expression of CXCR2 in rat cerebellar granules by immunocytochemistry and Western blot. Fig. 1 (A and B) indicates that granule cells positively stain for CXCR2, revealing the presence of the receptor over the whole cell surface. The specificity of the staining was verified by labeling the cultures with antibodies to CXCR2 preincubated with the CXCR2 peptide used as immunogen (Fig. 1, C and D). CXCR2 expression was further confirmed by Western blot analysis, that revealed the presence of a major protein band (40 kDa) recognized by a specific CXCR2 antibody, as shown in Fig. 2. Normal rabbit IgG was used for controls (not shown).

GROβ Activates PIP2–PLC Generating Cellular InsP3 and DAG—Fig. 3A shows the time course of InsP3 accumulation that peaks at 1 min after cell stimulation, reaching 197.5 ± 20.5% of control values, and declining thereafter. We have also investigated the accumulation of DAG in cells prelabeled with [3H]arachidonic acid. A similar kinetics of [3H]DAG accumulation was observed upon GROβ stimulation (Fig. 3B): after 1 min of GROβ treatment, [3H]DAG reached 171 ± 4% of control values, remaining above basal values for at least 5 min. Experiments were also performed to study the effect of U73122, a PIP2–PLC inhibitor, on GROβ-mediated InsP3 and [3H]DAG accumulation. Cell pretreatment for 30 min with 10 μM U73122 almost completely abolished measurable [3H]DAG accumulation over basal levels, and greatly reduced InsP3 formation after 15 s with GROβ (5 μM stimulation). When U73122 was used at a concentration of 20 μM, InsP3 accumulation was even more reduced (70% inhibition). The basal levels of InsP3 were comparable both in control and U73122-treated cells.

GROβ Mobilizes Intracellular Ca2+—We investigated the Ca2+-mobilizing properties of GROβ on cerebellar granule cells. When bath applied to neurons loaded with Ca2+-Green 1 AM fluorescent dye, GROβ produced an increase of basal
FIG. 1. Rat granule neurones express CXCR2. Immunofluorescence analysis of CXCR2 expression in cultured rat granule neurones. Confocal micrographs of cultured granules labeled with polyclonal antibody to CXCR2 preincubated (C and D) or not (A and B) with control CXCR2 peptide, and revealed with tetramethylrhodamine B isothiocyanate-conjugated goat anti-rabbit antibodies. A and B and C and D show, respectively, two optical sections of the same field, through the dendrites (A and C) and through the cell bodies (B and D). The images were recorded with the same laser intensity and amplification parameters to allow direct comparison of the fluorescence signal. Bar: 10 μm.

FIG. 2. Expression of CXCR2 in primary cultures of cerebellar granule cells. Western blot analysis, performed with a polyclonal antibody to CXCR2. Arrow indicates the putative CXCR2. Molecular weight markers are indicated on the right.

fluorescence with ΔF/F = 0.3 ± 0.01 (mean ± S.E.; 107 out of total 245 cells examined, 107/245). The fluorescence increase had a delay of 9 ± 0.5 s, and a time-to-peak, defined as the time from the baseline to peak amplitude, of 68 ± 4 s; the increase in [Ca2+]i was sustained for more than 500 s after chemokine withdrawal. A comparably long delay between CXCR2 stimulation and onset of the Ca2+ response was elsewhere described (20). Representative examples of the fluorescence transient induced by GROβ are shown in Fig. 4.

GROβ Induces SM Hydrolysis in Granule Cells—To investigate the possibility that GROβ induces the activation of SM hydrolysis, granule cells were prelabeled for 48 h with [14C]serine, and then exposed to GROβ (60 nM). The results are illustrated in Fig. 5. The hydrolysis of cellular [14C]SM begins within the first minutes of cell stimulation, and proceeds for about 90 min, diminishing thereafter (Fig. 5A). Accordingly, upon GROβ stimulation there was an increase in [14C]ceramide. Cellular [14C]ceramide reached 125.6 ± 3.7% of control values after 15 min of GROβ and returned to control levels after about 90 min (Fig. 5B). The levels of [14C]ceramide increase did not fully parallel the decrease in [14C]SM, probably because of its metabolism. When vehicle alone was given to cells neither SM nor ceramide levels were altered, for the time observed.

Activation of N- and A-SMases by GROβ—To assess whether the hydrolysis of SM induced by GROβ was carried out by more than one type of SMase, we measured the activity of both N- and A-SMases, with an in vitro assay that makes use of the exogenous substrate [14C]SM. As shown in Fig. 6, the N-SMase activity is maximal after 15 min stimulation with GROβ, persists for the following 15 min, and returns to basal levels within 60 min. The activity of the A-SMase was more prolonged, lasting for about 60 min of cell stimulation, and decreased thereafter. Since the A-SMase can be activated by DAG (21, 22), we hypothesized that the mechanism responsible for A-SMase activation upon GROβ stimulation is due to the accumulation of DAG that occurs during GROβ stimulation of rat granules. Accordingly, at concentrations between 20 and 30 μM, the PIP2-PLC inhibitor U73122 abolished GROβ-mediated A-SMase activation (Fig. 7), leaving unaffected the activation of the N-SMase. The polyether antibiotic monensin, an inhibitor of A-SMase because of its ability to alter the pH of endolysosomal compartments (22, 23), blocked completely GROβ-induced A-SMase activation at concentrations 0.5–1 μM (Fig. 7). Cell pretreatment for 2 h with imipramine (40 μM) or desipramine (20 μM), both inducing the degradation of cellular acid sphingomyelinase (24, 25), also inhibited the activation of A-SMase (Fig. 7). These effects were specific, because N-SMase was not affected by inhibitor treatment (not shown). In addition, neither monensin nor U73122 were effective on constitutive A-SMase activity (3 nmol of [14C]SM/mg of protein), while both imipramine and desipramine reduced basal activity (to 1.7 nmol of [14C]SM/mg of protein). Furthermore, monensin at 0.5–1 μM did not influence GROβ-induced InsP3 accumulation (not shown). Higher doses of monensin (5–10 μM) damaged cells, and were not used. When the effect of monensin and U73122 was investigated on GROβ-stimulated [14C]Serine-labeled cells, the variation in [14C]ceramide and [14C]SM levels were comparable to those observed in untreated cells (not shown).

GROβ Activates JNK1 and ERKs—JNK1 can be efficiently activated by exposing cells to a variety of perturbing agents like ionizing and ultraviolet radiations, peroxides, heat and osmotic shocks, as well as chemokines and inflammatory cytokines (26). We investigated JNK1 activation upon GROβ cell stimulation by following the phosphorylation of the fusion protein-specific substrate, GST-c-Jun (1–223). Fig. 8 shows that cell
Fig. 3. GROβ stimulation of granule neurones induces PIP2 hydrolysis, revealed as InsP3 and [14C]DAG accumulation. Granule neurones were incubated for 2 h in PBS and stimulated in this same buffer with 60 nM GROβ for the indicated times. A, reactions were blocked with ice-cold trichloroacetic acid and InsP3 mass was measured as described under “Experimental Procedures.” Results are expressed as percentage of control values (2.8 pmol/100 mg of protein, taken as 100%) and data reported represent the mean ± S.D. of five independent experiments. Statistical significance (Student’s t test p < 0.01) is indicated (*). B, reactions were blocked with ice-cold methanol and [14C]DAG was analyzed as described under “Experimental Procedures.” Data are expressed as percentage increase over control values (taken as 100%) and represent the mean ± S.D. of four separate experiments. Statistical significance (Student’s t test p < 0.01) is indicated (*).

Table:<br>treatment for 15 min with GROβ induces the activation of JNK1 as evidenced by GST-c-Jun phosphorylation (166 ± 18% of control value, mean of four different experiments in duplicate ± S.E. Student’s t test p < 0.02). A delayed rise was also observed at 60 min of cell stimulation when the phosphorylation of GST-c-Jun was 122.3 ± 3.8% of control value (mean of 20 separate experiments ± S.E. Student’s t test p < 0.0001). Recent evidence indicates that ceramide generated by different stressing signals can mediate JNK activation (27, 28). Accordingly, treating the granule cells for 30 min with 30 μM C6-ceramide induced JNK1 activation: 128 ± 6% (mean of five different experiments ± S.E., Student’s t test p < 0.01) of control value. When the cells were stimulated by 50 units/ml bacterial SMase for 30 min, the phosphorylation of GST-c-Jun was again increased over the control. These data would suggest that the effect of GROβ on JNK1 may be mediated by ceramide. Interestingly, we observed that neither U73122 (not shown) nor imipramine or desipramine treatment (Fig. 9A) could block GROβ-induced JNK1 activation, suggesting that the activation of A-SMase is not necessary for this activation. The study with monensin was hampered by its effect on the basal kinase activity, while U73122, imipramine, and desipramine did not significantly alter basal JNK activity (data not shown). The observed GROβ-induced JNK1 activation was comparable when granule neurones were stimulated in Locke’s medium or in basal Eagle’s medium containing 25 mM KCl and 10% fetal calf serum, further indicating that the observed kinase activation was not due to the stress of nutrient withdrawal (data not shown). The activation of JNKs was further confirmed following GROβ-induced JNK phosphorylation with a phospho-specific Ab (Fig. 9A). With the same approach we have also investigated the activation of ERKs and p38, and the results are shown in Fig. 9B. GROβ-induced ERK activation in rat granule cells had been already shown in a previous paper (12) with a kinase assay. Fig. 9B also shows that p38 is not activated by 15 min GROβ treatment. The same results were obtained when cells were stimulated for different times (from 5 to 60 min, data not shown).

DISCUSSION
Chemokines are an expanding family of leukocyte chemoattractants that act through specific receptors selectively expressed on well defined lymphoid cell populations (1). Furthermore, chemokines, and their receptors, have been found also in other tissues including the central nervous system (10, 29, 30). The role of chemokines in the central nervous system during normal and pathological conditions is only recently emerging, but already some close correlations between their expression levels and clinical signs have been traced (31). Studies with transgenic animals expressing N51/KC, a neutrophil-specific chemokine, established that chemokine expression in the central nervous system is responsible for leukocyte trafficking from the vascular compartment (32), and that CXCR4 expression is essential for a functional cerebellar development (14). In neural cells, CXCR4 also mediates human immunodeficiency virus type-1-induced apoptosis (33). In addition, we have re-
recently suggested additional functions for the CXC chemokines IL-8 and GROα in the central nervous system, namely, the modulation of the synaptic functions of cerebellar Purkinje neurones (11).

To better understand the effects of chemokines on neuronal function and development, it is crucial to describe the set of biochemical pathways activated by these molecules. Here we show that cerebellar granule cells express functional CXCR2 and we demonstrate, to our knowledge for the first time, that a chemokine, namely GROα, activates the SM pathway, with the involvement of both neutral and acid sphingomyelinases. This double activation, even though generating the same lipid second messenger ceramide, may subserve different cellular functions, as proposed for tumor necrosis factor-α and IL-1 receptor signaling (34, 35). Since we have found CXCR2, a GROβ receptor, on granule neurones, we speculate that this receptor mediates the activation of the SMases. Although SM hydrolysis has been recently described for endothelin-1-stimulated rat small arteries (36), the activation of the SM cycle has not been shown to be mediated by G protein-coupled receptors, and the mechanisms involved in SMases activation are not known. We also show here that the activation of the A-SMase is dependent on the accumulation of cellular DAG, as reported for tumor necrosis factor-α receptor (22), because it is inhibited by U73122, monensin, imipramine, and desipramine. Neurones were incubated for 1.5 h in PBS and incubated for further 30 min in the presence of either 30 μM U73122 or 1 μM monensin. Alternately, cells were incubated for 0.5 h in PBS and incubated for 2 h with 40 μM imipramine or 20 μM desipramine. Cells were then stimulated with 60 nM GROβ in the presence of the inhibitors for 30 min, and samples were processed as described in the legend to Fig. 6. Results are expressed as percentage of [14C]SM hydrolyzed by untreated cells (C, taken as 100%), and represent the mean ± S.D. of four independent experiments.
bacterial SmaSe induce JNK1 activation. Nevertheless, the observation that GROβ induces JNK1 activation even upon U73122, imipramine, and desipramine treatment, when A-SmaSe activity is inhibited, indicates that ceramide produced by this enzyme is not necessary to activate JNK1. Although the coordinated activation of the sphingomyelin pathway and JNK1 by stress induces apoptosis in a number of proliferating cell types (27, 28, 43), the effects of ceramide generation in primary cultures of postmitotic neurons seem to be rather distinct. Primary cultures of cortical neurons (44) are resistant to ceramide-induced apoptosis, and ceramide can be partially cytoprotective on sympathetic neurons (45), while both protective (46) and toxic (47) effects have been reported for cerebellar granule neurons. At low doses, ceramide potentiates elongation of neural processes (48, 49) and inhibitors of sphingolipid metabolism disrupt axon growth (50). Furthermore, JNK1 activation has been described in mature neuronal cells following injury, suggesting that JNK1 could play a role in the organization of a strong response to cell damage (51). In contrast, the activation of JNK and c-Jun phosphorylation have been shown to be essential for apoptosis of neurons deprived of growth factors (52–54). The effects of GROβ-induced SmaSe and JNK1 activation in rat granules is currently under investigation, since we observe that chemokine treatment modulates the survival of cerebellar granule neurons from cell death induced by potassium deprivation.2

Since recently ceramide has been also reported to modulate, in cultured oligodendrocytes, K+ channel, in a ras-dependent way (55), and since glial cells express several chemokine receptors, among which CXCR2, chemokine-mediated ceramide generation could also be relevant in mediating the regulation of synaptic transmission. Data reported in these paper describe a novel signal transduction pathway for chemokines, and offer new perspectives to understand the role played by chemokines in the central nervous system during development, and in the response to local inflammatory conditions, when their level can increase severalfold (30).

Acknowledgments—We thank Dr. Ricardo Miledi and Dr. Alfred Merrill for critical reading of the manuscript, and Dr. Loriana Castellani for confocal images.

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